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REVIEW

SUBJECT COLLECTION: CYTOSKELETON

Ena/VASP proteins in cell edge protrusion, migration and adhesion

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ABSTRACT

The tightly coordinated, spatiotemporal control of actin filament remodeling provides the basis of fundamental cellular processes, such as cell migration and adhesion. Specific protein assemblies, composed of various actin-binding proteins, are thought to operate in these processes to nucleate and elongate new filaments, arrange them into complex three-dimensional (3D) arrays and recycle them to replenish the actin monomer pool. Actin filament assembly is not only necessary to generate pushing forces against the leading edge membrane or to propel pathogens through the cytoplasm, but also coincides with the generation of stress fibers (SFs) and focal adhesions (FAs) that generate, transmit and sense mechanical tension. The only protein families known to date that directly enhance the elongation of actin filaments are formins and the family of Ena/VASP proteins. Their mechanisms of action, however, in enhancing processive filament elongation are distinct. The aim of this Review is to summarize our current knowledge on the molecular mechanisms of Ena/VASP-mediated actin filament assembly, and to discuss recent insights into the cell biological functions of Ena/VASP proteins in cell edge protrusion, migration and adhesion.

KEY WORDS: Ena/VASP proteins, Actin dynamics, Cell adhesion, Cell migration, Protrusion

Introduction

Members of the Ena/VASP protein family are structurally conserved and present in both multicellular animals and single-celled organisms such as *Dictyostelium* (Fig. 1A). *Drosophila* Enabled (Ena), the founding member of this family, was identified through its genetic interaction with Abl tyrosine kinase, and was later found to play an important role in axon guidance in the developing nervous system (Bashaw et al., 2000; Gertler et al., 1995). *C. elegans* also contains a single Enabled homolog known as UNC-34, which was shown to be crucial for the migration of cells and growth cones in the developing embryo (Fleming et al., 2010). Vertebrates express three Ena-related paralogs – vasodilator-stimulated phosphoprotein (VASP), mouse Ena (Mena, also known as ENAH), and Ena-VASP-like (Evl). VASP was originally isolated from human platelets (Reinhard et al., 1992) and recognized as major substrate for the cAMP- and cGMP-regulated protein kinases PKA and PKG (Lambrechts et al., 2000; Waldmann et al., 1987), but it is also widely expressed in other cells and tissues (Reinhard et al., 1992). Mena and Evl were subsequently identified by sequence similarity (Gertler et al., 1996).

Members of the Ena/VASP family of proteins encompass three highly conserved domains (Fig. 1B), an N- and C-terminal, homologous domain, thus termed Ena/VASP homology 1 (EVH1) and EVH2, respectively, and a central, proline-rich domain (PRD). The N-terminal EVH1 domain binds most commonly to the so-called FPPPP motif, found in cytoskeletal proteins such as vinculin (Brindle et al., 1996), zyxin (Drees et al., 2000), lamellipodin (Krause et al., 2004) and the *Listeria* surface protein ActA (Niebuhr et al., 1997; Pistor et al., 1995). However, other cytoskeletal proteins such as the WAVE regulatory complex (WRC) subunit Abi from *Drosophila* or the human testin LIM-domain protein (Tes) either bind to the EVH1 domain with proline-rich sequences that deviate from the core consensus sequence (W/F)PxφP motif (x, any residue; φ, hydrophobic amino acid) or even utilize an unrelated LIM domain for interaction (Acevedo et al., 2017; Ball et al., 2000; Boëda et al., 2007; Chen et al., 2014). The EVH1 domain has been proposed to mediate subcellular targeting of Eva/VASP proteins (Krause et al., 2003). Nevertheless, the EVH1 domain alone does not appear to be sufficient for robust targeting to all subcellular sites, despite its essential function (see below). The central PRD domain is the least conserved and is rich in clusters of proline residues. It binds to the actin-monomer-binding regulator profilin, and is primarily used in the cellular context for recruitment of profilin–actin complexes to fuel actin assembly (Ferron et al., 2007) and for mediating interactions with signaling proteins containing SH3- or WW-domains, such as FE65 or Irsp53 (also known as APBB1 and BAIAP2, respectively) (Ermekova et al., 1997; Jonckheere et al., 1999; Krugmann et al., 2001; Reinhard et al., 1995). The C-terminal EVH2 domain mediates interactions with monomeric and filamentous actin, as well as tetramerization (Bachmann et al., 1999; Hüttelmaier et al., 1999). The G-actin-binding site (GAB) within the EVH2 domain represents a WASP homology 2 (WH2) motif found in many other actin regulators (Dominguez, 2016; Paunola et al., 2002). The adjacent F-actin-binding site (FAB) has also been proposed to possess WH2-like properties (Dominguez, 2009; Ferron et al., 2007). The VASP tetramer contains long stretches of intrinsically disordered amino acid sequences and is an exceptionally flexible molecule, as revealed by electron microscopy and analytical ultracentrifugation (Breitsprecher et al., 2008) (Fig. 1C,D). The only structured regions of the molecule are the globular N-terminal EVH1 domains and a short right-handed coiled-coil tetramerization domain (Tet) at the C-terminus of the protein (Ball et al., 2000; Kühnel et al., 2004).

In this Review, we start by discussing requirements for subcellular positioning of Ena/VASP proteins followed by a brief summary of the initial difficulties and controversies of the biochemical properties of this fascinating family of actin polymerases, and then sketch our current understanding of the

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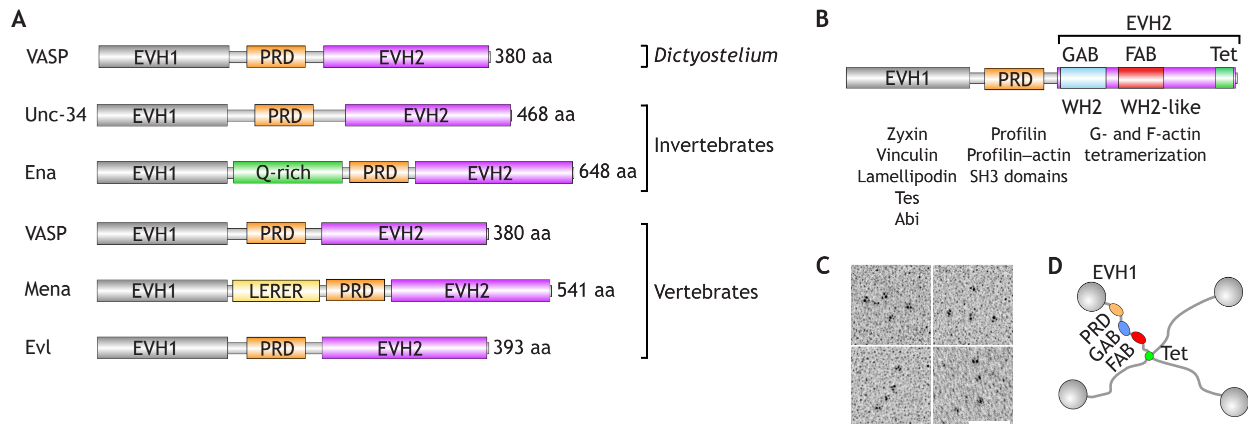

Fig. 1. Domain organization and structure of Ena/VASP proteins. (A) Representative Ena/VASP proteins from different organisms. All family members share a highly similar, tripartite domain organization. *Drosophila* Ena (Enabled) and vertebrate Mena (murine Ena) additionally contain a Q-rich region (green) or LERER repeats (yellow), respectively. Numbers indicate amino acid residues (aa). (B) Structure–function relationship of Ena/VASP proteins. The N-terminal EVH1 region contributes to subcellular targeting of Ena/VASP proteins predominantly through interaction with proteins containing FP_4 or related proline-rich repeats, but this requires EVH1 to be combined with C-terminal tetramerization (also see Fig. 3). The central proline-rich domain (PRD) of Ena/VASP binds to profilin and profilin–actin complexes, as well as SH3- and WW-containing proteins. The EVH2 domain at the C-terminus encompasses a WH2 type G-actin binding (GAB) motif (blue), a WH2-like F-actin binding module (FAB, red) followed by the tetramerization (Tet) domain (green) mentioned above. (C) Transmission electron microscope images of murine VASP tetramers after rotary shadowing, illustrating its highly flexible architecture (Breitsprecher et al., 2008). Scale bar: 50 nm. Images courtesy of Günther Resch and John Victor Small, Institute of Molecular Biotechnology (IMBA), Austria. (D) Cartoon illustrating a highly flexible VASP tetramer.

molecular mechanisms of Ena/VASP-mediated actin filament assembly *in vitro* and *in vivo*. This will lead us to elaborate on the functions of Ena/VASP proteins in cell edge protrusion, adhesive two-dimensional (2D)-migration and cell adhesion. We will also briefly highlight the potential relevance of Ena/VASP member phosphorylation, although concerning this topic and further detail, readers are mostly referred to several, excellent reviews (Blanchoin et al., 2014; Drees and Gertler, 2008; Krause and Gautreau, 2014; Romero et al., 2020; Trichet et al., 2008).

Subcellular positioning of Ena/VASP proteins

Our body cells can harbor dozens of distinct actin filament-containing structures, the relative extent of formation of which will of course depend on state of differentiation or cell-specific functions (Rottner et al., 2017). This means that even non-muscle cells will harbor contractile pseudo-sarcomeric structures in the form of stress fibers (SFs) and their anchorage sites, focal adhesions (FAs), and at the same time frequently highly dynamic protrusive structures at their motile fronts (see model in Fig. 2A). The most prominent, protrusive structures include the sheet-like lamellipodia and the finger-like, actin filament bundle-containing filopodia. If bundled arrays remain embedded into lamellipodial actin networks, they are termed microspikes (Small et al., 2002).

Ena/VASP proteins are long known to accumulate at all these sites of active actin assembly (Fig. 2A). Specifically, VASP, Mena and Evl have been shown to localize to FAs and SFs in a dotty fashion, to the protruding lamellipodial leading edge as a continuous line, and the distal tips of microspikes and filopodia in the form of bright spots (Damiano-Guercio et al., 2020; Gertler et al., 1996; Lanier et al., 1999; Reinhard et al., 1992; Rottner et al., 1999). Moreover, these proteins accumulate at the surfaces of bacterial pathogens, such as *Listeria monocytogenes* (Chakraborty et al., 1995), as well as in phagosomes (Coppolino et al., 2001), dorsal ruffles (Michael et al., 2010), invadopodia (Philippar et al., 2008) and in epithelial cell–cell contacts (Vasioukhin et al., 2000). For examples for most of these distinct localization patterns, see Fig. 2B–G.

The EVH1 domain has been proposed to mediate subcellular targeting of Eva/VASP proteins (Bear et al., 2000; Krause et al., 2003). Despite its essential function, however, the EVH1 domain alone does not mediate robust targeting to all subcellular sites. Although the VASP-EVH1 domain, for instance, has been reported to be sufficient at least for targeting to filopodial tips, it failed to robustly accumulate at the rim of lamellipodia and in FAs (Fig. 3; Applewhite et al., 2007). This is true both in the presence and absence of endogenous Ena/VASP proteins. Thus, additional domains of Ena/VASP proteins mediating interactions with actin or other actin-binding factors were hitherto concluded to be necessary for appropriate, subcellular targeting. However, the availability of true Ena/VASP family knockout cell lines now allows defining the minimal domains required for subcellular Ena/VASP targeting. Indeed, Fig. 3 (bottom row) exemplifies that the EVH1 domain alone can mediate all aspects of VASP subcellular distribution, as long as it is combined with the tetramerization domain to increase its functional affinity and residence time. This shows that, as opposed to previous assumptions (Loureiro et al., 2002), the F-actin-binding EVH2 domain is dispensable for proper subcellular targeting.

Ena/VASP recruitment to FAs requires mostly zyxin (Hoffman et al., 2006), with contributions from additional FPPPP motif-harboring proteins, such as vinculin or palladin. The latter has also been implicated in the recruitment of VASP to non-contractile, so called dorsal SFs (Gateva et al., 2014; Hotulainen and Lappalainen, 2006). An additional level of complexity comes from the EVH1 ligand Tes, which is recruited to FAs by zyxin, but can compete with canonical FPPPP-containing ligands for the interaction with Mena (Boëda et al., 2007; Garvalov et al., 2003). Another EVH1 interactor, proline-rich EVH1 ligand (PREL1; also known as Rap1-GTP-interacting adaptor molecule, RIAM), appears to trigger Ena/VASP accumulation in protrusions and FAs downstream of Rap/Ras signaling (Jenzora et al., 2005; Lafuente et al., 2004). The PREL/RIAM-related lamellipodin (Lpd; also known as RAPH1) has been proposed to be obligatory for Ena/VASP targeting to the leading edge of lamellipodia (Krause et al., 2004), but its genetic disruption recently disproved this view (Dimchev et al., 2020).

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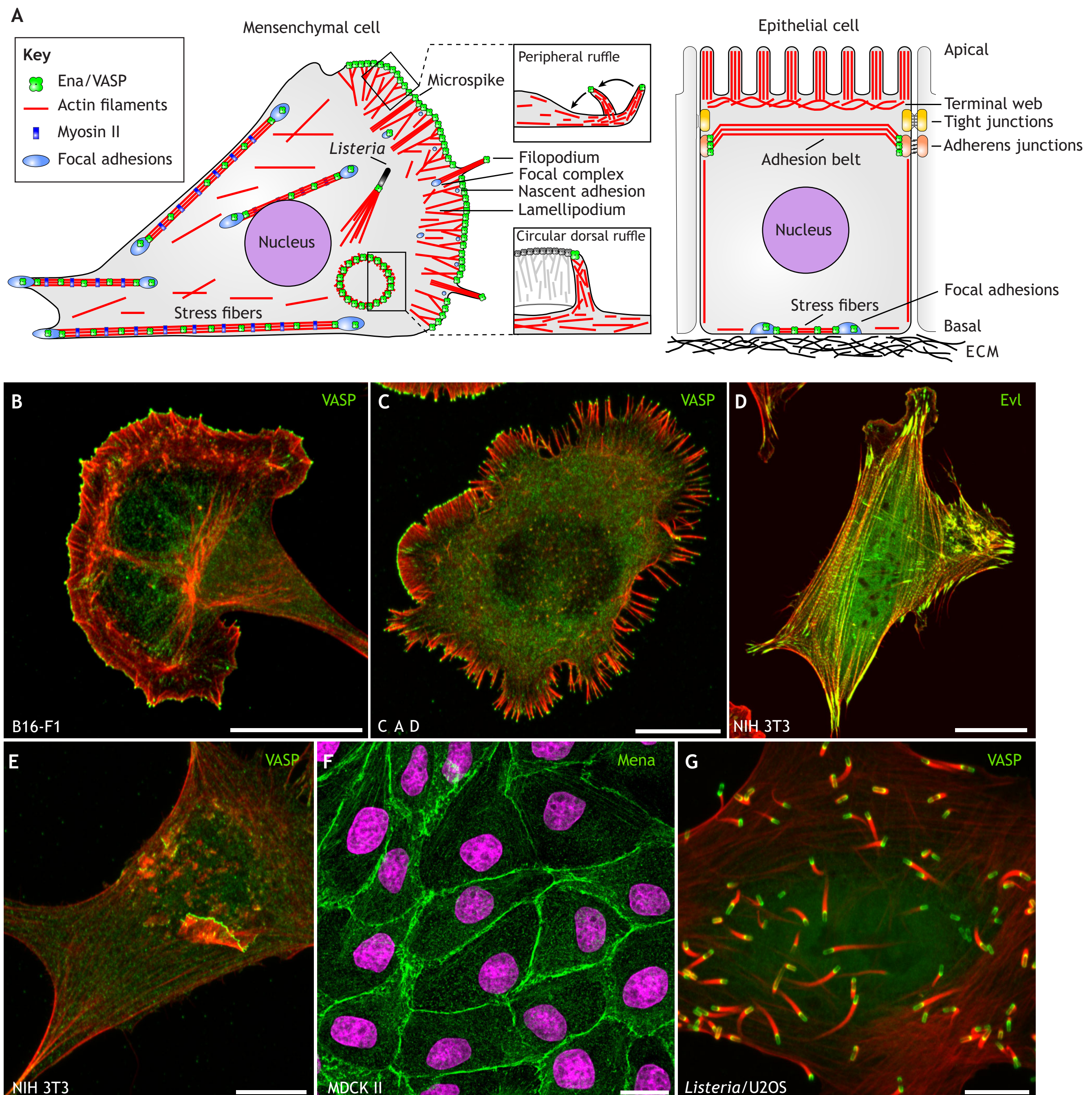

Fig. 2. Subcellular localization of Ena/VASP proteins in different cell types. (A) Schematic illustration of specific localizations of Ena/VASP proteins to most prominent actin-based structures of mesenchymal (left) and epithelial cells (right). Only ventral SFs are shown for clarity. (B) Endogenous VASP at the leading edge and tips of microspikes in B16-F1 mouse melanoma cells. (C) Endogenous VASP at the leading edge and tips of microspikes and filopodia in neuronal CAD cells. (D) Ectopically expressed Evl targeted to FAs and ventral SFs of NIH 3T3 fibroblasts. (E) VASP at the tip of a dorsal ruffle in an NIH 3T3 cell. (F) Mena accumulating in cell–cell contacts of an MDCK II monolayer. (G) VASP enriching at the rear surface of *L. monocytogenes*, which promotes its intracellular motility in infected U2OS ostersarcoma cells. Ena/VASP proteins are shown in green, F-actin in red and DAPI in magenta (in E). Scale bars: 20 µm (B–D,F); 10 µm (E,G).

The formation of lamellipodial actin networks occurs downstream of Rac-triggered activation of the Arp2/3 complex by the WRC (Rottner et al., 2021). In *Dictyostelium*, the latter was shown to operate upstream of Ena/VASP accumulation in pseudopods (Litschko et al., 2017). This finding was recently confirmed in mammalian cells lacking the WRC, but harboring non-canonical, lamellipodia-like structures (Kage et al., 2021 preprint). Interestingly, *Dictyostelium* and *Drosophila* Abi have been shown to bind to Ena/VASP through EVH1 (Chen et al., 2014; Litschko et al., 2017), thus likely mediating Ena/VASP accumulation in pseudopodia and lamellipodia. The precise recruitment features to additional subcellular structures, such as along SFs or to adherens junctions of epithelial or endothelial cells, remain to be established.

The molecular mechanism of Ena/VASP-mediated actin assembly

Previous biochemical analyses quickly established the function of individual domains of vertebrate Ena/VASP proteins in filament

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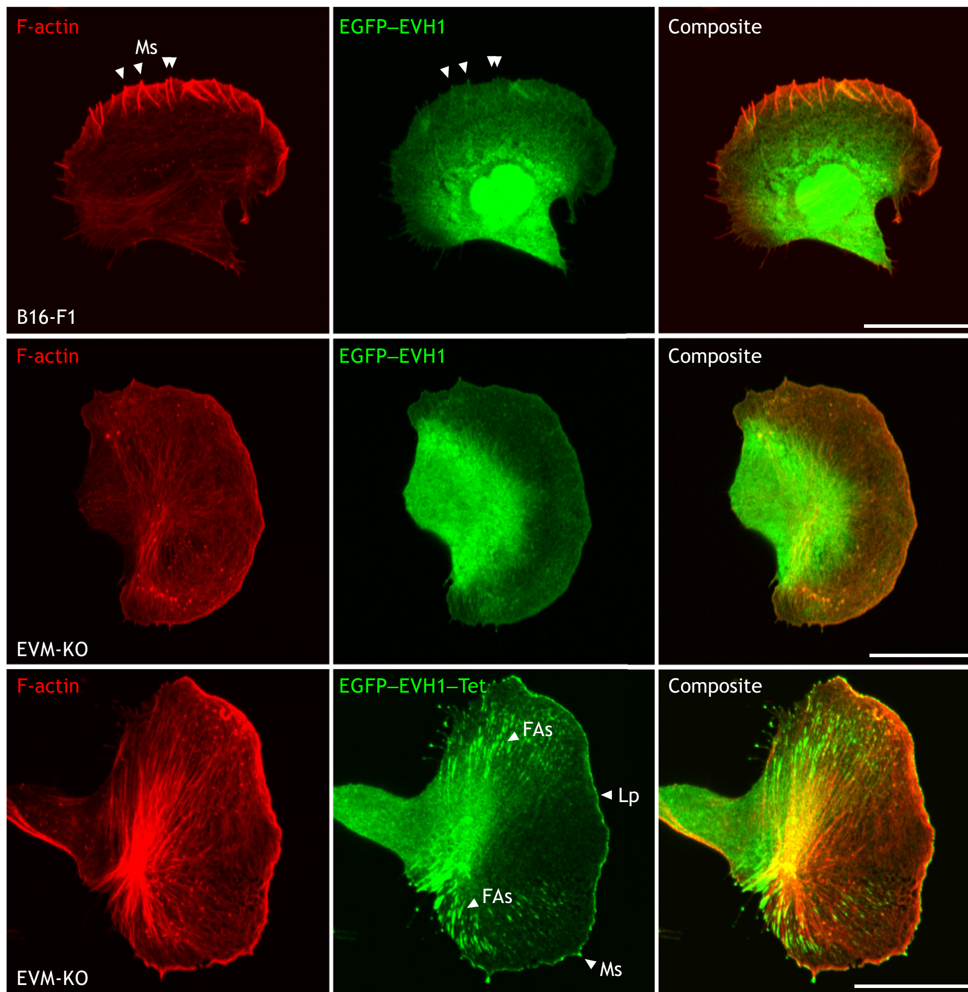

Fig. 3. The EVH1 and tetramerization domains are required for appropriate subcellular positioning of VASP. Representative images of B16-F1 and Ena/VASP-deficient EVM-KO cells (Damiano-Guercio et al., 2020) migrating on laminin, expressing indicated constructs (green) and counterstained for F-actin (red). The EVH1 domain alone fused to EGFP (EGFP–EVH1) accumulated only very weakly at the tips of microspikes (Ms) in B16-F1 wild-type cells (white arrowheads) and was not detectable at the tip of the lamellipodium (Lp) and stress fibers (SFs). Similar results were observed in Ena/VASP-deficient EVM-KO cells (EGFP–EVH1, middle panel), even though in addition, this cell line is virtually devoid of microspikes (also see Fig. 5). In stark contrast, the EVH1 domain combined with the short Tet motif (EGFP–EVH1–Tet, bottom panel), mediating tetramerization of the construct, was fully sufficient for its prominent accumulation at both leading edge and stress fibers in EVM-KO cells. Scale bars: 20 µm.

bundling, interaction with monomeric actin and tetramerization. However, owing to the rather low affinity of the vertebrate GAB motif for actin monomers (Breitsprecher et al., 2011), it is not surprising that earlier work reported conflicting results on the impact of Ena/VASP proteins on actin assembly, as relatively low concentrations of actin and Ena/VASP in buffers with different salt concentrations were used (Trichet et al., 2008). The situation changed upon analysis of *Dictyostelium* VASP, which harbors a high-affinity GAB (Breitsprecher et al., 2008, 2011). Using total internal reflection fluorescence (TIRF) microscopy with purified proteins, it could be shown that both human and *Dictyostelium* VASP are directly involved in accelerating filament elongation by delivering actin monomers to the growing barbed end (Breitsprecher et al., 2008). This study further revealed that, in bulk solution, *Dictyostelium* VASP accelerates actin filament elongation up to sevenfold as compared to the approximately twofold acceleration by human VASP, although both actin polymerases were readily inhibited by low concentrations of heterodimeric capping protein (CP; comprising e.g. human CAPZA1 and CAPZB), which binds tightly to barbed ends to terminate filament growth (Maun et al., 1996; Yamashita et al., 2003). In striking contrast, dense clustering of neighboring VASP tetramers in the range of 10 nm on functionalized beads enabled long-lasting processive filament elongation that became virtually insensitive even to high concentrations of CP (Breitsprecher et al., 2008) (Fig. 4A).

Based on thermodynamic data, the determination of the association rate constants of G-actin to human and *Dictyostelium* GAB, as well as TIRF microscopy, a quantitative mathematical model of VASP-mediated filament elongation could be developed (Breitsprecher et al., 2011). It is derived from the ‘actoclampin’ model of actin filament end-tracking proteins (Dickinson and Purich, 2002; Dickinson et al., 2004) in which processive elongation is achieved by alternating, multivalent and affinity-modulated interactions between the VASP tetramer and the filament barbed end (Fig. 4B–D). The model implies that, at steady state, only one polypeptide chain of the VASP tetramer is bound to the terminal filament subunit at any given time, leaving a number (N) of free GABs to recruit actin monomers from solution. Captured monomers can then be either released back into solution or are transferred onto the filament tip and irreversibly incorporated, so that the rate of VASP-mediated filament elongation scales with the saturation of GABs with actin monomers (Breitsprecher et al., 2011). This not only explains why *Dictyostelium* VASP carrying a high-affinity GAB is more efficient when compared with its human counterpart at the low actin concentrations used in *in vitro* assays, but also implies that, due to the much higher cellular actin concentration, all Ena/VASP proteins are fully saturated with actin *in vivo* and act as powerful actin polymerases (Breitsprecher et al., 2011). Global fitting of experimental data for filament elongation from beads coated with VASP mutants carrying different GAB domains yielded N=3, formally suggesting that a single VASP tetramer operates at a filament tip during filament elongation (Breitsprecher et al., 2011). In fact, processive, but relatively short-lived, filament elongation has also been shown with single

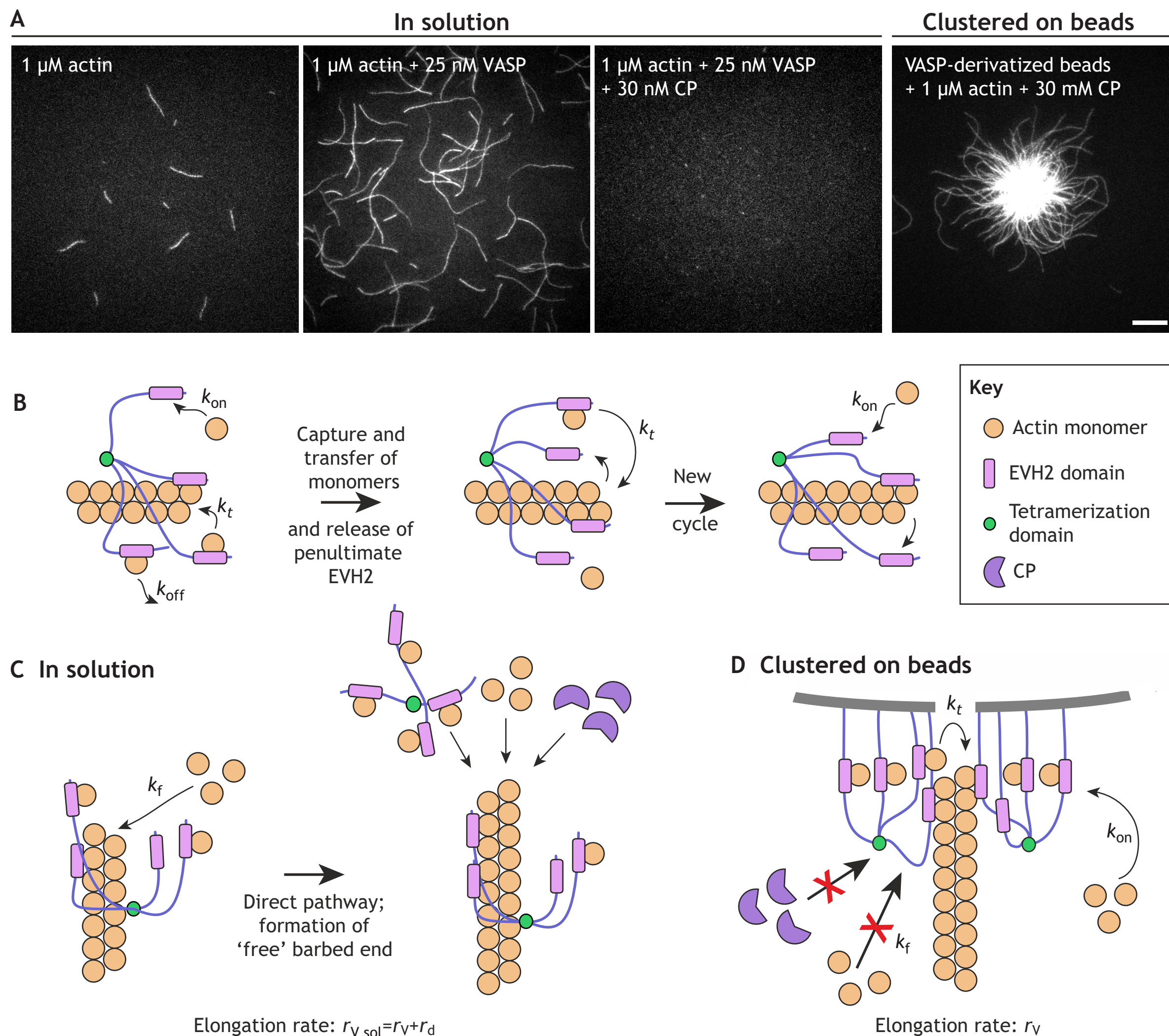

Fig. 4. Molecular mechanism of VASP-mediated filament elongation in solution and in clustered arrays. (A) Representative images from time-lapse movies illustrating acceleration of filament elongation by *Dictyostelium* VASP and its inhibition by CP in solution. In contrast, on VASP-derivatized beads, filament elongation becomes long-lasting and virtually resistant to CP. Scale bar: 10 µm. Image courtesy of Thomas Pokrant, Hannover Medical School, Germany. (B) General mechanism of VASP-mediated actin assembly. The VASP tetramer is continuously attached to the filament-barbed end by at least one EVH2 domain during filament elongation. Free EVH2 domains can capture actin monomers from solution with an on-rate constant k_{on}, and subsequently either transfer the monomers onto the barbed end with a transfer rate k_t to drive filament assembly, or release them back into solution with an off-rate k_{off}. Through this mode of action, the VASP tetramer is able to processively track and elongate growing barbed ends. (C) In solution, processive association of VASP with the barbed end is rather short lived, and therefore competes with the spontaneous addition of actin monomers to the barbed end through the direct pathway, with an independent transfer rate constant k_f. Free barbed ends are then accessible for either other VASP tetramers, actin monomers or capping protein (CP). The average elongation rate governing filament growth is therefore composed of the rates of VASP-mediated filament elongation (r_v) and of the direct pathway (r_d). (D) VASP clustering on surfaces, such as beads or the plasma membrane, leads to processive association of VASP with growing filament ends, efficiently blocking barbed end access for CP and the spontaneous addition of monomers. Thus, in clustered arrays, filament elongation is exclusively driven by actin monomers recruited and transferred by VASP. For more details, see Breitsprecher et al. (2011).

vertebrate and *Drosophila* Ena/VASP tetramers (Hansen and Mullins, 2010; Winkelman et al., 2014). Since single VASP tetramers are only transiently associated with barbed ends, monomers can also be added spontaneously to filament ends in a parallel pathway (termed the direct pathway). Thus, the total elongation rates observed with VASP in solution constitute the sum of the rate of VASP-mediated filament elongation and that resulting from the direct pathway (Breitsprecher et al., 2011). However, the underlying mechanism in clustered arrays is profoundly different, since in the latter, processive VASP-mediated filament elongation becomes exceptionally long-lasting and resistant against CP (Breitsprecher et al., 2008, 2011). Experimental testing of the model by variation of oligomerization states and increase of GAB numbers on individual polypeptide chains indeed revealed that – within a broad range – the rates of VASP-mediated actin filament elongation are directly proportional to the number of free GABs in solution (Brühmann et al., 2017). In contrast, in surface-bound VASP clusters, formed by the different oligomerization mutants, *N* is not only fixed to three free GABs, but additionally shows that VASP molecules from distinct oligomers or VASP monomers can synergize in the processive elongation of single actin filaments (Brühmann et al., 2017). Notably, processivity, which additionally correlates with the affinity of the FAB for filament side binding, increases continuously with Ena/VASP oligomerization (Breitsprecher et al., 2011; Brühmann et al., 2017). Thus, the VASP tetramer appears to be the evolutionarily optimal form

to drive processive actin assembly from clustered arrays in the presence of CP.

Regulation of Ena/VASP-mediated actin assembly by profilin

Actin bound to profilin is assumed to be the main polymerization-competent form of monomeric actin in cells (Kaiser et al., 1999; Pollard and Borisy, 2003). However, an open issue in the field is the question as to whether Ena/VASP utilizes profilin to accelerate actin assembly *in vivo*, as typically seen with formins (Kovar et al., 2006), or whether it might also operate with pure actin and if so to what extent. The co-crystallization of mammalian profilin–actin in complex with a peptide spanning the PRD and GAB of murine VASP actually suggests that this region has an important function in recruitment and subsequent delivery of monomeric actin to the GAB domain (Ferron et al., 2007). However, although one study found no positive effect of human or *Dictyostelium* profilin on VASP-mediated filament elongation *in vitro* (Breitsprecher et al., 2008), others have reported that VASP and profilin enhance the rate of actin filament elongation (Hansen and Mullins, 2010; Pasic et al., 2008). One important difference between these studies is how actin assembly rates were compared. While Breitsprecher et al. explored VASP-mediated actin assembly in the presence or absence of profilin, as typically done in the formin field, the latter studies only compared rates of actin filament elongation in the presence of VASP and profilin–actin with those observed with profilin–actin alone, precluding assessment of the direct effect of profilin on Ena/VASP-mediated actin assembly. In our more recent work, we also analyzed different profilin isoforms, and found that Ena/VASP proteins can indeed effectively assemble filaments from profilin–actin, albeit with approximately only half the rates than seen with pure actin alone (Brühmann et al., 2017). Unexpectedly, in a very recent study using profilin 1-deficient CAD cells, Ena/VASP was concluded to be non-functional despite its increased localization at the protruding cell edge (Skruber et al., 2020). However, given that at least *in vitro*, Ena/VASP proteins can use both pure actin and profilin-actin complexes for actin assembly, this conclusion, derived from mitochondrial Ena/VASP sequestration and lamellipodial F-actin intensity measurements, seems counterintuitive. Considering the established positive correlation between protrusion and Ena/VASP accumulation in wild-type lamellipodia (Rottner et al., 1999), the hypothesis put forward by Skruber et al. deserves to be revisited in more direct assays in the future.

Ena/VASP proteins in cell edge protrusion

Consistent with the positive regulatory role in both actin filament elongation *in vitro* and the efficiency of actin assembly-dependent motility of intracellular pathogens (see Box 1), Ena/VASP proteins were originally considered to be positive regulators and effectors of lamellipodia protrusion. Historically, for instance, VASP was the first actin-binding protein shown to dynamically accumulate at the edges of protruding lamellipodia in a fashion that linearly correlated with protrusion rates, and thus active actin polymerization (Rottner et al., 1999). This behavior had perfectly fitted earlier knowledge on the positive regulatory function of Ena/VASP proteins on the surface of *Listeria* (Sechi and Wehland, 2004). In other words, rapid protrusion perfectly correlated with maximal VASP accumulation at lamellipodial leading edges, and a halt in protrusion or lamellipodial retraction resulted in the complete vanishing of Ena/VASP from these sites (Rottner et al., 1999). Importantly, these activities are completely independent of association of lamellipodia with the substratum, which occurs through nascent adhesions or focal complexes that Ena/VASP members also associate with, or from their recruitment to other subcellular structures, such as cytoplasmic SFs (Rottner et al., 1999, 2001). Importantly, these Ena/VASP dynamics must also be distinguished from their general association with the entire plasma membrane, as mediated for instance by factors equipped with a C-terminal CAAX-box, originally identified in Ras or Rho GTPases, and destined for membrane association through prenylation (Roberts et al., 2008). Around the same time, in their seminal studies, Gertler and colleagues found, surprisingly, that effective fibroblast migration negatively correlated with Ena/VASP expression, mostly using Mena- and VASP-deficient cells that have been screened for lowest possible Evl expression, termed MV^{D7} (Bear et al., 2000). Aside from analyses using MV^{D7}, Ena/VASP overexpression or their inhibition by non-productive mistargeting, for instance to mitochondrial surfaces was also used (Bear et al., 2000). Consistent with the above results, this particular study concluded that Ena/VASP also inhibits rates of membrane extension and retraction (Bear et al., 2000), contradicting the view of positive regulatory roles on *Listeria* motility and lamellipodia protrusion (Rottner et al., 1999; Sechi and Wehland, 2004). One complication in the interpretation of this study is that MV^{D7}, unlike the original assumptions, later turned out not to be truly Ena/VASP-deficient, as they still expressed significant levels

Box 1: Ena/VASP functions in *Listeria* motility

Listeria monocytogenes, a food-born human pathogen, can cause serious medical problems, such as meningitis or miscarriage. The gram-positive bacterium hides within the cytoplasm of infected cells and from there can spread into neighboring host cells, thereby effectively escaping the humoral immune response. Spreading occurs through active actin polymerization that pushes the bacterium forward, accompanied by formation of a transient, actin filament comet tail (Welch and Way, 2013). Interestingly, *Listeria* binds both to the Arp2/3 complex (Domann et al., 1992; Kocks et al., 1992) and Ena/VASP (Chakraborty et al., 1995). Binding to the latter requires interactions between their EVH1 domains and the ActA consensus motif E/DFPPPPXD/E, which is repeated four times in ActA and also present in host cell vinculin and zyxin (Niebuhr et al., 1997). Although this domain can redirect VASP and actin accumulation to various subcellular compartments, including mitochondria (Fradelizi et al., 2001; Pistor et al., 1994), this activity only constitutes half of the fascinating story of *Listeria*-mediated actin comet formation. In addition, seminal studies by the groups of Mitchison and Carlier have established ActA as a key determinant of Arp2/3 complex-mediated *Listeria* motility (Loisel et al., 1999; Welch et al., 1998). The *in vitro* reconstitution of actin-based *Listeria* motility using purified proteins confirmed that although the Arp2/3 complex is obligatory for actin assembly on the bacterial surface, Ena/VASP family members are not essential, but increase the efficiency of the process (Loisel et al., 1999), which is highly analogous to their role in lamellipodia (Damiano-Guercio et al., 2020). The relevance of Ena/VASP proteins in *Listeria* motility was also explored in MV^{D7} cells lacking Mena and VASP (Bear et al., 2000; Geese et al., 2002). Although these studies could clearly establish the effects of Ena/VASP gene dose on *Listeria* motility and successfully explore the relevance of specific Ena/VASP domains, the reported data may have to be recapitulated, at least partly, since MV^{D7} cells still express Evl (Auerbuch et al., 2003; Damiano-Guercio et al., 2020). In addition, suggestions for how precisely Ena/VASP contribute to Arp2/3 complex-mediated actin assembly at the *Listeria* surface range from the promotion of Arp2/3-mediated branched nucleation (Skoble et al., 2001) to inducing the dissociation of ActA from the branch junction (Samarin et al., 2003). Considering the recently described promotion of WRC- and Arp2/3-dependent actin assembly by the Ena/VASP antagonist CP (Funk et al., 2021), and extrapolating this mechanism to ActA-dependent processes, we feel it will certainly be instrumental to tease out the precise functions of both CP and Ena/VASP at the *Listeria* surface in the future.

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of Evl (Damiano-Guercio et al., 2020). However, the original hypothesis of a negative effect of Ena/VASP on actin assembly at the leading edges of lamellipodia was subsequently corrected by the discovery of a positive role. The latter positive regulatory function, was concluded to counteract the slow and persistent advancement of the cell front presumed to accompany effective fibroblast migration (Bear et al., 2002). More specifically, lamellipodia that protruded too rapidly as a result of high activities of Ena/VASP were now proposed to be prone to rapid and frequent collapse, thus being incapable of mediating the slow but efficient leading edge advance in fibroblast migration. This view seemingly solved the original discrepancies that had arisen when collectively considering all those observations. Hence, since then, the consensus view in the field was that actin polymerases that act at lamellipodial leading edges, such as VASP or, for instance the formin and Cdc42 effector FMNL2, promote actin filament elongation, thereby increasing the spacing between Arp2/3-dependent branches in such actin networks. Of note, the continuous branch formation by Arp2/3 complex is considered to be an inherent feature of the formation of actin networks such as lamellipodia, which must therefore be coordinated with the activities of various actin regulators during actin-dependent protrusion (Dimchev et al., 2021; Krause and Gautreau, 2014). The increase of branch spacing at the individual filament level as caused by Ena/VASP would thus decrease the stability and protrusion persistence of these structures. Conversely, reduction of the activity of Ena/VASP or FMNL2 would enhance the density of Arp2/3-dependent branches, the persistence of protrusion and lamellipodial force development, which could indeed make a lot of sense as beautifully summarized about one decade later (Krause and Gautreau, 2014). However, subsequent studies by various labs clearly established that the relative dependencies of all those parameters, that is lamellipodium protrusion versus actin assembly rate versus cell migration, are far more complex than originally anticipated (Damiano-Guercio et al., 2020; Kage et al., 2017; Law et al., 2021).

Specifically, according to this simple model (Krause and Gautreau, 2014) and with respect to FMNL formin activities, the expected phenotypes of removal of FMNL2 and its related family member FMNL3, both of which accumulate at the tips of protruding lamellipodia (Kage et al., 2017), were not only a reduction in protrusion and polymerization rate of the network, but also an increase in actin filament densities, Arp2/3 complex incorporation and exerted protrusion strength. However, none of the expectations were correct, except for reduced protrusion rates; instead, FMNL2/3 KOs exhibited unchanged Arp2/3 complex densities and actin network polymerization rates, despite the decreased F-actin densities and exerted protrusion forces (Kage et al., 2017). Based on all these observations, the conclusion was that FMNL formins (as opposed to Ena/VASP) polymerize a subpopulation of lamellipodial actin filaments, thereby contributing to the overall F-actin concentration and thickness of the lamellipodium, as well as its protrusion efficiency and strength, without directly affecting the Arp2/3 complex-dependent branched actin network (Kage et al., 2017). These conclusions were also confirmed by mathematical modeling (Dolati et al., 2018). However, the story does not end here, as the complete removal of all three Ena/VASP family members, which was recently achieved through CRISPR/Cas9-mediated genome editing (Damiano-Guercio et al., 2020) led to a phenotype that significantly differed from that obtained upon formin depletion, and contradicted previous proposals and models (Bear et al., 2000; Krause and Gautreau, 2014). Upon complete loss of Ena/VASP, lamellipodia still formed and protruded and assembled actin more slowly and with increased Arp2/3 complex incorporation, as would be expected from previous models, but the cells displayed significantly reduced actin network densities with markedly perturbed actin filament architecture (Fig. 5), as well as an at best unchanged, but certainly not increased, lamellipodial persistence (Damiano-Guercio et al., 2020). In analogy, a more recent characterization of Nance–Horan syndrome-like 1 protein (NHSL1), an inhibitor of the WRC, the main Arp2/3 complex activator in canonical lamellipodia, revealed that increased lamellipodial Arp2/3 complex activities and F-actin intensities in lamellipodia do not necessarily give rise to reduced protrusion speed. More specifically, NHSL1-disrupted cell lines displayed unchanged protrusion rates in spite of increased Arp2/3 complex and F-actin densities, but compromised lamellipodia stability (Law et al., 2021). Likewise, removal of the prominent WRC antagonist CYRI (CYFIP-related Rac interactor) increased protrusion plasticity, i.e. the extent of protrusion and retraction (Fort et al., 2018), again distinct from expectations of a model, in which Arp2/3-dependent branching density would positively and negatively correlate with protrusion persistence and speed, respectively (Krause and Gautreau, 2014).

Together, these data show that the interrelationships of all these lamellipodial parameters are much more complex than previously anticipated. Indeed, despite the comparable activities of given actin-binding proteins at the single-filament level (such as the actin polymerase function of Ena/VASP and formins), the precise phenotypic outcome of interfering with the function of the different factors in cells can be surprisingly distinct. Thus, the mechanism of how Ena/VASP counteracts Arp2/3 activity and incorporation into lamellipodia deserves further in-depth analysis and elucidation.

Aside from all this, it should be additionally emphasized that increased protrusion activity, as mediated for instance by FMNL or Ena/VASP activity at lamellipodial edges, does not necessarily translate into abrogated 2D migration, as had been deduced from earlier observations (Bear et al., 2002). Furthermore, it is difficult to exclude cell-type-specific effects when studying highly complex processes such as migration, the efficiency of which depends on a myriad of parameters, but our recent studies have clearly established that reduced protrusion efficiency (e.g. upon Ena/VASP removal) can indeed positively correlate with reduced migration efficiency (Damiano-Guercio et al., 2020). As this positive correlation was observed in both B16-F1 melanoma cells and NIH 3T3 fibroblasts, it is unlikely that this discrepancy with earlier studies can solely be explained by cell-type-specific differences. Instead, we conclude that the efficiency of cell migration may differentially depend on the effectivity of lamellipodium protrusion in distinct cell types, so neither characteristic can be unambiguously deduced indirectly by just exploring the respective other. However, in highly motile cell types, such as the model cell line B16-F1 melanoma, we clearly and repeatedly see a positive correlation between capability or efficiency of lamellipodia protrusion and cell migration (Damiano-Guercio et al., 2020; Kage et al., 2017; Schaks et al., 2018).

Relative contributions of Ena/VASP proteins to lamellipodia protrusion versus microspike and filopodia formation

Genetic disruption of all Ena/VASP family members in B16-F1 mouse melanoma cells strongly decreased lamellipodial F-actin content and the width of lamellipodia, hence overall actin filament mass (Damiano-Guercio et al., 2020). However, the most striking phenotype was the virtual elimination of microspike bundles within the remaining lamellipodia (see Figs 3 and 5); in wild-type cells,

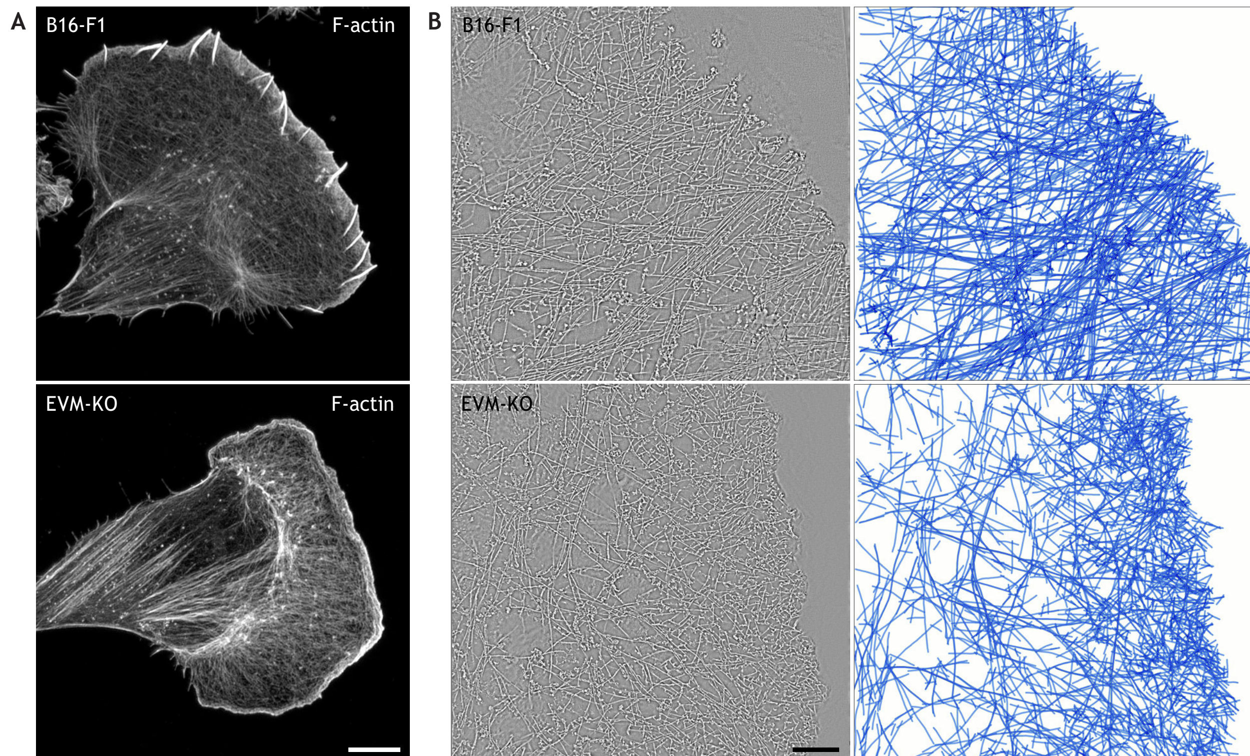

Fig. 5. Loss of Ena/VASP perturbs lamellipodia and abrogates microspike formation in B16-F1 cells. (A) Representative Airyscan confocal images of wild-type B16-F1 and EVM-KO cells allowed to spread and migrate on laminin and stained for the actin cytoskeleton. Scale bar: 5 µm. (B) Electron tomography of ultrastructural changes in lamellipodial actin networks upon loss of Ena/VASP family members. Transmission electron micrographs of negatively stained cytoskeletons of protrusive edges of a representative wild-type B16-F1 versus an EVM-KO cell (left). 2D projections of digital 3D tomograms of the same fields showing actin filament trajectories in blue (right). Scale bar: 100 nm. For further details see Damiano-Guercio et al. (2020). Images in B courtesy of Jan Müller, Maria Nemethova and Michael Sixt, Institute of Science and Technology (IST), Austria.

these consist of bundled lamellipodial filaments that appear to translocate laterally within lamellipodial actin networks driven by actin polymerization (Small et al., 2002). However, as other actin polymerases can also contribute to microspike formation, such as the FMNL subfamily of formins (Kage et al., 2017), it is not entirely clear why Ena/VASP removal had such a strong defect. One possibility is that microspike bundles, which can be distinguished from bona fide filopodia owing to their almost complete embedding into lamellipodial actin networks (see above), are triggered non-specifically by the sheer amount of lamellipodial filaments, so that a reduction of actin filament mass might already be sufficient for a decrease in microspike bundles. This would be consistent both with the correlation between reduced F-actin mass and microspike numbers upon FMNL2 and FMNL3 knockout (Kage et al., 2017), and explain the almost complete removal of these structures upon Ena/VASP disruption despite the assumed presence of FMNLs in the lamellipodia of these cells (Damiano-Guercio et al., 2020). However, even though it seems tempting to speculate that filament bundling into microspikes is abolished if F-actin mass in the lamellipodium falls below a certain threshold, this exclusive explanation is unlikely as FMNL overexpression – which is known to increase lamellipodial F-actin mass (Dimchev et al., 2017) – does not rescue microspike formation in EVM-knockout cells (EVM-KO) lacking all Ena/VASP family members (Damiano-Guercio et al., 2020). Thus, microspike formation appears to require specifically Ena/VASP activity, although the underlying molecular pathway has hitherto remained unclear.

On a different note, the loss of microspikes did not directly translate into similarly strong defects in the formation of canonical filopodia (Damiano-Guercio et al., 2020), which are capable of protruding beyond and independently of lamellipodial actin networks (Small et al., 2002). To our knowledge, this provides the first direct experimental evidence for the notion that filopodia and microspikes should indeed be considered as independent entities, and are not interchangeable, as proposed based on previous observations that suggested that filopodia can in principle emerge from microspikes (Svitkina et al., 2003). Yet, and partly consistent at least with previous observations of strongly diminished filopodia formation in VASP-null *Dictyostelium* mutants (Han et al., 2002), Ena/VASP-deficient cells displayed moderately reduced filopodia numbers as well, if induced, for instance, by Arp2/3 complex inhibition (Damiano-Guercio et al., 2020). In contrast, filopodia nucleated by constitutively active mDia2 (also known as DIAPH3), a Diaphanous-related formin, appeared to form completely independently of Ena/VASP activity (Damiano-Guercio et al., 2020). Of note, cells can be coerced to form filopodia by various means (see, for example, Rottner and Schaks, 2019), including the suppression of lamellipodia (as for instance by Arp2/3 complex inhibition, see above) or by employing constitutively active formins, in particular mDia2, which is particularly effective in this context (Block et al., 2008; Yang et al., 2007). It has hitherto remained unknown, however, what the obligatory factors are in filopodia formation or whether this means that filopodia could be formed by various redundant mechanisms. Notwithstanding this, Ena/VASP family members are surprisingly relevant for the formation of microspike bundles, and much less so for filopodia, irrespective of how they are formed. Given the fact that the literature uses the terms microspikes and filopodia in a largely synonymous fashion, the

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observation that VASP removal only affects microspikes but not filopodia, provides us with an experimental tool to distinguish between those related, yet clearly distinct, structures in the future.

Ena/VASP proteins in cell–matrix adhesion

FAs are the main cellular structures linking the intracellular cytoskeleton of adherent cells to the extracellular matrix (ECM) on rigid substrates. They are large macromolecular protein complexes, relevant for adhesion and cell migration by generating, transmitting and sensing mechanical tension (Burridge and Guilluy, 2016; Winograd-Katz et al., 2014; Zaidel-Bar et al., 2007). Proper FA formation and turnover is also crucial for efficient cell spreading, for example, after trypsinization of adherent cells. As revealed by super-resolution imaging, the architecture of FAs comprises stratified layers of distinct proteins that function together to transmit forces sensed by clustered, plasma membrane-spanning integrin receptors to actin filaments (Kanchanawong et al., 2010); here, VASP resides together with zyxin and α-actinin in the so-called ‘actin-regulatory layer’ in close proximity to SF ends. Although the prominent FA proteins zyxin and vinculin can both interact with Ena/VASP (Brindle et al., 1996; Drees et al., 2000), only zyxin-deficient fibroblasts were shown to exhibit severely reduced accumulation of VASP and Mena in FAs as compared to control (Hoffman et al., 2006). Notably, zyxin was also found to recruit Ena/VASP and α-actinin to sites of SF strain to mediate the repair of damaged bundles (Smith et al., 2010). Moreover, AMP-activated protein kinase (AMPK)-mediated phosphorylation of VASP has been proposed to terminate actin assembly in FAs, thereby increasing tension and driving conversion of transverse arcs and non-contractile, dorsal SF into mature contractile ventral SFs (Tojkander et al., 2015). Notwithstanding this, in spite of their prominent accumulation in FAs, the precise and specific roles of Ena/VASP proteins in cell substrate adhesion and FA formation have remained under debate, as the conclusions from multiple studies present an incoherent picture. For instance, VASP-deficient fibroblasts have been reported to have thicker and more stable SFs and enlarged FAs as compared to control cells (Galler et al., 2006), whereas others have reported that depletion of VASP impairs FA formation in LX2 liver myofibroblasts (Kang et al., 2010). Another study implicated VASP in the splitting of FAs during their maturation, which, interestingly, correlates with an increase in FA-associated tension (Young and Higgs, 2018). In spite of this, no conclusive evidence was found previously for changes in cell spreading or differences in the composition or appearance of FAs in fibroblasts that lacked Mena and VASP compared to controls (Bear et al., 2000). Thus, considering the high degree of functional redundancy within this protein family, the analyses of only partially depleted cells likely might have prevented the accurate assessment of the contributions of Ena/VASP proteins to integrin-dependent adhesion. Indeed, our analysis of CRISPR/Cas9-generated mutant cell lines devoid of all three Ena/VASP members first of all showed that Ena/VASP-deficient B16-F1 cells or fibroblasts exhibit severe spreading defects on laminin or fibronectin, respectively (Damiano-Guercio et al., 2020). Of note, in these experiments, Evl rescued the spreading defect more effectively than the other Ena/VASP family members. Complete loss of Ena/VASP in fibroblasts also noticeably perturbed FA formation and impaired integrin-mediated adhesion, as verified by markedly diminished traction forces (Damiano-Guercio et al., 2020). Interestingly, once again, Evl proved most effective in rescuing relevant adhesion parameters, such as adhesion size and the generation of traction forces exerted by these structures. The particularly important role of Evl in these processes is further supported by FRAP data showing that Evl is more stably associated with other FA components compared to VASP (Damiano-Guercio et al., 2020). This is also in line with earlier work demonstrating that Evl-mediated actin polymerization regulates cell matrix adhesion, maturation of FAs and mechanosensing during durotaxis (Puleo et al., 2019). By analysis of chimeric Ena/VASP proteins, the authors of this study further proposed that the EVH1 domain of Evl is uniquely required for its function at FAs. Even though the full range of molecular functions of Ena/VASP proteins in FAs is not yet sufficiently understood, the more robust attachment of Evl to zyxin could, at least in part, explain the specialized role of this family member in adhesion.

Ena/VASP proteins in cell–cell adhesion

Adherens junctions (AJs) mediated by cadherin adhesion receptors constitute important molecular assemblies that initiate and stabilize adhesion between neighboring cells. Proper regulation of these structures is crucial for various processes in tissues, including cell polarization, cytoskeletal regulation, migration, mechanotransduction and establishment of endothelial barriers (Bachir et al., 2017; Charras and Yap, 2018). Given their functional similarity, it is not surprising that FAs and AJs share many components, including Ena/VASP proteins, vinculin and zyxin (Hansen and Beckerle, 2006; Scott et al., 2006; Vasioukhin et al., 2000). Using super-resolution imaging, the highly organized protein organization within planar cadherin-based adhesions could be mapped at nanoscale resolution (Bertocchi et al., 2017). This revealed a compartmentalized architecture (distal to proximal relative to the plasma membrane), in which the cadherin–catenin compartment is located at the plasma membrane and followed by a central structure that contains vinculin connected to the innermost layer, which contains actin filaments and actin-regulatory proteins, including VASP and zyxin (Bertocchi et al., 2017). Although zyxin has also been implicated in recruiting Ena/VASP to AJs (Hansen and Beckerle, 2006; Nguyen et al., 2010), tyrosine phosphorylated (Y822) and mechanically stretched vinculin appears to act as the primary factor in recruiting VASP and Mena to cadherin junctions (Bays et al., 2014; Leerberg et al., 2014). Notably, vinculin is not tyrosine phosphorylated in cell–matrix adhesions, possibly explaining why it has little role in the recruitment of Ena/VASP to FAs (Bays et al., 2014). Interestingly, loss-of-function studies have revealed that Ena/VASP activity is necessary for tension-dependent actin polymerization in cadherin junctions of various cell types (Leerberg et al., 2014; Scott et al., 2006). Correspondingly, *Drosophila* Ena was previously shown to be required for actin assembly in adherens junctions within the follicle cell epithelium (Baum and Perrimon, 2001). And more recently, adhesive interactions between neighboring cells have been reported to promote compaction of small cadherin precursor assemblies into larger adhesive clusters during AJ maturation (Charras and Yap, 2018; Wu et al., 2015). It is tempting to speculate therefore that this tension-sensitive pathway may ultimately lead to Ena/VASP clustering, which, in turn, initiates junctional actin assembly, and drives actin remodeling and shapes adhesion architecture. The critical role of Ena/VASP proteins in maintaining a functional endothelium is further highlighted in mice lacking all three family members. These animals exhibit defects in acto-myosin contractility and structural integrity of endothelial barriers, leading to edema, hemorrhage and lethality (Furman et al., 2007). Nevertheless, our current knowledge regarding the specific functions of Ena/VASP proteins in these fascinating structures is far from complete and will therefore require further research.

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Ena/VASP functions in the context of additional actin regulators and post-translational modifications

Aside from their impact on actin remodeling based on their subcellular distributions and actin assembly activities, a precise understanding of Ena/VASP functions will also have to include a comprehensive dissection of post-translational modifications (PTMs), such as phosphorylation and indirect effects based on interactions or competition with other actin regulators. As one example, heterodimeric CP has recently turned out to promote Arp2/3-dependent actin network formation, as for instance in lamellipodia by competing off the non-productive, continuous association of WAVE proteins (e.g. WAVE1, also known as WASF1) with the growing barbed ends of filaments (Funk et al., 2021). However, CP is also a well-established antagonist of Ena/VASP family members, potentially explaining both CP and Arp2/3 complex accumulation at the periphery of Ena/VASP-deficient cells (Damiano-Guercio et al., 2020). Thus, we anticipate that comprehensive interpretation of gene removal phenotypes will increasingly have to consider such mechanistic connections between connected actin regulators. This is also of particular relevance in the context of the multiple direct interactions that have previously been described for Ena/VASP proteins with various actin regulators, most directly in this context with Arp2/3 complex activators such as WAVE or WASP (Castellano et al., 2001; Chen et al., 2014; Havrylenko et al., 2015).

VASP has also been shown to be phosphorylated on multiple sites, including both on serine/threonine sites and tyrosine residues (for details see Döppler and Storz, 2013). As an example, a tyrosine (Y39) within the EVH1 domain that is relevant for subcellular positioning (see above) has been implicated in both zyxin binding and FA targeting (Maruoka et al., 2012), but experiments with true KO cells will certainly help to quantify the precise relevance of this effect. Phosphorylation of other, further C-terminal sites, that is S239 and T278 were shown to inhibit VASP-dependent actin assembly (Benz et al., 2009; Harbeck et al., 2000), which – if occurring in FAs – was subsequently connected to the maturation of SFs and establishment of contractility (Tojkander et al., 2015). Clearly, such events will likely add to the fine tuning of activities of all family members and to the complexity of their functions, so will have to be explored comprehensively in the context of all family members and their relative abundances in different cell types and tissues.

Conclusion and future directions

Despite recent progress, much remains to be learned concerning the intricate specific functions of Ena/VASP family members and their coordination in distinct cell types and tissues. The availability of CRISPR/Cas9 technology will allow the generation and characterization of true KO cells and tissues, as well as enable rescue experiments with specific family members to obtain a more complete picture of their potentially distinct activities. Furthermore, it does remain puzzling why the field for so many years has believed in negative regulatory functions of Ena/VASP members in migration. Future studies should therefore also aim at providing convincing data to reconcile any previous discrepancies among the former and more recent work. For instance, such studies should validate the recently observed positive correlations between Ena/VASP activity, lamellipodium protrusion and efficiency of cell migration (Damiano-Guercio et al., 2020) in additional cell types, including the previously employed Rat2 fibroblasts (Bear et al., 2002). Finally, many open questions remain, which can now be addressed with modern technology and novel approaches, including but not restricted to the precise functions of phosphorylations, and the impact of the manifold direct and indirect interactions of Ena/VASP family members (see previous section). Elucidating the answers to all those questions will constitute major steps forward in our understanding of the fine tuning of actin remodeling in distinct cell types and tissues, and might harbor the potential to compensate for defects in actin-dependent disease.

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