The role of CD44v6 in RTK internalization and trafficking

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von

Diplom-Biochemiker Dieter-Augustin Malinger

aus

Temeschburg - Rumänien

Dekan: Prof. Dr. Martin Bastmeyer

Referent: PD Dr. Véronique Orian-Rousseau

Korreferent: Prof. Dr. Reinhard Fischer

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Karlsruhe 14.12.2012

Abstract

Receptor Tyrosine Kinases (RTKs) are cell surface receptors that control vital cellular functions including proliferation, migration and survival. The activation of RTKs is tightly controlled and only transient upon ligand binding under normal physiological conditions. Deregulated RTK signaling plays a causative role in tumor development and metastasis. Receptor internalization is inseparably connected with RTK signaling and required for the activation of endosome-specific signaling pathways and the correct distribution of signaling molecules for complex cellular responses such as cell migration. Several cell adhesion molecules have been shown to be important at different steps of activation, signaling and internalization of RTKs. Such a cell adhesion molecule is CD44v6 which has been identified as a marker for metastasis in a pancreatic cancer cell line and is able to confer metastatic behavior upon transfection into non-metastatic cancer cell lines. CD44v6 acts as a co-receptor for the RTKs Met and VEGFR-2 and is required for their activation and downstream signaling. Met and VEGFR-2 both play pivotal roles in tumor development and metastasis formation.

In my PhD thesis I demonstrate that Met internalization induced by HGF is also strictly dependent on CD44v6. Using immunofluorescence techniques, it was shown that HGF-induced Met internalization was blocked by a peptide mimicking an essential region within CD44v6 or by transfection of a CD44v6 cytoplasmic tail deletion mutant. Importantly, Ras signaling downstream of Met was also required for HGF-induced Met internalization. Furthermore, CD44v6 and Met were shown to traffic together through Rab5-positive endosomes upon HGF-induction, suggesting the possibility that internalized CD44v6 is required for Met signaling from endosomes.

Interestingly, Met and VEGFR-2 were found to be activated with strikingly different kinetics in the same endothelial cell line. This difference could be due to a different mechanism of internalization. Upon VEGF-A_{165}-induction, VEGFR-2 associates both with CD44v6 and another cell adhesion molecule, Nrp-1. Thus, CD44v6 and Nrp-1 might collaborate with each other to modify VEGFR-2 internalization and signaling.

In conclusion, my work shows new aspects on the regulation of RTK signaling by receptor endocytosis mediated by the cell adhesion molecule CD44v6.

Zusammenfassung

Rezeptor-Tyrosin-Kinasen (RTKs) sind Zelloberflächen-Rezeptoren, die lebenswichtige zelluläre Funktionen wie Proliferation, Migration und Überlebens-Signale kontrollieren. Die Aktivierung von RTKs wird streng reguliert und erfolgt unter normalen physiologischen Bedingungen nur transient nach Liganden-Bindung. Unkontrollierte RTK-Aktivierung spielt eine ursächliche Rolle bei der Entstehung von Tumoren und Metastasierungen. Die Rezeptor-Internalisierung ist untrennbar mit der RTK-Signaltransduktion verbunden und notwendig für die Aktivierung von Endosom-spezifischen Signalwegen und die korrekte Verteilung von Signalmolekülen, die wiederum für komplexe zelluläre Reaktionen wie Zellmigration nötig sind. Es wurde gezeigt, dass verschiedene Zelladhäsionsmoleküle eine wichtige Rolle bei der Aktivierung, Signalübertragung und Internalisierung von RTKs spielen. Ein solches Zelladhäsionsmolekül ist CD44v6, das als Marker für Metastasierungen in einer Bauchspeicheldrüsenkrebs-Zelllinie entdeckt wurde. Transfektion von CD44v6 in nicht-metastasierende Krebszelllinien befähigt diese zur Metastasierung. CD44v6 fungiert als Ko-Rezeptor für die RTKs Met und VEGFR-2 und ist nötig für deren Aktivierung und Signaltransduktion. Sowohl Met als auch VEGFR-2 sind entscheidend an der Krebsentstehung und Metastasierung beteiligt.

In meiner Doktorarbeit zeige ich, dass die HGF-induzierte Internalisierung von Met ebenfalls unbedingt von CD44v6 abhängig ist. Unter Anwendung von Immunfluoreszenz-Techniken wurde gezeigt, dass die HGF-induzierte Met-Internalisierung durch ein Peptid, das eine essentielle Region in CD44v6 imitiert, oder durch Transfektion einer CD44v6-Mutante ohne zytoplasmatischen Teil blockiert wurde. Ein wesentlicher Befund war auch dass die Met-vermittelte Aktivierung von Ras ebenfalls für die HGF-induzierte Internalisierung von Met nötig ist. Des Weiteren wurde gezeigt, dass CD44v6 und Met nach HGF-Induktion gemeinsam durch Rab5-positive Endosomen wandern, was die Möglichkeit andeutet, dass internalisiertes CD44v6 für die Met-Signaltransduktion von Endosomen benötigt wird.

Interessanterweise wurden Met und VEGFR-2 in einer endothelialen Zelllinie mit deutlich unterschiedlicher Kinetik aktiviert. Dieser Unterschied könnte durch einen unterschiedlichen Mechanismus der Internalisierung begründet sein. Nach Induktion mit VEGF-A_{165} interagiert VEGFR-2 sowohl mit CD44v6 als auch mit einem weiteren Zelladhäsionsmolekül, Nrp-1. Daher könnten CD44v6 und Nrp-1 zusammenarbeiten, um die Internalisierung und Signaltransduktion von VEGFR-2 zu regulieren.

Die vorliegende Arbeit zeigt neue Aspekte der Regulierung der RTK-Signaltransduktion durch Rezeptor-Internalisierung, die von dem Zelladhäsionsmolekül CD44v6 vermittelt wird.

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Abbreviations

Arp2/3	actin related protein2/3
CAM	cellular adhesion molecule
Cbl	casitas B-lineage lymphoma
CD44	cluster of differentiation 44
CD44s	CD44 standard
CME	clathrin-mediated endocytosis
ECM	extracellular matrix
EGF	epidermal growth factor
EGFP	enhanced green fluorescence protein
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
Erk	extracellular signal-regulated kinase
ERM	ezrin, radixin, moiesin
F-actin	filamentous actin
FGF	fibroblast growth factor
Gab1	Grb2-associated binding protein 1
GEF	guanidine exchange factor
GF	growth factor
Grb2	growth factor receptor bound protein 2
HA	hyaluronic acid
HGF	hepatocyte growth factor
HGFA	hepatocyte growth factor activator
HRS	HGF-regulated tyrosine kinase substrate
HUVEC	human umbilical vein endothelial cell
JNK	Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MBS	Met-binding site
Met	mesenchymal-epithelial transition factor
MMP	matrix metalloproteinase
mRFP	monomeric red fluorescent protein
MVB	multivesicular body
NRP-1	Neuropilin-1
N-WASP	neural Wiskott-Aldrich syndrome protein
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PTP	protein-tyrosine phosphatase
RIN1	Ras and Rab interactor 1
RTK	receptor tyrosine kinase
Shc	Src-homology 2 domain-containing transforming protein
SHP2	Src-homology 2 domain-containing phosphatase 2
STAT	signal transducers and activators of transcription
TGF	transforming growth factor
TIAM-1	T-cell lymphoma invasion and metastasis-inducing protein 1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

1. Introduction

Prologue

Receptor tyrosine kinases (RTKs) control important cellular processes such as proliferation, migration, survival and differentiation. The signaling of RTKs however has to be tightly regulated and aberrant RTK signaling plays a causative role in tumor development and progression in many cancer types. Receptor endocytosis is the most important means of RTK signaling regulation. It has been shown that it is not only a prerequisite for signal termination by receptor degradation in lysosomes. More and more evidence show that receptor internalization has tremendous importance in diversifying signals and specifying the signal output. On endosomes, cell surface receptors are brought together with additional signaling partners that are not available at the plasma membrane. Thus, receptor internalization is required for the activation of endosome-specific signaling pathways. Moreover, receptor internalization and intracellular trafficking is essential for locally restricted signaling that is needed during polarized cellular responses such as directed cell migration. Altogether, the intracellular trafficking route of the internalized receptors determines at the same time the duration of signaling, the association with signaling partners that are specific for different endosomal compartments, and the location where the signals are generated. Cell adhesion molecules that are also present at the cell surface, sense the microenvironment of the cell and modify RTK signaling in order to elicit appropriate cellular responses. They can do so by promoting or inhibiting RTK activation or by recruiting additional signaling partners. Furthermore, cell adhesion molecules can also modify RTK signaling by regulating the internalization and intracellular trafficking route of RTKs as it was shown for the cell adhesion molecule Nrp-1 (Neuropilin-1) and the RTK VEGFR-2. In this case, recruitment of Nrp-1 to VEGFR-2 leads to the trafficking of VEGFR-2 to recycling endosomes instead of lysosomes which is essential for pro-angiogenic signaling and the induction of angiogenesis by endothelial cells (Ballmer-Hofer et al, 2011). Therefore, RTK internalization and signaling are inseparably connected to each other and a better understanding of the mechanism and regulation of internalization is necessary to understand the complex signaling process of RTKs.

1.1 The biology of Met and its ligand HGF

In 1984, the fusion protein TPR-Met was discovered as an oncogene in a chemically transformed human osteogenic sarcoma cell line (Cooper et al, 1984). In TPR-Met, a dimerization domain encoded by TPR (translocated promoter region) is fused to the intracellular kinase domain of Met by chromosomal translocation (Park et al, 1986). The TPR-encoded motif (a leucine zipper motif) leads to constitutive dimerization and subsequently ligand-independent activation of the Met kinase domains by autophosphorylation (Rodrigues & Park, 1993). The corresponding proto-oncogene MET was subsequently identified as a receptor tyrosine kinase (Park et al, 1987), but its ligand was unknown at the time. In the 1980s, scatter factor (SF) was identified as a fibroblast-derived protein that could induce scattering of epithelial cells (Stoker et al, 1987). Independently, the hepatocyte growth factor (HGF) was characterized as a mitogen for hepatocytes (Nakamura et al, 1989), and it turned out that SF and HGF are the same protein (Gherardi & Stoker, 1990; Weidner et al, 1991) and as well the ligand for the receptor tyrosine kinase Met (Bottaro et al, 1991; Naldini et al, 1991). Since their discovery, a large number of reports have demonstrated the essential role of HGF and Met in embryonic development and tissue repair, but also their causative role in cancer development and progression of various cancer types (reviewed in (Birchmeier et al, 2003; Gherardi et al, 2012; Trusolino et al, 2010). Therefore, HGF and Met have become valuable targets for cancer treatment and there is a strong interest in further understanding the complex mechanism of Met signaling and regulation for the development of effective anti-cancer therapies.

1.1.1 The structure of Met

Met is a heterodimer that consists of an extracellular α-chain that is linked by a disulfide bond to a membrane-spanning β-chain composed of an extracellular part, a short transmembrane domain and a cytoplasmic domain that constitutes the complete intracellular part of Met (see Fig1.1). The heterodimer is generated from a single chain precursor by furin-mediated cleavage in the endoplasmic reticulum (Komada et al, 1993). The Met protein harbours several domains. The extracellular part starts with the Sema domain that is formed by the whole α-chain and the N-terminal part of the β-chain. This domain is also found in the semaphorins and plexins that are involved in axon guidance. The Sema domain is followed by

a short PSI domain (plexins, semaphorins and integrins share a similar domain) and finally four IPT domains (immunoglobulin-like fold shared by plexins and transcriptional factors) (Gherardi et al, 2003; Trusolino et al, 2010). A transmembrane helix connects the extracellular part of Met to its cytoplasmic region. The juxtamembrane sequence of the intracellular region contains two phosphorylation sites. Phosphorylation of Ser975 leads to the downregulation of Met kinase activity (Bardelli et al, 1994) whereas phosphorylation at Tyr1003 generates a binding site for the E3 Ubiquitin ligase c-Cbl (Casitas B lineage lymphoma). Binding of c-Cbl and subsequent ubiquitylation of Met results in Met internalization and degradation (Peschard et al, 2001). The intracellular region of Met further contains the Met kinase domain with the catalytic tyrosine residues (Tyr1230, Tyr1234, and Tyr 1235), and finally the unique bidentate docking site of Met comprising Tyr1349 and Tyr1356 (Bottaro et al, 1991; Gandino et al, 1990; Longati et al, 1994; Peschard et al, 2001; Ponzetto et al, 1994).

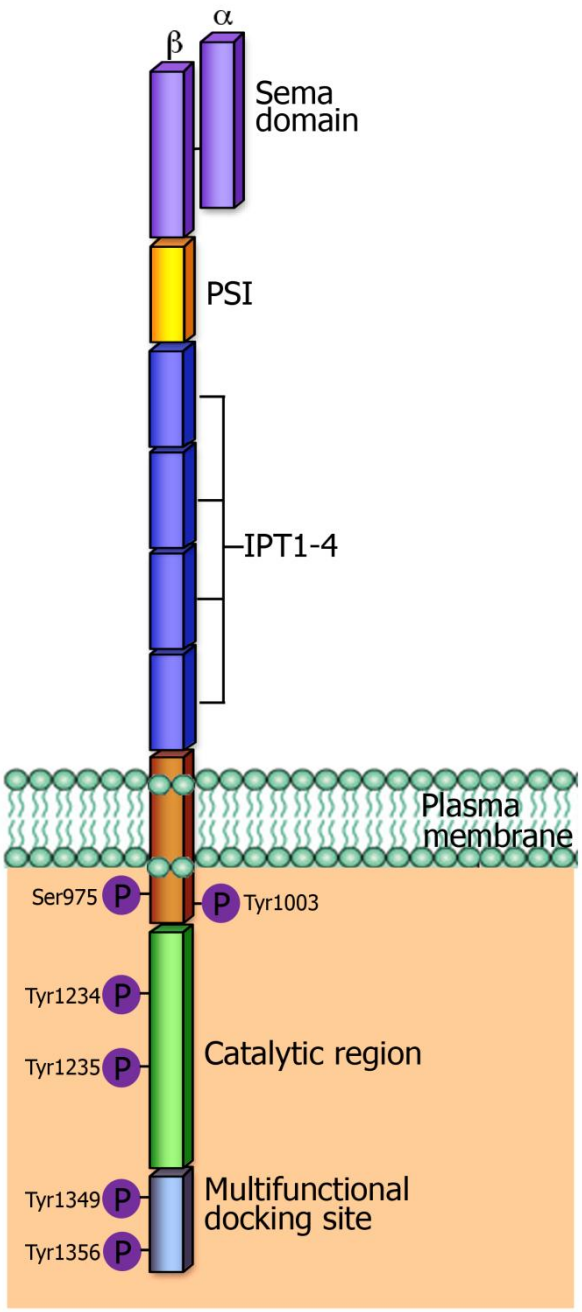

Fig.1.1 The domain structure of Met

1.1.2 The structure of HGF

The hepatocyte growth factor consists of six domains: an N-terminal hairpin loop (N), four kringle domains (K1-K4), and a C-terminal serine proteinase homology domain (SPH)

without enzymatic activity (Donate et al, 1994). HGF is related to the proenzyme plasminogen that is circulating in the blood and dissolves fibrin blood clots following its proteolytic activation to plasmin. Like plasminogen, HGF is also synthesized as a single-chain precursor (pro-HGF) and converted into its active form by proteolytic cleavage. The cleavage of pro-HGF occurs between the K4 and the SPH domain and gives rise to a disulfide-linked heterodimer consisting of an α- and a β-chain (Mizuno et al, 1992; Nakamura et al, 1987; Nakamura et al, 1989). The HGF β-chain contains a low affinity binding site for Met that binds to the Met Sema domain. This low affinity binding site is exposed only after activation of HGF by proteolytic cleavage (Stamos et al, 2004). Additionally, the HGF α-chain contains a high affinity binding site for Met that binds to a region in the IPT3 and IPT4 domains of Met as well in its inactive as in its active form (Basilico et al, 2008). HGF is secreted as an inactive precursor into the interstitial space and sequestered in its inactive form in the extracellular matrix (ECM) of most tissues by binding to heparin-like proteoglycans (Kobayashi et al, 1994; Lyon et al, 1994; Trusolino et al, 2010). Activation of HGF is mediated by several serine proteases such as matriptase, hepsin, and soluble HGF activator (Mizuno et al, 1994; Owen et al, 2010). Proteolytic activation of HGF is a step that also contributes to the regulation of HGF/Met signaling (see section 1.1.4).

1.1.3 The mechanism of Met signal transduction

Binding of HGF to the Met receptor causes receptor dimerization and subsequent autophosphorylation on three tyrosine residues in the kinase domain (Tyr1230, Tyr1234, and Tyr1235). This in turn leads to the phosphorylation of Tyr1349 and Tyr1356 in the multifunctional docking site of Met that then recruits various adaptor proteins and signaling partners (Longati et al, 1994; Ponzetto et al, 1994). This bidentate multifunctional docking site is unique to the Met receptor family (comprising Met, Sea and Ron). If this site is fused to the kinase domain of other RTKs, then these RTKs are able to elicit cellular responses that are specific for Met such as branching morphogenesis of epithelial cells (Ponzetto et al, 1994Sachs, 1996 #659).

The phosphorylated multifunctional docking site binds multiple substrates including PI3K (phosphatidylinositol 3-kinase) (Graziani et al, 1991:Ponzetto, 1994 #408), the protein tyrosine kinase Src (Ponzetto et al, 1994), PLCγ1 (phospholipase Cγ1) (Ponzetto et al, 1994), the adaptor proteins Grb2 (growth factor receptor-bound protein 2) (Ponzetto et al, 1996),

Gab1 (Grb2-associated-binding protein 1) (Weidner et al, 1996), and SHC (Src homology 2 domain-containing transforming protein) (Fixman et al, 1996; Pelicci et al, 1995), as well as the transcription factor STAT3 (signal transducer and activator of transcription 3) (Boccaccio et al, 1998; Zhang et al, 2002) and others (reviewed in (Birchmeier et al, 2003) and (Trusolino et al, 2010)).

A key player in Met signaling is the multi-adaptor protein Gab1 (Grb2-associated-binding protein 1). Gab1 can associate with tyrosine-phosphorylated Met directly through a unique 13 amino acid Met-binding site (MBS) or indirectly through Grb2 (Lock et al, 2000; Schaeper et al, 2000). The unique MBS of Gab1 allows a robust direct interaction between Gab1 and Met and a prolonged Gab1 phosphorylation upon HGF-induction. Such a prolongation cannot be achieved by other receptors such as the EGF-Receptor where Gab1 is only indirectly recruited through Grb2 (Birchmeier et al, 2003; Lock et al, 2000; Maroun et al, 1999). The essential role of Gab1 in Met signaling was shown in genetic studies with knockout mice where deletion of Gab1 resulted in a similar phenotype as does the deletion of Met or HGF (Sachs et al, 2000). Interestingly, both direct interaction of Gab1 with Met and indirect interaction through Grb2 are required for liver and placenta formation. In contrast, only one of these interactions, but not both, is required for limb muscle development (Schaeper et al, 2007). Thus, it is important in which manner Gab1 is recruited to the Met receptor, probably because this results in the formation of distinct signaling complexes.

Upon its phosphorylation by Met, Gab1 serves as an additional docking platform and recruits further signal transducers amongst which are SHC, PI3K, the protein tyrosine phosphatase SHP2 (Src homology 2 domain-containing phosphatase 2) (Schaeper et al, 2000), the adaptor protein CRK (CT10 regulator of kinase) (Garcia-Guzman et al, 1999), PLCγ1 (Gual et al, 2000), and the Ras-GAP (GTPase activating protein) p120 (reviewed in (Trusolino et al, 2010)).

Activation of Met leads to the activation of several downstream signaling pathways. These include the three MAPK (mitogen-activated protein kinase) subfamilies Erk (extracellular signal-regulated kinase), JNK (Jun N-terminal kinase), and p38 (Campbell et al, 1998; Recio & Merlino, 2002; Rodrigues et al, 1997), the PI3K-Akt pathway (Ponzetto et al, 1994; Royal et al, 1997), the STAT pathway (Boccaccio et al, 1998; Zhang et al, 2002), and NFκB (Fan et al, 2005; Muller et al, 2002). All these pathways control different cellular functions such as survival, proliferation, differentiation and migration and act in concert via cross-talk with each other to mediate the complex cellular processes described in section 1.1.5.

1.1.4 Regulation of HGF/Met signaling

HGF/Met signaling is regulated and modified at multiple levels. This regulation starts already before activation of Met by its ligand HGF. The level of HGF is regulated both on the transcriptional level as well as by proteolytic activation of the HGF precursor. As mentioned in chapter 1.1.2, HGF is produced as an inactive precursor and sequestered in the ECM. Proteolytic activation of HGF can then be mediated by one of three serine proteases which are the soluble HGF activator (HGFA) and the membrane-bound serine proteases matriptase and hepsin (Mars et al, 1993; Mizuno et al, 1992; Mizuno et al, 1994; Owen et al, 2010). HGFA itself is activated by thrombin which connects HGF/Met signaling to the coagulation cascade (Shimomura et al, 1993). Activation of HGF is further fine tuned by HGF activator inhibitors (Kawaguchi et al, 1997; Shimomura et al, 1997).

The levels of Met at the cell surface are determined by the shedding of the extracellular part of Met by ADAM (a disintegrin and metalloprotease) family members (Foveau et al, 2009; Schelter et al, 2010) as well as by ubiquitylation and subsequent internalization and degradation of the Met receptor (Hammond et al, 2001; Jeffers et al, 1997; Peschard et al, 2001).

Furthermore, Met signaling can be attenuated or modified by controlling the phosphorylation state of Met or its signaling partners by several protein-tyrosine phosphatases (PTPs). PTP1B and TCPTP (T cell phosphatase) for instance dephosphorylate the catalytic tyrosines of Met (Sangwan et al, 2008), whereas DEP1 dephosphorylates the tyrosine residues in the bidentate docking site of Met (Palka et al, 2003). The PTP SHP2 is a crucial signaling modifier of Met as Gab1 mutant mice where Gab1 is unable to bind SHP2 phenocopy the Met knockout mice (see section 1.1.5) (Schaeper et al, 2007). One important function of SHP2 is the dephosphorylation of the binding site for p120 Ras-GAP on Gab1 which inhibits the recruitment of p120 to Gab1 (Maroun et al, 2000). The p120 Ras-GAP enhances the intrinsic GTP-ase activity of Ras thereby promoting the conversion of active GTP-bound Ras into the inactive GDP-bound form. Inhibiting the recruitment of p120 Ras-GAP to Gab1 causes sustained Ras signaling which is required for HGF-induced epithelial morphogenesis (Maroun et al, 2000).

The complexity of Met signaling is even increased by the interaction of Met with several cell surface proteins (Bertotti & Comoglio, 2003; Comoglio et al, 2003). The α6β4 integrin for instance has been shown to associate with Met in carcinoma cells to intensify Met signaling. Upon HGF-induced Met activation, the β4-subunit becomes phosphorylated at

three tyrosine residues and recruits SHC, PI3K, and SHP2 to promote downstream signaling and invasive growth (Bertotti et al, 2005; Bertotti et al, 2006; Trusolino et al, 2001). The expression of α6β4 integrin expression correlates with carcinoma tumor progression and invasion (Rabinovitz & Mercurio, 1996) and the collaboration between β4 integrin and Met is involved in cell transformation (Bertotti et al, 2005).

Another interesting example of transmembrane proteins that regulate Met signaling are plexins. Plexins are the receptors of the semaphorins and are mainly involved in axonal guidance. Plexins were found to be widely expressed, but their function outside the nervous system and the mechanism of plexin-mediated signaling are not well understood. Plexins need to collaborate with RTKs to mediate downstream signaling (Conrotto et al, 2005; Swiercz et al, 2004:Giordano, 2002 #626). The semaphorin 4D (Sema 4D) receptor plexin B1 can activate Met in epithelial cells in response to Sema 4D, independently of HGF (Giordano et al, 2002). In cells expressing both Met and plexin B1, the plexin B1 ligand Sema4D leads to phosphorylation of both plexin B1 and Met and induces cellular responses similar to HGF. Cells that do not express Met cannot be induced with Sema 4D unless Met is ectopically expressed (Giordano et al, 2002). In endothelial cells, Sema 4D induces angiogenesis and this is also dependent on the collaboration of plexin B1 with Met (Conrotto et al, 2005).

The best characterized regulator of Met signaling however is CD44v6, an isoform of the CD44 family of cell adhesion molecules that will be described in chapter 1.4. The function of CD44v6 for Met signaling is twofold. The extracellular part of CD44v6 is required for the binding of HGF to the Met receptor and subsequent phosphorylation of Met. The cytoplasmic domain of CD44v6 binds to ERM (ezrin, radixin, moiesin) proteins that link the receptor complex to the actin cytoskeleton that is essential for downstream signaling from the Met receptor (Orian-Rousseau et al, 2002; Orian-Rousseau et al, 2007).

1.1.5 The role of Met and HGF in development and organ regeneration

Activation of Met by HGF induces several responses such as proliferation, migration, and survival in various cell types (reviewed in (Birchmeier et al, 2003)). A unique feature of HGF is the induction of scattering and invasive growth of epithelial cells (hence the alternative name scatter factor) which comprises epithelial to mesenchymal transition (EMT) and increased cell motility (Bardelli & Comoglio, 1997; Stoker et al, 1987). When epithelial cells grown on a collagen matrix are induced with HGF, they respond with migration and

invasion into the matrix (Weidner et al, 1990). A combination of all cellular responses induced by HGF is required for tube formation. This complex response of epithelial and endothelial cells elicited by HGF requires all known signaling pathways activated by Met (Birchmeier et al, 2003; Rosario & Birchmeier, 2003). Met-induced epithelial morphogenesis and tube formation is required during the development of several organs including liver, kidney, lung, and pancreas (Rosario & Birchmeier, 2003).

Genetic studies revealed the essential role of Met and HGF in embryonic development. Met or HGF knockout mice are embryonic lethal and die due to defects in placental development (Bladt et al, 1995; Uehara et al, 1995) and have a significantly reduced liver size (Schmidt et al, 1995). Furthermore, myogenic progenitor cells are not able to undergo EMT in these mice and cannot delaminate from the epithelial dermomyotome to migrate to the limb bunds. Therefore, Met or HGF knockout embryos cannot develop muscles in the limbs, tongue and diaphragm (Bladt et al, 1995; Uehara et al, 1995).

HGF/Met signaling also plays a central role during organ repair in the adult. It has been demonstrated that after liver damage, HGF levels are increased and Met is activated resulting in proliferation of hepatocytes and liver regeneration (Michalopoulos & DeFrances, 1997). An upregulation of HGF following injury has also been observed in other organs such as the kidney, lung, skeletal muscle, and the heart (reviewed in (Gherardi et al, 2012)).

Since Met or HGF total knockout mice die during embryonic development, conditional knockout mice were generated to study the role of HGF/Met signaling in specific tissues in the adult. Crossing of $Met^{fl/fl}$ transgenic mice with mice carrying the Mx-Cre transgene allows to specifically remove Met in single organs at a specific time. Importantly, when Met is specifically removed in the liver of adult mice, liver regeneration is impaired after injury by hepatoxic chemicals or hepatectomy, showing the essential role of Met in liver repair (Borowiak et al, 2004; Huh et al, 2004). An essential role of Met has also been demonstrated in wound repair in the skin (Chmielowiec et al, 2007).

1.1.6 The role of Met and HGF in cancer

Several steps in tumor progression and metastasis resemble the HGF/Met-mediated processes in embryogenesis and organ regeneration such as proliferation, epithelial to mesenchymal transition (EMT), delamination from the primary tumor, migration and invasion. Indeed, the assumption that HGF/Met signaling is hijacked by cancer cells to

acquire an invasive phenotype has been supported by many experimental and clinical studies and is now well established (reviewed in (Birchmeier et al, 2003; Gherardi et al, 2012; Trusolino et al, 2010).

Met and/or HGF are expressed in carcinomas and various other types of human tumors and overexpression of HGF and/or Met is correlated with poor prognosis (Birchmeier et al, 2003). Activating point mutations of Met were found in sporadic and inherited human renal papillary carcinomas (Danilkovitch-Miagkova & Zbar, 2002; Schmidt et al, 1997), hepatocellular carcinomas and other human solid cancers (Ma et al, 2008). Activating mutations of Tyr1230 and Tyr1235 in the kinase domain of Met were found to be clonally selected in the metastases of human head and neck cancers as the transcripts of the mutant alleles were highly expressed in the metastases, but barely detectable in the primary tumors (Di Renzo et al, 2000). This supports the notion that amplified Met signaling promotes tumor progression and metastasis.

Further support comes from experimental approaches demonstrating that human and mouse cell lines that ectopically overexpress HGF and/or Met become invasive and form metastases in athymic nude mice (Rong et al, 1994). Accordingly, downregulation of HGF or Met in human tumor cells attenuates their tumorigenicity (Abounader et al, 2002). Finally, mice that overexpress HGF or Met as a transgene develop several types of tumors and metastases (Takayama et al, 1997).

Not only activating mutations in the kinase domain of Met or overexpression of Met and/or HGF are tumorigenic, but also Met mutations that prevent efficient internalization and degradation of Met such as mutations in the c-Cbl-binding site of Met (Peschard et al, 2001).

1.2 The biology of VEGF and its receptors

Vascular endothelial growth factors (VEGFs) and vascular endothelial growth factor receptors (VEGFRs) regulate the development of the cardiovascular system, the lymphatic system, and the formation of new vessels from pre-existing ones (angiogenesis and lymphangiogenesis). In mammals, the VEGF family comprises five members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF (placenta growth factor)). In addition, there are structurally related proteins from the parapoxvirus (VEGF-E) and snake venom (VEGF-F). They bind in an overlapping manner to three different VEGFRs (VEGFR-1, VEGFR-2, and VEGFR-3) that are all RTKs and are primarily expressed on endothelial cells and cells of the

hematopoietic system (reviewed in (Olsson et al, 2006)). VEGFR-1 is expressed on hematopoietic stem cells, monocytes and macrophages and is required for their migration (Holmes et al, 2007). VEGFR-1 is also expressed on vascular endothelial cells and was proposed to act as a decoy receptor for VEGF-A, thereby decreasing VEGFR-2 signaling (Park et al, 1994). VEGFR-2 is expressed on vascular and lymphatic endothelial cells and is the major mediator of VEGF-A signals on endothelial cells. Finally, VEGFR-3 is predominantly expressed on lymphatic endothelial cells. It can only bind to VEGF-C and VEGF-D and is important for the regulation of lymphangiogenesis (Holmes et al, 2007; Olsson et al, 2006).

1.2.1 The ligands of VEGFR-2

VEGFR-2 can bind to VEGF-A, -C, -D, -E, and –F. The principal activator of angiogenesis is VEGF-A. VEGF-A exists in multiple isoforms that are generated by alternative mRNA-splicing of exons 6 and 7 of the 8 exons in the VEGF-A gene (Tischer et al, 1991). Exons 6 and 7 code for the heparin-binding region of VEGF-A. The resulting VEGF-A isoforms differ in their length and are named after the number of amino acids they contain. So far the isoforms VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{148}, VEGF-A_{165}, VEGF-A_{183}, VEGF-A_{189}, and VEGF-A_{206} have been identified (Harper & Bates, 2008). The different VEGF-A isoforms differ in their heparin-binding affinity. This determines their sequestration in the ECM or on cell surfaces, probably by binding heparin-containing proteoglycans. The shortest isoform, VEGF-A_{121}, does not bind to heparin and is freely diffusible. The largest isoforms VEGF-A_{189} and VEGF-A_{206} bind heparin with high affinity and are completely sequestered in the ECM (Houck et al, 1992). The predominant VEGF-A isoform is VEGF-A_{165}, an isoform that has intermediate heparin binding affinity. Thus, about 50-70% of the secreted VEGF-A_{165} remains associated to the ECM or cell surfaces (Park et al, 1993). The ECM-bound VEGF-A isoforms can be released upon plasmin-mediated cleavage at the C-terminus which generates bioactive and diffusible fragments (Houck et al, 1992).

In 2002, an additional isoform of VEGF-A_{165} was identified that is expressed in normal renal tissue, but downregulated in renal cell carcinoma (Bates et al, 2002). This isoform was termed VEGF-A_{165}b and differs in the sequence of the C-terminal six amino acids from the previously known VEGF-A_{165} isoform (now also called VEGF-A_{165}a). VEGF-A_{165}b was found to be an anti-angiogenic splice variant of VEGF-A_{165} since conditioned medium

containing this isoform significantly and dose dependently inhibited VEGF-A_{165}(a)-mediated proliferation and migration of endothelial cells (Bates et al, 2002). VEGF-A_{165}b is generated by selection of a distal splice site (DSS) in exon 8, whereas selection of the proximal splice site (PSS) leads to the generation of VEGF-A165(a). Remarkably, DSS selection leads to the disruption of the Nrp-1-binding site in VEGF-A_{165}. The role of the co-receptor Nrp-1 will be described in the next section (1.2.2). Anti-angiogenic analogues have also been identified for other VEGF-A isoforms and are termed VEGF-A_{xxx}b (reviewed in (Harper & Bates, 2008)).

1.2.2 VEGFR-2 signal transduction

VEGFR-2 is the principal mediator of the physiological and pathological effects of VEGF-A. Upon ligand-binding, VEGFR-2 undergoes dimerization and autophosphorylation on several tyrosine residues (reviewed in (Cross et al, 2003)). The most important phosphorylation site required for the VEGF-A induced physiologic effects is Tyr1175. Knock-in experiments in mice showed that mutation of this phosphorylation site (in mice Tyr1173) results in embryonic lethality between day 8.5 and 9.5 with similar defects to those observed in the VEGFR-2 knockout mice (see section 1.2.3) (Sakurai et al, 2005). Phosphorylation of Tyr1175 in VEGFR-2 leads to the recruitment of several adaptor proteins and signal transducers, including PLC-γ, Sck (Shc-like protein), and Shb (Src homology 2 domain containing adaptor protein B) and the activation of various signaling pathways such as Erk and p38 MAP Kinases and Akt (Holmes et al, 2007). Interestingly, the classical Grb2-Sos-Ras pathway is not activated by VEGFR-2, in contrast to most other RTKs including Met. Erk phosphorylation upon VEGFR-2 activation occurs instead in a Ras-independent manner via PLC-γ and PKC (Takahashi et al, 1999).

Importantly, VEGFR-2 signaling is strictly dependent on the collaboration with cell adhesion molecules as co-receptors. Nrp-1 for instance is essential for the development of the vascular system. Nrp-1 knockout mice displayed insufficient and disorganized vascularization and were embryonic lethal (Kawasaki et al, 1999). Nrp-1 was reported to enhance the binding of VEGF-A_{165} to VEGFR-2 and VEGF-A_{165}-mediated chemotaxis (Soker et al, 1998). Indeed, the anti-angiogenic VEGF-A isoform VEGF-A_{165}b which is unable to bind Nrp-1 induces only partial phosphorylation followed by rapid inactivation of VEGFR-2 (Kawamura et al, 2008). The cell adhesion molecule CD44v6 was shown to be essential for VEGFR-2 activation and downstream signaling in response to VEGF-A_{165} and VEGF-A_{121} (Tremmel et

al, 2009). For further information about the role of cell adhesion molecules in VEGFR-2 see section 1.4.5 and 1.5.4.

1.2.3 The role of VEGFR-2 signaling in health and disease

VEGFR-2 mediates VEGF-A induced proliferation, migration, survival, and permeability of endothelial cells and is essential for the development of the vascular system (reviewed in (Holmes et al, 2007)). VEGFR-2 knockout mice die *in utero* between day 8.5 and 9.5 due to a lack of vasculogenesis and the inability to develop yolk-sac blood-islands and organized blood vessels (Shalaby et al, 1995). In the adult, VEGFR-2 signaling is required for vascular homeostasis (Lee et al, 2007) and angiogenesis during wound healing and the female reproductive cycle (Hoeben et al, 2004).

However VEGFR-2 is also involved in pathological processes. Amplified VEGFR-2 signaling and uncontrolled neovascularization is associated with several diseases such as neovascular glaucoma, diabetic retinopathy and age-related macular degeneration that can lead to blindness due to uncontrolled vessel growth in the eye (Ferrara et al, 2003). The most important pathological function of VEGFR-2 signaling however is tumor angiogenesis. Tumor growth and progression is dependent on tumor vascularization for the supply with oxygen and nutrients. Indeed, the expression of pro-angiogenic VEGF-A isoforms was found to be increased in various cancer types (Harper & Bates, 2008).

1.3 Cell adhesion molecules function as co-receptors for RTKs

Receptor tyrosine kinases (RTKs) present at the cell surface are activated upon binding their ligands which leads to the activation of distinct sets of downstream signaling pathways. RTK signaling however is not only determined by the presence or absence of the respective ligand. Cell adhesion molecules which are also present at the cell surface act as co-receptors and modify the signals to adjust the cellular responses to their microenvironment. They do so in very different ways that is by recruiting additional signaling partners to the RTKs, amplifying or attenuating signaling or inducing or preventing RTK internalization (reviewed in (Orian-Rousseau & Ponta, 2008)). Members of several families of cell adhesion molecules have been shown to function as co-receptors for RTKs, for instance integrins, cadherins,

neuropilins, syndecans, plexins, and CD44 isoforms. One member of the CD44 family, CD44v6, has been shown to be of particular importance for Met and VEGFR-2 signaling.

1.4 The CD44 family of cell adhesion molecules

The CD44 family comprises a large polymorphic group of transmembrane glycoproteins. Different CD44 isoforms are expressed in a cell type specific manner on all vertebrate cells. CD44 was first discovered as an antigen recognized by a monoclonal antibody raised against human white blood cells (Dalchau et al, 1980). Later, various isoforms of CD44 with molecular weights ranging from 80 to 200kD were found on many other cell types. CD44 was independently identified by several groups and given different names depending on the context in which it was identified. Therefore, CD44 is also known as Pgp-1 (phagocyte glycoprotein-1) (Hughes et al, 1983), ECMR II (extracellular matrix receptor II) (Wayner et al, 1988), Hermes (Goldstein et al, 1989), HUTCH-1 (Gallatin et al, 1989), and HCAM (Goldstein & Butcher, 1990). All CD44 isoforms are encoded on a single, highly conserved gene and the huge variety of the proteins is generated by alternative splicing and post-translational modifications (Screaton et al, 1992:Naor, 2002 #718).

1.4.1 CD44 gene structure and alternative splicing

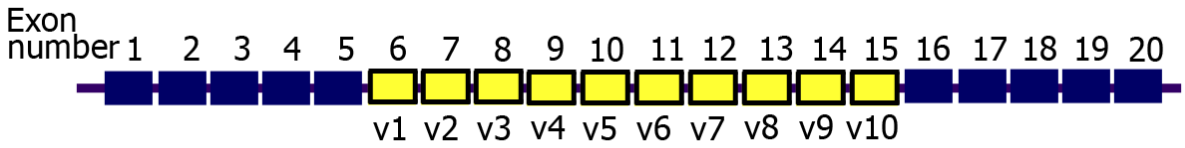

Fig.1.2 The gene structure of CD44

The CD44 gene consists of 20 exons. Ten of theses exons are variant exons that can be included by alternative splicing (exons 6-15, also known as v1-v10).

The CD44 gene encodes 20 exons. Exons 1-5 and 16-20 are the constant exons that are found in all CD44 isoforms. Exon 1-5 and 16-17 encode the constant part of the CD44 extracellular region, exon 18 a hydrophobic single-pass transmembrane domain, and exons 19

and 20 code for the cytoplasmic region. The other 10 exons (exons 6-15) can be excluded or included in various combinations by alternative splicing of the CD44 pre-mRNA (Screaton et al, 1993; Screaton et al, 1992; Tolg et al, 1993). Exons 6-15 are also called variant exons v1-v10. Exons 19 and 20 are also alternatively spliced (Goldstein & Butcher, 1990). Most CD44 isoforms contain exon 20 coding for a 73 amino acid cytoplasmic domain. Inclusion of exon 19 generates a very short cytoplasmic domain of only 5 amino acids (Goldstein et al, 1989). The physiological relevance of this shorter isoform is not yet known. In humans, the variant exon v1 contains a stop codon and is therefore never included (Screaton et al, 1993; Tolg et al, 1993).

1.4.2 CD44 protein structure

The smallest isoform of CD44, CD44 standard (CD44s), does not contain any variant exon and is ubiquitously expressed in vertebrates. It has an apparent molecular weight of approximately 85kD and consists of a 270 amino acid extracellular part (ectodomain), a 23 amino acid transmembrane domain, and a 73 amino acid cytoplasmic tail (Brown et al, 1991; Stamenkovic et al, 1989). The huge variety of CD44 is created by insertion of variant exons in different combinations into the extracellular part. In humans, the largest isoform including all variant exons (CD44v2-v10) was found in keratinocytes (Bloor et al, 2001).

The CD44 ectodomain

The extracellular part of CD44 consists of an N-terminal globular domain, a variable region, and a stem region. The N-terminal globular domain is encoded by exons 1-5 and contains the so-called link domain (exons 2 and 3) which is responsible for the binding of hyaluronic acid (HA), a major component of the ECM (Ponta et al, 2003; Thorne et al, 2004). The link domain is a stretch of 90 amino acids with high homology to the cartilage link protein and the proteoglycan core protein (Deak et al, 1986; Neame et al, 1986; Ponta et al, 2003). Two disulfide bonds formed by four highly conserved cysteine residues are crucial for the correct conformation of the link domain (Day & Sheehan, 2001). Two further cysteine residues flanking the link domain on either side mediate the correct folding of the N-terminal globular domain and are also essential for HA binding (Banerji et al, 1998). In the smallest isoform, CD44s, the N-terminal globular domain is followed by a short 46 amino acid stem region encoded by exons 16 and 17 that connects the ectodomain to the transmembrane

domain. The stem region contains proteolytic cleavage sites for metalloproteases and serine proteases (Bazil & Strominger, 1994; Okamoto et al, 1999) which can be used for the shedding of the CD44 ectodomain (Katoh et al, 1994). The subsequent intramembranous cleavage of the remaining membrane-bound C-terminal fragment of CD44 by γ-secretase gives rise to a CD44 intracellular domain (ICD) fragment that translocates to the nucleus and stimulates transcription (Okamoto et al, 2001).

The stem region can be enlarged by insertion of the variant-exon-encoded sequences. In humans up to 381 additional amino acids can be inserted (CD44v2-v10) and in mice up to 423 (CD44v1-v10) (Ponta et al, 2003; Screaton et al, 1992).

Further heterogeneity can be generated by posttranslational modifications of the ectodomain. These modifications are cell type specific and can modify the function of CD44 (English et al, 1998). The ectodomain contains several sites for N-linked and O-linked glycosylations and glycosaminoglycan modifications both in the variant and the constant sequences (Brown et al, 1991; Greenfield et al, 1999; Sleeman et al, 1997). Importantly, the variant exon v3 contains the only heparan sulphate addition site which enables v3-containing CD44 isoforms to bind heparan-sulphate-dependent growth factors (Bennett et al, 1995; Sherman et al, 1998; Yu et al, 2002).

The transmembrane domain

The single-pass transmembrane region consists of 23 hydrophobic amino acids and a cysteine residue which participates in clustering of CD44, thereby promoting the binding of HA (Liu & Sy, 1996). Posttranslational modification of the transmembrane domain by palmitoylation enhances the interaction of the CD44 cytoplasmic domain with ankyrin which has been implicated in hyaluronan-dependent cell adhesion and migration (Bourguignon et al, 1991; Lokeshwar et al, 1994).

The cytoplasmic domain

The cytoplasmic domain of CD44 mediates interactions with several intracellular proteins with important functions in cytoskeletal organization and signaling. The first one that was discovered was ankyrin which links CD44 to the cytoskeletal component spectrin and is involved in HA-induced cell adhesion and motility (Lokeshwar & Bourguignon, 1992; Lokeshwar et al, 1994). The cytoplasmic domain of CD44 also binds to ERM proteins (ezrin, radixin, moesin) with a basic amino acid sequence that is located between the transmembrane domain and the ankyrin-binding motif (Legg & Isacke, 1998; Yonemura et al, 1998). ERM

proteins connect CD44 (and other proteins) to the actin cytoskeleton through their C-terminus and are thought to play an important role in cell migration (Turunen et al, 1994). The tumor suppressor protein merlin is related to the ERM proteins and also binds to the ERM-binding motif of CD44. Merlin however does not link CD44 to actin as it lacks an actin-binding site. Merlin mediates contact inhibition of cell growth at high cell density by binding to CD44 (Morrison et al, 2001).

Furthermore it was shown in a human ovarian tumor cell line that upon HA-binding, CD44 associates with the actin nucleation factors N-WASP (neural Wiskott-Aldrich syndrome protein) and Arp2/3 (actin-related protein 2/3) which leads to actin polymerization and migration (Bourguignon et al, 2007).

The cytoplasmic domain of CD44 contains several highly conserved serine residues that can be phosphorylated and regulate the binding of CD44 to ERM proteins, merlin, and ankyrin. In resting cells, Ser325 in the cytoplasmic domain of CD44 is constitutively phosphorylated. HA-induced activation of PKC (protein kinase C) causes dephosphorylation of Ser325 and phosphorylation of Ser291 and results in increased binding of ezrin (Legg et al, 2002). This PKC-mediated switch of Ser-phosphorylation is essential for directional cell motility.

Ankyrin binding is also regulated by phosphorylation of the CD44 cytoplasmic domain. Rho kinase (ROK) activation by CD44-bound RhoA results in phosphorylation of the CD44 cytoplasmic domain promoting ankyrin binding to CD44 and tumor cell migration and metastasis formation in a breast cancer cell line (Bourguignon et al, 1999).

1.4.3 Physiological functions of CD44

The smallest isoform of CD44, CD44s, is ubiquitously expressed in vertebrate cells during development and in the adult (reviewed in (Naor et al, 1997)). The variant CD44 isoforms are only expressed in a few cell types, predominantly in proliferating cells such as activated immune cells and proliferating epithelial cells (Arch et al, 1992; Lesley et al, 1993; Stamenkovic et al, 1991). Interference studies with antibodies raised against CD44s (recognizing all isoforms) or CD44 variants revealed that CD44 participates in several cellular responses such as proliferation, migration and adhesion. CD44 is involved in various physiological and pathological processes such as embryonic development (Sherman et al, 1996), haematopoiesis (Miyake et al, 1990), lymphocyte homing (O'Neill, 1989; Shimizu &

Shaw, 1991)}, lymphocyte activation (Arch et al, 1992), inflammation (Mikecz et al, 1999; Wittig et al, 2000), and tumorigenesis (Orian-Rousseau, 2010).

Role of CD44 in embryonic development

CD44 has important functions during embryonic development. The isoform CD44v3-v10 is expressed in the apical ectodermal ridge and is involved in limb development. Through the heparan sulfated variant exon v3 it binds FGF-4 (fibroblast growth factor 4) and FGF-8 and presents the growth factors to the receptors on the underlying mesenchymal cells to induce proliferation and limb bud outgrowth (Sherman et al, 1998).

CD44 is also required for T cell migration and maturation. The migration of foetal liver cells to the thymus and subsequent development to mature T cells depends on CD44 and can be blocked with a CD44 antibody (Kawakami et al, 1999).

Application of an antibody recognizing all CD44 isoforms in pregnant rats caused delay in birth and enhanced rates of intrauterine abortions. An antibody directed only against CD44v6 hampered embryonic development until day 16-18 of gestation (Zoller et al, 1997).

CD44 is also highly expressed in the heart and the somites during embryonic development suggesting a role in organogenesis (Wheatley et al, 1993).

Despite the multiple functions of CD44 during embryonic development, CD44 total knockout mice are viable and show only a mild phenotype mainly in the development of the hematopoietic system. CD44 knockout mice in which the expression of all isoforms was abolished by deletion of the exons coding for the constant N-terminal region of CD44 had an increased number of myeloid progenitor cells in the bone marrow whereas the number was decreased in the spleen and the peripheral blood (Schmits et al, 1997), suggesting an impaired migration of these cells from the bone marrow. In another study, impaired lymphocyte homing to the thymus was observed in CD44-deficient mice (Protin et al, 1999).

In contrast to the total CD44 knockout, a more severe phenotype was observed in conditional CD44 knockout mice in which CD44 expression was only abrogated in keratinocytes of the skin by expression of CD44 antisense cDNA under the control of the keratin-5 promoter which is active since stage E11.5 of embryonic development. These mice displayed an abnormal thick skin, decreased skin elasticity, and an accumulation of HA in the skin. Furthermore, keratinocytes were unable to proliferate and wound healing was delayed as compared to wild type mice (Kaya et al, 1997). The drastic differences between the total knockout and the conditional knockout imply that the loss of CD44 expression at different stages of embryonic development leads to a different outcome. Several studies suggest that

loss of CD44 at early stages of embryonic development can be compensated by other proteins, whereas this is not possible at later stages. One such study showed that during limb bud development in wild type mice, mesenchymal cells require CD44 and FGF for proliferation. On the contrary, only FGF alone was required for the proliferation of mesenchymal cells in CD44 total knockout mice (Ponta et al, 2003). Thus, a substitute for CD44 should be present in the CD44 total knockout mice. Another study showed that the function of CD44v6 as a co-receptor for Met can be taken over by ICAM-1 (intercellular adhesion molecule 1) in the human hepatocellular carcinoma cell line HepG2 that does not express CD44v6. In these cells, Met can be activated by HGF in the absence of CD44v6 and Met activation can be blocked by antibodies against ICAM-1 (Olaku, 2008). Furthermore, CD44v6-specific antibodies could abrogate Met activation and Met-mediated liver cell proliferation after partial hepatectomy in wild-type mice, whereas Met activation and liver cell proliferation in CD44 knockout mice was blocked with antibodies against ICAM-1 (Olaku et al, 2011).

1.4.4 Role of CD44 in tumorigenesis

Expression of CD44 variants is found in several cancer types and has been associated to tumor initiation, progression, metastasis formation, tumor recurrence, and chemo- and radiotherapy resistance (Bourguignon, 2012; Ohashi et al, 2007; Seiter et al, 1993; Takeuchi et al, 1995; Tanabe & Saya, 1994; Yae et al, 2012).

A causative role of the CD44 variant CD44v4-v7 has been demonstrated in rat pancreatic cancer cells (Gunthert et al, 1991). It was shown that CD44v4-v7 was expressed in the metastasizing pancreatic carcinoma cell line BSp73ASML, but not in the non-metastasizing rat pancreatic carcinoma cell line BSp73AS or in normal tissue. Introduction of CD44v4-v7 into the non-metastasizing cancer cell line was sufficient to establish the full metastatic behavior. Moreover, metastasis formation of the metastatic cell line BSp73ASML and the transfected cells could be blocked by monoclonal antibodies recognizing the CD44 variant exon v6 (Seiter et al, 1993).

Recently, it has been demonstrated in an *in vivo* study that CD44 variant expression enhances lung metastasis formation of breast cancer cells. Orthotopic transplantation of CD44 variant isoform-expressing 4T1 breast cancer cells caused lung metastasis formation in mice. In contrast, 4T1 breast cancer cells lacking CD44 variant isoforms did not form lung metastases (Yae et al, 2012).

In a clinical study, it was shown that CD44v3, CD44v5, CD44v6, and CD44v7 were expressed in 90% of non-small cell lung carcinomas (NSCLCs) whereas there was no expression or only low expression of these variants in normal lung tissues. Moreover, high expression of the isoform CD44v6 correlated with lymph node metastasis formation (Miyoshi et al, 1997).

Expression of CD44 variant isoforms including v6 has been correlated with poor prognosis in various cancer types such as colorectal carcinoma, cervical cancer, non-Hodgkin´s lymphoma, vulvar cancer, acute myeloid leukemia, and non-small cell lung carcinoma (Hirata et al, 1998; Kainz et al, 1995; Legras et al, 1998; Naor et al, 1997; Ristamaki et al, 1997; Sleeman et al, 1996; Tempfer et al, 1998; Wielenga et al, 1998).

Not only CD44 variants have been associated with tumor progression, but also CD44s was shown to promote tumor development in some cancer types. Overexpression of CD44s in a human B cell lymphoma cell line increased tumor growth and metastasis formation (Sy et al, 1991). Furthermore, high expression of CD44s is associated with poor prognosis in human non-Hodgkin´s lymphoma (Pals et al, 1997).

While high expression of different CD44 isoforms is correlated with tumor progression and metastasis in several cancer types, this is not the case for all cancer types. In oral squamous cell carcinomas for instance, a low expression of CD44v6 correlates with lymph node metastasis formation (Kunishi et al, 1997) and overexpression of CD44v8-v10 in human B cell lymphoma cells impairs tumor formation (Sy et al, 1991; Sy et al, 1992).

1.4.5 Molecular mechanisms of CD44 function

The huge heterogeneity of CD44 that is generated by alternative splicing and post-translational modifications enables the CD44 proteins to take over very diverse functions and to participate in multiple physiological processes.

CD44 is a receptor for ECM components

CD44 binds several components of the ECM such as collagen, laminin, and fibronectin (Borland et al, 1998; Naor et al, 1997). The most prominent ligand for CD44 however is HA (Aruffo et al, 1990). HA is a high molecular-weight linear polysaccharide consisting of alternating repeats of D-glucuronic acid and N-acetyl-D-glucosamine. HA is ubiquitously expressed in the extracellular space with the highest concentration in soft connective tissues (Laurent & Fraser, 1992). HA-synthase-2 gene deletion in mice results in severe

cardiovascular defects and death at stage E9.5 of embryonic development. Furthermore, numerous defects were observed in the yolk sac, vasculature, and the structure of the ECM as well as defects in EMT and cell migration (Camenisch et al, 2000).

CD44 binds HA molecules with a length of at least 6 sugar residues (Underhill et al, 1983). A single HA molecule can contain up to 25,000 disaccharide units and cause clustering of CD44 (Yang et al, 2012). Binding of HA occurs in the link domain of the extracellular part of CD44 (He et al, 1992). The binding affinity of CD44 to HA can be altered by different N- or O- glycosylation of the CD44 ectodomain (Skelton et al, 1998). The HA-binding affinity of CD44 can also be regulated by phosphorylation of serine residues on the intracellular part of CD44 (Pure et al, 1995). A dynamic change between high and low binding-affinity conformations of CD44 are required for leukocyte rolling (Ogino et al, 2010). Different CD44 variant isoforms can bind to different repertoires of ECM components. CD44 variants containing variant exons v6 and v7 were shown to be able to bind to the glycosaminoglycans chondroitin sulfate, heparin, and heparin sulfate in addition to HA (Sleeman et al, 1997).

CD44 also mediates the metabolism of HA. One third of the total HA content of the body is turned over (degraded and newly synthesized) every day (Stern, 2004). This requires uptake of HA into the cells via endocytosis which is dependent on CD44 and can be blocked with CD44 antibodies (Culty et al, 1992; Hua et al, 1993). The importance of CD44-mediated HA-homeostasis was shown by CD44 antisense cDNA expression in the skin, which caused accumulation of HA and blistering (Kaya et al, 1997).

CD44 is a coordinator of growth factor- and MMP-activity

CD44 is able to bind numerous growth factors as well as matrix metalloproteinases (MMPs) and serves as a platform where MMPs are brought together with their substrates. CD44 promotes tumor cell invasion in a mouse mammary carcinoma cell line and a human melanoma cell line by recruiting MMP-9 to the cell surface of the cancer cells. MMP-9 subsequently degrades collagen IV and facilitates tumor cell invasion (Yu & Stamenkovic, 1999). MMP-9 recruitment to the cell surface also caused the proteolytic activation of the transforming growth factor (TGF)-β precursor. TGF-β activation was strictly dependent on MMP-9 binding to the cell surface by CD44 (Yu & Stamenkovic, 2000). Active TGF-β then induced tumor angiogenesis.

CD44 also coordinates the proteolytic activation of heparin-binding epidermal growth factor (HB-EGF) by MMP-7. This is however CD44 isoform dependent as the HB-EGF

precursor only binds to the heparan sulfate moiety of variant exon v3 (Yu et al, 2002). Activated HB-EGF on the cell surface then binds and activates its receptor ErbB4 which induces cell survival and tissue remodeling.

CD44 is a co-receptor for receptor tyrosine kinases

As described in chapter 1.3, RTK activation and signaling is not only dependent on the respective ligands, but RTKs are often associated with cell adhesion molecules that act as co-receptors and mediate receptor activation and downstream signaling (Orian-Rousseau & Ponta, 2008). Also different isoforms of CD44 act as co-receptors for different RTKs. CD44s promotes heterodimerization of the epithelial growth factor receptor (EGFR)-family members ErbB2 and ErbB3 in response to the ligand neuregulin in primary Schwann cells. Presence of CD44s enhanced ErbB2 activation and was necessary for Schwann cell-neuron interactions and Schwann cell survival (Sherman et al, 2000). CD44s also mediates the activation of ErbB2 in ovarian carcinoma cells (Bourguignon et al, 1997). ErbB4-activation by HB-EGF is dependent on the isoform CD44v3 (Yu et al, 2002) (see above).

The activation of Met by its ligand HGF is strictly dependent on CD44v6 in several cancer cell lines and primary epithelial and endothelial cells (Orian-Rousseau et al, 2002; Tremmel et al, 2009). The co-receptor function of CD44v6 for Met has been studied extensively and is so far the best-described function of CD44 for an RTK.

CD44v6 is an essential co-receptor for Met

In a rat pancreatic carcinoma cell line that expresses Met and CD44s, but no CD44 variant isoform, Met cannot be activated by HGF unless a v6-containing CD44 isoform is introduced by transfection (Orian-Rousseau et al, 2002). Introduction of a CD44 isoform containing all variant exons except exon v6 could not restore the ability to activate Met in response to HGF. Furthermore, treatment of cells with antibodies raised against the CD44 variant sequence v6 or v6-containing CD44 ectodomains could block HGF-induced activation and downstream signaling of Met (Orian-Rousseau et al, 2002). Further studies revealed that a three amino acid sequence within v6 is essential for Met activation. Interestingly, this sequence is species specific: EWQ in rats, GWQ in mice and RWH in humans. Peptides of at least 5 amino acids containing this sequence can block HGF-induced Met activation and cell migration (Matzke et al, 2005). These peptides were also able to block angiogenesis and metastasis formation *in vivo* (Matzke et al., submitted). The co-receptor function of CD44v6 for Met is twofold. The extracellular part of CD44v6 is required for the activation of Met in

response to HGF (Matzke et al, 2005; Orian-Rousseau et al, 2002). The function of the extracellular part of CD44v6 seems to be the binding of HGF and the presentation of HGF to Met. Evidence for this comes from FACS analyses of HGF-binding experiments. Biotinylated HGF binds to cells that express both CD44v6 and Met and also to cells that express only CD44v6 but not Met. Importantly, cells that express Met but not CD44v6 are not able to bind biotinylated HGF. However, introduction of CD44v6 into these cells by stable transfection conferred the ability to bind HGF (Matzke, 2006). These findings are in agreement with the observation that CD44v6, Met and HGF are found together in a ternary complex if HGF is present (Orian-Rousseau et al, 2002).

The cytoplasmic domain of CD44v6 binds to ERM proteins that link the receptor complex to the actin cytoskeleton. This link is necessary for the formation of a signaling complex to mediate downstream signaling from the activated receptor (Orian-Rousseau et al, 2002; Orian-Rousseau et al, 2007). In cells transfected with a truncated form of CD44v6 missing the cytoplasmic domain, Met is still activated in response to HGF, but activation of downstream targets such as Erk, Akt and JNK is inhibited. Fusion of the truncated CD44v6 mutant with constitutive active ezrin restored downstream signaling from Met in response to HGF (Orian-Rousseau et al, 2007). The link to actin was also shown to be necessary for downstream signaling from Met as it could be blocked with ezrin mutants lacking the C-terminal actin-binding motif or by interfering with actin polymerization (Orian-Rousseau et al, 2007).

CD44v6 is required for VEGFR-2 signaling and angiogenesis

Activation and signaling of VEGFR-2 in response to two different VEGF-A isoforms (VEGF-A_{165} and VEGF-A_{121}) were also shown to be dependent on CD44v6. The co-receptor function of CD44v6 in this case seems to be similar to that for Met. The extracellular part of CD44v6 is required for VEGFR-2 activation whereas the cytoplasmic domain mediates downstream signaling. Binding of ERM proteins and the link to the actin cytoskeleton are also necessary for downstream signaling from VEGFR-2. Activation of VEGFR-2 could be blocked with v6 peptides, a soluble CD44v6 ectodomain or v6-specific antibodies. Treatment with v6 peptides also significantly reduced angiogenic sprouting *in vitro*. Moreover, application of the v6 peptide in an orthotopic mouse model *in vivo* resulted in decreased tumor vascularization and tumor growth (Tremmel et al, 2009).

CD44v6 is required for InlB-induced Met internalization

Like all other RTKs, Met gets internalized into the cells by endocytosis following its activation. The bacteria *Listeria monocytogenes* makes use of this internalization step to invade Met-expressing eukaryotic cells. With its surface protein internalin B (InlB), it mimicks HGF and activates Met to become endocytosed together with Met. Interestingly, the activation of Met by InlB and entry of the bacteria into the cells is also dependent on CD44v6 (Jung et al, 2009).

1.5 RTK internalization

RTK activation is a transient process and the duration of RTK signaling is under tight control. The main way to terminate RTK signaling is the removal of the receptors from the cell surface via a process that is called receptor internalization. Several pathways of RTK internalization have been described, including clathrin-mediated endocytosis, caveolin-mediated endocytosis, and macropinocytosis. The most extensively studied and best-described pathway for RTK internalization however is the clathrin-mediated endocytosis. It is the major internalization pathway induced upon activation of RTKs and other surface receptor families (reviewed in (Sorkin & Von Zastrow, 2002; Sorkin & von Zastrow, 2009; Zwang & Yarden, 2009). However, it became evident that receptor endocytosis has a much greater impact on signaling than just terminating the signal. The output of any given signal depends on the duration and the location within the cell which is determined by receptor endocytosis and intracellular trafficking (reviewed in (Gould & Lippincott-Schwartz, 2009; Scita & Di Fiore, 2010; Sorkin & Von Zastrow, 2002; Sorkin & von Zastrow, 2009)). Most studies of the mechanism of clathrin-mediated endocytosis focused on the EGF-Receptor as a model system. The mechanism of clathrin-mediated endocytosis and receptor sorting is briefly described in the following sections.

1.5.1 The mechanism of clathrin-mediated receptor endocytosis

Formation of clathrin-coated vesicles

Ligand binding to RTKs simultaneously initiates the assembly of a signaling complex as well as the recruitment of components mediating receptor internalization and downregulation.

The formation of clathrin-coated pits is initiated by Eps15 (EGF-Receptor pathway substrate 15), intersectins and AP-2 (adaptor protein 2). Receptors are selected for clathrin-mediated endocytosis by AP-2 in collaboration with various cargo-specific adaptors (reviewed in (McMahon & Boucrot, 2011; Traub, 2009)). Clathrin is then recruited by AP-2 which leads to the formation of clathrin-coated pits (CCPs). CCPs are pinched off from the plasma membrane by the GTPase dynamin to generate clathrin-coated vesicles (CCVs). Once released from the plasma membrane, the clathrin coat is removed by auxilin and the ATPase HSC70 (heat shock cognate70) (Schlossman et al, 1984; Ungewickell et al, 1995).

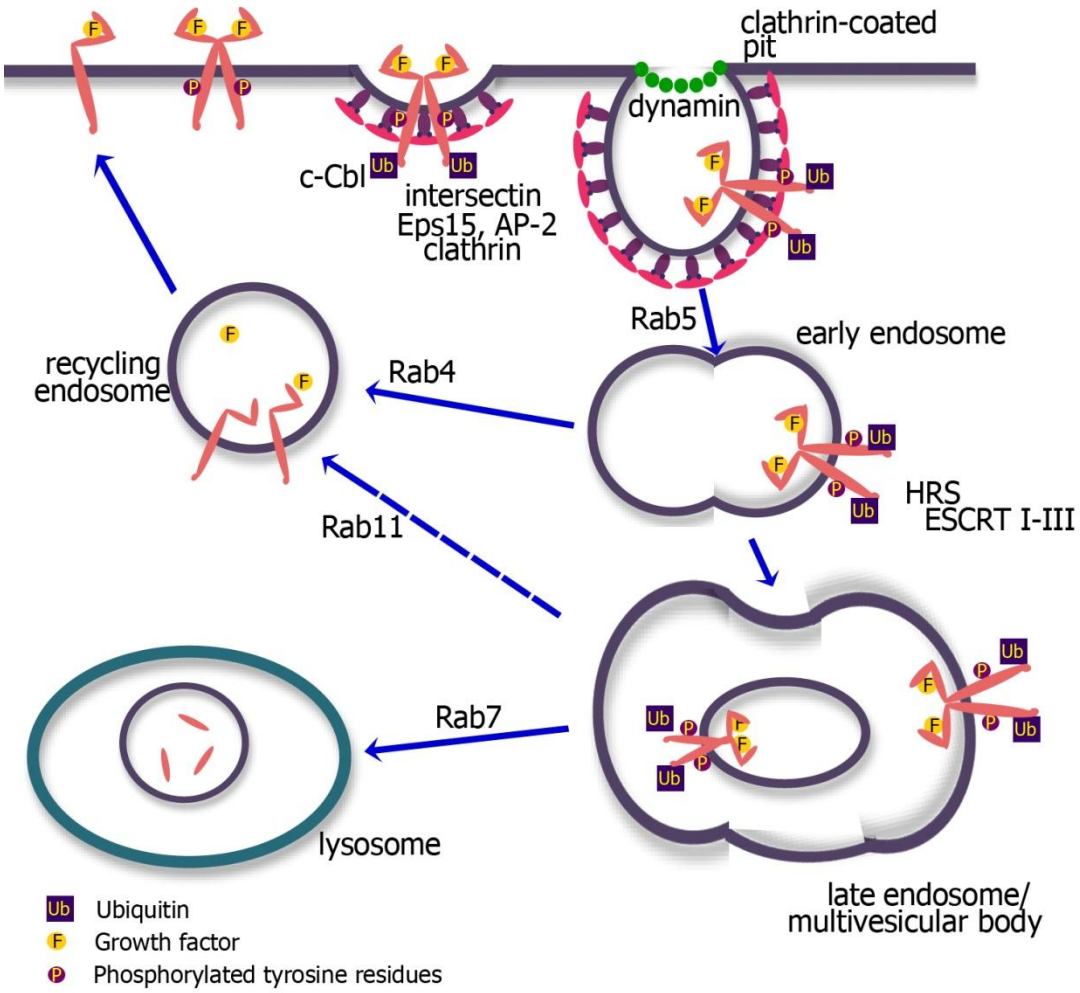

Fig.1.3 Scheme of clathrin-mediated endocytosis and intracellular trafficking of RTKs

Tyrosine-phosphorylated receptors are ubiquitylated by E3 ubiquitin ligases such as c-Cbl. Eps15, intersectins and AP-2 recruit clathrin and mediate clathrin-coated pit formation. Clathrin-coated vesicles are released from the plasma membrane by dynamin and transported to early endosomes by Rab5. Receptors are sorted by HRS and three ESCRT complexes which leads to receptor degradation or recycling to the plasma membrane (Rab4: fast recycling; Rab11: slow recycling). Receptors that are sorted for degradation are taken up into multivesicular bodies. Rab7 mediates the fusion of multivesicular bodies whith lysosomes where receptors are degraded.

The E3 ubiquitin ligase Cbl is also recruited to activated receptors at the plasma membrane through the interaction of its N-terminal tyrosine kinase binding domain (TKB) with specific phosphotyrosine residues in the activated receptors (Tyr1045 in the EGFR (Levkowitz et al, 1999) and Tyr1003 in Met (Peschard et al, 2001)). Cbl then mediates the ubiquitylation of the receptors (Thien & Langdon, 2001). Although several components of the endocytic machinery such as Eps15 and Epsin contain ubiquitin-interacting motifs (UIM) receptor ubiquitylation is not essential for receptor internalization (Abella & Park, 2009; Sorkin & von Zastrow, 2009; Zwang & Yarden, 2009). However, receptor ubiquitylation is crucial for the correct sorting of the receptors on endosomes to target them for lysosomal degradation (Abella et al, 2005; Duan et al, 2003; Huang et al, 2007).

Intracellular sorting and trafficking

The intracellular trafficking of the internalized receptors to different endosomal compartments is mediated by Rab GTPases. In humans, they comprise a family of more than 60 members that are located at distinct intracellular membrane compartments and coordinate most of the membrane trafficking processes (reviewed in (Stenmark, 2009; Stenmark & Olkkonen, 2001; Zerial & McBride, 2001)).

Activation of the EGF-Receptor results in the activation of the small GTPase Rab5 through a downstream signaling pathway that involves Ras (Barbieri et al, 2000; Tall et al, 2001). After the release of CCVs from the plasma membrane, activated (GTP-bound) Rab5 mediates the transport of the newly generated vesicles away from the plasma membrane and the fusion with early endosomes. Rab5 recruits several effectors to the new vesicles, including PI3-Kinase (Christoforidis et al, 1999b). PI3-Kinase converts phosphatidylinositol into phosphatidylinositol-3-phosphate (PI3P), which is necessary for the recruitment of FYVE finger domain (Fab1, YOTB, Vac1, EEA1)-containing proteins such as EEA1 (early endosomal antigen 1) and HRS (hepatocyte-growth-factor-regulated tyrosine-kinase substrate) (Komada & Soriano, 1999; Lawe et al, 2000; Stenmark & Aasland, 1999). EEA1 and Rab5 then promote the fusion of the vesicles with early endosomes where receptor sorting takes place (Christoforidis et al, 1999a; Simonsen et al, 1998). The receptors can either be dephosphorylated and deubiquitylated and recycled back to the plasma membrane through a fast recycling route that is driven by Rab4 (van der Sluijs et al, 1992) or they are sorted into late endosomes. From late endosomes, receptors still have the possibility to recycle back to the plasma membrane through a slow recycling route that is mediated by Rab11 (Ullrich et al, 1996). Receptors that are sorted for degradation are taken up into multivesicular bodies

(MVBs). Rab7 then mediates the fusion of the MVBs with lysosomes where the receptors are eventually degraded (reviewed in (Sorkin & von Zastrow, 2009; Zwang & Yarden, 2009)). How the decision between receptor recycling and receptor degradation is made, is not yet understood. In the case of the EGF-Receptor and the VEGF-Receptor 2, the fate of the receptor is ligand-dependent. Activation of the EGF-Receptor by EGF leads to lysosomal degradation of the receptor whereas activation by TGF-α (transforming growth factor-α) results in receptor recycling (Roepstorff et al, 2009). Similarly, activation of VEGFR-2 by the VEGF-A isoform VEGF-A_{165}a causes receptor recycling whereas binding of VEGF-A_{165}b leads to degradation (Ballmer-Hofer et al, 2011).

A crucial signal that determines receptor fate during the sorting process on early and late endosomes is receptor ubiquitylation. Ubiquitylated receptors are recognized by HRS through its UIM (Urbe et al, 2003). HRS then initiates the binding of ESCRT (endosomal sorting complex required for transport) proteins (Bache et al, 2003a; Bache et al, 2003b) that mediate the uptake of the receptors into MVBs which leads to their lysosomal degradation by acid-dependent proteases (reviewed in (Abella & Park, 2009; Gruenberg & Stenmark, 2004; Zwang & Yarden, 2009).

Receptor sorting can be regulated by various mechanisms. For example threonine phosphorylation of EGF-Receptor by protein kinase C (PKC) targets the receptor for recycling instead of degradation (Bao et al, 2000). Thus, receptor sorting and trafficking is not a passive process where the receptor simply acts as a passenger, but it is regulated by signaling pathways initiated from the receptor itself.

1.5.2 Signaling from the endosome

First evidence that signaling events occur from internalized receptors on endosomes came from the findings that RTKs are still tyrosine-phosphorylated and fully activated on the endosomes (Baass et al, 1995; Grimes et al, 1996; Sorkin et al, 1993). Moreover, many signaling proteins including Grb2, SOS, Shc, Ras, Raf, MEK, MAPK, and Src have been found on endosomes (Sorkin & Von Zastrow, 2002). Now it is well established that signaling continues on endosomes and is only terminated after the uptake of the receptors into MVBs where their catalytic active tyrosine kinase domains are isolated from the signal-transduction machinery in the cytosol (Futter et al, 2001; Lloyd et al, 2002; Sorkin & Von Zastrow, 2002). Endosomes can prolong signals initiated at the plasma membrane and serve as platforms for

the activation of signaling pathways that cannot be activated at the plasma membrane such as Rac (see below). Furthermore, locally restricted signals can be generated by transport of signaling proteins in endosomes to specific regions of the cell which is required for polarized cellular responses such as cell migration. The essential contribution of RTK internalization to the total signal outcome has been demonstrated in several experimental studies.

Receptor endocytosis is necessary for robust activation of Erk in response to EGF, NGF (nerve growth factor) and insulin since inhibition of RTK internalization by expression of dynamin mutants resulted in remarkably reduced Erk activation (Ceresa et al, 1998; Sorkin & Von Zastrow, 2002; Vieira et al, 1996; Zhang et al, 2000). Receptor endocytosis also mediates the distinction and differentiated response to NGF applied either to the cell body or distal axons of neuronal cells. NGF applied to distal axons results in internalization and transport of its receptor TrkA to the cell body wich is a prerequisite for activation of the MAP Kinase Erk5 and CREB (cAMP responsive element-binding protein) and neuronal survival. In contrast, NGF applied to the cell body of neuronal cells results in the activation of Erk1 and 2 but is not sufficient for CREB activation and neuronal survival (Watson et al, 2001).

Furthermore, internalization of the EGF-Receptor upon EGF-induction is essential for the activation of the Akt-GSK3β (glycogen synthase kinase 3β) pathway and regulation of gene expression by APPL (adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper-containing). APPL1 and APPL2 are recruited to early stages of early endosomes by activated (GTP-bound) Rab5 (Miaczynska et al, 2004). APPLs then mediate the binding of GSK3β and its activation by Akt on early endosomes. This is essential for cell survival during zebrafish development where downregulation of APPL1 by morpholinos caused widespread apoptosis. In contrast, the Akt-mTOR (mammalian target or rapamycin) axis does not require APPL1 and RTK internalization (Schenck et al, 2008; Sorkin & von Zastrow, 2009). Generation of PI3P by PI3-Kinase, which is also recruited to early endosomes by active Rab5 (see above), leads to further maturation of the early endosomes. As a consequence, the PI3P-binding protein EEA1 is recruited to early endosomes and displaces APPLs from endosomes (Zoncu et al, 2009). The released APPLs subsequently translocate to the nucleus and influence gene expression (Miaczynska et al, 2004).

In the case of Met, clathrin-mediated internalization has also essential implications for signaling. Internalization of active Met into Rab5-positive early endosomes is required for the interaction of the small GTPase Rac with its guanine exchange factor (GEF) TIAM1 (T-lymphoma invasion and metastasis gene 1). This leads to the activation of Rac on early endosomes. Subsequent recycling of active (GTP-bound) Rac to specific regions in the

plasma membrane in Arf6 (ADP ribosylation factor 6)-positive endosomes causes locally restricted actin remodeling required for HGF-induced cell migration. Downregulation of Rab5 with siRNA in HeLa cells inhibited Met internalization, Rac activation, and cell migration in response to HGF (Palamidessi et al, 2008). Nuclear translocation of STAT3 is as well dependent on the internalization of active Met. STAT3 signaling is crucial for HGF-induced tube formation by epithelial cells and is also required for Met-dependent cell transformation and tumorigenesis (Boccaccio et al, 1998; Lefebvre et al, 2012; Zhang et al, 2002). Kermorgant et al. demonstrated that upon HGF-induction Met and STAT3 co-localize on endosomes and traffic together to perinuclear compartments. Met internalization and trafficking as well as tyrosine kinase activity of Met on endosomes was necessary for the nuclear translocation of active STAT3 (Kermorgant & Parker, 2008).

Further examples of endosome-dependent signaling are reviewed in (Gould & Lippincott-Schwartz, 2009; Scita & Di Fiore, 2010; Sorkin & von Zastrow, 2009) and others.

1.5.3 Signaling regulates endocytosis

As described in the previous section, RTK internalization has a great impact on signaling. On the other hand, signaling is actively influencing RTK endocytosis and intracellular trafficking. Many components of the endocytic machinery are modified by different signal-transduction pathways. The clathrin heavy chain for example becomes tyrosine-phosphorylated by Src in response to EGF or NGF which results in an increased recruitment of clathrin to the plasma membrane (Beattie et al, 2000; Sorkin & von Zastrow, 2009; Wilde et al, 1999). The adaptor protein Eps15 which is a core component of clathrin-coated pits (see above) also becomes tyrosine-phosphorylated in response to EGF (Confalonieri et al, 2000) or HGF (Parachoniak & Park, 2009). Eps15-phosphorylation has been shown to be essential for the internalization of the EGF-Receptor in response to EGF. Eps15 that is mutated in the tyrosine-phosphorylation site (Eps15 Y850F) is still recruited to the plasma membrane upon EGF-induction, but expression of this mutant completely blocks the internalization of the EGF-Receptor in response to EGF. Internalization of the constitutively internalized transferrin receptor however was not affected by the Eps15 phosphorylation mutant suggesting that Eps15-phosphorylation is required for ligand-induced internalization (EGFR) but not for constitutive internalization (transferrin receptor)

(Confalonieri et al, 2000). Stress-induced activation of p38 MAP Kinase can even lead to ligand-independent receptor internalization (Cavalli et al, 2001; Zwang & Yarden, 2006).

PKC can rescue the EGF-Receptor from the degradative pathway and enhance EGF-Receptor recycling by phosphorylation of the receptor on a single threonine residue (Thr654) (Bao et al, 2000). Interestingly, PKC also regulates intracellular trafficking of Met. In contrast to the EGF-Receptor however, phosphorylation of Met by PKC does not trigger Met recycling but translocation of Met to perinuclear endosomes (Kermorgant et al, 2003; Kermorgant et al, 2004).

Further examples are described in (Sorkin & Von Zastrow, 2002; Sorkin & von Zastrow, 2009).

1.5.4 Adhesion molecules modify receptor endocytosis

As described in chapter 1.3 RTKs usually do not act alone but in collaboration with co-receptors such as cell adhesion molecules that sense the microenvironment and modify the signal according to additional cues that cannot be detected by the RTK itself. Together, they elicit an appropriate response of the cell to its environment. Given the tight and intertwined connection between signaling and endocytosis, it is not surprising that cell adhesion molecules can modify RTK endocytosis.

VEGFR-2 internalization is controlled by VE-cadherin (vascular endothelial). At high confluency, VE-cadherin gets engaged in adherens junctions and mediates contact inhibition by blocking the internalization of VEGFR-2. Inhibition of VEGFR-2 internalization reduces VEGF-induced VEGFR-2 phosphorylation and downstream signaling, probably by the recruitment of the density-enhanced phosphatase 1 (DEP-1) which dephosphorylates VEGFR-2 (Lampugnani et al, 2006). At lower confluency or in the absence of VE-cadherin, VEGFR-2 can be internalized upon VEGF-induction and signaling from endosomal compartments can take place and induce cell proliferation (Grazia Lampugnani et al, 2003; Lampugnani et al, 2006).

Intracellular trafficking and signaling of VEGFR-2 are also regulated by another cell adhesion molecule, neuropilin-1 (Nrp-1). Nrp-1 is internalized together with VEGFR-2 in response to VEGF-A_{165}a and is required for the trafficking of VEGFR-2 through Rab11 recycling endosomes and activation of the p38 MAP Kinase. Mutations in the cytoplasmic domain of Nrp-1 or induction with another isoform of VEGF-A that is unable to bind Nrp-1

(VEGF-A_{165}b) prevents the trafficking of VEGFR-2 through Rab11 endosomes and p38 MAP Kinase activation. VEGFR-2 is then sorted to the degradative pathway through Rab7 endosomes which results in a different signaling outcome and impaired angiogenic sprouting of endothelial cells (Ballmer-Hofer et al, 2011).

The internalization of the fibroblast growth factor 1 (FGFR-1) is regulated by the cell adhesion molecules E-cadherin (epithelial) and N-cadherin (neuronal). Upon fibroblast growth factor (FGF) induction, E-cadherin and FGFR-1 are internalized and traffic together through the same endosomal compartments (Bryant et al, 2005). Internalization is necessary for the nuclear translocation of FGFR-1 (Bryant et al, 2005) where it regulates genes that trigger cell proliferation (Reilly & Maher, 2001). However, when E-cadherin is stabilized at the plasma membrane by cell-cell contacts under high confluency, FGFR-1 internalization is blocked as well as nuclear translocation of FGFR-1 and MAP Kinase activation in response to FGF (Bryant et al, 2005). In contrast, association of FGFR-1 with N-cadherin inhibits FGF-induced internalization of FGFR-1 but results in sustained Erk MAP Kinase signaling from the plasma membrane, leading to MMP-9 expression and invasion of breast tumor cells (Suyama et al, 2002). Thus, cell adhesion molecules determine receptor signaling by regulating internalization as well as the assembly of signaling complexes at the plasma membrane and on endosomes.

1.5.5 Derailed endocytosis and cancer

Receptor endocytosis has a great impact on signaling and is also crucial for the most efficient and enduring way to terminate signaling, which is receptor degradation in the lysosomes. Therefore it is not surprising that aberrant endocytosis or intracellular trafficking of receptors is connected with cancerogenesis. In many tumors, the receptors escape from the endocytic downregulation which results in increased signaling and cell transformation (reviewed in (Abella & Park, 2009; Lanzetti & Di Fiore, 2008; Mosesson et al, 2008)). First evidence for this came from transformation studies with the retrovirus Cas NS-1. This virus causes B-cell lymphoma and myeloid leukemia in mice. The transforming agent of this virus was found to be viral Cbl (v-Cbl), a homologue to the mammalian Cbl family of E3 ubiquitin ligases. In contrast to normal Cbl proteins, v-Cbl binds to phosphorylated receptors but does not lead to ubiquitylation which prevents receptor degradation and signal downregulation (Langdon et al, 1989). Mutations in Cbl that impair its ubiquitin ligase activity have now also

been found in patients with acute myeloid leukemia (AML) (Caligiuri et al, 2007; Sargin et al, 2007).

Mutations in RTKs that prevent the binding of E3 ubiquitin ligases are also tumorigenic and have frequently been found in cancer (Abella & Park, 2009; Mosesson et al, 2008). A Met mutant that is missing the Cbl-binding site (Y1003F) cannot be ubiquitylated and has transforming activity (Abella et al, 2005; Peschard et al, 2001). This receptor is still internalized but escapes from receptor degradation which leads to sustained signaling (Abella et al, 2005). Similar mutations have been found in the EGF-Receptor and the c-Kit receptor (Masson et al, 2006; Waterman et al, 2002).

Cbl activity can also be decreased by Src kinases which are well-known to promote tumorigenesis (Biscardi et al, 1999; Tice et al, 1999). Active Src phosphorylates c-Cbl on tyrosine residues which triggers c-Cbl ubiquitylation and subsequent degradation (Bao et al, 2003).

In addition, aberrant expression of several other components of the endocytic machinery such as the adaptor proteins Eps15, endophilin II or Numb has also been found in human cancers, further supporting the causative role of aberrant receptor endocytosis in cancer development (reviewed in (Abella & Park, 2009; Mosesson et al, 2008)).

1.6 Aim

The cell adhesion molecule CD44v6 is an essential co-receptor for the RTKs Met and VEGFR-2. The extracellular part of CD44v6 is required for the activation of both receptors, probably by promoting the binding of growth factors to the receptors, whereas the cytoplasmic domain of CD44v6 is necessary to mediate downstream signaling from the activated receptors by providing a link through ERM proteins to the actin cytoskeleton. Biochemical assays revealed that CD44v6 is also involved in the internalization of Met upon HGF-induction.

The aims of my work are to elucidate the mechanism by which CD44v6 mediates HGF-induced Met internalization, and to study whether this internalization process influences Met signaling and reciprocally. An additional aim is to investigate whether VEGFR-2, which relies on Neuropilin-1 for trafficking, also needs CD44v6 for internalization and trafficking.

2. Material and Methods

2.1 Material

2.1.1 Chemicals and consumables

Name	Supplier
Acetic Acid	Merck, Darmstadt, Germany
Agarose	PEQLAB, Erlangen, Germany
Ampicillin	Roth, Karlsruhe, Germany
Ammonium Persulfate (APS)	Roth, Karlsruhe, Germany
Agar Agar	Otto Nordwald GmbH, Hamburg, Germany
Aprotinin	Sigma-Aldrich, Taufkirchen, Germany
Bovine Serum Albumine (BSA)	PAA Laboratories GmbH, Pasching, Austria
Bromphenol Blue	Roth, Karlsruhe, Germany
Cycloheximide	Serva, Heidelberg, Germany
Cytodex-3 beads	GE Healthcare, Freiburg, Germany
Dimethylsulfoxide (DMSO)	Fluka, Neu-Ulm, Germany
Dithiothreitol (DTT)	Roth, Karlsruhe, Germany
6x DNA Loading Buffer	Fermentas, St Leon-Rot, Germany
Dapi	Life Technologies, Darmstadt, Germany
Di-sodium tetraborate	Merck, Darmstadt, Germany
Draq5	Biostatus Limited, Shepshed, UK
Dulbecco's Modified Eagle Medium (DMEM)	Gibco/Invitrogen, Karlsruhe, Germany
ECL western blotting substrate	Thermo Scientific, Dreieich, Germany
Eagle´s Minimal Essential Medium (EMEM)	American Type Culture Collection (ATCC)/ LGC Standards, Wesel, Germany
EGM-2 BulletKit	Lonza, Basel, Switzerland
Ethylene glycol bis(2-aminoethyl) tetraacetic acid (EGTA)	Roth, Karlsruhe, Germany
Ethylenediamine tetraacetic acid (EDTA)	Roth, Karlsruhe, Germany

Ethanol (EtOH)	Roth, Karlsruhe, Germany
Ethidium bromide	Roth, Karlsruhe, Germany
EZ-Link Sulfo-NHS-SS-Biotin	Thermo Scientific, Dreieich, Germany
FCS (fetal calf serum)	PAA, Pasching, Austria
Fibrinogen Type I (from bovine plasma)	Sigma-Aldrich, Taufkirchen, Germany
Fluorescence mounting medium	Dako, Hamburg, Germany
Gel Extraction Kit	PEQLAB, Erlangen, Germany
Glucose	Roth, Karlsruhe, Germany
Glycerol	Roth, Karlsruhe, Germany
Glycin	Roth, Karlsruhe, Germany
Guanidine-HCL	Roth, Karlsruhe, Germany
HEPES	Roth, Karlsruhe, Germany
Hydrochloric acid (HCl), fuming	Merck, Darmstadt, Germany
Iodoacetamide	Sigma-Aldrich, Taufkirchen, Germany
Isopropanol	Roth, Karlsruhe, Germany
Leupeptin	Sigma-Aldrich, Taufkirchen, Germany
Kanamycin	Roth, Karlsruhe, Germany
Magnesium Chloride	Roth, Karlsruhe, Germany
Magnesium Sulfate	Sigma-Aldrich, Taufkirchen, Germany
MESNA (2-Mercaptoethanesulfonic acid sodium salt)	Sigma-Aldrich, Taufkirchen, Germany
Methanol (MeOH)	Roth, Karlsruhe, Germany
2-mercaptoethanol	Roth, Karlsruhe, Germany
N-ethylmaleimide	Sigma-Aldrich, Taufkirchen, Germany
n-Octyl-β-D-glucopyranoside	Calbiochem, Bad Soden, Germany
NeutrAvidin agarose beads	Thermo Scientific, Dreieich, Germany
Nonidet P-40 (NP-40)	Roth, Karlsruhe, Germany
Page Ruler Prestained Protein Ladder	Fermentas, St Leon-Rot, Germany
Paraformaldehyde	Merck, Darmstadt, Germany
peqGOLD DANN Ladder-Mix 100-10000bp	PEQLAB, Erlangen, Germany

pegGOLD Protein Marker IV	PEQLAB, Erlangen, Germany
Plasmid Maxi Kit	Qiagen, Hilden, Germany
Protein A Agarose beads	Calbiochem, Bad Soden, Germany
Protein G plus Agarose beads	Calbiochem, Bad Soden, Germany
Phosphate Buffered Saline (PBS) w/o $CaCl_2$ and $MgCl_2$	Gibco/Invitrogen, Karlsruhe, Germany
Phosphate Buffered Saline with $CaCl_2$ and $MgCl_2$ (PBS++)	Gibco/Invitrogen, Karlsruhe, Germany
PMSF (phenyl methanesulphonyl fluoride)	Sigma-Aldrich, Taufkirchen, Germany
Potassium Chloride (KCl)	Roth, Karlsruhe, Germany
PromoFectin transfection reagent	Promocell, Heidelberg, Germany
Rotiphorese® Gel30: Acrylamide/ bis-acrylamide (30%/0,8%)	Roth, Karlsruhe, Germany
Sodium Acetate (NaAc)	Roth, Karlsruhe, Germany
Sodium Chloride (NaCl)	Roth, Karlsruhe, Germany
Sodium Dodecyl Sulphate (SDS)	Roth, Karlsruhe, Germany
Sodium Fluoride (NaF)	Sigma-Aldrich, Taufkirchen, Germany
Sodium Hydroxide (NaOH)	Roth, Karlsruhe, Germany
Sodium Hydrogen Phosphat	Roth, Karlsruhe, Germany
Sodium Dihydrogenphosphat	Roth, Karlsruhe, Germany
Sodium orthovanadate	Sigma-Aldrich, Taufkirchen, Germany
Tetramethyl ethylen diamine (TEMED)	Roth, Karlsruhe, Germany
Thrombin	Sigma-Aldrich, Taufkirchen, Germany
Tris-base	Roth, Karlsruhe, Germany
Tris-HCl	Roth, Karlsruhe, Germany
Trypsin 0.25% (w/v) -EDTA	Gibco/Invitrogen, Karlsruhe, Germany
Trypton/Pepton	Roth, Karlsruhe, Germany
Triton-X-100	Roth, Karlsruhe, Germany
Tween® 20	Sigma-Aldrich, Taufkirchen, Germany
Yeast extract	Roth, Karlsruhe, Germany

2.1.2 Bacteria and eukaryotic cell lines

2.1.2.1 Bacteria

Name	Source and description
E. Coli DH5α	Recombination defect suppressive strain for plasmid expression. Genotype: *F-, Φ80ΔlacZ, ΔM15, Δ(lacZYA-argF), U169, endA1, hsd R17 (rk-, mk+), phoA, sup E44, thi-1, λ-, rec A1, gyr A96, relA1*. Obtained from Invitrogen (Karlsruhe, Germany).

2.1.2.2 Eukaryotic cell lines

Name	Source and description
HeLa	Human cervix adenocarcinoma cell line of epithelial origin containing the human papilloma virus-18 (HPV-18), derived from a 31-year-old woman of black ethnicity. Obtained from the American Type Culture Collection (ATCC)/LGC Standards (Wesel, Germany) in a cryopreserved form. Cell line designation: HeLa.
HUVEC	Human umbilical vein endothelial cells, pooled from several donors. Obtained from Lonza (Basel, Switzerland) in a cryopreserved form.
Human skin fibroblasts	Human skin fibroblasts, derived from a human female fetus of Caucasian ethnicity. Obtained from the American Type Culture Collection (ATCC)/LGC Standards (Wesel, Germany) in a cryopreserved form. Cell line designation: Detroit 551. Total life span 25 passages.

2.1.3 Primary Antibodies

IF=immunofluorescence; IP=immunoprecipitation: WB=western blot

Protein of interest	Name	Description	Application (Dilution)	Supplier
Akt	#9272	rabbit polyclonal antibody recognizing human, mouse, and rat Akt	WB (1:1000)	Cell Signaling, Beverly, England
P-Akt (S473)	#9271	rabbit polyclonal antibody recognizing Akt1 phosphorylated at serine 473 of human, mouse, and rat	WB (1:1000)	Cell Signaling, Beverly, England
CD44v6	BIWA	mouse monoclonal antibody recognizing the epitope encoded by variably spliced exon v6 of human CD44v6	IF (10µg/ml) IP (10µg/ml) WB (1:300)	Bender, Wien, Österreich
CD44v6 rat	1.1ASML	mouse monoclonal antibody recognizing the epitope encoded by variably spliced exon v6 of rat CD44v6	IF (1:1000) WB (1:1000)	
Eps15	C-20	rabbit polyclonal antibody recognizing an epitope at the C-terminus of human, mouse, and rat Eps15	IP (1:50) WB (1:500)	Santa Cruz, Heidelberg, Germany
Erk	K-23	rabbit polyclonal antibody recognizing human, rat, and mouse Erk MAPK	WB (1:1000)	Santa Cruz, Heidelberg, Germany

P-Erk (T202/Y204)	phospho-p44/42 MAPK	rabbit polyclonal antibody recognizing phosphorylated human, rat, and mouse Erk MAPK	WB (1:1000)	Cell Signaling, Beverly, England
GFP	B-2	mouse monoclonal antibody recognizing green fluorescent protein (GFP)	IP (4µg/ml) WB (1:1000)	Santa Cruz, Heidelberg, Germany
HA	12CA5	mouse monoclonal antibody recognizing the influenza hemagglutinin nonapeptide sequence YPYDVPDYA	IP (4g/ml) WB (1:1000)	Roche Diagnostics, Mannheim, Germany
HRS	958/3	rabbit antibody recognizing human HRS	IF (1:1000)	was a gift from Sylvie Urbé, University of Liverpool
Met	25H2	mouse monoclonal antibody recognizing an epitope in the intracellular part (surrounding Tyr1234) of human, mouse, and rat Met	IP (1:50) WB (1:500)	Cell Signaling, Beverly, England
Met	AF276	goat monoclonal antibody recognizing an epitope in the extracellular part of human Met	IF (10g/ml)	R&D Systems, Wiesbaden, Germany
P-Met (Y1234/1235)	D-26	rabbit polyclonal antibody recognizing tyrosine-phosphorylated human, rat, and mouse Met	WB (1:500)	Cell Signaling, Beverly, England

Neuropilin-1	C-19	goat polyclonal antibody recognizing an epitope at the C-terminus of human neuropilin-1	IP (1:50) WB (1:500)	Santa Cruz, Heidelberg, Germany
P-Tyrosine	4G10	mouse monoclonal antibody recognizing tyrosine-phosphorylated proteins from all species	WB (1:500)	Millipore, Schwalbach, Germany
Ubiquitin	P4D1	mouse monoclonal antibody recognizing ubiquitin, polyubiquitin, and ubiquitinated proteins from all species	WB (1:1000)	Cell Signaling, Beverly, England
VEGFR-2	A-3	mouse monoclonal antibody recognizing an epitope at the C-terminus of human, mouse, and rat VEGFR-2	IP (1:50) WB (1:500)	Santa Cruz, Heidelberg, Germany
P-VEGFR-2 (Y1175)	19A10	rabbit monoclonal antibody recognizing human and mouse VEGFR-2 only when phosphorylated at tyrosine 1175	WB (1:500)	Cell Signaling, Beverly, England

All isotype control antibodies (normal mouse IgG, normal rabbit IgG, normal goat IgG) were from Santa Cruz, Heidelberg, Germany.

2.1.4 Secondary antibodies

Name	Supplier
Goat anti-mouse HRP-conjugated	Dako, Hamburg, Germany
Goat anti-rabbit HRP-conjugated	Dako, Hamburg, Germany
Rabbit anti-goat HRP-conjugated	Dako, Hamburg, Germany
Donkey anti-goat Alexa Fluor 488	Invitrogen, Karlsruhe, Germany
Donkey anti-goat Alexa Fluor 546	Invitrogen, Karlsruhe, Germany
Donkey anti-mouse Alexa Fluor 488	Invitrogen, Karlsruhe, Germany
Donkey anti-mouse Alexa Fluor 633	Invitrogen, Karlsruhe, Germany
Donkey anti-rabbit Alexa Fluor 488	Invitrogen, Karlsruhe, Germany

2.1.5 Enzymes

Name	Supplier
SmaI restriction enzyme	Fermentas, St. Leon-Rot, Germany
Shrimp Alkaline Phosphatase (SAP)	Fermentas, St. Leon-Rot, Germany
T4 DNA Ligase	Fermentas, St. Leon-Rot, Germany
XhoI restriction enzyme	New England Biolabs, Frankfurt a. M., Germany

2.1.6 Growth factors

Name	Source and Description
Hepatocyte growth factor (HGF)	Recombinant human HGF was obtained from R&D Systems (Wiesbaden, Germany) and activated with 5% FCS overnight.
Vascular endothelial growth factor A_{165} (VEGF-A_{165})	Was a gift from Kurt Ballmer-Hofer, PSI Villigen, Switzerland.

2.1.7 Peptides

Name and Sequence	Source and Description
Human CD44v6 peptide KEQWFGN**RWH**EGYR	NMI Peptides, Reutlingen, Germany
Control peptide HNREQANLNSRTEETI	Was a gift from Jonathan Sleeman, KIT Karlsruhe, Germany

2.1.8 Plasmids

The following plasmids were used for cloning or transfection of eukaryotic cells:

Name	Source and description
hCD44v6-EGFP	The cDNA sequence of human CD44 containing the variant exon v6 was cloned into the multiple cloning site of the pEGFP-N2 fusion vector. For a detailed description of the cloning procedure, see *2.2.1.7*. This plasmid allows the expression of human CD44v6 with a fluorescent EGFP-tag at the C-terminus.

hCD44v6	The hCD44v6 expression plasmid was generated from the hCD44v6-EGFP plasmid by introducing a stop-codon (TAA) behind the sequence of CD44v6. This plasmid allows the expression of human CD44v6 without a fluorescent tag.
hCD44v6 tailless-EGFP	The cDNA sequence of human CD44 containing the variant exon v6, but missing the exons coding for the cytoplasmic part, was cloned into the multiple cloning site of the pEGFP-N2 fusion vector. For a detailed description of the cloning procedure, see *2.2.1.7*. The plasmid allows the expression of a tailless mutant of human CD44v6 with a fluorescent EGFP-tag at the C-terminus.
hCD44v6 tailless	The hCD44v6 tailless expression plasmid was generated from the hCD44v6 tailless-EGFP plasmid by introducing a stop-codon (TAA) behind the sequence of CD44v6. This plasmid allows the expression of a mutant form of human CD44v6 missing the cytoplasmic domain without a fluorescent EGFP-tag.
HA-Erk	Mammalian expression vector containing the cDNA of the MAP Kinase Erk with an HA-tag. Was a gift from A. Ulrich (Martinsried, Germany).
mEGFP-HRas S17N	mEGFP-HRas S17N was a gift of Karel Svoboda (Addgene plasmid #18665). The plasmid allows the expression of a constitutive inactive mutant of human HRas with a fluorescent EGFP-tag at the N-terminus.
Metastop (rat CD44v4-7 tailless)	The sequence of rat CD44v4-7 from the 5' untranslated region up to the transmembrane domain was cloned into the Topo-pCRII vector (Invitrogen) (Orian-Rousseau et al., 2007) and subcloned into the PSV7 vector.
PSV7	Was obtained by replacing the multiple cloning site in PSG5(Green et al, 1988) and contains the SV40 early promoter for expression.
pEGFP-N2	Mammalian expression vector containing EGFP under the control of the CMV promoter. Suitable for the generation of fusion proteins with a fluorescent EGFP-tag at the C-terminus. Obtained from Takara BioEurope/Clontech, St.-Germain-en-Laye, France.
Rab5a Q79L -GFP	Rab5a Q79L –GFP was a gift of Kurt Ballmer-Hofer, PSI Villigen, Switzerland. This plasmid allows the expression of constitutively active human Rab5a with a fluorescent GFP-tag.
mRFP-Rab5	mRFP-Rab5 was a gift of Ari Helenius (Addgene plasmid #14437). This plasmid allows the expression of human wildtype Rab5a with a fluorescent mRFP-tag at the N-terminus.

2.2 Methods

2.2.1 Nucleic acid techniques

2.2.1.1 Transformation of bacteria

For transformation, 200µl of chemically competent bacteria DH5α were incubated for 30-60min on ice with 5µl of a ligation reaction or with 1µl of a purified plasmid (500-1000ng plasmid DNA) containing a resistance gene for either ampicillin or kanamycin. Then the bacteria were heat-shocked at 42ºC for 1min and incubated on ice for 2min. Subsequently, 250µl of LB medium were added and the transformed bacteria were incubated for 60min at 37ºC on a shaker. An agar plate (1,5% agar agar in LB medium) containing either 100µg/ml ampicillin or 50µg/ml kanamycin (depending on the resistance gene of the respective plasmid) was warmed to RT and 50µl of the bacterial suspension were spread over the agar plate and incubated at 37ºC over night. In case of ligase reaction mix all bacterial suspension was spread over the agar plate.

2.2.1.2 Small-scale purification of plasmid DNA

For mini-preparation of plasmid DNA, a colony of transformed bacterial clones was picked from an agar plate, placed into 3ml LB medium containing either 50µg/ml ampicillin or 30µg/ml kanamycin (depending on the resistance gene of the respective plasmid) as a selection marker, and incubated at 37ºC over night with constant shaking at 180rpm. Subsequently, 1ml of the bacterial culture was transferred into an eppendorf 1.5ml reaction tube and centrifuged at 13000rpm for 30s at 4ºC (Eppendorf centrifuge 5415 R). For the isolation of the plasmid DNA, the solutions P1, P2, and P3 of the Plasmid Maxi Kit® (Qiagen, Hilden, Germany) were used. The supernatant was removed and the bacterial pellet was washed once with ice-cold TE buffer (10mM EDTA, 50mM Tris-HCl pH 8.0). Then, the bacteria were resuspended in 200µl resuspension buffer P1 (10mM EDTA, 50mM Tris-HCl pH 8.0 supplemented with 100µg/ml RNase A) and incubated for 5min at RT. The bacteria were lysed by adding 200µl lysis buffer P2 (200mM NaOH, 1% SDS) and incubating for 5min on ice. The lysis was stopped by adding 200µl cold neutralizing buffer P3 (3M Na

Acetate, pH 4.8) followed by vortexing and incubation for 5min on ice. Debris was spun down at 13000rpm for 15min at 4ºC (Eppendorf centrifuge 5415 R) and the supernatant was transferred into a new reaction tube. If necessary the centrifugation step was repeated to completely remove all debris. Then the DNA was precipitated by addition of 1ml pre-chilled ethanol (-20°C) to 400µl of the supernatant, incubation for 30min at -80ºC, and centrifugation at 12000rpm for 15min at 4ºC (Eppendorf centrifuge 5415 R). The supernatant was discarded and the DNA pellet was purified from residual salts by washing with 200µl 80% ethanol. After a final centrifugation at 12000rpm for 10min at 4ºC (Eppendorf centrifuge 5415 R), the supernatant was discarded and the residual ethanol was allowed to evaporate. The DNA was resuspended in 30µl TE buffer (10mM EDTA, 50mM Tris-HCl pH 8.0) and the DNA-concentration was determined using a NanoDrop® device.

2.2.1.3 Large-scale purification of plasmid DNA

For large-scale purification of plasmid DNA, the Qiagen Plasmid Maxi kit (Qiagen, Hilden, Germany) was used. Transformed bacteria were cultured in 300ml LB medium containing either 50µg/ml ampicillin or 30µg/ml kanamycin (depending on the resistance gene of the respective plasmid) at 37ºC over night with constant shaking at 180rpm. Then, the bacteria were centrifuged at 6000rpm for 15min at 4ºC (Beckmann J2-HS centrifuge, rotor JA-10) and the pellet was resuspended in 10ml resuspension buffer P1 (10mM EDTA, 50mM Tris-HCl pH 8.0 supplemented with 100µg/ml RNase A). Subsequently, alkaline lysis was performed by addition of 10ml lysis buffer P2 (200mM NaOH, 1% SDS). The solution was mixed gently and the reaction was allowed to proceed for 3min at RT. Then the lysis was stopped by adding 10ml ice-cold neutralizing buffer P3 (3M Na Acetate, pH 4.8) and gentle mixing. To remove debris, the lysate was centrifuged at 4000rpm for 40min at 4ºC (Heraeus, Megafuge 1.0 R). Thereafter, a Qiagen Tip 500 column was equilibrated with 15ml equilibration buffer QBT (700mM NaCl, 50mM MOPS pH 7.0, 15% isopropanol (v/v), 0.15% Triton X-100 (v/v)) and the lysate without debris was loaded on the column and allowed to flow through by gravity to enable maximum binding of the DNA to the column. Then the column was washed twice with 30ml wash buffer QC (1M NaCl, 50mM MOPS pH 7.0, 15% isopropanol (v/v)) and the DNA was eluted with 15ml elution buffer QF (125mM NaCl, 50mM Tris-HCl pH 8.5, 15% isopropanol (v/v)) and collected in a 50ml Falcon tube containing 11ml isopropanol. The solution was gently mixed and kept for 15min on ice or stored on -20°C to allow precipitation of the DNA. Thereafter, the DNA was spun down for

60min at 4000rpm at 4°C (Heraeus, Megafuge 1.0 R). The supernatant was discarded and the DNA pellet was dissolved in 400µl DNase free water and transferred into an eppendorf 1.5ml reaction tube. Then the DNA was again precipitated by adding 1ml pre-chilled ethanol (-20°C) and 40µl 3M NaAc pH4.9. The DNA was spun down at 12000rpm for 10min at 4°C (Eppendorf centrifuge 5415 R), and the DNA pellet was washed with 1ml 70% ethanol. After a final centrifugation at 12000rpm for 10min at 4ºC (Eppendorf centrifuge 5415 R), the supernatant was discarded and the residual ethanol was allowed to evaporate. The DNA was resuspended in 400µl TE buffer (10mM EDTA, 50mM Tris-HCl pH 8.0) and the DNA-concentration was determined using a NanoDrop® device.

2.2.1.4 Determination of DNA-concentrations

To determine the DNA-concentration and purity of a DNA-solution, the optical density (OD) was measured at 260, 230 and 280nm using a NanoDrop® device and the software ND-1000 (version 3.1.2). An OD_{260} of 1.0 corresponds to 50µg/ml of pure double-stranded DNA. To evaluate the purity of the DNA-solution, the ratios OD_{260}/OD_{280} and OD_{260}/OD_{230} were calculated. A ratio of OD_{260}/OD_{280} of 1.8 indicates that the solution is relatively free of protein contamination, whereas a ratio of OD_{260}/OD_{230} above 1.6 indicates that the solution is free of organic chemicals and solvents.

2.2.1.5 Separation of nucleic acids by agarose gel electrophoresis

DNA-fragments were separated according to their size by agarose gel electrophoresis with agarose gel concentrations ranging from 1 to 2% (w/v) depending on the size of the fragments that had to be separated. The required amount of agarose was dissolved in 150ml TAE buffer (0.04M Tris pH 7.2, 0.02 sodium acetate, 1mM EDTA) and dissolved by boiling. Then the agarose solution was cooled down to about 40ºC before the intercalating DNA-dye ethidium bromide was added to a final concentration of 0.4µg/ml and the solution was poured into a horizontal gel electrophoresis tank. A comb was placed into the agarose solution to form pockets in which the samples could be loaded and the solution was allowed to polymerize. Then the tank was filled with TAE buffer and the DNA-samples were mixed

with 6x DNA Loading Buffer (Fermentas, St. Leon-Rot, Germany) and loaded onto the gel. A standard DNA-ladder, the peqGOLD Ladder-Mix 100-10000bp (PEQLAB Biotechnologie, Erlangen, Germany), was also loaded on the gel and the fragments were separated at 80-120V. The different DNA-fragments could be visualized under UV-light and their size could be determined by comparison with the standard DNA-ladder.

2.2.1.6 Extraction of DNA from agarose gels

DNA-fragments were extracted from agarose gels using the peqGOLD Gel extraction Kit (PEQLAB Biotechnologie, Erlangen, Germany) according to the manufacturer's instructions. Briefly, the DNA-fragments were visualized under mild UV-light and gel pieces containing the DNA-fragments of the desired sizes were cut out with a scalpel. This had to be done relatively quickly to avoid point mutations in the DNA caused by the UV-light. The gel pieces were weighted and transferred into 1.5ml eppendorf reaction tubes. Then 100µl XP2 Binding Buffer were added for each 100µg of agarose gel and the gel pieces were dissolved in the buffer at 55°C for 5min with vortexing every 2-3min. When the agarose gel pieces had been completely dissolved, the pH was adjusted with approximately 5µl 5M NaAc pH 4.9 (until the solution turned from orange to yellow) and the solution was loaded onto a HiBind® DNA spin column and centrifuged at 10000rpm for 1min (Eppendorf centrifuge 5415 R). The flow-through was discarded and the column was washed once with 300µl XP2 Binding Buffer and twice with 750µl SPW Wash Buffer (completed with ethanol to 80% final ethanol concentration) by centrifugation at 10000rpm for 1min (Eppendorf centrifuge 5415 R). Then the column was dried by centrifugation at 10000rpm for 2min (Eppendorf centrifuge 5415 R) and the DNA was eluted from the column with 30µl Elution Buffer and collected in an 1.5ml eppendorf reaction tube by centrifugation at 10000rpm for 1min (Eppendorf centrifuge 5415 R).

2.2.1.7 Cloning of human CD44v6 and CD44v6 tailless

2.2.1.7.1 Design of the cloning insert

To generate the human CD44v6 full length and CD44v6 tailless constructs, the pEGFP fusion vector (Takara BioEurope/Clontech, St.-Germain-en-Laye, France) was chosen as a suitable mammalian expression vector. The sequence of the human CD44v6 gene was screened for restriction sites using the ApE software (A plasmid Editor by M. Wayne Davis) to find restriction enzymes that would not cut in the CD44v6 sequence, but that have single restriction sites in the multiple cloning site of the pEGFP fusion vector. Accordingly, XhoI, HindIII, and SmaI were chosen as suitable restriction enzymes for cloning of the human CD44v6 sequence into the pEGFP fusion vector. Then a cloning insert was designed that consisted of the following parts (from 5´ to 3´): XhoI restriction site – CD44v6 sequence without terminal stop codon – HindIII restriction site – flexible linker – SmaI restriction site.
The complete sequence of the human CD44v6 full length insert was:

CTCGAGATGGACAAGTTTTGGTGGCACGCAGCCTGGGGACTCTGCCTCGTGCCGCTGAGCCTGGCGCAGATC
GATTTGAATATAACCTGCCGCTTTGCAGGTGTATTCCACGTGGAGAAAAATGGTCGCTACAGCATCTCTCGGAC
GGAGGCCGCTGACCTCTGCAAGGCTTTCAATAGCACCTTGCCCACAATGGCCCAGATGGAGAAAGCTCTGAGC
ATCGGATTTGAGACCTGCAGGTATGGGTTCATAGAAGGGCACGTGGTGATTCCCCGGATCCACCCCAACTCCA
TCTGTGCAGCAAACAACACAGGGGTGTACATCCTCACATCCAACACCTCCCAGTATGACACATATTGCTTCAAT
GCTTCAGCTCCACCTGAAGAAGATTGTACATCAGTCACAGACCTGCCCAATGCCTTTGATGGACCAATTACCAT
AACTATTGTTAACCGTGATGGCACCCGCTATGTCCAGAAAGGAGAATACAGAACGAATCCTGAAGACATCTAC
CCCAGCAACCCTACTGATGATGACGTGAGCAGCGGCTCCTCCAGTGAAAGGAGCAGCACTTCAGGAGGTTACA
TCTTTTACACCTTTTCTACTGTACACCCCATCCCAGACGAAGACAGTCCCTGGATCACCGACAGCACAGACAGA
ATCCCTGCTACCACTCAGGCAACTCCTAGTAGTACAACGGAAGAAACAGCTACCCAGAAGGAACAGTGGTTTG
GCAACAGATGGCATGAGGGATATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGGGACAGCTGCAG
ACCAAGACACATTCCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACTCACATGG
GAGTCAAGAAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGGCTGATCATC
TTGGCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTCGAAGAAGGTGTGGGCA
GAAGAAAAAGCTAGTGATCAACAGTGGCAATGGAGCTGTGGAGGACAGAAAGCCAAGTGGACTCAACGGAG
AGGCCAGCAAGTCTCAGGAAATGGTGCATTTGGTGAACAAGGAGTCGTCAGAAACTCCAGACCAGTTTATGAC
AGCTGATGAGACAAGGAACCTGCAGAATGTGGACATGAAGATTGGGGTGAAGCTTTCCGGAGCTGCCGCTGC
AGGAGGCAGCCCCGGG.

The insert was then chemically synthesized at GenScript (Hong Kong) and cloned into the pUC57 cloning vector via the restriction enzyme EcoRI, the restriction site of which was added to both ends of the insert.

The CD44v6 tailless construct was generated by removing the sequence coding for the cytoplasmic tail of CD44.

The complete sequence of the human CD44v6 tailless insert was:
CTCGAGATGGACAAGTTTTGGTGGCACGCAGCCTGGGGACTCTGCCTCGTGCCGCTGAGCCTGGCGCAGATC
GATTTGAATATAACCTGCCGCTTTGCAGGTGTATTCCACGTGGAGAAAAATGGTCGCTACAGCATCTCTCGGAC
GGAGGCCGCTGACCTCTGCAAGGCTTTCAATAGCACCTTGCCCACAATGGCCCAGATGGAGAAAGCTCTGAGC
ATCGGATTTGAGACCTGCAGGTATGGGTTCATAGAAGGGCACGTGGTGATTCCCCGGATCCACCCCAACTCCA
TCTGTGCAGCAAACAACACAGGGGTGTACATCCTCACATCCAACACCTCCCAGTATGACACATATTGCTTCAAT
GCTTCAGCTCCACCTGAAGAAGATTGTACATCAGTCACAGACCTGCCCAATGCCTTTGATGGACCAATTACCAT
AACTATTGTTAACCGTGATGGCACCCGCTATGTCCAGAAAGGAGAATACAGAACGAATCCTGAAGACATCTAC
CCCAGCAACCCTACTGATGATGACGTGAGCAGCGGCTCCTCCAGTGAAAGGAGCAGCACTTCAGGAGGTTACA
TCTTTTACACCTTTTCTACTGTACACCCCATCCCAGACGAAGACAGTCCCTGGATCACCGACAGCACAGACAGA
ATCCCTGCTACCACTCAGGCAACTCCTAGTAGTACAACGGAAGAAACAGCTACCCAGAAGGAACAGTGGTTTG
GCAACAGATGGCATGAGGGATATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGGGACAGCTGCAG
ACCAAGACACATTCCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACTCACATGG
GAGTCAAGAAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGGCTGATCATC
TTGGCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAAGCTTTCCGGAGCTGCCGCTGCA
GGAGGCAGCCCCGGG.

2.2.1.7.2 Restriction digestion

The pUC57 cloning vectors containing the CD44v6 full length or CD44v6 tailless insert respectively, as well as the pEGFP mammalian expression vector were digested with the SmaI (Fermentas, St. Leon-Rot, Germany) and XhoI (New England BioLabs, Frankfurt a. M., Germany) restriction enzymes. As the SmaI restriction enzyme is temperature sensitive, the SmaI digestion was first carried out for 2h at 30°C and then the XhoI digestion was carried out at 37°C for 2h. The restriction digestion was stopped by heat inactivation of the restriction enzymes at 65°C for 20min. Then the DNA-fragments were separated by agarose gel electrophoresis as described under 2.2.1.5 and the desired DNA-fragments (pEGFP

expression vector at 4.7kb, human CD44v6 full length insert at 1.3kb, and human CD44v6 tailless insert at 1.1kb) were cut out and purified as described under 2.2.1.6.

The digested pEGFP expression vector was then treated with Shrimp Alkaline Phosphatase (SAP) (Fermentas, St. Leon-Rot, Germany) at 37°C for 30min to prevent religation of the vector without insert. After the treatment, the DNA was again purified using the peqGOLD Gel extraction Kit (PEQLAB Biotechnologie, Erlangen, Germany) as described in 2.2.1.6 and used for ligation.

2.2.1.7.3 Ligation of DNA fragments

The CD44v6 full length and CD44v6 tailless inserts were ligated with the pEGFP vector using 5U of T4 DNA Ligase (Fermentas, St. Leon-Rot, Germany). The respective insert and the digested and SAP-treated vector were mixed in a molar ratio of 1:1 or 5:1 in T4 DNA Ligase Buffer (50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM DTT, 1mM ATP, 25µg/ml BSA) supplemented with 5% PEG 4000 for blunt-end ligation. The ligation mix of 30µl total volume was incubated at RT for 2h and the reaction was stopped by heat inactivation of the enzyme at 65°C for 20min. As a control, the vector was incubated in a ligation mix without insert.

The obtained CD44v6 full length and CD44v6 tailless constructs carried a fluorescent EGFP-tag at the cytoplasmic C-terminus of the transmembrane proteins. As this EGFP-tag seemed to disturb the function of the CD44v6 full length protein, the expression of the EGFP-tag was repressed by insertion of a stop codon behind the CD44v6 sequence.

2.2.2 Cell culture and transfection

2.2.2.1 Culturing of eukaryotic cell lines

All mammalian cells were cultured under standard conditions (37°C, 95% humidity, 5% CO_2) in a cell culture incubator (Thermo Scientific, Wilmington, USA). The cells were grown in sterile Cellstar® cell culture dishes (Greiner Bio-One, Frickenhausen, Germany) of different sizes according to the experimental setup. HeLa cells were grown in DMEM (Gibco/Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (PAA, Pasching, Austria) and used up to passage 25. Human skin fibroblasts were cultured in EMEM (ATCC/LGC Standards, Wesel, Germany) supplemented with 10% fetal calf serum (PAA Pasching, Austria) and used up to passage 25. Human umbilical vein endothelial cells (HUVEC) were cultured in EGM-2 (Lonza, Basel, Switzerland) with supplements and used up to passage 12. For all cells, the medium was renewed every second day until they reached subculturing confluency.

2.2.2.2 Subculturing procedure

HeLa cells were passaged at 80% confluency. The cells were washed once with PBS, incubated for approximately 3min with 0,25% (w/v) Trypsin-0,5mM EDTA (Gibco/Invitrogen, Karlsruhe, Germany) at 37°C and when the cells had detached from the cell culture dish the reaction was stopped by adding fresh medium containing 10% FCS. The cells were centrifuged at 12000rpm for 3min (Heraeus Megafuge 1.0), resuspended in fresh medium and seeded at dilutions from 1:5 to 1:10 into new cell culture dishes.

HUVECs were grown to 80% confluency. Then the cells were washed once with PBS and incubated for approximately 5min with 0,25% (w/v) Trypsin-0,5mM EDTA at 37°C**.** When the cells had detached, the reaction was stopped by adding PBS containing 10% FCS and the cells were centrifuged for 5min at 800rpm. Then, the cells were resuspended in fresh endothelial cell growth medium and seeded at a 1:5 dilution into new cell culture dishes.

Human skin fibroblasts were grown to 100% confluency. Then the cells were rinsed once with 0,25% (w/v) Trypsin-0,5mM EDTA and incubated with 0,25% (w/v) Trypsin-0,5mM EDTA for approximately 5min at 37°C until the cells had detached from the cell

culture dish. The reaction was stopped by adding fresh medium containing 10% FCS and the cells were centrifuged for 5min at 800rpm (Heraeus Megafuge 1.0), resuspended in fresh medium and seeded at a 1:3 dilution into new cell culture dishes.

2.2.2.3 Freezing and thawing of cells

In order to generate a stock, cells of low passage numbers were frozen down in liquid nitrogen. When the cells had reached subculturing confluency, they were detached from the cell culture dishes as described under 2.2.2.2 and resuspended in cryoprotectant medium. For HeLa cells and human skin fibroblasts, the cryoprotectant medium was complete culture medium supplemented with 5% (v/v) sterile DMSO and for HUVECs the cryoprotectant medium was 10% (v/v) sterile DMSO in fetal calf serum. The cell suspension was transferred in 1ml aliquots into cryotubes (Nunc) and immediately placed into a propanol freezing canister at -80°C to allow a moderate temperature decline of 1°C per minute. On the next day, the cryovials were placed into liquid nitrogen for long term storage.

Cells were thawn by gently shaking the frozen cryotube in a 37°C water bath for approximately 1min until the cell suspension became liquid. Then the cell suspension was immediately pipetted into a fresh cell culture dish containing pre-warmed complete culture medium. The cells were allowed to attach to the cell culture dish over night and the medium was renewed thereafter.

2.2.2.4 Cell transfection

For transfection, HeLa cells were grown to 90-100% confluency and transfected with plasmid DNA using the PromoFectin transfection reagent (Promocell, Heidelberg, Germany) according to the manufacturer´s instructions, but using only 50% of the amount of DNA as this was found to result in better cell survival. Briefly, for one well of a 12-well plate, 1µg of plasmid DNA and 2µl PromoFectin transfection reagent were dissolved in 50µl serum free medium each at RT. When two plasmids were cotransfected, 0,75µg of each plasmid was used. Then the PromoFectin solution was added to the DNA solution at once, mixed, and

incubated for 20-30min at RT. The medium of the cells was replaced by fresh medium containing 10% FCS and the DNA/PromoFectin mix was added dropwise to the cells under constant swirling of the cell culture dish to allow instant distribution. For transfection in larger cell culture dishes, the amounts of DNA and transfection reagent were scaled up according to the manufacturer´s recommendations.

2.2.3 Protein biochemical methods

2.2.3.1 Preparation of cell lysates for protein analysis

For analysis of protein expression or activation of the MAP Kinase Erk or Akt, cells were washed with ice-cold PBS and lysed in 2x SDS sample buffer (120mM TrisHCl pH 6.8, 4% SDS, 20% glycerol, 0.01% bromphenol blue, 100mM DTT). Typically, cells in a well of a 6-well plate were lysed in 200-300µl 2x sample buffer and cells in a 10cm dish were lysed in 500µl 2x sample buffer. The lysates were transferred to a 1.5ml eppendorf reaction tube, boiled at 99°C for 5min, and vortexed immediately after boiling to ensure complete denaturation of the proteins.

2.2.3.2 Separation of proteins by SDS-PAGE

Proteins were separated according to their size by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Bio-Rad Mini-PROTEAN® Tetra System (Bio-Rad Laboratories, München, Germany). For separation of small proteins of less than 100kD, gels containing 10% polyacrylamide were used (4ml double distilled water, 3.33ml 30% acrylamide stock solution, 2.5ml 1.5M Tris pH 8.8, 50µl 20% SDS, 100µl 10% APS and 6µl TEMED for a final volume of 10ml). Larger proteins of up to 250kD were separated in gels containing 7,5% polyacrylamide (4,8ml double distilled water, 2.5ml 30% acrylamide stock solution, 2.5ml 1.5M Tris pH 8.8, 50µl 20% SDS, 100µl 10% APS and 6µl TEMED for a final volume of 10ml). The acrylamide solution was poured between two glass plates (a longer and a shorter one) that were separated by spacers and that had been inserted into a casting stand. The space between the glass plates was filled with the solution up to two cm from the top of the shorter glass plate and the acrylamide solution was overlaid with

ethanol and allowed to polymerize. After polymerization of the separation gel, the ethanol was discarded and the stacking gel (for 10ml final volume, 6.8ml double distilled water were mixed with 1.7ml 30% acrylamide stock solution, 2.5ml 0.5M Tris pH 6.8, 50 µl 20% SDS, 100µl 10% APS and 10µl TEMED) was poured on top of the separation gel. A comb was quickly inserted to form pockets in which the samples could be loaded. After polymerization of the stacking gel, the gel was inserted into a running chamber filled with SDS-PAGE running buffer (192mM glycine, 25mM Tris, 0.1% SDS). The protein samples were boiled in 2x SDS sample buffer at 99°C for 5min and loaded into the pockets of the stacking gel. A standard protein marker, the peqGOLD Protein-Marker IV (PEQLAB Biotechnologie, Erlangen, Germany), was also loaded and the samples were run at 90V into the stacking gel and at 150V in the separating gel until a satisfying protein separation was obtained (detected by the protein marker bands).

2.2.3.3 Western Blot transfer and protein detection

After electrophoresis, the proteins were transferred from the gel onto an Immobilion™ polyvinylidene fluoride (PVDF) membrane (Millipore, Schwalbach, Germany). The membrane was soaked in methanol for 1min and equilibrated with transfer buffer (192mM glycine, 20mM Tris, 10% methanol). Then the gel was placed onto the membrane and whatman papers soaked in transfer buffer were added to both sides of the sandwich. The transfer was performed in a blotting chamber filled with transfer buffer at 135V for 1.5-2h.

For protein detection with specific antibodies, the membrane was incubated in blocking solution (5% BSA in TBST (20mM Tris, 140mM NaCl, 0.2% Tween® 20, pH 7.6)) for 1h at RT to reduce unspecific binding of the antibodies to the membrane. Then the membrane was incubated with the respective primary antibody in blocking solution at 4°C over night (see table of antibodies for detailed information). Subsequently, the membrane was washed three times for 10min with TBST and incubated with a horseradish peroxidase-coupled secondary antibody diluted 1:2000 in blocking solution for 1h at RT. The secondary antibody was directed against the immunoglobulins of the host of the primary antibody. After washing the membrane again three times for 10min with TBST, the proteins were detected using the enhancer of chemioluminescence (ECL) western blotting substrate (Thermo Scientific, Dreieich, Germany). The signal was developed using Fuji Super RX 18x24 films (Ernst Christiansen GmbH, Planegg, Germany).

2.2.3.4 Membrane stripping

To use the membrane for protein detection several times, all bound antibodies had to be removed by membrane stripping. Therefore, the membrane was incubated in stripping solution (2%SDS, 63mM Tris-HCl pH6.8, 50mM DTT) at 55°C for 50min under constant shaking. After extensive washing with TBST (20mM Tris, 140mM NaCl, 0.2% Tween® 20, pH 7.6), the membranes could be reused for protein detection with another antibody.

2.2.3.5 Immunoprecipitation

For immunoprecipitation, cells on a 10cm cell culture dish were washed with ice-cold PBS and lysed in 500µl lysis buffer supplemented with protease and phosphatase inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 1mM sodium vanadate, 10mM sodium fluoride, 10µg/ml aprotinin, and 10µg/ml leupeptin). Lysis buffer A (25mM Tris-HCl pH 7.4, 150mM NaCl, 0,5% Triton X-100, 0.1% SDS) was used for immunoprecipitation of c-Met and HA-tagged proteins. Lysis buffer B (25mM HEPES pH 7.5, 100mM NaCl, 10mM $MgCl_2$, 1mM EDTA, 10% glycerol, 1% NP-40) was used for immunoprecipitation of c-Met, VEGFR-2, CD44v6, and Eps15. The lysates were transferred into 1.5ml eppendorf reaction tubes and incubated on a rotating wheel at 4°C for 30min to ensure complete cell lysis. Then the lysates were centrifuged at 12000g 4°C (Eppendorf centrifuge 5415 R) for 10min to remove cell debris. The supernatant was transferred into a new 1.5ml reaction tube and incubated with the respective antibody on a rotating wheel at 4°C over night (see table of antibodies for detailed information). Subsequently, 20µl each of agarose beads coupled to Protein A and Protein B respectively were added and incubated for 2h at 4°C on a rotating wheel. The precipitates were washed three times with the respective ice-cold lysis buffer, resuspended in 20µl 2x SDS sample buffer (120mM TrisHCl pH 6.8, 4% SDS, 20% glycerol, 0.01% bromphenol blue, 100mM DTT), boiled for 5min at 99°C and subjected to SDS-PAGE and Western Blot (see 2.2.3.2 and 2.2.3.3).

2.2.3.6 Co-immunoprecipitation

For co-immunoprecipitation of VEGFR-2, Neuropilin-1, and CD44v6, HUVECs were seeded on 15cm cell culture dishes and grown to 70% confluency. The cells were starved for 15-18h with 1% FCS in endothelial cell growth medium without further supplements and the cells were induced for 5min on 37°C with 40ng/ml VEGF-A_{165} or left untreated. Then the cells were washed once with ice-cold PBS and lysed in 1ml lysis buffer C (25mM Tris-HCl pH 7.6, 100mM NaCl, 0.5mM EGTA, 5% glycerol, 1% NP-40, 1mM PMSF, 1mM sodium vanadate, 10mM NaF, 10µg/ml aprotinin, and 10µg/ml leupeptin). The lysates were cleared by 10min centrifugation at 12000g 4°C (Eppendorf centrifuge 5415 R) and the supernatants were incubated with the respective primary antibodies (for VEGFR-2: 15µl/ml A-3; for CD44v6: 6µg/ml BIWA; for Neuropilin-1: 15µl/ml C-15) at 4°C over night on a rotating wheel. Then 30µl each of Agarose Beads coupled to Protein A or Protein B were added and incubated for 2h at 4°C on a rotating wheel. The precipitates were washed three times with ice-cold lysis buffer C, resuspended in 20µl 2x SDS sample buffer (120mM TrisHCl pH 6.8, 4% SDS, 20% glycerol, 0.01% bromphenol blue, 100mM DTT), boiled for 5min at 99°C and subjected to SDS-PAGE and Western Blot (see 2.2.3.2 and 2.2.3.3).

2.2.3.7 CD44v6 peptide blocking

HeLa cells were seeded in 6-well cell culture dishes, grown to 70% confluency, and starved for 24 hours with serum free medium. Cells were treated with either a CD44v6 specific blocking peptide or a control peptide (100ng/ml) for 10min at 37°C prior to induction with 25ng/ml HGF for the indicated time periods. Cell lysates were prepared as described in 2.2.3.1) and subjected to Western Blot analysis for phosphorylated Met (see 2.2.3.2 and 2.2.3.3).

2.2.3.8 Potassium-depletion assay

HeLa cells were seeded in 6-well cell culture dishes, grown to 70% confluency, and starved for 24 hours with serum free medium. Then all cells were washed three times with control buffer (10mM KCl, 20mM HEPES pH 7.4, 140mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$,

0.1% (w/v) glucose, 0,2% (w/v) BSA) and incubated in this buffer for 30min at 37°C. Subsequently the cells were washed three times with either control buffer or potassium free buffer (control buffer without KCl) and thereafter incubated with a hypotonic solution of the respective buffer (1:1 dilution of control buffer or potassium free buffer with H_2O) for 1-2min on 37°C. Then the cells were washed twice with control buffer or potassium free buffer and incubated in the respective buffer for 30min on 37°C. For activation of the receptor c-Met, HGF was added to the respective buffer to give a final concentration of 25ng/ml and the cells were incubated with the HGF-containing buffer on 37°C for the indicated time periods. To stop the induction, the cells were washed twice with ice-cold PBS, lysed in 300µl 2x SDS sample buffer (120mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.01% bromphenol blue, 100mM DTT), and the lysates were subjected to SDS-PAGE and Western Blot analysis (see 2.2.3.2 and 2.2.3.3).

Blocking receptor internalization by potassium depletion was confirmed by immunofluorescence. Therefore, HeLa cells were seeded on glass coverslips in 6-well plates and treated in the same way as described above. After HGF-induction, the cells were washed twice with ice-cold PBS and fixed immediately with pre-chilled Methanol at -20°C for 10min. Nuclei and c-Met were stained as described under 2.2.4.1 and the samples were analyzed at a Leica SPE confocal microscope using a 63x objective.

2.2.3.9 Ubiquitylation assay

$1x10^6$ HeLa cells were seeded on 10cm cell culture dishes and transfected with the tailless CD44v6 construct or the control vector. 24 hours after transfection, cells were serum starved over night and induced with 25ng/ml HGF on 37°C for the indicated time periods. Then cells were washed with ice cold PBS and lysed in 500µl lysis buffer A (25mM Tris-HCl pH 7.4, 150mM NaCl, 0,5% Triton X-100, 0.1% SDS, 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM sodium vanadate, 10mM sodium fluoride, 10µg/ml aprotinin, and 10µg/ml leupeptin) containing 2.5mg/ml deubiquitinase-inhibitor N-ethylmaleimide (Sigma-Aldrich, Taufkirchen). Cell lysates were cleared by 10min centrifugation at 12000g and an aliquot was taken to check for the expression of the transfectant. Met was immunoprecipitated by incubating the lysates with the anti-Met antibody 25H2 over night at 4°C and subsequent incubation with agarose beads coupled to Protein A/G for 2-3 hours at 4°C. Precipitates were washed four times with lysis buffer and bound proteins were separated by SDS-PAGE and

Western Blot (see 2.2.3.2 and 2.2.3.3). The western blot membrane was immediately denaturated with 6M Guanidin-HCl, 20mM Tris pH7.4, 1mM PMSF, and 5mM 2-Mercaptoethanol for 30min at 4°C, washed four times with TBST (20mM Tris, 140mM NaCl, 0.2% Tween® 20, pH 7.6) and blocked over night at 4°C with 5% BSA in TBST. Then, the membrane was incubated with the anti-ubiquitin antibody P4D1 (Cell Signaling, Beverly, England) diluted 1:1000 in blocking solution for 1 hour at RT, washed three times 10min with TBST, incubated with HRP-coupled secondary antibody and developed with ECL.

2.2.3.10 VEGF and HGF induction kinetics

To compare the kinetics of HGF- and VEGF-A_{165}- induction, HUVECs were seeded on 10cm cell culture dishes and grown to 70-80% confluency. Then the cells were starved for 15-18h with endothelial cell growth medium supplemented with 1% FCS but without any further growth factors. (Due to the sensitivity of HUVECs to starvation, 1% FCS was added to the starvation medium because this improved cell viability and inducibility.) After starvation, the cells were induced for the indicated time periods on 37°C with either 25ng/ml HGF or 40ng/ml VEGF-A_{165}. Thereafter the cells were washed once with ice-cold PBS and lysed in 500µl lysis buffer B (25mM HEPES pH 7.5, 100mM NaCl, 10mM $MgCl_2$, 1mM EDTA, 10% glycerol, 1% NP-40, 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM sodium vanadate, 10mM sodium fluoride, 10µg/ml aprotinin, and 10µg/ml leupeptin). The lysates were centrifuged for 10min at 12000g and 4°C to remove cell debris. To analyze the activation of the MAP Kinase Erk, an aliquot of 50µl was taken from the supernatants, diluted with 50µl 2x SDS sample buffer, boiled for 5min at 99°C and subjected to SDS-PAGE and Western Blot (see 2.2.3.2 and 2.2.3.3). The rest of the supernatants was taken for immunoprecipitation of VEGFR-2 or c-Met respectively as described under 2.2.3.5. The precipitates were subjected to SDS-PAGE and Western Blot, and the membrane was probed with specific antibodies for phospho-Met or phospho-VEGFR2.

2.2.4 *In vitro* experiments

2.2.4.1 Immunofluorescence

20mm glass cover slips were placed in 12-well cell culture dishes and $5x10^4$ cells were seeded in each well. When indicated, the cells were transfected as described under *2.2.2.4*. 24h after transfection, the cells were starved over night in serum free medium and 2h prior HGF-induction, 50µM cycloheximide was added to the medium to block de novo protein synthesis. When indicated, cells were treated with 100ng/ml of a CD44v6-specific blocking peptide or a control peptide for 10min at 37°C prior to HGF-induction. Subsequently, the cells were chilled on ice and incubated with 25ng/ml HGF in serum free medium for 1h on ice to allow binding of the growth factor to the receptors while preventing receptor internalization. Subsequently, the cells were incubated on 37°C for the indicated time periods to allow receptor internalization. To stop the internalization, cells were washed with ice-cold PBS and fixed immediately with pre-chilled methanol (-20°C) for 10min at -20°C. After fixation, cells were washed three times with PBS and incubated with blocking solution (5% FCS + 0,2% Triton X-100 + 0,05% Tween in PBS) for 1 hour at RT. When fluorescence-tagged proteins had been transfected, cells were fixed with 4% paraformaldehyde in PBS for 15min at room temperature, washed three times for 10min with PBS, and permeabilized with 0,3% Triton X-100 for 3min at room temperature prior to incubation with blocking solution. Subsequently, the cells were incubated with the respective primary antibodies in blocking solution over night at 4°C. After three washes with PBS, the respective Alexa Fluor labeled secondary antibodies were applied together with Draq5 or Dapi (for nucleus staining) in blocking solution for 1h at RT. Finally, the cells were washed three times with PBS and mounted on glass slides with fluorescent mounting medium (Dako, Hamburg, Germany). Samples were analyzed with a Zeiss LSM or Leica SPE confocal microscope using a 63x objective.

2.2.4.2 Fibrin bead assay

2.2.4.2.1 Coating of beads with HUVECs

For the fibrin bead assay, HUVECs of maximum passage 8 were grown on a 10cm cell culture dish, trypsinized as described under 2.2.2.2, and resuspended in complete endothelial

cell growth medium. The cells were counted in a Neubauer counting chamber, and $1x10^6$ cells were used for coating of 40µl Cytodex-3 bead suspension (GE Healthcare Europe, Freiburg, Germany). The beads were washed with complete endothelial cell growth medium and mixed with the HUVECs in a total volume of 4ml complete endothelial cell growth medium in a FACS-tube (polypropylene). The tube was then left for at least 4h in a cell culture incubator with shaking every 20min. Thereafter, the cell-bead-suspension was transferred into a 10cm cell culture dish with 5ml of complete endothelial cell growth medium and left in a cell culture incubator over night. Cells that had not attached to the beads were now attaching to the cell culture dish and could be separated from the coated beads. At the same day, a 10cm cell culture dish of confluent human skin fibroblasts was washed twice with PBS and incubated in complete endothelial cell growth medium over night.

2.2.4.2.2 Embedding of coated beads in fibrin gel

On the second day, a fresh 2,5mg/ml fibrinogen solution was prepared by dissolving the required amount of fibrinogen Type I (Sigma, Taufkirchen, Germany) in endothelial starvation medium (EGM-2 medium without VEGF, EGF, FGF, IGF and FBS). For one 96-well-plate 7ml fibrinogen solution was needed. Then the medium from the cell culture dish containing the coated beads was carefully discarded without detaching the beads and the dish was rinsed twice with 5ml endothelial cell growth medium to wash off the beads. The bead suspension was transferred into a 50ml Falcon tube and the beads were washed twice with PBS. The fibrinogen solution was sterile filterized, the beads were resuspended in the fibrinogen solution, and 25µl of a 4U/ml aprotinin solution were added per 1ml bead-fibrinogen-solution. Then 8µl of a 10U/ml thrombin solution were pipetted into each well of a 96-well-plate and 90µl of the fibrinogen-bead-suspension were added. The 96-well-plate was first incubated at RT for 5min and then transferred to 37°C into a cell culture incubator for 2-4h until the gel had polymerized.

2.2.4.2.3 Covering the gel with fibroblasts

After 2-4h, after the gel had become solid, the fibroblasts were seeded on top of the gel. It is essential to make sure that the gel has become solid before adding the fibroblasts, to

prevent that the fibroblasts will sink into the gel and cannot be distinguished from angiogenic sprouts. The human skin fibroblasts were trypsinized as described under 2.2.2.2, counted in a Neubauer counting chamber, and resuspended in endothelial starvation medium to a final concentration of 25000 cells/ml. Then 200µl of the cell suspension, corresponding to 5000 cells, were pipetted into each well and allowed to attach to the fibrin gel over night.

2.2.4.2.4 Induction of angiogenic sprouting

After the fibroblasts had attached to the fibrin gel, the medium was carefully discarded without damaging the gels and angiogenic sprouting was induced by adding 200µl endothelial starvation medium containing the indicated growth factors. Each condition was applied in at least three different wells. Complete endothelial cell growth medium was applied as a positive control, and endothelial starvation medium without any further growth factors was applied as a negative control. The medium was renewed every second day.

2.2.4.2.5 Fixation and staining

After one week the angiogenic sprouts were washed three times for 5min with PBS and fixed with 200µl 4% paraformaldehyde per well at 4°C over night. The next day the plate was washed three times with PBS and incubated with blocking solution (1% BSA + 0.2% Triton-X100 in PBS). Then nuclei were stained with Dapi (1:1000) and actin was stained with Alexa 546-phalloidin (1:500) diluted in blocking solution at 4°C over night. Finally, the plate was extensively washed with PBS and the angiogenic sprouts were imaged with an Olympus IX81 confocal microscope using a 2x objective.

3. Results

Met and VEGFR-2 are receptor tyrosine kinases (RTKs) that control cell proliferation, migration, survival and branching morphogenesis. Both receptors are crucial for embryonic development and tissue homeostasis in the adult. Both Met and VEGFR-2 have been shown to interact with CD44v6 upon induction with their respective ligands and activation of both receptors is strictly dependent on CD44v6 (Orian-Rousseau et al, 2002; Tremmel et al, 2009). Therefore, the mechanism of their activation might be similar. Like most other RTKs, Met and VEGFR-2 get only transiently activated upon ligand-binding and are internalized by clathrin-mediated endocytosis (Petrelli et al, 2002:Salikhova, 2008 #1241). Internalization of Met upon induction with HGF has recently been shown also to be dependent on CD44v6 using biochemical methods (Hasenauer, 2010). My PhD project is divided into two parts. In the first part, I analyzed the HGF-induced internalization of Met and CD44v6 using immunofluorescence techniques and studied the mechanism by which CD44v6 mediates Met internalization. In the second part, I investigated if there are differences in the activation and internalization between Met and VEGFR-2.

Part I. The role of CD44v6 in the internalization of Met

3.1 Visualization of Met internalization by immunofluorescence microscopy

To study the role of CD44v6 in the HGF-induced internalization of Met, we have chosen HeLa cells which express both CD44v6 and Met and in which the HGF-induced internalization of Met has been studied extensively (Abella et al, 2005; Hammond et al, 2001; Kermorgant et al, 2003; Kermorgant et al, 2004; Parachoniak et al, 2011; Parachoniak & Park, 2009; Peschard et al, 2001).

The first step of my studies consisted in the visualization of Met internalization into endosomes and subsequent intracellular trafficking upon HGF induction. To this aim, I used immunofluorescence microscopy which allows determining the intracellular localization of proteins with specific fluorescence-labeled antibodies. As a marker for endosomes, I used the

endosomal sorting protein HRS (hepatocyte-growth-factor-regulated tyrosine-kinase substrate) which binds to ubiquitylated proteins on early endosomes and mediates their sorting into recycling endosomes or into multivesicular bodies (MVBs) (Bache et al, 2003a; Gruenberg & Stenmark, 2004; Katzmann et al, 2002). HRS remains associated with the receptors during their trafficking through early and late endosomes.

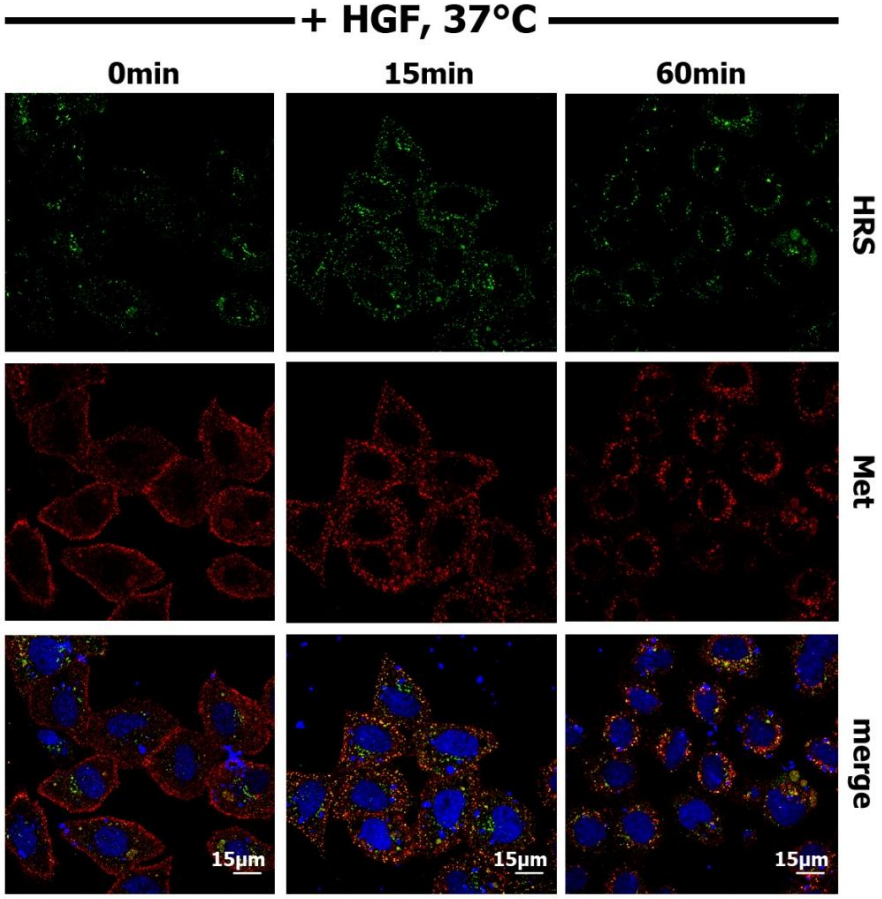

Fig.3.1 Met is internalized into HRS-positive endosomes upon HGF-induction.

HeLa cells were serum starved and subsequently treated with 50µM cycloheximide for 2h to block protein synthesis. Then the cells were incubated with 25ng/ml HGF for 1h on ice and shifted to 37°C for the time periods indicated to allow receptor internalization to take place. Finally, the cells were fixed, permeabilized, and stained for endogenous Met (red) and HRS (green) with specific primary antibodies and Alexa Fluor-labeled secondary antibodies. Nuclei were stained with the DNA-binding dye Draq5 and images were taken with a confocal microscope (Zeiss LSM510) using a 63x objective.

HeLa cells were serum starved and then treated with cycloheximide to block de novo protein synthesis in order to reduce intracellular staining of Met. Subsequently, the cells were incubated with HGF for 1h on ice, a step that allows the binding of growth factors to the

receptors but blocks internalization as the membranes become stiff at low temperatures (Gorden et al, 1978). This procedure is referred to as a cold start. Following the cold start, receptor internalization was allowed to take place by incubation of the cells at 37°C for different time periods. Cells were then fixed, permeabilized and stained with specific antibodies for Met and HRS.

Directly after the cold start, without subsequent incubation at 37°C, Met was exclusively located at the plasma membrane of the cells and was not co-localized with HRS (Fig.3.1). 15min after the temperature switch to 37°C, Met was located in dotted structures in the cell periphery close to the plasma membrane where it co-localized with the endosomal marker HRS. After 60min at 37°C, Met and HRS were still co-localized on dotted structures, but were now found close to the nucleus. This is in line with previously published data showing that Met is internalized into HRS-positive endosomes (Abella et al, 2005) and trafficks to perinuclear compartments (Kermorgant et al, 2003) upon HGF-induction. These results indicated that using confocal microscopy it is possible to study the internalization process of Met.

3.2 A CD44v6 peptide blocks HGF-induced Met internalization

Met-dependent metastasis of pancreatic carcinoma cells (Matzke et al., submitted) and Met-induced angiogenesis (Tremmel et al, 2009) can be blocked with a small peptide mimicking an essential sequence within the variant v6-region of CD44v6 (Matzke et al, 2005). We hypothesized that the v6 peptide binds to CD44v6, interfering with an intramolecular interaction within CD44v6. This might lead to the disruption of the CD44v6 structure and prevent the co-receptor function (Groner, 2009). To test whether the co-receptor function of CD44v6 is required for Met internalization, HeLa cells were incubated with a v6 peptide or a control peptide prior to the induction with HGF. The cells were fixed after different incubation periods at 37°C and the internalization of Met was analyzed with immunofluorescence microscopy. In the absence of HGF, Met was exclusively located at the plasma membrane of the cells both after treatment with the v6 peptide and the control peptide (Fig.3.2). After 30 and 60min incubation with HGF at 37°C, Met was found internalized into endosomal compartments in cells that had been treated with the control peptide. In contrast, Met internalization was inhibited and Met remained at the plasma membrane in cells that had been treated with the v6 peptide. The v6 peptide also interfered with the activation of Met as

shown in the Western Blot in Fig3.2. Therefore, the v6 peptide inhibits HGF-induced Met internalization by blocking the activation of Met by HGF.

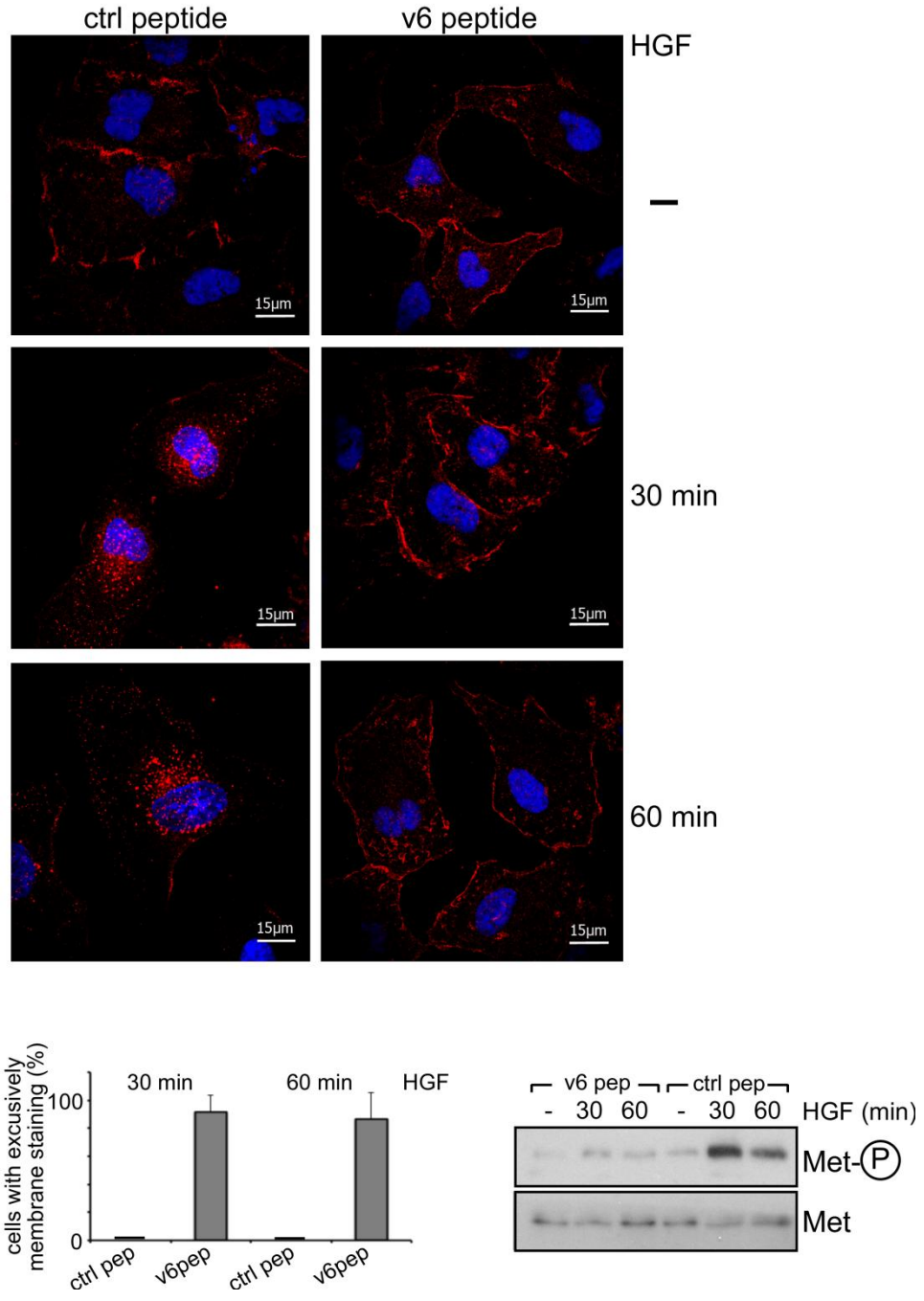

Fig.3.2 HGF-induced Met internalization can be blocked with a CD44v6 peptide.

Serum starved HeLa cells were treated with 50µM cycloheximide to block protein synthesis and incubated with a v6 peptide or a control peptide for 10min at 37°C. Cells were then induced with 25ng/ml HGF at 37°C for the indicated time periods. Subsequently, cells were either lysed and the lysates were subjected to Western Blot analysis for phospho-Met and Met (below) or cells were fixed and permeabilized and stained for Met (red) with specific antibodies (above). Nuclei were stained with Dapi and images were taken with a confocal microscope (Leica SPE) using a 63x objective. The quantification shows mean values of three independent experiments.

3.3 CD44v6 traffics together with Met upon HGF-induction

In order to address the possible role of CD44v6 in Met internalization and intracellular trafficking, I first tested whether CD44v6 traffics with Met through the same endosomal compartments upon HGF induction. Therefore, HeLa cells were starved, treated with cycloheximide, and induced with HGF for 1h on ice (cold start). After subsequent incubation at 37°C for the indicated time points, cells were fixed and endogenous Met and CD44v6 was stained with specific antibodies.

At the cold start, both Met and CD44v6 were exclusively located at the plasma membrane of the cells (see Fig.3.3A). After 15min incubation at 37°C, the membrane staining faded and CD44v6 and Met were co-localized on dotted structures in the cell periphery that resembled endosomes. Met and CD44v6 were still co-localized on these dotted structures after 30 and 60min at 37°C but had moved closer and closer to the nucleus over time.

Co-localization of CD44v6 and Met was quantified by calculating the mean Pearson Coefficient (PC) of six images per time point using the Imaris software. The value of the PC can range from 1 to -1 with 1 indicating complete co-localization and -1 exclusion of the pixels from two channels (Bolte & Cordelieres, 2006). For all time points the PC and the merged images indicated co-localization of CD44v6 and Met (Fig.3.3A). Therefore, CD44v6 seems to associate with Met at the plasma membrane and both proteins are internalized and traffic together upon HGF-induction.

The intensity profiles of the green (CD44v6) and the red (Met) signal were also measured for all time points (Fig.3.3B). At the cold start, the intensities of both signals were measured at the plasma membrane, for the time points after temperature shift the intensity profiles were measured across vesicles containing both CD44v6 and Met. In all cases the intensity profile of the red channel fitted to the intensity profile of the green channel, indicating co-localization. Furthermore, the peaks for 15, 30 and 60min at 37°C have a width that corresponds to the size of endosomes (0.5-1µm).

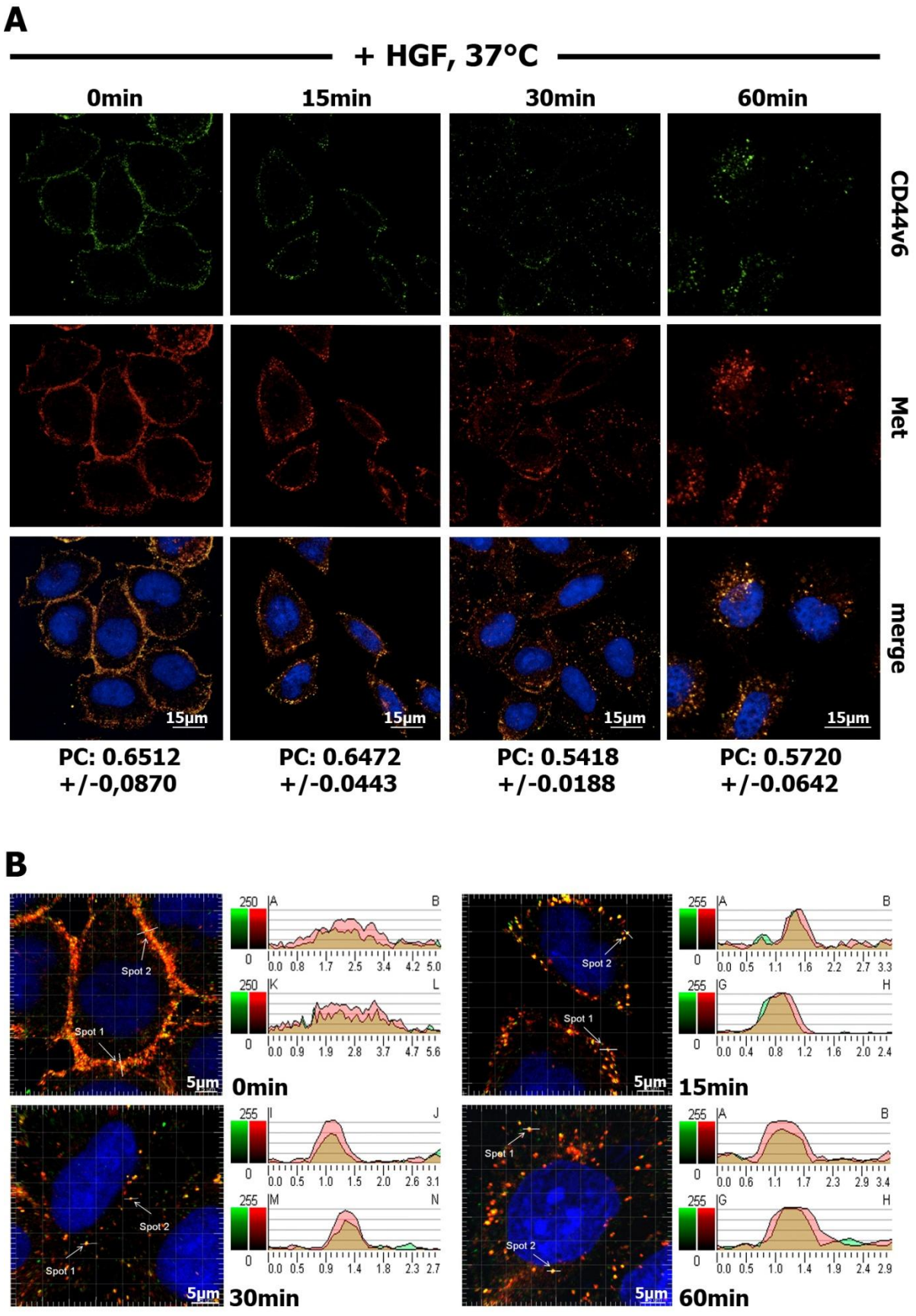

Fig.3.3 CD44v6 traffics together with Met upon HGF-induction.

A Serum starved HeLa cells were treated for 2h with 50µM cycloheximide, incubated with 25ng/ml HGF for 1h on ice (cold start), and then shifted to 37°C for the indicated time periods. After fixation and permeabilization, cells were stained for endogenous CD44v6 (green) and Met (red) with specific primary antibodies and Alexa Fluor-labelled secondary antibodies. Nuclei were stained with Draq5. Images were taken with a confocal microscope (Zeiss LSM510) using a 63x objective. For each time point, the mean Pearson Coefficient of six images was calculated using the Imaris software.
B Intensity profiles of the CD44v6 (green) and Met (red) signal were measured along lines drawn across the plasma membrane or vesicles containing CD44v6 and Met. The intensity of the respective emission wavelength is plotted against the distance in µm.

3.4 Met and CD44v6 traffic together through Rab5-endosomes upon HGF-induction

Receptors that are internalized via clathrin-mediated endocytosis enter the cells in clathrin-coated vesicles. The small GTPase Rab5 subsequently mediates the transport to early endosomes. Receptors can then be recycled back to the plasma membrane through a fast recycling pathway or a slow recycling pathway. Each recycling pathway is driven by another Rab family member: fast recycling from early endosomes is mediated by Rab4 and slow recycling from late endosomes is mediated by Rab11. If receptors are sorted for degradation, they are transported to lysosomes by Rab7 (reviewed in (Zwang & Yarden, 2009)). The exact trafficking route of internalized receptors can therefore be elucidated by immunofluorescence microscopy using different fluorescence-labeled proteins of the Rab family.
The trafficking of Met and CD44v6 to early endosomes was confirmed by transfection of mRFP (monomeric red fluorescence protein)-labeled wild type Rab5 (Vonderheit & Helenius, 2005) into HeLa cells. After transfection of mRFP-Rab5, the fixation with methanol that was used in the previous experiments could not be used since this results in the destruction of the mRFP protein structure and the loss of the fluorescent signal. Instead, paraformaldehyde fixation was applied which resulted in very high background staining with the CD44v6 antibody. To overcome this drawback, I expressed a human CD44v6 protein from an expression vector that was co-transfected with the mRFP-Rab5 construct. After transfection cells were starved, treated with cycloheximide and subjected to a cold start. Then the cells were fixed immediately or shifted to 37°C for 15min before fixation. Cells were thereafter permeabilized and stained with specific antibodies for Met and CD44v6 and images were taken at a confocal microscope.

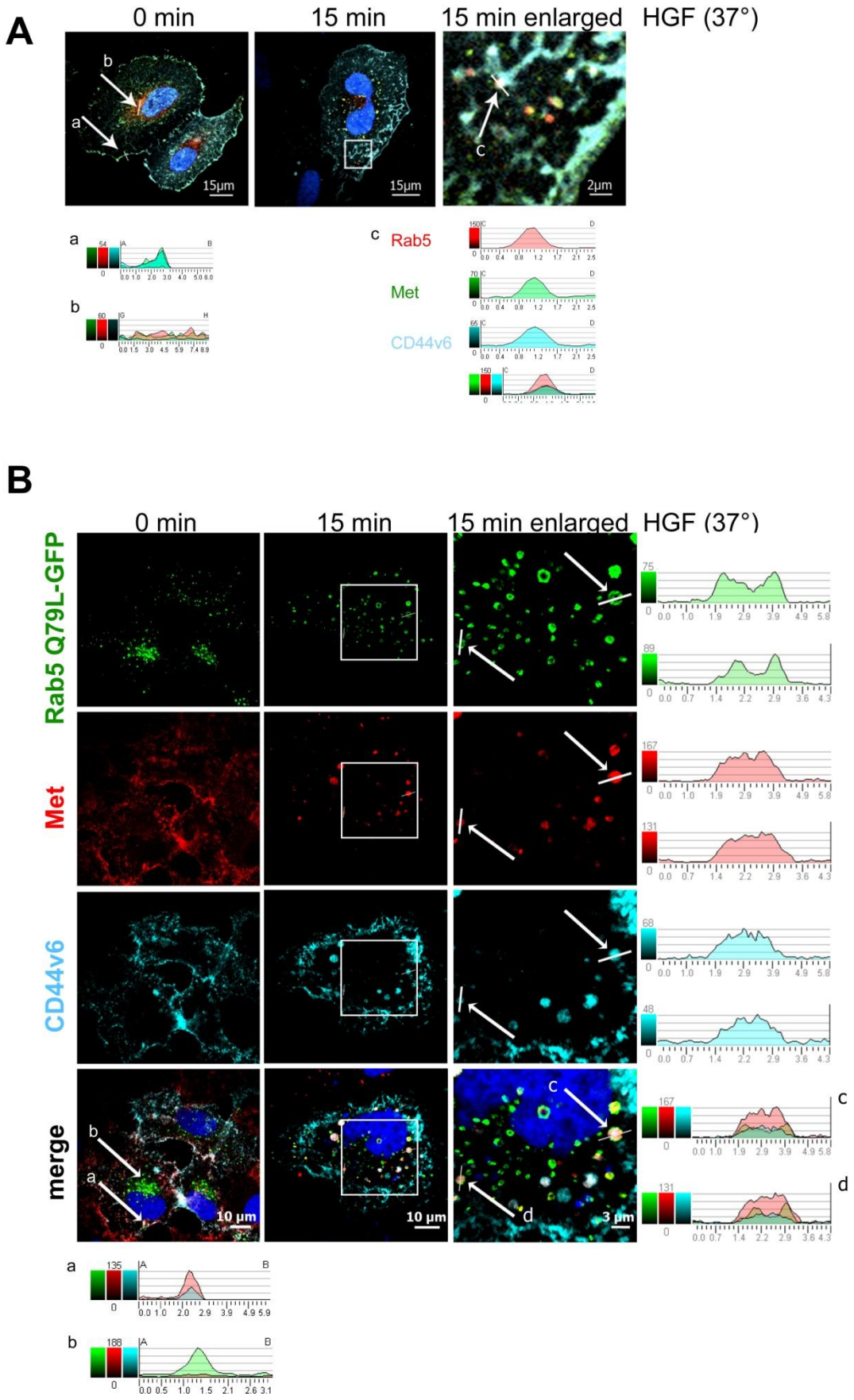

Fig.3.4 Met and CD44v6 traffic through Rab5-positive endosomes upon HGF-induction.

A HeLa cells were co-transfected with mRFP-Rab5 wild type and CD44v6. 24h after transfection, cells were serum starved over night and treated for 2h with 50µM cycloheximide. Cells were then incubated with 25ng/ml HGF for 1h on ice (cold start) and fixed immediately or shifted for 15min to 37°C before fixation. Thereafter, cells were permeabilized and stained for CD44v6 (cyan) and Met (green) with specific primary antibodies and Alexa Fluor-labeled secondary antibodies. Nuclei were stained with Dapi and images were taken with a confocal microscope (Leica SPE) using a 63x objective. Intensity profiles of the mRFP-Rab5 (red), Met (green), and CD44v6 (cyan) signals were measured along lines drawn across the plasma membrane (a) or across vesicles (b and c). The intensity of the respective emission wavelength is plotted against the distance in µm.
B HeLa cells were co-transfected with the constitutive active Rab5 construct (Rab5Q79L-GFP) and CD44v6 and treated as in A. Intensity profiles of the Rab5 Q79L-GFP (green), Met (red), and CD44v6 (cyan) signals were measured along lines drawn across the plasma membrane (a) or across vesicles (b, c, d).

The merged images demonstrate that Met and CD44v6 were not co-localized with Rab5 directly after the cold start without subsequent 37°C shift (Fig3.4A). Intensity profiles measured at the plasma membrane (Fig.3.4A a) and at Rab5-containing vesicles (Fig.3.4A b) show co-localization of Met (green) and CD44v6 (cyan) at the plasma membrane but no co-localization with Rab5 (red). After subsequent incubation of the cells at 37°C however, co-localization of Met, CD44v6 and Rab5 was observed on vesicular structures (see merged images and intensity profiles c in Fig.3.4A).

Endosomal vesicles containing mRFP-Rab5 wild type were rather small and difficult to detect. Constitutive active Rab5, where the intrinsic GTPase activity is lost due to a point mutation (Q79L), causes increased early endosomal vesicle fusion and results in the formation of large vesicles (Stenmark et al, 1994). Such a GFP (green fluorescent protein)-labelled constitutive active Rab5 construct (Rab5Q79L-GFP) was used to increase the size of the Rab5 endosomes for better detection. This construct has been used in previous studies for the investigation of the intracellular trafficking route of internalized receptors (Ballmer-Hofer et al, 2011). HeLa cells were co-transfected with the Rab5Q79L-GFP construct and the human CD44v6 construct. Then cells were treated as described for transfection with mRFP-Rab5 and images were taken with a confocal microscope. Directly after the cold start, Met (red) and CD44v6 (cyan) were located at the plasma membrane of the cells and there was no Met or CD44v6 staining on Rab5Q79L-containing vesicles (green) as shown in Fig3.4B (0min 37°C). This was confirmed by measuring the intensity profiles of each channel across the plasma membrane (Fig.3.4B a) and across Rab5 vesicles (Fig.3.4B b). Co-localization of Met, CD44v6 and Rab5Q79L was observed on vesicular structures upon subsequent temperature increase to 37°C for 15min (see images and intensity profiles on the right in Fig3.4B). So, the

results obtained with the constitutive active Rab5 confirmed the findings obtained with wild type Rab5.

In future experiments, transfections with fluorescence-labeled Rab4, Rab7, and Rab11 constructs will reveal the intracellular trafficking route of CD44v6 and Met upon HGF-induction.

3.5 The cytoplasmic domain of CD44v6 is required for HGF-induced internalization of Met

The cytoplasmic domain of CD44v6 mediates downstream signaling from the Met receptor by binding to ERM proteins that link the receptor complex to the actin cytoskeleton. The cytoplasmic domain of CD44v6 is however not required for the activation of Met by its ligand HGF which is only dependent on the extracellular part of CD44v6 (Orian-Rousseau et al, 2002; Orian-Rousseau et al, 2007).

In a first instance, I tested whether expression of a human CD44v6 protein with a deletion of the cytoplasmic part would alter the internalization or trafficking of Met upon HGF-induction. To do so, a truncated human CD44v6 construct missing the cytoplasmic domain was generated and transfected into HeLa cells. As a control, a full length human CD44v6 construct (used in the previously shown experiments for co-transfection with Rab5) was also generated. 24h after transfection with either the wild type (wt) or the tailless CD44v6 construct, cells were serum starved, treated with cycloheximide and subjected to a cold start. Then cells were incubated for the indicated time periods at 37°C, fixed, permeabilized, and stained with specific antibodies for Met and CD44v6. Both CD44v6 wt and CD44v6 tailless proteins can be detected with the v6-specific antibody as the v6-region is located in the extracellular part of the transmembrane protein.

Directly after the cold start, Met was exclusively located at the plasma membrane in both the CD44v6 wt-transfected cells and in the CD44v6 tailless-transfected cells (Fig3.5). At this time point both CD44v6 wt and CD44v6 tailless were co-localized with Met at the plasma membrane. 15min after the temperature switch to 37°C, Met could be detected on dotted structures resembling endosomes in cells that had been transfected with the wt CD44v6. These vesicles were found more close to the nucleus after 30 and 60min incubation on 37°C. In striking contrast, Met internalization was drastically reduced in cells transfected with the tailless mutant CD44v6 (also shown in the graph in Fig.3.5). In these cells Met was still

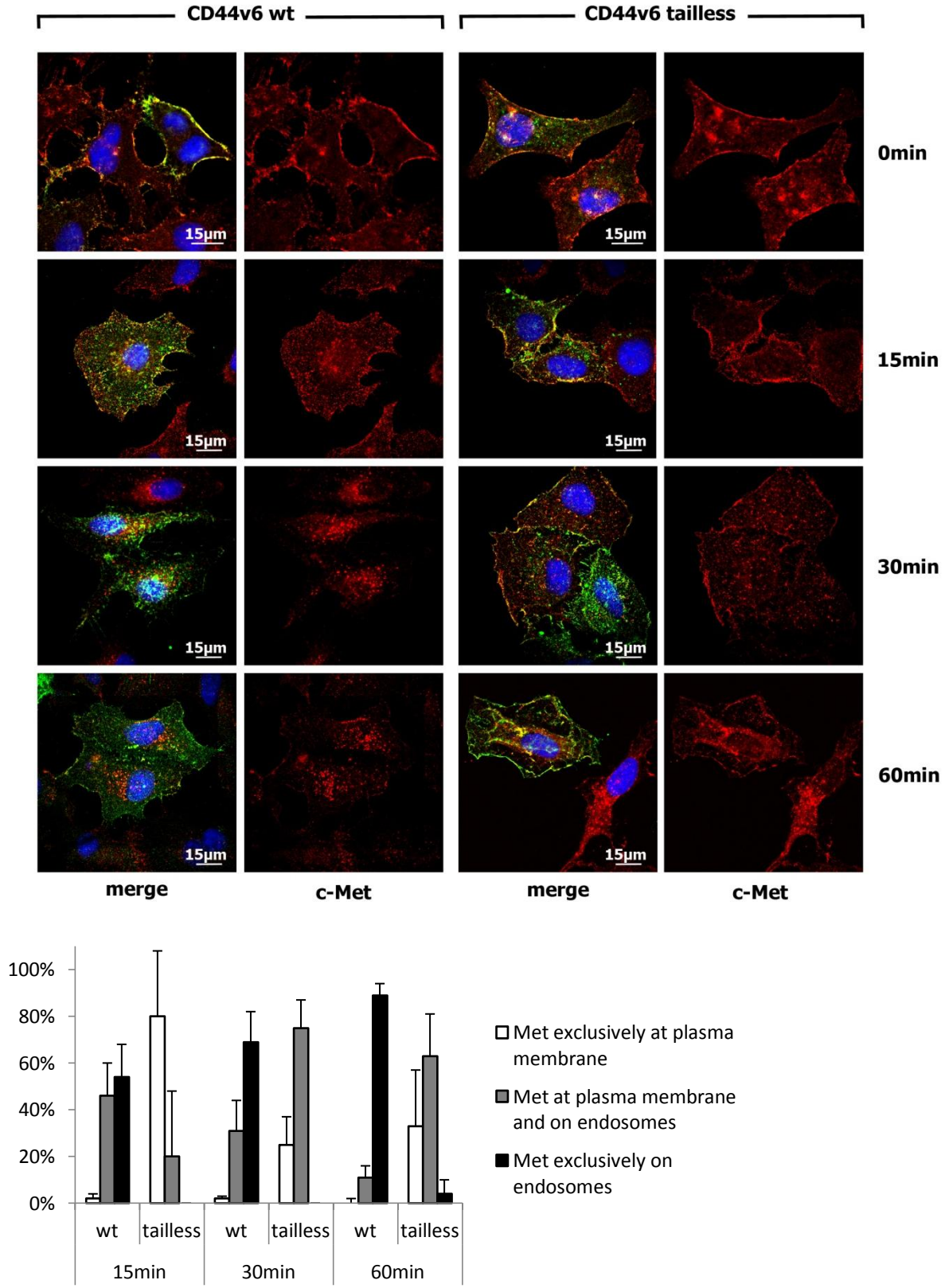

Fig.3.5 HGF-induced internalization of Met can be blocked with a tailless mutant of CD44v6.

HeLa cells were transfected either with wild type CD44v6 or with a CD44v6 tailless mutant. 24h post transfection, cells were serum starved over night and subsequently incubated for 2h with cycloheximide to block protein synthesis. Then cells were incubated with 25ng/ml HGF for 1h on ice (cold start) and shifted to 37°C for

the indicated periods. Thereafter, cells were fixed, permeabilized, and stained with specific antibodies for Met (red) and CD44v6 (green). Nuclei were stained with Dapi and images were taken at a confocal microscope (Leica SPE) using a 63x objective. For quantification, the percentage of transfected cells with Met exclusively at the plasma membrane or exclusively on endosomes or both at the plasma membrane and on endosomes was calculated for each time point. The results are shown as the mean value of three independent experiments.

absent from endosomes at 15min post temperature switch to 37°C. As a measure for the internalization I counted transfected cells in which Met was located exclusively at the plasma membrane or exclusively on endosomes or both at the plasma membrane and on endosomes (see graph in Fig.3.5 for quantification). After 60min at 37°C, Met was exclusively located on endosomes in ca. 89% of the CD44v6 wt-transfected cells in contrast to only ca. 4% of the CD44v6 tailless-transfected cells.

These results show that the cytoplasmic part of CD44v6 is indeed necessary for the internalization of Met. As the cells still contain endogenous CD44v6, the internalization of Met was drastically reduced but not completely blocked upon transfection with the tailless CD44v6 mutant. In addition to the cytoplasmic part of CD44v6, the link between ERM (ezrin, radixin, moiesin) proteins and the actin cytoskeleton has also been shown to be required for the HGF-induced internalization of Met. Analysis of Met internalization with a biochemical method that allows to detect the amount of internalized protein on a Western Blot revealed that transfection of HeLa cells with an Ezrin construct mutated in the actin-binding domain had a similar effect on HGF-induced Met internalization as transfection with a tailless CD44v6 mutant (Hasenauer, 2010).

3.6 Signaling mediates Met internalization

The cytoplasmic domain of CD44v6 as well as the link of CD44v6 to the actin cytoskeleton through ERM proteins has been demonstrated to be essential for HGF-induced downstream signaling from Met (Orian-Rousseau et al, 2007). The finding that the same components are essential for the internalization of Met raises the question whether internalization of Met is required to induce downstream signaling or whether it is on the contrary that CD44v6-mediated downstream signaling is needed to trigger internalization.

In order to find out whether Met internalization is required for HGF-induced downstream signaling, potassium (K^+) depletion was used to block clathrin-mediated

endocytosis. Potassium depletion causes the disassembly of the clathrin coat from clathrin-coated pits and specifically inhibits clathrin-mediated endocytosis (Larkin et al, 1983; Larkin et al, 1986). Therefore, K^+ depletion is one of the traditional methods to block clathrin-mediated endocytosis (McMahon & Boucrot, 2011; Sorkin & Von Zastrow, 2002).

HeLa cells were serum starved and treated with a K^+-free buffer or a K^+-containing control buffer (for a detailed description of the procedure see section 2.2.3.8). The cells were subsequently induced with 25ng/ml HGF in the respective buffer at 37°C for the indicated time periods. The inhibition of Met internalization by K^+ depletion was confirmed by immunofluorescence microscopy and activation of downstream signaling pathways was analyzed by Western Blot. We analyzed the activation of the PI3-Kinase – Akt pathway and the Ras – MAP Kinase Erk pathway since activation of these pathways has been demonstrated to depend on the Met-CD44v6-Ezrin-actin complex (Orian-Rousseau et al, 2002; Orian-Rousseau et al, 2007). For immunofluorescence microscopy, cells were fixed, permeabilized, and stained with specific antibodies for Met and images were taken with a confocal microscope. To detect the activation status of Akt and Erk, cells were lysed and subjected to Western Blot analysis for phospho-Akt, total Akt, phospho-Erk, and total Erk levels.

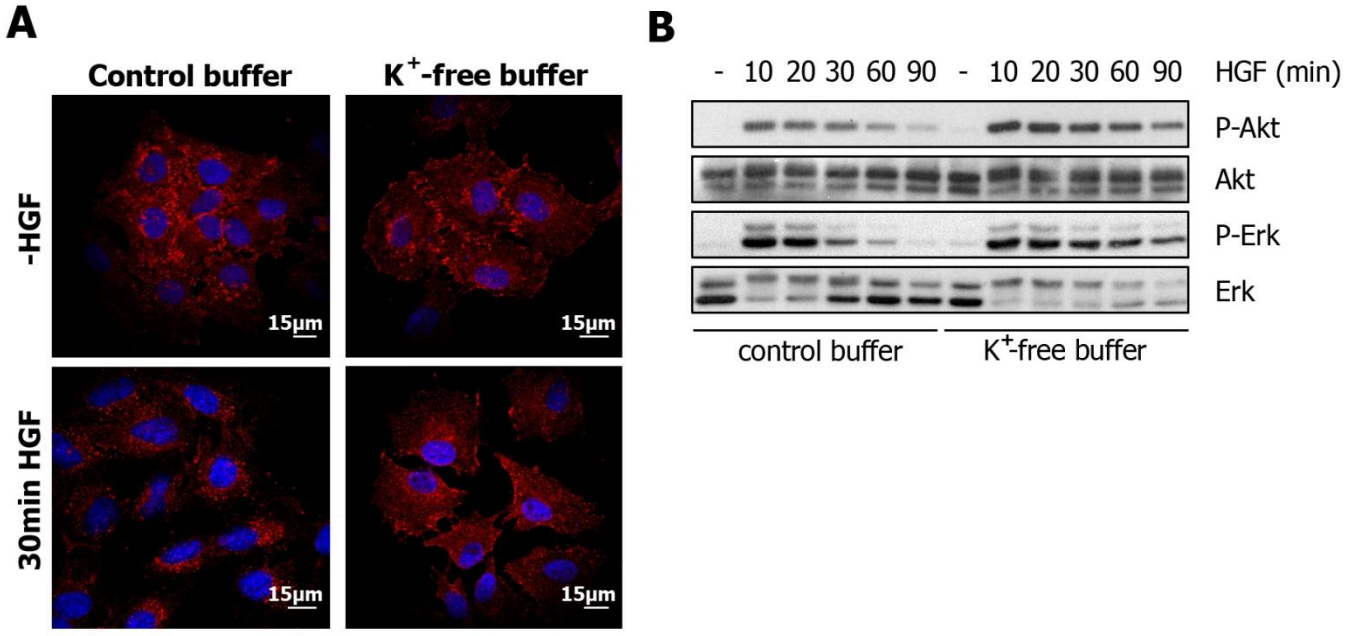

Fig.3.6 Potassium depletion blocks Met internalization but not activation of Akt and Erk.

HeLa cells were serum starved for 24h and subsequently treated with potassium-free buffer or control buffer. Cells were then stimulated with 25ng/ml HGF in the respective buffer at 37°C for the indicated periods.

A Cells were then fixed, permeabilized, and stained for Met (red) with specific antibodies. Nuclei were stained with Dapi and images were taken at a confocal microscope (Leica SPE) using a 63x objective.

B Cells were lysed immediately after HGF-stimulation and the lysates were subjected to Western Blot analysis for phospho-Akt, total Akt, phospho-Erk, and total Erk levels.

As shown in the immunofluorescence images in Fig.3.6A, HGF-induced Met internalization was indeed blocked upon treatment with the K^+-free buffer but was not inhibited after treatment with the control buffer. In the absence of HGF, Met was of course located at the plasma membrane in both cases. HGF-induced activation of Akt and Erk was not inhibited by the K^+-depletion and occurred in both cases as shown in the Western Blots in Fig.3.6B, suggesting that clathrin-mediated endocytosis of Met is not necessary for the activation of these pathways in response to HGF. However, the duration of activation of both Akt and Erk was remarkably prolonged in cells that had been treated with the K^+-free buffer as compared to cells that had been treated with the control buffer. Thus, inhibition of Met internalization does not block downstream MAP Kinase and PI3-Kinase signaling, but has a great impact on the activation kinetics.

As mentioned above, the cytoplasmic tail of CD44v6 is required for the activation of Akt and Erk as well as for the internalization of Met in response to HGF. Since inhibition of clathrin-mediated endocytosis did not block HGF-induced activation of Akt and Erk, this must be an event that is independent of Met internalization. However, Met internalization itself might require downstream signaling.

To investigate the latter possibility, we inhibited signaling and measured the impact on internalization. A dominant negative Ras mutant (EGFP-HRasS17N (Yasuda et al, 2006)) was transfected into HeLa cells to inhibit Ras activation. This construct carries an EGFP (enhanced green fluorescent protein)-tag which was used for detection by fluorescence microscopy. In order to confirm that Ras activation was indeed blocked with this mutant, activation of the Ras substrate Erk was assessed in HeLa cells transfected with either EGFP-HRasS17N or an EGFP control vector. Since the transfection efficiency with this construct was around 50%, Erk activation had to be determined only in transfected cells. Therefore, a hemagglutinin-tagged Erk construct (HA-Erk) was co-transfected and HA-Erk was immunoprecipitated with anti-HA antibodies. 24h after co-transfection of HA-Erk and either EGFP-HRasS17N or the control vector, cells were serum starved and subsequently induced with HGF for 10min at 37°C or left uninduced. Cells were lysed immediately thereafter and HA-Erk was immunoprecipitated. The levels of phospho-Erk and total Erk were analyzed by Western Blot. As shown in Fig.3.7A, Erk was only activated in response to HGF in the control vector transfected cells but not in the cells that had been transfected with the EGFP-HRasS17N mutant, suggesting that the Ras mutant efficiently blocked Ras signaling.

This construct was then used to investigate whether Ras signaling is required for HGF-induced Met internalization.

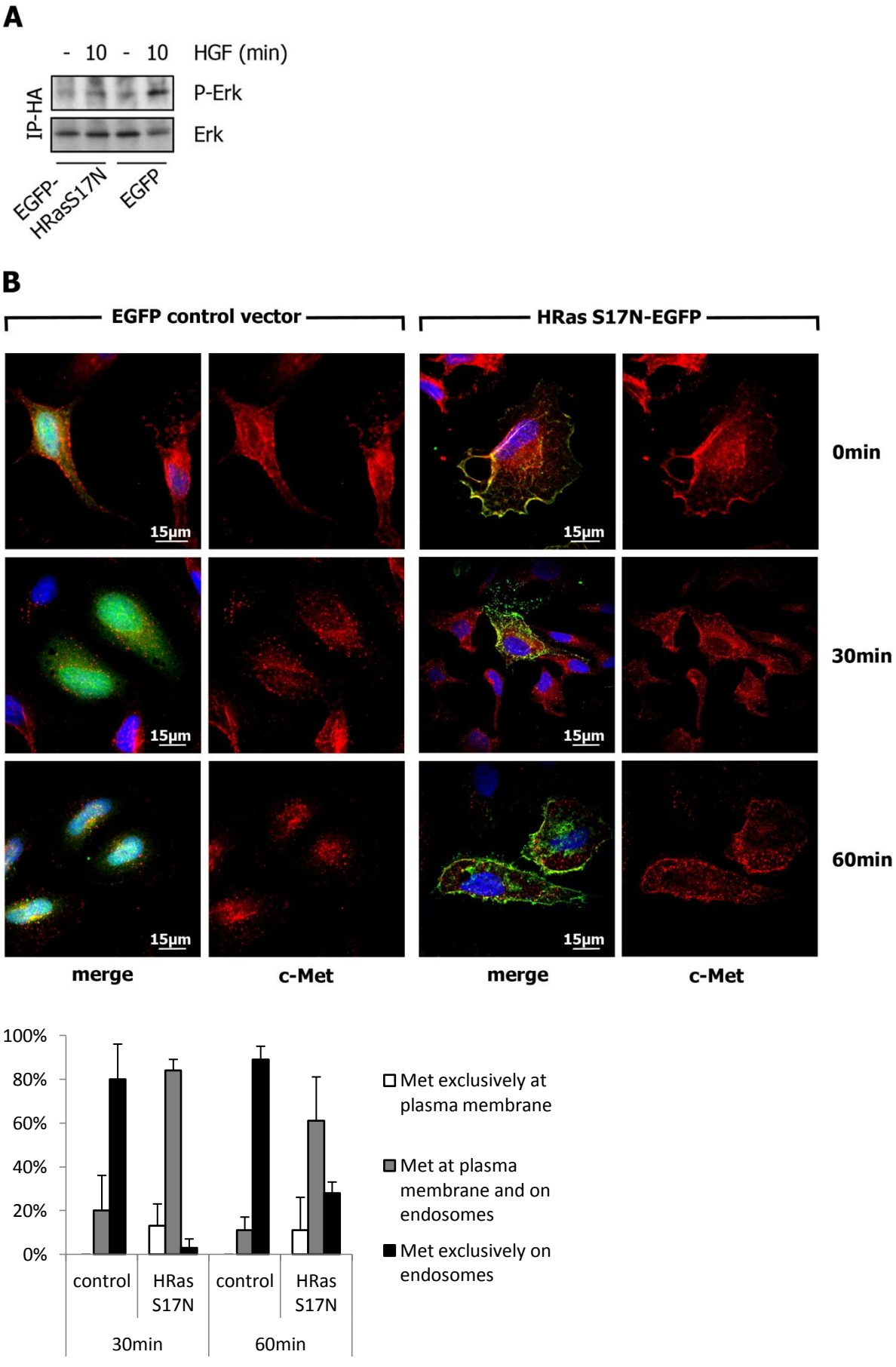

Fig.3.7 Ras signaling is required for HGF-induced Met internalization.

A HeLa cells were co-transfected with HA-Erk and either the control vector or the EGFP-HRasS17N vector. After 24h, cells were serum starved over night and stimulated with 25ng/ml HGF on 37°C for the indicated time periods. Cells were lysed and HA-Erk was immunoprecipitated with HA-specific antibodies to detect Erk only from transfected cells. Western Blot analysis was performed to assess phospho-Erk and total Erk levels.
B HeLa cells were transfected either with the control vector or with the EGFP-HRasS17N mutant. After 24h, the cells were serum starved, treated for 2h with 50µM cycloheximide, and subsequently incubated with 25ng/ml HGF for 1h on ice. Cells were shifted to 37°C for the indicated time periods, fixed, permeabilized, and stained with specific antibodies for Met (red). Nuclei were stained with Dapi and images were taken with a confocal microscope (Leica SPE) using a 63x objective. For quantification, the percentage of transfected cells with Met exclusively at the plasma membrane or exclusively on endosomes or both at the plasma membrane and on endosomes was calculated for each time point. The results are shown as the mean value of three independent experiments.

HeLa cells were transfected with either the EGFP control vector or with EGFP-HRasS17N. 24h after transfection, cells were serum starved and treated with cycloheximide for 2h to block protein synthesis. The cells were then subjected to a cold start and shifted to 37°C for the indicated time periods. Cells were fixed, permeabilized, and stained for Met with specific antibodies. Nuclei were stained with Dapi and images were taken with a confocal microscope (Leica SPE) using a 63x objective.

Directly after the cold start, Met was located at the plasma membrane in both the Ras mutant transfected cells as well as in the control vector transfected cell (Fig.3.7B). In the control vector transfected cells, Met was internalized and accumulated in perinuclear compartments 30 and 60min after the temperature switch to 37°C. In contrast, Met internalization was drastically reduced in cells that had been transfected with the dominant negative Ras mutant as shown in the immunofluorescence images and the quantification graph in Fig.3.7B. Therefore, HGF-induced activation of Ras downstream of Met is required to trigger the internalization of Met. Since Ras activation in response to HGF has been shown to be dependent on the cytoplasmic part of CD44v6 (Orian-Rousseau et al, 2007), this could explain how CD44v6 mediates both Met signaling and Met internalization.

3.7 HGF-induced ubiquitylation of Met is independent of the CD44v6 cytoplasmic domain

The internalization of RTKs is mediated by a complex endocytic machinery consisting of numerous components such as clathrin, adapter proteins, ubiquitin ligases and small GTPases (McMahon & Boucrot, 2011; Zwang & Yarden, 2009). Many of the components of the endocytic machinery need to be modified by phosphorylation or ubiquitylation in order to efficiently mediate receptor internalization (reviewed in (Abella & Park, 2009; Sorkin & von Zastrow, 2009; Zwang & Yarden, 2009). The E3 ubiquitin ligase c-Cbl is recruited to Met in response to HGF and subsequently ubiquitylates Met (Peschard et al, 2001). This has been shown to be essential for the intracellular sorting of Met to lysosomal degradation (Abella et al, 2005). A Met mutant that cannot bind to c-Cbl is not ubiquitylated and undergoes constant recycling which results in deregulated signaling and cell transformation (Abella et al, 2005; Peschard et al, 2001). CD44v6- and Ras-mediated internalization of Met may be promoted by the recruitment of E3 ubiquitin ligases, such as c-Cbl, to regulate ubiquitylation of Met.

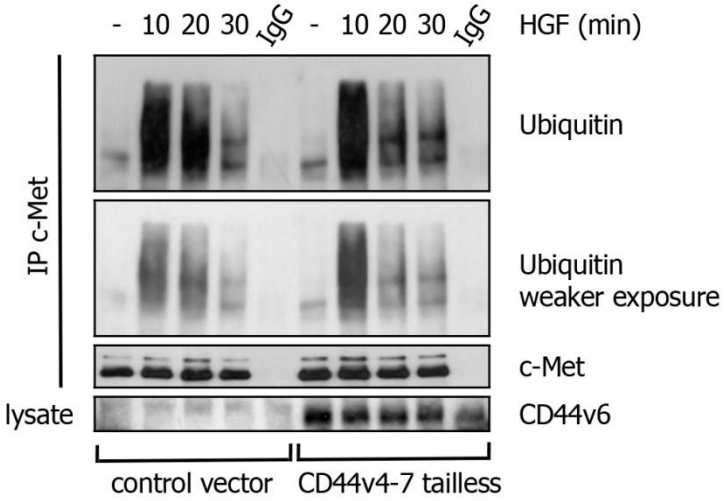

Fig.3.8 HGF-induced ubiquitylation of Met is not dependent on the cytoplasmic domain of CD44.
HeLa cells were transfected either with a control vector or the CD44v4-7 tailless mutant. 24h after transfection, cells were starved and subsequently stimulated with 25ng/ml HGF at 37°C for the indicated time periods. Cells were lysed and Met was immunoprecipitated. The precipitates were subjected to SDS-PAGE and transferred to a PVDF-membrane. Afterwards the membrane was denaturated and probed with specific antibodies for Ubiquitin and Met. Expression of the CD44v4-7 tailless mutant was confirmed by Western Blot analysis of the lysates with a CD44v6-specific antibody.

To find out whether the cytoplasmic domain of CD44v6 is needed for Met ubiquitylation, HeLa cells were transfected either with a control vector or a CD44v4-7 tailless mutant (containing variant exon v6). 24h after transfection, the cells were starved and induced with 25ng/ml HGF on 37°C for the indicated time periods. Thereafter, cells were lysed and Met was immunoprecipitated with specific antibodies. The precipitates were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. Subsequently the membrane was denaturated and Ubiquitin and Met were detected with specific antibodies. Ubiquitylation of Met could be detected by the appearance of a smear starting at the size of Met (140kD).

As shown in Fig.3.8, Met was not ubiquitylated in the absence of HGF but Met ubiquitylation occurred in response to HGF both in the control vector transfected cells and in the CD44v4-7 tailless mutant transfected cells. Thus, ubiquitylation of Met does not require the cytoplasmic domain of CD44v6 and therefore the mechanism by which CD44v6 mediates Met internalization does not involve Met ubiquitylation.

In the first part of my PhD project I have shown that Met internalization upon HGF-induction is strictly dependent on CD44v6. HGF-induced Met internalization could be blocked with a v6 peptide or by expression of a truncated form of CD44v6 that is missing the cytoplasmic domain. HGF-induced ubiquitylation of Met, which is known to promote Met degradation (Abella et al, 2005), was however not inhibited by expression of the CD44v6 tailless mutant. Furthermore, downstream signaling of Ras was also demonstrated to be necessary for HGF-induced Met internalization. Finally, CD44v6 was shown to traffic together with Met through Rab5-positive early endosomes and perinuclear compartment in response to HGF.

Part II. Comparison of the CD44v6 co-receptor function for Met and VEGFR-2

CD44v6 is a co-receptor for both Met and VEGFR-2. The activation of both receptors could be blocked with a CD44v6 peptide or anti-CD44v6 antibodies (Matzke et al, 2005; Orian-Rousseau et al, 2002; Tremmel et al, 2009). The co-receptor function of CD44v6 is similar for both receptors: in both cases the extracellular part of CD44v6 is required for the

activation of the receptors (Orian-Rousseau et al, 2002; Tremmel et al, 2009) by promoting the binding of the growth factors to the receptors (Volz, in preparation). Furthermore, the intracellular part of CD44v6 and the link through ERM proteins to the actin cytoskeleton are required for downstream signaling from both receptors (Orian-Rousseau et al, 2002; Orian-Rousseau et al, 2007; Tremmel et al, 2009). Since CD44v6 mediates the internalization of Met, it is possible that CD44v6 is also involved in the internalization of VEGFR-2. However, VEGFR-2 also employs another co-receptor, Nrp-1 (Ballmer-Hofer et al, 2011; Cebe Suarez et al, 2006; Soker et al, 2002), which has been demonstrated to determine the intracellular trafficking route of VEGFR-2 and thereby the final signaling outcome (Ballmer-Hofer et al, 2011). Therefore, the internalization of VEGFR-2 might be mediated by Nrp-1 in collaboration with CD44v6 or independently of CD44v6.

In the second part of my PhD project I investigated whether CD44v6 is involved in the internalization of VEGFR-2 and whether there are differences in the internalization process of Met and VEGFR-2.

For that purpose, the human umbilical vein endothelial cells (HUVECs) were chosen as a suitable cell line. HUVECs express VEGFR-2 and Met as well as CD44v6 and are therefore an ideal system to compare the internalization process of both receptors and the role of CD44v6 in this process.

3.8 The activation kinetics of Met and VEGFR-2 are strikingly different

I first compared the activation kinetics of Met and VEGFR-2 in response to their ligands HGF or VEGF-A_{165} respectively. If Met and VEGFR-2 are activated with the same kinetics, this would indicate that Met and VEGFR-2 might be activated and internalized similarly.

HUVECs were serum starved and induced with either HGF or VEGF-A_{165} at 37°C for the indicated time periods (Fig.3.9). Cells were then lysed and Met or VEGFR-2 respectively were immunoprecipitated. The precipitates were separated by SDS-PAGE and subjected to Western Blot analysis for phospho-Met and total Met or phospho-VEGFR-2 (Tyr1175) and total VEGFR-2 levels. The activation kinetics of their common downstream target Erk was also analyzed. For that purpose, whole cell lysates were separated by SDS-PAGE and subjected to Western Blot analysis for phospho-Erk and total Erk levels.

Interestingly, Met and VEGFR-2 were activated with remarkably different kinetics by their respective ligands HGF or VEGF-A_{165} (see Fig.3.9). Upon HGF-induction, Met became strongly phosphorylated for at least 30min and even after 60min, Met was not completely inactivated. In contrast, the activation of VEGFR-2 in response to VEGF-A_{165} was much more transient with the strongest phosphorylation as early as 5min upon induction and almost no phosphorylation was detected after 20min. These differences in the activation were also seen on the level of their downstream target Erk, which was robustly activated in response to HGF and only shortly upon induction with VEGF-A_{165}. Therefore, Met and VEGFR-2 are activated and/or downregulated by different mechanisms. This might be due to a different mechanism of receptor internalization.

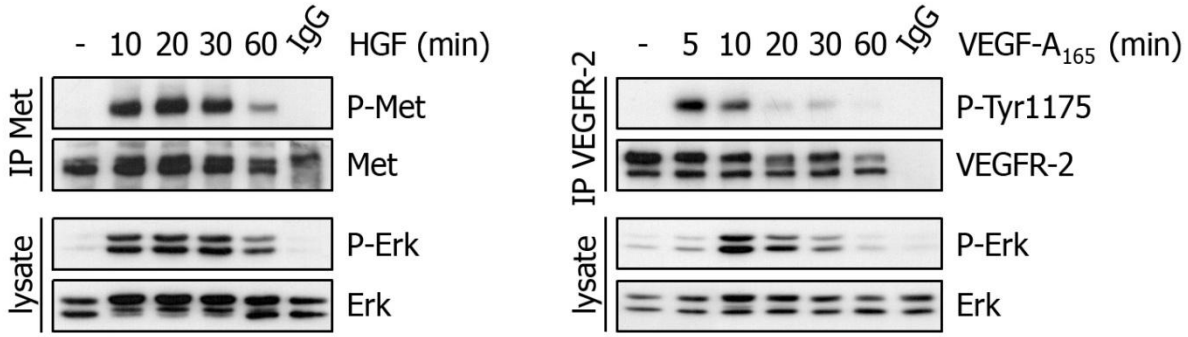

Fig.3.9 Met and VEGFR-2 are activated with strikingly different kinetics.

HUVECs were starved and subsequently stimulated either with 25ng/ml HGF (left side) or 40ng/ml VEGF-A_{165} (right side) for the indicated periods at 37°C. Cells were then lysed and Met (left side) or VEGFR-2 (right side) was immunoprecipitated and subjected to Western Blot analysis for phospho-Met and total Met or phospho-(Tyr1175)VEGFR-2 and total VEGFR-2, respectively. Whole cell lysates were subjected to Western Blot analysis for phospho-Erk and total Erk levels.

3.9 HGF and VEGF-A_{165} have different physiological effects in angiogenic sprouting

Since Met and VEGFR-2 were activated with such strikingly different kinetics, we investigated whether Met- and VEGFR-2- activation might have different physiological effects. Both HGF and VEGF-A have been demonstrated to induce endothelial cells to form blood vessels, a process that is called angiogenesis (Bauters et al, 1994; Bussolino et al, 1992; Grant et al, 1993)). To study the physiological effects of Met and VEGFR-2 activation in

HUVECs an *in vitro* angiogenesis assay was used, namely the fibrin-bead assay that consists in the formation of three-dimensional blood vessels in 96-well plates.

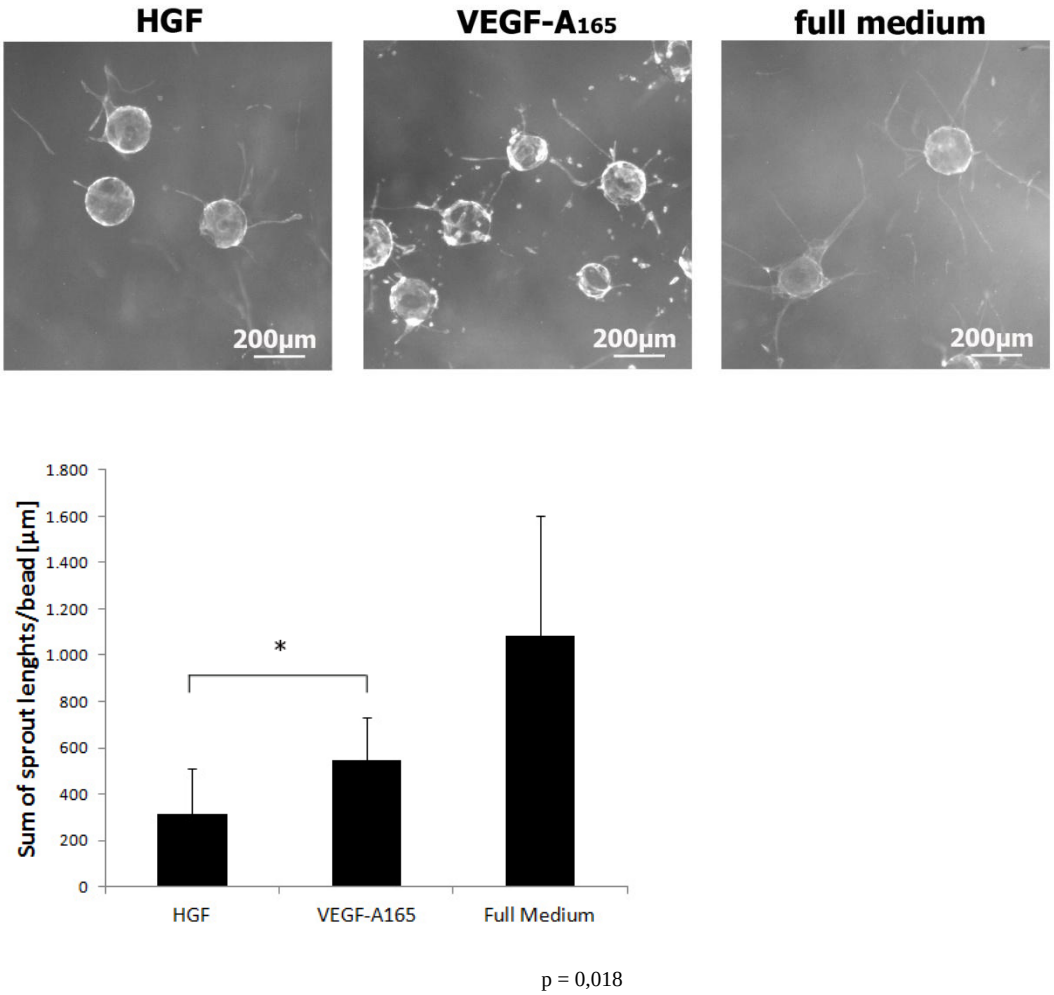

Fig.3.10 Angiogenic sprouting induced by HGF or VEGF-A_{165}.
HUVECs were coated on Cytodex-3 beads and embedded in fibrin gels in 96-well plates. The gels were covered with human skin fibroblasts and sprouting was induced either with 5ng/ml VEGF-A_{165} or 10ng/ml HGF or a combination of both. Full endothelial cell growth medium served as a positive control. The medium was renewed every other day for one week. Then the sprouts were fixed, permeabilized, and actin was stained with phalloidin-rhodamin. Images were taken at a high throughput confocal microscope system (Olympus IX81) using a 2x objective and sprouts were analyzed with ImageJ. For quantification, the sprout lengths were measured and the sum of sprout lengths was calculated for each bead. The values represent the mean of at least 15 beads per condition in two independent experiments ±SD (* p = 0,018).

Cytodex-3 beads were coated with HUVECs and embedded in fibrin gels in 96-well plates (Material and Methods). The gels were covered with human skin fibroblasts that

provide essential growth factors and angiogenic sprouting was induced by addition of serum-free endothelial cell medium containing either VEGF-A_{165} or HGF. As a positive control, angiogenic sprouting was induced with endothelial cell growth medium containing a combination of several growth factors. The medium was changed every second day for one week and the formation of sprouts was observed. At the end of the experiment, the sprouts were fixed, the cells were permeabilized, and actin was stained with phalloidin-rhodamin for visualization of the sprouts by fluorescence microscopy. Images were taken with a high throughput confocal microscope system (Olympus IX81) and sprouting was analyzed with the ImageJ software. For quantification, the lengths of all sprouts were measured and the number of sprouts/bead was counted. Furthermore, the average sprout length and the sum of sprout lengths/bead were calculated for each condition.

As shown in Fig.3.10, angiogenic sprouting could be induced as well with VEGF-A_{165} or HGF. VEGF-A_{165} however induced the formation of longer sprouts than HGF (with an average length of 170µm as compared to 118µm). The average number of sprouts/bead was also increased upon VEGF-A_{165}-induction as compared to HGF-induction (see Table3.1).

Table3.1 HGF and VEGF-A_{165} induce angiogenic sprouting with a different morphology.

Angiogenic sprouting was induced in the fibrin bead assay as described above. Sprout lengths were measured with ImageJ and the number of sprouts was counted for each bead. The sum of sprout lengths/bead and the average sprout length were calculated for each condition. The values represent the mean of at least 15 beads per condition in two independent experiments.

	HGF	**VEGF-A_{165}**	**full medium**
sum sprout lengths/bead [µm]	318 ± 200	548 ± 185	1083 ± 518
average length/sprout [µm]	118 ± 48.6	170 ± 30	250 ± 64.7
average number sprouts/bead	2.7 ± 1.3	3.2 ± 0.8	4.3 ± 2.0

3.10 CD44v6 is found in a complex with VEGFR-2 and Neuropilin-1

As mentioned above, VEGFR-2 signaling in HUVECs is strictly dependent on CD44v6 (Tremmel et al, 2009), but VEGFR-2 also recruits Nrp-1 as a co-receptor (Ballmer-Hofer et al, 2011; Cebe Suarez et al, 2006; Soker et al, 2002), which determines the intracellular trafficking route of VEGFR-2 and the final signal outcome (Ballmer-Hofer et al, 2011). To find out whether CD44v6 and Nrp-1 interact simultaneously with VEGFR-2, co-immunoprecipitation experiments were performed for the three proteins.

HUVECs were serum starved and induced with 40ng/ml VEGF-A_{165} for 5min at 37°C or left uninduced. Cells were then lysed and VEGFR-2 or Nrp-1 or CD44v6 was immunoprecipitated as indicated with specific antibodies. The precipitates were separated by SDS-PAGE and subjected to Western Blot analysis for CD44v6, VEGFR-2 and Nrp-1 (Fig.3.11).

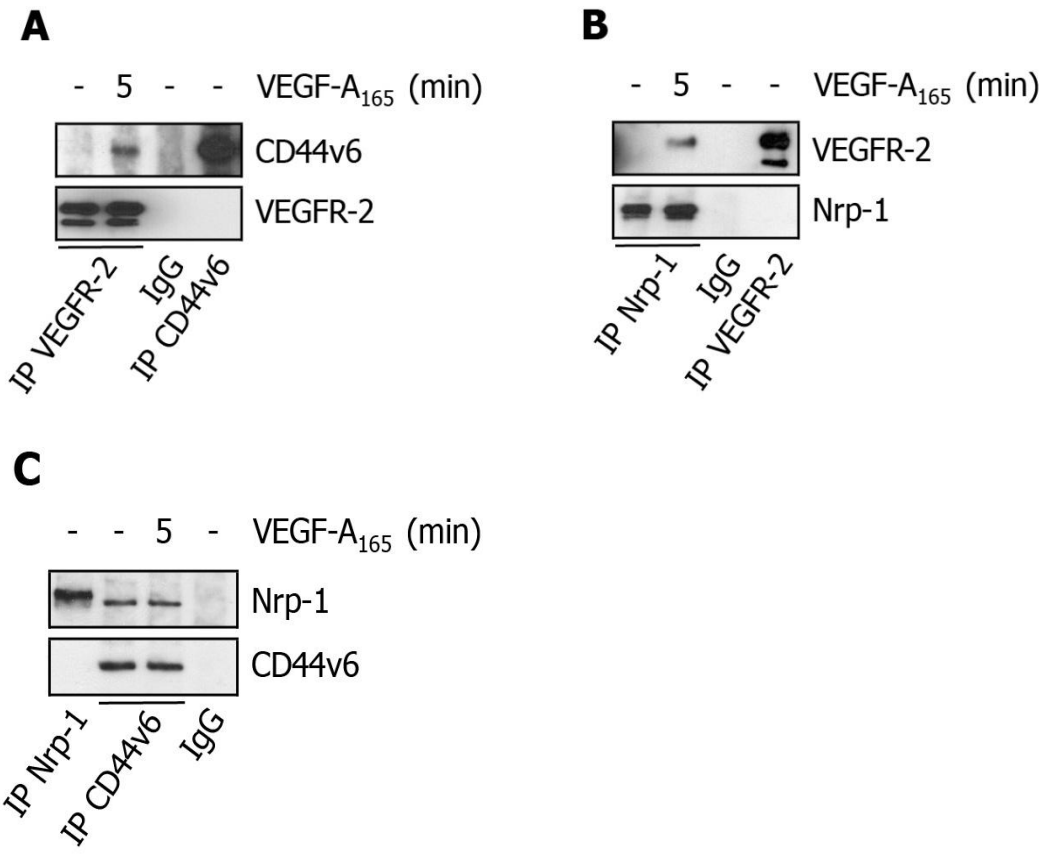

Fig.3.11 VEGFR-2 interacts with both CD44v6 and Nrp-1 upon induction with VEGF-A_{165}.
HUVECs were starved and subsequently induced with 40ng/ml VEGF-A_{165} for 5min or left uninduced. **A** Cells were lysed and VEGFR-2 or CD44v6 was immunoprecipitated. The precipitates were separated by SDS-PAGE

and subjected to Western Blot analysis for CD44v6 and VEGFR-2. **B** Cells were lysed and Nrp-1 or VEGFR-2 was immunoprecipitated. The precipitates were separated by SDS-PAGE and subjected to Western Blot analysis for VEGFR-2 and Nrp-1. **C** Cells were lysed and CD44v6 or Nrp-1 was immunoprecipitated. The precipitates were separated by SDS-PAGE and subjected to Western Blot analysis for Nrp-1 and CD44v6.

Upon VEGF-A_{165}-induction, an interaction was observed between VEGFR-2 and CD44v6 as well as between VEGFR-2 and Nrp-1. Both interactions were inducible and did not occur in the absence of VEGF-A_{165} (see Fig.3.11 A and B). An interaction was also observed between CD44v6 and Nrp-1 (Fig.3.11 C). However, this interaction seemed to be constitutive and occurred also in the absence of VEGF-A_{165}. These results suggest that CD44v6 is already associated with Nrp-1 in a pre-formed complex and upon VEGF-A_{165}-induction, both co-receptors are recruited to VEGFR-2. Thus, the internalization and intracellular trafficking of VEGFR-2 could be mediated by both CD44v6 and Nrp-1 together. These findings, however, have to be confirmed with other methods such as immunofluorescence microscopy. Further experiments will reveal the contribution of CD44v6 and Nrp-1 in the internalization of VEGFR-2.

In summary, despite a similar function of CD44v6 for the activation and downstream signaling of Met and VEGFR-2, both receptors are activated with strikingly different kinetics and have different physiological effects in angiogenesis. The different kinetics imply a different mechanism of Met and VEGFR-2 activation and/or internalization and downregulation. This might be due to different functions of CD44v6 or due to additional co-receptors such as Nrp-1. Both CD44v6 and Nrp-1 were shown to interact with VEGFR-2 in response to VEGF-A_{165}.

4. Discussion

CD44v6 is a co-receptor for the RTKs Met and VEGFR-2. CD44v6 is required for both the activation of the receptors by their respective ligands HGF or VEGF-A_{165} and for the downstream signaling from the activated receptors by providing a link through ERM proteins to the actin cytoskeleton (Orian-Rousseau et al, 2002; Orian-Rousseau et al, 2007; Tremmel et al, 2009). My work shows in addition that HGF-induced internalization of Met is also strictly dependent on CD44v6. Indeed, iternalization of Met upon HGF-induction could be inhibited with a v6 peptide or by transfection of a cytoplasmic tail deletion mutant of CD44v6. Importantly, Ras signaling downstream of Met was also found to be necessary for HGF-induced Met internalization. Finally, CD44v6 itself was shown to be internalized together with Met upon HGF-induction and to traffic together with Met through Rab5-positive endosomes to perinuclear compartments.

Although the function of CD44v6 for the activation and signaling of Met and VEGFR-2 is similar (Orian-Rousseau et al, 2002; Orian-Rousseau et al, 2007; Tremmel et al, 2009), both receptors are activated with strikingly different kinetics and have distinct physiological effects. This implies a different mechanism of Met and VEGFR-2 activation and/or downregulation. The different mechanisms might be due to different functions of CD44v6 or due to additional co-receptors such as Nrp-1 that collaborate with VEGFR-2. An inducible interaction of VEGFR-2 with both CD44v6 and Nrp-1 in response to VEGF-A_{165} could also be shown in my work.

Using immunofluorescence microscopy, it is shown here that both the treatment with a v6 peptide and the transfection with a CD44v6 cytoplasmic tail deletion mutant interfere with HGF-induced Met internalization. The CD44v6 ectodomain promotes the binding of the ligand HGF to the Met receptor (Volz, in preparation) which is required for the activation of Met. The v6 peptide interferes with the function of the CD44v6 ectodomain and represses Met activation. The repression of internalization of Met by the v6 peptide is in agreement with the fact that activation of RTKs is a prerequisite for their internalization. The CD44v6 tailless mutant does not block Met activation, but signaling downstream of Met (Orian-Rousseau et al, 2002). The effect of the tailless mutant suggests a role of the CD44v6 cytoplasmic domain and signaling in internalization.

As mentioned above, the cytoplasmic domain of CD44v6 is not required for Met activation but it is required for the binding to ERM proteins such as Ezrin which link the receptor complex to the actin cytoskeleton, a step that is essential for downstream signaling from Met. Indeed, transfection of a dominant negative Ezrin mutant with a truncated F-actin (filamentous actin) binding site or inhibition of actin polymerization abrogates activation of the Ras/Erk pathway in response to HGF without affecting the phosphorylation of Met (Orian-Rousseau et al, 2007). We have shown that the binding of Ezrin to the actin cytoskeleton is a prerequisite for the HGF-induced internalization of Met (Hasenauer&Malinger et al., Plos One, under revision). The requirement of Ezrin for the internalization process has also been demonstrated for several other membrane receptors. Internalization of the LDL (low density lipoprotein)-Receptor could be blocked with a dominant-negative Ezrin mutant in somatolactotropic GH(3) cells (Smith et al, 2004). Similarly, a dominant-negative Ezrin mutant inhibited the internalization of the β2 adrenergic receptor in human embryonic kidney cells (Cant & Pitcher, 2005). That means that the binding of Ezrin to CD44v6 and to the actin cytoskeleton is required both for Met signaling and Met internalization. The requirement of ezrin raises the question whether the activation of signaling pathways downstream of Met are required to trigger Met internalization.

My work shows that a dominant negative Ras mutant indeed blocks HGF-induced Met internalization. Thus, Ras signaling is vital for the internalization of Met in response to HGF.

Ras activation has previously been shown to trigger the ligand-dependent internalization of another RTK, namely the EGF-Receptor. Ras signaling in response to EGF results in the activation of RIN1 (Ras and Rab interactor 1) which is a guanine exchange factor (GEF) of Rab5. Activated RIN1 then mediates the activation of Rab5 which is required for the EGF-induced internalization of the EGF-Receptor (Tall et al, 2001). Whether the internalization of Met in response to HGF is also mediated by this mechanism has to be investigated in further experiments. This could be done by transfection of a dominant negative Ras mutant and subsequent analysis of Rab5 activation in response to HGF. Rab5 activation can be analyzed by immunoprecipitation with specific antibodies that detect only GTP-bound Rab5.

However, signaling promotes clathrin-mediated receptor internalization not only by the activation of GEFs. Also the adaptor protein Eps15 which is essential for clathrin-coated pit formation has to be phosphorylated to mediate the internalization of the EGF-Receptor in response to EGF (Confalonieri et al, 2000). Eps15-phosphorylation occurs as well upon Met activation in response to HGF (Parachoniak & Park, 2009). Preliminary data of my own show

that Eps15-phosphorylation occurs upon induction with HGF for 1h on ice (Fig.4.1). Under these conditions, Met can be activated but internalization is blocked since membranes become stiff at low temperatures. Hence, Eps15-phosphorylation in response to HGF is an early event during the Met internalization process that occurs at the plasma membrane and not after the internalization of Met. Consequently, Eps15-phosphorylation could also be a prerequisite for the initiation of Met internalization. Whether Eps15-phosphorylation is dependent on the cytoplasmic domain of CD44v6 will be investigated by assessing HGF-induced Eps15-phosphorylation after transfection of the CD44v6 tailless mutant.

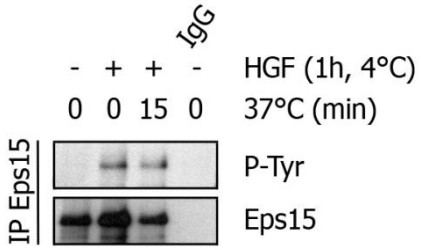

Fig.4.1 Eps15-phosphorylation occurs at the plasma membrane.
HeLa cells were serum starved for 24h and where indicated, stimulated with 25ng/ml for 1h on ice (cold start). Cells were then shifted to 37°C for the indicated time periods and lysed immediately thereafter. Eps15 was immunoprecipitated and subjected to Western Blot analysis for phospho-tyrosine and total Eps15.

The activation of Rab5 and Eps15 downstream of the EGF-Receptor are not the only cases where signaling regulates receptor endocytosis or intracellular trafficking in endosomes (reviewed in (Sorkin & Von Zastrow, 2002; Sorkin & von Zastrow, 2009)). Several other proteins of the endocytic machinery have to be phosphorylated for efficient receptor internalization (reviewed in (Sorkin & Von Zastrow, 2002)). Phosphorylation of the E3 ubiquitin ligase c-Cbl for instance is required for the activation of its ligase activity which leads to receptor ubiquitylation and degradation (Levkowitz et al, 1998). Interestingly, the specific phosphorylation of c-Cbl on Tyr371 by the Src kinase triggers c-Cbl degradation and thereby increased receptor recycling as shown for the EGF-Receptor (Bao et al, 2003). PKC can also rescue the EGF-Receptor from degradation and target it for recycling. In this case the phosphorylation target is Thr654 on the receptor itself (Bao et al, 2000).

CD44v6 could mediate HGF-induced Met internalization merely by allowing downstream signaling from Met. In addition however, CD44v6 might also have a mechanical role in Met internalization. The link of CD44v6 through Ezrin to the actin cytoskeleton could

be important for the release of Met-containing clathrin-coated vesicles from the plasma membrane and the inward movement of the released vesicles. Actin polymerization has been shown to occur at sites of clathrin-coated pit formation and to precede the detachment of clathrin-coated vesicles from the plasma membrane (Merrifield et al, 2002). Moreover, inhibition of actin polymerization with latrunculin A abrogates receptor endocytosis in mammalian cells (Lamaze et al, 1997).

Ezrin, recruited to Met by CD44v6, might also promote actin polymerization. Indeed, Ezrin on purified phagosomes was shown to recruit the actin-filament nucleators N-WASP (Neuronal Wiskott-Aldrich Syndrome Protein) and ARP 2/3 (actin-related protein 2/3) thereby stimulating F-actin assembly (Marion et al, 2011).

Ample examples in the literature show the interdependence between signaling and internalization (Scita & Di Fiore, 2010; Sorkin & Von Zastrow, 2002; Sorkin & von Zastrow, 2009). Signaling proteins influence the internalization and intracellular trafficking processes at multiple points as described above, and conversely has receptor internalization a great impact on signaling processes. Not only is receptor internalization the prerequisite for efficient downregulation of RTK signaling by receptor degradation in lysosomes, but it is as well crucial for the activation of certain signaling pathways initiated on endosomes or the correct distribution of signals within the cell.

I investigated whether Met internalization is required for HGF-induced activation of Akt and Erk. Activation of these pathways in response to HGF is strictly dependent on the cytoplasmic domain of CD44v6 (Orian-Rousseau et al, 2002). Met internalization was blocked by inhibiting clathrin-mediated endocytosis (CME) by potassium depletion. The result was that both Akt and Erk were still activated in response to HGF. However the duration of Akt and Erk activation was remarkably prolonged in comparison to control cells where CME was not inhibited. These findings suggest that Met internalization is not required for Akt and Erk activation, but rather for promoting their downregulation. Kermorgant et al. showed however that HGF-induced Erk activation is dependent on Met internalization and can be blocked by expression of a dominant-negative dynamin mutant (K44A) (Kermorgant et al, 2004). This difference to my result might be due to the different experimental approaches that were used to block CME. Further experiments with alternative methods to interfere with CME such as clathrin downregulation with siRNA will help to clarify the dependence of Erk activation on Met internalization.

However, Met internalization upon HGF-induction has been shown to be crucial for the activation of other signaling pathways than Akt and Erk. HGF-induced cell migration requires the internalization of active Met into early endosomes which is mediated by Rab5. The activation of the small GTPase Rac then specifically occurs on early endosomes as Rac is brought together with its GEF TIAM1 (T-cell lymphoma invasion and metastasis-inducing protein 1) exclusively on this compartment. Activated Rac is subsequently recycled to distinct regions at the plasma membrane where it mediates actin remodeling that is required for cell migration. Consequently, inhibition of Met internalization by downregulation of Rab5 prevented Rac activation and HGF-induced migration of HeLa cells (Palamidessi et al, 2008). Interestingly, TIAM1 and CD44v3 interaction is required for the activation of Rac1 and HA-induced migration of metastatic breast tumor SP1 cells (Bourguignon et al, 2000). Met internalization and trafficking to perinuclear compartments has also been reported to be required for nuclear translocation of activated STAT3 in response to HGF (Kermorgant & Parker, 2008). Thus, Met internalization is of vital importance for several Met-induced signaling pathways.

Other receptors than Met have also been found to depend on internalization and endosomal signaling events for the correct signal outcome. Internalization of the EGF-Receptor is necessary for the activation of GSK3β and nuclear translocation of APPLs which in turn is required for cell survival during development of zebrafish (Miaczynska et al, 2004; Schenck et al, 2008). Neuronal cell survival induced by NGF on distal axons requires the internalization of the NGF-Receptor TrkA and the trafficking of TrkA along microtubules to the neuronal cell body. This is essential for the activation of Erk5 and CREB in the cell body and subsequent nuclear translocation of active CREB for anti-apoptotic gene regulation (Watson et al, 2001). Activation and nuclear translocation of SMADs in response to TGFβ (transforming growth factor β) requires the internalization of the TGFβ-Receptor. SARA (SMAD anchor for receptor activation) interacts with the TGFβ-Receptor on early endosomes and recruits SMAD2. This interaction on early endosomes is required for activation of SMAD2 by TGFβ-Receptor and TGFβ-induced SMAD signaling (Hayes et al, 2002; Tsukazaki et al, 1998).

During the study of the dependency of HGF-induced Met endocytosis on CD44v6 it was unraveled that CD44v6 becomes co-internalized with Met in response to HGF and traffics with Met through early endosomes to perinuclear compartments. The fact that CD44v6 traffics with Met to internal compartments might indicate that Met requires CD44v6 for

signaling from endosomes. As mentioned above, the activation of Rac by TIAM1 is an event that happens exclusively on endosomes and CD44 interacts with TIAM1. Whether internalized CD44v6 is indeed involved in endosomal signaling of Met will be very interesting to study. However, this is a challenge since CD44v6 is needed for the internalization of Met and downregulation of CD44v6 or deletion of either the ectodomain or the cytoplasmic tail abrogates Met translocation to endosomes. CD44 internalization has also been reported following the activation of ErbB receptors in response to the ligands EGF or heregulin (Palyi-Krekk et al, 2008).

CD44v6 is required for the activation of both Met and VEGFR-2 by their ligands HGF and VEGF-A_{165}, respectively. However, I could show during my PhD that both receptors are activated with strikingly different kinetics in the same endothelial cell line. VEGFR-2-activation in response to VEGF-A_{165} was much more transient than activation of Met in response to HGF. Induction with HGF or VEGF-A_{165} also had different physiological effects in angiogenic sprouting of endothelial cells. Initial experiments with biochemical methods to analyze the endocytosis of Met and VEGFR-2 in response to their respective ligands showed that the internalization kinetics of both receptors correspond well to their activation kinetics since VEGFR-2 is much faster internalized than Met. Thus, the different activation kinetics might be due to a different mechanism of receptor internalization.

Indeed, VEGFR-2 has been shown to rely on another cell adhesion molecule for trafficking and signaling which is Nrp-1. Nrp-1 is responsible for the different signal outcome of angiogenesis-activating and inhibitory isoforms of VEGF-A. Nrp-1 is internalized together with VEGFR-2 in response to the angiogenesis-activating isoform VEGF-A_{165}a. Co-internalization of Nrp-1 with VEGFR-2 is required for the trafficking through Rab11-positive recycling endosomes and the activation of p38 MAP Kinase. Induction with the angiogenesis-inhibitory isoform VEGF-A_{165}b which lacks the Nrp-1-binding site prevents the trafficking of VEGFR-2 through Rab11 endosomes and results in receptor sorting of VEGFR-2 to Rab7 endosomes and lysosomal degradation. This leads to a different signaling outcome as p38 MAP Kinase is not activated and angiogenic sprouting of endothelial cells is inhibited (Ballmer-Hofer et al, 2011).

The different mechanism of VEGFR-2 internalization upon VEGF-A_{165}-induction could be mediated by Nrp-1 in collaboration with CD44v6 or independently of CD44v6. In my work I could show hat VEGFR-2 interacts with both CD44v6 and Nrp-1 in response to VEGF-A_{165}. Further experiments will reveal the roles of Nrp-1 and CD44v6 in the VEGF-

A_{165}-induced internalization of VEGFR-2. For instance, the internalization of VEGFR-2 could be analyzed after downregulation of Nrp-1 by siRNA to find out whether VEGFR-2 is still internalized. Downregulation of CD44v6 however is not possible since CD44v6 is necessary for the activation of VEGFR-2 by VEGF-A_{165}. Instead the CD44v6 tailless mutant could be used to find out if the internalization of VEGFR-2 also requires the cytoplasmic part of CD44v6. Furthermore, it would be interesting to investigate whether CD44v6 is internalized together with VEGFR-2 as it has already been shown for Nrp-1 (Ballmer-Hofer et al, 2011).

CD44v6 is a promising target for anti-cancer therapy since CD44v6 is required for the activation and signaling of two different RTKs that both have prominent roles in cancer development and progression. Amplified Met signaling leads to cell transformation and is closely associated with cancerogenesis and metastasis formation. Interestingly, aberrant receptor endocytosis has been found in many cancers. In several cancers, receptors escape from degradation by different mechanisms that interfere with receptor ubiquitylation which is required for the sorting of the receptors to lysosomal degradation (reviewed in (Abella & Park, 2009; Mosesson et al, 2008). For the Met receptor, a single mutation in the binding site of the E3 ubiquitin ligase c-Cbl (Y1003) prevents Met ubiquitylation and confers transforming activity (Abella et al, 2005; Peschard et al, 2001). This Met mutant is still internalized but is not sorted for degradation which results in sustained signaling, cell transformation, and tumor development (Abella et al, 2005). This is of clinical relevance since different Met mutations that result in the deletion of the c-Cbl binding site were found in several lung cancers (Kong-Beltran et al, 2006; Ma et al, 2005; Onozato et al, 2009). Similar mutations were also found in the Cbl binding site of the EGF-Receptor, c-Kit, and CSF-1R (colony stimulating factor-1 receptor) and are associated with various cancer types such as glioblastoma, gastrointestinal stromal tumors, and acute myeloid leukemia (AML) (reviewed in (Abella & Park, 2009)). Mutations in Cbl that impair its ubiquitin ligase activity have been found in patients with AML (Caligiuri et al, 2007; Sargin et al, 2007). Furthermore, aberrant expression of numerous other components of the endocytic machinery has been identified in human cancers (reviewed in (Abella & Park, 2009; Mosesson et al, 2008)). The utmost importance of receptor endocytosis for signal downregulation has been demonstrated in a study with Tpr-Met. Tpr-Met is an oncogenic fusion protein of the Tpr dimerization domain and the intracellular kinase domain of Met (Park et al, 1986). Tpr-Met is constitutive active and cannot be targeted for degradation by endocytosis as this protein is cytosolic and lacks the

c-Cbl binding site. Introduction of a c-Cbl binding site into Tpr-Met is not sufficient to block its transforming activity as the cytosolic protein is still not accessible for receptor endocytosis. Only the targeting of Tpr-Met to the plasma membrane allows its internalization and attenuates its transforming activity (Mak et al, 2007).

However, Met internalization can also promote tumor progression and metastasis. Different constitutive active Met mutants (M1268T and D1246N) undergo internalization but escape from receptor degradation and are instead recycled back to the plasma membrane. Internalization of these mutants was required to induce actin remodeling and cell migration. Furthermore, inhibition of receptor endocytosis by short hairpin RNA-knockdown of the clathrin heavy chain blocked metastasis formation *in vivo* (Joffre et al, 2011).

Tumor growth is also dependent on tumor vascularization for the supply with oxygen and nutrients. HGF can directly induce the formation of new blood vessels (Bussolino et al, 1992; Grant et al, 1993), but the principal inducer of angiogenesis is VEGFR-2 (Ferrara et al, 2003; Harper & Bates, 2008; Holmes et al, 2007). Indeed, anti-angiogenic therapy is frequently used for the treatment of various cancer types. However, anti-angiogenic therapy can result in tumor hypoxia which upregulates Met expression and promotes Met-induced invasive growth and metastatic spreading in various cancer types and leads to poor prognosis for the patients (Hara et al, 2006; Ide et al, 2006; Pennacchietti et al, 2003; Scarpino et al, 2004). Blocking the function of CD44v6 could overcome this problem since this approach simultaneously interferes with Met and VEGFR-2 signaling.

For the development of an efficient anti-cancer therapy based on CD44v6, the function of CD44v6 in the signaling process has to be studied extensively. The role of CD44v6 in the internalization process is an important aspect in this regard since receptor endocytosis and signaling are inseparably connected with each other.

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Lebenslauf

Persönliche Daten
Dieter-Augustin Malinger
Rastatterstr.10
69126 Heidelberg
E-Mail: dietermalinger@gmail.com
geboren am 03.08.1980 in Temeschburg, Rumänien

Schulbildung

1991-2000	Helmholtz-Gymnasium Heidelberg
21.06.2000	*Abitur, Abschlussnote: 1,8*

Wehrdienst

07/2000-04/2001	Sanitäter, Raketenartillerie-Bataillon 122, Walldürn

Studium

04/2002-02/2009	Biochemie, Eberhard-Karls-Universität Tübingen
16.02.2009	*Diplom in Biochemie, Abschlussnote: 1,3* Thema der Diplomarbeit: Optimierung und Anwendung eines Modellassays zur Untersuchung des Einflusses von metabolisierten Testverbindungen auf ihre Wirksamkeit in vitro am Beispiel von p38 MAP Kinase Inhibitoren Note: 1,3 Studienschwerpunkte: Immunologie, Pharmazeutische Chemie
09/2009-02/2013	Promotion, Karlsruher Institut für Technologie *Doktor der Naturwissenschaften, Abschlussnote: 1,0* Thema der Doktorarbeit: The role of CD44v6 in RTK internalization and trafficking Note: 1,0
02/2006-06/2006	Auslandssemester an der University of Auckland, Neuseeland
07/2006-12/2006	Praktikum am Auckland Cancer Society Research Centre, Neuseeland Aufgaben: Identifizierung der molekularen Targets eines neuartigen Vascular Disrupting Agent Charakterisierung Tumor-infiltrierender Leukozyten in Dickdarm-Tumoren aus Mäusen

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[§] these authors contributed equally
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