Universidade de Aveiro, Departamento de Química

2017

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A vimentina interage com a via de sinalização Akt/mTOR e medeia o crescimento celular

Vimentin interacts with the Akt/mTOR pathway mediating cell growth

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Dissertation presented to Universidade de Aveiro and Åbo Akademi University to obtain the degree of MSc in Biochemistry, Clinical Biochemistry field, made under the supervision of Doctor John Eriksson Professor of Cell Biology, Director of Centre for Biotechnology, Chair of Turku Bioimaging, Faculty of Science and Engineering of Åbo Akademi University and Doctor Brian Goodfellow, Auxiliary Professor of the Chemistry Department of Universidade de Aveiro.

To my family, my most precious gift, for all the support and caring…

Juri

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Acknowledgements

Five years have passed. A journey full of lessons and people to whom I am very thankful. To professor John Eriksson, thank you so much for the opportunity, for believing in me, for the trust and kindness. To professor Brian Goodfellow, for the guidance and nice discussions during this last year. To Emilia Peuhu for kindly revising this thesis. To the proteomics facility of Turku Centre for Biotechnology, especially Arttu Heinonen for all the help with mass spectrometry. To my professors of the University of Aveiro, for all the lessons, discussions and patience, thank you, I hope we can keep in touch in the future. To my family from Aveiro, Antoine, Sofia, Sónia, Ana, Mariana, Vivien, Pedro, Henrique, Daniel, Marta, Catarina and Rafael for always being there, for the studying sessions, for the nights out, for the good times, for the love and laughter, I can't thank you enough, I love you guys so much. To my old friends back home, Ana Maria, Ana Catarina, Inês, Vânia, Cristiano, Gabriel, Rui, Xavier, it amazing how we rarely see each other during the semester but still every time we meet it is just like high school again, like we are together every day, laughing at each other's silly jokes. To Johannes, my absolute favourite german guy, for all the jokes, for keeping in touch, for being a great friend, I am sure we will see each other very soon. To my lab colleagues and friends in Biocity, for making me feel home and welcome in a strange northern country. Arun thank you for all the help, especially during the first two weeks, you are a true friend. Ponnuswammy, thank you for the guidance, and for always being in a good mood, unlike me. Alia, for being my friend, for the statistics, for the sports, the football evenings, the adventures in remote places, you are amazing. To Preethy, thank you for sharing your office and for all the nice conversations that always make me smile. Elena, thank you, for the image J skills that I have now, for all the coding, the ice skating, the billiard, the foosball, but mostly for being you. Maria, for the breakfasts, lunches and dinners, the jazz sessions and for being my first friend from Turku. Alejandro, you are such a wonderful person, so full of joy, you are that person that always makes me smile just by saying *olá prima*, even when I am walking around Biocity feeling sad and frustrated, thank you! Diosa and Jens, for tricking me to go ice swimming, for the games, movies and dinners, for the great times still to come, I am very thankful for having you guys in my life. Elnaz, I don't even have words for you dear, thank you for everything, I love you. To my family, to whom I owe everything I am and have today, for the opportunity, for the patience, for the love, for saying that it ok to be away even though it is so hard. Mãe e pai, vocês são os melhores e os mais malucos de todos, obrigado por me ensinarem que tudo se consegue. Avós, obrigado por tudo, vocês são maravilhosos, nunca vou conseguir agradecer o tanto que fizeram por mim. Aos meus irmãos, Dalila e Leonardo, por me ensinarem o que é amar incondicionalmente alguém, por aturarem a chata da mana mais velha que está sempre a estudar. Aos meus tios pelo mimo e por me darem priminhas e priminhos dos quais morro de saudades. To my Thunder, for being the best buddy and destroying my notebooks, preventing me from studying. Last but not least, to Michael, for being my home and my love always. Thank you sharing your life with me, for taking care of me and putting up with my panic attacks, thank you for the 5 years of adventures we had and the ones that are still to come, I love you.

Palavras-chave Vimentina, filamentos intermédios, crescimento cellular, via de sinalização Akt/mTOR, síntese proteica

Resumo

A vimentina é uma proteína da classe III dos filamentos intermédios que promove processos tais como proliferação, migração e invasão celular através da interação com diferentes vias de sinalização. No entanto, o papel da vimentina no crescimento celular é ainda pouco conhecido. Neste estudo, observamos que fibroblastos isolados de embriões de ratinhos sem vimentina (*Vim* -/- MEFs) eram mais pequenos que o tipo normal (WT). Assim, o objetivo deste estudo era entender de que forma a vimentina regula o crescimento celular. Com recurso a modelos *in vitro,* técnicas de microscopia e técnicas bioquímicas descobrimos que *Vim* -/- MEFs tinham menor volume e concentração de proteínas quando comparadas com WT MEFs. Adicionalmente, a síntese proteica e ativação de mTORC1 estavam significativamente reduzidas em *Vim* -/- MEFs. Através de co-imunoprecipitação, descobrimos que a vimentina interage com os complexos mTORC2 e TSC. Assim, postulamos que a vimentina regula o crescimento celular por interação com proteínas da via de sinalização AKT/mTOR.

Keywords Vimentin, intermediate filaments, cell growth, Akt/mTOR pathway, protein synthesis

Abstract Vimentin is a type III intermediate filament protein that takes part in cell proliferation, migration and invasion, by acting as a signalling scaffold. The role of vimentin in cell growth, however, is poorly understood. We observed that vimentin knockout mouse embryonic fibroblasts (*Vim* -/- MEFs) were smaller than the wild type (WT). Therefore, this work aimed to understanding how vimentin regulates cell growth. Using *in vitro* models, imaging techniques and biochemical approaches, we have found that the volume and protein concentration of *Vim* -/- MEFs is lower when compared to WT MEFs. Further, protein synthesis and mammalian target of rapamycin complex 1 (mTORC1) activation was attenuated in *Vim* -/- MEFs. By co-immunoprecipitation we found that vimentin interacts with mammalian target of rapamycin complex 2 (mTORC2) and tuberous sclerosis protein complex (TSC) after insulin stimulation. Consequently, we postulate that vimentin regulates cell growth by interacting with proteins of the AKT/mTOR pathway.

Contents

List of Tables

List of Figures

Acronyms and abbreviations

-/-	Knockout
4E-BP1	Eukaryotic translation initiation factor 4E binding protein 1
ACN	Acetonitrile
Bad	Bcl-2-associated death promoter
BSA	Bovine serum albumin
Co-IP	Co-immunoprecipitation
Cdk5	Cyclin dependent kinase 5
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
eIF4E	Eukaryotic translation initiation factor 4E
EMT	Epithelial to mesenchymal transition
Erk	Extracellular signal–regulated kinase
ESI	Electrospray ionization
FA	Formic acid
FCS	Fetal carf sérum
Foxo	Forkhead box O
GAP	GTPase activating protein
GTP	Guanosine triphosphate
HDFs	Human dermal fibroblasts
HE	Haematoxylin-eosin
HIF-1	Hypoxia initiation factor 1
IFs	Intermediate filaments
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IkB	Inhibitor of kB
Ins	Insulin
LC	Liquid chromatography
MAPK	Mitogen activated protein kinase
MEF	Mouse embryonic fibroblasts
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2

MRI	Magnetic resonance imaging
NF-kB	Nuclear factor kB
p70S6K	Ribosomal protein S6 kinase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK1	Pyruvate dehydrogenase kinase 1
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3 kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PP2A	Protein phosphatase 2 A
PRAS40	Proline-rich Akt substrate 40
PTEN	Phosphatase and tensin homolog
RAPTOR	Regulatory associated protein of target of rapamycin
Rheb	Ras homolog enriched in brain
Rho-A	Ras homolog gene family member A
RICTOR	Rapamycin insensitive companion of target of rapamycin
ROCK	Rho-A associated protein kinase
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SFM	Serum free medium
TBS	Tris buffered saline
TGF-β	Transforming growth factor β
Tris	Tris hydroxymethyl aminomethane
TSC	Tuberous sclerosis protein complex
Vim	Vimentin
WB	Western Blotting
WT	Wild type

1 Introduction

The cytoskeleton is a fibrous network that maintains the function, shape and motility of the cell, supports the organelles, participates actively in the transport of cargo and in various signalling pathways. It is comprised of three major classes of proteins: microtubules, microfilaments and intermediate filaments (IFs). Microtubules are the largest type of filaments, about 25 nm in diameter. They are polarised structures composed of tubulin that provide organisation and organelle positioning. Microfilaments, on the other hand, are made of actin and have 6 nm in diameter. Microfilaments are involved in muscle contraction by interaction with myosin, but they also play a role in cell division and migration. Finally, intermediate filaments are medium sized filaments (10 nm in diameter) composed of more than 70 proteins, organised in 6 classes, which are expressed in a tissue specific manner. Unlike the other members of the cytoskeleton, IFs are not polarised structures and they have numerous roles besides structural support that include migration, proliferation, growth and they can act as signalling scaffolds. Therefore, the idea of IFs being static structures has changed. Now, it is known that they are essential for many cellular processes, including regulation of key signalling pathways. Vimentin, a very versatile type III IF, is essential and has a very complex signalling pattern, interacting with specific kinases and regulating their function. It is highly expressed in proliferative cells, such as cancer and mesenchymal cells. This protein is associated with cancer progression and metastasis, since it can induce epithelial to mesenchymal transition (EMT) and promote invasion. Cell growth is essentially achieved by synthesis of macromolecules, such as proteins, lipids and nutrients, which is regulated by the Akt/mTOR pathway. This dissertation will be focused on understanding the mechanisms by which vimentin affects the cell growth.

2 Review of literature

2.1 Overview of Intermediate Filaments

It took decades to understand that the cytoskeleton is comprised of three quite distinct groups of proteins. A long time was also needed to find out that IFs are actually a big family of proteins with different roles and expression patterns (1). The interest began with studies of hair fibers, but it was only years after, that IFs were considered a separate class of

cytoskeleton proteins, instead of coupled to the other classes (2). Afterwards, many studies were published describing the various classes of IFs (3,4). According to their molecular features, IFs can be divided in six distinct classes, present in a tissue specific manner: class I and II IFs are keratins, found in epithelial cells; class III covers desmin and vimentin, existing in muscle and mesenchymal cells, respectively; nestin and neurofilaments, present in neurons and stem cells, form class IV of IFs proteins; class V includes lamins, which are associated to the nuclear membrane and therefore present in all cell types; finally, class VI IFs, phakinin and filensin, are expressed in eye lenses (table 2.1) (5,6).

2.1.1 Structure and assembly of intermediate filaments

IFs are elastic structures, resistant to stress, allowing them to maintain cell integrity. This major class of cytoskeletal proteins is essential for the structure, motility and survival of the cell. They are 10-12 nm in diameter and a well conserved structure: a rod alpha-helical domain, important for polymerization, flanked by non-helical N and C terminus with high degree of variability (1,5). This feature allows specific interactions between IFs and other proteins, not only related to mechanical support, but also related to signalling pathways, where they mainly act as scaffolds, since they lack enzymatic activity (5).

The assembly of IFs begins with the interaction between two rod domains, causing parallel dimerization of the filaments. Dimers can then associate and form antiparallel tetramers, which will then assemble in octamers and form complex structures (figure 2.1; (7)). Interestingly, some IFs can polymerise with each other and form homopolymers, while others need to assemble with a different type of IF, forming an heteropolymer (8,9), which gives even greater versatility to these filaments. In addition, IFs lack polarity and they can assemble without the use of energy, which is another characteristic that separates them from microfilaments and microtubules. Nevertheless, the spatial assembly of IFs is dependent on other members of the cytoskeleton (8,10).

2.1.2 Function and Expression of intermediate filaments

In the early times of IFs research, the major function of IFs was considered to be structural support, but now it is known that they have a wide list of functions that also include organelle positioning, roles in migration, adhesion, inflammatory responses and cell stress (reviewed

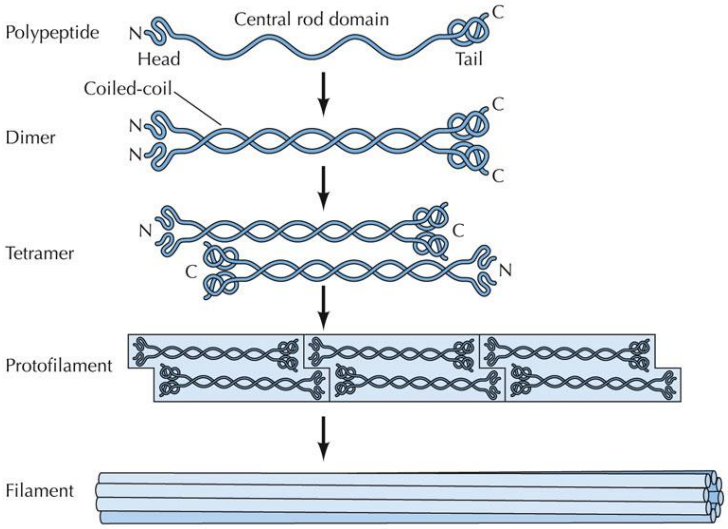

Figure 2.1 Assembly of intermediate filaments. Interaction between two rod domains causes parallel dimerization of the filaments. Dimers can then associate and form antiparallel tetramers, which will then assemble in protofilaments and further to a complete filament (11).

in (12)). Studies on mice with mutations in genes coding for IFs proteins have shown major signalling problems (5), suggesting that IFs play a role in signalling as well.

The reason for the versatility and the wide range of unique characteristics of IFs is mainly because of their peculiar expression patterns. IFs are expressed in a tissue specific manner but their expression pattern is also dependent on development (13). For example, vimentin is expressed in cells of mesenchymal origin, while keratins are present in epithelial cells. Nestin is expressed only during development in the nervous system, kidney and muscle. Then, with differentiation, it is eventually replaced by other classes of IFs (14). Moreover, the extracellular matrix (ECM) can also play a role in the expression of these proteins, because differential IFs expression can be seen in the same cell type in different ECM environments, enhancing the role of the surrounding environment in these cases (15).

2.1.3 Regulation by post-translational modifications

While microfilaments and microtubules are mostly regulated by their associated proteins, IF regulation occurs by post-translational modifications (PTMs) such as phosphorylation, glycosylation and sumoylation, among others, that affect the function and dynamics of the network (16,17). Sumoylation, addition of a small protein called SUMO to lysine residues, is considered to be a conservative modification on IFs, essential for their assembly and solubility, which are key properties of these proteins (18). Glycosylation, on the other hand, is thought to be regulating IF phosphorylation, as it occurs close to the phosphorylation sites

Table 2.1 Tissue distribution of the various classes of intermediate filaments proteins. Adapted from Pallari and Eriksson, 2006 (5).

Intermediate Filament	Protein	Tissue distribution	Examples of related diseases
Type I	Keratins 9-20	Epithelial cells	Epidermolysis bullosa simplex, liver diseases
Type II	Keratins 1-8	Epithelial cells	
Type III	Vimentin	Mesenchymal cells Muscle cells	Cataract
	Desmin		Myopathy
Type IV	Nestin	Myoblasts Neuroepithelial stem cells	Not reported
	Neurofilaments	CNS neurons	Neurodegenerative diseases
Type V	Lamins	Widely distributed	Laminopathies
Type VI	Filensin	Eye lenses	Cataracts
	Phakinin		

of the proteins (19). Phosphorylation is the most relevant modification in IFs and it occurs in the head or tail domains of the proteins, at serine, threonine and tyrosine residues. Therefore, IFs are the target of a wide range of protein kinases and protein phosphatases, enhancing the diversity and versatility of these proteins as well. The most classical examples of kinases that phosphorylate IFs are protein kinase C (PKC) for lamins and stress-activated proteins kinases for keratins. Rho kinase is also known to phosphorylate many IFs and Cdk5, during development, phosphorylates nestin (reviewed in (6)). This whole picture can get even more complex because many PTMs can be present at the same time, crosstalking to regulate the function of IFs (18). Studying the role of IFs in cells is a challenging task, and there still are lots of questions regarding this subject. Nevertheless, since signalling pathway malfunction is the main reason for the onset and progression of certain diseases, and provided that IFs interact with a wide range of signalling molecules, it is imperative to understand the interactions between these proteins and cell signalling.

2.1.4 Intermediate filaments and disease

IFs have been shown to be related to disease already in the early days of research, but nowadays, a wide range of diseases are known to be associated with IFs (table 2.1.). These conditions arise from problems in the assembly, organisation and regulation of IFs, which lead to signalling abnormalities (20). Keratins, for example, are associated with many skin and liver diseases (reviewed in (21)). Keratins are perhaps the most studied IFs in terms of diseases, since mutations in keratins are directly associated with various pathological conditions. Vimentin and type VI IFs, on the other hand, are known to be linked with cataract

development, even though vimentin can be indirectly related to many disorders (22,23). Further, studies on nestin showed that it is overexpressed in Alzheimer's disease (24). IFs with obvious relations with diseases, such as keratins, are extensively studied, therefore the knowledge on these is quite broad. However, research on IFs related to diseases indirectly, such as vimentin, is very poor still, and more studies are needed to understand their functions.

2.2 Vimentin as a scaffold protein

2.2.1 Structure and function

Vimentin is a type III IF with 466 amino acids and a very well conserved structure. It possesses four alpha-helixes in total, flanked by a N (head) and a C (tail) non-alpha-helix terminus, that associate in parallel forming homo and heterodimers (17). The heads are presented in a symmetric way and have numerous phosphorylation sites, allowing vimentin to be regulated by PTMs (16,17). Vimentin is expressed in highly proliferative cells, such as cancer and mesenchymal cells, including fibroblasts, endothelial cells lining blood vessels, renal tubular cells, macrophages, neutrophils and leukocytes (25).

As an IF protein, one of the functions of vimentin is structural support, which is supported by mice studies showing that lack of this protein leads to defects in the morphology of glial cells (26). Nevertheless, vimentin can also regulate the recycling mechanisms of the cell, cell adhesion, cell division, cell death, leukocyte transmigration, inflammation, participate in organelle trafficking and positioning, interacting with microtubules, and play a role in lipid homeostasis (27–30). Vimentin was shown to form a cage around lipid droplets and proven to be a facilitator for adipogenesis (27). Studies on vimentin knockout fibroblasts showed impaired migration and motility which resulted in defects in wound healing (31,32). Interestingly, *Vim* -/- mice were seen to possess a normal phenotype, showing that vimentin is not a critical protein for survival (33). However, these mice showed wound healing defects, reduced blood flow during vessel dilation, decreased renal mass and tended to perish from renal failure (30,31,34). These observations indicate that vimentin is essential for wound healing mechanisms and also modulation of the responses to blood flow in arteries. Other studies have shown that vimentin is a marker of epithelial to mesenchymal transition (EMT), a cellular reprogramming process in which epithelial cells acquire mesenchymal phenotype, altering their shape and motility. These alterations are associated with the

expression of vimentin, which is normally mainly expressed in mesenchymal cells (35–37). Since EMT is a crucial event in wound healing processes, these observations strongly support the previous *Vim* -/- mice studies (31). Increased expression of vimentin was also observed in many tumour cell lines and it was related to increased motility and metastasis (38–40), linking EMT to cancer. Furthermore, the hypoxia initiation factor (HIF-1), a regulator of the cell response to hypoxia, was demonstrated to regulate vimentin expression (41). Since hypoxia can be a driver of metastasis, HIF-1 can promote EMT by regulating vimentin's expression. Further, a study reported that vimentin could interact with p53, a known tumour suppressor (42). This study showed that vimentin was capturing p53 in response to survival signals and releasing it in the presence of death signals, thereby enhancing the role of vimentin in cell death and survival (42). Interestingly, vimentin was also demonstrated to be a possible transcription regulator of p53 (42). Studies of the molecular mechanisms underlying all these functions have revealed that vimentin plays a role in many signalling pathways related to cell adhesion, migration, proliferation and survival (43).

2.2.2 Regulation by Post-translational modifications

The extensive range of roles of vimentin in the cell is mainly achieved by its regulation by PTMs. PTMs allow interactions with a wide number of proteins and consequent participation in signalling cues. A study showed that vimentin can be sumoylated, but the significance of this modification is still poorly understood (44). Glycosylation is known to widely occur on the same residues as phosphorylation, working like a competition mechanism. This PTM is thought to have complementary roles with phosphorylation, regulating the function of vimentin itself (45,46). Phosphorylation, made by kinases on serine, tyrosine and threonine residues, is the most relevant PTM occurring in vimentin, since it regulates its association with other proteins. More than 20 phosphorylation sites have been identified at the N-terminus of vimentin, most of them on serines. The most commonly phosphorylated serine is S38, which is the target of several kinases. In order to reverse phosphorylation and keep homeostasis, vimentin interacts with protein phosphatases that remove these PTMs (17).

If one thinks about the cell processes in which vimentin is involved – cell migration, growth, proliferation, organelle trafficking, scaffolding – phosphorylation can be considered

the main driver of all these. *In vivo* phosphorylation of vimentin by protein kinase A (PKA) on S38 and S72 phosphosites has resulted in assembly of vimentin filaments in BHK-21 cells (17). Furthermore, multiple phosphorylation by p21 was also seen, regulating structural reorganization, as well as phosphorylation by Aurora B, causing segregation of the filaments during cytokinesis (47). On the other hand, vimentin interaction with phosphorylated extracellular signal-regulated kinase (Erk), was shown to prevent it from being degraded therefore keeping it functional in promoting cell proliferation (48). RhoA-binding kinase (ROCK) was proven to be a very efficient vimentin kinase causing the collapse of vimentin network (49). Vimentin association with protein phosphatase 2A (PP2A) prevented phosphorylation of vimentin *in vitro* (50). Moreover, phosphorylation of vimentin on S38 by Akt makes vimentin filaments interact with each other, which protects vimentin from caspase-induced proteolysis, enhancing cell motility and promoting tumour metastasis (51). Vimentin interaction with Scrib, a protein implicated in cell migration, was demonstrated to drive cell migration and increase the invasive capacity (52). PKC was shown to promote interaction between vimentin and 14-3-3 (53). In addition, vimentin was reported to interact with 14-3-3 in different scenarios, regulating signalling pathways related to autophagy inhibition and tumorigenesis. One study showed that vimentin is limiting 14-3-3, instead of 14-3-3 preventing vimentin binding to other proteins (54). Thus, vimentin could be acting as a modulator of the signalling pathways requiring 14-3-3, such as the Akt/mTOR pathway (55). Even though vimentin can interact with many protein kinases, it does not necessarily mean that it is phosphorylated by them. Vimentin can interact with PKC on membrane vesicles but no phosphorylation occurs (56). Moreover, vimentin can be associated with Raf-1 kinase and it is not phosphorylated by it (57). Taken together, these observations suggest that vimentin is an essential protein in many distinct signalling pathways, mainly related to cell growth, proliferation and motility and that phosphorylation is essential for these functions to be conducted. Nevertheless, phosphorylation is not mandatory for vimentin to interact with protein kinases.

2.2.3 Vimentin and disease

Mutations that cause a single amino acid change can be the reason for the arise of diseases. One study showed that a vimentin mutation that caused a change in one amino acid is involved in rheumatoid arthritis (58). In addition, cataract formation is also associated with

this sort of mutations, even though vimentin is involved in cataracts in many different ways (59). Vimentin can also be related to other disorders in a more indirect way, such as neuropathies and cancer. Vimentin aggregation is seen in a neuropathy caused by mutations in a E3 ligase adaptor protein (60–62). Hence, here, the problem is not vimentin itself, but a mutated protein that affects the function of vimentin, although the mechanism is still poorly understood. As mentioned before, studies on *Vim* -/- mice showed that vimentin is not essential for normal development and survival but these mice were demonstrated to have severe wound healing defects (30,31). Even though wound healing defects might not be pathogenic, the mechanisms of wound healing and the mechanisms of cancer invasion can be linked by EMT (35). Therefore, since vimentin plays a role in key processes of the cell, further studies on the molecular mechanisms by which vimentin regulates cell functions are needed.

2.3. Cell growth regulation

To divide and proliferate, the cell firstly needs to grow. This is mainly achieved by synthesis of macromolecules, such as proteins and lipids, upon stimulation. The regulation of the size of a cell is essential to keep proper development and function in a multicellular organism context, where growth and division are coordinated in proliferating cells, so that a doubling in mass occurs for each division (63). Nevertheless, cell cycle is independent from cell growth, and cells can grow without dividing (64–66).

There are various pathways involved in the regulation of cell size, and these can interact with each other, acting in agreement with the necessities of the multicellular organisms. This regulation, unlike unicellular organisms, in which growth depends mainly on the amount of nutrients available, is achieved by a balance between synthesis and degradation of macromolecules, in response to different stimuli, integrating nutrients and growth factors (63,67). Mitogens can activate the transcription factor Myc that will increase ribosomal RNA levels and protein synthesis, leading to an increase of the cell size and cell cycle progression (68). Cells overexpressing *Myc* were seen to be much larger than the wild type ones. On the other hand, studies with *Drosophila* have shown that partial loss of *Myc* resulted in miniature flies (69).

A second way of cell size regulation is the Hippo pathway. It comprises a group of tumour suppressors that can control cell number by promoting apoptosis and inhibiting proliferation

(70). Mutations in any of the implicated genes of the Hippo pathway showed exaggerated cell growth. Furthermore, the heads of adult flies with Hippo mutations were larger when compared to the wild type ones (71–73). Interestingly, studies have shown the existence of a feedback loop where Myc can regulate cell growth by affecting the activity of the Hippo pathway (68). Moreover, it was demonstrated that the expression of *Myc* can be actually controlled by yes-associated protein (Yap), a transcription factor that is part of the Hippo pathway (68). Both pathways can also interact with the one that might be the major regulator of cell size in most types of cells - the Akt/mTOR pathway, a signalling cascade activated by insulin and other growth factors that ultimately leads to protein synthesis.

2.3.1. The Akt/mTOR pathway

Insulin, along with other growths factors, can trigger the insulin receptor and the insulin like growth factor (IGF) receptor and regulate cell growth by controlling the Akt-mTOR pathway (figure 2.2). Activation of the receptor causes activation of pyruvate dehydrogenase kinase 1 (PDK-1) by phosphoinositide 3 kinase (PI3K), which in turn activates Akt, also called protein kinase B (PKB). Akt will then phosphorylate TSC, causing disassembly of the TSC inhibitory complex from mTORC1. mTOR is a serine threonine protein kinase crucial for several survival cues in the cell, and it can form two different complexes: mTORC1, referred above, which is mainly characterised by regulatory associated protein of target of rapamycin (RAPTOR), proline-rich Akt substrate 40 (PRAS40) and the common subunits; and mTORC2, comprised by rapamycin insensitive companion of target of rapamycin (RICTOR) and the common subunits. TSC complex functions as a GTPase activating protein (GAP) for Ras homolog enriched in brain (Rheb), which, when bound to guanosine triphosphate (GTP), interacts with mTORC1 and increases its kinase activity. Thus, removal of TSC complex promotes GTP binding to Rheb, activating mTORC1, which will then phosphorylate eukaryotic translation initiation factor 4E binding protein (4E-BP1) and S6 kinases (ribosomal proteins; reviewed in (74)). While 4E-BP1 prevents translation, phosphorylated 4E-BP1 causes release of eIF4E, leading to protein synthesis (75,76). Furthermore, mTORC2 can be regulated by PI3K and fully activate Akt by phosphorylation of Akt on Ser473 (77). The Akt/mTOR pathway is functioning in the presence of nutrients, and, during fasting, the pathway is supressed. This pathway ultimately leads to the regulation

of protein synthesis (figure 2.2) (74), as well as nutrient uptake and lipid synthesis, all of which drive cell growth.

2.3.1.1. Insulin receptor signalling

The Akt/mTOR pathway is triggered by insulin and IGFs by binding to insulin and IGF receptors, which are highly homologous receptor tyrosine kinases (RTK). These are composed by two alpha extracellular subunits and two beta transmembranar subunits, both generated from the same precursor that suffers alternative splicing (78). The insulin receptor family includes various forms of the receptors, which can have different outcomes and are tissue specific (79). These receptors are activated mostly by insulin and IGF-1 and they can initiate a cascade of phosphorylation that controls many aspects of the metabolism. Although insulin and IGF-1 prefer to bind to their own receptor, they can also bind to each other's receptor with lower affinity (80). Studies on knockout mice for different insulin receptor ligands showed distinct phenotypes and abnormalities between them (81,82), suggesting that distinct processes can be affected depending on which ligand is binding, even though they possess similar motifs. The main pathway activated by insulin receptors is the Akt/mTOR,

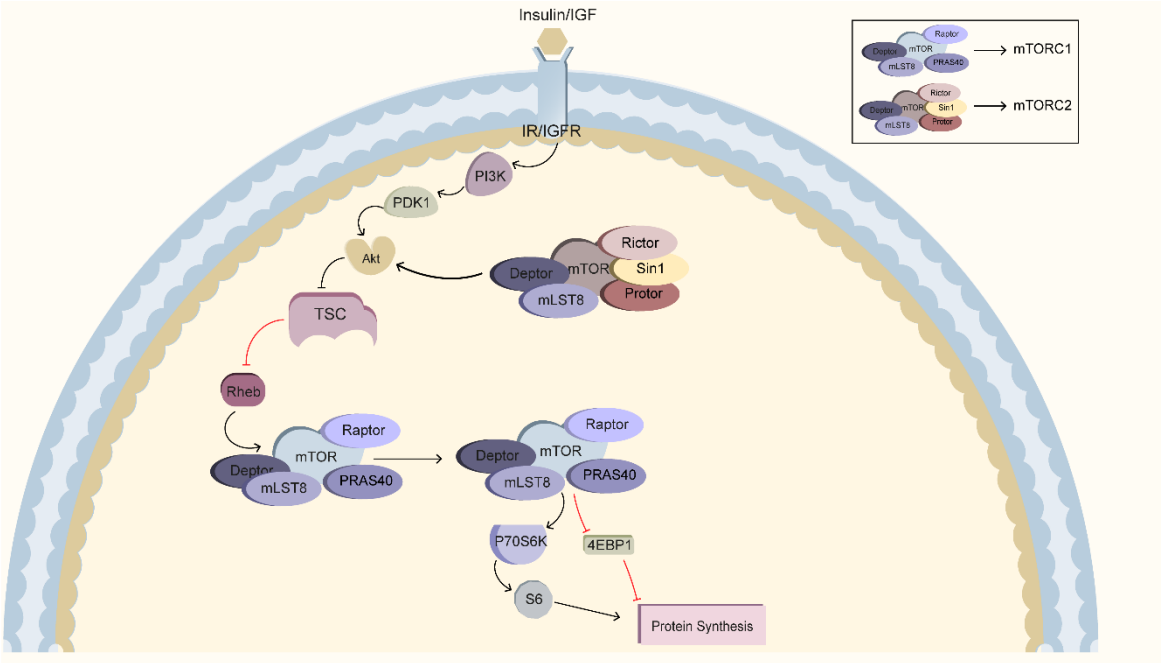

Figure 2.2 Simplified model of the Akt/mTOR pathway. Activation of the receptor causes phosphorylation of PDK-1 by PI3K, which in turn activates Akt. Akt will then phosphorylate TSC, causing disassembly of the TSC inhibitory complex from mTORC1. This promotes GTP binding to Rheb, thus activating mTORC1, which will then phosphorylate 4E-BP1 and S6 kinases. This pathway ultimately leads to the regulation of protein synthesis and cell growth by controlling gene expression.

via PI3K-PDK-1, as referred above, but PKC is also activated by these receptors and it has a very important role on glucose transport and regulation of lipid synthesis (83). In addition, the mitogen activated protein kinase (MAPK) pathway can also be triggered by insulin receptors and affect cell proliferation by controlling gene expression (84,85). Neither MAPK nor PKC pathways depend on Akt/mTOR function. Akt activation, besides triggering the whole Akt/mTOR pathway referred above, also leads to other secondary pathways that complement each other. For example, Akt can phosphorylate forkhead box O (Foxo), regulating lipid synthesis and gluconeogenesis (86). Akt can also phosphorylate murine double minute 2 (mdm2), the inhibitor of p53, inhibiting apoptosis (87). Still related to cell survival, Akt phosphorylates caspase 9 and Bcl-2-associated death promoter (Bad), inhibiting apoptosis through a different pathway (88–90). In addition, Akt can trigger the nuclear factor kB (NF-kB) signalling pathway by phosphorylating the inhibitor of kB (IkB) kinase (91). Further, Akt can be involved in vasodilation, glucose uptake, fatty acid oxidation, autophagy control, among other cell functions, by phosphorylating additional proteins and starting different cascades (77,92,93). One study showed that Akt could inhibit beclin-1 and inhibit autophagy, enhancing tumorigenesis. In fact, Akt was observed to inhibit beclin-1, which was interacting with both 14-3-3 and vimentin, suggesting that a protein complex composed by these four proteins can be promoting tumorigenesis (94).

Uncontrolled activity of insulin receptor signalling pathways can lead to major unbalances in metabolism and tumorigenesis. Various protein tyrosine phosphatases attenuate the signal and reduce the activity of the receptor, keeping homeostasis (95). It is necessary to consider that all these pathways can crosstalk, and the information must be combined to keep homeostasis.

2.3.1.2. Crosstalk to regulate cell growth

As stated in the previous sections, insulin receptor signalling triggers a significant part of the key processes in the cell, and most of them involve Akt. Interestingly, all outcomes from insulin receptor signalling promote cell survival, growth and proliferation. Activation of the MAPK pathway by growth factors leads to activation of *Myc*, a very important transcription factor in the cell (84). Some of the targets of *Myc* are translation initiation factors, which are downstream mTORC1 (96,97). Even further, *Myc* can affect directly the TSC complex, a key complex of the Akt/mTOR pathway, by binding to the promoter of TSC (98). These

findings point to a crosstalk between distinct pathways that can be activated by the same family of receptors and act in agreement to control cell growth and proliferation through a complex network of feedbacks (figure 2.3).

The Hippo pathway can coordinate cell growth and affect cell number and it was demonstrated to be interacting with these pathways, affecting essential proteins upstream of Akt. It was reported that the Hippo pathway is able to regulate IGF-1, which is an activator of insulin receptor signalling (99). On the other hand, members of the Hippo pathway were shown to inhibit phosphatase and tensin homolog (PTEN), an inhibitor of PI3K. This way, the loss of members of the Hippo pathway was shown to cause activation of mTORC1, leading to increased cell growth (100–102). Furthermore, since PI3K is directly activated by the insulin receptor, the whole downstream signalling is affected by upregulation of PI3K. These findings point for a crosstalk between the different pathways controlling cell size and provide evidence that the Hippo pathway can control the outcomes of insulin receptor signalling.

2.3.1.3. Mutations in the Insulin/Akt/mTOR pathway lead to size defects

To understand the outcomes of the Akt/mTOR pathway, several studies have been conducted with knockout animal models. Studies in *Drosophila* have shown that deletion of p70S6K reduces cell and body size (103). Similar results were found with mice lacking PDK-1, that the mice themselves were smaller in body size (104).

In addition, isolated mouse embryonic fibroblasts (MEF) from PDK-1 null mice were about 30% smaller than the wild type ones (104). In agreement with the other results, depletion of PI3K in cells caused impaired growth, whereas overexpression of this protein resulted in abnormally big cells (105,106). Even further, knock-out of insulin receptor ligands in mice resulted in growth retardation (revised in (107)). A study made with *Drosophila*, to evaluate the effect of the loss of dTOR, the homolog of mTOR in the flies, showed that the mutant cells were dramatically reduced in size, enhancing the role of this protein in cell size control (108). All these studies support the idea that the Akt/mTOR pathway is a key pathway controlling cell size and growth.

2.3.1.3.1. Pathological conditions associated

The overall mechanisms regulated by insulin receptor signalling, are related to cell survival and proliferation and the Akt/mTOR pathway can participate in controlling all these

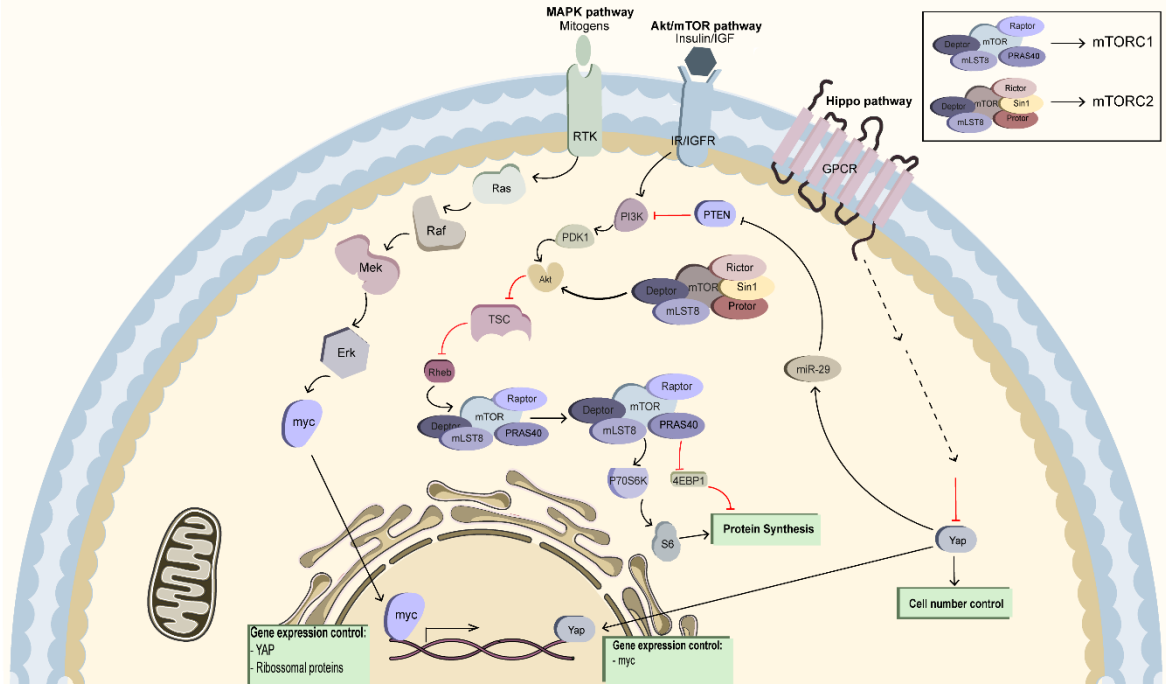

Figure 2.3 Crosstalk between Hippo, MAPK and Akt/mTOR pathways. The Hippo pathway can inhibit PTEN, keeping PI3K active. The myc pathway can regulate the other two pathways by gene expression regulation.

processes. The knockout animal models studies referred above showed, consistently, the defects on growth. On the other hand, studies have shown that mutations that increase the activity of the PI3K/Akt/mTOR signalling pathway reduce autophagy (revised in (85)). As examples, common cancer mutations, such as on the tumour suppressor p53, are able to promote constitutive activation of mTORC1 (109), explaining the increased growth and reduced organelle recycling in cancer. Another typical cancer mutation is the loss of the tumour suppressor PTEN, which results in increased activity of the Akt/mTOR pathway, because PI3K becomes continuously active (110). Also, other diseases can be associated with the Akt/mTOR pathway. A rare genetic disease caused by mutations in TSC results in the development of non-malignant tumours in many organs (111). Furthermore, given the relationship between this pathway and lipid synthesis, it can also be linked to metabolic diseases such as obesity, fatty liver diseases, insulin resistance and type II diabetes (revised in (112)). Therefore, an extensive range of disorders can either arise from, or be affected by the Akt/mTOR pathway.

3 Aims and outline

Vimentin, a type II IF, was shown to be involved in various signalling pathways related to cell migration and proliferation. Although the role of vimentin in cell proliferation and migration are widely studied, the mechanisms by which vimentin regulated cell growth remain unknown. The regulation of cell growth is done by a balance between synthesis and degradation of macromolecules, such as proteins. We hypothesised that vimentin regulates cell growth. Consequently, this dissertation has as main aim to understand if vimentin regulates cell growth and as second aim unravel the underlying molecular mechanism. To do this, a series of *in vitro* experiments, namely cell volume, protein amount and protein synthesis were performed with mouse embryonic fibroblasts, to discuss whether vimentin is responsible for the size differences. Vimentin rescue experiments were also conducted to see if the normal phenotype could be restored. mTOR activation was then studied to understand the differences in the Akt/mTOR pathway between WT and *Vim* -/- MEFs, since this is the pathway responsible for protein synthesis. In parallel, a series of mouse experiments were made to investigate the influence of vimentin on body size. Using co-immunoprecipitation with human dermal fibroblasts, the role of vimentin in cell growth was studied by checking whether it interacted with proteins of the cell growth pathway. Finally, by means of mass spectrometry, the candidates for this phosphorylation dependent interaction were studied.

4 Materials and Methods

4.1 Animal Experiments

WT and *Vim* -/- mice generated from the heterozygous 129/SvJ x C57BL/6 were used in all experiments.

4.1.1 Genotyping

Ear samples taken from both WT and *Vim* -/- mice were heated at 55 ºC with proteinase K (0.2 mg/ml) and 400 µl of lysis buffer (10 mM Tris pH 8.0, 200 mM NaCl, 5 mM EDTA and 0.2 % SDS). When the ear pieces were completely dissolved, the samples were centrifuged for 3 minutes at 5000 rpm and the supernatant was put into new tubes. The tubes were incubated at room temperature for 5 minutes with 500 µl of isopropanol, followed by centrifugation. The supernatants were, then, discarded and 1 ml of 70 % ethanol was added, followed by vortexing and 3 minutes of incubation at room temperature. The samples were centrifuged; the supernatant was discarded and they were left to dry. Then, 100 µl of sterile water was added to each tube and the polymerase chain reaction (PCR) was prepared with 2 µl of sample, 0.2 µM of each primer (forward primer for WT sequence: 5' TGT CCT CGT CCT CCT ACC GC 3'; forward primer for *Vim* -/- sequence: 5' AGC TGC TCG AGC TCA GCC AGC 3'; common reverse primer: 5' CTG TTC GCC AGG CTC AAG GC 3') and 21.5 µl of Taq Ready Mix (GE Healthcare, UK). The PCR conditions are described in table 4.1. Next, the samples were resolved in a 2 % agarose gel.

Table 4.1 PCR conditions used for genotyping.

Step	Temperature (ºC)	Time	Cycles
Denaturation	95	3 minutes	1
Denaturation	94	1 minute	35
Annealing	65	20 seconds	
Extension	72	30 seconds	
Final extension	75	5 minutes	1
Hold	4	infinite	-

4.1.2 Body weight

To evaluate body weight, WT and *Vim* -/- male mice with of three and six months old were weighted.

4.1.3 Organ weight and Size

To evaluate organ size, WT and *Vim* -/- male mice with the same age were sacrificed by cervical dislocation. The organs of interest, kidney and heart, were collected, photographed and weighted. The organs were fixed with 3 % paraformaldehyde (PFA) for further experiments.

4.1.4 Tissue sectioning and staining

The organs of interest, liver, kidney, intestine and adipocyte, of the sacrificed mice were fixed with PFA and embedded paraffin. The sectioning and haematoxylin-eosin staining was carried out by Loinais-Suomen pathology laboratory. The sections were imaged using Panoramic Slide Scanner (3DHISTECH, Hungary) and Image J was used for analysis.

4.2 Cell lines and cell culture

All the cell lines were cultured in Dulbecco's modified medium (DMEM) supplemented with 10 % fetal calf serum (FCS), 2 mM of L-glutamine, 100 U/ml of penicillin, 100 µg/ml of streptomycin at 37 ºC in a 5% CO_2 incubator and passaged when they were about 80 % confluent.

Immortalized mouse embryonic fibroblasts (MEF), wild type and *Vim* -/- were used (113). Human dermal fibroblasts (HDF) were bought from American Type Culture Collection (ATCC).

4.1.1 Transfections

Vim -/- MEFs were seeded in 6-well plates on the day prior transfection. The cells were transfected with 10 µg of pCMV script-vimentin and untransfected cells were used as a control. The transfection method that was used was Xfect^{TM} (Clontech Laboratories, USA), according to the instructions of the manufacturer. This chemical method uses a biodegradable polymer has a transfection agent and it has very low cytotoxicity and it is very efficient. These transfection experiments aimed to understand whether the WT phenotype and signalling could be rescued by inserting vimentin plasmid into *Vim* -/- MEFs. Therefore, these transfected cells were used for volume analysis.

4.1.2 Treatments

Human dermal fibroblasts were starved with serum free medium (SFM) on the day prior to co-immunoprecipitation. The next day they were treated either with 5 % FCS or 100 nM of insulin for 10 minutes and cells with no treatment were used as control. An additional 10 minute treatment with 10 nM calyculin A, a phosphatase inhibitor, was made for the co-IP of TSC2 as a positive control and, once again, cells without this treatment were used as control. These treatments allowed stimulation of the insulin-Akt-mTOR pathway so that the signalling could be evaluated. The same treatment was applied to MEFs before evaluating mTORC1 activation by SDS-PAGE and Western Blotting, but 10 nM of insulin was used instead. A 1 nM thymidine treatment of 24 hours was made to the cells before performing cell volume assays to ensure that the cells were all at the same cell cycle stage (G1/S phase) and exclude volume differences related to cell cycle.

4.2 Assays

4.2.1 Cell volume assay

In order to evaluate the cell volume, WT and *Vim* -/- MEFs were grown in 6-well plates overnight. The samples were trypsinised and fixed with 3 % PFA for 20 minutes in the dark and then stained with 2 µM of CellTracker^{TM} fluorescent probes (ThermoFisher Scientific, USA), which pass through the cell membrane freely, become impermeant and are well retained by the cell.

Leica TCS SP5 Matrix confocal microscope was used to image the samples and to take a z-stack to be able to calculate the cell volume. The results were analysed using Imaris® software 8.1 (BITPLANE, Switzerland). Three biological replicates were performed for this experiment.

4.2.2 Total protein concentration assay

In order to evaluate the protein concentration, WT and *Vim* -/- MEFs were seeded in 6-well plates and incubated overnight. On the next day, the cells were trypsinised and $3{,}0x10^5$ were lysed for 1 hour in a buffer containing 150 mM NaCl, 1 % Triton X-100, 0.2 % SDS, 50 mM Tris pH 8.0 and 0.5 % of sodium deoxycholate at 4 ºC. Then a BCA assay (ThermoFisher Scientific, USA) was performed according to manufacturer's instructions. This colorimetric assay is based on the reduction of copper by protein in an alkyne medium

(biuret reaction), forming a purple complex, which absorbance can be measured at 562 nm. Three biological replicates were made for this experiment.

4.2.3 Protein synthesis assay

In order to evaluate protein synthesis rate, WT and *Vim* -/- MEFs were seeded in 96-well plates and starved overnight. The assay was carried out using the Click-iT® HPG Alexa Fluor® Protein Synthesis Assay Kits (ThermoFisher Scientific, USA), which uses an analogue of methionine containing an alkyne that is incorporated during protein synthesis and can be detected by a chemoselective reaction between the alkyne and an azide present in the fluorescent dye (Alexa Fluor 488) to quantify protein synthesis. The medium was changed to methionine free with 50 µM of the methionine analogue. The cells were treated with 100 nM of insulin for 30 minutes and cells with no treatment were used as control. This was followed by PBS wash, 20 minutes of 3 % PFA fixation, 5 minutes of PBS 0,3 % Triton X permeabilization and 1 hour of blocking at room temperature with PBS 0,3 % Tween 3 % BSA. Then, the Click-it reaction was prepared according to the instructions and added to the cells for 30 minutes in dark. The cells were washed and kept in PBS. The imaging was performed with Cell-IQ (CM Technologies Oy, Finland) using the green channel and 10 X plan, 1392x1040 pixels. The analysis of the images was carried out with the Cell-IQ Analyser software by measuring the intensity. Three biological replicates were performed.

4.3 Co-Immunoprecipitation

Three groups of starved HDF cells were used for this experiment, in which cells were either treated with 5 % FCS or 100 nM of insulin for 10 minutes, and cells with no treatment were used as control. The cells were washed with PBS and lysed with a lysis buffer containing 0.3 % CHAPS, 40 mM HEPES, 120 mM NaCl, 1 mM EDTA, phosphatase inhibitor and proteinase inhibitor and then collected to tubes. The samples were shaken for 1 hour at 4 ºC, while the magnetic beads were being washed. The samples were centrifuged, the supernatant was collected and the protein concentration was measured using a BCA assay. New tubes with 500 µg of protein were incubated with 0.20 µg of magnetic beads at 4 ºC for 1 hour. The samples were placed on the magnet; the supernatant was collected to new tubes and 1 ng of antibody (table 4.2) was added for incubation during 1 hour at 4 ºC. IgG was used as a negative control. Then, 0.25 µg of magnetic beads was added to each tube,

followed by 1 hour incubation at 4 ºC. The samples were finally washed with lysis buffer and boiled for 10 to 15 minutes at 98ºC with 1x laemmli buffer (62.5 mM Tris-HCl pH 6.8, 1 % SDS, 10 % glycerol, 0.005 % bromophenol blue and 1 % beta-mercaptoethanol).

4.4 Immunostaining

4.4.1 Volume analysis

The MEF WT and *Vim* -/- cells and rescued cells were trypsinised and centrifuged. The samples were fixed with 3 % PFA and incubated for 20 minutes in the dark. After centrifugation, the samples were washed three times with PBS and permeabilised for 5 minutes with PBS 0.3 % tween. The blocking was performed for 1 hour using PBS 0.3 % tween 3 % BSA. Vimentin primary antibody (Biolegend, USA) was diluted 1:1000 in PBS 0.3 % tween 3 % BSA. The samples were incubated with the antibody overnight at 4 ºC. After washing the samples three times with PBS 0.3 % tween, they were incubated with the secondary antibody (chicken, Invitrogen, UK), diluted 1:500 in PBS 0.3 % tween 3 % BSA, for 1 hour at room temperature, in the dark. The samples were then washed once again and kept in PBS at 4 °C in the dark for further analysis.

4.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting

The samples were prepared by heating for 10-15 minutes at 98 ºC with 1x laemmli buffer, and then resolved in a SDS-PAGE gel (6-15 %) with a buffer containing 250 mM glycine, 25 mM Tris-base and 0.1 % SDS. The gels were run for 30 minutes at 80 V, followed by 90 minutes at 120 V. The resolved samples were transferred to a nitrocellulose membrane using wet transfer, with 400 mA for 1 hour. The buffer used contained 192 mM glycine, 24 mM Tris-base and 20 % methanol. Next, the membranes were blocked with 5 % milk in PBS 0.3 % Tween and then incubated with the desired primary antibody (table 4.2), diluted 1:1000 in 3 % BSA in PBS 0.3 % Tween, overnight at 4 ºC. On the next day, the membranes were washed with PBS 0.3 % Tween and incubated with the appropriate secondary antibody (table 3.3.), diluted 1:10000 with 5 % milk in PBS 0.3 % Tween, for 1 hour at room temperature.

The membranes were washed once again and exposed with enhanced chemiluminescence (ECL) western blotting substrate (ThermoFisher Scientific, USA).

4.6 Microscopy

4.6.1 Cell Volume

The ©Leica TCS SP5 Matrix confocal microscope was used to measure 3D cell volume with 20X plan, 1024x1024 pixels, with both red and green channels. A z-stack was taken to all samples for further volume analysis.

4.6.2 Protein synthesis

The imaging was performed with Cell-IQ (CM Technologies Oy, Finland) using the green channel and 10 X plan, 1392x1040 pixels. The analysis of the images was carried out with the Cell-IQ Analyser software by measuring the intensity of the fluorescence.

Table 4.2 List of primary antibodies and respective secondary antibodies used for WB and Co-IP.

Primary Antibody	Used for	Company	Secondary Antibody	Company
S6	WB	Cell Signalling Technology, USA	Rabbit	Promega, USA
p70S6K	WB	Cell Signalling Technology, USA	Rabbit	Promega, USA
p-S6	WB	Cell Signalling Technology, USA	Rabbit	Promega, USA
p-p70S6K	WB	Cell Signalling Technology, USA	Rabbit	Promega, USA
Insulin Receptor	WB	Cell Signalling Technology, USA	Rabbit	Promega, USA
HSC70	WB	Enzolifesciences, USA	Rat	GE Healthcare, UK
RICTOR	WB	Bethyl, USA	Rabbit	Promega, USA
RICTOR	IP	Millipore, UK	Mouse	Proemga, USA
TSC2	WB, IP	Cell Signalling Technology, USA	Rabbit	Promega, USA
Vimentin	WB	Cell Signalling Technology, USA	Rabbit	Promega, USA
Vimentin 9	WB	Sigma-Aldrich, USA	Mouse	Promega, USA
IgG	IP	Millipore, UK	Mouse	Promega, USA
IgG	IP	Cell Signalling Technology, USA	Rabbit	Promega, USA

4.7 Mass spectrometry analysis

The samples used for mass spectrometry were prepared from a co-IP of RICTOR as described previously. Then the samples were run in a precast 10 % SDS-PAGE gel (Bio-rad, USA) for 1 hour at 200 V, followed by silver staining. To identify the phosphorylation sites, we performed in-gel alkylation and digestion with trypsin, C18 reverse phase chromatography and LC-MS/MS using a Q Exactive (ThermoFisher Scientific, USA), a ESI-hybrid-quadrupole-orbitrap. The mobile phase was water 0.1 % formic acid (FA) (v/v) (solvent A) or acetonitrile (ACN)/water 80:20 (v/v) 0.1 % FA (v/v) (solvent B) at a flow rate

of 300 nl/min. The peptides were separated by gradient elution from 5-43 % solvent B in 18 minutes, followed by 2 minutes of 43-100 % (v/v) solvent B and 5 minutes of 100 % solvent B. Before running the samples, they were dissolved in 0.1 % FA. The scan range was set from 300-17500 m/z with HCD (higher energy collisional dissociation) fragmentation and up to 10 data-dependent MS/MS spectra were fragmented in each scan with 10 seconds' dynamic exclusion. An inclusion list of theoretical tryptic peptides was made by *in-silico* digestion using Swiss-Prot (*Homo Sapiens* vimentin) in Skyline (MacCoss Lab Software, USA) to increase the probability of identification of the peptides. The analysis of the results was made using Proteome Discoverer 2.1 (ThermoFisher Scientific, USA) and the database search was made using Mascot 2.5.1 (Matrix Science, USA), using phosphorylation and methylation as variable modifications and carbamidomethylation as fixed modification with 10 ppm of mass tolerance for precursors and 0.2 Da for fragment ions with $m/z \geq +2$. It was allowed one miss-cleavage and the validation was made using decoy database search with target false discovery rates (FDR) of 0.01 (strict) and 0.05 (relaxed); and ptmRS with a confidence threshold set to $p<0.05$.

4.8 Statistical Analysis

All the statistical analysis was carried out using the GraphPad Prism 7 (GraphPad Software Inc., USA). An unpaired non-parametric student's t-test was conducted for all the data with $n \geq 3$ biological replicates. The data is presented in the form of mean ± standard error of the mean (SEM).

4.9 Figure panels and illustrations

All illustrations were made with Adobe Illustrator and using Servier Medical Art. All figure panels were made using CorelDRaw X8 (Corel, Canada).

5 Results

5.1 Loss of vimentin reduces cell size

Vimentin is an IF protein that plays a role as a scaffold in many cell signalling pathways related to proliferation, migration and survival. Initially, MEFs lacking vimentin were observed to be smaller in size, when compared to WT MEFs (figure 5.1A). This lead us to hypothesise that vimentin plays a role in growth regulation. To test this hypothesis and to confirm that the observed difference was not caused by differential cell spreading, we quantified the cell volume of wild type (WT) and vimentin knockout (*Vim* -/-) MEFs. The cells were trypsinised, fixed and stained with a dye for the whole cell. Using confocal microscopy (figure 5.1B) and imaging tools, the 3D cell volume was measured (figure 5.1C). We found that *Vim* -/- MEFs were 20-25 % smaller than WT MEFs (figure 5.1D). The same experiment was conducted using cells treated with thymidine for 24 h, which synchronised the cells in G1/early S phase to exclude differences due to cell cycle progression. We observed a similar difference in cell volume (figure 5.1E), supporting the previous experiment. Then, we wanted to confirm whether vimentin was responsible for this phenotype or not, so we transfected *Vim* -/- MEFs with 10 µg of pcMV-script vimentin and we performed the same cell volume experiment. Indeed, an increase in cell volume was observed after rescuing the cells (figure 5.1F), suggesting that the lack of vimentin makes the cells smaller than WT. However, this experiment lacked an empty vector control and it will be performed again. To further confirm these results, we lysed both WT and *Vim* -/- MEFs and we measured protein amount using a BCA assay, since cell volume is directly related with the amount of protein in the cells. With this experiment, we observed that protein amount in *Vim* -/- MEFs was significantly lower than in WT MEFs, supporting the previous results on cell volume (figure 5.1G). Cell growth is tightly regulated by insulin and other growth factors that lead to protein synthesis. Since the protein concentration was lower in *Vim* -/- MEFs, we then performed a protein synthesis assay using a fluorescent marked methionine protein synthesis assay kit. The relative level of protein synthesis was significantly lower in *Vim* -/- MEFs, which suggests that the difference seen in the phenotype could be due to a malfunction in the protein synthesis pathway, caused by the lack of vimentin. Further, insulin stimulation after cell starvation lead to an increase in cell volume in WT MEFs but not in *Vim* -/- MEFs (Mohanasundaram *et al.* unpublished), suggesting that loss of vimentin disrupts insulin dependent signalling in MEFs.

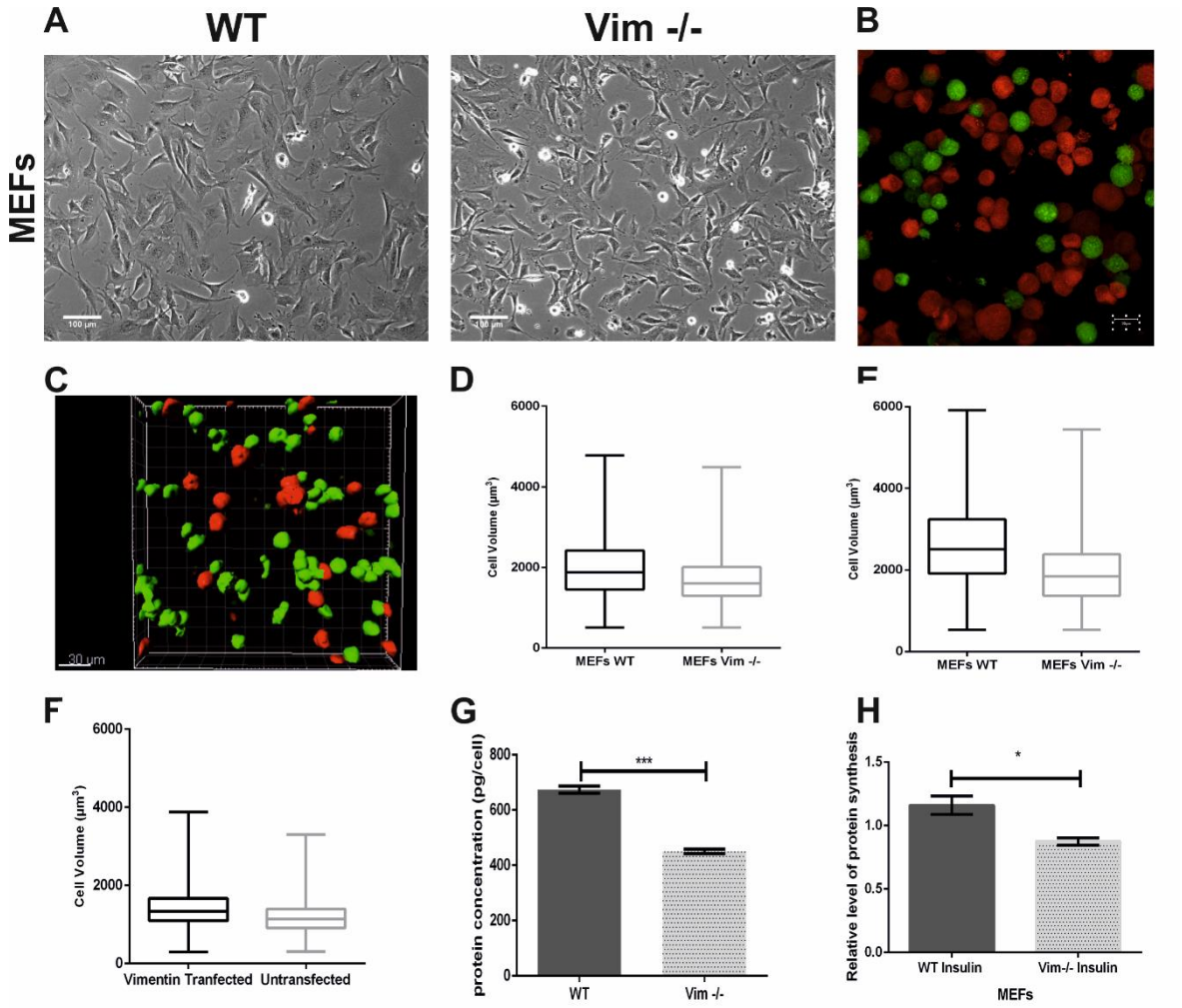

Figure 5.1 *Vim* -/- MEFs are smaller than WT. a. Phase contrast representative image of WT and *Vim* -/- MEFs showing that *Vim* -/- MEFs are smaller than WT. **b.** Maximum projection of WT MEFs shown in red and *Vim* -/- MEFs in green after a z-stack taken with a confocal microscope. **c.** Representative 3D reconstruction made with Imaris software (BITPLANE) of a z-stack taken to the MEFs which was used to estimate the volume of the cells **d.** Cell volume of *Vim* -/- MEFs is lower than WT. **e.** Cell volume of *Vim* -/- MEFs is lower than WT after a 24 h thymidine treatment. **f.** Rescued MEFs show an increase in cell volume when compared with untransfected *Vim* -/- MEFs. **g.** *Vim* -/- MEFs have lower protein amount. The same number of WT and *Vim* -/- MEFs were lysed for 1 hour at 4ºC and then a BSA assay was conducted. **h.** Fluorescent based protein synthesis assay shows decreased protein synthesis in *Vim* -/- MEFs upon insulin stimulation. The results are presented in the form of mean ± standard deviation of the mean of three biological replicates (n=3) and a student t-test was performed. *** $p<0.001$; *$p<0.05$.

Interestingly, studies in mice showed that *Vim* -/- mice weight 20-25 % less than WT mice (figure 5.2A) and fellow lab colleagues demonstrated that the body mass index (mass/height^2) of *Vim* -/- mice was significantly lower than WT mice (Mohanasundaram *et al.* unpublished). In addition, kidneys and hearts isolated from *Vim* -/- mice were not only lighter than the WT organs (figure 5.2B) but also smaller (figure 5.3C-D). As a support, we sectioned various tissues from the mice. Using haematoxylin-eosin (HE) staining, followed by imaging and cell size quantification, we found that adipocytes from *Vim* -/- mice were

significantly smaller than the WT adipocytes (figure 5.2E-F). Other sections were made for kidney, liver and smooth muscle but the size analysis was not successful because these

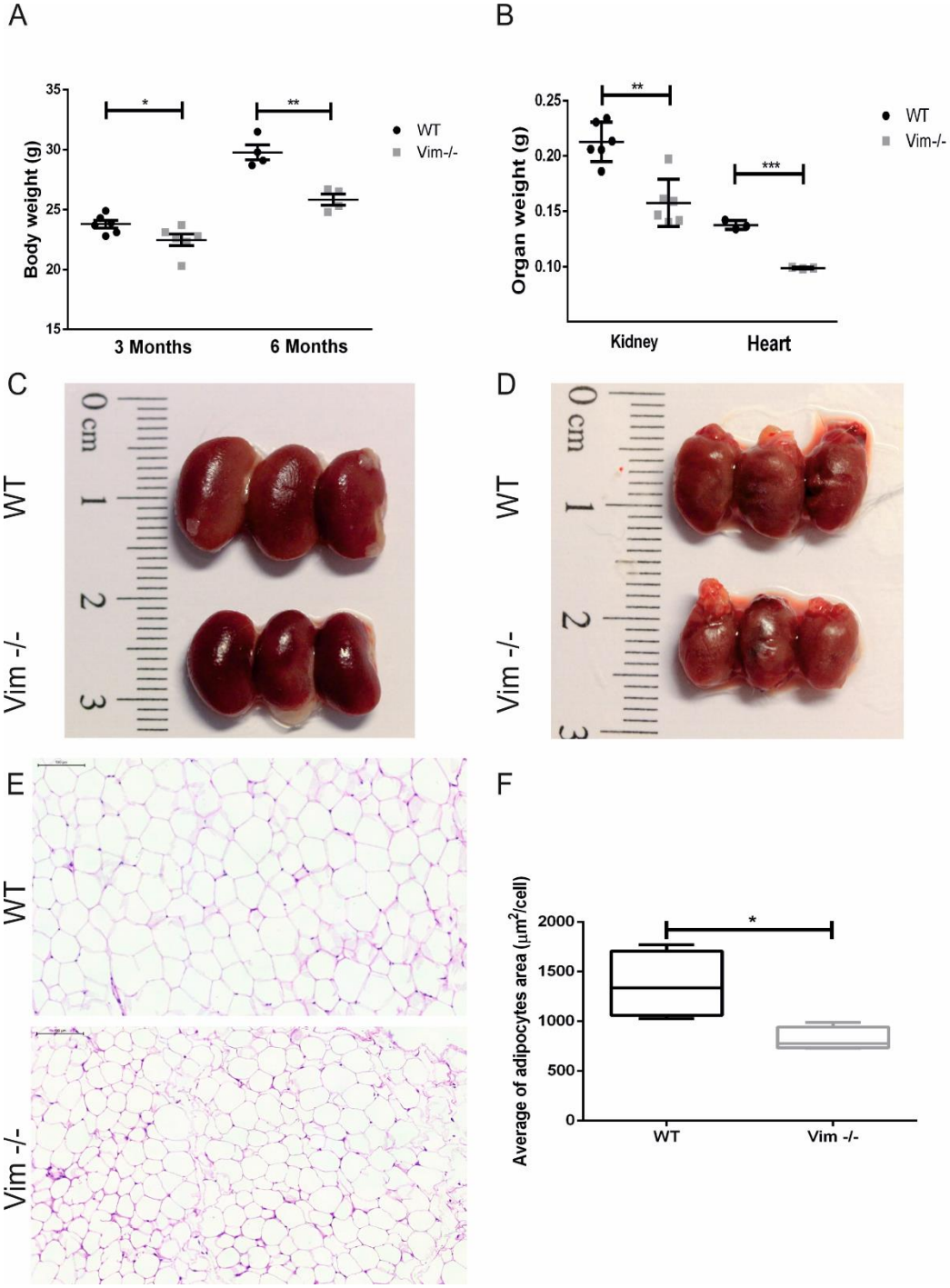

Figure 5.2 *Vim* -/- mice are smaller than WT. a. body weight of a group of WT and *Vim* -/- male mice of 3 months old (n=6) and a group of WT and *Vim* -/- male mice of 6 months old (n=4), showing decreased body weight in about 20 % in *Vim* -/- mice. **b.** kidney (n=6) and heart (n=3) organ weight of male mice of 4 months old, showing decreased organ weight in *Vim* -/- mice. **c.** *Vim* -/- kidneys are smaller than WT. **d.** *Vim* -/- hearts are smaller than WT. **e.** Adipocytes show decreased cell size in *Vim* -/- mice. **f.** The area of the adipocytes was quantified using ImageJ tools and a student t-test was used to evaluate the significance of the results. Sections of WT and *Vim* -/- (n=4) mice with 3 months old were used. The results are presented in the form of mean ± standard deviation of the mean of the biological replicates. *p<0.05; **p<0.01.

tissues are very dense, compact and complex. Supporting results were obtained by fellow lab colleagues by EcoMRI, which showed that *Vim* -/- mice are much leaner that WT mice (Mohanasundaram *et al.* unpublished).

5.2 Vimentin regulates cell size through insulin/Akt/mTOR signalling

The main pathway regulating protein synthesis production is the Akt/mTOR pathway, triggered by insulin and other growth factors, by binding to insulin and IGF receptors. Therefore, the next step was to evaluate differences in the Akt/mTOR pathway between WT and vim –/- MEFs, using biochemical techniques. We starved WT and *Vim* -/- MEFs overnight and stimulated them with insulin or FCS for 10 minutes. Then, we harvested the cell lysates with laemlli buffer and boiled the samples for 15 minutes at 98 ºC. We performed SDS- PAGE and western blotting and we found that the phosphorylation of p70S6K on T389 and S6 on S234/236 were attenuated in *Vim* -/- MEFs (figure 5.3A). These proteins are located immediately downstream mTORC1 and p70S6K is directly phosphorylated by it. Therefore, these proteins are widely used to check mTORC1 activation. These results suggest that the activation of mTORC1 is dysregulated in the absence of vimentin, since there is less phosphorylation of p70S6K and S6 in *Vim* -/- MEFs after stimulation. Further, there was no significant difference in insulin receptor expression (figure 5.3B), excluding differences related to the number of receptors in the cells. Taken together, these data point to an interaction between vimentin and upstream mTORC1 regulators. To further investigate this, Co-IP studies were conducted to evaluate protein interactions.

5.3 Vimentin interacts with the Akt/mTOR pathway after stimulation

Akt must be phosphorylated twice for full activation, once by PDK1 and a second one by mTORC2. Since mTORC1 activity was lower in *Vim* -/- MEFs, one explanation could be that mTORC2 was not phosphorylating Akt preventing it from being fully active. Therefore, we decided to investigate whether mTORC2 interacted with vimentin. To do so, HDF cells were starved with serum free medium the day before the co-IP. Then, they were treated either with FCS or insulin for 10 minutes and cells with no treatment were used as control. We performed co-IP of RICTOR, a subunit that is specific for mTORC2, which allowed us to evaluate the interactions with other proteins. Then SDS-PAGE and western blotting was

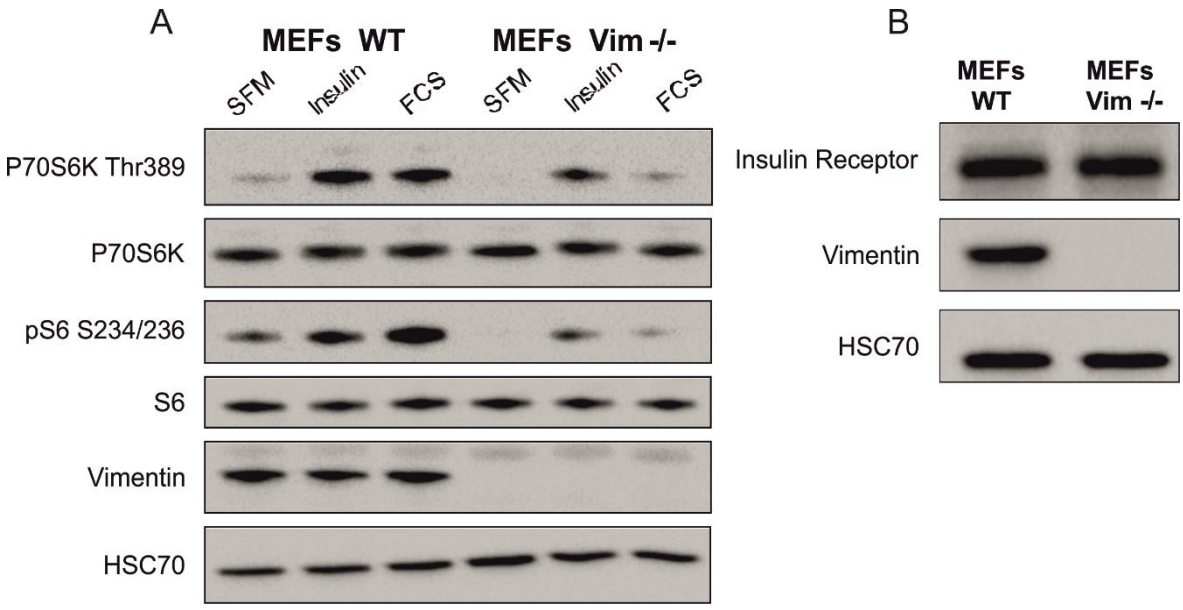

Figure 5.3 mTORC1 activation is lower in Vim -/- MEFs. a. Phosphorylation of p70S6K and S6, the proteins immediately downstream mTORC1, is lower in Vim -/- MEFs, indicating that the mTORC1 activation is also reduced. The cells were starved with serum free medium (SFM) the day before the assay. Then, they were treated either with 5 % fetal calf serum (FCS) or 10 nM of insulin for 10 minutes and cells with no treatment were used as control, followed by SDS-PAGE and western blotting. **b.** No difference was seen in insulin receptor expression, excluding changes due to the number of receptors available. HSC70 was used as loading control. Three independent trials were conducted for this experiment.

interaction between vimentin and RICTOR was seen upon stimulation of the pathway (figure 5.4A). This suggests that vimentin might enhance mTORC1 activation, possibly by promoting further activation of Akt. Phosphorylation of Akt upon activation of the pathway leads to phosphorylation of the TSC complex, releasing and activating mTORC1. Since mTORC1 activation was previously shown to be lower in *Vim* -/- MEFs, we also checked interactions between vimentin and the TSC complex by co-IP. Our preliminary data suggests that vimentin interacts with TSC2 after stimulation (figure 5.4B), as a vimentin band is seen upon insulin or FCS stimulation upon reblotting the TSC nitrocellulose membrane for vimentin. In addition, the interaction between vimentin and TSC in the serum starved condition of calyculin A treatment suggests that this interaction occurs upon phosphorylation, since calyculin A is a phosphatase inhibitor, so the proteins will be in their phosphorylated state. Taken together, these data point to an- interaction between vimentin and both mTORC2 and TSC2 that could be in a phosphorylation-dependent event. Therefore, we then decided to do mass spectrometry to see which sites were phosphorylated in each of the treatments. To do so, we performed co-IP of RICTOR as done before, using the same conditions, we ran a SDS-PAGE gel and isolated the vimentin band after silver-

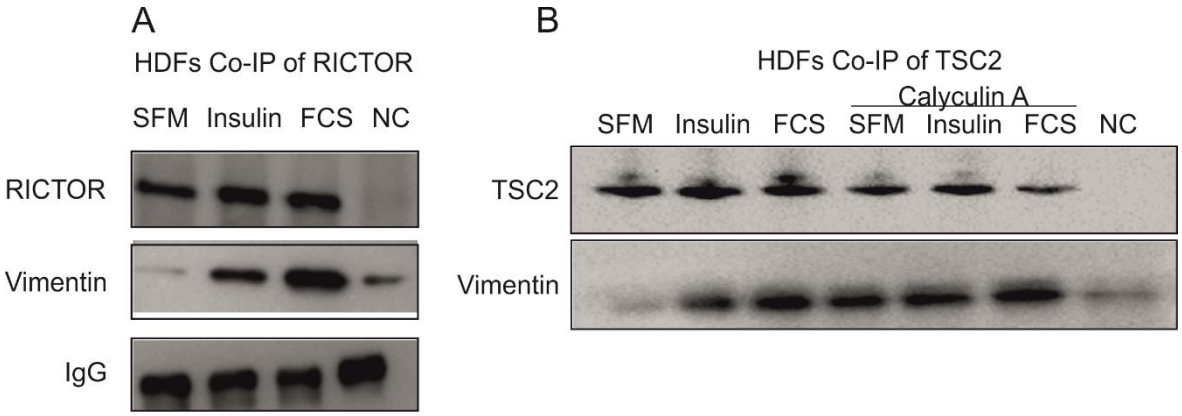

Figure 5.4 Vimentin interacts with the Akt/mTOR pathway after stimulation. a. Representative of three independent experiments of co-immunoprecipitation of RICTOR, one of the specific subunits of mTORC2. Vimentin interacts with RICTOR upon stimulation with either insulin or fetal calf serum (FCS) suggesting that vimentin contributes to the complete activation of Akt, promoting cell growth. **b.** The interaction seen after stimulation suggests that vimentin could be enhancing protein synthesis by sequestering the TSC2 inhibitory complex. In addition, the interaction in the serum starved condition of calyculin A treatment suggests that this interaction occurs upon phosphorylation. HDF cells were starved with serum free medium the day before the co-IP. Then, they were treated either with 5 % FCS or 100 nM of insulin for 10 minutes and cells with no treatment were used as control. For TSC2, an additional 10 minute treatment with calyculin A was done to the cells and cells without this treatment were used as control. The co-IP was performed with magnetic beads, using IgG as a negative control (IgG) The co-IP was followed by SDS-PAGE and western blotting.

staining. We digested the samples, separated them by liquid chromatography (LC) and performed a data dependent run using a ESI-hybrid-quadrupole-orbitrap mass spectrometer. The results are presented in table 5.1 where phosphorylated sites are green and non-phosphorylated ones are red. The vimentin phosphorylated sites upon insulin or FCS treatment that are not phosphorylated in SFM cells, such as Y150, S278 and Y383 are good candidates for the possible phosphorylation-dependent interaction between vimentin and RICTOR seen with the co-IP results.

Table 5.1 Summary of the results obtained from the co-IP of RICTOR with mass spectrometry in a data dependent run. The first column indicates the treated samples and the first row the phosphorylation sites. The colour code indicates if the site was found to be phosphorylated with high confidence (green) or not phosphorylated at all (red). After the treatment, co-IP of RICTOR was performed, followed by SDS-PAGE. The band corresponding to vimentin was cut and digested for each sample. The samples were separated by LC and they were run in a ESI-hybrid-quadrupole-orbitrap mass spectrometer. Only one experimental replicate was performed for this experiment.

	S5	S22	S29	S39	S56	S83	T101	Y150	T165	T202	S226	S214	Y276	S278	S299	S316	S325	T336	Y358	Y383	S412	T426	S430	T458	S459
SFM																									
INS																									
FCS																									

6 Discussion

The existing knowledge on the roles of vimentin in the cell is much greater than when vimentin was seen only as a cytoskeletal protein. Today, we understand that this protein promotes key processes in the cell, such as proliferation, migration and invasion. This study began by identifying the difference in cell size and volume between WT and vim -/- MEFs, as well as protein content. These results showed that vim -/- MEFs are not only smaller, with lower volume, but have also lower protein amount. Therefore, the differences in size are not because of the lack of a cytoskeletal protein but due to overall lower protein mass in vim -/- MEFs. Cell size changes depending on which cell cycle phase the cells are. So, we performed a 24 hour treatment with thymidine, which allowed us to exclude size differences related to cell cycle because this compound synchronizes the cells in G1/S phase (114,115). Then the rescuing experiment showed an increase in cell volume after vimentin transfection into vim -/- MEFs, suggesting that it is the lack of vimentin that causes the smaller phenotype. However, this experiment lacked an empty vector control because we didn't have the right one, but it will be included in the future. Also, protein content and protein synthesis experiments should be performed with the transfected cells to complement the results, along with SDS-PAGE and western blotting of the proteins of the Akt/mTOR pathway. The lower relative level of protein synthesis allowed us to link the size differences with the Akt/mTOR pathway. In addition, these results are supported by previous unpublished results in lab that showed that after starvation for 36 hours, WT MEFs increase their size after stimulation with insulin, unlike vim -/- MEFs, demonstrating very clearly a problem in insulin dependent signalling. This phenotype described here agrees with the mice data that demonstrated the lower body weight, organ size and smaller adipocytes in vim -/- mice. We had several other tissue sections, including smooth muscle, kidney and liver but we were not able to successfully study the cell size difference because unlike adipose tissue, these tissues are very dense and complex, so it is very challenging to measure, we would need higher resolution. Our lab has skin sections where there is also a difference in smooth muscle, and Eco-MRI results show that the overall fat and muscle tissue is lower in vim -/- mice. If it was possible to successfully section skeletal muscle, one would expect a difference, as it is a tissue that responds to insulin, but that is challenging to do because the tissue would lose its stretched shape, so the analysis would not be as accurate. The difference in organ size could be due to less collagen deposition as well because our lab has previously published

that vim -/- MEFs produce less collagen when compared to WT MEFs (36). Nevertheless, we plan to perform the cell volume analysis on primary cells isolated from these tissues, which would be a better way to compare the results.

In general, the results summarized so far are in agreement with what has been described in the literature about mutations in the Akt/mTOR pathway (103–108). Indeed, we observed less mTORC1 activation in vim -/- MEFs after stimulation, as well as lower protein synthesis, suggesting a dysregulation in the Akt/mTOR pathway due to the absence of vimentin. This is one of the major pathways in the cell; it not only leads to proteins synthesis but its members also control proliferation, autophagy, glucose uptake, lipogenesis, motility and many other processes (77,92–94). Furthermore, vimentin has already been linked to some of these processes, namely, proliferation, autophagy and motility (25,36,94). Therefore, here we provide a novel role for vimentin, regulation of cell growth. It would be interesting to evaluate the expression and activation of more proteins in the pathway to fully understand this mechanism. The co-IP of RICTOR showed an interaction with vimentin. RICTOR is part of mTORC2, a complex that can phosphorylate and fully activate Akt. It was recently published that mTORC2 can activate the insulin receptor itself (116). It was demonstrated that mTORC2 has tyrosine kinase activity and it contributes to activation of the insulin receptor, suggesting that instead of the known autophosphorylation of the RTK, it would be a combined effort of autophosphorylation and phosphorylation by mTORC2. Also, Akt was demonstrated to be able to phosphorylate mTORC2 and promote its activation (117). These two feedbacks contradict the known inhibition of the insulin receptor by mTORC1 (118,119). However, studies have shown that muscle specific knockout of RICTOR does not abolish Akt T308 or S473 phosphorylation (120), which then means that mTORC2 is not required for full activation of Akt. On the other hand, another study has shown that knockout of RICTOR affects negatively on both Akt phosphorylation sites (121) and a recent study has that demonstrated that mTORC2 inhibition leads to suppression in Akt T308 phosphorylation (122). Contradictory results by another group state that there is an increase of Akt T308 phosphorylation in RICTOR -/- mice (123). These discrepancies might be because of the existence of compensatory mechanisms or the use of different models for the studies. Nevertheless, here we provide a new step in signalling with our Co-IP results: vimentin interacts with mTORC2 and regulates cell growth. This is in agreement with the previous studies that showed that mTORC2 contributes to the full activation of the

Akt/mTOR pathway (116,117,121,122). It was previously published that Akt phosphorylates vimentin leading to cell motility (51). This Akt-mediated phosphorylation could be one explanation for cell growth as well, but by controlling different downstream proteins. Upon insulin receptor activation, Akt phosphorylates mTORC2, TSC and vimentin causing them to interact and contribute to the full activation of Akt (figure 6.1.). Supporting this claim, there is a study showing that the TSC inhibitory complex is required for proper activation of mTORC2 and that these two complexes physically interact (124). Although, for this claim, more studies of interactions and kinase activity need to be conducted. Our preliminary data of the co-IP of TSC2 suggested an interaction between vimentin and TSC2 after stimulation, possibly in a phosphorylation dependent manner. However, to support this claim, kinase inhibitors should be used. Our mass spectrometry data provided us with a series of candidates of vimentin phosphosites that could be involved in the interaction between vimentin and RICTOR. The phosphorylated sites after insulin and FCS stimulation that are not phosphorylated in the serum starved condition and vice versa, such as S278, Y150 and Y383 need to be further studied and validated. First, because the mass spectrometry data presented is the result of one trial only and second, this phosphosites need to be validated either by using phosphomutants or by heavy peptides in mass spectrometry, run along with the samples.

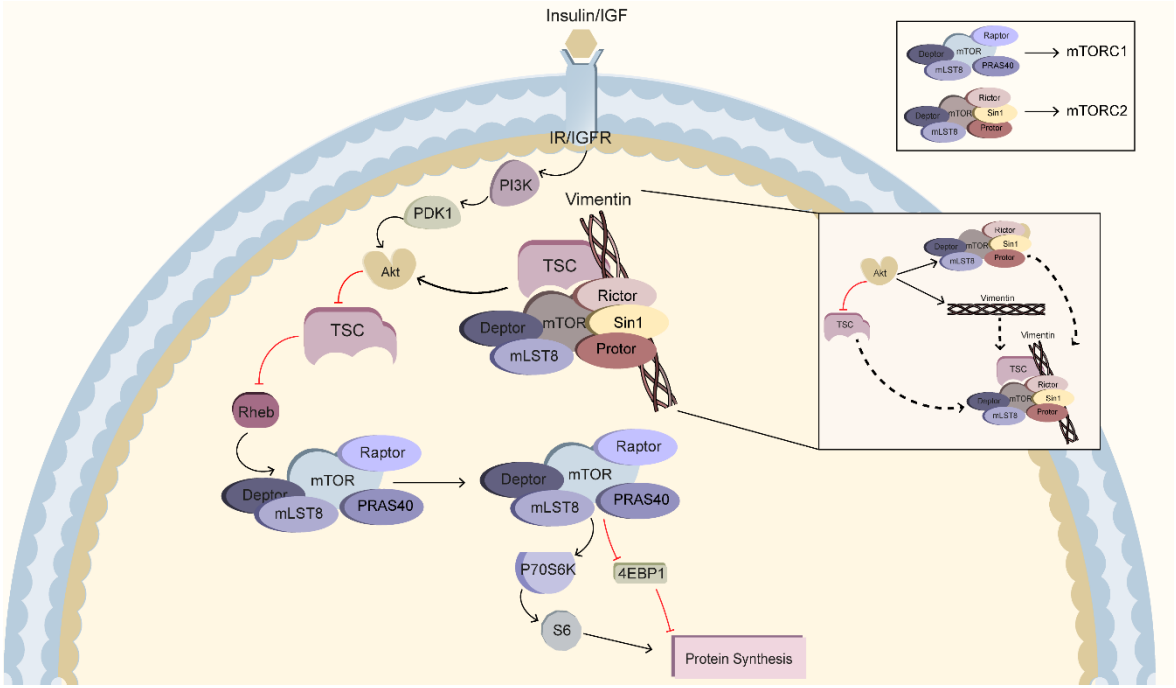

Figure 6.1 Overview of the proposed model. Upon activation of the pathway, Akt phosphorylates vimentin, TSC and mTORC2, causing them to interact to fully activate Akt and lead to protein synthesis.

Cell growth is an essential step for cell proliferation and full body homeostasis. The Akt/mTOR pathway is a key process in the cell and comprises a group of proteins that can provide all sorts of cues related to cell survival by interacting with different proteins. As an example, Akt provides for proliferation and growth, but also in glucose uptake and for lipid metabolism (77,93). mTORC2 has been shown to be essential for skeletal growth and bone formation in mice (125) as well as required for lipogenesis and hepatic glucose metabolism (126). Further, it has been shown to sustain thermogenesis via Akt-induced glucose uptake and glycolysis in brown adipose tissue (127), which is an anti-obesity mechanism. In fact, mice lacking p70S6K also show a lean phenotype and are protected against diet induced obesity (128). In addition, diet induced obese mice show higher activity of mTORC1 (129). Similarly, prolonged inhibition of mTORC1 with rapamycin in mice improved body composition, metabolism and lipid profile (130). Also, in both cases, p70S6K and mTORC1 downregulation increases lifespan in mice, due to a better metabolism (131). In agreement, our vim -/- mice have smaller adipocytes and are much leaner than WT. The lack of vimentin causes lower activity of the Akt/mTOR pathway and reduces the likelihood of the mice to develop diet induced obesity, therefore these models could be used to understand how to overcome this condition.

Several studies have shown that genes coding for proteins of the Akt/mTOR pathway can be amplified or overexpressed in cancer. Further, the proteins of the Akt/mTOR pathway can also be hyperactivated (reviewed in (132)). EMT is a key step in metastasis because it allows epithelial cells to acquire mesenchymal features, increasing their motility (reviewed in (133)). Studies have showed that the activation of the Akt/mTOR pathway is required for EMT (134–137). In NMuMG cells, the activation of the transforming growth factor β (TGFβ) induced PI3K/Akt (137) activation as well as mTORC2, which activation was shown to be required for EMT (135). The authors also referred that the activation of the TGFβ pathway was accompanied by an increase in cell size and protein content (134). Further, using rapamycin, an inhibitor of mTORC1, impaired the invasive behaviour of the cells (134). Studies with a different cell line showed less collagen deposition upon knockdown of RICTOR (136). Taken together, these studies highlight an interaction between EMT and the Akt/mTOR pathway in cancer. Vimentin has already been linked to EMT and collagen deposition (35,36) and it was reported to be an EMT marker. Therefore, an increase in cell motility is expected when cancer cells start expressing vimentin and

undergoing EMT. The present work provides a link between vimentin and Akt/mTOR signalling, showing that vimentin promotes protein synthesis and cell growth by interacting with members of the Akt/mTOR pathway. Cell growth is also seen upon EMT activation by TGFβ signalling. Therefore, vimentin could be the scaffold protein bringing these pathways together and contribute to EMT by integrating these two complex signalling pathways.

7 Concluding remarks and future perspectives

The knowledge related to vimentin and its roles in the cell has evolved a great deal in the past years. Our laboratory showed very recently that vimentin is involved in cell proliferation and the present work shows that vimentin plays a role in cell growth regulation in MEFs by interacting with the Akt/mTOR pathway. This work first showed that *Vim* -/- MEFs have lower volume and protein amount, as well as lower protein synthesis, when compared to WT MEFs and we demonstrated that the WT phenotype could be restored by transfecting vimentin plasmid into *Vim* -/- MFEs. Also, we showed that the activation of mTORC1 was attenuated in *Vim* -/- MEFs, upon insulin or serum stimulation. With co-IPs, we showed an interaction between vimentin and mTORC2. By mass spectrometry we discovered several candidates for this interaction, which need to be further studied. In addition, an interaction between vimentin and TSC that seems to be in a phosphorylation dependent manner was also demonstrated. Therefore, combining these results with what was described in the literature, we postulated that upon activation of Akt, it would phosphorylate TSC, vimentin and mTORC2, leading to the formation of a complex that would contribute to the full activation of Akt and promote protein synthesis. Cell growth, a process highly dependent on the synthesis of macromolecules such as proteins, is an essential step for proliferation, development and full body homeostasis. Full activation of Akt, that seems to be deficient in vim -/- MEFs, is a crucial step for not only this pathway but also other cues that rely on Akt, such as glucose uptake, lipid metabolism and autophagy. Therefore, since vim -/- are leaner and have smaller adipocytes, the lack of vimentin reduces the likelihood of these mice developing diet induced obesity and that this model could be used to understand how to overcome this condition. For the future, we plan to isolate primary cells from different mouse tissues to perform the same cell volume analysis as we have shown here. We also want to continue the mass spectrometry experiments and proceed with the validation of these results using phosphomutants. In addition, we will try to fully understand the differences in the Akt/mTOR pathway in MEFs by checking the expression and activation of other proteins and validate the preliminary data of the co-IP of TSC2. We will also perform protein kinase activity assays to show the activity of the proteins involved in the interactions that we saw. Stimulated emission depletion microscopy (STED), a high-resolution technique will be used to show the interactions of vimentin with the proteins of the Akt/mTOR pathway and complement the results.

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