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MAKING ENDOTHELIAL CELLS MOVE – A STUDY OF ANGIOMOTIN AND BINDING PARTNERS

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Institutet**

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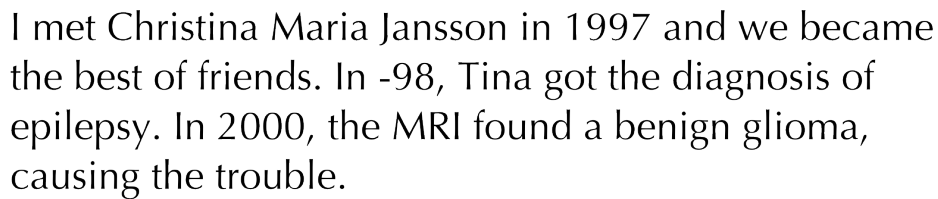
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Cover photo: Blood vessels invading into a bFGF-stimulated matrigel plug. Red: Blood vessel (CD31). Green: Pericytes (NG2). Staining and design by Nathalie Luna Persson. Microscopy by Sara Hultin.

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Still, she kept her spirits up.

Tina died on a Sunday, 23 years old. The anaplastic pleomorphic xanthoastrocytoma had taken its' toll.

You are my inspiration and I will miss you, always.

Populärvetenskaplig sammanfattning

Blodkärl finns i hela kroppen. De syns för det mesta som turkosa strängar under huden när vi ser efter. De är oerhört viktiga för vår överlevnad och bildandet av nya blodkärl är som allra viktigast när våra sår läker, under menstruation och graviditet samt självklart under fosterutvecklingen i mammans mage. Lagom är bäst tycker kroppen och därför ställer överdriven eller otillräcklig blodtillförsel till med problem och sjukdom. I den här avhandlingen har jag fokuserat på sjukdomen cancer.

Tumörer kan inte överleva och växa bortom ett knappnålshuvuds storlek utan syre och näring. Därför åker de snålskjuts på kroppens eget maskineri och lockar till sig blodkärl. I och med detta kan tumören inte bara växa utan har också införskaffat ett sätt att sprida sig på, metastasera. Sammantaget är detta anledningen till att hög blodkärlsnärvaro i tumörer inte är bra när man talar om patienters prognos.

Blodkärl består av speciella celler, så kallade endotelceller. Nya blodkärl kan bildas (förgrenas) från ett befintligt kärl genom att endotelcellerna, likt en ny kvist på en gren, börjar växa, dela sig och röra sig från det befintliga blodkärl (grenen) mot t.ex. tumören. Detta kallas angiogenes. Det finns andra sätt för blodkärl att bildas på, men detta är det i särklass vanligaste sättet som det sker på i en tumör.

Jag har studerat proteinet Angiomotin, ett protein som vi har visat är viktigt för endotelcellernas rörelse. Man har visat att Angiomotin binder till speciella, kolesterolrika fettmolekyler, till proteiner viktiga för celldelning och till så kallade polaritetsproteiner. De sistnämnda ger cellen en känsla för vad som är fram och bak när den ska röra sig. När celler tappat den förmågan blir de antingen stillasittande eller rör sig helt slumpartat åt olika håll.

Forskargruppen jag tillhört har visat att vaccination mot Angiomotin både skyddar möss mot tumörutveckling och att befintliga tumörer hämmas i sin tillväxt. En potentiell terapeutisk antikropp mot Angiomotin som vi, tillsammans med läkemedelsföretaget BioInvent, tagit fram har visat på kraftigt minskad kärlinväxt i tumörer och tumörsimulerande material. Mer arbete med att utveckla antikroppen kommer dock att behövas för att ge den en tillräckligt bra livslängd i kroppen för att vara praktiskt användbar. I ett hitintills opublicerat arbete har jag bidragit till att försöka öka livslängden på antikroppen och har varit med när vi gjort försök att kunna använda den för diagnostiska ändamål.

I den här avhandlingen visar jag *hur* Angiomotin binder till sina olika partners och *varför* detta påverkar endotelcellernas rörelse. Påståendet att Angiomotin ska vara ett mål att attackera för potentiella läkemedel får ytterligare tyngd i och med de fynd som gjorts. Vi har till exempel sett att tillväxten av neurofibromatos, en sjukdom där tumörer växer längs med hjärnans och ryggmärgens nerver, till stor del är beroende av Angiomotin. Jag hoppas att mitt arbete och det som kommer ske efter att jag lämnat denna forskargrupp bidrar till nya behandlingssätt mot cancer.

Abstract

Angiogenesis is a process crucial for tumor growth and metastasis formation and encompasses the control of endothelial directional migration, anastomosis and lumen formation. We have previously shown that Angiomotin (Amot), a membrane-associated scaffold protein, plays an essential role in controlling endothelial cell migration and cell shape. Amot is critical for normal development as more than 75% of *amot* deficient mouse embryos die *in utero* due to vascular defects.

In this thesis, the vascular system of the mice that survive gestation are studied. These mice are apparently normal in regards of body weight, kidney function and reproduction. Further, the vascular system did not exhibit any changes in perfusion or vascular density. However, the growth of Lewis Lung Carcinoma tumor xenograft was significantly impaired in *amot* deficient mice as compared to sibling wt mice. Analysis of the tumor vasculature revealed a marked change in vessel morphology and decreased vascular density and perfusion. These data argue that Amot, besides its role in development, plays a critical role in pathological angiogenesis. Gene expression analysis shows that the loss of *amot* expression leads to indirect effects, which activates NF- κ B regulated genes and results in an inflammatory phenotype *in vitro* and *in vivo*.

As the thesis title implies, the function of Amot in endothelial cell migration has been investigated by identifying and studying its binding partners and their functions. The polarity proteins Patj and Mupp1 (plus Pals1) are presented as binding partners to the Amot PDZ-binding motif. This is of interest since establishing cell polarity is a key step in directional endothelial migration. Migration also depends on correct actin polymerization, driven by Rho GTPases. We show that Amot is essential for the localization of RhoA activity at the leading edge of a migrating cell and that the Rho GEF (activator of RhoA) Syx1 also associates to Patj/Mupp1. Finally, we show that the interaction of the PDZ-binding motif to Patj/Mupp1/Syx1 is essential for vessel migration in developing zebrafish embryos.

Furthermore, tight junction-associated tumor suppressor Merlin (*nf2*) is identified as a binder to the Amot coiled-coil domain. Rich1, a negative regulator of Rac1 (also a RhoGTPase), has been shown to bind to Amot through the same domain. We show that Amot regulates Rac1 activity and subsequent MAPK pathway activation through Merlin and Rich1 and that Merlin and Rich1 compete for the same binding site on Amot, resulting in Rac1 regulation. We hypothesize that Rich1 is inhibited by its binding to Amot, leaving Rac1 in its active state, able to polymerize actin. Upon binding to Merlin, Amot releases Rich1 and Rac1 is inhibited. Finally, we show that Schwann cell tumors, caused by loss of *nf2* expression, exhibit a decreased growth rate upon loss of *amot* expression, leading to prolonged mouse survival. This further emphasizes the potential for Amot as a drug target and indicates that anti-Amot therapy could be used to treat Schwannoma patients.

Amot localizes to the lamellipodia of migrating cells, a region of the cell membrane with specific lipid composition. The Amot coiled-coil domain has been shown to be a conserved lipid-binding domain. I propose a model where migratory cues trigger the change in lipid composition of the cell membrane, concentrating lipids to which Amot binds to the lamellipodia. Amot brings with it the complex of Patj/Mupp1/Syx1, localizing RhoA activity and thus actin polymerization at the leading edge.

List of Publications

- I. The Amot/Patj/Syx signaling complex spatially controls RhoA GTPase activity in migrating endothelial cells.** Mira Ernkvist, Nathalie Luna Persson, Stéphane Audebert, Patrick Lecine, Indranil Sinha, Miaoliang Liu, Marcus Schlüter, Arie Horowitz, Karin Aase, Thomas Weide, Jean-Paul Borg, Årindam Majumdar, Lars Holmgren. Blood. 2009. Jan 1; 113(1):244 – 253.
- II. A Tight Junction-Associated Merlin-Angiomotin Complex Mediates Merlin's Regulation of Mitogenic Signaling and Tumor Suppressive Functions.** Chunling Yi, Scott Troutman, Daniela Fera, Anat Stemmer-Rachamimov, Jacqueline L. Avila, Neepa Christian, Nathalie Luna Persson, Akihiko Shimono, David W. Speicher, Ronen Marmorstein, Lars Holmgren, Joseph Kissil. Cancer Cell. 2011. April 12; 19(4): 527 – 540.
- III. The Role of Angiomotin in Pathological Angiogenesis.** Nathalie Luna Persson, Liping Yang, Maddalena Arigoni, Raffaele Calogero, Guido Forni, Staffan Nyström, Federica Cavallo, Lars Holmgren. 2012. Manuscript.

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List of Abbreviations

¹¹ C	Carbon-11
¹²⁴ I	Iodine-124
AAMP	Angio-Associated Migratory Cell Protein
ABD	Albumin-Binding Domain
ACCH	Amot Coiled-Coil Homology
Amot	Angiomotin
AmotL1	Angiomotin-like 1
AmotL2	Angiomotin-like 2
Arg1	Arginase 1
ASM	Aggressive Systemic Mastocytosis
BAR	Bin/Amphiphysin/Rvs
Baz	Bazooka
BBB	Blood-Brain Barrier
bFGF	Basic Fibroblast Growth Factor
CAF	Cancer-Associated Fibroblast
CNS	Central Nervous System
Crb3	Crumbs homology domain 3
CTL	Cytotoxic T-Lymphocyte
DC	Dendritic Cell
DLAV	Dorsal Longitudinal Anastomosis Vessel
EC	Endothelial Cell
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
FACS	Fluorescent Activated Cell Sorting
FDG	Fluoro-2-deoxy-D-glucose
FSP-1	Fibroblast-Specific Protein-1
GEF	Guanine Nucleotide Exchange Factor
HER-2	Human Epidermal Growth Factor Receptor 2
INADI	InaD-like protein
ISV	Inter-Somitic/Segmental Vessel
JAM	Junctional Adhesion Molecule
Lats 1/2	Serine/threonine-protein kinase Lats 1/2
Lin7C	Protein lin-7 homolog 3
MDSC	Myeloid-Derived Suppressor Cell
Mer	Merlin
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
MO	Morpholino
MOB1A/B	Mob kinase activator 1 A/B
MPDZ	Multi-PDZ domain protein
MPP5	MAGUK p55 subfamily member 5-A
Mst 1/2	Serine/threonine-protein kinase 3/4
NF-2	Neurofibromatosis-2

NG2	Nerve/Glial antigen 2
NK Cell	Natural Killer Cell
Pals1	Protein Associated to Lin 7
Par-3	Partitioning defective 3 homologue
Patj	Pals-1-Associated to Tight Junction protein
PDGF	Platelet-Derived Growth Factor
PDGFR- β	Platelet-Derived Growth Factor Receptor- β
PEG	Polyethylene Glycol
PET	Positron Emission Tomography
Plekhg5	Plextrin homology domain 5
PLGF	Placental Growth Factor
PmT-EC	Polyma Middle-T Endothelial Cell
Rich-1	RhoGAP Interacting with CIP4 Homologues -1
RTK	Receptor Tyrosine Kinase
α -SMA	ALPHA-Smooth Muscle Actin
Std	Stardust
Sec	Selenocystein
Sel-tag	Selenocystein tag
SMC	Smooth Muscle Cell
T _{reg}	T-Regulatory Cell
TAM	Tumor-Associated Macrophage
TAZ	Transcriptional co-activator with PDZ-binding motif
TEF	Transcriptional Enhancer Factor
VEGF	Vascular Endothelial Growth Factor
WW45	Protein salvador homolog 1
YAP	Yes-associated protein
ZF	Zebrafish
ZO	Zona Occludens

1. The Hallmarks of Cancer and Cancer Microenvironment

Cancer is a heterogeneous disease that comes in many shapes, sizes, origins, causes, compositions, behaviors and most importantly, outcomes. Unlike e.g. chicken pox, cancer is not a single disease; it is the collective name for illnesses that share some common characteristics, the main one being continuous, abnormal growth of cells. In turn, 'breast cancer' is a collective term for tumors originating from or growing in breast tissue, sharing some traits but still differing immensely amongst themselves.

Tumors start out as benign and may progress into malignancy, meaning that it has become more aggressive, is growing outside its original compartment and is spreading. This change, called tumor progression, occurs as the tumor cells undergo dynamic genetic changes, leading to the selection and clonal expansion of those cells that have gained advantageous characteristics. In 2000, Douglas Hanahan and Robert Weinberg came up with the term 'The Hallmarks of Cancer' Figure 1 [1, 2]. They state that all the tricks we see cancer cells (of any kind) perform boil down to eight essential alterations in cell physiology:

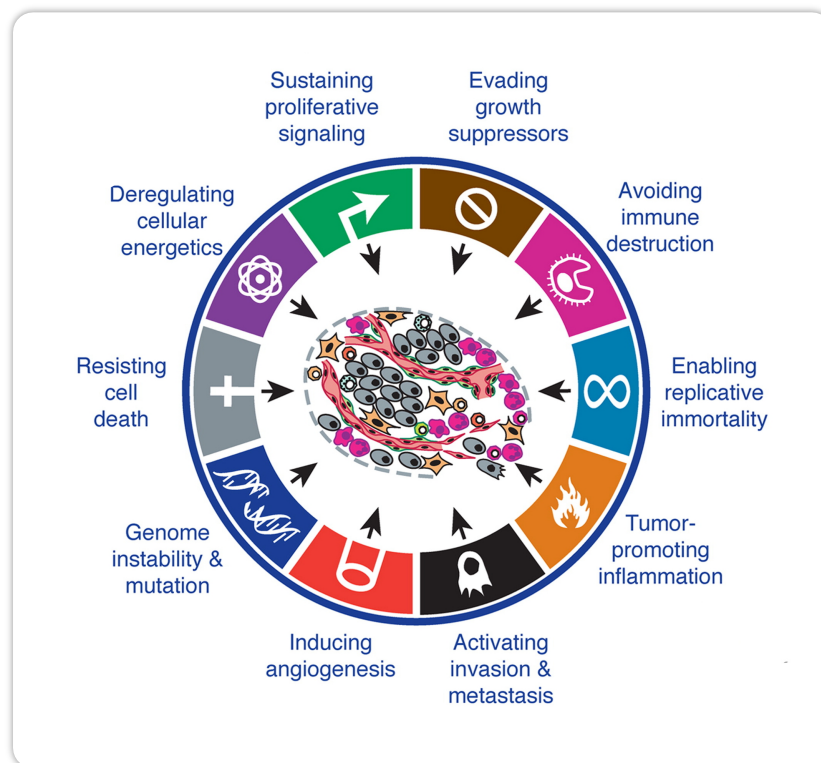


Figure 1. The Hallmarks of Cancer - The Next Generation. Modified from Hanahan & Weinberg, 2011. Cell.

- Self-sufficiency in growth signals
- Insensitivity to growth-inhibitory (antigrowth) signals
- Evasion of programmed cell death (apoptosis)
- Limitless replicative potential
- Sustained angiogenesis
- Tissue invasion and metastasis
- Evading immune destruction
- Deregulating cellular energetics

‘Genome instability & mutations’ and ‘Tumor-promoting inflammation’, in Figure 1, are not Hallmarks *per se*, but are events that drive tumor progression and facilitate acquisition of the Hallmarks.

During the last decades, major research efforts have focused on understanding the mechanisms underlying cancer development. With time the way of viewing cancer has changed, which is clearly shown in Figure 2. Instead of studying the mere tumor cells, the tumor is now considered to be as intricate as any organ, with all the added complexity of interacting cell types that comes with it [3-8]. I will focus the remainder of this section on the other cell types within and surrounding the tumor, the so-called tumor microenvironment, and how they contribute to the ever-mounting complexity of cancer.

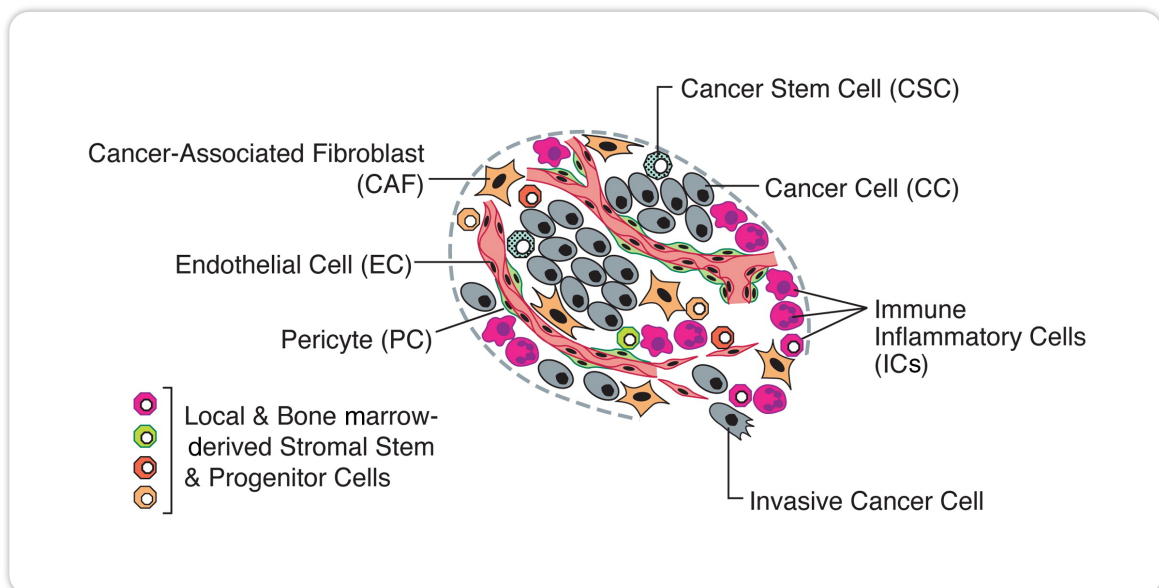


Figure 2. The Tumor Microenvironment. Modified from Hanahan & Weinberg 2011, Cell.

1.1 Fibroblasts

Fibroblasts are a kind of cell found most abundantly in connective tissue. They produce the Extracellular Matrix (ECM), the fundamental framework that all cells utilize for adhesion and migration. They also modulate the

surrounding cells by secreting growth factors and cytokines [9]. Fibroblasts are heterogenous in morphology, changing appearance depending on their localization [10].

Cancer associated fibroblasts (CAFs) is a term that encompasses a very heterogenic group of fibroblasts, sharing their morphological appearance as fibroblasts, their localization in the tumor stroma and expression of a few surface markers: α -smooth muscle actin (α -SMA), platelet-derived growth factor (PDGF) receptors and Fibroblast specific protein (FSP)-1 [11]. Some claim CAFs to originate from bone-marrow progenitor cells, others say they are local tissue-derived fibroblasts and there are also reports of transdifferentiating epithelial cells [12-15].

Studies on CAFs have mostly been conducted by co-injecting them together with tumor cells into mice and subsequent observation of the altered characteristics of the tumor cells. The collective conclusion is that CAFs have a cancer-initiating capacity, stimulate tumor cell proliferation and stimulate angiogenesis. This is done by providing the ECM for the endothelial cells to migrate and by expressing the essential growth factors needed for the survival and stimulated migration of vascular endothelial cells [11]. The net effect is that CAFs facilitate the initiation, growth, invasion and metastasis of tumors [9, 16]. Moreover, it has been shown that CAFs mediate drug resistance to therapies such as anti-angiogenic drugs and tyrosine kinase inhibitors [11] [17]. Therefore, the notion of using CAF gene signatures both for prognosis and response-prediction to therapy is being studied, with promising results [11, 18].

1.2 Immune Cells

The inflammatory system is a complex and fascinating system. Not only can it rid us of diseases, adapt and learn it can cause havoc in the shape of allergies, autoimmune diseases and cancer. The latter happening as chronic inflammation drives tumor progression into a more aggressive disease [19, 20]. As a token of their omnipresence in our lives tumor-infiltrating immune cells are a frequent finding when studying patient tumor biopsies. However, unlike the presence of blood vessels, which is unanimously considered poor for patient prognosis immune cells have conflicting functions meaning that their general presence is ambiguous [20-22].

There is an ever-growing list of tumor-promoting molecules secreted by inflammatory cells. On the list are growth factors such as Epidermal Growth Factor (EGF), Vascular Endothelial Growth Factor (VEGF) and basic Fibroblast Growth Factor (bFGF), chemokines, cytokines and also pro-angiogenic and pro-invasive factors such as Matrix Metalloproteinase (MMP)-9 and proteases [23, 24]. As a result, infiltrating immune cells may

promote tumor proliferation, spur angiogenesis and facilitate invasiveness/metastasis.

Tumors try to evade the anti-tumorigenic part of the immune system, like the Cytotoxic T-Lymphocytes (CTLs) e.g. by downregulating receptors that may trigger immune responses, like Major Histocompatibility Complex (MHC) class I [25, 26]. They secrete anti-inflammatory factors to throw the attacking immune cells off their scent and to recruit tumor-promoting immune cells. It is a delicate balance. While the presence of Natural Killer (NK) cells and CTLs indicates anti-tumorigenic activity and thus is beneficial for patients, these cells may be rendered anergic. The combination of factors secreted by the tumor and the cells recruited, can result in an unfavorable milieu. Bone-marrow-derived myeloid cells differentiate into several cell types in response to tumor-secreted factors. They become Tumor Associated Macrophages (TAMs), Myeloid Derived Suppressor Cells (MDSCs) and Tolerogenic Dendritic Cells (DCs). The latter stimulates the differentiation of bone-marrow-derived progenitor cells into Regulatory T Cells (T_{regs}). Just as they would in a healthy individual T_{regs} suppresses immune response and maintains the tolerance to self-antigens. This means the double presence of CTLs and T_{regs} results in no killing of tumor cells [22]. To add insult to injury both MDSCs and TAMs secrete e.g. Arginase 1 (Arg1), digesting L-arginin needed by the CTLs and as a result causing anergy and a lack of tumor killing [27]. As a result, knowledge of both environment and cell types present is needed in order to make an assessment if the tumor is being helped or fought by the immune system in a given tumor.

1.3 Pericytes

Charles Rouget unknowingly described the first pericytes as far back in time as the late 19th century. He observed cells with distinct finger-like cytoplasmic projections that wrapped themselves around capillaries [28-30]. Today, the envelopment of capillaries still is the common trait of all pericytes (see Figure 3). They form a continuous sheath around endothelial tubes, with smooth muscle cells replacing them on larger vessels. The number of pericytes per vessel varies depending on localization in the body and the characteristics of the vessel to envelope [31].

Pericytes are related to smooth muscle cells and differ in morphology depending on their origin or their microenvironment, i.e. the vascular bed and their position along the arterio-venous axis. [32] [31] [33]. Pericytes stem from different origins: proliferative expansion of pre-existing local pericyte-pools (longitudinal recruitment) [34] [35, 36] and differentiation from mesenchymal cells (fibroblasts) [37]. In the Central Nervous System (CNS) pericytes originate from both mesoderm-derived mesenchymal stem cells and neuroectoderm-derived neural crest cells, depending on the

location within the developing cerebrovascular tree [38]. Mesoderm-derived bone marrow progenitor cells have also been shown to contribute to the CNS pericyte population after ischemia [39, 40]. This differs from endothelial cells, which are thought to be of uniform origin, with the exception of tumor endothelial cells.

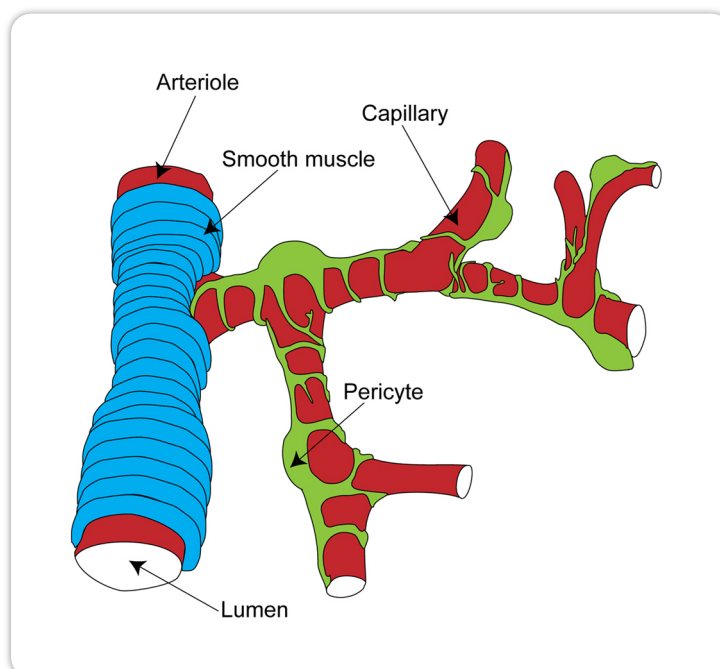


Figure 3. Smooth muscle cell coverage of arterioles and pericyte coverage of capillaries. Modified from Hamilton et al. 2010. Front Neurogenetics.

In turn, pericytes may differentiate further into smooth muscle cells and macrophages. In a kind of limbo between the cell types, pericytes may acquire macrophage-like phagocytotic qualities that enable them to clear toxic substances and byproducts [38, 41]. Again, this feature is most important and mostly studied, in the CNS.

Despite the heterogeneity pan-pericyte markers can be found. Pericytes of different kinds express non-muscle myosin, tropomyosin desmin, nestin, platelet-derived growth factor receptor β (PDGFR- β), aminopeptidase A, aminopeptidase N (CD13), sulfatide, α -smooth muscle actin (ACTA2/ α -SMA) and nerve/glial antigen 2 proteoglycan (NG2) [42]. In quiescent tissue the common denominator for pericytes is that they all express PDGFR- β , NG2 and Desmin. The expression of α -SMA appears to differ depending on what pericyte-type is observed, where it resides and which state it is in. This has given rise to a debate as to whether or not α -SMA is a pan-pericyte marker [38]. However, in tumor vasculature α -SMA is expressed.

As a relative to smooth muscle cells pericytes have contractile capabilities, making it possible to constrict blood vessels, regulating blood flow, in

response to neuronal signals. This is most essential for the functionality of the Blood-Brain Barrier (BBB), where the constriction of the cerebral capillaries allows/disallows access for larger proteins and particles to the brain. As a consequence of this necessity CNS vasculature has significantly higher pericyte coverage than do peripheral tissues [31, 38].

Pericytes are interconnected with endothelial cells both mechanically through their shared, commonly secreted, basement membrane and through shared ions and molecules via gap junctions [43]. These bi-directional junctions enable the cells to communicate and influence each other's behavior. The Endothelial Cells (ECs) secrete soluble factors such as PDGF and basic Fibroblast Growth Factor (bFGF) that promote pericyte proliferation and migration. Studies on mouse genetic models show that the recruitment of pericytes to normal and tumor vessels are dependent on the PDGFR- β signaling pathway [35].

In turn, the pericytes secrete multiple factors that affect the ECs. Angiopoietin (Ang)-1 is secreted by pericytes and it binds to Tyrosine kinase with immunoglobulin-like and EGF-like domains (Tie)-2 receptors expressed on the surface of endothelial cells. In the angiogenic vessel, endothelial Tie-2 expression is localized behind the leading edge, coexisting with the location of vessel maturation [44]. In turn, Ang-2 is expressed by endothelial cells, located at the leading edge of sprouting vessels [45]. Studies show that Ang-2 competes with Ang-1 for Tie-2. Also pericytes express Tie-2 and in the presence of high levels of Ang-2 (binds to Tie-2) pericytes dissociate from the endothelial cells and this results in the destabilisation of the vessel, an event occurring during angiogenesis (see Section 2.2).

Vascular Endothelial Growth Factor (VEGF) is secreted by sprouting endothelial cells and leads to the expression of Ang-2 and the suppression of PDGFR signaling. This, together with MMP degradation of the shared basal membrane, can explain the loss of pericytes during the initial phase of angiogenesis. In the context of pericytes VEGF has multiple functions. Under hypoxic conditions VEGF directly induces proliferation and migration of pericytes [46]. Pericytes also secrete low levels of VEGF that acts as a survival mechanism for endothelial cells in a juxtacrine/paracrine manner and maintains endothelial homeostasis [34, 47]. Other pathways, ligands and receptors are also important for the functionality of pericytes eg. Notch-3 and Ephrin-B2 [48].

In tumors, the role of pericytes is somewhat unclear. It abides to logic that the function of pericytes in tumors would be to stabilize the newly forming tumor blood vessels, sending survival signals to the endothelial cells. However, studies show that pericyte coverage of tumor vasculature is variable, ranging all the way down to 10% in eg. Glioblastoma [49]. Overall, factors secreted by tumors appear to be sufficient for endothelial survival.

All in all, pericytes are an important part of the machinery governing vessel homeostasis and blood brain barrier integrity. However, their role in tumor vasculature remains to be elucidated.

1.4 Endothelial cells

Endothelial cells constitute the innermost layer of all blood vessels in the body and are the cells in focus for this thesis. Upon initiation of migration they exhibit protruding, growth factor seeking filopodia and a ruffled membrane at the leading edge called lamellipodia *in vitro* [50-52]. Endothelial cells constitutively express multiple markers, shown in Table 1, but other markers exist, expressed upon induction or on organ-specific endothelia [53].

During embryonic development, blood vessel formation is one of the earliest events of organogenesis. Mesodermal angioblasts are endothelial cell precursors that differentiate into endothelial cells in response to e.g. VEGF [54]. This is a process called vasculogenesis, further described in Section 2.1. From this nascent primitive plexus more vessels are sprouted through angiogenesis, further described in Section 2.2.

Endothelial cells show remarkable heterogeneity for being a single cell kind. The surrounding cells and matrix shape the ECs and their behavior. Examples of ECs that have gained very "organ"-specific characteristics are the ECs of the High Endothelial Venules (HEVs), the bone marrow endothelium and the cerebral endothelium. Also, these specialized ECs have been showed to secrete factors important for certain organs to develop properly, such as the pancreas [55].

Table 1. Endothelial Markers. From Garlanda & Dejana, *Arterioscler Thromb Vasc Biol.* 1997.

	Species ¹	Cell Type ²	Reference
Factor VIII-related antigen	h/m	ECs (irregularly expressed by capillaries and tumor vessels), platelets, megakaryocytes	Belloni and Tressler, 1990
CD31/PECAM-1	h/m	ECs, platelets, megakaryocytes, B and T lymphocyte subsets, monocytes, neutrophils	DeLisser et al, 1994, Vecchi et al, 1994
Angiotensin-converting enzyme	h/m	ECs, epithelial cells, monocyte-macrophages, T lymphocytes	Belloni and Tressler, 1990
Type I scavenger receptor (acetylated-LDL uptake)	h/m	ECs, macrophages, SMCs, pericytes, fibroblasts	Voyta et al, 1984
<i>Ulex europaeus</i> I agglutinin binding/O(H) blood-type antigen	h	ECs, erythrocytes	Jackson et al, 1990
<i>Bandeirea simplicifolia</i> lectin binding	m	ECs	Belloni and Tressler, 1990
<i>Griffonia simplicifolia</i> agglutinin binding	m	ECs	Sahagun et al, 1989
Weibel-Palade bodies	h/m	ECs	Weibel and Palade, 1964
Vascular endothelial cadherin	h/m	ECs, trophoblasts, PLN sinus macrophages	Lampugnani et al, 1992
CD34	h/m	ECs, hemopoietic precursors	Krause et al, 1996
CD102/ICAM-2	h/m	ECs, lymphocytes, monocytes, platelets	Springer, 1990
CD51/61 (vitronectin receptor)	h/m	ECs (overexpressed in tumor ECs), platelets, megakaryocytes, osteoclasts, mast cells, B lymphocytes	Brooks et al, 1996
CD105/endoglin	h	ECs (overexpressed in tumor ECs), monocyte-macrophages, B lymphocytes, syncytiotrophoblasts	Gougos and Letarte, 1988
CD36	h	Microvascular ECs, monocyte-macrophages, erythroid cells, platelets, megakaryocytes	Greenwalt et al, 1992
CD73/VAP-2	h	ECs, T and B lymphocytes, tonsillar epithelium	Airas et al, 1993
S-ENDO 1/MUC18	h/m	ECs, SMCs, dendritic cells, leukocytes, melanoma cells, carcinoma cells	Bardin et al, 1996
Thrombomodulin	h/m	ECs, SMCs	Esmon, 1995
HEMCAM	m	Microvascular ECs, hemopoietic progenitors	Vainio et al, 1996
Sca-1	m	ECs, hemopoietic precursors	Yamamoto et al, 1996
AAMP	h	ECs, cytotrophoblasts, mononuclear inflammatory cells, melanoma cells, adenocarcinoma cells	Beckner et al, 1995

- Endothelial markers are those most commonly used and/or relatively restricted to the endothelial lineage. Most of them are not expressed by all kinds of vessels or in all tissues; markers ubiquitously expressed are marked as EC. Other antibodies nonspecifically recognizing ECs are described in Reference 30.
- h indicates human; m, murine; AAMP, angio-associated migratory cell protein; SMC, smooth muscle cell, and PLN, peripheral lymph nodes.
- 1 Expression by h or m cells only has been considered.
- 2 For some markers, tissue distribution analysis is incomplete; only the most common expression pattern has been considered.

1.4.1 Endothelial cells in this thesis.

Mouse Aortic Endothelial (MAE) cells are spontaneously immortalized murine endothelial cells of the aorta [56]. Our MAE cells have a C57/B6 background and are described in Levchenko et al 2003 [57]. MAE cells express no (by Western Blot) detectable levels of Amot [58].

Bovine Capillary Endothelial (BCE) cells are, as the name implies, capillary endothelial cells of bovine origin. We have used them both with and without being immortalized with hTERT [59, 60]. BCE cells express both isoforms of Amot [61, 62].

Mouse (MS)-1 cells (ATCC: CRL-2279) are SV40 Large T transformed murine pancreatic islet endothelial cells, also from a C57/B6 background [63]. MS-1 cells express primarily the p80-Amot isoform [64].

Polyoma Middle T Endothelial Cells (PmT-ECs) have been described in publications by our group and are used in Paper I and III of this thesis [61, 65-67]. It is a stable *amot* negative murine endothelial cell line with a C57/B6 background. It was procured from embryoid bodies of wild-type or *amot* negative mice that had been differentiated into ECs and were subsequently immortalized with Polyoma Middle T [61, 68]. Wild-type PmT-ECs express both isoforms of Amot [61, 67].

2 Blood vessels

In a healthy individual, blood vessels follow a hierarchy where larger vessel like arteries and veins branch into smaller arterioles and venules, which in turn branch out into capillaries. Capillaries, the smallest blood vessels in the body, consist of several layers. Surrounding the circulating erythrocytes and plasma is the innermost layer, the endothelial cells. Each EC connects to the other via Tight Junctions (TJs) and Gap junctions [32, 43]. The ECs secrete a basal membrane on which they are attached. Sharing, and secreting, the same membrane are the pericytes, sheathing and stabilizing the endothelial tube. Inter-neurons, regulating vascular tone, are connected and in the case of cerebral vasculature, astrocytes encase the capillary. Apart from the shared basement membranes, gap junctions between the cells allow for inter-cell communication.

Tumor vessels are abnormal in both architecture and structure. Ignoring the hierarchy of the vascular system they also tend to be dilated, tortuous, and hyperpermeable [69].

2.1 Vasculogenesis

The process of vasculogenesis (Figure 4 and 8b) in mice is very well described in a review article by Chung & Ferrara, published last year [70]. It describes the mesodermal-derived hemangioblast, which migrates from the primitive streak into e.g. the yolk sac and the intra and extra embryonic ectoderm. As enough cells have aggregated into blood islands they join to form a primary capillary plexus. In parallel, the dorsal aorta and cardinal vein are formed and together they form a primitive vascular network, further branched and matured via angiogenesis (see Section 2.2). The blood vessels undergo maturation and stabilization upon the recruitment of mural cells. This process is illustrated in more detail in Figure 4 below.

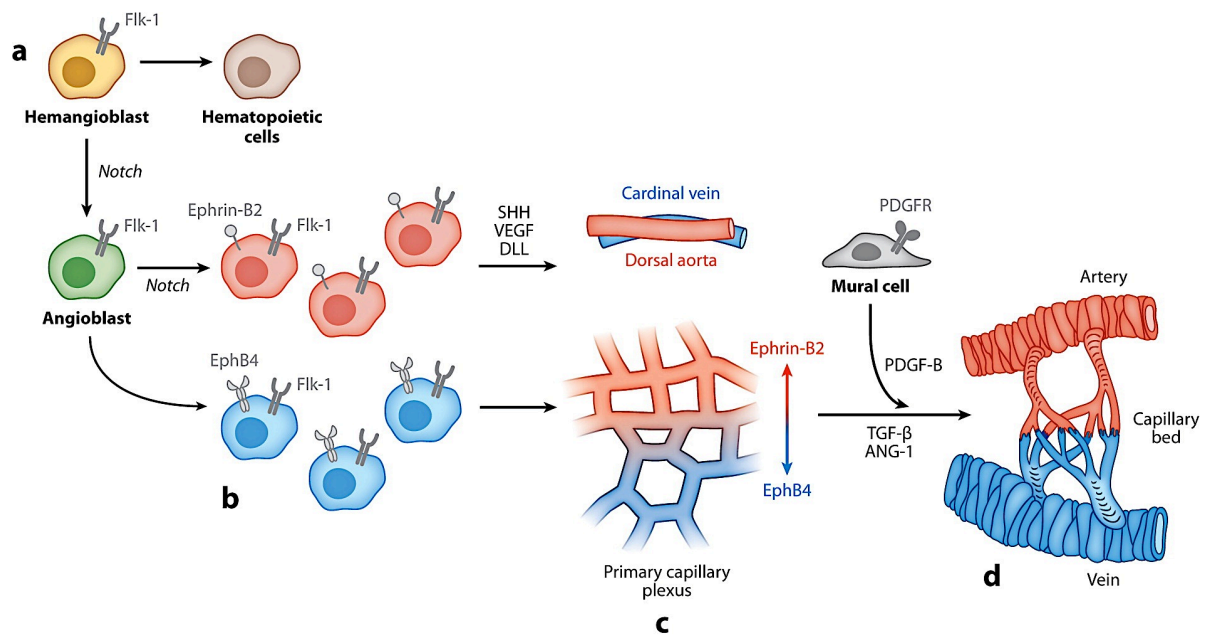
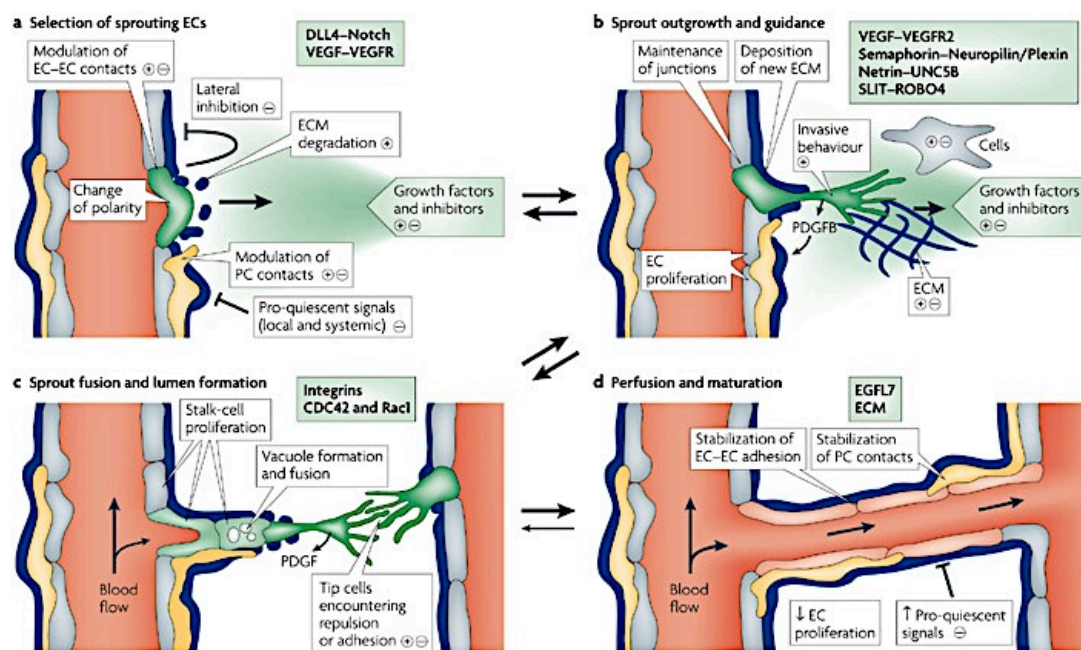


Figure 4. Developmental vasculogenesis and angiogenesis, as described by Chung & Ferrara. 2011. *Annu Rev Cell Dev Biol*: "(a) In the embryo, the hemangioblast precursor characterized by Flk-1 expression can give rise to cells of the hematopoietic lineage and to Notch signaling–specified angioblasts. (b) Notch signaling further contributes to arterial-venous (A-V) fate specification by promoting arterial cell fate. (c) Intraembryonic angioblasts aggregate directly into the dorsal aorta or cardinal vein, a process mediated by vascular endothelial growth factor (VEGF), Sonic hedgehog (SHH), and Notch signaling. In the yolk sac, angioblasts fuse in formation of a vascular plexus; expression of arterial Ephrin-B2 and venous EphB4 function in A-V specification. (d) Following vascular remodeling, mural cells are recruited for stabilization and maturation of nascent vessels; this process is mediated by platelet-derived growth factor B (PDGF-B), transforming growth factor β (TGF- β), and angiopoietin-1 (ANG-1) signaling."

2.2 Angiogenesis

The process of angiogenesis occurs when new blood vessels are sprouted from a pre-existing vessel (Figure 8a and in more detail in Figure 5). In response to external angiogenic stimuli (e.g. Ang-2) mural cells and pericytes let go of the ECs. They start secreting proteases like MMPs to

digest the surrounding extracellular matrix (ECM). The angiogenic factors surrounding the vessel and released by the ECM degradation triggers a dilation of the vessel and the TJs on the ECs are gradually lost. The resulting leakage of plasma proteins from the vessels into the ECM deprived, angiogenic area, functions as a provisional fluid matrix on which the ECs can migrate. As the ECs start migrating toward a gradient of growth factors such as VEGF and bFGF one EC takes the lead, probing the way [71-73]. This tip cell is unique, with several differences both in morphology and markers. Trailing behind are the stalk cells, which proliferate continuously in order to maintain the connection between the mother vessel and the sprout [71, 74]. As the sprout reaches another blood vessel (or the same one further up or downstream) the vessels fuse through the process of anastomosis [71]. Upon the initiation of blood flow the ECs resume their quiescent state and switch to secreting factors attracting pericytes and allow for the vessel to mature. In the absence of circulation after anastomosis, the sprouted vessel will not survive. This process, called vessel pruning/regression, is caused by mechanisms that still remain to be elucidated [75, 76].



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Figure 5. Sprouting angiogenesis in more detail. From Adams & Alitalo. 2007. *Nat Rev Mol Cell Biol*.

2.2.1 Physiological angiogenesis

After the primary vascular plexus (see Section 2.1) has been formed angiogenesis is the main process through which the capillary network is branched and fully developed during embryogenesis [77].

Angiogenesis is primarily active to expand the vascular network in growing tissues. However, angiogenesis is still important during; wound healing, the ovarian cycle and endometrial renewal prior to menstruation. During pregnancy is also essential for the correct formation of the placenta and the growth and vascularization of the uterus [78, 79].

2.2.2 Angiogenesis in cancer/Pathological angiogenesis

Blood vessels are essential for tissue survival; hence insufficient angiogenesis is associated with pathological conditions. Excessive angiogenesis is also a problem and is usually coupled to proliferative diseases such as cancer. The word cloud in Figure 6 shows diseases associated with either side of pathological angiogenesis imbalance, black representing Excessive angiogenesis and blue insufficient [80].

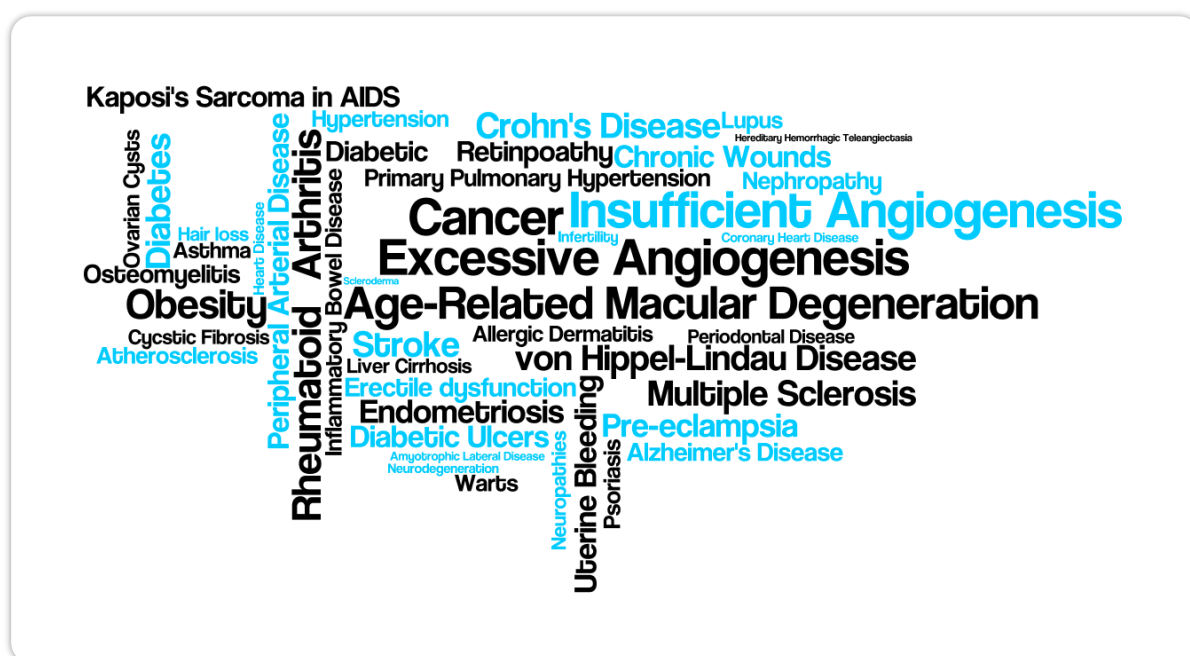


Figure 6. Wordcloud on angiogenesis-associated diseases, excessive (black), insufficient (blue).

2.3 Intussusception

Intussusception is a process of blood vessel formation with many names; non-sprouting angiogenesis, longitudinal splitting, luminal division and splitting angiogenesis. As the names indicate it is the process when one capillary splits into two, see Figure 7 and 8c. Endothelial cells of the capillary wall invaginate into the luminal space of the vessel and form islands of endothelial cells, spreading along the vessel direction forming 'tissue pillars' and ending in splitting the vessel along the longitudinal axis

[55, 76, 81]. Intussusception has been found in a multitude of physiological processes (like the formation of the pulmonary capillary bed) and also during pathological conditions [82].

The advantages of intussusception over vasculogenesis and angiogenesis is that blood vessels are formed more rapidly and in a more economic manner (energetically and metabolically) since the need for proliferation, basement membrane degradation and invasion into surrounding tissues is not as extensive [55].

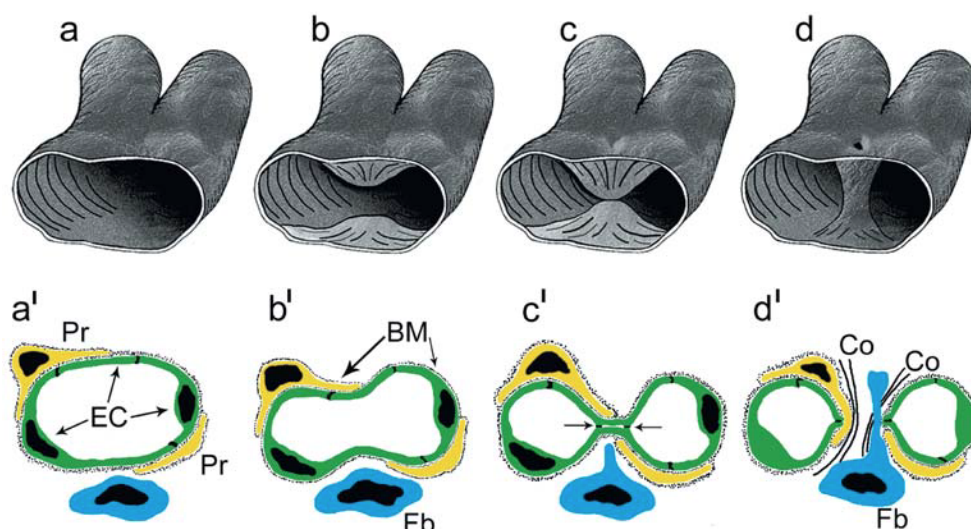


Figure 7. The formation of new blood vessels through intussusception. From Djonov et al. 2003. *Cell Tissue Res.*

2.4 Other means of blood vessel formation

Vasculogenic mimicry (Figure 8e) is a process where tumor cells line part of a blood vessel, but examples of complete tubular and patterned matrix type of blood containing vessel have been reported. This type of vascularity has been seen in several cancer types such as melanomas, breast carcinomas, ovarian and prostatic carcinoma and many more [76, 81].

Vessel co-option (Figure 8d) is not a process of blood vessel formation per se. However, it is a solution for tumors to attain nutrients and oxygen without the need of de-novo formation of vessels. This is accomplished by the tumors growing along existing vessels, relying on diffusion of gases and molecules to sustain them. Logically, vessel co-option is therefore mostly seen in well-vascularized tissues like brain and lung [81]. Since this way of sustaining tumor growth is independent of angiogenic triggers, tumors relying mostly on vessel co-option do not respond to anti-angiogenic therapy.

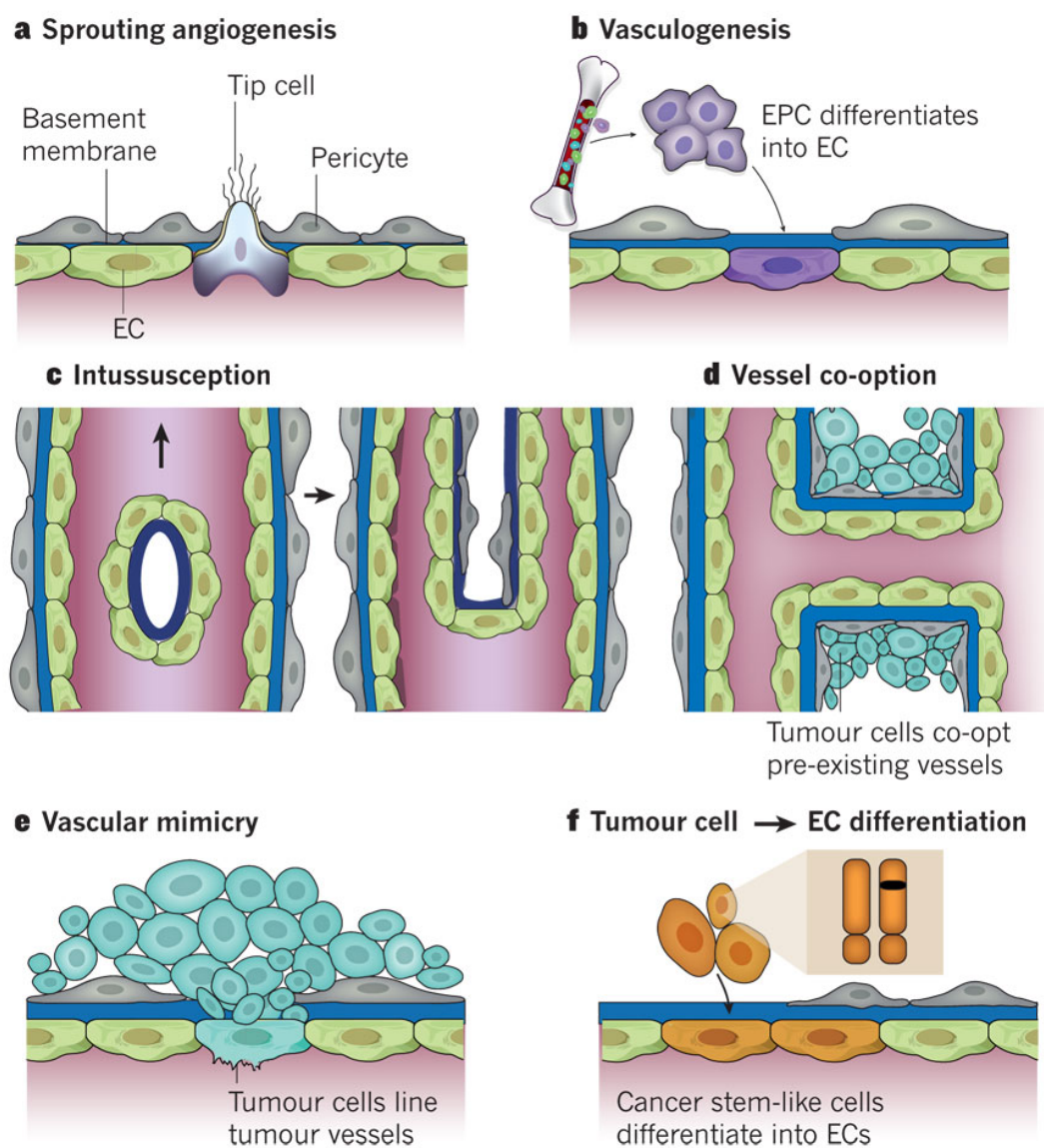


Figure 8. The different means of blood vessel formation. From Carmeliet & Jain. 2011. Nature.

3 Angiomotin

Angiomotin (also known as KIAA1071, AF286598) was discovered in the Holmgren lab in 2001 through a yeast-two-hybrid screen for novel Angiostatin receptors [83]. The protein was found to be expressed in blood vessels of different cancer-types and to be important for angiogenesis occurring during embryonic development [61, 68]. Upon overexpression, *the gene* caused increased migration in micro capillary cells. Logically, it was named Angiomotin from the latin word *angio* for vessel and *motus* for motility.

The Motin family of proteins consists of Angiomotin (Amot) [84], [85] [65], [86], [61], [87], [88], [67], [62], [58], [83], Angiomotin-like 1 (AmotL1) [64], [89], [90],

[62],[91] and Angiomotin-like 2 (AmotL2) [92], [93], [94], [95]. Our and other labs have shown that Angiomotin exists in two different isoforms, called p80- and p130-Amot [86], [67], [96]. The same holds true for the rest of the family, bringing the total number of proteins in the family up to six, in humans. Whether these isoforms are formed due to alternative splicing or have completely different promoters is currently under discussion and investigation, but to date the splice isoform hypothesis remains, as reported by Moreau et al in 2005.

3.1 General structure of the Motin family

Structure-wise, the common denominator for the family is that all members have a conserved coil-coiled domain and a PDZ-binding domain, as illustrated in Figure 9. The longer isoforms also sport an N-terminal proline- and glutamine-rich domain, containing a PPXY-motif [89], [67], [95]. Angiomotin is the only member that also contains an Angiostatin-binding domain (see Figure 9). These domains all play a role in interactions, effects and localizations of the proteins. I will briefly summarize the importance of each domain in this section, but more detailed information on interacting partners can be found in Section 4.

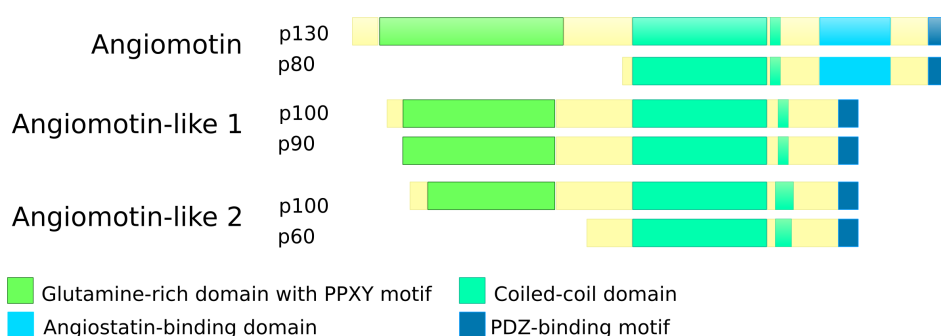


Figure 9. Schematic structure of the Motin family.

For the remainder of this section, the data discussed is focused upon Amot. However, considering the high conservation of the sequences and the similarities in binding proteins, the data in this section is also of interest for AmotL1 and AmotL2.

3.1.1 The Coiled-coil Domain

An intra-membrane localization of Angiomotin has long been debated back and forth. The Holmgren lab argues for a part of the protein being exposed on the cell surface, whereas structure prediction analysis tools (and believers thereof) state that the amino acid sequence does not comprise a trans-membrane domain [95]. The reasoning behind the

existence of an extracellular domain on Amot is that; 1) It binds to Angiostatin, a secreted, extracellular protein. Upon binding to Amot, the complex is internalized, a phenomenon not seen in the control experiments [83], 2) Anti-Amot antibodies can bind to the cell surface [62, 88, 97], 3) When biotinylating all surface proteins, Amot is biotinylated [62] and 4) Amot is degraded by extracellular trypsin, while intracellular proteins are not [62]. Since the both the PPXY-motifs (N-terminal) and the PDZ-binding motif (C-terminal) are needed for some of Amot's intracellular protein-protein interactions (more on this in Section 4) they need to be located intra-cellularly [65, 67]. The conclusion in the Holmgren lab is that the exposed part of Amot may span from the coiled-coil domain to the Angiostatin binding domain.

While trying to crystallize Amot, alone and with binding partners, multiple truncated variants of Amot were produced. Amongst them was the isolated coiled-coil domain, which proved to be the most stable and soluble part of the protein. From studies conducted by the Holmgren group in collaboration with the Swedish company BioInvent, I had access to a limited amount an anti-Angiomotin IgG as well as a control [97]. Exploratory gel filtration showed that the anti-Angiomotin IgG bound to the Amot coiled-coil domain, whereas the control did not (unpublished data, Figure 10). This data shows the binding domain of the anti-Amot antibody and may be interpreted as some part of the coiled-coil domain being exposed on the cell surface. We therefore moved on to looking more closely at the coiled-coil domain. We (unpublished) and Wells et al (2006) have found that Angiomotin has predicted structural similarities to the (Bin/Amphiphysin/Rvs) (BAR) domain of Amphiphysin, which is best visualized when Amot is modeled upon the structure of Amphiphysin (see Figure 11) [87]. In short, BAR domains either binds to curved membrane structures or bends the associated membrane to a curve of its liking [98]. BAR domains are also common for interaction with other BAR domain proteins and this is also the case for Amot, a topic covered in Section 4. After further investigation, Wells et al now call the Amot coiled-coil domain for Amot coiled-coil homology (ACCH) and declares it a conserved lipid-binding domain, concluding that the coiled-coil domain of Amot binds to cholesterol-rich membranes [99].

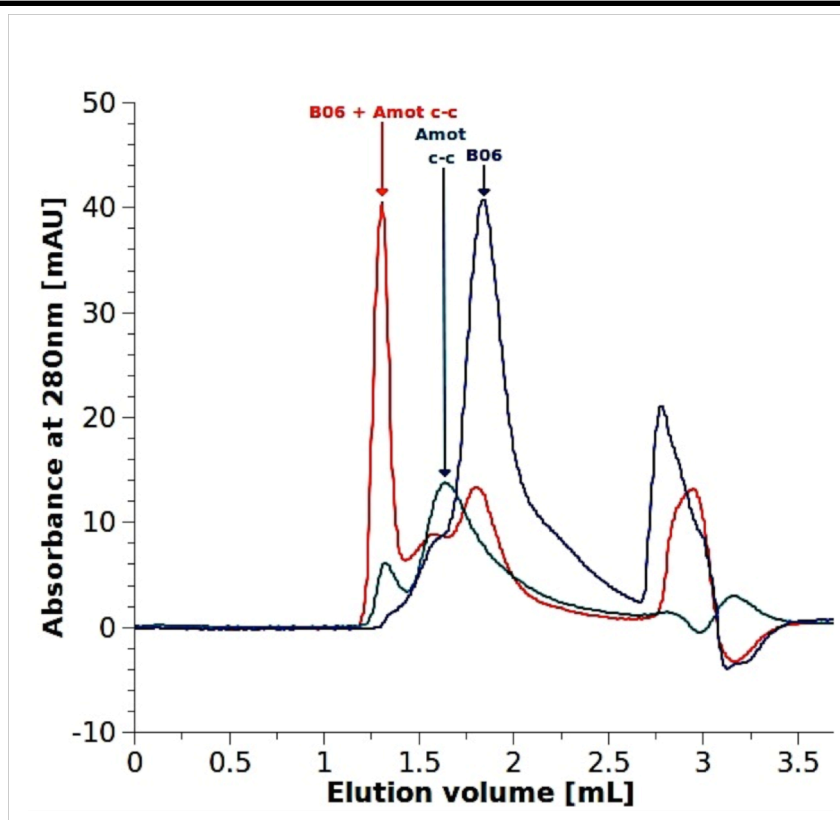


Figure 10. Exploratory gel filtration on purified Amot coiled-coil domain and the anti-Amot IgG. Arrows indicate sample peak, showing that the combination of Amot and the antibody elutes later than either alone, suggesting that they form a larger complex. Unpublished data.

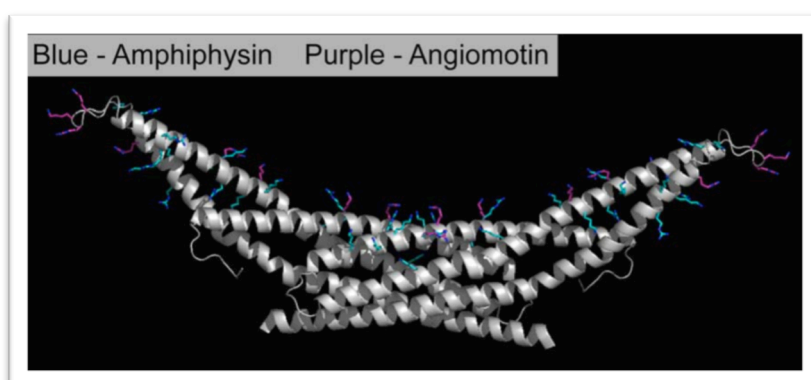


Figure 11. Overlay of Amot amino acid sequence on the crystal structure of Amphiphysin BAR domain. From Wells et al. 2006. Cell.

3.1.2 The PDZ-binding motif

In general, PDZ binding motifs have one goal in life: to bind a PDZ-domain and vice versa, meaning that they are certain protein:protein interaction points. More specifically, PDZ domains are shown to mediate the formation of larger protein complexes and to be crucial for correct localization of membrane proteins [100, 101]. The PDZ-binding motif of Amot is a short four amino acid sequence at the C-terminus; EYLI (AmotL1; EVLI, AmotL2; EILI). We have shown that the PDZ-binding motif of Amot is essential for endothelial cell migration and that the loss of the motif leads to inhibition of cellular migration, embryonic lethality and impaired endothelial migration during zebrafish vascular development [65], [58], [57]. Interestingly, the motif is essential for the TJ localization of p80-Amot but not for p130-Amot [86].

3.1.3 The N-terminal domain

As mentioned above the N-terminal domain is a proline- and glutamine-rich domain unique to the longer isoforms of the Motin family. It contains a PPXY-motif, capable of interacting with WW domains of other proteins, like those of; MAGI, NEDD4 and YAP/TAZ [62, 102-104]. Unlike the PDZ-binding motif, the N-terminal domain of p130-Amot is essential for its localization to TJs and interaction with actin fibers [67].

3.2 The two isoforms: p80- and p130-Amot

When Angiomotin was first discovered it was the p80-Amot isoform that was characterized. Troyanovsky et al. found that the protein localizes to the lamellipodia of migrating cells and later it was also shown to be present in cell-cell contacts, overlapping with tight junction proteins like ZO-1 [62, 83, 87]. The p130-Amot isoform was also characterized in the Holmgren lab [67, 86], with a 409 amino acid (aa) long N-terminal domain added to what was known as p80-Amot (Figure 9) [62, 67, 86]. Eventhough both isoforms localizes to TJs (but are not essential for TJ formation) p130-Amot does so to a larger extent but also forms punctated patterns throughout the cytoplasm upon IF staining, colocalizing with actin [67]. As more data compiled, multiple differences between p80-Amot and p130-Amot were seen; (1) p130-Amot has an actin binding N-terminal domain, p80-Amot does not, (2) Overexpression of p130-Amot leads to flatter ECs than p80-Amot, (3) p80-Amot ECs respond to anti-migratory signals by Angiostatin while p130-Amot ECs do not [67, 86].

By co-immunoprecipitation Ernkvist et al. found that the two isoforms can homo-oligomerize and also form hetero-oligomers with each other through their mutual coiled-coil domains [86]. This piece of information was of particular interest when they found that the relative expression levels of p80-Amot to p130-Amot dictate cell behavior. In an overexpression setting *in vitro*, induced expression of p80-Amot removes p130-Amot from cell-cell junctions, thus destabilizing the junctions and promoting a migratory phenotype. This indicated that the hetero-oligomer does not seem to function in the tight junctions, possibly due to steric hindrance or blocking of a binding site by the oligomerization. Taken together, the data shows that the ratio of the isoforms dictates cell phenotype and that p80-Amot function is dominant over the function of p130-Amot. Differential expression of the isoforms can be seen in several situations, such as during retinal angiogenesis after birth in mice and during embryonic development. Retinal angiogenesis occurs postnatally in mice and studies show a divergence in expression the two Amot isoforms p80 and p130. p80-Amot is expressed during post-natal day (P)3-P7 whereas p130-Amot is expressed at P7-9 and in adult, fully developed, retinas [86]. During embryonic development p80-Amot is expressed during E5-19 while p130-Amot does not appear to be expressed at all. However, in the placenta p130 is expressed at E13-16, while p80-Amot is constitutively expressed [67].

3.3 Angiomotin in Angiogenesis

Angiogenesis depends on endothelial cell migration and as such we have studied Angiomotin *in vitro* and *in vivo* in order to understand its function and importance.

The Holmgren lab has used the embryoid body model system to study tube formation through both vasculogenesis and angiogenesis. Embryonic stem cells from *amot* negative and wild-type mice were allowed to differentiate and form a primitive vascular plexus and initial capillaries. *amot* negative EB ECs did not sprout in response to VEGF stimulation, indicating either a loss of VEGF responsiveness or malfunctioning EC migration [61].

As mentioned in Section 1.4.1, a stable *amot* negative endothelial cell line was procured from the EBs, called PmT-EC. These cells exhibit a disorganized actin cytoskeleton, disrupted focal adhesions and a loss of polarity. These results coincide with what we observe in both MS-1 and BCE cells upon *amot* mRNA expression knock-down with siRNA [61, 66]. Furthermore, the *amot* negative PmT-ECs display a significant increase in membrane protrusions rapidly forming and retracting as well as several lamellipodia and a depolarized phenotype. In Paper I, this phenomenon is explained by a misslocalization of RhoA activity leading to actin being polymerized at random throughout the membrane [65]. Both the stable

and siRNA treated *amot* negative ECs show a marked decrease in directional migration in response to growth factor stimuli, both as a sheath and as single cells [61, 62, 64, 83].

To date we have studied the loss of *amot* expression in both zebrafish (zf) and mice. In zf, the antisense morpholino (MO) technique has been utilized in order to block and degrade *amot* mRNA. In mice, we have used a conventional knock-out mouse strain, which has now been replaced by a conditional knock-out strain currently being bred to be studied [61, 68].

In the conventional *amot* negative mouse model (C57/B6 backcross) loss of *amot* expression leads to dilation of cranial vessels and an insufficient vascularization of the intersomitic region [61]. As a result 75% of the conventional *amot* negative mice die *in utero* at E11-E11.5 indicating that *amot* plays an important role during embryonic vascular development [61, 68].

As shown in Aase et al 2007 and Ernkvist et al 2009 the loss of *amot* mRNA expression in zebrafish embryos leads to malformation of the inter-somitic/segmental vessels (ISVs, see Figure 13 for anatomy) [61, 65, 68]. The ISVs sprout from the dorsal aorta, but come to a halt, instead of continued dorsal migration all the way between the somites and subsequent anastomosis to form the Dorsal Longitudinal Anastomosing Vessel (DLAV). Also, a dilation of the cranial arteries is observed, corresponding to our findings in mice.

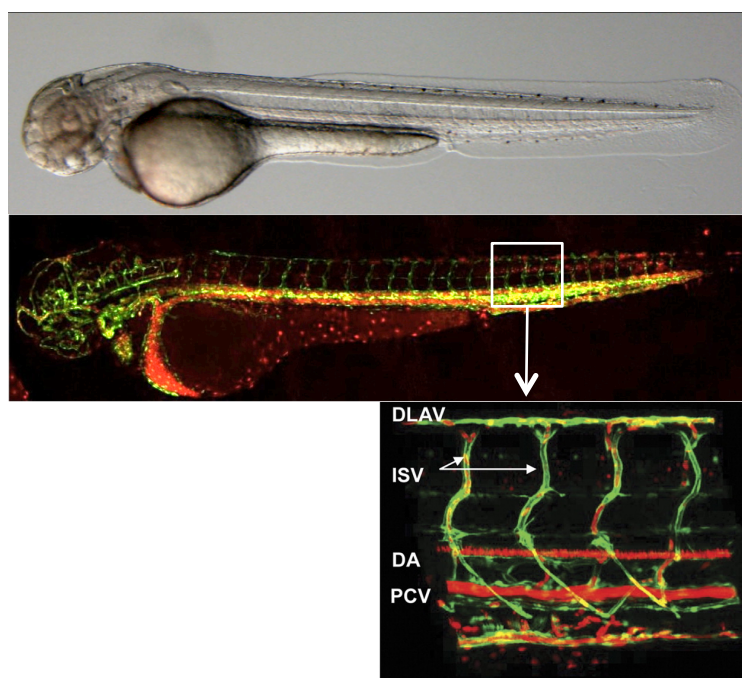


Figure 12. Anatomy of the zebrafish embryo. Top: Brightfield image. Middle: Fluorescence image of transgenic zf. Green: ECs expressing GFP under the *fli* promotor. Red: Erythrocytes. Bottom: Fluorescence image zoomed in on somites. Vessels indicated.

In 2006 and 2012 the Holmgren, Kiessling and Cavallo labs show that DNA vaccination against human p80-Amot in both transplanted xenograft breast cancer tumors and in spontaneously developed mammary carcinomas in mice (Balb/C background), leads to both tumor protection and a marked reduction in tumor growth in already established tumors [84, 88]. Furthermore, in Paper III, we show that loss of *amot* expression leads to impaired pathological angiogenesis [66].

3.4 Tissue expression

Using western and northern blot analysis the expression of Amot has been detected in various tissues. In mice Amot has been found to be expressed in the developing embryo and in the retina of neonatal pups [68, 86]. More specifically Shimono and Behringer found that: “*amot* is expressed in a dynamic, region-specific pattern in the Visceral Endoderm (VE) just before and during gastrulation. Our findings suggest that *amot* is required in the Anterior VE for movement away from the epiblast and association with the proximally located extra-embryonic ectoderm”. As mentioned in Section 3.2, Amot expression has also been seen during retinal angiogenesis in neonatal mouse pups and in placenta (cytotrophoblasts). In the adult, Amot expression has been reported in brain, heart and retina, but is also expressed to a lesser degree in liver and lungs [67]. In humans, Amot expression was studied in fetal and adult tissues and found to be high in fetal kidneys, placenta and brain and lower in lungs. In adults Amot expression was high in muscle tissue, placenta, brain and kidneys while lower in the heart and pancreas [83].

Eventhough most data is collected in blood vessels and endothelial cells, Amot expression is not restricted to endothelial tissue. In Aase et al. 2007, Amot expression was also found in Rathke’s pouch, a collection of epithelial cells destined to become the anterior pituitary (murine)[61]. Heller et al (2010) state: “Although Amot expression is low in most differentiated epithelial cells, it is strongly up-regulated during epithelial migration programs during developmental processes, such as trophoblast extension and anterior visceral endoderm formation.”[99] Expression in epithelial cells has also been reported in other articles (e.g. Paper II), indicating that Amot is not a protein unique to the endothelium but remains a significant part of endothelial signaling [85, 87, 105].

Earlier data from The Human Protein Atlas project (protein expression analysis on human tissue; www.proteinatlas.org) showed that Amot was preferentially expressed in blood vessels and in brain tissue, corroborating our findings. However, on the current webpage, all data on Amot has been removed, pending new analysis. In order to get more, independent tissue expression data on Amot one must turn to other sources. Today, the

Internet harbors several ambitious databases. One such is GeneSapiens.org (FIMM, Turku, Finland). It collects human microarray gene expression data from cell lines, healthy tissue as well as patient material. *amot* expression in healthy tissue is depicted in Figure 13.

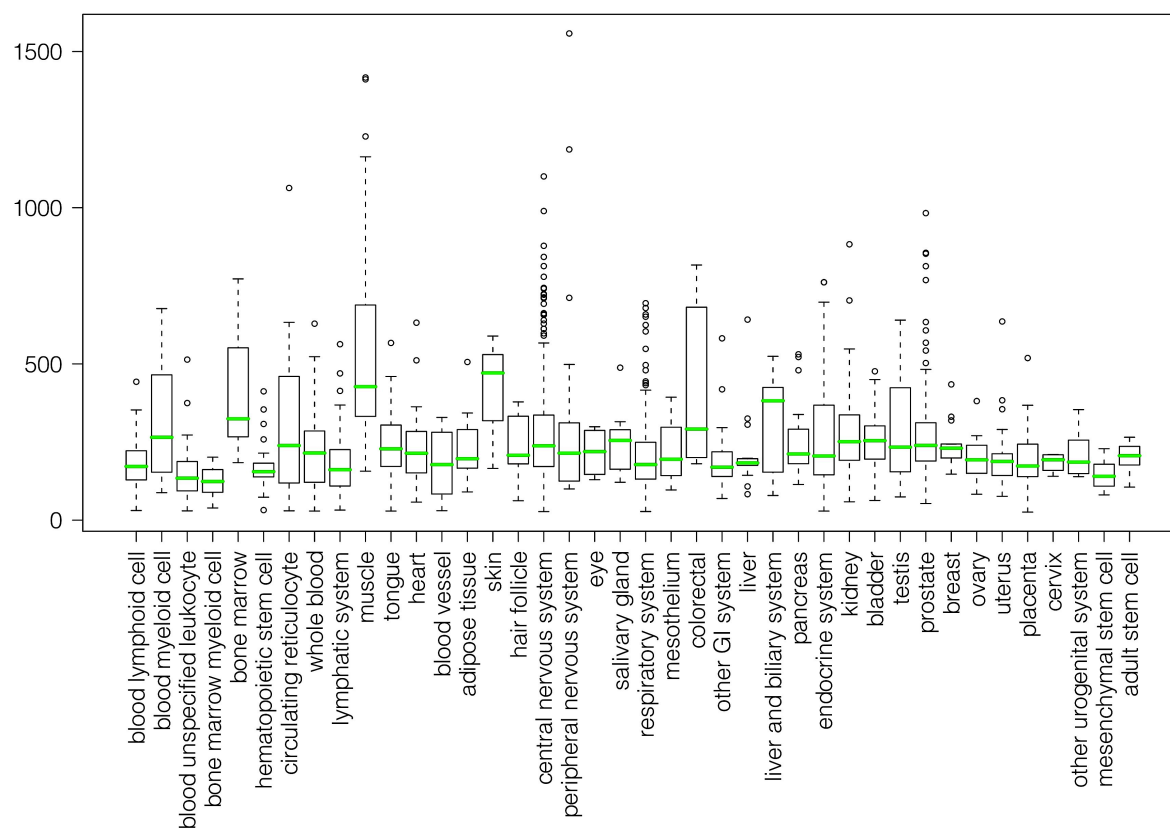


Figure 13. The relative *angiomin* mRNA expression in samples from different tissues. Gene array results pooled and compiled by GeneSapiens.org for AMOT/ENSG00000126016, 2012-07-31

Searching literature on *Amot* expression in pathologies reveals that Kaposi's sarcoma tumor vessels express *Amot* but the vessels of normal tissue do not and that Peripheral Blood Mononuclear Cells (PBMCs) of Rheumatoid Arthritis patients express *Amot* at a higher level than osteoarthritis patients [83, 106]. Also, upon looking at *amot* gene expression in tumor tissue, Jiang et al found that *amot* is expressed at significantly higher levels in human breast carcinomas compared to normal breast tissue [107]. In mice, *Amot* expression was found in the blood vessels of spontaneously formed lobular mammary carcinomas in transgenic BALB/c mice, but not in normal breast tissue [88]. Lastly, GeneSapiens.org can also provide expression data on pathological conditions, depicted in Figure 14.

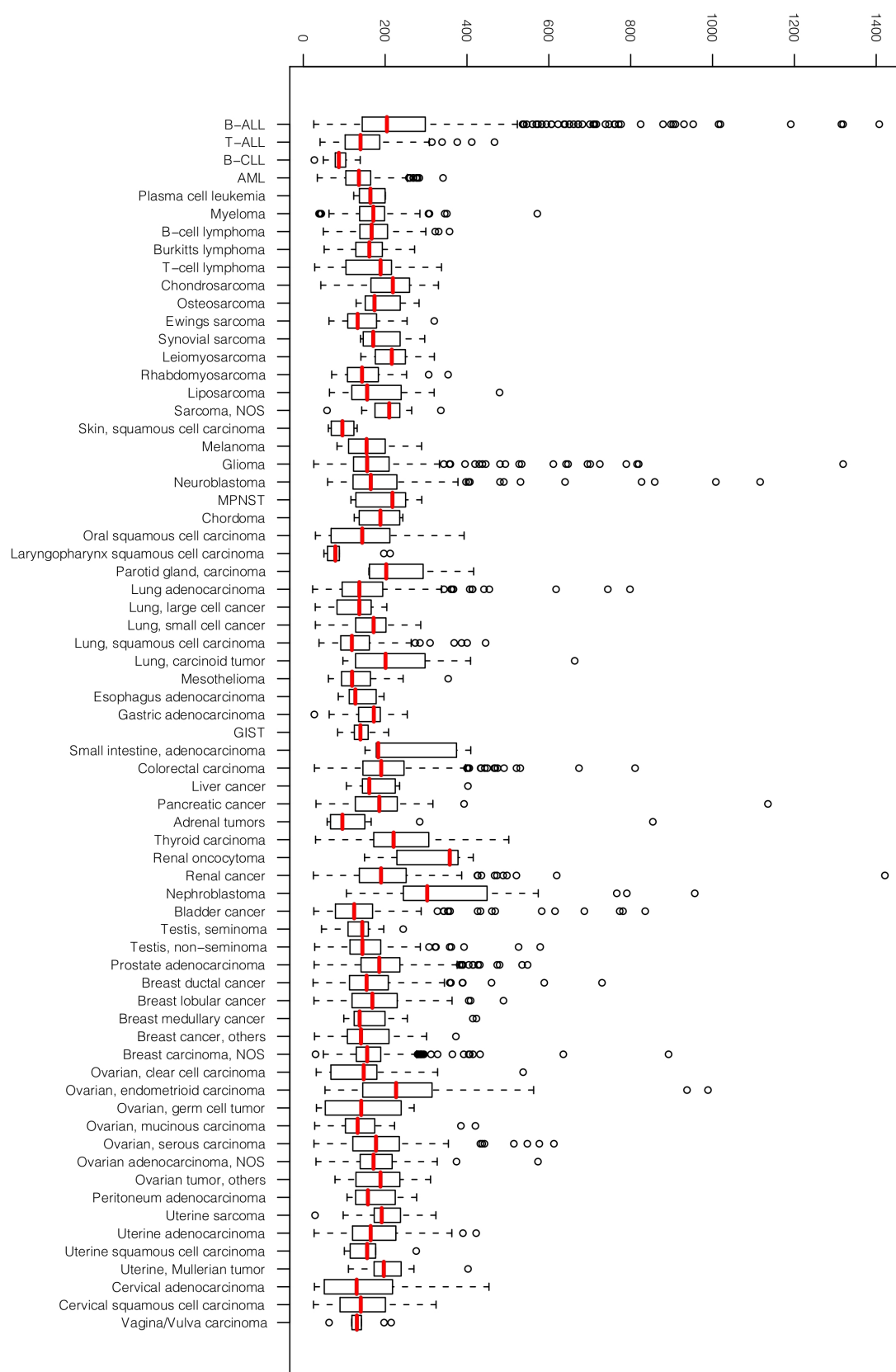


Figure 14. The relative *angiomin* mRNA expression in samples from different pathological conditions. Gene array results pooled and compiled by GeneSapiens.org for AMOT/ENSG00000126016, 2012-07-31.

Taking all of this data together, Amot is postulated to be useful as a prognostic marker for aggressive disease and patient survival as well as a therapeutic target. It follow suit that anti-Angiomotin therapy should be attempted. More on that in Section 6.

4 Angiomotin Binding Partners

The proteins found to bind to Amot are all somehow involved in cell polarity and migration and sometimes also proliferation, mostly fitting the known function of Amot. Cell polarity has mostly been studied using epithelial cells and *Drosophila Melanogaster* (fruit fly) or *Caenorhabditis Elegans* (nematode) as model systems *in vitro* and *in vivo*. As a consequence, nomenclature can be a jungle. When drawing conclusions, I will try to keep to the official mammalian/human names of the proteins, however, when stating what has been found both unofficial names and fly names of the orthologs might frequent the text. To make inter-species translation quicker, please refer to POST-IT number 1. Apart from the proteins mentioned in this section the PDZ-binding motif of Amot has been reported to bind Filamin A and PTN13 [65].

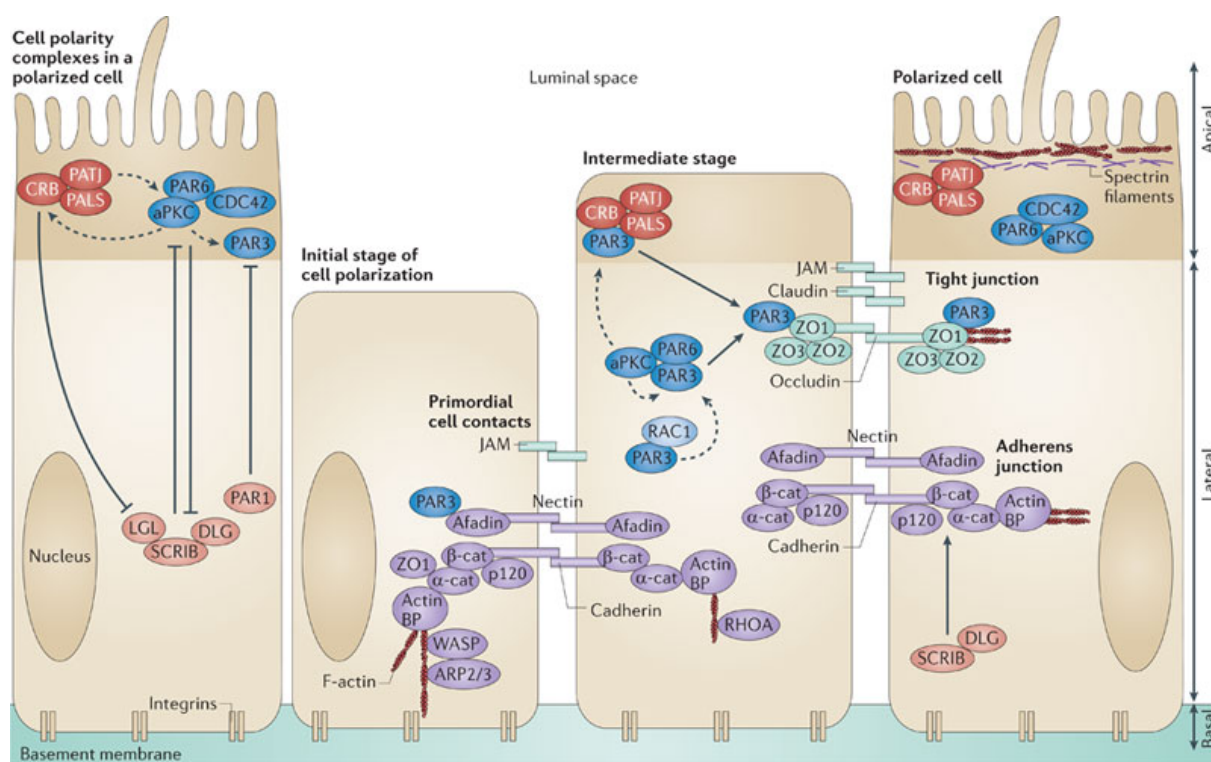
Inter-species Translation POST-IT 1	
- Amot Binding Partner Orthologs	
Mammals	D. Melanogaster
(INADI/PATJ) InaD-like protein	Discs Lost (Dlt)
(MPDZ/MUPP1) Multi-PDZ domain protein	Patj (Patj)
(Plekhg5/Syx1) Plextrin homology domain 5	CG42674
(MPP5/Pals1) MAGUK p55 subfamily member 5-A	Stardust (Std)
(Crb3) Crumbs homology domain 3	Crumbs (Crb)
(Par-3) Partitioning defective 3 homolog	Bazooka (Baz)
(Lin7C) Protein lin-7 homolog 3	Veli (Veli)
(NF-2) Neurofibromatosis-2/Merlin	Merlin (Mer)

4.1 Cell polarity

As most topics in this thesis, cell polarity could fill a few theses on its own. Cell polarity has mostly been studied in epithelial cells, which have very distinct polarity compartments; Basal (facing ECM, the bottom), basolateral/lateral and Apical (facing the lumen, the top). Eventhough

endothelial cells lack these exact compartments, conclusions from epithelial cells can be translated into ECs. As such, the data presented on the functions of Amot binding partners will most often come from epithelial cell studies.

Three major polarity complexes participate in the establishment of apical-basal polarity; The Crumbs Complex, the PAR complex and the Scribble complex (Figure 15) [108]. The Crumbs complex (see Section 4.2.3.) is required for the establishment of the apical membrane and comprises Crumbs homology domain 3 (Crb3), MAGUK p55 subfamily member 5-A (MPP5, also known as Pals1) and InaD-like protein (InaDI, also known as Patj). The PAR complex is more involved in the lateral compartment and consists of partitioning defective 3 (Par3), Par6, atypical Protein Kinase C (aPKC) and Cell division control 42 (Cdc42). The Scribble complex is the union of Scribble (Scrib), Lethal Giant Larvae homologue (LGL) and Discs Large homologue (DLG). The complex defines the basolateral domain of epithelial cells. Together these three complexes interact with each other, adhesion proteins and to the actin cytoskeleton to organize and maintain junctional complexes, polarity and, to some extent, migration [108, 109].



Nature Reviews | Cancer

Figure 15. The major polarity complexes in epithelial polarity. From Martin-Belmonte & Perez-Moreno. *Nature Reviews Cancer*. 2012.

4.2 The CRB3 Complex

4.2.1 PATJ and MUPP1

Pals-1 associated tight junction protein (Patj) and Multi PDZ domain protein 1 (Mupp1) are homologous and possibly paralogous multi-PDZ domain proteins where the PDZ domains (10 and 13 respectively) are positioned like beads on a string after an L27 domain (Figure 16). They are scaffolds, bringing together proteins with PDZ-binding motifs and L27 domains into larger complexes.

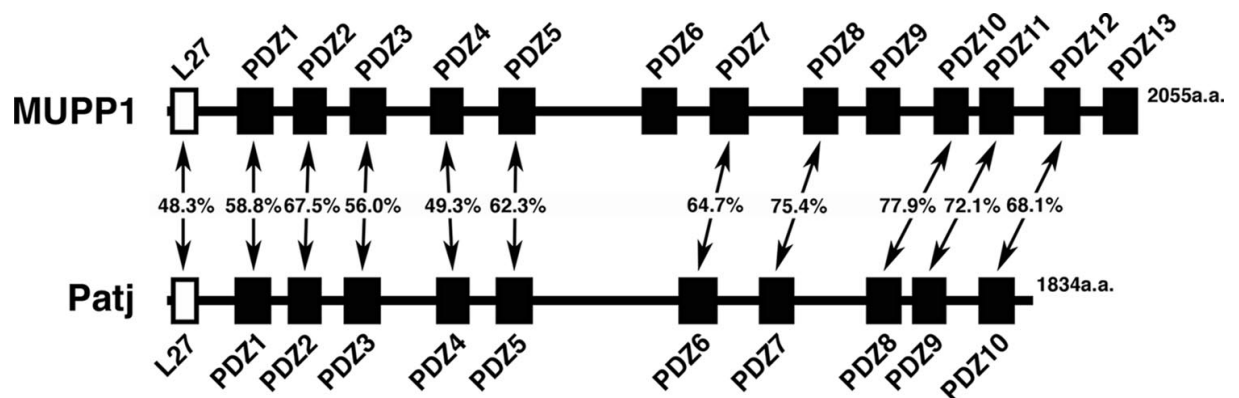


Figure 16. Domain structure of Mupp1 and Patj. Percentages indicate level of identity between domain sequences. From Adachi et al. 2009. Mol Cell Biol.

The L27 domain of Patj and Mupp1 interacts with the L27 domain of Pals1, which in turn binds to Protein Lin 7 homology 3 (Lin7) and Crb3 [110]. The various PDZ domains have been mapped for binding to several polarity TJ and adherens junction (AJ) proteins like Claudin-1, Junctional Adhesion Molecule-1 (JAM-1), Zona Occludens (ZO)-1 and -3, nectins and Par-6 [111, 112].

Shin et al (2007) speculate that Patj and accompanying complex localizes to the leading edge of a migrating cell and then recruits atypical Protein Kinase C (aPKC) and Par3, thereby redirecting microtubule formation. This theory is similar to the one we draw in Paper I [113].

Since Patj and Mupp1 have matching domain structures, binding partners and cellular expression but somewhat diverging function, the difference between the two has been investigated. Adashi et al. attempt to explain this by dissimilar affinities for the different binding partners [111].

In Paper I (and confirmed in Wells et al 2006 in epithelial cells) we identify Patj and Mupp1 as binding partners of Amot. Further investigation showed that Amot bound to the third PDZ domain (PDZ3) of Patj and that AmotL1

and AmotL2 bound to PDZ 1 and 2, respectively [65]. We further show that PDZ10 binds to the RhoGEF Plexstrin homology domain 5 (Plekhhg5, also known as Syx1) [114]. Taken together with the findings of a disruption of the complex during migration, we propose a model where Amot and Patj are localized to the leading edge of a migrating cell, recruiting Syx1 and actin polymerization to the correct place for generating movement.

4.2.2 PALS1

Pals1, called Nagie oko in ZF, consists of: two L27 domains, a PDZ domain, an SH3 domain, a band 4.1-binding domain, and a GUK domain. The two L27 domains mediate the interaction with Patj and Lin7. The PDZ domain binds Crb3 and within the N-terminus hides an internal motif (U1 domain) binding to Par6, connecting the Crumbs complex to the Par complex [115-117].

4.2.3 CRB3

Crumbs homology domain 3 or Crb3 consists of a large extracellular domain, a transmembrane domain and an intracellular domain containing one FERM and one PDZ-binding motif. The function of the extracellular domain remains to be elucidated, but appears to be of lesser importance since only the intracellular domain is enough to rescue the loss of polarity caused by *crb3* knock-out [118]. The function of the FERM motif remains to be elucidated. Unlike the extracellular domain, the FERM domain is of greater importance for Crb3 function, as mutation of the domain results in severe tight junction disruption [119]. The same holds true for the PDZ-binding motif, which interacts with Pals1, connecting Crb3 to Patj.

Crb3 is localized to the apical membrane of mammalian epithelial cells and is concentrated to TJs. Overexpression of Crb3 leads to an expansion of the apical domain and concomitant reduction of the basolateral domain. Loss of Patj expression leads to the misslocalization of Crb3 and a disruption of TJs, indicating that Crb3 is important for TJs formation and that its localization is dependent on Patj and most possibly Pals1 [120]. Interestingly, whereas Patj is required for epithelial migration and is localized at the leading edge of a migrating cell, Crb3 is not [113]. This suggests the entire polarity complex is not present during actual cell migration.

4.3 Rho GTPase modulators

The Rho GTPases; Rho, Rac and Cdc42, coordinate the machinery of cell migration. They modulate the actin cytoskeleton by regulating actin polymerization, depolymerization and the activity of actin-associated myosin [121]. The RhoGTPases are regulated through cycling between GDP-bound (inactive) and GTP-bound (active) state. This binary switch is regulated by: Guanine Nucleotide Exchange Factors (GEFs), GTPase Activating Proteins (GAPs) and Guanine Nucleotide Dissociation Inhibitors (GDIs). GEFs activate the RhoGTPase by exchanging GDP for GTP, GAPs inactivate by controlling the shift from GTP to GDP and GDIs interact with the GDP-bound and prenylated form of the GTPase, inhibit the dissociation of the GDP and control the cycling between the cell membrane and the cytosol (See Figure 18) [122].

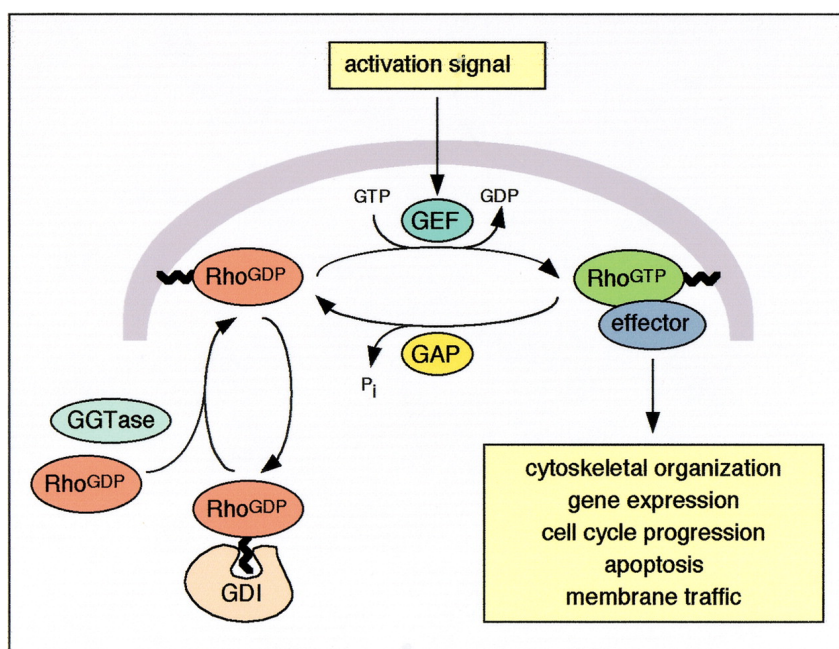


Figure 17. The regulation of RhoGTPases. From Schmidt & Hall 2002. *Genes & Dev.*

4.3.1 Plekhg5/Syx1

In Paper I, we find that correctly localized RhoA activity is dependent upon Amot expression. We show that Amot binds to Patj/Mupp1, which in turn binds to the RhoGEF Plekhg5/Syx1 [65]. Syx1 has been reported to be expressed at high levels in brain and heart, two highly vascularized tissues, overlapping with that of Amot. We and Garnaas et al. show that *syx1* is important during developmental angiogenesis in zfish and mice and that loss of gene expression leads to impaired blood vessel formation, similar to that of *amot* [65, 114]. We show that Syx1 interacts with the PDZ domains on

Patj, indicating the involvement of the Syx1 PDZ-binding motif. Horowitz et al. show that high Syx1 expression increases endothelial cell migration and tube formation and that this effect depends upon the PDZ-binding motif [123]. Taken together, we propose that Amot localizes to the lamellipodia at the leading edge of a cell, bringing with it Patj and Syx1 and by these interactions localizes RhoA activity and promotes endothelial cell migration.

4.3.2 ARHGAP17/Rich-1

ARHGAP17 or RhoGAP Interacting with CIP4 Homologues (Rich-1) is a RhoGAP found to regulate the activity of both Cdc42 and Rac1, the other two members of the RhoGTPase family, promoting the formation of filopodia and lamellipodia, respectively [124]. Rich-1 contains a coiled-coil domain encompassing a BAR domain, a RhoGAP domain, a proline-rich domain and a C-terminal PDZ-binding motif [125]. Wells et al found that Rich1 is important for epithelial cell TJ formation by showing that loss of Rich1 expression leads to the disruption of TJs. They also find an interaction between the BAR domain of Rich1 to the coiled-coil/Bar-domain of Amot [87]. In mammals, Rich1 exists in several isoforms, one of which is Rich-1B. It has been suggested that Rich-1B controls vesicle formation or membrane trafficking [124]. This is of interest since BAR domain proteins may directly regulate vesicle formation and since Amot has been shown to localize to vesicular structures [83, 126]. This, together with the TJ localization and association of both proteins has led to the theory of Amot and Rich1 to function as a means for shuttling tight junctions proteins from the golgi to the cell membrane or from the membrane into endosomes [127].

4.4 Merlin

Apart from being the name of a legendary wizard, Merlin (NF-2, Mer in *Drosophila*) is also the name of an Ezrin, Radixin, Moesin (ERM)-related protein [128]. As do most proteins, Merlin has several aliases, the best known being Neurofibromatosis type 2 (NF-2), which is also the name of the gene encoding the protein. Merlin is a tumor suppressor (TS) and is unique in that no other ERM has been shown to mediate contact inhibition of proliferation.

Being a relative to the ERM-proteins, Merlin shares most of their structural characteristics; an N-terminal FERM domain and a coiled-coil domain. In contrast to ERM-proteins Merlin lacks the C-terminal, hydrophilic, actin-binding motif. Also, Merlin has two additional motifs, missing in other ERM proteins: a conserved Blue box motif within the FERM domain and an N-

terminal segment. Most TS act through catalytic or DNA-binding domains but since Merlin lacks both, the action of Merlin must come through binding and modulating other proteins and pathways.

ERM proteins function through conformational changes. In a non-phosphorylated state the FERM domain and the C-terminus of the ERM-protein are interacting, forming what in literature is termed the “closed” state. Upon Rho kinase phosphorylation this head-to-tail interaction is lost (“open” state) and the FERM domain is free to associate to the cytoplasmic tails of adhesion molecules such as ICAM-1 and CD44. In turn, the C-terminal domain is free to interact with actin filaments, making ERM proteins (e.g. ezrin) key players in regulating cortical actin homeostasis.

Phosphorylated (Ser518) Merlin binds to and forms heterodimers with ERM-proteins like ezrin and localizes to the cell cortex. This phosphorylation state is most commonly seen in subconfluent, dividing cells. The phosphorylation is mediated by p21-activated kinase (PAK) as a consequence of integrin and receptor tyrosine kinase (RTK) signaling. Just like with ERM-proteins the phosphorylation of Ser518 is thought to affect the interaction between the C-terminus and FERM-domain of Merlin and result in the “open” state, mentioned above.

Unphosphorylated is the state at which Merlin mediates contact inhibition of proliferation. The dephosphorylation and thus activation (“closed” state) of Merlin is mediated by the myosin phosphatase MYPT1–PP1d. Explained by its structural composition Merlin localizes to sites of adherens molecules and pools of actin. In confluent cells it accumulates in adherens junctions, TJs and lamellipodia, a pattern similar to Amot [129]. In subconfluent cells it displays a more nuclear localization [130]. In *Drosophila melanogaster*, Merlin interacts with the protein called Expanded (downstream of the protocadherin Fat) to inhibit proliferation through the Hippo Pathway (see Section 4.5). In mammals, the full story needs yet more research in order to be coherent. Merlin activates the Hippo pathway and is associated with both TJ and AJ proteins but the exact mechanotransductive signals remain to be elucidated [129, 131].

The interaction between Merlin and Amot is explained in Paper II, where we show that Amot and Merlin interact through their mutual coiled-coil/BAR domains [132]. The same domain used for interacting with Rich1 (see Section 4.3.2). We postulate that Merlin and Rich1 compete for the same binding site on Amot and that this is a regulatory machinery for Rac activity. Furthermore, we show that the MAPK pathway activation seen by Merlin is dependent on Amot function and interaction.

Contact-mediated inhibition of proliferation is crucial for tissue organization, and is one of the mechanisms contributing to the development of tumors and cancer. As mentioned in Section 1, it is one of the hallmarks of cancer. The anti-migratory phenotype seen in cells

overexpressing Merlin is also of interest. In Paper II, we report the association of Merlin and Amot in the context of cancer. Cells lacking *nf2* expression developed aggressive tumors. However, upon parallel loss of *amot* expression tumors become less aggressive, explained by reduced proliferation *in vitro* [132]. Merlin has been reported to associate with the plasma membrane by binding to phosphoinositides, particularly PIP, through six charged residues within the FERM domain that are conserved in other ERM proteins [133]. This coincides with lipid affinity of Amot coiled-coil domain [133]. The interaction of Merlin with phosphoinositides is dispensable for Ser 518 phosphoregulation, but it seems necessary for Merlin's localization to the plasma membrane and for some aspects of growth suppression [129].

Studies show two more functions of Merlin that I found of interest. One is in promoting the endocytosis of signaling receptors and the other is in inhibiting Axl at a transcriptional level [128, 129]. In Paper III, Axl is presented to be regulated by *amot* expression and to have an inverse relationship with *amot*, most easily illustrated by the correlation plot between *amot* and *axl* in blood vessels, from GeneSapiens.org (Figure 19) [66].

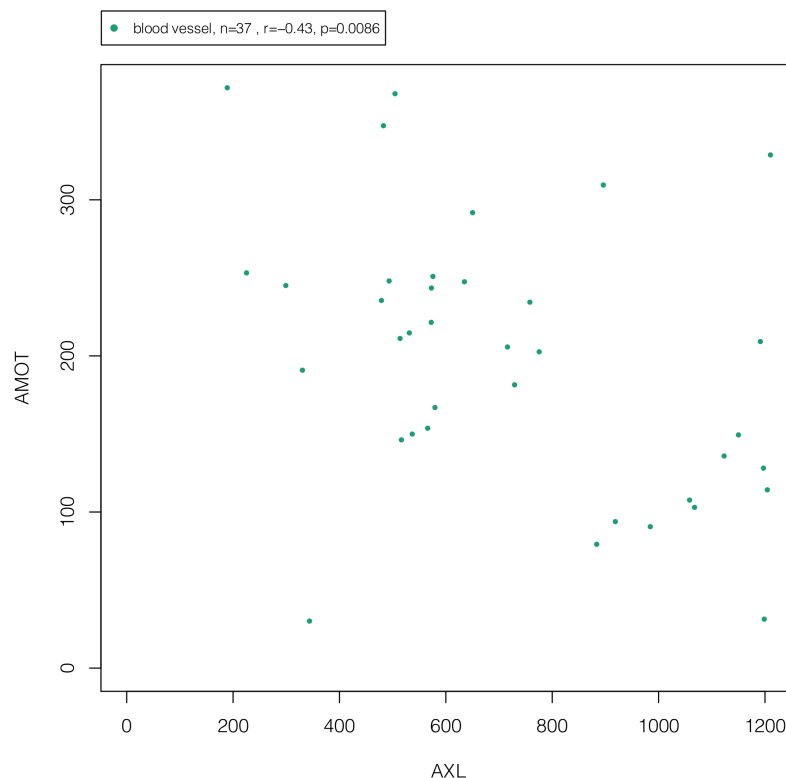


Figure 18. Gene expression plot of the correlation of *axl* and *amot* expression. High *amot* expression correlated to low *axl* expression and vice versa. From GeneSapiens.org 2012-08-01.

4.5 The Hippo Pathway. Of Flies and Men.

I will dedicate this subsection, to very briefly, introduce the Hippo Pathway. The Hippo pathway is a tumor suppressor kinase cascade, which controls cell survival and proliferation in *Drosophila melanogaster*. It is a cascade that is conserved and can be found also in mammals. As you may know, nomenclature for mammalian and fly proteins differs greatly and for your convenience, I have added Post-IT number 2, summarizing the different names.

Inter-species Translation POST-IT 2	
- Hippo Pathway Orthologs	
Mammals	Drosophila
(Mst1/2) Serine/threonine-protein kinase 3/4	Hippo (Hpo)
(WW45) Protein salvador homolog 1	Salvador (Sav)
(Lats1/2) Serine/threonine-protein kinase Lats1/2	Warts (Wts)
(YAP) Yes-associated protein	Yorkie (Yki)
(TAZ) Transcriptional co-activator with PDZ-binding motif	Yorkie (Yki)
(MOB1A/B) Mob kinase activator 1 A/B	Mats (Mats)
(TEF-1) Transcriptional Enhancer Factor-1	Scalloped (Sd)
(FDM6) Expanded equivalent	Expanded (Ex)

The Hippo Pathway consists of Hippo, which is a member of the Ste20 family of kinases [134]. Hippo binds to and brings together the proteins Salvador (Sav) and the kinase complex of Warts and Mats. Upon Hippo pathway activation, Warts phosphorylates the transcriptional co-activator Yorkie. This leads to the cytoplasmic retention of Yorkie and a resulting expressional suppression of Cyclin E and inhibitor of apoptosis (IAP). Thus, activation of the Hippo pathway leads to a reduction in proliferation and an increase of apoptosis. In fly, this pathway is crucial during development. Consequently, suppression of the hippo pathway leads to neoplasia and tumor development [135-137].

In the context of Amot, the hippo pathway is of interest due to publications showing that the PPXY motif in the N-terminal domain of p130-Amot binds to the WW domain of Yap/Taz (equivalent of Yorkie) and sequesters it in the cytoplasm [102, 103, 137, 138]. This hinders Yap/Taz from translocating to the nucleus and there exert its gene regulatory function.

Furthermore, Merlin, an Amot binding partner (see Section 4.4) is also involved in the hippo pathway [128, 139-147]. Boggiano and Fehon postulate that Yap is recruited to the plasma membrane by AJ proteins like α -actinin and to TJ by Amot:Crb3:Patj:Pals1, sequestering it at either location and thereby inhibiting its oncogenic activity (Figure 19) [147]. The full connection between Merlin, Amot and the Hippo pathway and how this may be interconnected with the MAPK signaling pathway, presented in Paper II, needs to be further investigated.

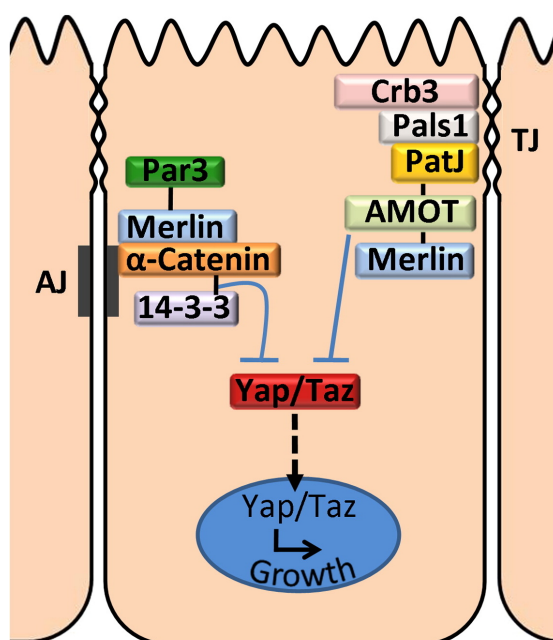


Figure 19. The Hippo pathway regulation by AJ and TJ as proposed by Boggiano and Fehon 2012. Dev. Cell. Yap/Taz is recruited to AJs by α -actinin and Merlin and by the Crb3:Pals1:Patj:Amot:Merlin complex at TJs.

5 Anti-angiogenic therapy

One of the problems with designing targeted drugs for the actual tumor cells is that the tumors cells are very prone to genetic mutation. These rather frequent changes both in genotype and phenotype makes it hard to target both a protein that is not prone to change and still not attack the surrounding normal tissue. While there still are a few successful targeted drugs against e.g. HER-2 in the clinic, other possibilities are needed. As mentioned in the earlier sections, tumors need to hook up to the vascular network in order to grow beyond 1mm in diameter [148]. The hope with anti-angiogenic therapy was to target the process of blood vessel formation, kill the tumor vessels and starve the tumors to death. If not that, one hoped to at least stop the tumors from growing further and to block metastasis. This has been attempted mainly by targeting the pro-angiogenic pathway of VEGF, but also HIF1- α [149].

5.1 Endogenous inhibitors

5.1.1 Thrombospondin

Thrombospondin is a secreted anti-angiogenic protein expressed by a variety of cells, including tumor cells. Thrombospondin binds to surface receptors, like CD95, CD36 and CD47 and induce apoptosis. It is a common finding that upon removal of a primary tumor either by surgery or irradiation, the small, inactive metastases start to grow [150]. By combining surgery/radiation with Thrombospondin treatment, metastatic growth has been shown to be halted [151]. The clinical future of Thrombospondin as a drug remains to be seen. However, multiple studies are being conducted on Thrombospondin analogues and inhibitors of the different Thrombospondin receptors [152-154].

5.1.2 Angiostatin

Angiostatin was described by the Folkman lab in 1994 [155]. It was first isolated from the urine of tumor bearing mice and was found to inhibit both tumor growth and endothelial cell proliferation [156]. Angiostatin is a proteolytic fragment of plasminogen fragment and can be even further cleaved into smaller components, with different binding partners and function. Angiostatin binds to several cell surface receptors apart from Angiomotin; ATP-synthase, integrins, annexin II, C-met receptor NG2 proteoglycan, tissue-type plasminogen activator, chondroitin sulfate proteoglycans, and CD26. To test its potential as an anti-angiogenic drug, Angiostatin was produced in yeast and showed promise in *in vivo*-studies. However, the poor circulatory half-life, proved to be its downfall. Further examination of its receptors/binding partners (like Angiomotin, see Section 6) continue to this day.

5.1.3 Endostatin

Endostatin is one of many derivatives of collagen (arresten, canstatin, tumstatin, restin etc.) that has anti-angiogenic capabilities. Endostatin originates from Collagen XVIII and inhibits tumor angiogenesis by blocking endothelial cell proliferation and tube formation [4, 157, 158]. This is accomplished by interaction with several receptors and pathways. In the clinic several clinical trials have been conducted with endostatin and endostatin analogues, e.g. Endostar has been approved in China for treatment of patients with non-small-cell lung cancer [159, 160]. Gene therapy regimens like RetinoStat have also been developed, where both angiostatin and endostatin expression is induced in the eyes of patients with age-related macular degeneration [161].

5.2 Inhibitors of angiogenic pathways

5.2.1 The VEGF Pathway

The VEGF pathway is mainly utilized by endothelial cells, but has an effect on other cells types, like renal epithelial cells and monocytes [162, 163]. In endothelial cells the VEGF pathway is responsible for cell survival, proliferation and migration, making it a key regulator of vasculogenesis and angiogenesis [164, 165]. As such, the VEGF pathway has become the main target of anti-angiogenic drugs of varying formats and modes of action.

There are the anti-VEGF antibodies, like *Bevacizumab (Avastin, Genentech/Roche)*, a humanized, monoclonal antibody which binds to VEGF and blocks it from binding to its receptor. This drug was the first of its kind and has since been approved for multiple cancerous diseases and various other disease, like age-related macular degeneration [166]. Following in the footsteps of Avastin, there is *Ranibizumab (Lucentis, Genentech/Novartis)*, the F'ab fragment version of the antibody mostly used in ophthalmologic disorders [167, 168].

Apart from antibodies binding and blocking VEGF, there are molecules, like *Aflibercept (Zaltrap/Eylea, Sanofi-Aventis/Regeneron Pharmaceuticals)*, which bind to VEGF-A, -B and placental growth factor (PlGF) and prevent them from binding and activating their receptors. It has been approved for the treatment of wet macular degeneration and shows promise in phase III trials for both prostate and metastatic colorectal cancer [169].

Another way of attacking the VEGF pathway is to target the receptors. There are anti-VEGFR2 antibodies e.g. *Ramucirumab (ImClone Systems Inc.)* that bind to the receptor and inhibit further signaling [170]. Other ways of blocking VEGF receptor signaling is to block the intracellular kinase domains, which can be done by small molecular inhibitors targeting these domains. *Imatinib/Imatinib mesilate (Gleevec/Glivec, Novartis)* is one such drug and it binds to the kinase domain of the receptor tyrosine kinases (RTKs) (like VEGF-receptor) and blocks their function by inhibiting substrate binding and receptor activation [171]. More small-molecular inhibitors of RTKs are e.g.: *Sunitinib (Sutent, Pfizer)*, formerly known as SU11248, *Sorafenib (Nexavar, Bayer/Onyx Pharmaceuticals)* and *Vatalanib (Bayer Schering and Novartis)* [172, 173]. All of these drugs do not only have VEGF-receptors as targets but also affect multiple kinases, e.g. PlGF and PDGF-Rs too. These drugs have all been approved for the treatment of various aggressive carcinomas.

5.3 Adverse effects

As mentioned earlier, angiogenesis is not active in the adult, apart from wound healing and processes within the female reproductive organs. Most endothelial cells are quiescent and as such adverse effects of drugs targeting angiogenic, proliferating, endothelial cells were not expected to be many nor severe. However, like any drug, anti-angiogenic drugs have their share of adverse effects. The nature of these adverse effects range from benign to life-threatening conditions. Several parameters affect the severity the individual patient will experience; dosage, combination with other drugs/chemotherapy, the location of the tumor/s, the specificity of the drug and the state and medical history of the patient [174]. However, so far the exact parametric combination leading to a specific patient response does not exist.

Anti-VEGF/VEGFR antibodies are mostly correlated with clotting disorders, resulting in wound complications, thrombosis or hemorrhage. Added to the list are also, hypertension, proteinuria (or worse), bowel perforation and reversible posterior leukoencephalopathy syndrome, to name a few more. RTK inhibitors present somewhat different adverse effects, due to the fact that they also target other molecules, not directly in the VEGF-pathway [174]. These effects are in part explained by the great variety of functions that are conducted by VEGF, which are thrown off balance upon anti-VEGF treatment [175].

The adverse effect above are acute, short-term effects. As Cook et al. state in their review of anti-angiogenic drugs, the long-term effects of these therapies are not yet fully known, since most drugs are so new on the market. [149].

5.4 Resistance

Eventhough anti-angiogenic drugs have been beneficial for patients, the positive effect on cancer is mostly transient. During the course of treatment, or even before, patients stop responding to therapy. They have become resistant to the treatment. G. Bergers and D. Hanahan divide tumor resistance mechanisms to anti-VEGF drugs into two compartments: Adaptive (evasive) and intrinsic (pre-existing) non-responsiveness [176]. As a result of the VEGF blockade of the drugs, the evasive non-responsiveness starts with the upregulation of alternative signaling pathways. Bone marrow-derived pro-angiogenic cells are recruited to the tumor site in order to continue vascular remodelling and to further increase the VEGF-independence pericyte coverage is increased. This mediated endothelial cell survival in the absence of VEGF. Lastly, the evasive responsiveness is characterized by the enhancement of tissue invasion and metastasis, a

means for the starved tumor cell to find a better vascularized milieu in which to grow.

The intrinsic (pre-existing) non-responsive tumors/tumor vessels already exhibit some of the traits mentioned above prior to anti-angiogenic treatment. This is thought to be caused by the selective pressure for that specific tumor. Tumors utilizing vessel co-option also fall into this category, as they do not rely upon neovascularization. This phenomenon explains why some patients do not respond to the treatment at all.

The major drawback with anti-angiogenic therapy has not been so much the drug resistance per se, which occurs with other treatments as well, but the change in tumor aggressiveness [177]. In responding patients, this is explained by defense mechanisms triggered by oxygen deprivation, i.e. the last evading resistance mechanism described above. As a result, a more aggressive tumor type is selected and the patients end up with a rapidly progressing disease.

By targeting multiple pathways, resistance may be overcome or delayed. This makes combining therapies an important notion also for anti-angiogenic therapies. I hope that even more targeted therapy will help abolish the worst adverse effects and help in plugging the holes through which tumors continue to escape death.

6 Anti-Angiomotin therapy

Considering the development seen with anti-VEGF therapy, mentioned in the above section, the continued work on targeted drugs and analogs of endogenous inhibitors is most important. Taking all the data presented on Amot so far, I hope that the clinical potential of an anti-Amot treatment has become clear. And, considering the notion that an anti-Amot molecule is mimicking the effect of Angiostatin, I deem that the concept of anti-Amot therapy needs no further presentation. In this section, I present the collected findings of the Holmgren group and collaborators on anti-Angiomotin antibodies, to give a coherent image of the work done and what stands to be done on this matter.

6.1 Patient generated antibodies – DNA Vaccination

Some of the problems with endogenous inhibitors such as angiostatin are short circulatory half-life and expensive production. To circumvent this problem, a DNA vaccination approach was attempted. The rationale behind the project was that the vaccination would result in the patients themselves producing the anti-Angiomotin antibodies that would inhibit the pathological angiogenesis and thus halt tumor growth.

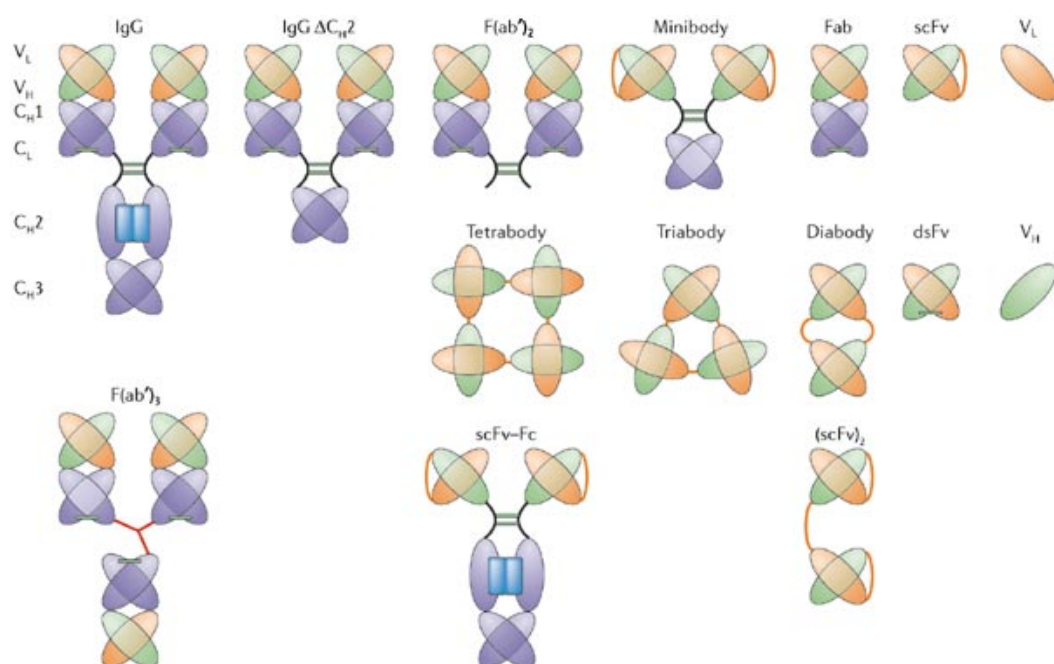
As always, the “patients” initially had to be mice, in this instance BALB-NeuT mice. Holmgren et al 2006 show that a co-vaccination against human Angiomotin and the extracellular and transmembrane domains of Human Epidermal Growth Factor Receptor 2 (Her-2) prevents tumor (TUBO) formation for more than 70 weeks in 80% of the mice [88]. Vaccination against Angiomotin inhibited angiogenesis, as shown through the lack of blood vessel ingrowth in the matrigel plug assay (microtumor/bFGF-induced angiogenesis) and delayed the onset of disease [84, 88]. The latter finding is remarkable, considering that vaccination against Neu did not elicit as efficient protection [84]. High, specific anti-Amot antibody titers were received in both BALB-NeuT and –PyMT mouse models, indicating a good immune response and that the effects seen most probably are due to anti-Amot antibodies. Interestingly, vaccination against Amot resulted in an otherwise ineffective single dose of doxorubicin to take effect and either delay tumor growth or lead to tumor rejection [84].

This shows a great potential for using anti-Angiomotin antibodies for treating highly vascularized tumors. Today, projects are still ongoing trying to modulate this vaccination (intra-muscular injection of plasmids followed by electroporation) approach into even better results, hopefully good enough to argue for clinical trials.

6.2 The B06 antibody

In collaboration with the pharmaceutical company BioInvent International (Lund, Sweden) a therapeutic anti-Amot antibody has been developed, intended for clinical use. The BioInvent way of antibody development is to use phage-display to screen the target protein (in this case Amot) against a single chain antibody fragment (scFv, see Figure 20) library called n-CoDeR® [178]. The selected candidates are tested for affinity and biological function. The best candidate is then produced at large scale in eukaryote cells and the scFv fragment is coupled to a human IgG backbone, a so-called humanized antibody.

In the case of the anti-Amot scFv, Boyden chamber migration assay was used *in vitro* and the matrigel plug assay *in vivo* to test functionality [97]. In the following section, multiple antibody formats will be presented, as illustrated by Figure 20.



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Figure 20. Antibody formats. From Carter. 2006. Nature Reviews Immunology.

6.2.1 The Single Chain Format

The single-chain variable fragment (scFv) is an antibody fragment of approximately 30kDa consisting of the variable heavy and light chain domain joined by an amino acid linker. As a result of their monovalency and small size, scFvs clear quickly from the bloodstream via kidney excretion and have a half-life of 0.5 - 2 hours [179].

The B06 scFv was functional for Fluorescent Activated Cell Sorting (FACS) analysis to verify Amot expression in cells and was able to stain blood vessel in sections from matrigel plus (bFGF-induced angiogenesis). Treatment of cells with the antibody resulted in inhibited endothelial cell migration and tube formation *in vitro*. This coincided with almost complete inhibition of angiogenesis in matrigel plugs *in vivo* and with the inhibition of retinal angiogenesis in neonatal mouse pups [97].

However, with the low biological half-life, the scFv format of the B06 antibody is not eligible for clinical trials as a therapeutic antibody. In upcoming sections this antibody has been put to other use.

6.2.2 The IgG-Format

This version of B06 is a full antibody, IgG. It is composed of two synthetic scFv, coupled a human IgG backbone. The molecular weight of an IgG of approximately 150 kDa results in long residence in circulation for up to 3 weeks [180]. This makes the IgG format suitable for therapeutic purposes as it results in longer intervals between doses. This is the format BioInvent works with and wished to develop.

However, the work and results on the IgG format are mostly unpublished and the rights owned by BioInvent, so I will continue to describe other formats.

6.2.3 The PEGylated F'Ab

In the context of drug development, one way of circumventing the problem of short biological half-life is to conjugate the drug with polyethylene glycol (PEG) polymer chains. This process, called PEGylation renders the protein/drug larger, thus increasing the half-life, increasing water solubility and masking/ reducing immunogenicity of the drug in question [181]. This was done the F'ab format of the B06 antibody with a 20kDa PEG-maleimid compound and resulted in a reduction in the number of matrigel plug-invasive vessels (i.p. injection) (Figure 21) [97].

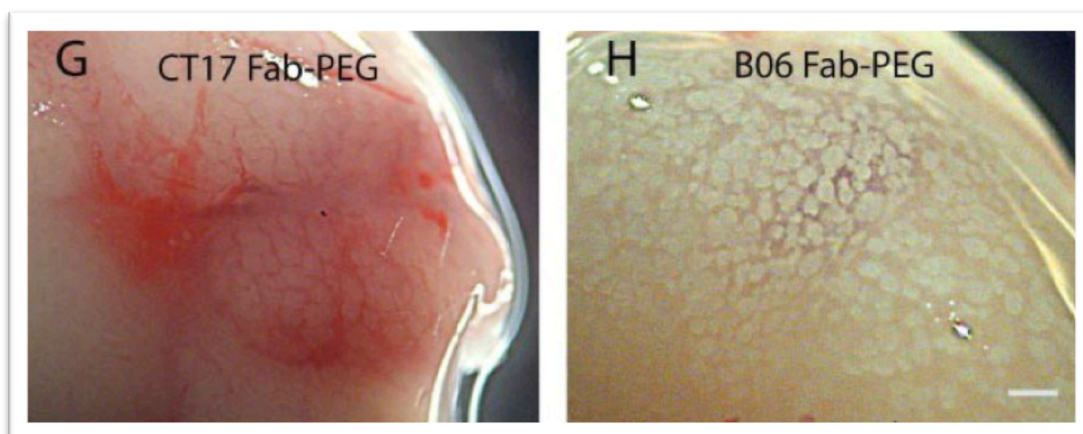


Figure 21. The effect of i.p. injected, PEGylated B06 F'ab antibody into TUBO-stimulated matrigel plugs (panel H), compared to control F'ab antibody (panel G). From Levchenko et al. 2008. FASEB J.

However, despite the fact that PEGylation is generally accepted in the pharma industry the technology for efficient production has not yet been developed. Consequently, PEGylation is costly therefore commercially unattractive.

6.2.4 The Imager

Having a molecule with high specificity and affinity for its target, but a short half-life is not bad. For applications like immuno-*in vivo* imaging this is a desired characteristic. When it comes to immuno-positron emission tomography (immuno-PET), a short half-life means minimizing the time the substance resides within the patient and as a result reduces the radiation experienced by the patient and also the people in his/her close proximity. Also, long biological half-life brings with it logistical dilemmas for both caretakers and patients, since additional appointments may be needed (one for injection of tracer, one for imaging). As mentioned previously a full antibody (~150kDa) may remain up to 3 weeks in circulation, while the ScFv (~25kDa) clears quickly from the bloodstream in a matter of hours.

Biomolecules such as proteins, e.g. antibodies, are ideal for targeted imaging. However, this technique is not widely used in the clinic due to the lack of efficient labeling procedures. Before injection, the biomolecule needs to be radio-isotope labeled, i.e. a radionuclide needs to be incorporated in the protein's chemical composition. This can be accomplished by producing the protein in media containing radiolabeled amino acid, by inducing the exchange of normal for radiolabeled molecule using an enzymatic reaction or by changing the nucleophilic properties of antibody amino acid residues, making them susceptible to uptake of the radionuclide [182]. The group of E. Arnér has developed a method of efficient, residue-selective labeling and subsequent re-purification of proteins by using a so-called selenocystein (Sec) tag (Sel-tag) [183, 184]. In collaboration with Dr. Arnér a Sel-tag was added to the C-terminal of the B06 ScFv and the protein was labeled with carbon 11 (^{11}C). As a tracer, ^{11}C is used in the context of ^{11}C -choline or ^{11}C -methionine for PET-imaging [185]. It has a half-life of 20.38 min and gives rise to beta-irradiation during its decay [186]. Together with Dr. Sharon Stone-Elander the ^{11}C - B06 ScFv was injected into both healthy and tumor bearing mice.

Figure 22 shows the kinetics and biodistribution of the B06 ScFv during 60min of imaging in the PET-camera. Initially, the antibody goes to all major organs and later concentrates at the kidneys, for excretion, and at the tumor site. Figure 23 shows a PET-image of the antibody (tracer) uptake and shows the antibody targeting the tumor (MAE), comparing it to the current standard tracer for tumor sugar metabolism, the glucose analogue Fluoro-2-deoxy-D-glucose (FDG).

These results are most interesting, but no study is complete without a negative control. Studies have shown that at early time-points after injection, small molecules and proteins are readily infiltrating the tumor from the vasculature, independent of specific binding [187]. Upon running Sel-tagged GFP we confirm that this is indeed the case. The GFP also localizes to the mouse tumors, similarly to the B06 ScFv, during this time

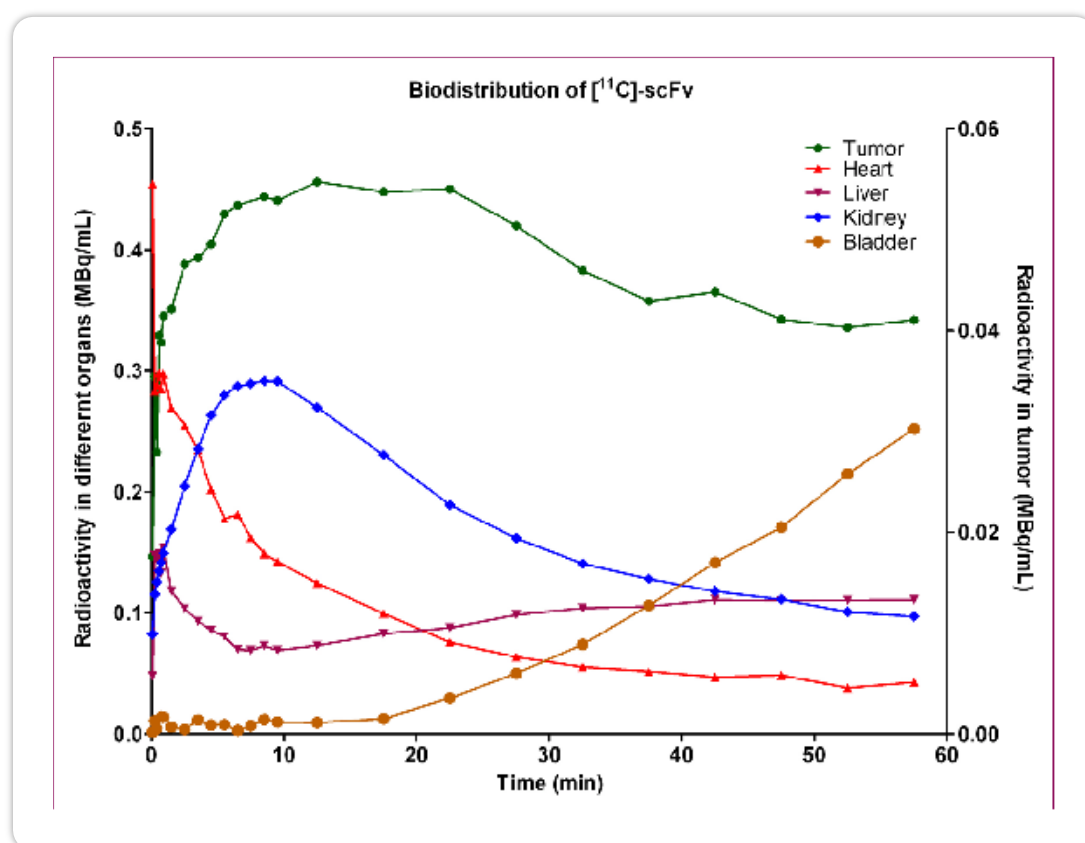


Figure 22. Kinetics analysis of ^{11}C -labeled B06 ScFv in a tumor-bearing mouse. Unpublished data.

frame. According to literature the phenomenon of unspecific tumor localization time-limited [187]. There is a steady efflux of the non-binding protein from the tumor to the vasculature and later to the kidneys for excretion. This means that, given enough time the non-binding protein will be cleared. In contrast a protein specifically targeting an epitope within the tumor will reside much longer than the non-binder. Consequently, the distribution of the antibody and control should be compared at a later time-point. As shown in Tijink et al 2009 waiting up to 48h post-injection of the tracer yielded images with good background to signal ratios [188]. “A candle in the dark”, the signal quality to aim for in order to be able to use this technique for clinical practice.

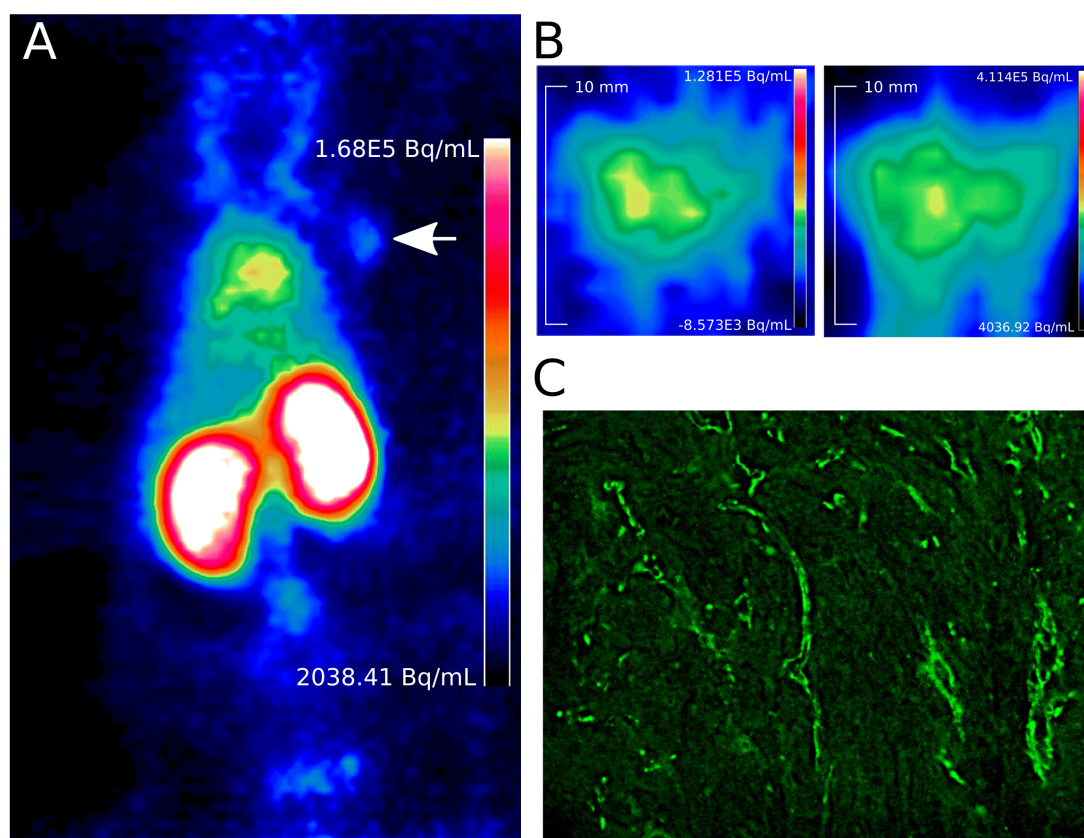


Figure 23. PET-image showing ^{11}C -B06 scFv injected into tumor-bearing mouse. (A) Coronal section of whole mouse at 5-10 post injection. Head up. Tumor indicated by arrow. (B) Transverse section of tumor. Left: ^{11}C -B06 scFv. Right: ^{18}F -FDG. (C) Sectioned tumor, stained for B06 scFv, showing the vascular targeting of the antibody.

Potential anti-angiogenic drugs struggle in early clinical trials, since their effect is hard to measure [189]. The hope and aim for this version of the B06 antibody is to be able to determine whether or not the patient is responding to therapy by looking at blood vessel density by PET-imaging. By having a fast read-out this would help other drugs to reach the market and to further individualize therapy.

In the future, I hope the use of more stable radio-isotopes with longer half-life i.e. ^{124}I will decide the fate of the B06 scFv as an immune-PET tracer and a potential diagnostic tool.

6.2.5 The Diabody

In the spectra of alternative antibody formats we also have the multivalent variants of the scFv: Dimeric (diabody), trimeric (Triabody) and tetrameric

(Tetrabody). In the quest of increasing biological half-life without increasing the size to the extent of losing biological activity, we chose to produce the B06 antibody as a diabody. As the name implies, a diabody is a dimer of two scFvs. Not only does this give rise to a larger molecular mass (~55kDa) and longer biological half-life (3-7 hours), it also opens up for the possibility of having a bispecific antibody [190]. Two scFv with different ligands. In the case of the B06 antibody, we chose to have a homodimeric diabody. Also, diabodies have been shown to have up to 40-fold lower dissociation constants, giving them a much higher affinity to their target [191, 192].

Diabodies can form spontaneously, depending on the characteristics of the scFv. However, the linker-region between the heavy and light chain of the scFv can be modified to induce non-covalent multimer formation. The B06 scFv readily formed multimers over time, as the purified protein was stored. In order to force controlled dimerisation only, we therefore re-cloned and modified the linker region to 5 amino acids only. This renders the scFv unable to fold properly and allow the VL domain to bind the VH domain. In order to get VL:Vh binding, the short linker scFv dimerizes. Triabodies and tetrabodies can be forced to form in the same fashion; by reducing the linker region size to less than three amino acid residues [193, 194].

By dimerizing the B06 scFv antibody we hope to increase the half-life up to 7 hours. This format is the one to be used as a means of transporting toxic substances into the tumor and tumor vasculature, a technique called vascular targeting.

6.2.6 The Trojan Horse/Vascular Targeting

The Trojan horse was the means for the Greeks to, after a 10-year siege, enter Troy and end the war. They created a wooden horse, in which they hid a host of soldiers. Afterwards they sailed away, pretending to forfeit. As a trophy to their victory, the Trojans pulled the horse, and soldiers, inside the barricades and consequently lost the war. We hope to “build” our own Trojan horse in the shape of the B06 diabody, to gain access to the tumor tissue and angiogenic vessels. Instead of soldiers inside, we aim to couple it to toxic substances and kill off the angiogenic vessels and tumor cells, thus coming a way to winning the war, and siege, of cancer.

Vascular targeting is not a new concept and several potential drugs are undergoing clinical trials. However, choosing the most suitable agent for killing off angiogenic vessels at minimal risk for healthy tissue (in this case, especially kidneys) will take careful planning.

6.2.7 The Albumin Binding Domain

The concept of an Albumin binding domain (ABD) to prolong half-life is not new [195, 196]. The rationale is that the ABD is incorporated into the DNA and amino acid sequence of an antibody, or smaller antibody fragment. Upon injection into a blood stream, the ABD will bind to serum albumin and thus increase dramatically in size and be protected from kidney excretion. The bond between the ABD and serum albumin is non-covalent and has a low affinity compared to the affinity of the antibody to its ligand. At any given time within the body, a certain amount of albumin-bound and unbound antibody will exist. The albumin-unbound antibody will readily bind to its ligand and the bound may exchange the albumin for the ligand instead.

We have coupled the B06 scFv to an ABD N-terminally and produced it in bacteria in small, medium and larger quantities. We are currently testing the different bonds; ABD-antibody:ligand, albumin:ABD-antibody and albumin:ABD-antibody:ligand to know if the functionality of the B06 scFv is compromised by the binding to albumin. Experiments in the nearby future will determine whether or not the ABD is truly prolonging serum-half life in mice.

7 This Thesis

Insufficient or exaggerated angiogenesis is a trait found in many diseases common in man. Looking beyond cancer, angiogenesis is affected in obesity, retinopathies, macular degeneration, hair loss, psoriasis, arthritis, endometriosis, Alzheimer's disease, multiple sclerosis, stroke, erectile dysfunction and many more. Current anti-angiogenic therapies have been successful in treating some of these ailments. However, in the context of cancer the current therapies have mixed results. As patients become resistant they relapse and develop an even more aggressive disease. As mentioned in earlier paragraphs most current drugs are broad, targeting families of receptors involved in many other cellular functions and existing on more cells than endothelial cells.

The aim of this thesis is to contribute to the understanding of angiogenesis by studying the function of Angiomotin. In parallel, drug development has been on the agenda, further developing a potential anti-Angiomotin drug for both therapeutic and diagnostic purposes. Surrounding mechanistic work has been paramount in trying to find other potential drug targets in the same functional pathway. All in all, this thesis aimed to find a new, specific angle from which to strike pathological blood vessels in the hope of aiding patients and further individualizing their therapy.

7.1 Results and Discussion

Paper I. The importance of the PDZ-binding motif in connecting Angiomotin to polarity proteins and affecting cell migration.

We have previously shown that cells expressing a C-terminal Amot mutant lacking the last three amino acids, the PDZ-binding motif, exhibit migratory defects [57]. In this paper we wanted to identify the proteins binding to the PDZ-binding motif of Angiomotin and what their functions are in terms of regulating cellular migration.

All members of the Motin family of proteins contains PDZ-binding motifs, hence Amot, AmotL1 and AmotL2 underwent the peptide pull-down experiments identifying their binding PDZ-binding domain partners. We show that all members bind to polarity and TJ proteins, indicating similar functions for all family members. We show that Angiomotin binds to the multi-PDZ domain containing protein Patj and Mupp1 and associates to their associated protein complex of Pals1 and Lin-7. This data was supported by two papers published while this article was under development [87, 90].

We also found a connection between Angiomotin and a protein called Syx1. The connection between Amot and Syx1 is thought to be indirect, as

both proteins have PDZ-binding domains but no PDZ-domains [95, 123]. Another protein is most likely modulating the connection between the two. Further assays identified Mupp1/Patj as a partner to Syx1, also in the context of the Pals1:Lin-7 complex.

Syx1 is a RhoGEF, a protein exchanging GDP-RhoA for active GTP-RhoA. In this paper we perform a FRET-analysis using a RhoA-probe, showing localization of active RhoA in a cell. In the presence of Angiomotin, RhoA-activity was concentrated to the leading edge of a migrating cell, similar to the localization of Amot. However, upon loss of *angiomotin* expression RhoA-activity was dispersed throughout the cell edges, resulting in the extensive protrusion activity, published earlier [61].

These data indicate that Angiomotin localizes RhoA-activity and concomitant actin polymerization at the right place and time during cellular migration. We therefore conclude that upon migratory stimuli, Angiomotin is localized to the leading edge of a cell, associating to polarity proteins and recruiting the RhoGEF Syx1, thereby localizing and concentrating RhoA activity at the leading edge. This is accomplished by the interaction of Angiomotin with the polarity complex Patj/Mupp1:Pals1:Lin-7, where both Angiomotin and Syx1 bind to Patj/Mupp1. Supporting our theory that Syx1 is indeed a protein acting downstream of Amot in the same signaling pathway is the data showing that Morpholino knock-down of *syx1* mRNA expression phenocopies the loss of *angiomotin* mRNA expression.

Conclusions from Paper I:

- The Amot PDZ-binding domain is crucial for binding to polarity proteins and the Rho-GEF Syx1.
- The miss-localization of the Rho-GEF Syx1 may explain the migratory dysfunction seen upon loss of Amot expression.
- Morpholino knock-down (MO) of *syx1* mRNA expression in zebrafish phenocopies that of MO of *amot*; impaired migration of intersegmental vessels.

Paper II. The coiled-coil domain of Angiomotin binds to Merlin and Rich1, further connecting Angiomotin to the Rho-family of proteins and tumor development.

In this paper the association between Angiomotin and Merlin was studied. We found that pull-down of Merlin resulted in the capture of Amot, AmotL1, Patj and Pals1. Further analysis shows that Merlin does not interact with Patj nor Mupp1, but does interact directly with Amot (both isoforms) through their mutual coiled-coil domains.

Both Angiomotin and Merlin have been reported to localize to AJs and TJs. Calcium switch studies show that Angiomotin localizes to junctions several hours after the switch back to high calcium containing medium whereas Merlin localizes to primordial junctional structures already after 15 min. Further findings suggest that neither protein is dependent upon the other for its initial junctional localization. However, mutated and miss-localized Angiomotin dissociated Merlin from the TJs. This indicates that eventhough Angiomotin is not involved for the initial junctional localization of Merlin, it mediates the retention of Merlin at mature TJs.

Angiomotin has been reported to interact with Syx1 and Rich1 (also known as ARHGAP17 or Nadrin), modulators of three members of the Rho-family of proteins: RhoA [65], Rac1 [61] and cdc42 [87].

Wells et al show that Angiomotin negatively regulates the GAP-activity of Rich 1 and consistent with that data we show that loss of *angiomotin* mRNA expression reduces the amount of active Rac1. In contrast, Merlin has been reported as a negative regulator of Rac1 and affecting the Ras-MAPK pathway.

ShRNA knock-down of *angiomotin* mRNA expression led to a decrease in the phosphorylation of several MAPK pathway activated molecules; c-Raf (S338), MEK (S298), ERK (T202/204), p95RSK (S380) and BAD (S112). However, phosphorylation of BAD (S136), a result of PI3K-AKT pathway signaling, was unaffected. Taken together these data show that Angiomotin regulates Rac1-Pak-MAPK signaling in an opposite manner from Merlin and Rich1.

Our data suggests that both Angiomotin and Merlin require Rich1 to affect the MAPK-pathway. After further investigation we found that Rich1 was dissociated from Angiomotin by introduction of Merlin in a dose-dependent manner. This indicates that Merlin and Rich1 may compete for the same binding site on Angiomotin and that the affinity for Merlin to Angiomotin is higher. This hypothesis was deemed plausible considering the finding by Wells et al. in 2006, where they found that Angiomotin and Rich1 interact through their mutual coiled-coil domains, similar to that of Angiomotin and Merlin. The exact cue from the extracellular environment and subsequent molecular mechanisms controlling this switch remain to be elucidated.

Patients with mutations in the *nf2* gene develop primarily Schwann cell tumors of peripheral nerves. In mice we show that the loss of *nf2* expression in Schwann cells in combination with a downregulation of *amot* mRNA expression leads to marked decrease in tumor growth rate and a prolonged mouse survival. This shows that Angiomotin is required for Schwannoma development due to loss of Nf2. It also emphasizes the potential for Angiomotin to be used as a drug target.

Conclusions from Paper II:

- Merlin and Angiomotin interact directly through their mutual coiled-coil domains.
- Merlin and Rich1 compete for binding sites on Angiomotin. During mitogenic signaling, Angiomotin binds to and sequesters Rich1, leading to an increase in Rac1 activity. During growth suppressive conditions Merlin binds to Angiomotin and Rich1 is free to inhibit Rac1 signaling through Ras-MAPK mitogenic pathways.
- Angiomotin expression is required for schwannoma development in *nf2*-deficient Schwann cells.

Paper III. Identification of genes affected by the loss of *angiomotin* expression.

We have previously reported that more than 75% of all *amot* negative mice die *in utero* at E11. Analysis of embryos at E9-11 showed severe vascular defects such as insufficient vascularization of the intersomitic region as well as dilation of cranial vessels [61].

In this paper we study the surviving few mice but initially fail to find any apparent abnormalities. Upon studying organ vascularization standard histology examination and specific blood vessel staining reveal no detectable difference between wt littermates and *amot* negative mice. However, upon tumor xenograft implantation of Lewis Lung Carcinoma cells, the mice responded differently.

We observe paler tumors in the *amot* negative mice and after inspection of tumor sections under microscope we conclude that this is caused by a marked reduction in vascularity. Perfusion studies revealed that not only did the *amot* negative mice exhibit fewer tumor blood vessels; the vessels present were poorly perfused.

We show that even though these mice found a means to circumvent the effects of absent *angiomotin* expression during embryonic development, they were unsuccessful during pathological conditions. This supports the theory that there is a difference between physiological and pathological angiogenesis and indicates that Angiomotin plays an important role in this process.

Gene expression analysis using microarray revealed that the loss of *angiomotin* mRNA/gene expression leads to effects on genes important for cellular migration and inflammation. We observed an involvement of the NF- κ B pathway and a marked increase in gene expression of inflammatory receptors such as Immuno Cell Adhesion Molecule (ICAM)-1 and P-Selectin (SELP).

In parallel with our finding that loss of *angiomotin* expression e.g. leads to effects on (software classification) “Cellular Movement” and “Cardiovascular System Development” we also found effects on “Organismal Survival” and “Cell Cycle”. However, when looking closer upon the genes regulated no coupling to the Hippo Pathway could be found, which was unexpected. More time needs to be spent looking at these results to see which pathway may be affected in this context.

The changes in expression were confirmed both on mRNA and protein level using quantitative Polymerase chain reaction (qPCR), Fluorescence Activated Cell Sorting (FACS) analysis and ImmunoFluorescence (IF) on cells treated similarly to the microarray batches. The increased ICAM-1 expression was also confirmed on blood vessels in tumor tissue from *amot* negative mice.

It has been shown that cytoskeletal disruption can trigger an NF- κ B pathway response [197-201]. Loss of Amot expression leads to a marked effect on cytoskeletal integrity. We suggest that these cytoskeletal alterations may trigger an NF- κ B pathway response both *in vitro* and *in vivo*, leading to the upregulation of inflammatory receptors like ICAM-1.

In summary, we have identified a set of angiomotin-regulated genes involved in angiogenesis. Further studies are necessary to understand more of the mechanisms associated to these genes. We show that *angiomotin* expression is one component driving pathological angiogenesis and appears to be involved in tumor vessels maturation and subsequent perfusion. The loss of angiomotin is associated with an NF- κ B pathway involvement and an upregulation of inflammatory receptors. Future studies will reveal if a specific population of immune cell is recruited to the tumor.

Conclusions from Paper III

- Adult angiomotin negative mice experience reduced pathological angiogenesis, combined with a low vessel perfusion.
- Loss of angiomotin is associated with an NF- κ B response and an up-regulation of inflammatory receptors such as ICAM-1 and P-Selectin both *in vivo* and *in vitro*.

7.2 My Conclusions & Future Perspectives

I think a wider approach is needed to map the function of a given protein. For us, this means that we, at every experiment, analyze the localization and levels of each Motin and their corresponding binding partner. Given the homology between the Motins, the overlap in function and tissue expression, there is a chance that the levels of the one will affect the function of the other. I believe this is the only way to accumulate enough data about the Amot, AmotL1 and AmotL2 trio to draw larger conclusion as to how they affect endothelial cells and an organism as a whole.

When it comes to Amot in the context of migration I hypothesize that migratory cues such as high VEGF concentration attract chemical sensors associated to lipid rafts. The rafts accumulate, attracting lipid-binding proteins like BAR-domain containing proteins like Amot. Amot follows the rafts, bringing with it proteins like Patj:Pals1 and Syx1. In parallel, p80-Amot expression is upregulated and p130-Amot is sequestered from the TJs, thus weakening the junction. Functional TJs need the entire Crb3:Pals1:Patj complex and the removal of Patj:Pals1 causes TJ disruption. Crb3 is internalized to endosomes, disbanding the TJs completely. The localization of Amot at the leading edge brings with it double effects on the actin cytoskeleton, one being the localization of Rho activity via Syx1, the other being removing the inhibition of Rac1 by sequestering Rich1 either at the leading edge or in the vesicles now containing p130-Amot in the cytoplasm.

The way I see it there are several points of interest to study in order to sort out and eliminate hypotheses like mine.

I. The MAP kinase pathway and contractility. The association of Amot and Merlin or Rich1 and subsequent activation/inhibition of the MAP kinase (MAPK) pathway (Pak1/2-MEKK1-MEK1/2-ERK—TF—Gene expression) needs to be investigated further.

If we believe that both the findings from Paper I and Wells et al 2006 are correct then we need to find out in which context the one and/or the other is true. Amot binds Syx1, localizing ROCK activity and subsequent actomyosin contraction. Amot also binds to Rich1 and while Rich1 is bound Rac is active, free to activate PAK. PAK phosphorylates and inactivates Myosin Light Chain Kinase (MLCK) and leads to a decrease in contractility. This scenario seems unlikely to me: both promoting and inhibiting contractility at the same time. However, PAK has been reported to directly phosphorylate Myosin Regulatory Light Chain (MRLC), thus promoting contractility. The immediate question I think of are: *Is Amot associated to other proteins in TJs versus in lamellipodia? Which ones? What decides when Merlin/Rich1 binds to Amot? Is Merlin constitutively expressed in both senescent, dividing, migrating and stationary cells, or is the expression timed with a certain cellular response? Or, is the expression of Merlin constant but the protein occupied*

elsewhere in the TJs, only free to bind Amot and release Rich1 at a certain time-point of cellular life?

The reason I write “if” we believe the in the findings is because it has also been shown that the PDZ-binding motif of Amot, which is not essential for the Rich1 interaction, is essential for the migratory function of p80-Amot [57].

In cells expressing the PDZ-binding motif mutant form of p80-Amot, p80-Amot is misslocalized (cytoplasmic), which would result in subsequent misslocalization of both Rho and Rac induced migration [86]. This could indicate that the proper localization of p80-Amot is dependent upon the PDZ-binding motif, in turn implying that localization is dependent on Patj/Mupp1. However, since Syx1 is misslocalized this cannot be. It could be that binding to Amot causes a conformational change in Patj/Mupp1, enabling Syx1 binding, but it seems unlikely. It can be that a series of partners are needed in order for Rich1 to bind the coiled-coil domain and that those partners need the PDZ-binding motif. No matter what, this phenomena needs more investigation.

II. IL-8. Related to point I is the topic of IL-8. Taking into consideration our findings in Paper III of the increased expression of inflammatory receptors and NF- κ B response upon loss of *angiomotin* expression together with the MAPK pathway, IL-8 emerges. Firstly, the MAPK pathway may trigger NF- κ B signaling. Secondly, expression of IL-8 can be triggered through both the MAPK and NF- κ B pathway [202]. Thirdly, endothelial cells express IL-8. In fact, they even have IL-8-filled vesicles, ready to disperse the content into the extracellular space upon the correct cue [203]. The effect of IL-8 on ECs is the increase of proliferation, cell survival and the stimulation of several angiogenic properties, like MMP secretion [204]. In the gene expression analysis of Paper III we find no direct evidence of this. However, not enough time has been spent analysis the data in the MAPK context to be certain. Taken together this may explain why we see an involvement of “Cell cycle”, “Cell survival” and “Organismal survival” as parallel results when analyzing the microarray data from *amot* negative endothelial cells. We need to answer questions like: *Is there a difference in IL-8 production in amot negative cells/mice?*

III. Isoform specificity and kinetics. We also need to pinpoint when and where Amot is interacting with all of its published partners. As I mentioned in Section 3.2 our lab has published that overexpression of p80-Amot redistributes the localization of p130-Amot from TJs to the cytoplasm, resulting in a migratory phenotype [86]. This raises the question of what function each isoform is performing, in the context of binding partners. *At confluency in TJs, which Amot isoform is interacting with Merlin? With Patj? Where is each isoform localized at different stages of cell life/movement and what is it interacting with? Where do Plekhg5 and Rac1 fit in all of this? Where is Crb3 localized in migrating ECs? Where is p130-Amot localized at the same time? Where is Merlin localized in amot knock-outs? How does Axl expression fit into all of this?*

IV. Lipid rafts and the lipid composition of a cell. Lipid rafts are distinct subdomains of the plasma membrane, islands containing high concentrations of cholesterol and glycosphingolipids. Apart from lipids they also contain a variety of proteins, especially those involved in cell signaling. The immunological synapse is

one example of their importance. The coiled-coil domain of Angiomotin is a BAR-domain specifically binding to lipids. According to Heller et al 2009, this domain (called ACCH) preferentially binds cholesterol rich membranes, more specifically membranes enriched in phosphoinositide-3-phosphate (PI(3)P) and PI(4)P. Upon polarization and migration cellular membranes demonstrate a distinct lipid composition, e.g. review by Wang and Margolis (2007). The lamellipodia (to where Amot localizes upon migration) consists mainly of PI(4,5)P₂. This is inconsistent with the published findings by Heller. However, considering that the studies were only conducted on artificially constructed vesicular structures some binding data may have been missed. The Amot BAR-domain may still bind to a specific composition of phosphoinositides during “physiological” conditions in the cell. *What is the lipid composition of lamellipodia of migrating ECs? Does Amot coiled-coil domain bind to this composition of lipids? If so, what is the relation of these rafts and Amot during different time-points in the cell? Does the one co-localize with the other?* This may shed light upon how Amot is relocalized to the lamellipodia upon EC migration. If there is a connection between the two, these rafts may be targets for drug design. E.g. Glucocorticoids may alter lipid raft composition and change cell behavior [205]. *Can we remove amot from lamellipodia by removing/blocking a certain protein?*

Secondly, I believe that the findings of Heller et al should be looked into in more detail. PI(3)P and PI(4)P are mostly substrates for other PIs but (especially in the case of PI(3)P) are part of early endosome (EE) membranes and the multivesicular body (MVB) compartment. This, together with our findings that especially AmotL2 localizes to endosomal structures calls for further investigation. *Are PI(3)/(4)P important for AmotL2 function?* Since the EEs have been implied in receptor recycling and Angiostatin is internalized upon binding to its receptor: *Is Amot, like Merlin, involved in receptor recycling?*

V. The mechanism of anti-Amot therapy. The work with the B06 anti-antibody is ongoing and I am excited to hear of the outcome. Apart from answering the questions raised in Section 6: *“Will it work for imaging tumor vessels? Did we manage to prolong halftime and retain biological function?”* there are still some that would be nice to answer. I failed to crystalize the Amot coiled-coil domain, with and without the antibody. It would have been a nice way of seeing if the antibody affects the coiled-coil domain structurally. One hypothesis of how the B06 antibody (and most probably angiostatin) exerts its anti-Amot function is that it causes a conformational change, causing a blockade of other binding sites for other partners. Another hypothesis, now made more explainable due to the EEs and BAR-domain mentioned earlier, is the one of internalization and sequestration. *How does the B06 antibody inhibit Amot function?* The answer to this question I believe will help us understand more of Angiomotin signaling and yield more drug targets, all in order to fight off cancer and angiogenesis-associated diseases.

VI. Prevention. However much I would love for anti-angiogenic therapy to be the solution to the global health problem of cancer I cannot help to feel that one of the best solutions to the problem is to not get cancer in the first place. Cancer prevention is therefore an important field of research, where I believe that we, as

angiogenic researchers, may contribute greatly. It has been shown that a lot of both women and men carry *in situ* tumors in breast and prostate without developing cancer [206]. In order to develop into lethal disease these dormant tumors need other stimuli. One such factor can be the deregulation of endogenous angiogenesis inhibitors, caused by genetic mutations, dietary factors or other environmental factors. Personally, I find the notion of being able to halt tumor development by changing my diet, very interesting. However, I will continue dreaming about helping those who already have caught this dreadful disease.

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