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Review

A new front in cell invasion: The invadopodial membrane

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ABSTRACT

Invadopodia are F-actin-rich membrane protrusions that breach basement membrane barriers during cell invasion. Since their discovery more than 30 years ago, invadopodia have been extensively investigated in cancer cells *in vitro*, where great advances in understanding their composition, formation, cytoskeletal regulation, and control of the matrix metalloproteinase MT1-MMP trafficking have been made. In contrast, few studies examining invadopodia have been conducted *in vivo*, leaving their physiological regulation unclear. Recent live-cell imaging and gene perturbation studies in *C. elegans* have revealed that invadopodia are formed with a unique invadopodial membrane, defined by its specialized lipid and associated protein composition, which is rapidly recycled through the endolysosome. Here, we provide evidence that the invadopodial membrane is conserved and discuss its possible functions in traversing basement membrane barriers. Discovery and examination of the invadopodial membrane has important implications in understanding the regulation, assembly, and function of invadopodia in both normal and disease settings.

Contents

1. Introduction

Directed vesicle trafficking to and from the plasma membrane facilitates the generation of membrane extensions, specialized secretion sites, and rapid delivery and removal of specific proteins from cell membranes. Examples of directed membrane trafficking include neurite outgrowth (Hausott and Klimaschewski, 2016), neuronal and immunological synapse function (Gonnord et al., 2012), wound healing (Abreu-Blanco et al., 2011), cell division (Shuster and Burgess, 2002), and cell migration (Maritzen et al., 2015). Targeted vesicle delivery requires a source of internal vesicles/membrane, exocytic trafficking machinery, and when vesicles are dynamically recycled, endocytic recycling components (Grant and Donaldson, 2009). This review will highlight recent studies in *C. elegans* that indicate a role for vesicular trafficking in the regulation of invadopodia, specialized F-actin-rich surface structures that mediate cell invasion through extracellular matrix barriers. Invadopodia in *C. elegans* undergo dynamic addition of a specialized invadopodial membrane. The invadopodial membrane is specifically associated with invadopodia and contains unique lipid and protein components distinct from the surrounding plasma membrane. During invadopodia breakdown, the invadopodial membrane lipid and protein components are rapidly recycled through endolysosomal vesicles then delivered back to the plasma membrane to form new invadopodia. In this review we will provide a brief history of invadopodia, discuss evidence for the conservation of the invadopodial membrane and focus on the regulation of traf-

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ficking and possible functions of the invadopodial membrane. The identification and investigation of this unique membrane is providing a deeper mechanistic understanding of invadopodia formation and cell invasion during normal development and diseases such as cancer.

2. Background: invadopodia are specialized subcellular structures that mediate basement membrane and interstitial matrix invasion

Basement membranes are dense, sheet-like forms of extracellular matrix that underlie all epithelia and endothelia and surround muscle, fat, and Schwann cells (Halfter et al., 2015; Yurchenco, 2011). Independent polymeric laminin and type IV collagen networks as well as a number of associated proteins including perlecan and nidogen contribute to basement membrane composition (Yurchenco, 2011). Functionally, basement membranes create tissue barriers, provide structural support, and facilitate filtration, as well as harbor cues for cell differentiation, polarization, and growth (Breitkreutz et al., 2013; Hay, 1981; Poschl et al., 2004; Rasmussen et al., 2012; Suh and Miner, 2013; Yurchenco, 2011). During development and immune cell surveillance, specialized cells acquire the ability to invade basement membrane barriers to allow cell movement into and out of tissues (Kelley et al., 2014; Madsen and Sahai, 2010; Rowe and Weiss, 2008). Misregulation of invasion through basement membranes underlies the pathology of developmental diseases, immune disorders, and cancer (Barsky et al., 1983; Hagedorn and Sherwood, 2011). Given the importance of basement membrane invasion in development, immune function, and human health, there has been great interest in understanding how cells transmigrate basement membrane barriers.

In 1989 Wen-Tien Chen used the term invadopodia to name highly protrusive, matrix-degrading membrane structures, composed of actin regulators and proteases found in transformed embryonic chicken fibroblasts plated on glass slides with a thin coating of matrix—a surface that mimics the 2D topography of basement membranes (Chen, 1989; Even-Ram and Yamada, 2005; Genot and Gligorijevic, 2014; Murphy and Courtneidge, 2011). Since Chen's initial description, invadopodia have been observed in many metastatic cancer cell lines (Hoshino et al., 2013) and emerged as one of the key subcellular structures that invasive cells use to breach basement membrane barriers (Lohmer et al., 2014; Schoumacher et al., 2013; Schoumacher et al., 2010). Invadopodia also appear to mediate invasion through the more porous type I collagen rich interstitial matrices that reside between cells and tissues. Imaging of invasive cells in *in vitro* 3D type I collagen matrices has revealed that invadopodia (also referred to as "invadopodia equivalents") in these environments take on the morphology of long, thin filopodial structures (Li et al., 2010; Tolde et al., 2010; Wolf et al., 2009). Podosomes are another F-actin based membrane-associated structure similar to invadopodia, but are generally not protrusive and are most often associated with non-transformed cells that mediate matrix remodeling events, such as dendritic cells, osteoclasts, macrophages, and vascular smooth muscle cells (Davies and Stossel, 1977; Gawden-Bone et al., 2010; Hoshino et al., 2013; Linder et al., 2011; Murphy and Courtneidge, 2011; Seano et al., 2014; Zambonin-Zallone et al., 1988). In some culture conditions, however, podosomes extend long protrusions that degrade extracellular matrix, suggesting a possible close relationship between podosomes and invadopodia (Gawden-Bone et al., 2010). To help account for such findings, the term invadosomes has recently been adopted to incorporate both structures (Destaing et al., 2011; Linder, 2009; Linder et al., 2011; Saltel et al., 2011), proposing that invadopodia, podosomes, and possibly other actin-based cellular protrusions that bind and degrade extracellular matrix represent a spectrum of molecularly related structures that may adapt and even interchange in response to the microenvironment (Di Martino et al., 2016; McNiven, 2013). In this review, we will be consistent with the bulk of previously published work that defines invadopodia as highly protrusive invasive structures (Linder et al., 2011; Lohmer et al., 2014). We include within this definition invadopodia observed during developmental and normal physiological invasion events, recognizing that invadopodia are likely a component of a normal invasion program co-opted by tumor cells (Lohmer et al., 2014; Murphy and Courtneidge, 2011).

Through candidate gene approaches, proteomic analysis, and more recent *in vivo* genetic screens, approximately 100 genes have been associated with invadopodia formation, function, and breakdown (see Table 1 and references therein). This includes well-studied actin regulators, matrix metalloproteinases (MMPs), signaling pathways, and integrins, as well as genes involved with glycolysis, metabolism, protein degradation, chaperone activity, and protein synthesis for which an exact role in invadopodia formation has not been determined (Attanasio et al., 2011; Hoshino et al., 2013; Lohmer et al., 2016). The breadth of gene families associated with invadopodia likely reflects the complexity and intricate regulation of invadopodia and suggests that many aspects of their function and control remain unknown.

Although most studies have examined invadopodia in cancer cells *in vitro*, recent imaging advances in *ex vivo* and *in vivo* settings are establishing their existence and physiological importance in basement membrane invasion in both normal and disease settings (Di Martino et al., 2016; Genot and Gligorijevic, 2014; Lohmer et al., 2014). These studies include examination of cancer cell invasion on isolated rat peritoneum basement membranes (Schoumacher et al., 2010), imaging of vascular invasion by cancer cells in mouse and chicken embryos (Gligorijevic et al., 2012; Leong et al., 2014; Roh-Johnson et al., 2014), examination of intestinal epithelial cell invasion in a reactive oxygen species (ROS) disease model in zebrafish (Seiler et al., 2012), and visualizing anchor cell invasion during organogenesis in *C. elegans* (Hagedorn et al., 2013). Studying invadopodia in native contexts is not only confirming the relevance of these structures for cell invasion through basement membrane, but also is revealing new aspects of invadopodia biology. One fascinating example comes from the discovery of the invadopodial membrane in the anchor cell of *C. elegans*.

3. Invadopodia in *C. elegans* are formed from a recycling invadopodial membrane

The *C. elegans* anchor cell is a specialized uterine cell that initiates uterine-vulval attachment following invasion through underlying basement membrane (Sherwood and Sternberg, 2003). Anchor cell invasion is facilitated by dynamic and highly protrusive F-actin-rich invadopodia that localize to the anchor cell-basement membrane interface (the invasive cell membrane). The basement membrane in *C. elegans* is highly conserved and all major basement membrane components and receptors found in vertebrates are also present in *C. elegans* (Kramer, 2005). A suite of unique attributes of *C. elegans* as a model organism—including fluorescently tagged basement membrane components, anchor cell specific expression of fluorescently tagged proteins, the highly stereotyped nature of invasion, and genetic analysis—have allowed detailed experimental dissection of invadopodia *in vivo* (Hagedorn et al., 2014; Hagedorn et al., 2013; Lohmer et al., 2016; Lohmer et al., 2014).

Similar to tumor progression, where cancer cell invasion is promoted by signals from neighboring cells such as tumor associated macrophages (Noy and Pollard, 2014; Roh-Johnson et al., 2014), anchor cell invasion is stimulated by the underlying vulval cells (Sherwood and Sternberg, 2003). The vulval cells direct

Table 1
Proteins associated with invadopodia formation. (Dorfleutner et al., 2008; Hagedorn et al., 2014; Yamaguchi et al., 2005; Lanier et al., 2015; Mukhopadhyay et al., 2009; Lohmer et al., 2016; Nakahara et al., 2003; Hagedorn et al., 2014; Yamaguchi et al., 2005; Hoffmann et al., 2016; Lizarraga et al., 2009; Ayala et al., 2008; Semprucci et al., 2015; Vignjevic et al., 2006; Takkunen et al., 2010; Oikawa et al., 2008; Md Hashim et al., 2013; Gryaznova et al., 2015; Bravo-Cordero et al., 2011; Nakahara et al., 1998; Bravo-Cordero et al., 2011; Lin et al., 2014; Bravo-Cordero et al., 2011; Visser et al., 2013; Branch et al., 2012; Garcia et al., 2016; Md Hashim et al., 2013; Harper et al., 2010; Harper et al., 2010; Yamaguchi et al., 2010; Crowley et al., 2009; Hoshino et al., 2009; Ayala et al., 2009; Stylli et al., 2009; Yamaguchi et al., 2005; Jerrell and Parekh, 2016; Vishnubhotla et al., 2007; Beaty et al., 2014; Mueller et al., 1992; Goertzen et al., 2016; Choi and Lim, 2013; Hashimoto et al., 2004; Razidlo et al., 2014; Beaty et al., 2013; Hagedorn et al., 2009; Mueller and Chen, 1991; Rey et al., 2011; Badowski et al., 2008; Bowden et al., 1999; Liu et al., 2009; Liu et al., 2009; Scott et al., 2012; Yamaguchi et al., 2005; Rajadurai et al., 2012; Hagedorn et al., 2013; Eckert et al., 2011; Diaz et al., 2009; Gianni et al., 2009; Chen, 1989; Eckert et al., 2011; Pignatelli et al., 2012; Lucien et al., 2011; Sun et al., 2014; Sun et al., 2014; Sutoh Yoneyama et al., 2014; Moshfegh et al., 2014; Schoumacher et al., 2010; Hwang et al., 2012; Hoskin et al., 2015; Moshfegh et al., 2014; Nakahara et al., 2003; Ziel et al., 2009; Chen and Kelly, 2003; Pignatelli et al., 2012; Deryugina et al., 2002; Nascimento et al., 2010; Nakahara et al., 1997; van Hinsbergh et al., 2006; McLaughlin et al., 2014; Busco et al., 2010; Bowden et al., 1999; Eckert et al., 2011; Furmaniak-Kazmierczak et al., 2007; Gianni et al., 2010b; Nakahara et al., 2003; Yamaguchi et al., 2011; Chen, 1996; Monsky et al., 1994; Valenzuela-Iglesias et al., 2015; Lohmer et al., 2016; Rajadurai et al., 2012; Jacob et al., 2013; Garcia et al., 2012; Vikesaa et al., 2006; Zhao et al., 2016; Marchesin et al., 2015; Grass et al., 2012; Miyazawa et al., 2013; Sakurai-Yageta et al., 2008; Bravo-Cordero et al., 2007; Bravo-Cordero et al., 2011; Williams et al., 2014; Steffen et al., 2008; Williams et al., 2014; Hoshino et al., 2013b; Lovett et al., 2010; Wiesner et al., 2010; Wiesner et al., 2010; Chevalier et al., 2016; Lorenz et al., 2004; Yamaguchi et al., 2005; Artym et al., 2006; Bowden et al., 1999; Clark et al., 2007; Macpherson et al., 2014; Beaty et al., 2013; Smith-Pearson et al., 2010; Sun et al., 2009; Williams and Coppolino, 2014; Williams et al., 2014; Abram et al., 2003; Chuang et al., 2004; Sharma et al., 2013; Martin-Villar et al., 2015; Neel et al., 2012; Schoumacher et al., 2010; Hauck et al., 2002; Vitale et al., 2008; Alexander et al., 2008; Mukhopadhyay et al., 2009; Bharti et al., 2007; Creed et al., 2015; Cortesio et al., 2008; Furmaniak-Kazmierczak et al., 2007; Cortesio et al., 2008; Gianni et al., 2010a; Liu et al., 2010; Ward et al., 2015; Williams and Coppolino, 2014; Albrechtsen et al., 2011; Lauzier et al., 2012; Caldieri et al., 2009; Yamaguchi et al., 2009; Baldassarre et al., 2003; Yamamoto et al., 2011; Monteiro et al., 2013; Frittoli et al., 2014).

Gene	Role in invadopodial life cycle	References
AFAP1	Actin dynamics	(Dorfleutner et al., 2008)
ENAH/VASP**	Actin dynamics	(Hagedorn et al., 2014)
ARP2/3**	Actin dynamics	(Yamaguchi et al., 2005)
CARMIL2	Actin dynamics	(Lanier et al., 2015)
CALD1	Actin dynamics	(Mukhopadhyay et al., 2009)
CDC42**	Actin dynamics	(Lohmer et al., 2016; Nakahara et al., 2003)
ADF/CFL1**	Actin dynamics	(Hagedorn et al., 2014; Yamaguchi et al., 2005)
CRP2	Actin dynamics	(Hoffmann et al., 2016)
DRF1-3	Actin dynamics	(Lizarraga et al., 2009)
ERK	Actin dynamics	(Ayala et al., 2008)
ETAR	Actin dynamics	(Semprucci et al., 2015)
FSCN1	Actin dynamics	(Vignjevic et al., 2006)
FLNA	Actin dynamics	(Takkunen et al., 2010)
GRB2	Actin dynamics	(Oikawa et al., 2008)
HIF1A	Actin dynamics	(Md Hashim et al., 2013)
ITSN1/2	Actin dynamics	(Gryaznova et al., 2015)
ARHGAP35	Actin dynamics	(Bravo-Cordero et al., 2011; Nakahara et al., 1998)
ARHGEF28	Actin dynamics	(Bravo-Cordero et al., 2011)
PODXL	Actin dynamics	(Lin et al., 2014)
RHOC	Actin dynamics	(Bravo-Cordero et al., 2011)
TRMP7	Actin dynamics	(Visser et al., 2013)
VCL	Actin dynamics	(Branch et al., 2012)
WICH/WIRE	Actin dynamics	(Garcia et al., 2016)
ARHGEF7	Actin dynamics	(Md Hashim et al., 2013)
ATX	Actin dynamics	(Harper et al., 2010)
EPAC	Actin dynamics	(Harper et al., 2010)
PIP5KIα	Actin dynamics	(Yamaguchi et al., 2010)
SVIL	Actin dynamics	(Crowley et al., 2009)
LAMTOR1	Actin dynamics	(Hoshino et al., 2009)
FGD1	Actin dynamics	(Ayala et al., 2009)
NCK1	Actin dynamics, Matrix degradation	(Stylli et al., 2009; Yamaguchi et al., 2005)
ROCK1/2	Actin dynamics, Matrix degradation	(Jerrell and Parekh, 2016; Vishnubhotla et al., 2007)
TLN1	Actin dynamics, Matrix degradation	(Beaty et al., 2014; Mueller et al., 1992)
KISS1R	Actin dynamics, MMP activation	(Goertzen et al., 2016)
TIS21	Actin dynamics, regulation of ROS	(Choi and Lim, 2013)
ARF6	Cargo transport	(Hashimoto et al., 2004)
VAV1	Cdc42 activation	(Razidlo et al., 2014)
β1 and β3 Integrin**	ECM attachment, actin dynamics	(Beaty et al., 2013; Hagedorn et al., 2009; Mueller and Chen, 1991)
HDAC6	EGF signaling, hypoxic conditions	(Rey et al., 2011)
PXN	Erk activation	(Badowski et al., 2008; Bowden et al., 1999)
EXO70	Exocyst complex, actin dynamics	(Liu et al., 2009)
SEC8	Exocyst complex, actin dynamics	(Liu et al., 2009)
ACC1	Fatty acid synthesis and de novo lipidogenesis	(Scott et al., 2012)
EGF	Induction	(Yamaguchi et al., 2005)
MET	Induction	(Rajadurai et al., 2012)
NTN1	Induction	(Hagedorn et al., 2013)
PDGF	Induction	(Eckert et al., 2011)
ROS	Induction	(Diaz et al., 2009; Gianni et al., 2009)
SRC	Induction	(Chen, 1989)
TGF-β	Induction	(Eckert et al., 2011; Pignatelli et al., 2012)
RPS6KA1	Induction, hypoxic conditions	(Lucien et al., 2011)
ORAI1	Invadopodia assembly and Matrix degradation	(Sun et al., 2014)
STIM1	Invadopodia assembly and Matrix degradation	(Sun et al., 2014)
PLEC	Invadopodia stabilization	(Sutoh Yoneyama et al., 2014)
PAK1	Invadopodial disassembly	(Moshfegh et al., 2014)
VIM	Invadopodial elongation	(Schoumacher et al., 2010)
Microtubules	Invadopodial elongation	(Schoumacher et al., 2010)
IMP3	Invadopodial formation, multiple mechanisms	(Hwang et al., 2012)
EZR	Invadopodial turnover	(Hoskin et al., 2015)
RAC1**	Invadopodial turnover	(Moshfegh et al., 2014; Nakahara et al., 2003; Ziel et al., 2009)
DPP4	Matrix degradation	(Chen and Kelly, 2003)
HIC-5	Matrix degradation	(Pignatelli et al., 2012)
MMP2	Matrix degradation	(Deryugina et al., 2002)
MMP9	Matrix degradation	(Nascimento et al., 2010)
MT1-MMP (MMP14)	Matrix degradation	(Nakahara et al., 1997; van Hinsbergh et al., 2006)
NEDD9	Matrix degradation	(McLaughlin et al., 2014)
NHE-1	Matrix degradation	(Busco et al., 2010)
PRKD1	Matrix degradation	(Bowden et al., 1999)
TWIST1	Matrix degradation	(Eckert et al., 2011)
uPAR	Matrix degradation	(Furmaniak-Kazmierczak et al., 2007)
NOX1	Matrix degradation	(Gianni et al., 2010b)
PI3K3CA	Matrix degradation	(Nakahara et al., 2003; Yamaguchi et al., 2011)
FAP	Matrix degradation, Protease docking, localization	(Chen, 1996; Monsky et al., 1994)
PFN1	Membrane composition	(Valenzuela-Iglesias et al., 2015)
GDI1	Membrane trafficking	(Lohmer et al., 2016)
GAB1	Met signaling	(Rajadurai et al., 2012)

Table 1 (*Continued*)

RAB40B	MMP trafficking	(Jacob et al., 2013)
WIP	MMP trafficking, actin dynamics	(Garcia et al., 2012)
CD44	MT1-MMP recruitment	(Vikesaa et al., 2006; Zhao et al., 2016)
JIP3/4	MT1-MMP trafficking	(Marchesin et al., 2015)
CD147	MT1-MMP trafficking	(Grass et al., 2012)
CDCP1	MT1-MMP trafficking	(Miyazawa et al., 2013)
IQGAP1	MT1-MMP trafficking	(Sakurai-Yageta et al., 2008)
RAB8A	MT1-MMP trafficking	(Bravo-Cordero et al., 2007)
RHOA	MT1-MMP trafficking	(Bravo-Cordero et al., 2011)
STX4	MT1-MMP trafficking	(Williams et al., 2014)
VAMP7	MT1-MMP trafficking	(Steffen et al., 2008; Williams et al., 2014)
ZF21	MT1-MMP trafficking	(Hoshino et al., 2013b)
YB-1	MT1-MMP trafficking	(Lovett et al., 2010)
KIF3A/B	MT1-MMP trafficking	(Wiesner et al., 2010)
KIF5A	MT1-MMP trafficking	(Wiesner et al., 2010)
TOM1L1	MT1-MMP trafficking	(Chevalier et al., 2016)
N-WASP**	MT1-MMP trafficking, actin dynamics	(Lorenz et al., 2004; Yamaguchi et al., 2005)
CTTN	MT1-MMP trafficking, actin dynamics	(Artym et al., 2006; Bowden et al., 1999; Clark et al., 2007)
CLIC3	MT1-MMP trafficking, cargo selection	(Macpherson et al., 2014)
ABL1/2	MT1-MMP trafficking, EGF signaling	(Beaty et al., 2013; Smith-Pearson et al., 2010; Sun et al., 2009)
SNAP23	MT1-MMP trafficking, Src and EGFR trafficking	(Williams and Coppolino, 2014; Williams et al., 2014)
TKS4/5	MT1-MMP trafficking, Src signaling	(Abram et al., 2003)
SYNJ2	RAC1 activity	(Chuang et al., 2004)
SHIP2	Regulates invadopodia $PI(3,4)P_2$ levels	(Sharma et al., 2013)
PDPN	Regulator of Invadopodia maturation	(Martin-Villar et al., 2015)
RALB/RALBP1	Required, unknown mechanism	(Neel et al., 2012)
PLS1	Required, unknown mechanism	(Schoumacher et al., 2010)
FAK	Src signaling	(Hauck et al., 2002; Vitale et al., 2008)
P130CAS	Src signaling	(Alexander et al., 2008)
TP53	Src signaling	(Mukhopadhyay et al., 2009)
ASAP1	Src signaling	(Bharti et al., 2007)
ADRB2	Src signaling	(Creed et al., 2015)
CAPN2	Src signaling	(Cortesio et al., 2008)
ERK1/2	Src signaling	(Furmaniak-Kazmierczak et al., 2007)
PTP1B	Src signaling	(Cortesio et al., 2008)
NOXA1	Src signaling	(Gianni et al., 2010a)
Laminin-332	Src signaling	(Liu et al., 2010)
Ggi2	Src trafficking to invadopodia	(Ward et al., 2015)
STX12	Src, EGF trafficking	(Williams and Coppolino, 2014)
ADAM12	Src/EGF signaling, hypoxic conditions	(Albrechtsen et al., 2011)
TGM1/2	TGF-β signaling	(Lauzier et al., 2012)
CAV-1	Vesicle / membrane trafficking	(Caldieri et al., 2009; Yamaguchi et al., 2009)
DNM2	Vesicle trafficking	(Baldassarre et al., 2003)
FBP17	Vesicle trafficking	(Yamamoto et al., 2011)
WASH	Vesicle trafficking	(Monteiro et al., 2013)
RAB4	Vesicle trafficking	(Frittoli et al., 2014)
RAB5A	Vesicle trafficking	(Frittoli et al., 2014)

The proteins listed were identified in numerous cancer cell types and model systems. Rows shaded grey represent proteins with direct roles in invadopodial membrane formation and function.
**Identified in numerous species, indicating a conserved mechanism.

invasion by generating a diffusible cue that activates the Rho GTPase CDC-42 within the anchor cell (Lohmer et al., 2016). Active CDC-42 promotes robust invadopodia formation along the anchor cell-basement membrane interface. These invadopodia breach the basement membrane shortly after secretion of the vulval cue in the early-to-mid L3 larval stage (Hagedorn et al., 2013; Lohmer et al., 2016). Interestingly, several genes encoding G-protein signaling components were identified in the sensitized invadopodial screen that isolated CDC-42 (Lohmer et al., 2016), suggesting that the vulval cue might act through a G-protein coupled receptor pathway.

Anchor cell invadopodia undergo rapid turnover, with an average lifespan of 45 s. In contrast, invadopodia seen in cancer cells in culture exist for minutes to hours (Li et al., 2010; Sibony-Benyamini and Gil-Henn, 2012). Invadopodia observed *in vitro* may lack the appropriate microenvironment required for rapid formation and turnover, or developmental invasion events may be more swift and efficient than cancer cell invasion (Di Martino et al., 2016; Genot and Gligorijevic, 2014). Similar to invadopodia in cancer cell lines, anchor cell invadopodia are dependent on integrin for their formation (Destaing et al., 2010) and are composed of F-actin and a number of actin regulators, including the ADF/cofilin ortholog UNC-60A, the WASP ortholog WSP-1, and the Ena/VASP ortholog UNC-34 (Hagedorn et al., 2014; Hagedorn et al., 2013; Lohmer et al., 2016).

Anchor cell invadopodia have a specialized membrane that is molecularly distinct from the rest of the anchor cell plasma membrane, and is enriched in the phospholipid $PI(4,5)P_2$, the membrane associated Rho GTPases MIG-2 (RhoG), and CED-10 (Rac) (Hagedorn et al., 2014). The invadopodial membrane is highly dynamic as invadopodia depress, penetrate, then cross the basement membrane with a single long protrusion (Hagedorn et al., 2013). This behavior may be common during invasion, as similar single protrusions have been observed when tumor cells transmigrate basement membranes (Hotary et al., 2006; Leong et al., 2014; Schoumacher et al., 2010).

Live-cell imaging and gene perturbation studies have revealed that the anchor cell's invadopodial membrane is rapidly recycled through the endolysosomal system and targeted back to the invasive cell membrane to form new invadopodia (Fig. 1 and Supplementary Video 1 in the online version at DOI: 10.1016/j.ejcb.2016.06.006). Evidence of this active trafficking is particularly apparent after loss of UNC-60A (cofilin), a key actin regulatory protein that disassembles F-actin filaments (Blanchoin et al., 2014). UNC-60A localizes to invadopodia and is required for anchor cell invasion. Consistent with its role in disassembly of F-actin, loss of UNC-60A leads to a large buildup of F-actin at the invasive cell membrane (Fig. 1). Loss of UNC-60A also dramatically disrupts the invadopodial membrane (Supplementary Video 2 in the online version at DOI: 10.1016/j.ejcb.2016.06.006), as invadopodial membrane components are no longer found at the invasive cell membrane and instead localize with static endolysosome vesicles within the anchor cell. As cofilin is known to break apart cortical F-actin to facilitate the trafficking and exocytosis of vesicles to the plasma membranes of cells (Lee et al., 2009; Mahaffey et al., 2013), these observations suggest that UNC-60A may disassemble cortical F-actin to allow targeting of the invadopodial membrane from the endolysosome to nascent invadopodia at the cell membrane. The endolysosome marker LAMP-1 localizes to invadopodial membrane at the cell surface, and fluorescence loss in photobleaching (FLIP) experiments revealed that LAMP-1 molecules undergo rapid recycling from the invadopodial membrane and through the endolysosome system in less than 5 min (Hagedorn et al., 2014). The

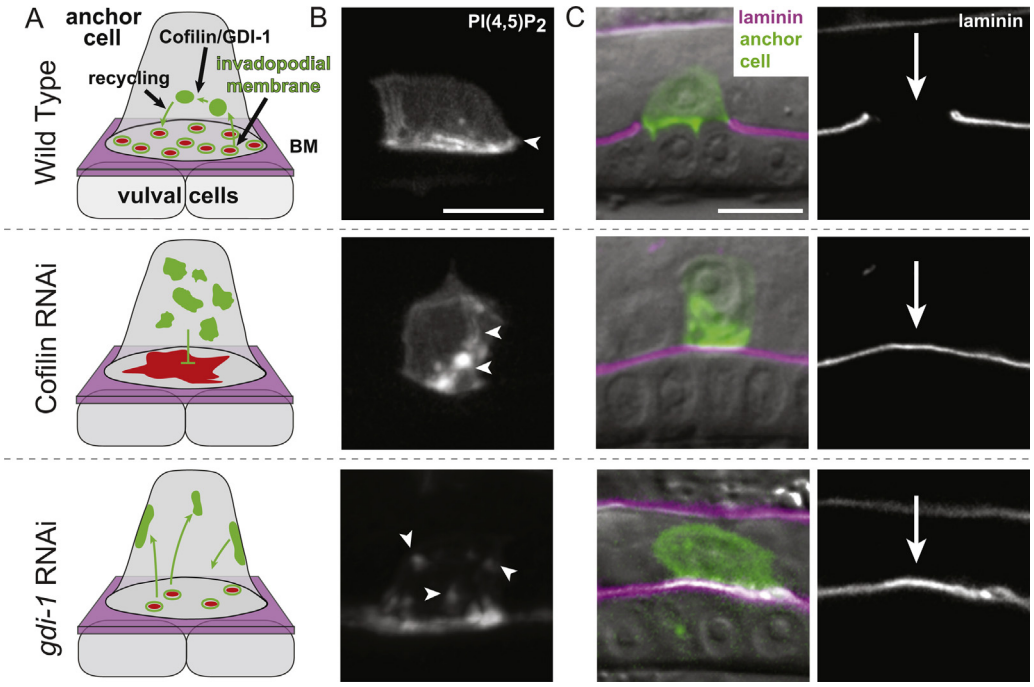

Fig. 1. Models of Cofilin and GDI-1 function in invadopodial membrane trafficking in the *C. elegans* anchor cell.
(A) Schematic images of invadopodial membrane trafficking within the anchor cell during anchor cell invasion into the vulval cells. F-actin and actin regulators (red) are coupled with invadopodial membrane trafficking (green) to form invadopodia at the anchor cell-basement membrane (BM, shown in magenta) interface prior to invasion. Both cofilin (encoded by the *C. elegans unc-60a* gene) and GDI-1 regulate trafficking of the invadopodial membrane through the endolysosomal system. RNAi-mediated knockdown of cofilin results in a static, non-trafficked invadopodial membrane that is localized internally, an accumulation of stagnant F-actin along the invasive cell membrane, and an absence of invadopodia formation. Loss of GDI-1 results in mis-targeting of invadopodial membrane to all cell membranes and a reduction in the number of invadopodia. (B) A grayscale image of the invadopodial membrane observed with mCherry:PLCδ^{PH}, which binds to the invadopodial membrane component PI(4,5)P_2 (highlighted by white arrows). In wild-type animals, the invadopodial membrane is localized along the invasive cell membrane. Loss of cofilin (through cofilin RNAi) results in a static, non-trafficked internal membrane and reduction of GDI-1 protein (through *gdi-1* RNAi) causes the invadopodial membrane to be trafficked to all cell membranes. (C) Left, RNAi-mediated loss of cofilin or GDI-1 results in invasion defects as seen by an inability of the anchor cell (green—visualized with either the invadopodial membrane probe mCherry:PLCδ^{PH} or the F-actin probe mCherry:MoeABD) to breach the basement membrane (magenta; visualized with laminin:GFP) overlaid on a DIC image. Right, a grayscale image of laminin:GFP. Arrow shows position of basement membrane breach in a wild type animal and a lack of breach after loss of cofilin or GDI-1. Bars, 5 μm.

rate of cycling is consistent with the rapid invadopodia turnover rate observed in the anchor cell. Additional evidence of active recycling and targeting of the invadopodial membrane comes from the identification of GDI-1 (Rab GDP dissociation inhibitor) as a regulator of invadopodia and anchor cell invasion (Lohmer et al., 2016). Rab GDIs have a high affinity for GDP-bound Rab proteins and deliver Rabs to specific membrane compartments to help regulate membrane trafficking (Pfeffer, 2013). Loss of GDI-1 in the anchor cell results in dynamic mis-targeting of invadopodial membrane to the apical and lateral membranes of the anchor cell (Fig. 1, Supplementary Video 3 in the online version at DOI: 10.1016/j.ejcb.2016.06.006). Importantly, other trafficking events in the anchor cell—including integrin receptor localization to the invasive cell membrane, EGF-like ligand (LIN-3) secretion, and deposition of the matrix component hemicentin into the basement membrane—are normal in the anchor cell after loss of UNC-60A (cofilin) or GDI-1 (Hagedorn et al., 2014; Lohmer et al., 2016). These observations suggest that the invadopodial membrane is uniquely trafficked and recycled through the endolysosome and targeted to the cell-basement membrane interface to form invadopodia.

4. The specialized invadopodial membrane may be conserved in other invasive cells

There is convincing evidence suggesting that the recycling of the invadopodial membrane observed in *C. elegans* is a shared feature of invadopodia. When Chen first characterized invadopodia, he noted the “highly motile membrane” and with transmission electron microscopy (TEM) visualized vesicles associated with invadopodia (Chen, 1989). Subsequent EM studies in melanoma cells also revealed many membrane surface protrusions associated with invadopodia (Baldassarre et al., 2003), and similar internal vesicles (thought to be endosomes) were observed within invadopodia in breast cancer cells (Schoumacher et al., 2010). Live-cell, high-resolution total internal reflection fluorescence (TIRF) microscopy visualized dynamic ruffling and undulation of the invadopodial membrane in breast cancer cells (Artym et al., 2011). Notably, podosomes and integrin based focal adhesions are not associated with similar membrane processes, highlighting the distinct nature of the invadopodial membrane (Artym et al., 2011). In addition to comparable membrane dynamics and vesicle trafficking, PI(4,5)P_2, a component of the *C. elegans* anchor cell invadopodial membrane, also localizes at invadopodia and in vesicular structures surrounding invadopodia in breast cancer cells (Yamaguchi et al., 2010). Furthermore, invadopodia in breast cancer cells are enriched in lipid raft membranes, which are actively trafficked to and from invadopodia (Yamaguchi et al., 2009). Finally, studies on the trafficking of the transmembrane matrix metalloproteinase MT1-MMP, a key protease associated with invadopodia in numerous cancer cell lines, has indicated that MT1-MMP localizes to the invadopodial membrane and is recycled through the endolysosome. MT1-MMP is dynamically internalized by both clathrin- and caveolar-mediated endocytosis and directed via a Rab5 early endosome vesicle trafficking pathway to either a Rab4 fast recycling exocytosis pathway (thought to be a minor component of MT1-MMP recycling) or through Rab7 delivery to late endosome/endolysosome where most MT1-MMP accumulates. Following accumulation in the late endosome/endolysosome, MT1-

MMP is trafficked and exocytosed back to invadopodia through an Arf6, endosomal WASH protein, exocyst complex, and V-SNARE protein VAMP-7 mediated mechanism (Frittoli et al., 2014; Linder, 2015; Marchesin et al., 2015; Monteiro et al., 2013; Poincloux et al., 2009; Remacle et al., 2003; Williams and Coppolino, 2011). MT1-MMP is stabilized at invadopodia in the plasma membrane by a direct interaction with invadopodial F-actin, providing a link between the F-actin core of invadopodia and the invadopodial membrane (Yu et al., 2012). MT1-MMP recycling takes approximately one hour in Madin-Darby canine kidney and fibrosarcoma cells, matching the turnover rates of invadopodia in cancer cells (Wang et al., 2004; Williams and Coppolino, 2011). Together these studies suggest that the invadopodial membrane is a unique membrane domain, and that its trafficking through the endolysosome might be a shared feature of invadopodia in normal development and cancer progression.

5. Function of the invadopodial membrane

Perturbations in the invadopodial membrane impede or block the ability of the anchor cell to invade through the basement membrane, offering strong evidence for the essential function of the invadopodial membrane during cell invasion (Hagedorn et al., 2014; Hagedorn et al., 2013; Lohmer et al., 2016). A key feature of invadopodia that distinguishes them from podosomes is their highly protrusive nature and membrane ruffles (Artym et al., 2011). Active recycling of the invadopodial membrane through the endolysosome may provide a source of new membrane, allowing the invadopodia to rapidly extend into and protrude through basement membrane and interstitial matrices. In other biological events, addition of membrane from the endolysosome is used to mediate plasma membrane repair and neurite outgrowth (Arantes and Andrews, 2006; Reddy et al., 2001). Furthermore, endocytic trafficking is used to add membrane during cytokinesis (Grant and Donaldson, 2009). Adding weight to the notion that the endolysosome delivers membrane to invadopodia, the GTPase Arf6 together with its effectors JIP3 and JIP4 mediate the delivery of endosomes for membrane addition during cytokinesis and regulate exocytosis of MT1-MMP (Marchesin et al., 2015; Montagnac et al., 2009). Thus, the molecular machinery of the vesicular trafficking system is present for rapid membrane addition to invadopodia to allow for its protrusive activity.

The invadopodial membrane may also deliver and concentrate proteases at invadopodia (Frittoli et al., 2011; Scita and Di Fiore, 2010; Trimble and Grinstein, 2015). Many studies in cancer cell lines have focused on the targeted delivery of MT1-MMP to invadopodia, which is thought to be required for basement membrane break down and breach (see Table 1 for genes associated with MT1-MMP trafficking) (Hotary et al., 2006). Interestingly, transmembrane MMPs such as MT1-MMP might be a deuterostome innovation, as protostomes such as *C. elegans* and *Drosophila* do not encode these genes (Fanjul-Fernandez et al., 2010). However, *C. elegans* and *Drosophila* do encode membrane anchored glycosyl-phosphatidyl inositol (GPI) MMPs (Altincicek et al., 2010; Page-McCaw, 2008), which could be directed to invadopodia via the invadopodial membrane. Vertebrate GPI-anchored MMPs are overexpressed in cancer and strongly implicated with cancer progression, but their trafficking and subcellular localization is poorly understood (Sohail et al., 2008). Whether GPI-anchored MMPs are trafficked to invadopodia is an important unanswered question. Genetic loss of the sole *C. elegans* GPI-anchored MMP *zmp-1*, however, only slightly delays anchor cell invasion (Sherwood et al., 2005), strongly suggesting that the invadopodial membrane has additional functions outside of delivering MMP proteases during basement membrane invasion.

Other functions of the invadopodial membrane are also possible. For example, the invadopodial membrane may have a fundamental role in invadopodia assembly. The unique lipid composition of the invadopodial membrane may act as a platform that recruits and activates the various signaling, actin regulatory, and adhesion proteins required for invadopodia formation and turnover (Moshfegh et al., 2014; Yamaguchi and Oikawa, 2010). Endocytic vesicles also are emerging as key signaling centers (Scita and Di Fiore, 2010). Signaling from endosomes that originate from endocytic events at invadopodia might allow rapid feedback and adaption during invasion. Consistent with this possibility, endocytosis of collagen has been observed in macrophages, fibroblasts, and hepatic stellate cells (a liver cell that maintains extracellular matrix homeostasis during liver damage) (Bi et al., 2014). Further, in fibrosarcoma cells fibronectin is internalized into the endolysosome, the same compartment as MT1-MMP (Sung et al., 2011). Intriguingly, collagen endocytosis in hepatic stellate cells leads to the transcriptional upregulation of MMP-9, consistent with a signaling function of endocytosed collagen (Bi et al., 2014). It is thus possible that the invadopodial membrane has numerous essential functions in invadopodia formation, function, and adaptation to the microenvironment.

6. Summary and outlook

The complexity of F-actin and membrane structures within invadopodia observed by TIRF microscopy lead to invadopodia being referred to as “invasive superstructures” (Artym et al., 2011). Given its impressive and complex structure, it should come as no surprise that we are only beginning to understand the intricacies of invadopodia regulation and function. The discovery of an actively recycled invadopodial membrane reveals another layer of regulation to these fascinating cellular drill bits that breach basement membrane barriers.

To advance our understanding of the invadopodial membrane, it will be important to determine how invadopodial lipids and membrane components beside MT1-MMP are restricted to the invadopodial membrane at the plasma membrane. Targeted exocytosis and endocytosis may play a role in maintaining the invadopodial membrane at the cell surface (Trimble and Grinstein, 2015). In addition, F-actin, which can limit protein and lipid diffusion, is a strong candidate for preventing diffusion of invadopodial membrane components into the broader plasma membrane (Kusumi et al., 2012; Trimble and Grinstein, 2015). Indeed, actin filaments attached to the plasma membrane can even promote ordered lipid raft domains in cells, suggesting that the F-actin core of invadopodia may not only play a role in maintaining, but also in organizing the invadopodial membrane (Dinic et al., 2013). It will also be crucial to examine the trafficking of other invadopodial membrane components in cancer cell lines beyond MT1-MMP and to carefully examine the initial stages of endocytic trafficking of invadopodial constituents in *C. elegans* (*i.e.* early endosomes) to determine if the lipids and proteins of the invadopodial membrane are trafficked together as a unit through a complete cycle of endocytic recycling or if they coalesce at the endolysosome (where all components are present in *C. elegans*) from different routes prior to reaching the cell surface to form invadopodia. Notably, the protease MT3-MMP is co-trafficked with MT1-MMP in Madin-Darby canine kidney cells, supporting the possibility that invadopodial membrane proteins are trafficked jointly (Wang et al., 2004). Rigorous live-cell image analysis of invadopodial membrane component trafficking will also be necessary to further dissect invadopodial membrane regulation. Interestingly, recent live-cell imaging work using TIRF microscopy in human macrophages has revealed that MT1-MMP remains at the mem-

brane in small islets after dissolution of podosomes, and that these islets seed the reemergence of podosomes at these sites (El Azzouzi et al., 2016). Whether a similar memory mechanism is used to direct waves of invadopodia to specific extracellular matrix sites to facilitate invasion will be fascinating to explore. Finally, biochemical isolation of the invadopodial membrane will be key in further elucidating invadopodial membrane composition (Asano et al., 2009). Together, these efforts should ultimately lead to a deeper mechanistic understanding of invadopodial membrane trafficking and function. As evidence in *C. elegans* suggests that invadopodial membrane recycling is uniquely regulated from other secretion and vesicle trafficking events, this analysis will not only increase our understanding of invadopodia, but also may reveal specialized features of the invadopodial membrane that could be to targeted with novel cancer therapeutics to halt cell invasive behavior.

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