

Cell motility through plasma membrane blebbing

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Plasma membrane blebs are dynamic cytoskeleton-regulated cell protrusions that have been implicated in apoptosis, cytokinesis, and cell movement. Influencing Rho-guanosine triphosphatase activities and subsequent actomyosin dynamics appears to constitute a core component for bleb formation. In this paper, we discuss recent evidence in support of a central role of nonapoptotic membrane blebbing for cell migration and cancer cell invasion as well as advances in our understanding of the underlying molecular mechanisms. Based on these studies, we propose that in a physiological context, bleb-associated cell motility reflects a cell's response to reduced substratum adhesion. The importance of blebbing as a functional protrusion is underscored by the existence of multiple molecular mechanisms that govern actin-mediated bleb retraction.

Cell protrusions

In response to intra- and extracellular cues, remodeling of the submembranous cytoskeleton constantly reorganizes the plasma membrane (PM) of eukaryotic cells. These cytoskeletal rearrangements are controlled by the Rho family of small GTPases and their downstream signaling cascades, resulting in distinct types of actin-rich invaginations or protrusions such as filopodia, lamellipodia, invadopodia, podosomes, phagocytic cups, and uropods that serve specialized biological functions (for review see Chhabra and Higgs, 2007). In addition to these classical and well-studied PM protrusions, cells display structures referred to as PM blebs. Blebs expand up to 2 μ m from the PM and are defined by a bulky, rounded morphology. Observed under varying experimental conditions, all blebs follow with remarkable precision a similar, highly dynamic life cycle that roughly lasts 1 min: rapid bleb expansion, a short static phase, and low retraction of the bleb to the exact PM position where it originated (Fig. 1 A; Cunningham, 1995; Charras et al., 2005; Tournaviti et al., 2007).

Blebbing is initiated by a combination of events that involve local disruption of membrane-actin cortex interactions, leading to rapid protrusion of the PM as a result of the cell internal hydrostatic pressure (Trinkaus, 1973; Charras et al., 2005). Disruption of the membrane-actin cortex can also be caused by a local increase in cortical contractility of the actomyosin gel (Paluch et al., 2006). Importantly, initial powering of bleb expansion does not involve actin polymerization events, which distinguishes PM blebs from all other known cell protrusions. Although the expanding bleb PM is not coupled to an actin cortex, actin is subsequently polymerized at the bleb cortex to halt bleb expansion, and actomyosin contractility is generated to retract the bleb (Cunningham, 1995; Sheetz et al., 2006; Charras et al., 2007). Therefore, dynamic PM blebbing critically depends on filamentous actin integrity, whereas in most cases, microtubules are not essential for this process.

PM blebs were observed as early as 1919 (Hogue, 1919) and were described as hyaline blisters or bubbles (Holtfreter, 1943). This was followed by numerous studies investigating dividing or spreading cells as well as cancer cells (Zollinger, 1948; Landau and McAlear, 1961; Taylor, 1961; Gustafson and Wolpert, 1967; Price, 1967; Trinkaus and Lentz, 1967). Early work already indicated a link between PM blebbing and cell movement and also included imaging of blebs on living cells such as fibroblasts (Boss, 1955) or during spreading of human conjunctiva cells on glass surfaces (Taylor, 1961). Later, Trinkaus analyzed PM blebs in great detail during early *Fundulus heteroclitus* blastula and gastrula development. In a series of publications, he and his colleagues defined some of the kinetics and many physicochemical characteristics of blebbing activity in migrating *F. heteroclitus* deep cells during embryogenic movement (Trinkaus and Lentz, 1967; Tickle and Trinkaus, 1973, 1977). Despite these observations, PM blebbing was subsequently primarily viewed as a by-product of apoptotic and necrotic processes, during which cells display prominent blebs that are not related to cell locomotion (Lewis, 1923; Torgerson and McNiven, 1998; Coleman et al., 2001; Sebbagh et al., 2001). However, blebbing is not essential for the execution of cell death programs (Coleman et al., 2001; Shiratsuchi et al., 2002). Thus, the correlation between these protrusions and various types of cell movement regained increasing attention in recent years. Consequently, most of the recent progress in our understanding of membrane blebbing was made by studying nonapoptotic blebs, often in 3D environments. Therefore, this review will focus on membrane blebbing involved in cell motility. Although not decisively analyzed in all studies on PM blebs, the survival of blebbing cells over several

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Abbreviations used in this paper: GPCR, G protein-coupled receptor; PM, plasma membrane; ROCK, Rho-associated kinase.

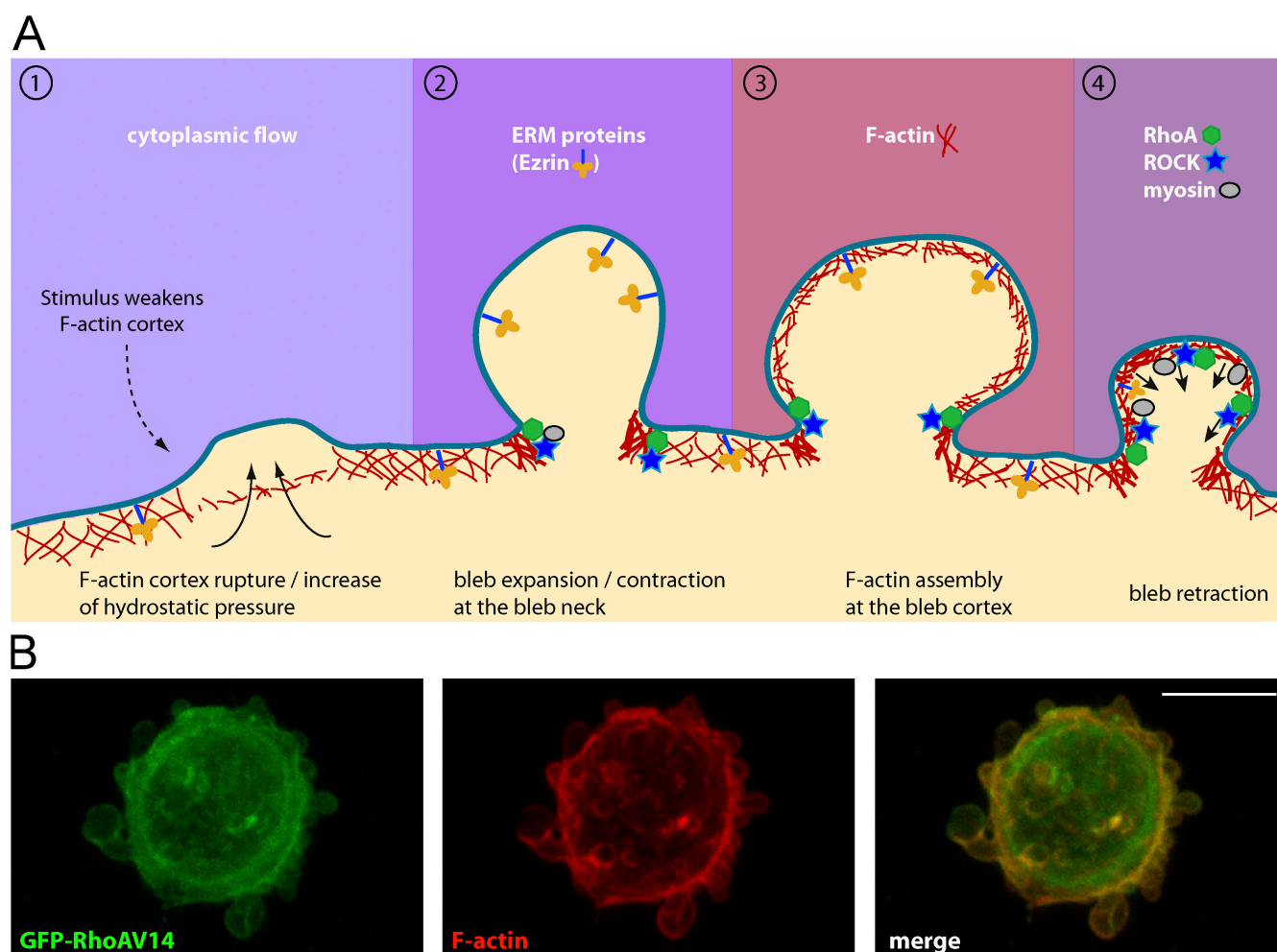


Figure 1. Molecular requirements for bleb formation and retraction. (A) Schematic presentation of a PM bleb life cycle. Many of the molecular details depicted refer to the scenario in filamin A-deficient M2 melanoma cells (Cunningham, 1995; Charras et al., 2006). Because not all molecular players detected in blebs are directly involved in blebbing and localization of some operating components is not detected upon overexpression of epitope-tagged proteins (Charras et al., 2006; Tournaviti et al., 2007), only components with documented localizations and functional relevance are indicated. Blebbing is initiated by extracellular triggers, causing localized destabilization or depolymerization of the cortical actin meshwork (1). Local disruption of the cortex-membrane interaction leads to the rapid formation of a bulky PM protrusion promoted by the cytoplasmic hydrostatic pressure (cytosolic flow; Trinkaus, 1973). The expanding bleb PM is not coupled to an actin cortex but is coated by actin-membrane cross-linker proteins of the ERM family such as ezrin (2). Actin is subsequently polymerized at the bleb cortex (3) by mechanisms that are still unknown, leading to a halt in bleb expansion (static phase). Increased actin filament assembly, recruitment of myosin to the bleb lumen, and local activity of RhoA-ROCK generate contractility that consequently retracts the bleb (4; Cunningham, 1995; Sheetz et al., 2006; Charras et al., 2007). (B) Maximum projection from confocal z stacks of a GFP-RhoA-V14 (green) expressing the MDA-MB-435 tumor cell contacting matrigel. The cell shows numerous PM blebs with constitutively active RhoA (RhoA-V14) partially visible in the bleb cortex. Filamentous actin was visualized using rhodamine-phalloidin (red). Bar, 20 μ m.

days, the lack of apoptotic markers and nuclear fragmentation, as well as the reversible nature of blebbing generally serve to confirm the nonapoptotic nature of such blebs.

Membrane blebbing in cell movement and invasion

The interest in a potential connection between PM blebbing and cell movement was rekindled when blebbing was detected in various human cancer cell lines such as M2 melanoma or migrating Walker carcinoma cells (Cunningham et al., 1992; Keller and Bebie, 1996). These studies were subsequently expanded to more physiological scenarios to study cell migration through 3D matrices (Even-Ram and Yamada, 2005). Cancer cells that crawl through a 3D matrix can be morphologically distinguished by two modes of invasion: one that appears as a mesenchymal

cell movement that relies on proteolytic degradation of the surrounding matrix and another that adopts a rounded, more amoeboid mode of motility that frequently is accompanied by cell blebbing (Sahai, 2005). In an exciting study, Wolf et al. (2003) showed that cancer cells invading through 3D collagen under conditions in which matrix degradation is blocked efficiently use an amoeboid mode of invasion with the formation of bleb-like constriction rings. Consistent with this, some cancer cells preferentially use the bleb-associated amoeboid mode of invasion to bypass the requirement for extracellular matrix proteolysis or simply to switch modes of motility as an escape mechanism (Sahai and Marshall, 2003; Friedl, 2004). Furthermore, experimental induction of PM blebs in noninvasive cells promotes their ability to invade into 3D matrices (Gadea et al., 2007; Tournaviti et al., 2007). Together, these studies strongly

Table I. Experimental systems initiating PM blebbing and their key regulators

Nonapoptotic bleb formation caused by	Proposed function	Rho/ROCK	Myosin	Formin	Src	References
Protease inhibition in 3D collagen converts spindle-shaped HAT1080 and MDA-MB231 cells into spherical cell shape by remodeling of contractile actin cortex	Tumor cell invasion					Wolf et al., 2003
A375 cells in 3D matrigel leads to cell rounding and remodeling of contractile actin cortex	Tumor cell invasion	+				Sahai and Marshall, 2003
p53 ^{-/-} MEFs and A375 cells have elevated RhoA activity leading to cell rounding	Cell invasion	+				Gadea et al., 2007
MDA-MB-435 cells in 3D matrigel leads to cell rounding and remodeling of the contractile actin cortex	Tumor cell invasion	+	+	hDia1	–	Kitzing et al., 2007
SH4 domain overexpression causes spontaneous disruption of actin cortex	Cell invasion	+	+		+	Tournaviti et al., 2007
Filamin A deficiency in M2 cells leads to spontaneous disruption of actin cortex	ND	+	+			Cunningham et al., 1992; Charras et al., 2005
Talin deficiency in megakaryocytes	ND					Wang et al., 2008
Overexpression of RhoA Q63L in epithelial cells	Cell dissemination	+				Vasiliev et al., 2004
Overexpression of active DIP	ND	+		mDia2		Eisenmann et al., 2007
Rac1 deficiency in mouse fibroblasts	Cell proliferation and morphology					Vidali et al., 2006
Overexpression of FilGAP causes Rac inhibition and suppresses integrin-mediated cell spreading	Cell polarity	+				Ohta et al., 2006
RacL61A37 overexpression	ND					Schwartz et al., 1998
Overexpression of PM-anchored MARCKS	Reduced adhesion					Myat et al., 1997
Disruption of membrane–cytoskeleton interaction by reduced PM PIP2 levels as a result of experimental depletion or PIP5K γ deficiency	ND					Raucher et al., 2000; Wang et al., 2008
Migration of primordial germ cells toward SDF1 α in zebrafish	Chemotaxis	+	+			Blaser et al., 2006
Cell migration of <i>Dictyostelium</i> toward cAMP	Chemotaxis		+			Langridge and Kay, 2006; Yoshida and Soldati, 2006
Exposure of neuroblastoma cells to lysophosphatidic acid	ND		+			Hagmann et al., 1999
Cholecystokinin stimulation of pancreatic acini	ND		+		+	Singh and McNiven, 2008
Extracellular ATP via P2X7 surface receptors in various cell types	ND	+	+			MacKenzie et al., 2001; Verhoef et al., 2003; Panupinthu et al., 2007
Vaccinia virus presenting phosphatidylserine as pseudoapoptotic signal	Vaccinia virus entry	– (Rac)	+			Mercer and Helenius, 2008
<i>Pseudomonas aeruginosa</i> contact with epithelial cells	Pathogen motility					Angus et al., 2008

suggest that PM blebbing enables cells to gain directed motility in 3D environments.

Actin-membrane interactions in membrane blebbing

The use of 3D invasion models will continue to yield crucial information on the physiological roles exerted by blebs. However, these experimental systems are relatively difficult to access for molecular manipulation. So far, insights into the molecular mechanisms that govern PM blebbing are thus mostly derived from 2D standard cell cultures that display blebbing under certain conditions. Consistent with an early study that demonstrated blebbing after the addition of serum (Taylor, 1961), many investigators subsequently revealed that contractility for bleb retraction is provided by signaling through Rho-ROCK-

myosin and defined this module as essential core machinery common to virtually all types of blebs analyzed so far, including apoptotic and nonapoptotic blebs as well as cancer cells using a blebbing motility mode (Sahai and Marshall, 2003; Charras et al., 2005; Gadea et al., 2007; Kitzing et al., 2007; Tournaviti et al., 2007). In this cascade, GTP-loaded Rho activates its effector kinase Rho-associated kinase (ROCK) that directly phosphorylates myosin light chain, thereby inducing actomyosin contraction. Evidence is accumulating that blebbing can result from targeting this cascade at different levels, suggesting the existence of distinct types of blebs that derive from individual mechanisms (Table I).

One mode of regulation occurs directly at the level of Rho, where activation of Rho causes PM blebbing. This can be mediated by extracellular signals (see the next section) or by intracellular

signaling cascades, such as the up-regulation of RhoA in the absence of the tumor suppressor p53 (Gadea et al., 2007). PM blebbing as a result of Rho activation can also occur indirectly via the Rac GTPase, whose activity is tightly balanced with that of Rho (Sander et al., 1999). In one such example, expression of FilGAP suppresses the activity of Rac, leading to cross talk regulation of RhoA and subsequent membrane blebbing (Ohta et al., 2006). Similar events likely explain extensive PM blebbing after overexpression of an effector loop mutant of active Rac1 (Schwartz et al., 1998) or *Dictyostelium discoideum* RacB (Lee et al., 2003) as well as the complete lack of Rac1 expression (Vidali et al., 2006). As indicated by the potent suppression of PM blebbing by specific inhibitors of ROCK (Table I), regulation is also possible at the level of this Rho effector. This mechanism is likely physiologically relevant because Pinner and Sahai (2008) recently unraveled that in A375 melanoma cells, Rho-ROCK activity is directed toward the induction of PM blebbing by association with PDK1, which displaces the negative regulator RhoE from ROCK to allow sustained PM localization of active ROCK.

In addition to direct effects on the Rho-ROCK cascade, mechanical disruption of the actin cortex–membrane interaction represents another important trigger for PM blebbing. This was first revealed by the best-studied 2D model system of PM blebbing, the melanoma cell line M2 that is deficient for the actin-binding protein filamin A and displays constant PM blebbing (Cunningham et al., 1992; Cunningham, 1995). Revealing the complexity of the membrane–cortex interaction, deficiency in another actin–membrane linker protein, talin, also results in pronounced membrane blebbing (Wang et al., 2008). Given the importance of cortex–membrane interactions, it is not surprising that besides the protein-binding partners involved, the lipid composition of the membrane bilayer also has great impact on the rigidity of the interaction. Presumably as a result of its ability to regulate actin remodeling at the PM, the phosphoinositol PIP2 has proven critical importance in this context. Indeed, experimental or genetic depletion of PM PIP2 leads to vigorous PM blebbing (Raucher et al., 2000; Wang et al., 2008). Thus, sequestering and/or saturation of PM PIP2 might explain why efficient loading of the cytoplasmic leaflet of the PM with specific targeting signals such as palmitoylated MARCKS (myristoylated alanine-rich PKC substrate) proteins (Myat et al., 1997) or SH4 membrane-targeting domains (short motifs that mediate PM localization of Src kinases; Tournaviti et al., 2007) results in the efficient generation of dynamic PM blebs. Rho-ROCK-myosin signaling is probably essential for blebbing dynamics in all of these cases. How precisely this cascade is initiated by these different causes of membrane–cortex disruption needs to be resolved in the future.

Blebs may also be distinguishable based on the involvement of the machines for actin polymerization. Local actin polymerization is governed by so-called actin nucleators, including the Arp2/3 multiprotein complex that generates branched filamentous actin meshworks and nucleators such as the formin family of proteins, which promote the assembly of linear actin filaments through their formin homology 2 domain (Evangelista et al., 2003). Analyzed for three examples, no evidence was found for a direct involvement of Arp2/3 in blebbing, suggesting that

this nucleator is dispensable (Charras et al., 2006; Langridge and Kay, 2006; Eisenmann et al., 2007). In contrast, the formin family of actin nucleators has attracted attention in regard to their involvement in blebbing. These studies have focused in particular on the Diaphanous formins, proteins that are tightly regulated through physical and functional interactions with Rho-GTPases (Faix and Grosse, 2006). Beyond this ability to nucleate actin filaments, some diaphanous formins can also regulate bundling of actin filaments (Faix and Grosse, 2006; Goode and Eck, 2007) or amplify Rho-ROCK signaling (Kitzing et al., 2007), functions that could be at work during bleb contraction. In support of a role for diaphanous formins in dynamic PM remodeling, blebbing cell motility of human MDA-MB-435 cancer cells in 3D matrices relies on the action of the RhoA interaction partner and regulator Dia1, whereas Dia2 was found to be dispensable (Kitzing et al., 2007). A function similar to that of Dia1 may be exerted by the diaphanous formin FHOD1 in cells that display PM blebbing and enhanced invasiveness as a result of the overexpression of SH4 domains (Tournaviti et al., 2007; unpublished data). Interestingly, expression of the Diaphanous-interacting protein, a negative regulator of mouse Dia2, causes blebbing in an mDia2-dependent manner (Eisenmann et al., 2007). Thus, positive and negative regulation of different formins can result in PM blebbing, probably as a result of different endogenous Rho-GTPase effector coupling. A full understanding of this complexity will require identification of the specific actin nucleators involved in blebbing initiated by different triggers and their precise localization during the bleb life cycle.

Extracellular cues that initiate membrane blebbing

Although the role of blebbing in cancer invasion has been recognized, and we have learned a lot about the molecular events occurring during bleb formation and retraction, we know very little about the extracellular signals or ligands that initiate this type of 3D cell motility and induce blebbing in a physiological context. Some of the early experiments studying PM blebbing on human conjunctiva cells were performed under serum-stimulating conditions in which blebbing was, in fact, dependent on the presence of FCS in the cell culture medium (Taylor, 1961). Given the players involved such as Rho, ROCK, and myosin, this does not seem surprising, as serum is a well-known activator of these signaling components. Apparently consistent with this is the finding that NB2a rat neuroblastoma cells undergo vigorous and transient blebbing after exposure to physiological concentrations of the serum component lysophosphatidic acid (Hagmann et al., 1999), which is a ligand for G protein–coupled receptors (GPCRs). In addition, the neurotransmitter and peptide hormone cholecystokinin was found to induce reversible PM blebs in rat pancreatic acinar cells accompanied by myosin II reorganization to the bleb neck (Torgerson and McNiven, 1998; Singh and McNiven, 2008). The concept that soluble ligands could initiate PM blebbing is further supported by recent observations that the chemoattractant cAMP elicits blebs in *D. discoideum* cells (Langridge and Kay, 2006) that occur at the leading edge of migrating cells toward the

cAMP source (Yoshida and Soldati, 2006), whereas during zebrafish primordial germ cell migration, the chemokine SDF-1 α , a ligand for the GPCR CXCR4, promotes bleblike protrusions through the local increase of intracellular calcium and subsequent actomyosin contractions at the leading edge (Blaser et al., 2006). Thus, some GPCR ligands seem to function as external cues for bleb-associated cell movement. This principle may extend beyond GPCRs, as stimulation of the ATP-gated ion channel P2X7 by extracellular ATP also induces potent membrane blebbing (MacKenzie et al., 2001; Verhoef et al., 2003). The functional consequences of these blebs, including a potential role in cell motility/chemotaxis, remain to be defined. Thus, our current understanding of signal-induced bleb formation, particularly in mammalian cells, is still very fragmentary, as is the relation between soluble and matrix adhesion factors for this specialized mode of motility.

Blebbing induced by alterations in cell adhesion

In addition to blebbing induced by extracellular ligands, other stimuli may be essential for membrane blebbing in physiological settings. Interestingly, MDA-MB-435 cancer cells display a mesenchymal phenotype when plated on plastic dishes or under rigid surface adhesion but change into a round and blebbing morphology when contacting matrigel (Kitzing et al., 2007). This indicates that changes in cell adhesion as well as the “softness” of adhesion function as a motility or differentiation signal that promotes PM blebbing during tumor progression. In line with this scenario, studies of early embryonic movements of *F. heteroclitus* blastoderm suggested that blebs serve as specialized protrusions for directional cell locomotion searching for adhesive substratum (Tickle and Trinkaus, 1973; Trinkaus, 1973). Indeed, disintegration experiments of ectoderm cells from amphibian gastrulae revealed that these cells undergo morphological changes reminiscent of blebbing (Holtfreter, 1943). Thus, loss of cell–cell contacts or changes in cell adhesion properties might promote bleb formation during early development (Shook and Keller, 2003). Consistent with this idea is the observation that rat liver epithelial cells stably expressing a constitutively active (Q63L) version of RhoA can undergo detachment from their epithelial sheets, displaying a rounded bleb-associated mode of motility, which may resemble a mechanism of cancer cell dissemination (Vasiliev et al., 2004). Based on these observations, one can conclude that under conditions in which cell adhesion is relatively strong, such as on a rigid matrix, contractile force generates tension within the cell. In contrast, under reduced adhesion, such as during cell division or migration through a matrigel, cell contraction can initiate events of actin cortex–membrane destabilization that are additionally driven by the increased hydrostatic pressure during cell rounding. Collectively, experimental findings so far emphasize the idea that cell matrix adhesion and interaction may generally control PM blebbing, particularly in 3D environments.

Conclusions/future directions

From the currently available data, it is evident that blebs use Rho-ROCK-myosin as common machinery to provide contractility.

However, blebs can be initiated by a variety of experimental and physiological triggers that disrupt actin–membrane interactions or induce Rho-ROCK signaling, and individual blebs likely differ in the actin nucleators involved in bleb retraction. 2D culture systems have been useful for the initial molecular characterization of blebbing; however, much remains to be learned about the detailed composition and regulation of the blebbing machinery. Although alterations of adhesion properties emerge as a critical switch of bleb regulation in 3D environments, further identification of the extracellular factors and their ligands that trigger blebbing is a major challenge for the field. Another burning question relates to the mechanism by which blebbing facilitates cell motility. Conceivably, polarized blebbing may generate force that facilitates directed movement. However, in several experimental settings, nonpolarized blebbing also correlates with enhanced cell invasion (Gadea et al., 2007; Kitzing et al., 2007; Tournaviti et al., 2007), suggesting that blebs exert additional functions beyond force generation to ensure pathfinding of invading cells (Sahai and Marshall, 2003; Wolf et al., 2003). Such an activity was recently exemplified by PM blebs that serve as preferred entry portals for pathogen invasion (Angus et al., 2008; Mercer and Helenius, 2008). The reappraised role of blebs in cell movement warrants increased efforts to unravel the interplay between the cellular machines involved and the pathophysiological relevance.

We are grateful to Freddy Frischknecht for critical reading of the manuscript. We also thank B. Di Ventura and T. Kitzing for help with the figure.

Our research is funded by grants from the Deutsche Forschungsgemeinschaft (Fa 378/6-1 and GRK1188 to O.T. Fackler and GR 2111/1-3 to R. Grosse). O.T. Fackler and R. Grosse are members of the excellence cluster Cellular Networks.

Submitted: 14 February 2008

Accepted: 13 May 2008

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