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HYPOXIA-INDUCED AMOEBOID CANCER CELL MIGRATION: PRINCIPLES, MOLECULAR MECHANISMS AND PLASTICITY

Veronika Boekhorst

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HYPOXIA-INDUCED AMOEBOID CANCER CELL MIGRATION: PRINCIPLES, MOLECULAR MECHANISMS AND PLASTICITY

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HYPOXIA-INDUCED AMOEBOID CANCER CELL MIGRATION: PRINCIPLES, MOLECULAR MECHANISMS AND PLASTICITY

A

DISSERTATION

Presented to the Faculty of

The University of Texas

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Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

Veronika te Boekhorst, M.S.

Houston, Texas

December 2017
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DEDICATION

I dedicate this dissertation work to all young women, men and children from any minority group or family background: Be strong, work diligently and never give up being who you are and who you want to become.
ACKNOWLEDGEMENTS

With great appreciation, I would like to acknowledge all people who have made my education and training a great and unforgettable experience.

I deeply express my gratitude to my mentor and Ph.D. advisor Dr. Peter Friedl. His guidance, support and excellence has tremendously shaped my scientific and personal development.

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Last, but not least, I thank all the special people in my life, my family and friends for their support and believe in me.
Cancer invasion and metastasis can occur through single cell and collective strategies with different consequences for cell and tissue patterning and metastatic outcome. Tumor hypoxia, by elevating hypoxia-inducible factors (HIF), is an established inducer of metabolic and invasive reprogramming of cancer cells, such as the induction the epithelial to-mesenchymal transition (EMT). However, how hypoxia/HIF impacts on established epithelial cancer cell migration programs and which mechano-signaling adaptations are induced upon hypoxic stress, remains unknown.

Using epithelial breast cancer (BCC) and head and neck squamous carcinoma (HN-SCC) spheroids in 3D fibrillar collagen, we characterized the invasion patterns as well as mechano-signaling requirements of hypoxia-induced cancer cell migration modes. While epithelial cancer cells migrated collectively under normoxic conditions, hypoxia (0.2% O₂) or pharmacological stabilization of HIF-1α by the prolyl hydroxylase inhibitor dimethyloxalylglycine (DMOG), induced the transition of collective-to-single cell migration. Besides mesenchymal-like movement, most epithelial cells converted to amoeboid migration with distinctive blebby protrusions or, less frequently, with actin-rich pseudopodal protrusions towards the direction of migration. Both bleb-based and pseudopodial protrusions supported polarized interactions with collagen fibrils, allowing amoeboid cells to move equally fast (0.1-0.5 µm/min) and persistently (confinement ratio: 0.1-0.6) as elongated, mesenchymal-like single cell migration modes. HIF-induced blebby amoeboid migration was dependent on (i) RhoA/ROCK mediated actomyosin...
contraction for efficient bleb formation and migration, (ii) reduced β1 integrin-mediated matrix interactions, and (iii) independent on matrix-metalloproteinase-mediated collagen remodeling. As central mechanism underlying phenotypic conversion towards blebby amoeboid migration, HIF stabilization upon hypoxia or DMOG treatment upregulated calpain-2 expression and enzymatic activity, which, by cleavage of focal adhesion protein talin-1, limited β1 integrin function, which was rate-limiting for the adaptation to and maintenance of amoeboid movement. Accordingly, interference with calpain function by pharmacological inhibitors or RNA interference prevented talin-1 cleavage, restored β1 integrin surface activity, downstream Src signaling, and reverted blebby amoeboid migrating cells towards spindle-shaped, mesenchymal-like phenotypes. Likewise, ectopic expression of a calpain-uncleavable talin protein restored active β1 integrin expression and converted amoeboid blebby migrating cells towards elongated migration morphologies.

Consistent with elevated calpain activity observed during 3D spheroid collagen migration in vitro, calpain activity was elevated in roundish, amoeboid disseminating single cells observed at the tumor-stroma interface in human HN-SCC tumor xenografts models. Calpain function was further required for round blebby amoeboid migration after HIF-stabilization in vivo.

Together, our data establish HIF signaling as microenvironmentally regulated pathway for cell reprogramming which induces blebby amoeboid migration in cancer cells. Our data further identify calpain-2 as master regulator upstream of β1 integrin shutdown, via talin-1 cleavage, which induces and maintains the conversion between elongated and amoeboid tumor cell dissemination. The HIF induced amoeboid migration program represents a migratory escape strategy in epithelial tumor cells to abandon metabolically perturbed tissue regions, and drives molecular and functional plasticity of cancer cell dissemination.
TABLE OF CONTENT

Approval Sheet.......................................................................................................................... i
Title Page...................................................................................................................................... ii
Copyright.................................................................................................................................... iii
Dedication..................................................................................................................................... iv
Acknowledgements...................................................................................................................... v
Abstract ....................................................................................................................................... vii
Table of Content.......................................................................................................................... ix
List of Illustrations ....................................................................................................................... xiii

Chapter 1: Introduction and Background.................................................................................... 1

1. Introduction .............................................................................................................................. 1

2. Cancer types and their migration modes .................................................................................. 4
   2.1 Amoeboid migration........................................................................................................... 4
   2.2 Mesenchymal migration .................................................................................................. 6
   2.3 Collective cell migration ................................................................................................. 7
   2.4 Diversity of metastatic evasion ...................................................................................... 9

3. Determinants controlling cancer cell migration modes .......................................................... 13
   3.1 Cellular determinants ..................................................................................................... 13
   3.2 Microenvironmental determinants ............................................................................... 22

4. Plasticity of cancer cell migration programs .......................................................................... 26
   4.1 Plasticity of single cancer cell migration ....................................................................... 27
   4.2 Collective to single cell transition ................................................................................ 28
   4.3 Collective plasticity ......................................................................................................... 31

5. Multiscale mathematical modeling of cancer cell migration and plasticity ......................... 32
   5.1 Mathematical tool box to model cell migration ............................................................. 32
   5.2 Virtual assembly of moving cells .................................................................................. 33
Hypoxia and/or HIF-1α-induced switch from collective to single-cell migration ........................................53
Hypoxia does not impair cell viability ........................................................................................................58
Hypoxia-induced collective-to-single cell transition depends on HIF-1α..................................................... 60
Hypoxia induces a stable and highly migratory blebby ameboïd migration mode................................. 62
HIF-induced ameboïd cells display polarized interactions with collagen fibrils ............................... 65
Roundness is a stable feature of blebby ameboïd migration in different collagen densities. 66
Blebby ameboïd migration is a stable and efficient migration mode......................................................... 68
Chapter 4: Specific Aim 2 ......................................................................................................................... 72
Results: Molecular mechanisms underlying hypoxia-induced migration modes................очной 72
Hypoxia-induced MMP-independent ameboïd movement ............................................................... 72
Cell contractility as driving force underlying hypoxia ameboïd blebby migration .......................... 76
Hypoxia/HIF-stabilization reduce β1 integrin activity and signaling .................................................. 82
Diminished β1 integrin activity is rate-limiting for the phenotypic switch towards ameboïd
blebby migration........................................................................................................................................... 86
β1 integrin regulation in response to HIF stabilization is controlled by calpain-2 activity ........ 89
Elevated calpain-2 function mediates blebby round ameboïd migration........................................... 91
Calpain-2 activation mediates talin-1 cleavage and lowers β1 integrin signaling ......................... 99
Friction and cell contractility combined mediate ameboïd round migration morphologies .......... 104
HIF-induced ameboïd transition is independent of collagen substrate dimensionality ........... 108
Chapter 5: Specific Aim 3 ......................................................................................................................... 111
Results: Validation of hypoxia-induced cellular and molecular migration phenotypes
in tumor xenografts and orthotopic human tumor models in vivo ......................................................... 111
Elevated calpain activity in round, ameboïd invading single cells in orthotopic human
HN-SCC xenografts.................................................................................................................................... 111
HIF-induced calpain-2 mediates blebby ameboïd migration in vivo................................................. 113
Chapter 6: Discussion, Significance and Future Directions ......................................................................... 117
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bibliography</td>
<td>130</td>
</tr>
<tr>
<td>Vita</td>
<td>163</td>
</tr>
</tbody>
</table>
LIST OF ILLUSTRATIONS

Chapter 1. Introduction and Background

Figure 1. Single-cell and collective migration modes .......................................................... 11
Figure 2. Cellular modules determining migration modes ..................................................... 21
Figure 3. Plasticity of cell migration programs ..................................................................... 30
Figure 4. Mathematical modelling of cell migration modules and modes ............................. 35

Chapter 3: Characterize principles and plasticity of epithelial cancer cell migration
in response to hypoxia

Figure 5. Hypoxia induced HIF-1 dependent plasticity of cancer cell migration .................. 55
Figure 6. HIF-induced single cells are low in membranous E-cadherin expression .............. 56
Figure 7. Hypoxia induced plasticity of cancer cell invasion: impact on cell viability ......... 58
Figure 8. Hypoxia-induced plasticity and regulation by HIF-1 ........................................... 60
Figure 9. Hypoxia/HIF-induced amoeboid blebby migration ............................................. 63
Figure 10. Cellular, cytoskeletal and protrusion analysis of HIF-induced amoeboid migration
modes ..................................................................................................................................... 65
Figure 11. Elongation analysis and kinetics of HIF-induced single cell migration morphologies
................................................................................................................................................ 67
Figure 12. Stability and switching behavior of HIF-induced single cell migration phenotypes 69
Figure 13. Migration speed and directionality of HIF-induced single cells migrating into
collagen ..................................................................................................................................... 70

Chapter 4: Identify molecular mechanisms underlying hypoxia-induced migration
modes

Figure 14. Hypoxia and/or HIF-induced MMP-independent amoeboid migration ............ 74
Figure 15. Elevated Rho/ROCK-induced myosin activity under hypoxia and/or after HIF-
stabilization with DMOG ................................................................................................. 78
Figure 16. Dependence of HIF-induced amoeboid blebby migration on ROCK and myosin activity ................................................................. 80
Figure 17. Reduced β1 integrin activity and downstream signaling under hypoxia and/or after HIF stabilization by DMOG ................................................................. 84
Figure 18. Dependence of HIF-induced amoeboid blebby migration on β1 integrin function .......... 87
Figure 19. Calpain-2 expression and functional correlation with active β1 integrin status .......... 90
Figure 20. Calpain expression and activity in 2D monolayers ............................................. 92
Figure 21. Calpain activity during amoeboid migration in 3D spheroid culture ....................... 94
Figure 22. Calpain inhibition reduced calpain activity and phenotypic reversion of amoeboid blebby cells .............................................................................................................. 95
Figure 23. Reversion of the blebby amoeboid to elongated phenotype after interference with calpain-2 function ........................................................................................................ 97
Figure 24. Elevated calpain-mediated talin-1 cleavage .......................................................... 99
Figure 25. Reversion of blebby amoeboid to spindle-shaped migration morphology after expression of calpain-uncleavable TalinL432G ........................................................................ 102
Figure 26. Effect of cell contractility and friction forces on cell length (roundness) of migrating single cells by the mathematical subcellular element model ........................................... 106
Figure 27. Spheroid model for analyzing HIF-induced single cell migration behavior on 2D collagen ...................................................................................................................... 109

Chapter 5: Validate hypoxia-induced cellular and molecular migration phenotypes in tumor xenografts and orthotopic human tumor models in vivo

Figure 28. Elevated calpain activity in human HN-SCC oral cancer tumor model in mice ........ 112
Figure 29. HIF-induced blebby amoeboid migration requires calpain function in vivo .......... 114
Figure 30. Correlation of morphologies and protrusion types of HIF-induced blebby amoeboid migration after calpain inhibition in vivo ........................................................................ 116

Chapter 6: Discussion, Significance and Future Directions
Figure 31. Proposed mechanism underlying hypoxia/HIF-induced blebby amoeboid cancer cell migration.................................121
CHAPTER 1: INTRODUCTION AND BACKGROUND

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1. Introduction
Cell migration is an evolutionarily conserved, multi-faceted process which allows individual cells and cell groups to move, change position and build or maintain tissues and organs for embryonic development as well as homeostasis and regeneration (Sonnemann & Bement, 2011; Weijer, 2009). Virtually any nucleated cell, after promigratory stimulation, is able to transiently or continuously migrate, including stem cells, epithelial and endothelial cells, stromal
and neuronal cells, and leukocytes (Friedl, 2004). In progressing cancer disease, tumor cells can acquire similar migration ability, which enables tumor cells to change position within a given tissue and spread from a tumor mass (Friedl & Alexander, 2011; Massagué & Obenauf, 2016; Sahai, 2007). Common to all cell types, cell migration results from an integrated multi-step process mediated by molecular and physical programs, including cytoskeletal polarity and dynamics, cell-cell and cell-matrix adhesion and pericellular proteolysis (Friedl & Wolf, 2003). Moving cells are responsive and adjust to external environmental cues, such as tissue dimension, structure and substrate type, and molecular triggers, including ECM-abundant cytokines and chemokines (Friedl & Wolf, 2010). In addition, by mechanically and chemically modifying structural proteins of tissues, moving cells can mediate structural alterations and shaping of tissue structures and thereby, depending on context, contribute to tissue formation or degeneration.

Both, the primary tumor and metastatic lesions can release disseminating cells which penetrate local tissues, enter blood vessels and circulate systemically (Fidler, 2003). The movement of cancer cells and metastatic dissemination occur from early to late stages of cancer disease (Hüsemann et al., 2008; Klein, 2009). Thus, integral to cancer progression, metastatic evasion, seeding and re-seeding all contribute to the initiation and amplification of systemic metastasis (Wan, Pantel, & Kang, 2013). As an underlying mechanism, cancer metastasis depends upon the ability of cancer cells to switch from migratory, hence disseminating to sessile, hence locally growing state, likely in response to microenvironmental cues.

To initiate and complete the metastatic cascade, two principal types of migration programs are required, (i) the ability to cross tissue barriers, such as traversing across basement membranes (BM) and vascular walls, and (ii) interstitial invasion, by movement along tissue structures and through gaps and spaces present in connective (Alexander, Weigelin, Winkler, & Friedl, 2013; Fidler, 2003; Valastyan & Weinberg, 2011). Traversing across tissue
barriers is required for migration between tissue compartments, such as penetration through the BM underling the epithelium, from which epithelial tumors originate, or tumor cells intra- and extravasating blood vessels (Glentis, Gurchenkov, & Vignjevic, 2014). For interstitial invasion, after BM penetration or when tumors primarily originate in the interstitium (e.g. sarcoma, hematopoietic and glial tumors), cells directly engage with interstitial matrix and move along tissue interfaces and cell surfaces (Alexander et al., 2013). For systemic metastasis, moving cells undergo alternating phases of tissue barrier penetration, interstitial migration and migration arrest, which indicates a high degree of versatility and flexibility of migration abilities as well as responsiveness to tissue cues. Thus, each step of the metastatic cascade requires different mechanical and chemical activities of the tumor cell in a spatiotemporal-controlled manner, including adhesion and mechanotransduction, proteolysis and cell deformation (Chambers, Groom, & MacDonald, 2002; Nguyen, Bos, & Massagué, 2009).

Depending on the tumor type, stimulation and tissue context, tumor cells apply a range of molecular and mechanical mechanisms to move and integrate tissue-derived signals (Friedl & Wolf, 2003; Sahai, 2007). Tumor cells can either disseminate as individual cells, via mesenchymal or amoeboid single cell migration, or by collective migration, whereby cell-cell adhesion and cooperation remain intact (Friedl and Wolf 2003). Induced by environmental triggers, tumor cells may additionally adapt and change their migration mechanisms and transit between migration programs, including collective-to-single cell or mesenchymal-to-amoeboid transitions (Friedl & Alexander, 2011). Such plasticity of migration modes represents a putatively important coping strategy, which allows tumor cells to retain migration capability and escape from regions of environmental challenge. Such adaptation (“plasticity”) of cancer cell migration strategies likely recapitulate mechanical and molecular programs engaged in stem cells and primordial tissue dynamics during embryonic development (Peter Friedl & Gilmour, 2009; Paluch & Raz, 2013).
2. Cancer types and their migration modes

Cancer cells exploit various strategies to invade tissue, including collective cell migration, in which cells migrate as multicellular groups mediated by strong cell-cell adhesion e.g. by expression of E-cadherin; or single cell migration, including amoeboid or mesenchymal migration, where no cell-cell junctions are present and cancer cells migrate individually (Friedl and Wolf, 2010) (Fig. 1).

The types and molecular programs underlying cancer cell migration, originally identified using experimental 2D and 3D live-cell migration assays in vitro, have been confirmed by in vivo imaging in animal models and validated by inference with the histological patterns of invasion zones and organization of circulating tumor cells (CTCs) in clinical specimens. In aggregate, these analyses suggest that both, tumor type and its differentiation state determine adaptive migration programs by which cancer cells navigate through each step of the metastatic cascade.

2.1 Amoeboid migration. Amoeboid single tumor cell migration is classified from roundish but highly deformable cell morphology typically detected in myeloid leukemia and lymphoma cells, small-cell lung carcinoma and small cell prostate cancer, as well as induced experimental models of melanoma and epithelial tumors, such as breast cancer (Condeelis & Segall, 2003; Madsen & Sahai, 2010; Pinner & Sahai, 2008). Histologically, amoeboid movement is characterized by roundish single cells, which individually and often diffusely infiltrate the tissue. In leukemia, amoeboid tumor cells recirculate in large numbers and represent the predominant cell type in the blood (Hutchinson et al., 2014).

Amoeboid movement is initiated and maintained by asymmetric cell interactions with tissue structures via membrane protrusions at the leading edge (Fig. 1a1). Subsequently, amoeboid translocation is mediated by relatively low-adhesive and poorly proteolytic cell-ECM/substrate interactions, which are maintained by dynamic shape change and cell contractility. Thereby, amoeboid movement mediates particularly flexible cell-tissue interactions
which depend on shape change and intercalation for propagation through tissue gaps and discontinuities ("elbowing") (Friedl, Entschladen, Conrad, Niggemann, & Zänker, 1998; Lorentzen, Bamber, Sadok, Elson-Schwab, & Marshall, 2011; Paluch & Raz, 2013; Wyckoff, Pinner, Gschmeissner, Condeelis, & Sahai, 2006). The roundish shape, types of cell protrusions and cytoskeletal flexibility are jointly regulated by the small GTPases RhoA and Rac (Fig. 2a, c).

Both cell polarization towards direction of migration and retraction of the cell rear depend upon cortical actomyosin contractility, controlled by RhoA via ROCK signaling and myosin II activity (O’Connor & Chen, 2013; Paluch & Raz, 2013). Cortical actin network contraction additionally increases hydrostatic pressure in the cell which triggers bleb-like outward protrusion of the plasma membrane ("blebbing") through which cells protrude towards and intercalate surrounding 3D tissue structures (Blaser et al., 2006; Paluch & Raz, 2013; Paluch, Piel, Prost, Bornens, & Sykes, 2005). Other protrusions at the leading edge, including pseudopods, lamellipods and filopods, depend upon actin polymerization mediated by local Rac activity and provide polarized adhesion to 2D and 3D substrates (Sanz-Moreno & Marshall, 2010). Blebby and podal protrusions, well defined in amoeboid movement, are likely relevant in other migration modes as well. While the cell front is engaged with substrate, lateral and rear regions of the cell undergo contraction controlled by RhoA/ROCK and myosin II activity, which mediate cell contraction along the longitudinal axis (Bastounis et al., 2014), shorten the cell rear, and carry the nucleus forward (Renkawitz et al., 2009; Wyckoff et al., 2006).

Thus, shape adaptation mediated by Rho/ROCK-driven actin contraction and deformability are key mechanisms enabling amoeboid migrating tumor cells (Sanz-Moreno & Marshall, 2010; Wyckoff, Pinner, Gschmeissner, Condeelis, & Sahai, 2006). In addition, the adaptive shape change enables cells to move along and between tissue discontinuities without proteolytic degradation of extracellular matrix (Wolf et al., 2013). Amoeboid migration arguably represents the simplest, most flexible and therefore potentially the most efficient strategy to
move through tissues and between tissue barriers.

2.2 Mesenchymal migration. Mesenchymal movement is often found in sarcomas, gliomas and epithelial cancer cells after undergoing epithelial to mesenchymal transition (EMT), and further is present in tumor types of generally low differentiation (“spindle-cell tumors”) (Cates et al., 2008; Chanrion et al., 2014). Histologically, mesenchymal movement can be inferred from elongated, spindle-like cell shape with oval nuclear morphology (Moreno-Bueno et al., 2009). Often mesenchymal tumor cells express EMT markers, including nuclear Oct-4, Twist, Snail/Slug and cytoplasmic vimentin, which allow their detection in tissue samples as grouped or individual cells infiltrating remodeled, often desmoplastic tissue (Zeisberg & Neilson, 2009).

During migration, mesenchymal tumor cells maintain an elongated, polarized cell shape, with a protruding pseudopod and/or multiple filopods, which adhere to and pull on ECM substrate and determine the direction of migration (Starke, Maaser, Wehrle-Haller, & Friedl, 2013) (Fig. 1a). At the leading edge, Rac-induced actin assembly and integrin binding to the substrate induce cell polarization and protrusion (Geiger, Spatz, & Bershadsky, 2009a). Concomitantly with integrin engagement, FAK and Src kinases induce maturation of focal ECM adhesion and mechanotransduction, which, together with contractile cortical actin, mediates high contractile tension and pulling forces towards ECM structures (Fig. 2a3, c) (Guarino, 2010; Lawson et al., 2012; S. J. Parsons & Parsons, 2004). At the rear, RhoA-induced actomyosin contractility coincides with turnover of integrin mediated focal adhesions to the ECM, releasing anchorage of the cell rear and leading to forward sliding of the cell body (Huveneers & Danen, 2009; Sadok & Marshall, 2014). Thus, alternating Rac-induced cell elongation at the leading edge and Rho-mediated rear retraction enables cycles of cell-matrix adhesion, pulling and relaxation on tissue structures (Starke et al., 2013; van Helvert & Friedl, 2016). Adjacent to focal interactions to ECM, surface matrix metalloproteases (MMPs) remove ECM structures and barriers, such as collagen fibers, which leads to tissue remodeling and the generation of
spatially widened tissue tracks along the migration path (Fig. 2d) (Friedl et al., 1997; Wolf et al., 2007).

In 2D and 3D migration assays, integrin adhesion receptors equip cancer cells with a repertoire of substrate-recognition and attachment abilities, thus providing flexibility in substrate choice. As examples, α2β1, α1β1, and α11β1 integrins mediate binding to collagen and αVβ3, αVβ5 and α5β1 binding fibronectin during migration (Hayri E Balcioglu, van Hoorn, Donato, Schmidt, & Danen, 2015; Hynes, 2002). This ability to recognize different substrates is likely critical for cancer cell dissemination and metastasis, as series of different ECM interaction systems engage with different tissue components. Examples include cell-binding to interstitial collagens when invading interstitial tissue, laminins when moving along basement membranes, or fibronectin when entering inflamed microenvironments, with concomitant co-engagement of cell-ECM adhesion systems in complex environments (Gritsenko, Ilina, & Friedl, 2012; Kaplan et al., 2005; Lu, Weaver, & Werb, 2012; Weigelin, Bakker, & Friedl, 2012).

Mesenchymal migration as a molecularly and mechanistically complex mechanotransduction program thus supports both cell migration and tissue remodeling in a unified process. As a consequence of coordinated adhesion and proteolysis systems, mesenchymal movement allows tumor cells to even penetrate tissues of very high density (Wolf et al., 2013) as well as the passage of vascular basement membranes (Vitale, Avizienyte, Brunton, & Frame, 2008; Wang & McNiven, 2012).

2.3 Collective cell migration. Collective tumor cell migration is a key invasion strategy in most epithelial tumors such as breast cancer, squamous cell carcinoma, colon cancer and others, as well as in mesenchymal tumors (Friedl and Gilmour 2009; Friedl et al. 1995; Cheung et al. 2013). Histologically, collective invasion is characterized by direct, next-neighbor position of tumor cells which have reached interstitial stroma outside the tumor core but retain cell-cell junction markers, including cadherin receptors and membrane-localized β-catenin (Cheung et
al., 2013; Friedl, Locker, Sahai, & Segall, 2012). Collective migration is best identified by careful 3D reconstruction of histological samples (Bronsert et al., 2014) but also can be inferred from the presence of roundish cell nests and elongated strands in conventional histology (Friedl et al., 2012).

During collective migration in experimental 2D and 3D assays, cell-cell contacts coordinate and polarize migrating tumor cells into a multi-cellular functional unit (“super cell”) (Friedl et al., 2012; Khalil & Friedl, 2010). Distinct levels of actin dynamics, integrin-mediated substrate adhesion, and proteolytic ECM remodeling characterize leader and follower cell behaviors (Fig. 1b). At the invasion front, particularly polarized and migratory leader cells engage with surrounding tissue structures via Rac-driven filopodal protrusions and integrin-mediated substrate adhesion, while remaining connected to follower inner and outer strand cells (Yamaguchi, Mizutani, Kawabata, & Haga, 2015). Cell-cell connections depend on sufficiently stable cell-cell adhesion to follower cells to withstand dragging forces generated by leader cells and Rho-mediated actin contraction across multiple cell bodies (Bazellières et al., 2015; Reffay et al., 2014). Guided by cell-cell contacts, inner and outer strand cells follow along the same path of the leading front (Cheung & Ewald, 2014). Both, leader and follower cells engage integrins and/or other adhesion systems to generate force, and exert proteolytic matrix remodeling, particularly through MMPs, to digest tissue barriers and generate a path of least resistance along which the cells move forward (Hegerfeldt et al., 2002; Mayor & Etienne-Manneville, 2016; Wolf et al., 2007). Cell-cell contacts are typically formed by cadherins (e.g. E-, N-, P-cadherin) and cortical actin strings, which together form and mediate the stability of adherens junctions (Haeger, Wolf, Zegers, & Friedl, 2015; Meng & Takeichi, 2009). Other cell-cell adhesion systems include immunoglobulin superfamily members and ephrins/EpH receptor adhesions that mediate more labile or transient cell-cell interactions (Kania & Klein, 2016; Wai Wong, Dye, & Coombe, 2012), as well as connexins, which enable communication through gap
junctions and signal transduction between connected tumor cells (Li, Zhou, & Donahue, 2008).

Collective invasion is adaptive, in a cell-type and tissue-responsive manner (Fig. 1b). Tumor cells either locally invade as (1) collective sheets or strands while remaining attached to the tumor lesion, typically detected in epithelial tumors, such as breast or squamous cell carcinoma (Bronsert et al., 2014); (2) isolated clusters detached from the primary/metastatic lesion such as epithelial tumors and melanoma (Friedl et al., 1995); (3) neuronal-like networks of connected cells, detected in neuroectodermal tumors, such as glioblastoma (Osswald et al., 2015); or (4) as “jammed” collective cohorts induced by spatially narrow tissue boundaries (“confinement”) of otherwise transiently/loosely connected (single) cells, e.g. in melanoma and sarcoma cells (Haeger, Krause, Wolf, & Friedl, 2014).

Compared to single cell migration, collectivity of migrating cancer cells likely bears advantages for surviving the metastatic cascade, including: (i) a highly pro-migratory and pro-survival environment by secretion of growth factors, chemokines and proteases; (ii) the displacement of poorly mobile or even immobile but potentially highly proliferative cells inside the strands by highly mobile neighbor or leader cells; and (iii) protection of cells located in inner regions of groups from environmental assault, such as immune cell attack or shear stress in the tissue or vasculature (Denais et al., 2016; Peter Friedl & Gilmour, 2009).

2.4 Diversity of metastatic evasion – individual and clustered circulating tumor cells.

Clinically, indications for individual as well as collective metastasis can be detected at all stages of disease progression, evidenced by histologically diverse patterns of local tissue invasion and the circulation of both individual and clustered tumor cells (Aceto et al., 2014; Liotta, Saidel, & Kleinerman, 1976; Massagué & Obenauf, 2016). In primary lesions of epithelial tumors, collective invasion is very prominent (Peter Friedl et al., 2012), but only a minority of clustered and a majority of individual CTCs are detected in peripheral blood of epithelial cancer patients (Kraan et al., 2011). Thus, the most prevalent migration mode in tissue may differ from the most
likely strategy for entering and surviving the circulation.

Given their distinct mechanisms for propagating through tissue, individual or collective tumor cell migration likely generate distinct routes of entry into the circulation and strategies for metastatic organ colonization (Aceto et al., 2014; Cheung et al., 2016), and several facts argue in favor for clinical relevance of each dissemination program. Clustered CTCs are many-fold more likely to initiate organ colonization compared to individually circulating cells (Aceto et al., 2014; Cheung & Ewald, 2016; Liotta et al., 1976). Alternatively, in epithelial tumors single CTCs may express EMT and stemness markers (Armstrong et al., 2011; Satelli et al., 2015), suggesting a mesenchymal route for hematogenous spread and survival. In hematological neoplasias, including lymphoma and leukemia, amoeboid single-cell migration coincides with anchorage-independent survival and proliferation and an ability for particularly efficient systemic dissemination and recirculation (Trendowski, 2014), not unlike recirculating leukocytes (Hutchinson et al., 2014). Thus, the likelihood for individual versus collective metastasis may originate from distinct mechanisms, types and phases of disease. The migration mode(s) most suited to enter and support survival in the circulation, and particular niches in the tumor stroma, vascular beds and distant organs most favorable for each migration mode and resulting metastatic efficacy remain to be identified.
Figure 1. Single-cell and collective migration modes. (a) Transition from non-migrating to single-cell migration states and their relation to cell-matrix interaction strength. (b) Different
modes of collective cell migration, determined by the morphology and strength of cell-cell interactions. Figure content is published (te Boekhorst, Preziosi, & Friedl, 2016).
3. Determinants controlling cancer cell migration modes

How efficiently and by which mode cancer cells migrate is determined by several cellular and tissue-intrinsic properties which adapt and respond to local and global mechanical and molecular signals. In cooperation, multiple properties not only define whether, how fast and to which direction a cancer cell migrates, but also which migration mode is maintained in a particular tissue context and for which purpose.

3.1 Cellular determinants

**Cytoskeletal organization.** The cytoskeleton including actin filaments, microtubules and intermediate filaments jointly define and adjust cell shape and contractility during cell migration (Fig. 2a). Cytoskeletal dynamics include actin assembly into linear or branched filaments to generate membrane protrusions and adhesion dynamics as well as actomyosin contractility to create cell tension and pull on extracellular cues (Gardel et al., 2008; Pollard & Borisy, 2003; Roca-Cusachs et al., 2013). By adjusting the organization and kinetics of actin filaments, a rich armatorium of cytoskeletal substructures is formed. At light-microscopical resolution, three organization types of actin filaments support cell migration, including cortical distribution without foci, cortical distribution with short-lived and focalized adhesion sites, and strongly focalized adhesions with inserting stress fibers (Fig. 2A).

Cortical, non-focalized actin is a dynamic sheet-like structure, composed of branched actin filaments in parallel to the plasma membrane which forms and dissolves locally (Fig. 2a1). Cortical actin networks together with non-polar, low-adhesive interactions with surrounding substrate are typically present in stationary cells (Lin, Risbood, Jain, Vacanti, & Lee, 2004). Cortical, non-focalized actin networks further align along cell-cell junctions and provide largely stable cell integration between neighbors (Wu et al., 2014). When cortical actin is locally
ruptured, as consequence of the GTPase RhoA which induces myosin-mediated contraction of actin filaments or increases intracellular hydrostatic pressure, a local bleb-like membrane protrusions forms and, within seconds to minutes, is stabilized by new cortical actin filaments (E. K. Paluch & Raz, 2013). Blebs typically form towards the leading edge, engage with surrounding tissue structures, move laterally and rearward, and resolve within minutes by cortical actomyosin mediated retraction, which drives single cells slowly forward (Goudarzi, Banisch, Mobin, Maghelli, Tarbashevich, Strate, van?den?Berg, et al., 2012; E. K. Paluch & Raz, 2013). Bleb-like protrusions mediate amoeboid migration in Dictyostelium discoideum, leukocytes and some cancer cells moving in 3D environments (Bergert et al., 2015; P Friedl, Entschladen, Conrad, Niggemann, & Zänker, 1998b; Liu et al., 2015a) or germ cells migrating in the zebrafish embryo (Goudarzi, Banisch, Mobin, Maghelli, Tarbashevich, Strate, van den Berg, et al., 2012). Migration driven by cortical actin flow and blebs arguably represents the least complex cytoskeletal mechanics involved in cell migration.

When cortical actin flow is combined with local enrichment of actin-rich substructures, cell protrusions provide diversification of cell polarization and shape change (Fig. 2a2). Actin-rich focal protrusions consist of actin filaments organized in parallel by cross-linking proteins, including fascin, filamin, the spectrin superfamily (e.g. α-actinin, spectrin, dystrophin) and I-BAR family of proteins (Bin/Amphipysin/Rvs) (Blanchoin, Boujemaa-Paterski, Sykes, & Plastino, 2014; M. Parsons & Adams, 2008). Specialized membrane protrusions, mediated by membrane-microfilament binding proteins (ERM proteins, ankyrin, dystrophin, spectrin), include lamellipodia, filopodia, podosomes and invadopodia during migration across 2D surfaces, and, additionally, lobopodia during migration trough 3D matrices/tissues, often associated with ECM remodeling capabilities (Blanchoin et al., 2014; Petrie & Yamada, 2012; Wolf & Friedl, 2009). Alternatively, actin-rich small adhesions are formed by transient clusters of adhesion receptors and adaptor proteins connecting with cortical actin filaments (Swaminathan, Fischer, & Waterman, 2016).
Actin focalization without protrusion typically occurs at local adhesion sites, either as short-lived focal contacts with transient aggregation of adhesion receptors, altered membrane composition, or mature focal adhesions, with or without inserting stress fibers to adhere and pull on the counterpart substrate (H. E. Balcioglu, van Hoorn, Donato, Schmidt, & Danen, 2015; Case et al., 2015; Steinwachs et al., 2016). Forming and resolving these actin-rich substructures provide mechanically and molecularly distinct types of local polarization, adhesion and mechanocoupling towards environmental structures (Plotnikov et al., 2012; Renkawitz et al., 2009).

When cell-matrix interactions are strong, actin-rich cell-matrix adhesions and stress fibers consisting of thick actin bundles and myosin-II transmit high force and contractility towards the substrate (Fig. 2a3) (H. E. Balcioglu et al., 2015; Case et al., 2015; Chrzanowska-Wodnicka & Burridge, 1996). Filopodia and lobopodia transmit moderate force and bleb-like interactions are largely non-adhesive and generate little traction force but, due to their stiffness, provide non-adhesive intercalation and friction to surrounding tissue (E. K. Paluch & Raz, 2013; Petrie & Yamada, 2012). Because of their defined shape and function, these principal actin organizations together with cell shape are useful classifiers inferring the mode and mechanics of individually migrating cells (Peter Friedl & Wolf, 2010; Petrie & Yamada, 2012; Starke, Wehrle-Haller, & Friedl, 2014).

**Cell-ECM adhesion.** The actin cytoskeleton is coregulated with the distribution and function of transmembrane adhesion receptors. Adhesion regulation in moving cells includes non-, poorly or strongly focalized adhesions, which show different molecular composition, mechanocoupling and speed of adhesion turnover (Fig. 2b1-3) (Bergert et al., 2015; Chrzanowska-Wodnicka & Burridge, 1996; Gad et al., 2012; Liu et al., 2015; Renkawitz et al., 2009). With increasing focalization, cell-matrix adhesions become assembled at the leading edge and disassembled towards the rear to mediate adhesion-based forward movement.
Such adhesions are predominantly provided by integrins (Geiger, Spatz, & Bershadsky, 2009; Maaser et al., 1999; Schmidt & Friedl, 2010), with modulation by co-signaling molecules with ECM-binding ability, including cell surface proteoglycans (Couchman, 2010; Geiger et al., 2009), CD44 (Kim & Kumar, 2014), and discoidin domain receptors (DDRs) (Shintani et al., 2008; H. Xu et al., 2012). Focal adhesion turnover at the cell rear is mediated by calpain-2 induced proteolysis of focal adhesion adapter proteins, including talin, paxillin and FAK (Neil O Carragher & Frame, 2002; Franco et al., 2004; Franco & Huttenlocher, 2005). Based on the type and amount of available adhesion receptors, cells choose and prioritize substrate for migration, with intermediate interaction strength providing highest migration rates (Fig. 2b4).

Integrins are cell surface receptors composed of α and β integrin chains with differential substrate binding strength. α2β1 and α3β1 integrins preferentially engage with fibrillar type I and III collagens; αVβ3, αVβ1, α5β1 prioritize fibronectin; and α3β1 and α6β1 connect to laminins (Iain D Campbell & Humphries, 2011). Through integrins, moving cells sense and regulate adhesion and cytoskeletal contractility through adaptor proteins and cytosolic kinases, which regulate adhesion sites and their connection to the cytoskeleton, with varying size, molecular complexity, mechanotransduction capability and lifetime (Fig. 2b1-3) (Plotnikov et al., 2012). Non- or weakly adherent cell-matrix interactions provide a physical interface between cell body and substrate which supports mechanical friction and cell intercalation between tissue layers; however, the precise interaction mechanism and molecular types of low-force interactions remains unclear (Fig. 2b1) (Bergert et al., 2015; Renkawitz et al., 2009). Nascent adhesions or focal complexes at the leading edge generate small transient forces mediating initial substrate grab of forward moving of cells (Fig. 2b2) (Changede, Xu, Margadant, & Sheetz, 2015; Swaminathan et al., 2016). First, talin is engaged in nascent adhesions, followed by recruitment of additional cytoskeletal adaptors (kindlin, paxillin), and mechanosensing modulators (vinculin, p130Cas), by which the adhesion grows in size and stability (Bachir et al.,
2014; Changede et al., 2015). When myosin-IIA additionally engages, nascent adhesions maturate into focal adhesions and support cell contractility (Kubow et al., 2013). At the high end of size and strength, contractile stress fibers connect with mature focal adhesions, provide stable anchorage to the surrounding substratum, transmit traction force, and maintain integrin activation and focal adhesion signaling (Fig. 2B3) (Beningo, Dembo, Kaverina, Small, & Wang, 2001; Geiger et al., 2009). High force reinforces the downstream intracellular signaling through focal adhesion kinase (FAK), Src and the small GTPases Rac and Rho and increases size, shape and lifetime of focal and adhesion strengths (Geiger et al., 2009; Grashoff et al., 2010). Consequently, high cell contractility is required for movement with low-adhesion, to control cortical actin and hydrostatic pressure, as well as high adhesion by coupling actin filaments to focal adhesions and generating the force for rear retraction (Fig. 2b4).

Besides integrin-mediated mechanotransduction, weaker and less well-defined adhesion mechanisms are provided by cell surface proteoglycans, including syndecans, glypicans and neuropilin, which interact with ECM substrates through sugar moieties (Mythreye & Blobe, 2009; Schmidt & Friedl, 2010). When co-engaged in parallel, adhesion systems and growth factor receptor signaling cooperate by converging signaling through PKC and Src, and thereby support integrin-mediated mechanocoupling (Couchman, 2010; Moon et al., 2005); however, their role in cell migration remains unclear.

**Cell contractility.** Distinct migration types underlie different molecular control of cytoskeletal dynamics. Small Rho GTPases, including Rac1 and RhoA, are central in the regulation of actin cytoskeletal dynamics during cell migration, and their relative expression level and spatial distribution at the rear or leading edge of the cell determine the migration mode by which cancer cells invade (Fig. 2c) (Hinde et al., 2013). Mesenchymal migration is driven by high Rac1 levels at the leading edge, which promotes actin polymerization and leading edge protrusion, as well as high RhoA levels at the rear of the cell, which induces actomyosin
contraction resulting in detachment and forward movement of the cell (Friedl and Wolf, 2010; Murali and Rajalingam, 2014). Rho-mediated myosin motors further contribute to focal adhesion maturation and stabilize focalized adhesive interactions to the ECM. In contrast to mesenchymal migration, amoeboid movement is predominantly mediated by RhoA/ROCK regulated actomyosin contractility, while Rac levels are low (Sahai and Marshall, 2003). In this signaling cascade, GTP-bound (active) RhoA activates its effector kinase ROCK, which either directly phosphorylates myosin light chain (MLC) or inhibits the MLC phosphatase, resulting in actomyosin contraction (Murali and Rajalingam, 2014). Elevated RhoA and myosin activity at the cell rear and front elevate intracellular hydrostatic pressure, which generates propulsive motion and induces blebby membrane protrusions, defining their three life phases: initiation, expansion, and retraction (Guillaume T. Charras, Coughlin, Mitchison, & Mahadevan, 2008).

**Cell-cell adhesions.** Cell-cell contacts determine whether cells migrate individually or as cohesive group, by regulating cell-cell adhesion and multicellular coordination (Peter Friedl et al., 2012). Cell-cell interactions are supported by overlapping adhesion systems of different strength and functional context, including cadherins, members of the immunoglobulin superfamily of adhesion molecules (IgCAM) and connexins (Ilina & Friedl, 2009). In addition, adhesion strength is modulated by ephrins and the erythropoietin-producing hepatocellular (Eph) receptors, which may cause both, adhesion and repulsion between cells (Batlle & Wilkinson, 2012).

Cadherins mediate homo- or heterophilic intercellular adhesions, including relatively strong adherens junctions formed between classical cadherins (E-, N- or P-cadherin, cadherin 7, cadherin-11 and cadherin-13), less strong desmosomal cell-cell junctions between atypical desmosomal cadherins and regulation of classical cadherin- mediated adherens junctions by atypical proto-cadherins, Fat and Dachsous cadherins and Flamingo/Celsr (Pokutta & Weis, 2007)(Meng & Takeichi, 2009) (Harris & Tepass, 2010). Classical cadherins provide cell-cell
contact stability by connecting to the actin and microtubule cytoskeleton via adaptor and signaling molecules, including α-catenin, β-catenin, p120-catenin, and co-engagement of small GTPases RhoA and Rac1 and downstream Src activation mediating cytoskeletal dynamics (Harris & Tepass, 2010). Thus, cadherin function defines the quality and stability of junctions between moving cells (Peter Friedl et al., 2012).

In addition to cadherins, IgCams, including L1CAM, EpCAM, NCAMs and ALCAM, support transient cell-cell binding between moving cells and towards cells encountered in tissues (Wai Wong et al., 2012). IgCams mediate hemophilic and heterophilic interactions between cells by connecting to the actin cytoskeleton via adaptor proteins (e.g. α-actinin, ankyrin, ezrin). They cooperate with integrins through Src, ERK or ILK, and thereby increase migration capability through cell-cell adhesion as well as cell-substrate interaction, with particular relevance when classical cadherin function is low. In cooperation with cadherins, Eph receptors and their respective Ephrin ligands provide bidirectional signaling between cells, which modulates actomyosin contractility via Src and FAK signaling and locally affects protrusions and cell-cell junctions by pro- or anti-adhesive signaling (Halloran & Wolman, 2006; Kania & Klein, 2016; Rohani, Parmeggiani, Winklbauer, & Fagotto, 2014).

Depending on tissue context, at least three types of intercellular junctions are formed, which vary in kinetics and life-times (Fig. 1b). Weak cell-cell adhesions are mediated by IgCAMs, which transiently allow cell attachment and trigger intracellular signaling (Haeger et al., 2014; Wai Wong et al., 2012). These junctions support neuronal and leukocyte cell-cell interactions, and individually moving cells under confluence (Cayrol et al., 2008; Haeger et al., 2014). Transient junctions consist of cadherins and Ephrin/Eph receptors which together balance pro-adhesive intercellular forces with repulsion signals, respectively (Halloran & Wolman, 2006). Transient junctions bind moving neural crest cells, which migrate in network formations mediated by intercellular adhesion simultaneously with local contact inhibition of locomotion and retraction ("kiss and run"). This contact mode contributes to moderately
adherent collective migration and multicellular streaming, where cells can oscillate between 
individual and collective behaviors (Halloran & Wolman, 2006; Scarpa & Mayor, 2016).

Stringent cadherin-based adherens junctions are mediated by classical cadherins, via 
connection to contractile cortical actin filaments, often in cooperation with desmosomal and 
tight junctions and in absence of repulsion signals (Peglion, Llense, & Etienne-Manneville, 
2014; Wu et al., 2014). Stable cell-cell adhesions connect moving epithelial and endothelial 
cells during cohesive collective migration, which depend upon stringent junctions that mediate 
strong mechanical adhesion between cells (Bazellières et al., 2015; Peter Friedl et al., 2012).

Thus, depending upon adhesion molecules engaged, very weak to strong cell-cell 
interactions can be maintained by moving cells, resulting in a range of interaction kinetics.
Figure 2. Cellular modules determining migration modes. (a) Organization of the actin cytoskeleton, including diffuse (1), cortical-focal (2) and focal with stress fibers (3). (b) Adhesion regulation. (1) Low adhesion exerted by integrins diffusely distributed in non-clustered adhesion domains. (2) Intermediate adhesion resulting from clustered integrins and locally focalized cortical actin cytoskeleton (focal contacts). (3) Strongly adherent mature focal adhesions with...
focalized actin filaments and insertion of contractile stress fibers containing myosin-II. (4) Migration speed as function of adhesion strength in different 2D and 3D environments. Numbers denote low (1), intermediate (2) and strong (3) adhesion types. (c) Conversion of protrusion type depending on the balance and location of active Rac, Cdc42 and Rho and related strength of cell-substrate adhesion. (d) Pericellular proteolysis, through cell-contact dependent targeting of surface proteases (1) or secreted proteases (2) resulting in confined ECM reorganization (1) or diffuse lysis (2). Figure content is published, adapted from (te Boekhorst et al., 2016).

3.2 Microenvironmental determinants

Migrating tumor cells integrate structural and chemical signals from the tumor microenvironment and adjust their migration strategy accordingly. Virtually any mechanical and/or signaling molecule cue encountered in tissues can modulate how cancer cells migrate.

**ECM organization.** Interstitial tissues and organ sites represent complex microenvironments and interfaces, with a high variety of structural, mechanical and molecular properties that guide, rather than impede, cancer cell migration before or during tumor-induced microenvironmental perturbation (Gritsenko, Ilina, & Friedl, 2012). Tissue-intrinsic structural and mechanical properties include geometry, spacing, degree of ECM fiber orientation, and ECM stiffness (te Boekhorst, Preziosi, Friedl, 2016). Tissues comprise 2D surfaces (e.g. basement membranes), random or aligned 3D fibrillar collagen in respectively loose or dense interstitial tissue, and 3D space bordered by 2D surfaces of varying width, textures and composition, such as tissue tracks along muscle fibers, nerves and blood vessels (Alexander, Weigelin, Winkler, & Friedl, 2013; Friedl & Wolf, 2010; Joyce & Pollard, 2009). These molecular and mechanical cues generate different degrees of barriers and guidance and in cooperation
impact on migration programs, by either reinforcing an ongoing migration strategy or inducing modulation followed by a plasticity response (Table 1) (Kumar, Kapoor, Desai, Inamdar, & Sen, 2016; Liu et al., 2015; Wolf et al., 2013; Xu, Boudreau, & Bissell, 2009). For example, space confinement together with low ECM adhesion supports amoeboid migration of cells that on unconfined 2D substrate migrate by adhesive, mesenchymal mechanisms (Liu et al., 2015). Alternatively, aligned matrix fibers, which provide a track of low resistance, support cancer cell migration independent of proteolytic ability, whereas migration in dense tissue with very small pores is protease-dependent (Kumar et al., 2016; Wolf et al., 2013).

In progressing tumors, the organization of the peritumoral stroma underlies significant structural and molecular remodeling induced by both cancer cells and tissue-resident cells, which can influence invasion programs. Cancer-associated fibroblasts (CAFs) with generally high proteolytic ability, can generate tissue tracks which support collective migration of tumor cells (Gaggioli et al., 2007). Moreover, by deposition, stretching and crosslinking of structural matrix proteins (e.g. FN, collagen type I and III), CAFs can promote a stiffness-related invasion response in many cancer types (Lu, Weaver, & Werb, 2012; Paszek et al., 2005). Thus, by combined mechanical, spatial and associated signaling parameters, the tumor stroma coevolves an inductive scaffold and reciprocally controls the type, extent and plasticity response of cancer invasion (Egeblad, Rasch, & Weaver, 2010; Provenzano et al., 2006).

**Growth factors and cytokines.** Besides ECM guidance cues, the activated tumor stroma expresses a variable repertoire of cytokines and chemokines which, by diffusion or as ECM-tethered immobilized ligands, impact cancer cell invasion and dissemination programs (Lu, Weaver, & Werb, 2012). Cytokines, including HGF, VEGF, IGF, TGFβ, and FGF as well as chemokines, including CXCL10, CXCL12, CCL21, or CCL25, among many other factors, induce promigratory chemotactic signaling as well as EMT and other plasticity programs in tumor cells (Friedl & Alexander, 2011; Goel & Mercurio, 2013; Hollier, Kricker, Van Lonkhuyzen,
Leavesley, & Upton, 2008; Kermorgant, Aparicio, Dessirier, Lewin, & Lehy, 2001; Roussos, Condeelis, & Patsialou, 2011; Wells, Chao, Grahovac, Wu, & Lauffenburger, 2011). As example, cancer-associated fibroblasts release TGFβ and PDGF which induce EMT and promote invasion of cancer cells (Matise et al., 2012). Cancer-activated adipocytes secrete cytokines (e.g. TNF-α, leptin, and IL-6) which induce EMT (Wolfson, Eades, & Zhou, 2015), upregulate the release of proteases (e.g. MMP-2, MMP-2, MMP-11) and thereby promote both migration plasticity and invasion in breast cancer cells (Dirat et al., 2011; Fujisaki et al., 2015; Lee, Jung, & Koo, 2015). Extracellular growth factors and chemokines induce signaling in both migrating cancer cells and stromal cells, including MAPK, cMET, JAK/PI3K/JNK, mTOR, Src family kinase and Rho/Rac signaling, which lead to the multi-parameter reprogramming of signaling networks and, accordingly, cell functions (Donà et al., 2013; El Haibi et al., 2010; Friedl & Alexander, 2011; Heit, Tavener, Raharjo, & Kubes, 2002). IGFR signaling, for example, induces EMT in breast cancer cells via the induction of TGFβ (Walsh & Damjanovski, 2011), and induces protease expression via PI3K signaling which supports a pro-invasive behavior in lung carcinoma cells (Zhang & Brodt, 2003). Macrophage-induced paracrine loops between SDF-1 and EGF signaling guides the invasion of breast cancer cells to nearby blood vessels (Goswami et al., 2005; Wyckoff et al., 2004) or promotes breast cancer invasion via alternate signaling routes, e.g. CXCR2/FGF and Wnt signaling (Bohrer & Schwertfeger, 2012; Pukrop et al., 2006).

Growth factor and chemokine repertoires as well as their receptors are not stable, but transient and adaptive parameters. ECM-bound chemokines and growth factors and their receptors can be proteolytically cleaved and either liberated and activated, or degraded, and further regulation occurs by internalization from the membrane and recycling (Kessenbrock, Plaks, & Werb, 2010; Murphy, 2008). In addition, varying degrees of tumor-associated inflammation modulate pro-inflammatory cytokine pools (Landskron, De la Fuente, Thuwajit, Thuwajit, & Hermoso, 2014; López-Novoa & Nieto, 2009).
Metabolic control and tumor hypoxia as key microenvironmental condition.

Metabolic perturbation of the tumor and its microenvironment strongly impact the migratory behaviors and adaptation of cancer cells (Clark & Vignjevic, 2015; López-Novoa & Nieto, 2009; Lu & Kang, 2010). As key microenvironmental condition during solid tumor growth, low oxygen tension (hypoxia) induces the cellular reprogramming of tumor cells to adapt to hypoxic stress, altering cell metabolism, proliferation, survival and invasion behavior (Benita et al., 2009; Majmundar, Wong, & Simon, 2010).

Cells, including cancer cells, sense oxygen levels by the regulation of HIF1α degradation and activation (Hirota & Semenza, 2005; Panu Jaakkola et al., 2001; Masoud & Li, 2015a). Under normoxia, HIF-1α is hydroxylated by oxygen sensitive prolyl hydroxylases (PHDs) and ubiquitinated by von-Hippel Lindau-containing E3 ubiquitination-protein ligases, leading to rapid proteasomal degradation (Hirota & Semenza, 2005; Panu Jaakkola et al., 2001). Conversely, when oxygen levels are low, PHD hydroxylation activity is reduced and HIF-1α is stabilized, heterodimerizes with the constitutively expressed HIF-1β, translocates to the nucleus and upon binding to coactivators, i.e. p300/CPB, and induces the transcription of the numerous hypoxia-responsive elements (HRE)-bearing genes (Benita et al., 2009; Panu Jaakkola et al., 2001; G. L. Wang & Semenza, 1995).

Hypoxia/HIF induced metabolic reprogramming induces cancer cells to switch from mitochondrial oxidative phosphorylation towards glycolysis for energy (ATP) generation (Warburg effect) (Hammoudi, Ahmed, Garcia-Prieto, & Huang, 2011) (Rankin & Giaccia, 2016). Next to, or inherent with metabolic adaptations, hypoxia activates migration programs in cancer cells, often via EMT signaling (Webb, Chimenti, Jacobson, & Barber, 2011). Upon hypoxia induced induction of EMT characteristic transcription factors, such as Twist, Snail, Zeb, E-cadherin and vimentin (Liao, Corle, Seagroves, & Johnson, 2007; Nieto, 2011; M.-H. Yang et al., 2008a), EMT signaling leads to the downregulation of adherens junctions, upregulation of
migration-enhancing vimentin, and activation of Rac GTPase (Gilkes, Semenza, & Wirtz, 2014; McInroy & Määttä, 2007; Vuoriluoto et al., 2011). As example, through EMT, stationary epithelial cells lose their polarity and cell-cell adhesion due to decreased E-cadherin expression, convert to a more aggressive mesenchymal phenotype through vimentin upregulation, and eventually become migratory (Krishnamachary et al., 2006; Nieto, 2011).

Hypoxia-induced reprogramming of cancer cell invasiveness likely also promotes the plasticity of tumor cell migration, including collective to single-cell transition (Zhang et al., 2013). Thus, hypoxia is an established inducer of cancer cell invasion and metastasis (Krishnamachary et al., 2006; McKeown, 2014; Taddei et al., 2013; Yang et al., 2008). However, how hypoxia and HIF signaling impact on cancer cell migration beyond or besides EMT, including kinetic, phenotypic and molecular reprogramming towards plasticity of migration programs, remains unclear.

Together, the combined action of tissue organization, composition and molecular cross-talk between growth factor and cytokine signaling, protease activity and metabolic reprogramming progress over weeks and months and impose longitudinal molecular adaptation in cancer cells, which directly and indirectly diversifies strategies of metastatic dissemination over time. However, which migration programs are microenvironmentally regulated and how they impact on different steps of the metastatic cascade remains to be determined.

4. Plasticity of cancer cell migration programs

Cancer cell invasion is a heterogeneous and adaptive process, modulated by reciprocal reprogramming of both cancer cells and the microenvironment. The adaptation and interconversion of ongoing migration strategies is regulated by the induction of different signaling programs, here summarized as plasticity of cancer cell dissemination (Fig. 3).
Such adaptive responses reflect/equip tumor cells with a versatility and inventory to integrate and cope with extracellular regulations. By which strategy and how efficiently cancer cells move, and whether or not tumor cells transition between migration modes depends on molecular and physical input moving cancer cells receive from their environment.

4.1. Plasticity of single cancer cell migration. Known transitions between single cell migration programs include the interconversion between mesenchymal and amoeboid migration and difficult-to-classify, intermediate states between amoeboid migration types. Molecular interference has revealed that the transition from mesenchymal to amoeboid migration (MAT) (Fig. 3a) is favored by a range of experimental conditions, including (1) reduced cell-ECM adhesion, (2) loss of ECM proteolysis, (3) enhanced RhoA- and myosin II-mediated actomyosin contractility, (4) microtubule de-stabilization, and, consequently, resulting in increased ability of cell-shape adaptation (Belletti et al., 2008, 2010; Berton et al., 2009; Sahai & Marshall, 2003; Sanz-Moreno et al., 2008; Taddei et al., 2011; Wolf et al., 2003). Particularly loss of cell-ECM adhesion, increased actomyosin contractility and diminished ECM degradation support the morphological adaptation from an elongated to a roundish-ellipsoid cell body and transition of migration mode. By gaining amoeboid features, the increase in deformability and shape change, despite reduced proteolytic ECM clearance, enables efficient passage and circumvention of tissue barriers (Wolf et al., 2003).

In a reverse process, amoeboid-migrating cells may convert to mesenchymal migration (amoeboid-to-mesenchymal transition, AMT) when (1) ECM adhesion, (2) proteolytic ECM remodeling, and (3) Rac-induced actin assembly and protrusion formation are increased (compare Fig. 3a) (Panková, Rösel, Novotný, & Brábek, 2010; Sanz-Moreno et al., 2008). As a consequence of these molecular changes, AMT can be identified by a morphological transition from round, spherical to elongated, spindle-cell shape.

Amoeboid interconversion (AI) includes a transition between amoeboid-blebby and
amoeboid-filopodial states. Both states may be transient, based on oscillatory engagement of Rho and Rac at the leading edge, and result in difficult-to-categorize phenotypes. When Rac is active, filopodal membrane protrusions predominate, while dominant RhoA activity supports protrusive blebs at the leading edge (Fackler & Grosse, 2008; Petrie & Yamada, 2012). These different protrusions likely generate a range of distinct interaction types to tissue components; the differential mechanics and consequences remain to be clarified.

Interconversions of migration programs likely provide flexibility for moving cells when confronted with different types of tissues, vascular walls and basement membranes. MAT, AMT and AI may further be associated with the regulation of stemness and altered ability of metastasis formation (Taddei et al., 2014), yet their relative contributions to any step of the metastatic cascade remain to be clarified.

4.2 Collective to single cell transition. The transition from collective to single-cell migration occurs frequently during the progression of epithelial tumors when (1) cell-cell adhesion systems are downregulated or (2) when tissue confinement is relieved during transit from dense to lose tissue topology.

The epithelial to mesenchymal transition (Fig. 3b, EMT) is the result of a signaling program that downregulates epithelial features, particularly cell-cell junctions and apicobasal anchorage to basement membranes, and upregulates stromal-type adhesion systems and cytoskeletal dynamics (Polyak & Weinberg, 2009; J. Xu, Lamouille, & Derynck, 2009). EMT results in the progression of many, if not all, epithelial cancers, including colon, lung, prostate and breast cancer (Savagner, 2010), and strongly enhances cancer invasion, metastatic progression, morphological heterogeneity, and correlates with poor prognosis (Polyak & Weinberg, 2009). With EMT, cells weaken or fully resolve cell-cell junctions, including adherens junctions, desmosomes and tight junctions, upregulate stromal protease as well as integrin adhesion systems (from β1 to β3 switching, αv signaling), and shift Rho-mediated actomyosin
contractility from cell-cell junctions towards cell-matrix interactions (Mamuya & Duncan, 2012; Parvani, Galliher-Beckley, Schiemann, & Schiemann, 2013). These molecular reprogramming events result in deregulated cell-cell contacts, loss of apicobasal but gain of front-rear polarity, and ultimately favor the transition from epithelial or collective to mesenchymal features (Bousquet et al., 2016; Grünert, Jechlinger, & Beug, 2003; Moustakas & Heldin, 2007). Jointly, altered integrin-mediated focal adhesion dynamics, cytoskeletal reorganization and enhanced ECM deposition facilitate cell dispersal and invasive migration into tissues (Xu et al., 2009). In addition to cell individualization, recent observational and modeling work indicates a high likelihood for mixed behaviors after EMT, including intermediate (e.g. metastable or hybrid) phenotypes, such as detached collective or loosely connected migrating groups (Jolly et al., 2015; Savagner, 2010). With such EMT-associated reprogramming, or partial EMT, emerging moving cell clusters may still maintain cell-cell contacts but already alter their differentiation state and gain pluripotent potency (Jolly et al., 2015; Savagner, 2010). Thus, EMT and EMT-like processes reflect molecular plasticity with direct and indirect, reversible or irreversible impact on cancer cell migration modes. The relevance of EMT-like processes in evoking intermediate phenotypes in cancer progression, e.g. circulating cluster formation and survival, remains to be determined (Jolly et al., 2015).

Besides EMT, the downregulation of cell-cell adhesions alone can cause collective-to-single cell transition. As examples, experimental interference with β1 integrin, which lowers both cell-matrix and cell-cell adhesion, leads to disintegration of melanoma clusters and transition to amoeboid single cell migration (Hegerfeldt et al., 2002). Similarly, enhancing EGF signaling promotes cell evasion from the solid tumor followed by amoeboid dissemination in vivo (Wyckoff et al., 2004). In addition, growth factor signaling can cause single cell detachment independent of E-cadherin regulation. As example, HGF-induced signaling can weaken cell-cell junctions and promotes single cell dissemination in the absence of EMT (de Rooij, Kerstens, Danuser, Schwartz, & Waterman-Storer, 2005).
Biophysical mechanisms controlling collective-to-single cell transitions depend upon the space and geometries encountered by moving cells in different tissues. Collectively moving mesenchymal cells undergo conversion to single-cell dispersal when encountering loosely organized tissue, allowing migrating cells to abandon weak cell-cell interactions and disperse individually, a process termed “unjamming” (Haeger, Krause, Wolf, & Friedl, 2014). In vivo, unjamming occurs when cancer cells migrate from confined into open space, e.g. from dense collagen meshworks into loose interstitial tissue (Weigelin et al., 2012; Weigelin, Bakker, & Friedl, 2014).

Figure 3. Plasticity of cell migration programs. (a) Mesenchymal-to-amoeboid transition, resulting from lowering adhesion to substrate pericellular proteolysis and leading edge protrusion, or from increasing Rho-mediated actomyosin contractility, either independently or jointly. (b) Collective-to-amoeboid or -mesenchymal single-cell transition, mediated by molecular programs which lower cell-cell adhesions or strengthened cell-matrix interactions. Figure content is published (te Boekhorst et al., 2016).
4.3 Collective plasticity. Plasticity of collective migration modes occurs frequently in epithelial cancers and describes different cohesive and structural organizations of invading cell groups (Friedl & Gilmour, 2009). Different collective modes include highly differentiated epithelial cohorts with conserved lumen formation; collective strands without lumen but with cohesive multicellular organization; small clusters after detachment or cell aggregation, and chain migration in strand-like fashion with preserved but minimal cell-cell contact (Friedl et al., 2012).

Collective plasticity is a consequence of varying degrees of apicobasal polarity within the group as well as varying strength of intercellular adhesion. High apicobasal polarity leads to inner lumen formation in collective epithelial strands, often accompanied by secretion and interaction with basement membrane proteins at the basal side; this recapitulates tubulogenesis such as during glandular duct formation of otherwise quiescent and immobilized epithelia (Cheung & Ewald, 2014). Intercellular adhesion strength can be modified by up- or downregulation of adherens junctions, e.g. by partial EMT, which may coincide with different levels of cohesive behavior (Batlle & Wilkinson, 2012; Bazellières et al., 2015; Peglion, Llense, & Etienne-Manneville, 2014). Collective plasticity likely underlies the formation of circulating tumor cell clusters (“microemboli”) after collective transmigration of the vessel wall and intraluminal disintegration of the cluster by molecular regulation or shear force exerted by the blood stream (Armstrong et al., 2011; Cheung & Ewald, 2016). A morphological framework for the plasticity of collective invasion and its implications for transitions between tissues remain to be established.

The ability of tumor cells to switch transiently or permanently between molecular pathways that determine migration ability and strategy, i.e. pericellular proteolysis, adhesion to ECM, and cell-cell interaction, represents an array of coping strategies that enable tumor cells to engage with virtually any tissue type and respond to numerous microenvironmental challenges.
5. Multiscale mathematical modeling of cell migration and plasticity

The range of plasticity programs in biological contexts, and the wealth of underlying molecular and physical mechanisms, provide a fascinating range for multi-parameter stimulation and data analysis; it also causes significant challenge due to an ever increasing complexity, with often multiple levels of control signals and feedback loops (Peter Friedl & Wolf, 2010). Thus, besides cell-based in vitro and in vivo analysis, identifying cause-consequence relationships requires additional computational analysis and mathematical modeling.

5.1 Mathematical toolbox to model cell migration. The cell-based analysis of plasticity of cell migration in vitro and in animal models is limited by the number of physical and chemical parameters that can be probed simultaneously and in context. Parameters represent the properties that influence the spatial distribution and temporal evolution of the state variables, including chemical affinities, reaction rates, chemotactic sensitivity, cellular stiffness, adhesiveness, and cell traction force. To enrich wet-lab analysis, many cooperating modules, including cell adhesion, cytoskeletal function, and cell-tissue-interaction, can be probed simultaneously by mathematical modeling (DiMilla, Stone, Quinn, Albelda, & Lauffenburger, 1993; Palsson & Othmer, 2000). Starting from parameters and response patterns identified by wet-lab analyses, simulation tools and algorithms are used to mechanistically link multiple inputs by intra- and intercellular signal processing and gene activation to migration outputs to predict how signals control a migration mode and its adaptation responses (Danuser, Allard, & Mogilner, 2013).

Considering a cell or a sub-cellular element as an information processing unit, input/output is the communication between an information processing system (cell) and the outside world (tissue, other cells). Input is the ensemble of signals the system is exposed to and output is the product which is generated. As first step, mathematical modeling aims to repeat known behaviors established by wet-lab research. Then, modeling allows to extract mechanisms and
identify which migration modules cooperate and are critical for the response of an individual cell and cell ensembles. Modelling can further identify unexpected “outlier behavior” as new phenotype, which in wet-lab experimentation is commonly interpreted as irreproducibility and thus escapes in-depth analysis. A mathematical model thus creates a virtual reality for cell behaviors, by examining ensembles of inputs and their connectivity over ranges that exceed experimental wet-lab possibilities. This aspect gains relevance considering the high speed and moderate cost of computational approaches compared to wet-lab experimental analyses.

The execution of a set of interconnected mathematical modules depicts a migrating cell and its environment as a complex system which reproduces the multi-scale nature of biological features. A complex system consists of multiple connected parameters and variables interacting with each other and with feedbacks. Its overall behavior cannot be expressed by the behaviors of its single components, but reaches higher-order, non-linear outcome and variable degree of order and robustness, including positive, negative, and mutually inhibitory feedback loops in a network of interactions. Results from multi-parametric computation exceed the sum of effects from each individual parameter. To combine input variables, a multi-scale model defines a reception fingerprint (Fig. 4a) which feeds into the intracellular processing machinery (Fig. 4b). The relevance of particular modules, both interfacial and sub-cellular (“molecular”), can be tested by virtual expression regulation, interference or deletion, thus recapitulating genetic, protein expression, or signaling profiles characteristic for cell activation or disease states.

5.2 Virtual assembly of moving cells. The combined activation of several protein cascades downstream of the sensory apparatus are interlinked and mediate an integrated cell response. The reception fingerprint representing multiple stimuli which vary in strength and duration is integrated by mathematical strategies to define a cell function response (Fig. 4c). Since each parameter generally depends on state variables which are not constant but time-dependent,
landscapes change over time and reflect the evolution of parameter ensembles. Multi-dimensional landscapes, which cannot be represented in a single diagram, are typically displayed by a series of 3D diagrams or multi-parametric heat maps obtained by fixing the other parameters (Lomakin et al., 2015; Shafqat-Abbasi et al., 2016; Tozluolu et al., 2013).

For example, migration efficacy of tumor cells in 3D space depends upon EGF stimulation, fibronectin and matrigel concentration and stiffness, and available integrin receptors, and peak speed is determined by intermediate matrigel concentration but maximum integrin availability (Zaman et al., 2006). The availability of such landscapes allows predictions on critical steps of decision making to guide biologists to conditions of interest for refined wet-lab experiments. Thus, mathematical models support the understanding of how complex reception fingerprints and the individual responsiveness repertoire cooperate and generate n-dimensional migration footprints.
Figure 4. Mathematical modelling of cell migration modules and modes. (a) Modeling of input parameters of adhesion and growth factor signaling (“receptor fingerprint”). (b) Modeling of intracellular signal transduction and gene expression, including positive and negative feedback loops and interference approaches, including knockdown and ablation strategies. The cartoon represents mathematical connections between modules. (c) Multi-parametric integration of multiple input parameters and intracellular processing. The output is represented as 3D landscape (blue surface) defined by the state of each function module, the kinetic evolution of which determines adaptation of migration mode. Due to stochasticity, output is probabilistic with a range of possible responses in cell ensembles (inset, red line). The cartoon represents graphically how multiple inputs are combined by linear and non-linear mathematical
operations to reach a complex output, which is delivered by computational analysis. Figure content is published (te Boekhorst et al., 2016).
6. Specific Aims

Tumor hypoxia, by elevating hypoxia-inducible factors (HIF), is an established inducer of cancer cell migration and metastatic progression of cancer disease. However, how hypoxia/HIF impacts on established epithelial cancer cell migration programs and which mechano-signaling adaptations are induced upon hypoxic stress, remains unknown. We hypothesize that hypoxia/HIF induces cellular and molecular reprogramming of cancer cells, involving adaptations in cell-cell adhesions, ECM remodeling, cell-matrix interactions and cytoskeletal actin dynamics, and thereby drives plasticity of established epithelial cancer cell migration strategies.

1. Characterize principles and plasticity of epithelial cancer cell migration in response to hypoxia
   a. Determine hypoxia/HIF-induced migration modes and efficiencies into 3D collagen
   b. Describe cytoskeletal actin dynamics and protrusion types underlying HIF-induced migration phenotypes
   c. Investigate the variability and stability of hypoxia-induced migration programs

2. Identify molecular mechanisms underlying hypoxia-induced migration modes
   a. Investigate molecular mechanisms underlying cellular regulators of induced migration programs
   b. Determine the dependency of hypoxia-induced migration modes on cellular determinants (cell contractility, cell-ECM adhesion and cell-ECM remodeling)

3. Validate hypoxia-induced cellular and molecular migration phenotypes in tumor xenografts and orthotopic human tumor models in vivo
   a. Determine HIF-induced migration modes in human tumor xenografts
   b. Validate underlying molecular mechanisms in human orthotopic tumor models
CHAPTER 2: MATERIALS AND METHODS

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Cell culture
Murine 4T1 cells (CRL-2539, ATCC) were cultured in RPMI (RPMI1640; Sigma), containing 10% fetal bovine serum (FBS; Sigma), penicillin (100 U/ml) and streptomycin (100 µg/ml; PAA) and sodium pyruvate (1mM; Thermo Fisher Scientific). Human oral UT-SCC38 cells and UT-SCC58 cells as well as UT-SCC38 Lifeact-GFP and UT-SCC58 Lifeact-GFP cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Sigma), supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich), penicillin (100 U/ml) and streptomycin (100 µg/ml; PAA), and sodium pyruvate (1mM; Gibco).

HNSCC cell lines Detroit 562 (purchased from the American Type Culture Collection) and HN31 (provided by Dr. John Ensley, Wane State University, Detroit, MI) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% FCS (Sigma). Stable Detroit 562 cell lines expressing control vector for short hairpin RNAs (shRNAs) were established as described previously (G. Zhou et al., 2014).
Antibodies and reagents

The following antibodies and dyes were used for Western blotting and immunofluorescence staining: mouse monoclonal anti-E-cadherin (clone 36/E-cadherin; BD Biosciences), rabbit polyclonal anti-HIF1α (Novus Biologicals), rabbit polyclonal anti-COL3/4 (Immunogloblue), rabbit polyclonal anti-MT1-MMP (ab38971; Abcam), mouse monoclonal anti-β-tubulin (Abcam), rabbit polyclonal anti-Phospho-Myosin Light Chain 2 (Thr18/Ser19) (Cell Signaling), rabbit polyclonal anti-Myosin Light Chain (Cell Signaling), mouse monoclonal anti-RhoA (Santa Cruz Biotechnology), mouse monoclonal anti-Rac1 (BD Biosciences), rabbit polyclonal anti-GAPDH (Cell Signaling), mouse monoclonal anti-human CD29 (clone HUTS-4; Millipore Sigma), rat anti-mouse CD29 (clone 9EG7; BD Biosciences), mouse anti-human CD29 (clone TS2/16, BioLegend), mouse monoclonal anti-human CD29 (clone 4B4, Beckman Coulter), rabbit polyclonal anti-Calpain 2 Large Subunit (Cell signaling), rabbit polyclonal anti-calpastatin (Cell signaling), rabbit polyclonal anti-Talin-1 (Abcam), rabbit polyclonal anti-phospho-v-Src Family (Tyr416, corresponding Tyr419 of human Src) (Cell signaling), rabbit polyclonal anti-Src (Cell signaling), purified mouse anti-p190 (BD Biosciences), FITC-labeled rat anti-CD44 (eBioscience), FITC-labeled Armenian hamster anti-CD29 (eBioscience), rabbit anti-cleaved caspase-3 (Cell signaling), chicken polyclonal anti-β-actin (ab13822; Abcam), secondary goat anti-rabbit/mouse/chicken antibodies conjugated to horseradish peroxidase (Jackson), secondary pre-absorbed goat anti-rabbit/mouse IgGs coupled to AlexaFluor fluorescence dyes (Alexa-Fluor-488, 568, 633 or 647; Invitrogen), Alexa Fluor-546/633 conjugated phalloidin (Invitrogen), DAPI (Invitrogen).

For flow cytometry, the following antibodies were used: mouse monoclonal anti-human CD29 (clone HUTS-4; Millipore Sigma), purified mouse IgG2b isotype control (Clone 27-35; BD Biosciences), IgG from mouse serum (I5381; Sigma), purified rat anti-human CD29 (clone MAB13, BD Bioscience), rat anti-mouse CD29 (clone 9EG7; BD Biosciences), purified rat IgG2a isotype control (Clone R5-95; BD Biosciences), FITC-conjugated armernian hamster anti-
mouse/rat CD29 (Clone HMβ1-1; BioLegend), FITC-conjugated armenian hamster IgG isotype control (CLoneHTK888; Biolegend), rabbit polyclonal anti-Calpain 2 Large Subunit (Cell signaling), rabbit IgG isotype control (Cell Signaling), AlexaFluor 488-conjugated anti-mouse IgG and -rat IgG, AlexaFluor 647-conjugated anti-rabbit IgG.

The following inhibitors and reagents were used: GM6001 (CalBiochem), Y-27632 (Y; Sigma), Blebbistatin (Sigma), Dasatinib (Sigma), calpeptin (Calbiochem), PD150606 (PD; Cayman Chemicals), CMAC t-BOC-Leu-Met (7-Amino-4-Chloromethylcoumarin, t-BOC-L-Leucyl-L-Methionine amide) (CMAC; Thermo Fisher Scientific), Dimethyl oxalylglycine (DMOG; Cayman Chemicals), Dimethyl sulfoxide (DMSO; Sigma), trypsin-EDTA (Sigma), methyl cellulose (Sigma), collagen I Rat Tail (Corning), bovine collagen solution (Advanced Biomatrix), Paraformaldehyde solution (PFA; Affymetrix).

**Hypoxia culture and DMOG treatment**

For cell function studies, cells were cultured as 2D monolayers or spheroids in 3D collagen lattices in normoxia (21% O2) or hypoxia (0.2% O2; CB53 incubator, Binder GmbH) for 72 hr (4T1) or 96 hr (UT-SCC38), unless indicated otherwise. For pharmacological activation of HIFs, cells were treated with DMSO (0.1%; solvent control) or the prolyl-hydroxylase inhibitor DMOG (1 mM) for 72 hr (4T1) or 96 hr (UT-SCC38), unless indicated otherwise.

**Cell isolation from 2D culture**

Subconfluent monolayers were grown under DMSO or DMOG for 72 hr (4T1) or 96 hr (UT-SCC38). After gentle removal of supernatant medium, low-adhesive cells were harvested by manual horizontal circular washing with warm PBS (100 cycles, 37 °C). Cells remaining plate-attached after washing (i.e. the highly adhesive population) were detached using trypsin-EDTA (2mM, 37C). Individual populations were subjected to cell viability analysis, flow cytometry analysis, or pooled for Western Blot analysis.
Spheroid migration assay on 2D collagen surface

To test cell migration behavior on 2D surfaces, multicellular spheroids were generated by the hanging drop method, as described below (4T1, 500 cells/spheroid). Spheroids were washed twice in medium and seeded for 6 hr onto culture vessels coated with 2mg/ml rat tail collagen I diluted in 100% methanol. Attached spheroids were treated with DMSO or DMOG (1mM) dissolved in respective culture media for 72 hr (4T1) at 37°C, 5% CO₂. Migration morphologies and efficacy were monitored by bright-field time-lapse microscopy (Leica) or endpoint analysis after 72 hr (4T1).

Spheroid invasion assay in 3D collagen matrices

Multicellular spheroids were generated from subconfluent cells using the hanging-drop assay (20% methylcellulose; 500 cells/25 µl drop; overnight spheroid assembly) (Korff & Augustin, 1998). Spheroids were embedded in intermediate dense non-pepsinized rat-tail collagen type I solution 2.5 mg/ml, avg. pore size 4 µm²) or in low-dense bovine collagen (1.7 mg/ml, avg. pore size 20 µm²) or in highly dense (4 mg/ml, avg. pore size 1 µm²) prior to collagen polymerization (37°C, 10-20 min) (Wolf et al., 2013). After polymerization, 3D cultures were treated with DMOG (1 mM) or DMSO (0.1%; vehicle control) suspended in respective incubation media. Migration morphology and efficacy were monitored by bright-field time-lapse microscopy (Leica) or endpoint analysis after 72 hr (4T1) or 96 hr (UT-SCC38).

Pharmacological treatment of migration cultures

The molecular dependencies of cell migration modes and efficacy of individual cells after detachment from the spheroid were tested by adding biologically active compounds to 3D collagen cultures, including: ROCK inhibitor Y-27632 (Y, 20 µM), myosin II inhibitor blebbistatin (20 µM), β1 integrin blocking antibody 4B4 (0.5 µg/ml), β1 integrin activating antibody TS2/16
(20 µg/ml, UT-SCC38), β1 integrin activating antibody 9EG7 (5 µg/ml, 4T1), SFK inhibitor Dasatinib (5 nM), calpain inhibitor PD150606 (50 µM) after 48 hr (4T1) or 72 hr (UT-SCC38) of culture in the presence of DMOG or DMSO. Spheroid cultures in 3D collagen were preincubated with respective antibodies or inhibitors for 1 h, and invasion morphology and efficacy were monitored by bright-field time-lapse microscopy (Leica) or image analysis at the endpoint after 72 hr (4T1) or 96 hr (UT-SCC38). All antibodies and inhibitors used in live-cell assays were azide-free (as purchased or generated by dialysis followed by sterilization).

**Analysis of cell viability in 2D and 3D cultures**

Cell viability of cells cultured as monolayers was quantified after detachment by Trypsin/EDTA (0.05 %) using staining of dead cells with propidium iodide (2.5 µg/ml) and detection by flow cytometry (FACS Calibur, BD). Data were analyzed by FCS Express 4 Plus Research Edition (De Novo Software). To assess apoptosis in 3D collagen-based cultures, spheroids were fixed with PBS-buffered PFA (4%, 15 min, 21 °C), stained with DAPI and the number of cell death events was determined as the fraction of nuclei with apoptotic/necrotic morphology (fragmentation, condensation, ballooning) for every second confocal slice from 3D stacks (5 µm inter-slice distance) per cumulative nuclear area determined on thresholded DAPI images by Fiji/ImageJ. The following numbers of spheroids were analyzed from two independent experiments: 4T1 normoxia, n=14; 4T1 hypoxia, n=11; UT-SCC38 normoxia, n=7; UT-SCC38 hypoxia, n=7. This method is published content (Lehmann et al., 2017). To evaluate apoptosis induction prior to nuclear fragmentation, fixed spheroids were stained for DAPI, Phalloidin, and cleaved-caspase 3.

**Bright-field time-lapse microscopy**

3D spheroid cultures were placed in wax-sealed migration chambers filled with containing medium and kept at 37°C using heat-lamps (Efbe-Schott, IR812) during entire imaging sessions. Bright-
field time-lapse microscopy was performed using DM IL LED microscopes (Leica Microsystems), equipped with STC-405 cameras (Sentech). Image acquisition was controlled using the Time Controlled 16-Channel Recorder software (SVS-Vistek GmbH 2004). Images were taken at 4min-intervals for 24h or 48h starting at 48 hr (4T1) or 72 hr (UT-SCC38) after DMOG or DMSO treatment, using 10x/0.22 or 20x/0.30 HI Plan objectives (Leica Microsystems).

**Calpain activity assays in 2D monolayers, 3D spheroid cultures and human tumor xenografts**

For detection of calpain activity in subconfluent monolayers, cells were detached with trypsin-EDTA (2mM), PBS washed, incubated in the dark with LIVE/DEAD Far red dye (Thermo Fisher Scientific) together with CMAC (10 µM, 20min, 37 °C) in PBS, then washed with PBS and fixed (0.5% PFA in PBS, ON, 4°C) or incubated with calpain inhibitor PD150606 (100 µM, 2hr, 37°C in medium) prior to fixing. Cleaved CMAC substrate (Thermo Fisher Scientific; excitation/emission peaks at 351/430 nm) was measured by flow cytometry (LSRII, BD) using 355nm laser excitation and detected through a 440/40 nm filter. Quantification of mean fluorescent intensity (MFI) was performed after linear subtraction of MFIs of unstained control samples using FlowJo (Version 10.3.).

Calpain activity was detected in 3D spheroid cultures, 3D spheroids migrating on 2D collagen or in human tumor xenografts using multiphoton microscopy. Spheroid collagen cultures were washed with PBS, incubated with CMAC (20 µM, 30min, 37°C), and fixed in PFA (2% PFA/PBS, 5 min, 37°C followed by 4 % PFA, 10 min, 37°C). For calpain inhibition, 3D cultures were incubated with calpain inhibitor PD150606 (100 uM, 37°C for 20hr and refreshed 2hr before fixation. For morphology analysis, fixed-CMAC incubated 3D cultures were incubated with AlexaFluor 546-conjugated phalloidin in PBS (1 hr, RT). For detection of calpain activity in human tumor sections, fresh-frozen tumors were cryo-sectioned, thawed for 5 min at RT, incubated with CMAC (100 µM, 45 min, 37 °C), washed with PBS and fixed in PFA (2% PFA,
5 min, 37°C followed by 4% PFA, 10 min, 37 °C). Samples were imaged by multiphoton microscopy (LaVision BioTec) using a 25x NA 1.05 water objective (Olympus) as 3D stacks with 2 μm inter-slice distance. Cleaved and uncleaved CMAC substrate was excited using the Titanium Sapphire laser at 730 nm with 20 mW and detected with a customized 427/10 filter (Semrock) and below 405 nm (Semrock), respectively. Optimal excitation wavelength at 730 nm for cleaved and uncleaved CMAC was identified with a wavelength scan for an excitation range from 710 nm to 1090 nm. No correlation for cleaved and uncleaved CMAC signal intensities was observed. AlexaFluor 546-conjugated phalloidin was excited with a titanium sapphire OPO laser at 1090nm at 40mW and detected with a 620/50 filter. Collagen fibers were detected by second harmonic generation (SHG; 1090nm, BP 525/50). Image analysis was performed in Fiji/ImageJ as described below.

**Cell tracking analysis**

Migration paths and speed of single cells were digitized using Autozell (software version 080912; Center for Computing and Communication Technologies [TZI], University of Bremen, Bremen, Germany). The average migration speed was calculated by the length of the xy-path divided by the time. Only viable cells were included in the analysis.

**Immunostaining and confocal microscopy**

For immunofluorescence staining, 3D collagen cultures (100 µl gels) were fixed (2% PFA, 5 min, 37°C followed by 4 % PFA, 10 min, 37 °C), washed (PBS, 3x10 min), permeabilized and blocked (0.1 % PBS/BSA/10% normal goat serum /0.3% Triton X-100 for 1 hr at RT and incubated with primary antibody in PBS/BSA (0.1 %)/Triton X-100 (0.3 %) for 18h at 4°C. Cultures were thoroughly washed (PBS, 8 x 1 hr), incubated with DAPI and secondary antibodies (18h, 4 °C) in PBS/BSA (0.1%)/Triton X-100 (0.3%), washed again (PBS, 3x10 min), and imaged as whole-mount 3D samples. Confocal fluorescence and reflectance microscopy
was performed by sequential single-channel confocal scanning (TCS SP5 Confocal Microscope, Leica Microsystems) at an inter-slice distance of 2 µm. The following objectives were used: ACS APO 20x NA 0.60 IMM CORR, CS APO 40x NA 1.15 and 63x NA 1.30 oil CS (Leica Microsystems). For detection of pMLC by immunofluorescence, PBS-PFA was supplemented with 10% (v/v) phosphatase inhibitor (PhosSTOP, Roche) during fixation. Image processing and single-cell cytometry of fluorescence intensities in 3D samples were performed using Fiji/Image J as described below.

**Image analysis**

All bright-field, confocal and multiphoton microscopy images were analyzed and processed using Fiji/ImageJ software. Unless stated otherwise, morphology analysis was performed from bright-field microscopy images obtained with a 20x NA xx objective. Dead (cellular fragmentation or condensation), mitotic and post-mitotic cells (cell swelling, proximate round or oppositely polarized cell doublets) were excluded from all analyses.

**Analysis of E-cadherin intensities.** Regions of interest (ROIs) were defined in strands or single cells from multichannel overlays to mark the whole-cell area, as described (Lehmann et al., 2017). In short, total signal intensities from each ROI were quantified from every second confocal slice from 3D stacks and divided by the DAPI-positive nuclei count/ROI. To take into account intensity variation caused by imaging depth and different laser settings between spheroids, the average intensity of the 3 lowest measured ROI values (assumed constant for all samples) of a given spheroid was calculated and used as internal reference to normalize signal intensities between spheroids and independent samples. This method is published content (Lehmann et al., 2017).

**Migration morphology analysis.** Analysis of migration morphologies and classification of phenotypes was performed on z-series obtained by imaged bright-field microscopy from non-
overlapping spheroid regions (2-10 regions per spheroid/condition; 5-10 spheroids/experiment). Scoring was performed by two independent people, in part using double-blinded analysis.

**Protrusion analysis.** Protrusion types and amount of individual migrating cells were scored on phalloidin-stained (F-actin) samples from confocal maximum intensity z-projections (40x NA xx objective). Dead and mitotic and post-mitotic cells were excluded from the analysis based on fragmented nuclear morphology.

**Elongation kinetics and factors.** Elongation kinetics of individual cells migrating after HIF-stabilization with DMOG was performed on time-lapse movies or fixed samples at the end-point. Time-resolved elongation was measured as the ratio of the maximum cell length over the maximum cell width every 1 hr over 8 to 24h time periods, depending on the time a cell was present in the field of imaging.

**Survival analysis and phenotype outcome.** Stability of migration modes and phenotype outcome was obtained from time-lapse movies as the time until the first morphological switch into another mode was noted, based on the following criteria. A switch was considered stable when the acquired shape was persistent for more than 3 hrs. Only cells that were recorded for an entire 48 hr period were included in the analysis.

**Nuclear diameter analysis.** Cell deformation was measured as the minimum diameter of deformed DAPI-positive nuclei from the in-focus z-slice of 3D confocal stacks, as described (Wolf et al., 2013).

**Analysis of calpain activity in invading single cells.** Single cell cytometry of fluorescence intensity of cleaved CMAC substrate (calpain activity) was analyzed from maximum intensity projections using the shape tool. Cleaved CMAC substrate intensities per cell were calculated as corrected total cell fluorescence (CTCF), defined as Integrated Density minus the product of the area of a selected cell and the mean fluorescence of background readings, or as Integrated Densities corrected for the background, as indicated. The CTCF per cell was correlated to the
elongation (maximum cell length/ maximum cell width) of the same cell obtained from the phalloidin channel.

**Immunoblotting**

Western blot analysis of protein expression was performed using subconfluent 2D monolayer cultures. Cells were washed twice with ice-cold PBS, followed by protein extraction with lysis buffer (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0; 150 mM NaCl; 0.1% NP-40), supplemented with a protease inhibitor cocktail (Roche, Cat. 11 836 145 001) shortly before cell lysis. For analysis of HIF-1α and Twist, nuclear extraction was performed using a nuclear lysis buffer (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0; 400 mM NaCl), supplemented with protease inhibitor cocktail. For analysis of active β1 integrin epitope expression, cells were lysed under non-reducing conditions. Protein concentration was determined by the Bradford assay (Biorad), lysates loaded on a 12% SDS-PAGE gel or on Novex 4-20% Tris-Glycine Mini Gels (Thermo Fisher Scientific) and transferred using the iBLOT system (Thermo Fisher Scientific) or by electroblotting (120 V, 1 h) in transfer buffer (28 mM Tris-HCL; 39 mM Glycine; 20% methanol v/v) onto PVDF membranes (Thermo Fisher Scientific or Milipore). Immunodetection was performed using the following primary antibody dilutions: anti-HIF1α, 1/1000 or 1/500; anti-MT1-MMP, 1/2000; anti β-actin, 1/1000. IR dye coupled secondary antibodies (dilution 1/1000) were used for detection of primary antibody complexes by the Odyssey reader (Licor). Alternatively, HRP conjugated secondary antibodies (anti-rabbit IgG, dilution 1/2000; anti-chicken IgG, dilution 1/10000; anti-mouse IgG, dilution 1/5000) in combination with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and CL-Xposure films (Thermo Fisher Scientific) were used for protein detection.

Densitometry analysis of pMLC/total MLC active β1 integrin/total b1 integrin, pSrc/total Src, p190RhoGap was performed after normalization to the β-actin loading control for each protein.
followed by normalization to total protein expression levels using Fiji/Image J. This method includes published content (Lehmann et al., 2017).

**GTPase pull down assay**

4T1 cell monolayers were treated with DMSO or DMOG for 72 hr. For generation of cell lysates, subconfluent cells were washed on ice twice in cold PBS and lysed in gold lysis buffer (1% Triton X-100, 20mM Tris-HCl pH 8.0, 500mM NaCl, 10mM MgCl₂, 15% glycerol) containing 0.5mM DTT (Sigma) and 0.5ml EDTA-free protease inhibitor cocktail (cOmplete, Roche). Glutathione S-transferase (GST)-rhotein (RhoA-binding domain) or GST-Pak3 (Rac1-binding domain) fusion proteins were generated in E. coli bacteria (plasmids were kindly provided by M. Zegers (Dept. of Cell Biology, Radboudumc Nijmegen, The Netherlands) and bound to glutathione sepharose 4B beads (GE Healthcare Life Sciences). Active (GTP-bound) RhoA or Rac1 were precipitated from cell lysates at 4°C for 30 min, eluded in western blot lysis- buffer, and analyzed by Western blotting as described above.

**Flow cytometry**

For detection of β1 integrin levels, cells were cultured as monolayers to 80% confluency and detached by trypsin-EDTA (2 mM), washed in PBS, and stained with LIVE/DEAD fixable Aqua stain (Thermo Fisher Scientific; 30 min, 4°C). Unfixed viable 4T1 cells or fixed UT-SCC38 cells (2% PFA in PBS, 10 min, RT) were stained with primary antibodies and matching IgG controls dissolved in FACS buffer (2% FBS/2mM EDTA in PBS) for 1 hr at 4 °C, washed with FACS buffer, incubated with secondary antibody dissolved in FACS buffer for 0.5 - 1 hr at 4 °C, and analyzed by flow cytometry (BD FACS CANTO II, BD Biosciences).
For detection of intracellular calpain-2 expression levels, cells were fixed (2% PFA in PBS, 10 min, RT), permeabilized (0.2 % Triton-X100 in PBS; 5 min, RT), and then subjected to primary and secondary antibody incubations as described above.

Quantification of MFI was calculated by linear subtraction of IgG control MFIs followed by total β1 integrin content and/or normalization to MFIs of DMSO, as indicated. All data analysis was performed using FlowJo (Version 10.3.).

**Transient transfection of cell monolayers and 3D spheroid cultures**

Transient calpain-2 knockdown in UT-SCC38 cells was performed using MISSION siRNA targeting human CAPN2 (NM_001748, siRNA ID: SASI_Hs01_00059356, Sequence start 2234, Sigma Aldrich) and MISSION siRNA universal negative control (SIC001, Sigma Aldrich). For ectopic expression of a calpain-uncleavable talin protein, plasmid DNA constructs GFP-TalinL432G (26725, addgene) and pEGFP N1 (vector control, Clontech Laboratories) were used to transfec 4T1 and UT-SCC38 cells.

For transfection of cells cultured as monolayers, a transfection mixture containing 100 µM of mission-siRNA (siCAPN2 or siControl) and 128 µl MISSION siRNA transfection reagent (Sigma Aldrich) in 1.2 ml serum-free medium was added to 6.8 ml complete medium of UT-SCC38 cells (200K cells, 10cm² dish), or containing 6 µg of plasmid DNA (GFP-TalinL432G or pEGFP) and 15 µl Lipofectamine 2000 (Thermo Fisher Scientific) in 500 µl OptiMEM (Thermo Fisher Scientific) was added to 9 ml complete medium of 4T1 and UT-SCC38 cells (75K cells, 10cm² dish) for 20 hr. Then medium was changed and transfected cells were treated as indicated.

For transfection of 3D spheroids, a transfection mixture containing 100 µM of mission-siRNA (siCAPN2 or siControl) and 32 µl MISSION siRNA transfection reagent (Sigma Aldrich) in 200ul serum-free medium or containing 4 µg of plasmid DNA (GFP-TalinL432G or pEGFP) and 10 µl Lipofectamine 2000 (Thermo Fisher Scientific) in 75 µl OptiMEM (Thermo Fisher Scientific) was added to 4T1 and UT-SCC38 cells (50K cells, suspended in aggregation medium containing
20 % methylcellulose). Cells suspended in transfection solution including RNAi were plated as hanging drops (25 µl; 500 cells per drop) for spheroid formation (18 hr, 37 °C), washed with medium, embedded in collagen and treated as indicated.

**Stable down-regulation of HIF-1α by shRNA**

U6 promoter-driven short hairpin RNA (shRNA) expression vectors targeting HIF-1α (Mission shRNA, NM_010431.1-1864s21c1) (5'-CCGGTGATGCTATTGTCATGCTGACCTGGATTGACATGCTATCCATTCTTTTTG-3') for mouse HIF1a, NM_001530.x-1048s1c1(5'CCGGGTGATGAAAGAATTACCGAATCTCGAGATTCGGTAATTCTTTCATCAC TTTTT-3') for human HIF1a; Sigma Aldrich) or non-targeting control vector (pKLO.1) were expressed in HEK293T to produce lentiviral pseudotyped particles using the ViraPower lentiviral expression system (Thermo Fisher Scientific). Mouse 4T1 cells and human UT-SCC38 cells were infected and pooled populations obtained by purmoycin (4 µg/ml) selection over 10 days (Lehmann et al., 2017). Efficiency of HIF-1α down-regulation was analyzed by Western blotting. This method is published content (Lehmann et al., 2017).

**Orthotopic human HN-SCC xenograft model**

Animal experiments were approved by the institutional Animal Care and Use Committee (IACUC) of the University of Texas MD Anderson Cancer Center (00001148-RN00 and 00001522-RN00). Orthotopic human HN-SCC xenografts in nude mice were established as described (Myers, Holsinger, Jasser, Bekele, & Fidler, 2002). HN-31 and Detroit 562 cells were injected (5 x 10^4 in 30 µl serum-free DMEM; Gibco) into the dorsal part of the tongue of 6- to 8-week-old male athymic nude mice (ENVIGO). Mice were euthanized by CO₂ asphyxiation 30
days post-injection or when losing more than 20% of their pre-injection body weight. Tongues containing primary tumors were resected at the endpoint and cryopreserved.

**Subcellular element model for the simulation of cell length of single cell migration**

For mechanical simulation of single cell migration behavior and cell length, a subcellular element method model, originally designed for collective cell migration (Basan, Elgeti, Hannezo, Rappel, & Levine, 2013; Zimmermann, Camley, Rappel, & Levine, 2016), was adopted for modeling of single cell migration adjusting the intercellular adhesion value to 0.1. In this model, each virtual cell is represented by two particles, the front particle and the rear particle, and the length between the two particles determines the cell length. The following input parameters were modeled independently when simulating the emergent behavior of single cells: (i) contractility force (defined as the force between the two particles within one cell), (iii) self-propulsion force (for single cells the front particle and the rear particle have a persistent force in opposite direction along the cell, the force of the front particle is larger than the force of the rear particle; for collective cells the self-propulsion force is subject to contact inhibition of locomotion, which will affect both the direction and magnitude of the self-propulsion force, see more details in [1], (iii) the friction between the particle and the substrate. We assumed a dependence between the self-propulsion force and the friction. For each friction value (3.0, 1.0, 0.3), the following corresponding self-propulsion forces values were: 1.5/1.3, 0.84/0.73, 0.3/0.26 (front/rear). The outputs of the simulation are: (i) the total number of single or collective cells, (ii) cell length and (iii) migration speed. All movies for the simulations were generated by VMD 1.9.3 for Unix (Humphrey, Dalke, & Schulten, 1996).

**Tumor cell implantation in vivo and intravital multiphoton imaging**

Animal experiments were approved by the institutional Animal Care and Use Committee (IACUC) of the University of Texas MD Anderson Cancer Center (00001002). The morphology
of intradermally migrating H2B-mcherry/Lifeact-GFP expressing UT-SCC38 or H2B-mcherry/Lifeact-GFP expressing UT-SCC58 was analyzed after intradermal injection (i.d.) into a dorsal skin-fold chamber window transplanted onto mice. Apoptotic cells (nuclear fragmentation) and non-migrating cells (cells lacking actin protrusions) were excluded from the analysis. Prior to i.d. injection, cells were cultured in the presence of DMOG (1 mM) with or without calpain inhibitor PD150606 (100 μM) for 48 hr. DMOG and PD150606 were refreshed after every 20 hr. For i.d. injection, cells were detached using trypsin-EDTA (2 mM), washed in PBS, filtered through a FACS mesh sieve and injected as individual cells (1x10⁴ cells/mouse) into 8-to 10-week old male athymic nude mice (Experimental Radiation Oncology, MD Anderson Cancer Center). 4 hr post-injection, the morphologies of the cell body and nucleus was obtained from 3D stacks with 2 μm inter-slice distance from at least 3 regions/animal (1 animal/group; 1 mouse per timepoint) using intra-vital multiphoton imaging using the following excitation and detection settings: Lifeact-GFP (920 nm, 20mW, BP 525/50); H2B-mCherry (1090 nm, 50mW, BP620/50); second harmonic generation (SHG; collagen) (1090 nm, 50mW, BD 525/50), third harmonic generation (1180 nm, 70mW, BP 387/15). Image analysis, 3D reconstruction and maximum intensity projections were performed using Fiji/ImageJ.

**Statistical analysis**

Data represent either the mean and standard deviation (bar graphs) or individual cell or individual spheroid data points (scatter dot plots) with median and interquartile range (25%-75%) of individual representative or pooled experiments, as indicated. Statistical analysis was performed using two-tailed non-paired non-parametric Mann-Whitney U (two groups) or Kruskal Wallis Multiple Comparison with Dunn’s correction (more than two groups) using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA), unless otherwise stated.
CHAPTER 3: SPECIFIC AIM 1

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Specific Aim 1: Characterize principles and plasticity of epithelial cancer cell migration in response to hypoxia

Results

Hypoxia and/or HIF-1-induced switch from collective to single-cell migration

Hypoxic tumor regions are not only found in perinecrotic regions of the tumor core but also at tumor edges and the invasive front. Thus, tumor cells will encounter hypoxia before and during invasion (Büchler et al., 2004; Lehmann et al., 2009). To test how hypoxic stress affects incipient epithelial cancer invasion from multicellular spheroids, we used 4T1 murine breast carcinoma cells as competent model for local invasion and distant metastasis (Pulaski, & Ostrand-Rosenberg, 2001) and partial activation of epithelial-to-mesenchymal transition (EMT) programs (J. Yang et al., 2004), and fully epithelial but only locally invasive UT-SCC38 human head and neck squamous carcinoma cells isolated from a patient without metastatic disease
Spheroids were implanted into 3D fibrillar collagen matrices and subjected to normoxia (21% O₂) or severe hypoxia (0.2% O₂) for up to 4 days (Fig. 5a). In normoxia, both cell types showed effective collective invasion, with tip cells leading finger-like strands and only a few spontaneously detaching single cells (Fig. 5b, c), consistent with collectively moving epithelia and carcinoma (Nguyen-Ngoc et al., 2012). In contrast, hypoxia induced plasticity of cancer cell migration modes: 4T1 cells retained a mixed phenotype, consisting of collective invasion with intact propagation of tip cells and additionally enhanced single-cell dissemination, whereas the collective phenotype of UT-SCC38 cells was reinforced, through formation of an increased number of tip cells and enhanced branching (Fig. 5b, c). The non-invasive cell fraction in the core (not shown) and in collectively invading strands retained high levels of E-cadherin, whereas E-cadherin levels were low in detached 4T1 and UT-SCC38 single cells (Fig. 6). This validates the induction of predominantly single cell invasion under hypoxia.
Figure 5. Hypoxia induced HIF-1 dependent plasticity of cancer cell migration. (a) Tumor spheroid generation and collagen-based 3D culture. BF, bright-field; IF, immunofluorescence; ff, and the following. (b, c) Morphology (b) and quantification (c) of collective invasion (determined by the number of tip cells) and detached single cells from multicellular spheroids cultured in 3D collagen lattices. Images show endpoints after 72 hr (4T1) or 96 hr (UT-SCC38). Box and whisker plots represent the medians ± minimum/maximum values. White arrowheads, collective strands; yellow arrowheads, detached single cells. N, normoxia; H, hypoxia; V, vehicle (DMSO); D, DMOG. Scale bars, 100 μm (overviews), 50 μm (insets). "Reprinted from

**Figure 6. HIF-induced single cells are low in membranous E-cadherin expression.** Topography of E-cadherin and resulting intensity distributions (bottom histograms) in detached 4T1 and UT-SCC38 cells. White arrowheads, detached cells. Images show maximum-intensity projections. Scale bars, 100 µm (overviews), 20 µm (insets). “Reprinted from Hypoxia Induces a HIF-1-Dependent Transition from Collective-to-Amoeboid Dissemination in Epithelial Cancer
Hypoxia does not impair cell viability

Hypoxia did not compromise cell viability of either cell type in 2D monolayers (Fig. 7a), but resulted in moderately increased apoptosis in the core of 3D spheroids (Fig. 7b), whereas moving single cells were without signs of apoptosis (nuclear or cellular fragmentation) or apoptosis induction (cleaved caspase-3 epitope expression) (Fig. 7c). Thus, besides initiating cancer cell migration in non-invasive cultures (Pennacchietti et al., 2003), hypoxia diversifies ongoing invasion.

Figure 7. Hypoxia induced plasticity of cancer cell invasion: impact on cell viability (a)
Cell viability of 4T1 and UT-SCC38 cells cultured as monolayers for 96hr, determined by PI staining and FACS analysis. N, Normoxia, H, Hypoxia. (b) Number of apoptotic nuclei 22
(determined through nuclear morphological changes associated with apoptosis including nuclear condensation and fragmentation) per cumulative nuclear area derived from confocal stacks (DAPI channel) of spheroids cultured in normoxia or hypoxia for 72 hr (4T1) or 96 hr (UT-SCC38). “Reprinted from Hypoxia Induces a HIF-1-Dependent Transition from Collective-to-Amoeboid Dissemination in Epithelial Cancer Cells. Current Biology, 27(3), 392–400. Lehmann, S., te Boekhorst, V., Odenthal, J., Bianchi, R., van Helvert, S., Ikenberg, K., … Friedl, P. Copyright 2017, with permission from Elsevier.” (c) Detection of cleaved caspase-3 during collagen invasion of UT-SCC38 cells for 96 hr after DMSO or DMOG treatment for 96 hr with or with pre-treatment with Etoposide (100 μg/ml) for 1 hr before fixing.
Hypoxia-induced collective-to-single cell transition depends on HIF-1α

As a generic signaling hub induced by hypoxia, hypoxia-inducible factor 1 (HIF-1) was upregulated in hypoxic 4T1 and UT-SCC38 cells (Fig. 8a). Consistently, dimethyloxalylglycine (DMOG), an inhibitor of HIF prolylhydroxylases that stabilizes HIFs (P. Jaakkola et al., 2001), enhanced invasion and imposed plasticity with strongly enhanced single-cell detachment from both 4T1 and UT-SCC38 spheroids. Stable downregulation of HIF-1a (Fig. 8b) reverted single-cell detachment during hypoxia (4T1) and after DMOG treatment (UT-SCC38) towards predominantly collective invasion (Fig. 8d). HIF-1 downregulation was tolerated by 4T1 cells but compromised the viability of UT-SCC38 cells during hypoxia (Fig. 8c), which allowed meaningful analysis of migration in UT-SCC38 cells only after DMOG/DMSO treatment conditions (Fig. 8d). In conclusion, by converting collective to single-cell migration, hypoxia via HIF-1 signaling induces rapid plasticity of 3D invasion programs.
Figure 8. Hypoxia-induced plasticity and regulation by HIF-1. (a) Western blot of HIF-1 levels in cells cultured as monolayers, exposed to hypoxia (24 hr). (b) Immunoblot analysis of HIF-1α levels in shHIF-1α or shcontrol 4T1 and UT-SCC38 monolayers cells after 24 hr (4T1) or 8 hr (UT-SCC38) of culture. (c) Viability of shRNA-mediated knockdown cells cultured as monolayers under normoxia or hypoxia for 96 hr. Means ± SD are shown (normoxia, triplicates; hypoxia, quadruplicates). (d) Morphology of 4T1 and UT-SCC38 spheroids expressing shHIF-1α or shcontrol RNA after 72 hr of culture. Floating bars indicate the median ± min/max values. Scale bars, 100 μm (overviews), 50 μm (insets 4T1), 20 μm (insets UT-SCC38). “Reprinted from Hypoxia Induces a HIF-1-Dependent Transition from Collective-to-Amoeboid Dissemination in Epithelial Cancer Cells. *Current Biology*, 27(3), 392–400. Lehmann, S., te Boekhorst, V., Odenthal, J., Bianchi, R., van Helvert, S., Ikenberg, K., … Friedl, P. Copyright 2017, with permission from Elsevier.”
Hypoxia induces a stable and highly migratory blebby amoeboid migration mode

Single-cell migration comprises at least two subtypes, including spindle-shaped and amoeboid migration (Veronika te Boekhorst et al., 2016). To evaluate the characteristics of HIF-induced single cell migration types, based on morphology, topologic scoring of roundness and protrusion type was performed. While both cancer models display a variety of single cell invasion strategies during collagen invasion at normoxic (21% O2) levels, the population of single cells detached from spheroids in hypoxia revealed mixed morphologies including a minority of elongated, spindle-like cells and a majority (70%–80%) of rounded cells lacking long extensions (Fig. 9a; elongation index range 1 to 4). This rounded-shape pattern is reminiscent of amoeboid migration in highly invasive tumor cells after inhibition of matrix metalloproteinases (MMPs) or activation of Rho/ROCK (Rho associated protein kinase) signaling (Sanz-Moreno et al., 2011; Wolf et al., 2003). This suggests a HIF-specific induced amoeboid-migrating phenotype.

HIF-induced individual amoeboid moving cells comprised bleb- (65-80%) or pseudopod-based (20-30%) subtypes, whereas the DMSO control cells comprised a lower fraction of bleb- (30-50%) or pseudopod-based (10-30%) amoeboid migration subtypes (Fig. 9b, c).
Figure 9. Hypoxia/HIF-induced amoeboid blebby migration. (a) Morphological classifiers and frequency distributions of elongation indices (cell length/width; right) detected in single cells after detachment from spheroids in 3D collagen culture after 72 hr (4T1) or 96 hr (UT-SCC38) of hypoxia. “Reprinted from Hypoxia Induces a HIF-1-Dependent Transition from Collective-to-Amoeboid Dissemination in Epithelial Cancer Cells. Current Biology, 27(3), 392–400. Lehmann, S., te Boekhorst, V., Odenthal, J., Bianchi, R., van Helvert, S., Ikenberg, K., … Friedl, P. Copyright 2017, with permission from Elsevier.” (b) Representative bright field images of 3D spheroid cultures invading into collagen after HIF stabilization with DMOG. Scale bars, 100 μm (overviews), 10 μm (insets). (c) Quantification of migration morphologies (E, elongated; P pseudopodial; B, blebby) (left panel) after HIF stabilization with DMOG compared to DMSO treatment of individual migrating 4T1 and UT-SCC38 cells after detachment from 3D spheroids in collagen and fold-change (right panel) of amoeboid blebby phenotype induced by DMOG. V, vehicle (DMSO); D, DMOG. Data represent the mean ± SD, n = 3 independent experiments.
HIF-induced amoeboid cells display polarized interactions with collagen fibrils

Both bleb-based and pseudopodal amoeboid migration supported polarized interactions with collagen structures in direction of migration (Fig. 10a). In contrast to pseudopodial amoeboid migration, where cells display longitudinal polymerized actin in elongated protrusions, HIF-induced blebbby amoeboid migration was characterized by actin-rich blebs in direction of migration, typically present during bleb retraction and indicating active blebbing dynamics (Fig. 10a, b). Occasionally bleb-mediated and pseudo-/ filopodial protrusions were simultaneously present in the same cell (Fig. 10b).

**Figure 10.** Cellular, cytoskeletal and protrusion analysis of HIF-induced amoeboid migration modes. (a) Confocal analysis of cellular and cytoskeletal amoeboid morphology by immunofluorescence and confocal microscopy. Maximum projections, s z-slice distance 2 μm. Scale bars, 10 μm. White arrows, filopods; yellow arrows, pseudopods; red arrows, blebs. (b) Quantification of protrusion types of detached UT-SCC38 cells invading collagen after HIF-stabilization with DMOG. Data from individual cells with their respective amount and type of protrusions.
Roundness is a stable feature of blebby amoeboid migration in different collagen densities

While elongated cells showed an elongation-index (EI, i.e. elongation factor EF) ranging from 5 to 15, pseudopodal and blebby amoeboid cells showed respective EF ranges of 3 to 5 and 1 to 3 after HIF-stabilization (Fig. 11a). The EF range of each HIF-induced migration phenotype was unaffected when cells moved in different collagen densities (Fig. 11a), indicating a cell-intrinsic mechanism inducing this range of migration modes. These data establish the 3D collagen model as a valid tool for assessing subtype dynamics and kinetics over time. The identification of an EF range underlying each migration mode, further allowed analysis of intrinsic kinetics versus switching behavior between single cell migration modes during migration into collagen.

To evaluate the stability (reciprocal switching between migration modes) of HIF-induced amoeboid blebby migration compared to pseudopodal and elongated migration types, we measured the elongation indices over time of HIF-induced single cell migration modes during invasion into 3D collagen after detachment from the spheroid. Round shape remained stable in blebby amoeboid cells that migrated for several hours within collagen (EF 1 to 2) (Fig. 11b). In contrast, pseudopodal (EF 2 to 5) and elongated, spindle-like cells (EF 5 to 15) displayed wider variability in elongation kinetics (Fig. 11b), inherent to stronger extension-retraction cycles (Starke et al., 2013).

Notably, whereas the blebby amoeboid phenotype – once adopted - remained stable over time, elongated or pseudopodal-amoeobid moving cells were less stable and tended to switch from elongated (EF 3 to 10) towards a roundish morphology (EF 1 to 2) (Fig. 11b, Switching category).
Figure 11. Elongation analysis and kinetics of HIF-induced single cell migration morphologies. (a) Elongation analysis of assigned 4T1 single cell migration phenotypes into collagen with low and high pore sizes after HIF stabilization with DMOG for 72 hr. EF, elongation factor. (b) Elongation kinetics during collagen invasion of individual cells detached from the spheroid after HIF-stabilization with DMOG were assigned to classified migration modes or switching behavior and represented as a heat map (E, elongated; P pseudopodial; B, blebby).
Blebby amoeboid migration is a stable and efficient migration mode

Relative survival analysis of cell migration modes after HIF stabilization showed that blebby amoeboid was the most stable migration mode (Fig. 12a). Conversely, pseudopodial amoeboid and predominantly elongated migrating cells were less stable, with approximately 20% and 50% of cells abandoning their primary migration mode, respectively (Fig. 12a). As converging phenotypic outcome after switching, both pseudopodial amoeboid and elongated migrating cells transitioned to a blebby round migration mode without reverting over hours (Fig. 12b). In addition, despite reduced division rates compared to elongated cells, stable blebby amoeboid cells were proliferating during collagen migration and continued in their amoeboid mode after cell division (Fig. 12c). Together, this suggests that HIF-stabilization favors blebby amoeboid migration mode, either directly or after varying transition time.

To compare the migration efficiency between HIF-induced stable single cell migration modes to each other as well as with DMSO control cultures, we analyzed the average cell migration speed and directionality of each mode during migration into collagen. Blebby amoeboid cells reached variably fast (0.1-0.5 µm/min) and persistent (confinement ratio: 0.1-0.6) movement in collagen (Fig. 13a, d). When comparing migration efficiencies and persistence between migration modes under HIF, migration speed of blebby amoeboid cells was highly comparable with pseudopodial amoeboid and elongated, spindle-like cells, and did not significantly differ in migration speeds and directionality from cells moving in DMSO control cultures (Fig. 13a, b, c). This consistency was not affected by varying collagen density (Fig. 13d). Together, this suggests that hypoxia and HIF induce a stable amoeboid blebby migration mode with round morphology.
Figure 12. Stability and switching behavior of HIF-induced single cell migration phenotypes. (a) Survival curve of 4T1 single cell migration modes invading into collagen recorded for 48hr after HIF-stabilization with DMOG for 48 hr. Log-rank test, **p-value=0.0019. (b) Phenotypic switching behavior and quantification of phenotype outcome post-switching of 4T1 single cells that switched shape within the first 24hr after HIF-stabilization with DMOG for 48hr. (E, elongated; P pseudopodial; B, blebby). (c) Mitosis of a blebby amoeboid 4T1 cell migrating in collagen at 72 hr after DMOG treatment. Scale bars, 10 μm.
Figure 13. Migration speed and directionality of HIF-induced single cells migrating into collagen. (a, b) Migration speed and directionality of single cells after DMSO or HIF-stabilization with DMOG treatment by time-lapse microscopy and automated cell tracking analysis. Data are represented as median with interquartile range, n=3. (c) Migration paths of DMOG-induced 4T1 migration morphologies in low dense 1.7 mg/ml bovine collagen, time (h:min). Scale bars, 10 μm. (d) Migration velocities of 4T1 single cells in low, intermediate and
highly dense collagen after HIF-stabilization with DMOG. E, elongated; P pseudopodial; B, blebbby. Data are represented as box and whisker plots with median ± min/max from three independent experiments. No statistically significant difference in speed between different phenotypes in different collagen densities (data shown) as well as within each phenotypic group in different densities (data not shown).
CHAPTER 4: SPECIFIC AIM 2

Specific Aim 2: Identify molecular mechanisms underlying hypoxia-induced migration modes

Results

Hypoxia-induced MMP-independent amoeboid movement

Amoeboid migration can be classified in a leukocyte-like nonproteolytic and MMP-dependent migration in other cell types (Peter Friedl & Wolf, 2003). To assess whether the hypoxia-induced amoeboid migration mode is dependent on MMP activity, we analyzed the extent of collagen degradation and the regulation of MT1-MMP (matrix metalloproteinase 14), the central enzyme degrading fibrillar type I collagen (Sabeh, Li, Saunders, Rowe, & Weiss, 2009). In normoxic control cultures, significant levels of collagen 3/4 fragment (col3/4) degradation epitope, emerging after MMP-mediated cleavage of collagen (Wolf et al., 2007), surrounded collective invasion zones, whereas single cells under hypoxia showed negligible pericellular collagenolysis in both 4T1 and UTSCC38 cultures (Fig. 14a). Active MT1-MMP protein levels were consistently decreased in both cell types in hypoxia and, albeit less pronounced in 4T1 cells, after DMOG treatment (Fig. 14b). The broad-spectrum MMP inhibitor GM6001, added to the spheroids at the onset of invasion, did not affect the migration speed of individually moving cells (Fig. 14c), but compromised the efficacy of collective invasion in both normoxic and hypoxic conditions (Fig. 14d). Thus, hypoxia-induced amoeboid movement of single cells occurred through MMP-independent mechanisms. In 3D confined space, MMP-independent amoeboid movement is biophysically dependent on the deformability of the nucleus (Wolf et al., 2013). Strongly deformed nuclei (smallest nuclear diameter <4 mm) were more frequent in hypoxic versus normoxic single cells (Fig. 14e). In addition, the average nuclear diameter was
significantly lower in disseminating single cells compared to remnant collective strands despite hypoxic conditions (Fig. 14f), indicating increased cell deformation during MMP-independent single-cell dissemination. Together, this indicates that hypoxia-induced ameboid cancer cell migration occurs largely independent of MMP-mediated collagen degradation.
Figure 14. Hypoxia and/or HIF-induced MMP-independent amoeboid migration. (a) Topography (maximum-intensity projections, top images) col3/4 degradation epitope staining.
as normalized values per cell in collective strands or detached cells after migration in 4mg/RT collagen. (b) Western blot analysis of MT1-MMP levels after 72 hr (4T1) or 96 hr (UT-SCC38) culture under normoxia (N) or hypoxia (H), or after treatment with DMSO (V) or DMOG (D) for 48 hr (4T1) or 72 hr (UTSCC38). (c) Migration speed of individualized 4T1 cells under hypoxia in the presence or absence of GM6001 (GM) obtained by single-cell tracking from time-lapse movies (45 cells [DMSO] or 47 cells [GM6001] were analyzed). Bars, means ± SD. (d) Length of collective strands (measured from spheroid edge towards the most distal tip of a strand) in normoxic and hypoxic 4T1 spheroids in the presence or absence of GM6001. Box and whisker plots represent the medians ± min/max values. (F) Nuclear morphologies under normoxia or hypoxia. Yellow arrowheads, significant deformation. (G and H) Frequency distribution of the smallest nuclear diameters in single cells in normoxia or hypoxia (G) and smallest nuclear diameters in collective (cc) and single (sc) cells in hypoxia (H). Black bars, means ± SD. Scale bars, 20 µm (a) and 10 µm (e). “Reprinted from Hypoxia Induces a HIF-1-Dependent Transition from Collective-to-Amoeboid Dissemination in Epithelial Cancer Cells. Current Biology, 27(3), 392–400. Lehmann, S., te Boekhorst, V., Odenthal, J., Bianchi, R., van Helvert, S., Ikenberg, K., … Friedl, P. Copyright 2017, with permission from Elsevier.”
Cell contractility as driving force underlying hypoxia amoeboid blebby migration

Cell shape and sphericity as well as bleb-mediated amoeboid movement are typically mediated by cortical and front-rear cell contractility (E. K. Paluch & Raz, 2013; E. Paluch et al., 2005). Actomyosin contraction generates contractile forces at the rear, transcellular hydrostatic pressure and bleb protrusions at the leading edge (E. K. Paluch & Raz, 2013). To test whether hypoxia- or HIF-induced conversion from elongated to blebby amoeboid migration underlies elevated cell contractility, we analyzed first the expression and activation levels of myosin light chain (MLC), the regulator subunit of myosin motor proteins (Amano et al., 1996). After culture at 0.2% oxygen or incubation with DMOG, 4T1 and UT-SCC38 cells were harvested and both total and active MLC$^{\text{pT18/S19}}$ protein levels were analyzed. Active MLC$^{\text{pT18/S19}}$ epitope was consistently increased (2- to 4-fold) in both cell types under hypoxia or after HIF stabilization with DMOG, compared to normoxic and DMSO-treated control cells (Fig. 15a). To verify myosin activation during amoeboid blebby migration, we determined MLC$^{\text{pT18/S19}}$ signal localization in migrating UT-SCC38 cells in 3D collagen culture, after detachment from the spheroid. Consistently, MLC$^{\text{pT18/S19}}$ predominantly localized at the rear and within bleb protrusions at the leading edge in HIF-induced amoeboid blebby migrating cells (Fig. 15b).

RhoA GTPase signaling via Rho kinase (ROCK) is a key regulatory signaling pathway inducing actomyosin contraction (Abele et al., 2006). In this signaling pathway, active (GTP-bound) RhoA induces ROCK which mediates activating phosphorylation of MLC at Thr18/19 (Amano et al., 1996). To address whether elevated MLC$^{\text{pT18/S19}}$ levels were ROCK-mediated, we determined MLC$^{\text{pT18/S19}}$ levels of 2D cultures after HIF-stabilization and interference with ROCK inhibition by Y-27632. ROCK inhibition significantly reduced MLC$^{\text{pT18/S19}}$ levels despite HIF-stabilization, indicating that MLC activation by HIFs was highly ROCK dependent (Fig. 15c). Concomitantly, upstream active (GTP-bound) RhoA levels were strongly elevated in 2D cultures, while active (GTP-bound) Rac-1 levels remained unchanged compared to DMSO control cultures (Fig. 15d). This suggests that HIF-signaling engages the
RhoA/ROCK/actomyosin signaling pathway, whereas Rac1-mediated actin dynamics remain unaffected.

When evaluating the dependence of HIF-induced blebby amoeboid migration on myosin activity, both blebby protrusions and migration speed were inhibited by myosin-II inhibitor blebbistatin (Fig. 16a). Blebbing activity of amoeboid single cells was significantly diminished during migration into collagen (Fig. 16d), along with a diminished average migration speed from 0.25 μm/min to 0.05 μm/min (medians), while round morphology was maintained after myosin inhibition (Fig. 26c). Likewise, interference with ROCK activity by Y-27632 abolished amoeboid migration efficiency to similar levels, while amoeboid typical round morphology remained unaffected (Fig. 16b). Together, this indicates that elevated ROCK/myosin signaling is required for bleb formation and that efficient amoeboid migration after HIF-stabilization is bleb-mediated. Importantly, abrogation of ROCK/myosin function was not sufficient to revert HIF-induced amoeboid round migration towards a pseudopodal round or elongated migration mode, suggesting that further independent mechano-signaling components of the migration machinery, required for phenotypic switching, are likely affected by HIF signaling.
Figure 15. Elevated Rho/ROCK-induced myosin activity under hypoxia and/or after HIF-stabilization with DMOG. (a) Representative Western blot and densitometric analysis of pMLC (T18/S19) over total MLC levels after 2D culture for 72 hr (4T1) or 96 hr (UT-SCC38) for indicated conditions. Normalized densitometry values show the mean of three independent experiments. (b) Visualization of activated MLC (pT18/S19) in blebby membrane protrusions (visualized by CD44 cell surface marker serving as membrane label) of UT-SCC38 blebby amoeboid cells migrating in 1.7mg/ ml bovine 3D collagen after treatment with DMOG for 96 hr. Lined arrow heads, pore between bleb and cytosol; filled arrow heads, pMLC in bleb. Scale
bars 10 μm (overview), 5 μm (zoom). (c) Immunoblot analysis and densitometry analysis of pMLC (T18/S19) over total MLC levels after DMSO or DMOG treatment and ROCK inhibition with Y-27632 for 2 hr prior to cell harvesting. n = 1. (d) Pull-down of active (GTP-bound) RhoA and active (GTP-bound) Rac1 levels in 4T1 monolayer cells after treatments with DMSO or DMOG, n = 2. N, normoxia; H, hypoxia; V, vehicle (DMSO); D, DMOG; Y, Y-27632.
Figure 16. Dependence of HIF-induced amoeboid blebby migration on ROCK and myosin activity. (a, b) Migration speed of individual UT-SCC38 and 4T1 cells from 3D spheroid culture after interference with (a) ROCK signaling (Y-27632 inhibitor; Y) and (b) myosin activity (Blebbistatin; BS). Values represent the median ± min/max values. n=3. (c) Representative migration paths and single cell morphologies (insets) of UT-SCC38 single cells after detachment from 3D spheroid culture under DMOG treatment with or without interference of myosin II function with myosin inhibitor Blebbistatin. Scale bars 100 µm (overview) and 10 µm (insets). (d, e) Analysis of single cell migration morphology (E, elongated; R, round) (d) and
membrane blebbing activity (e) of round UT-SCC38 single cells migrating in collagen recorded by time-lapse microscopy for 24 hrs after DMOG treatment with or without interference with Blebbistatin (BS) (E, elongated; R, round). Data show the mean ± SD (d) or individual data points representing the means of single cells analyzed from 5 spheroid regions (e) of three independent experiments, t-test. The amount of round and elongated cells after DMOG treatment ± BS was tested as not significant. N, normoxia; H, hypoxia; V, vehicle (DMSO); D, DMOG; BS, Blebbistatin.
Hypoxia/HIF-stabilization reduce β1 integrin activity and signaling

Amoeboid movement lacks focal adhesions and does not generate strong traction force towards collagen fibers, but instead is mediated by low-adhesive interactions and intercalation of the cell body between ECM structures (Bergert et al., 2015). Because contractility was required for kinetic bleb formation and migration (compare Fig. 16), we hypothesized that the phenotypic switch from elongated towards round morphology underlies HIF-induced alterations in the cellular adhesome.

To test whether hypoxia or HIF stabilization lead to dampened adhesion to ECM, we analyzed the expression levels and activation state of β1 integrin, which comprises the central collagen type I adhesion receptors α1β1 and α2β1 (I. D. Campbell & Humphries, 2011). After culture at 0.2% O₂ or incubation with DMOG, 4T1 and UT-SCC38 cells were analyzed for both total and active β1 integrin levels, using epitope-specific antibody staining. Active β1 integrin epitope levels were consistently reduced (0.1- to 0.4-fold) in both cell types when cultured as 2D monolayers under hypoxia or after HIF stabilization with DMOG, compared to normoxic and DMSO-treated control cells (Fig. 17a, c). In other cell models, experimental interference with β1 integrin binding to ECM substrate causes the loss of focal adhesion sites and, concomitantly, lowers Src and focal adhesion kinase activity followed by activation of the Rho/ROCK pathway and actomyosin contraction (Abele et al., 2006). As consequence of reduced adhesion and cell spreading along substrate, cells adopt a roundish shape. Similarly, after HIF stabilization, the activity state and expression of integrin downstream effectors was reduced, including active Src (pSrcT419) and p190 (RhoGAP) expression (Fig. 17b). This suggests that both hypoxia and HIF stabilization limit integrin activity as well as integrin downstream signaling.

To test whether reduced β1 integrin activation scales with lower adhesion of the cells, we established a cell isolation assay based on shear force which discriminates loosely from and firmly adherent cells in 2D monolayer culture (Fig. 17d). The adhesion status of cells harvested in this assay did not affect cell viability (Fig. 17e), establishing the differential cell assay as valid.
tool for comparing β1 integrin activity levels with adhesion status. After two days of culture in DMOG and differential cell separation, loosely adherent cells showed reduced (0.5-fold) active β1 integrin with no change in total β1 integrin surface levels, while more strongly attached cells retained or occasionally upregulated (1.4-fold) high active β1 integrin status (Fig. 17f). To verify the active β1 integrin status during collagen invasion, we determined the active β1 integrin 9EG7 epitope levels in migrating 4T1 cells, after detachment from the spheroid. Consistently, HIF-induced individual cells moving in the collagen compartment displayed reduced levels of active β1 integrin, whereas DMSO control cultures retained high levels of active β1 integrin during migration into collagen (Fig. 17g).
Figure 17. Reduced β1 integrin activity and downstream signaling under hypoxia and/or after HIF stabilization by DMOG. (a, b) Representative Western blot and densitometric analysis of active and total β1 integrin content (a) and activated Src (pTyr419), total Src and...
p190 (RhoGAP) content in pooled low- and high-adhesive cells cultured as 2D monolayer for 48 hr (4T1) and 72 hr (UT-SCC38) for indicated conditions. Normalized densitometry values show the means from three independent experiments. (c) Flow cytometry analysis and MFI of active β1 integrin expression in UT-SCC38 after treatment with DMSO or DMOG for 72 hr. (d) Workflow for harvesting and analyzing pooled or separated high- and low-adhesive cells in 2D culture. (e) Viability analysis of 4T1 low and high adhesive cells of 2D monolayer cultures treated as indicated. (f) Flow cytometry analysis and MFI of active β1 integrin surface expression in the loosely adhesive 4T1 cells after treatment with DMSO or DMOG for 48 hr. (g) Detection of primed/active β1 integrin epitope (mAb 9EG7) on 4T1 cells in 3D spheroid invasion culture after treatment with DMSO or DMOG for 72 hr. Scale bars, 100 μm (overview), 10 μm (inset). N, normoxia; H, hypoxia; V, vehicle (DMSO); D, DMOG.
Diminished β1 integrin activity is rate-limiting for the phenotypic switch towards amoeboid blebby migration

To test whether HIF-induced blebby amoeboid migration was dependent on β1 integrin function, we assessed the migration efficiency after β1 integrin blockade by mAb 4B4 added to cultures after detachment from the spheroid, when amoeboid single cell migration was established. After HIF stabilization, the migration speed of UT-SCC38 cells was partially inhibited by adhesion-perturbing anti-β1 integrin antibody 4B4 in a dose-dependent manner (Fig. 18a), while bleb-rich rounded cell morphology was unaffected. A low dose (0.5 μg/ml) of mAb 4B4 reduced the average speed from 0.125 μm/min, towards 0.75 μm/min, while a 10-fold higher dose (5 μg/ml) of mAb 4B4 further reduced the speed to a residual movement of 0.025 μm/min (Fig. 18a). This indicates that HIF-induced migration is partially β1 integrin dependent.

To test whether diminished β1 integrin function was required for the phenotypic switch towards amoeboid blebby migration, migration phenotypes were evaluated after β1 integrin activation by mAb TS2/16 (UT-SCC38) and mAb 9EG7 (4T1) added to cultures after cell detachment from the spheroid. Stabilization of β1 integrin-collagen interactions converted (-0.4-fold in 4T1, 0.4-fold in UT-SCC38 compared to IgG treated DMSO control baseline) the roundish morphology and bleb protrusions towards elongated, spindle-shaped cell shapes with pointed pseudopodal and filopod-like protrusions, despite ongoing HIF stabilization (Fig 18b). Conversely, lowering β1 integrin-mediated adhesion by mAb 4B4 or downstream Src signaling by Dasatinib induced (1.2-fold compared to treatment control) amoeboid blebby migration in otherwise pseudopodal phenotypes in UT-SCC38 cells present in DMSO-treated control culture (Fig. 18c, d). Together, these data suggest that active β1 integrin shutdown is required for inducing and sustaining blebby amoeboid migration in response to HIF stabilization.
Figure 18. Dependence of HIF-induced amoeboid blebby migration on β1 integrin function. (a) Migration paths and trajectories (left panel) and migration speed (right panel) of
individual UT-SCC38 cells in 1.7mg/ml bovine 3D spheroid culture after interference with β1 integrin function (mAb 4B4). Scale bars, 100 μm. (b, c, d) Morphology analysis of single cells after 3D spheroid collagen invasion culture with DMSO or DMOG and interference with β1 integrin activation (mAb 9EG7, 4T1, n=2; mAB TS2/16, UT-SCC38, n=2) (b), β1 integrin function (mAB 4B4, UT-SCC38, n=2) (c), and SFK activity (Dasatinib, UT-SCC38, n=2) (d). Values represent the mean +/s SD (top panels). Fold change values of the blebby phenotype are shown for one representative experiment (5 spheroid quarters with 2 regions per quarter/condition, 4T1; 5 spheroids/condition, UT-SCC38). Columns, mean values; dots, fold change of spheroid quarters (4T1) or spheroids (UT-SCC38) analyzed (lower panels). Scale bars, 100 μm (overviews), 10 μm (insets). N, normoxia; H, hypoxia; V, vehicle (DMSO); D, DMOG.
β1 integrin regulation in response to HIF stabilization is controlled by calpain-2 activity

Calpain 2 is an important regulator of integrin function in focal adhesion complexes. By cleaving the adaptor proteins talin-1 and paxillin which anchor integrins to the actin cytoskeleton, calpain-2 weakens β1 integrin-mediated ECM adhesion and facilitates the sliding of the cell rear (Franco & Huttenlocher, 2005). Thus, we hypothesized that calpain-2 is a potential master regulator for dampened β1 integrin function and transition to blebby amoeboid migration under hypoxia. To test this hypothesis, we analyzed first whether low active β1 integrin status correlates with calpain-2 levels in low adhesive 4T1 cells. HIF-stabilization with DMOG significantly increased the amount of weakly adhesive 4T1 cells which were both active β1 integrin low and calpain-2 high, compared to DMSO treated control cells (Fig. 19a).

To test whether low active β1 integrin status depends on calpain-2 activity, we tested whether calpain-2 inhibition with the selective and cell permeable calpain inhibitor PD150606 re-installs active β1 surface levels of weakly adhesive 4T1 cells after HIF stabilization with DMOG. Consistently, calpain inhibition restored active β1 integrin levels, while total β1 integrin levels remained unchanged (Fig. 19b). This indicates that calpain-2 is engaged and required for reducing active β1 integrin levels after HIF-stabilization.
Figure 19. Calpain-2 expression and functional correlation with active β1 integrin status.

(a) Correlation of calpain-2 expression and active β1 integrin status in low adhesive 4T1 cells. Values represent the mean +/- SD, n=2. (b) Representative flow cytometry histogram and MFI analysis of active over total β1 integrin content in low and high adhesive 4T1 cells after DMOG treatment with or without calpain inhibition (PD150606). Values represent the normalized MFI values of the experiment shown, n=2. N, normoxia; H, hypoxia; V, vehicle (DMSO); D, DMOG.
Elevated calpain-2 function mediates blebby round amoeboid migration

To investigate whether calpain-2 function underlies HIF-induced amoeboid migration, we determined whether calpain-2 expression and regulation are elevated under hypoxia and HIF stabilization by DMOG. In DMOG-treated 2D cultures, calpain-2 protein expression was moderately increased compared to DMSO control cultures (Fig. 20a, b), and calpain-2 expression was particularly elevated in low adhesive cells rather than in the high adhesive cell fraction (Fig. 20c).

To determine whether elevated calpain expression coincides with elevated calpain activity, the calpain activity sensor CMAC, t-BOC-Leu-Met (CMAC) was used for measuring calpain activity in 2D monolayer cells by flow cytometry. With an excitation/emission maximum at ~330/403 nm in its uncleaved substrate form, CMAC shifts to blue fluorescence with an excitation/emission maximum at ~350/430 nm upon peptidase cleavage, and thus can be used to measure peptidase activity (Maryam Niapour & Berger, 2007; Noma et al., 2009). Calpain activity (cleaved CMAC mean fluorescence intensity) was increased under hypoxia and after HIF-stabilization with DMOG in total cell populations (1.5 fold under hypoxia, 0.6-fold after DMOG treatment, compare mean MFI values), and in both low adhesive and high adhesive cells (1- to 1.2-fold, compare mean MFI values) (Fig. 20d, e), and were largely reverted to normoxic or DMSO control levels after calpain inhibition with PD150606 (100 μM) (Fig. 20d, e). Consistently with elevated calpain activity, expression levels of the endogenous calpain inhibitor calpastatin were decreased under hypoxia and DMOG-mediated HIF stabilization (Fig. 20f).
Figure 20. Calpain expression and activity in 2D monolayers. (a) Immunoblot analysis of calpain-2 expression in 2D monolayers after 72 hr (4T1) and 96 hr (UT-SCC38) for conditions indicated, n=3. (b, c) Representative flow cytometry histograms and MFI values of calpain-2 expression levels in UT-SCC38 (b) and low- and high-adhesive 4T1 2D cultures (c) for conditions indicated. MFI values indicate the means from the shown experiment, representing three independent experiments. (d, e) Representative flow cytometry histograms and MFI values of calpain activity (cleaved CMAC substrate) in 4T1 2D cultures (d) and in low- and high-adhesive cells (e) for conditions indicated, n=3. MFI values, mean values of the experiment shown. (f) Representative Western blot and densitometry analysis of calpastatin expression in
4T1 2D cultures after 48 hr for indicated conditions. Normalized densitometry values show the mean of three independent experiments. N, normoxia; H, hypoxia; V, vehicle (DMSO); D, DMOG; PD, PD150606.

To verify elevated calpain activity during collagen invasion, we determined the levels of cleaved CMAC substrate in migrating 4T1 and UT-SCC38 cells in 3D spheroid collagen culture. Both, hypoxia and HIF-stabilization by DMOG treatment induced calpain activity in both collective invasion strands and detached single cells, whereas DMSO treated 3D control cultures showed low signal intensities (Fig. 21a, b). Calpain activity was significantly elevated (CTCF > 5x10^6) in the majority of individual cells that displayed round morphologies (EF <2) after HIF-induced detachment from spheroid culture (Fig. 21c, d), while a small subset of round cells was low in calpain activity. This indicates that calpain activity largely correlates with amoeboid migration after HIF stabilization. As technical control, calpain inhibition reduced calpain activity in detached single cells (Fig. 22a, c). Calpain inhibition further significantly reduced the number of round cells (EF <2) which were high in CMAC activity (CTCF > 5x10^6) and induced predominantly elongated migration morphology, which was prevalent in cleaved CMAC^dim cells (CTCF < 5x10^6), whereas DMOG-only treated control cultures showed predominantly round morphologies which were cleaved CMAC^bright (EF <2 and CTCF > 5x10^6) (Fig. 22b, d).
Figure 21. Calpain-2 activity during amoeboid migration in 3D spheroid culture. (a) Detection of calpain activity (cleaved CMAC substrate) in 3D spheroid collagen invasion cultures of 4T1 and UT-SCC38 cells after 72 hr (4T1) and 96 hr (UT-SCC38) in collagen culture for conditions indicated (maximum intensity projections). Scale bars, 100 μm; n = 2 independent experiments. (b, c, d) Quantification of corrected total cell fluorescence (CTCF) for cleaved CMAC substrate intensities (b), cell elongation by elongation factor analysis (EF) (c) and CTCF/EF correlation (d) of 4T1 single cells invading into collagen after detachment from the spheroid for conditions indicated. Data points represent individual cells with median ± interquartile range. V, vehicle (DMSO); D, DMOG.
Figure 22. Calpain inhibition reduced calpain activity and phenotypic reversion of amoeboid blebby cells. (a) Detection of calpain activity (cleaved CMAC substrate) in 3D spheroid collagen invasion cultures of 4T1 cells for conditions indicated. Endpoint images, Maximum intensity projections, Scale bars 100 μm, n=2. (b, c, d) Quantification of corrected total cell fluorescence (CTCF) for cleaved CMAC substrate intensities (c), cell elongation by elongation factor analysis (EF) (b) and CTCF/EF correlation (d) of 4T1 single cells invading into collagen after detachment from the spheroid for conditions indicated. Data points represent individual cells with median ± interquartile range. D, DMOG; PD, PD150606.
To test whether calpain-2 is required for blebby amoeboid migration, we assessed migration phenotypes after interference with calpain function with calpain inhibitor PD150606 and specific siCAPN2-mediated calpain-2 knockdown. Calpain inhibition and transient calpain-2 knockdown both reduced blebby round migration after HIF-stabilization with DMOG and induced a phenotypic switch from round towards elongated migration morphology (median EF ~ 4.5) (Fig. 23a, b). Thus, calpain-2 activity is required for HIF-mediated blebby amoeboid migration.

Elongated, spindle-like migration is typically maintained by high ECM-adhesion forces with moderate cell contractility (Starke et al., 2013; Veronika te Boekhorst et al., 2016). Thus, we evaluated whether calpain, besides β1 integrin activity, also controls actomyosin activity after HIF-stabilization. Calpain inhibition partially reverted hypoxia- and DMOG-induced MLC activation (phosphorylation at T18/S19) (Fig. 23c), suggesting that calpain-2 supports both β1 integrin shutdown and increased myosin contractility.
Figure 23. Reversion of the blebby amoeboid to elongated phenotype after interference with calpain-2 function. (a) Representative endpoint images (left panel) and morphology analysis (right panel) of 4T1 single cells after 3D spheroid collagen invasion culture with DMSO (V) or DMOG (D) with or without interference with calpain function (PD, PD150606). Values represent the mean ± SD from two independent experiments (top panel). Fold change values
of the blebby phenotype are shown for one representative experiment (5 spheroid quarters with 2 regions per quarter/experiment). Columns, mean values; dots, fold change of spheroid quarters analyzed. Scale bar 100 μm. (b) Representative immunoblot analysis of calpain-2 expression and densitometry analysis (left panel) and migration morphology analysis of UT-SCC38 single cells after 3D spheroid collagen invasion culture with DMSO (V) or DMOG (D) with or without siRNA-mediated calpain-2 knockdown (right panel). Values represent the mean ± SD from two independent experiments (top panel). Fold change values of the blebby phenotype are shown for one representative experiment (5 spheroids/experiment). Columns, mean values; dots, fold change of spheroids (lower panel). (c) Immunoblot analysis of pMLC (T18/S19) and MLC expression of 2D monolayer cells under normoxic or hypoxic conditions with or without calpain inhibition (PD, PD150606), n=2. N, normoxia; H, hypoxia; V, vehicle (DMSO); D, DMOG.
Calpain-2 activation mediates talin-1 cleavage and lowers β1 integrin signaling

Calpain-2 has been shown to control focal adhesions by cleaving submembraneous adhesome proteins, including talin, paxillin and FAK, with talin as major focal adhesion complex protein for generating mature focal adhesions (Franco et al., 2004; Franco & Huttenlocher, 2005) (compare Fig. 2b). To understand the underlying mechanism by which calpain-2 mediates β1 integrin regulation and blebby amoeboid migration, we evaluated whether calpain-2 modulates talin-1 by proteolysis under hypoxia. Both hypoxia and DMOG treatment increased proteolytic talin-1 cleavage and the occurrence of talin-1 fragment of 190kDa, which is a characteristic product after calpain-2 mediated cleavage of full-length talin-1 (Franco et al., 2004; Franco & Huttenlocher, 2005) (Fig. 24a). Talin cleavage was reduced in 2D cultures after calpain inhibition by approximately 80-95% compared to hypoxia and DMOG control cultures, despite HIF-stabilization (Fig. 24b).

**Figure 24. Elevated calpain-mediated talin-1 cleavage.** (a) Representative Western blot of talin-1 content including full length (270 kDa) and cleavage fragment (190kDa) in 4T1 and UT-SCC38 cells at 24 hr (4T1) and 72 hr (UT-SCC38) after 2D culture for indicated conditions.
Densitometry analysis, mean values, n=3. (b) Representative Western blot of inhibited talin-1 cleavage in 2d monolayer 4T1 and UT-SCC38 cells after DMSO, DMOG with or without calpain inhibition for 24 hr (4T1) and 72 hr (UT-SCC38) (PD150606, PD, 4T1; calpeptin, CP, UT-SCC38). Densitometry analysis, mean values, n=2. N, normoxia; H, hypoxia; V, vehicle (DMSO); D, DMOG; PD, PD150606.
To verify that the calpain-2/talin axis is required for mediating blebby amoeboid migration, we addressed migration morphologies after expression of a calpain-uncleavable talin construct GFP-TalinL432G, which functionally resembles wildtype-talin function and stabilizes focal adhesions, but cannot be cleaved by calpain-2 for focal adhesion turnover (Franco et al., 2004). Transient transfection of 2D monolayers and 3D spheroid cultures migrating into collagen consistently showed GFP-TalinL432G expression (Fig. 25 a, b). Under DMOG conditions, calpain-uncleavable talin reverted amoeboid-rounded cell morphology towards an elongated, spindle-shape like phenotype, whereas GFP-transfected control cells or GFP-talinL432G transfected cells after DMSO treatment retained spherical shapes (Fig. 25c). In elongated single cells, GFP-TalinL432G was enriched in focal adhesion points towards collagen fibers (collagen reflection not shown), typically at the tip and along pseudopodal protrusions of elongated cells, and around sites of elevated cell width, such as the cell rear including the nucleus (Fig. 25d, overview (left), middle panel, arrow heads), where focal adhesions form larger clusters and become internalized (I. D. Campbell & Humphries, 2011; Roca-Cusachs et al., 2013). In round blebby cells which did not convert to elongated morphology, GFP-TalinL432G localized uniformly distributed at cortical actin that lacked focal enrichments, while at subcellular level blebby protrusions remained largely GFP-TalinL432G free (Fig. 25d, right panel, arrow heads). Together, these data show that calpain-mediated talin cleavage is required for limiting β1 integrin function and maintains the blebby amoeboid-moving phenotype under hypoxia.
Figure 25. Reversion of blebby amoeboid to spindle-shaped migration morphology after expression of calpain-uncleavable TalinL432G. (a) Immunoblot analysis for GFP expression in 4T1 cells 72 hr after transient GFP and GFP-TalinL432G transfection in 2D culture for conditions indicated, n=2. (b) Maximum projections of 4T1 cells in 3D spheroid collagen invasion culture 72 hr post-transfection during hanging drop formation. Scale bars 100 μm. (c) Morphology images (left panel) and analysis of single cell migration morphology as well as fold change in amoeboid blebby phenotype after DMSO (V) and DMOG (D) treatment combined with transient expression of EGFP or calpain-uncleavable GFP-TalinL432G. Data represent the mean ± SD (top panels), n=2. Fold change values of the blebby phenotype are shown from one representative experiment (5 spheroid quarters with 2 regions per quarter/experiment, 4T1; 5 spheroids/experiment, UT-SCC38); Columns, mean values; dots, fold change of spheroids. Scale bar, 100 μm. (d) Subcellular localization of GFP-TalinL432G in collagen invading transfected 4T1 cells after HIF-stabilization with DMOG. White-filled arrow heads, blebs; white-lined arrow heads, focalized clusters. Scale bars, 100 μm (left, overviews), 50 μm (middle panel, elongated cell), 25 μm (right image, blebby cell). V, vehicle (DMSO); D, DMOG.
Friction and cell contractility combined mediate amoeboid migration morphologies

The aggregated experimental data have shown that HIF-induced phenotypic switching from elongated to blebby amoeboid migration is induced by calpain-2 activation, which, via talin cleavage, reduces β1 integrin engagement and downstream Src and p190RhoGap signaling. Concomitantly, bleb-formation and the resulting blebby amoeboid migration were mediated by elevated RhoA/ROCK/myosin activity. To test the individual and combinatorial effect of friction and cell contractility on roundness, we adapted a subcellular element model (Zimmermann et al., 2016) for the simulation of cell length of single cell migration modes (Fig. 26a). This model describes each virtual cell as two particles, resembling the front and the rear of one moving cell. The distance between the particles represents the elongation of a moving cell. Input parameters include cell-cell adhesion strength, cell-ECM adhesion (friction), propulsion forces and cell contractility (Fig. 26a). Output parameters include the amount of single or collective migrating cells, migration speed and cell length. To test the validity of this model for predicting a hypoxic response, we first simulated “normoxic” and “hypoxic” conditions with input parameters altered for friction (reciprocal cell-ECM adhesion) and cell contractility (Fig. 26, normoxic and hypoxic conditions). When simulating hypoxic conditions, by reducing the friction from 3.0 to 1, and increasing cell contractility from 2 to 2.5, the amount of single cell migration was enhanced (Fig. 26 b, c), and single cells migrated faster reaching a longer distance from the base, compared to the collective sheet (Fig. 26b, migration paths). Furthermore, individually migrating cells showed a decrease in cell length (average cell length 0.6, compared to the “normoxic” control conditions (average cell length 0.9), while the cell speed was not altered (Fig. 26 d). These in silico results recapitulate key features of the hypoxia-induced calpain-2/β1-integrin/ Rho axis pathway for transition to amoeboid single cell migration behavior observed in 3D collagen invasion culture.

To understand the interplay between friction and cell contractility on cell length of migrating single cells, we performed a multi-combinatorial simulation for low to high friction and
contractility values (Fig. 26e). A step-wise reduction from high to low cell friction, when cell contractility was constant, resulted in a step-wise, however only moderate, reduction in the cell length of migrating single cells (Fig. 26 e). Likewise, a step-wise increase in cell contractility, when friction values were constant, resulted in a moderate step-wise reduction in cell length. Notably, when a gradual decrease in cell friction was combined with a gradual increase contractility, the cell length of individually migrating cells was reduced even stronger, i.e. a transition from elongated towards roundish morphology, compared to the change in cell length by decreasing friction only or increasing contractility only (Fig. 26 e). This suggests that enhanced cell contractility in low-adhesive amoeboid cells enhances/locks the phenotypic outcome to a round migration morphology,
Figure 26. Effect of cell contractility and friction forces on cell length (roundness) of migrating single cells by the mathematical subcellular element model. (a) Schematic representation of the subcellular element model where a virtual cell is represented by two particles (details are outlines in Chapter 2: Materials and methods). (b) Baseline model for simulation of collective sheet and single cell migration with moderate intercellular adhesion, high friction and medium contractility forces representing parameters observed for 4T1 and UT-
SCC38 migration under normoxic conditions (left panel, S1; simulation 1), as well as simulation for single cell migration behavior after parameter adjustment characteristic for a response to hypoxia, including medium/low friction and high contractility forces (right panel; S2, simulation 2). Images show endpoints of time-resolved simulations (middle panel) with zoomed detail of paired-particle (two particles represent one cell) migration (top panel), overviews (middle panel) and migration trajectories of maximum intensity projections of the entire movie (bottom panel). (c, d) Model output for the number of single cells (c) and their cell length as well as cell speed (d) for simulation S1 (“normoxia”) and S2 (“hypoxia”). (e) 3D landscape for the impact of cellular friction and contraction forces (input parameters) on the median cell length (outcome). Each column (color) represents a different combination of input parameters for friction and contractility strength, indicated by colors illustrated in (a). Column height, median cell length for each respective simulation.
HIF-induced amoeboid transition is independent of collagen substrate dimensionality

To validate these in silico findings and predictive strength of the subcellular element model, where single cell migration was model on a 2D substrate, we established a model where 3D spheroids attach, flatten out, and migrate on a 2D collagen surface (Fig. 27a). As predicted by the subcellular element model, HIF-stabilization with DMOG induced a collective-to-single cell transition at the invasion front (Fig. 27b, c). Notably, the majority of migrating single cells displayed round migration morphologies when migrating on 2D collagen, HIF-stabilization induced a conversion to blebby round migration, and reduced elongated migration phenotypes, compared to DMSO control cultures. These findings are comparable with the phenotypic switch induced during 3D collagen invasion (compare Fig. 9). Elongation analysis (heatmap representation further shows that DMOG-induced blebby and pseudopodal cells were more round (smaller EF) compared to DMSO control cells, indicated by a predominantly red (blebbby) and orange (pseudopodal) color code, compared to a mixed red/orange (blebby) and orange/yellow color code for DMSO control cells, respectively (Fig. 2e). Lastly, calpain activity was elevated in collective 2D strands and in migrating single cells with round morphology after HIF-stabilization with DMOG, compared to DMSO treated control cells (Fig. 27f). Together, these data show that the adaptation to a round amoeboid morphology are largely independent of the dimensionality of the collagen environment, indicating a cell-intrinsic molecular reprogramming underlying amoeboid adaptation. In addition, these data further strengthen the biochemistry, and flow cytometry data acquired from cells in 2D culture.
Figure 27. Spheroid model for analyzing HIF-induced single cell migration behavior on 2D collagen. (a) Schematic overview of a spheroid migration assay of 4T1 cells on a 2D collagen surface with endpoint images taken by bright-field microscopy. Scale bars, 1mm
(overview), 10 μm (zoom). (b) 4T1 single cells detached from a collective sheet on 2D collagen after DMSO or DMOG treatment for 72 hrs. Endpoint images, Scale bar 100 μm. (c, d, e, f) Number of detached 4T1 single cells (median values) (c), their migration morphology (d), elongation factors (e) and calpain activity measured by CMAC incubation (f) from 3D spheroids after culture on 2D collagen and HIF stabilization with DMOG (D) or DMSO (V) control treatment for 72 hrs. n=2. SC, single cells. Scale bars, 100 μm.
CHAPTER 5: SPECIFIC AIM 3

Specific Aim 3: Validate hypoxia-induced cellular and molecular migration phenotypes in tumor xenografts and orthotopic human tumor models in vivo

Results

Elevated calpain activity in round, amoeboid invading single cells in orthotopic human HN-SCC xenografts

To evaluate the relevance of calpain-mediated blebby amoeboid migration during local tumor growth and tissue invasion, we detected calpain activity in fresh-frozen sections from human head and neck squamous carcinoma (HN-SCC) tumor tissues orthotopically grown in nude mice. HN-31 and Detroit562 human HN-SCC tumors predominantly invaded collectively, observed as collective cell groups after vertical tumor sectioning (Fig. 28a, lined arrow heads). At the tumor stromal interface, individually migrating tumor cells were observed, which displayed round morphologies, indicative of amoeboid migration (Fig. 28a, filled arrow heads). Cleaved CMAC intensities were significantly high in the tumor compartment, compared to the surrounding stroma (Fig. 28b). Consistent with elevated calpain activity observed during 3D collagen migration in vitro, calpain activity was elevated in both, collectively and single migrating tumor cells, which displayed round amoeboid-like migration morphologies (EI range 1 to 2.2) (Fig. 28b, c). Thus, the range of high calpain activity in NHSCC tumor nests and disseminating single cells as well as a small subset of calpain-low disseminating cells was consistent between 3D spheroid culture and human orthotopic xenografts.
Figure 28. Elevated calpain activity in human HN-SCC oral cancer tumor model in mice.
(a) Maximum projections of ex-vivo detected calpain activity (cleaved CMAC) in human HN-SCC tumor tissue sections of human HN-31 and human Detroit256 tumors grown in athymic nude mice (1 tumor/cell line). Filled arrow heads, invading single cells with round morphology positive for cleaved CMAC; lined arrow heads, collective cell groups/nests. Scale bars, 100 μm (overviews), 10 μm (insets). (b) Quantification of cleaved CMAC intensities (calpain activity) in
HN-31 stroma and tumor compartments. Each data point represents a single cell or fiber. (c) Elongation factor (index) analysis of single HN-SCC cells invading the tumor stroma.

**HIF-induced calpain-2 mediates blebby amoeboid migration in vivo**

To address whether hypoxia or HIF-stabilization promotes blebby amoeboid migration in vivo, locally invasive but non-metastatic UT-SCC38 and invasive and metastatic UT-SCC58 cells were treated in vitro with DMOG to induce HIF stabilization and downstream signaling, with or without calpain inhibition (Fig. 29a, b). After 2 days of culture, preconditioned cells were transplanted orthotopically into the collagen-rich deep dermis of mice bearing a skin window (Fig. 29a, b). Migration phenotypes together with fibrillar matrix were coregistered by intravital multiphoton microscopy four hours post-implantation. DMOG-only pretreated UT-SCC38 and UT-SCC58 cells displayed predominantly blebby round migration morphologies in dermal collagen (Fig. 29c). When calpains were inhibited additionally, both cell lines showed significantly elongated morphologies, and bleb-like protrusions were largely diminished, compared to cells treated with DMOG alone (Fig. 29c, d, e). Cell elongation and reduction of blebby protrusions in the same cell were correlated after calpain inhibition, and consistently, blebs were predominantly features of roundish cells (Fig. 30a). When monitored by time-lapse microscopy, blebby round cells displayed intercalating movement through existing pores between collagen fibers (Fig. 30b, upper panel), while movement of elongated cells showed no circumpassing but displayed focal adhesion points to collagen structures (Fig. 30b, lower panel). Together, this suggests that calpain is required for round blebby amoeboid migration after HIF-stabilization in vivo.
Figure 29. HIF-induced blebbly amoeboid migration requires calpain function in vivo. (a) Model for intra-dermal tumor cell implantation into the dorsal skin-fold chamber window for
intravital monitoring of cell migration morphologies in vivo. (b) Workflow of the experimental approach, including pre-treatment, injection and imaging of HN-SCC migration morphologies in the mouse dermis. (c, d, e) Maximum intensity projections (c), morphology (d) and protrusion type analysis (e) of Lifeact-GFP expressing UT-SCC38 and Lifeact-GFP expressing UT-SCC58 cells migrating in dermal collagen after pre-treatment with DMOG with or without calpain inhibition (PD150606) prior to intradermal injection into mice. Values represent individual cells; horizontal lines, medians. 1 mouse per group, 4 regions per mouse. Scale bars, 50 μm (overviews), 10 μm (insets).
Figure 30. Correlation of morphologies and protrusion types of HIF-induced blebbby amoeboid migration after calpain inhibition in vivo. (a) Correlation of cell elongation factors with bleb-based protrusions of human UT-SCC cancer models in vivo after intradermal injection with or without calpain inhibition (PD150606). Data points represent individual cells with median ± interquartile range. D, DMOG; PD, PD150606. (b) High-resolution z-representation of single z-slices displaying interactions with collagen fibers of a round cell with blebby protrusions (DMOG, top panel) and an elongated cell with podal protrusions (DMOG combined with calpain inhibition (lower panel), arrow heads, collagen interactions. Scale bars, 10 μm. B, Blebby; E, elongated.
CHAPTER 6: DISCUSSION, SIGNIFICANCE AND FUTURE DIRECTIONS

Beyond its established functions in triggering cancer cell migration and metabolic reprogramming, we here identify HIF signaling as driver of plasticity of cancer cell migration programs promoting a conversion from epithelial collective towards amoeboid single cell migration mechanisms. HIF-induced amoeboid migration depends upon reduced β1 integrin mediated cell-ECM adhesion and increased Rho/ROCK/myosin II activity and is MMP-independent. As a master regulator induced by HIF, calpain-2 controls bleb-mediated amoeboid migration strategies by limiting β1 integrin functions through talin-1 cleavage. Through β1 integrin shutdown, HIF induces an amoeboid migration mode that may serve as a migratory escape strategy in metabolically challenged tumor cells and drive molecular diversity of cancer cell dissemination.

Molecular programs induced by hypoxia/ HIF converging towards amoeboid cell functions

Hypoxia occurs when tumor growth exceeds blood vessel supply, such as when blood vessels are malfunctioning, preferentially in the tumor core, at necrotic areas but also at peripheral tumor zones proximal to stromal interfaces. Thus, cancer cells encounter hypoxic/HIF challenge before and during invasion (Büchler et al., 2004; Lehmann et al., 2009). HIF is an established inducer of EMT, by upregulation of Snail and Twist for repression of E-cadherin, and thus a well-established inducer for the onset and reprogramming of cell migration (Imai et al., 2003; M.-H. Yang et al., 2008). In this study using epithelial cancer models, we have shown that hypoxia induces a switch from collective to single cell dissemination into 3D collagen matrices with amoeboid round type of motion as predominant outcome (Lehmann et al., 2017). At least a subset of amoeboid-moving cells contained elevated twist as molecular trait of EMT in 4T1 breast cancer cells (Lehmann et al., 2017); thus, amoeboid moving cells may retain molecular
programs which were previously ascribed to mesenchymal cells and phenotypes. On the other hand, the majority of HIF-induced 4T1 amoeboid cells and all amoeboid UT-SCC38 head and neck cancer cells lacked molecular features of EMT reprogramming, including the lack of twist, snail and vimentin upregulation (Lehmann et al., 2017). These data suggest that HIF induces other, EMT-independent molecular programs which control the functional transition from epithelial to amoeboid migration.

To induce HIF signaling, we exposed cancer cells to severe hypoxic conditions (0.2% O₂) and drug-mediated inhibition of PHD function (Panu Jaakkola et al., 2001). Our data identify HIF as a master regulator for cell reprogramming towards amoeboid cell functions, and suggests HIF-induced low proteolytic and low adhesive amoeboid migration as a cellular program to cope with metabolic challenge. Which metabolic adaptations in mitochondrial or glycolysis pathways and overall energy requirements occur in amoeboid-moving cells compared to other invasion strategies remains to be identified.

We here evaluated HIF-induced cellular reprogramming in the context of hypoxic challenge. Considering that HIF stabilization can be induced by a range of microenvironmental conditions, HIF-induced amoeboid migration may represent as an overarching program involved in cell escape when confronted with diverse microenvironmental challenges. As example, in response to growth factor signaling or cellular transformation, oncogenic PI3K/Akt/mTOR and MEK/ERK signaling modulate HIF expression levels, stability and transcriptional activity (Masoud & Li, 2015). Similarly, tumor acidosis by elevated lactate or pyruvate production and redox stress are well established inducers of HIF-signaling (Chiche, Brahimi-Horn, & Pouysségur, 2010). Whether oncogenic transformation or other metabolic challenges manifest in amoeboid escape from metabolically perturbed microenvironments remains subject of further investigation.
Integrated mechanochemical strategies used by hypoxia/ HIF-induced amoeboid moving cells

Blebby amoeboid migration is a fundamental and evolutionary conserved cell migration program, characterized for the social amoeba *Dictyostelium discoideum* i.a. under nutrient starvation, or for migrating germ cells in the zebrafish embryo (Goudarzi, Banisch, Mobin, Maghelli, Tarbashevich, Strate, van den Berg, et al., 2012; te Boekhorst et al., 2016). In cancer disease, mesenchymal-to-amoeboid conversion was observed by a range of experimental manipulation, including ECM remodeling abilities, confinement, adhesion to ECM ligands and Rho/Rac mediated actin-based protrusion and contractility regulation in (Bergert et al., 2015; Liu et al., 2015; Wolf et al., 2003). However, a (patho-) physiological natural stimulus inducing blebby amoeboid migration remained unknown. We here describe hypoxia-induced HIF stabilization as a patho-physiologically relevant stimulus for molecular reprogramming of epithelial cancer cells towards a low-proteolytic, low-adhesive and contraction-driven blebby amoeboid migration.

HIF-induced blebby amoeboid migration was observed as viable and proliferating migration mode under hypoxic/HIF challenge. Blebby amoeboid cells displayed asymmetric interactions with collagen fibers mediated by bleb protrusions towards and lateral to the direction of migration. Other conditions naturally associated with bleb formation in cells were excluded, particularly pre-apoptotic and apoptotic cell rounding and unpolarized blebbing of the entire cell body (Barros et al., 2003; G T Charras, 2008). Cells moving with blebs lacked signs of apoptosis, such as nuclear and cellular fragmentation, induction of apoptosis, such as caspase-3 activation, or cessation of migration followed by fragmentation of the cell or its nucleus. HIF-induced amoeboid migrating cells showed reduced proliferation, but retained the principal ability to divide, and when injected into the venous blood into mice, their ability for metastatic seeding was comparable with control cells (Lehmann et al., 2017). Thus, the
amoeboid blebby program reflects a physiological cell condition and is associated with survival strategies rather than cell death. However, the relevance of the amoeboid metastatic cascade as well as whether amoeboid switching is a prevalent feature during primary tumor and/or metastasis development remains unknown.

In both 4T1 and UT-SCC38 cell models studied in this thesis, HIF-induced blebby amoeboid migration was observed as a stable migration program, without phenotypic reversion towards elongated migration morphology during HIF-stabilization within several days of observation. In contrast, most, less-frequent elongated, spindle-like cells as well as pseudopodal amoeboid phenotypes were transient states under HIF challenge and, within hours, reverted towards the blebby amoeboid migration program. This indicates an early-onset diversity of migration programs induced by hypoxia, which eventually converge towards blebby amoeboid migration as a probabilistic process. However, a small subset of elongated cells stably retained the initial migration program for the time observed, which suggests hypoxia as inducer of general cell migration plasticity. The relevance of each migration program, including adaptive amoeboid blebby or adaptation-resistant spindle-like migration for metastatic disease progression remains unclear. In addition, the stability of amoeboid migration after reoxygenation remains to be identified.

As a central mechanochemical mechanism, HIF-induced adaptions towards amoeboid migration included reduced β1 integrin activity, elevated Rho/ROCK/myosin activity and very low to absent ability to proteolytically remodel the collagen matrix by MT1-MMP (Fig. 31). Jointly, these molecular states are reminiscent of amoeboid migration and experimentally induced molecular reprogramming from mesenchymal-to amoeboid migration in cancer cells (Hegerfeldt et al., 2002; Sahai & Marshall, 2003b; Taddei et al., 2014; V. te Boekhorst & Friedl, 2016), as well as in other models of cell migration, such as during integrin-independent leukocyte migration (P Friedl et al., 1998b; Lämmermann et al., 2008). Consistent with the concept of a low-adhesive migration strategy which further depends upon actomyosin
contractility, HIF stabilization reduces integrin function and induces Rho/ROCK signaling as central adaption events towards blebby round morphology. Thus, we here identified a HIF-induced natural cell-autonomous process for lowering integrin functions and increasing actomyosin contractility to reach bleb-based amoeboid movement in concert with reduced MT1 expression and proteolytic activity as an integrated program. These data integrate independently established conceptual modules of amoeboid cell migration as coexisting network to induce and maintain this particular migration mode. Further work should address the interdependency between these cellular determinants, such as whether elevated RhoA signaling is a downstream event of reduced β1 integrin signaling via Src and p190RhoGAP.

**Figure 31. Proposed mechanism underlying hypoxia/HIF-induced blebby amoeboid cancer cell migration.** HIF-stabilization under hypoxia or after DMOG treatment induces calpain-2 activity, which via talin-1 cleavage, reduces β1 integrin activity, which leads to weakened ECM adhesion and adaptation to a roundish shape. Reduced β1 signaling, via pSrc and p190, induces RhoA/ROCK signaling which activates actomyosin and regulates uropod retraction at the cell rear and blebby protrusions at the leading edge for efficient blebby amoeboid cell migration.
In our study, in silico analysis using a mathematical subcellular element model confirmed that both, a combination of cell contractility and cell friction can concert sheet-like migration towards individualization and reduce cell elongation towards roundish migration morphology. Both, friction and cell contractility were identified as rate-limiting for adaptation to a round migration mode. This slightly contradicts our wet-lab findings where reduced β1 integrin activity (friction) was identified as rate-limiting for phenotypic conversion, while cell contractility rather mediated efficient bleb formation and locked cells in a round state. The current state-of-the-art subcellular element model simulates both contraction and friction as independent parameters (Zimmermann et al., 2016), while regulation of cell contractility and friction are likely interdependent and coregulated. Thus, future work should aim to model both parameters interdependently to simulate signaling dynamics between contractility and friction in response to HIF.

**Calpain-2 as master regulator of integrin function and phenotypic switching towards amoeboid migration**

Calpains, including calpain-1 (µ-calpain) and calpain-2 (m-calpain), regulate cell migration and invasion by regulating cytoskeletal remodeling, invadopodia formation, protrusion dynamics and rear contraction (Franco & Huttenlocher, 2005; Sarah J. Storr, Carragher, Frame, Parr, & Martin, 2011). While calpain-1 predominantly regulates protrusion dynamics at the leading, Calpain-2 is an established local regulator of focal adhesions by inducing focal adhesion turnover which lowers integrin functions in subcellular regions and supports cell migration by facilitating uropod retraction (Franco & Huttenlocher, 2005; Sarah J. Storr et al., 2011). The data presented in this thesis reveal an additional global function of calpain-2 affecting the entire cell and the mode of active migration. Here, calpain-2 induces roundness and controls blebby protrusions by reducing β1 integrin activation and inducing actomyosin activity (cell contractility). Calpain-2 and its gene CAPN2 have been previously shown to be upregulated
under hypoxia by HIF transcriptional activity (Cui, Zhou, Dehne, & Brüne, 2015; Edelstein et al., 1996; Zheng et al., 2014). Our data show a moderate increase in calpain-2 expression, consistent with calpain-2 being a HIF-target. This suggests that calpain-2 is a master regulator of phenotypic switching from elongated towards amoeboid blebby migration programs after HIF stabilization. Whether calpain-1, typically involved in mediating protrusion dynamics at the leading edge of migrating cells, is engaged by HIF-induced calpain-mediated cellular reprogramming remains unclear.

Talin-1 is the major focal adhesion adaptor protein for maturing β1 integrin-mediated focal adhesion contacts to the ECM and a particularly relevant calpain-2 target involved in focal adhesion turnover (Critchley & Gingras, 2008). We identified that dampened β1 integrin function underlying HIF-induced blebby amoeboid conversion was mediated by calpain-2, as calpain inhibition as well as siRNA interference of calpain-2 restored β-integrin levels reverted amoeboid towards spindle-like migration. In addition, cleavage of talin-1 by calpain-2 is central to maintain blebby amoeboid migration given that ectopic expression of uncleavable talin reverted the phenotypic conversion and re-induced cell elongation. This suggests that the calpain/talin axis is sufficient for inducing interconversion of migration programs. In addition to talin-1, calpain-2 mediated focal adhesion turnover typically also includes the cleavage of other focal adhesion adapter molecules, including paxillin, vinculin and FAK as well as β1 integrin (Franco & Huttenlocher, 2005). Whether this broad range of target proteins is reached by calpain-2 regulation induced by HIF signaling remains to be determined.

Using the CMAC t-BOC-Leu-Met fluorescent reporter, we identified consistently elevated calpain activity in 2D monolayer cells. Consistent with other studies, calpain activity detected with CMAC was sensitive to calpain inhibition with PD150606 (Maryam Niapour & Berger, 2007; Noma et al., 2009). To link calpain activity to migration programs, we here established a novel protocol for using CMAC t-BOC-Leu-Met for detection of calpain activity of tumor spheroids within 3D collagen culture and in tumor xenografts. Calpain activity was
predominantly high in amoeboid round cells compared to elongated cells, and in the collectively migrating cohort during 3D collagen invasion and in HN-SCC tumor xenografts. Accordingly, calpain inhibition with PD150606 reduced calpain activity and reverted cell rounding to cell elongation. Thus, given this robust and consistent activity induction, calpain-2 likely engages with further targets. Besides regulation of the adhesome, calpain-2 regulates numerous other receptors relevant for cell migration, such as cadherins and β-catenin (Franco & Huttenlocher, 2005). The observed elevated calpain activity in the collective invading cell fraction thus indicates functions for calpain-2 prior to detachment of or beyond amoeboid migration regulation. However, the role of calpain activity in collective invasion and collective-to-amoeboid transition, as well as which other cell programs are controlled by calpain-2 during HIF-induced cell plasticity remains unclear.

Whether the transcriptional upregulation alone contributes to the profound increase in calpain activity, or whether calpain activity regulation by upstream effectors is sufficient to induce amoeboid phenotype remains unclear. Calpain activity is regulated by multiple factors, most importantly by its small regulatory subunit calpain S1 or S2 with which calpains form a heterodimer, by intracellular calcium levels, by its endogenous inhibitor calpastatin, and by post-translational activating or inhibiting phosphorylation (Franco & Huttenlocher, 2005). In the 4T1 breast cancer model, we observed a consistent downregulation of calpastatin protein levels under hypoxia and after HIF stabilization, which may contribute to calpain activation. However, in other cell models calpastatin expression level alone was not sufficient to activate or inactivate calpain, and likely rather functions as attenuator of calcium-activated calpain than as an inhibitor (Franco & Huttenlocher, 2005). In addition, contrasting findings have been reported on calpastatin regulation under hypoxia, with either up- or downregulation in response to hypoxic stress in a cell-type dependent manner (Blomgren et al., 1999; M Niapour, Farr, Minden, & Berger, 2012). Thus, how and whether HIF stabilization alters calpastatin levels in the HN-SCC cell model, and whether calpastatin expression levels contribute to elevated calpain activity
under hypoxia and HIF signaling remains to be tested. Interestingly, hypoxia has been shown to induce intracellular calcium levels in different cell types (Arnould, Michiels, Alexandre, & Remacle, 1992; Gusarova et al., 2011). Thus, further investigation will be needed to clarify hypoxia/HIF-induced calpain-2 activity regulation in our cancer cell models, including investigation of m-RNA levels, post-translational modification and the contribution of other calpain-2 regulators.

It remains to be clarified whether calpain activity is monophasic or cycling. In migrating single cells, calpain activity was predominantly observed in amoeboid round migration phenotypes. However, in a minority of round single cells low calpain-2 activity was observed. Variability in calpain activity could suggest that calpain activity is monophasic, and returns to baseline levels during cell migration, or is periodically engaged and cycling. In addition, parallel calpain-dependent and -independent mechanisms to lower cell adhesion may coexist and drive amoeboid migration under hypoxia even after calpain-2 activity has reverted to baseline activity. Cycling behavior could be explained by regulatory feedback loops along the HIF/ calpain-2/ integrin/ Src/ RhoA axis, which modulate signaling intensity of both upstream inducers and downstream effectors of calpain-2. For example, calpain can degrade HIF-1α (Nanduri et al., 2009; J. Zhou, Kö, Herr, Frank, & Brü, 2006), and potentially induce its own negative regulation; thus, under sustained hypoxia cyclic behavior could be maintained. A positive feedback from RhoA to HIF, from HIF to RhoA, and from Src to calpain has been described as well (Carragher et al., 2002; Gilkes, Xiang, et al., 2014; Hayashi et al., 2005; Matoba et al., 2013). The magnitude of such feedback loops may contribute to either continuous or discontinuous calpain-2 expression and activity, likely on single cell level. Thus, mathematical cellular signaling models should be used to identify signaling dynamics which predict calpain-2 cycling behavior and whether calpain-2 hysteresis is present in the amoeboid system. This will clarify whether a calpain-2 low activity status can still co-occur with a RhoA high (contractile) and low β1 integrin
low (low adhesive) state, under which cells display round morphologies and which is required for amoeboid migration.

**Calpain-2 as biomarker and molecular target in cancer**

Calpains, calpain-1 and calpain-2, have been implicated as regulators of cytoskeletal dynamics underlying cancer cell migration and metastasis (Leloup & Wells, 2011; Sarah J. Storr et al., 2011). Aberrant activity and expression levels of the calpain system deregulates adhesion, cytoskeletal organization and promotes invasion in several human cancers, including bladder, prostate, breast and lung cancer (Leloup & Wells, 2011). Beyond their contribution to cell migration and invasion, calpains further regulate oncogenic transformation, proliferation, apoptosis, survival and angiogenesis in numerous cancer types (Leloup & Wells, 2011; Sarah J. Storr et al., 2011). Therefore, targeting calpains may inhibit cancer cell dynamics, transformation, growth, and survival simultaneously. However, deregulation of the calpain system is highly diverse among different cancer types and can involve abrogated expression and/or activity of calpain-2, calpain-1, calpastatin, calpain small regulatory subunits S1 and/or S2, dependent on cancer type and progression state (Leloup & Wells, 2011; Sarah J. Storr et al., 2011). Thus, multiple inhibitors or interference strategies might be required to target deregulated calpain functions. In addition, due to their numerous functions, calpains exert pro-apoptotic but also pro-survival functions in a cancer subtype dependent manner. Thus, to evaluate whether calpains serve as reliable prognostic marker for disease progression and prognosis of patient survival and whether calpains will be suited as molecular target, will require careful stratification of their function and contribution in different cancer types.

In a set of specific cancers, including colorectal cancer, HN-SCC head and neck cancers and subtypes of breast cancer, specifically calpain-2 expression and activity levels are elevated in the primary tumor, lymph node metastasis and distant metastasis, compared to normal tissue (Lakshmikuttyamma, Selvakumar, Kanthan, Kanthan, & Sharma, 2004; Leloup &
Wells, 2011; Spirina et al., 2013; S J Storr et al., 2012; Sarah J. Storr et al., 2014). As calpain activity was measured by molecular enzyme assays from bulk biopsies, it remains unclear in which tumor compartments, including tumor stroma vs tumor mass, or non-invasive vs invasive tumor fraction, calpain-2 activity was elevated. Across these tumor types, elevated calpain activity correlates with accelerated disease progression, and with poor patient survival in basal-like and triple negative breast-cancer (S J Storr et al., 2012). We here established a histochemical assay for detection of calpain activity combined with morphometric analysis by 3D reconstruction of tumor xenografts. Consistent with other findings in HN-SCC cancer, calpain activity levels were high in HN-SCC tumor xenografts. Sub-compartmental analysis shows that calpain activity levels are predominantly elevated in the invasive tumor fraction, including collective and individual amoeboid cells with high calpain activity, compared to the surrounding cell- and collagen rich stroma. This delivers a proof-of-concept for the role of calpain activity in mediating amoeboid migration in other HN-SCC tumor models. Whether the highly calpain active collective invading groups reflect a stressed niche that reacts to redox challenge and serves as a reservoir for amoeboid cell detachment remains to be determined.

**Further therapeutic implications**

We here identified a HIF-induced calpain-2/integrin/Src/RhoA/ROCK axis for cellular reprogramming towards amoeboid migration under hypoxic challenge. To delineate therapeutic implications for the targeting of this newly identified pathway for successful interference of migration and its contribution to metastasis, it will be essential to identify (i) whether HIF-induced amoeboid migration contributes to metastatic progression, (ii) which migration program present under hypoxic challenge, including elongated versus amoeboid migration, is the most relevant phenotype for metastatic disease progression.

However, the ability to transition between both, mesenchymal and amoeboid as well as
collective and single cell migration modes, equips moving cancer cells with a versatile repertoire of dissemination and adaptation strategies. Such plasticity responses in cancer often reflect migratory escape and therapy-resistance niches, and contribute to poor efficacy and limited treatment outcome in the clinics (te Boekhorst & Friedl, 2016). Thus, mechanistic insight in regulatory pathways that control plasticity responses is valuable for estimating and/or predicting treatment outcome.

Likely, hypoxia-induced amoeboid adaptation represents a challenging niche for therapeutic targeting. As example, the amoeboid switch towards protease-independent migration allows migratory escape to treatment strategies aimed to prevent cancer cell migration and metastasis by targeting cell proteolysis and ECM remodeling (Wolf et al., 2013). Likewise, integrin targeting with RGD-related peptides or integrin blocking antibodies might be ineffective to abrogate low-adhesive amoeboid migration. On the contrary, interference with integrin function or downstream Src signaling might induce amoeboid migration in otherwise normoxic cell populations, and promote migratory escape (Hegerfeldt et al., 2002).

Our data revealed drug-based inhibition of calpain activity as well as mAb-based integrin activation as effective strategies to prevent the calpain-2 mediated amoeboid switch under HIF-challenge. Whether cytotoxic responses can be yield at higher doses of calpain inhibition, beyond low-dose mediated inhibition of phenotypic switching, remains to be investigated. In addition, interference with ROCK/myosin activity highly impairs the efficiency of hypoxia-induced amoeboid migration. Notably, compromising actomyosin contractility, ROCK inhibitors not only successfully inhibit amoeboid migration, but also mesenchymal and collective migration programs (Sadok et al., 2015). Thus, ROCK inhibition, currently awaiting evaluation in clinical trials for new generation ROCK inhibitors, may be particularly suited to limit (patho-) physiologically- or therapy-induced plasticity and efficiency of cancer cell migration.
Conclusive summary

Using a combinatorial approach of in vitro 2D and 3D culture, ex vivo tumor xenografts, in vivo migration models and in silico mathematical modelling, we here establish HIF signaling as microenvironmentally regulated pathway for cell reprogramming towards blebby amoeboid migration in cancer cells. Our data identify calpain-2 as master regulator upstream of β1 integrin shutdown, via talin-1 cleavage, which induces and maintains the conversion from elongated to blebby amoeboid migration strategies. The HIF induced amoeboid migration program represents a migratory escape strategy in epithelial tumor cells to abandon metabolically perturbed tissue regions, and drives molecular and functional plasticity of cancer cell dissemination. The relevance of this cell biological program for metastatic disease progression and/ or therapeutic intervention requires further investigation.
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