UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS QUÍMICAS

TESIS DOCTORAL

Role of the vimentin C-terminal domain in filament reorganization during cell división

Papel del dominio C-terminal de vimentina en la reorganización de filamentos durante la división celular

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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CENTRO DE INVESTIGACIONES BIOLÓGICAS MARGARITA SALAS (CSIC)

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Abbreviations

aa	Amino acid
ACR	Acrolein
AF	Actin Filament(s)
AGEs	Advanced glycation end products
ATP	Adenosine triphosphate
BAEC	Bovine aortic endothelial cells
Bleb	Blebbistatin
Cdk1	Cyclin dependent kinase 1
cyPG	Cyclopentenone prostaglandin
CytB	Cytochalasin B
DAPI	4',6'-diamino-2-fenil-indol
DIC	Differential interference contrast
EMT	Epithelial mesenchymal transition
F-actin	Filamentous actin
GDP	Guanosine diphosphate
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HNE	4-hydroxy-2-nonenal
HRP	Horseradish Peroxidase
IF	Intermediate filament(s)
Jasp	Jasplakinolide
LatA	Latrunculin A

MCV	Mutated citrullinated vimentin
MDA	Malondialdehyde
MT	Microtubule(s)
NFs	Neurofilaments
NHMC	Human mesangial cells
NO_2-POPC	Nitrated 1-palmitoyl-2-oleyl-phosphatidyl choline
Noc	Nocodazole
PBS	Phosphate buffered saline
PGA_1	Prostaglandin A_1
PKC	Protein kinase C
PTM	Post-translational modification(s)
PUFA	Polyunsaturated fatty acids
ROI	Region of interest
RCS	Reactive carbonyl species
RFP	Red fluorescent protein
STED	Stimulated emission depletion
ULF	Unit length filament
wt	Wild-type

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Summary in English

Role of the vimentin C-terminal domain in filament reorganization during cell division

The cell cytoskeleton is constituted by three main groups of structures, the actin filaments, microtubules and the intermediate filaments. The interplay between the three main cytoskeletal networks is determinant for cellular processes such as cell migration, signaling and mechanics, as well as during cell division. Vimentin is a type III intermediate filament protein that is broadly distributed. Vimentin forms an extended and dynamic filament network that plays diverse functions in a great variety of tissues and is involved in the pathogenesis of numerous diseases.

The vimentin monomer is constituted by three domains, a α-helical rod domain flanked by the disordered N-terminal and C-terminal domains. The N-terminal domain of vimentin is essential for filament assembly, yet the role of the C-terminal domain is still controversial. Nevertheless, the C-terminal domain is known to mediate the interaction with other proteins and divalent cations.

In this work, the importance of the vimentin tail domain in the distribution of vimentin filaments, both in resting cells and in mitosis has been addressed. Moreover, the interaction of vimentin with the actin cortex during mitosis as well as the structural elements necessary for this interaction have been studied.

The importance of the vimentin C-terminal domain was evaluated by generating a vimentin mutant that lacks the entire tail domain, vimentin (1-411). In vimentin-deficient cells, vimentin wt formed extended filaments, while vimentin (1-411) formed perinuclear bundles. Moreover, an overexpression of this truncated form disrupted the normal vimentin extended network. In mitosis, vimentin wt distributed towards the cell periphery whereas vimentin (1-411) stayed in close proximity of the dividing chromosomes and sometimes entangled them. This abnormal distribution induced alterations in cell division, such as asymmetric partition of vimentin and cell death, which were not observed in cells expressing vimentin wt. Increases in the proportions of mitotic cells showing lagging chromosomes and of cells with aberrant nuclei were also observed. Thus, the vimentin C-terminal domain is essential for normal filament distribution and mitotic progression.

Interestingly, the peripheral distribution of vimentin during mitosis takes place in a variety of cell types, including primary cells and cancer cell lines. Moreover, disruption of the microtubule or actin networks showed that vimentin peripheral redistribution is actin-

dependent and requires the integrity of the actomyosin cortex and the presence of the C-terminal domain.

Super-resolution microscopy analysis confirmed that vimentin is in very close proximity of the actin cortex in mitotic SW13/cl.2 and Vero cells, showing a high percentage of colocalization with actin at the cell periphery.

Importantly, vimentin was found to play a modulatory role in actin distribution and characteristics during mitosis. In vimentin-deficient cells, the f-actin signal showed to be more irregular than in vimentin-positive cells. Moreover, the presence of vimentin filaments at the bottom of the cell was associated with a lower content of basal f-actin.

Gradual removal of certain amino acid sequences from the C-terminal domain of vimentin progressively impaired normal filament elongation in interphase cells and peripheral distribution in mitosis. In particular, a drastic impairment in cortical association was observed upon deletion of the segment located between residues 424 and 448. Nevertheless, certain elongation-deficient vimentin mutants were able associate with the actin cortex in mitosis, thus indicating that formation of full filaments is not required for mitotic redistribution. However, the tail domain alone was not sufficient to induce cortical association of fluorescent chimeric proteins in mitosis.

Certain approaches mimicking pathophysiological conditions, including transfection with the HIV protease and treatment with electrophilic lipids, induced a marked rearrangement of vimentin filaments in interphase cells, promoting a condensation of vimentin close to the cell nucleus. In mitosis, a reduction of cortical vimentin was observed, causing the accumulation of vimentin close to the dividing chromosomes, which was associated with mitotic defects, such as cell death and asymmetric vimentin partition. Human vimentin possesses a conserved cysteine residue, C328 that is important for the effect of oxidants and for vimentin reorganization in response to electrophilic compounds in cells. A vimentin C328S mutant was more resistant to lipoxidation both in interphase and mitotic cells.

In conclusion, it is shown that vimentin filaments redistribute to the cell periphery in mitosis in a tail domain-dependent manner. The reorganization of vimentin during cell division suggests interplay of vimentin filaments with the actomyosin cortex in certain cell types. Moreover, pathophysiological conditions can be associated with altered vimentin distribution. Finally, the single cysteine residue of vimentin seems to play an important role in the response of vimentin to agents causing lipoxidation, thus potentially acting as a redox sensor.

Summary in Spanish

Papel del dominio C-terminal de vimentina en la reorganización de filamentos durante la división celular

El citoesqueleto está constituido por tres grupos principales de estructuras, los filamentos de actina, los microtúbulos y los filamentos intermedios. La vimentina es una proteína perteneciente a la familia de filamentos intermedios de tipo III que tiene una amplia distribución en el organismo. La vimentina forma una red celular de filamentos extendidos y dinámicos que desempeña diversas funciones en una gran variedad de tejidos y está involucrada en la patogénesis de numerosas enfermedades. En varios procesos celulares, como la migración, la señalización y la mecánica celular, se ha descrito que la vimentina interactúa con otras proteínas del citoesqueleto.

El monómero de vimentina consta de tres dominios, un dominio alfa helicoidal flanqueado por los dominios N-terminal y C-terminal. El dominio N-terminal de vimentina es esencial para el ensamblaje de los filamentos, mientras que se ha descrito que el dominio C-terminal participa en la interacción con otras proteínas y con cationes divalentes.

Por lo tanto, en este trabajo hemos estudiado la importancia del dominio C-terminal de la vimentina en la distribución de filamentos tanto en interfase como en mitosis. Además, también se han estudiado tanto su importancia en la interacción con la corteza de actina durante la mitosis como los elementos estructurales necesarios para dicha interacción.

La importancia del dominio C-terminal de vimentina se evaluó generando un mutante que carece de todo el dominio de la cola, la vimentina (1-411). En las células deficientes en vimentina, la vimentina wt formó filamentos extendidos, mientras que la vimentina (1-411) formó agregados perinucleares. Además, una sobreexpresión de esta forma truncada impidió la formación normal de la red extendida de vimentina. Durante la mitosis, la vimentina wt se distribuyó hacia la periferia celular y la vimentina (1-411) se mantuvo cerca de los cromosomas en división. Esta distribución anormal provocó problemas en la división celular, como la división asimétrica y la muerte celular, que no se observaron en las células con vimentina wt. También se observó un aumento de cromosomas mal distribuidos en células en anafase y de células en interfase con núcleos aberrantes. Por lo tanto, el dominio C-terminal de la vimentina es esencial para la distribución normal de filamentos y para la progresión mitótica.

Curiosamente, la distribución periférica de vimentina se observó en varios tipos celulares, como células tumorales y células de cultivos primarios. Además, la distribución periférica de vimentina mostró ser dependiente de la actina y requirió la integridad del cortex de actomiosina así como de la presencia de la cola de vimentina.

El análisis por microscopía de superresolución confirmó que la vimentina está muy próxima a la corteza de actina en las células SW13/cl.2 y Vero en mitosis, mostrando una alta colocalización con actina en la periferia celular.

Los resultados obtenidos indican que la vimentina desempeña un papel importante en la distribución y características de la actina durante la mitosis. En células deficientes en vimentina, la señal de f-actina fue más irregular que en las células con vimentina. Además, la presencia de filamentos de vimentina en la parte basal de la célula se asoció con una disminución de la f-actina en esa zona.

Una eliminación gradual de secuencias del dominio C-terminal de la vimentina perjudicó progresivamente la extensión de los filamentos y su distribución periférica durante la mitosis. En particular la eliminación de la secuencia entre los aa 424 y 448 mostró los efectos más drásticos. No obstante, algunas construcciones de vimentina deficientes en ensamblaje se asociaron con el cortex de actina en mitosis, indicando que la formación de filamentos extendidos no es necesaria para su distribución hacia la periferia de la célula. Sin embargo, la cola de la vimentina por sí sola asociada a proteínas fluorescentes no fue suficiente para promover la asociación cortical durante la mitosis.

Tratamientos que mimetizaron condiciones fisiopatológicas, incluyendo la transfección con la proteasa del VIH y la incubación con lípidos electrófilos, indujeron una reorganización de los filamentos de vimentina en las células en interfase, promoviendo una condensación de la vimentina cerca del núcleo celular. En mitosis, se observó una disminución de la vimentina cortical y una acumulación de vimentina cerca de los cromosomas en división. Esta distribución causó defectos mitóticos, como la muerte celular y la división asimétrica. La vimentina humana posee un residuo de cisteína conservado, la Cys328, que es importante para el efecto de oxidantes y para la reorganización de la vimentina en respuesta a compuestos electrófilos en las células. El mutante de vimentina C328S se mostró más resistente a la lipoxidación tanto en interfase como en las células mitóticas.

En conclusión, los resultados obtenidos muestran que los filamentos de vimentina se redistribuyen hacia la periferia celular durante la mitosis de manera dependiente del dominio C-terminal. La reorganización de la vimentina durante la división celular sugiere

una interacción con la corteza de actomiosina en algunos tipos celulares. Además, condiciones fisiopatológicas pueden estar asociadas a la alteración de la distribución de vimentina. Finalmente, el único residuo de cisteína de la vimentina parece tener un papel importante en la respuesta de la vimentina a agentes lipoxidantes, actuando así como potencial sensor redox.

Introduction

1. Cytoskeletal proteins

The cell cytoskeleton is considered the machinery that regulates cell shape and mechanics. This machinery has an enormous molecular complexity and is constituted by distinct functional systems that differ in size and protein composition. It comprises three main groups of structures, actin filaments (AF), microtubules (MT) and intermediate filaments (IF) (Fig. 1). The three different networks are in a constant interplay which enables specific cellular functions [1].

Actin is one of the major cytoskeletal proteins of eukaryotic cells. It is located mainly beneath the plasma membrane and in the cell cytoplasm as stress fibers and contributes to cell shape, mechanical support and movement [2]. Actin monomers, globular actin (G-actin), interact head to tail and polymerize in the form of filaments (f-actin). This process requires ATP hydrolysis and polar filaments are formed with the appearance of a double-stranded helix [3]. Actin filaments are semi-flexible polymers which adapt to external stress and show bending and fluctuation properties [4], and are considered the thinnest of all the three systems, with approximately 7 nm of diameter.

Microtubules are the thickest polymers with approximately 24 nm of diameter. They extend from the juxtanuclear area, namely, from the microtubule-organizing center (mTOC), to the cell edge. The tubulin unit is a dimer, consisting of one α- and one β-subunit, that has a binding site for GTP in the β-subunit. These dimers bind end to end to form protofilaments and these organize into polar cylindrical microtubules. The plus end of microtubules, where the subunit exchange is faster, is rich in β-subunits. Microtubules enable the transport of different cargo proteins through the cell and are crucial in cell division [5].

IF constitute the third cytoskeletal system and are present both in the cytoplasm and in the nucleus of the cells. These proteins have an important structural role, providing mechanical strength to cells and tissues, but are also involved in cell signaling and interactions, acting as integrators of cytoskeletal responses. IF are non-polar and are made of filamentous proteins that do not require nucleotide hydrolysis for assembly into long filaments. The diameter of IF can vary from 3-4 nm, in the case of nuclear lamins, to approximately 12 nm in the case of cytoplasmic IF. Cytoplasmic IF extend from the perinuclear region towards the cell periphery, as observed with microtubules [6].

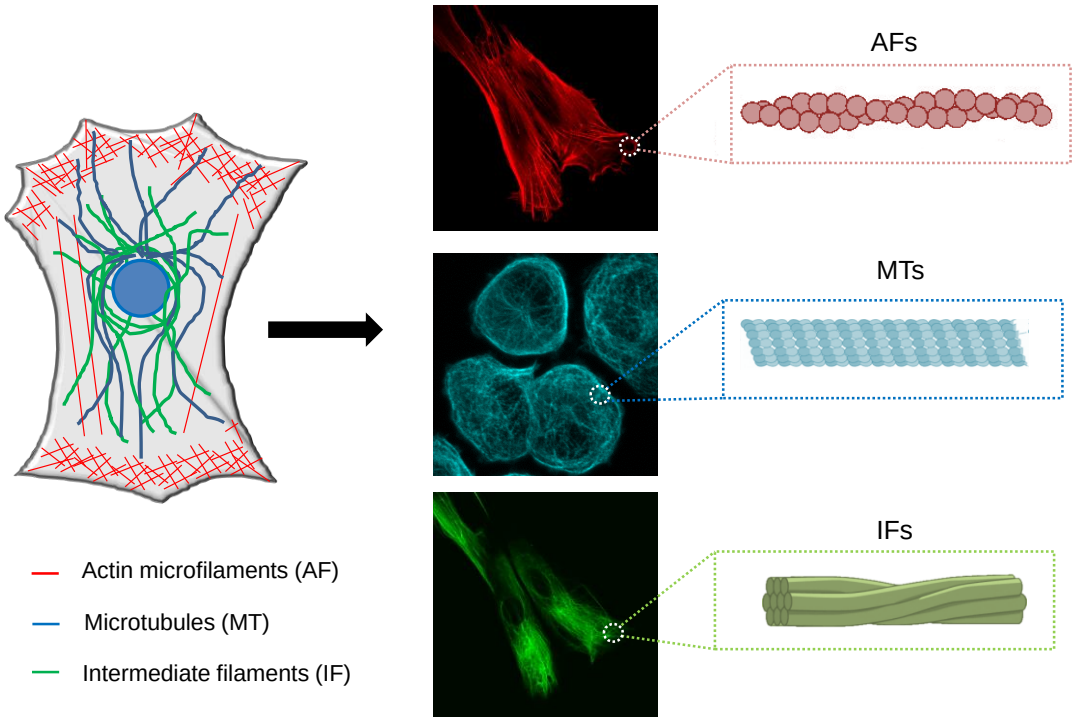


Figure 1 – **Distribution of the cytoskeletal structures in cells**. The left image shows a schematic view of the distribution of cytoskeletal proteins in cells. Actin filaments (red) are present mainly at the cell periphery and in the form of stress fibers in the cytoplasm. Microtubules (blue) and intermediate filaments (green) extend from the center of the cell towards the cell edge. Middle panels, show immunofluorescence images of actin, microtubules and vimentin filaments (from top to bottom) and the corresponding polymeric structures of each network are shown in the right panels. Adapted from [1].

1.1. Intermediate filaments

Intermediate filament proteins are present in all vertebrate cells and in some bacterial species, which express intermediate filament-like proteins [7]. The human genome encodes around seventy different IF proteins [8], that are divided into six different groups depending on the similarity of their sequence [9]. The most abundant are the type I and type II keratins, which are mainly present in epithelial cells and are responsible for cell integrity and tissue elasticity. Type III intermediate filaments are found in various tissues with different functions. This sub-class includes four proteins: vimentin, glial fibrillary acidic protein (GFAP), desmin and peripherin. Type IV comprises the neurofilaments (NFs), nestin, α-internexin, synemin and syncoilin, which are expressed mainly in neuronal cells. Type V includes the lamins, which are present exclusively in cell nuclei. Finally, type VI

proteins are called “orphan proteins” because they are more distant from the rest of IF, and include the lens proteins filensin and phakinin [10].

Intermediate filaments have several common properties. These proteins are characterized by their alpha helical central rod domain which is flanked by two non-helical domains, the N-terminal (head) and C-terminal (tail) domains, of variable size [11]. Essentially, some parts of the rod domain of IF proteins are conserved among the different types and are crucial for the formation of the initial blocks for IF elongation. The presence of the head domain is essential for proper filament formation, while the role of the tail domain is thought to be involved in lateral association and organization of the filaments [11]. Assembly of cytoplasmic IF is similar between the different classes. Briefly, the rod domain, due to its hydrophobicity, allows the interaction between monomers and consequently the formation of dimers and tetramers. The tetramers associate laterally and form unit length filaments (ULF’s), which then associate end to end and form long filaments of approximately 10-12 nm of width (Fig. 2) [12]. The assembly of intermediate filaments does not require energy or intervention of other proteins and it can be performed in vitro [13].

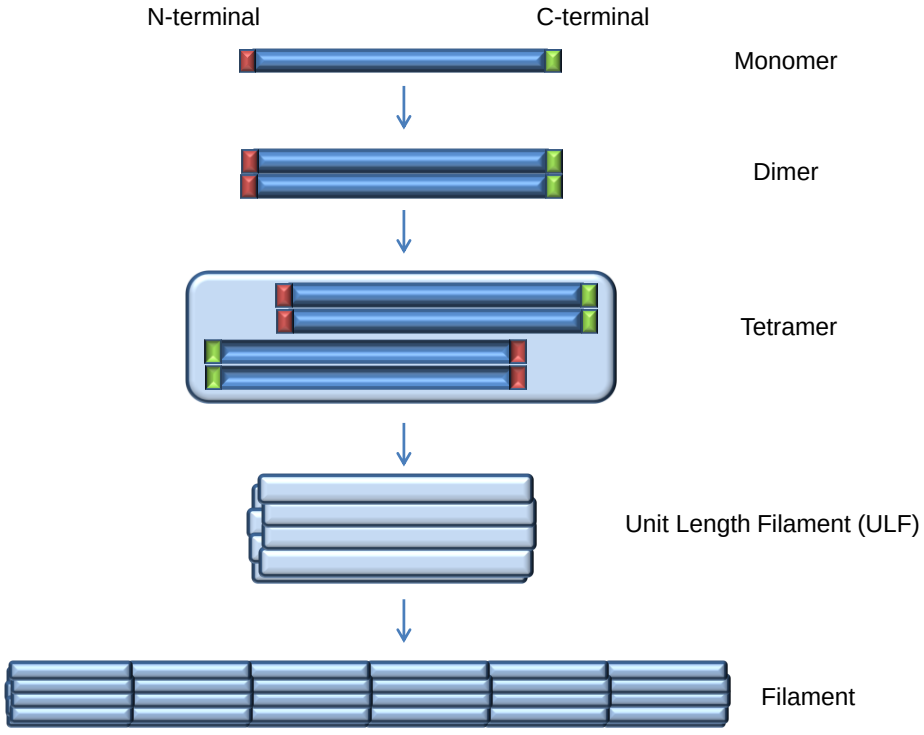


Figure 2- Schematic representation of the assembly process of cytoplasmic intermediate filaments. The assembly process starts with the formation of a parallel dimer that assembles in anti-parallel tetramers. The tetramers then associate laterally to form unit length filaments (ULF’s) that then anneal end to end to form long filaments.

Intermediate filaments are very stable, remaining intact even in the presence of high concentrations of salts, while microfilaments and microtubules are depolymerized under these conditions. Nevertheless, IF are highly dynamic and can rearrange and exchange subunits without compromising filament integrity [14]. There is a constant interplay between the soluble subunits and the filaments in cells which can be regulated by different factors, such as post-translational modifications [15] and the interaction with other cytoskeletal proteins [10]. Thus IF are considered more flexible and stretchable than other cytoskeletal structures and confer the elasticity and rapid rearrangement required for different cellular processes. These proteins are essential for maintaining cellular integrity and are implicated in cell migration, cell growth, apoptosis and cell response to stress [10]. Therefore, due to their diversity and variable disordered domains, IF can provide a unique cytoskeletal architecture in different cell types [16].

1.2. Type III intermediate filaments

Type III intermediate filament proteins include vimentin, desmin, peripherin and GFAP. Desmin is expressed mainly in muscle cells, GFAP in astrocytes and glial cells and peripherin in neurons. Vimentin is the most broadly distributed among all type III IF and is found in mesenchymal and fully differentiated cells such as blood and lens cells [17]. Type III intermediate filaments can exist as homopolymers, in contrast to keratins or neurofilaments, which form obligatory heteropolymers. However, type III proteins can also form heteropolymers with other type III or type IV proteins [18].

IF have been associated with different pathologies that can be related to alterations in gene splicing, mutations or sequence variants. In the case of type III proteins, pathologies like Alexander disease and desminopathies have been described. Alexander disease is a fatal neurodegenerative disease caused by mutations of the GFAP protein, leading to the formation of protein aggregates in glial cells and to astrocyte dysfunction [19]. Similarly, mutations and posttranslational modifications in desmin can cause different myopathies and desminopathies [20]. Regarding peripherin, an overexpression of this protein can cause neurodegeneration or even neuronal death [21]. This protein has also been associated with sporadic and familial amyotrophic lateral sclerosis (ALS) [22]. Vimentin has been linked with different pathologies such as cataracts, cancer and inflammatory diseases [23-25]. Since this work is mainly focused on vimentin, its role in the different cell processes and pathophysiological conditions will be considered in further detail.

1.3. Vimentin

The vimentin sequence was first described in 1988. The protein consists of 466 amino acids, with a molecular weight of around 53 kDa [26], and its sequence is highly conserved between species. As described for IF, the vimentin monomer has a tripartite structure with a α-helical rod domain of 317 amino acids, a disordered N-terminal domain constituted by 95 amino acids and an unstructured C-terminal segment with a 54 amino acid-long sequence [27]. The rod domain of vimentin is divided into four α-helical regions that are separated by flexible linkers (Fig. 3) [6, 28]. The crystal structure of full-length vimentin is challenging to obtain due to the self-assembly of the filaments into non-soluble structures. Nevertheless, the crystal structures of different parts of the rod domain of vimentin have been already obtained [9, 29]. Vimentin possesses a conserved cysteine residue, C328 in human vimentin, that is important for the effect of oxidants and for vimentin reorganization in response to electrophilic compounds in cells [30].

In cells, vimentin particles fuse into short squiggles that assemble into elongated filaments, forming a cytoplasmic filamentous network that extends from the nucleus towards the cell edge. Nevertheless, vimentin exists both as soluble structures and polymerized filaments which are constantly exchanging subunits [14].

Vimentin plays an important role in the mechanical support of cells and regulates the position and function of cellular organelles. Its interaction with several different proteins underlies its different roles in cells such as cell response to stress, migration, cell growth and apoptosis [10].

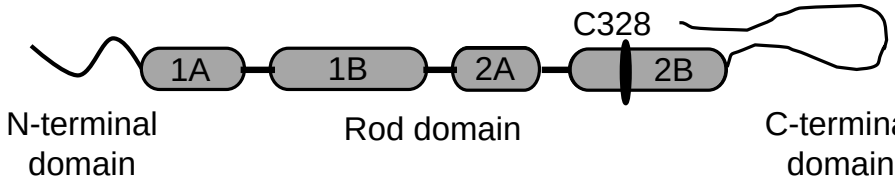


Figure 3 – **Schematic representation of the vimentin monomer**. Vimentin is constituted by an alpha-helical central rod domain, integrated by four regions, flanked by the N-terminal (head) and C-terminal (tail) domains. The position of C328 in the 2B coil is indicated.

1.3.1. Vimentin assembly – roles of the N- terminal and C-terminal domains

The role of the N-terminal domain of vimentin in filament formation has been widely studied. A peptide from the N-terminal domain of vimentin was found to be essential for

filament assembly, since the removal of these amino acids promoted the precipitation of the protein and the formation of aggregates in vitro and in cells [31, 32]. Moreover, the removal of the entire N-terminal domain completely abolished filament assembly [11].

The role of the C-terminal domain of vimentin in filament assembly is more controversial. In vitro, tailless vimentin (vimentin (1-411)), in which the entire tail domain was removed, is able to form filaments but with a wider and a more heterogeneous diameter than filaments formed by vimentin wild type (wt) [11]. Therefore, the vimentin tail was reported to be involved in lateral packing of filaments and in the control of filament thickness [33]. Moreover, deletion or mutations of a conserved region of the C-terminal domain, the RDG motif, altered filament morphology [34]. Additionally, the vimentin tail has been suggested to undergo conformational changes during in vitro assembly [35] and to interact with divalent cations [36, 37].

In cells, the vimentin tail acts as a cytoplasmic retention signal [38, 39] and promotes filament stability [40]. Nevertheless, tailless vimentin has been shown to form either normal extended filaments or aggregates depending on the experimental system [38, 40]. Moreover, the removal of the RDG motif was reported to prevent the formation of extended filaments in cells lacking vimentin filaments [41]. However, the factors that determine these different patterns and the consequences of the expression of tailless vimentin in cells are not fully understood.

1.3.2. Vimentin in cell dynamics

Early studies pointed to vimentin as a regulator of cell mechanics and shape. Vimentin-deficient embryonic fibroblasts were found to be more fragile and mechanically unstable than wt cells [42]. Moreover, reduced mechanical stability and resistance upon shape alteration were observed in cells lacking vimentin filaments [43], which could underline the delayed in vitro wound healing observed in these cells [43, 44].

Vimentin is a known epithelial mesenchymal transition (EMT) marker and player. EMT is characterized by an increase in cell motility, increased focal adhesion dynamics and loss of desmosomal contacts [45]. Thus, the expression levels of vimentin have been associated with the mesenchymal cell shape and motility, whereas silencing vimentin causes mesenchymal cells to adopt an epithelial shape [45, 46]. Consequently, vimentin knockout reduced the migration of several distinct cellular types, including fibroblasts [45], leukocytes, astrocytes [47] and several cancer cells [46].

Cell migration also requires vimentin dynamics. Different assembly states of vimentin regulate the formation of lamellipodium, suggesting a role of vimentin in the regulation of cell polarity and motility [48]. Longer vimentin filaments associate with the cell surface and inhibit the formation of lamellipodia, while disassembly of vimentin into short filaments has the opposite effect [48]. Moreover, dynamic vimentin organization and assembly are associated with the rapid morphological changes in B-cells upon antigen recognition [49] and with regulation of the invasive capacity of lung fibroblasts [50].

Vimentin also plays an important role in cell adhesion, since the turnover of focal adhesions seems to be dependent on the presence of vimentin. In MCF7 cells transfected with vimentin, there was an increased association of vimentin with focal adhesions and its presence led to a higher turnover rate of paxillin [45]. Moreover, vimentin knockdown in cells led to a downregulation of β1-integrin and vinculin [51]. Furthermore, the presence of vimentin at focal adhesions increased the adhesion strength [52].

1.3.3. Vimentin in organelle positioning

As a cytoplasmic protein, vimentin is vastly associated with organelle distribution and movement within the cytoplasm. In vimentin-deficient cells, the intracellular glycolipid synthesis seems impaired due to a defect in the transport between the endosomal/lysosomal pathway and the Golgi apparatus [53]. Additionally, a direct interaction between the formiminotransferase cyclodeaminase (FTCD), a protein from the Golgi apparatus, and vimentin was described in fibroblast-like kidney cells. This interaction is important for the control and remodeling of the Golgi complex [54]. The interaction of vimentin with the adaptor complex AP-3, involved in protein traffic, was reported to control the position and subcellular distribution of endocytic organelles [55]. The movement of melanosomes in cells has also been reported to be vimentin dependent [56]. Moreover, the vimentin single cysteine residue showed to be determinant in the position of lysosomes and aggresome formation [30].

Vimentin is also associated with the position and function of mitochondria, providing partial protection from oxidative damage and favoring the connection between mitochondrial respiratory chain and oxidative phosphorylation [57]. Moreover, the knockdown of vimentin induced alterations in mitochondria, such as fragmentation and disorganization. Vimentin filaments colocalize and interact with mitochondria, regulating the interaction of this organelle with other cytoskeletal proteins [58]. Additionally,

interaction of mitochondria with the N-terminal domain of vimentin regulates their movement by anchoring them in the cytoplasm [59].

The nucleus has also correlations with vimentin, and its position in migrating cells and astrocytes is perturbed by the absence of vimentin filaments [60]. Other studies have also pointed to vimentin as a modulator of nuclear morphology: cells that express vimentin have a more regular nuclear morphology than vimentin-negative cells [61].

Besides its function in organelle positioning and movement, vimentin has been associated with the regulation of autophagy [50], which increases upon vimentin depletion [62]. Moreover, vimentin participates in the process of degradation of misfolded proteins. The vimentin filaments form a cage surrounding damaged proteins, leading to the formation of aggresomes that localize in the perinuclear area and are destined for degradation [63].

In summary, vimentin has an important impact in the mechanical properties of the cell by increasing the cytoplasmic elastic properties and reducing the intracellular movement [64]. The intracellular processes and cellular behavior in which vimentin plays a role are depicted in Fig. 4.

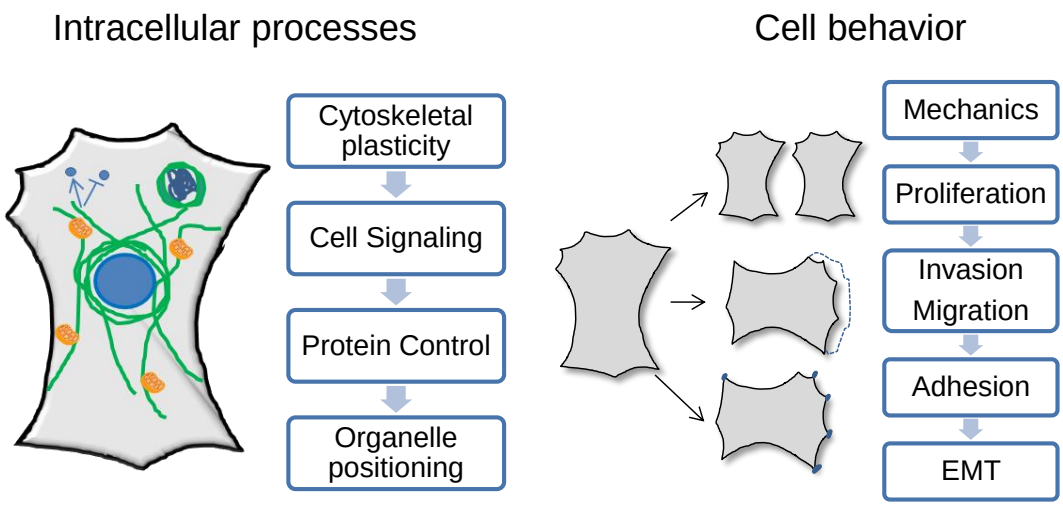


Figure 4 – Vimentin roles in cells. Vimentin participates in different intracellular processes such as cell signaling, protein quality control and organelle positioning and is involved in cytoskeletal plasticity. The presence of vimentin in cells regulates processes such as cell mechanics, proliferation, invasion, migration, adhesion and EMT.

1.3.4. Vimentin in pathophysiology

Vimentin has been related with several pathological conditions, including cancer, cataracts, inflammation, heart and vascular system diseases.

As referred above, vimentin has a determinant role in EMT, which links this protein to different types of cancer. Thus, the malignancy, migration and the spread of cancer cells are related to vimentin expression. In lung cancer, vimentin was associated with tumor progression and metastasis [65, 66]. A similar behavior was observed in breast cancer cells [67] and melanoma [68], in which vimentin was related to the invasive properties of the malignant cells. It has also been shown that vimentin expression in prostate cancer leads to high tumorigenesis activity [69], whereas in gastrointestinal cancer high vimentin levels were related with malignancy and drug resistance [23].

Nevertheless, the high expression of vimentin in such different types of cancer opens the way for new therapeutic and prognostic approaches. In melanoma metastasis vimentin is upregulated and thus can be used as a predictor for high risk patients [68]. In metastatic prostate tumors, vimentin interacts with integrins, and blocking this interaction can be used as a potential therapy [70]. On the contrary, in lung carcinomas a decrease in glycosylated vimentin was observed, which could be a potential biomarker for treatment and diagnosis [66].

Vimentin has also been involved in the pathogenesis of immune-related diseases, such as Crohn's disease and rheumatoid arthritis. In Crohn's disease, an inflammatory disease of the gastrointestinal tract, there is an upregulation of vimentin [25]. Moreover, EMT might be involved in the pathogenesis of intestinal fibrosis in this pathology [71].

In rheumatoid arthritis there is a production of antibodies that target a mutated citrullinated vimentin (MCV). Increases in anti-MCV immunoglobulins are detected at early stages of the disease and constitute sensitive and specific diagnostic parameters [72, 73]. Moreover, antibodies against citrullinated proteins, including vimentin, are believed to play a role in rheumatoid arthritis pathogenesis [73].

Vimentin has been reported to play a role in the internalization and replication of several viruses, such as human immunodeficiency virus (HIV) type I, human papillomavirus and influenza A virus [74-76]. Interestingly, in vitro studies have shown that the HIV protease cleaves vimentin in the C-terminal domain [76], whereas in cells it promotes the accumulation of vimentin in the juxtanuclear area [77]. Moreover, recent observations show that modulating vimentin levels in cells can inhibit HIV replication, thus being a possible therapeutic target [78].

Vimentin mutations have also been associated with pathological conditions, such as cataracts. Vimentin filaments are known to maintain the structural integrity of eye lens. In fact, the vimentin mutation E151K in coil 1B resulted in the formation of misfolded vimentin that led to cataract formation [24]. Interestingly, higher levels of vimentin have been found in diabetic cataract, suggesting the involvement of EMT in this disease [79]. Consequently, controlling vimentin levels could be a possible therapeutic strategy for this condition [79].

Table 1 outlines the pathogenic and diagnostic or therapeutic implications of vimentin expression or mutation in different pathological conditions.

Table 1 – Implications of vimentin expression in different pathologies.

Pathological condition	**Consequences**	**Diagnosis/Drug target**
Lung cancer	• Tumor progression and metastasis	• Disease prediction • Biomarker (glycosylated vimentin)
Breast cancer	• Invasiveness	
Prostate cancer	• Tumorigenesis activity	
Gastrointestinal cancer	• Malignancy and drug resistance	
Melanoma		• Predictor for high risk patients
Cataracts	• Vimentin aggregates	
Crohn's disease	• Pathogenesis of intestinal fibrosis	
Rheumatoid arthritis	• Increased citrullinated vimentin. Immune reaction	• Biomarker for diagnosis (anti-MCV antibodies)
HIV	• Vimentin cleavage and aggregation	• Therapeutic target

As stated above, vimentin can be used as a biomarker for the diagnosis and treatment of several pathologies, which vary from cancer to infectious and inflammatory diseases. Importantly, there are already some drugs that are known to target vimentin and can be used as potential therapies. Recently, withaferin-A was proposed to target vimentin by covalent binding to its cysteine residue [80]. In fact, the beneficial properties and

anticancer activity of withaferin-A were already reported in pancreatic cancer cells [81]. Moreover this drug showed anti-tumor and anti-metastatic properties in mouse models of breast cancer [82]. However, in spite of its beneficial effects, this compound does not seem to target vimentin or its cysteine residue specifically [83]. In addition, silibinin, a drug used in liver diseases, was shown to reverse EMT by suppressing vimentin expression and to inhibit prostate tumor growth and progression [84, 85]. Statins, which are known for their role in cholesterol reduction, also target cells expressing vimentin promoting cancer cell death [86].

In summary, vimentin is associated with different pathologies, either through its increased expression or point mutations in its sequence. Therefore, this protein can be both a drug target and a biomarker for different diseases.

1.3.5. Post-translational modifications of vimentin

The vimentin network is highly dynamic and reorganizes in different essential cellular processes, including cell division and cell growth. This reorganization is mainly regulated by post-translational modifications (PTM), such as phosphorylation, glycosylation, ubiquitylation and lipoxidation. Phosphorylation is by far the most studied covalent modification on intermediate filament proteins [15].

Vimentin is a substrate for several different kinases, such as cyclin-dependent kinase (Cdk1), protein-kinase C (PKC), Rho kinase and Aurora B, which associate vimentin to signal transduction in cells [87, 88]. The head and tail domains of vimentin contain more than 35 residues susceptible to phosphorylation [89], mainly serine and threonine residues, which are crucial for maintaining cell integrity, mitosis and filament dynamics [87]. Early evidence suggested that phosphorylation of the head domain of vimentin could block polymerization in vitro and elicit the disassembly of existing filaments [90, 91]. Moreover, the disassembly of vimentin increases the solubility of the protein [87].

Vimentin phosphorylation can also regulate its interaction with other proteins. For instance, vimentin phosphorylation is required for its interaction with 14-3-3 proteins [92] and PKC-regulated phosphorylation of vimentin is required for the recycling of integrin traffic in cell [70].

Additionally, the interaction of vimentin with other proteins can increase its phosphorylation. AKT1 binding to vimentin promotes S39 phosphorylation and enhances the ability of vimentin to induce motility and invasion of cells, while protecting vimentin from

caspase-induced proteolysis [93], and filamin A is required for PKC-mediated vimentin phosphorylation and for regulation of cell spreading [94].

Regarding other modifications, it was recently proposed that SUMOylation of vimentin is required for proper cell migration and proliferation [95], whereas O-GlcNAcylation is important for protein-protein interactions [96]. Moreover, during mitosis, the interplay between GlcNAcylation and phosphorylation is important for the distribution of vimentin in the two daughter cells [97].

Vimentin PTM in pathology have also been reported. An increased phosphorylation of vimentin can be used as a marker for different infiltrative and non-infiltrative tumors [98]. Citrullinated vimentin in rheumatoid arthritis is involved in the pathology of the disease [73]. Moreover, vimentin is the main target of SUMOylation upon PIAS3 stimulation in glioma cells [99]. Vimentin acetylation, regulated by SIRT5, plays an important role in hepatocellular carcinoma migration [100]. On the other hand, USP14 decreases the levels ubiquitinated vimentin thus leading to an increase of the malignant behavior of gastric cancer cells [101].

Vimentin is a major target of advanced glycation end products (AGEs). Vimentin is the preferential target for N-(carboxymethyl)lysine modification in human fibroblasts and this modification reduces the contractile capacity of these cells [102]. Furthermore, site-specific O-GlcNAcylation of vimentin influences the assembly and/or disassembly of the filaments [96], as reported for phosphorylation. Besides, this modification is used by the intracellular pathogen *Chlamydia trachomatis* to promote its own replication [96]. Moreover, high glucose levels induce O-GlcNAcylation of vimentin and consequently increase protein stability and promote migration and invasion of cholangiocarcinoma cells [103].

Vimentin is also a substrate for several proteases. Calpains cleave vimentin at the N-terminal domain, leading to defects in the in vitro polymerization [104]. Moreover, the soluble calpain cleavage products bind to phosphorylated-ERK, being involved in axonal regeneration [105]. Likewise, caspases cleave vimentin at different sites in vitro and in cells and cleavage at D85 induces pro-apoptotic effects [106]. The C-terminal domain also contains sites for viral protease cleavage. The HIV-type I protease has been reported to cleave vimentin between residues L423 and R424 (considering the initial M) [76], whereas the Moloney mouse sarcoma virus cleaves vimentin at several points of the tail domain [107]. Interestingly, the chemical gambogic acid promotes the cleavage of vimentin in cells by unknown proteases, leading to products that were cleaved near S51 and E425 [108].

Oxidative modifications of vimentin can also occur; however the oxidation pattern of vimentin is still poorly understood. Nevertheless several works have highlighted the importance of vimentin lipoxidation, which induces a severe IF reorganization [30]. Therefore, a detailed overview of protein lipoxidation will be given in the following section.

In summary, vimentin is a target of numerous post-translational modifications that alter its structural and functional properties. Consequently, these modifications control vimentin network assembly and cell homeostasis and, in some cases, contribute to pathological conditions. Nevertheless, vimentin modifications can be used as therapeutic strategies or biomarkers in some diseases [109].

2. Cytoskeletal crosstalk in different cellular processes

Cytoskeletal proteins have different functions in cells that are determined by their structural and physical properties. Nevertheless, the interplay of the three networks is important in different cellular processes and usually the perturbation of one of the networks affects the other two systems [16]. The interaction of the three different systems can occur at multiple levels: biochemical signaling, direct binding and through crosslinking and motor proteins [1].

2.1. Vimentin interplay with microtubules

The first evidence of the interaction between microtubules and intermediate filaments arose from electron microscopy studies of different cell types. The two cytoskeletal structures were found in the cell cytoplasm in parallel arrays [110]. Moreover, coalignment of vimentin intermediate filaments with detyrosinated MT was observed in fibroblasts [111]. Additionally, microtubule disrupting agents promoted a condensation of vimentin filaments in the perinuclear region [110, 112, 113] and an impaired movement of vimentin dots [114], suggesting that vimentin movement and extension of vimentin filaments towards the cell edge is microtubule-dependent [113, 115].

The unidirectional movements of vimentin raised the possibility that vimentin interacted with the plus end of microtubules. In fact, it was observed that vimentin structures colocalized with kinesin, a microtubule motor protein, and in the absence of this protein the formation of an extended vimentin network was blocked [114]. This suggests that the interaction of intermediate filaments and kinesin is required for the anterograde (plus end–directed) movement of vimentin and the assembly of extended filaments.

Although the cytoplasmic IF movements are towards the cell periphery, the movements of various forms of IF are bidirectional, suggesting that the MT motor dynein may also be involved in the regulation of IF motility. Immunofluorescence and electron microscopy assays showed that NFs and vimentin localized with dynein and dynactin [116, 117]. Moreover, normal vimentin filament organization in cells requires the presence of these proteins. Consequently, a balance between the two motor proteins is necessary for the assembly and maintenance of vimentin filaments in interphase cells [117].

Importantly, vimentin filaments can also have a role in microtubule maintenance. The absence of vimentin filaments alters microtubule organization and perturbs cell polarity in mouse fibroblasts [118]. Moreover, after disassembly with nocodazole, newly polymerized microtubules follow the vimentin network, suggesting that vimentin can stand as a template for microtubule organization [119].

These observations show that the interaction of vimentin with microtubules occurs mainly through crosslinking or motor proteins. Moreover, the presence of each one of the cytoskeletal systems affects the distribution of the other. The interaction of these proteins regulates cell migration and polarity; nevertheless, no evidence of their interaction in mitosis has been described yet.

2.2. Interplay of the vimentin and actin networks

The evidence of the crosstalk between vimentin and actin is vast and is described in processes such as cell migration, signaling and mechanics [120]. It is known that vimentin and actin affect each other's subcellular localization and motility, although they are not necessary for each other's assembly [121]. However, few studies have addressed the direct interaction of these proteins. The first evidence of a direct interaction between vimentin and actin was obtained through microinjection studies, which showed that a peptide from the vimentin tail colocalized with actin structures in cells [122]. Later, in vitro studies suggested that the two proteins interacted with each other through the C-terminal domain of vimentin [123].

Early evidence showed that vimentin filament organization is dependent on the actomyosin cortex contraction [112]. Additionally, the integrity of actin arcs is necessary for the correct perinuclear localization of vimentin [121]. The interplay of these proteins is also required for the position of the nucleus and its movement across the cytoplasm [60].

Remarkably, the transport of vimentin particles through microtubules is controlled by actin filaments. The interaction with actin restricts ULF transport along microtubules and

treatment of cells with an actin depolymerizing agent increases the movement of vimentin particles. These results suggest the importance of the actin cytoskeleton in the regulation of vimentin transport and in the stabilization of the network [124].

In turn, the actin cytoskeleton can be regulated by vimentin in several ways. Vimentin depletion leads to an increase of stress fiber assembly and cell contractility [125]. Moreover, vimentin particles are detected in lamellipodia of migrating cells and the disassembled vimentin filaments facilitate the actin-based protrusion of these structures [48]. Additionally, the increase in vimentin expression, regulated by kinases and growth factors, promotes lamellipodia formation [126], whereas vimentin depletion decreases the lamella in mesenchymal cells [121].

Vimentin has been related with actin structures mainly during cell migration, and adhesion and several proteins are known to mediate the crosstalk between the two proteins, particularly in adhesion-dependent processes [127]. Fimbrin, an actin crosslinking protein, colocalizes with vimentin in structures such as podosomes, filopodia and retraction fibers [128]. The focal adhesion protein, αvβ3-integrin, connects actin with vimentin filaments in endothelial cells [52, 129] and its vimentin interaction regulates focal contact size and helps stabilize cell-matrix adhesions [130]. Moreover, the recruitment of vimentin to focal adhesions in endothelial cells requires β3 integrin and plectin [52]. Recently, CARMIL2 (capping protein, Arp2/3, myosin-I linker 2) has been described to behave as a linker between vimentin and the actin capping protein, playing a role in invadopodia formation and cell migration [131].

Vimentin was found to be present in several actin structures in interphase cells; nevertheless, their interaction in mitosis, to the best of our knowledge, has not been explored before this work was undertaken. A colocalization of atypical actin conformations with phosphorylated vimentin has been recently reported [132]. However, the consequences of this interaction have not been addressed.

2.3. Cytoskeletal reorganization during mitosis

During cell division cells have to go through a dynamic process that requires deep cytoskeletal rearrangement. The microtubules form the mitotic spindle that allows the separation of the chromosomes, and actin forms several structures including the rigid actomyosin cortex. Information on microtubule and actin reorganization in mitosis is extensive and will be only briefly summarized in the following sections. IF are thought to be disassembled in many cell types through phosphorylation or remain as polymerized

structures. Fig. 5 represents the organization of the different cytoskeletal proteins both in interphase and in mitotic cells.

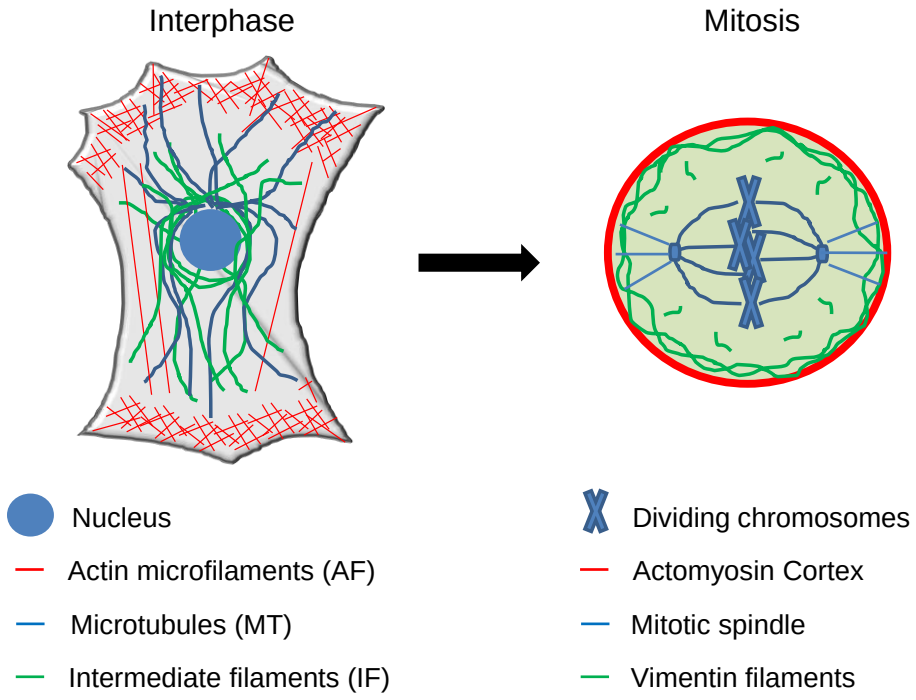


Figure 5 – Cytoskeletal reorganization during mitosis. Left image shows the cytoskeletal proteins distribution in interphase cells. Right image shows the distribution of the cytoskeletal proteins in mitosis. Actin filaments form the mitotic cortex at the cell periphery, microtubules form the mitotic spindle and the vimentin network is either disassembled or it remains in filaments giving space to the dividing chromosomes.

2.3.1. Vimentin regulation during mitosis

The dynamic changes of vimentin filaments are known to occur during different stages of the cell cycle. The main mechanism described for vimentin reorganization during mitosis is phosphorylation-dependent disassembly. A higher vimentin kinase activity is observed in mitotic cells compared with non-mitotic cells [133].

Vimentin phosphorylation occurs mainly in its N-terminal domain [134]. S55 was one of the first residues found to be specifically phosphorylated during mitosis. This residue is phosphorylated by Cdk1 in different cell types [135, 136]. The dephosphorylation of this residue occurs during cytokinesis, indicating a specific regulation of vimentin phosphorylation in different cell cycle phases [137]. Other residues were also found to be phosphorylated during mitosis in the N-terminal domain of vimentin, namely: S6, S24, S33, S46, S50, S65, S82 and S6 [138].

Phosphorylation of the C-terminal domain of vimentin, at T457, S458 and S459, has also been found to occur in mitotic cells. Nevertheless, these sites do not seem to play a role in vimentin disassembly [139]. In spite of this, phosphorylation of vimentin on S459 by polo-like kinase 1 (Plk1) is necessary for the inhibition of endocytic vesicle fusion and integrin trafficking towards the cleavage furrow during cytokinesis [140].

Cytokinesis is one of the processes in which IF phosphorylation is more tightly regulated [141]. Different kinases regulate the phosphorylation of vimentin at the cleavage furrow. Rho-kinase specifically phosphorylates vimentin at S38 and S71 and Aurora-B at S72 [142]. Moreover, PKC also plays a role in vimentin regulation and separation during cytokinesis [143]. Synergistic effects are also reported in the formation of the IF bridge during cytokinesis, the phosphorylation promoted by Cdk1 recruits Plk1 and facilitates vimentin phosphorylation at S82 [144]. Recently, it was proposed that O-GlcNAcylation also regulates vimentin phosphorylation at S71, indicating that a combination of different PTM can regulate the progression of mitosis [145].

Correct mitotic progression is dependent on vimentin disassembly/assembly during the different stages of mitosis. Indeed, a vimentin mutant in which several N-terminal serine residues that are phosphorylated during mitosis, were substituted by alanine residues (SA mutant), impairs cytokinesis and leads to cataract formation [146]. Nevertheless, hyperphosphorylation can also result in mitotic defects. A small molecule recently found to inhibit cell cancer growth, the FOXC2-inhibiting vimentin effector 1 (FiVe1), induces the phosphorylation of S56 and consequently the appearance of multinucleated cells and cell cycle arrest [147].

The reorganization of vimentin during mitosis can also be directly or indirectly regulated by its interaction with other proteins. In fact, the presence of nestin, a type IV IF protein [10], regulates the disassembly of vimentin filaments in a process dependent on the phosphorylation of vimentin at S55 [148]. Moreover, alpha-B-crystallin colocalizes with vimentin in mitotic cells, contributing to the remodeling of intermediate filaments during cell division. However, this interaction seems to be cell-type dependent and appears to play a role in mediating the aggregation state of IF during mitosis [149].

Overall, the residues of the N-terminal domain of vimentin are determinant for vimentin phosphorylation and organization during mitosis. Additionally, the interaction of vimentin with other proteins may also determine the structural changes observed during cell division. Yet, the reorganization of IF depends on the cell type.

2.3.2. Microtubules reorganization during mitosis

Microtubules are critically reorganized during the different mitotic phases and their distribution is regulated by various MT associated proteins. The changes in MT dynamics during mitosis has to be accompanied by the inactivation of the microtubule stabilizing proteins [150]. During the first mitotic phases, MT form the mitotic spindle that interacts with the kinetochore, the protein structure that links the duplicated chromatids, allowing the movement and alignment of the chromosomes [151].

The mitotic spindle is constituted by three different MT subclasses, the interpolar and the astral MT and the kinetochore-fibers (K-fibers). The astral MT connect the centrosomes to the cell cortex and have a role in centrosome separation and spindle position [152]. The interpolar MT are the most dynamic; they arise from the centrosome and extend towards the center of the spindle, having the function of maintaining the spindle bipolarity. During anaphase, these MT rearrange and become the major component of the central spindle [153]. Lastly, the K-fibers are the less dynamic MT and are responsible for attaching the chromosomes to the spindle poles and for the proper segregation of the chromatids to the daughter cells.

MT stabilizing or depolymerizing agents constitute valuable tools in the study of the microtubule cytoskeleton. In particular, the synthetic compound nocodazole causes microtubule depolymerization by binding to the free tubulin dimers, and preventing their incorporation into the network [154]. Moreover, due to its characteristics it has been used to block cells in mitosis by preventing the formation of the mitotic spindle [155].

2.3.3. Reorganization of actin in mitosis

The actin network suffers a drastic rearrangement when cells enter mitosis and generates the rigid and spherical actomyosin cortex [156], which implies a cortical enrichment of f-actin and myosin [157].

Recent studies have described that the cell cortex of mitotic cells is thinner than the cortex of interphase cells. In fact, Chugh et al. have observed that the cortex thickness of mitotic cells is around 200 nm, almost half of the thickness observed in interphase [158]. Interestingly, inhibition of myosin II did not profoundly affect the cortex thickness, but the proteins that control actin filament length markedly influenced cortex properties [157, 158].

Cell rounding and actin mechanics during mitosis are complex processes that require a coordination of several signaling pathways and proteins. Moesin, a protein that promotes

the interaction of actin with the plasma membrane, also controls the establishment of the rounded cell cortex during mitosis, starting the formation of the rigid cortex and promoting the relaxation during anaphase. Besides, a constant cooperation with myosin II is necessary to maintain cell shape and spindle morphogenesis [156]. Moreover, Cdk1 seems to be necessary for maintaining the levels of cortical myosin II, which is essential for keeping cortical tension and intracellular pressure during mitosis [157]. Ect2, a Cdk1 substrate, is also activated during prophase inducing the formation of the rounded cortex. Nevertheless, this protein also regulates the formation of the actomyosin ring during anaphase. Thus, Ect2 is able to reshape the mitotic cell cortex in different mitotic phases [159]. In turn, RhoA was also found to be essential for the enrichment of cortical f-actin and myosin II and for cortical retraction and rigidity during cell rounding [157, 160].

During mitosis, other actin structures have been described as necessary for normal mitotic progression and spindle assembly and position. For instance, Mitsushima et al. observed the formation of a cytoplasmic amorphous actin structure that appeared during prometaphase and moved around the nucleus of cells, in a Cdk1 and Arp3 dependent manner [161]. Later, in mouse zygotes the formation of a cytoplasmic f-actin ring was observed, which was surrounding the chromosomes and was only visible in mitotic cells [162].

2.3.4. Cytoskeletal crosstalk during mitosis

Recently, there is a growing interest in the study of the cytoskeletal interplay during mitosis, mainly in the interaction of tubulin with actin [163, 164]. However, the association and the role of intermediate filaments are not well described.

Chromosome segregation in mitosis requires a coordination of the microtubule spindle and the actin cell cortex. Actually, the actin cytoskeleton has been directly related with spindle formation [165]. Depolymerization of the actin network with low concentrations of actin polymerization inhibitors, which apparently did not affect cortical actin, led to the displacement of the mitotic spindle from the center of the cell. This indicates that the intact actin network is necessary for central spindle positioning during mitosis [162]. Furthermore, some authors suggest that the cytoplasmic f-actin ring formed during mitosis regulates microtubule dynamics and thus spindle orientation [166]. The inhibition of actin or tubulin polymerization altered the formation of the ring-like f-actin structure and consequently the spindle position and led to asymmetric division [166]. The role of f-actin

and myosin in spindle position and function is reviewed in Sandquist et al., [165]. Recently, it was shown that actin filaments are necessary for the formation of centrosomes, and individual actin filaments precede the formation of the K-fibers [164]. Moreover, actin was recently found as an essential constituent of the human oocyte spindle and also important for microtubule dynamics during this process [163].

Thus, a full characterization of cytoskeletal interactions in mitosis will require substantial efforts. The most obvious crosstalk mechanisms affect actin and tubulin systems. Nevertheless, given its intricate connections with the other two systems, the interaction of cytoplasmic IF with tubulin and actin during mitosis deserves detailed study. Prior to the publication of our work, no interaction of IF with actin or microtubules in mitosis had been reported, to our knowledge. This will be an important aspect of the results described in the present work.

3. Lipoxidation

The balance between oxidants and antioxidants determines cell damage by oxidant species. A decrease in the antioxidant defenses can favor the accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The increase of these species under oxidative stress conditions, leads them to interact with distinct biomolecules, specifically with unsaturated membrane phospholipids, forming a huge variety of reactive lipids [167]. In several diseases and pathologies that involve inflammation and the release of reactive species, lipid peroxidation is known to occur [168]. The reactive lipids can then interact with proteins causing their modification. Indeed, vimentin is an important target for lipoxidation as it will be detailed below.

3.1. Formation of electrophilic lipids

Reactive lipid species can be generated through the contribution of enzymatic and non-enzymatic processes. Enzymatic pathways include enzymes such as cytochrome P450, lipoxygenases, cyclooxygenases and prostaglandin synthases [169]. Non-enzymatic lipid peroxidation leads to the formation of different oxidized lipids, including short-chain and long-chain products, some of which can exert adverse and/or beneficial biological effects. Phospholipids bearing polyunsaturated fatty acids (PUFA) are important targets for these chain reactions and can be modified by oxygen or nitrogen reactive species leading to new electrophilic molecules [170].

Most of the lipid species generated by enzymatic and/or non-enzymatic pathways are reactive and electrophilic species carrying carbonyl groups (aldehydes or ketones) or α, β-unsaturated moieties (Fig. 6) [171]. The most reactive and most studied lipid peroxidation products are malondialdehyde (MDA), acrolein (ACR), 4-hydroxyhexanal (4-HHE) and 4-hydroxy-2-nonenal (HNE), which are also formed in higher levels compared with other products [172]. Among these, HNE is by far the most investigated electrophilic lipid and its levels are known to increase in several diseases such as Alzheimer disease, atherosclerosis and rheumatoid arthritis, among others [173].

Moreover, electrophilic eicosanoids, as cyclopentenone prostaglandins (cyPG) and isoprostanes can be also formed during these reactions [174]. CyPG are normally associated with the inflammatory response and are considered potential therapeutic compounds and with anti-inflammatory properties. Nevertheless, some studies have reported that cyPG may have a double role and promote either pro- or anti-inflammatory effects in several diseases [175]. Therefore, these compounds may block or potentiate tumor growth, depending on the experimental model, and induce protein aggregation resulting in neurodegenerative symptoms in mice [176].

Nitrated phospholipids (NO_2-PL) comprise a recently identified class of electrophilic lipids that have been detected in cardiomyoblasts subjected to ischemia and starvation and in cardiac mitochondria from diabetic rats [177, 178]. Nevertheless, the information about the biological actions of NO_2-PL is scarce. These lipids can act as radical scavengers in vitro and in cells, and they have been proposed to display anti-inflammatory actions [179]. Moreover, our group recently found that nitrated 1-palmitoyl-2-oleyl-phosphatidyl choline (NO_2-POPC) can act as a potential novel electrophilic lipid mediator that interacts in vitro with known protein targets of lipoxidation [180].

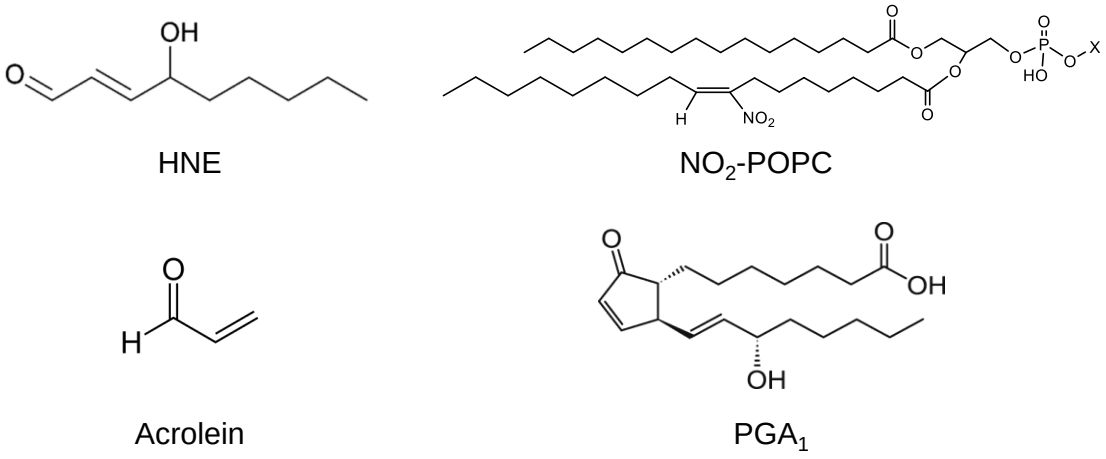


Figure 6 - **Structure of some electrophilic lipids**. Examples of short-chain and long-chain lipid peroxidation products: 4-hydroxy-2-nonenal (HNE), nitrated 1-palmitoyl-2-oleyl-phosphatidyl choline (NO_2-POPC), acrolein and prostaglandin A_1 (PGA_1).

3.2. Protein modification by lipoxidation

The modification of proteins by electrophilic lipids, forming covalent adducts, is called lipoxidation. The products resulting from lipid peroxidation, which contain carbonyl groups or α, β-unsaturated moieties, can react with nucleophilic residues of proteins [181], such as cysteine, histidine or with amino acids bearing an amino group, as is the case of lysine and arginine [182]. These adducts can be formed through different chemical reactions, rendering Schiff adducts and/or Michael adducts. Schiff base adducts are covalent adducts that are formed due to the reaction between free amino groups of proteins and aldehydes or ketones, forming a Schiff base. Michael adducts result from attack of the nucleophilic residues of proteins (mainly amino sulfhydryl groups) to the carbon in the β position of the α,β-unsaturated carbonyl group (Fig. 7). Both adducts are reversible, nevertheless Schiff adducts are more unstable. On the other hand, Michael adducts with cysteine residues of proteins are considered to be virtually irreversible under physiological conditions, although retro-Michael reactions can also occur [181, 183, 184].

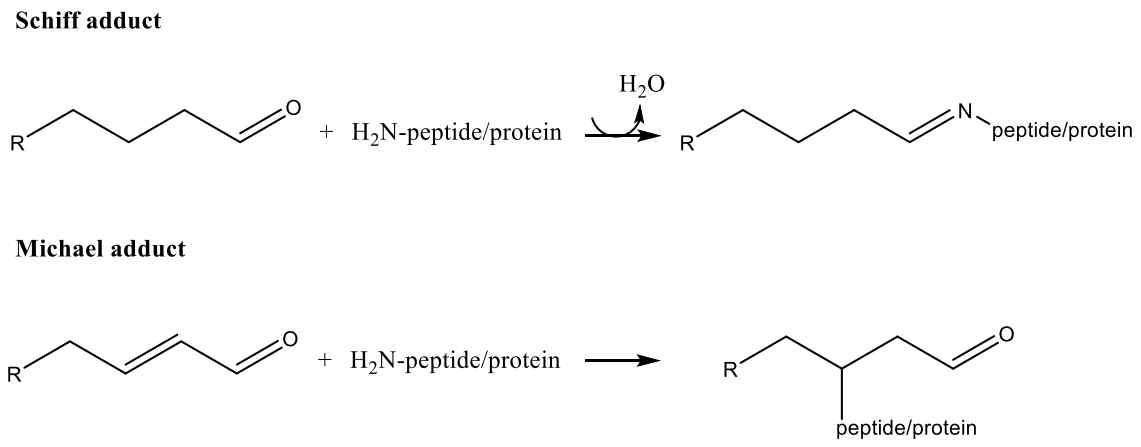


Figure 7 - **Representation of Schiff and Michael adducts of electrophilic lipids with peptides/proteins**. The electrophilic lipids used as an example are an aldehyde (upper panel) and a lipid with a α,β-unsaturated carbonyl group (lower panel) (adapted from [181]).

The modification of proteins by electrophilic lipids can lead to changes in their conformation and assembly properties and alter their interaction with other proteins and their cellular localization. These effects can lead to relevant changes in the function of the proteins susceptible to modification and contribute to pathogenic mechanisms [181].

The modification of proteins by lipids is not random, lipoxidation occurs in a restricted set of the proteome. However, the highly reactive species are normally less specific than the less reactive species. Moreover, the less reactive compounds primarily react with cysteine residues and are normally associated with signaling mechanisms or activation of stress pathways [185]. Apart from reactivity and specificity, other aspects influence the reaction of electrophilic species with proteins, such as the site of production and cellular localization [167].

The most abundant and reactive proteins towards reactive carbonyl species (RCS) have been proposed to act as scavengers of electrophilic species, thus protecting other proteins in which a covalent adduction would lead to damage. As a matter of fact, albumin [186], the chaperones Hsp70 and Hsp90 [187] and cytoskeletal proteins [188] came up as targets of oxidation/lipoxidation and potential scavengers of reactive species, since these proteins are normally present at high levels in comparison with other possible protein targets. Consequently, actin and albumin can act as detoxifying agents of RCS, due to the presence of several highly nucleophilic residues in their sequence. Therefore, the

susceptibility to oxidation of some of these targets may be related with defense mechanisms [186, 188].

There is a growing interest in the study of lipoxidation both in physiology and pathophysiology and some protein-lipid adducts can even be used as biomarkers of aging, damage and inflammation [186]. Nevertheless, protein lipoxidation by electrophilic lipids can have dual effects: at low concentrations it can induce protective and antioxidant pathways while at higher concentrations can lead to excessive oxidation and cell death [189, 190].

3.3. Lipoxidation in (patho)physiological conditions

Evidence of lipoxidation in vivo both in healthy and in damaged tissues has accumulated in recent years. The formation of reactive species is increased in pathological conditions, which leads to a rise of lipoxidation in some diseases. In fact, protein-HNE adducts have been reported in cancer, neurodegenerative and chronic inflammatory diseases [191].

Lipoxidation has a particular relevance in aging and there is evidence of many kinds of adducts both during healthy aging and in some age-related diseases [192, 193]. Recently, a proteomic study of the human cerebral cortex revealed an increase of lipoxidized proteins in older individuals, and suggested that protein vulnerability to oxidation is related with the cellular localization [194]. The authors found modifications in proteins involved in energy metabolism, protein homeostasis and neurotransmission, as well as in cytoskeletal proteins [194].

Additionally, the role of protein lipoxidation in neurodegenerative and cardiovascular diseases, and in particular, of protein modification by HNE has received much attention. For instance, an increase in HNE-modified proteins was found in the cytoplasm of neuronal cells from Alzheimer's disease patients compared with control samples [195]. Aldehyde adducts in the atherosclerotic plaques and adducts of ApoB-100 with oxidized phosphatidylcholine have also been evidenced [196]. These modifications appear to work as biomarkers and can be used to predict the disease [197].

Cytoskeletal proteins can also be related with redox control in several diseases and during life span [194, 198]. These proteins are known targets for different lipids, as is the case of cyPG and HNE [30, 175]. Neurofilaments are the major neuronal targets of HNE in axons [198, 199]. Moreover, tubulin, GFAP and neurofilaments were found to be lipoxidized in different parts of the brain and the modifications increased with age [194].

Actin has also been extensively studied and the cysteine residue at the position 374 is reported to be modified by different electrophilic lipids [152, 188]. Moreover, actin has been reported to act as carbonyl scavenger and to serve as a potential marker for carbonylation damage [188]. Vimentin is also widely studied as a lipoxidation target, and it is known that cyPG bind selectively to its single cysteine residue (C328), causing the rearrangement of vimentin filaments [30, 200, 201]. Vimentin lipoxidation is considered with more detail in the next section.

3.4. Lipoxidation of vimentin

Vimentin is a widely distributed protein and it is known to be a target for several oxidants and electrophilic lipids. Interestingly, the species reported to modify vimentin continue to expand.

One of the first electrophilic lipids found to interact with vimentin was 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) [200]. In fact, this molecule is known to form covalent adducts with thiol groups of proteins such as GFAP [202], peroxisome proliferator–activated receptors (PPAR) and several other signaling proteins [203]. The modification of vimentin by this lipid endorsed a rearrangement of the vimentin network, by promoting an accumulation in the perinuclear area [200]. Moreover, in vitro studies have identified another prostaglandin, PGA_1, which formed adducts with vimentin, suggesting that the interaction required the thiol group of C328 for PGA_1 addition [204].

Other reactive lipids, such as HNE [205] and MDA [206] have also been found to covalently bind to vimentin. Using mass spectrometry approaches, the modification of vimentin by these two lipids was found to occur at its single cysteine residue. Additionally, a vimentin mutant on the cysteine residue showed more resistance to lipoxidation in cells, suggesting that this residue is the main functional target for lipoxidation and works as an important redox sensor [30, 201]. Conversely, in vitro assays showed that HNE also modified the vimentin C328S mutant, suggesting that other nucleophilic residues are susceptible to modification [201].

The relevance of these lipoxidative modifications in the vimentin network is not entirely understood. Interestingly, recent work from our group has pointed out the importance of vimentin lipoxidation in filament formation and dynamics [201]. On the other hand, vimentin is known to participate in the response to misfolded proteins upon lipoxidation. There is a reorganization of the vimentin network in the perinuclear area surrounding the damaged proteins. Thus, vimentin reorganization can act as a potential mechanism to help the cell

to isolate the modified proteins [63]. Besides, the presence of vimentin in cells protected mitochondria from oxidative stress [207]. Therefore, lipoxidative modifications of vimentin can contribute to the maintenance of cell homeostasis and participate in the response to cell damage.

In summary, vimentin is a target for multiple post-translational modifications that can alter the dynamic properties of the protein and regulate its interaction with other proteins. Moreover, the N- and C-terminal domains are important targets for these modifications and are determinant in the structural and functional properties of the protein. Therefore, the study of the importance of these domains will help to elucidate their role in filament distribution and in the cell response to stress.

Aims and Objectives

Vimentin is a type III intermediate filament protein that forms an extended network in cells. This protein plays distinct roles in different cellular processes, which require the presence of the different domains. The role of the N-terminal domain of vimentin in filament assembly and distribution is well known, yet, the role of the C-terminal domain is still controversial. Nevertheless, the tail domain of vimentin has been proposed to interact with other cytoskeletal proteins. Indeed, vimentin involvement in cytoskeletal crosstalk is described in processes such as cell migration, adhesion and mechanics, but in mitosis this interplay is still poorly understood.

Cytoskeletal proteins are known targets of PTM that can alter protein-protein interactions in different cellular processes. Notably, vimentin is the target for diverse oxidative and electrophilic modifications in which its single cysteine residue plays an important role.

Thus, there is a need for the study of the role of the vimentin tail domain in filament distribution in cells and in the interaction with cytoskeletal proteins during cell division. Moreover, it is important to unveil the role of the cysteine residue in pathophysiological conditions during mitosis and its function in filament distribution.

Therefore, the following objectives were proposed:

- To study the importance of the vimentin C-terminal domain in filament distribution in interphase and in mitotic cells.
- To assess the impact of vimentin aggregates or bundles on mitotic progression.
- To evaluate the effects of actin and tubulin disrupting agents in vimentin distribution during mitosis.
- To explore the interplay between vimentin and actin in mitosis and the effect of vimentin filaments on the actin cortex properties.
- To assess the effects of experimental conditions mimicking pathophysiological situations in vimentin distribution during mitosis.
- To study the importance of the single cysteine residue in vimentin distribution upon lipoxidation.

Materials and Methods

1. Materials

1.1. General reagents

1.1.1. Cell culture and treatments

Cell culture medium DMEM, DMEM F12 and RPMI 1640, newborn calf serum, penicillin/streptomycin, geneticin G-418 and trypsin-EDTA were from Gibco Life Technologies. Fetal bovine serum was from Sigma or Biowest. Sterile plastic material for cell culture was obtained from Falcon (Beckton Dickinson). For cell imaging of live cells and visualization of mitotic cells, 35-mm glass bottom dishes (p35) were from MatTek Corp.

C3 transferase toxin was from Cytoskeleton. Latrunculin A and jasplakinolide were from Santa Cruz Biotechnology. 4-hydroxynonenal (HNE) and prostaglandin A_1 (PGA_1) were from Cayman Chemical. Blebbistatin, nocodazole, dimethyl sulfoxide (DMSO), cytochalasin B and ritonavir were from Sigma. Lipofectamine 2000 was from Thermo Scientific. Nitrated POPC (NO_2-POPC) was a gift from Dr. Rosário Domingues (University of Aveiro, Portugal) and was prepared as previously described [177].

1.1.2. Electrophoresis and western blotting reagents

Protein separation was performed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Acrylamide, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS) and glycine were from Bio-Rad. Tris(hydroxymethyl)aminomethane was from Merck. Agarose D2 for DNA electrophoresis was from Pronadisa.

Protein concentration in cell lysates was determined using the bicinchoninic acid (BCA) protein assay kit from Pierce. Polyvinylidene fluoride (PVDF) Immobilon-P membranes were from Merck Millipore and the Enhanced Chemiluminescence (ECL) western blotting detection reagents were from GE Healthcare.

1.1.3. Other reagents

High purity salts, buffers and other reagents used for preparing solutions were mainly from Sigma and Merck. Milli-Q distilled water was from Millipore. Restriction enzymes, buffers and LigaFast™ rapid DNA ligation system were from Promega. 4,6-diamidino-2-phenylindole (DAPI) was from Sigma.

1.2. Antibodies

Anti-vimentin antibodies were: mouse monoclonal V9 clone (sc-6260) and its Alexa-488 conjugate from Santa Cruz Biotechnology, mouse anti-vimentin monoclonal antibody (V5255) from Sigma. Anti-actin (A2066) and anti HSP70 were from Sigma and anti-α-tubulin (ab52866) from Abcam. Secondary anti-mouse and anti-rabbit immunoglobulins conjugated to horseradish peroxidase (HRP) were from Dako.

1.3. Plasmids

Plasmids used were either commercial or in-house generated or modified. All plasmids were amplified in competent E. Coli strains (XL10-Gold ultracompetent cells from Agilent Technologies) in the presence of the corresponding selection antibiotic (Sigma). Small-scale DNA preparation was achieved using the High Pure Plasmid Isolation Kit from Roche. Large-scale plasmid isolation was performed with the EndoFree Plasmid Maxi Kit from Biotool. All plasmids were sequence-verified at the DNA Sequencing Service, Secugen S.A. at CIB-CSIC. Electrophoretic separation of the intact plasmids or digestion products was performed on 1.2% (w/v) agarose gels with 5 ng/ml ethidium bromide in Tris-acetate-EDTA (TAE) buffer. Visualization of the gels was performed under UV light on a Gel-Doc XR imaging system (Bio-Rad).

1.3.1. Previously described plasmids

The bicistronic plasmids RFP//vimentin wt and RFP//vimentin C328S, coding for the red fluorescence protein DsRed-Express2 (RFP) and wt human vimentin or C328S vimentin as separate products, and the GFP-fusion constructs, GFP-vimentin wt and GFP-vimentin C328S have been previously described by our group [30]. Briefly, full-length human cDNA vimentin (Origene) was cloned into the EcoRI and BamHI sites of pEGFP-C1 and pIRES2 DsRed-Express2 vectors (Clontech) to obtain GFP-vimentin wt and RFP//vimentin wt, respectively. Cysteine 328 was mutated to serine using the Quickchange XL site-directed mutagenesis kit from Agilent and primers, forward 5'-GGTGCAGTCCCTCACCTCTGAAGTGGATGCCC-3' and reverse, 5'-GGGCATCCACTTCAGAGGTGAGGGACTGCACC-3' to obtain GFP-vimentin-C328S or RFP//vimentin C328S.

1.3.2. Plasmids generated for this work

All primers used for mutagenesis and PCR were synthetized at the Protein Chemistry Facility at CIB-CSIC in an oligonucleotide synthesizer (Applied Biosystems 3400). QuikChange XL Site-Directed Mutagenesis Kit from Agilent Technologies was used for point mutations with the designed primers.

The different tail truncated mutants, vimentin (1-411), (1-423), (1-448) and (1-459) were generated by introduction of stop codons at positions 412, 424, 449 and 460, respectively, by site directed mutagenesis of the parent plasmids, RFP//vimentin wt or GFP-vimentin wt. Truncated vimentin constructs showed the expected mobility in SDS-PAGE gels as well as immunoreactivity (Fig. 21). Thus, all constructs used were recognized by an antibody raised against full-length vimentin, which recognizes the N-terminus of the protein (anti-vim N-term), whereas an antibody against the beginning of the tail (clone V9) recognized all constructs except tailless vimentin (1-411).

The GFP-vimentin (412-466) construct was generated in two steps. First, an EcoRI site was introduced in the GFP-vimentin wt plasmid at position 1692 of the vimentin mRNA sequence (accession number NM_03380.4). Then, the plasmid was digested with EcoRI and re-ligated with the Ligafast system from Promega, eliminating the amino acid residues from 1 to 411 of the vimentin sequence. The GFP-vimentin (316-466) and GFP-vimentin (362-466) were generated following the strategy used for GFP-vimentin (412-466). For the GFP-vimentin (316-466) the EcoRI site was introduced at position 1408 of the vimentin mRNA sequence and for the GFP-vimentin (362-466) at position 1542 (accession number NM_03380.4). The GFP-vimentin G452V plasmid was generated by site-directed mutagenesis using the Nzytech mutagenesis kit.

The oligonucleotides used for the generation of the mutant constructs described in this section are specified in table 2.

Table 2 – Sequence of the oligonucleotides used for mutagenesis. The mutated nucleotides appear in bold.

Protein	Accesion No.	Mutant construct	Oligonucleotide sequence
Human vimentin	NP_003371	Vimentin (1-411)	5'-GGCGAGGAGAGCAGGATTT**AA**CTGCCTCTTCCAAACTTTTCC-3'
"	"	Vimentin (1-423)	5'-CCTCCCTGAACCTG**TA**GGAAACTAATCTGGATTCACTCC-3'
"	"	Vimentin (1-448)	5'-GGACACTTCTGATTAAGACGGTTGAA**TGA**AGAGATGGACAGGTTATC-3'

Human vimentin	NP_003371	Vimentin (1-459)	5'-GGTTATCAACGAAACTTCT**T**AGCATCACGATGACC-3'
"	"	Vimentin EcoRI(1692)	5'-GGCGAGGAGAGCAGGA**A**TTCTCTGCCTCTTCCAAACTTTTCC-3'
"	"	Vimentin EcoRI(1542)	5'-GCCGTTGAAGCTGCTAACTACCAAGA**GAATT**CTATTGGCCGCCTGCAGG ATGAGATTC-3'
"	"	Vimentin EcoRI(1408)	5'-CTGCGCCAGGCAAAGCAGGAG**AAT**TCCACTGAGTACCGGAGACAG-3'
"	"	Vimentin G452V	5'-CGGTTGAAACTAGAGATG**T**ACAGGTTATCAACGAAACTTCTC-3'

1.3.3. Commercial plasmids

The plasmid pCDNA3/GFP-PR (GFP-PR), encoding a fluorescent construct of the type I HIV protease, was a gift from Nico Dantuma (Addgene plasmid #20253) and was previously reported [208]. The mCherry-vimentin wt plasmid was from Genecopoeia. The pmCherry-C1, pEGFP-C1 and pIRES2 DsRed-Express2 plasmids were from Clontech.

The constructs coding for the vimentin tail domain fused through a six glycine linker to the mCherry fluorescent protein, either directly or through a peptide with predicted coiled coil structure (EIAALKQEIAALKQEIAANKQEIAALKQE) [209], were generated by Genewiz Inc. (South Plainfield, NJ) by oligonucleotide synthesis and cloning into the parent vector. Briefly, the desired sequences, shown below, were introduced into the pmCherry-C1 plasmid, between the EcoRI and BamHI sites (in bold). The sequence of the glycine spacer (6Gly) is displayed in green, that of the coiled coil in red and of and the vimentin tail in black:

- **mCherry-6Gly-vimentin(412-466)**:

  GAATTCTGGCGGCGGCGGCGGCGGCTCTCTGCCTCTTCCAAACTTTTCCTCCCTGAA CCTGAGGGAAACTAATCTGGATTCACTCCCTCTGGTTGATACCCACTCAAAAAGGACA CTTCTGATTAAGACGGTTGAAACTAGAGATGGACAGGTTATCAACGAAACTTCTCAGCA TCACGATGACCTTGAATAA**GGATCC**

- **mCherry-6Gly-coiled coil-vimentin(412-466)**:

  GAATTCTGGCGGCGGCGGCGGCGGCGAGATCGCCGCCCTGAAGCAGGAGATCGCC GCCCTGAAGCAGGAGATCGCCGCCAACAAGCAGGAGATCGCCGCCCTGAAGCAGGA GTCTCTGCCTCTTCCAAACTTTTCCTCCCTGAACCTGAGGGAAACTAATCTGGATTCAC TCCCTCTGGTTGATACCCACTCAAAAAGGACACTTCTGATTAAGACGGTTGAAACTAGA GATGGACAGGTTATCAACGAAACTTCTCAGCATCACGATGACCTTGAATAA**GGATCC**

A summary of all plasmids used in this study is shown in table 3.

Table 3 – Plasmids used in this work.

	Name	Abbreviation	Nature	Products expressed	Tag
1	pIRES-DsRed-Express2	RFP//	Bicistronic vector	RFP	NA
2	pIRES-DsRed-Express2-vimentin wt	RFP//vim wt	Bicistronic	RFP and vimentin aa 1 to 466	None
3	pIRES-DsRed-Express2-vimentin (1-411)	RFP//vim (1-411)	Bicistronic	RFP and vimentin aa 1 to 411	None
4	pIRES-DsRed-Express2-vimentin (1-423)	RFP//vim (1-423)	Bicistronic	RFP and vimentin aa 1 to 423	None
5	pIRES-DsRed-Express2-vimentin (1-448)	RFP//vim (1-448)	Bicistronic	RFP and vimentin aa 1 to 448	None
6	pIRES-DsRed-Express2-vimentin (1-459)	RFP//vim (1-459)	Bicistronic	RFP and vimentin aa 1 to 459	None
7	pEGFP-C1	GFP	Vector	GFP	NA
8	pEGFP-C1–vimentin wt	GFP-vim wt	Fusion	GFP fused to vim wt	GFP
9	pEGFP-C1–vimentin(1-411)	GFP-vim(1-411)	Fusion	GFP fused to vim aa 1 to 411	GFP
10	pEGFP-C1–vimentin(1-423)	GFP-vim(1-423)	Fusion	GFP fused to vim aa 1 to 423	GFP
11	pEGFP-C1–vimentin(1-448)	GFP-vim(1-448)	Fusion	GFP fused to vim aa 1 to 448	GFP
12	pEGFP-C1–vimentin(1-459)	GFP-vim(1-459)	Fusion	GFP fused to vim aa 1 to 459	GFP
13	pEGFP-C1–vimentin C328S	GFP-vim C328S	Fusion	GFP fused to vim C328S	GFP
14	pEGFP-C1–vimentin G452V	GFP-vim G452V	Fusion	GFP fused to vim G452V	GFP
15	pEGFP-C1–vimentin(412-466)	GFP-vim(412-466)	Fusion	GFP fused to vim aa 412 to 466	GFP
16	pEGFP-C1–vimentin(362-466)	GFP-vim(362-466)	Fusion	GFP fused to vim aa 362 to 466	GFP
17	pEGFP-C1–vimentin(316-466)	GFP-vim(316-466)	Fusion	GFP fused to vim aa 316 to 466	GFP
18	pmCherry-C1- vimentin wt	mCherry-vim wt	Fusion	mCherry fused to vim wt	mCherry
19	pmCherry-C1-6Gly-vimentin (412-466)	mCherry-6Gly-vim(412-466)	Fusion	mCherry fused to a glycine spacer and vim aa 412 to 466	mCherry
20	pmCherry-C1-6Gly-coiled coil-vimentin (412-466)	mCherry-6Gly-coiled coil-vim(412-466)	Fusion	mCherry fused to a glycine spacer, a coiled coil structure and vim aa 412 to 466	mCherry

2. Methods

2.1. Cell culture

The following cell lines were used in this work:

SW13/cl.2 human adrenocarcinoma vimentin-deficient cells were a generous gift from Dr. A. Sarriá (University of Zaragoza, Spain). SW13 parental cells (mixture of vimentin-positive and negative cells) were acquired from Sigma, from The European Collection of Authenticated Cell Cultures (ECACC 87031801). Fibroblast-like African green monkey kidney cells (Vero) were from the collection of Centro de Investigaciones Biológicas Margarita Salas (CSIC, Madrid). Bovine aortic endothelial cells (BAEC, BW-6001) were from Lonza. Primary human dermal fibroblasts from an adult donor (ref: AG10803) were obtained from the NIA Aging Cell Repository at the Coriell Institute for Medical Research (Camdem, NJ). MCF7 (HTB-22™) humam breast carcinoma cells, HeLa and U-251 MG human glioblastoma astrocytoma cells (formerly known as U-373 MG, HTB-17™) were originally from ATCC and were authenticated by microsatellite amplification (short tandem repeat (STR)-PCR profiling), in Secugen, S.L., Madrid, Spain. Human mesangial cells (NHMC) were from Clonetics. HAP1 parental and vimentin-deficient cells (HAP vim–) (HZGHC003297c010) were from Horizon Genomics, GmbH (Vienna, Austria).

SW13/cl.2, SW13 parental cells, Vero, HeLa, MCF7, U-251 MG, and AG107803 were cultured in DMEM with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37ºC, in a humidified atmosphere with 5% CO_2. BAEC and NHMC were cultured in RPMI1640 with 10% (v/v) newborn calf serum or fetal bovine serum, respectively, with antibiotics, at 37ºC and 5% CO_2. HAP vim– were cultured in DMEM F12 with FBS and antibiotics. All cell lines were passaged by trypsin-EDTA detachment, followed by complete medium wash and centrifugation at 1000 x g for 5 min. Cells were suspended in fresh medium and plated at the desired dilution. For maintenance of stably transfected cells, 500 µg/ml final concentration of G-418 (Invitrogen) was added to the culture medium.

2.2. Cell treatments

Cell treatments with the different agents were performed in serum free medium, unless otherwise specified. An equivalent amount of the corresponding vehicle was added to control cells. Acute microtubule disruption was achieved by treatment with 5 µM

nocodazole for 30 min, whereas for cell cycle synchronization (mitotic arrest) cells were treated with 0.4 μM nocodazole for 20 h in complete medium. Disruption of f-actin was performed by treatment with 10 μM cytochalasin B, 2.5 μM latrunculin A or 50 nM japlakinolide for 30 min, 2 μg/ml C3 toxin for 3.5 h or 20 μM blebbistatin for 1 h. For treatment with electrophilic lipids, cells were incubated in the presence of 10 μM HNE for 4 h, 20 μM PGA_1 for 20 h or 10 μM NO_2-POPC for 6 h. Inhibition of transfected HIV protease was achieved by addition of 10 μM ritonavir in serum-containing medium, immediately after transfection. For removal of the inhibitor, cells were washed three times with fresh medium with serum, without antibiotics.

2.2.1. Detection of lagging chromosomes

For assessment of lagging chromosomes, cells transfected with RFP//vimentin wt or (1-411) were treated with 0.4 μM nocodazole to induce mitotic arrest, after which, cells were washed twice with complete medium for nocodazole removal and further incubated in fresh medium for 100 min to increase the proportion of cells in anaphase. Cells were subsequently fixed and stained with DAPI. Cells with chromosomes at mid-distance between the two groups of separating chromosomes were considered positive for this mitotic defect.

2.3. Transient transfections

Transient transfections were carried out with cells at 70% confluence using Lipofectamine 2000, following the manufacturer instructions. Briefly, for single transfections, 1 μg of DNA and 3 μl of Lipofectamine were used per p35 dish. For live visualization of vimentin filaments in vimentin-deficient cells, cells were transfected with 0.8 μg of the corresponding bicistronic RFP//vimentin plasmid plus 0.2 μg of GFP-vimentin plasmid. For overexpression of RFP//vimentin wt or RFP//vimentin (1-411) in Vero, SW13 parental or U-251 MG astrocytoma cells, 2 μg of DNA and 4.5 μl of Lipofectamine were used. Transfections were carried out in medium without antibiotics for 5 h and cells were allowed to recover for 48 h before imaging or treatment.

For expression of increasing amounts of vimentin (1-411) in SW13/cl.2 cells the following plasmid quantities were used: for expression of 0.2 μg, 0.1 μg of RFP//vim (1-411) + 0.1 μg GFP-vim (1-411); for 0.6 μg, 0.4 μg RFP//vim (1-411) + 0.2 μg GFP-vim (1-411); and for 0.8 μg, 0.6 μg RFP//vim (1-411) + 0.2 μg GFP-vim (1-411).

For expression of different proportions of vimentin wt and tailless (1-411), the following plasmid amounts were used: 0.8 µg RFP//vim wt + 0.2 µg GFP-vim wt (wt:tailless, 10:0); 0.8 µg RFP//vim wt + 0.2 µg GFP-vim(1-411) (wt:tailless, 8:2); 0.4 µg RFP//vim wt + 0.4 µg RFP//vim(1-411) + 0.2 µg GFP-vim(1-411) (wt:tailless, 4:6); 0.2 µg RFP//vim wt + 0.6 µg RFP//vim(1-411) + 0.2 µg GFP-vim(1-411) (wt:tailless, 2:8). Routinely, cells were visualized 48 h after transient transfection.

2.4. Immunofluorescence

Cells were fixed with 4% (w/v) paraformaldehyde for 25 min at r.t., permeabilized with 0.1% (v/v) Triton-X100 in PBS and blocked with 1% (w/v) BSA in PBS. HeLa cells were available as fixed preparations from a previous work from our group [210]. Antibodies were used at 1:200 (v/v) dilution in blocking solution. For experiments involving detection of vimentin (1-411), the monoclonal antibody recognizing the vimentin N-terminus was used for all conditions. For experiments involving selective detection of full-length vimentin or not requiring a comparison with vimentin (1-411), the V9 antibody was employed. F-actin was stained with Phalloidin-Alexa568 (Molecular Probes) or Phalloidin-Alexa488 (Molecular Probes), following the manufacturer's instructions. Nuclei were counterstained with DAPI (3 µg/ml).

2.5. Fluorescence microscopy

Cells transfected with the various constructs were visualized live by confocal microscopy on Leica SP2 or SP5 microscopes. Images were acquired every 0.5 µm and single sections or overall projections are shown, as indicated. Signal specificity was confirmed by using controls of cells not immunostained or stained only with secondary antibodies. Samples of experimental conditions that needed to be compared were processed in parallel. Microscope settings were saved for use in the following experiments without major adjustments, and the LUT command was used to guarantee non-overexposed image acquisition. Direct visualization on glass-bottom culture dishes was found optimal for imaging mitotic cells, in order to preserve their spherical shape. Unless otherwise stated, images shown are single channels or overlays of single Z-sections.

Super-resolution microscopy was performed with a confocal multispectral Leica TCS SP8 system equipped with a 3X Stimulated Emission Depletion (STED) module. Vimentin was detected with anti-vimentin V9 conjugated to Alexa488 and actin with Phalloidin-

Tetramethylrhodamine B isothiocyanate from Sigma at 0.25 µg/ml, final concentration. Since DAPI interferes with STED, mitotic cells were spotted by observation under bright field as round cells without nuclear limit and with the typical pattern of condensed chromosomes. Images were acquired with a confocal multispectral Leica TCS SP8 system.

For live cell visualization, time-lapse microscopy was carried out in a multidimensional microscopy system Leica AF6000 LX in a humidified 5% CO_2 atmosphere at 37ºC. Usually, green fluorescence and differential interference contrast (DIC) images were recorded. Cells were randomly selected, and images were acquired every 15 min.

2.6. Image analysis

The area occupied by vimentin filaments was quantified by comparing the region with detectable vimentin fluorescence with the total cell area. The region of interest (ROI) was selected by hand, delimiting the fluorescence signal of the channel of interest, using Image J (FIJI) program. Depending on the experimental conditions, the cell area was obtained by the red background (RFP signal derived from the DsRed-Express2 bicistronic plasmid) or by f-actin staining.

3D-reconstructions were obtained with FIJI, Imaris or Leica software. Fluorescence intensity profiles and measurements of mean fluorescence intensity and standard deviation of pixel brightness values, to illustrate the dispersion of f-actin signal intensity, were obtained with FIJI.

The percentage of vimentin in the daughter cells was quantified using Image J software, from images obtained from the time-lapse videos. The duration of mitosis was obtained from the videos.

Colocalization analysis of vimentin and actin in cells imaged by STED was performed with LAS-AF software from Leica on single z-sections of images. Colocalization rates (expressed as percentages) and Pearson coefficients were calculated. Whole-field images or regions of interest were delimited prior to image analysis of both transfected cells and non-transfected cells. Results are presented as mean colocalization rates or Pearson coefficients ± standard error of mean (SEM). For quantitation of the proportion of cortical vimentin, a cortical ROI was defined by manual selection of the inner edge of the actin cortex and automatic outward extension of the selection thickness to 1 µm. In addition, the

proportion of cortical vimentin colocalizing with actin was assessed within this ROI using the JacoP´s plugin and Pearson and Manders coefficients.

Orthogonal projections were obtained with Leica software. 2D maps from image stacks were obtained by FIJI and the free Map3-2D software developed by Sendra el al., [211], which unfolds surface information onto a single map, using a sphere adjustment. The proportion of basal f-actin or vimentin with respect to the total cellular content was quantitated from image stacks acquired every 0.2 μm as the ratio of sum of the integrated intensity of the 3 basal planes vs the sum of all planes, using FIJI.

For quantitation of impaired vimentin peripheral distribution induced by electrophilic lipids, the proportion of vimentin fluorescence present in the central area of mitotic cells (central circle comprising 60% of the total cell diameter in a single section at mid-cell height), with respect to the total area was measured. For all other measurements of peripheral vimentin, a cortical ROI of approximately 1.5 μm thickness was manually defined with FIJI.

Quantification of the ratio of cortical/cytoplasmic vimentin of the different GFP constructs fused to different segments of the vimentin tail was obtained with FIJI program. ROI with the same dimensions were manually drawn on the cortical region and on the cytoplasm and the ration between their fluorescence intensities calculated. Two different ROI from each location were selected for each cell. Due to the diffuse background of these constructs the ratio between the two areas is approximately 1.

2.7. Cell lysis and western blot (WB)

For the preparation of protein samples, plates were washed with cold PBS, and lysis buffer containing 50 mM Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% (w/v) SDS, 0.1 mM β-mercaptoethanol, 50 mM sodium fluoride and 0.1 mM sodium orthovanadate with 2 μg/ml of the protease inhibitors leupeptin, aprotinin and trypsin inhibitor as well as 1.3 mM Pefabloc, was added to the cells. Cells were homogenized by forced passes through a syringe with needle (0.45 mm (26G) x 12.7 mm) from BD Plastipak^{TM} on ice. Protein concentration was estimated by the BCA method.

Cell lysates were denatured at 95ºC for 5 min in Laemmli sample buffer (80 mM Tris-HCl, pH 6.8; 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.15% (w/v) bromophenol blue), prior to separation by SDS-PAGE. Aliquots of denatured lysates containing 30 μg of protein were run on 10% (w/v) polyacrylamide gels and transferred to

Immobilon-P membranes (Millipore) in a semi-dry unit from Bio-Rad, using a Tris-glycine methanol three-buffer system following the instructions of the manufacturer. Membranes were blocked with 2% (w/v) non-fat dried milk in T-TBS (Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% (v/v) Tween-20). For antibody incubation, primary antibodies were used at 1:500 dilution and HRP-conjugated secondary antibodies at 1:2000 dilution. The signal of interest was visualized using a chemiluminiscence detection system (ECL) and exposure to Agfa autoradiography film.

2.8. Statistical analysis

All experiments were repeated at least three times with similar results and measurements from different samples were always taken. Results are presented as average values ± standard error of mean (SEM), unless otherwise stated. Statistical analysis was performed with GraphPad Prism. Statistical differences were evaluated by the unpaired two-tailed Student's t-test and were considered significant when $p<0.05$, which is represented in graphs by symbols. When indicated, ANOVA followed by Tukey post-test for multiple dataset comparisons, was used. Significance levels for every experiment are given in the figure legends. In some cases, values are grouped for conciseness.

Results

1. The vimentin tail is necessary for correct filament distribution in cells

The vimentin C-terminal domain remains less characterized than the other vimentin domains regarding its structure and interaction with other proteins. Functionally, it has been shown that this domain is dispensable in vitro, but its role in cells is still controversial. Consequently, the importance of the tail domain of vimentin in protein distribution and mitotic progression as well as its role in the interaction with cytoskeletal proteins during mitosis will be explored in this chapter.

1.1. Vimentin (1-411) forms curly bundles in interphase cells

The role of the vimentin C-terminal domain in filament assembly and distribution was assessed using different strategies and several cell lines. Initially, vimentin-deficient cells were used to express both vimentin (1-411) and vimentin wild-type (wt). For this, SW13/cl.2 cells, which lack any type of cytoplasmic intermediate filaments [212], and the vimentin knockout haploid fibroblast cell line, HAP vim– were used. These cells allow studying the effects of vimentin mutations without having the interference from the endogenous protein. For live-cell visualization of vimentin network, we used a combination of two different plasmids, namely, the bicistronic plasmid RFP//vimentin wt or RFP//vimentin (1-411) which encode the red fluorescence protein (RFP) and untagged vimentin as separate proteins, and a plasmid that encodes the green fluorescence protein (GFP) fused to vimentin (GFP-vimentin wt or (1-411)) (Fig. 8a). The transfected cells were identified by the red diffuse background, arising from RFP, and the vimentin network visualized by the GFP vimentin construct [30].

In both vimentin-deficient cell types vimentin wt formed filaments extended throughout the cytoplasm (Fig. 8a, upper panels), while vimentin (1-411) had the tendency to form curly bundles near the center of the cell (Fig. 8a, lower panels). Quantitation of the percentage of the total cell area occupied by vimentin in each case demonstrated the impaired extension of the vimentin (1-411) filament network (Fig 8a, graph).

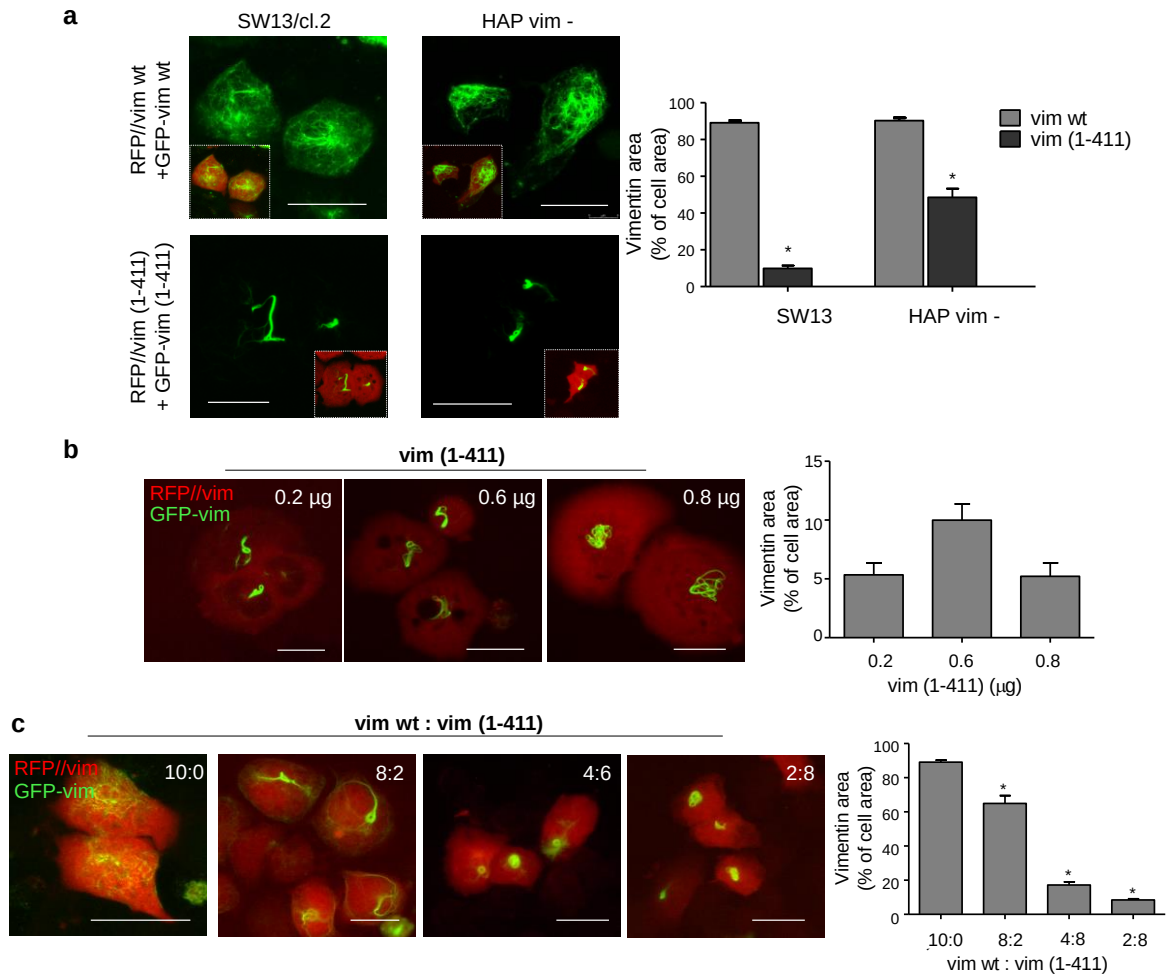


Figure 8 – Tailless vimentin forms curly bundles in vimentin-deficient interphase cells. (a) SW13/cl.2 and HAP vim– cells were transiently transfected with 0.8 µg of the bicistronic plasmids RFP//vimentin wt (RFP//vim wt) or RFP//vimentin (1-411) (RFP//vim (1-411)) plus 0.2 µg of the fusion construct GFP-vim wt or vim (1-411) and observed live 48 h after transfection. The graph depicts the percentage of the cell area occupied by each vimentin construct. Results are average values of at least 20 determinations from three experiments (*p<0.0001 vs vim wt). (b) SW13/cl.2 cells were transiently transfected with the following amounts of the indicated vim (1-411) plasmids to reach the total DNA quantities specified in the figure; from left to right, RFP//vim+GFP-vim, 0.1+0.1; 0.4+0.2 and 0.6+0.2 µg. The graph shows the area occupied by vimentin. (c) SW13/cl.2 cells were transiently transfected with different proportions of constructs coding for vim wt or vim (1-411) (as detailed in Materials and Methods), and the cell area covered by vimentin is represented in the graph. The experiment was repeated three times and at least 20 cells were quantified per experimental condition (*p<0.001 vs vim wt:vim (1-411) 10:0). Scale bars, 20 µm. Vim, vimentin.

The formation of vimentin accumulations or bundles was also observed upon transfection of low amounts of vimentin (1-411) (Fig. 8b). Expressing different amounts of vimentin (1-411), 0.2 µg, 0.6 µg and 0.8 µg in a combination of RFP//vimentin (1-411) and GFP-vimentin (1-411), promoted the same type of protein aggregates. Thus, the

impairment in network extension does not appear to depend on the amount of plasmid transfected (Fig. 8b, see graph).

The distribution of vimentin was also assessed by expressing both vimentin (1-411) and vimentin wt in different proportions. Interestingly, a gradual condensation of vimentin filaments was observed in the presence of increasing amounts of vimentin (1-411) (Fig. 8c). Taken together, these results indicate that vimentin (1-411) polymerization is not inhibited, yet it has an altered cellular organization. Moreover, the presence of vimentin (1-411) leads to an alteration of the organization of full-length vimentin.

1.2. Expression of vimentin (1-411) disrupts the endogenous vimentin network

The effect of vimentin (1-411) on network organization was evaluated in cells that express an endogenous vimentin network. Three different cell lines that express vimentin together with other intermediate filament proteins were used. U-251MG astrocytoma cells express several different cytoplasmic intermediate filaments such as GFAP and nestin in addition to vimentin [213, 214]. SW13 parental cells express only vimentin as a cytoplasmic intermediate filament. In turn, fibroblast-like Vero cells express other cytoplasmic intermediate filaments, such as keratins [215]. These cells were transfected with either RFP//vimentin wt or RFP//vimentin (1-411) in excess, and transfected cells were identified by the red background (Fig. 9). Visualization of the vimentin network was performed by immunofluorescence with the V9 antibody which is raised against the C-terminal domain of vimentin, and therefore, only recognizes full-length vimentin [216].

In cells transfected with an excess of vimentin wt no apparent alteration in filament distribution was observed (Fig. 9a, upper panels). In contrast, the presence of vimentin (1-411) in excess promoted a collapse of the full-length vimentin network, that is, endogenous vimentin, in all three different cell types (Fig. 9a, lower panels). The alteration of the endogenous vimentin network was characterized by a marked retraction of the filaments and an accumulation at the perinuclear region, which was demonstrated by a significant decrease in the full-length vimentin area compared with the total cell area in cells transfected with vimentin (1-411) (Fig. 9b). These results show that the absence of the tail of vimentin leads to aberrant filament distribution and that an excess of this truncated construct in cells is able to disrupt the normal vimentin filament network.

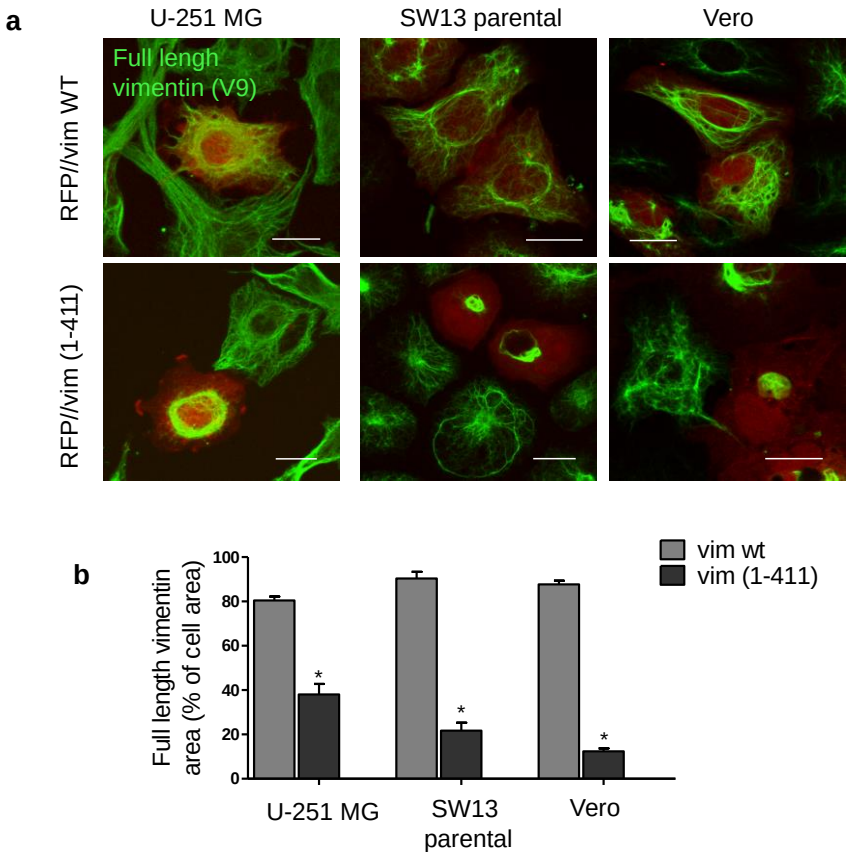


Figure 9 – Vimentin (1-411) disrupts vimentin full-length (wt) distribution. (a) U-251 MG astrocytoma, SW13 parental and Vero cells were transiently transfected with RFP//vim wt or RFP//vim (1-411) in excess (as described in Materials and Methods). Full-length vimentin condensation was assessed by immunofluorescence with V9 anti-vimentin antibody, which recognizes the tail domain of vimentin (green). (b) The graph shows the vimentin full-length area in comparison with total cell area in the cells transfected with the indicated constructs. Results are average values of three experiments and at least 20 cells were analysed per experimental condition (*p<0.0001 vs vim wt).

1.3. Vimentin (1-411) distributes close to the dividing chromosomes during mitosis

Given the aberrant organization of vimentin (1-411) in interphase cells and the proximity of the aggregates or bundles to nuclear structures, we found it interesting to study the distribution of this truncated form during mitosis (Fig. 10).

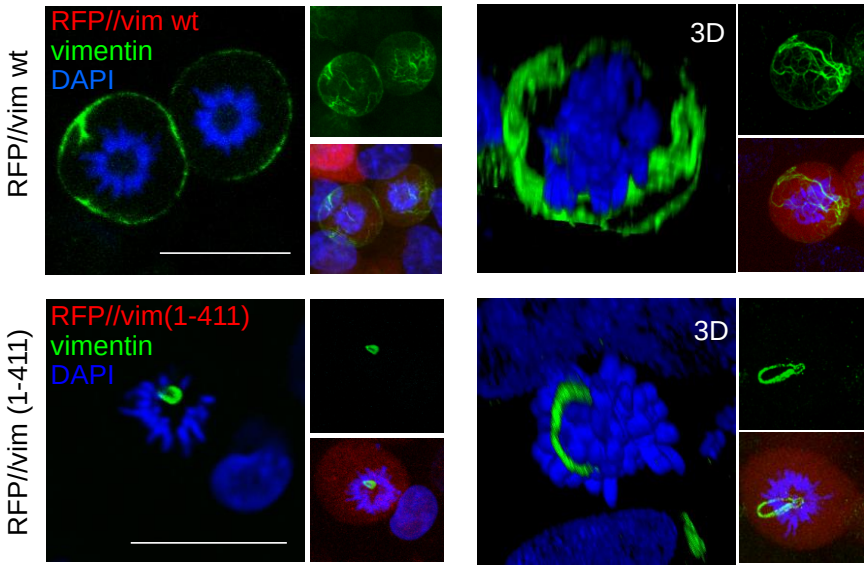


Figure 10 – Vimentin (1-411) interferes with the dividing chromosomes in mitosis. SW13/cl.2 cells were transiently transfected with RFP//vim wt or RFP//vim (1-411) and vimentin distribution was observed by immunofluorescence. Single sections taken at mid-cell height (left images) and 3D-reconstructions (right images) are shown. Images in small panels depict overall projections for vimentin alone (above) or the merge of the three channels (vimentin, RFP and DAPI, lower image). Scale bars, 20 μm.

Although in numerous cell types vimentin filaments have been reported to disassemble in mitosis [148, 217], we found that in SW13/cl.2 vimentin wt filaments were preserved and distributed towards the periphery of the cell (Fig. 10, upper panels). In sharp contrast, vimentin (1-411) bundles remained very close to the dividing chromosomes (Fig. 10, lower panels). 3D reconstructions obtained from mitotic cells (Fig. 10, right panels) showed that vimentin wt formed a cage surrounding the dividing chromosomes, while vimentin (1-411) stayed in close contact with chromosomes and in some cases entangled them. These results raised the possibility that the presence of vimentin (1-411) bundles close to the dividing chromosomes could lead to complications during cell division.

1.4. Vimentin (1-411) leads to mitotic defects

The effects of vimentin (1-411) bundles during mitosis were studied by performing time-lapse experiments. For this, SW13/cl.2 cells transiently transfected with RFP//vimentin wt or (1-411) together with a small amount of the corresponding GFP-fusion construct, GFP-vimentin wt or (1-411), were employed for directly monitoring vimentin filaments in live cells. The cells were visualized by differential interference contrast (DIC)

microscopy and some of those presenting green fluorescence were randomly selected (Fig. 11).

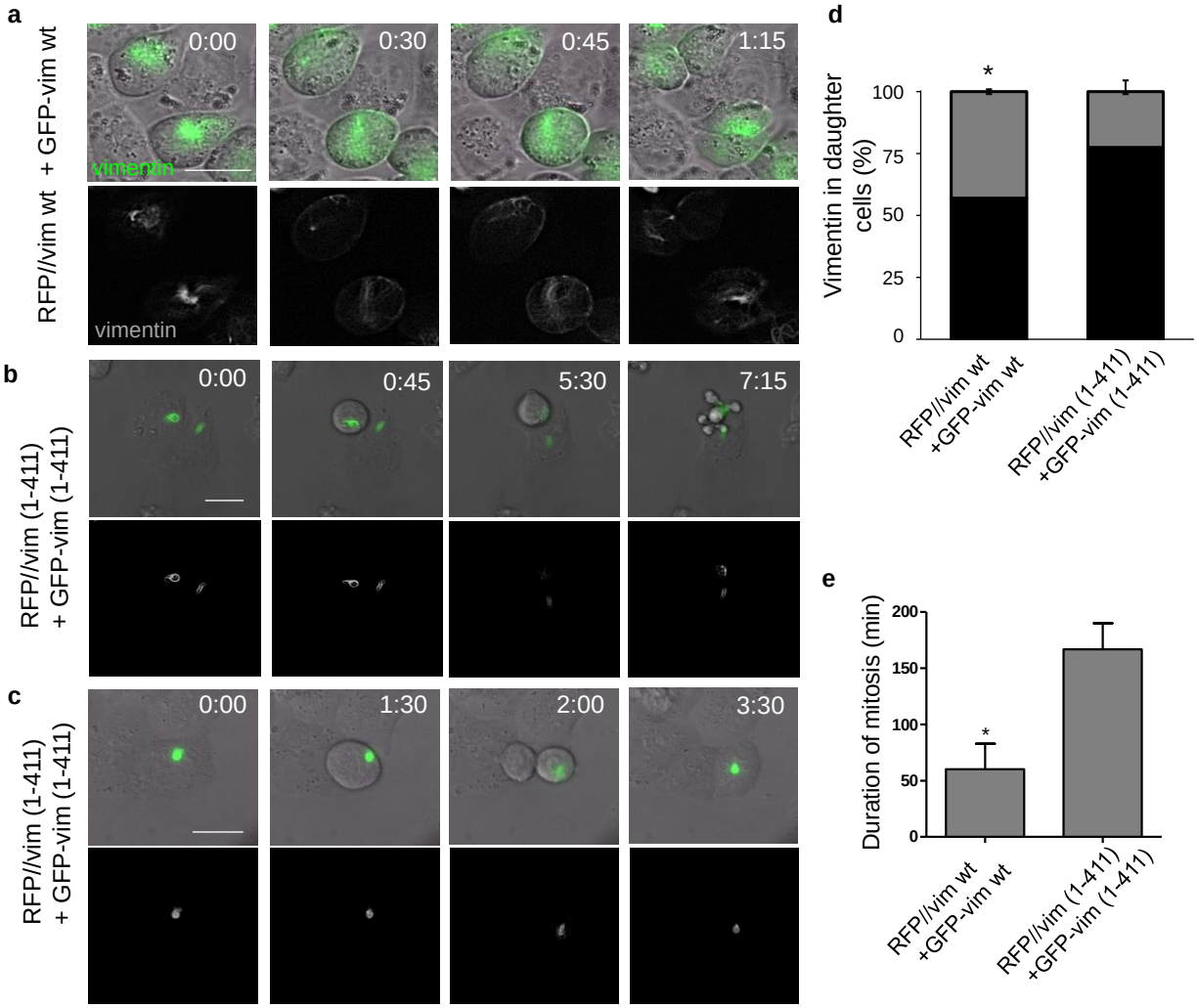


Figure 11 – Monitoring of cells expressing vimentin wt or vimentin (1-411) during mitosis. (a, b, c) SW13/cl.2 cells were transiently transfected with RFP//vim wt plus a tracer amount of GFP-vim wt, or the equivalent constructs for vim (1-411), as indicated, for live cell monitoring. Transfected cells were randomly selected, and images were acquired every 15 min. Representative images of the overlays of DIC and green fluorescence (upper panels) and green fluorescence only (lower panels, in gray scale), at the indicated time points, in hours:minutes, are shown. (d) Graph represents the distribution of vimentin between the two daughter cells as percentage of the vimentin levels in each cell with respect to the total amount. Results are average values of three experiments and at least 16 cells were analyzed per experimental condition (*p<0.0001). (e) Duration of mitosis considered from the time of cell rounding to the separation of daughter cells, in cells expressing vim wt or vim (1-411). Results are average values of three experiments and at least 33 cells were analyzed per experimental condition (*p< 0.02 vs vim(1-411)).

Cells expressing vimentin wt showed mainly extended filaments and a peripheral enrichment of vimentin could be appreciated in dividing cells (Fig. 11a, lower panels). In addition, vimentin distributed approximately evenly between the two daughter cells (Fig. 11a). Typically, the time from cell rounding to daughter cell separation was from 1 h to 2 h. In contrast, cells expressing vimentin (1-411) suffered distinct fates. Some cells attempted to divide, but the incapacity to complete mitosis led to membrane blebbing and then to cell death (Fig. 11b). On the other hand, some cells were able to complete cell division but with asymmetric distribution of vimentin between the two daughter cells, with one cell keeping the vimentin aggregate and the other staying, initially, free of it (Fig. 11c). Quantification of the percentage of vimentin inherited by each daughter cell showed a clear misdistribution of vimentin (1-411), in comparison with the more uniform distribution of vimentin in cells harboring vimentin wt (Fig. 11d). The duration of mitosis was quantified in cells harboring vimentin (1-411) that were able to complete mitosis, showing that they needed a significantly longer time to divide than cells expressing vimentin wt (Fig. 11e). These results indicate that the tail domain of vimentin is essential for the correct progression of mitosis and filament reorganization during cell division.

Then, to assess the possible consequences of the cytokinetic defects observed, the appearance of multinucleated cells or cells with aberrant nuclei, as well as chromosome segregation defects, were analyzed.

In cells expressing vimentin wt, most of the nuclei had a normal appearance and only a small proportion of cells showed multiple nuclei (Fig. 12a, left panel). In contrast, cells harboring a vimentin (1-411) displayed vimentin filaments that tied up the nuclei and showed aberrant or multiple nuclei in a higher proportion (Fig. 12a, right panel). Furthermore, to study potential chromosome segregation defects, cells were transiently arrested in mitosis with a low concentration of nocodazole, the treatment was then released and cells in anaphase were analyzed. A higher number of cells expressing vimentin (1-411) showed lagging chromosomes in comparison with cells expressing vimentin wt (Fig. 12b).

These results suggest that the mitotic defects observed in cells holding vimentin tailless could lead to chromosome segregation defects and the appearance of cells with aberrant nuclei.

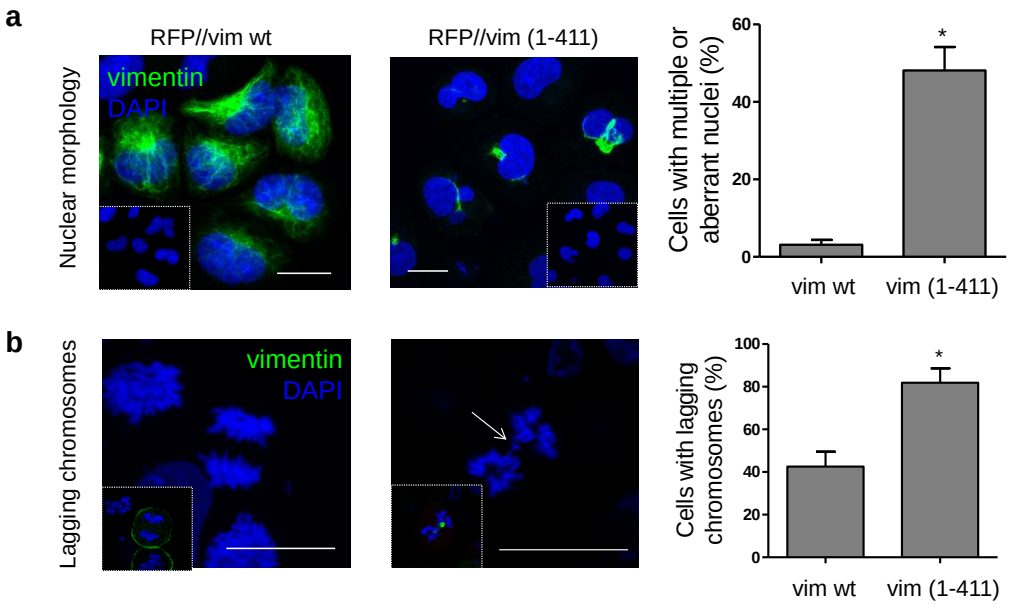


Figure 12 – Vimentin (1-411) promotes nuclear alterations and chromosome segregation defects. SW13/cl.2 cells were transiently transfected with RFP//vim wt or vim (1-411). (a) Nuclear morphology was assessed by DAPI staining and vimentin distribution by immunofluorescence. The percentage of cells showing multiple or aberrant nuclei is depicted in the graph. Results are from three determinations totaling at least 200 cells per experimental condition (*p<0.002). (b) Cells were synchronized by mild nocodazole treatment, as described in Materials and Methods. Cells in anaphase were monitored 100 min after nocodazole removal and the proportion of cells showing lagging chromosomes was assessed by DAPI staining. The graph on the right depicts results from four determinations totaling at least 60 cells per experimental condition (*p<0.01). Arrow points to the lagging chromosomes.

1.5. Interaction of vimentin with cytoskeletal proteins during mitosis

Cytoskeletal proteins are in a constant crosstalk, which is necessary to maintain cell shape and mechanics [1]. The interaction of vimentin with other cytoskeletal proteins, namely with tubulin and actin is well known. However, in mitosis their interplay is not completely understood. Therefore, the interaction of vimentin with tubulin and actin during mitosis and the importance of the tail domain in this interaction were addressed next.

1.5.1. Vimentin distribution in mitosis apparently does not require microtubule integrity

Vimentin distribution is known to be dependent on microtubules, since short vimentin filaments move along these structures to form extended filaments [218]. The effect of microtubule disruption on vimentin in interphase cells is reported; nevertheless in mitosis the interplay of these proteins is not described. The effect of microtubule depolymerization

on the distribution of the vimentin network in SW13/cl.2 cells was assessed in cells transfected with RFP//vimentin wt or RFP//vimentin (1-411) (Fig. 13).

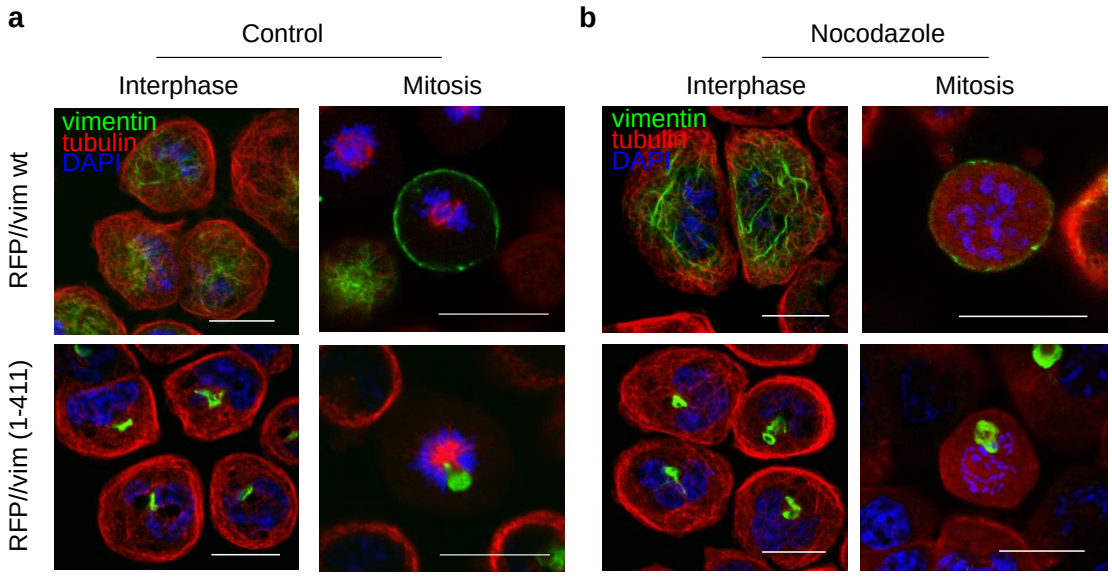


Figure 13 – Distribution of vimentin wt and vimentin (1-411) upon microtubule disruption. SW13/cl.2 cells were transiently transfected with RFP//vim wt or RFP//vim (1-411) and treated with vehicle DMSO (0.2%, v/v) (a) or with 5 μM nocodazole for 30 min in serum-free medium (b). Cells were fixed and the distribution of vimentin (green) and tubulin (red) was assessed by immunofluorescence in interphase and mitotic cells. Nuclei were stained with DAPI. Results are representative from at least three experiments with similar results. Images represent the overlay channels from a single section at mid-cell height. Scale bars, 20 μm.

Under our experimental conditions, vimentin wt and tubulin showed a filamentous distribution in interphase cells. In dividing cells tubulin formed the mitotic spindle and vimentin wt distributed at the cell periphery (Fig.13a, upper panels). Nevertheless, vimentin (1-411) aggregates localized near the mitotic spindle and the dividing chromosomes (Fig. 13a, lower panels). The inhibition of microtubule polymerization with nocodazole did not drastically change the distribution of vimentin and tubulin filaments in interphase (Fig. 13b, left panels). However, in mitosis it led to a diffuse tubulin pattern and completely blocked the formation of the mitotic spindle. This promoted a disordered organization of the chromosomes but did not affect vimentin wt peripheral distribution. In turn, vimentin (1-411) bundles remained close to the dividing chromosomes (Fig. 13b, right panels). Therefore, vimentin distribution in mitosis does not seem to be dependent on microtubule integrity.

1.5.2. Vimentin distributes towards the cell periphery in mitosis in an actin-dependent manner

The interaction of vimentin and actin has been extensively studied, although in mitosis this interaction has not been explored. During cell division, actin forms the rigid cell cortex underneath the plasma membrane, which is responsible for cell rounding, cortical stiffness and spindle positioning [219]. Several proteins are known to localize at the cell cortex with actin, including crosslinking proteins (actinin and filamin) and proteins involved in contractility, such as myosin [4]. Given the marked peripheral distribution of vimentin wt in mitosis, its potential colocalization with actin during cell division was studied (Fig. 14).

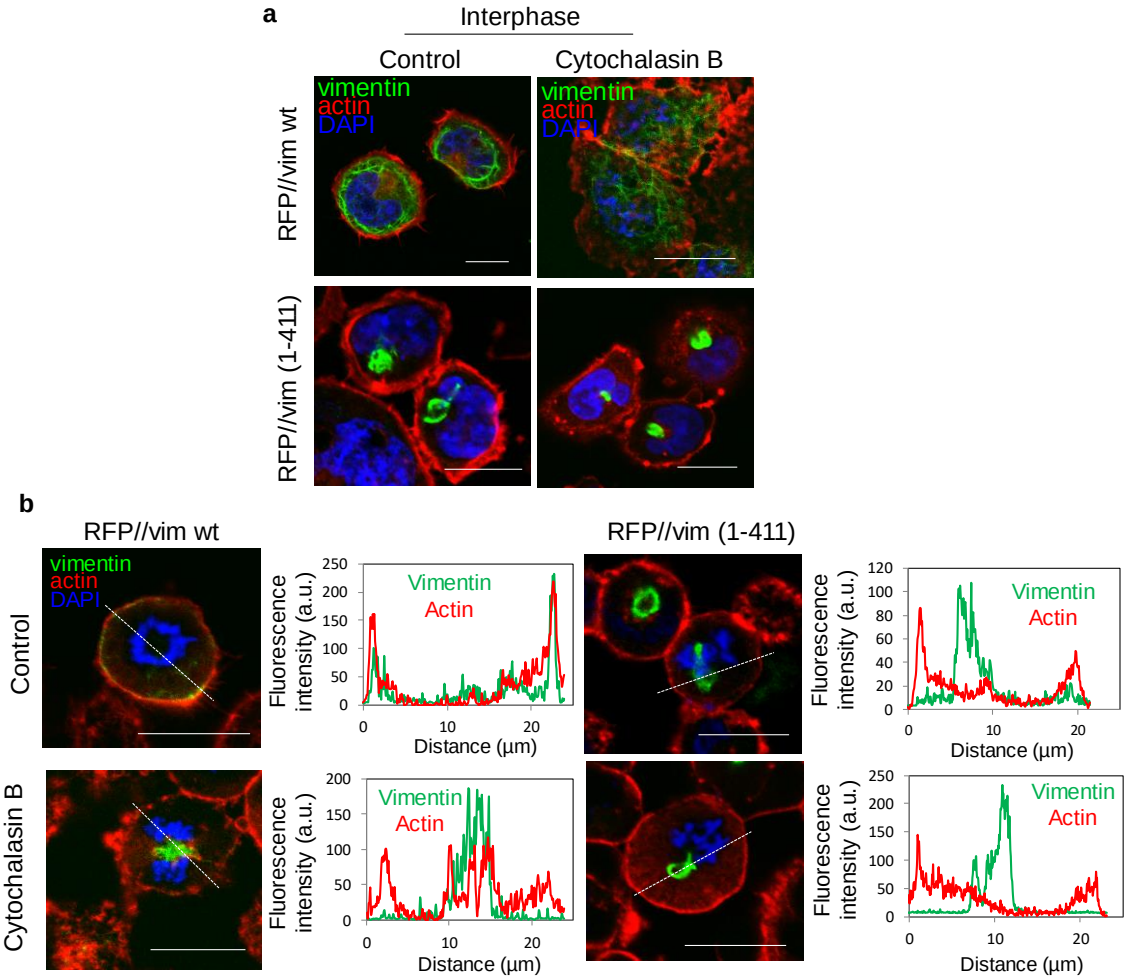


Figure 14 – Effect of actin disruption on vimentin distribution. SW13/cl.2 cells were transfected with the indicated plasmids and cultured in the absence or presence of 10 µg/ml cytochalasin B for 30 min in serum-free medium, in interphase (a) and mitotic cells (b). Vimentin was visualized by immunofluorescence (green), f-actin was stained with Phalloidin (red) and nuclei with DAPI. Images shown are overlay channels of single sections. Fluorescence intensity profiles (b) of vimentin and actin along the dotted lines are shown in the right panels. The experiment was repeated at least 3 times and representative images are shown. Scale bars, 20 µm.

In interphase cells, while vimentin wt presented a filamentous cytoplasmic distribution, actin showed a distribution both at the cell cortex and in short cytoplasmic fibers. In contrast, vimentin (1-411) showed the typical appearance at the center of the cell, in close contact with the cell nucleus (Fig. 14a, left panels). In cells treated with cytochalasin B (Cyt B), an inhibitor of actin polymerization [220], an irregular distribution of actin was observed but vimentin wt maintained its cytoplasmic filamentous distribution and vimentin (1-411) was not altered either (Fig. 14a, right panels).

Interestingly, in mitosis, vimentin wt was in very close proximity to the actin cortex, which is illustrated in the fluorescence intensity profile. On the other hand, in cells treated with Cyt B, cortical actin was disrupted and vimentin wt displaced from the cell periphery accumulating close to the dividing chromosomes (Fig. 14b, left panels). In contrast, vimentin (1-411) stayed at the center of the cell, as clearly demonstrated in the fluorescence intensity profile, which also showed the absence of vimentin coinciding with f-actin at the cell cortex. Moreover, vimentin (1-411) bundles did not undergo any significant change upon treatment (Fig. 14b, right panels).

Taken together, these results suggest that vimentin redistribution to the cell periphery in mitosis is actin-dependent and that the tail domain of vimentin is required for this process.

1.5.3. Alteration of actin polymerization with various agents affects vimentin wt peripheral distribution in mitosis

A more detailed analysis of the role of f-actin in vimentin distribution in mitosis was carried out by using other agents known to alter actin polymerization, such as latrunculin-A (LatA), which inhibits [221], and jasplakinolide (Jasp), which promotes [222], actin polymerization. Treatment of cells with LatA elicited a marked diminution of f-actin staining. In interphase cells, only disperse f-actin aggregates were observed, while vimentin filament distribution was not severely affected. Treatment with Jasp provoked the accumulation of actin filaments, which overlapped with vimentin at some points (Fig.15a, upper panels). In mitosis, cortical actin was entirely lost with Lat A treatment, and cortical vimentin was reduced by 40%. With Jasp, cortical actin was not completely lost, but vimentin distribution showed a mixed pattern, with some cells preserving cortical vimentin distribution while others showed filaments dislodged from the periphery (Fig. 15a, lower panels).

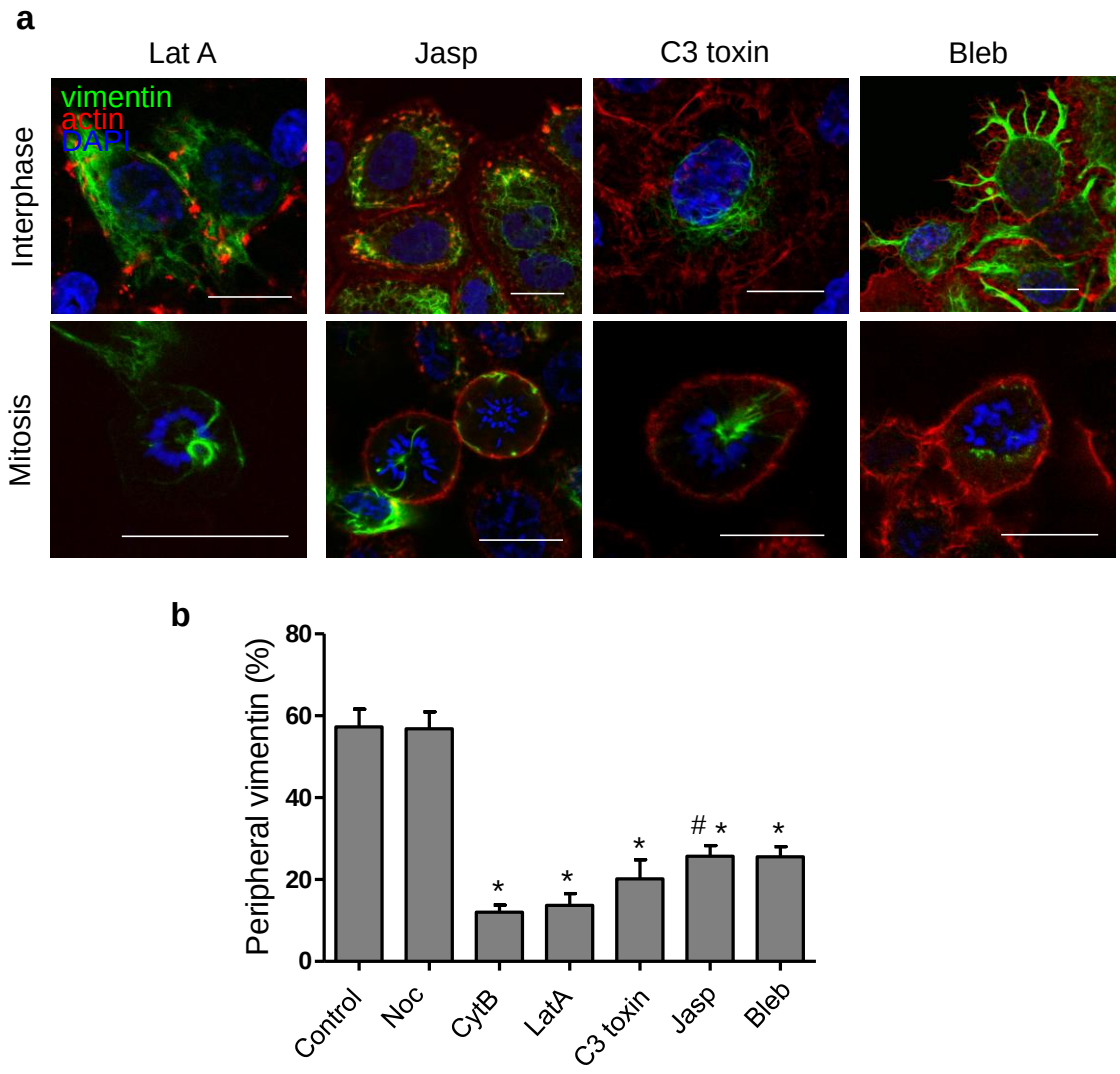


Figure 15 – Vimentin cortical distribution is impaired by f-actin disruption. (a) SW13/cl.2 cells stably transfected with RFP//vim wt were treated with 2.5 μM latrunculin A (LatA) or 50 nM jasplakinolide (Jasp) for 30 min in serum-free medium; C3 toxin (2 μg/ml) was used for 3.5 h and 20 μM blebbistatin (Bleb) for 1h. (b) The percentage of peripheral vimentin is shown in the graph in control and treated cells (*vs control and Noc, # vs CytB, using Turkey's multiple comparison test, ($p<0.05$)).

The effect of other agents that interfere with proteins responsible for the formation of the actin cortex was also evaluated. Namely, inhibition of Rho proteins, which are responsible for actin stability and cortex assembly [157, 223], with C3 toxin, or inhibition of the myosin II specific ATPase with blebbistatin [224, 225] also induced a dislodgement of vimentin from the cell periphery, although cortical actin was not completely lost with these treatments (Fig. 15a, right panels). The graph in Fig. 15b depicts the percentage of cortical vimentin with the different disrupting agents. The

results obtained with nocodazole and Cyt B are also included for comparison. As observed, all the agents used to disrupt the actin cortex caused a reduction of the percentage of peripheral vimentin, whereas nocodazole did not alter vimentin distribution. With these results we can conclude that alteration of actin promotes a significant change in vimentin distribution in mitosis. The effects of the different agents used are summarized in table 4.

Table 4 – Agents used to disrupt cytoskeletal structures.

Agent	Target	Action	Effect on cortical vimentin
Nocodazole	Tubulin	Polymerization inhibition	No effect
Cytochalasin B	Actin	Polymerization inhibition	Dislodgement
Latrunculin A	Actin	Polymerization inhibition	Dislodgement
C3 toxin	Rho A →→ actin	Polymerization inhibition	Dislodgement
Jasplakinolide	Actin	Promotion and stabilization of f-actin	Partial dislodgement
Blebbistatin	Myosin	Inhibition of myosin II ATPase	Partial dislodgement

Altogether, these results point to the importance of the tail domain on vimentin distribution in interphase and in mitotic cells. Moreover, the peripheral distribution of vimentin during mitosis is dependent on the integrity of the actomyosin cortex. The interplay of vimentin and actin will be studied in more detail in the next chapter.

1.6. Cortical distribution of vimentin in mitosis occurs in several cell types

Intermediate filaments are highly regulated in mitosis, although the structural changes depend on the cell type and on the IF protein. During mitosis vimentin can be disassembled or remain in filaments, which is mainly regulated by post-translational modifications, particularly phosphorylation [146, 226, 227].

The distribution of vimentin in different cellular types from diverse origins, including both tumoral and non-tumoral cells and cells from primary cultures was analyzed, both in interphase and in mitosis (Fig. 16).

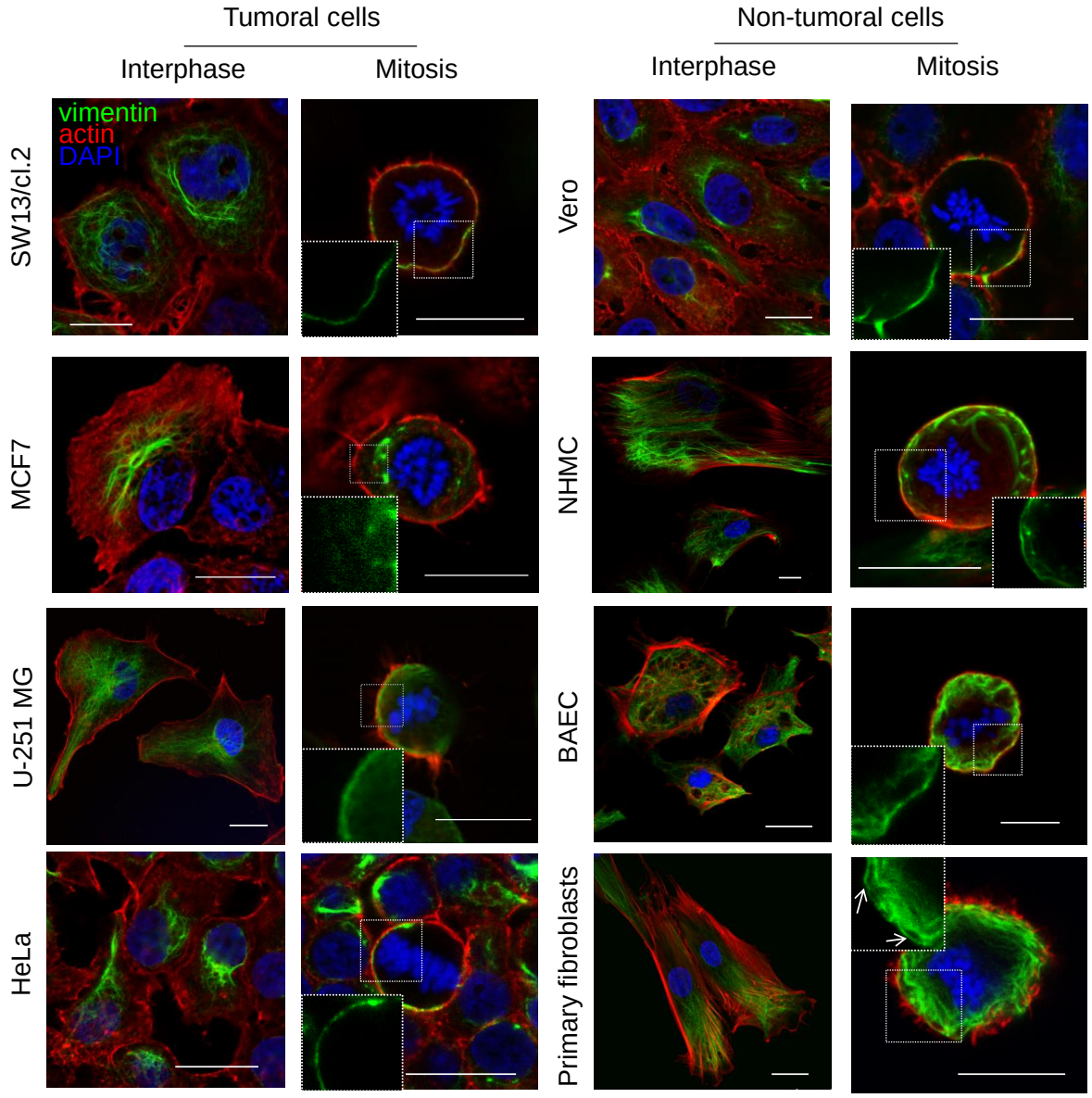


Figure 16 – Cortical association of vimentin in several cell types. The distribution of vimentin and f-actin in interphase and mitotic cells was analyzed in several different cell types. SW13/cl.2 and MCF-7 cells were previously transfected with RFP//vim wt. The additional cellular types hold and endogenous vimentin network. Insets depict enlarged areas of the cell periphery showing vimentin distribution. Representative single sections at mid-cell height are shown. Cells were fixed and stained with Alexa488-conjugated V9 vimentin antibody (green), f-actin with Phalloidin (red) and nuclei with DAPI. Scale bars, 20µm.

As vimentin-positive, non-tumoral cells, bovine aortic endothelial cells (BAEC), primary human dermal fibroblasts (AG10803), fibroblast-like cells (Vero) and human

mesangial cells (NHMC) were used. Human glioblastoma astrocytoma (U-251 MG) and HeLa cells were used as vimentin-positive tumoral cells, and SW13/cl.2 and human breast carcinoma cells (MCF7) as vimentin-negative cells. Vimentin distribution in interphase cells was similar in all cell types, showing the typical cytoplasmic distribution, although with a variable degree of filament extension (Fig. 16, left images)

It was observed that, in addition to SW13/cl.2, vimentin filaments persisted in mitosis in Hela, Vero, NHMC, BAEC and primary fibroblasts, whereas they were mainly disassembled in MCF7 cells and in U-251 MG cells the pattern observed was mixed. Nevertheless, except in MCF7, a certain degree of cortical localization was observed in most cells (Fig. 16, insets).

Thus, these results show that vimentin distribution during mitosis is cell type dependent, and that association with cortical actin occurs in several cell types [228].

1.7. Cortical distribution of vimentin is mitosis-dependent

The cortical distribution of vimentin could be also a consequence of cell rounding. To discard this possibility, the distribution of vimentin and actin was evaluated in newly-plated, round cells.

In these cells, vimentin presented a perinuclear distribution that was not related to actin (Fig 17, inset). Orthogonal projections confirmed the perinuclear distribution of vimentin and its absence from the basal layer of the cell.

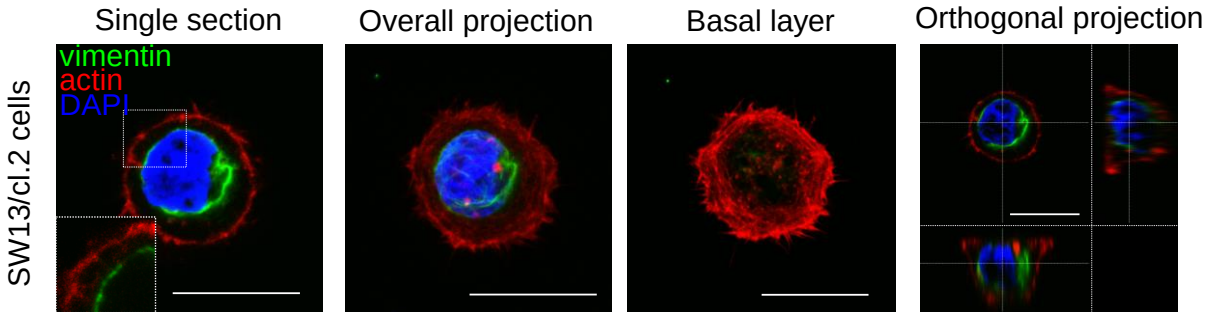


Figure 17 – Vimentin distribution in newly-plated cells. SW13/cl.2 cells stably transfected with RFP//vim wt were detached by trypsinization and plated in a new dish. Cells were fixed 10 min after plating and the distribution of vimentin, f-actin and nuclei was assessed as above. Scale bars, 20µm.

These results confirm that vimentin cortical distribution is not a consequence of cell rounding and is mitosis-dependent.

2. Vimentin-actin interplay in mitotic cells

The results shown above illustrate that in SW13/cl.2 cells transfected, vimentin distributes towards the cell periphery during mitosis in an actin-dependent manner. This interaction at the cell periphery points to intermediate filaments as new players in cell cortex structure and dynamics. Super-resolution microscopy and time-lapse experiments were employed to study the relative distribution of these two proteins in more detail. Additionally, studies were performed to determine the sequence of the C-terminal domain of vimentin responsible for the peripheral distribution and the interaction with the actin cortex.

2.1. Vimentin is in close proximity of actin at the cell cortex in mitosis

The study of the interaction of vimentin and actin in mitosis was performed by super-resolution microscopy, using stimulated emission depletion (STED). For this, Vero cells, which possess an endogenous vimentin network, and SW13/cl.2 cells stably transfected with vimentin wt were used. The proportion of cells in mitosis was increased by treatment with low concentrations of nocodazole. Mitotic cells were identified using DIC by its rounded shape and by the star-like pattern of the dividing chromosomes (Fig. 18a). Middle cell sections were selected to be analyzed by STED microscopy.

In Vero cells, vimentin distributed very close to the actin cortex and is localized at or immediately bellow the actin layer (Fig. 18b). In some SW13/cl.2 cells it was possible to detect vimentin between two actin layers (Fig. 18c, upper panels) or entangled with actin, going in and out of the actin cortex (Fig. 18c, lower panels). The proximity of vimentin and actin was highlighted by the colocalization mask.

A 3D reconstruction of vimentin in SW13/cl.2 cells (Fig. 18d) showed the robust vimentin filaments in diverse orientations. Fig. 18e depict a 3D reconstruction of vimentin and f-actin at the bottom half of the same cell, showing that f-actin forms a sphere constituted by a patchy pattern of structures perpendicular to the cell surface. Penetration sites of vimentin in the actin structure, concordant with the pattern observed at middle sections by STED were also detected.

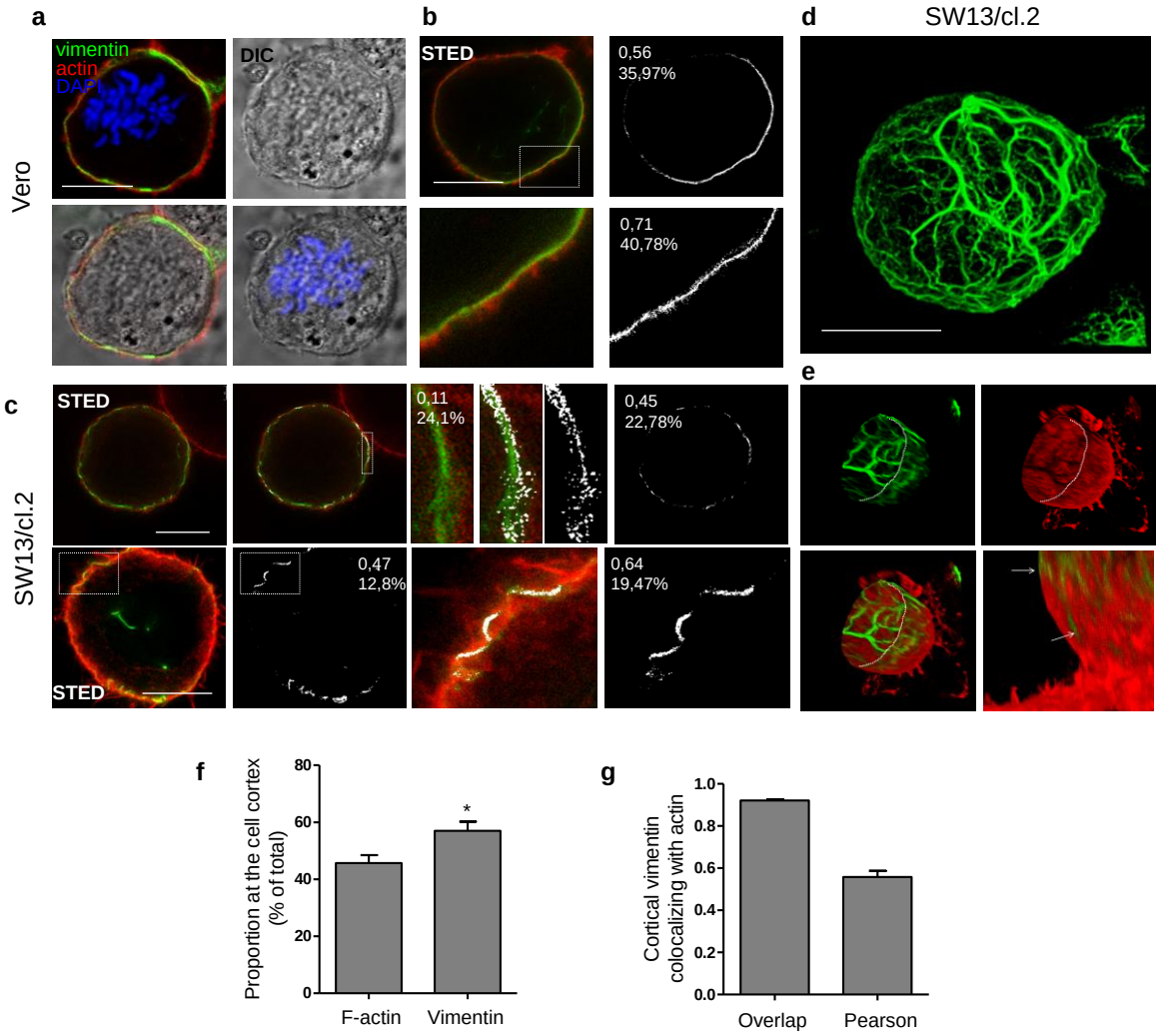


Figure 18 – STED super-resolution microscopy of vimentin and f-actin in mitosis. (a) Confocal microscopy images illustrating the identification of mitotic Vero cells by the typical aspect of dividing chromosomes in DIC, confirmed by DAPI staining in merged images. The distribution of f-actin and vimentin was observed by TRITC-Phalloidin staining and immunofluorescence with Alexa488-conjugated V9 antibody, respectively. (b) Mitotic Vero cells were analyzed by STED and images of one cell are shown. Colocalization masks (in white) of the whole section or the area enlarged, delimited by the dashed rectangle, are shown to the right. Colocalization analysis was performed with Leica software, LasX. Numbers in insets represent the Pearson's coefficient and the percentage of colocalization for the selected regions. (c) SW13/cl.2 cells stably transfected with RFP//vim wt were treated with 0.4 µM nocodazole overnight to increase the proportion of mitotic cells. Vimentin and f-actin were detected as above. STED images of several cells are shown. Colocalization masks (white) or enlargements of areas delimited by dashed rectangles are shown to the right of the corresponding images. Numbers in insets represent the Pearson's coefficient and the percentage of colocalization for the selected regions. (d) 3D reconstruction of vimentin organization in a mitotic SW13/cl.2 cell. (e) 3D reconstruction of the basal half section of the same cell, to show the inside and outside of the sphere. Single channels and overlay channels (lower panels) are shown. Deconvolution of the green channel was performed using Imaris software for one representative cell treated as in (c). The arrows point at protrusions of vimentin through the actin cortex. (f) Proportion of f-actin or vimentin located at the cell cortex (*p<0.02). (g) Colocalization of cortical vimentin with f-actin by overlap and Pearson coefficients for the cortical ROI. Scale bars, 10 µm.

Lastly, the proportion of cortical f-actin and vimentin was quantified at middle sections of the cells, f-actin showed a 45,7% of cortical distribution, while 57% of vimentin was localized at the cell periphery. The calculated overlap and Pearson coefficients of 0.92 and 0.56, respectively, represent the cortical vimentin colocalizing with actin at the cell cortex. These results indicate that vimentin closely interacts with the actin cortex during mitosis.

2.2. Vimentin affects cortical actin distribution in mitosis

The cell cortex properties are regulated by different proteins, mainly crosslinking or actin binding proteins. For instance, myosin [157] and Arp2/3 complex proteins [229] regulate the heterogeneity of the actin cortex and the formation of actin filaments, consequently affecting cortex thickness and cortical tension [158]. Therefore, a potential effect of vimentin in the properties of the actin cortex was studied.

2.2.1. F-actin distribution is more irregular in vimentin-deficient cells

A more detailed analysis of the vimentin-actin interplay at the cell cortex was performed by obtaining 3D reconstructions of vimentin and f-actin in vimentin-positive and negative cells (Fig. 19). From the 3D reconstruction, a 2D map of the cell periphery was obtained to analyze and quantify the cell cortex properties [211]. Analysis of the 2D projections confirmed that vimentin, at some points, was outside the actin layer (Fig 19, upper panels). Fluorescence intensity profiles obtained from the 2D map revealed a more irregular pattern of the f-actin layer in non-transfected cells and cells expressing the RFP// empty plasmid than in cells expressing vimentin (Fig. 19c).

Additionally, an alternate distribution of vimentin and f-actin signals was observed in the fluorescence intensity profile of the RFP//vimentin wt cells, indicating that the presence of vimentin may interfere with f-actin distribution. To confirm whether vimentin affected the uniformity of actin distribution, the standard deviation of the f-actin pixel signal from the 2D projection was calculated. A significantly higher standard deviation of f-actin signal was obtained in cells lacking vimentin filaments, indicating larger variations of f-actin distribution (Fig. 19d).

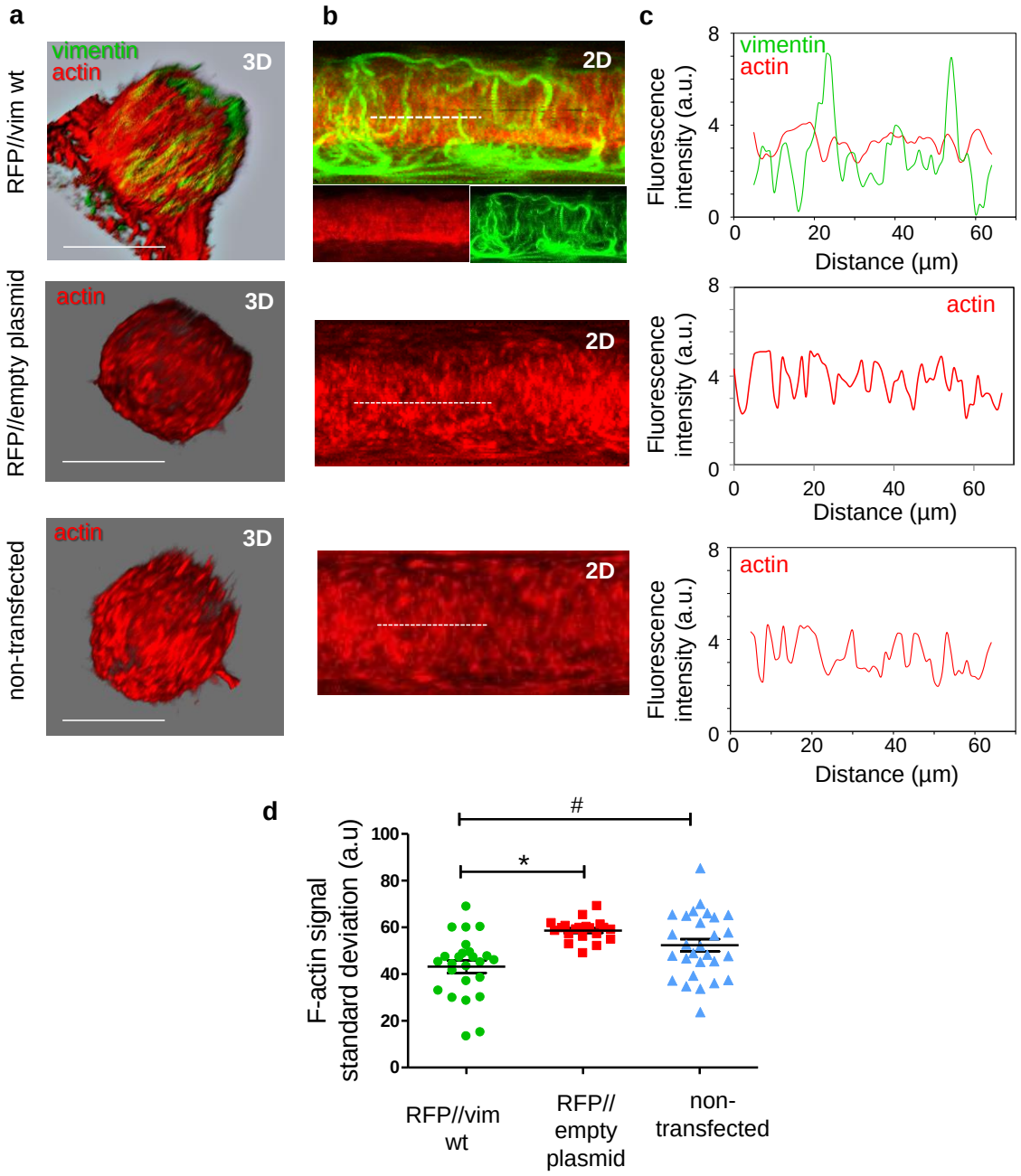


Figure 19 – Vimentin affects the properties of f-actin in mitosis. Vimentin was detected by immunofluorescence and f-actin by Phalloidin staining. XY image stacks were used to obtain 3D-reconstructions of mitotic cells and 2D-projections. (a) 3D-reconstruction of SW13/cl.2 cells, stably expressing vimentin wt (top), RFP from the bicistronic pIRES DsRed-Express2 empty plasmid (RFP// empty plasmid, middle), or non-transfected (bottom). (b) 2D-maps from the same cells (for cells expressing vimentin wt, merged and single channels are shown). (c) Fluorescence intensity profiles along the dotted lines on the 2D maps. (d) Graph depicting the standard deviation values of f-actin from the 2D-maps. Images are representative of three experiments and at least 20 cells were measured per experimental condition. (*p<0.001, #p<0.02 vs the indicated experimental condition). Scale bars, 20 µm.

2.2.2. The proportion of f-actin at the basal region of the cell is affected by the presence of vimentin

In orthogonal projections, vimentin wt was detected both at the top and at the basal layers of the cell (Fig. 20a, upper panels). At the bottom of the cell, vimentin wt formed a net of robust filaments. In contrast, in vimentin (1-411) expressing cells low levels of vimentin were detected at the basal and top layers, due to its accumulation at the center of the cell and in some cases close to an inner actin ring, similar to the one described as an important structure for spindle positioning and orientation [166]. Interestingly, the basal f-actin signal was less intense in cells expressing vimentin wt than in non-transfected cells or cells expressing vimentin (1-411) (Fig. 20a, lower panels).

In addition, the proportions of basal vimentin and f-actin with respect to the total signal intensity for each cell were quantified. Consistent with the above results, cells expressing vimentin wt displayed a higher proportion of vimentin at the basal layers (Fig. 20b). In turn, f-actin showed reciprocal changes, with basal levels being significantly higher in cells lacking vimentin filaments and in cells expressing vimentin tailless (Fig. 20c).

Overall, these results suggest that vimentin exerts an important effect on actin distribution and characteristics during mitosis. These effects may have an impact on cell cortex dynamics during cell division and in mitotic progression.

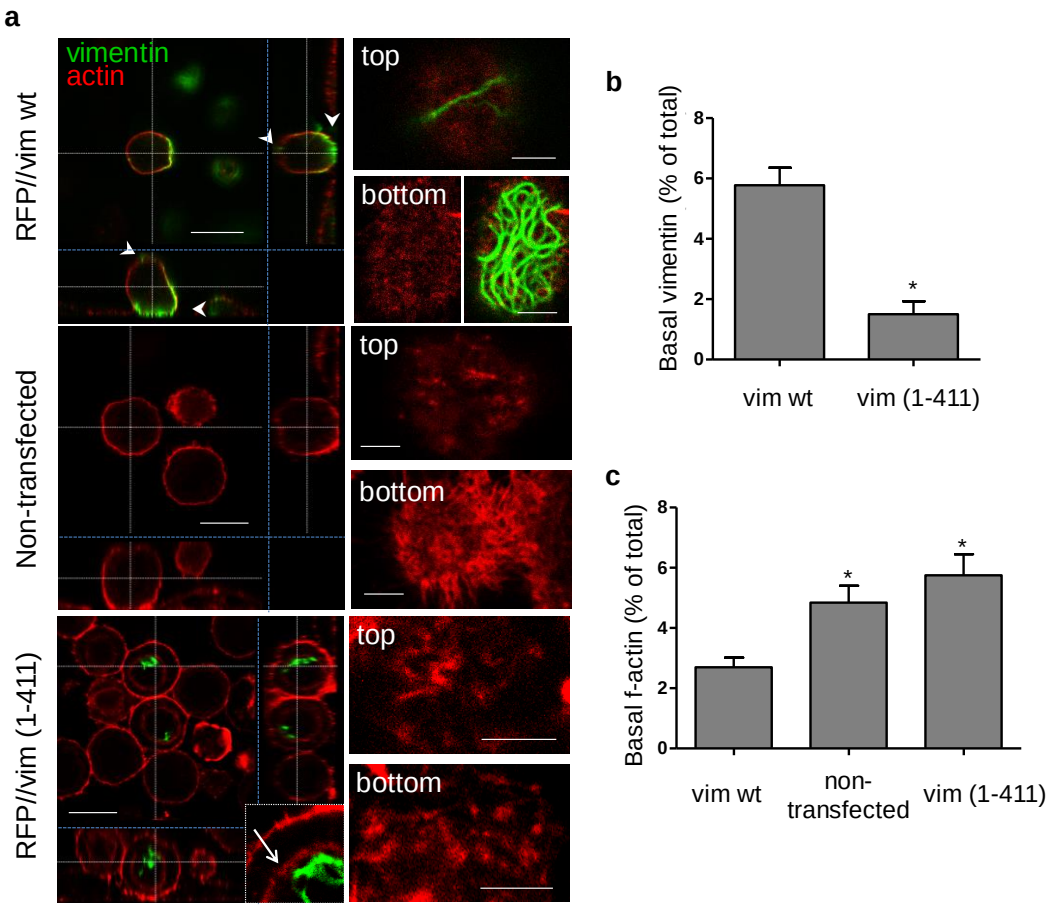
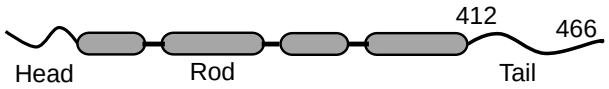


Figure 20 – Basal f-actin is affected by the presence of vimentin. (a) Single sections and orthogonal projections along the marked axes of SW13/cl.2 cells transfected with RFP//vim wt, RFP//vim (1-411) or non-transfected. The projections illustrate the position of the vimentin constructs in the cell. Scale bars, 20 μm. Arrowheads mark the presence of vimentin wt at the top and bottom of the cell. Inset at the bottom right of the RFP//vim (1-411) panel shows the position of vimentin (1-411) with respect to the cytoplasmic f-actin ring (arrow). Right panels show enlarged images of the bottom and top sections of one cell. Scale bars in right panels, 5 μm. (b) Basal levels of vimentin wt and (1-411) are represented in the graph with respect to the total cellular content, obtained from at least 19 determinations (*p<0.0001 vs vim wt). (c) The graph depicts the percentage of f-actin present at the basal layer with respect to the total content, obtained from at least 18 determinations (*p<0.002 vs vim wt).

2.3. Impact of C-terminal deletions on vimentin peripheral distribution

The results shown above demonstrated that vimentin was in close contact with the actin cortex and that the tail domain was important for vimentin peripheral distribution in mitosis. Nevertheless, the structural elements required for this interaction are not known. Previous evidence suggested that the C-terminal domain of vimentin was important for the cellular location of the protein [33]. Therefore, we generated several vimentin mutants lacking different parts of the tail domain for expression in SW13/cl.2 cells (Fig. 21a).

a



Vimentin construct	Vimentin tail sequence
Full length vimentin	SLPLPNFSSLNLRETNLDSLPLVDTHSKRTLLIKTVETRDGQVINETSQHHDDLE
Vimentin (1-459)	SLPLPNFSSLNLRETNLDSLPLVDTHSKRTLLIKTVETRDGQVINETS
Vimentin (1-448)	SLPLPNFSSLNLRETNLDSLPLVDTHSKRTLLIKTVE
Vimentin (1-423)	SLPLPNFSSLNL
Vimentin (1-411)	-

b

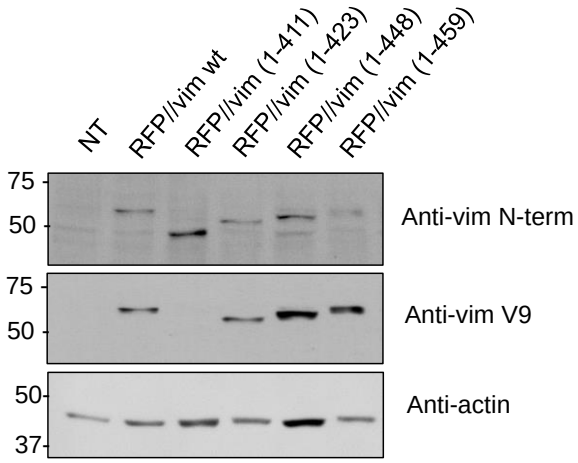


Figure 21 – Electrophoretic mobility of vimentin truncated constructs. (a) Scheme of the vimentin monomer and sequence of the different vimentin C-terminal truncated constructs. (b) SW13/cl.2 cells were transfected with the indicated constructs coding for vimentin wt or the various truncated forms. Cell lysates were analyzed by western blot with antibodies against the N-terminus (upper panel) or the C-terminus of vimentin (middle panel, V9 monoclonal antibody). The same membrane was incubated with anti-actin antibody as a control (lower panel). NT, non-transfected.

The electrophoretic mobility of these constructs present in lysates from transfected cells was assessed using two different antibodies, namely, an antibody raised against the N-terminal domain of the protein, and the V9 antibody which is raised against the C-terminal domain [216]. With the N-terminal antibody all the constructs were detected with the expected mobility. The V9 antibody failed to detect vimentin (1-411), consistent with the absence of its epitope in this construct. The signal given by an anti-actin antibody was used as protein loading control (Fig. 21b).

The first mutant generated was vimentin (1-423) which lacks the last 43 amino acids of the vimentin sequence. This mutant mimicked a previously described cleavage product of vimentin by the HIV-type I protease [76]. SW13/cl.2 cells were transfected with

RFP//vimentin (1-423) (untagged vimentin) alone or RFP//vimentin (1-423) plus a small amount of GFP-vim (1-423). In both cases, vimentin (1-423) adopted the same distribution in interphase cells forming aggregates similar to those observed with vimentin (1-411) that also localized close to the cell nucleus (Fig. 22a-c). In mitosis, those aggregates stayed near the dividing chromosomes (Fig. 22c and d), not showing any peripheral distribution or association with the actin cortex (see fluorescence intensity profile).

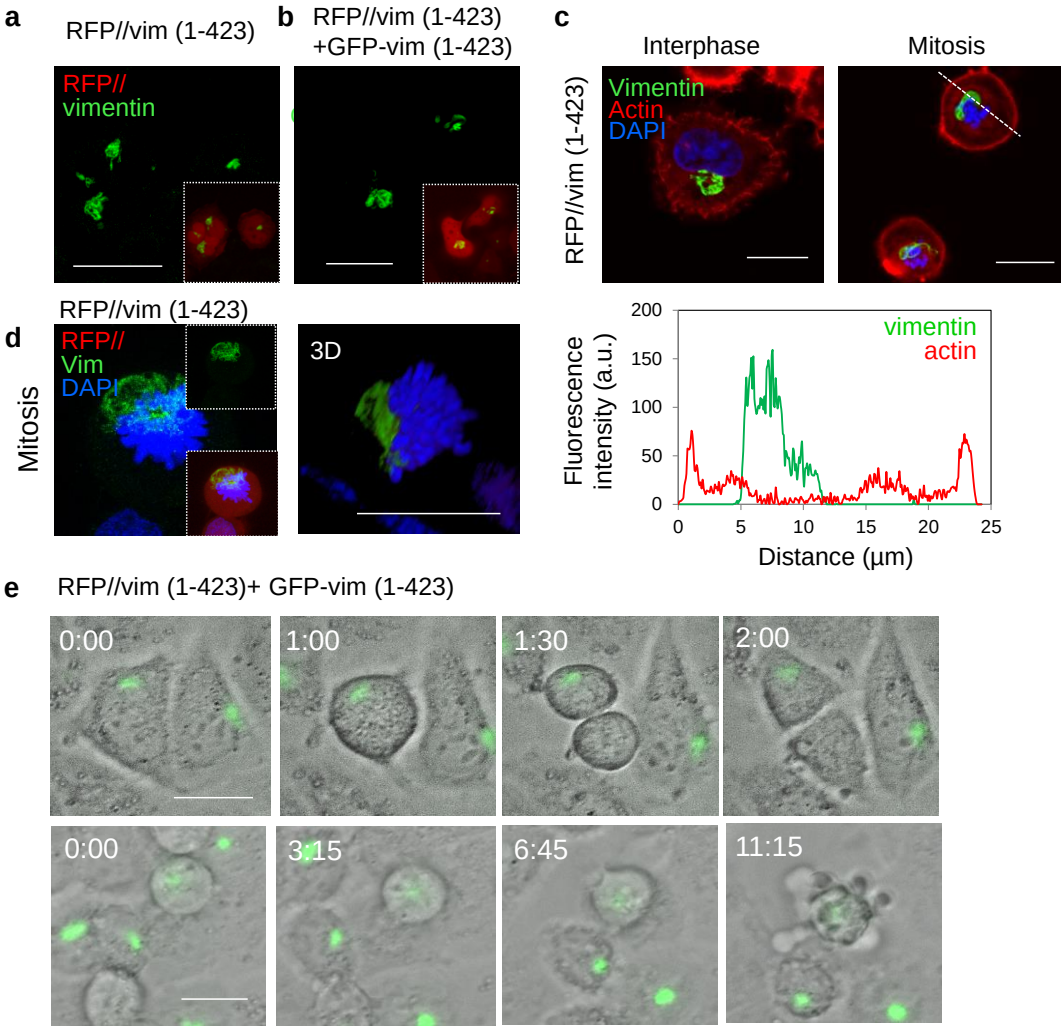


Figure 22 – Distribution of vimentin (1-423) in interphase and mitotic cells. SW13/cl.2 cells were transiently transfected with RFP//vim (1-423) (a, c, d) or with RFP//vim (1-423) + GFP-vim (1-423) (b, e). (a) Overall projections of interphase cells, showing immunofluorescence of vimentin or (b) GFP-vim fluorescence and the cytoplasmic RFP fluorescence (insets). (c) Interphase cells (left panel) and mitotic cells (right panel) are shown. Vimentin was visualized by immunofluorescence, f-actin by Phalloidin staining and nuclei by DAPI, and single sections are depicted. The fluorescence intensity profiles of vimentin and f-actin signals along the dotted line in the mitotic cell are represented in the graph. (d) Left panel, overall projections of vimentin and DAPI, vimentin alone (green) or overlays of vimentin, DAPI and RFP fluorescence (inset). The right panel displays the 3D projection of the same cell showing vimentin and DAPI. (e) Time-lapse experiments of cells expressing vimentin (1-423) show asymmetric partition of vimentin (upper panels) and cell death (lower panels).

A 3D reconstruction clearly illustrated vimentin (1-423) in contact with the dividing chromosomes (Fig. 22d), which may suggest cytokinetic defects. Time-lapse experiments showed mitotic alterations (Fig. 22e), which included delayed mitosis, vimentin asymmetric partitioning (upper panels) and cell death (lower panels). Most of the cells that were able to divide underwent asymmetric partitioning of vimentin.

The vimentin (1-448) mutant, lacking the last 18 amino acids of the vimentin sequence, was generated to remove the RDG motif, which has been reported to be necessary for normal filament assembly [34, 41].

In non-dividing cells, vimentin (1-448) showed extended filaments together with some protein accumulations (Fig. 23a and b). The filaments persisted in mitosis and a certain degree of cortical localization along with actin was observed (Fig. 23c, fluorescence intensity profile). However, in some cells vimentin accumulations persisted at the basal layers or at the cell periphery as observed in the 3D reconstruction (Fig. 23d). Time-lapse experiments showed that some cells completed division by asymmetric partitioning (Fig. 23e, upper panels), whereas others presented delayed separation which resulted in cell death (Fig. 23e, lower panels).

Thus, the vimentin (1-448) construct showed a more extended filament network, which was not observed with vimentin (1-411) (Fig. 1) or vimentin (1-423) (Fig. 22); nevertheless it still displayed some defects during cell division.

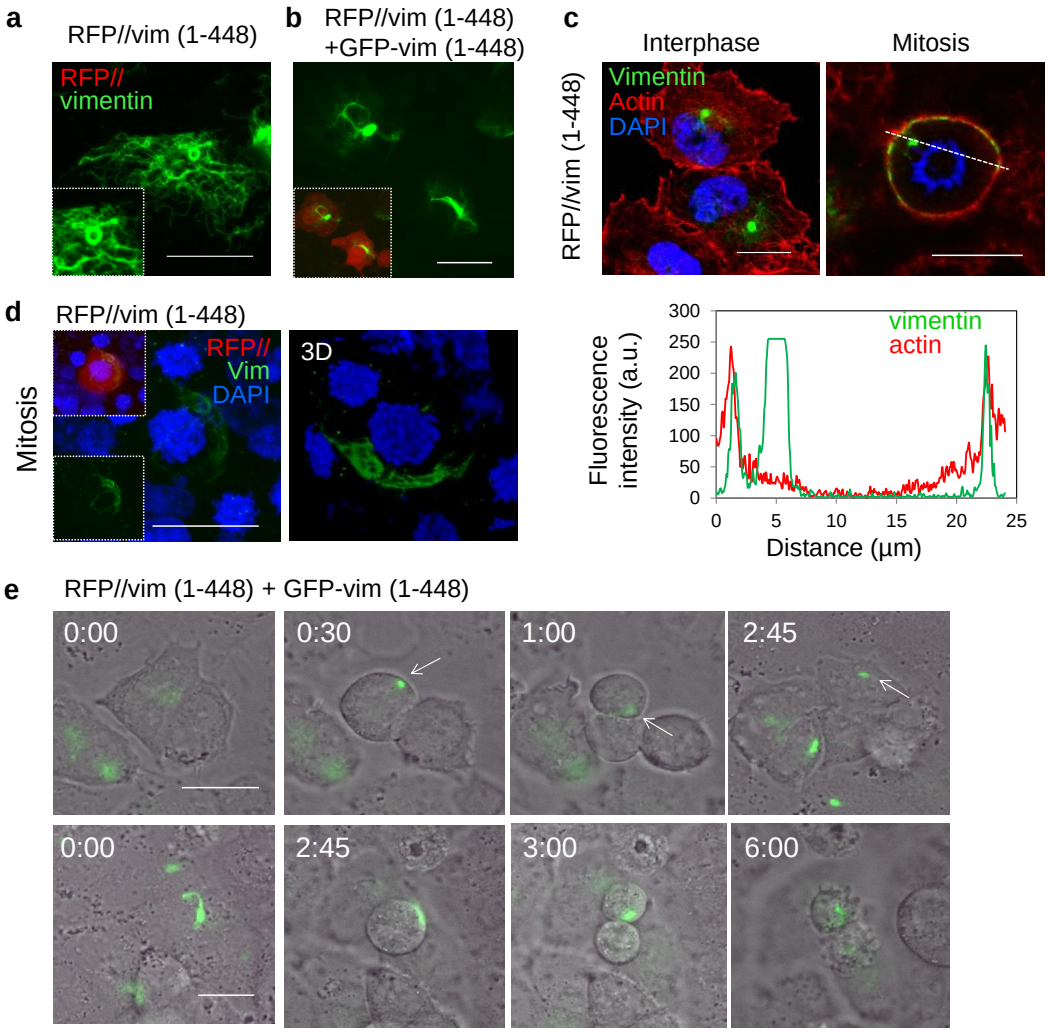


Figure 23 – Distribution of vimentin (1-448) in interphase and mitotic cells. SW13/cl.2 cells were transiently transfected with RFP//vim (1-448) (a, c, d) or with RFP//vim (1-448) + GFP-vim (1-448) (b, e). (a) Overall projections of interphase cells, showing immunofluorescence of vimentin or (b) GFP-vim fluorescence and the cytoplasmic RFP fluorescence (insets). (c) Interphase cells (left panel) and mitotic cells (right panel) are shown. Vimentin, f-actin and nuclei were stained as above. The fluorescence intensity profiles of vimentin and f-actin signals along the dotted line in the mitotic cell are represented in the graph. (d) Left panel, overall projections of vimentin and DAPI, vimentin alone (green) or overlays of vimentin, DAPI and RFP fluorescence (inset). The right panel displays the 3D projection of the same cell showing vimentin and DAPI. (e) Time-lapse monitoring of cells expressing vimentin (1-448) show asymmetric partition of vimentin (upper panels) and mitotic failure (lower panels).

The last C-terminal truncated mutant generated was vimentin (1-459), lacking only the last seven amino acids. This construct formed long cytoplasmic filaments, similar to those observed in vimentin wt, leading to an extended network. Yet, 18% of the cells showed bundles or accumulations (Fig. 24a and b). In mitotic cells, vimentin (1-459) adopted mostly a peripheral distribution, maintaining a close interaction with the actin cortex, as

illustrated in the fluorescence intensity profile (Fig. 24c). Nevertheless, some cells harbored vimentin filaments in contact with the dividing chromosomes (Fig. 24c and d). Time-lapse experiments showed that the majority of the cells divided normally, with nearly uniform distribution of vimentin between the two daughter cells (Fig. 24e, upper panels). Notably, the cells that held vimentin bundles went through partial asymmetric distribution of vimentin, with one of the cells keeping most of the protein (Fig. 24e, lower panels).

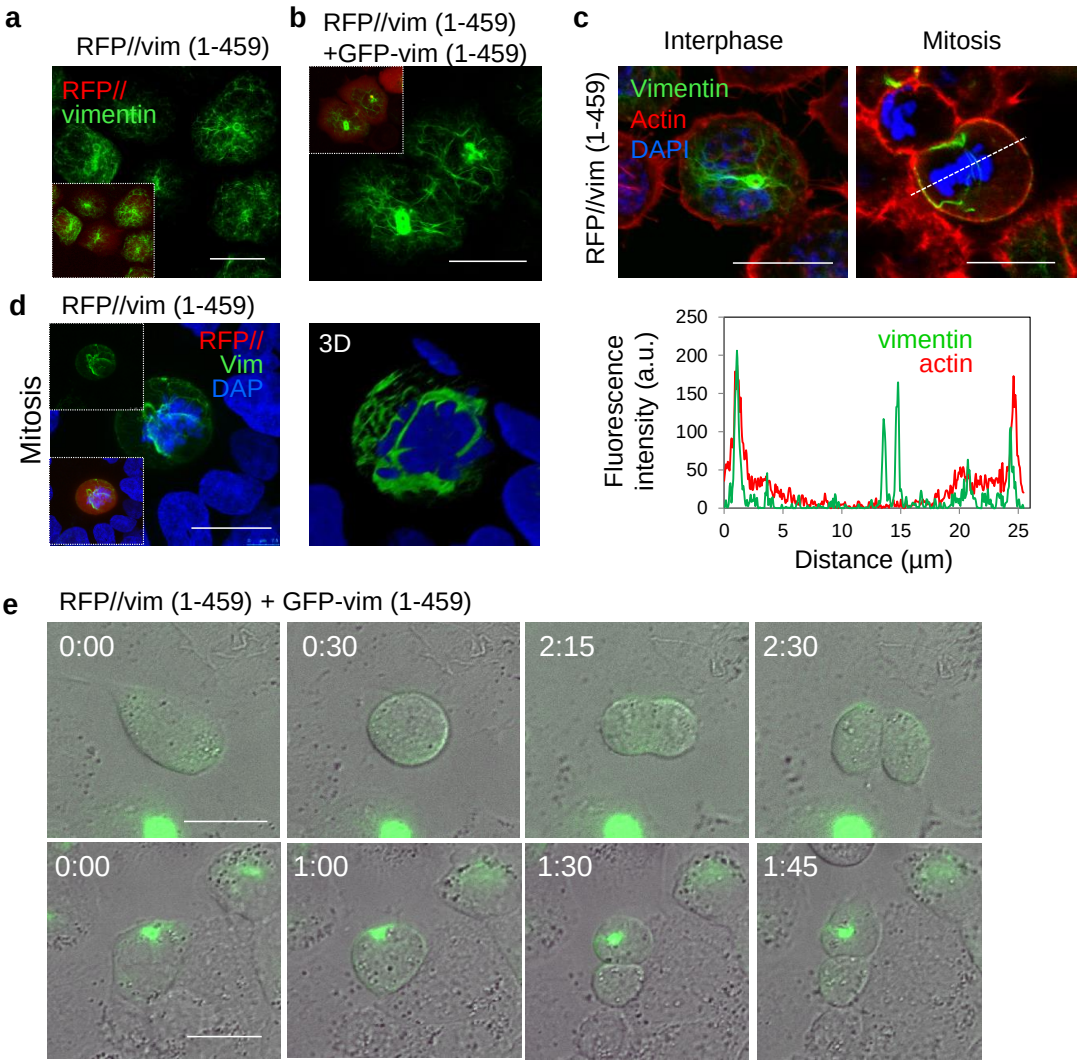


Figure 24 – Distribution of vimentin (1-459) in interphase and mitotic cells. SW13/cl.2 cells were transiently transfected with RFP//vim (1-459) (a, c, d) or with RFP//vim (1-459) + GFP-vim (1-459) (b, e). (a) Overall projections of interphase cells, showing immunofluorescence of vimentin or (b) GFP-vim fluorescence and the cytoplasmic RFP fluorescence (insets). (c) Interphase cells (left panel) and mitotic cells (right panel) are shown. Vimentin, f-actin and nuclei were visualized as above. The fluorescence intensity profiles of vimentin and f-actin signals along the dotted line in the mitotic cell are represented in the graph. (d) Left panel, overall projections of vimentin and DAPI, vimentin alone (green) or overlays of vimentin, DAPI and RFP fluorescence (inset). The right panel displays the 3D projection of the same cell showing vimentin and DAPI. (e) Time-lapse monitoring of cells expressing vimentin (1-459) show normal cell division with even distribution of vimentin (upper panels) and asymmetric partitioning of vimentin (lower panels).

Overall, different fates in cell division and interaction with actin were observed with the different tail domain truncations. Fig. 25 summarizes the observations from Figs. 21-24, showing the different parameters related with vimentin distribution and cell division. In interphase cells, a decrease in the cell area occupied by the vimentin constructs was observed that correlated with the length of the tail domain deletion (Fig. 25a). Moreover, in cells harboring the shortest constructs there was a tendency towards the appearance of aberrant nuclei (Fig. 25b). In mitotic cells, vimentin (1-423) remained near the chromosomes in a higher proportion than the other two constructs (Fig. 25c). In fact, cells expressing this construct needed longer times to divide (Fig. 25d). These results suggest the existence of a correlation between the presence of vimentin near the chromosomes and the duration of mitosis, and potentially other mitotic alterations.

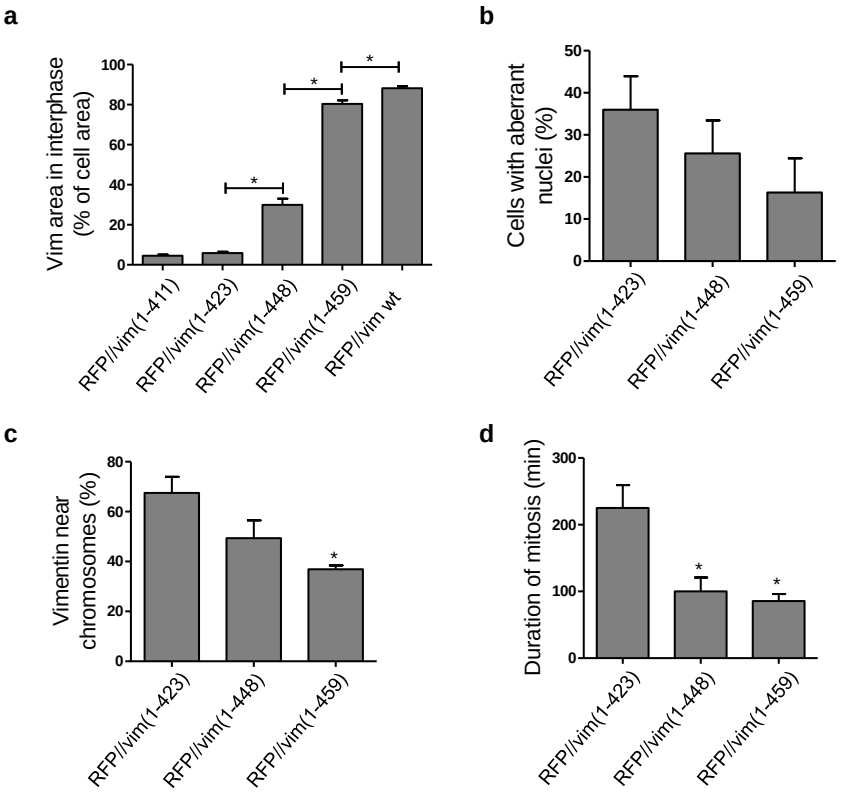


Figure 25 – Distribution and effects of vimentin wt and the C-terminal truncated constructs in cells. SW13/cl.2 cells were transfected with the indicated constructs coding for vimentin wt or the various truncated forms. (a) The ability of the different vimentin constructs to form an extended network was analyzed by the percentage of the cell area occupied by each of them. Results are from three different experiments and at least 20 cells per experimental condition were analyzed (*$p<0.001$ between the indicated conditions). (b) Proportion of cells harboring aberrant nuclei. Results are average values of three experiments totaling at least 150 cells per experimental condition. (c) Percentage of mitotic cells with vimentin near the chromosomes (*$p<0.01$ vs vim (1-423)). Results are average values of three experiments totaling at least 50 cells per experimental condition (d) Duration of mitosis (*$p<0.02$ vs vim (1-423)). Results are average values of at least two experiments totaling at least 14 cells per experimental condition.

Altogether these results indicate that a gradual removal of amino acids from the C-terminal domain of vimentin impairs normal filament extension and peripheral distribution in mitosis. The most drastic effects were observed in cells transfected with the mutants which lacked a major part of the vimentin tail, namely, vimentin (1-411) and (1-423). Additionally, vimentin residues between 424 and 448 seem to be important for the interaction with actin and mitotic distribution.

2.4. The formation of a full filament network is not required for cortical association of vimentin

To discern between the possibilities that the distribution of vimentin towards the cell periphery in mitosis required the formation of full filaments or was impeded by intense bundling, the distribution of GFP-vimentin constructs, which do not reach the stage of fully extended filaments [30], was studied next.

SW13/cl.2 vimentin-deficient cells were transiently transfected with GFP-vimentin fusion constructs with different vimentin sequences, including GFP-vimentin wt and all the truncated mutants. Moreover, to rule out the possibility that impairment of cortical localization could be due to defective elongation, two GFP-vimentin mutants described as being elongation defective were used. The first one is a vimentin mutant at the cysteine residue (GFP-vimentin C328S) which was already described by our group [30] and forms dots in interphase cells. The second one is the mutant G452V. This mutation is located within the 450RDG452 sequence, which has been described as important for filament assembly [34, 41]. The distribution of GFP-vimentin mutations and truncated variants was assessed first in interphase cells (Fig. 26).

GFP-vimentin wt formed short squiggles that distributed throughout the cytoplasm. Shorter constructs, GFP-vim (1-411) and GFP-vimentin (1-423) did not reach the squiggle state and formed only dots in the case of vimentin (1-411), and dots or small aggregates in the case of vimentin (1-423). The GFP-vimentin (1-448) construct formed small aggregates/dots together with some squiggles whereas the longer construct, GFP-vimentin (1-459), formed squiggles very similar to those observed in GFP-vimentin wt (Fig. 26a).

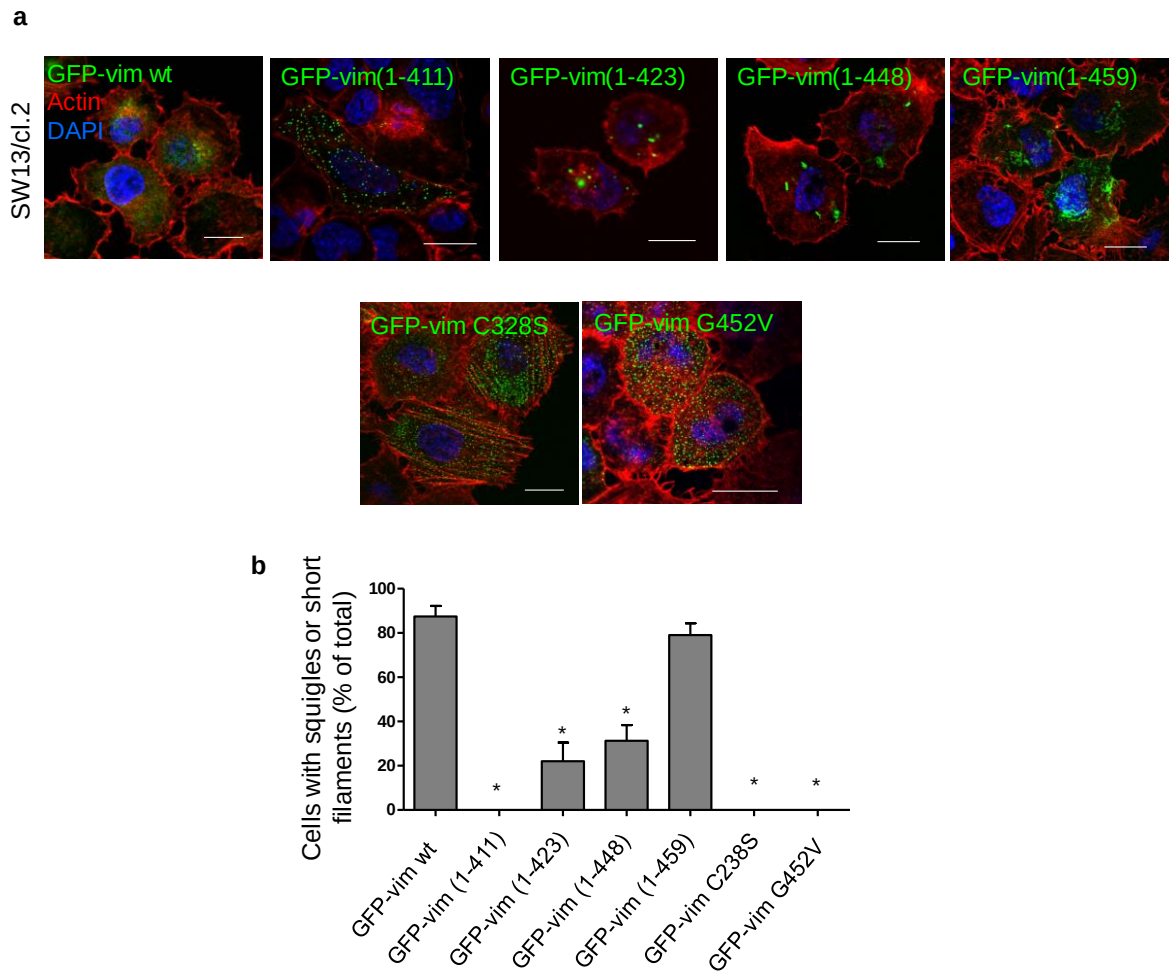


Figure 26 – Distribution of different GFP-vimentin fusion constructs in non-dividing cells. (a) SW13/cl.2 cells were transiently transfected with the indicated constructs and their distribution was evaluated in interphase cells. Cells were fixed and stained with Phalloidin for f-actin visualization and DAPI for nuclei and vimentin was visualized by the GFP fluorescence. Scale bars, 20 µm. (b) The percentage of cells harboring squiggles or short filaments is shown. Results are average values from three experiments and at least 14 determinations per experimental condition (*p<0.0001 vs wt).

Under our conditions, the GFP-vimentin G452V mutant, also failed to assemble into filaments, forming dots in interphase SW13/cl.2 cells (Fig. 26a, lower panel). The percentage of cells presenting squiggles or short filaments upon transfection with the different constructs is represented in Fig. 26b. A significant decrease in the formation of squiggles was seen in the shorter and mutant constructs with respect to GFP-vimentin wt, with the exception of GFP-vimentin (1-459).

The distribution of these constructs was then analyzed in mitotic cells (Fig. 27).

GFP-vimentin wt and GFP-vimentin (1-459) squiggles distributed mainly at the cell periphery colocalizing with f-actin, as shown in the fluorescence intensity profiles. In contrast, GFP-vimentin (1-411) and GFP-vimentin (1-423) dots/aggregates showed a disperse distribution and did not associate with the actin cortex. GFP-vimentin (1-448), which showed a mixture of dots and squiggles, presented a predominant peripheral distribution with some dots in the cell cytoplasm. The vimentin mutants GFP-vimentin C328S and G452V were able to relocate to the cell periphery, despite their inability to reach the squiggle state.

The proportion of dots associated with the cell periphery was quantified for all the constructs that did not reach the squiggle state and is displayed in Fig. 27b. A significant decrease in the association of the constructs GFP-vim (1-411) and (1-423) with the cell cortex in comparison with GFP-vim C328S and G452V was observed. Moreover, the percentage of peripheral vimentin during mitosis for all the constructs was determined (Fig. 27c). In general, GFP-vimentin constructs showed a more diffuse pattern and lower association with the cell periphery in mitosis than untagged vimentin. Nevertheless, a significant decrease of cortical vimentin was observed in GFP-vimentin (1-411) and (1-423) compared to GFP-vimentin wt. In addition, a significant decrease in cortical association was observed between GFP-vimentin (1-459) and GFP-vimentin C328S, probably due to the more diffuse pattern observed in the case of GFP-vimentin C328S.

These results showed that shorter truncated constructs did not reach the cell periphery in mitosis. Moreover, correct filament elongation was not necessary for the interaction with the actin cortex, since the mutants that failed to elongate displayed a clear association with the actomyosin cortex. Moreover, these results highlight the importance of the segment 424-448 for vimentin distribution in mitosis.

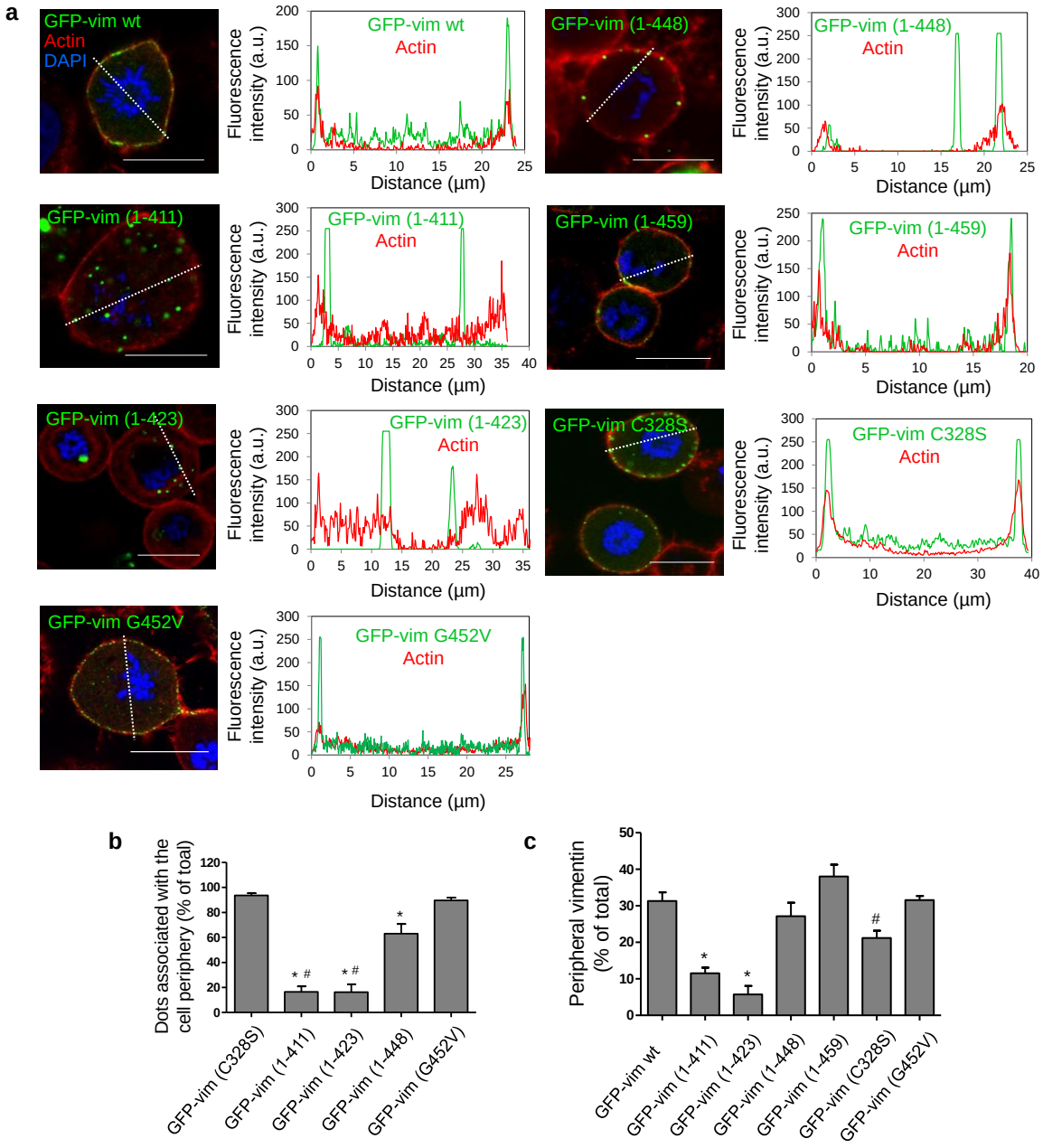


Figure 27 – Distribution of GFP-vimentin fusion constructs in mitosis. (a) SW13/cl.2 cells were transiently transfected with the indicated truncation/mutation constructs. Cells were treated with 0.4 µM nocodazole to increase the proportion of mitotic cells. Cells were fixed and stained as above. Single sections at mid-cell height are shown. Fluorescence intensity profiles represent vimentin and f-actin distribution along the dotted lines. (b) The percentage of dots at the cell periphery was quantified for the cells expressing the constructs that failed to elongate (*p<0.05 vs GFP-vim C328S and #p<0.05 vs GFP-vim G452V by Anova Tukey multiple comparison test). (c) The percentage of peripheral vimentin was analyzed for all different constructs (*p<0.05 vs vim wt, #p<0.05 vs vim (1-459) by Anova Turkey multiple comparison test). Results are average values from three experiments and at least 10 determinations per experimental condition.

2.5. The vimentin tail is not sufficient for the interaction with the actin cortex in mitosis

Additional studies to explore the association of the tail domain of vimentin with the actin cortex in mitosis were performed. For this, two different strategies were devised based on the use of fusion constructs with fluorescent proteins.

2.5.1. GFP fusion constructs of the vimentin tail do not distribute to the actin cortex in mitosis

Several GFP fusion constructs, containing several segments of the C-terminal end of vimentin, were generated and transiently transfected into SW13/cl.2 cells for analysis of their association with the actin cortex as above (Fig. 28). As a control, the distribution of GFP alone, both in interphase and in mitosis was studied.

In interphase cells (Fig. 28b, left images), GFP showed a diffuse pattern and it was detected both at the nucleus and the cytoplasm of the cell. Furthermore, in the GFP-vimentin (412-466) construct, that contains only the C-terminal domain of vimentin, the same diffuse pattern was observed but no fluorescence was detected in the nucleus (inset). GFP-vimentin (362-466) and GFP-vimentin (316-466), which contain also a part of the rod domain of the protein, in interphase cells displayed a diffuse cytoplasmic pattern with some small aggregates. In mitotic cells (Fig. 28b, right panels), GFP, GFP-vimentin (412-466) and GFP-vimentin (362-466) showed a diffuse pattern with no enrichment of fluorescence at the cell periphery (insets and fluorescence intensity profiles). Contrarily, GFP-vimentin (316-466) showed a small, but detectable, degree of cortical association, as displayed by the fluorescence intensity profile and quantification in Fig. 28c.

Taking together these results suggest that the GFP-vimentin fusion constructs with C-terminal segments of vimentin yield mainly a green diffuse background. Nevertheless, the GFP-vimentin (316-466) construct that bears a longer part of the rod domain of the protein showed points of colocalization with actin.

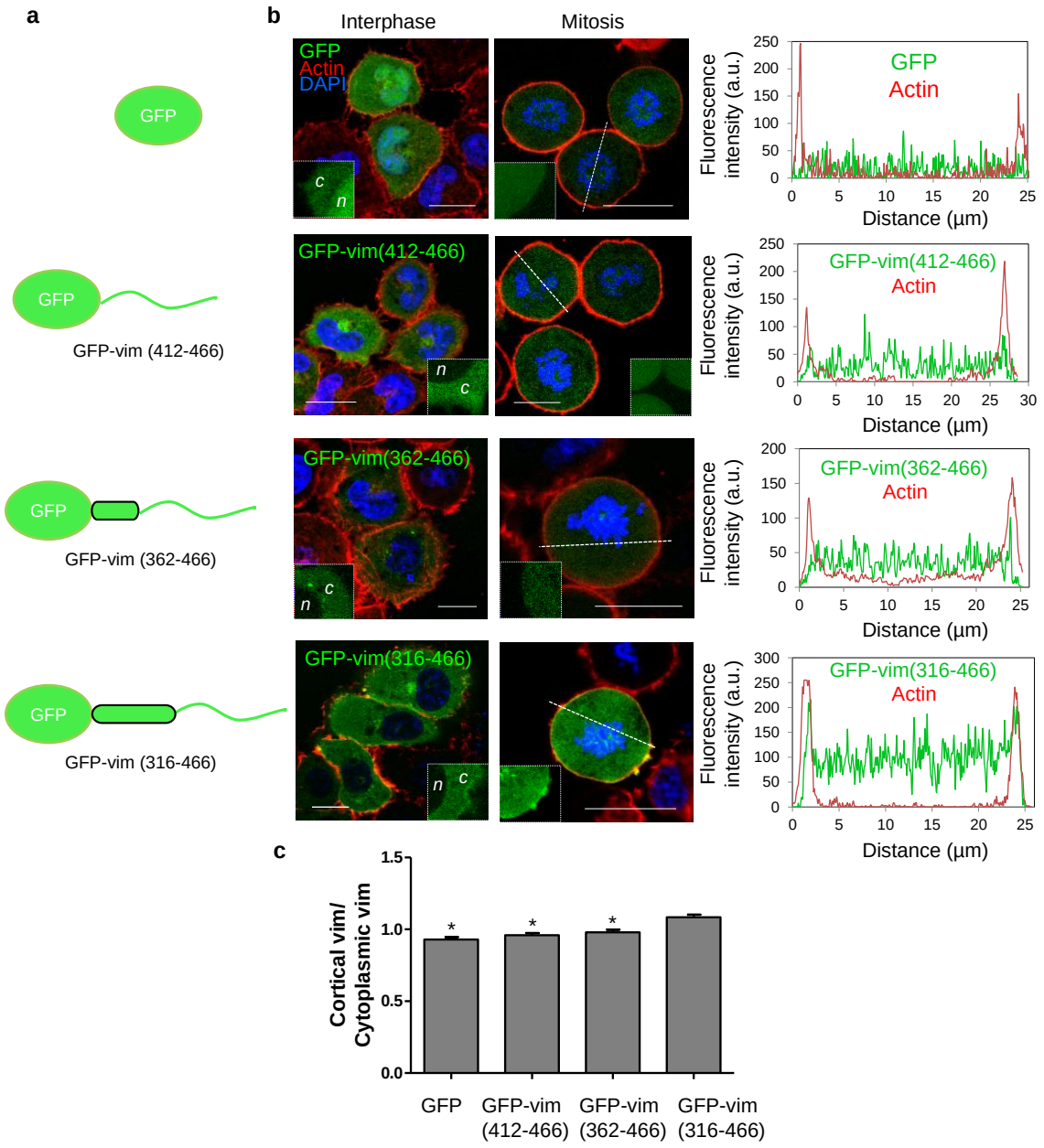


Figure 28 – Localization of chimeric vimentin constructs. (a) Schematic representation of chimeric constructs containing GFP and various segments of the vimentin C-terminal end. (b) SW13/cl.2 cells were transfected with the indicated constructs and the localization of the resulting proteins and of f-actin was assessed as above in interphase (left images) and dividing (right images) cells, after mild nocodazole treatment. Single sections at mid-height of the cell are shown. Insets depict enlarged areas of interest of the green channel to highlight the cytoplasm(*c*)-nuclear(*n*) boundary in interphase cells or the periphery of mitotic cells to show the cortical region (right). Fluorescence intensity profiles for vimentin and f-actin along the dotted lines in dividing cells are shown. (c) Graph displays the ratio between the peripheral and cytoplasmic vimentin signal. Results are average values from three experiments and 15 cells were analyzed per experimental condition (*p<0.0001 vs GFP-vim (316-466)).

2.5.2. Chimeric constructs of the vimentin tail domain fused to mCherry do not localize at the cell cortex in mitosis

The results described above indicate that the vimentin tail alone is not able to elicit a significant cortical translocation of chimeric constructs in mitotic cells, which could require other structural elements. The GFP protein is known to form dimers when in high concentrations and is a large protein compared with the segments of vimentin that were fused to it.

Therefore, we employed other strategies using the monomeric fluorescence protein mCherry. Two fusion constructs of this protein and the C-terminal domain of vimentin were generated. The first construct, mCherry-6Gly-vimentin (412-466), holds a six glycine spacer (6Gly) between the vimentin tail and the mCherry protein, whereas the second one, mCherry-6Gly-coiledcoil-vimentin (412-466), holds the 6Gly spacer plus the sequence of a peptide with predicted coiled coil structure [209] (Fig. 29a). The α-helical coiled-coil domain was introduced in an attempt to obtain a more favorable orientation or oligomerization state of the vimentin tail domain, by providing a sequence with secondary structure more similar to that of the C-terminal end of the rod domain. The distribution of these C-terminal constructs was analyzed in SW13/cl.2 and compared with the distribution of mCherry-vimentin wt both in interphase and in mitotic cells (Fig. 29).

The distribution of mCherry-vimentin wt in interphase cells was similar to that of GFP-vimentin wt, showing short filaments or squiggles. The mCherry-6Gly-vimentin (412-466) and the mCherry-6Gly-coiledcoil-vimentin (412-466) displayed a diffuse pattern in interphase cells with small vimentin aggregates close to the cell nucleus (Fig. 29b, left panels). In mitosis, mCherry-vimentin wt was mostly diffuse but some enrichment at the cell cortex was observed (Fig. 29b, fluorescent intensity profile). The other two constructs did not show any cortical enrichment of vimentin, which indicates that the presence of a coiled coil structure is not sufficient to elicit the interaction of the vimentin C-terminal domain with the actin cortex (Fig. 29b, right panels).

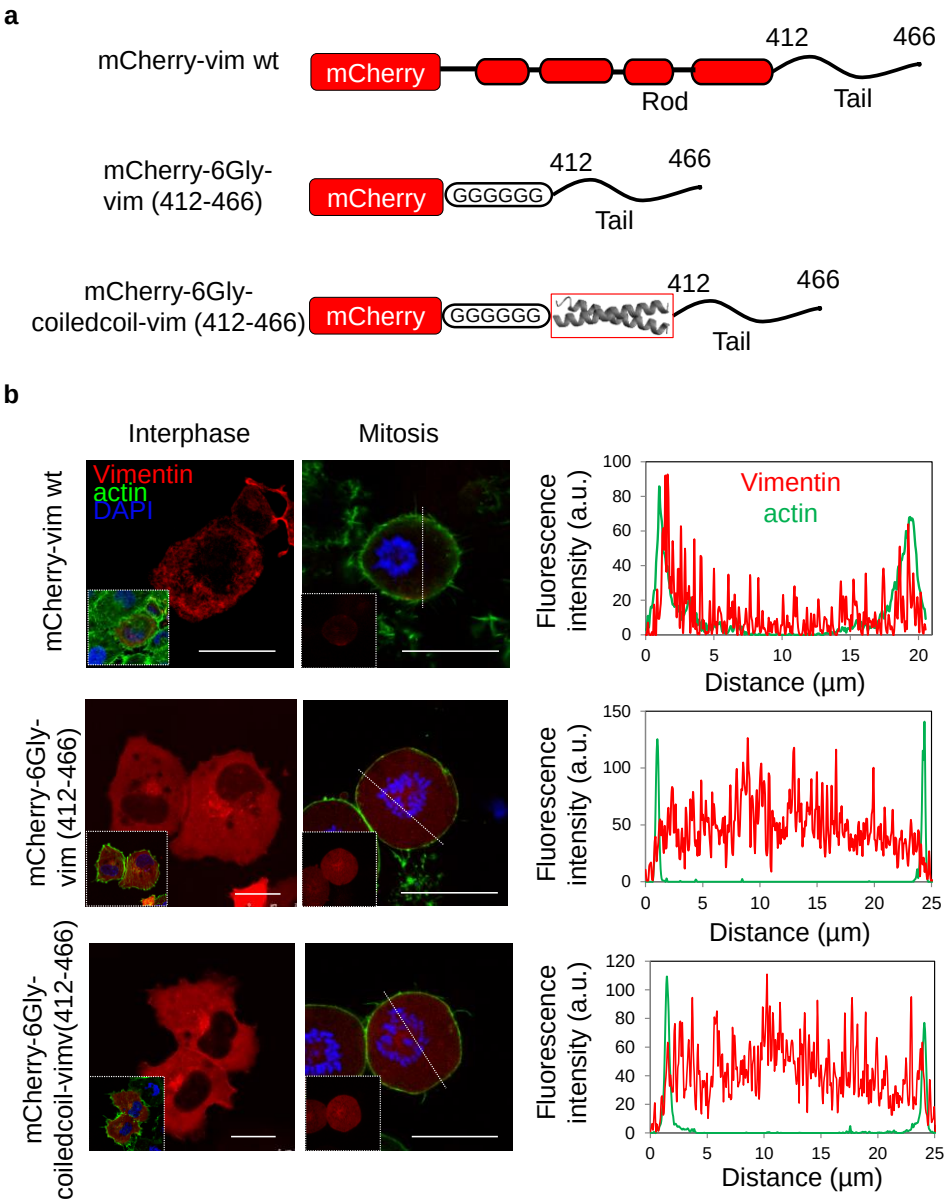


Figure 29 – Distribution of monomeric fluorescent proteins bearing the vimentin tail domain. (a) Schematic representation of the mCherry fusion constructs. (b) SW13/cl.2 cells were transfected with the indicated constructs. Cells were fixed 48 h after transfection and the distribution of vimentin was assessed in interphase and mitotic cells. F-actin was stained with Phalloidin (green) and the nuclei were stained with DAPI. Representative images and the fluorescence intensity profiles along the dotted lines are shown. Scale bars, 20 µm.

3. Vimentin distribution in experimental conditions mimicking pathophysiological situations

Vimentin has been associated with different diseases such as cancer and inflammatory diseases [230]. As described above, disruption of vimentin distribution can lead to mitotic alterations. Therefore, the effect of pathogenic agents known to alter vimentin in interphase cells on vimentin cortical association was studied [30, 76].

3.1. Vimentin distribution is altered by the HIV protease

Early studies in cells microinjected with the HIV protease suggested that vimentin was cleaved preferentially between the residues L423 and R424 (considering the initial M), which led to a collapse of the protein [76]. Here, the effect of the HIV protease was studied in SW13/cl.2 and Vero cells, which were transfected with a fluorescent HIV-protease construct (GFP-PR).

Live cell visualization of SW13/cl.2 cells co-transfected with RFP//vimentin wt, mCherry-vimentin wt and GFP-PR showed that the presence of the protease induced the reorganization of vimentin filaments promoting a collapse near the nucleus of the cell (Fig. 30a). The HIV protease inhibitor Ritonavir blocked the effect of the protease preserving vimentin filaments (Fig. 30a, lower left panel). However, when the protease inhibitor was removed, vimentin filaments retracted from the cell periphery and accumulated at the center of the cell (Fig. 30a, lower right panel). Few mitotic cells could be spotted under these conditions; nevertheless in the cells that persisted, vimentin was accumulated near the dividing chromosomes (Fig. 30b).

The presence of the HIV-protease (green fluorescence) in Vero cells also altered the endogenous vimentin network. In some cells accumulation of vimentin close to the cell nucleus was observed (Fig. 30c, upper panels), whereas in other cases vimentin was totally absent, which may suggest that it was completely degraded (Fig. 30c, lower panel).

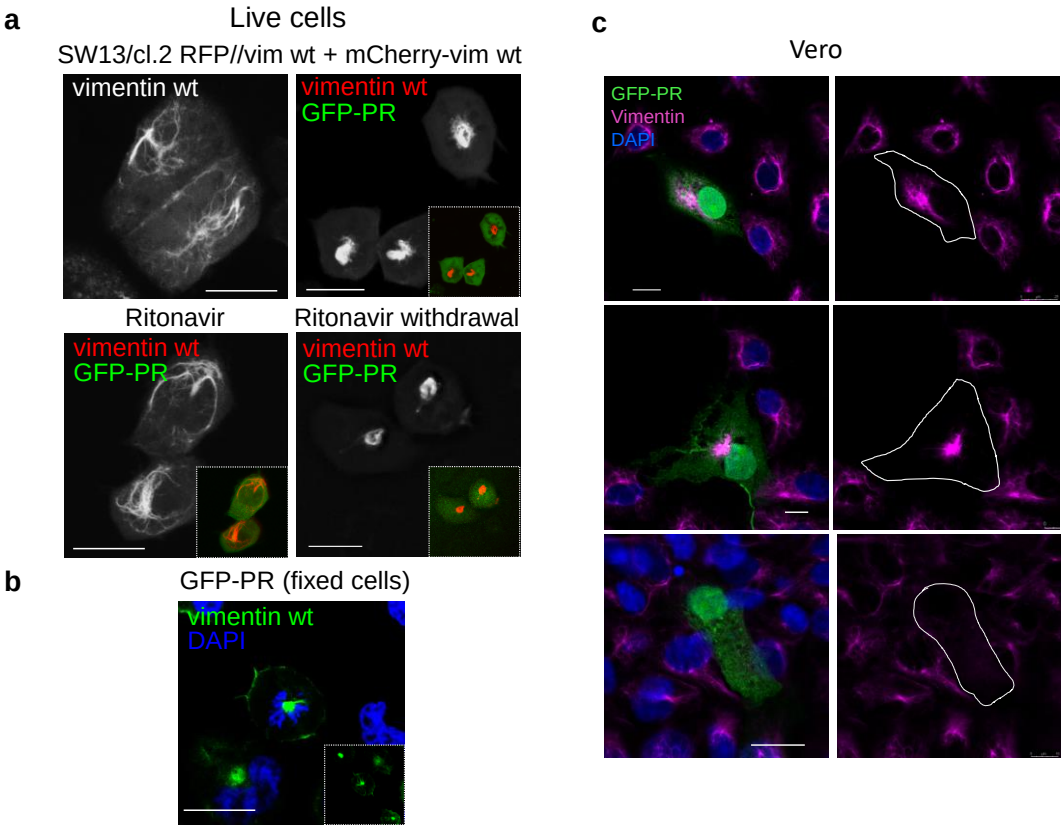


Figure 30 – Effect of HIV protease on vimentin distribution. (a) SW13/cl.2 cells were co-transfected with RFP//vim wt (80%) and mCherry-vim wt (20%), to monitor vimentin filaments (gray scale, red in insets) in live cells, together with a fluorescent HIV-type I protease construct (GFP-PR, green fluorescence in insets). Upper panels, cells were imaged 24 h after transfection; lower panels, the HIV inhibitor ritonavir was added immediately after transfection and cells were visualized 24 h later (left panel). The lower right panel depicts cells after 5 additional hours cultured in the absence of the inhibitor. Overall projections are shown. (b) Cells were fixed and stained with DAPI to identify mitotic cells. Vimentin is represented in green. Overlay channels of a single section are shown. (c) Vero cells were transfected with GFP-PR (green) and fixed 24 h hours later, and vimentin was detected by immunofluorescence (magenta). Left images show the merged channels, right images show vimentin distribution. Results are representative from three experiments with similar effects. Images are overlay channels of single sections. Scale bars, 20 μm.

3.2. Electrophilic lipids alter vimentin cortical distribution in mitosis

Cytoskeletal proteins are known targets of electrophile addition and are important in the redox control of several diseases and during life span. Recent studies from our group described the modifications of vimentin and GFAP by different electrophilic lipids, which promoted the alteration of the intermediate filament network. These modifications occurred preferentially on the single cysteine residue of both proteins [30, 202]. Here the effect of different electrophilic lipids, prostaglandin A_1 (PGA_1), 4-hydroxynonenal (HNE) and a nitrated phosphatidylcholine (NO_2-POPC), on the distribution of vimentin was studied both in interphase and mitotic cells.

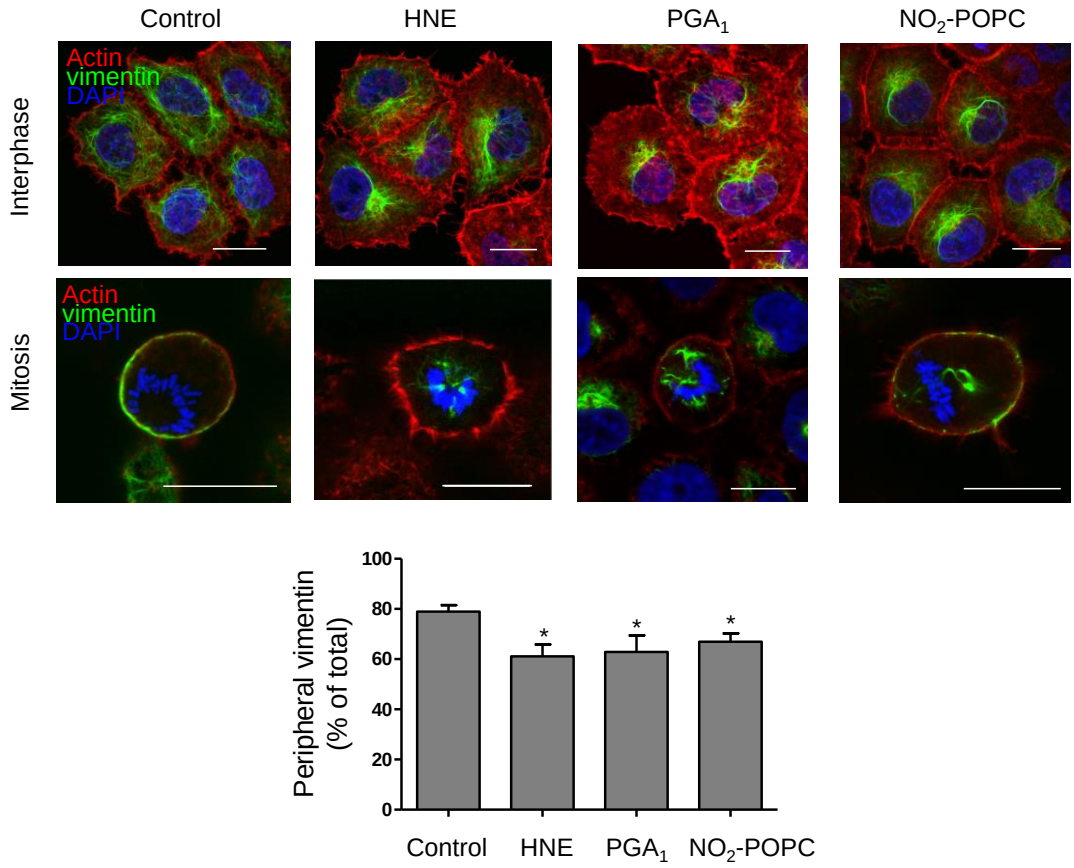


Figure 31 – Electrophilic lipids impair vimentin distribution in interphase and mitotic cells. SW13/cl.2 cells stably transfected with RFP//vim wt (untagged vimentin) were treated with vehicle DMSO (control) (0.1% v/v) or the electrophilic lipids, 4-hydroxynonenal (HNE) at 10 μM for 4 h, prostaglandin A_1 (PGA_1) at 20 μM for 20 h or nitrated phospholipid (NO_2-POPC) at 10 μM for 6 h. Cells were fixed after treatments and vimentin, f-actin and DAPI staining was performed as above. The graph shows the proportion of peripheral vimentin in mitotic cells upon treatment with the various agents. Results are average values from three different experiments totaling at least 11 determinations (*p<0.03 vs control).

In interphase cells, the vimentin network retracted from the cell periphery and condensed close to the cell nucleus in the presence of the various lipids (Fig. 31, upper panels). However, no apparent alteration of the f-actin network was observed. Then the effect of these lipids was assessed in mitotic cells. In control cells, vimentin presented the typical peripheral distribution very close to the actin cortex. Interestingly, in cells treated with electrophilic lipids vimentin cortical association significantly diminished and accumulation near the dividing chromosomes was observed (Fig. 31, lower panels). Nevertheless, cortical actin appeared to be preserved under these conditions.

These results indicate that electrophilic lipids alter vimentin distribution in mitosis, leading to its partial detachment from the actin cortex. This effect could be due to the direct modification of vimentin, although the possibility of an indirect effect cannot be excluded.

3.3. Mitotic progression is affected by PGA_1

Cyclopentenone prostaglandins are involved in several biological processes such as inflammation, proliferation, tumorigenesis and apoptosis [231]. Several works from our laboratory have described the modification of different proteins by these molecules [175, 232, 233]. Since, as shown above, PGA_1 alters vimentin distribution in mitosis, the impact this lipid on mitotic progression was explored next (Fig. 32).

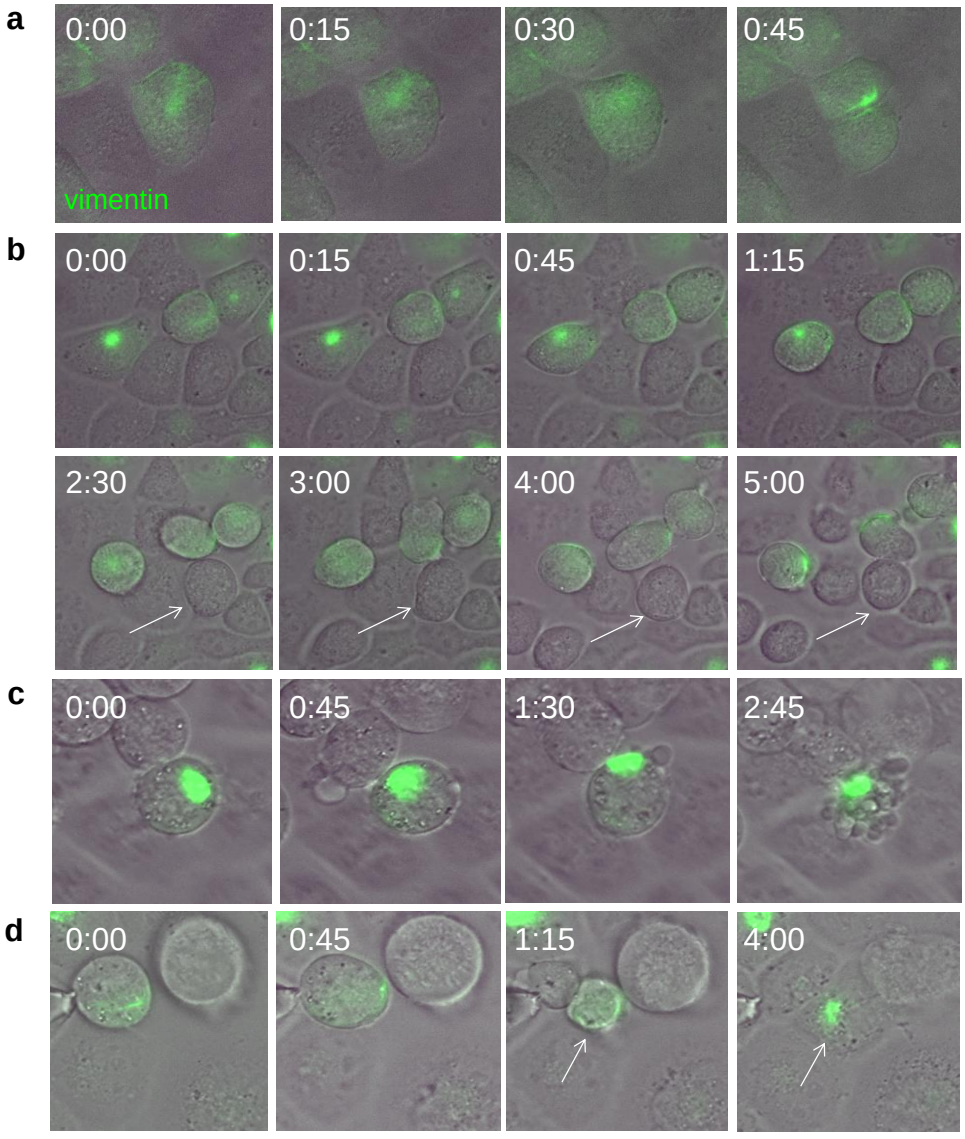


Figure 32 – Prostaglandin A_1 induces mitotic alterations. SW13/cl.2 cells were transiently transfected with RFP//vim wt (80%) plus a small amount of GFP-vim wt (20%) and treated overnight with vehicle DMSO (a) or 20 μM prostaglandin A_1 (PGA_1) 24 h after transfection (b, c, d). Transfected cells were selected and images were acquired every 15 min at the indicated time points in hours:minutes. Representative images of the overlays of DIC and green fluorescence are shown.

Time-lapse experiments showed that control cells treated with vehicle, DMSO, divided similarly to RFP//vimentin wt cells without any treatment (Fig. 32a). Cells treated with PGA_1, which frequently showed vimentin accumulations, suffered some mitotic errors. Consequently, some cells attempted to divide for several hours but did not complete mitosis (Fig. 32b) or died during this process (Fig. 32c). Nevertheless, other cells were able to complete mitosis, undergoing asymmetric distribution of vimentin (Fig. 32d). However, it should be noted that non-transfected cells also failed to divide in some cases (Fig. 32b, arrow). This could be due to PGA_1-induced alterations in the cell cycle, as it has been observed in other cell types [234, 235].

Thus, although PGA_1 altered mitotic progression, this could be due to the direct modification of vimentin or to modifications or effects on other proteins or other cellular structures.

3.4. The vimentin C328S mutant is more resistant to lipoxidation

Previous works from our laboratory pointed to the only cysteine residue of vimentin as the main functional target of lipoxidation. Therefore, the effect of electrophilic lipids on the distribution of a vimentin mutant which lacks the cysteine residue was explored next. For this, SW13/cl.2 stably transfected with vimentin wt or with vimentin C328S were used. The cells were treated with two different electrophilic lipids, PGA_1 or NO_2-POPC and the distribution of vimentin was evaluated both in interphase and mitotic cells (Fig. 33).

The vimentin C328S mutant was described formerly by our group [30]. Although this mutant was able to form full-length filaments in cells, the area covered by vimentin C328S was slightly narrower than that occupied by the wt (Fig. 33a).

In interphase cells, treatment with either lipid elicited the accumulation of vimentin wt close to the cell nucleus, while in cells expressing vimentin C328S the effect of the electrophilic lipids was significantly attenuated (Fig. 33a). Thus, a 20 % to 30% reduction in the vimentin wt area was observed upon treatments, whereas the reduction in vimentin C328S area was less than 10% in the case of PGA_1 and non-detectable in the case of NO_2-POPC (Fig. 33a, graph).

In mitosis, vimentin C328S adopted a peripheral distribution, although the percentage of cortical vimentin was lower than that of vimentin wt (Fig. 33b, graph). As shown above, treatment with PGA_1 induced a decrease of 20% in peripheral vimentin wt, remarkably, it did not affect the percentage of peripheral C328S vimentin.

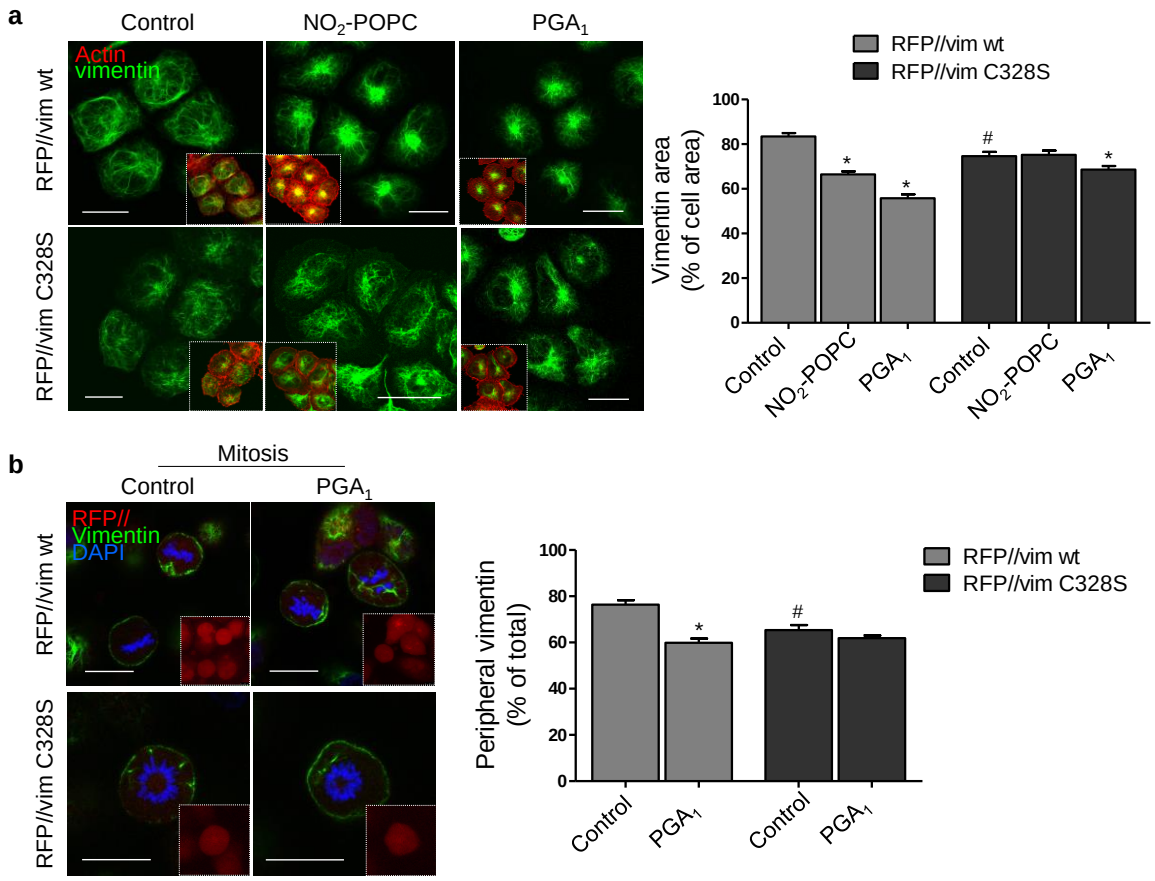


Figure 33 – Vimentin C328S is less susceptible to lipoxidation. (a) SW13/cl.2 cells stably transfected with RFP//vimentin wt or RFP//vimentin C328S were treated with vehicle, DMSO 0.1% (v/v), 20 µM prostaglandin A_1 (PGA_1) for 20 h or nitrated phospholipid (NO_2-POPC) at 10 µM for 6 h. Cells were fixed and stained as above. The graph represents the vimentin area with respect to the total cell area. At least 50 cells were monitored per experimental condition (*p<0.02 vs control; #p<0.01 vs control wt). (b) SW13/cl.2 cells were transfected as above, treated with vehicle DMSO, or 20 µM PGA_1 for 20 h and processed as above. Red fluorescence (insets) represents the transfection control. The graph shows the percentage of peripheral vimentin in mitotic cells. Results are average values of at least 14 determinations per experimental condition from three independent experiments. (*p<0.0001 vs the corresponding control; #p<0.001 vs control wt).

These results suggest that the cysteine residue of vimentin could be important for redistribution of the protein in mitosis. On the other hand, vimentin C328S suffers milder alterations upon treatment with electrophilic lipids than vimentin wt, suggesting that C328 is an important target of lipoxidation. Nevertheless, modification of other cellular proteins or indirect modifications of the cysteine residue could contribute to these effects.

Taken together these results show that the vimentin network undergoes important reorganizations in response to electrophilic lipids and a mutant at the cysteine residue is more resistant to these alterations, both in interphase and in mitosis. Moreover, they support the role of vimentin as a redox sensor and the importance of its distribution for normal mitotic progression.

Discussion

1. The vimentin tail domain is necessary for the correct filament distribution in cells

Vimentin is a structural protein that forms extended filaments in the cell cytoplasm and is essential for several cellular functions, from organelle positioning to mechanical stability [236]. The vimentin monomer is constituted by three different domains that play specific roles in filament assembly. It is known that the N-terminal domain of vimentin is essential for filament formation, both in vitro and in cells [11, 31, 32]. In turn, the C-terminal domain is not essential for in vitro filament assembly and its role in cells is more controversial. Thus in this work, the role of the C-terminal of vimentin in filament distribution in several cell types has been studied in detail.

1.1. Vimentin (1-411) forms aggregates or bundles in interphase cells and disrupts the endogenous vimentin network

In vitro, studies have shown that the removal of the entire vimentin tail domain does not abolish the filament formation [11]. Nevertheless, it is thought that the tail domain is necessary for filament lateral packing [33], and a conserved region located in this domain, the RDG motif, has been suggested to interact with the rod domain leading to conformational changes important for filament formation in vitro [34].

The RDG motif had also been previously reported to be important for vimentin filament assembly into extended filaments in SW13/cl.2 cells [41]. Furthermore, human tailless vimentin showed a collapsed network in vimentin-deficient MCF7 cells [40]. However, *Xenopus laevis* tailless vimentin formed either short structures or aggregates in vimentin-free epithelial cells [36], or extended filaments in MCF7 cells [38]. Therefore, there were considerable discrepancies about the importance of this domain depending on the experimental system.

Here, we showed that vimentin (1-411) formed bundles that accumulated at the center of the cell in different vimentin-deficient cells lines, namely, SW13/cl.2 and HAP vim–. Moreover, this distribution was not dependent on the amount of plasmid used, since cells transfected with lower amounts of this construct showed the same pattern (Fig. 8). Furthermore, our results showed that in vimentin-deficient cells the vimentin tail domain was necessary for the correct cellular organization of the filaments and their extension towards the cell periphery. Nevertheless, it seems that polymerization itself is not inhibited since thick filament bundles are observed.

Importantly, tailless mutants of other intermediate filament proteins were reported to display an impaired distribution in cells. Tailless α-internexin, a type IV intermediate filament, formed abnormal thick filaments in SW13/cl.2 cells [237] and the same was observed with tailless GFAP [238] and tailless neurofilaments [239].

Thus, it can be hypothesized that the defective cellular distribution of vimentin (1-411) could derive from the incapacity of filaments to elongate or the impaired interaction with other proteins. We have confirmed these observations using different types of human vimentin-deficient cells. In fact, the previously reported discrepancies could be due to the use of cells from different species or to the presence of other cytoplasmic intermediate IF in the cell types used. In our experimental system, using SW13/cl.2 cells, which do not contain other cytoplasmic intermediate filament proteins [212], vimentin (1-411) formed bundles. Nevertheless, a tailless mutant GFAP was able to form a more extended network in the presence of vimentin filaments [238]. In addition, the intermediate filament protein phakinin, which is a tailless protein, needed filesin, another intermediate protein, to form an extended filament network in cells [240].

There were previous reports of the incorporation of IF proteins, lacking different domains, into a full filament network. For instance, NF-M and α-internexin can form an normal filament network if only one of the proteins lacks the head or the tail domain [237]. Additionally, co-transfecting a tailless NF protein with full-length vimentin, allowed the formation of extended filaments [239]. Therefore, it could be expected that tailless vimentin could incorporate, at least to a certain degree, into filaments in cells expressing full-length vimentin. Nevertheless, it was of interest to evaluate if vimentin (1-411) could alter full-length vimentin distribution.

This was explored by co-transfecting vimentin (1-411) and vimentin wt in different proportions. As displayed in Fig. 8, when vimentin (1-411) was transfected in a higher proportion compared to vimentin wt, it led to the condensation of vimentin filaments. Moreover, a retraction of the filaments from the cell periphery by expressing an excess of vimentin (1-411) in cells holding an endogenous vimentin network was observed (Fig. 9), while vimentin wt did not reproduce the same effects. As a matter of fact, similar observations had been described for GFAP. GFAPδ, which has a C-terminal domain different from GFAPα, impaired the formation of GFAP wt filaments in vitro when present in higher proportions. Likewise, in cells GFAPδ incorporated into the endogenous GFAP network when expressed at low levels. However, higher levels disrupted the normal

network and led to the formation of cytoplasmic aggregates [241]. Similarly, the proportion of full-length and tailless vimentin appears to determine the effect on the filament network.

The tail domain of intermediate filaments has been involved in longitudinal elongation and lateral packing [242], possibly to maintain the IF network architecture, preventing filament bundling and thickness. Thus, deletion of the tail domain could determine the state of filament elongation in cells and impair the normal vimentin extended distribution if present at higher levels.

1.2. Vimentin (1-411) promotes nuclear alterations and leads to mitotic defects

During cell division, the distribution of vimentin filaments is highly regulated by phosphorylation. In fact, the best characterized reorganization of vimentin during mitosis is phosphorylation-induced disassembly, which is brought about by the coordinated action of various kinases. The degree of vimentin filaments disassembly in mitotic cells was shown to be dependent on several kinase activities and on the cell type. For instance, Cdk1 activity could either lead to the disassembly of the filaments or the formation of vimentin perinuclear aggregates [137]. Additionally, Aurora-B and $p34^{cdc2}$ kinases also contribute to the reorganization and disassembly of vimentin filaments [136, 138, 139].

Our results showed that in SW13/cl.2 cells vimentin wt had a peripheral distribution in mitosis, leaving space for the dividing chromosomes, while vimentin (1-411) bundles stayed in very close proximity of the chromosomes, sometimes entangling them (Fig. 10). This filament distribution could have consequences for mitotic spindle organization. In fact, cells harboring vimentin wt divided regularly, faster and with a similar distribution of vimentin between the two daughter cells. On the contrary, cells expressing vimentin (1-411) displayed mitotic defects, including cell death and asymmetric division (Fig. 11).

In cells, protein aggregates can suffer different fates, and either can be degraded by autophagy or lead to the formation of aggresomes [243]. When the proteasomal degradation is compromised, misfolded proteins form cytoplasmic aggregates that converge towards the cell center forming aggresomes. However, if the degradation of the aggregate fails, this could lead to cell death and problems in cell division. Actually, different pathologies are related with the accumulation of protein aggregates that are responsible for the disease consequences. In Alexander disease, the expression of mutated GFAP results in the formation of Rosenthal fibers that accumulate at the cell cytoplasm and potentially lead to cell death [244]. Consequently, the asymmetric

distribution of the vimentin (1-411) aggregate can be related with the mechanism of cells to self-renew from damaged proteins. Moreover, the cell death observed in cells expressing vimentin (1-411) could derive from the presence of vimentin large aggregates. However, further studies are necessary to analyze the consequences of vimentin aggregates during cell division. As future work, it would be interesting to study the effect of phosphorylation in the distribution of vimentin wt and vimentin (1-411).

The vimentin network has been involved in the maintenance of nuclear morphology [61] and in the regulation of nuclei position [60]. Our results showed that in interphase cells the proportion of cells with aberrant nuclei was higher in cells transfected with vimentin (1-411) than in cells harboring vimentin wt (Fig. 12). Similar results were observed in Alexander disease. The presence of the Rosenthal fibers was associated with altered nuclear morphology and interference with the normal mitotic progression [244]. In addition, long-term aggresome accumulation increases the incidence of abnormal nuclear morphology, cell cycle arrest and apoptosis [245].

Interestingly, in cells resuming mitosis the frequency of lagging chromosomes was significantly higher in the presence of vimentin (1-411) than of the wt. Chromosome segregation errors could lead to severe consequences, such as aneuploidy and polyploidy or even cell death. Importantly, defects in chromosome separation can also have implications in human health, including different types of trisomy or increased cancer predisposition [246]. This raises potential cytotoxic implications of vimentin (1-411) bundles during mitosis.

In summary, these results showed that vimentin (1-411) aggregates lead to mitotic catastrophe and asymmetric division, raising the possibility of cytotoxic effects during mitosis (Fig. 34). Moreover, the presence of this truncated vimentin form increases the proportion of cells with lagging chromosomes during cell division and altered nuclear morphology in interphase cells. Remarkably, the vimentin “SA mutant”, which several serine residues known to be phosphorylated in mitosis have been substituted by alanine residues, induced mitotic defects such as cytokinetic failure, aneuploidy and appearance of multinucleated cells [146].

Taken together, these observations strengthen the importance of vimentin reorganization for the normal progression of mitosis. Although we have focused on the organization of vimentin in cells in which filaments persist in mitosis, it would be interesting to explore the fate of vimentin (1-411) in cells in which the protein typically disassembles during cell division.

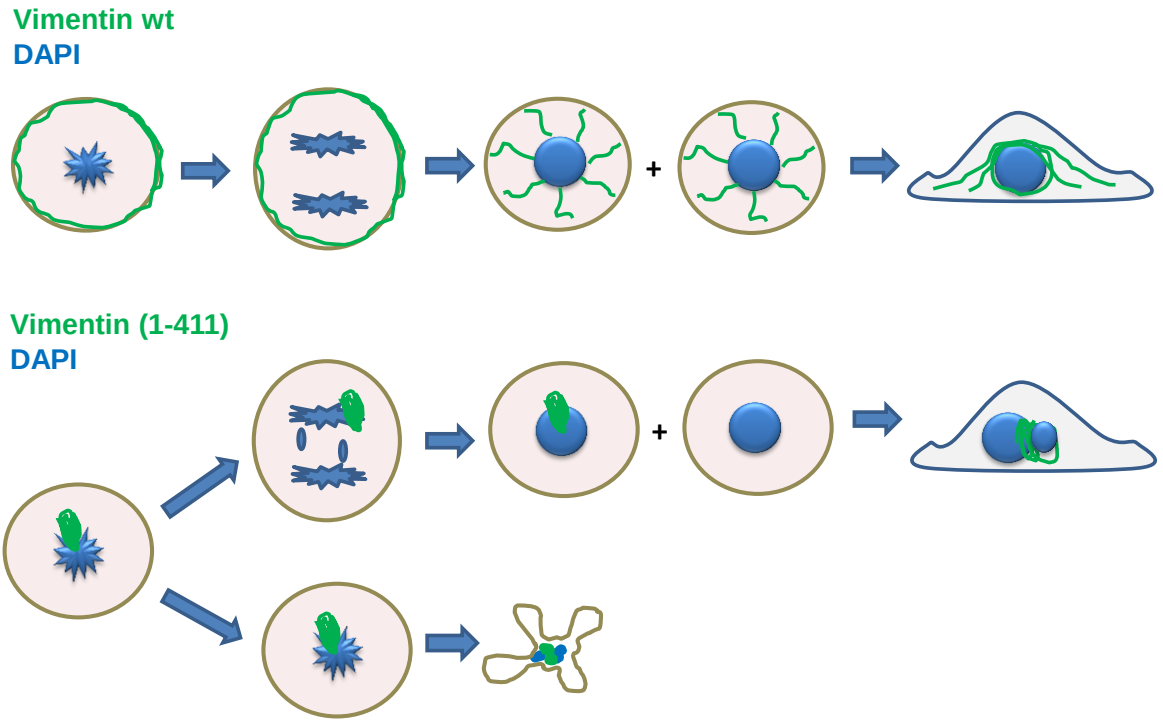


Figure 34 – Effect of vimentin wt and (1-411) in cell division. In some cell types vimentin wt is not disassembled in mitosis but distributes at the cell periphery, leaving space for the dividing chromosomes. Vimentin wt divides nearly equally between the daughter cells which show normal nuclear morphology. Vimentin (1-411) accumulates at the center of the cell interfering with the dividing chromosomes, leading to the appearance of lagging chromosomes, asymmetric division or cell death, and may alter nuclear morphology.

1.3. Vimentin mitotic distribution is not tubulin dependent

Vimentin and microtubules are found in the cytoplasm of interphase cells in parallel arrays [110]. Moreover, the extension of vimentin filaments is known to be microtubule-dependent [113], and when microtubules are altered, a condensation of the vimentin network is observed [113], together with reduced movement of vimentin particles [114]. Nevertheless, under our conditions, the low concentration of nocodazole employed did not induce evident alterations of vimentin distribution in interphase SW13/cl.2 cells (Fig. 13).

Remarkably, to the best of our knowledge, the interplay between vimentin and tubulin in mitosis has not been described. We have observed that vimentin wt underwent peripheral redistribution and tubulin formed the mitotic spindle. The rate of microtubule disassembly is known to be cell-type specific and time dependent [247]. Consistently, we observed that the effect of nocodazole was dependent on the cell type and on the concentration used. In SW13/cl.2 cells the acute disruption of microtubules with nocodazole completely blocked the formation of the mitotic spindle and caused

misorientation of the dividing chromosomes (Fig. 13). However, under these conditions, the distribution of vimentin wt and vimentin (1-411) were not affected.

Unpublished results from our group showed that low concentrations of nocodazole, used to induce cell cycle synchronization, affected more severely primary fibroblasts and mesangial cells than SW13/cl.2 cells. Therefore, the use of this agent was restricted to SW13/cl.2 cells.

Taken together, these results suggest that the distribution of vimentin in mitosis is not dependent on microtubule integrity, at the range of concentrations of nocodazole used.

1.4. Vimentin distributes towards the cell periphery in an actin-dependent manner

The interaction of vimentin and actin is well described in processes such as cell migration and mechanics [120]. Although the interplay between these proteins is well studied in interphase cells, in mitosis is poorly understood.

The disordered C-terminal domain of vimentin has been proposed to participate in protein-protein interactions, including actin [123], and to interact with divalent cations [37]. Our results indicate that, if not disassembled, vimentin filaments should undergo mitotic cortical translocation to facilitate mitosis progression. This is supported by the behavior of vimentin (1-411), which does not reach the actomyosin cortex and interferes with the dividing chromosomes causing aberrant mitosis (Fig.11) [228]. Consequently, these results unveil a possible interaction of the C-terminal domain of vimentin with the actin cortex during cell division.

In interphase cells, the perinuclear localization of vimentin is dependent on the integrity of actin arcs [121]. Moreover, actin disruption promotes changes in the vimentin network. For instance, vimentin organization is dependent on the actomyosin cortex contraction [112]. Accordingly, during mitosis the integrity of the actin cortex could also influence the vimentin distribution.

Typically, the actin cytoskeleton rearranges during mitosis to generate a rigid and rounded actomyosin cortex, which is constituted mainly by an f-actin network together with myosin and actin-binding proteins [248]. Cytochalasin B is a cell-permeable mycotoxin that is known to depolymerize f-actin filaments [220]. Treatment of cells with this compound provoked a remarkable reorganization of the vimentin wt network, leading to its accumulation at the cell center, mimicking the distribution of vimentin (1-411) (Fig.14).

Subsequently, other agents known to alter actin filaments were also used. While latrunculin A disrupts the actin network [221], jasplakinolide stimulates the polymerization of actin filaments and stabilizes them [249]. The effect of Lat A led to the loss of cortical f-actin signal, promoting a dislodgment of vimentin similar to that elicited by Cyt B (Fig.15). On the other hand, with jasplakinolide treatment, the actin cortex was apparently preserved but vimentin showed a mixed pattern: either maintained the peripheral distribution or had some filaments at the center of the cell.

Different regulators have been identified to contribute to the remodeling of the actomyosin cortex at mitotic entry. In cells, activation of Ect2/Pbl via RhoA initiates the mitotic rounding [159]. Likewise, a Rho-dependent activation of myosin-II promotes actin stability and cortex assembly [157, 160]. Blebbistatin is a known myosin II specific ATPase inhibitor that blocks myosin function in several cellular processes, including cell division [225, 250]. In turn, C3 toxin is an inhibitor of Rho proteins, which are essential for the formation of the cortex [157]. Treatment of cells with either of these two compounds also led to a dislodgment of vimentin from the cell periphery (Fig.15). These results suggest that the interference with the mechanisms involved in cell cortex formation and maintenance also affects vimentin distribution, even if the cell cortex is not totally disrupted. Thus, the possibility of interaction of vimentin with other proteins responsible for the formation or the stability of the actin cortex cannot be excluded.

Consequently, the integrity of the actomyosin cortex seems to be essential for peripheral distribution of vimentin (Fig. 35). However, further studies are necessary to determine if the altered vimentin distribution upon cortex disruption is due to the direct interaction with actin or with other proteins present at the cell cortex, such as plectin, as suggested recently [251].

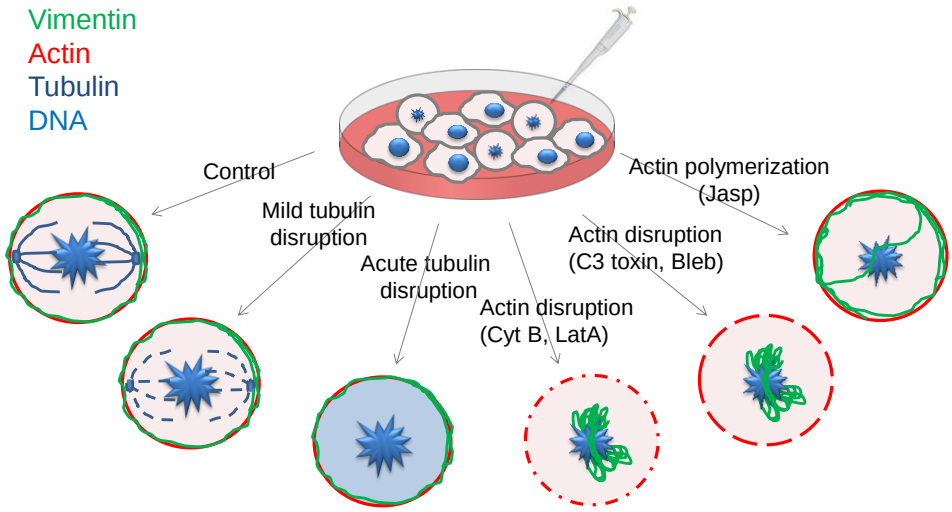


Figure 35 – Schematic representation of the influence of tubulin and actin disrupting agents in mitotic vimentin distribution. Control mitotic cells showed intact actin at the cell periphery and tubulin forming the mitotic spindle (left). Treatment of cells with low or high concentrations of nocodazole showed a partial or total disruption of the mitotic spindle, respectively, but did not affect vimentin peripheral distribution. The disruption of the actin network with distinct agents affected cortical actin and consequently vimentin distribution, eliciting a dislodgment from the cell cortex of different degrees. Therefore, vimentin peripheral distribution during mitosis seems to be actin-dependent.

1.5. Cortical distribution of vimentin in mitosis occurs in several cell types

Vimentin organization in mitosis is cell-type dependent and can either form a filament cage surrounding the mitotic spindle or disassemble. Vimentin disassembly during mitosis is mostly regulated by phosphorylation of the N-terminal residues in combination with protein-protein interactions. More than 11 residues located at the N-terminal of vimentin are known to be phosphorylated during mitosis in a controlled manner [146]. Although phosphorylation of the C-terminal domain has also been reported in mitosis, these phosphorylation sites do not seem to play a role in vimentin disassembly [139]. The presence of nestin has also been reported to facilitate the disassembly of vimentin in a process dependent on S55 phosphorylation [148].

Here it is shown that robust vimentin filaments are preserved in mitosis in several cell types (Fig. 16). In dividing SW13/cl.2 cells, which are nestin negative [212], vimentin filaments persist in mitosis. On the contrary, in nestin-positive MCF7 cells [252] vimentin filaments were partially disassembled. Other cell types, such as U-251 MG [18] or BAEC [253], which express variable nestin levels and other intermediate filament proteins, showed mixed patterns. Thus, there seems to be a correlation between nestin expression

and vimentin disassembly in the different cell types. However the majority of the cell types used preserved the filamentous vimentin network during mitosis. Curiously, even in the cell types that showed more cytoplasmic vimentin, some cortical colocalization with f-actin was observed, as is the case of primary fibroblasts and BAEC cells (Fig.16, insets).

The persistence of intermediate filaments during mitosis has been previously described in different cell types. In endothelial cells, filaments of approximately 10 nm of width were found to form a cage around the mitotic apparatus and no major disassembly was observed during mitosis [254]. Moreover, in HeLa, CHO and PtK2 cells these filaments were identified as vimentin [255, 256].

Additionally, in newly-plated cells, which showed a spherical shape, vimentin did not translocate to the cell periphery, but showed a perinuclear distribution (Fig.17). This indicates that cortical distribution of vimentin occurs selectively in mitosis and is not a consequence of cell rounding.

2. Vimentin-actin interplay in mitotic cells

Vimentin-actin interplay occurs in interphase cells at several organization levels [1] and it is known that the two proteins affect each other's localization and motility [121]. Vimentin restricts the retrograde movement of actin arcs and restrains actin polymerization and stress fiber assembly [125]. In turn, actin limits the transport of vimentin particles along microtubules [124] and actomyosin arcs interact with vimentin and drive their retrograde movement, promoting vimentin perinuclear localization [121]. In mitosis, no evidence of vimentin-actin interplay was available and thus, the importance of this interaction was evaluated in this work.

2.1. Vimentin is in close contact with the actin cortex and affects its distribution in mitosis

The results described above show that vimentin distributes towards the cell periphery in different cell types and in an actin-dependent manner. Nevertheless, a more detailed analysis of the vimentin-actin interaction was performed by using super-resolution microscopy by stimulated emission depletion (STED). This showed that vimentin was in close contact with the actin cortex, localizing immediately bellow the actin cortex or entangled to it (Fig. 18).

The use of super-resolution microscopy allows having a better resolution due to the selective deactivation of fluorophores. This technique minimizes the area of illumination at the focal point, providing an improved determination of the desired molecules [257]. Therefore, by using colocalization analysis of the vimentin and actin signals, a close proximity between the two structures was observed [132]. Determination of colocalization coefficients, the Pearson coefficient and the overlap coefficient, evidenced a high coincidence of vimentin and f-actin at the cell periphery. Interestingly, in SW13/cl.2 cells the proportion of cortical vimentin was higher than that of cortical f-actin (Fig. 18).

In mitotic SW13/cl.2 cells, the presence of vimentin was associated with a lower standard deviation of the f-actin signal in global 2D-projections [211] (Fig. 19). Moreover, in vimentin-positive cells, vimentin was abundant at the basal layers of the cell whereas basal f-actin displayed lower intensity (Fig. 20). Thus, the presence of vimentin influences actin distribution apparently exerting a negative feedback on the formation of defined f-actin structures. Therefore, these results suggest a vimentin-actin interplay affecting the properties of the actomyosin cortex in mitosis (Fig. 36). Given the importance of the actin cortex in the properties of mitotic cells, more detailed studies are needed to evaluate the implications of vimentin in the stiffness and contractility of the cell cortex and also in cell rounding and spindle orientation [258].

The roles of different proteins in regulating the cortex properties have been widely studied. Myosin [157], Arp2/3 complex proteins [229] and crosslinking proteins [259, 260] are described to be responsible for the heterogeneity of the actin cortex, regulating the cortical density and tension and the formation of actin filaments of different sizes. Moreover, Chugh et al. [158] showed that proteins that affect cortex thickness or actin filament length also alter the cortical tension. In vitro studies have also showed that if the actin conformation is modified, the contractile properties of the network could change drastically [259]. The heterogeneity of the actin cortex was studied recently by super-resolution microscopy, which showed an asymmetric distribution of the density of cortical actin that in some cellular regions was closer to the cell membrane [261]. Consequently, the presence of vimentin at the cell cortex could lead to alterations in f-actin, thus modulating the cell cortex properties such as tension and thickness.

The impact of vimentin on the actin network could result from the direct interaction with actin or through crosslinker proteins. For instance, vimentin and actin are known to interact with plectin [262], chaperones or actin associated proteins, such as fimbrin [128], integrins [52] and capping proteins [131]. Filamin A, an actin crosslinking protein that is reported to

provide mechanical stability to cells and stabilize the plasma membrane [263] also interacts with vimentin filaments [94]. In fact, this protein is described as a linker between actin and intermediate filaments [94] and also localizes at the cell cortex during mitosis [264]. Some of these proteins could be involved in the peripheral distribution of vimentin in mitosis; however this could also happen through the interaction with the cell membrane or with membrane proteins. Early studies showed that vimentin N-terminal domain interacts with membrane lipids [265, 266]. In fact, we have observed that vimentin protrudes through the actomyosin cortex at some points (Fig.18). Therefore, the interaction with membrane lipids or proteins cannot be excluded.

Consequently, these results show the importance of the study of vimentin and intermediate filament proteins as players in the dynamics of the cell cortex in mitosis. Moreover, further studies need to be performed to determine how vimentin and actin interact, and if this interaction happens directly or through other linker protein(s).

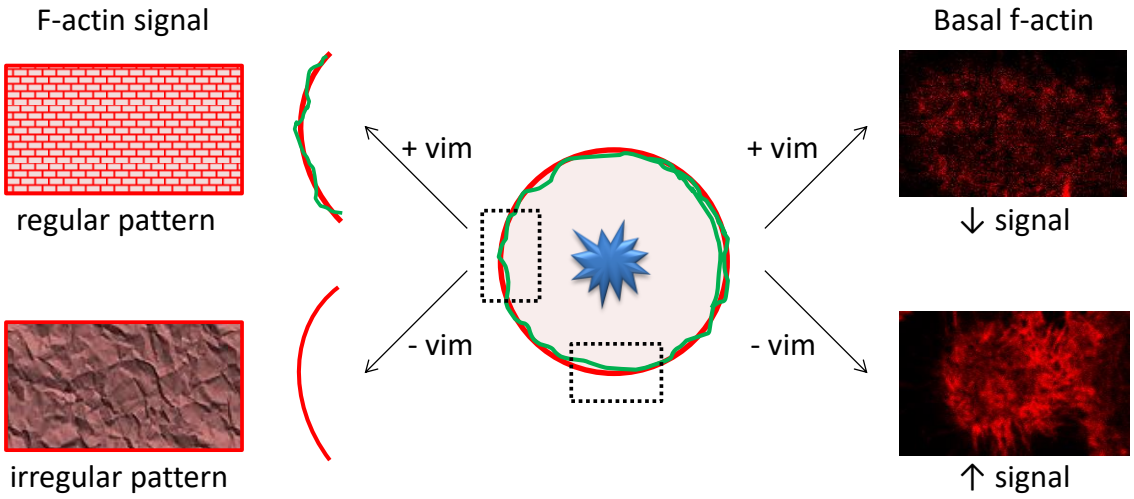


Figure 36 – Vimentin affects f-actin distribution in mitosis. Left panels show that the presence of vimentin at the cell cortex is associated with a more regular pattern of f-actin signal. In contrast, a more heterogeneous pattern of the f-actin layer in cells lacking vimentin filaments was observed. Right panels show that the presence of vimentin at basal layers of the cell affected basal f-actin, which appear to be more intense in cells lacking vimentin filaments.

2.2. Impact of C-terminal deletions on vimentin peripheral distribution

The structural determinants allowing vimentin to reach the cell periphery in mitosis were explored by using several truncated vimentin constructs (Fig. 21). Vimentin tail integrity showed to be determinant for cortical association. It was observed that both

untagged and GFP fusion vimentin constructs exhibited a gradual impairment of cortical association upon serial tail truncations, the strongest impact occurring after deletion of the 43 distal residues, which resulted in the construct vimentin (1-423) (Fig. 22). This construct mimicked the reported cleavage product of HIV type I protease, which showed a similar distribution in cells [76, 77]. Vimentin (1-423) formed curly bundles that entangled with the chromosomes, as observed with vimentin (1-411) (Fig. 10) and did not interact with the actin cortex. On the other hand, vimentin (1-448) displayed a heterogeneous pattern consisting both in accumulations and extended filaments (Fig. 23). Moreover, vimentin (1-448) filaments showed some colocalization with the actin cortex in mitotic cells together with some cytoplasmic aggregates. The construct lacking only the last six amino acids, vimentin (1-459), formed filaments with a similar morphology and extension than those of vimentin wt and showed high colocalization with the actin cortex (Fig. 24). Therefore, the sequence containing the residues 424–448 appears to comprise important determinants for mitotic redistribution (Fig. 37).

The vimentin tail structure is the most heterogeneous and flexible region of the vimentin sequence [35]. Moreover, this region contains the RDG sequence, comprising the residues 444 to 452. We have observed that the truncated C-terminal mutants that lack this region formed more aggregates and associated less with the actin cortex. Actually, the segment containing the RDG motif was proposed to form a loop that would project from filaments and participate in protein-protein interactions [33, 267]. This region is conserved and is also present in other intermediate filament proteins, such as desmin. For desmin, mutations in this region can be related with the appearance of myopathies [268]. Our results indicate that the sequence containing the RDG motif seems to play a role in cortical association, yet filaments were still formed in cells expressing the vimentin (1-448) construct that lacks this region (Fig. 23), and cortical association of a point mutant in this sequence, G452V (see below), is not impaired. Therefore, further studies are needed to unveil the mechanisms and structural determinants implicated in the distribution of vimentin during mitosis.

2.3. The formation of a full filament network is not required for cortical association of vimentin

In cells, vimentin filaments form an extended network that undergoes constant and rapid alterations due to its dynamic properties. Vimentin can exist both in soluble

tetrameric complexes and in polymerized filaments that are constantly exchanging subunits [269, 270]. Therefore, to study if the cortical association of vimentin only occurs when it is organized in filaments, two vimentin mutants that fail to elongate or assemble were used.

GFP-vimentin C328S, in which the cysteine residue was substituted by a serine, was already described by our group [30] and formed only dots in cells. The GFP-vimentin G452V mutant has a mutation located within the 450RDG452 sequence. It was previously observed that a G452V mutant failed to form filaments in vitro [34]. In cells, a vimentin mutant lacking all three amino acids of this sequence was assembly-incompetent [41]. In our experimental conditions, the GFP-fusion construct of this mutant also formed only dots in cells. Interestingly, both mutants failed to elongate into extended filaments but effectively associated with the cell periphery (Fig. 27).

In fact, other vimentin mutants with single point mutations were described as being elongation deficient and have been used in different works to study vimentin dynamics [270] and to evaluate the effect of vimentin on the actin network [121, 125]. For example, vimentin Y117L is unable to form extended filaments in cells [271], but it has been reported to interact with other cytoskeletal structures [125]. Based on in vitro characterization [27], it was suggested that the dots formed by this vimentin mutant in cells were blocked at the ULF stage of vimentin assembly [270]. However, under our experimental conditions we cannot ascertain the state of assembly of the dots formed by GFP-vimentin C328S or GFP-vimentin G452V. Nevertheless they appear to undergo some degree of assembly since the proteins showed a particulate pattern. Therefore, it would be interesting to assess whether the better characterized Y117L mutant interacts with the actin cortex in mitosis.

Taken together, these results suggest that the assembly in fully extended filaments is not necessary for the association of vimentin with the actomyosin cortex, since even small assemblies with lower degrees of organization are able to interact with cortical structures.

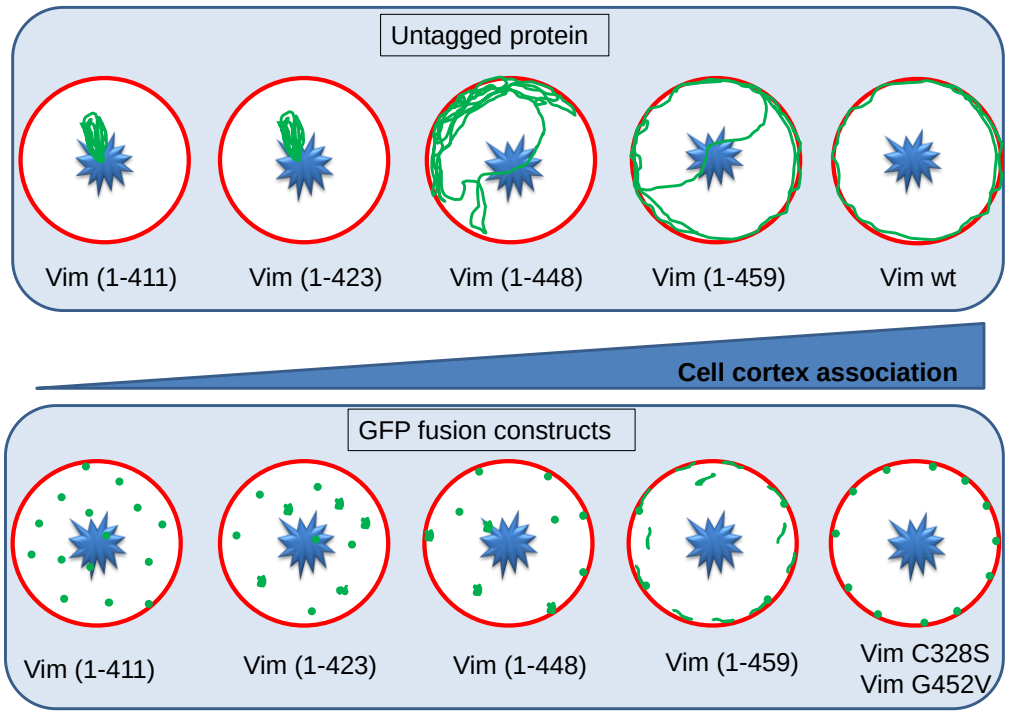


Figure 37 – Schematic representation of the importance of the vimentin C-terminal domain in cortical association in mitosis. The vimentin C-terminal domain is required for distribution of the protein in mitosis. Peripheral distribution of vimentin is impaired progressively by serial tail deletions. A higher colocalization of vimentin with the actin cortex is observed in the constructs that bear a longer part of the vimentin tail and in the single point vimentin mutants, for both the untagged proteins and for the GFP fusion constructs.

2.4. The vimentin tail domain is not sufficient for cortical association

The results described above showed that the C-terminal domain of vimentin was necessary for vimentin association with the actin cortex. Therefore, the ability of this region to interact with the actin cortex was explored. Different constructs bearing the C-terminal domain of vimentin alone or with small segments of the rod domain of the protein were used. All the different GFP-fusion constructs generated appeared as diffuse proteins that did not undergo cortical association. However, GFP-vimentin (316-466), the construct that bears a longer part of the rod domain of vimentin, showed some degree of coincidence with the actin cortex (Fig. 28).

Interestingly, the vimentin tail has been proposed to form dimeric structures protruding from the filament and participate in protein-protein interaction “in pairs” [122]. This is also supported by electron paramagnetic resonance (EPR) studies, which have proposed that vimentin tails self-associate during filament assembly [267]. GFP is known to form dimers

at high concentrations, in which the monomers interact in an anti-parallel manner [272]. If the studies mentioned above are correct, this characteristic could affect the orientation of the C-terminal tail domains and hamper their interaction with other proteins, due to the possibility of being oriented in opposite directions [273].

Therefore, we have used another fluorescent protein that is known to be monomeric, the mCherry protein. In this case we have used the mCherry fused to the vimentin tail through a Glycine spacer, or through a spacer containing also a peptide with coiled-coil structure. The α-Helical coiled-coil domain is intended to mimic the influence of the rod domain by providing a dimerization-prone structure. The presence of the coiled-coil structure was described to potentiate the oligomerization state of large proteins domains [209]. Therefore, our aim was to favor the dimerization of the vimentin C-terminal to study its possible interaction with the actin cortex. Nevertheless, no cortical association was observed with either of the constructs (Fig. 29).

This may indicate that deletion of parts of the vimentin sequence could also influence the structure or assembly of the vimentin C-terminal domain. For instance, the absence of the rod or the N-terminal domain of the protein could have an impact on the assembly or structure of the C-terminal domain, consequently affecting the interaction with other proteins. It is described that only the mutation of one amino acid, Y117, alters the stability of the vimentin C-terminus of coil 2B [274]. Moreover, the stability of the N- and C-terminal domains is necessary for filament formation. Therefore, if most of the vimentin sequence is deleted, the vimentin C-terminal may lack contact sites that are essential for the filament, or even ULF, structure or assembly [274].

In summary, although vimentin polymerization into full filaments is not necessary for cortical localization, a certain level of organization appears necessary since soluble vimentin forms do not undergo cortical association, for which integrity of the N-terminus could also play a role [228]. These results suggest that vimentin cortical association needs the contribution from several domains of vimentin to achieve a certain level of organization and/or to adopt a certain conformation. Alternatively, other parts of the protein could directly participate in the interaction with or targeting at the actin cortex. Thus, the tail domain is essential, but not sufficient, for vimentin cortical association in mitosis, and other structural or conformational factors appear necessary.

3. Vimentin distribution in experimental conditions mimicking pathophysiological situations

Vimentin has been linked to a wide variety of pathological conditions, including cancer, inflammatory diseases and viral infections. Although most pathologies are associated with an increase in vimentin expression [236], PTM or mutations can also lead to pathological conditions. For instance, citrullinated vimentin is associated with the pathogenesis of rheumatoid arthritis [109] and a mutation in the vimentin monomer induces misfolding that leads to the appearance of cataracts [24]. Vimentin is a target for several oxidants and electrophilic lipids, nevertheless its oxidative pattern is still poorly understood [15].

3.1. Vimentin distribution is altered by the HIV protease

The distribution of vimentin is described to be altered in cells microinjected with the HIV protease. The protease is reported to cleave vimentin in the C-terminal domain, between the residues L423 and R424 (including the initial M). Similar vimentin degradation patterns were observed in cell lysates from HIV-infected patients [275]. Apparently, this proteolytic cleavage promotes a collapse of the protein close to the nucleus of the cell [76]. We have observed that cell transfection with a fluorescent construct of the HIV-type I protease (GFP-PR) induced the condensation of vimentin filaments (Fig. 30). Moreover, these protein accumulations persisted in mitosis and localized with the dividing chromosomes in SW13/cl.2 cells. In Vero cells, no cells in mitosis transfected with the GFP-PR were detected, which could suggest that the expression of the HIV protease impaired mitosis or cell survival. Besides, if the HIV protease altered vimentin distribution in interphase cells, it would be possible that vimentin cleavage products had toxic effects in cells.

Vimentin has been associated with different viral infections, in which some virus need the intact vimentin network to replicate, while others promote the vimentin collapse in the perinuclear area. In parvovirus infection the vimentin IF network is rearranged to the perinuclear area, whereas in cells lacking vimentin or with disrupted vimentin the virus failed to replicate [276]. Vimentin also plays a role in the vesicular membrane trafficking and acidification of endosomes and thus in the release of the influenza virus genome into the cell nucleus. Besides, vimentin-deficient cells are resistant to this virus [75]. In the infection of human papillomavirus 16 vimentin levels modulate virus internalization in cells [74]. In Moloney mouse sarcoma virus infection, vimentin is cleaved in a similar way as in

the HIV infection, producing vimentin fragments lacking all or part of the C-terminal domain [107]. Accordingly, vimentin can play different types of roles in viral infections depending on the type of virus, either favoring viral infection or its toxic consequences.

Our results raise the potential cytotoxic implications of vimentin cortical dislodgment in HIV infection, although the cleavage or damage of molecules other than vimentin could also contribute to these effects.

3.2. Electrophilic lipids alter vimentin cortical distribution in mitosis and lead to mitotic defects

The vimentin network is highly dynamic and can suffer extensive reorganization in response to several types of stress. The consequence of the exposure to electrophilic lipids in vimentin distribution was studied by using HNE, PGA_1 and NO_2-POPC. The electrophilic lipids induced a reorganization of the vimentin network both in resting and mitotic cells, inducing an accumulation close to the nucleus in interphase cells and near the dividing chromosomes in mitosis (Fig. 31).

In fact, both actin and vimentin are targets for several electrophilic modifications, as well as for thiolation and nitrosylation [277, 278]. The vimentin reorganization upon electrophilic modification was previously described [30], however the consequences of these aggregates are not known.

In biological systems the discovery of different reactive species is constantly increasing. Electrophilic lipids and their different by-products are known as the resulting products of lipid peroxidation and are beginning to be considered as important signaling molecules relevant in physiology and pathophysiology [181, 189]. The biological actions of these lipids depend on their structure, levels and the place where they are generated, and so, these aspects influence the type of target proteins on which they can act. The antioxidant and detoxifying mechanisms regulate the action of these electrophilic lipid mediators [279, 280]. Moreover, due to the fact that these molecules may target selectively certain proteins depending on their structure, they have been studied as potential therapeutic agents [233]. In fact, cyclopentenone prostaglandins and NO_2-FA have been used in clinical trials for several diseases, such as cardiovascular diseases and cancer [203, 281].

HNE is the most investigated electrophilic lipid and a major lipid peroxidation product generated by oxidation of lipids containing polyunsaturated ω-6 fatty acids [282]. HNE-

protein adducts have been linked to cytotoxic effects. For instance, in Alzheimer disease there is an increase of HNE modified proteins in the cytoplasm of neuronal cells [195]. Moreover, several cytoskeletal proteins have been identified as targets of this lipid, such as neurofilaments [199], GFAP [194], actin [152] and vimentin [205]. NO_2-POPC and PGA_1 have also been related with protein modification. We have previously found that NO_2-POPC can act as electrophilic lipid mediator that can interact in vitro with proteins [180]. Furthermore, covalent adduction of cyclopentenone prostaglandins have been described in vimentin, tubulin and actin [283].

During mitosis, we have observed that treatment with PGA_1 induced mitotic errors similar to those observed in vimentin (1-411), which included asymmetric division, cell death and delayed mitosis (Fig. 32). In fact, these results could be related to the ability of the cyclopentenone ring in the prostaglandin structure to induce cell cycle arrest [284]. The prostaglandin 15d-PGJ_2 induces cell cycle block at G_2-M phase in breast cancer cells and human neuroblastoma cells [234, 235] and PGA_1 promotes the same effect in lung carcinoma cells [233]. Nevertheless, the role of vimentin modification in these effects deserves further study.

Here, we have shown that the formation of vimentin aggregates by electrophilic lipids could lead to mitotic catastrophe and asymmetric division of vimentin (Fig. 32). This raises potential cytotoxic implications of vimentin cortical dislodgement in pathophysiological settings, as observed upon lipoxidation and HIV protease, although damage or cleavage of macromolecules different than vimentin could contribute to these effects. Furthermore, the cortical displacement of vimentin might also be due to the modification of other proteins that are known to interact with vimentin.

In addition, treatment of cells with electrophilic lipids could also induce changes in the cell membrane or membrane proteins. Electrophilic lipids have been described to interact with membrane proteins and receptors [175, 285]. Moreover, the incorporation of these modified lipids in the biological membranes can affect the physical properties and functions of the membrane by inducing the modification of other unsaturated fatty acids. This can result in changes in cell membrane hydrophobicity and membrane integrity [286]. Therefore, the reorganization of vimentin induced by the presence of electrophilic lipids could also be a consequence of changes in the cell membrane properties. Nevertheless, the study of vimentin mutants less susceptible to lipoxidation can shed light onto the role of these modifications in vimentin redistribution.

3.3. The vimentin C328S mutant is more resistant to lipoxidation

Vimentin possesses a single cysteine residue (C328) which has been shown to be a target for nitrosylation [278] and lipoxidation [30, 200]. Previous studies have proposed that the substitution of the cysteine residue by a serine increased the resistance of vimentin to lipoxidation [30, 200]. However, the importance of the cysteine residue in filament distribution and in response to stress in mitosis has not been addressed. Our results showed that vimentin C328S was more resistant to the modification by PGA_1 and NO_2-POPC in interphase cells and to PGA_1 in mitotic cells (Fig. 33). This indicates that the cysteine residue is an important target for the functional consequences of electrophile modification.

The high reactivity of the cysteine residue could also be a consequence from the fact that this residue is flanked by acidic and hydrophobic residues that can increase its susceptibility to modification [278]. Indeed, electrophilic lipids such as CyPG modify cysteine residues selectively, depending on their reactivity, as it has been reported not only for vimentin [287] but for other and proteins such as Ras and the transcription factor NF-κB [175]. Besides, in vitro studies have identified vimentin as a target of prostaglandin PGA_1, showing that the interaction required the thiol group for PGA_1 addition [204].

Recently, it was also proposed that the cysteine residue has an important role in filament assembly, since several electrophilic agents induce vimentin remodeling in cells or alterations of vimentin filament assembly in vitro in a manner dependent on the presence of C328 [201]. Here, we have shown that in mitosis the vimentin C328S had a decreased association with the actin cortex (Fig. 33). These results could suggest the importance of the cysteine for the correct filament distribution and also for protein-protein interactions.

The vimentin cysteine residue showed to be a main target for vimentin lipoxidation, although the modification of other resides cannot be excluded. Nevertheless, the cysteine residue can be a direct target for the modification by these lipids, thus explaining the protective effect of the mutation to a serine residue. Although vimentin is particularly susceptible to electrophilic species, many other protein targets can act as hot spots for reactive lipids [30]. Thus, several potential mechanisms for vimentin disruption by HIV-PR or lipoxidation are summarized in Fig. 38.

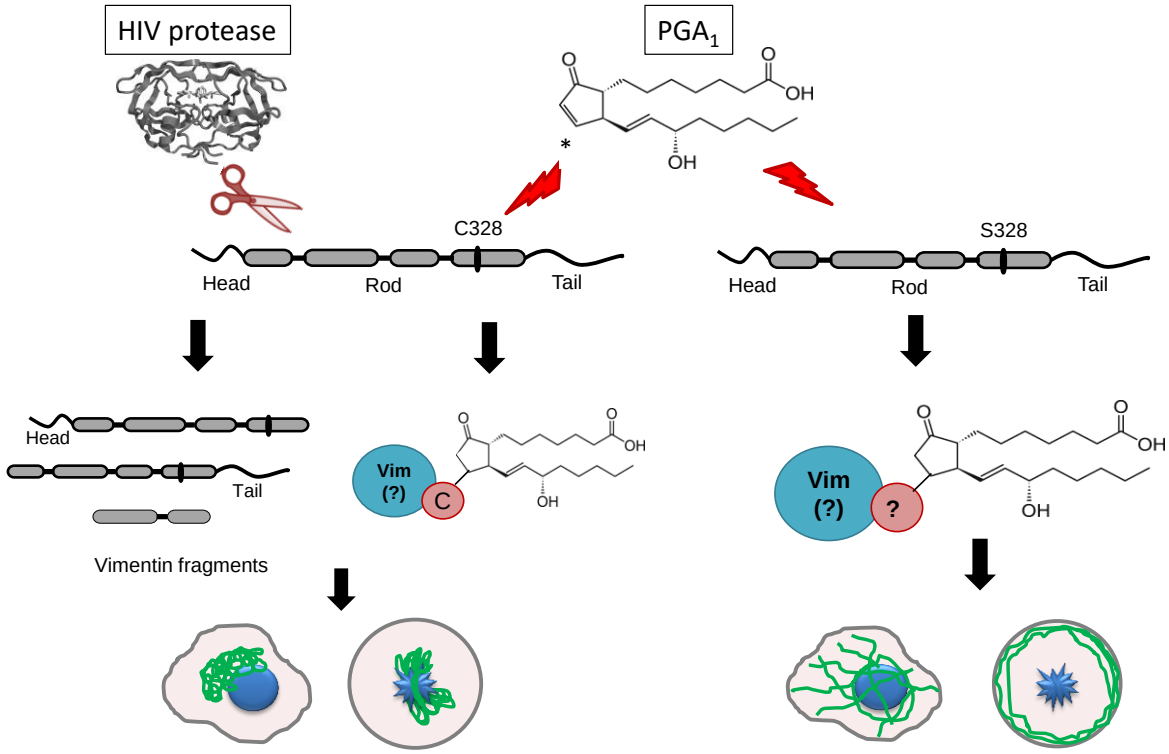


Figure 38 – Effects of HIV protease and electrophilic lipids on vimentin distribution. Conditions mimicking pathophysiological situations affected vimentin distribution. The cleavage by the HIV protease and the modification by electrophilic lipids promoted an accumulation of vimentin close to the cell nucleus and the dividing chromosomes. The reorganization induced by electrophilic lipids required the cysteine residue. Treatment of vimentin C328S mutant with PGA_1 showed that this mutant is more resistant to lipoxidation. However vimentin or other proteins can be either non-modified or modified in residues other than the cysteine and play indirect effects on vimentin distribution and/or cortical association in mitosis.

In conclusion, our results show that vimentin filaments redistribute to the cell periphery in mitosis in a tail domain-dependent manner. Vimentin reorganization in mitosis suggests the importance of the interplay with the actomyosin cortex and the dynamics of intermediate filaments during cell division. Thus, our results open the way for the search of strategies modulating these interactions. Moreover, vimentin distribution can be affected by pathophysiological conditions, including electrophile addition and protease cleavage. The cysteine residue of vimentin seems to be a key redox sensor and important for normal filament distribution in mitosis, since mutants in this residue showed suboptimal cortical association but increased resistance to lipoxidation.

Conclusions in English

As a result of the work developed during this thesis, on the importance of the C-terminal domain of vimentin in the distribution of the protein and in the cortical association with the actomyosin cortex during mitosis, we can conclude that:

1. Vimentin (1-411) displays severe defects in cellular organization and is able to disrupt the normal vimentin network.
2. Vimentin (1-411) interferes with the dividing chromosomes during mitosis, leading to asymmetric division or cell death.
3. In several cell types vimentin filaments persist in mitosis and associate with the actin cortex.
4. The integrity of the actomyosin cortex is determinant for vimentin distribution in mitosis.

These results unveil the importance of the vimentin C-terminal domain in appropriate filament distribution in interphase and in mitotic cells.

5. Vimentin is in intimate contact with the actomyosin cortex in mitotic cells.
6. The presence of vimentin at the cell cortex affects the distribution of f-actin in mitosis.
7. The intact vimentin C-terminal domain is necessary for the correct mitotic distribution and interaction with the actomyosin cortex.
8. Full-filament assembly is not essential for vimentin cortical association.
9. The vimentin tail domain is not sufficient for the interaction with the actin cortex in mitosis.

Taken together, these results define the structural requirements for vimentin cortical association and unveil a novel role of this protein in the organization of the cell cortex in mitosis.

10. In some cell types the HIV protease induces a perinuclear condensation of vimentin filaments, which persist near the dividing chromosomes during mitosis.
11. Electrophilic lipids impair the distribution of vimentin filaments in interphase cells and lead to mitotic defects.
12. The vimentin C328S mutant is less susceptible to lipoxidation-induced alteration in interphase and in mitosis.

These results disclose the importance of the distribution of vimentin during mitosis and the role of the cysteine residue as a sensor for electrophiles.

Conclusions in Spanish

Como resultado de este trabajo, donde se ha estudiado la importancia del dominio C-terminal de vimentina en su distribución y en su asociación con la corteza de actina durante la mitosis, concluimos que:

1. La vimentina (1-411) exhibe defectos en la organización celular y puede alterar la distribución de los filamentos de vimentina silvestre.
2. La vimentina (1-411) interfiere con los cromosomas en división durante la mitosis, lo que puede dar lugar a una división asimétrica o a muerte celular.
3. Los filamentos de vimentina persisten en mitosis en varios tipos celulares y se asocian con la corteza de actina.
4. La integridad de la corteza de actomiosina es determinante para la distribución de vimentina en la mitosis.

Estos resultados revelan la importancia del dominio C-terminal de vimentina en la correcta distribución de filamentos en células en interfase y en mitosis.

5. La vimentina está en íntimo contacto con la corteza de actomiosina en las células mitóticas.
6. La presencia de vimentina en la corteza celular afecta la distribución de f-actina en mitosis.
7. La correcta distribución de vimentina en la mitosis y su interacción con corteza de actina requiere en dominio C-terminal completo.
8. El ensamblaje de la vimentina en filamentos no es esencial para la asociación con la corteza en mitosis.
9. El dominio C-terminal de vimentina por sí solo no es suficiente para la interacción con la corteza de actina durante la mitosis.

En conjunto, estos resultados desvelan los requerimientos estructurales para la asociación cortical de vimentina y sugieren un nuevo papel de esta proteína en las propiedades de la corteza celular.

10. En algunos tipos celulares, la proteasa del VIH induce una condensación perinuclear de los filamentos de vimentina, que persiste cerca de los cromosomas durante la división celular.
11. Los lípidos electrófilos pueden alterar la distribución de los filamentos de vimentina en las células en interfase y provocar defectos mitóticos.

12. El mutante de vimentina C328S es más resistente a la lipoxidación en interfase y en mitosis.

Estos resultados muestran la importancia de la distribución de vimentina durante la mitosis y el papel del residuo de cisteína como sensor de respuesta a agentes electrófilos.

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Related publications

- Sofia Duarte, Álvaro Viedma-Poyatos, Elena Navarro-Carrasco, Alma E. Martínez, María A. Pajares, Dolores Pérez-Sala. **Vimentin filaments interact with the actin cortex in mitosis allowing normal cell division**. Nat Commun. 2019 Sep 13;10(1):4200. https://doi.org/10.1038/s41467-019-12029-4

- Sofia Duarte, Tânia Melo, Rosário Domingues, Juan de Dios Alché, Dolores Pérez-Sala. **Insight into the cellular effects of nitrated phospholipids: Evidence for pleiotropic mechanisms of action.** Free Radic Biol Med. 2019 Nov 20;144:192-202. https://doi.org/10.1016/j.freeradbiomed.2019.06.003

- Andreia Mónico, Sofia Duarte, María A. Pajares, Dolores Pérez-Sala. **Vimentin disruption by lipoxidation and electrophiles: Role of the cysteine residue and filament dynamics.** Redox Biol. 2019 Jan 8;101098. https://doi.org/10.1016/j.redox.2019.101098.

- Giulia Coliva, Sofia Duarte, Dolores Pérez-Sala, Maria Fedorova. **Impact of inhibition of the autophagy-lysosomal pathway on biomolecules carbonylation and proteome regulation in rat cardiac cells**. Redox Biol. 2019 Jan 24; 101123. https://doi.org/10.1016/j.redox.2019.101123.

- Silvia Zorrilla, Andreia Mónico, Sofia Duarte, Germán Rivas, Dolores Pérez-Sala, María A.Pajares**. Integrated approaches to unravel the impact of protein lipoxidation on macromolecular interactions**. Free Rad Biol Med. 2019 Apr 13; 0891-5849. https://doi.org/10.1016/j.freeradbiomed.2019.04.011.