Phosphorylation as a mediator of protein function in cell migration, differentiation, and death – Vimentin, AATF, and Par-4 in the spotlight

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To my family

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I. Isoniemi KO, Mohanasundaram P, Hyder CL, Cheng F, Vihinen H, Jokitalo E, Eriksson JE. "Phosphorylation of a vimentin N-terminal serine cluster regulates cellular vimentin network assembly" Manuscript

II. Hyder CL*, Kemppainen K*, Isoniemi KO, Imanishi SY, Goto H, Inagaki M, Fazeli, E, Eriksson JE and Törnquist K 2015 "Sphingolipids inhibit vimentin-dependent cell migration" Journal of Cell Science: 128: 2057-69

III. Sjöqvist M, Antfolk D*, Isoniemi KO*, Cheng F, Antila C, Niemi R, Björk N, Eriksson JE, and Sahlgren C. "Vimentin modulates Notch signaling dynamics and development of the embryonic vasculature" Submitted Manuscript

IV. de Thonel A, Hazoumé A, Kochin V, Isoniemi KO, Jego G, Fourmaux E, Hammann A, Mjahed H, Filhol O, Micheau O, Rocchi P, Mezger V, Eriksson JE, Rangnekar VM, and Garrido C. 2014. "Regulation of the proapoptotic functions of prostate apoptosis response-4 (Par-4) by casein kinase 2 in prostate cancer cells" Cell Death & Disease 5: e1016

V. Ferraris SE, Isoniemi KO, Torvaldson ES, Anckar J, Westermarck J, and Eriksson JE. 2012. "Nucleolar AATF regulates c-Jun-mediated apoptosis" Molecular Biology of the Cell 21: 4323-32

* Equal contribution

ABBREVIATIONS

A Alanine
AATF Apoptosis antagonizing transcription factor
ABC ATP-binding cassette
ADAM Disintegrin and metalloproteinase
ADP Adenosine diphosphate
AP-1 Activator protein 1
AP1 Adaptor protein-1 complex
AP3 Adaptor protein-3 complex
ARF1 ADP-ribosylation factor 1
AFT Activating transcription factor
ATM Ataxia telangiectasia mutated
Bfsp1 Beaded filament structural protein 1
Bfsp2 Beaded filament structural protein 2
C5a Complement component 5
CAM Chorion allantois membrane
CCL2 C-C motif chemokine 2 precursor
CCR7 C-C chemokine receptor type 7
CD47 Cluster of differentiation 47
Cdk5 Cyclin-dependent protein kinase 5
Chk2 Checkpoint kinase 2
CK2 Casein kinase 2
CLEM Correlative electron microscopy
CNS Central nervous system
CSL CBF1, Suppressor of Hairless, Lag-1
D Aspartate
DCM Dilated cardiomyopathy
Dll Delta-like ligand
DNA Deoxyribonucleic acid
DRM Detergent-resistant membrane
DSL Delta/Serrate/LAG-2
E Glutamic acid
EBS Epidermolysis bullosa simplex
ECM Extracellular matrix
EGF Epidermal growth factor
EMT Epithelial-mesenchymal transition
ER Endoplasmic reticulum
ERK Extracellular-signal-regulated kinase
FAK Focal adhesion kinase
FasL Fas ligand
FRAP Fluorescence recovery after photobleaching
FTCD Formiminotransferase-cyclodeaminase
FTY720 Fingolimod
G Glycine
GFAP Glial fibrillary acidic protein
GFP Green fluorescent protein
GPCR G-protein-coupled receptor
GRP78 78 kDa glucose-regulated protein
GTP Guanosine triphosphate
HDAC Histone deacetylase
HDL High-density lipoprotein
HIF1α Hypoxia-inducible factor 1 α
HIPK2 Homeodomain-interacting protein kinase 2
HMCV Human cytomegalovirus
HUVEC Human umbilical vascular endothelial cell
IAP Integrin-associated protein
ICAM-1 Intercellular adhesion molecule 1
IF intermediate filament
IGF Insulin-like growth factor
Jag1 Jagged 1
JNK c-Jun N-terminal kinase
K Lysine
K1…86 Keratin 1…86
KASH Klarsicht, ANC-1, syne homology
LAMP-2 Lysosome-associated membrane protein
MAGE Melanoma-associated

antigen

MAPK Mitogen-activated protein kinase

MDB Mallory-Denk body

MEF Mouse embryonic fibroblast

MET Mesenchymal-epithelial transition

MICAL Molecule interacting with CasL

MK2 Mitogen-Activated Protein Kinase-Activated Protein Kinase 2

MT Microtubule

MT1-MMP Membrane type-1 matrix metalloproteinase

mTOR Mammalian target of rapamycin

NECD Notch extracellular domain

NF Neurofilament

NF-κB Nuclear factor kappaB

NF-H Neurofilament heavy

NF-L Neurofilament light

NF-M Neurofilament medium

NICD Notch intracellular domain

NMJ Neuromuscular junction

NRAGE Neurotrophin receptor-interacting MAGE homolog

P Proline

Par-4 Prostate apoptosis response-4

PCD Pre-coil domain

PDGF Platelet-derived growth factor

pERK Phosphorylated extracellular-regulated kinase

PI 3-kinase Phosphoinositide 3-kinase

PIN1 Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1

PKA Protein kinase A

PKC Protein kinase C

PP2A Protein phosphatase 2

pRb Retinoblastoma protein

PTEN Phosphatase and Tensin homolog

PTM Post-translational modification

R Arginine

RACK1 Receptor for activated C kinase 1

Raf Rapidly accelerated fibrosarcoma

RhoA Ras homolog gene family, member A

ROCK Rho-associated protein kinase

S Serine

S1P Sphingosine-1-phosphate

S1PR Sphingosine-1-phosphate receptor

SDF1 Stromal-derived growth factor

SPC Sphingosylphosphorylcholine

SphK Sphingosine kinase

STAT3 Signal transducer and activator of transcription 3

SUMO Small ubiquitin-like odifier

TG2 Tissue transglutaminase

TRAF TNF receptor-associated factor

TRIM32 Tripartite motif -containing protein 32

TSG101 Tumor susceptibility gene 101

ULF Unit-length filament

UV Ultraviolet

VAM Vimentin-mediated adhesion

VCAM-1 Vascular cell adhesion protein 1

VE Vascular endothelial

VEGF Vascular endothelial growth factor

VEGFR Vascular endothelial growth factor receptor

WFA Withaferin A

ABSTRACT

Protein phosphorylation is a major regulator of cellular signaling. It functions when a kinase joins a phosphate group to a protein, or when a phosphatase strips off a phosphate group from serine, threonine, or tyrosine residue of the protein. Protein phosphorylation is a major regulator of cellular signaling, and offers fast, dynamic, and reversible means of regulation.

Intermediate filaments (IFs) are a protein family with about 70 members, which are part of the cytoskeleton – a filamentous 3D meshwork inside the cells that engages in support, transport, and several other functions. IFs play a role in establishing and maintaining cellular organization and tissue integrity through forming cytoplasmic and nuclear filamentous structures. IFs provide structural support and assist in mechanical cellular functions. In addition, IFs work as signaling scaffolds. Through these activities, IFs regulate cellular functions such as motility, stress and apoptosis, cell growth and division, cellular differentiation, and homeostasis. Intermediate filament structure consists of conserved central rod domain and more variable N-terminal head and C-terminal tail domains. A multitude of post-translational modification (PTM) sites resides mostly in the head and tail domains. The most important of these PTMs is likely phosphorylation. Phosphorylation is known to regulate IF assembly and disassembly, and in many cases allows IFs to function as a dynamically adjustable signaling scaffold.

Vimentin is the most abundant IF, and it shows the highest expression in cells originating from mesenchyme. Vimentin is a canonical marker, and one of the causative factors of epithelial-mesenchymal transition (EMT), in which epithelial cells become more migratory and invasive, typically during development or cancerogenesis.

Our research group previously identified all the interphase in vivo serine/threonine phosphorylation sites. In our first study (I), by using phosphorylation on/off status mimicking mutagenesis, we demonstrate that from multiple sites, only phosphorylation of vimentin serines 7, 8 and/or 9 are critical to vimentin filamentous structure, and S6 and S71 and/or 72 have a lesser effect. Some phosphorylation of S7, 8 and/or 9 may be needed to stabilize or generate vimentin structure. Our results show that phosphorylation of serines 6, 7, 8 and/or 9 may affect cytoskeletal interactions, and has a major effect in increasing the tetrameric soluble pool of vimentin incrementally based on phosphorylation amount. Based on the previous research also, solubility increase is most likely caused by breakage of vimentin head-rod helix 2B interaction and warrants further investigation.

S1P and SPC are sphingolipids, which regulate processes like cellular migration, for example. In our second study (II), we demonstrate how S1P and SPC cause a restructuring of vimentin and regulate thyroid and breast cancer cell chemotactic migration via vimentin serine 71 phosphorylation. S1P receptor 2 and Rho-associated protein kinase (ROCK) appear to mediate these effects.

In the third study (III), we demonstrate how vimentin balances Notch signal-

ing pathway by causing an increase in Jagged 1-Notch signaling at the expense of Delta-like 4-Notch signaling in angiogenesis. When vimentin is absent, Jagged 1 accumulates at the cell membrane and loses its transactivation capability. Vimentin aminoterminal PCK-regulated phosphorylation sites have a role in this. We demonstrate that this signaling effect causes weaker vasculature in the chorion allantois membrane (CAM) model, in the human umbilical vascular endothelial cell (HUVEC) angiogenesis assay, in aortic ring sprouting angiogenesis assay, and finally in vimentin knockout mouse embryos.

Par-4, CK2, AATF, and c-Jun are important regulators of cellular apoptosis and survival machinery. These proteins regulate others or are regulated by phosphorylation – CK2 being a kinase. Par-4 and CK2 function especially prominently in prostate cancer. AATF is an important component in DNA damage response.

In the fourth study (IV), we demonstrate how CK2-mediated phosphorylation of Par-4 impairs pro-apoptotic functions of Par-4. This effect involves prevention of caspase cleavage of Par-4 by Par-4 S124 phosphorylation in rats, or effects independently of cleavage by Par-4 S231 phosphorylation in humans. We also show that this phosphorylation has relevance in prostate cancer cells.

In the fifth study (V), we demonstrate previously unknown pro-apoptotic function of AATF after UV stress. We show that this mechanism requires AATF translocation from the nucleolus to nucleoplasm and interaction with transcription factor c-Jun, as well as activation of the pro-apoptotic function of c-Jun.

These studies reveal new phosphorylation-mediated mechanisms in the regulation of vimentin structure via phosphorylation of the N-terminal serine cluster, and separately via a sphingolipid-mediated pathway that also inhibits chemotactic cancer cell migration through vimentin phosphorylation. Only little studied vimentin effect on angiogenesis is described further including previously unknown effect of vimentin on Notch signaling possibly via PKC phosphorylation sites on vimentin. Our results strengthen the view of vimentin as a multifunctional cellular scaffold, and provide information about vimentin function especially in the context of cancer. Two separate studies on apoptosis describe phosphorylation-controlled CK2-Par-4-connection as potentially important in prostate cancer survival and AATF behaving pro-apoptotically after UV stress by controlling c-Jun activation by phosphorylation. Together, the results demonstrate the operational modalities of some phosphorylation-dependent switches and how these types of switches may regulate cellular fate and function in different ways.

SAMMANFATTNING

Proteinfosforylering är en viktig regulator av cellulär signalering. Det fungerar när ett kinas ansluter en fosfatgrupp till ett protein eller när ett fosfatas frigör en fosfatgrupp från protein serin, treonin eller tyrosin-rest. Proteinfosforylering är en viktig regulator av cellsignalering och utgör ett snabbt, dynamiskt och reversibelt sätt att ändra en signalmekanism från en funktion till en fullständing annan funktion.

Intermediära filament (IF) är en proteinfamilj med cirka 70 medlemmar som utgör en del av cytoskelettet. IF har roller i att upprätta och upprätthålla cellulär organisation och vävnadsintegritet genom att de bildar cytoplasmiska och nukleära filamentära eller trådlika strukturer. Intermediära filament ger strukturellt stöd och bistår cellers mekaniska funktioner. Dessutom kan IF fungera som byggställningar för signalering. Via dessa roller reglerar IF cellfunktioner som rörlighet, stress och apoptos, celltillväxt och delning, cellulär differentiering och homeostas. IF har en struktur med en konserverad central domän och mer variabla N-terminala och C-terminala domäner. De N- och C-terminala domänerna har ett stort antal ställen för posttranslationella modifieringar av vilka fosforylering utgör den viktigase. Fosforylering är känd för att reglera IF polymerisering och depolymerisering och ger i många fall möjlighet för IF att fungera som en dynamiskt omformbar byggställning för signalering .

Vimentin är mest allmänt förekommande IF-protein som uttrycks i de flesta celler av mesenkymal härstamning. Vimentin är en kanonisk markör och en av orsaksfaktorer för den s.k. epitel-mesenkymala transitionen (EMT), där epitelceller blir övergår till att bli mer rörliga och invasiva i samband med differentiering och cancer.

Vårt labb har tidigare karakteriserat alla interfasspecifika in vivo serin / treonin fosforyleringsställen. Vi kunde visa att genom specifik mutagenes att som imiterar fosforylerat och defosforylerat tillstånd att utav alla vimentinets otaliga fosforyleringsställen är det endast fosforylering av vimentinet seriner 7, 8 och 9 som är av helt avgörande betydelse för att uppnå den fintrådiga struktur som vimentin normalt uppvisar. S6 och S71 och samt 72 uppvisade mindre effekt. Fosforylering av S7, 8 och / eller 9 kan behövas för att stabilisera eller bilda vimentin struktur. Fosforylering av seriner 7, 8 och / eller 9 kan även påverka cytoskeletala interaktioner och initiera depolymerisering av vimentin.

S1P och SPC är sfingolipider, som reglerar t.ex. cellulär migration. I en annan studie visar vi hur S1P och SPC via en ny signalväg kan omstrukturera vimentin och reglera kemotaktisk migration hos sköldkörtel- och bröstcancerceller via vimentinets serin-71-fosforylering. Dessa effekter förmedlas av S1P-receptor 2 och Rho-associerat proteinkinas (ROCK).

I den tredje studien visar vi hur vimentin balanserar Notch signalvägen genom att orsaka ökning av Jagged 1-Notch-signalering på bekostnad av Delta-like 4-Notch signalering i angiogenes. När vimentin är frånvarande, ackumuleras

Jagged 1 i cellmembranet och förlorar sin trans-kapacitet. Vi visar att denna signaleffekt ger upphov till defekta och svagare kärl i fyra modeller: en modell där celler får växa på den inre ägghinnan, d.v.s. chorionallantois membranet (CAM), i en modell där vaskulära endotelceller från människans navelsträng (HUVEC) genomgår angiogenes och den spirande angiogenes analyseras, i analys av aortaringar och slutligen analys av vasulaturen i vimentin knockout musembryon.

Par-4 och CK2 är båda viktiga regulatorer av prostatacancer apoptos och överlevnad. I den fjärde studien visar vi hur CK2-medierad fosforylering av Par-4 försämrar Par-4 proapoptotiska funktioner. Denna effekt beror på att CK2 specifikt inhiberar kaspasberoende klyvning av Par-4 eller verkningar oberoende av klyvning. Vi visar också att denna fosforylering har relevans i prostatacancer.

I den femte studien visar vi en proapoptotisk funktion av AATF efter UV-stress. Vi visar att denna mekanism kräver att AATF utlokaliseras från nukleolerna till nukleoplasman samt en interaktion med transkriptionsfaktorn c-Jun som i sin tur aktiverar c-Juns proapoptotisk funktion.

Tillsammans har dessa studier visat nya fosforylering-medierade mekanismer för reglering av vimentinets struktur, för reglering av en sfingolipid-medierad uppströms signalväg som i sin tur reglerar vimentinets struktur och därmed dess effekter på kemotaktisk migration, samt för att reglera de effekter vi demonstrerat gällande vimentinets förmåga att påverka angiogenes i musembryo och cellmodeller via reglering av Notch-ligander. Våra resultat stärker synen på vimentin som en multifunktionell cellulär byggnadsställning för signalering och ger information om vimentin funktion speciellt i samband med cancer. Vi har också utforskat en annan fosforyleringsberoende räcka där CK2hämmar apoptos i prostatacancer via fosforylering och hämning av pro-apoptotiska Par-4 och . En liknande omkopplare mellan överlevnad och apoptos i prostatacancer upptäckte vi i i samband med att nukleolärt AATF efter UV-strålning påverkade aktiveringen och fosforyleringen av c-Jun genom en tillsvidare oupptäckt translokation av AATF från nukleoler till nukleoplasman. Sammantaget belyser våra resultat ett antal olika sätt med vilka fosforyleringsberoende omkopplare kan reglera cellens ödesbeslut.

INTRODUCTION

For an organism to develop correctly, survive, and reproduce, the building blocks of organisms, cells, need to respond correctly to extra- and intracellular cues. These responses are regulated by a multitude of signaling networks, and lead to cellular responses such as changes in cell shape, cellular and tissue organization, cell motility, cell division and death, maintaining of homeostasis, and further signaling events. Many cellular signaling events are dependent on the addition or removal of post-translational modifications to/from specific amino acids in proteins. The most studied and perhaps the most important of these is phosphorylation.

Entire cells can move by swimming through aqueous solution with the help of beating cilia, flagella, etc. Another form of cell motility is crawling motility. Crawling motility is divided into amoeboid motility and cell migration with lamellipodia. The cytoskeleton, including intermediate filaments, is a crucial part of cell migration. Crawling motility is important in various steps of both development and wound healing, and when misregulated, it can, for example, cause cancer progression by allowing cancer cells to spread to surrounding tissues and to form metastases.

Blood vessels are formed either by vasculogenesis or angiogenesis. Angiogenesis occurs when new blood vessels form from pre-existing ones. Angiogenesis is one of the crucial processes in growth, development, and wound healing. When angiogenesis activates in tumors, oxygen can reach the inside of tumors. This event allows tumors to grow much larger and potentially become malignant. Cell-to-cell-contact-based Notch signaling is a critical regulator of angiogenesis.

Apoptosis is an intracellular death program. It is used by an organism to eliminate unneeded or severely damaged cells. Casein kinase 2 (CK2), Par-4, AATF, and c-Jun are proteins that can, depending on the context, induce apoptosis (Par-4, c-Jun, AATF) or protect from apoptosis (CK2, c-Jun, AATF) through apoptotic signaling circuitry.

When cells harbor specific mutations, their growth becomes uncontrollable. Such uncontrolled growth can cause cancer. There are many barriers, including angiogenesis, metastasis, and apoptosis avoidance, which cancer cells must overcome to become lethal.

One of the important regulators of cellular signaling and mechanics are the third and least studied part of the cellular cytoskeleton: intermediate filaments. Intermediate filaments (IFs) consist of ~70 members of network-forming proteins that express and function in a tissue-specific manner.

Vimentin is the most abundant of the IFs. Its expression occurs in cells originating from mesenchyme. Although vimentin knockout mice created in the 1990s had a very mild phenotype, new interest in vimentin has arisen recently due to vimentin representing a potential target for cancer metastasis treatment, and as influential for organisms under stress. Vimentin is a well-known marker of epithelial-mesenchymal transition (EMT), in which cells lose their epithelial phenotype

and become more migratory and invasive. EMT is thought to be an important mechanism in cancer. Vimentin forms filaments inside cells and also has a more freely-moving soluble pool. Vimentin network structure affects cell mechanics, and acts as a signaling scaffold. Soluble subunits of vimentin have several signaling functions. In this thesis, we examine mechanisms of how vimentin affects cell migration downstream of sphingolipids sphingosine-1-phosphate and sphingosylphosphorylcholine, how it affects angiogenesis via Notch signaling, and how vimentin structure is affected by specific phosphorylation sites. Also, the antiapoptotic effect of CK2 via Par-4 phosphorylation and the apoptotic effect of AATF via c-Jun after UV stress are demonstrated.

REVIEW OF THE LITERATURE

1. Post-translational modifications

Post-translational modifications (PTMs) occur in protein biosynthesis after translation during the lifetime of the protein. Post-translational modifications increase the diversity of the proteome by covalent addition of functional groups or proteins, by cleaving regulatory subunits proteolytically, or by degrading entire proteins. Estimates show that 5% of the proteome consists of enzymes that can perform approximately 400 types of post-translational modifications (Khoury et al., 2011; Snider and Omary, 2014a; Walsh, 2006). Some of the typical modifications are: phosphorylation, ubiquitination, glycosylation, methylation, nitrosylation, lipidation, acetylation, and proteolysis (Khoury et al., 2011). Post-translational modifications are crucial components of all cell signaling pathways, often affecting protein structure and interactions in a critical way. They affect almost all aspects of normal cell biology and pathogenesis, including processes relevant to this thesis: cell migration, differentiation, and death. Often post-translational modifications offer a fast, dynamic, and usually reversible way to alter protein function (Walsh, 2006).

1.1. Mechanisms of post-translational modifications

Post-translational modifications can be added or removed in any step during the life cycle of a protein with the help of a specific enzyme. For example, many proteins are modified after translation completes. Modification may mediate proper protein folding or stability, or guide the new protein to a distinct cellular compartment (Bauer et al., 2015; Braakman and Bulleid, 2011; Nalivaeva and Turner, 2001). After folding and proper localization, the protein can be modified by PTMs, which, for example, may regulate protein enzymatic activities, target proteins for degradation, or cleave the protein. Sometimes, post-translational modifications occur in a step-wise fashion to mature or activate a protein. Some modifications, such as phosphorylation and acetylation, are reversible, and some, such as proteolytic cleavage and sulfation, are not. A single protein can have a multitude of different modifications, which act in a variety of ways (Dai and Gu, 2010; Nalivaeva and Turner, 2001; Walsh, 2006).

1.2. Protein phosphorylation and dephosphorylation

All the articles in this thesis examine the effects of protein phosphorylation. Reversible phosphorylation is the most abundant post-translational modification in cellular proteins. At least one third of proteins go through reversible phosphorylation by phosphorylating kinases and dephosphorylating phosphatases. Protein kinases are one of the largest gene families in living organisms. In humans, there are 518 different kinases and 106 inactive pseudogenes (Roskoski, 2015). There are two large kinase families: histidine autokinases and eukaryotic protein kinases. Eukaryotic kinases catalyze the phosphorylation of serine, threonine, or tyrosine residues, or in some cases all of the listed amino acids (Marks et al., 2009; Roskos-

ki, 2015). The number of protein phosphatases is close to 200 (Almo et al., 2007). Recently, it has been found that arginine, histidine, and lysine residues may also be phosphorylated yielding acid-labile phosphoramidates (Cieśla et al., 2011).

The phosphate group carries two negative charges in a neutral medium and can form four hydrogen bonds via its four oxygen atoms. Adding this structure to an amino acid may cause substantial changes in protein structure. Phosphorylation yields highly substrate-specific effects. It can activate or deactivate protein function, provide new interaction interfaces, or affect protein stability or localization (Nishi et al., 2014). Many factors, including cellular stresses, nutrition status, or activation of signaling pathways, can influence the activity of a kinase. Also, tissue-specific expression of kinases or subcellular localization of them, and the specificity to particular substrate sequences, allows control over when and where specific substrates become phosphorylated (Marks et al., 2009).

2. Cell migration

Cell migration is important both during the development and maintenance of the homeostasis of multicellular organisms. Immune responses, tissue formation in embryonic development, and wound healing require organized cell movement to certain locations (Alberts et al., 2007; Weinberg, 2013). Aberrantly regulated cell migration can produce a progression of cancer, e.g. during metastasis (Yilmaz and Christofori, 2010). Cell migration can occur as mesenchymal or amoeboid migration (Bear and Haugh, 2014; Guck et al., 2010; Lämmermann and Sixt, 2009). Typical in amoeboid migration is the formation of blebs, which are produced by actomyosin contractility (Lämmermann and Sixt, 2009). Mesenchymal migration is characterized by the formation of lamellipodia at the front leading edge of the cell, and filopodia extending beyond lamellipodia. All the components of the cytoskeleton have important roles in regulating cell migration (Bear and Haugh, 2014; Bisi et al., 2013; Machesky, 2008; Mattila and Lappalainen, 2008).

The main processes included in cell migration are the formation of cell polarity, protrusion formation, cell adhesion, translocation of the cell body, and the retraction of the rear end, all of which are accompanied by signal integration and regulation (Vicente-Manzanares and Horwitz, 2011). Cell polarity forms through molecular and functional differences between the front and rear of the migrating cell (Macara and McCaffrey, 2013). Protrusion formation involves the extension of cellular membranes. Leading protrusion usually points in the direction of cell movement. Protrusions usually form in response to chemoattractive signals and their gradients, which cause cells to move in the correct direction. However, some cells extend protrusions in an exploratory manner without directional stimulation. Formation of protrusions requires several cellular processes. Actin cytoskeleton polymerization pushes the cell membrane facing towards movement forward, and the cell membrane expands under tension when endocytosed vesicles fuse to the cell front. Protrusions must also adhere to the surrounding substrate to allow the rest of the cell to be pulled toward protrusion (McNiven, 2013; Petrie and Yamada,

2012; Ridley, 2011). Cell adhesion occurs between adjacent cells or between the cell and the extracellular matrix (ECM). Cell-matrix adhesion is the most thoroughly-studied form of adhesion mediating cell migration. Cell-matrix adhesions are sites of convergence between the actin cytoskeleton and ECM fibrils. There are several types of adhesions: nascent adhesions, focal complexes, focal adhesions, podosomes and invadopodia with different distributions, molecular compositions, and roles in migrating cells (Parsons et al., 2010; Ridley, 2011). Cell body translocation immediately follows protrusion. This movement is caused by coordinated contraction of the actomyosin cytoskeleton, and depends on myosin II. Translocation of the nucleus is controlled by actin-myosin contraction and microtubule motors. Cell rear retraction is accomplished by coordinated contraction of the actin cytoskeleton and disassembly of the adhesions at the trailing edge (Cramer, 2013). Multiple intertwined signaling networks control cell migration. Signaling can be initiated through G-protein-coupled receptors (GPCRs), integrins, growth factors, and other receptors, and continues to propagate along one or more signaling branches of complex interconnected networks. Small Rho (Ras-homology) GTPases are important regulators of migration. Migration-related signaling often converges to small Rho GTPases. One of them is RhoA, which can relay signals downstream (among others) to Rho-associated protein kinases (ROCKs). Small Rho GTPase signaling results in relatively few varieties of cellular response, including the aforementioned processes (Alberts et al., 2007; Bear and Haugh, 2014; Devreotes and Horwitz, 2015; Hasan and Siekmann, 2015; Weinberg, 2013).

Intermediate filaments vimentin and sphingolipids S1P and SPC are non-essential regulators of cell migration. In this thesis, we examine how the effects of sphingolipids S1P and SPC on migration are connected to IF vimentin effect on migration through S1P receptor 2 (S1PR2) and ROCK.

3. Angiogenesis

Angiogenesis is a process in which new vasculature forms from pre-existing blood vessels. The process is crucial to organism development and growth, wound healing and granulation tissue formation, but it is also critical to the formation of larger, malignant, tumors. Early embryonic mesoderm gives rise to blood vessels and lymphatic vessels, for example. Blood supply depends on endothelial cells, which form the lining of blood vessels. During tissue growth and repair, endothelial cells extend and remodel existing vasculature. There are two forms of angiogenesis: sprouting and intussusceptive. In intussusceptive (splitting) angiogenesis a new blood vessel is formed when an existing blood vessel splits in two. This study mainly concerns sprouting angiogenesis. In the sprouting angiogenic process, an endothelial cell starts to extend and proliferate toward the surrounding matrix from the wall of an existing blood vessel. An endothelial tip cell, with many filopodia, leads the advance of each capillary sprout by invading surrounding tissue. Behind the tip cell, endothelial stalk cells form and divide, and start to form a hollow capillary sprout that becomes a new blood vessel tube. Notch signaling is essential

to the decision of which cells become tip or stalk cells. Angiogenesis has many similarities and signaling mechanisms in common with lymphangiogenesis and neuronal growth (Alberts et al., 2007; Krishna Priya et al., 2016; Weinberg, 2013).

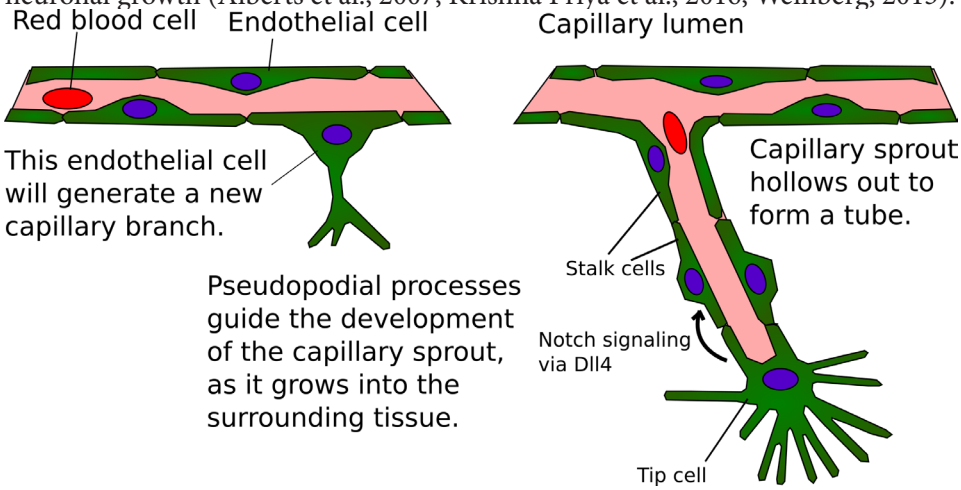


Figure 1: Basic mechanisms of angiogenic sprouting. Adapted from (Alberts et al., 2007).

Angiogenesis is controlled by the production of hypoxia-inducible factor 1 α (HIF1α) when tissues face low oxygen levels. HIF1α stimulates transcription of vascular endothelial growth factor (VEGF) (Ahluwalia and Tarnawski, 2012). VEGF secretes from cells and stimulates nearby endothelial cells to proliferate, invade through basal lamina of the parent capillary or venule, and form sprouts. The tip cells of the sprouts detect the VEGF gradient and move toward it. When the oxygen concentration in the tissue becomes normal, expression of HIF1α and VEGF is downregulated ending the angiogenic response (Alberts et al., 2007; Carmeliet, 2005; Cébe-Suarez et al., 2006; Kiselyov et al., 2007; Weinberg, 2013).

Notch signaling mediates angiogenesis by regulating one of the main processes of angiogenesis: endothelial cells signaling to each other and the decision of which endothelial cells become tip cells and which become stalk cells. This effect is regulated by specific Notch ligand, Delta-like 4, which is expressed in tip cells and signals to neighboring stalk cells, activating their Notch signaling. This causes neighboring cells to downregulate their VEGF receptors causing them to become unresponsive to VEGF, and thus preventing the formation of too many sprouts (Alberts et al., 2007; Benedito and Hellström, 2013; Bridges et al., 2011; Garcia and Kandel, 2012; Kangsamaksin et al., 2014a; Liu et al., 2014; Weinberg, 2013). In this thesis we further examine the newly found connection between vimentin cytoskeleton and angiogenesis, and specifically focus on how vimentin affects the signaling from the Notch ligands Jagged1 and Delta-like.

4. Apoptosis

Multicellular organisms require tight control of their cell populations, since overall cell numbers and cell types, as well as cell locations, need to be correct in

order to produce a healthy and functional organism. Cell numbers are controlled by their rate of proliferation, but also by cell death. This ratio must be balanced to keep an organism the correct size. Cells can undergo apoptosis or necrosis. Cells that die as a result of acute injury typically undergo necrosis: they swell and burst. Unnecessary or sufficiently damaged cells are removed by programmed cell death, the most common form of which is apoptosis (Edinger and Thompson, 2004; Nicotera et al., 1999; Nikoletopoulou et al., 2013). Apoptosis is apparent during development. For example, in order to form separate and correctly shaped digits, numerous cells between the digits must be removed. During neuronal development, many neurons die in order to match the number of target cells that require innervation. Also, in adult humans, billions of cells in the bone marrow and intestines die each day. Apoptosis also occurs during DNA damage and other stresses, if the damage incurred is substantial enough. Apoptosis allows the organism to eliminate nonfunctioning cells, and prevents the development of cancer by removing cells with potentially cancer-promoting mutations (Alberts et al., 2007; Krishna Priya et al., 2016).

In apoptosis, cells die without bursting or causing inflammatory reactions. Cells undergoing apoptosis shrink and condense; the cytoskeleton collapses, the nuclear envelope disassembles, and the nuclear DNA breaks into fragments. The cell surface also changes, sending signals to nearby cells or macrophages to phagocytose the apoptotic cell (Alberts et al., 2007; Moffitt et al., 2010).

Apoptosis is carried out primarily by an intracellular proteolytic cascade, mediated by caspases. During apoptosis, inactive procaspases are activated to caspases, often initially in distinct activation complexes: DISC or apoptosome. Cells have at least two distinct pathways for apoptosis. The extrinsic pathway activates by extracellular ligands binding to death receptors. The intrinsic pathway is activated by intracellular signals in response to stresses. Extracellular signal proteins and Bcl2 and IAP proteins are major regulators of apoptosis, only allowing apoptosis to occur when it is beneficial for the organism (Alberts et al., 2007; Moffitt et al., 2010).

In this thesis, we examine how protein casein kinase 2 (CK2), a pro-survival factor, inhibits pro-apoptotic effects of prostate apoptosis response-4 (Par-4) via direct phosphorylation that is likely functioning in prostate cancer.

5. Cytoskeleton

The cytoskeleton is a scaffolding structure in the cellular cytoplasm. The cytoskeleton provides structure and shape for the cell. The cytoskeleton consists of three different protein families: microfilaments, microtubules, and intermediate filaments (IFs) (Huber et al., 2013). Microfilaments, consisting of actin, are around 6 nm thick, and are flexible and relatively strong filaments. Microfilaments are highly versatile, and function in cytokinesis, cell motility, and changes in cell shape. Actin filaments can provide force for a cell with actin-attaching myosin molecular motors, which can be applied for pushing the cell membrane or moving vesicles inside the cell, for example (Sackmann, 2015). Actomyosin-driven

contractile molecular motors also drive muscle contraction (Chalovich, 1992). Microtubules form from α- and β-tubulin with an average width of 24 nm. Microtubules can form cilia and flagella, and also form platforms for intracellular transport. Microtubules participate in movements of secretory vesicles, organelles, and intracellular substances. Dynein and kinesin motor proteins move along microtubules. During mitosis, microtubules form mitotic spindles, which are crucial for pulling apart eukaryotic chromosomes. Microtubule networks protrude from central locations called microtubule organizing centers (MTOC) (Alberts et al., 2007; Vasiliev and Samoylov, 2013; Wade, 2009).

6. Intermediate filament protein family

Intermediate filaments are a protein family consisting of about 70 members, and the third member of the cytoskeleton. They are found in metazoan cells. Intermediate filaments form flexible filamentous networks inside the cell. Most of IFs are mainly cytoplasmic, except the lamins, which reside mainly in the nucleus (Eriksson et al., 2009).

Intermediate filaments get their name from being "intermediate" in width (about 10 nm), as compared to other cytoskeletal components, namely microtubules and actin. The IF networks are flexible and give mechanical strength to the cell. Intermediate filament networks protect cells from mechanical stress, provide cells with much of their shape, and some IFs are significant for cell motility (Eriksson et al., 2009; Goldman et al., 2008; Herrmann et al., 2007). Intermediate filaments are expressed tissue-specifically and also at certain times, such as during wound healing or embryonic development (Pallari and Eriksson, 2006).

Intermediate filaments are divided into six distinct subcategories based on their sequences. Type I and II IFs are formed by acidic and basic keratins – mixing in a 1:1 ratio. Keratins are expressed in epithelial cells and consist of 54 members in humans (Moll et al., 2008; Oshima, 2007; Toivola et al., 2015). Group III IF proteins consist of vimentin, desmin, glial fibrillary acidic protein (GFAP), and peripherin, of which vimentin is the subject of three articles presented in this thesis. These proteins can form homo- or heteropolymeric filaments. Type IV IFs consist of neurofilaments (NFs) NF-L, NF-M, and NF-H, synemin, α-internexin, nestin, and syncoilin (Moorwood, 2008; Omary, 2009; Xie et al., 2015; Yuan et al., 2012). Type V IFs consist of lamins. They reside mostly on the nuclear membrane (Gruenbaum and Foisner, 2015; Omary, 2009; Osmanagic-Myers et al., 2015; Yoon et al., 2012). Intermediate filament type VI consists of two proteins: beaded filaments Bfsp1 and Bfsp2. They are expressed in eye lenses (FitzGerald, 2009; Omary, 2009). A summary of the IFs and the tissues they are expressed in is shown in Table 1.

Tissue	Intermediate filaments expressed
Lens	Bfsp1 (VI); Bfsp2 (VI); Vimentin (III)
Nervous system	Neurofilaments NFH, NFM, NFL (IV); α-internexin (IV); Nestin (IV); Peripherin (III); GFAP (III)
Skin	Epidermal keratins K1, K2, K5, K6 (II), K9, K10, K14-K17, K19 (I)
Lungs, Liver, Intestine, Gut	Simple epithelial keratins K7, K8 (II), K18-K20, K23 (I)
Muscle (cardiac, skeletal, smooth)	Desmin (III); Syncoilin (III); Synemin (IV)
Blood vessel/other mesenchyme	Vimentin (III)
Nucleus	Lamin A/C (V); Lamin B1, B2 (V)

Table 1: Intermediate filament tissue distribution. The group of the IF is shown in brackets (I-VI). Adapted from (Omary, 2009).

6.1. Intermediate filament structure

All IFs share a similar structure. In the center of the amino acid sequence there is a conserved rod domain, which is crucial for filament assembly and forms α-helixes. In the ends of an IF, there is an N-terminal head domain and a C-terminal tail domain. These domains are more variable in sequence, and contain multiple post-translational (most importantly phosphorylation) modification sites that affect IF structure and function (Hyder et al., 2008a). The N- and the C-termini likely cause the unique characteristics of each IF protein (Parry, 2005). The rod domain forms the core of the filament. The N- and the C-termini reside on the filament surface. Type I and II epidermal keratins, and type III IF N-terminal head domains exhibit a flexible structure, which can interact with the rod domain (Block et al., 2015; Godsel et al., 2008; Lowery et al., 2015). In both cases, head domain is needed for in vitro filament assembly (Herrmann et al., 1996; Wilson et al., 1992). C-terminus is not required for filament assembly but produces an increase in the mass-per-length ratio (Herrmann et al., 1996). Exposure of N- and C-termini on the filament surface leaves them free to associate with other filaments and cellular structures in vivo, and affect filament structure or assembly through these associations (Block et al., 2015; Godsel et al., 2008; Lowery et al., 2015).

Intermediate filaments are long and flexible structures. Therefore, it is challenging to produce crystal from them to use for structural analysis with X-ray diffraction. By crystallizing partial filaments, an almost complete structure of IF vimentin rod domain (which has a relatively stable structure) dimer has now become known (Chernyatina et al., 2012, 2015). However, ascertaining how the much more freely

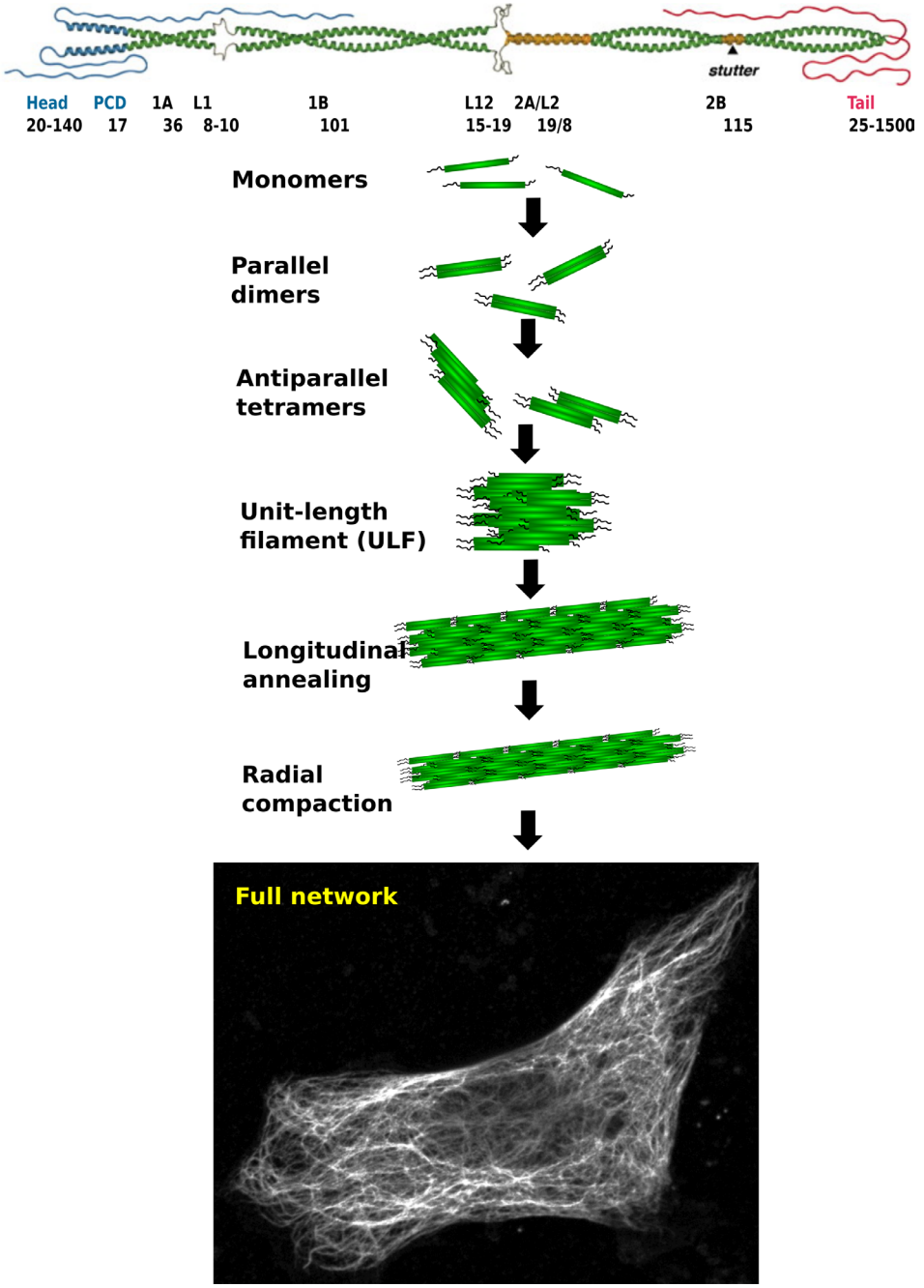
mobile head and tail domains, and their post-translational modifications, contribute to the overall IF structure cannot be determined by crystallization and X-ray diffraction analysis. As a result, there is a need for alternative approaches.

6.2. Intermediate filament assembly

Intermediate filament assembly starts with the formation of dimers. In dimers, α-helical rod domains of two proteins are wound together in a coiled-coil form in a parallel fashion. After dimer formation, dimers assemble into tetramers. Tetramers form when two dimers associate in an antiparallel form (Mücke et al., 2004; Sokolova et al., 2006). This tetrameric subunit is a form of filament, in which most of the soluble non-filamentous vimentin resides in a cell. This form is apolar, and complete filaments are apolar as well. This stands in contrast to actin and microtubules, which form filaments with distinct plus and minus ends (Cooper, 2000).

Tetramers associate into higher-order oligomers to form unit-length filaments (ULFs). ULFs are around 60 nm long. In these polymers, filaments can associate in antiparallel formations in a number of ways. ULFs elongate longitudinally to form immature IFs. Immature 16 nm-wide filaments radially shrink into a more compact, 10-12 nm-wide structure in order to form mature filaments. This step has been suggested as occurring via lateral arrangements of filaments so that the mass or the filament length does not change (Godsel et al., 2008; Herrmann et al., 1996, 2007; Parry et al., 2007; Sokolova et al., 2006).

Figure 2 (next page): Structure and assembly of IFs. Vimentin as an example. A) The molecular structure of vimentin dimer. Intermediate filament structural domains and their lengths in amino acids. Intermediate filaments consist of a variable head and tail domains and a more conserved rod domain. The rod domain consists of α-helical, more rigid, coils A1, B1, A2 and B2 and more flexible linkers L1, L12, and L2 (present knowledge identifies A2 and L2 as structurally paired). B) Different assembly stages of IFs. C) Full vimentin network in MEF cells. Adapted from (Minin and Moldaver, 2008a; Parry et al., 2007).



IF networks are stable under normal conditions. Their half-time of recovery is typically in the order of minutes (e.g. for vimentin t½ this is about six minutes) (Yoon et al., 1998). When cells migrate, divide, or spread, or when stresses such as shear flow or stretching affect cells, IFs undergo structural rearrangements. During these events, largely unknown post-translational modifications and inter-

acting proteins regulate IF assembly states (Snider and Omary, 2014a). Intermediate filaments elongate from the filament ends, and they are capable of exchanging subunits along their length (Colakoğlu and Brown, 2009).

Lateral association of protofibrils into protofilaments is likely regulated by the IF head domain. The termination of filaments after they have grown to approximately 10 nm is regulated by the tail domain (Heins et al., 1993). Phosphorylation is a critical effector of these events. Vimentin and desmin in vitro phosphorylation, which is caused by protein kinase A or C, disassemble pre-existing filaments and block subunit polymerization (Eriksson et al., 2004a; Geisler and Weber, 1988; Inagaki et al., 1987). This observation suggests that site-specific phosphorylation of the amino-terminal head domain of IF proteins induces the disassembly of IFs (Sihag et al., 2007a). Phosphorylation of vimentin head domain drives specific head domain regions away from each other (Aziz et al., 2010). Phosphorylation of some of the vimentin head domain amino acids 35-75 has been hypothesized to be active in the assembly and breakdown of vimentin filaments. This hypothesis derives from studies that show filament assembly requires some parts of the head domain (Gill et al., 1990; Heins et al., 1993; Sihag et al., 2007a). It is also known that the large middle part of the vimentin N-terminus (amino acids 40-95 or 25-38) and the entire C-terminus are not essential for vimentin filament formation (K R Rogers, 1995; Shoeman et al., 2002). Nonapeptide motif near the N-terminus of vimentin head is essential for filament formation (Beuttenmüller et al., 1994; Herrmann et al., 1992). Vimentin disassembly by phosphorylation produces tetrameric subunits with specific functional significance as a soluble vimentin pool in cells. Therefore, the critical step of filament assembly that is regulated by phosphorylation may be protofilament assembly from tetramers (Eriksson et al., 2004a). The IF assembly stage has a critical effect on the signaling events it regulates (in addition to mechanical properties) and therefore on the cellular functions of IFs.

6.3. Phosphorylation and other post-translational modifications of intermediate filaments

Intermediate filaments go through a multitude of post-translational modifications, including phosphorylation, farnesylation, ubiquitylation, sumoylation, glycosylation, acetylation, transamidation, and ADP-ribosylation. It is important to characterize post-translational modifications' on-off mechanisms, crosstalk, and utility as biomarkers for IF-related diseases (Snider and Omary, 2014a). IF PTMs have a multitude of different cellular functions. Often, the effects are based on PTMs affecting which molecules can interact with IF surfaces, or by affecting IF networks or their assembly itself. Most PTMs reside in the tail and head domains, which have better access for interacting with the surrounding molecules and which are also the least conserved parts of IFs, likely contributing the most to unique functions of different IFs.

IFs have similar structures and variably conserved sequences. Therefore, specific PTM in one IF may have similar PTM site and corresponding effects in other IFs also. The most well-known PTM of IFs is phosphorylation. Phosphorylation

was first observed to regulate IF reorganization, increase dynamic subunit exchange by ser/thr-kinases, and to promote IF solubility (Sivaramakrishnan et al., 2009a; Snider and Omary, 2014a; Woll et al., 2007a). Effects of tyrosine phosphorylation are less known, but keratin 8 rod domain Y267 phosphorylation is known to promote keratin insolubility (Snider et al., 2013). Several sites, mostly in the IF head and tail domains, regulate IF reorganization. IF subunits can dynamically join IF filaments anywhere along the length of IF structures. This subunit exchange is heavily regulated by phosphorylation. Usually, when affecting solubility, serine or threonine phosphorylation increases IF solubility. Other PTMs are also likely to affect solubility, and rod domain PTMs are likely increased by some head and tail domain PTMs when the rod domain becomes more accessible to modifying enzymes (Hyder et al., 2008b; Sihag et al., 2007b; Sivaramakrishnan et al., 2009b; Woll et al., 2007b).

Neurofilaments NF-M and NF-H have high phosphorylation of their tail domain lys-ser-pro (KSP) repeat motifs. This phosphorylation is caused by proline-directed kinases. KSP-phosphorylation regulates axonal caliber by cross-bridging NF sidearms, and also affects axonal stability and transport (Ackerley et al., 2003; Nixon et al., 1994; Snider and Omary, 2014a). NF tail domain is typically highly phosphorylated in the axons. On the other hand, phosphorylation of the head domain is relatively low in the neuronal cell bodies. NF phosphorylation is deregulated in several neurodegenerative diseases (Binukumar et al., 2013).

Another important role of IF phosphorylation is the regulation of stress response. Keratin phosphorylation may serve as a phosphate "sponge" in protecting hepatocytes during liver injury. Keratins act as phosphorylation targets that become phosphorylated instead of the pro-apoptotic proteins, which are activated by phosphorylation (Ku and Omary, 2006; Snider and Omary, 2014a). Phosphorylation-preventing mutations of K8 S74 and K18 S54 are known to increase apoptosis and liver injury in mice (Ku and Omary, 2006; Ku et al., 1998). K4-K6 also become phosphorylated like K8 during various stresses and disease, indicating a possible similar role (Toivola et al., 2002). Since IFs are one of the most abundant proteins, this kind of functionality, which requires large quantities of protein to absorb extra phosphate groups or immobilize other molecules, is possible for them.

Lamin A/C disassembly during mitosis is regulated by its serine 22 phosphorylation by Cdk1. This same pathway is used by cytomegalovirus (HCMV) by its kinase UL97, along with possibly PKC and Pin1 acting as mediators, in order to accomplish HCMV nuclear egress (Hamirally et al., 2009; Milbradt et al., 2010). This mechanism likely functions in a different context in the regulation of the transport of endogenous ribonucleoprotein particles to the cytoplasm. When this mechanism is disrupted by Lamin C muscle knockout in D. Melanogaster, neuromuscular junction (NMJ) defects are observed (Speese et al., 2012).

Cell process	IFs found to be involved	Details
Axonal properties	NF-H, NF-M	Stability, caliber, and transport – KSP repeats
Cell growth	K5, K17, K18, vimentin	Skin wound healing, liver regeneration, autophagy, interaction with 14-3-3
Cell migration	K8, vimentin	K8 inhibits, vimentin increases
Cell stress	K8-K18	Inhibits hepatotoxicity and apoptosis
Disease states	phospho-IF accumulation	pNF in neurons in axon and also in cell body, loss of pIF polarity
Muscle atrophy	Desmin	Ubiquitylation and breakdown of thin filaments
Neuromuscular junction development	Nestin	Disperses AchR cluster
Nuclear egress	Lamin A/C	Herpesvirus or ribonucleoprotein release from nucleus by disassembled lamina

Table 2: A list of cell processes where phosphorylation of different IFs plays an important role (Adapted from (Snider and Omary, 2014a))

Nestin phosphorylation is significant in NMJ (Yang et al., 2011). Cdk5 phosphorylates nestin T316 during myoblast differentiation causing nestin disassembly (Sahlgren et al., 2003). Nestin also has a scaffolding property for Cdk5 and p35 at NMJ synapses (Yang et al., 2011). Phosphorylation of nestin causes Cdk5 dissociation and activation, which affect acetylcholine-induced dispersal of acetylcholine receptor clusters (Lin et al., 2005). Without the aforementioned mechanism, nestin-null mice exhibit impaired motor coordination. During muscle atrophy, desmin S28, 32, and 68 phosphorylation helps with the breakdown of thin filaments

and Z-band components via ubiquitin ligase Trim32 in mature myofibers (Cohen et al., 2012).

An important signaling function for IFs is associations with 14-3-3 proteins. For example, K18 S33 phosphorylation has been shown to regulate 14-3-3ζ binding. Adaptor protein 14-3-3 binds to some IF proteins, including K5, K17, K18, and vimentin, in a Ser/Thr-phosphorylation-mediated way. The interaction affects cell growth and tumorigenesis (Liao and Omary, 1996). K18 S34 and K17 T9 and S44 phosphorylation create the 14-3-3 binding site. This interaction stimulates mTOR activation and cell growth during, for example, wound healing in skin epithelia (Kim et al., 2006; Ku et al., 2002). With K18, it also affects 14-3-3ζ availability in the nucleus to bind Cdc25, and aids mitotic progression (Pallari and Eriksson, 2006). 14-3-3σ stabilizes a complex of soluble actin and cytokeratin, increasing breast tumor invasion. Stabilization is dependent on PKCξ-mediated phosphorylation (Boudreau et al., 2013).

Vimentin and many vimentin phosphorylation sites affect cell migration (presented in more detail later in this text). K8 S432 phosphorylation had a context-specific effect with increased migration of pancreatic and gastric cells and decreased migration of squamous carcinoma cells (Alam et al., 2011; Busch et al., 2012).

Intermediate filament phosphorylation occurs in many diseases. Advances in proteomic capabilities may allow pinpointing specific IF PTMs to indicate different diseases, and IF PTMs may become a potential target for IF disease treatment (Omary et al., 2004; Snider and Omary, 2014a; Szeverenyi et al., 2008).

Other PTMs of IFs are less studied than phosphorylation and have a variety of effects. Sumoylation of IFs is known to regulate filament formation and solubility, and be modified in some IF mutant diseases and stresses, mainly in keratins and lamins. Vimentin$_{354}$ sumoylation has been observed to affect migration. Acetylation of K8 K207 reduces solubility. K18 glycosylation can increase cell survival. Lamin A farnesylation affects its own association with the nuclear membrane and plays a role in the aging syndrome progeria. Lamin B farnesylation is crucial to neuronal development. Ubiquitination of keratins marks them for degradation. Transamidation of keratins is important for skin barrier function and formation of ´MDBs. ADP-ribosylation inhibits at least desmin filament formation (Snider and Omary, 2014b).

Intermediate filament PTMs show complex crosstalk in modulating protein properties and cellular functions. For example, sumoylation of keratins is strongly increased by keratin phosphorylation (Snider and Omary, 2014a; Snider et al., 2011). Increase in sumoylation is partially dependent on keratin 8 S74 phosphorylation and following sumoylation of keratin 8 K285 and K364 (Daub et al., 2008; Dephoure et al., 2008). In neurofilaments and keratins, there is an inverse correlation of phosphorylation and glycosylation. This occurs, for example, when NF-H hyperphosphorylates, and has lower glycosylation during aging due to decreased PP2A activity. It also occurs in Alzheimer-diseased brains when NF-M has increased phosphorylation and decreased glycosylation (Deng et al., 2008; Veeranna

et al., 2011). Keratins show similar behavior in a mouse liver injury model. Blockade of K18 glycosylation increases K8 S74 phosphorylation (Ku et al., 2010). K8 lysine acetylation may function as a sensor to link metabolic status to K8 phosphorylation and filament reorganization. K8 K207 acetylation promotes insolubility and increases phosphorylation of K8 S74 and aggregation (Snider et al., 2013). Ubiquitination takes place when IFs are hyperphosphorylated (Ku and Omary, 2000). Intermediate filament ubiquitination also occurs with proteasomal inhibition and formation of IF inclusions, such as Mallory-Denk Bodies (MDBs) found in liver cells during some diseases (Ku et al., 2007a; Tang et al., 2010). MDB formation is also associated with transamidation and stress-induced keratin phosphorylation (Kwan et al., 2012). The mechanisms of IF crosstalk are largely unknown. Specific PTMs influence especially the immediate surroundings of the PTM and potentially (possibly by protein folding) regions further away in the IF molecule wherein the PTM is located. In addition, PTM can potentially block or free sites on nearby molecules in higher-order IF filamentous structures.

6.4. Intermediate filaments in cell mechanics, stress, and disease

Intermediate filaments can provide protection from many types of stresses. Intermediate filaments have cytoprotective roles in the inhibition of apoptosis, organelle homeostasis, and scaffolding. The best known of the cytoprotective mechanisms is the protection from mechanical stress (Pekny and Lane, 2007; Toivola et al., 2010). Mechanical forces are absorbed by the flexible, crosslinked, filamentous structures formed by IFs.

Intermediate filaments have important functions in regulating cell mechanics. Cytoplasmic IFs, especially keratin, vimentin, and desmin, strongly affect cell stiffness (Charrier and Janmey, 2016; Gruenbaum and Aebi, 2014; Kiss et al., 2006; Nolting et al., 2014; Schopferer et al., 2009; Wang and Stamenovic, 2002a). In the nucleus, lamins regulate nuclear mechanics and mechanotransduction (Davidson and Lammerding, 2014; Lammerding et al., 2006). Intermediate filaments are apolar networks and are in constant interaction with other cytoskeletal networks: microtubules, microfilaments, and possibly other IFs (Gruenbaum and Aebi, 2014). Intermediate filaments connect to other cytoskeletal networks directly, and for example through plectin molecules. During cell contraction or externally applied stresses, plectins potentially transmit considerable quantities of forces among the three cytoskeletal filament systems (Castañón et al., 2013; Foisner et al., 1988).

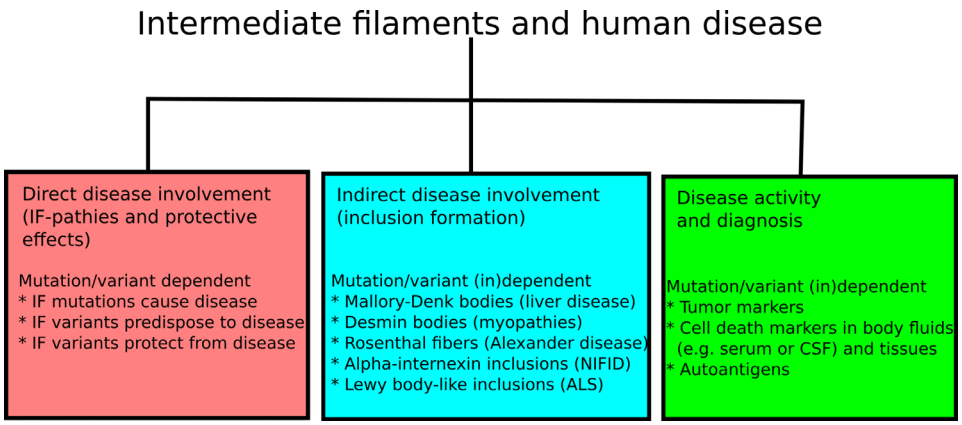


Figure 3: The involvement of IFs in human diseases. Adapted from (Omary et al., 2009).

Specific mutations in most of the IFs can either cause or result in predisposal to disease states. To date, only a few IFs are not linked to diseases. Some IFs are also involved mutation-independently in human diseases, leading to the formation of characteristic cytoplasmic inclusions. The effect of these inclusions on the disease progression is not clear, but they are nevertheless histopathologic and diagnostic hallmarks of these diseases. IF-protein inclusions may be caused by overexpression of particular IFs in specific diseases (Omary, 2009). Mutation typically in keratins K5 and K14 can cause epidermolysis bullosa simplex (EBS) (Coulombe et al., 2009; Müller et al., 1998). Keratins K8, K18, and K19 can also be the causative agents in liver diseases. K8 and K18 are the major constituents and essential for the formation of MDBs, which are associated with several liver diseases (Ku et al., 2007b; Strnad et al., 2012; Zatloukal et al., 2007). Desmin mutations can cause skeletal and cardiac myopathies, of which there are over 20 different types (Goldfarb and Dalakas, 2009). Neuronal type III and IV IFs can be involved in several neurodegenerative disorders and in the formation of inclusions. Neurodegenerative Alexander disease is almost always caused by dominantly acting mutations in GFAP gene, and initially defects in astrocytes expressing GFAP (Li et al., 2002; Quinlan et al., 2007). In many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and motor neuron diseases, there are large accumulations of neuronal IFs in the neurons: neurofilaments, α-internexin, and peripherin. Mutations in NEFL gene (coding NF-L) are one cause of the inherited peripheral neuropathy Charcot-Marie-Tooth disease (Kazamel and Boes, 2015; Saporta, 2014). Mutations in NF-H, NF-M, and peripherin are weakly linked to amyotrophic lateral sclerosis (commonly known as ALS), that degrades motor neurons (Liem and Messing, 2009; Riva et al., 2016). Mutations in nuclear lamins can cause laminopathies. Laminopathies range from muscular dystrophy and cardiomyopathy to partial lipodystrophy and progeroid syndromes. Numerous mutations in lamin A and C (coded by LMNA gene), or less rarely in lamin B1 or B2, can cause these diseases (Maraldi et al., 2011; Worman, 2012). Beaded filament proteins Bfsp1 and Bfsp2

and vimentin mutations have also been found to cause cataracts by changing the optical properties of the eye lens (Song et al., 2009). Vimentin absence does not cause cataracts since vimentin knockout mice do not have cataracts. Vimentin with assembly disrupting-mutation R113C or phospho-deficient mutations in mitotic phosphorylation sites cause cataract formation in mouse models. In humans, one individual with E151K vimentin assembly-disrupting mutation had a cataract (Bornheim et al., 2008; Colucci-Guyon et al., 1994; Matsuyama et al., 2013; Müller et al., 2009).

In summary, the multitude of diseases associated with IFs highlights the important role of these abundant proteins in the main tissues they are expressed in. Many of the diseases in which abnormal IFs are causative agents relate to the significant role of IFs in the protection from stress.

7. Vimentin

Vimentin belongs to group III IFs. Vimentin is expressed mainly in cells of mesenchymal origin. In murine development, vimentin expression is highest during the primitive streak stage starting from embryonic day 8.5 (Franke et al., 1982). In adults, there is less vimentin expression, and it is localized to connective tissue mesenchymal cells, the central nervous system and muscle (Larsson et al., 2004). Vimentin expresses in many cell types, including pancreatic precursor cells, neuronal precursor cells, sertoli cells, fibroblasts, trophoblastic giant cells, renal tubular cells, endothelial cells lining blood vessels, mesangial cells, macrophages, astrocytes, leukocytes, and renal stromal cells (Carter et al., 2005; Cochard and Paulin, 1984; Evans, 1998; Ko et al., 2004; Mahrle et al., 1983; Satelli and Li, 2011a; de Souza and Katz, 2001). Vimentin likely functions in many of these cell types in similar ways, such as by providing mechanical stability. However, vimentin also affects several physiological processes that are often specific to cell type. Still, specific functions of vimentin are known only in a few of the cell types in which vimentin is expressed. Vimentin, like all other IFs, is composed of central α-helical rod domain, flanked by distal head and tail-regions (Parry, 2005). Vimentin functions in the cell's biological and mechanical activities, including regulation of cell migration, adhesion, contractility, stiffness, stiffening, and proliferation (Wang and Stamenovic, 2002b). These processes concerning single, sometimes specific types of cells typically affect more expansive processes in the multicellular organism, such as wound healing, immune response, angiogenesis during implantation and embryonic development, lymphangiogenesis, etc. The effects vimentin has/may have on these multicellular processes, possibly under stress, have not yet been completely determined.

Knockout models of vimentin did not reveal any apparent defects and showed virtually normal phenotypes. Therefore, vimentin is not critical for the survival of mice under normal physiological conditions (Colucci-Guyon et al., 1994). Still, further research has shown that vimentin functions as a regulator of fundamental physiological processes, and can have a major impact on the survival of an organ-

ism during stress (Ivaska et al., 2007a). Later studies involving more detailed evaluation showed that vimentin knockout mice had impaired wound healing in both embryonic and adult stages due to weak and severely disabled fibroblasts incapable of migrating (Eckes et al., 2000). Vimentin knockout mice exhibit morphological changes in glia cells, decreased dilation of resistance arteries under flow, disturbed leukocyte homing to lymph nodes, and vascular endothelium with less integrity (Colucci-Guyon et al., 1999; Eckes et al., 1998; Henrion et al., 1997; Nieminen et al., 2006). Vimentin knockout mice also show impaired motor coordination and cerebellar defect (Colucci-Guyon et al., 1999).

Vimentin, like other IFs, functions as scaffolding and signaling platforms for signaling molecules. Intermediate filament scaffolding abilities can regulate intracellular distributions of signaling proteins and thereby their abilities to phosphorylate specific targets. On the other hand, IF-associated signaling molecules can participate in the regulation of their own scaffold (Ivaska et al., 2007a; Kim and Coulombe, 2007). Vimentin, for example, affects Erk-signaling scaffold to activate Slug – one of the EMT key proteins (Virtakoivu et al., 2015). Vimentin-deficient fibroblasts exhibit slower cell proliferation due to a lower level of DNA synthesis (Wang and Stamenović, 2000). Citrullination of vimentin is involved in rheumatoid arthritis pathology, and is a candidate for diagnostic testing (Kuna, 2012).

Recently, vimentin has been associated with inflammation and reactive oxygen species (ROS) production. Vimentin downregulates activation of NLRP3 inflammasome attenuating pathophysiologic events of acute lung injury (dos Santos et al., 2015). Vimentin has been shown to negatively affect bacterial killing and ROS production in murine colitis models (Mor-Vaknin et al., 2013). Vimentin and GFAP also negatively affect neurogenesis increased endocytosis of Jagged1 in astrocytes (Wilhelmsson et al., 2012).

7.1. Vimentin phosphorylation

Recently identified functions of vimentin establish it as an organizer of both structural, and signaling-associated proteins. Interactions with different partners are highly dynamic and require a fast turnover. Phosphorylation is a key regulator of IF dynamics. Vimentin has a highly complex phosphorylation pattern in its N-terminal head and C-terminal tail (Eriksson et al., 1992, 2004a; Kochin et al., 2006). Vimentin phosphorylation motifs are highly conserved, and many of these sites still lack an identified partner or function. It is presumed that numerous phosphorylation sites of vimentin regulate numerous protein-protein interactions and through them cellular processes, which vimentin participates in (Ivaska et al., 2007b). As with other IFs, phosphorylation modulates the organization of the vimentin network and the subcellular distribution of vimentin, for example by affecting vimentin solubility. Vimentin phosphorylation sites and their corresponding kinases have specific functions in different cellular states, such as mitosis, differentiation, and stress (Omary et al., 2006). In addition to phosphorylation, vimentin has been observed to be at least sumoylated, ADP-ribosylated, glycosylated, and acetylated (Choudhary et al., 2009; Icenogle et al., 2012; Rho et

al., 2009; Wang et al., 2010b). There is far less information regarding the sites and effects of these modifications as compared to phosphorylation.

	Phosphorylated serine residues in mouse vimentin head domain and their in vitro kinases																	
	6	**8**	**9**	**20**	**24**	**25**	**28**	**33**	**38**	**41**	**46**	**50**	**55**	**64**	**65**	**71**	**72**	**82**	**86**
A kinase	X				X		X		X		X	X			X		X		
C kinase	X	X	X	X	X	X	X	X	X	X		X			X				
CaM kinase									X									X	
PAK						X			X			X			X		X		
Cdk1										X			X						
Rho-kinase									X							X			
Aurora-B	X				X				X		X			X	X		X		X
Plk1																		X	

Table 3: Mouse vimentin head domain phosphorylation sites and their in vitro kinases. Modified from (Izawa and Inagaki, 2005). The phosphorylation sites involved in vivo in mitosis are marked in yellow.

Phosphorylation of vimentin during mitosis causes vimentin disassembly and is required for normal mitosis. This process is well studied and includes multiple kinases and phosphorylation sites. During mitotic vimentin filament disassembly, vimentin serine 55 was observed to be hyperphosphorylated, and p34cdc2-kinase was responsible for disassembly (Chou et al., 1990, 1991). Other interphase-specific high-turnover sites have also been identified, among them serine 38 and serine 72, which are phosphorylated by PKA in vitro (Inagaki et al., 1987). Phosphorylation of these sites causes decelerated filament formation in vivo (Eriksson et al., 2004a). It has also been shown that vimentin disassembly increases from serine 56 phosphorylation, which is mediated by PAK (Li et al., 2006). In addition, novel functions of vimentin in mitosis, differentiation, and stress are regulated by phosphorylation (Chou et al., 1990, 1991; Gard and Lazarides, 1982; Goto et al., 1998, 2003; Stefanovic et al., 2005; Takai et al., 1996; Yamaguchi et al., 2005).

Vimentin functions as a regulator of 14-3-3. 14-3-3 binds to vimentin phosphorylation-dependently. Phosphorylation-dependent binding to 14-3-3 can conformationally change the target protein, shut out a specific region, or change the subcellular localizations of targets, thereby modulating their functions (Bridges and Moorhead, 2005). Head-domain phosphorylation of vimentin has been shown to play a crucial role in 14-3-3 binding. In this way, vimentin can scaffold a portion of 14-3-3, limiting the ability of 14-3-3 to bind to other target molecules such as Raf (Tzivion et al., 2000). Later observation has shown that at least the binding of 14-3-3 to vimentin inhibits autophagy and promotes Akt-mediated tumorigenesis. In this case, 14-3-3 serves as a linker between phosphorylated vimentin and auto-

phagy-initiating Beclin 1 (Wang et al., 2012).

Phosphorylation of numerous sites of vimentin is usually associated with increased migration (Chang et al., 2012; Eriksson et al., 2004a; Helfand et al., 2011; Ivaska et al., 2005a).

Observed vimentin post-translational modification sites, their regulators and cellular function.			
N-terminus			
S4,6,7,8,9	Phosphorylation	β1-integrin cycling and cell motility regulation	PKC epsilon-mediated (Ivaska et al., 2005a)
	Phosphorylation	Macrophage transendothelial migration regulation	PI3Kgamma-mediated (Barberis et al., 2009)
S38	Phosphorylation	Slowing of assembly kinetics	PKA (Eriksson et al., 2004a)
	Phosphorylation	Phosphorylation at the cleavage furrow in cytokinetic event	Rho-kinase (Goto et al., 1998)
	Phosphorylation	Phosphorylation-mediated by Rac1 during lamellipodia formation	p21 (Helfand et al., 2011)
	Phosphorylation	Increased breast cancer cell motility and invasion, and vimentin protection from caspases	AKT1 (Zhu et al., 2011)
R44	ADP-ribosyla-		(Icenogle et al., 2012)
R49	ADP-ribosyla-		(Icenogle et al., 2012)
S50	Phosphorylation		
S55	Phosphorylation	Filament disassembly in early G_2/M phase	CDK1 (Chou et al., 2003; Tsujimura et al., 1994)
	Phosphorylation	Reorganization of filaments during smooth muscle contraction	PAK-1 (Tang et al., 2005)

	Phosphorylation	Regulation of the dissociation of Crk-associated substrate from vimentin	PAK-1 (Wang et al., 2007)
	Phosphorylation	GTP-induced secretion in neutrophils.	Cdk5 (Lee et al., 2012)
	Phosphorylation	Cdc42GAP activation decreases	PAK-1 (Li et al., 2009)
S71	Phosphorylation	Phosphorylation at the cleavage furrow in cytokinetic event	Rho-kinase (Goto et al., 1998)
	Phosphorylation	Filament remodeling in neurites	Rho-kinase (Nakamura et al., 2000)
	Phosphorylation	GlcNAc-induced, increase in disassembly to tetramers	Rho-kinase (Komura et al., 2012)
S72	Phosphorylation	Decelerated assembly kinetics	PKA (Eriksson et al., 2004a)
	Phosphorylation	Phosphorylation at the cleavage furrow in cytokinetic event	Aurora-B (Goto et al., 2003; Yasui et al., 2001)
S82	Phosphorylation	Sequential phosphorylation after CDK1 phosphorylation on S55 in mitosis. Crucial for filament segregation between daughter cells. Works in co-ordination with S72 phosphorylation by Aurora-B kinase	Plk-1 (Yamaguchi et al., 2005)
	Phosphorylation	Formation of a vimentin cage during African Swine Fever virus infection.	CamKII (Stefanovic et al., 2005)
	Phosphorylation	Memory phosphorylation site in astrocytes. Suggested to decrease vimentin disassembly threshold after additional phosphorylation	CamKII (Oguri et al., 2006)

	Phospho-rylation	Enterovirus 71 VP1 binding site in phosphorylated form	CamKII (Haolong et al., 2013)
	Phospho-rylation	Increase during IbeA+ E. Coli K1 invasion	CamKII (Chi et al., 2010)
Rod domain			
S354	Sumoyla-tion	Inhibits glioma cell migra-tion	PIAS-mediated (Wang et al., 2010b)
C-terminus			
S418	Phospho-rylation	Unknown function	
S429	Phospho-rylation	Unknown function	
S457	Phospho-rylation	Unknown function	p37 (Chou et al., 1996)
S458	Phospho-rylation	Unknown function	p37(Chou et al., 1996)
	Phospho-rylation	Decreases endocytic vesicle fusion in mitosis, required for integrin trafficking to-ward cleavage furrow during cytokinesis	Plk1 (Ikawa et al., 2014)

Table 4: Post-translational modifications of vimentin and their cellular functions. Modified from (Hyder et al., 2008a).

7.2. Vimentin in cell migration, adhesion, and EMT

Vimentin is considered a canonical marker for epithelial-mesenchymal transition (EMT). EMTis a cellular re-programming process wherein epithelial cells acquire a mesenchymal phenotype that causes them to dramatically alter their shape and exhibit increased motility (Gonzalez and Medici, 2014; Thiery, 2002). Epithelial-mesenchymal transition normally occurs during embryonic development and wound healing, but it can also appear during pathological processes, most importantly cancer, wherein EMT increases cancer cell invasiveness and metastasis (Ivaska, 2011; Lamouille et al., 2014; Moustakas and Heldin, 2007). During EMT, epithelial cells which normally express only keratin IFs, start to, express vimentin, fibronectin, N-cadherin, Twist, and Snail and decrease expression of keratins and E-cadherin (Gonzalez and Medici, 2014; Ivaska, 2011). There is also a reverse process called mesenchymal to epithelial transition (MET), wherein cells begin acquiring the epithelial phenotype characterized by reverse expression changes compared to EMT and lower motility rates (Chaffer et al., 2006; Yao et al., 2011).

Intermediate filaments have variable effects on cell migration and can have different effects in different cell models. Keratins have variable, but usually reductive, effects on cell migration depending on the context and keratin type, but vimentin always stimulates cell migration (Chung et al., 2013; Leduc and Etienne-Manneville, 2015). Vimentin expression has been especially associated with increased migration of immune and cancer cells, whereas keratin expression usually decreases cancer and epithelial cell migration (Eckes et al., 1998; Fortier et al., 2013; Ivaska et al., 2007a; Iyer et al., 2013; Nieminen et al., 2006; Rotty and Coulombe, 2012; Schoumacher et al., 2010; Seltmann et al., 2013). Intermediate filament type is not the only variable for the effects on migration. For example, the expression level of IFs and their interaction partners, intracellular organization, and covalent modifications all affect cell migration (Chung et al., 2013; Leduc and Etienne-Manneville, 2015). The mechanisms by which vimentin affects cell migration are not completely known, but some studies have shown that there are multiple mechanisms for both structural and signaling functions of vimentin, involving mature network and soluble subunits. Vimentin has been shown to affect cell polarity determination, the formation of adhesions, lamellipodia formation, and cell migration-affecting signaling protein transport and localization (Chernoivanenko et al., 2013; Ivaska et al., 2007a).

Vimentin is the main IF present in cell types in which migration is most often studied: fibroblasts and leukocytes. Leukocyte migration affects leukocyte extravasation when leukocytes home to tissues, and therefore affects immune resistance. Leukocytes can migrate through cell junctions (paracellular route) or have the opportunity to migrate directly through endothelial cells (transcellular route) (Dejana, 2006). Vimentin affects both of these processes and indeed, in a vimentin-deficient mouse model, both lymphocyte extravasation to tissues and the lymphatic system are impaired (Nieminen et al., 2006). Fibroblast migration is a major effector of wound healing. Vimentin knockout mice have slower wound healing response, and fibroblasts from these mice exhibit less directional migration and contraction (Eckes et al., 1998). Vimentin has been observed to retract from fibroblast lamellipodia during migration, which allows directional migration response (Helfand et al., 2011). Retraction is mediated by actomyosin arcs and plectin crosslinkers (Jiu et al., 2015). Also, vimentin expression can appear in many cell types after wounding, as a major positive contributor to migration in these cases (Eckes et al., 2000).

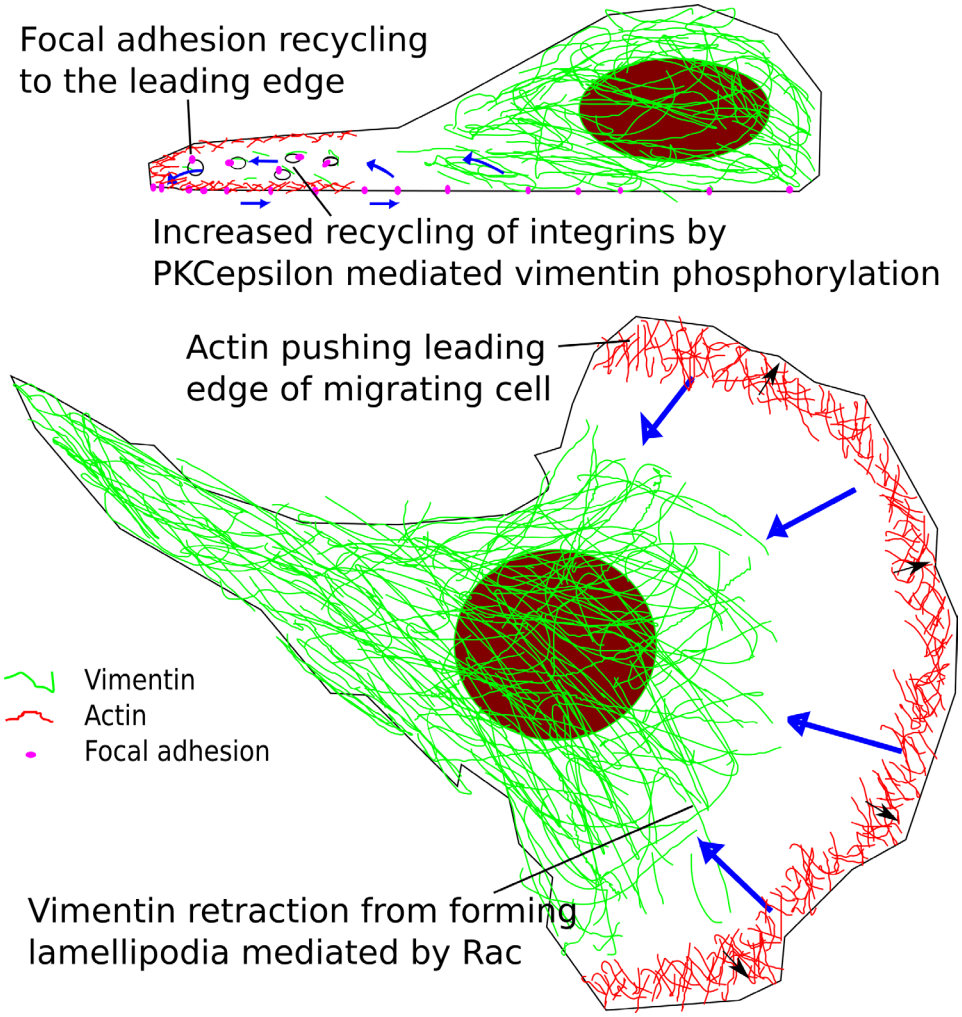


Figure 4: Important cell migration mechanisms and the major mechanistic roles of vimentin in them. Adapted from (Alberts et al., 2007; Helfand et al., 2011; Ivaska et al., 2005a).

Vimentin is expressed widely during embryogenesis, but its role in cell migration in embryogenesis is less studied. Nevertheless, embryogenesis progresses almost normally in a vimentin-deficient mouse model, indicating that vimentin may not be needed for cell migration during embryogenesis, perhaps due to slow speed requirements for cells (Chernoivanenko et al., 2013). However, vimentin plays an important role in cancer cell migration (Hendrix et al., 1996; Leduc and Etienne-Manneville, 2015; Mendez et al., 2010; Wei et al., 2008).

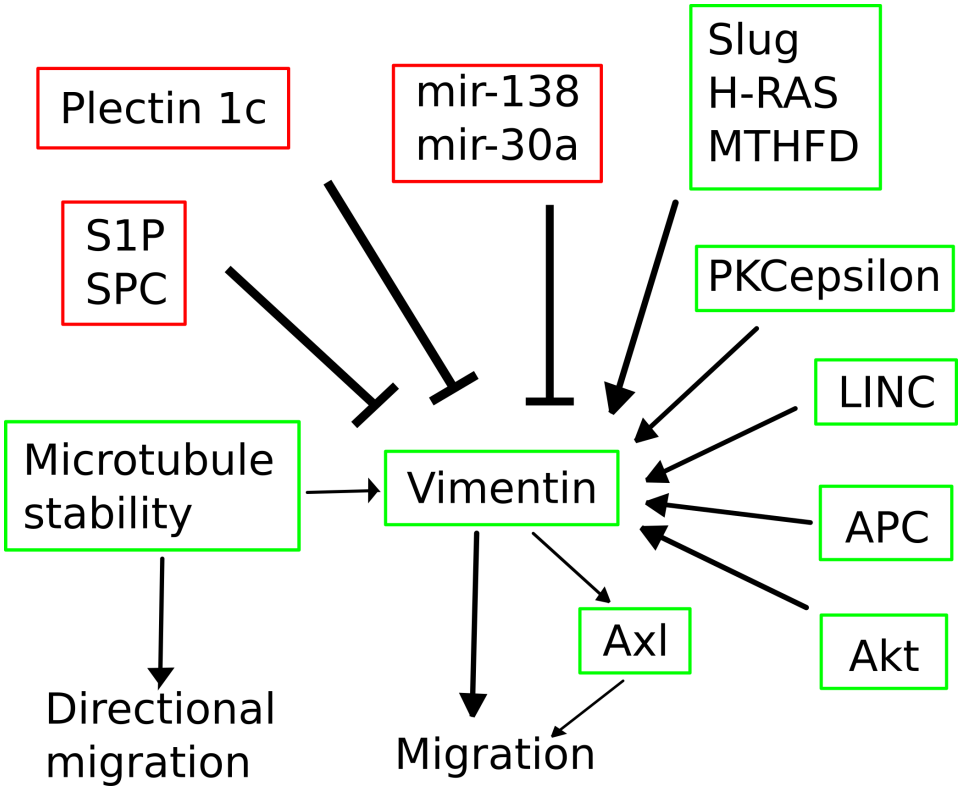


Figure 5: Some of the signaling molecules that affect migration through vimentin. Modified from (Chung et al., 2013).

An important migration regulator, Rho-associated protein kinase 1 (ROCK1), has been shown to phosphorylate serines 38 and 71 in vimentin N-terminus (Goto et al., 1998; Kumar et al., 2007; Sin et al., 1998). ROCK1 was shown to interact directly with vimentin, most likely on the head domain of vimentin. When RhoA was activated, the result was the collapse of the vimentin network in a ROCK1-dependent way, while at the same time, ROCK1 was detached from vimentin and traveled to the cell periphery (Sin et al., 1998).

Cell adhesion is a crucial process in cell migration and signal transduction. Vimentin regulates cell adhesion mainly via surface adhesion receptors (Ivaska et al., 2005b, 2007a). Vimentin-negative endothelium has a reduced number of adhesion receptors ICAM-1 and VCAM-1, and lymphocytes have a lower level of integrin β1, which also contributes to adhesion. In vimentin negative mice, endothelium is leaky, which reflects defects in cell-cell adhesion and/or cell interactions (Nieminen et al., 2006). Vimentin regulates membrane trafficking of integrins via PKCepsilon-containing vesicles. PKC-mediated phosphorylation of vimentin serines 4,6,7,8 and/or 9 regulates vimentin association and disassociation to integrin and PKCε-containing vesicles, and allows recycling of integrins back to the plasma membrane in fibroblasts affecting directional migration (Ivaska et al., 2005a). This effect is regulated by vimentin binding to filamin A and filamin A binding to

PKCε, which allows vimentin phosphorylation by PKCε (Kim et al., 2010). Also, PI3Ky-mediated (PI3K protein family member, which relays G-protein binding receptor signals to PI3K signaling pathways) phosphorylation of vimentin serines 4,6,7,8 and/or 9 regulate transcellular migration (Barberis et al., 2009).

Vimentin affects focal adhesions; structures that assemble clearly in cultured cells and have less clear counterparts observed in in vivo. In focal adhesions, integrins bridge the extracellular matrix with the cellular cytoskeleton and recruit a complex network of signaling and scaffolding proteins (Geiger et al., 2001). In vimentin-mediated matrix adhesion (VAM) vimentin binds directly to α2β1-integrin in endothelial cells. α2β1-integrin in turn binds laminin and collagen in a cell-dependent manner (Kreis et al., 2005; Wallez and Huber, 2008). Vimentin is connected to stable hemidesmosome-like structures by α6β4-integrin (Homan et al., 1998). Outside focal adhesions on the inside of plasma membrane, vimentin filaments link to integrins through IAP (integrin-associated protein, CD47) and PLIC-linker proteins (Lindberg et al., 1993; Wang and Frazier, 1998). Vimentin also associates with lipid rafts in neutrophils and may mediate integrin trafficking in detergent-resistant membrane rafts (DRMs) (Feuk-Lagerstedt et al., 2007). In addition to participating in the above-mentioned integrin-mediated cell-matrix contacts, vimentin can be part of cell-cell contacts. Endothelial cells have specialized desmosome-like junctions, called complexus adherens, which anchor both actin and vimentin IFs to VE-cadherin-based plasma membrane domains (Calkins et al., 2003; Green et al., 2005; Kowalczyk et al., 1998). Vimentin has also been reported to reside in filopodia and podosomes of adherent macrophages. Non-filamentous vimentin associates with flexible crosslinking protein fimbrin in these structures. When observed, this association occurred through the vimentin N-terminus and seemed to be dependent on vimentin phosphorylation (Correia et al., 1999).

7.3. Vimentin in cell mechanics

Vimentin filaments, among other IFs, are critical regulators of intracellular cell mechanics. The vimentin network increases mechanical integrity of the cells and localizes and stabilizes intracellular organelles (Buehler, 2013; Gruenbaum and Aebi, 2014; Guo et al., 2013). Vimentin filaments also affect cell shape and the organization of other cytoskeletal networks (Mendez et al., 2010; Wang and Stamenovic, 2002b). The organization of the GFAP network is disrupted in vimentin-deficient astrocytes and later restored when vimentin is transfected back to cells (Galou et al., 1996). In vimentin-deficient mice, arterial dilation is impaired, and arterial remodeling altered (Henrion et al., 1997). Another role of vimentin in cell mechanics is increased mechanosensing of the cells through focal adhesion, anchored to (via plectin) vimentin IFs. Major mechanosensor focal adhesion kinase (FAK) has been shown to regulate vimentin targeting to focal adhesions (Gregor et al., 2014). Endoplasm, which is an organelle and microtubule-rich region at the center of the cell, is needed to transport cargos to the cell periphery and develop long-range forces. Cells that express polymerization-deficient vimentin

variant have deficient endoplasmic spreading and defects in focal adhesion growth (Lynch et al., 2013; Tsuruta and Jones, 2003). It has been postulated that endoplasmic spreading requires the coalescence of vimentin IFs at force-bearing focal adhesions (Lynch et al., 2013).

Vimentin polymer gels show stress-strain relationships, which are highly non-linear. Low strain causes very low stiffness, but high strain causes relatively much higher stiffness. This non-linear relationship is called strain-stiffening or hardening behavior (Janmey et al., 1991). Strain-stiffening occurs in live cells making IFs major determinants of cell stiffness cell type-dependently. Cells without vimentin are less stiff, and stiffen less than wild-type cells under increasing shear stress (Wang and Stamenovic, 2003). In endothelial highly-spread cells, the effect is even more prominent. Vimentin network deforms rapidly under physiological fluid shear stress (Helmke et al., 2000). Through vimentin network's connections to the nucleus, cell junctions, and other cytoskeletons, vimentin transmits mechanical stress and maintains interactions with the other cytoskeletal components, the nucleus, and nearby cells (Helmke et al., 2000). Vimentin-deficient cells have reduced contractility, and cell-cell interactions are dramatically decreased in these cells (Eckes et al., 1998). Vimentin filaments are required for the mechanical strength of the cytoskeleton, and protect the structural integrity of the cell. Cells without vimentin are more prone to plasma membrane deformation and rupture than wild-type cells when cell surface receptors are pulled (Maniotis et al., 1997).

Vimentin IFs provide stabilization for microtubules. Continuous lateral support from IFs reduces the critical buckling length of microtubules and thus maintains their stability during cell contraction (Goldman et al., 1996; Tang et al., 2008). In fibroblasts and endothelial cells IF contribution to cell stiffness is about 20-40%, but in T lymphocytes rigidity depends mainly on vimentin, whereas microtubules play a minor role in T cell rigidity (Brown et al., 2001; Wang and Stamenović, 2000). Structural alterations in vimentin IFs in circulating T lymphocytes may be important in transendothelial migration of these cells (Wang and Stamenovic, 2002b).

7.4. Vimentin in vesicle and molecule trafficking and organelle positioning

Vimentin filaments associate with microtubule and actin networks and the motor proteins connected to these networks. Via these connections, vimentin filaments can regulate membranous organelle transport (most prominently endo-lysosomal system), the positioning of membranous organelles, and molecule trafficking. Intermediate filaments, vimentin included, have multiple links to vesicular membrane transport machinery. Vimentin also affects the function of some cell organelles, for example, endosomes and mitochondria (Margiotta and Bucci, 2016; Minin and Moldaver, 2008b; Styers et al., 2004).

Vimentin polymers are highly dynamic and exchange subunits between the polymer and soluble subunits. Non-filamentous IF precursors, which are short filaments termed "squiggles", are transported along microtubules with the help of

motor proteins kinesin and dynein. Transported IF subunits are hypothesized to help transport specific interacting proteins by linking them to microtubule motor complexes (Helfand et al., 2002, 2003, 2005). One notable example of this occurs during sciatic nerve damage. Damage induces vimentin expression and cleavage by calpains. Soluble vimentin fragments link phosphorylated Erk1 and Erk2 kinases to importin β and dynein-mediated transport to nerve cell body. Phosphorylated Erk kinase can activate signaling that results in neuronal regeneration (Perlson et al., 2005). Vimentin also protects active Erks from phosphatases by steric hindrance (Perlson et al., 2006). These functions at least partially explain why vimentin is re-expressed upon injuries, and they provide a model of how vimentin can function as a long-distance messenger (Ivaska et al., 2007b).

Vimentin is active in vesicle trafficking, mainly through its effects on receptors and interactions with vesicle trafficking components. Vimentin interacts with AP-3 adapter protein complexes through its domain consisting of β3A and β3B subunits. AP-3 regulates vesicle transport between endosomal and lysosomal compartments, and affects transport to hematopoietic granules, melanosomes, and synaptic vesicles. AP-3 or vimentin absence in fibroblasts leads to drastic zinc ion decrease, especially in vesicles, indicating a role in zinc transport (Minin and Moldaver, 2008a; Styers et al., 2004). Zinc also stabilizes vimentin structure via vimentin C328 (Pérez-Sala et al., 2015).

Disruption of IF keratin 8 assembly leads to defects in autophagosomal vacuole formation in hepatocytes. Vimentin, on the other hand, inhibits autophagy through sequestration of Beclin 1 (Blankson et al., 1995; Holen et al., 1992; Parsons et al., 2002; Wang et al., 2012). Vimentin binding to AP-3 may also affect autophagosome formation via LAMP-2 (lysosome-bound membrane protein) (Shintani and Klionsky, 2004). Regulation of vimentin in membrane trafficking also works the opposite way: when membrane traffic is inhibited by mutant forms of ARF1 (a small GTPase that functions in vesicle budding) or Brefeldin A (which inhibits vesicle trafficking from Golgi to the cell surface) vimentin filaments are disrupted, and the membrane adaptor complexes AP-1 and AP-3 translocate on vimentin filaments. Microtubules also become more bundled by induced vimentin disruption (Perlson et al., 2005; Styers et al., 2006).

Mitochondria localize around IFs in many cell types. Motility of mitochondria in cultured fibroblasts is reduced by vimentin (Nekrasova et al., 2011). Vimentin IFs also increase mitochondrial membrane potential (Chernoivanenko et al., 2015). Vimentin interacts with Golgi apparatus. This interaction is mediated by formiminotransferase cyclodeaminase (FTCD), M130, MICAL (molecule interacting with CasL) and GTPase Rab1, which participates in the ER-Golgi transport (Gao and Sztul, 2001; Minin and Moldaver, 2008a; Suzuki et al., 2002).

The localizations of the cell nuclei are regulated by IFs and other components of the cytoskeleton. Intermediate filaments connect to the nucleus via plectin and the protein families KASH and SUN (Starr, 2007). In the case of vimentin, in vimentin-free SW13 cells, nuclear membrane invaginations were observed along with

other distortions (Minin and Moldaver, 2008a; Sarria et al., 1994).

7.5. Vimentin in angiogenesis

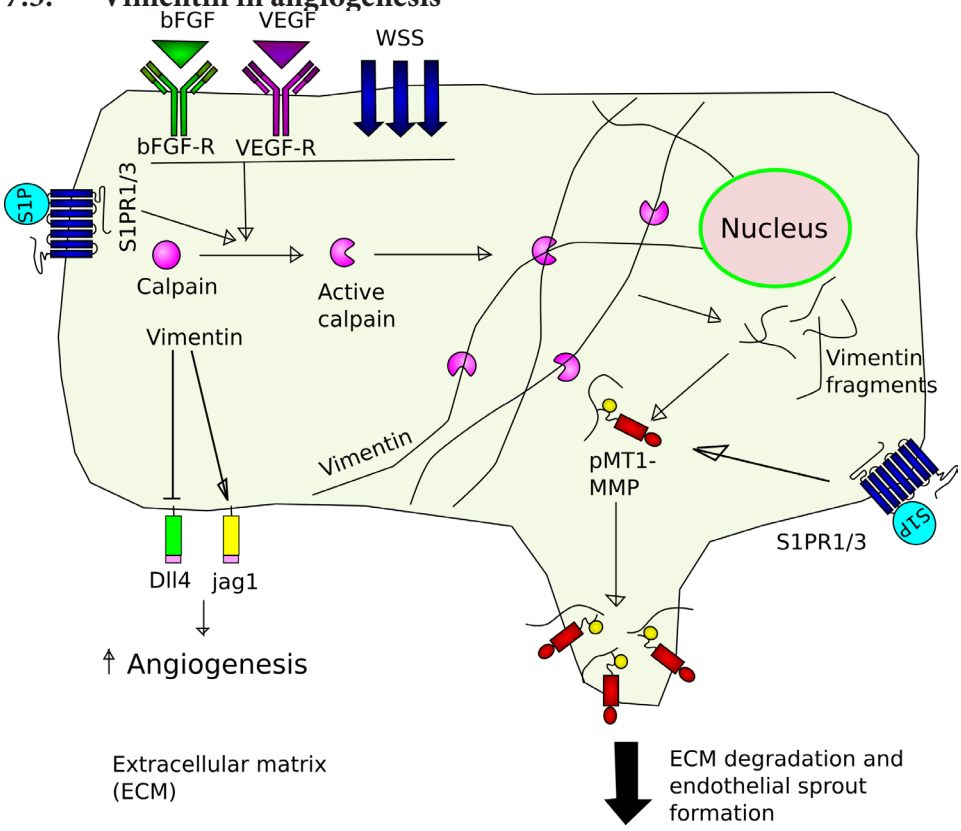


Figure 6: Role of vimentin in angiogenesis (adapted from (Dave and Bayless, 2014a)).

Vimentin also affects the angiogenic processes. Angiogenesis has a critical significance in several processes such as pregnancy, ovulation, wound healing, and tumor invasion, and vimentin may affect these processes partly through its effects on angiogenesis (Carmeliet, 2003; Folkman and D'Amore, 1996). Angiogenesis occurs in several steps: basement membrane degradation, sprout initiation, migration, lumen formation, and stabilization, and vimentin likely affects more than one of these processes (Dave and Bayless, 2014a; Iruela-Arispe and Davis, 2009). The first possible link between vimentin and angiogenesis was identified when it was demonstrated that vimentin silencing resulted in a lag in granulation tissue formation (Eckes et al., 1998). Another study indicated that deficiency of vimentin causes increased fragility of the retina under stress and impaired angiogenesis under oxygen-induced retinopathy (Lundkvist et al., 2004). Vimentin is a substrate of calpain. Calpain cleavage of vimentin produces soluble vimentin. This soluble vimentin binds directly to the cytoplasmic tail of MT1-MMP. When calpain or vimentin are silenced, the result is impaired surface localization of MT1-MMP. Less MT1-MMP on the cell surface leads to significantly reduced endothelial sprouting

responses (Kang et al., 2011; Kwak et al., 2012).

7.6. Vimentin in cancer

Vimentin is overexpressed in various epithelial cancers, including prostate cancer, gastrointestinal tumors, CNS tumors, breast cancer, malignant melanoma, and lung cancer. Overexpression of vimentin correlates highly with tumor growth, invasion, and poor prognosis (Hendrix et al., 1996; Leduc and Etienne-Manneville, 2015; Mendez et al., 2010; Wei et al., 2008). Many tumors originating from epithelia start to express vimentin during metastasis (Satelli and Li, 2011a). Vimentin expression in cancer cells was shown to correlate with the cells ability to migrate and invade surrounding tissue (Gilles et al., 1996; Santini et al., 1996; Sommers et al., 1992). The enhancing effect of vimentin on processes such as cell migration and invasion, EMT, and perhaps less importantly cell proliferation and angiogenesis, at least partially explains why vimentin hastens cancer progression. However, many of the detailed mechanisms involved in how vimentin affects cancer are still unknown (Chernoivanenko et al., 2013; Dave and Bayless, 2014a; Kidd et al., 2014; Thiery, 2002). Because knocking out vimentin results in only mild phenotype in mice, and because even heavy vimentin aggregation in mice only causes cataracts, vimentin is a possible molecular target for slowing tumor progression without significant side effects (Bornheim et al., 2008; Colucci-Guyon et al., 1994; Müller et al., 2009; Satelli and Li, 2011a). Migration of cancer cells is important for metastatic properties. Around 90% of cancer deaths are not caused by a primary tumor, but developed metastases (Mehlen and Puisieux, 2006). Vimentin inhibition could potentially be useful in inhibition of cancer metastases for a variety of cancers (Satelli and Li, 2011a).

Withaferin A (WFA) is a bioactive compound isolated from Withania Somnifera – a plant from the nightshade family. It has been shown to bind tetrameric vimentin in a binding pocket between the pair of head-to-tail α-helical dimers (Bargagna-Mohan et al., 2007). Apoptosis caused by WFA is more pronounced in cancer cells containing vimentin. This apoptosis promotion was suggested as resulting from vimentin degradation upon binding to WFA (Lahat et al., 2010). WFA has also been shown to reduce lung metastatic nodules in breast cancer mouse models in low doses (Thaiparambil et al., 2011). WFA was also shown to be rather unspecific in inhibiting vimentin, and affected other IF networks as well, meaning it may have debilitating organism-wide effects (Grin et al., 2012). Silibinin is a flavolignan isolated from milk thistle. Silibinin has strong chemopreventative and anticancer properties. Silibinin inhibits cell migration and invasion by downregulation of vimentin and MMP-2 in prostate cancer cells, and upregulation of E-cadherin indicating inhibition of EMT in a mouse model (Singh et al., 2008; Wu et al., 2009). Also, antibiotic salinomycin induces mesenchymal-to-epithellian transition (MET, the opposite of EMT) in colorectal cancer cells (Kopp et al., 2014). MicroRNAs miR-200 and miR-30 were also shown to cause MET in thyroid carcinomas (Braun et al., 2010; Satelli and Li, 2011b).

8. Sphingolipids

Sphingolipids are a class of lipids. They are amphipathic in nature. A hydrophobic end consists of a sphingoid base linked to a fatty acid, and a hydrophilic end consists of hydroxyl groups, phosphates, and sugar residues. Sphingolipids are important for the structure of the plasma membrane, as well as in different signaling pathways (Gault et al., 2010). They affect many biological processes such as adhesion, migration, cell death and survival, proliferation, angiogenesis and embryogenesis (Pitson, 2011). Aberrant sphingolipid metabolism, often degradation and accumulation defect, can cause sphingolipidoses, for example, Tay-Sachs, Niemann-Pick, Fabry's or Gaucher's diseases, or metachromatic leukodystrophy, depending on the specific metabolic defect (Sandhoff, 2013).

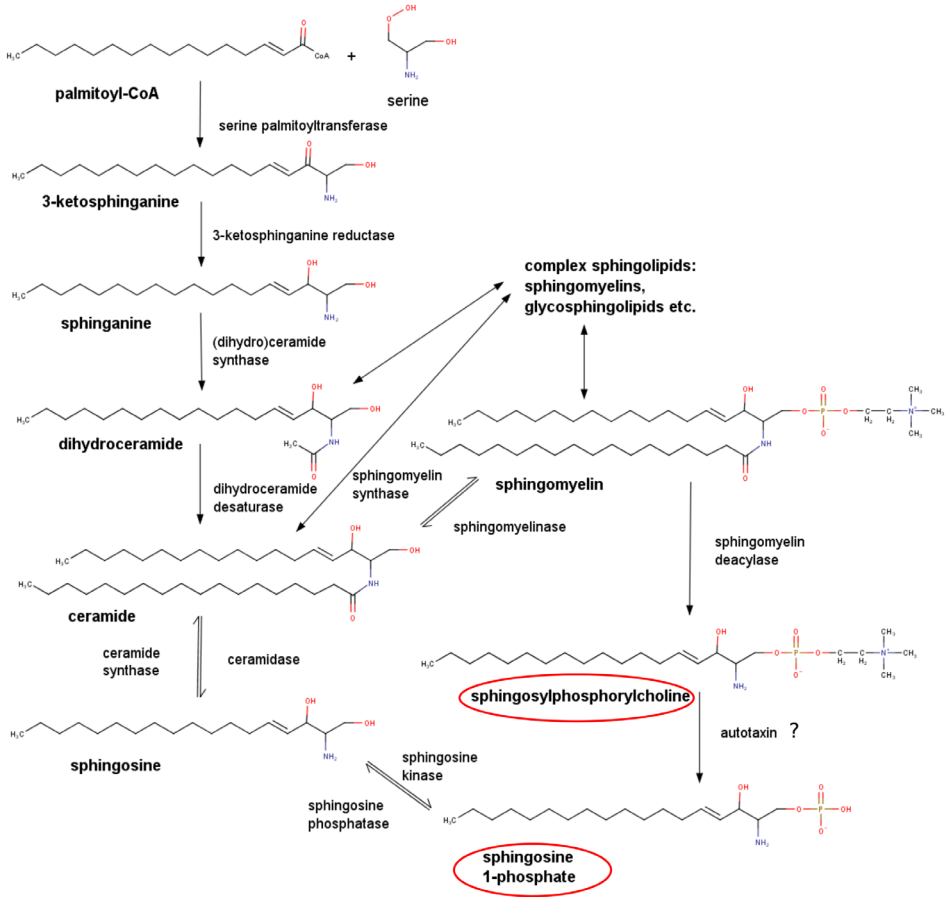


Figure 7: De novo sphingolipid synthesis pathways, including potential sphingosylphosphorylcholine (SPC) metabolism and possible reaction into sphingosine-1-phosphate (S1P). Adapted from (Bertea et al., 2010; Nixon et al., 2008).

Sphingolipids are divided into different groups. Simple sphingolipids include sphingoid bases and ceramides. All sphingolipids derive from ceramide group and/or sphingoid bases. Complex sphingolipids include glycosphingolipids and

sphingomyelins. Glycosphingolipids contain glucose or the galactose sugar group. Glycosphingolipids are divided into cerebrosides, gangliosides, and ceramide oligosaccharides. Molecules in the sphingomyelin group contain phosphorus (D'Angelo et al., 2013; Gault et al., 2010; Goñi et al., 2014; Peter Slotte, 2013).

Sphingomyelins are synthesized from ceramide and phosphorylcholine derived from phosphatidylcholine. They are the only sphingolipids that contain phosphorus. Sphingosine-1-phosphate (S1P) and sphingosylphosphorylcholin (SPC), which are of interest in this thesis, belong to sphingomyelin group. Sphingomyelins are most abundant in nervous tissue, especially in the myelin sheath of nerves, but they also reside in the blood (Goñi and Alonso, 2006; Karlsson, 1998; Merrill et al., 1997; Peter Slotte, 2013; Ramstedt and Slotte, 2002; Slotte, 2013).

8.1. Sphingosine-1-phosphate and sphingosylphosphorylcholine

Sphingosylphosphorylcholine (SPC) and sphingosine-1-phosphate (S1P) are the two structurally related lysosphingolipids belonging to sphingomyelin group. They affect cellular functions through signals transduced by either activation of G-protein coupled receptors or possibly by acting intracellularly. S1P has been intensely studied, and SPC is the less studied of these two, although SPC has also been the subject of much recent interest. The S1P and the proposed SPC synthesis pathway is shown in Fig. 7 (Alewijnse and Michel, 2006; Mendelson et al., 2014; Nixon et al., 2008; Pyne et al., 2016; Yue et al., 2015).

Sphingosine-1-phosphate is a major regulator of cellular responses, including proliferation, survival, motility, angiogenesis, and inflammation. Angiogenesis, cardiogenesis, neurogenesis, and limb development during embryogenesis are all affected by S1P (Alewijnse and Michel, 2006; Mendelson et al., 2014; Pyne et al., 2016). S1P has also an important role in leukocyte trafficking (Aoki et al., 2016a). S1P resides in animal cells in relatively small amounts. Insects, yeasts, and plants also contain S1P (Mendelson et al., 2014; Pyne et al., 2016). Five different S1P receptors (S1PR1-5) activate various signaling routes and different functional outcomes in cells. Distinct G-protein families partner in particular combinations with different S1P receptors. Ras, Rho, and Rac are commonly activated later. Signaling molecules further downstream include adenylate cyclase, phospholipase C, PI-3-kinase, protein kinase C, and calcium (Lee et al., 2001; Mendelson et al., 2014; Ryu et al., 2002; Sugimoto et al., 2003). Two sphingosine kinases, SphK1 and SphK2, convert sphingosine to S1P, and are essential for S1P metabolism. They are common in the cytosol and endoplasmic reticulum. They are expressed tissue-specifically in unique cellular locations and have distinct functions (Mendelson et al., 2014; Pyne et al., 2016).

SPC naturally resides in plasma and, in higher amounts, in serum. SPC is also an important constituent of lipoproteins. SPC has cell type-specific functions in major tissues, such as in the heart, blood vessels, skin, brain, and immune system. As S1P, SPC affects cardiac and blood vessel functions. Also, SPC can act as a mitogen in several different cell types and, in certain circumstances, may also be a pro-inflammatory mediator (Alewijnse and Michel, 2006; Nixon et al., 2008).

The effects of SPC are regulated through a variety of different intracellular signaling cascades, also dependent on cell type. SPC synthesis and breakdown pathways are less well understood than S1Ps (Nixon et al., 2008). SPC synthesis increases in some pathological conditions, including cancer (Nixon et al., 2008; Yue et al., 2015). Some of the effects of SPC occur via plasma membrane receptors (e.g. S1P2 and S1P3) used by S1P. SPC binds to these with significantly lower affinity than S1P (An et al., 1997; Liliom et al., 2001; Okamoto et al., 1999). SPC-specific receptor signal transduction mechanisms have not been found, although SPC has some novel effects on cells compared to S1P, suggesting that there could be novel SPC receptors (Alewijnse and Michel, 2006; Nixon et al., 2008). SPC has been reported to increase calcium levels in numerous cell types by cell type-specific mechanisms (Afrasiabi et al., 2006; Calcerrada et al., 1999; Orlati et al., 1998). SPC is known to often activate mitogen-activated protein kinases (MAPK) and sometimes, like S1P, monomeric G-proteins (Nixon et al., 2008). Several effects of SPC are blocked by inhibiting RhoA or Rho-kinase (Afrasiabi et al., 2006; Orlati et al., 1998; Todoroki-Ikeda et al., 2000).

S1P has several crucial functions in tissues. Immune cell trafficking, vascular development, and the stability of blood vessels are affected by S1P receptor expression and localization dynamics (Mendelson et al., 2014). Through S1PR1 S1P maintains flow-dependent vascular network stability. S1PR1 deletion causes endothelial hypersprouting by increasing VEGFR2 activation and poor vessel function via destabilization of VE-cadherin. This results in hypoxia and increased VEGFA expression and hypersprouting (Gaengel et al., 2012; Jung et al., 2012; Liu et al., 2000). S1P also regulates tissue inductive events. S1P regulates mesenchymal and/or endothelial cell differentiation into pancreatic tissue (Serafimidis et al., 2011). In limb development, S1P affects skeletal patterning, vascularization, and digit sculpting (Chae et al., 2004a). These effects may function via HIF1α and VEGF (Ben Shoham et al., 2012). Neuronal tissue contains S1P receptors in especially high levels (Chae et al., 2004b). Mice without Sphk isoenzymes exhibit neuronal cell loss likely through apoptosis and decreased cell division (Mizugishi et al., 2005). S1P is also a regulator in neural progenitor cell recruitment (Callihan and Hooks, 2012; Mendelson et al., 2014).

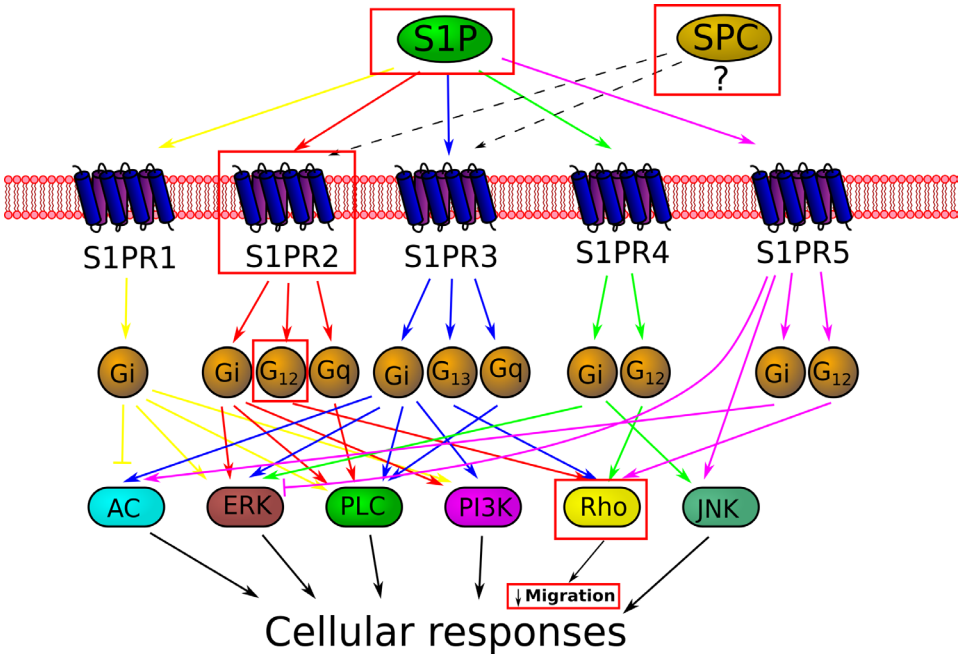


Figure 8: Different sphingosine-1-phosphate (S1P)-induced signaling pathways through S1P-receptors (S1PR1-5). The signaling pathway studied in this thesis is denoted by red boxes (Rho activation has several possible outcomes). Adapted from (Nagahashi et al., 2014).

The functions of SPC in tissues are less known. Based on the results from in vitro cell models, SPC may induce differentiation of mesenchymal stem cells to smooth muscle cells in vascular development (Jeon et al., 2006; Nixon et al., 2008). SPC has pro-inflammatory effects in various contexts (Wirrig et al., 2011). In disrupted endothelial layer, platelets are attracted to the injury site and secrete, among other factors, SPC and S1P. In these conditions, SPC can increase inflammation by regulating inflammatory cytokine tumor necrosis factor α and ICAM-1 (Imokawa et al., 1999; Nixon et al., 2008; Okamoto et al., 2003). SPC can sometimes act as a chemoattractant for natural killer cells, potentially contributing to inflammation (Jin et al., 2005). In the skin, SPC increases wound healing in in vivo models (Sun et al., 1996). SPC increases interleukin-6 expression, which causes an increase in fibronectin expression. An increase in fibronectin expression increases collagen deposition during wound healing (Gailit and Clark, 1994; Suhr et al., 2003). SPC also increases connective tissue growth factor expressions (Zhu et al., 2005). In atopic dermatitis epidermal lesions, SPC concentration is four-fold compared to the normal epidermis. In neurons, SPC increases calcium levels and can increase nerve growth factor synthesis (Furuya et al., 1996; Huang and Chueh, 1996). SPC effects in neurons may involve neural cell maintenance and survival. Numerous sphingomyelinase gene mutations can cause neurodegenerative Niemann-Pick disease types A and B (Simonaro et al., 2002). In A-type disease, SPC levels in

the cerebral cortex are increased 50- to 100-fold (Rodriguez-Lafrasse and Vanier, 1999). SPC may contribute to neurodegeneration by inducing astrocytes to release glutamate, which increases calcium in adjacent neurons or via tumor necrosis factor secretion, which causes inflammation (Chiulli et al., 2007; Nixon et al., 2008).

S1P and SPC have common effects, but also some differences in their effects on cells. For example, SPC has been shown to decrease heart rate species-specifically in guinea pigs, possibly via the muscarinic receptor-activated K+ channel, but S1P causes this effect in both humans and mice (Orlati et al., 1998; Todoroki-Ikeda et al., 2000). SPC causes hypertrophic growth in neonatal rat cardiomyocytes in vitro, whereas S1P does not (Sekiguchi et al., 1999). SPC and S1P generally cause vasoconstriction in individual blood vessels mostly via an increase in calcium and activation of the RhoA/Rho-kinase (ROCK) pathway, but the effects of SPC on blood vessels can be variable (Nixon et al., 2008). In intact blood vessel endothelium, specifically HDL-binding SPC, like S1P, may prevent cardiovascular disease (Kimura et al., 2001; Nofer and Assmann, 2005; Sachinidis et al., 1999).

S1P and SPC can affect the IF cytoskeleton. SPC can cause reorganization of keratin 8 and 18 filaments into perinuclear space, increased keratin phosphorylation, and higher cell elasticity in tumor cells originating from human epithelia. These effects are largely independent of microtubules and F-actin (Beil et al., 2003). SPC-induced phosphorylation of keratins regulates keratin reorganization and cell motility in a substantial manner (Busch et al., 2012; Fois et al., 2013; Park et al., 2011). S1P also causes reorganization of vimentin. S1P causes vimentin to form complexes with FAK and RACK1 in invading endothelial cells. Complex formation affects focal adhesion formation (Dave et al., 2013; Sin et al., 1998).

8.1.1. S1P and SPC in cell migration

S1P affects cell migration in several concepts through various mechanisms. The effects of S1P to migration occur mainly through S1PR1-3. While S1PR1 and S1PR3 typically induce migration, when activated, S1PR2 typically inhibits migration (Adada et al., 2013a).

In vascular sprouting, endothelial cells show collective migration patterns. The collective migration patterns are regulated by S1P and S1PR1-3 (Gaengel et al., 2012; Jung et al., 2012). Also, S1P helps the movement of immune cells from lymphatic organs to blood circulation by forming a gradient at the boundary between the tissues and the vasculature (Cyster and Schwab, 2012; Pappu et al., 2007). This gradient forms with the help of spinster 2 (a sphingosine-1-phosphate transporter), cell-surface lipid phosphate phosphatase 3, and sphingosine kinases. Egress-promoting receptors, e.g. S1PR1, together with retention promoting receptors, e.g. CCR7, on lymphocytes recognize the gradient and activate a suitable chemotactic response (Bréart et al., 2011; Cyster and Schwab, 2012; Fukuhara et al., 2012; Pham et al., 2008).

S1P affects cell-cell and cell-matrix adhesion mainly through G-protein coupled S1P-specific S1P1-5 receptors and in much smaller amounts via intracellular targets, e.g. HDAC1 and 2, TRAF2, and prohibitin (Alvarez et al., 2010; Chun

et al., 2010; Hait et al., 2009; Strub et al., 2011). The adhesion effect can consequently affect cell migration and invasion, cell growth and survival, differentiation and cytokine production. S1P receptor 1 activation can induce the formation of VE-cadherin-containing adherens junctions in vascular endothelial cells. S1PR1 also influences adhesion between pericytes and endothelial cells via N-cadherin-dependent junctions (Lee et al., 1999; Paik et al., 2004). S1PR1 and 3 can activate integrins and focal contact formation in vascular endothelial cells (Paik et al., 2001). Microtubule polymerization at the cell cortex is regulated by S1PR1 and Rac (Paik et al., 2004).

S1PR2 signaling disrupts adherens junctions via Rho, Rho-kinase (ROCK), and PTEN, and inhibits specific growth factor or chemokine-induced cell migration by inhibiting Rac (Michaud et al., 2010; Sanchez et al., 2005; Sugimoto et al., 2003). S1P induces actin stress fiber assembly through S1PR2 and Rho GTPase, and cortical actin assembly through S1PR1 and Rac GTPase, (Lee et al., 2001; Ryu et al., 2002; Sugimoto et al., 2003). S1PR2 signaling typically inhibits cell migration through Rho and ROCK signaling or PTEN signaling that leads to inhibition of Akt (Adada et al., 2013a). Insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF) are known to be affected by S1PR2 and S1P can inhibit via S1PR2 chemokines C5a, CCL2, and stromal-derived growth factor 1 (SDF1, CXCL12), which all are regulators of cell migration in specific contexts (Michaud et al., 2010; Sugimoto et al., 2003).

During organism development, S1P is crucial for heart cell precursor migration. In zebrafish, S1PR2 and spinster 2 mutations cause the formation of two hearts (Kupperman et al., 2000). This defect may be tracked to observable defects in endodermal sheets. S1P affects via S1P2/G13/Rho integrins and fibronectin. The effect may cause myocardial precursor cell migration guidance defect (Zhang et al., 1999). Prechordal plate migration is regulated by S1P (Kai et al., 2008). S1P regulates migration and clustering of pancreatic endocrine precursor cells during development (Mendelson et al., 2014).

8.1.2. S1P and SPC in cancer

S1P plays an important role in inflammatory disease, cancer, and Alzheimer's disease. Recently developed sphingosine analog Fingolimod (FTY720), which affects S1P signaling, has been shown to be effective in treating multiple sclerosis and has potential uses in cancer treatment (Aoki et al., 2016b; Patmanathan et al., 2015; White et al., 2016). Fingolimod becomes phosphorylated to its active form in the body. The active form can bind S1P1, 3, 4, and 5 receptors. The binding causes inhibition of lymphocyte egress from lymphoid organs (Aoki et al., 2016b). SphK1 and SphK2 are predictive markers in inflammatory diseases and cancer. Sphingosine kinases are potential targets for cancer treatment. Additionally, there is evidence of S1PR and sphingosine kinase 1 interaction, which can amplify cancer cell growth (Pyne et al., 2014). The stimulative or inhibitory effect of S1P on cancer cells depends on the cell type. The effect may be dependent on S1P receptor composition (Adada et al., 2013b; Meyer zu Heringdorf and Jakobs, 2007). Recent-

ly, SPC has raised interest in cancer research due to high levels of SPC in cancer patients' malicious ascites, and because of SPC's effect on cancer cell proliferation and migration in pancreatic cancer cells and epithelial ovarian carcinoma cells, among others (Yue et al., 2015).

9. Rho-associated protein kinase (ROCK)

Rho-associated coiled coil kinase (ROCK) is a major signaling protein relaying signals from small GTPase RhoA. ROCK family consists of two isoforms; ROCK1 and ROCK2. ROCK is a considerable regulator of actin cytoskeleton organization and it affects, for example, contraction, adhesion, migration, proliferation, and apoptosis. ROCK affects several processes of cancer malignancy, such as tumorigenicity, tumor growth, metastasis, angiogenesis, tumor cell apoptosis and survival and chemoresistance, more often by hastening cancer progression, but sometimes in an inhibitory manner (Wei et al., 2016a). ROCK effect on cell migration is usually increasing, but sometimes inhibitory, for example, in the case of MCF-7 breast cancer cells, SW480 and – only in three-dimensional matrix – SW620 colon cancer cells (Loirand, 2015; Wei et al., 2016b).

10. Notch signaling

Notch signaling is an important regulator in cell fate determination. Notch signaling is activated by direct contact of the signal-sending cell to the signal-receiving cell (Hori et al., 2013). Differentiation, apoptosis, and proliferation are influenced by Notch signaling depending heavily on the cellular context. The Notch signaling pathway exists in a conserved fashion in most multicellular organisms (Artavanis-Tsakonas et al., 1999; Bray, 2006; Kopan and Ilagan, 2009). Many diseases that present changes in cell fate and proliferation, such as cancer, are affected by aberrant Notch signaling (Louvi and Artavanis-Tsakonas, 2012).

Notch receptors localize mainly on the cell surface within the plasma membrane in a processed heterodimer form. Furin-dependent cleavage (S1 cleavage) occurs in the Notch extracellular domain (NECD), during travel of Notch receptor through the Golgi complex (Logeat et al., 1998). During Notch synthesis and secretion, O-linked glycosylation takes place in the NECD. Post-translational modifications help Notch receptors fold correctly and, in the right conformation, interact with their ligands DSLs (Delta, Serrate, Lag-2) (Rana and Haltiwanger, 2011). The glycosylation is regulated by Fringe-gene, which encodes a glycosyltransferase. The glycosyltransferase transfers N-acetylglucosamine to fucose on Notch epidermal growth factor-like (EGF) repeats (Brückner et al., 2000; Moloney et al., 2000). Notch receptor in the signal-receiving cell binds ligands directly onto the signal-sending cell surface. Mammals have four Notch receptors: Notch 1, Notch 2, Notch 3, and Notch 4, and five ligands: Jagged 1, Jagged 2, Delta-like 1, Delta-like 3, and Delta-like 4 (Bray, 2006; Hori et al., 2013; Kopan and Ilagan, 2009). Metalloproteinase ADAM performs second NECD cleavage (S2 cleavage) when a receptor binds to a ligand on a signal-sending cell. This event facilitates

an S3 cleavage event by presenilin-containing γ-secretase complex within the Notch transmembrane domain (Brou et al., 2000; Struhl and Greenwald, 1999). At this point, Notch intracellular domain (NICD) is free to move off the membrane, and the NECD is trans-endocytosed inside the signal-sending cell still bound to its ligand (Gordon et al., 2008). Eventually, NICD travels to the nucleus. Inside the nucleus NICD forms a transcriptional complex with Mastermind (Mam), DNA-binding protein CSL (CBF1, Lag1, Suppressor of Hairless), and transcriptional co-activators allowing Notch target gene synthesis. Without NICD, CSL interacts with several co-repressors, causing transcriptional inhibition of Notch target genes (Bray, 2006; Kopan and Ilagan, 2009).

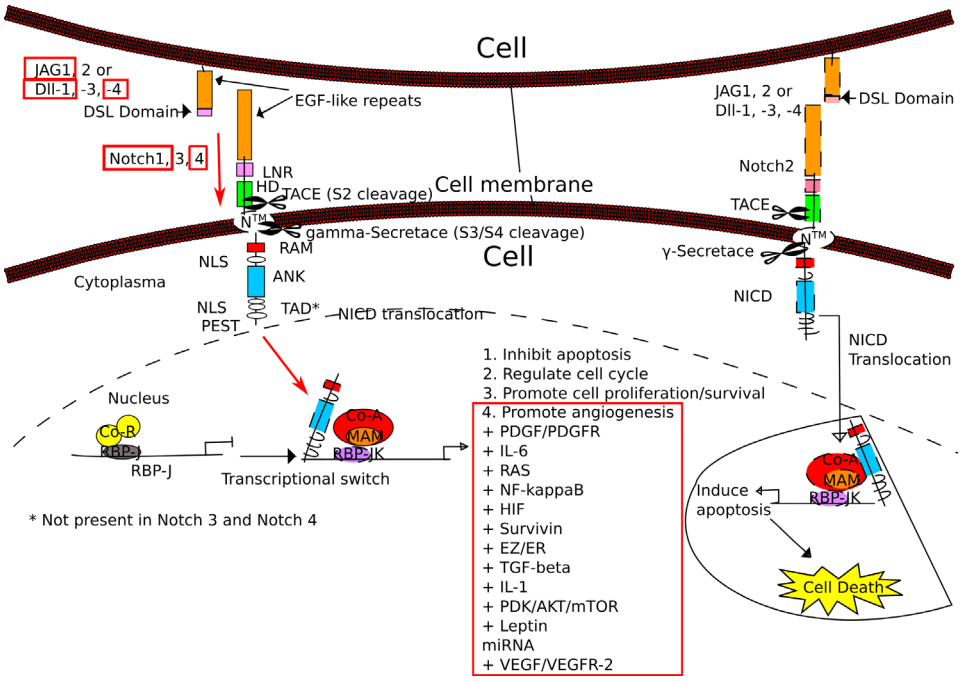


Figure 9: The mechanisms of Notch signaling in human cancers and angiogenesis. Notch signaling can lead to several outcomes depending on the context and signaling pathway. The angiogenic route is shown in detail. Adapted from (Zhou et al., 2013).

Notch ligands can bind Notch receptors in the same cell in a cis manner. Cis-interactions inhibit Notch signaling, whereas trans-interactions between cells enhance Notch signaling (del Álamo et al., 2011; Sprinzak et al., 2010). The interplay between these interactions is a crucial factor for distinguishing the signal-receiving cell from the signal-sending cell. The ratio of receptor-competent ligands to ligand-competent receptors on the cell surface is also a factor that affects cell fate determination. Ligand recycling critically regulates Notch signaling activity by controlling cell surface ligand accumulation. Trans-endocytosis of the ligands with the Notch receptor is considered a prerequisite for fully active Notch signaling. Binding of a ligand to a receptor on an adjacent cell and the following endocytosis

has been suggested to produce mechanical pulling force. The force causes a conformational change that reveals the S2 Notch receptor cleavage site and allows proteolytic cleavage of NICD, which is required for Notch signaling (Meloty-Kapella et al., 2012). Notch signaling activity is also regulated in a complex way by endocytosis and endosomal processing during various stages of Notch signaling (Fortini, 2009; Yamamoto et al., 2010). Endocytic trafficking has been shown to have a role in Notch signaling induced by ligands (Coumailleau et al., 2009). Under specific circumstances, the receptor can be activated in endocytic compartments without ligand activation (Hori et al., 2011; Vaccari et al., 2008, 2009; Wilkin et al., 2008).

10.1. Notch signaling in angiogenesis

Notch signaling is important in angiogenesis in development and pathological conditions, as well as for maintaining vascular homeostasis. In development, Notch functions in arterial differentiation, lymphatic development, and sprouting- and lymphangiogenesis. Notch ligands and receptors express in endothelial and perivascular cells (e.g. vascular smooth muscle cells, pericytes, and macrophages), and also in tumor endothelial cells. Defective expression of Notch ligands or Notch receptors during development causes embryonic lethality due to vascular defects (Krebs et al., 2000; Limbourg et al., 2005; Xue et al., 1999).

Notch signaling is critical in sprouting angiogenesis. In sprouting angiogenesis, there is communication between two cell types: tip and stalk cells. Tip cells protrude filopodia into the surrounding tissue, and stalk cells follow and proliferate to form the trunk of the new vessel sprouts (Patel-Hett and D'Amore, 2011; Ribatti and Crivellato, 2012). Notch signaling determines the formation of tip and stalk cells (Ribatti and Crivellato, 2012). When cells become stimulated by VEGF, cells with a higher VEGFR2 signal upregulate Dll4 and form tip cells (Tung et al., 2012). Dll4 then activates Notch signaling in adjacent endothelial cells to reduce their expression of VEGFR2 and VEGFR3. Reduction in VEGFR2 and VEGFR3 expression promotes the stalk cell phenotype (Ribatti and Crivellato, 2012; Tung et al., 2012). If Notch signaling is depressed, endothelial cells continue to form more sprouts in response to VEGF signaling (Benedito et al., 2009; Li et al., 2007; Napp et al., 2012; Ridgway et al., 2006; Siekmann and Lawson, 2007; Suchting et al., 2007; Thurston et al., 2007). Jagged 1 plays a proangiogenic role in mice by antagonizing Dll4-Notch signaling in cells, which express Fringe family glycosyltransferases (Benedito et al., 2009). Fringe family of glucosaminyl transferases modify Notch receptors in a way that enhances Dll4-signaling but suppresses Jagged1 signaling (Benedito et al., 2009). Notch signaling also regulates the expressions of endothelial growth factor receptors 1, 2, and 3 in a complicated way (Phng and Gerhardt, 2009).

Jagged1 and Dll4 have opposing effects on vascular formation, but how this signaling is balanced is widely unknown. Dll4-mediated activation of Notch signaling suppresses angiogenesis, and Jagged1 antagonizes Dll4 and promotes angiogenesis and sprouting (Benedito et al., 2009; Pedrosa et al., 2015; Trindade et al., 2008; Xue et al., 1999). Both Jagged1 and Dll4 regulate tip and stalk cell selection, and the

initiation of new branches in the branching endothelium (Benedito et al., 2009; Phng and Gerhardt, 2009). Dll1 functions as an inhibitor of angiogenesis when expressed by vasculature, but enhances angiogenesis when expressed by surrounding tissue. Dll1 maintains VEGF expression later in angiogenesis (Kume, 2009; Napp et al., 2012; Sörensen et al., 2009). How ligand-receptor signaling specificity is achieved is not clearly known.

If delta-like 4 (Dll4) is blocked, hyperbranching of blood vessels occurs when more tip cells are created. These vessels are leaky and less functional. Dll4 overexpression causes attenuated vessel formation in developing mouse embryos, and haploinsufficiency causes embryonic lethality (Gale et al., 2004; Trindade et al., 2008). Balanced regulation of ligands is crucial for functional vasculature formation (Andersson et al., 2011; Artavanis-Tsakonas et al., 1999).

Mice lacking Jagged1 are early embryonic lethal due to hemorrhages (Hansson et al., 2010; Xue et al., 1999). Jagged1 deregulation is associated with vascular injuries and observed in pulmonary arterial hypertension patients (Lindner et al., 2001; Yamamura et al., 2014). Jagged1-mediated Notch activation causes increased retinal vascular density and vascular smooth muscle recruitment to retinal arteries (Kangsamaksin et al., 2014b). Increased glucose causes Jagged1 overactivation and pathological angiogenesis, including increased sprouting and vessel destabilization (Yoon et al., 2014). The effect may be explained by the downregulation of soluble decoy VEGFR1 (antagonist of VEGFR2) expression by Jagged-Notch signaling. This leads to an increase in VEGFR2 signaling (Kangsamaksin et al., 2014b).

10.2. Notch signaling in cancer

Notch signaling promotes cell survival, angiogenesis, differentiation, and treatment resistance in many cancer types. Notch signaling also has crosstalk with other critical cancer-promoting signaling pathways. Notch signaling is, therefore, a target candidate for cancer drugs. Today there are several gamma-secretase inhibitors in clinical trials, but other Notch signaling molecules may also be potential targets (Purow, 2012). Current Notch inhibitors produce considerable side effects, rendering them unusable treatments (van Es et al., 2005; Wong et al., 2004; Yan et al., 2010). In a recent preclinical article, Notch decoy receptors that selectively block Delta-like- or Jagged-Notch signaling inhibited mouse xenograft tumor angiogenesis without considerable side effects (Kangsamaksin et al., 2014b).

11. Par-4

Par-4 is a pro-apoptotic protein that is especially active in prostate cancer. Par-4 can inhibit NF-kappaB and simultaneously activate FasL and Fas. These activation level changes can induce cancer cell apoptosis and tumor regression. They can selectively induce or sensitize cancer cells to apoptosis without affecting normal cells. Par-4 can induce apoptosis both intracellularly and extracellularly, and can be excreted spontaneously. Systemic Par-4 induces apoptosis via cell surface GRP78 receptor/caspases pathway (Bhattarai and Rangnekar, 2010; Burikhanov et al., 2009; Zhao et al., 2011). The in vivo delivery of Par-4 with adenovirus or nano-

liposomes to HT29- or PC-3-cell-derived tumor in mice has been shown to cause tumor regression or sensitization to therapeutic agents (Chakraborty et al., 2001; Kline et al., 2009). Par-4 has been observed to be downregulated in breast cancer, lymphoma, and pancreatic and renal carcinoma. The Par-4 gene that resides in chromosome 12 can often undergo deletion in gastric and pancreatic cancers, and in tumors of male germ cells (Boehrer et al., 2001; Cook et al., 1999; Johnstone et al., 1998; Nagai et al., 2010; Schneider et al., 2003). Par-4 is a major factor in breast cancer recurrence (Alvarez et al., 2013). Mice without Par-4 develop tumors in several tissues spontaneously (García-Cao et al., 2005). Par-4 induces apoptosis via its caspase-cleaved form, and by inhibiting survival factors NF-κB, PKCζ, and AKT kinase (Diaz-Meco et al., 1999; Díaz-Meco et al., 1996; Lee et al., 2010; Wang et al., 2010a). Phosphorylation of different sites of Par-4 can inhibit or activate Par-4-mediated apoptosis (Goswami et al., 2005; Gurumurthy et al., 2005).

12. CK2

Casein kinase 2 (CK2) is a serine/threonine protein kinase with amino acid consensus motif 'S/T-X-X-Asp/Glu/pSer' recognition property. It forms tetramers that consist of two subunits with a kinase catalytic domain (α and/or α′ in different combinations) and two β-subunits. β-Subunits mediate interactions between CK2 catalytic subunits and their substrates (Allende and Allende, 1995; Meggio and Pinna, 2003). Casein kinase 2 has global roles in cell growth (increase), cell proliferation (increase), and cell death (protect), and also regulates processes such as cell cycle control, DNA repair, and circadian rhythm (Volodina and Shtil', 2012). CK2 inhibition is presently under intense study, because CK2 activity and/or expression is increased in colon, prostate, lung, and head and neck cancers, among others (Daya-Makin et al., 1994; Gapany et al., 1995; Münstermann et al., 1990; Yenice et al., 1994). Inhibition of CK2 may affect cell growth, proliferation, and apoptosis, ubiquitously resulting to toxicity (Trembley et al., 2010). CK2 activity increases expression/activity of pro-survival proteins Wnt, NF-κB, and Akt, inhibits pro-apoptotic genes by preventing caspase cleavage of them, and inhibits mitochondrial apoptotic pathways (Ruzzene and Pinna, 2010; Wang et al., 2006a; Yenice et al., 1994). CK2 inhibition can predispose cancer cells to apoptosis, and overexpression of CK2 increases cancer cell survival (Desagher et al., 2001; Guo et al., 2001; Izeradjene et al., 2005; Ravi and Bedi, 2002; Wang et al., 2005, 2006b).

13. AATF

Apoptosis antagonizing transcription factor (AATF, also known as Che-1) is a transcriptional cofactor that is an important cofactor of DNA damage response machinery. This signaling network initiates DNA repair and prevents tumorigenesis by causing growth arrest or apoptosis when DNA damage occurs. AATF also affects various signaling pathways that regulate proliferation and survival in both physiological, and pathological conditions (Iezzi and Fanciulli, 2015). AATF is ubiquitously expressed in tissues. In cells, AATF usually shows nuclear and nucle-

olar localization, but a cytoplasmic localization has also been reported in specific cells (Iezzi and Fanciulli, 2015).

AATF is an essential component or an organism, since AATF knockout mice are embryonically lethal at the preimplantation state. When AATF in mice is mutated and not completely functional, embryos have reduced number of cells highlighting AATFs role in cell proliferation (Thomas et al., 2000).

The gene that produces AATF is located on chromosome 17. Its protein product consists of 558 amino acids and is highly conserved among eukaryotes. AATF protein negatively regulates its own coding (Monaco et al., 2003). AATF consists of an N-terminal acidic domain, a canonical leucine zipper, and three LXXLL motifs for nuclear receptor binding. It has one nuclear and two nucleolar localization signals (Fanciulli et al., 2000; Lindfors et al., 2000; Scott et al., 2011).

AATF is part of DNA damage response machinery and is phosphorylated and activated after DNA damage by MK2, ATM, and Chk2 checkpoint kinases (Bruno et al., 2006; Ciccia and Elledge, 2010; Höpker et al., 2012). After DNA damage, one option for AATF function is that MK2 phosphorylates AATF T366, which allows AATF to move from the cytoplasm, where it can be sequestered by MRLC3, to the nucleus (Höpker et al., 2012). ATM and Chk2-mediated AATF phosphorylation reduces AATF degradation, causing stabilization and accumulation of AATF (Bruno et al., 2006). These modifications also affect AATF interactions and cause a different cellular response; AATF effect changes from cell cycle progression to cell cycle arrest and survival. ATM-Chk2 phosphorylation moves AATF from E2F1-dependent promoters to checkpoint activation gene promoters (for example TP53 and p21) inhibiting them and the cell cycle, and promoting AATF interaction with p53 (Bruno et al., 2006; Desantis et al., 2015). ATM and Chk2 phosphorylation of ATF also occurs after DNA-damaging agent exposure (mild DNA damage) and promotes antiapoptotic factor XIAP transcription (Bruno et al., 2008). PolyADP-ribosylation of AATF occurs after DNA damage and increases AATF stability (Bacalini et al., 2011).

During DNA damage AATF may accumulate into centrosomes. AATF is part of the spindle assembly checkpoint: it prevents the formation of multiple centrosomes and multipolar spindles and enables mitotic arrest and apoptosis after mildly genotoxic treatments (Lara-Gonzalez et al., 2012; Sorino et al., 2013; Zhang et al., 2007). In apoptotic DNA damage AATF gets degraded by HIPK2-mediated phosphorylation of T144, promoting a conformational change which allows ubiquitination to occur (De Nicola et al., 2007, 2014). AATF promotes p53 transcription upon DNA damage. Transcription promotion also happens with mutant p53s, which are often found in cancers. If AATF depletes, then mutant p53 is not transcribed inducing apoptosis via tumor suppressor protein p73 (Bruno et al., 2010).

In addition to DNA damage, AATF participates in many types of stresses, including ER stress, hypoxia, hyperosmotic stress, and glucose deprivation. ER stress induces AATF which activates AKT1 through direct interaction with STAT3 (Ishigaki et al., 2010). AATF has been shown to protect from apoptosis induced by

ionizing radiation, hypoxia, or glucose deprivation by inducing autophagy (shown to protect from metabolic stress) via inhibition of mTOR (Desantis et al., 2015).

AATF is usually related to antiapoptotic activity (Iezzi and Fanciulli, 2015). AATF interacts with and antagonizes proapoptotic Dlk ZIP kinase (Page et al., 1999). In neural tissue AATF has antiapoptotic effects in physiological and pathological conditions. AATF interacts with neurotrophin receptor-interacting MAGE homolog (NRAGE), which functions in neuronal cell death during development and counteracts NRAGE's effect (Di Certo et al., 2007). AATF's antiapoptotic effect also functions in neurodegenerative processes. AATF can counteract neurodegeneration by interacting with Par-4, preventing its effect on producing a neurotoxic peptide (Guo and Xie, 2004; Xie and Guo, 2004). AATF is also a substrate, and interacts with Cdk5, whose activity is deregulated in neurodegenerative diseases (Buontempo et al., 2008). In kidney proximal tubule cells AATF has been shown to preserve mitochondrial function and reduce oxidative damage, consequently counteracting apoptotic cell death (Xie and Guo, 2006). Although AATF is mainly a pro-survival protein in several contexts, in this thesis, we describe a pro-apoptotic function for AATF, which activates after UV damage (Ferraris et al., 2012).

During proliferation, AATF promotes cell cycle progression by inhibiting pRb-regulated growth suppression. AATF binds to pRb and removes HDAC1 from the Rb E2F-complex, which allows transcription and S-phase entry. AATF is hyperphosphorylated during G1/S transition (Bruno et al., 2002). AATF also localizes to centrosomes during interphase and regulates centrosome duplication and spindle formation (Sorino et al., 2013). Therefore, AATF affects cell cycle progression with more than one mechanism.

Due to AATF's effects on, for example, cell cycle, cell survival, autophagy, and p53, AATF affects tumor cell survival. Cancer cell transformation has been shown to be affected by AATF, and increased expression of AATF may be crucial for the survival of tumor cells (Iezzi and Fanciulli, 2015). In one screening, mutations of AATF were not associated with breast cancer (Haanpää et al., 2009). AATF has been found to be upregulated in many leukemia cell lines (Bacalini et al., 2012; Kaul and Mehrotra, 2007). In neuroblastoma patients, AATF was overexpressed and predicted poor prognosis (Höpker et al., 2012). AATF downregulation leads to enhanced cytotoxicity from DNA-damaging chemotherapy, and tumor cells with mutant p53 were more likely to induce apoptosis (Bruno et al., 2006, 2008, 2010; Höpker et al., 2012). A functional AATF inhibitor has not yet been developed (Iezzi and Fanciulli, 2015). Therefore, AATF is a potential cancer treatment target, although this is still mainly theoretical.

AATF interacts with at least several proteins in several contexts. These interactions form the basis of AATF cellular functions. The interactions are often regulated by post-translational modifications that allow fast and dynamic responses (Iezzi and Fanciulli, 2015). It has been shown that AATF interacts with nuclear hormone receptors in vitro, and enhances transactivation of several steroid hormone receptors sometimes cooperating with acetyltransferase p300 (Leister et al., 2003).

AATF enhances androgen receptor-mediated transcription, and TSG101 binding to AATF regulates this transcription (Burgdorf et al., 2004). AATF interacts with pRb, p65, and STAT3 transcription factors (Bruno et al., 2002, 2006; Ishigaki et al., 2010). AATF can bind hRBP11 subunit in RNA polymerase II, and rat ortholog exhibits transactivation (stimulate transcription by binding to DNA) activity (Fanciulli et al., 2000; Page et al., 1999). Since the hRBP11 subunit exists in various forms in C-termini of other proteins of this protein family, AATF may have a wider effect via this interaction (Benga et al., 2005; Grandemange et al., 2001).

14. c-Jun

c-Jun is a member of the AP-1 transcription factor family. The AP-1-family consists of Jun, ATF, and Fos subfamilies. Different members of families stand out with particular DNA-binding specificities, dimerization partners, and posttranslational modifications. AP-1 family members primarily affect cell proliferation, survival, and death (Devary et al., 1991; Dhanasekaran and Reddy, 2008; Herrlich et al., 2008; Kasibhatla et al., 1998; Le-Niculescu et al., 1999). c-Jun usually acts antiapoptotically, but c-Jun deficiency has also been observed to protect fibroblasts from UV- or alkylating agents-induced apoptosis (Kolbus et al., 2000; Shaulian et al., 2000). Prolonged c-Jun activation has been shown to precede apoptosis (Kasibhatla et al., 1998; Raivich, 2008). Apoptosis occurs when a certain threshold of pro-apoptotic signals is reached when c-Jun induces at least TNFα, FasL, and Bim (Devary et al., 1991; Dhanasekaran and Reddy, 2008; Herrlich et al., 2008; Kasibhatla et al., 1998; Le-Niculescu et al., 1999). c-Jun is activated by phosphorylation induced by c-Jun N-terminal kinases (JNKs) (Johnson and Nakamura, 2007).

OUTLINE OF THE STUDY

The aim of this research was to study the effects of phosphorylation primarily in vimentin structure and function, and also phosphorylation-mediated functions of Par-4 and AATF in the apoptosis context.

Earlier, our lab identified several vimentin in vivo serine and threonine phosphorylation sites. Most of the functions of these phosphorylation sites are still unidentified. We aimed to characterize how phosphorylation of most of the vimentin serine and threonine in vivo phosphorylation sites affected mainly vimentin network structure with phospho-deficient and phospho-mimicking point mutations.

Sphingolipids S1P and SPC are known regulators of cellular migration and affect keratin 8 and 18 phosphorylation and network, but their effects on the vimentin network are not known. Our aim was to examine how vimentin organization and ultimately cell migration via vimentin phosphorylation was affected by sphingolipid S1P and SPC signaling in primarily cancer cell models.

There is limited information about the effects of IFs on Notch signaling, even though Notch signaling and vimentin share several similar functions in cells. Our aim, initially, was to examine how vimentin-influenced Notch signaling from the ligand side of the signaling pathway, and ultimately how vimentin affects angiogenesis via Notch ligand regulation.

Par-4 is a pro-apoptotic protein highly expressed in prostate cancer, which preferentially switches on apoptosis in cancer cell lines as compared to normal cells. CK2 also regulates apoptosis by inhibiting it, and is an important kinase in prostate cancer. Although these proteins affect the same processes in the same tissue, how Par-4 signaling connects to CK2 signaling has not yet been described. There were potential conserved sites for CK2 phosphorylation in Par-4. Our aim was to describe how CK2 signaling affects Par-4 signaling and ultimately apoptosis by direct phosphorylation.

AATF is a transcription factor protein described as promoting proliferation and inducing cell cycle arrest. The function of AATF has not been defined widely under stress conditions. Our aim was to describe how AATF functions after UV stress, and ultimately to describe the mechanism by which AATF induces apoptosis after UV stress.

EXPERIMENTAL PROCEDURES

More detailed information on the materials and methods used can be found in the original publications and manuscripts. Publications, where used, in parentheses.

1. Cell lines

MEF vimentin -/- and wild-type (I-III)
MCF7 (I)
HeLa (I)
MDA-MB-435S (II)
C643 (II)
SW13 (II)
293 FLN1 (III)
3T3J (III)
HTC116 (IV)
PC3 (IV)
LnCap (IV)
PNT2C2 (IV)
PrEC (IV)
COS7 (IV)
HEK293 (V)
MEF c-Jun -/- and WT (V)

2. Plasmids

pcDNA4-vimentin (WT, S4A&D, S4,6A&D, S6A&D, S4,6,7,8,9A&D, S7,8,9A&D, S419A&D) (I)
pcmv-vimentin (WT, S38A&D, S50A&D, S55A&D, S71,72A&D, S429A&D, TS457,T458A&D) (I)
GST-Par-4 (Filhol et al., 2003)we fused its catalytic alpha and regulatory beta subunits with green fluorescent protein (GFP (IV)
GFP-CK2α & β (Filhol et al., 2003)we fused its catalytic alpha and regulatory beta subunits with green fluorescent protein (GFP (IV)
pcDNA3.1 TOPO GFP-Par-4 (124A223A, 124D,223D, 124A, 124D, 223A, 223D, 123A), Flag or GFP rat Par-4 Δ124-332 (IV)
pcDNA3.1 Myc-Par-4 (WT, 231A, 231D) (IV)
GFP-AATF (V)
HA-c-Jun (Gal4-cJunwt, Gal4-cJunAA, Gal4-cJun-Δ) (Westermarck et al., 2002; Weiss et al., 2003) (V)

3. Antibodies

Vimentin (clone V9, Sigma-Aldrich) (I-III)
Vimentin (clone D21H3, Cell Signaling) (I-III)

Vimentin chicken polyclonal (Covance) (I-III)
Alexafluor488-conjugated phalloidin (Invitrogen) (I-II)
β-tubulin rabbit polyclonal (Abcam) (I)
Notch 1 (Cleaved-Val-1754 rabbit, Sigma Aldrich) (III)
β-actin (rabbit, Cell Signaling Technology) (III)
Cleaved Notch1 (rabbit Val1744, Cell Signaling Technology) (III)
CD31 (PECAM-1, BD Biosciences) (III)
Notch 1 (C20 rabbit, Santa Cruz Biotechnology) (III)
Jagged1 (rabbit 28H8, Cell Signaling Technology) (III)
Jagged1 (C20, Santa Cruz Biotechnology) (III)
Delta (C20 rabbit, Santa Cruz Biotechnology) (III)
Anti-Dll4 (rabbit) (Sigma-Aldrich) (III)
AATF (mouse, Santa Cruz Biotechnology) (V)
c-Jun (rabbit, Santa Cruz Biotechnology) (V)
Nucleolin (rabbit, Santa Cruz Biotechnology) (V)
c-Jun phS73 (rabbit, Cell Signaling Technology) (V)
actin (mouse, Sigma-Aldrich) (V)
phJNK (rabbit, Cell Signaling Technology) (V)
GFP (rabbit, Living Colors, Clontech) (V)

4. siRNAs

siControl 5'-CCUACAUCCCGAUCGAUGAUG(dTdT)-3' (Högel et al., 2011) (II)
siS1P$_2$ 5'-CUACAAAGCCCACUACUUU(dTdT)-3' (Högel et al., 2011) (II)
siCK2T (Thermo Fisher Scientific) (IV)
siPar-4T (Thermo Fisher Scientific) (IV)
siCK2AB (Ambion, Life Technologies) (IV)
siPar-4T (Thermo Fisher Scientific) (IV)
siPar-4sc (Santa Cruz, Tebu-Bio) (IV)
Scr siRNA fluorescently labeled with FAM (Santa Cruz) (IV)
AATF siRNA, scrambled siRNA (Qiagen) (V)

5. Complete methods list

Cell culture and treatments (I-V)
Plasmid constructs, molecular cloning and antibodies (I-V)
Transient transfections (I-V)
Microscopic analyses (I-V)
- Immunofluorescence labeling (I-V)
- Immunohistochemistry (III)
- Confocal microscopy (I-V)
- Colocalization analysis (III)
- Correlative electron microscopy (CLEM) and electron microscopy (I)
- Fluorescence recovery after photobleaching (FRAP) analysis (II)

- Vesicle tracking (III)
- Cell shape measurements (II)
- Vimentin distribution analysis (I)
- Fingerprint assay (III)

SDS-PAGE and western blotting (I-V)
Recombinant protein purification (IV)
Immunoprecipitation (IV-V)
Apoptosis assays (IV-V)
- Caspase cleavage assay (IV)
- Measure of CK2 activity (IV)
- Measure of apoptosis and caspase-3 activity (IV)
- UV irradiation and cell death analysis (V)

Phosphorylation assays (II, IV)
- Tryptic digestion, two-dimensional separation and Edman degradation (IV)
- *In vitro* kinase assay (IV)
- Mass Spectrometry (II)

Intermediate filament solubility assays (I-II)
- Sequential centrifugation (II)
- Assay of vimentin subunit composition (I)
- Crosslinking assay (I)
- Triton X-100 extraction (I)

Migration assays (II)
- Chemotactic migration with Boyden chambers (II)
- Wound healing with Incucyte ZOOM (II)
- Wound healing, single-cell motility imaging, and analysis with Cell-IQ (II)

Angiogenesis assays (III)
- Aortic ring protocols (III)
  - Aortic ring assay (III)
  - Vimentin Null Aortic Rings with Notch Ligands (III)
  - Quantification of Aortic Ring Sprouting Responses (III)
  - Immunostaining of Aortic Rings (III)
- Chorion-allantois membrane (CAM) angiogenic assay (III)
- 3D Matrigel angiogenesis assay (III)

Surface protein detection by biotinylation (III)
Recycling assay (III)
Luciferase reporter assays (III-IV)
Activation of Notch signaling by immobilized recombinant Notch ligands (III)
Receptor binding and ligand trans-endocytosis (III)
Quantitative real-time PCR (III-IV)

6. Selected methods

Some methods were used in more than one publication with slightly different protocols, but only one protocol is shown here. Methods that the author of this

thesis performed himself are preferentially shown here.

Methods that the author of this thesis performed himself for the publications:

I) Everything, except part of the cloning, epon block cutting, EM imaging and Suppl. Fig. 1B.
II) Fig. 2B, C (Cell shape measurements), Suppl. Fig. 2 (FRAP), Suppl. Movies 1,2 (FRAP).
III) Fig. 5A, B, C (Vesicle tracking), Fig. 6A, B (Fingerprint assay), Suppl. Fig. 6A, B, C (Colocalization analysis), Suppl. Fig. 8 (tissue mRNA databank correlation analysis).
IV) Fluorescence microscopy with Par-4 GFP constructs (with CK2a and TRAIL). Not in the publication.
V) Fig. 4D, E (Immunofluorescence).

6.1. Transient transfections (I-V)

Transient transfections for wild-type MEF and vimentin -/- MEF, MCF7, and HeLa cells were done with Lipofectamine LTX (Cat# 15338-100, Invitrogen, UK) according to the manufacturer's instructions for MCF7, HeLa, and MEF cell lines. For solubility assay MEF vim-/- cells were transfected by electroporation in Optimem I (Cat# 31985-047, Invitrogen, UK). Electroporation settings were 300 volts, 1,07 mF in a 400 µl cuvette with Gene Pulser II (Bio-Rad, UK).

6.2. Immunofluorescence (I-V)

I) Cells were grown on glass coverslips. Adhered cells were transfected as described. Cells were fixed two days after transfection for five minutes with 3% PFA in PBS. Cells were stained with one of the following protocols: Protocol 1: cells were permeabilized with 0.1% Triton X-100 in PBS for five minutes at room temperature (RT), blocked with 1% BSA in PBS for one hour at RT. Then, coverslips were incubated with primary antibody in 1% BSA in PBS for one hour, washed three times, for five minutes each in PBS, incubated for one hour at RT with secondary antibody, washed three times, for five minutes each in PBS, rinsed in distilled H2O and mounted with Mowiol mounting media. Protocol 2: Cells were incubated for five minutes in 100 mM glycine in PBS at RT, blocked with blocking buffer: 10% fecal calf serum (FCS), 0.2% saponin, 0.5% fish skin gelatin in PBS for one hour at RT, incubated with primary antibody in blocking buffer, rinsed three times in washing buffer (blocking buffer without serum), incubated with secondary antibody for 45 minutes in blocking buffer, rinsed three times in washing buffer and once in distilled H2O, finally and mounted on microscope slides with Mowiol mounting media.

6.3. Confocal microscopy (I-V)

Cells were imaged with Leica SP5 laser scanning confocal microscope (Leica; Wetzlar, Germany) using 60X /1.4 plan-apochromat oil immersion objective or Zeiss LSM510 META laser scanning microscope (Oberkochen, Germany). Correlative electron microscopy samples were imaged with Zeiss LSM510 META laser scanning confocal microscope using 100X /1.4 plan-apochromat oil immersion objective. The widefield mCherry transfection images were acquired with Leica

DMRE 20x magnification objective.

6.4. SDS-PAGE and western blotting (I-V)

I, II) Cells were scraped from the cell culture plate with a rubber scraper or treated otherwise, as described earlier. All the samples were dissolved in laemmli buffer (4% SDS, 20% glycerol, 120 mM pH 6.8 Tris-HCl, 0.02% bromophenol blue) containing 3% mercaptoethanol. Samples were treated for five minutes at 95 °C. Proteins were resolved by SDS–PAGE, and transferred to a nitrocellulose membrane (Protran nitrocellulose; Schleicher & Schuell, Dassel, Germany) using a wet transfer apparatus (GE Healthcare, USA). Membranes were blocked with 5% nonfat milk in PBS containing 0.3% Tween-20 for one hour at RT, and incubated overnight at 4 °C with primary antibody: vimentin (Cat# 5741, clone D21H3, Cell Signaling, USA). After washed three times, for five minutes each with PBS/0.3% Tween-20, membranes were probed with horseradish peroxidase–conjugated secondary antibodies purchased from Promega, Southern Biotechnology, and GE Healthcare. Proteins were visualized using the ECL detection kit (GE Healthcare, Amersham, Buckinghamshire, UK).

6.5. Correlative electron microscopy (CLEM) and electron microscopy (I)

Transiently transfected cells that were grown on plates with gridded glass bottom (Cat# P35G-2-14-C-GRID, MatTek Corporation, Massachusetts, USA) were fixed in PBS with 3% PFA. Plates were washed briefly two times with PBS. Cells were immunostained corresponding to immunostaining protocol described above. After confocal microscope imaging and noting cell positions, cells were fixed in 2.5% glutaraldehyde in cacodylate buffer (50 mM sodium cacodylate, 50 mM KCl, 2.5 mM MgCl, pH 7.8). Then, plates were rinsed in cacodylate buffer and osmicated for one hour in cacodylate buffer with 1% OsO_4. Plates were washed twice for three minutes each time in cacodylate buffer, and afterwards three times for five minutes each in distilled water. Then, samples were treated for one hour at 4°C with 1 % uranyl acetate, 0.3 M sucrose in distilled water. The plates were washed three times for three minutes each in distilled water. The plates were dehydrated in a 70 %, 96 %, and two times absolute ethanol series for one minute each. After this, almost all ethanol was removed, and some epon was dropped immediately onto the sample and the epon capsule put on top of the marked cells. Coverslips were incubated for two hours at room temperature and then baked for 14 hours at 60°C. Samples were transferred directly from oven to hot plate, and coverslips removed carefully. Samples were cut with a diamond microtome and previously confocal microscope imaged cells were imaged with an FEI Tecnai 12 transmission electron microscope. For pure electron microscopy samples, coverslips were used, and sample preparation was conducted as described for CLEM, with the exception of no immunostaining and confocal imaging done, and with sample preparation initiated from glutaraldehyde fixation. The dehydration step included a rinse in acetone at the end, and epon treatment was done on an aluminium planchette.

6.6. Colocalization analysis (III)

For colocalization analysis MEF WT and vim-/- cells were seeded on 24-well plates with glass coverslips and transfected with Rab4-GFP or Rab11 constructs with Lipofectamine LTX according to the manufacturer's instructions. Two days after transfection, cells were fixed and labeled according to immunofluorescence protocol. Samples were imaged with a Leica SP5 laser scanning confocal microscope (Wetzlar, Germany) using 60X /1.4 plan-apochromat oil immersion objective. Colocalization analysis of jagged1 and Rab4 or Rab11 was done using the Fiji ImageJ software and associated plugins. 3072x3072 resolution images with 80 nm pixel size were used for the analysis. The subtract background function was used with 200-pixel rolling ball radius for each image. Images showing colocalization and Manders colocalization coefficients were calculated with Colocalization Threshold. Pseudocolor images showing colocalization amounts were created from Colocalization Threshold colocalization images by removing green and red, transforming the image into grayscale and changing the look-up table to rainbow. Zero-zero pixels were not included in the threshold calculation. Pearsons colocalization coefficients were calculated using the Colocalization Test.

6.7. Fluorescence recovery after photobleaching (FRAP) analysis (II)

II) FRAP was carried out with Zeiss 780 confocal microscope (Carl Zeiss AG; Jena, Germany) with 60x water immersion objective. 50,000 MDA-MB-435S cells were plated on each 35 mm glass-bottomed dish (MatTek Corporation; Ashland, MA, USA). Cells were transfected with the mCherry-vimentin WT construct the following day with Lipofectamine LTX (Life Technologies) according to the manufacturer's instructions. The next day, the cells were lipid-starved O/N. FRAP imaging was performed 48 hours post-transfection. Bar-shaped 2 μm wide regions were bleached, and recovery was imaged for 15 minutes at 30-second intervals. The FRAP-images were analyzed afterward with ImageJ (measurement of the intensity of the bleached spot and control area) followed by FCalc (fitting of the recovery curve and calculation of half-recovery and mobile fraction values). The analysis was done only for spots where individual filaments could be followed from start to finish, and the analysis region was moved over time, if cell or the analyzed intracellular region moved uniformly. One function fit and correction for bleaching were used for FRAP calculation. The data for the recovery curves were normalized (deduction of background noise, normalization of all experiments to 0-1 scale). GraphPad Prism was used for the fitting of the exponential one-phase association recovery curve.

6.8. Vesicle tracking (III)

Immunofluorescence tracking of vesicles was performed on WT and KO MEFs. Cells were cultured in DMEM (Sigma-Aldrich, St Louis, MO). The media was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 units/ml both penicillin and streptomycin (all from Life Technologies, Carlsbad, CA). Jagged1 was over expressed in both cell types. 50,000 cells were plated onto 35 mm glass-bottomed plates and transfected with Jagged1 after 24 hours using

Lipofectamine LTX according to the manufacturer's instructions. Before labeling, human Alexa 488 secondary antibody was incubated with rN1ECD as described above, and the cells were simultaneously blocked with DMEM containing 10% goat serum and 1% BSA for 45 minutes at +37°C. Next, the cells were incubated for two hours at 37°C with medium containing the rN1ECD-Alexa 488 complex, to allow internalization. Culture media with the label was extracted and DMEM with supplements was added onto the cells. Alexa 488 signal from the brighter Jagged1 overexpressing cells were imaged with Leica SP5 confocal microscope with 63x oil objective. Images taken were in 512x512 resolution at 600 ms time intervals. Images were analyzed with CellProfiler software with an LAP tracking algorithm. Statistics were further analyzed, and movement track-images were produced with R statistics software.

6.9. Cell shape measurements (II)

The C643 cells were labeled with CFSE CellTracker dye according to the manufacturer's instructions prior to sphingolipid treatments. Cells were fixed, mounted, and entire cells were imaged with Leica SP5 or Zeiss LSM 780 confocal microscope with 20x air objective. For cell volume analysis with BioImageXD (Kankaanpää et al., 2012), images were converted to 512x512 resolution and stacks were segmented, in order to identify individual cells and measure volumes. Objects with less than 500 voxels were removed from the analysis. To measure cell perimeter, 3D image stacks were made into maximum projections and cell perimeter was analyzed with CellProfiler (Carpenter et al., 2006).

6.10. Vimentin distribution analysis (I)

Image quantitation for filament mixing experiment in Fig. 3 was conducted with imageJ software. Cell areas where vimentin intensity in the cell was measured were labeled manually. Quantitation was initiated by turning images into 8-bit grayscale images and auto thresholding them. Results for pseudocolor images were obtained using the following formula: P=(X/2+127)-(Y/2) where P is pixel intensity, X is green channel intensity (both vimentins), and Y is red channel intensity (human mutant vimentin). Results for histogram quantitation were calculated from areas after auto thresholding with corresponding formula P=Y/X.

6.11. Fingerprint assay (III)

The 96-well glass-bottom microplates (Sensoplate, #655892, Greiner bio-one, Austria, Kremsmünster) were coated overnight at RT with 50 µg/ml protein G in PBS. Wells were washed three times with RT PBS. After this, the wells were blocked with 10 mg/ml BSA in PBS for one hour at RT. The wells were washed three times with PBS at RT. After this, the wells were incubated with 1 µg/ml NotchECD-FC in 0.1 % BSA in PBS for two hours at RT. Then, the wells were washed three times with PBS at RT. The wells were washed once in the cell culture medium. 10,000 MEF WT of MEF vim-/- cells per well were plated. Cells were allowed to attach overnight. During the following day, the wells were washed three times in HBSS with magnesium and calcium. The wells were incubated with 3.3-dithio-bis-succinimidylproprionate at 37°C for 30 minutes in HBSS. The crosslinking reaction

was quenched with 50 mM Tris-HCl (pH 7.5) for 10 minutes. Cells were extracted with 0.1 % (w/v) SDS in PBS for five minutes at RT. The wells were washed with PBS. The wells were briefly rinsed with acetone, and then fixed for five minutes in acetone at RT. Then, the wells were washed three times in PBS at RT, and this was followed by immunofluorescence staining of jagged1 and NotchECD.

6.12. Assay of vimentin subunit composition (I)

The 150 mm plates of MEF vim-/- cells was transfected with vimentin mutants with electroporation. After two days, cells were scraped off into 200 µl IF fractionation buffer per plate (pH 7.5, 25 mM HEPES, 100 mM NaCl, 5 mM MgCl2, 5 mM EGTA, 0.5% Triton X-100 with protease inhibitors). Lysis was continued on ice for 30 minutes. Lysate fraction was collected, and the rest of the lysate was centrifuged in 15,000xG for 45 minutes at 4°C. Supernatant from centrifugation product is fractionated filament-fraction and pellet pellet-fraction (Eriksson et al., 2004b). Part of the fractionated filaments-fraction was further centrifuged in 200,000xG for 30 minutes at 4°C. Produced supernatant is soluble fraction, with only truly soluble vimentin subunits. Samples were analyzed by western blotting.

6.13. Statistical analyses (I-III)

Results are expressed as means±S.E.M. from at least three independent experiments. Values were analyzed using the Student's *t*-test, or one- or two-way ANOVA. Or, where appropriate, either Dunnett's or Bonferroni's *post hoc* tests were used for statistical analysis. All *P*-values were obtained using the two-tailed tests, and error bars in the graphs represent 95% confidence intervals. Quantitative data was analyzed using the GraphPad Prism program (La Jolla, CA, USA). Blots were treated by Photoshop (San Jose, CA, USA) and quantified by ImageJ (Bethesda, MD, USA). Statistical significance, $P<0.05$ and $P<0.01$, is denoted with single and double asterisks (* and **), respectively.

RESULTS AND DISCUSSION

1. Vimentin phosphomutants (I)

Phosphorylation is the most important post-translational modification regulating intermediate filament function. Our lab previously identified several serine and threonine phosphorylation interphase in vivo sites in vimentin. In the previous study, the in vivo sites were identified in baby hamster kidney (BHK-21) cells during interphase under Calyculin A (serine/threonine phosphatase inhibitor) treatment. It was observed that the phosphorylation-induced disassembly did not affect which sites were phosphorylated, but only the extent of phosphorylation of all the sites. Hamster vimentin sequence is very close to human vimentin (Eriksson et al., 2004a; Kochin et al., 2006). Our goal, which had already been set before the author arrived at the laboratory, was to create phospho-mimicking and phospho-deficient point mutations of these sites, and to study the structural and functional effects of blocked or constantly activated phosphorylation of single sites or small phosphorylation clusters.

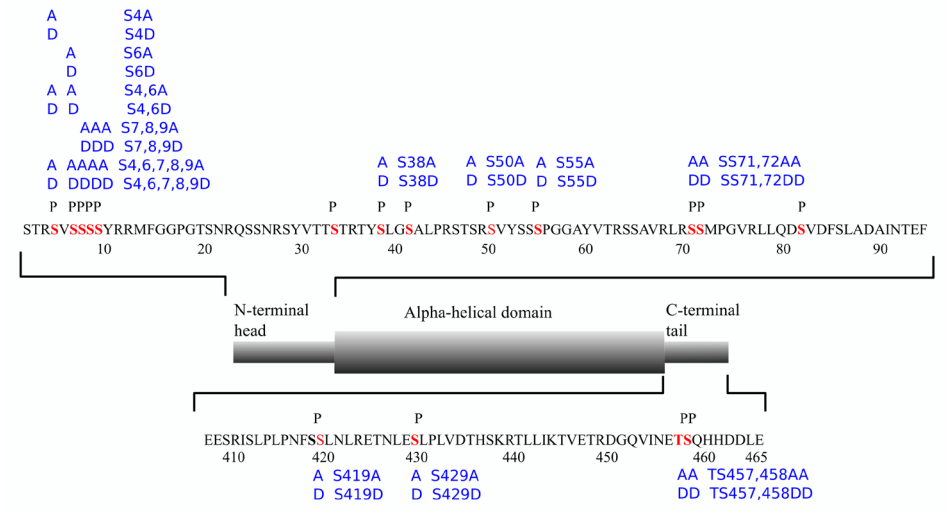


Fig. 10: Created phospho-deficient and phospho-mimicking mutants of vimentin (blue) and observed vimentin in vivo serine/threonine phosphorylation sites (red).

In this technique, phosphorylated amino acid residues (serine or threonine in this case) are mutated to similar residues that do not become phosphorylated in live cells (e.g. serine to alanine), or to residues that mimic the phosphorylated state of a phosphorylated residue most closely (e.g., serine to aspartic acid or glutamic acid). Generally, mutated proteins in which the phosphorylation of certain residues is blocked function similarly as wild-type protein with the unphosphorylated state of this residue. However, mutations that mimick phosphorylated state of a residue can in some cases function similarly as the wild-type with the phosphorylated state of this residue, but in some cases very differently. The reason for this

is, specifically, the different charge of aspartic or glutamic acid compared to phosphorylated serine or threonine (-1 versus -2), and slightly different structure. Also, many phosphorylation sites typically have a low amount of phosphorylation in cells, so having a mutant where a certain residue in every molecule is in the phosphorylated state may yield different results than normal quantities of phosphorylation (Dephoure et al., 2013; Paleologou et al., 2008). Also, the mutation of multiple adjacent sites together with some mutants in this study increases the probability that phosphomimicking or phosphodeficient mutants do not produce the same effects as the real mimicked structures and produces a some doubt to reliability of many of the findings.

The problem for functional studies with our created vectors was that the expression levels were very variable and typically low, even after transfection optimization in vimentin negative MEF and MCF7 cell lines. The expression problems made a functional comparison of vimentin phosphomutants difficult. Overexpression screening in mammalian cells in the cell lines, which are not the most easily transfectable, have been done with virus vectors (Arnoldo et al., 2014; Škalamera et al., 2011). Virus vector choice was, however, rejected. Because of the expression problems, analysis of the vimentin phosphomutants was mostly limited to methods, where the whole cell population was not analyzed.

1.1. Vimentin N-terminal serines 7, 8, and 9, and serines 71 and 72 are the key phosphorylation sites affecting vimentin organization

We did an immunofluorescence analysis of vimentin phosphomutants transfected to endogenously vimentin negative MCF7 breast cancer cells. Of all the phosphorylation site mutations observed, the most dramatic effects on vimentin structure were caused by phospho-mimicking and phospho-deficient mutations of vimentin N-terminal serine cluster and phospho-deficient mutations of serines S71 and 72. No clear changes in vimentin phenotype with vimentin S38, S55, S419, S429, and TS457,458 alanine and aspartate and S71,72 aspartate mutations were observed (not shown). However, vimentin S38D, S55D, and S71,72D mutants exhibited a rare (under 1% of transfected cells) phenotype with some aggregates and network disruption (not shown). We observed that vimentin S4,6,7,8,9A and S4,6,7,8,9D mutants completely and S71,72A partially disassembled vimentin network (and later described partial mutations of these sites usually had similar effects), indicating that these are the phosphorylation sites with the strongest effect on vimentin structure. The large majority of other sites did not have observable structural effects (I Fig. 2).

Vimentin S4,6,7,8,9D mutant had only soluble vimentin in about 60% of the transfected cells (quantitated later), while the other transfected cells also had round aggregates from small to massive, and often radially distributed (I Fig. 2, 3C). 3D-projection showed soluble vimentin distribution better (I Fig. 3B).

Vimentin S4,6,7,8,9A produced smaller, brightly stained, round aggregates nearer to the center of the cell. Some cells had thick, sparse, filamentous structures instead (I Fig. 2). With stimulated emission depletion (STED) imaging, which al-

lows immunofluorescence imaging up to 50 nm resolution with the microscope we used, we discovered that some cells had some normal-looking filaments connected to aggregates (I Fig. 3A).

Vimentin S71,72A mutant disassembled vimentin completely into aggregates in some cells, and some had an almost intact filamentous network. Aggregates produced by S71,72A mutant were more indeterminate in shape, varied in size, and usually filled the entire cytoplasm (I Fig. 2, 3A).

We did partial mutations of N-terminal serine cluster and observed that S7,8,9A and D exhibited corresponding phenotypes as S4,6,7,8,9A and D. Vimentin S4A&D, S6A&D and S4,6A&D had normal vimentin networks, but with vimentin S6D and S4,6D part of the transfected cells had aggregates in addition to the filamentous network (I Fig. 2, 3C). We quantitated the amount of aggregate-containing cells in transfected cells and discovered that vimentin S7,8,9D and S4,6,7,8,9D had both aggregates in about 40% of transfected cells, while S6D and S4,6D had aggregates in about 10% (I Fig. 3C). Results indicate that phosphorylation state of vimentin S7, 8 and/or 9 have (at least when taken together) a significant effect on vimentin structure, while S6 alone has a minor effect, and phosphorylation of S4 has no observable effect. The intracellular expression level of a protein is likely to increase aggregation when the protein is expressed over its natural expression level (Tartaglia et al., 2007). Transfected plasmid amount had a significant effect on aggregate formation frequency, suggesting that higher expression level inside the cells produces aggregates more frequently (I Fig. 3 D). This may indicate that in normal expression levels and phosphorylation amounts phosphorylation of vimentin S4,6,7,8,9D is likely to produce fewer aggregates or may not produce aggregates at all and that there is likely a threshold of vimentin concentration, above which aggregates start to form.

We transfected the mutants with phenotypic changes to HeLa cells to determine if the effects were dominant. When we imaged fluorescently labeled mutants in HeLa cells with existing endogenous vimentin, we observed major vimentin network disruptions with vimentin S4,6,7,8,9A and D, and S71,72A mutants, indicating the effects were dominant. Produced phenotypes were similar to mutant phenotypes observed in MCF7 cells. Most of the endogenous vimentin collapsed in cells in which aggregates were observed (I Fig. 3E). Endogenous vimentin collapse suggests that only a fraction of vimentin with certain phosphorylation state in these dominant sites may affect the whole network. Vimentin S6D mutant did not show aggregates and did not have a dominant effect (not shown).

Vimentin mutant	Aggregates in % transfected	Filament disruption	Phenotype dominance	Normal phenotype
S4A	No			X
S4D	No			X
S6A	No			X
S6D	10	No	No	
S4,6A	No			X
S4,6D	10	No	No	
S7,8,9A	100	Complete	Partial	
S7,8,9D	40	Complete	Partial	
S4,6,7,8,9A	100	Complete	Partial	
S4,6,7,8,9D	40	Complete	Partial	
S38A	No			X
S38D	<1	No	No	
S50A	No			X
S50D	No			X
S55A	No			X
S55D	<1	No	No	
S71,72A	30	Partial	Partial	
S71,72D	<1	No	No	
S419A	No			X
S419D	No			X
S429A	No			X
S429D	No			X
TS457,458AA	No			X
TS457,458DD	No			X

Table 5: Observed vimentin phosphomutant phenotypes.

The most striking phosphomutant phenotypes were produced by mutations of vimentin S7, 8 and 9. Vimentin S8 and 9 belong to nonapeptide motif SSYRRIF-GG of vimentin (amino acids 8-16). This motif is critical for tetramer assembly into higher vimentin structures. Desmin has a similar motif (Gohara et al., 2008; Herrmann et al., 1992). Many of the residues in this motif are crucial for filament formation. The end serines can be removed without breaking filaments, but the role of phosphorylation of the serines is still unclear (Beuttenmüller et al., 1994;

Gohara et al., 2008; Herrmann et al., 1992). Desmin S13, which corresponds to vimentin S9 in the conserved nonapeptide sequence, has been observed to accumulate desmin and cause desmin myopathy when mutated to F. Since vimentin S7,8,9D accumulates similarly, vimentin S9 would be a good candidate to be a major effector in our observed vimentin phosphomutant phenotypes (Pica et al., 2008; van Tintelen et al., 2009). Serine 9 has been observed to be partially phosphorylated in interphase and highly phosphorylated by PKC in vitro, and S6 and 8 are also PKC targets (Eriksson et al., 2004, Geisler et al., 1989; Inagaki et al., 1997). Vimentin serine 9 A and D mutants and possibly antibody against phosphorylated vimentin serine 9 (there is already phospho-vimentin S6 available) would provide good tools to study the mechanisms of phosphorylation of this cluster further, if deemed important enough.

In vitro vimentin can assemble without post-translational modifications (Ip et al., 1985). Our results indicate that in vivo phosphorylation (or other modifications) at vimentin S7,8,9 may be needed to stabilize vimentin in cell models. This observation is very likely not caused by the structural difference between serine and alanine, but likely by lack of phosphorylation, since phenotype corresponding to vimentin S7,8,9A had been observed with wild-type vimentin with PKC inhibition and vimentin S4,6,7,8,9A (Ivaska et al., 2005a). We also observed vimentin S4,6,7,8,9A to have a few complete filaments, although most of the vimentin were collapsed, suggesting that vimentin S4,6,7,8,9A mutant is capable of forming filaments. If in vitro assembly of vimentin S4,6,7,8,9A were comparable to WT, this would provide even stronger proof that in cells, for extensive vimentin networks to form, some phosphorylation of vimentin S4,6,7,8,9 is needed. Because S4,6,7,8,9 are not very highly phosphorylated during interphase (Eriksson et al., 2004a; Kochin et al., 2006; Snider and Omary, 2014a), only a fraction of S7,8,9 may have to be phosphorylated in order to stabilize vimentin structure. Earlier zinc ions have been observed to stabilize vimentin structure through vimentin C328 in living cells (Pérez-Sala et al., 2015).

Vimentin residues 40-95 or 25-38 are not needed for filament formation, and the middle section of the vimentin head has been hypothesized as forming a protruding loop (Shoeman et al., 2002). Therefore, changed interaction to a stabilizing protein, and not to the filament structure itself, may explain our observation of filament disruption by S71,72A mutant and is an interesting open question.

1.2. Vimentin phosphorylation does not affect vimentin distribution within endogenous network

We tested if mutant vimentins distribute differently than endogenous vimentin. We conducted the test using untagged human constructs in MEF cells by staining with an antibody that stains only human vimentin, and an antibody that stains both human and mouse vimentins, and then used image analysis to calculate the signal ratios of different antibodies in various parts of the cell. No clear changes in vimentin phenotype with vimentin WT, S38, S55, S419, S429 or TS457,458 alanine or aspartate or with S71,72 aspartate mutations were observed (I Fig. 4A, not

shown). The observed minor changes in mutant versus EG&mutant signal ratios (for example WT pseudocolor image in I Fig. 4A) are reasonably explained by the lack of sensitivity of the method. We discovered that aggregates of vimentin S4, 6, 7, 8, 9D or vimentin S71, 72A contained comparably more human vimentin than vimentin S4, 6, 7, 8, 9A mutant. This may indicate a weaker interaction between mutant and wild-type vimentin. Otherwise, there were no clear differences in the network distribution between endogenous and mutant vimentins in any of the mutants (I Fig. 4A, B, not shown). Quantitation of more cells per sample would bring more confidence to the result, since only a few cells (5 spots/cell) were quantitated. Our results indicate that phosphorylation of individual sites does not create preferences for vimentin localization inside the cells (other than solubility) or affect vimentin distribution inside the existing vimentin network.

1.3. Aberrant vimentin phosphorylation may lead to aggregation

To determine what the observed structures were, we conducted correlative light electron microscopy (CLEM) with assembly-disrupting mutations. We found out that the abnormal structures caused by vimentin S4,6,7,8,9A and D, S71,72A and S6D mutants were aggregates of slightly different morphology (I Fig. 6A, B). We quantitated the textures of the aggregates. We discovered that, for example, the SumAverage texture measure was significantly different in vimentin S4,6,7,8,9D aggregates than in aggregates caused by other mutants. The SumAverage measure is not directly transferable to human perception, but coarseness of the texture, for example, has an effect on the value (Haralick et al., 1973). The result highlights the major effect of the phosphorylated form of vimentin S4,6,7,8,9 has on vimentin structure compared to the unphosphorylated form. When we saw the large vesicular-like structures, our initial thought was that they were vesicles. It appeared likely that vimentin antibody did not penetrate larger aggregates, and only the surface was stained. We found structures colocalizing with various endosomal Rab-GFP markers, but CLEM confirmed that GFP in the endosomal constructs was accumulating inside aggregates (not shown). The result nevertheless indicates that the aggregates do not consist solely of vimentin.

Aberrant phosphorylation of IFs are related to aggregate formation, but which of these is the causative factor is not clear (Dale and Garcia, 2012; Negron and Eckert, 2000; Snider and Omary, 2014a). Heavy vimentin aggregation has been shown to cause cataracts and aggregated vimentin has been observed for example in giant axonal neuropathy patients cells (Bornheim et al., 2008; Bousquet et al., 1996; Müller et al., 2009; Schietke et al., 2006). Our results indicate that aberrant phosphorylation of vimentin at specific sites may be able to cause aggregation, but an open question does these situations arise in live cells.

1.4. Phosphorylation of vimentin N-terminal serine cluster affects vimentin cytoskeletal associations

Since aggregate localizations of described vimentin phosphomutants were different, we conducted immunofluorescence analysis to determine if their associations with other cytoskeletal components – actin and microtubules – were

different. Actin and microtubules, along with IFs, are the main proteins affecting localization of molecules and structures inside cells. Vimentin S4,6,7,8,9D mutant aggregates colocalized more with actin (on the cell edges) and microtubules (around larger aggregates) than with vimentin S4,6,7,8,9A mutant, indicating that phosphorylation of these sites may have effects on vimentin cytoskeletal interactions (I Fig. 5A, B). This observation would have also benefited from quantitation, since only some of the vimentin S4,6,7,8,9D aggregates were clearly covered with microtubules and only some of the vimentin S4,6,7,8,9D in the cell edges was clearly colocalizing with actin.

Vimentin links to myosin 10, kinesin, and dynein, of which, for example, myosin 10 has also been associated with integrin trafficking and migration, similarly as vimentin S4,6,7,8,9 phosphorylation (Barberis et al., 2009; Helfand et al., 2002; Ivaska et al., 2005a; Menko et al., 2014; Prahlad et al., 1998; Zhang et al., 2004). Unstructured vimentin is localized via microtubule networks, and soluble vimentin affects the transport of, for example, phosphorylated ERK and the transport of vesicles via multiple connections (Helfand et al., 2002; Margiotta and Bucci, 2016; Menko et al., 2014; Prahlad et al., 1998). The localization differences and observed effects of vimentin S4,6,7,8,9A and D on microtubule and actin association may be related to solubilized vimentin transport via microtubule and actin networks, and changed associations to cytoskeleton-associated motor proteins. Myosin 10 and 9 interaction of vimentin S4,6,7,8,9D was also suggested by our preliminary mass-spectrometry identification, which however could not be confirmed.

1.5. Vimentin serines 6,7,8,9 controls vimentin solubility with threshold mechanism

Since we observed unstructured, likely soluble, vimentin with vimentin S4,6,7,8,9D and S7,8,9D mutants in immunofluorescence images (I Fig. 2, 3B), we performed solubility assays to prove the increased solubility. We did Triton X-100 extraction and solubility assays to test detergent solubility. We noted that aggregates formed by mutants were not detergent-soluble, but S6D mutant produced more a fragmented network than WT, indicating increased solubility (I Fig. 7A). To obtain more quantitative results, we performed solubility assay with centrifugal fractionation, separating lysates into fractions containing vimentin in different forms: soluble (supernatant from 200000*g centrifugation), fragmented filaments and soluble subunits (supernatant from 15000*g centrifugation), and full filaments (pellet from 15000*g centrifugation). Results indicate that vimentin S4,6,7,8,9D mutant caused a major solubility increase compared to wild-type, and S6D caused a minor solubility increase. Vimentin S4,6,7,8,9A had less fragmented filaments than WT (I Fig. 7B). We conducted crosslinking assay with glutaraldehyde from the vimentin S4,6,7,8,9D soluble fraction. It proved identical to control, which had vimentin solubilized mainly to tetrameric form with calyculin A-treatment from HeLa cells (I Fig. 7C). Observations indicate that vimentin S4,6,7,8,9D soluble fraction is mainly in tetrameric formation.

Formation of higher than soluble tetramer vimentin structures needs vimentin

head domain interaction with helix 2B at the carboxyl end of the rod domain. Phosphorylation of vimentin (by PKA) can disrupt this interaction (Gohara et al., 2001). Vimentin head also interacts with rod domain helix 1A (Aziz et al., 2009). Interacting locations in the head domain are not known, but nonapeptide motif has been hypothesized to interact with one of these sites likely by having anionic rod domain parts binding to cationic arginines in vimentin (Gohara et al., 2008). Phosphorylation of vimentin does not affect head-rod 1A interaction (Aziz et al., 2009). Logically, combined with our observation, it is likely that phosphorylationof vimentin S7,8,9, by introducing repelling anionic molecules, breaks the head domain interaction with rod domain 2B, although direct interaction studies would be needed to confirm this. Also, it is an open question if the disassembly of vimentin by S7,8,9A-mutant could be caused by lack of binding dynamics or too tight binding to 2B-domain. A hypothesised model of vimentin N-terminal serine cluster phosphorylation function is proposed in Fig. 11.

Phosphorylation of vimentin S4,6,7,8,9 increases integrin recycling (via endosome regulation), which is active in directional motility, and transmigration (Barberis et al., 2009; Ivaska et al., 2005a). Serine 6 was more enriched in vimentin pool dissociating from PKCε-containing vesicles, and therefore was speculated to have an effect on vimentin disassembly (Ivaska et al., 2005a). In this previous study conducted by Ivaska et al., vimentin S4,6,7,8,9D was observed to have normal, filamentous, phenotype, vimentin S4,6,7,8,9A had a similar phenotype to our study and no solubility differences with vimentin S4,6,7,8,9A or D were observed (Ivaska et al., 2005a). These observations were from PKCε-reconstituted MEFs (our from MCF7s without endogenous vimentin). The results may indicate that endogenous vimentin negates the solubility difference, and PKCε-overexpression may rescue vimentin S4,6,7,8,9D aggregate-phenotype or, for example, expression levels were lower and cells with aggregate-phenotypes were not shown due to lower amounts of these.

In an earlier article from our lab, we noticed that PKA phosphorylation of vimentin at serines 38 and 72 increases vimentin solubility by transforming vimentin tetramers to higher off-rate in association with filamentous network, and predisposes further vimentin phosphorylation at S4,6,7,8,9. Also, part of vimentin had high phosphorylation at S4,6,7,8,9, and these serines had complex phosphorylation patterns (Eriksson et al., 2004a). Our results show that vimentin S(6,)7,8,9 are likely more critical to solubility than than S38 and 72 based on observed phenotypes. S38 and 72 phosphorylation may allow more S4,6,7,8,9 to be phosphorylated possibly by making easier for nonapeptide motif to detach from the rod domain and allowing kinases to act on it leading to further solubility. Vimentin S7,8,9 have not been identified as mitotic phosphorylation sites (Matsuyama et al., 2013), indicating that S7,8,9 are not critical for the solubilization of vimentin, at least during mitosis.

Serine and threonine phosphorylation sites are often found in clusters and these often interact with each other. Also, one kinase often phosphorylates more than one

clustered phosphorylation sites and the phosphorylation sites often interact with each other (Schweiger and Linial, 2010). Phosphorylation clusters tend to exhibit threshold-like behaviour instead of switch-like. In threshold-like behaviour above certain threshold of phosphorylation effect may switch to completely active or more often increase gradually (Gunawardena, 2005). Based on our results, vimentin N-terminal serine cluster seems to exhibit gradual threshold-like behaviour, since at least two phosphorylation sites regulate solubility (and produce aggregation). On the other hand, phosphorylation of S7,8, and 9 seems to produce switch-like behaviour (although this may be caused by threshold-like behaviour of the three phosphorylation sites) with complete filament breakdown and scattered phenotype and molecules, which spend large majority of time not connected to the network.

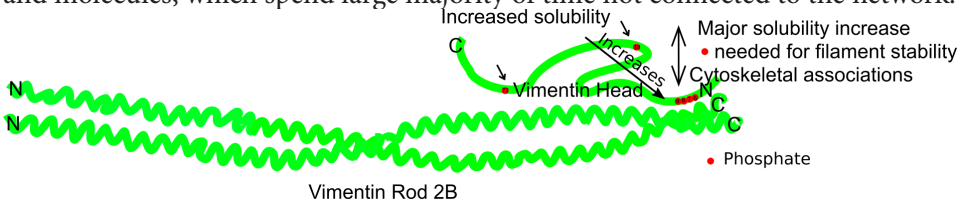


Fig 11. Hypothesized model for vimentin solubilization by phosphorylation, based on previous knowledge and our results. Some phosphorylation of vimentin S(6,)7,8,9 head domain may be needed for filament stabilization, but in higher amounts S(6,)7,8,9 phosphorylation may disturb intermolecular vimentin head-rod 2B domain interaction, heavily limiting vimentin mostly to tetrameric state. Phosphorylation sites further in the head domain have a lesser solubilization effect.

In summary, our results of human vimentin mutant phenotypes suggest that at least in MCF7 and MEF cells: 1) Vimentin S71 and/or S72, vimentin S6, and most critically vimentin S7, S8 and/or S9 are the only phosphorylation sites in which phosphorylation state observably affects vimentin structure; 2) Some phosphorylation of vimentin S7,8 and/or 9, and less critically of S71 and/or 72 may be needed for normal assembly of vimentin in live cells; 3) Phosphorylation of vimentin S6,7,8 and/or 9 may be a major regulator of vimentin solubility with gradual threshold mechanism of multiple sites, and, based on earlier research also, is hypothesized to disrupt vimentin head connection to the rod domain helix 2B; 4) Aberrant phosphorylation of vimentin S6,7,8 and/or 9, and S71 and/or 72 may cause vimentin aggregation; 5) Vimentin S4,6,7,8,9 D-mutant aggregates colocalize more than corresponding A-mutant with actin and microtubules in specific locations, suggesting that phosphorylation of these sites may also affect associations with other cytoskeleton.

The results suggest that phosphorylation of vimentin serines 6,7,8,9 may be a critical regulator of vimentin structure in interphase cells and would be interesting to study further, for example, during major restructuring events in interphase or, possibly, as a vimentin population with separate functions.

2. Sphingolipids regulate vimentin assembly and migratory properties (II)

2.1. Sphingolipids regulate vimentin organization

The initial result for this project occurred when another lab in our department accidentally noticed a change in vimentin solubility with sphingolipid treatment. Sequential fractionation confirmed this, and immunofluorescence images showed a vimentin restructuring into the perinuclear space (II Fig. 2A). FRAP analysis showed a decrease in the vimentin subunit exchange rate with S1P treatment (not shown).

This phenomenon was tested in three cell lines and was consistent in all of them. Only the time frame for vimentin restructuring was different. We continued to use C643 thyroid carcinoma cell line and MDA-MB-435S breast cancer cell line. Typical physiological concentrations of S1P and SPC are 10-100 nM. Concentrations we used were optimized for the lowest concentrations showing the effect. We also observed cell shape changes with both cell lines after SPC and S1P treatment (II Fig. 2A, B, not shown). C643 cell volume and circumference both decreased after S1P and SPC treatment (II Fig. 2B, C).

2.2. S1P and SPC inhibit migration through vimentin serine 71 phosphorylation

We conducted mass spectrometry to identify which sites of vimentin become phosphorylated with S1P and SPC treatment. Results showed that vimentin serines 38, 41, and 71 became phosphorylated (II Fig. 1D, not shown). From these, we tested 38 and 71, since we had phosphorylation-specific antibodies for those sites. We were able to confirm an increase in serine 71 phosphorylation most clearly with an increase by approximately three times with S1P, and 1.5 times with SPC treatment (II Fig. 1E&F). We concentrated on the S71 phosphorylation site.

Sphingolipids and vimentin are both regulators of migration. First, we tested to determine if SPC and S1P affect C643 and MDA-MB-435S migration. We observed 80-90% inhibition in migration with both cell lines with both treatments (II Fig. 1A&B). We also tested to determine whether migration effect of SPC and S1P occurs via vimentin in vimentin wild-type and vimentin knockout MEFs. SPC and S1P both had a non-significant effect on vimentin KO MEF migration, while WT MEF migration was significantly inhibited (II Fig. 6B).

We hypothesized regarding whether the phosphorylation of vimentin serine 71 contributes to migration defect. We used mCherry-tagged vimentin S71A mutant and vimentin WT and empty vector (EV) in the same backbone as controls. Filament formation of mCherry-vimentin was normal with endogenous vimentin background (not shown). We were able to rescue the SPC- and S1P-induced migration defect partially with vimentin S71A in Boyden chamber assay using MDA-MB-435S cells (II Fig. 6D). The result indicates that vimentin serine 71 phosphorylation regulates chemotactic migration. The reason for rescue being only partial may indicate that endogenous wild-type vimentin in the cells caused some migra-

tory inhibition via S71 phosphorylation. Vimentin S71A somewhat increased cell migration without treatment when compared to WT vimentin. S1P and SPC treatment also tend to round cells. Vimentin S71A mutant did not prevent this rounding in MDA-MB-435S cells (II Fig. 6F) indicating that vimentin S71 phosphorylation does not cause cell rounding. Also, WT MEF treatment caused vimentin S71 phosphorylation, but not cell rounding. We also attempted wound healing assay with C643 cells and vimentin S71A mutation. The vimentin S71A mutant did not affect cell motility, and it did not rescue migration defect during S1P or SPC treatments (not shown). In Boyden chamber, there is a serum gradient that induces chemotactic migration. The chemotactic gradient is not present in wound healing assay. The absence of the gradient may explain the difference, and suggests that vimentin phosphorylation affects chemotactic sensing in C643 cells. Adhesion receptor localization on the cell surface is known to be regulated by vimentin (Ivaska et al., 2007b). Our findings indicate that the organization of other receptors on the cell surface, especially those involved in chemotactic signaling, may be affected by vimentin phosphorylation. Single-cell motility analysis provided results that show a substantial decrease in cell movement caused by S1P and SPC (II Fig. 4D&E).

An earlier study indicates that keratin is a crucial component in cell rounding, which is induced by SPC (Beil et al., 2003). Sphingolipids, however, regulate many pathways that regulate actin polymerization and actomyosin contractility. Therefore, we used myosin IIA and IIB inhibitor blebbistatin to prevent actomyosin contractility. Blebbistatin rescued cell rounding effect induced by S1P and SPC (not shown). The rescue indicates that in our cell models, which are devoid of keratins, cell motility defects caused by S1P and SPC are dependent on both actin and vimentin.

2.3. S1P and SPC affect vimentin through the S1P2 receptor

Sphingolipids signal predominantly through G-coupled receptors S1PR1-5. S1PR2 is the most highly expressing receptor of these in MDA-MB-435S and C643 cells (Balthasar et al., 2006). While, for example, S1PR1 and S1PR3 typically induce migration, signaling via S1PR2 has been associated with migration inhibition (Adada et al., 2013b; Lepley et al., 2005). Therefore, we tested if S1PR2 inhibitor JTE013 would rescue sphingolipid-induced effects on migration, vimentin organization, and cell morphology. S1PR2 inhibition rescued cell velocities back to control levels (II Fig. 4D&E). Rac is a downstream signaling molecule of S1PR2 and is a major regulator of cell motility (Okamoto et al., 2000; Steffen et al., 2013; Sugimoto et al., 2003). S1PR2 has been shown to cause inhibition of Rac activation, explaining the effect. S1PR2 inhibition also rescued vimentin morphology changes, cell rounding effect, and vimentin serine 71 phosphorylation (II Fig. 4A&B&C). These effects were also, importantly, rescued with S1PR2 downregulation. We conclude that S1P and SPC signal through S1PR2 to phosphorylate vimentin serine 71 in C643 and MDA-MB-435S cells.

2.4. Vimentin serine 71 phosphorylation and sphingolipid effects on migration are prevented by Rho-associated kinase (ROCK)

ROCK has been shown to phosphorylate vimentin serine 71 in vitro. Also, in COS-7 cells, ROCK activation induces vimentin serine 71 phosphorylation (Goto et al., 1998). RhoA-ROCK-cascade is also known to be activated by sphingolipids, and is a regulator of cell migration (Lepley et al., 2005). We tested if sphingolipid affects vimentin serine 71 phosphorylation, cell morphology, and migration signals via ROCK, using ROCK inhibitor Y27632. Rock inhibitor rescued effects caused by S1P and SPC in all three cases (II Fig. 5A&B&C). ROCK has been associated in several studies as the only kinase that affects vimentin serine 71 phosphorylation. In one study, ROCK and vimentin have been shown to colocalize with vimentin, but when vimentin structure is disrupted, ROCK travels to the cell periphery. In this study, S1P was used as a ROCK activator, and it caused vimentin collapse and ROCK translocation to the cell periphery, although this was not studied in further detail (Sin et al., 1998).

Kinases, which phosphorylate certain amino acids, interact at least transiently with these sites. Therefore, in order to provide more evidence that ROCK would directly phosphorylate vimentin serine 71, we tried to analyze whether ROCK associates or interacts with vimentin. With immunofluorescence colocalization analysis we disovered that both SPC and S1P cause increased colocalization of ROCK with vimentin in C643 cells, particularly at the 30-minute time point. We checked if this was caused by cells shrinking and molecules coming closer to each other. After taking into account that cell volume does not decrease with SPC treatment but decreases with S1P treatment, only one timepoint with SPC treatment seemed to reliably indicate increased colocalization (data not shown). We tried co-IP, dot-blot assay, and proximity ligation assay to confirm the interaction, but with no success. Difficulties caused by vimentin becoming too insoluble after S1P and SPC treatment for immunoprecipitation, and perhaps interaction being too transient may have caused negative results. With a few attempts at proximity ligation assay, we were unable to eliminate the background signal sufficiently (not shown).

We did not examine how vimentin S71 phosphorylation inhibits migration. ROCK inhibits or induces migration context-dependently. The tissue-specific downstream signaling events of ROCK migration inhibition are not known (Wei et al., 2016c). Our results suggest that vimentin S71 phosphorylation is a key player in downstream in ROCK inhibition of chemotactic migration. Vimentin is associated with chemotactic regulation (Eckes et al., 1998). It has been proposed that vimentin maintains the directionality of the migrating cell (Chernoivanenko et al., 2013). Vimentin disassembly can induce membrane ruffling and change of migration direction (Mendez et al., 2010). Our results propose that vimentin S71 phosphorylation may be important for chemotactic migration.

Sphingolipids have varied signaling roles and usually pro-oncogenic effects. These effects signal mostly via five different G-protein-coupled S1P receptors. Among the identified downstream participants of S1P and SPC treatments are vi-

mentin and keratin IFs, both of which dramatically reorganize after sphingolipid treatment. Sphingolipid-induced keratin network reorganization is crucially mediated by keratin phosphorylation (Beil et al., 2003; Busch et al., 2012; Park et al., 2011). C643 and MDA-MB-435S cells do not express keratins and express desmin only weakly (not shown, (Alix-Panabières et al., 2009; Iyer et al., 2013; Sellappan et al., 2004)). Therefore, the observed effects of S1P and SPC through vimentin are not influenced by other IFs. Because both S1P and vimentin affect migration, we tested to determine if sphingolipid migration effect occurs via vimentin. We found that S1P and SPC have an effect on C643, and MDA-MB-435S cancer cell migration takes place in the following order: via an S1P2 receptor, ROCK activation, and finally phosphorylation of vimentin serine 71. Another effect of S1P and SPC was actin-dependent cell rounding, which may have an effect on migration. There are many missing details about the mechanisms by which vimentin affect migration. This study highlights one particular signaling pathway, where extracellular signaling can affect IF organization and cell motility.

3. Vimentin regulates Notch signaling dynamics and development of the embryonic vasculature (III)

Vimentin effect on Notch signaling is not known. Vimentin-deficient mice have a mild phenotype, but vimentin deficiency has been connected to defects in the modulation of vascular tuning and integrity, endothelial sprouting, flow-induced arterial remodeling, wound healing, and fibroblast migration (Dave and Bayless, 2014b; Ivaska et al., 2007a). Similar phenotypes are linked to aberrant Notch signaling. Jagged1 (one of the ligands that can activate Notch) deficiency causes early mice embryos to die from hemorrhages. Notch antisense mice (which have, as a consequence, reduced levels of Notch) have delayed wound healing. Also, Jagged-ligand deregulation leads to a predisposition to vascular injuries and vascular remodeling in patients and mice with pulmonary hypertension (Hansson et al., 2010; Krebs et al., 2000; Limbourg et al., 2005; Lindner et al., 2001; Xue et al., 1999; Yamamura et al., 2014). Vimentin is a known regulator of focal adhesions and affects recycling of integrins. Vimentin is already known to regulate several surface molecules (Kidd et al., 2014; dos Santos et al., 2015; Virtakoivu et al., 2015; Vuoriluoto et al., 2011). Based on these studies, we hypothesized that vimentin also may have some effect on Notch signaling.

3.1. Vimentin affects Jagged1 localization, expression, and signaling

When we blotted for Notch ligands in vimentin positive and negative SW13 adrenal carcinoma cells, we found that vimentin negative SW13s had manyfold expression of Jagged1 protein compared to vimentin positive cells (not shown). In vimentin-depleted HUVEC cells, Jagged1, and Notch1 intracellular domain (N1ICD), and less significantly Dll4, expression was elevated (III Fig. 2A). We discovered that Jagged1 had accumulated on the surface of vimentin negative cells. Expression levels of Jagged1 were about equal in vimentin negative and positive satellite cells, and in MEFs, but in both vimentin negative SW13 and MEF cells we

observed Jagged1 surface accumulation (III Fig. 6A). Jagged1 mRNA levels were reduced in vimentin-deficient MEF cells (not shown).

We used Jagged1 fingerprint assay to test if Jagged1 was still binding to Notch receptors. In this assay, coverslips were coated with Notch1 extracellular domain (ECD) or control, cells were grown on the coated coverslips, and crosslinking agent was used to link Notch1 ECD to ligands. Cells were extracted with SDS detergent, and the Jagged1 that was left on the coverslips was stained. Results showed that the Jagged1 levels bound to Notch1EC (III Fig. 6B&C) were somewhat similar to the surface levels of Jagged1 in vimentin negative versus positive MEFs (III Fig. 6A). The ratio indicated that Jagged1 binding to Notch1EC was not significantly affected by the absence of vimentin.

3.2. Vimentin decreases Notch signaling activity

Next, we tested if vimentin-deficient cells have a distinct Notch signal-sending potential to adjacent cells. 293 HEK-cells with luciferase reporter system for quantitation of Notch signaling were co-cultured with vimentin positive or negative SW13 cells. Both short- and long-term Notch signaling potential was higher in vimentin negative cells. However, when this higher signaling potential is compared to higher Jagged1-levels on the cell surface in vimentin negative cells, it is clear that only a fraction of Jagged1 is in active signaling state (III Fig. 7B). Jagged-mediated pulling force/Jagged1 surface expression ratio also indicated that Jagged1 signaling is more efficient in vimentin-containing MEF cells (III Fig. 7C).

Next, we measured Notch1EC internalization. Extracellular Notch is usually internalized in the signal-sending cell. Soluble Notch1EC was conjugated to a fluorophore and Notch1EC uptake was measured by FACS. In vimentin negative cells, we observed increased internalization of Notch1EC compared to wild-type cells (III Fig. 4 A). The comparably mild increase of internalization suggests that Notch1EC ligand binding and internalization are not radically affected, despite the fact that Jagged1 levels on the surface are radically higher. Internalized Notch1EC-vesicles had a different distribution in vimentin negative cells. When Notch1EC was attached to beads in order to mimic mechanical strain caused by Notch1EC binding described as pulling force, no differences in Notch1EC internalization were detected, despite higher surface levels of Jagged1 in vimentin negative cells (not shown).

We analyzed the movement of Notch1ECD inside cells. We fed fluorophore-labeled Notch1ECD into vimentin WT and KO MEFs and imaged internalized Notch1ECD in live cells with a confocal microscope. The Notch1ECD-containing vesicle tracks were analyzed. We found that the linearity of the vesicle tracks was lower in vimentin KO cells, but the vesicle speeds were unchanged (III Fig. 5 E&F).

We tested recycling amount of Jagged1 with surface biotin labeling and stripping. The small and similar amount of Jagged1 recycled in WT and vimentin KO MEFs, but Jagged1 recycling was faster in KO cells, indicating that vimentin may balance Jagged1 levels on the cell membrane. We did immunofluorescence colocalization analysis of Jagged1 with Rab4 and Rab11 recycling endosome GFP con-

structs and found that Jagged1 colocalizes less with both markers in vimentin KO cells (not shown). Lower colocalization indicates that Jagged1 may spend less time in recycling endosomes in KO cells and therefore recycles faster.

3.3. Vimentin phosphorylation potentiates Jagged-mediated Notch signaling activation

Phosphorylation of vimentin by PKC has been known to regulate intracellular trafficking. We observed that Jagged1 levels were decreased during PKC inhibition by BIM-I and increased during PKC activation by PMA in Jagged1 overexpressing 3T3 mouse fibroblast cells (III Fig. 8A). This experiment could also be conducted with WT and vimentin KO cells to obtain stronger evidence of the vimentin-specificity of the effect. Pretreatment of 3T3J cells with PMA also increased Notch signaling potential when 3T3J cells were co-cultured with 293FLN1 cells containing Notch activation luciferase reporter (III Fig. 8B). We used phospho-mimicking vimentin S4,6,7,8,9A and D mutants to determine the effects of these phosphorylation sites on Notch signaling. We discovered that endocytosis of Notch1EC is enhanced in cells that contain the phospho-mimetic mutant, and both mutants slightly enhanced Notch activation in adjacent cells (III Fig. 8C&D).

Fringe is a significant regulator of Notch signaling. Fringe increases Dll-mediated signaling and decreases Jagged signaling to adjacent cells. We found that vimentin deficiency increased Fringe expression (not shown). The expression change indicates that vimentin-deficient cells favor Delta-like-mediated signaling instead of Jagged-mediated, possibly because of the effects vimentin has on Fringe expression.

Since Notch is a known angiogenic regulator and because vimentin has been associated with endothelial sprouting, we tested how vimentin and Notch inhibition interact in the chorion allantois membrane (CAM) model (not shown). Vimentin inhibitor withaferin A (WFA) caused inhibition of new branching, but gamma-secretase inhibitor DAPT (which inhibits Notch signaling) caused new branches to form. Next, we tested the effects of Notch ligands and vimentin inhibition in HUVEC sprouting assay (not shown). Bead-immobilized Notch ligands can activate Notch signaling. WFA inhibited tube sprouting substantially, but adding immobilized Jagged1- or Dll1-ligand rescued this effect (III Fig. 3B, not shown). This provides more evidence that vimentin mainly affects Notch signaling through Jagged1- or Dll1-ligands.

3.4. Deletion of vimentin delays angiogenesis in mice

Next, we tested if vimentin negative mice embryos and yolk-sac embody changes in their vasculature. We used whole-mount immunostaining for PECAM-1. We found that vimentin negative embryos and surrounding yolk sacs at E11.5 have disturbed vascular patterning. Embryos had smaller blood vessels and less branching (III Fig 1A&B). This regression of vascular formation is similar to what has been reported in mice expressing non-functional Jagged1 ligands.

To further test the effect of vimentin on angiogenesis in mouse tissues, we did aortic rings assay with vim-/- and WT mice aortic rings embedded in a growth

factor-containing collagen matrix, which was optimized to induce sprouting. Endothelial sprouting response of aortic rings to VEGF was significantly weaker in vim-/- aortic rings than in WT (III Fig 1C). The number and length of sprouts were reduced, and sprouts were negative to endothelial cell markers PECAM-1 and VE-cadherin (III Fig. 1c and not shown).

Vimentin is known to affect endothelial sprout initiation via MT1-MMP (Kwak et al., 2012), and also regulates growth factor-dependent endothelial cell invasion via FAK (known to be important in angiogenesis) activity by forming complexes with RACK1 and FAK (Dave et al., 2013; Tavora et al., 2010). Our results show that another way that vimentin affects angiogenesis is by balancing Notch signaling in sprouting angiogenesis.

Notch is known to interact with LAMP-1, and Notch receptor cleavage is facilitated on late endosomal/lysosomal membranes by their acidity (Sjöqvist et al., 2014). Vimentin interacts with AP-3, a protein that regulates sorting of LAMP-1 and -2 positive vesicles, and vimentin-influenced endosome/lysosome acidity (Potokar et al., 2010; Styers et al., 2004). Therefore, vimentin depletion may enhance Notch processing in endo-lysosomal compartments. Protein trafficking is an important regulator of Notch signaling. Vimentin has multiple contacts to endocytic machinery, and the vimentin network may function as a passageway or barrier for vesicles. Our results show that Notch ligand endocytosis, recycling, routing, and movement is altered in vimentin-deficient cells. In summary, vimentin may function in Notch quality control in vesicles and/or the vimentin network may affect vesicle movements by steric hindrance. Jagged-mediated transactivation potential was impaired in vimentin-deficient cell. This may be caused by changes in actin polymerization-requiring pulling force. Pulling force may be affected by vimentin, since vimentin has been shown to be involved in cytoskeletal tension and integrin-mediated mechanotransduction (Gregor et al., 2014). Vimentin is known to interact with membrane proteins through PDZ-domains (Phua et al., 2009) and Jagged1, Dll1, and Dll4 have possibly signaling-affecting PDZ-domains (Ascano et al., 2003; Kolev et al., 2005). However, we have not been able to confirm interaction of vimentin with Notch ligands.

In summary, our results show that vimentin suppresses Notch signaling in endothelial cells and enhances angiogenesis. Vimentin suppresses Dll4/Notch signaling and enhances Jagged1/Notch transactivation potential. Jagged is an important regulator in angiogenesis, cancer, and regenerative processes in the brain. Our results show an important role for vimentin in Jagged modulation in angiogenesis and vascular integrity, highlighting Jagged as a potential therapeutic target in regenerative and cancer settings.

4. Casein kinase 2 (CK2) regulation of prostate apoptosis response-4 (Par-4) pro-apoptotic functions (IV)

4.1. Par-4 is a substrate for CK2

Par-4 and CK2 regulate the same cellular function, apoptosis, but have op-

posing effects on it. For both proteins, these effects are particularly prominent in prostate cancer. With the phosphorylation motif search program (http://scansite3.mit.edu/) we discovered that Par-4 contains potential CK2 phosphorylation sites, which are evolutionarily conserved, and we hypothesized that CK2 may phosphorylate Par-4 in living cells.

Therefore, we studied whether Par-4 is a target for CK2. We did immunoprecipitation by co-transfecting GFP-tagged rat Par-4 and hemagglutinin (HA)-tagged CK2 subunits (CK2α and/or CK2β of human origin) to COS cells. Immunoprecipitation was done both ways by pulling GFP or HA tag from the solution. We found that Par-4 interacted specifically with CK2α subunit but not with CK2β (IV Fig. 1A&B). Similar interaction often occurs with CK2 substrates (Allende and Allende, 1995). With purified proteins, we determined that the interaction was direct (not shown).

Next, we determined whether CK2 could phosphorylate Par-4 in vitro. We did in vitro kinase assay with immunoprecipitated GFP-tag CK2α and CK2β subunits. or recombinant CK2 with human GFP-Par-4. We found that Par-4 is indeed a CK2 kinase target (IV Fig. 1C). Catalytically inactive CK2β subunit was also (like with the majority of CK2 targets) required for Par-4 phosphorylation. Protein phosphatase 1 (PP1) is known to dephosphorylate some CK2 substrates (Popescu et al., 2009). We tested whether PP1 can dephosphorylate CK2-phosphorylated Par-4 in vitro by using recombinant PP1 and phosphatase inhibitors, and found this was the case (not shown).

We wanted to identify the serine/threonine residues in Par-4, which CK2 can phosphorylate. To do this, we produced a tryptic phosphopeptide map of rat Par-4-GST, which was in vitro-phosphorylated beforehand by CK2. Three major CK2-specific 32P-labeled spots were identified from the analysis of a thin layer chromatography plate, wherein tryptic peptides were separated into two dimensions (IV Fig. 2A). Matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) analysis of these spots, and later manual Edman degradation were performed to identify phosphorylated peptides. The results revealed that serines 124 and 223 are the target sites of CK2 phosphorylation in Par-4 (IV Table 1).

To determine whether Par-4 serines 124 and 223 have any functional relevance, we constructed phospho-deficient and phospho-mimicking GFP-tagged mutants of the following sites: GFP-Par-4 S124,223A, GFP-Par-4 S124,223D, and the corresponding single mutants of these sites. In vitro CK2 phosphorylation assay with GFP-Par-4 S124,223A revealed that the mutant Par-4 was not phosphorylated, indicating that these sites were indeed CK2 targets. S124D and S223D single mutants both had decreased phosphorylation compared to Par-4 wild-type, indicating that these sites are both targeted by CK2 (IV Fig. 2B).

4.2. CK2 phosphorylation of Par-4 inhibits its pro-apoptotic functions

Par-4 and CK2 are both regulators of anti-apoptotic NF-κB and pro-apoptotic

caspases, albeit in opposite ways (Chakraborty et al., 2001; Desagher et al., 2001; Nalca et al., 1999; Wang et al., 2006a). Therefore, we studied if Par-4 phosphomutants had altered effects on these processes. We studied how Par-4 pro-apoptotic functions changed with these mutations in PC-3 cells. With luciferase reporter assay, which indicated NF-κB transcriptional activity, we found that Par-4 wild-type and S124,223A had an inhibitory effect on NF-κB transcriptional activity, while S124,223D had no effect (not shown).

Next, we studied how Par-4 S124,223D mutant performs when apoptosis is induced in PC-3 prostate cancer cells with death ligand TRAIL or with chemotherapeutic drug Paclitaxel. We found that the cells transfected with Par-4 S124,223D mutant were resistant to apoptosis while wild-type or S124,223A mutant sensitized the cells to apoptosis (IV Fig. 3A). Par-4 S124,223D mutant caused absence of 50-kDa cleaved form of Par-4 (IV Fig. 3C). This cleaved form exists in human cells during apoptosis induced by cisplatin or Fas (Chaudhry et al., 2012; Leroy et al., 2005). The results indicate that phosphorylation of CK2 phosphorylation sites on Par-4 protects from apoptosis. This suggests that CK2 may inhibit the pro-apoptotic function of Par-4.

4.3. CK2 rat Par-4 S124 phosphorylation prevents Par-4 caspase cleavage and protects from apoptosis

In the pro-apoptotic functions of Par-4, Par-4 cleavage by caspases is a critical step. In humans, caspases cleave Par-4 to a fragment containing amino acids 132-340, and in rats to Par-4 124-332. Cleaved forms have full pro-apoptotic functions. Included SAC (selective for apoptosis in cancer cells, amino acids 137-195) core domain is crucial for the pro-apoptotic functions. Par-4 S124 is close to D123, an ortholog of human Par-4 D131, which is a caspase cleavage site (Chaudhry et al., 2012). Therefore, we tested whether rodent Par-4 S124 phosphorylation protects from caspase cleavage. We first confirmed that rodent Par-4 was also cleaved by caspases (IV Fig. 4A). Then, we used zVAD-FMK, a pan-inhibitor of caspases, to confirm that caspases caused the cleavage: there was no cleavage product with caspase inhibition (not shown). We then confirmed that Par-4 D123A (a potential rat caspase site) was not cleaved by caspases, and that this mutant also impaired Par-4 pro-apoptotic function like human equivalent Par-4 D131A had earlier been reported as doing (not shown).

Then, we tested whether CK2-mediated S124 phosphorylation regulates rat Par-4 cleavage by in vitro caspase assay using recombinant caspases and Par-4 wild-type and mutants. The assay showed that Par-4 wild-type, S124A, and S124,223A were cleaved by caspase-3 and caspase-8, but Par-4 S124D and S124,223D mutants were not. The results indicate that caspase cleavage is inhibited by S124 phosphorylation (IV Fig. 4A&B). We confirmed the result using in vitro CK2-phosphorylated recombinant rat Par-4-GFP in in vitro caspase 3-assay and observed that CK2 phosphorylated Par-4 was not cleaved (Fig. 4C). This effect was limited to rodent Par-4, because when we repeated the same assay with human Par-4, the Par-4 was cleaved.

We observed that rat Par-4 S124D and D123A mutants both had the same effects on Par-4 pro-apoptotic function, indicating that in rat phosphorylation of Par-4 S124 inhibits Par-4 pro-apoptotic function by preventing caspase-mediated Par-4 cleavage (not shown). CK2 phosphorylation of Par-4 D131 does not affect Par-4 proteolytic cleavage in humans. Phosphorylation of Par-4 by CK2, therefore, seems to have different effects in different species. CK2 phosphorylation has also been shown to inhibit proteolytic cleavage of caspase-9 in rodent cells (McDonnell et al., 2008). Max, Bid, or caspase recruitment domain-containing apoptosis repressors are also targets of CK2 phosphorylation in both species. In these cases, CK2 phosphorylation also inhibits caspase cleavage, and with Bid prevents the creation of a pro-apoptotic cleaved product (Desagher et al., 2001; Krippner-Heidenreich et al., 2001). Therefore, the role of CK2 in inhibition of its substrates across species has different mechanisms, and these mechanisms are dependent on the species.

Par-4 caspase-cleaved form is known to have pro-apoptotic abilities (Chaudhry et al., 2012). Therefore, we tested if the caspase-cleaved fragment retained Par-4 apoptotic properties. We observed that rat Par-4 (124-332) cleaved form caused cells to be even more sensitive to apoptosis induced by TRAIL than the full-length wild-type Par-4 (IV Fig. 4D).

Both normal and cancer cells can secrete Par-4 in cell cultures. Like intracellular Par-4, secreted Par-4 can also induce apoptosis in the surrounding cells by activating caspases (Burikhanov et al., 2009). For this reason, we tested whether the cleaved form of Par-4 could be secreted. We transfected COS cells with GFP, GFP-Par-4 wild-type, or Par-4 (124-332), and analyzed the cell media with western blotting. We detected both wild-type Par-4 and Par-4 (124-332), but not GFP alone in the culture media (not shown). To study the function of the secreted forms, we added the concentrated media containing secreted Par-4 wild-type or Par-4 (124-332) onto PC-3 cells in a culture medium for a 24 hour duration. We quantitated apoptosis and noticed that Par-4 (124-332) had a stronger apoptosis-inducing effect than wild-type Par-4 (not shown). According to our results, pro-apoptotic functions of rodent Par-4 are inhibited by CK2 through phosphorylation of Par-4 S124. The mechanism for this effect is the prevention of the caspase cleavage. The apoptotic process may further be amplified by secreted caspase-cleaved Par-4, which produces a "bystander" effect that increases apoptosis of the nearby cells.

4.4. Human Par-4 S231 phosphorylation by CK2 protects prostate cancer cells from apoptosis

Sequence comparison between human and rodent Par-4 showed that in humans, S231 corresponds to the S223 phosphorylation site in rodents, but S124 did not have an ortholog. The S231/S223 site is conserved across species. In leukemia cell lines this site was detected in earlier mass spectrometry studies, suggesting that this site may have functional significance (www.phosphosite.com).

We performed in vitro kinase assay of CK2 with human Par-4 wild-type and Par-4 S231D. Par-4 S231D had less phosphorylation than wild-type, indicating

Par-4 S231 is a target site of CK2 (IV Fig. 5B).

We produced an antibody that recognizes the phosphorylated form of human Par-4 serine 231 to help characterize the function of the phosphorylation site. In western blot, prostate cancer PC-3 cell lysate displayed a band corresponding to phosphorylated Par-4 (IV Fig. 6A). When a blocking peptide was added, this band was not detected, indicating the specificity of the antibody. When PC-3 cells were treated with TRAIL, we detected an increase in the Par-4 S231 phosphorylation (IV Fig. 5C). We tested the effect of Par-4 S231 phosphorylation on apoptosis by transfecting HCT116 cells (which allow higher transfection efficiency than PC-3s) with Par-4 S231A or S231D, and used TRAIL to induce apoptosis. We observed that with the S231A mutant, the cells were sensitized to TRAIL, but with S231D mutant the cells were less sensitive to TRAIL. Apoptosis induction was detected with caspase-8, PARP-cleavage, and DAPI staining (IV Fig. 5D-F). The results indicate that phosphorylation of Par-4 S231 by CK2 strongly (even more so than rat Par-4 S223 phosphorylation) reduces Par-4 apoptosis-inducing properties.

Increased CK2 activity has been observed in prostate cancer cells (Yenice et al., 1994). Therefore, we tried to find a correlation between Par-4 phosphorylation and CK2 activity and expression in normal PrEC and PNT2C2 cells, and in PC-3 and LNCaP prostate cancer cells. We learned that the cancer cells had a higher Par-4 phosphorylation at CK2 site Par-4 S231, and that cancer cells also had a higher CK2 activity (IV Fig. 6A). We used CK2 siRNA to study if CK2 depletion reduces Par-4 S231 phosphorylation, and found that this was the case (Fig. 6B). Both the CK2 expression levels and the Par-4 S231 phosphorylation were about 50% reduced, suggesting a coordinated regulation. When CK2 inhibitor 4,5,6-tetrabromobenzotriazole was used instead of siRNA, the results were similar (Ruzzene et al., 2002; Sarno et al., 2001). We did not describe the mechanism by which CK2 phosphorylation of Par-4 S231 regulates apoptosis in this article. S231 is in the C-terminal domain of Par-4. Par-4 C-terminal domain is known to regulate interactions between Par-4 and its partners, e.g. NF-κB and PKCζ. We hypothesize that phosphorylation of Par-4 S231 may affect these interactions.

Cells without CK2 are more prone to apoptosis (Di Maira et al., 2007; Izeradjene et al., 2004; Slaton et al., 2004; Wang et al., 2005). We tested whether the PC-3 cells become more apoptotic during TRAIL treatment when CK2 siRNAs are transfected to them. When both Par-4 and CK2 were downregulated, the PC-3 cells again became more resistant to TRAIL-induced apoptosis (IV Fig. 7A-D). The results show that the phosphorylation of Par-4 and the inhibition of its pro-apoptotic properties reside downstream from CK2 antiapoptotic signaling.

Our results indicate that in human prostate cancer cells Par-4 S231 has considerably more phosphorylation than in normal prostate cells, suggesting that CK2 phosphorylated Par-4 is potentially a biomarker for prostate cancer. The results also show that in prostate cancer cells CK2 has an antiapoptotic role via direct phosphorylation of Par-4, providing new significance for Par-4 as a key target for cancer therapeutics in prostate cancer.

5. Nucleolar AATF regulates c-Jun-mediated apoptosis

5.1. AATF upregulates UV-mediated cell death

AATF has been shown to affect cell growth and proliferation by interacting directly with the survival machinery components (Bruno et al., 2006; Fanciulli et al., 2000). We investigated whether AATF produces a response when the cells are under stress. We tested whether AATF overexpression affects MEFs and HEK293 (human embryonic kidney-derived cell line) cell response to UV irradiation. We found that AATF overexpression produces more detached and dead cells in response to UV irradiation (IV Fig. 1A). To make these findings more convincing, we quantitated active caspase-3 in AATF-GFP or GFP-expressing cells with flow cytometry. AATF-GFP-expressing cells contained more active caspase-3, indicating that more apoptotic cells were present (V Fig. 1B). We also measured apoptosis with annexin-V and propidium iodide (PI) staining, which stain cells in the early and later stages of apoptosis. In AATF overexpressing cells, with PI staining we observed an increased amount of cells with lower than G1-phase DNA content (IV Fig. 1C). The western blotting analysis of active caspase-3 and cleaved PARP-1 (caspase-3 substrate) showed the same results, indicating that AATF promotes UV-induced apoptosis (V Fig. 1D, not shown).

5.2. AATF activates c-Jun

Apoptosis can be induced through different signaling pathways. c-Jun has been identified as a critical mediator of UV-induced apoptosis (Kasibhatla et al., 1998; Kolbus et al., 2000; Shaulian et al., 2000). Therefore, we studied whether AATF activates c-Jun. GFP/GFP-AATF-expressing cells were predisposed to UV irradiation, harvested, and active c-Jun (indicated by phosphorylation at serine 73 (Pulverer et al., 1991)) was measured. The results showed that UV irradiation increased c-Jun activity, and AATF overexpression increased this even further, indicating that AATF regulates c-Jun activity (IV Fig. 2A). We also tested other separate AP-1 transcription family member ATF-2 activity, which also mediates UV-induced apoptosis. We found that AATF did not further increase ATF-2 activity, suggesting that the effect was a c-Jun-specific (not shown). AATF-specific siRNA also downregulated c-Jun activity (V Fig. 2D).

The length of the c-Jun activation also matters. More transient activation of c-Jun often results in cell cycle arrest and suppression of apoptosis under UV stress, while more persistent c-Jun activation more likely results to apoptosis (Devary et al., 1991; Shaulian and Karin, 1999, 2001; Shaulian et al., 2000). We tested whether AATF overexpression could activate c-Jun for a longer period by following c-Jun activation after UV exposure. The results showed that AATF overexpression can maintain high c-Jun activity at least for two days (V Fig. 2C). AATF downregulation, on the other hand, greatly reduced c-Jun activation (V Fig. 2D). Our results show that c-Jun AATF activity is increased (indicated by serine 73 phosphorylation) after UV stress. This increase is long-term, and likely causes AATF to participate in the c-Jun-regulated cellular processes, e.g. apoptosis.

c-Jun can dimerize and bind DNA. Therefore, we examined whether AATF-me-

diated activation of c-Jun is dependent on these properties by using constructs with c-Jun DNA-binding domain replaced by yeast Gal4-transcription factor. Gal4-transcription factor activates luciferase reporter expression. These constructs are functional and independent of c-Jun dimerization or DNA-binding (Weiss et al., 2003). We observed that AATF could activate transfected Gal4-c-Jun (control Gal4 DNA-binding domain) (V Fig. 2E). Therefore, AATF affect c-Jun activation functions by stimulating c-Jun transactivation capacity.

To eliminate the possibility that AATF effects c-Jun via p53, we tested AATF-mediated c-Jun activation in p53 negative PC3 and K562 cell lines. We found that c-Jun activation by AATF was normal, and therefore does not require p53 and is not specific to cell type (not shown).

We tested whether AATF can activate c-Jun under other stresses than UV. We used TNFα and serum withdrawal to induce c-Jun activation. In both cases, AATF overexpression strongly increased c-Jun activation, suggesting there is a much wider relevance for this signaling pathway (not shown).

We tested whether c-Jun-activation is noticeable in the c-Jun target gene expression with semi-quantitative RT-PCR. c-Jun target genes TNFα and FasL mRNA levels were both strongly upregulated in AATF-GFP-transfected UV-treated cells and downregulated in AATF siRNA-transfected UV-treated cells, indicating a consequently changed c-Jun transcriptional activity (V Fig. 2F&G).

To determine whether c-Jun target genes were induced c-Jun-dependently, we used wild-type and c-Jun KO MEFs. AATF-GFP overexpression without stress stimuli was enough to produce increased TNFα expression, but this was not seen in c-Jun KO MEFs. When c-Jun was reintroduced with AATF into c-Jun KO MEFs, TNFα expression was induced, but AATF or c-Jun transfection alone did not induce TNFα, indicating further confirmation that AATF induces TNFα via c-Jun (V Fig. 2H&I).

5.3. AATF induces apoptosis in a c-Jun-dependent manner

Next, we tested whether apoptosis induced by AATF functioned c-Jun-dependently. We overexpressed AATF in WT and c-Jun KO MEFs with UV irradiation. Only in WT MEFs did AATF produce a remarkable increase in apoptosis, indicating that AATF-mediated apoptosis functions in a c-Jun-dependent manner (V Fig. 3A&B).

Since AATF regulated c-Jun after UV treatment, we questioned whether AATF interacts with c-Jun after UV treatment. In HEK293 cells, we performed immunoprecipitation in both ways. We found that there was a low-stoichiometry interaction between AATF and c-Jun without UV treatment, and this dramatically increased after UV treatment (V Fig. 4A&B).

Previously, it has been observed that UV-mediated c-Jun activation can induce transcription of pro-apoptotic TNFα and FasL, among others (Dhanasekaran and Reddy, 2008; Herr et al., 2000; Kasibhatla et al., 1998; Le-Niculescu et al., 1999). Our results show that AATF directly activates the transcriptional activity of c-Jun, and that AATF is the first-identified regulator of c-Jun necessary for c-Jun-mediat-

ed apoptosis. Our results demonstrate that the amount of AATF in the cytoplasm positively correlates with c-Jun-mediated apoptosis induction.

c-Jun is important when cells try to adapt to environmental changes (Jochum et al., 2001; Wisdom, 1999). c-Jun also facilitates oncogenic transformation (Behrens et al., 2000). Different responses from c-Jun activity to different signals are often based on various protein complexes formed around c-Jun. The different complexes allow highly flexible responses for c-Jun target gene regulation. AATF interaction with c-Jun after UV stress seems to precede c-Jun phosphorylation. This may indicate that AATF tags the amount of c-Jun that can be activated for apoptosis induction and, therefore, functions as a permissive or limiting factor.

5.4. UV irradiation promotes AATF translocation from the nucleus to the nucleolus

One of the regulatory mechanisms of signaling pathways and forming signaling complexes includes subcellular localization of signaling molecules. The nucleolus acts as a safe to store molecules, which can be released to different compartments (e.g. to the nucleoplasm) to initiate different cellular functions. The nucleolus also works as an organelle, which often responds to stress (Mayer and Grummt, 2005; Pederson and Tsai, 2009; Stark and Taliansky, 2009). AATF mostly localizes to the cell nuclei, but has also been found in the cytoplasm and the nucleolus (Andersen et al., 2002, 2005; Fanciulli et al., 2000; Thomas et al., 2000). Therefore, we used immunofluorescence to image AATF localization before and after UV treatment. Our observation was that AATF localized to the nucleolus in the non-treated cells, but after the UV treatment completely moved to the nucleoplasm over time (V Fig. 4D). We also stained c-Jun to see whether it is the same compartment as AATF after UV treatment. We found that c-Jun remained in the nucleoplasm (but not in the nucleolus) both before and after UV treatment, and could be in contact with AATF in the nucleoplasm after UV treatment (V Fig. 4E). After UV treatment, AATF and c-Jun could also be seen in nuclear dot-like structures (V Fig. 4E). Our results suggest that AATF promotes UV-induced apoptosis by direct interaction with c-Jun and by promotion of c-Jun's transcriptional activity.

Protein sequestration is a common regulatory mechanism controlling protein signal-specific activity. Relocalization of several proteins to the subnuclear structures have been observed during stress (Boulon et al., 2010; Carmo-Fonseca et al., 2000; Pederson and Tsai, 2009; Stark and Taliansky, 2009). This translocation also occurs specifically during stress from the nucleolus to nucleoplasm (Mayer et al., 2005; Szymański et al., 2009; Westermarck et al., 2002; Wu et al., 2002; Yang et al., 2008). Nucleolar sequestration and its release under UV stress seem to be the main mechanism to determine the amount of AATF available to target c-Jun.

5.5. Identification of AATF domains regulating AATF localization, c-Jun activity, and apoptosis

To discover a more detailed mechanism of this phenomenon we created AATF deletion mutants 1-198, 1-494, 199-526 and Δ125-246. By performing co-immunoprecipitation analysis, we discovered that the c-Jun binding region of AATF is

within amino acids 199-454 (V Fig. 5A). Next, we examined which parts of AATF are necessary to activate c-Jun. We used luciferase-based AP-1 reporter assay in HEK 293 cells. For stimulation of c-Jun transcriptional activity, C-terminal amino acids 456-526 of AATF were necessary. We also found a potential inhibitory domain within amino acids 125-246 (V Fig. 5B). When we examined with confocal microscopy localization of these mutants within cells, we found that C-terminal fragment 456-526 was needed for nucleolar localization. AATF 1-198 fragment localized to the cytoplasm, but the 1-454 fragment localized to the nucleus. Still, according to our results, 1-454 fragment did not stimulate c-Jun (V Fig. 5D).

We tested what effects deletion mutants had on apoptosis induction in WT and c-Jun KO MEFs with AATF deletion mutants using PI/annexin-V staining. 199-526 and Δ125-246 mutants had the strongest positive effect on apoptosis induction. This happened only in the WT MEFs, not in the c-Jun KO MEFs. 1-198 and 1-454 mutants did not further stimulate apoptosis (V Fig. 5C). These results indicate that AATF enhances apoptosis induced by UV through its interaction between AATF's C-terminal tail and c-Jun. Relocalization of AATF to nucleoplasm allows this interaction.

Our results strongly indicate that c-Jun promotes apoptosis when nucleolar cofactors associate with c-Jun after stress stimulus and form a c-Jun-containing functional signaling complex. Since AATF also binds another master regulator besides c-Jun, p53, we can hypothesize that AATF, p53, and c-Jun could form a complex together under stress stimuli, and modulate promoter binding affinities of each other.

CONCLUDING REMARKS

Phosphorylation is so far the most studied post-translational modification and essential for cell signaling circuitry.

This thesis describes new phosphorylation-mediated mechanisms in cell migration, angiogenesis, and apoptosis contexts. Intermediate filament vimentin is involved in three of the articles having, at least, a partial phosphorylation theme. Also, two other articles describe two new apoptosis signaling mechanisms in which phosphorylation plays a key role or is involved. The main molecules studied in this thesis affect many cellular processes. They each have connections to cancer, and they are currently, at least in theory, potential targets for cancer treatments. The articles also examine more details of these molecules' effects on processes relevant for cancer: cell differentiation in angiogenesis, cell migration, and cell death.

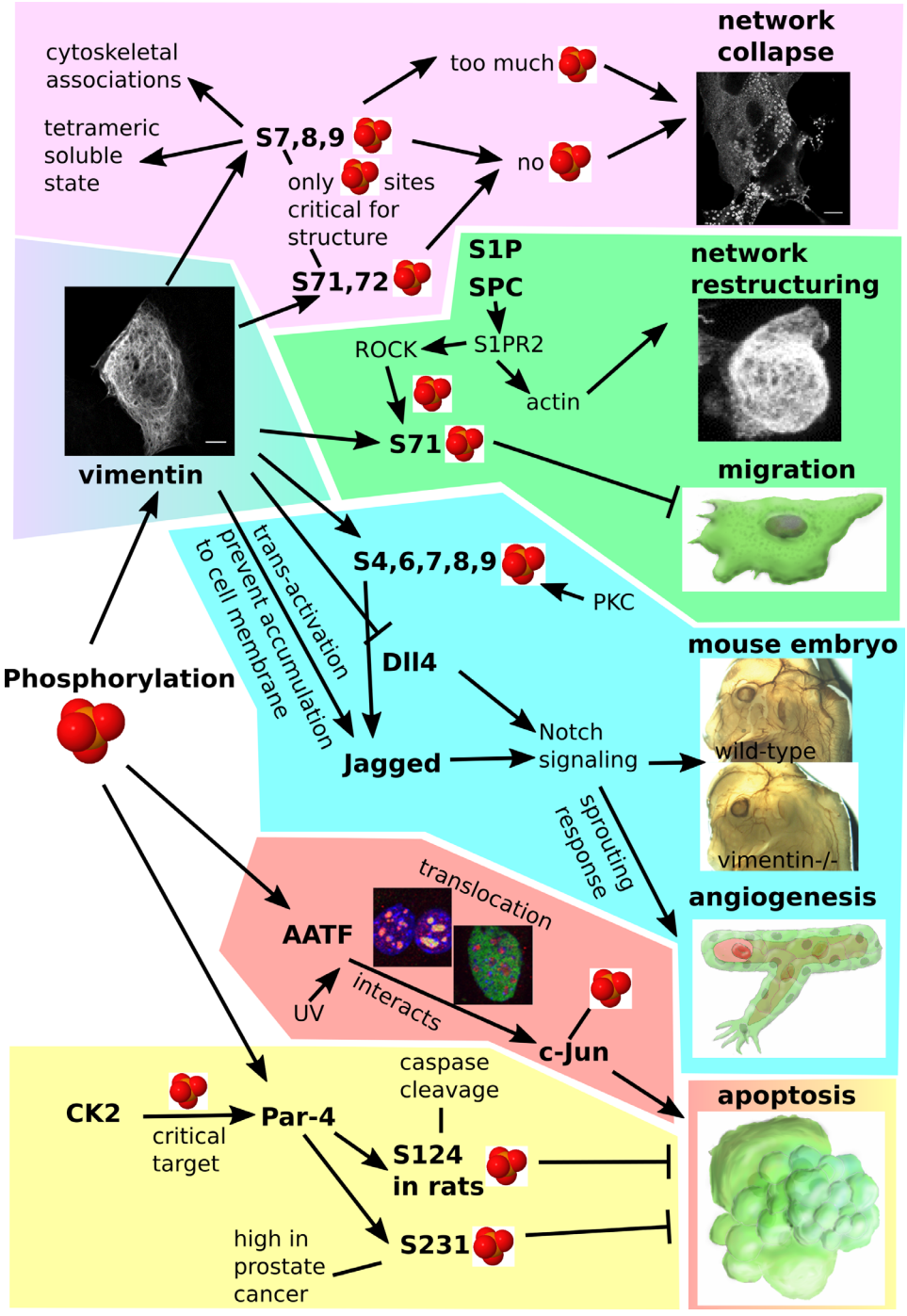
Interest in vimentin research has recently increased because it regulates numerous cellular processes in subtle ways that may be observed when an organism is stressed. Vimentin is a marker of EMT and has a major impact on increasing cell migration and invasion, and also increases angiogenesis and proliferation. It is likely that due to these facts, vimentin is overexpressed in many epithelial cancers. Due to its filamentous structure, vimentin offers a large surface area for other molecules to interact with and affect cellular processes with various mechanisms. Many these interactions are likely regulated by post-translational modifications, of which phosphorylation is the most, but still largely incompletely, studied. This thesis includes a systematic charting of almost all the in vivo-identified serine/threonine interphase phosphorylation site phospho-deficient and -mimicking mutants and their effects on vimentin filamentous structure. The analysis shows that only a few sites have critical and notable effects on vimentin filament network structure including, most importantly, serines 6, 7, 8 and 9. Phosphorylation of these residues is potentially important for vimentin solubilization in interphase, and some phosphorylation of these sites may be needed for network stabilization. Two other articles show among their results how vimentin effect to angiogenesis, that is barely researched, in development can be seen in vimentin negative mouse embryos that have a weakened vascular phenotype, and describe mechanisms by which individual phosphorylation site regulate longer known and more researched vimentin migration effect. The articles describe two new signaling pathways affecting (S1P/SPC and S1PR2) or affected by (signaling from Notch ligands) vimentin, and describe critical phosphorylation sites for these effects in vimentin. Our results strengthen the view of vimentin as a multifunctional signaling scaffold, and provide information of phosphorylation sites regulating some of these functions.

AATF, Par-4, and CK2 are proteins that are all primarily involved in regulation of cell survival or apoptosis. AATF has been known for its role as a transcriptional cofactor, for promoting cell cycle progression, for functioning as an antiapoptotic factor and for its role in DNA damage response. We show that AATF can also function pro-apoptotically by activating c-Jun, and demonstrate this role after UV

stress. This mechanism includes previously unknown AATF relocalization from the nucleolus to the nucleus, which allows fast and efficient signaling triggered by intracellular signals. Par-4 and CK2 have been known to be important especially for prostate cancer. We demonstrate that their pathways connect: CK2 can phosphorylate Par-4, inhibiting its pro-apoptotic effect in species-dependent mechanisms. We demonstrate that CK2 antiapoptotic effect functions largely through Par-4 and is active in prostate cancer, further indicating Par-4 as a potential target for prostate cancer therapies.

Knowing the functions and mechanisms of individual phosphorylation sites may, eventually, lead to, for example, applications in diagnostics with phosphorylation state-specific antibodies or therapeutics by alteration of phosphorylation.

Figure 12 (next page): Summary of the findings in this thesis. Background colors according to an article in which corresponding results are described (purple: I, green: II, blue: III, red: IV, yellow: V).


cytoskeletal associations
tetrameric soluble state
S7,8,9
too much
no
only sites critical for structure
network collapse
S71,72
S1P
SPC
ROCK
S1PR2
actin
network restructuring
vimentin
S71
migration
trans-activation prevent accumulation to cell membrane
S4,6,7,8,9
PKC
Phosphorylation
Dll4
Jagged
Notch signaling
mouse embryo
wild-type
vimentin-/-
sprouting response
translocation
AATF
UV
interacts
c-Jun
angiogenesis
caspase cleavage
CK2
critical target
Par-4
S124 in rats
S231
high in prostate cancer
apoptosis

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