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GETTING IN AND OUT OF SHAPE:

RELEVANCE FOR ORGAN FORMATION AND CANCER

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Karolinska Institutet

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Yo Adrian, I DID IT !!!
/Rocky Balboa

To my love Jessica and my Family

Getting in and Out of Shape: Relevance for Organ Formation and Cancer
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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ABSTRACT

A central question during development is how single cells form functional multi-cellular organ structures. The high reproducibility indicates intricate synchronization of cellular behaviors such as migration, proliferation and cell shape changes. How mechanical signals or forces regulate cell shape changes is less studied, however several reports indicate a role of mechanical forces in malignancy Increased force or stiffness in the matrix cause loss of tissue architecture associated with tumor progression. Important hallmarks of advanced cancerous tumors are the loss of epithelial character from the original tissue and the appearance of more mesenchymal-like cells, especially at the periphery, where the tumor cells are in contact with surrounding stromal cells. Typical of this epithelial–mesenchymal transition (EMT) is the loss of cell–cell adhesion and apical–basal cell polarity as well as the increased motility of tumor cells. Although the importance of EMT for tumor progression is widely accepted much less is known about the relationship between cell polarity and early events in carcinogenesis. The aim of this thesis was to study the role of AmotL2 during blood vessel formation and tumor progression.

In this thesis it is demonstrated that angiomotin-like 2 is expressed as two isoforms with distinct functions. The longer isoform p100 AmotL2 is localized to cell-cell junctions, and associates to the VE-cadherin complex where it couples adherent junctions to contractile actin fibers. Using gene inactivation strategies in zebrafish, mouse and endothelial cell culture systems, we show that inactivation of p100 AmotL2 dissociates VE-cadherin from cytoskeletal tensile forces that affect endothelial cell shape. We report that AmotL2 is essential for vascular lumen expansion, by transmission of junctional force between cells. We propose a novel mechanism for which transmission of mechanical force is essential for the coordination of cellular morphogenesis.

This thesis also provides data regarding p60 AmotL2, the shorter isoform. We show that hypoxic stress activates c-Fos dependent transcription of p60 AmotL2 resulting in disruption of apical basal polarity. Activation of p60 AmotL2 results in formation or of large intracellular vesicles that sequester Crb3 and Par3 polarity complexes inside the cell. In human tumors from breast and colon cancer patient's p60 AmotL2 expression correlates with loss of tissue architecture and cell polarity. Furthermore we provide data showing that p60 AmotL2 acts as a p100 AmotL2 antagonist sequestering p100 into intracellular vesicles. This results in alterations in actin reorganization and weakened cell-cell adhesions. These data point to a novel pathway, which controls metastatic spread.

LIST OF SCIENTIFIC PAPERS

I. **AmotL2 links VE-cadherin to contractile actin fibres necessary for aortic lumen expansion.**

Sara Hultin, Yujuan Zheng, **Mahdi Mojallal**, Simona Vertuani, Christian Gentili, Martial Balland, Rachel Milloud, Heinz-Georg Belting, Markus Affolter, Christian S.M. Helker, Ralf H. Adams, Wiebke Herzog, Per Uhlen, Arindam Majumdar*, Lars Holmgren*. Nat Commun. 2014 May 7;5:3743

II. **AmotL2 disrupts apical-basal cell polarity and promotes tumour invasion.**

Mahdi Mojallal*, Yujuan Zheng*, Sara Hultin, Stéphane Audebert, Tanja van Harn, Per Johnson, Claes Lenander, Nicolas Fritz, Christin Mieth, Martin Corcoran, Marja Hallström, Johan Hartman, Nathalie Mazure, Thomas Weide, Dan Grandér, Jean-Paul Borg, Per Uhlén, and Lars Holmgren. Nat Commun. 2014 Aug 1;5:4557

III. **p60 AmotL2 acts as a molecular clutch by disengaging E-cadherin from radial actin filaments.**

Mahdi Mojallal, Aravindh Subramani, Shigeaki Katani, Xiaofang Cao, Jose Nobre, Staffan Johansson, Panos Anastasiadis, Per Uhlen and Lars Holmgren. *Manuscript.*

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LIST OF ABBREVIATIONS

α-catenin	Alpha catenin
AJ	Adherent junction
Amot	Angiomotin
AmotL1	Angiomotin-like 1
AmotL2	Angiomotin-like 2
AP-1	Activator protein 1
aPKC	Atypical protein kinase c
ARNT	Aryl hydrocarbon receptor nuclear translocator
β-catenin	Beta catenin
β1-integrin	Beta 1 integrin
bZIP	Basic leucine zipper
Caco2	Human colonic adenocarcinoma cells
CAF	Cancer Associated Fibroblast
CAIX	Carbonic Anhydrase IX
CD44	Cell adhesion 44
CDC42	Cell division control protein 42
cDNA	Complementary DNA
COP II	Coat protein II
COPI	Coat protein I
Crb	Crumbs
DA	Dorsal aorta
dEP1	Density-enhanced phosphates 1
Dlgl	Disc large
DNA	Deoxyribonucleic acid
E-cadherin	Epithelial cadherin
E3 ligase	Ubiquitin ligase
EC	Endothelial cell
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
EphA4	Ephrin type-a receptor 4
EPLIN	Epithelial protein lost in neoplasm
ER	Endoplasmatic reticulum
ERK	Extracellular signal-regulated kinases
F-actin	Filamentous actin
FDA	Food and drug administration
FGF	Fibroblast growth factor
G-actin	Globular actin
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factors
GLUT1	Glucose transporter
GTP	Guanosine triphosphate
GUK	Guanlyate kinase
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HRE	Hypoxia responsive element
Jak/STAT3	Janus Kinase/Signal Transducer and Activator of Transcription
Lgl	Lethal giant larvae
LOX	Lysyl oxidase
MAF	Musculoaponeurotic fibrosarcoma

MAGI1	Membrane associated guanylate kinase
MAPK	Mitogen-activated protein kinases
MDCK	Madine Darby canine kidney
MET	Mesenchymal to epithelial transition
miR-491-5p	MicroRNA-491-5p
mRNA	Messenger RNA
MS1	Mammalian endothelial 1
N-cadherin	Neural cadherin
NF2	Neurofibromatosis type II
NSCLC	Non small cell lung carcinoma
Pals 1	Protein associated with Lin-7 (Pals)1
Pard	Partitioning defective 3
Patj	Pals1-associated tight junction
PB1	Phox Beam 1
PDZ	PSD-95-Discs Large-zona occludens-1
PECAM	Platelet endothelial cell adhesion molecule
PI3K	Phosphatidylinositol-4, 5-bisphosphate 3-kinase
Prrx1	Paired related homeobox 1
PTP	Protein-tyrosine phosphates
Rac	Ras-related C3 botulinum toxin substrate
Ras	Rat sarcoma viral ongogene homolog
RASIP 1	Ras interacting protein 1
RNA	Ribonucleic acid
Scrib	Scribble
siRNA	Small interfering RNA
SMC	Smooth muscle cell
SV40	Simian virus 40
Syx	Synectin-binding guanine exchange factor
TGF-β	Transforming growth factor beta
TGN	Trans golgi network
TIC	Tumor initiating cell
TJ	Tight junction
TPA	Tumor promoter 12-O-tetra- decanoylphorbol-13-acetate
TRAPP	Transport protein particle
TRE	Tpa responsive element
Twist	Twist-related protein
Ve-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	Von hippel lindau
YAP	Yes associated protein

1 INTRODUCTION

1.1 FOREWORD

The appearance of cells and the ability to change structure and shape has been well studied and documented during the 20th century. As far back as 1917, D'Arcy Thompson published a paper *'On growth and form'* in an attempt to explain cell shape changes during morphogenesis by using simple mathematical and physical formulations.

In this thesis I will touch upon mechanisms that is needed to establish and maintain cellular architecture, but also how these traits are then deregulated during malignant transformation. I believe that in order to be able to understand a disease, one must first understand how cells interact under normal conditions. The getting in and out of shape concept reflects on cells ability to change structure and shape when it comes to for example forming a tube or an organ, and how this organized structure is lost during tumor progression. Important hallmarks of advanced tumors are the loss of structure and character from the original tissue, and the appearance of more mesenchymal-like structure.

2 TISSUE DEVELOPMENT AND HOMEOSTASIS

Cells are the building blocks of multicellular organisms. Cells make tissues and tissues make organs, which provide functionality to the living creature. The more complex the organism the more organized and specialized are the cells.

There are many different types of cells, but all have the same basic structure. All cells consist of a **cell membrane** that envelops the cell, regulating what moves in and out. Cells also have a **cytoskeleton** that organizes and maintains shape. All cells contain two different kinds of genetic material, **DNA** and **RNA**. **Organelles** analogous to the organs of the human body are also present in cells. The organelles in the eukaryotic cells consist of a **nucleus**, where all the genetic material is stored, **mitochondria** which generates the cells energy, **endoplasmatic reticulum** that with the **Golgi apparatus** forms the cell packing system, **centrosome** the cytoskeleton organizer and **lysosomes** that contain digestive enzymes that breaks down excessive or worn out organelles.

During embryogenesis, the inner cell mass of the egg consist of a single layer of cells that migrate through the primitive streak turning single layer, into two and later into three layers of cells. These layers are called the germ layers and give rise to all the tissues in the body.

Each layer forms specific tissues and organs in the embryo. The outermost layer, the **ectoderm** differentiates to form the nervous system. The middle layer, the **mesoderm** forms connective tissue and muscle tissue in the body. And the inner layer, the **endoderm** gives rise to epithelium of the digestive system and the respiratory system.

For cells to be able to organize into tissues various biological processes are essential. Cells must be able to sense their environment, including where they are in relation to their neighbours. This is mediated by direct interaction of cells with the ECM through various receptors, such as integrins (Yu, Datta et al. 2005). Cells will also sense and alter the assembly, stiffness and composition of the ECM (Kass, Erler et al. 2007). Communication with other cells through the adhesion molecules, such as the cadherins (Gumbiner 2005), these cues provide instructions that enable cells to begin to assemble into groups. In a forming tissue, cells must coordinate asymmetrical distribution of polarity proteins (Wang and Nathans 2007). As a consequence the cytoskeleton and the cell trafficking machinery organize asymmetrically (Goldstein and Macara 2007).

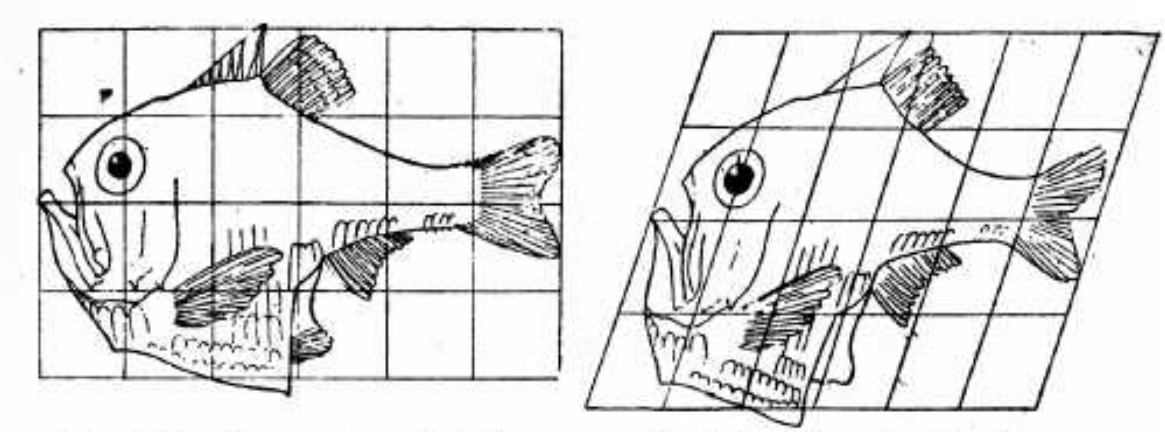

Fig. 517. *Argyropelecus Olfersi.* Fig. 518. *Sternoptyx diaphana.*

Figure 1. Example of Thompsonian transformation grids. Here illustrating transformation of *Argyropelecus olfersi* into *Sternoptyx diaphana* by applying a 20° shear mapping. Figure taken from Wikipedia.com

2.1 CELL POLARITY

Cell polarity is a fundamental feature that describes the ability of the cell to create order and organization. The organization seen at a macro-molecular level (organs, tubes and sheets of cells) is a reflection of organization observed at a micro-molecular level (the individual building blocks of the cell).

Almost every eukaryotic cell is asymmetric or polarized. For individual cells to form a tissue, polarity must be arranged in space and time (O'Brien, Zegers et al. 2002). Biological processes such as cell division, cell death, shape changes, cell migration and differentiation, must be coordinated with the polarity requirements of a tissue to form an organ (Nelson 2003).

There are different types of polarity (figure. 2) that are thought to regulate morphogenesis of epithelial structures: **planar cell polarity** which is the polarization of a field of cells within the plane of the same sheet, **front-rear polarity** used by migrating cells to maintain directionality, **mitotic-spindle polarity** used for positioning of daughter cells during cell division and **apical-basal polarity** which is the asymmetrical distributions of proteins and organelles along the apical-basal axis.

In this thesis I focus on Apical-basal polarity due to its prominent role in the acquisition of cell shape and directional transport.

The establishment of apical –basal polarity allows for changes in cellular structure and positioning of cell organelles in order to maintain asymmetry. The existence of non-coalescent apical and basolateral plasma membrane domains, which have a differential composition of proteins and lipids, is a feature of polarization allowing for directional transport of molecules across the epithelial sheet. Differences in membrane composition was shown back in 1982 when MDCK cells infected with viruses budding from either the apical or basolateral membrane had unique phospholipid compositions (van Meer and Simons 1982). In epithelial structures, such as the intestinal lining the apical membrane faces the luminal space and is distinguished by the presence of microvillus. The basal compartment is attached to the basement membrane. The asymmetric composed membranes has a polarized trafficking machinery that is composed of secretory organelles (the endoplasmic reticulum (ER) and Golgi complex) and endosomal compartments that are required for the generation and maintenance of the asymmetric distribution of plasma membrane proteins. Another feature of polarization is the correct positioning of the cell nucleus resulting in asymmetric cell division.

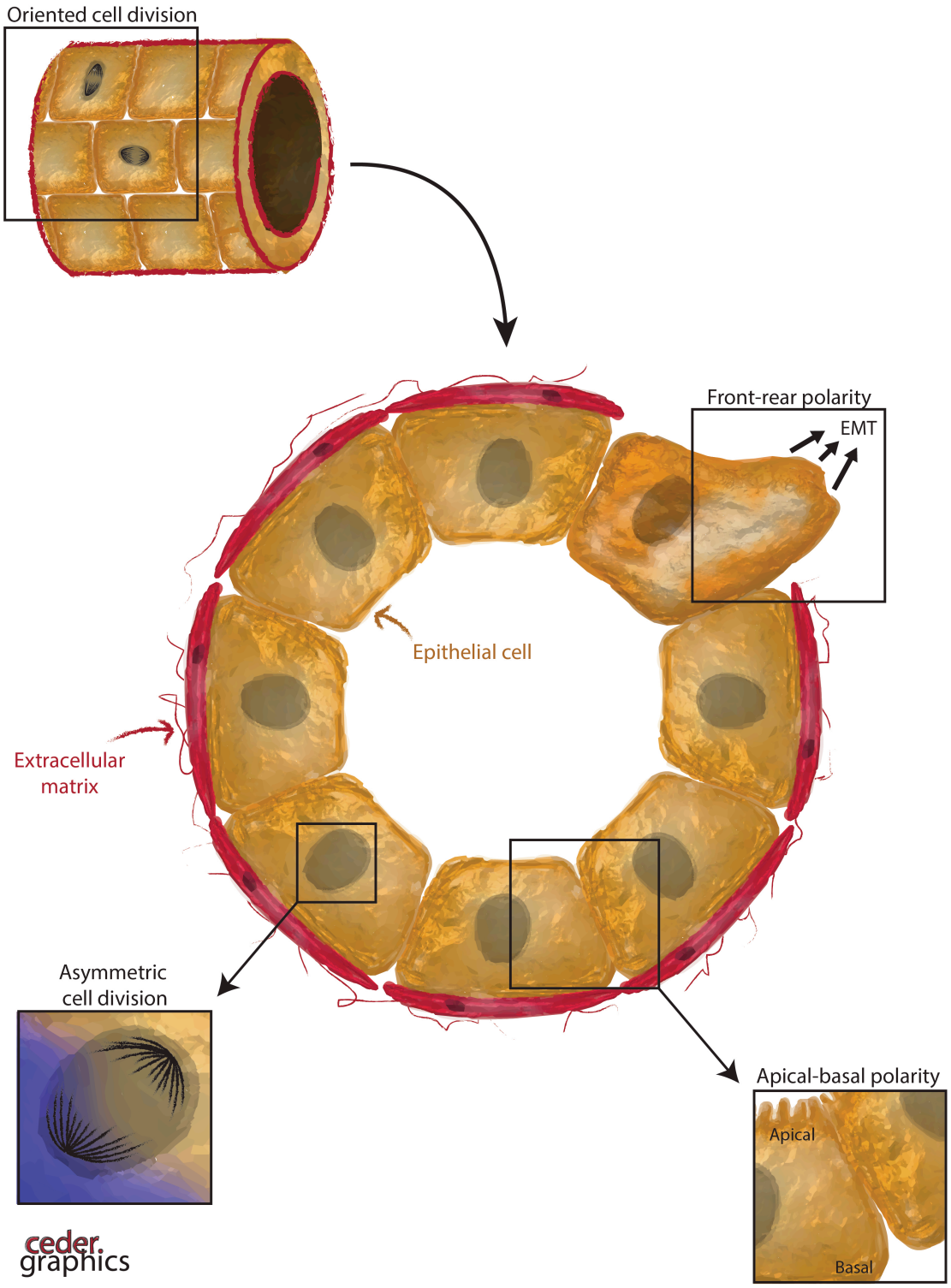

Figure 2. Different types of polarity that regulates morphogenesis. *Adapted from Muthuswamy et al 2012, Annu. Rev.Cell. Dev Biol.*

The general steps of cell polarity including its establishment and maintenance are highly conserved from Drosophila to humans. Mechanisms that promote the establishment of

apical-basal polarity vary between different model organisms and mammalian epithelial cells. However in epithelia, the formation of polarized cell layers depends on cell adhesion complexes and polarity protein complexes. The adherent and tight junctions contribute to the formation and maintenance of the apical and basolateral domains mainly through two adhesive complexes: cadherin–catenin and nectin–afadin (Niessen and Gottardi 2008). Studies conducted in late 1980's showed that the apical pole is independent of junction formation while formation of a basolateral membrane requires cell-cell interactions (Vega-Salas, Salas et al. 1987). The ECM, in particular the basement membrane also plays an important role in orienting apical-basal polarity. More specifically, the integrins have been recognized as essential mediators of apical-basal polarity. Genetic deletions of the β1 integrins have revealed that they have a central role in establishing cell polarity (Lee and Streuli 2014).

The polarity proteins that regulate apical-basal polarity are grouped into protein complexes according to their known localization: the subapically localized PAR (Cdc42/Par3/Par6/aPKC) and Crumbs (Crb/Pals1/PATJ) complexes and the basolaterally localized Scribble complex (Scrib/Lgl/Dlg) (Bryant and Mostov 2008). During the establishment and maintenance of cell polarity, the polarity complexes interact with each other. For example Scribble restricts the activity of the Crumbs complex to the apical domain (Bilder and Perrimon 2000) and thus the polarity complexes antagonize each other.

To gain further insight into how apical-basal polarity is regulated a short summary of the protein complexes involved follows.

2.1.1 PAR Complex

The PAR (*partitioning defective)* genes was first identified as proteins needed for asymmetric division during early embryogenesis in *C.elegans* as mutations in the PAR genes lead to defects in cell cleavage (Kemphues, Priess et al. 1988). In mammalian epithelium PARD3, PARD6 and atypical protein kinase C (aPKC) form a Par complex that localizes to apical tight junctions and regulates apical junction formation (Hirose, Izumi et al. 2002). PARD6 binds to aPKC through its PB1 domain and functions as an inhibitor of aPKC kinase activity and as a targeting subunit to recruit aPKC substrates, including PARD3 (Hirano, Yoshinaga et al. 2005).

Upon phosphorylation, PARD3 is lost from the apical membrane and localizes to adherent junctions in which it functions independently from PARD6 and aPKC (Morais-de-Sa, Mirouse et al. 2010). The Par complex proteins have also been shown to be deregulated during transformation. PARD6 is amplified and overexpressed in breast cancer (Nolan, Aranda et al. 2008). In contrast loss of Par3 has been reported to promote breast tumorigenesis and metastasis (McCaffrey, Montalbano et al. 2012; Xue, Krishnamurthy et al. 2013).

2.1.2 Crumbs Complex

Another complex located at the apical membrane is the Crumbs complex. Crumbs is a transmembrane protein, Crumbs (isoforms CRB1-3) is associated with Pals1 (MPP5), and PATJ (INADL) scaffold proteins. During polarization, the Crumbs complex establishes apical identity by recruiting actin cytoskeletal regulators such as moesin and βH-spectrin (Medina, Williams et al. 2002). In Drosophila embryos, *crumbs* mutation fail to assemble or stabilize junctions, which leads to breakdown of epithelial structure and extensive cell death (Tepass, Theres et al. 1990). Down-regulation of CRB3 in immortal embryonic mouse kidney cells promote tumor progression and metastasis (Karp, Tan et al. 2008).

2.1.3 Scribble complex

The Scribble complex is localized at the basolateral domain and is required for restricting the apical domain by antagonizing the Par complex (St Johnston and Sanson 2011). Scribble complex consists of three members, Scribble (SCRIB), Discs large (isoforms, Dlg 1-5) and Lethal giant larvae (isoforms, LLGL1-2). Scribble associates indirectly with Dlg via a linker protein, guanylate kinase (GUK), but binds directly to LLGL2 through its LRR (leucine-rich repeat) domain. In Drosophila mutations of *Scrib* cause defects in epithelial organization that results in embryonic lethality (Bilder and Perrimon 2000). It has also been proposed that Scrib could act as a tumor suppressor by regulating epithelial cell adhesion and migration as Scrib depletion disrupts E-cadherin mediated cell-cell adhesion (Qin, Capaldo et al. 2005).

2.2 CELL-CELL JUNCTIONS

Cell junctions are multi-protein complexes that connect neighbouring cells. They allow for the preservation and proper functioning of epithelial sheets. These junctions are also important in organization of tissues where cells can only adhere to other cells from the same tissue. Cell junctions are divided into four groups: *tight junctions* (TJ), *adherent junctions* (AJ), *desmosomes* and *gap junctions.*

2.2.1 Adherent Junctions

Adherent junctions (AJ) serve as points of cell-cell attachment that also anchor the actin cortex to the apical circumference of the cell, allowing contractile forces to be transmitted between cells (Niessen, Leckband et al. 2011). AJ's in epithelial cells contain the homophilic cell adhesion molecule E-cadherin whereas endothelial cells express VE-cadherin. E-cadherin is an epithelium-specific cadherin that is required for the development and maintenance of the normal function of all epithelial cells in tissues. The loss or down-regulation of E-cadherin is a key event in the process of tumor invasion and metastasis (*the deregulation of E-cadherin and its consequence is further discussed in paragraph 3.3*).

VE-cadherin is an endothelium-specific cadherin, which plays a relevant role in vascular homeostasis. It has been demonstrated that VE-cadherin is required for normal vasculogenesis, angiogenesis, and for the maintenance of vascular integrity (Dejana, Orsenigo et al. 2008). Loss of VE-cadherin results in apical-basal polarity and cytoskeletal defects (Carmeliet, Lampugnani et al. 1999; Strilic, Kucera et al. 2009; Harris and Nelson 2010).

The extracellular domain of VE/E-cadherin mediates homotypic interactions with molecules on neighbouring cells whereas its intracellular tail interacts with the actin cytoskeleton through binding β-catenin and α-catenin. Because α-catenin binds actin filaments, the VE/E-cadherin–β-catenin–α-catenin complex is thought to link adherens junctions to the actin cortex. Although biochemical studies suggest that mammalian α-catenin cannot bind β-catenin and F-actin at the same time (Drees, Pokutta et al. 2005; Yamada, Pokutta et al. 2005). The E-cadherin complex might be linked to F-actin via additional proteins, such as EPLIN (Abe and Takeichi 2008) or vinculin (Yonemura, Wada et al. 2010).

2.3 INTEGRINS AND THE EXTRACELLULAR MATRIX

The extracellular matrix (ECM) consists of tissue-specific proteins such as collagen, laminin and fibronectin and non-matrix proteins such as phosphatases, kinases and growth factors (Naba, Hoersch et al. 2012). Adhesion complexes at the cell surface physically link the ECM to the cytoskeleton. For example, focal adhesions (FAK), comprised of integrins, talin, vinculin and other proteins, connect the ECM to actin filaments (Brakebusch and Fassler 2003). Besides physically connecting cells within tissues, ECM's act as elastic scaffolds that resist cell-traction forces and thereby regulate tissue development by modifying physical force distributions, changing the cellular force balance, and altering cell shape (Huang and Ingber 1999; Beloussov, Louchinskaia et al. 2000). Changes in cell shape can alter diverse biological functions including: cell migration, growth, apoptosis, differentiation, contractility and cellular self-assembly (Chen, Mrksich et al. 1997; Engler, Sen et al. 2006; Alcaraz, Xu et al. 2008; Guo, Ouyang et al. 2012).

Epithelial branching morphogenesis is an example in which ECM remodeling play a central role in regulating final tissue form, as well as in amplifying the total tissue surface area available for molecular exchange in organs, such as lung, kidney, pancreas and mammary gland. These epithelial tissues exhibit 3D treelike structures that are built through budding and branching, which are controlled through mechanical interplay between cells and their ECM scaffolds. For example, during the formation of epithelial branching patterns in the embryonic mouse lung, a mechanical balance between cell-generated traction forces and differences in the ability of the underlying ECM to resist these stresses in different regions of the growing organ has been observed. Budding and cell proliferation are enhanced in regions of ECM thinning that exhibit increased flexibility, whereas growth is suppressed in regions that exhibit a thicker and more rigid basement membrane (Moore, Polte et al. 2005).

Cells can through their integrins sense the rigidity of the ECM, and adjust their tension accordingly (Saez, Buguin et al. 2005). When cells attach to stiff matrices they form large clusters of focal adhesions, binding more stress fibers leading to higher tension. As a consequence cell-cell adhesions weakens, resulting in disruption of the actin adhesion belt (Tsai and Kam 2009; DuFort, Paszek et al. 2011). However when cells are attached to softer matrices, integrin clusters are fewer. Also the binding of integrins to stress fibers is less, resulting in lower tension and stable adherent junctions (DuFort, Paszek et al. 2011). The cadherins located at the cell adhesion sites have mechanosensing properties. VE/E cadherin can through their binding to actin transmit force.

2.4 CELL SHAPE

The paragraphs thus far have described features essential for tissue morphogenesis and regulation of cell shape and size. Another important feature is the contractile actin cytoskeleton. The following paragraphs will give a general overview of the cytoskeleton, but will mostly focus on the actin cytoskeleton since it is believed to be the main mediator of mechanical force during morphogenesis and homeostasis.

2.5 THE CYTOSKELETON

The cytoskeleton is the framework of intra-cellular filaments that provide support for cellular shape, motility and contraction. Eukaryotic cells contain three main cytoskeletal filaments: *microfilaments* (actin filaments), *intermediate filaments* and *microtubules*. Microtubules are considered to be the largest type of filament, with a diameter of about 25 nanometers (nm), and they are composed of a protein called tubulin (Fletcher and Mullins 2010). Actin filaments are the smallest type, with a diameter of about 6 nm, they are made of a protein called actin. Intermediate filaments, as their name suggests, are mid-sized, with a diameter of about 10 nm (Munjal and Lecuit 2014). Unlike actin filaments and microtubules, intermediate filaments are constructed from a number of different subunit proteins. The intermediate filaments are in general polymers that are subdivided into six types depending on the protein structure. Keratins, lamins, desmin and vimentin are all part of the intermediate filaments (Fletcher and Mullins 2010).

2.5.1 Actin Cytoskeleton

The actin filaments are grouped into higher-order structures, forming bundles or three-dimensional networks with the traits of semisolid gels. Actin-binding proteins, which are critical components of the actin cytoskeleton, are crucial for the assembly and disassembly of actin filaments, they're cross-linking into bundles and networks, and their association with other cell structures.

Actin molecules are proteins of 375 amino acids. Binding sites present at each actin monomer (**globular G actin**) allows for head-to-tail interactions with other actin monomers to form filaments of actin **(filamentous F actin**) (Giganti, Plastino et al. 2005; Winder and

Ayscough 2005). Each monomer is rotated by 166 degrees in the filaments, which therefore have the appearance of a double-stranded helix. All the actin monomers are oriented in the same direction allowing the actin filaments to have a distinct polarity, and their ends (plus and minus ends) are distinguishable from one another.

Contractile microfilaments, composed of aligned bundles of actin and myosin II filaments, form a contractile cytoskeletal network that spans from the nucleus to the cell cortex, where it links to the cytoplasmic face of transmembrane integrin receptors and cadherins on the plasma membrane (Verkhovsky, Svitkina et al. 1995; Verkhovsky, Svitkina et al. 1997; Backouche, Haviv et al. 2006). These traction forces lead to patterns of mechanical contraction, which coordinates various transformations in cell and tissue shape. In developing epithelium, the mechanical linkage between the cytoskeletons of neighbouring cells occurs primarily at the apical adherent junctions (Gates and Peifer 2005; Halbleib and Nelson 2006), in which the intracellular domains of transmembrane cadherin proteins form an anchoring complex with β-catenin and its actin-binding partner, α-catenin (Ozawa and Kemler 1992). Although the nature of the connection between E-cadherin and the actin cytoskeleton remains unclear (Drees, Pokutta et al. 2005), genetic studies suggests that catenins mediate the mechanical linkage between neighbouring cells (Gates and Peifer 2005; Cavey, Rauzi et al. 2008).

Coupling of cell-generated mechanical forces through cell-cell adhesions results in an apical constriction of epithelial cells, which reduces the size of the apical surface of each cell relative to its base (Sawyer, Harrell et al. 2010). This physical constriction is used to deform the cells and thereby generate a variety of epithelial patterns during morphogenesis including folding, pitting, and tubing (Colas and Schoenwolf 2001; Leptin 2005).

2.6 VESICULAR TRAFFICKING

Molecules and most proteins are often too large to pass directly through membranes. Large molecules are instead packed into small membrane-wrapped containers called vesicles. Vesicles are constantly forming at the plasma membrane, the endoplasmatic reticulum (ER), and the Golgi. When formed, vesicles deliver their contents to destinations within or outside the cell.

mRNA is translated into protein at the ribosomes. Proteins that will be secreted contain a signal sequence that directs ribosomes to bind to the ER, the growing protein chain passes

into the lumen of the ER. As they move through the ER, proteins are subjected to post-translational modifications including refolding, disulfide bond formation and glycosylation. The proteins are pinched off in the ER membrane and transported through COP II vesicles to the Golgi. In the Golgi apparatus proteins undergo further modifications including glycosylation, phosphorylation and sulfation. At the outer layer of the Golgi, proteins are sorted into COPI vesicles for transport to specific destinations.

Vesicles are transported along microtubules to the cell membrane, releasing their contents in the process of exocytosis. However proteins can also be engulfed by the cell through the endocytic pathway, often large proteins that cannot pass through the cell membrane are transported through the endocytic pathway. The endocytic pathway can be divided into four categories: clathrin mediated endocytosis, caveolae, macropinocytosis and phagocytosis.

Vesicular trafficking has also been shown to be crucial for maintenance and assembly of cell polarity (Qi, Kaneda et al. 2011). The vesicular trafficking system helps sorting of apical and basolateral proteins through the production and fission of different types of post Golgi carrier vesicles. Vesicular fission seems to be mediated through dynamin 2, in apical routes (Salvarezza, Deborde et al. 2009), and PKD has a role in basolateral routes (Yeaman, Ayala et al. 2004). Myosin 2 and myosin 6 mediate basolateral transport from the TGN (Musch, Cohen et al. 1997; Au, Puri et al. 2007), whereas myosin 5 mediates apical transport (Roland, Bryant et al. 2011).

2.6.1 The TRAPP complexes

The TRAPP (Transport protein particle) complex was first characterized as a large protein complex functioning in the later stages of ER-to-Golgi traffic in yeast (Sacher, Jiang et al. 1998; Sacher, Barrowman et al. 2001). TRAPP consists of two distinct complexes, TRAPP I is ~300 kDa in size and contains seven subunits, whereas TRAPP II is ~1000 kDa and contains an additional three subunits Trs65p, Trs120p and Trs130p (Sacher, Barrowman et al. 2001). The yeast subunits of TRAPP and their corresponding mammalian othologues can be found in table 1.

The different subunits share some sequence similarity, six of the smallest subunits are categorized into two families of three (Bet3p, Trs33p and Trs31p; and Bet5p, Trs20p and Trs23p) (Sacher, Barrowman et al. 2000). TRAPP I and II both co-function with an early Golgi marker and are present in the cytosolic pool. The association to the Golgi network is

stable, due to Bet3p does not relocate to the ER when ER-to-Golgi traffic is blocked, unlike components that cycle between the ER and Golgi, and the complex remains stable under these conditions (Barrowman, Sacher et al. 2000).

Yeast TRAPP	*Mammalian orthologue*
Bet5p	TRAPPC1
Trs20p	TRAPPC2
Bet3p	TRAPPC3
Trs23p	TRAPPC4
Trs31p	TRAPPC5
Trs33p	TRAPPC6a, b
Trs65	None
Trs85p	None
Trs120p	TRAPPC9
Trs130p	TRAPPC10

Table 1. Complete list of TRAPP proteins and their mammalian orthologues.

It has been suggested that the TRAPP complex acts as a GEF (Cai, Zhang et al. 2005; Liang, Morozova et al. 2007). Ypt/Rab GTPases are conserved key controllers of the different protein transport steps in all eukaryotic cells. They switch between the GTP-bound 'on' and the GDP-bound 'off' states with the help of upstream regulators. Guanine nucleotide exchange factors (GEFs) activate GTPases by accelerating their intrinsic GDP release and GTP uptake reactions. While in the on state, Ypt/Rabs interact with several

effectors that supply the various substeps of vesicular transport, from vesicle formation through their motility, tethering and fusion.

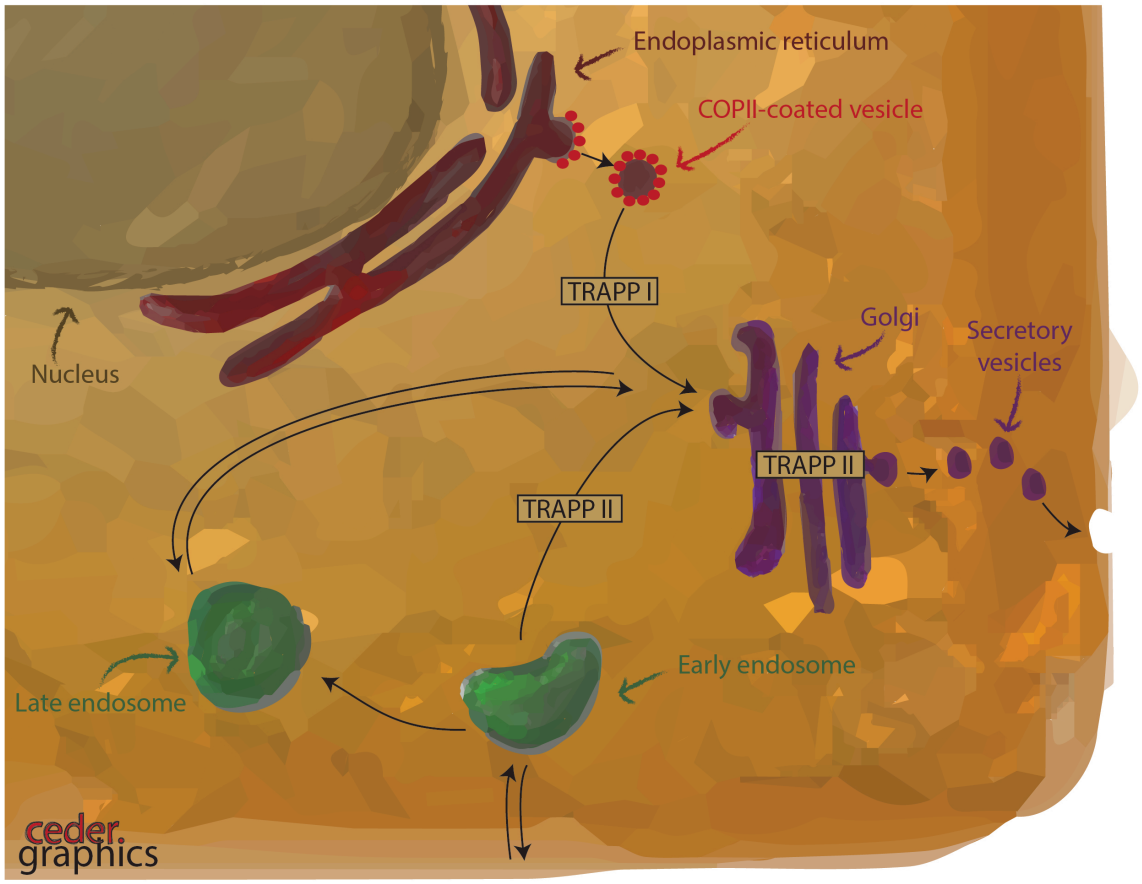

Figure 3. The TRAPP vesicular pathway in yeast. *Figure adapted from Barrowman et al 2010, Nat Rev Mol Cell Biol.*

In yeast, mutations in the two essential TRAPP II-specific subunits, Trs120p and Trs130p, abolish the Ypt31/32p GEF activity of TRAPP while increasing the Ypt1p GEF activity (Morozova, Liang et al. 2006). Mutations in Trs130 blocks vesicle trafficking through or from the Golgi, however mutations in Trs120 results in membrane structures that resemble Berkley bodies (large vesicles) and disrupts trafficking of proteins that recycle through the early endosome (Cai, Zhang et al. 2005).

The GEF activity of mammalian TRAPP has not been shown yet. However the mammalian TRAPPII complex has been suggested to function as a tethering complex that binds to Golgi-derived (COPI coated) vesicles to mediate Golgi traffic and has been implicated in the

selective transport of proteins to the plasma membrane in polarized cells (Qi, Kaneda et al. 2011; Westlake, Baye et al. 2011).

2.7 MESENCHYMAL TO EPITHELIAL TRANSITION

Transitions between epithelial and motile mesenchymal states are important during embryogenesis and organogenesis. They allow cells to alter their morphology and to change interactions with neighbouring cells and the microenvironment. These processes include epithelial to mesenchymal transition (EMT) (which is discussed in segment 3.3) and mesenchymal to epithelial transition (MET). MET occurs during embryogenesis giving rise to the trophoectoderm generating the first embryonic epithelium (Larue and Bellacosa 2005). At the early stage, MET is thought to be regulated by EphA4 by modulating cell shape changes in preparation for epithelialization (Barrios, Poole et al. 2003).

There is growing evidence supporting the fact that MET is crucial for later stages of cancer progression, metastasis. In colon carcinoma, recent reports show that the primary tumor and its corresponding liver metastasis have a mixed population of epithelial-mesenchymal phenotype whereas the center of the tumor retained epithelial characteristics. However, tumor cells located at the invasive front had acquired mesenchymal traits (Brabletz, Hlubek et al. 2005).

2.8 TUBULOGENESIS

Tube formation is a common feature of many developing organs such as lung, kidneys, mammary glands as well as the blood circulatory network. The function of these tubular structures is to transport gases, liquids and waste products and thus plays an essential role in organ function. Although the morphology of tubes may differ between tissues they still have some features in common. For example, established cellular tubes form apical and basal membrane domains, inter-cellular junctions form a basal membrane. There are clearly molecular mechanisms in common in tube formation, as for example Par3 controls tube formation in the neuro-ectoderm of the zebrafish, vessel lumen formation in mice as well as epithelial lumen formation in human cells (Bryant and Mostov 2008). The loss of polarity is also important for the expansion and formation of new tubes. In the case of endothelial cells,

migrating cells form anterior posterior polarity in which the leading cell, the tip cell, form filopodia which guides the migration and consequent anastomosis of neighbouring capillaries. Loss of polarity is also of importance in the context of cancer. Cancers such as colorectal, breast and prostate form polarized glandular tubular structures in the benign state but loose polarity during invasion and metastasis. Knowledge regarding the process of tube formation may provide not only important clues on how organs are formed but also how a malignant phenotype develops.

2.9 Blood vessel formation

The vascular system in higher animals consists of a highly organized network of arteries and veins. The vascular system permits blood to circulate and transport nutrients, oxygen, carbon dioxide and hormones to and from the cells in the body helping to maintain homeostasis. The vascular system is divided into blood and the lymphatic network, both being lined by endothelial cells (EC's). The arteries and the arterioles help transporting oxygenated blood from the heart to tissues in the body. The blood is then carried back through the veins and venules, being removed of waste products. Arteries and veins differ in the degree of blood pressure and the SMC'coating, but also their EC's and SMC have distinct identity and origin (Carmeliet 2003). It has been hypothesized that differences in blood flow between the arteries and veins were assumed to be responsible for arterial-venous specification (Chung and Ferrara 2011). However studies in the zebrafish has demonstrated that *Notch* signaling restricts angioblasts to either arterial fate, by expression of Ephrin B2 or a venous fate marked by EphB4 (Zhong, Childs et al. 2001).

The vascular system is crucial for development and is therefore also among the first to develop during embryogenesis (Chung, Lee et al. 2010; Chung and Ferrara 2011). Upon deregulation, the formation of blood vessels can contribute to malignant disorders such as retinopathy, cancer and rheumatoid arthritis.

During embryonic development in vertebrates, individual angioblasts migrate to the embryonic midline and differentiate into EC. These cells start to express typical EC markers such as PECAM-1 and VEGFR-2, which guides the cells to the midline. Upon reaching the midline cells elongate to form parallel cords, followed by the formation of a central lumen allowing for the initiation of circulation (Strilic, Kucera et al. 2009; Zeeb, Strilic et al. 2010; Herbert and Stainier 2011; Xu and Cleaver 2011).

Blood vessel formation is divided into *vasculogenesis* and *angiogenesis*. Vasculogenesis is the formation of blood vessels from differentiating hemangioblasts (formation of blood vessels when no pre-existing ones). The term angiogenesis however is the formation of new blood vessels from pre-existing ones.

2.9.1 Angiogenesis

After the formation of a primitive vascular system through vasculogenesis the blood vessel network expand through the process of angiogenesis. This term reflects the formation of new vessels from pre-existing ones. Physiological angiogenesis occurs also in adults during wound healing, menstrual cycle, pregnancy and skeletal muscle growth. Pathological angiogenesis promotes diseases such as obesity, rheumatoid arthritis, cancer and psoriasis.

Pathological and physiological angiogenesis are both initiated in response to low levels of oxygen and nutrients. The organization of the vasculature in a healthy organism is maintained by a balance between pro-angiogenic and anti-angiogenic factors (Reinacher-Schick, Pohl et al. 2008). This balance is lost during pathological angiogenesis promoting uncontrolled expansion of blood vessel networks.

Sprouting angiogenesis occurs in a stepwise fashion (figure 4.). In some tissues, such as brain and retina it is initiated by hypoxia. Which up-regulates the expression of genes such as VEGF, needed for vessel formation. At the tip of the sprouts are the tip cells responsible for vessel guidance (Gerhardt, Golding et al. 2003), the stalk of the sprout consists of cells proliferating and forming the vessel lumen in order to support the elongation of the stalk. Tip cells extend filipodial protrusions, which guides them towards angiogenic signals.

Sprouts anastamose and branches with other sprouts to form vascular loops and networks. The newly formed tubes are then covered by mural cells, pericytes and SMC, stabilizing the vessel. However, vessels not covered by mural cells regress. Finally blood flow is established in the new vessels (Carmeliet 2000; Jain 2003; Adams and Alitalo 2007).

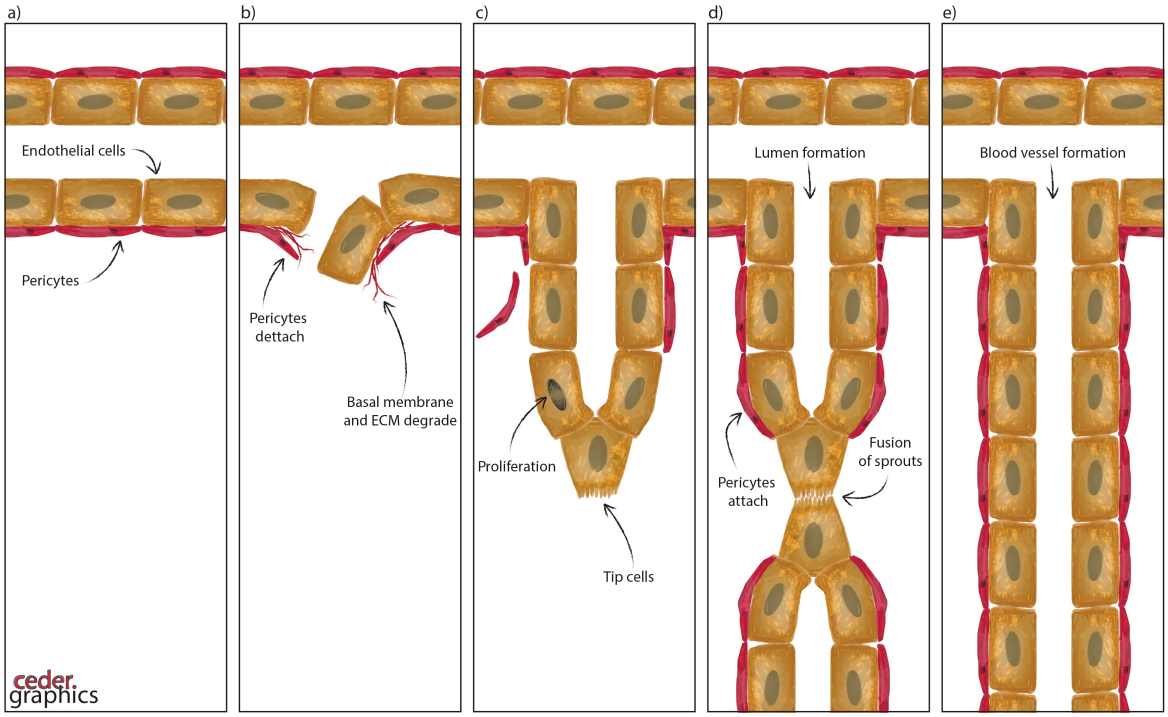

Figure 4. Different steps of sprouting angiogenesis. a) Blood vessel consisting of EC and pericytes. b) ECM is degraded, pericytes detach and EC's start to migrate. c) Tip cells guide the sprout, EC's proliferate in stalk. d) Tip cells adhere and form a lumen. e) The new tubes are covered by mural cells. *Figure adapted from Bergers and Benjamin 2003.*

2.9.2 Vascular lumen formation

The formation of a hollow vascular lumen is essential and needed for the transport of blood to any given tissue. The mechanism to how the vascular lumen is formed has been under intense investigation.

Two different mechanisms for lumen formation have been proposed recently: cord hollowing and cell hollowing (Folkman and Haudenschild 1980; Davis and Camarillo 1996; Kamei, Saunders et al. 2006). According to the cell hollowing principle intracellular vacuoles fuse together to form the vascular lumen. *In vitro* studies also showed that the vacuoles formed through pinocytosis (Davis and Camarillo 1996). More recent studies have however, suggested that cord hollowing is more likely to be the common mechanism for vascular lumen formation (Parker, Schmidt et al. 2004; Herbert, Huisken et al. 2009; Strilic, Kucera et al. 2009). However, different blood vessels form via a range of different cellular mechanisms. It is not completely unexpected, as endothelium is known to display a high level of heterogeneity across different tissues (Aird 2012). I expect that other mechanisms

of lumen formation will likely be identified as the vasculature of different organs are more extensively examined and understood.

Studies conducted in the past several years have shed light on the molecular mechanism of aortic lumen formation (Iruela-Arispe and Davis 2009; Xu and Cleaver 2011; Axnick and Lammert 2012). *In vitro* studies have suggested cell-cell adhesions, the cytoskeleton and apical-basal polarity being crucial for lumen formation (Davis and Senger 2005). Investigations performed on mice have further strengthened this notion, showing that inactivation of cytoskeletal (RASIP1), polarity (beta1 integrin and Par3) and cell-cell adhesion (VE-cadherin) proteins cause luminal defects in the dorsal aorta (DA) (Carmeliet, Lampugnani et al. 1999; Gory-Faure, Prandini et al. 1999; Zovein, Luque et al. 2010; Xu, Sacharidou et al. 2011).

3 LOSS OF TISSUE INTEGRITY AND STRUCTURE-CANCER

Tumors are no longer considered to be a collection of homogenous cancer cells, as they are recognized as organs, whose complexity might even exceed that of normal tissue (Hanahan and Weinberg 2011).

Cancer is often described as uncontrolled growth of cells with the ability to spread and colonize other parts of the body. Nearly 80 % of tumors arise from organized epithelial tissues. Histological analyses have revealed that tumors have a tissue architecture that is less organized and structured compared to that of normal tissue. This suggests that loss of architecture and structure is part of or a step towards malignant progression (Hansen and Bissell 2000).

The abnormal growth pattern of cancer develops progressively and is a multi-step process. As the tumor grows and progressively evolves away from normal towards malignant architecture, there are distinct stopping points along the way, normal → hyperplastic → dysplastic → neoplastic → metastatic (figure 5.).

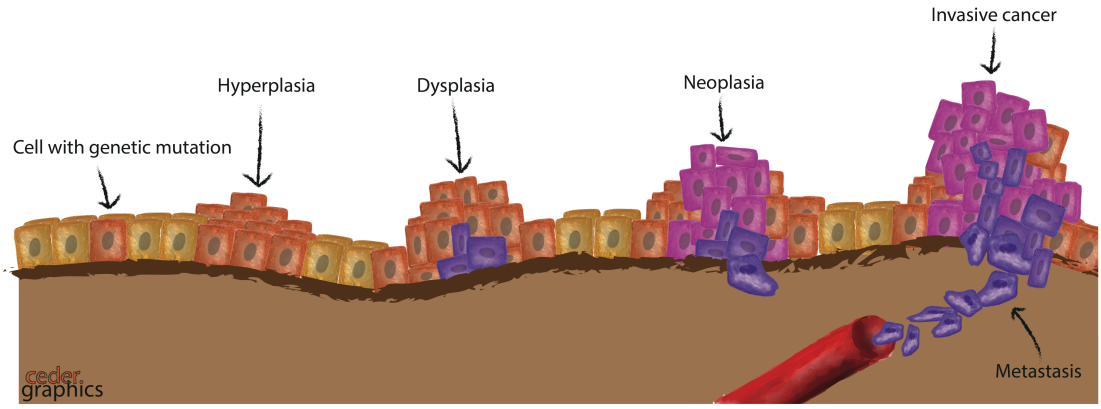

Figure 5. Different steps of malignant progression. *Figure adapted from /www.ndhealthfacts.org/*

Hyperplastic tissues deviate only minimally from those of normal tissue, as they only contain excessive amounts of cells.

Dysplastic tissue is more abnormal, the appearance of individual cells is no longer normal. Cytological changes include variability in nuclear size and shape, increased mitotic activity.

Neoplasia is a further degree of abnormality as tumor cells start to invade into underlying tissues.

Cells formed in a primary tumor may seed new tumor colonies at distant sites in the body, a process known as **metastasis**.

3.1 THE HALLMARKS OF CANCER

Douglas Hanahan and Robert Weinberg wrote in the year 2000, a review article proposing six biological traits that needs to be acquired for cancer to develop. These were termed The Hallmarks of Cancer (Hanahan and Weinberg 2000), six traits that contributes to the transformation of normal cells to malignant. The hallmarks are:

- **Resisting cell death**
- **Sustaining proliferative signaling**
- **Evading growth suppressors**
- **Activating invasion and metastasis**
- **Enabling replicative immortality**
- **Inducing angiogenesis**

In their updated Hallmarks of cancer: the next generation, the writers proposed two new hallmarks: **abnormal metabolic pathways** and **evading the immune system** (Hanahan and Weinberg 2011).

Drugs that will target the individual traits (the hallmarks) required for tumor progression have been developed and are in clinical trials, in some cases used for treating patients with malignant disease. Drugs targeting angiogenesis, VEGF inhibitors such as, Avastin has been approved by the FDA for colon and glioblastoma treatment. Or drugs targeting c-met /HGF to inhibit invasion and metastasis, Onartuzumab that has entered phase III in clinical trials for treatment of Non-small cell Lung carcinoma (NSCLC).

Larger parts of this thesis has focused on one of these traits; ***activating invasion and metastasis****, we have discovered a novel mechanism for which the cancer cell during hypoxic conditions deregulates apical basal polarity promoting invasion and metastasis.*

The deregulation of apical-basal polarity during tumor progression has been well studied and documented. The following paragraph will give further insight into how deregulation of apical –basal polarity contributes to tumor progression.

3.2 Loss of apical basal polarity and tumor progression

A strong correlation between malignancy and loss of epithelial architecture/organization has been documented for most cancers which may be due to loss of polarity (Bilder 2004). The deregulation of apical-basal polarity is often depicted as a hallmark of cancer although direct evidence whether this is a cause or consequence of tumor progression is not yet clear (Lee and Vasioukhin 2008; Royer and Lu 2011). A growth suppressive role of cell polarity stems from findings in Drosophila where the polarity proteins such as Crumbs, Scribble, Dlg and Lgl act as tumor suppressor genes (Bilder 2004). Furthermore, recent work point to the existence of a similar role in mammalian cells as loss of expression of either Crumbs/Crb3 or Scribble promotes tumor progression in mouse tumor model systems (Karp, Tan et al. 2008; Pearson, Perez-Mancera et al. 2011).

In epithelial stem cells, polarity proteins are also in control of asymmetric cell division, by regulating the polarized localization of cell fate determinants and the correct orientation of mitotic spindles. As a result, asymmetric cell division has a fundamental role in the control of progenitor or stem cell numbers and differentiation (Knoblich 2010).

This is of interest as a shift from asymmetric division of epithelial stem cells or cancer-initiating cells to symmetric divisions would result in dedifferentiation and an increase in cancer-initiating cells. Therefore, a defect in asymmetric division could contribute to tumor progression. In epithelial stem cells asymmetrical cell division is crucial for the maintenance of epithelial tissue homeostasis. When a stem cell divides asymmetrically it gives rise to two daughter cells, one with a identical cell fate, the other with a different one (Lee and Vasioukhin 2008; Knoblich 2010). Studies performed in *Drosophila* have indeed confirmed that asymmetric division functions as a mechanism of tumor suppression (Januschke and Gonzalez 2008).

Loss of apical-basal polarity also affects cell-cell contacts and has been associated with *epithelial to mesenchymal transition,* EMT. It has been shown that depletion of Par3 disrupts tight junction formation (Chen and Macara 2005). Moreover, siRNA experiments showing that PATJ is needed for proper polarization and tight junction formation in epithelial cells (Shin, Straight et al. 2005). Core polarity proteins are important for the maintenance and the formation of the apical junctional complex, it has been proposed that loss of polarity would induce or contribute to EMT. TGF-β stimulation has been shown to down-regulate Par3 expression through miR-491-5p in rat epithelial cells, suggesting that TGF-β may induce disruption of cell polarity (Zhou, Fan et al. 2010).

Loss of Par3 in mammary tumor models has been shown to promote invasion and metastasis through an aPKC-dependent activation of Jak/Stat3 signaling (McCaffrey, Montalbano et al. 2012). Mutushwamy and co-workers also found that loss of Par3 in breast cancer promotes invasion and metastasis, the increased invasive potential was due to reduced adhesive activity of E-cadherin mediated through constitutive expression of Rac GTPases (Xue, Krishnamurthy et al. 2013).

3.3 EPITHELIAL TO MESENCHYMAL TRANSITION

EMT was first discovered as a feature of embryogenesis (Kong, Li et al. 2011). EMT and mesenchymal to epithelial transition (MET), its reverse process, are crucial for the formation of several organs and tissues in the developing embryo and during numerous embryonic events such as gastrulation, neural crest formation and heart valve formation (Thiery, Acloque et al. 2009).

Upon initiation of EMT cellular junctions are deconstructed and junctional proteins are re-localized or degraded. E-cadherin is cleaved at the plasma membrane and degraded (Yilmaz and Christofori 2009). The loss of epithelial junctions during EMT results in the loss of apical-basal polarity (Huang, Guilford et al. 2012). After EMT is induced the expression of polarity proteins such as Crb3 and LGL2 is also repressed (Moreno-Bueno, Portillo et al. 2008).

During EMT cells reorganize their cortical actin cytoskeleton so that it enables cell elongation and directional migration (Thiery and Sleeman 2006; Yilmaz and Christofori 2009; Yilmaz and Christofori 2010). EMT promotes increased cell contractility and actin stress fiber formation. Moesin is believed to be responsible for these dynamic changes in actin reorganization (Haynes, Srivastava et al. 2011). However, the molecular mechanism controlling actin dynamics during EMT remains to be elucidated.

Transcriptional regulation of EMT includes micro-RNA's and several transcription factors such as Snail, Slug, Twist, Zeb1 and Prrx1. Signaling pathways such as *transforming growth factor beta* (TGF-β) can induce EMT through induction of for example Zeb1 expression (Gregory, Bert et al. 2008).

Throughout cancer progression it is believed that epithelial cells undergo a mesenchymal transition, during which they lose apical-basal polarity, intercellular adhesions. During epithelial to mesenchymal transition (EMT) cells reorganize their cytoskeleton, undergo a change in the signaling program that define cell shape and reprogram gene expression, this increases the motility of individual cells and enables the development of an invasive phenotype (Thiery and Sleeman 2006). Cells express a set of mesenchymal specific genes such as N-cadherin and Vimentin and become highly migratory (Yang and Weinberg 2008; Scheel and Weinberg 2012). Mesenchymal type of cells lack apical-basal polarity, have a spindle shaped morphology and interact with each other through focal points (Thiery and Sleeman 2006).

Mesenchymal like tumor cells penetrate through the basement membrane and enter the lymphatic system or the bloodstream, through which they rapidly disseminate to ectopic sites in the body. The cells extravasate and colonize surrounding tissue to form metastases.

Some studies have implicated EMT with the generation of cancer stem cells or tumor initiating cells (TIC's) (Mani, Guo et al. 2008). The TIC's are considered to be responsible for the outgrowth of metastasis and are very resistant to chemotherapy. Slug has been shown to induce a TIC phenotype (Guo, Keckesova et al. 2012). Breast cancer TIC's has been shown to express miR-200, which promotes stem cells features (Shimono, Zabala et al. 2009).

3.4 ECM REMODELING AND CANCER PROGRESSION

The extracellular matrix is essential for establishment and the maintenance of tissue polarity and architecture. The β1- integrin has for example been shown to be crucial for maintaining tissue polarity in the mammary gland (Akhtar, Marlow et al. 2009). However, abnormalities in ECM dynamics can affect the integrity of the basement membrane as a physical barrier and promote EMT, which together facilitates tissue invasion (Song, Jackson et al. 2000; Duong and Erickson 2004; Radisky and Radisky 2010).

The remodeling of the ECM during development is tightly regulated by expression or activities of multiple ECM enzymes. The remodeling enzymes are regulated at transcriptional, translational and posttranslational levels through inhibiting prodomains and selective protease inhibitors (Page-McCaw, Ewald et al. 2007; Aitken and Bagli 2009). The activities of the ECM remodeling enzymes are deregulated with age and under disease

conditions. In disease, the dynamics of the ECM may become abnormal leading to disorganization and changes in essential ECM properties. The main contributors to the altered activities of the ECM enzymes are the stromal cells, including cancer-associated fibroblasts (CAFs) and immune cells (Bhowmick, Neilson et al. 2004; Orimo, Gupta et al. 2005). But also other cell types such as epithelial cells and mesenchymal stem cells may also be involved in late stages of tumor progression (Quante, Tu et al. 2011; Singer and Caplan 2011).

Abnormal ECM dynamics are part of the Hallmarks of cancer (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). Excess ECM production or reduced ECM turnover is essential in tissue fibrosis of many organs (Frantz, Stewart et al. 2010). For example, various collagens show increased deposition during tumor formation (Zhu, Risteli et al. 1995; Kauppila, Stenback et al. 1998).

Changes in ECM topography may promote cancer cell invasion. The thickening and linearization of collagen fibers are found in tumors, in areas where tissue invasion and tumor vasculature are observed (Condeelis and Segall 2003; Provenzano, Eliceiri et al. 2006; Levental, Yu et al. 2009), suggesting that they play a role in cancer cell invasion. Live imaging studies have indeed shown that cancer cells migrate rapidly on collagen fibers in areas enriched in collagen (Wang, Wyckoff et al. 2002; Condeelis and Segall 2003; Wyckoff, Wang et al. 2007).

Moreover other ECM components and their receptors such as heparan sulfate proteoglycans and CD44 that promote growth factor signaling are often overproduced in cancer (Kainz, Kohlberger et al. 1995; Stauder, Eisterer et al. 1995). Thus abnormal changes in the amount of the ECM can potentiate oncogenic effects and promote malignant transformation.

An abnormal ECM can also promote crucial steps during tumor progression. Increase in collagen deposition or ECM stiffness, up-regulates integrin signaling and can promote cell survival and proliferation (Wozniak, Desai et al. 2003; Paszek, Zahir et al. 2005). Increased collagen cross-linking and ECM stiffness promotes focal adhesion assembly, activation of ERK and PI3K signaling, contributing to oncogenic transformation (Levental, Yu et al. 2009).

In conclusion, the deregulation of ECM dynamics disrupts tissue polarity and promotes cell invasion. Cells are directly affected by deregulated ECM dynamics contributing to transformation and metastasis. Part of this deregulation of ECM components can be attributed to hypoxia induced lysyl oxidase (LOX) (Erler, Bennewith et al. 2006; Barker, Chang et al. 2011). Excessive expression of LOX results in cross-linking of collagen fibers and other

ECM components, it also increases ECM stiffness and promotes metastasis and cancer progression (Levental, Yu et al. 2009).

3.5 HYPOXIA

Oxygen is required and essential for the growth and development of multicellular organisms. Aerobic organisms are in need of oxygen to produce energy, therefore oxygen deprivation creates stress in living cells. During low conditions of oxygen, hypoxia, cells activate a number of adaptive responses to match oxygen supply with redox, bioenergetic and metabolic demands.

For cells to adapt rapidly to hypoxic conditions, they must be able to sense changes and thereafter respond. The initial response is rapid and involves transcriptional and posttranscriptional mechanisms. During adaptation to low oxygen pressure there is a shift towards activation of certain genes regulating cellular functions such as angiogenesis, cell survival and metabolism (Harris 2002; Gordan and Simon 2007). Cells arrest in the cell cycle, reduce energy consumption and shift towards anaerobic metabolism and secrete pro-angiogenic and survival factors.

Similar to mammalian tissue, tumors need oxygen to grow. Tumors, however become hypoxic due to an imbalance between tumor cell oxygen consumption and vascular supply, which is caused by uncontrolled proliferation (Brown and Wilson 2004). This in turn means that tumor cells have adapted and are able to grow in hypoxic conditions.

In tumors hypoxia has been associated with increased malignancy, poor prognosis and resistance to radiotherapy and chemotherapy (Semenza 2003; Bertout, Patel et al. 2008; Vaupel and Mayer 2014). Hypoxia can also cause loss of specialized function, tumor cells de-differentiate and gain a stem-cell like phenotype as reported in neuroblastoma and breast cancer (Jogi, Ora et al. 2002; Helczynska, Kronblad et al. 2003; Lofstedt, Jogi et al. 2004). Low stages of differentiation in these tumor types have been shown to be associated with poor prognosis and disseminated disease (Giaccia, Siim et al. 2003; Brown and Wilson 2004).

Hypoxia has also been linked with EMT and loss of E-cadherin. HIF has been shown to decrease the expression of E-cadherin, the first evidence was shown in ovarian carcinoma, where nuclear HIF-1α localization correlated with loss of E-cadherin (Imai, Horiuchi et al. 2003). The mechanism for this action has been suggested to be by activation of Lysil oxidase-

2 (LOX 2), which in turn activates Snail, repressing E-cadherin (Peinado, Del Carmen Iglesias-de la Cruz et al. 2005).

A key modulator of transcriptional response upon hypoxic stress is the hypoxia-inducible factors (HIF's). The HIF's are heterodimeric transcription factors that mediate the primary transcriptional response to hypoxic stress in normal and transformed cells. The HIF's are basic helix-loop-helix proteins that are composed of a α-subunit and a stable β-subunit (ARNT) (Kaelin and Ratcliffe 2008). Together these subunits bind *hypoxia-responsive elements* (HRE's) that contain a conserved RCGTG core sequence. During normal conditions the α-subunit is degraded, however during hypoxic stress the α-subunit is stabilized so that the HIFα protein level and overall HIF transcriptional activity increases as the cells become more hypoxic (Majmundar, Wong et al. 2010).

The mechanisms for protein stabilization of HIF1-α and HIF-2α are similar. Posttranslational modifications carried out by the HIF-specific prolyl hydroxylase-domain proteins (PHD 1, 2, and 3) are responsible for controlling protein stability of the HIF-α subunits. The prolyl hydroxylated HIF-α subunits are recognized by the von-Hippel Lindau (VHL) tumor suppressor protein which is part of a multiprotein E3 ubiquitin-ligase that polyubiquitylates and targets HIF-α for proteasomal degradation (Metzen and Ratcliffe 2004; Kaelin 2005).

In mammals there are 3 isoforms of HIFα, HIF-1α and HIF-2α that are structurally similar and best characterized. The third isoform HIF-3α exists as a splice variant antagonizing HIF-1α and HIF-2α in a dominant negative way (Kaelin and Ratcliffe 2008).

Back in 1995, HIF-1α was first described by Semenza and colleagues (Wang, Jiang et al. 1995), and was shown to have a central role in mediating oxygen dependent transcriptional responses. HIF-2α was identified in 1997 by several independent groups (Ema, Taya et al. 1997; Flamme, Frohlich et al. 1997; Tian, McKnight et al. 1997). Increased expression of both HIF-1α and HIF-2α have been reported in a broad range of cancer cell types and have been associated with poor prognosis. Intense research has been focused on renal cell carcinomas (RCCs), approximately 90% of which lose the function of VHL (Kaelin 2008). The general view is that expression of the HIFα proteins in tumor cells drives tumor growth and progression by regulating the expression of crucial target genes.

3.6 The AP-1 transcription factor

The activator protein 1 (AP-1) transcription factor is a dimeric complex that comprises members of JUN, FOS, ATF (activating transcription factor) and MAF (musculoaponeurotic fibrosarcoma). The AP-1 complex can form many different combinations of heterodimers or homodimers, different combinations determine the genes that are regulated by AP-1 (Chinenov and Kerppola 2001; Wagner 2001; Vogt 2002). AP-1 is activated and regulates gene expression in response to a variety of stimuli such as: growth factors, stress signals, cytokines, bacterial or viral infections (Hess, Angel et al. 2004).

The AP-1 proteins are basic leucine-zipper (bZIP) proteins, they dimerize through a leucine zipper motif and contain a basic domain for interaction with the DNA backbone (Wagner 2001). In mammals the main AP-1 proteins are JUN and FOS.

Upon activation AP-1 up-regulates genes containing the TPA responsive element (TRE), so called because it is strongly induced by the tumor promoter 12-O-tetra- decanoylphorbol-13-acetate (TPA). The TRE element was first discovered in the promoter and enhancer elements of metallothionein I gene and simian virus 40 (SV40) (Angel and Karin 1991).

FOS and JUN were first discovered as viral oncoproteins v-FOS and v-JUN in the Finkel-Biskis – Jinkins osteosarcoma virus and avian sarcoma virus 17 (Vogt 2002). Several of the AP-1 proteins such as c-FOS, c-JUN and FOSB can transform cells in culture very efficiently, all have very potent transactivation domains (Jochum, Passegue et al. 2001). When overexpressed in mice, c-Fos causes osteosarcoma formation by transformation of chondroblasts and osteoblasts (Wang, Grigoriadis et al. 1991; Grigoriadis, Schellander et al. 1993).

AP-1 has also been shown to regulate genes that are required for apical-basal polarity, both c-Fos and c-Jun can depolarize epithelial cells and induce an invasive phenotype (Reichmann, Schwarz et al. 1992; Fialka, Schwarz et al. 1996). However only c-Fos can induce invasion in collagen gels when being overexpressed, indicating a more important role during late-stage tumorigenesis than c-Jun (Reichmann, Schwarz et al. 1992). *In vivo* the progression of chemically induced papillomas to invasive squamous-cell carcinomas is impaired in c-Fos-deficient mice, further supporting the importance of c-Fos in tumor invasion (Saez, Rutberg et al. 1995). Several reports have linked hypoxia to c-Fos, showing that hypoxia triggers activation of c-Fos in a calcium dependent manner (Muller, Krauss et al. 1997; Premkumar, Adhikary et al. 2000).

3.7 The MET tyrosine kinase receptor

During embryogenesis, cells bud off from forming tissues and migrate outwards to form the complex architecture of future organs (Bryant and Mostov 2008). Same process occurs in adult animals during wound healing and tissue repair, cells migrate into injury sites in an effort to recreate the pre-existing structures (Gurtner, Werner et al. 2008). Pathological processes of tumor invasion strongly resemble normal phases of embryogenesis or regeneration in that, similar to the wound edge, cells in the tumor front disrupt cell-contacts and infiltrate into the surrounding tissue where they resist cell death and grow before further dissemination (Schafer and Werner 2008). One major inducer of invasive growth is the hepatocyte growth factor (HGF), which also is the ligand for the MET tyrosine kinase receptor (Giordano, Ponzetto et al. 1989; Bottaro, Rubin et al. 1991; Naldini, Weidner et al. 1991).

In vitro, HGF has been shown to induce tubulogenesis in polarized epithelial cells (Montesano, Matsumoto et al. 1991; Montesano, Schaller et al. 1991). In addition HGF induced morphogenesis has been shown to act without the disruption of tight junctions in Madine Darby –canine kidney epithelial cells (Pollack, Apodaca et al. 2004). A part of the polarity machinery, Scrib has also been shown to play an essential role for HGF mediated morphogenesis. Depletion of Scrib in epithelial cells blocks HGF mediated extensions and morphogenesis (Eastburn, Zegers et al. 2012).

MET is required for various morphogenetic events in both embryonic and adult life (Birchmeier and Gherardi 1998; Birchmeier, Birchmeier et al. 2003) and is believed to drive tumor progression (Trusolino and Comoglio 2002; Lai, Abella et al. 2009). Downstream signaling of the MET receptor are stereotypical modules that are shared among different tyrosine kinase receptors such as: *The MAPK cascades* (Johnson and Lapadat 2002), *PI3K-Akt pathway* (Ponzetto, Bardelli et al. 1994) and *The STAT pathway* (Boccaccio, Ando et al. 1998), all of these pathways positively control MET-dependent proliferation, survival and migration.

Upon HGF binding the kinase activity of the MET receptor is activated by receptor dimerization and *trans*-phosphorylation of two catalytic tyrosine residues (Tyr1234 and Tyr1235) within the kinase activation loop. Next, phosphorylation of two additional docking tyrosines in the carboxy-terminal tail (Tyr1349 and Tyr1356), and when phosphorylated, these tyrosines act as a degenerate motif for the recruitment of many signal relay molecules (Ponzetto, Bardelli et al. 1994).

MET also acts as a substrate for several protein-tyrosine phosphatases (PTPs), including the receptor PTPs density-enhanced phosphates 1 (dEP1) and the non- receptor PTPs PTP1b (also known as PTPN1) (Machide, Hashigasako et al. 2006; Sangwan, Paliouras et al. 2008). These kind of phosphates oppose MET receptor signaling by triggering dephosphorylation of either the catalytic tyrosines (Sangwan, Paliouras et al. 2008) or the docking tyrosines (Palka, Park et al. 2003).

In tumors, MET is induced transcriptionally by hypoxia, inflammatory cytokines or pro-angiogenic factors (Pennacchietti, Michieli et al. 2003; Bhowmick, Neilson et al. 2004). For the effects of MET-dependent invasive growth to be fully executed combined activation of multiple pathways is necessary. For example Ras signals are primarily involved in MET-triggered cell proliferation, whereas PI3K recruitment is required for the induction of cell motility and invasion, however, a fully metastatic phenotype can be induced only when both effectors are associated with MET (Giordano, Bardelli et al. 1997; Bardelli, Basile et al. 1999).

Interfering with MET signaling appears to be a promising therapeutic approach, currently MET kinase inhibitors, HGF inhibitors and antibodies acting as antagonists are in human clinical trials. Active immunotherapy against MET expressing tumors have been tested and have been approved by the American FDA, for treatment of renal cell carcinoma and metastatic melanoma which suffers from deregulated MET activity (Reang, Gupta et al. 2006).

4 THE ANGIOMOTIN PROTEIN FAMILY

The angiomotin protein family consists of three members (Bratt, Wilson et al. 2002), Angiomotin (Amot), Angiomotin-like 1 (AmotL1 or JEAP) and Angiomotin-like 2 (AmotL2 or MASCOT). Each member of the protein family consists of 2 isoforms that is believed to be formed through alternative splicing (Moreau, Lord et al. 2005; Ernkvist, Aase et al. 2006; Zheng, Vertuani et al. 2009), however in *paper II* we show that the p100 and p60 AmotL2 have different promoter regions. The family members all share common structural characteristics. The N-terminal domain is conserved in the full-length protein of Amot, AmotL1 and AmotL2 and is composed of glutamine- rich domains, LPxY and PPxY motifs (Wang, An et al. 2012; Yi, Shen et al. 2013). All three members interact with MAGI1, YAP and E3 ligase NEDD4 through the N-terminal domain (Patrie 2005; Wang, Huang et al. 2011; Wang, An et al. 2012).

The shorter isoforms lack the N-terminal domain, however they are composed of the coiled-coiled domain and the c-terminal PDZ binding domain (Troyanovsky, Levchenko et al. 2001; Bratt, Wilson et al. 2002). The coiled-coiled domain serves as an oligomerization site, allowing members of the protein family to either form homo-oligomers through self-association, or hetero-oligomers with other family members (Patrie 2005; Ernkvist, Birot et al. 2008; Zheng, Vertuani et al. 2009).

The Amot protein family has been suggested to play a crucial role in regulating cell polarity. The members of the protein family have been shown to associate to Crb3 and Par3 polarity protein complexes via the c-terminal PDZ domain (Wells, Fawcett et al. 2006; Ernkvist, Luna Persson et al. 2009). Binding to Syx, a RhoA GTPase exchange factor is also mediated through the c-terminal PDZ domain and is essential for endothelial cell migration (Ernkvist, Luna Persson et al. 2009).

Amot, the founding member of the protein family was first identified in 2001 through a yeast-two hybrid screen of a human placenta cDNA library, using the kringle domains 1–4 of angiostatin as bait (Troyanovsky, Levchenko et al. 2001). It was named Angiomotin, meaning *motility* in latin, due to its migratory effects (Troyanovsky, Levchenko et al. 2001).

Amot knock-out mice displayed early lethality, mice died after gastrulation due to migration defects (Shimono and Behringer 2003). Further investigations in mice and zebrafish indicated that Amot plays a vital role in blood vessel formation, through the regulation of endothelial cell migration and polarity (Aase, Ernkvist et al. 2007). Amot has

also been reported to bind to the tumor suppressor Merlin via its coiled- coil domain resulting in inhibition of Rac and Ras-MAPK pathways. Depletion of Amot in Schwann cells prevents cell proliferation and tumorigenesis *in vivo*, suggesting Amot as a potential target for NF2 derived cancers (Yi, Troutman et al. 2011). Targeting of Amot has been shown to be a promising approach for inhibition of pathological angiogenesis (Holmgren, Ambrosino et al. 2006; Levchenko, Veitonmaki et al. 2008; Arigoni, Barutello et al. 2012).

AmotL1 was first discovered in a screen for novel tight junction associated proteins, and due to similarities to Amot named AmotL1 (Bratt, Wilson et al. 2002; Nishimura, Kakizaki et al. 2002). Amot and AmotL1 bind via the N-terminal domain to F-actin, promoting actin cytoskeleton remodeling (Ernkvist, Aase et al. 2006; Gagne, Moreau et al. 2009). Although Amot and AmotL1 have similar effects on endothelial migration and tight junction formation, Amot appears to be essential for the polarity of vascular tip cells whereas AmotL1 mainly affects the stability of cell-cell junctions of the stalk cells (Zheng, Vertuani et al. 2009).

The third member of the angiomotin protein family, AmotL2 has been reported to co-localize with the junctional protein MAGI-1 in epithelial cells, an interaction mediated through the N-terminal domain (Patrie 2005). AmotL2 is the only member of the motin protein family reported to be responsive to hypoxia, further suggesting a role in stress response (Mondon, Mignot et al. 2005; Liu, Laurell et al. 2007).

AmotL2 has been reported to be an FGF-responsive gene crucial for cell movements in zebrafish embryos (Huang, Lu et al. 2007). Recent work suggested that AmotL2 regulates postsynaptic differentiation in skeletal muscles by interacting with LL5β, a podosome component (Proszynski and Sanes 2013). Furthermore, evidence proposes that AmotL2 and other members of the protein family are involved in regulating the HIPPO pathway. Amot proteins negatively regulate the HIPPO pathway by associating with YAP1, and thereby sequestering it in the cytoplasm (Paramasivam, Sarkeshik et al. 2011; Wang, Huang et al. 2011; Zhao, Li et al. 2011).

5 AIMS OF THIS THESIS

The general aim of this thesis was to characterize the function of Angiomotin-like 2 (AmotL2) during blood vessel formation and cancer progression.

Specific aims of the papers:

I. **To elucidate the role of AmotL2 in vascular lumen formation.**

II. **To investigate the function of hypoxia induced p60 AmotL2 during tumor progression.**

III. **To asses whether p60 AmotL2 can antagonize the p100 isoform.**

6 RESULTS AND DISCUSSIONS

Paper I

The overall aim of this paper was to study if AmotL2 is required for vascular development, and to investigate its function at a cellular level. To do so we used two *in vivo* models, zebrafish, conditional knock out mice and *in vitro* studies using endothelial cells.

In zebrafish the genome has been duplicated throughout evolution, so there are two *amotl2* genes, *amotl2a* and *amotl2b*. In order to determine if *amotl2* is involved in vascular development we started off by individually targeting *amotl2a* and *amotl2b* functions in zebrafish using paralogue-specific morpholinos. We used double transgenic embryos where erythrocytes express dsRed (Tg(ga- ta1:dsRed)sd2) and the vascular endothelial network is visualized via EGFP (Tg(kdrl:EGFP). As previously reported (Huang, Lu et al. 2007) depletion of *amotl2a* resulted in gastrulation defects, however at a lower morpholino concentration embryos went through gastrulation but suffered

from pericardial oedema starting at 28h post fertilization (h.p.f.). Depletion of *amotl2b* also resulted in pericardial oedema but did not affect gastrulation. In morphant embryos an accumulation of erythrocytes was observed in areas of oedema, thus no circulation could be detected. In both the individual *amotl2a* and *amotl2b* knockdown embryos, and especially in the *amotl2* MO (double morphants), constrictions within the dorsal aorta (DA) were observed. The pericardial vein (PCV) was however not affected. So to rule out that the DA defects were not secondary to the loss of blood circulation, we targeted embryos with morpholinos against *silent heart /tnnt2*. These morphants lack heartbeat and vascular flow and did not exhibit any DA luminal defects, thus concluding that the DA defects are due to depletion of *amotl2*.

We further investigated the effects of amotl2 inactivation in mice using a tamoxifen-inducible conditional knockout mouse model. Endothelium specific depletion of amotL2 resulted in aortic constrictions. Similar to our observations in zebrafish, endothelial cells of the aorta in amotL2-mutant mice exhibited a rounded cell shape.

So what is causing the DA defects?

We took a closer look by injecting a fli1a:mCherry containing plasmid into Tg(fli1a:EGFP) zebrafish embryos, and found that the aortic endothelial cells had flattened out and elongated in the *amotl2* morphants. The length to width and the length to height ratios had significantly changed. The phenotype could be restored by co-injection of human AMOTL2 messenger RNA (mRNA). We could further show by using transgenic fish expressing Lifeact, that the cell shape changes was due to perturbations in cytoskeletal architecture. The *in vivo* experiments indicated a specific defect in the actin filaments anchored in the cellular junctions, whereas junction formation and the establishment of apical–basal polarity appeared intact.

In vitro studies using mammalian endothelial (MS1) cells showed that amotL2 was located to areas where actin fibers anchor, in the VE-cadherin-positive endothelial junctions. AmotL2 depleted cells did not form actin filaments that connect to the cell–cell junctions, whereas circumferential actin was maintained. Furthermore, calcium depletion studies showed that upon depletion of amotL2 by siRNA, VE-cadherin was still present at cell–cell junctions, but its connection to actin fibers were lost.

We and others have shown that the Amot family of proteins associates to the scaffold junctional protein MAGI1 via a WW protein interaction motif (Bratt, Wilson et al. 2002; Patrie 2005). It has further been reported that MAGI1 associates to VE-cadherin

via binding to B-catenin (Dobrosotskaya and James 2000; Sakurai, Fukuhara et al. 2006). So to further investigate if amotL2 functions together with VE-cadherin in controlling cytoskeletal architecture we performed co-immunoprecipitation studies showing that MAGI1 and amotL2 bind VE-cadherin. However, the binding of MAGI1 to VE-cadherin was lost in amotL2 siRNA-depleted cells, suggesting that amotL2 couples MAGI1 and actin fibers to the VE-cadherin junctional complex mediating mechanical force. This notion was further strengthen by using traction force microscopy, measuring tension between pairs of endothelial cells. We could show that AmotL2 was essential for the transmission of junctional force between endothelial cells *in vitro*. *In vivo,* blebbistatin treatment, targeting contractile actin fibers, between 18–20h.p.f. caused loss of circulation and reduced length to width and length to height ratios mimicking the effects of amotl2 MO in zebrafish.

In summary we have identified a novel mechanism for aortic lumen expansion by which force can be transmitted across cells via adherent junctions and actin fibers.

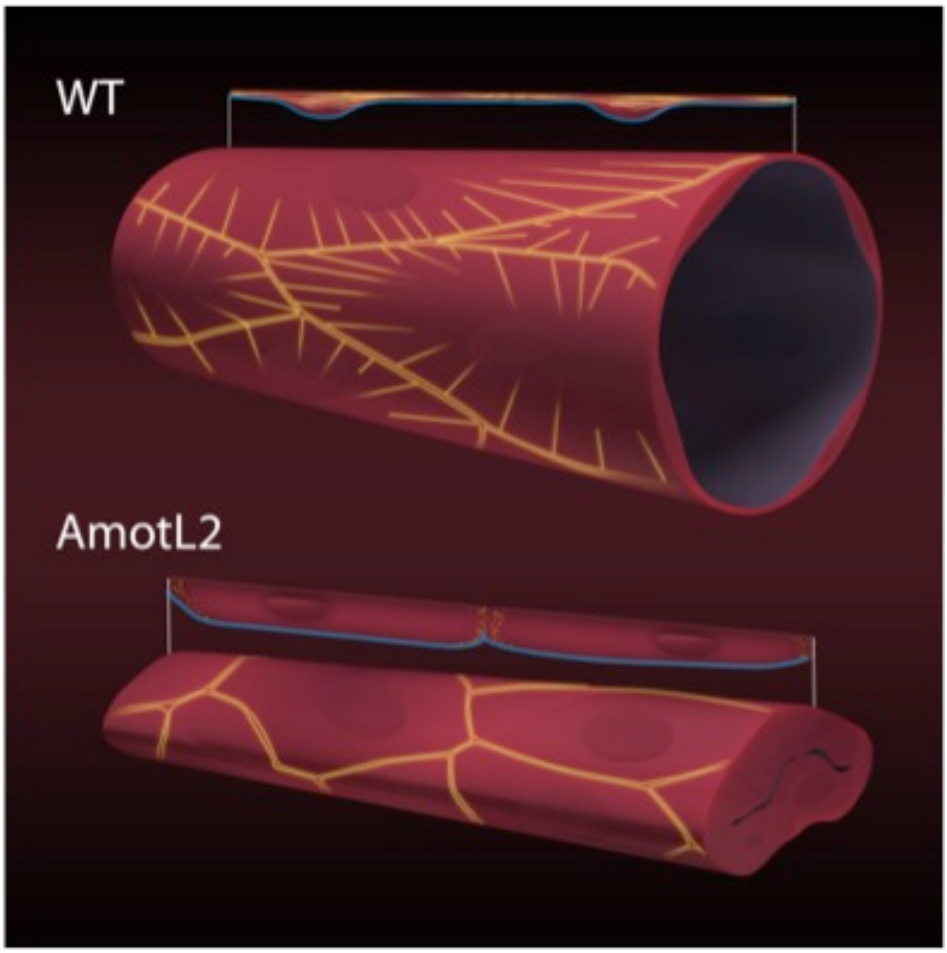

Figure 6. Schematic figure illustrating findings from paper I.

Paper II

The aim of this paper was to investigate the role of hypoxia induced AmotL2 during tumor progression.

The hypoxic regulation of AmotL2 prompted us to analyze the expression pattern of AmotL2 in human cancers. Paraffin-embedded sections from colon and breast cancer patients were stained for AmotL2. Normal breast epithelia was negative for AmotL2, however, the depolarized DCIS (ductal carcinoma in situ) showed strong positivity. Also tumor cells that had infiltrated the surrounding stroma were AmotL2 positive. Similar to breast tissues, AmotL2 staining was negative in normal colon epithelia. Polarized villous structures in the center of the tumors were AmotL2 negative, whereas the budding cells located in the tumor front were AmotL2 positive. The staining pattern suggested that AmotL2 was localized at large intracellular vesicles. Approximately 65 % of breast and colon (Dukes B-C) tumors were positive for AmotL2 staining. Furthermore, Kaplan-Meier analysis of AmotL2 expression and patient survival data showed that AmotL2 expression correlates with poor prognosis in colon cancer. AmotL2 encodes for two isoforms with a molecular mass of 100 kDa and 60 kDa. Both breast and colon tumor tissues showed negative staining when using a p100 AmotL2 specific antibody, indicating a correlation between p60 AmotL2 vesicles and malignancy.

We further went on to analyze if AmotL2 positive staining would overlap with hypoxia markers carbonic anhydrase IX (CAIX) or the glucose transporter 1 (Glut1). Immunofluorescent staining of cryosections from colon cancer patients showed a clear overlap between AmotL2 and CA9, and Glut1. We further showed *in vitro* that hypoxia would induce p60 AmotL2 in HeLa cells, giving rise to intracellular large vesicles. *In vitro* studies also indicated that hypoxic regulation of p60 AmotL2 is mediated by c-Fos binding to the *p60 AMOTL2* promoter. c-Fos is an oncogene that is part of AP-1 transcriptional complex. The AP1 complex has mainly been shown to be involved in proliferation, cell death and cellular transformation.

Mass spectrometry data helped us identify the large vesicular structures formed when p60 AmotL2 was expressed. Transport protein particle II complex (TRAPPII) were among the proteins that associated to the AmotL2 PDZ-binding motif, this complex associated specifically to p60 AmotL2 after 8 h of hypoxia. The TRAPPII is a highly conserved tethering complex that binds to Golgi-derived (coat protein complex 1-coated) vesicles to mediate Golgi traffic and has been implicated in the selective transport of proteins to the

plasma membrane in polarized cells (Qi, Kaneda et al. 2011; Westlake, Baye et al. 2011). Components of the TRAPP complex, TRAPPC9 and TRAPPC10 colocalized with p60 AmotL2 but not with junctional p100 AmotL2.

Previous work from our lab and others had shown that the Amot protein family interacts with PATJ/ Pals1/ Lin7 apical polarity protein complex (Wells, Fawcett et al. 2006; Sugihara-Mizuno, Adachi et al. 2007; Ernkvist, Luna Persson et al. 2009). This interaction is mediated by binding to the PDZ-binding motif of AmotL2. The PATJ/ Pals1/ Lin7 complex is characteristically associated to the apical membrane protein Crb3. Cytoplasmic domain of Crb3 could be immunoprecipitated with AmotL2, and endogenous levels of Crb3 colocalized with p60 AmotL2 in vesicles. Mass spectrometry analysis together with yeast two-hybrid screening revealed that also Par3 was directly associated with the PDZ-binding motif of AmotL2. Activation of p60 AmotL2 resulted in redistribution of Crb3 and Par3 from the apical membrane to intracellular vesicles. However, deletion of the PDZ-binding motif by which Par3 and Crb3 interact with p60 AmotL2 did not affect the subcellular localization of these proteins. Depletion of endogenous levels of p60 AmotL2 in 66c14 cells resulted in re-localization of Par3 and Crb3 to apical junctions and cilia. Sequestration of Par3 into intracellular vesicles resulted in altered Par3 dynamics, delayed tight junction formation, and delayed transport of E-cadherin to cell junctions.

We further went on to asses if sequestration of Par3 and Crb3 into intracellular vesicles would affect apical-basal polarity. MDCK as well as Caco-2 cells forms polarized cysts when grown in 3D matrices consisting of matrigel or collagen. Induction of p60 AmotL2, but not p100 AmotL2 or p60 AmotL2 ΔILI mutant, inhibited lumen formation and resulted in loss of apical–basal polarity. This affect was reversible. AmotL2 expression also correlated with loss of polarity *in vivo* in colon cancer patients.

Expression of p60 AmotL2 also caused scrambling of apical and basal membrane markers, interestingly also scrambling of c-Met from lateral junctions to apical and basolateral compartments. The hepatocyte growth factor (HGF) signaling via its receptor, c-Met, is closely associated to tumor invasion and metastasis (Boccaccio and Comoglio 2006). To further study if p60 AmotL2 would promote invasion, dox-regulated p60 cells were embedded in matrigel. p60 expression was not sufficient to induce invasion, however addition of HGF to p60 AmotL2-depolarized cells induced a robust invasive response in both MDCK and Caco-2 cysts. Depletion of p60 AmotL2 in the metastatic cell line 66c14 abrogated its invasive phenotype. Stimulation of the 66c14 cells with HGF increased invasion of the control cells, whereas AmotL2-depleted cells remained unresponsive.

To investigate if AmotL2 would induce invasion *in vivo,* control or p60 AmotL2 expressing Caco-2 cells were injected into mice. Histological analysis showed that control tumors grew as polarized glandular epithelia, whereas these structures were completely lost in p60 AmotL2 expressing tumors. The p60 AmotL2 expressing tumors had infiltrated into the surrounding stroma. By silencing endogenous levels of AmotL2 in 66c14 cells we inhibited dissemination and tumor growth *in vivo*.

In summary we present a novel molecular mechanism how cells from the invasive front of metastatic tumors respond to stress signals causing scrambling of polarity complexes. We have identified a novel pathway promoting plasticity independent of epithelial-mesenchymal transition.

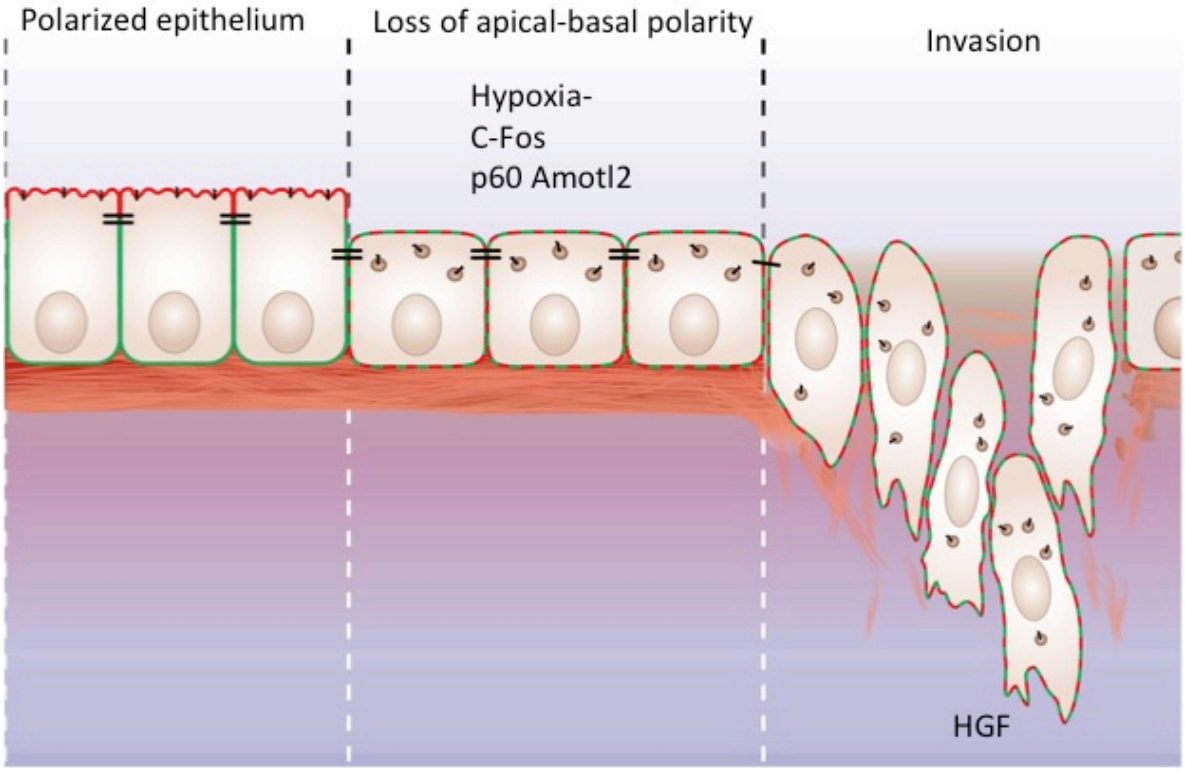

Figure 7. Schematic figure illustrating the findings in paper II.

Paper III

The aim of this paper was to investigate if p60 AmotL2 can antagonize the full-length isoform of AmotL2 and affect the connection of radial actin fibers to E-cadherin.

We have reported in Paper I that AmotL2 acts as a linker connecting radial fibers to VE-cadherin in the endothelium. Ongoing work in the group has shown that AmotL2 associates

to E-cadherin in the epithelium. We have shown that depletion of full length AmotL2 affects packing of the epithelial-cell layer and three-dimensional morphogenesis (Hildebrand et al submitted).

As previously reported the members of the Amot protein family form oligomers via the association of the coiled-coil domains (Ernkvist, Birot et al. 2008; Moleirinho, Guerrant et al. 2014). Different isoforms of AmotL2 have distinct promoters whereupon p100 AmotL2 is constitutively expressed, whereas p60 AmotL2 is induced by hypoxia (as shown in paper II). In order to test if p60 could associate to p100 AmotL2 and interfere with its normal function, we performed immunoprecipitation and immunofluroscent studies using antibodies directed against the p100 AmotL2 specific N-terminal domain. We could conclude that p60 and p100 AmotL2 forms oligomers, resulting in delocalization of p100 AmotL2 to intracellular vesicles.

We further investigated whether relocalization of p100 Amotl2 upon p60 activation would affect binding partners. Co-immunoprecipitation studies showed that the association of p100 AmotL2 to its binding partners was lost in the presence of p60 AmotL2. The disassociation of MAGI1 from AmotL2 resulted in perturbed cytoskeletal architecture. Phalloidine visualization revealed a specific loss of radial actin fibers, similar to what we have previously observed in p100 depleted cells (Hildebrand et al unpublished data).

Furthermore, the activation of p60 disassociated p100 AmotL2 from E-cadherin affecting epithelial organization and cell-cell cohesions. Live-cell imaging studies revealed that p60 AmotL2 expressing cells showed increase in filopodial extensions in a confluent epithelial layer, which could be due to decreased cohesiveness. Suggesting that p60 affects E-cadherin based tension from the adherent junctions.

In conclusion, we present data showing that p60 AmotL2 promotes invasion in two ways, inhibition of apical-basal polarity (paper II) and destabilization of E-cadherin mediated adhesions. In paper II we show that p60 AmotL2 activation results in delocalization of E-cadherin from lateral junctions to apical and basal compartments and delayed junction formation. In this paper we extend our findings showing that not only does p60 affect E-cadherin localization, but also its connection to actin filaments. The effect on the actin filaments phenocopy depletion of p100 AmotL2 indicating that p60 AmotL2 may act as a p100 AmotL2 antagonist.

7 CONCLUDING REMARKS

In this thesis I have studied the role of AmotL2 in vascular development and cancer progression. When I started this work it was not much known about AmotL2, most efforts had been focused on Amot or AmotL1.

This thesis provides evidence showing that AmotL2 is essential for vascular lumen expansion, by transmission of junctional force between cells. We report a novel mechanism for which transmission of mechanical force is essential for the coordination of cellular morphogenesis. Future studies may show if this mechanism is important for lumen formation in other tubular epithelia and ductal organs.

This thesis also provides evidence showing that AmotL2 plays a very critical role in deregulating apical basal polarity in invasive tumor cells. Here we identified hypoxia-induced p60 AmotL2 as a negative regulator of cell polarity during tumor progression. Furthermore we provide data showing that p60 AmotL2 affects E-cadherin based tension, promoting cell plasticity. We believe that we have discovered a novel pathway that contributes to metastatic spread, independent of EMT, and hope that this could add more insight to how cells are driven towards a more metastatic phenotype.

I hope with data presented in paper II, that p60 AmotL2 can be considered as a prognostic marker for metastatic disease due to its expression pattern. In paper II we also demonstrate that depletion of p60 AmotL2 inhibits tumor growth and invasion, which suggests for p60 AmotL2 to be a good target for metastatic disease. Recent publications demonstrating the importance of cell polarity during tumor progression suggests that perhaps deregulation of polarity can be added to the list of hallmarks in the future.

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