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NEW INSIGHTS INTO THE ROLE OF THE CENTROSOMAL MARK4 KINASE IN REGULATING THE DYNAMICS AND REMODELLING OF CYTOSKELETON FROM OVEREXPRESSION STUDIES OF ITS TWO ISOFORMS IN NORMAL AND TUMOR CELLS

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Abstract

MAP/Microtubule Affinity Regulating Kinase 4 (MARK4) belongs to a highly conserved family of serine–threonine kinases (MARKs) that are able to phosphorylate the microtubule associated proteins (MAPs), and cause these proteins to detach from microtubules (MTs) increasing microtubules dynamics. MARKs kinases represent the mammalian homologues of PAR-1, a protein involved in establishment of the cell shape and polarity in lower eukaryotes.

The *MARK4* gene is located at 19q13.2 and encodes at least two alternatively spliced isoforms, MARK4L and MARK4S, which have an identical protein structure apart from the C-terminal region. The two isoforms are differentially expressed in human tissues, particularly in the central nervous system (CNS). Several studies reported that MARK4S is expressed in normal brain tissue and neurons, suggesting that this isoform has a role in neuronal differentiation. Conversely, MARK4L is up-regulated in glioma and neural progenitor cells, pointing to a possible role of this isoform in cell proliferation. Recently, we highlighted an increasingly subverted MARK4L/MARK4S ratio, with prevalence of MARK4L, in glioblastoma and glioblastoma-derived cancer stem cells, that recapitulate the expression profiling of neural stem cells. These findings suggest that the expression of the two MARK4 isoforms is tightly regulated during the proliferation/differentiation of neural stem cells and changes in their expression levels may be a molecular marker of tumour transformation.

Unlike the other members of the family (MARK1, MARK2 and MARK3), that exhibit uniform cytoplasmic localisation, both MARK4 isoforms localise at the centrosomes and in the midbody, supporting their involvement in mitotic division and cytokinesis.

To elucidate the role played by MARK4 isoforms in cell cycle progression and in the regulation of the cytoskeleton, we monitored the activation status of MARK4 during the cell cycle and performed overexpression experiments in fibroblasts and glioma cell lines.

We showed that despite MARK4 is expressed across all the cell cycle phases, its active form, phosphorylated at the Thr214 residue, is prevalent in mitosis. Phospho-MARK4 is detected in centrosomes at all mitotic stages and in the midbody during cytokinesis. Conversely, only a fraction of interphase centrosomes show phospho-MARK4 positive signals.

Overexpression experiments on fibroblasts and glioma cell lines demonstrated the role of MARK4 in the regulation of cytoskeleton dynamics. Indeed overexpression of MARK4L or MARK4S led to a sharp decrease in microtubule density in both the cell systems, as monitored by immunofluorescence experiments. By contrast, overexpression of catalytically inactive MARK4L/MARK4S mutants, did not affect the microtubule network, indicating that the effects on MTs are dependent on the kinase activity of MARK4 and likely linked to MAPs phosphorylation.

Besides the effect on MT array, overexpressed MARK4L in fibroblasts showed a filamentous staining pattern, co-localising with vimentin, the core component of cytoskeleton intermediate filaments. In contrast, overexpressed MARK4S co-localised with vimentin to a lesser extent and only in few cells.

The MARK4L-vimentin co-localisation was particularly evident in the perinuclear zone and in some overexpressing cells, the filaments appeared reshaped as compared to those in GFP-transfected cells, and showed the formation of bundle structures. These alterations seem to be due to the kinase activity of MARK4L since overexpression of kinase dead mutants did not remodel the intermediate filaments.

The overall data highlight MARK4 as a key component in the regulation of microtubules dynamics, and indicate vimentin as a plausible target of MARK4L activity, suggesting a wide-ranging influence of MARK4 on cytoskeleton. Moreover the dynamic involvement of active MARK4 in structure like centrosomes and midbody, crucial for mitosis and cytokinesis, point to a fundamental role for this kinase in the cell cycle.

Introduction

1.MARK4: a member of the AMPK kinase family

AMP-activated protein kinase (AMPK) is a conserved serine-threonine kinase involved in the regulation of cellular and whole-body energy homeostasis (Bright *et al.*, 2009). AMPK is activated by different physiological and pathological stimuli that decrease cellular energy levels, increasing the AMP/ATP ratio (Hardie 2003).

The activation of AMPK needs phosphorylation of threonine residue 172, located in the activation loop of the catalytic domain. Three upstream AMPK kinases have been identified: the *Liver Kinase B1 (LKB1)*, a tumour suppressor protein kinase associated with Peutz-Jeghers Syndrome, the Ca^{2+}/calmoduline-dependent protein kinase β and the transforming growth factor-β-activated kinase 1 (TAK1). In addition to phosphorylation, AMPK is activated allosterically by AMP.

Besides its effects on energy homeostasis, AMPK plays many different functions including regulation of mitochondrial biogenesis, cell polarity and cell growth and proliferation.

Twelve kinases (BRSK1, BRSK2, NUAK1, NUAK2, SIK1, SIK2, SIK3, MARK1, MARK2, MARK3, **MARK4** AND MELK), show high sequence homology with the AMPK kinase domain, and have been identified and grouped in the AMPK-related kinase family (Bright *et al.*, 2009). These protein kinases have a similar structural organisation, and with the exception of MELK, can be activated by LKB1 upon phosphorylation of a conserved threonine.

AMPK-related kinases are expressed in different tissues and are involved in the regulation of diverse processes including metabolism, cell polarity, cell cycle, insulin signalling (Figure 1).

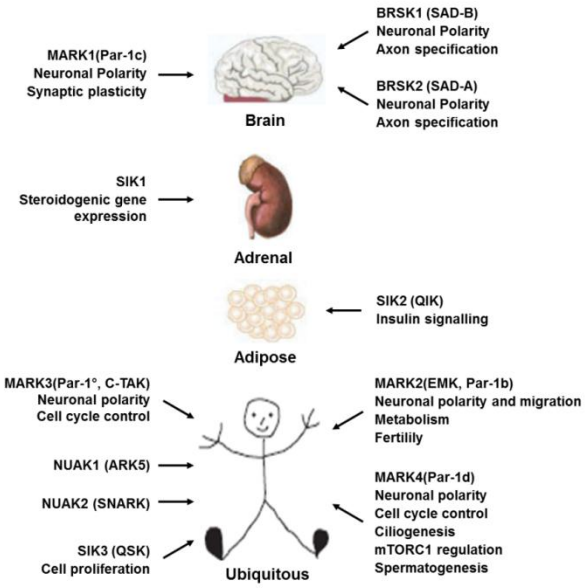


Figure 1. Tissue expression and functions of AMPK-related kinases (Modified from Bright *et al.*, 2009).

Among the AMPK-related kinases, substantial evidences have been acquired on the important roles played by MARK proteins in crucial processes like cell polarity and cell division.

2.The MARK protein family

The MARK proteins (Microtubule associated protein/Microtubule affinity regulating kinases) belong to a family of serine/threonine kinases that were originally identified by their ability to phosphorylate microtubule associated proteins (MAPs) (including tau, MAP2, MAP4 and doublecortin) at a specific site (serine in the KXGS motifs) in the microtubule binding repeats. As consequence of phosphorylation, MAPs decrease their affinity for microtubules (MTs), detach from them and increase their dynamics (leading to MT network destabilisation) (Drewes *et al.*, 1997).
In mammals the MARK family consists of four members (MARK1, MARK2, MARK3 and MARK4) and additional isoforms arise by alternative splicing.

2.1 Protein Structure

All the four MARK proteins have a conserved domain organisation, similar to the other AMPK-related kinases, and can be divided into six sequence segments, shown in Figure 2 (Timm *et al.*, 2008; Marx *et al., 2010*).

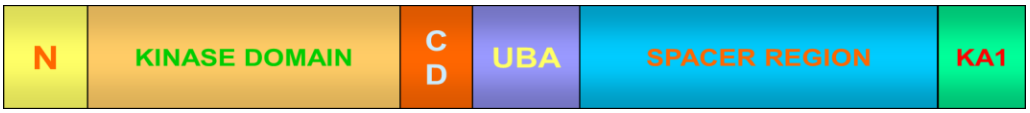


Figure 2. Schematic representation of MARK protein structure.

From the N to the C terminus they are:

- The ***N-terminal header*** (N), which function is still unknown. This domain is usually different for each MARK protein and is missing or considerably reduced in size in some isoforms.
- The ***catalytic domain***, the region with the highest homology among the four MARK proteins (~90%), presents a bi-lobal structure typical of protein kinases, with an active site cleft between the two lobes. The smaller N-terminal lobe consists of five β-strands and a single α-helix, while the larger C-terminal lobe is mostly folded into an α-helical structure. Both lobes contribute structural elements to the active site, consisting of: the P-loop (phosphate-binding), the catalytic loop and the activation loop (also called T-loop). The P-loop helps to position the ATP γ-phosphate to be transferred to the substrate's phosphorylation sites. The catalytic domain presents an RD motif, consisting of a catalytically active aspartate, preceded by an arginine residue. The T-loop contains a conserved sequence (LDTFCGSPP) where the threonine and serine residues are the

phosphorylation target. When MARK proteins are inactive, the T-loop is disordered, and folds over the cleft, blocking the access of ATP and substrate proteins. Phosphorylation of the conserved threonine in the T-loop, activates the protein, triggering its reorientation and stabilisation. The activation loop folds out of the catalytic cleft, that becomes thus accessible to the substrate(s) and ATP.

- The ***linker*** is a short stretch of amino acids, that starts with a charged four residues motif that resembles the common docking (CD) site in MAP kinases. This region may bind interactors or co-factors.
- The ***ubiquitin associate domain*** (UBA) is a small and globular region, consisting of three α-helices folded in a characteristic helical bundle. The domain is present in all the AMPK-related kinases, and has sequence homology with ubiquitin-associated proteins, but at difference of them, it is differently and unusually folded and probably does not bind ubiquitin. It has been suggested that the UBA domain of AMPK-related kinases has evolved from canonical UBA domains and has then lost the capacity of ubiquitin binding in favour of the ability to interact with the kinase domain (Murphy *et al.,* 2007). Indeed it has been demonstrated that UBA binds the N-lobe of MARK catalytic domain, locking it in an open inactive conformation (Panneerselvam *et al.,* 2006). Based on these features the interaction of UBA could have two different consequences on MARK activity. First, UBA can exert an autoinhibitory role maintaining the open conformation that prevents ATP and substrates binding (Panneerselvam *et al.,* 2006). On the other hand this conformation could increase the accessibility of the activation loop for activating or deactivating kinases (Murphy *et al.,* 2007). Accordingly it has been found that UBA is necessary for LKB1- mediated activation, despite not being the docking site for this upstream kinase (Jaleel *et al.,* 2006). In addition an intact UBA appears to be required for reaching full activity even if MARK protein is already phosphorylated at the threonine in the T-loop (Jaleel *et al.,* 2006).
- The ***spacer*** is the most variable region among the MARK kinases, varying in size between 290 and 330 residues. This domain maintains a native unfolded status and is important for the regulation of MARK activity, as it contains sites that are target of different kinases.
- The ***C-terminal tail domain*** is constituted by 100 amino acid residues and comprises the ***Kinase associated domain 1*** (KA1), that is conserved among the AMPK-related kinase family. This region has a hydrophobic, concave surface surrounded by positively charged amino acids. It has been suggested that this highly conserved surface may interact with negatively charged sequences present in the MARK catalytic domain or CD region, regulating the MARK activity (Tochio *et al.,* 2006). The C-terminal tail domain could be

another autoinhibitory domain, in addition to UBA. It has been also proposed that the KA1 domain is involved in protein localisation (Tochio *et al.*, 2006).

2.2 Regulation of MARK proteins

MARK proteins, as above outlined, present different domains, that are important for the regulation of kinase activity through multiple pathways. In particular MARK activity, is finely tuned by various post-translational modifications including phosphorylation and ubiquitination and by interaction with other proteins (Figure 3).

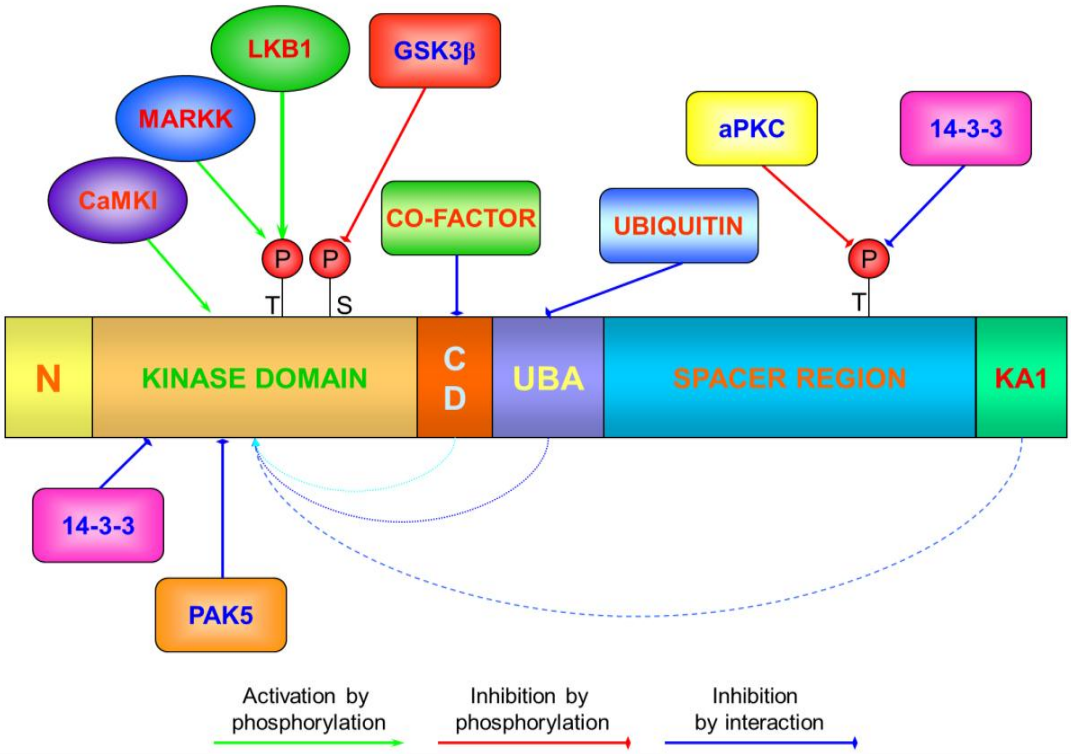


Figure 3. Schematic representation of regulatory pathways of MARKs.

2.2.1 MARKs regulation by phosphorylation

The activity of MARK proteins can be enhanced or reduced by the phosphorylation of specific threonine or serine residues, present in either the kinase domain and the spacer region (Matenia & Mandelwkow 2009).

The activation of MARK protein requires the phosphorylation of a conserved threonine present in the T-loop (T215 in MARK1, T208 in MARK2, T211 in MARK3 and T214 in MARK4) by two different upstream activating kinases: LKB1 (Lizcano *et al.*, 2004) and MARK kinase (MARKK/TAO1) (Timm *et al.*, 2003).

LKB1 is a tumour suppressor gene, which loss-of-function mutations are the molecular cause of Peutz–Jeghers syndrome (OMIM#175200), an autosomal dominant disorder characterised by the predisposition to a wide spectrum of benign and malign tumours, in particular gastrointestinal polyps and colon and breast cancers (Alessi *et al.,* 2006). Many different studies have demonstrated that LKB1 is able to interact, phosphorylate and activate various kinases including AMPK and all AMPK-related kinases apart from MELK. In mammalian cells LKB1 activity and localisation is regulated through its interaction with two proteins: the pseudokinase STE20-related adaptor (STRAD), and mouse protein 25 (MO25) (Baas *et al.,* 2003; Boudeau *et al.,* 2003; Brajenovic *et al.,* 2004).

MARKK is a kinase, that belongs to the STE20 kinase family and shows high homology with the TAO-1 (thousand-and-one amino acid) protein (Timm *et al.,* 2003).

It has been demonstrated that the activated Calcium/calmodulin-dependent protein kinase I (CaMKI) interacts with the kinase domain of MARK2, phosphorylating it at a novel site (Uboha *et al.,* 2007).

The activity of MARKs can be also negatively regulated by different kinases phosphorylating specific residues.

The glycogen synthase kinase 3β (GSK3β) phosphorylates a conserved serine present in the T-loop three amino acids after the threonine activation site (S219 in MARK1, S212 in MARK2, S215 in MARK3 and S218 in MARK4) (Timm *et al.,* 2008). The phosphorylation of this residue causes the inhibition of MARK activity, even when the conserved threonine is phosphorylated by LKB1 or MARKK. It has been shown that this particular serine is fundamental for the stabilisation of the activation loop. In the inactive state, the T-loop is folded into the catalytic cleft, obstructing the entry of both ATP and substrate. Following the phosphorylation of the conserved threonine, the activation loop folds out, opening the cleft for the substrate; in this configuration, the serine establishes hydrogen bonds with the catalytic aspartate. These bonds together with the interaction of the phospho-threonine, block the T-loop in the open conformation, which is crucial for the kinase activity. The phosphorylation of the conserved serine disrupts the interaction that stabilises the molecules, and the same result is achieved when this residue is mutated to alanine (Timm *et al.,* 2008).

The MARK proteins also interact with the atypical Protein Kinase C (aPKC), a kinase involved in the regulation of cell polarity. aPKC phosphorylate MARK2 at T595 in the spacer region, enhancing the interaction with 14-3-3/Par-5 protein. This modification down regulates MARK activity and stimulates its dissociation from the lateral membrane in polarised cells (Suzuki *et al.,* 2004; Hurov *et al.,* 2004). aPKC is also able to phosphorylate MARK3 at an analogous site,

suggesting that the MARK isoforms might have redundant functions in the cells (Hurov *et al.* 2004). It has been demonstrated that MARK4 interacts with PKC-λ, another kinase involved in cell polarity (Brajenovic *et al.,* 2004).
The human PIM 1 kinase, a member of CaMK superfamily, inhibits MARK3 activity by phosphorylating a serine residue in the catalytic domain (Bachmann *et al.,* 2004).

2.2.2 Ubiquitination

Based on the presence of the UBA domain, it has been suggested that binding of ubiquitin can regulate MARKs activity. Recently it has been shown that MARK4 and NUAK2 (an AMPK-related kinase) can be polyubiquitinated *in vivo*, and are deubiquitinated through the interaction with the deubiquitinating enzyme USP9X (Ubiquitin Specific Protease-9). Ubiquitin modifications were thought to be involved in the regulation of phosphorylation and activation by LKB1, because MARK4 and NUAK2 mutants which do not bind USP9X are hyperubiquitinated, unphosphorylated and thus inactive (for more details see chapter 3.3 Regulation of MARK4) (Al-Hakim *et al.,* 2008).

2.2.3 Regulation of MARK activity by protein interaction

Besides phosphorylation and ubiquitination the interaction between MARKs and other proteins contributes to modulating its kinase activity.
It has been demonstrated that the adaptor proteins 14-3-3 (Par-5) can interact with MARK through two different ways: 14-3-3 can bind the catalytic domain independently of the phosphorylation status (Benton *et al.,* 2003) or alternatively this protein can interact with the spacer region following aPKC phosphorylation (Hurov *et al.,* 2004). These interactions inhibit MARK activity, probably by stabilising the interaction between the tail domain with the amino terminal or the kinase domain. In addition the binding of 14-3-3 proteins regulates MARK spatially, altering its localisation.
The kinase activity of MARK2 is also inhibited by the interaction with the ste20-kinase PAK5. The PAK (p21-activated kinases) proteins are serine/threonine kinases involved in the regulation of different processes including cell cycle progression and cytoskeleton dynamics. PAK5 is able to inhibit MARK2 activity by the interaction of their respective catalytic domains being MARK phosphorylation status irrelevant for this binding (Matenia *et al.,* 2005).
The CD domain, present in all MARK isoforms, could act as site for the binding of various effectors similarly to what observed in MAP kinases (Tanoue *et al.,* 2003).

2.2.4 Other mechanisms of MARK regulation

As previously introduced, the UBA and KA1 domains can be involved in the auto-regulation of MARK activity, by interacting with the catalytic domain.

Dimerization is a novel possible mechanism for autoregulation of MARK activity, as observed for many different kinases (Marx *et al.,* 2010). MARK proteins crystallise *in vitro* as dimers with the two molecules covalently linked by disulphite bridges between T-loops. In this conformation the proteins are inactive, due to the open conformation assumed by the catalytic domain. Despite dimerization could occur also in living cells evidence for *in vivo* MARK dimerization is still lacking (Marx *et al.,* 2010).

2.3 MARK orthologous genes in lower eukaryotes

The mammalian MARK proteins show significant homology with numerous kinases present in lower eukaryotes, and in particular with that encoded by the *C. elegans PAR-1* gene. *PAR-1* is one of the six *partitioning defective* genes (PAR 1-6), which mutation disorganises the asymmetric cell division of the zygote (Drewes *et al.,* 1998). The PAR proteins cooperate in the formation of a polarised cell axis, which is essential for establishing the embryonic body axis. In particular the PAR-1 proteins localise in the posterior cortex, that develops into the germ cell line, and its kinase activity is fundamental for the distribution of the P granules. These particles are initially distributed evenly throughout the cytoplasm, but, before the first zygote cleavage, they move into the posterior pole always ending up in the germline in the following asymmetric cell divisions (Guo & Kemphues 1996).

With the only exception of *PAR-2*, *PAR* orthologous genes have been found in *Drosophila* (Table 1), where they are involved in the oocyte specification and in establishing the anterior- posterior polarity of the embryo (Riechmann *et al.,* 2001).

PAR orthologous genes have been discovered in mammals, and in particular four orthologous genes of *C.elegans PAR-1* have been identified (Table 1) (Inglis *et al.,* 1993; Drewes *et al.,* 1997; Espinosa *et al.,* 1998; Peng *et al.,* 1998; Kato *et al.,* 2001; Muller *et al.,* 2001).

C. elegans	*D. melanogaster*	*Mammals*	*Domain/function mammals)*
PAR1	PAR1	MARK1/PAR1c MARK2/PAR1b MARK3/PAR1a MARK4/PAR1d	Serine/Threonine kinases involved in microtubule dynamics
PAR2	Not identified	Not identified	
PAR3	PAR3/Bazooka	PAR3/ASIP PAR3β	PDZ domain PAR6, aPKC, JAM-1binding Necessary to form tight junctions
PAR4	dLKB1	LKB1	Serine/Threonine kinase PAR-1 phosphorylation/activation
PAR5	14-3-3 14-3-3ξ	14-3-3 ξ	14-3-3 domain
PAR6	PAR6	PAR6a PAR6b PAR6c PAR6d	PDZ and CRIB domainsPAR3 and aPKC binding Cdc42 Crumbs-PALS-PA7J binding Necessary to form tight junctions

Table 1. *PAR* orthologous genes in *Drosophila* and mammalians (Modified from Baas *et al.,* 2004).

Orthologs of PAR-1/MARK protein have been identified back to fission yeast where they are called kin1. These proteins play a role in the regulation of cell polarity. In particular, in *Schizosaccharomyces pombe* kin1 is involved in establishing the rod-shaped morphology of yeast cells. Cells in which *kin1* is deleted are still viable but are impaired in growth and round up. Interestingly these alterations can be in part rescued by the expression of human MARK, which re-establishes bipolar growth (Drewes & Nurse 2003).

The significant degree of sequence homology of proteins of the MARK-PAR-1-kin1family across evolutionarily distant organisms, suggests that these proteins operate in conserved signalling pathways controlling cell polarity.

2.4 Localisation and function of MARK proteins

It has been shown that MARK1, MARK2 and MARK3 present an uniform cytoplasm localisation, and are associated with the intracellular network. In addition MARK2 can be linked to the plasma membrane component (Drewes *et al,.* 1997; Trinczek *et al.*, 2004).

As described above the MARK proteins phosphorylate MAPs, regulating their affinity for MTs and thereby controlling microtubule dynamics. Based on this observation, MARK proteins can be

implicated in the control of many different cellular processes involving the microtubules network, such as cell polarity, centrosome formation, chromosome segregation and cytokinesis.

Microtubule-dependent transport. It has been reported that MARK proteins can be involved in the regulation of microtubule-dependent transport. MAPs can compete with the motor proteins kinesin and dynein for the MT binding, thus inhibiting the transport of vesicle and organelles. MARK proteins, phosphorylating MAPs, cause their detachment from MTs and thereby facilitate the transport of particles (Mandelkow *et al.,* 2004). Furthermore, it has been demonstrated that MARKs co-localise withe the clathrin-coated vesicles (CCV), highlighting a function of MARKs in regulating microtubule-dependent transport of CCV during endocytosis (Schmitt-Ulms *et al.* 2009).

Cell polarity. MARK proteins are asymmetrically localised in mammalian epithelial cells (Bohm *et al.,* 1997), and in particular MARK1 and MARK2 are necessary for polarisation of kidney or liver cells (Bohm *et al.,* 1997; Cohen *et al.,* 2004). MARK2 is involved in the polarisation of hippocampal neurones, by regulating the axon outgrowth. Indeed it has been found that MARK2 silencing promotes the growth of multiple axons in neurons, whereas overexpression inhibits axon and dendritic formation (Chen *et al.,* 2006; Terabayashi *et al.,* 2007). MARK2 activity is inhibited by the PAR-3/PAR-6/aPKC complex, that promotes the axon establishment (Chen *et al.* 2006; Terabayashi *et al.* 2007).
The role of MARK proteins in cell polarisation is also inferred by the observation that the *Helicoibacter pylori* CagA peptide interacts with MARK proteins, mimics their substrates and occupies the catalytic cleft inhibiting the kinase activity (Nesic *et al.,* 2009). This leads to the alteration of cell polarity and the destruction of epithelial architecture.

Neuron migration. The doublecortin (Dcx) is a microtubule associated protein, that can be phosphorylated by MARK proteins at the same KXGS motif shared by other MAPs. This protein is highly enriched in the leading processes of migrating neurons and the growth cone region of differentiating neurons. Dcx phosphorylation by MARK, regulating doublecortin affinity for microtubules, affects neuronal migration (Schaar *et al.,* 2004). Indeed after MARK2 silencing, neurons of the developing neocortex fail to migrate beyond the intermediate zone, and do not acquire a bipolar morphology (Sapir *et al.,* 2008).

Cell Cycle control, cell signalling and subcellular localisation. Phosphorylation is a common mechanism to control the activity and to change the cellular localisation of proteins. 14-3-3 are phospho-binding proteins that can interact with different partners, regulating several essential

processes. MARK kinases, phosphorylating their target proteins, are involved in the regulation of several of them by creating a binding site for 14-3-3 proteins.

MARK3 can phosphorylate the cell cycle regulatory phosphatase CdC25, mediating the binding between Cdc25 and 14-3-3 proteins, thus precluding Cdc25 activation of Cdc2/cyclin B complex by dephosphorylation, which is required for mitotic entry (Peng *et al.,* 1998). Moreover MARK3 phosphorylation and subsequently inhibition by Pim-1 promote cell cycle progression at the G2/M transition (Bachmann *et al.* 2004).

Phosphorylation of the kinase suppressor of Raf-1 (KSR1) by MARK3 causes the binding of 14-3-3 proteins, thus contributing to the regulation of the Ras-MAPK pathway (Muller *et al.,* 2001).

Class IIa histone deacetylases (HDACs) are present in both the cytoplasm and the nucleus, where they are involved in the repression of several developmental genes. MARK2 and 3 can phosphorylate HDACs, on their 14-3-3 binding site, and neutralise their repressive activity by 14-3-3-mediated nuclear exclusion (Dequiedt *et al.,* 2006). Similarly, MARK3 phosphorylates plakophilin 2 (PKP2), generating a 14-3-3 binding site, that drives PKP2 to the nucleus (Muller *et al.,* 2003).

Other physiological functions. Evidence based on experiments using MARK2 knockout mice prove that this kinase is involved in a variety of physiological functions like immune system homeostasis, learning and memory, fertility, growth and regulation of metabolism (Bessone *et al.,* 1999; Hurov *et al.* 2001; Hurov & Piwnica-Worms 2007; Segu *et al.,* 2008).

The MARK4 functions and cell localisation are reported in detail in the following paragraph.

3. MAP/Microtubule Affinity-Regulating Kinase 4 (MARK4)

MARK4 is the less characterised member of the MARK kinase family. The *MARK4* gene was discovered by Kato and colleagues in 2001, by analysing genes regulated by β-catenin/Tcf complex in a human hepatoma cell line (Kato *et al.*, 2001). Based on its homology to MARK3, the gene was named *MARKL1* (MARK1-like 1).

3.1 MARK4 gene, alternative transcript and protein structure

The *MARK4* gene is located on chromosome 19q13.2, and consists of 18 exons. The MARK4 transcripts can undergo alternative splicing, giving origin to two mature mRNA, that differ for the presence of exon 16 (Kato et al. 2001). They are:

- the MARK4S (short) mRNA, the native isoform, that contains all the 18 exons, and is 3609 bp long. This transcript encodes a 688 amino acid long protein, which molecular weight is around 75 kiloDalton (kDa). Due to the presence of exon 16 the stop-codon falls inside exon 18, making the encoded protein to contain all the classical MARK domains, with the exception of the C-terminal KA1 domain (Figure 4).

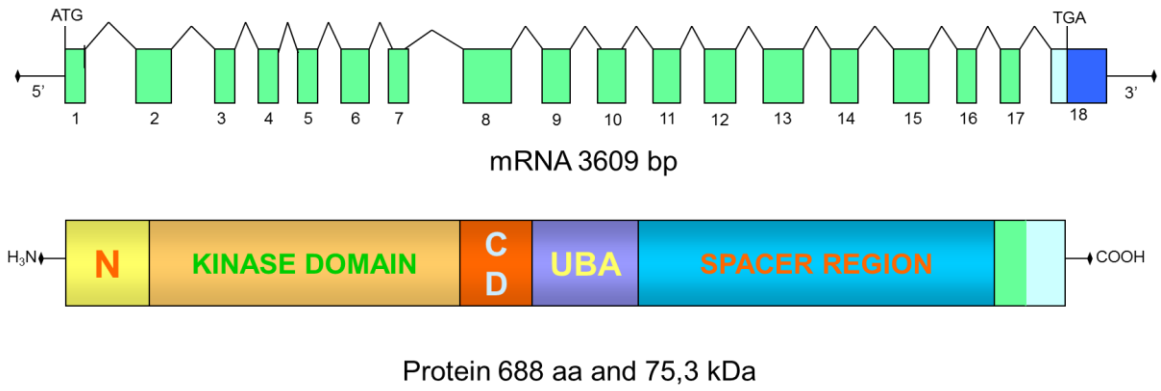


Figure 4. Schematic representation of MARK4S mRNA and protein. mRNA contains all exons but the stop codon is inside exon 18 leads to MARK4S isoform, that lacks the C-terminal KA1 domain.

- MARK4L (long) mRNA, is 3529 bp long, and arises by the skipping of exon 16. The lack of exon 16 causes the shift of the reading frame, that changes the stop codon determining the synthesis of a longer protein (752 amino acids), which predicted molecular weight is 82,5 kDa. The MARK4L protein presents all the typical MARK domains (Figure 5).

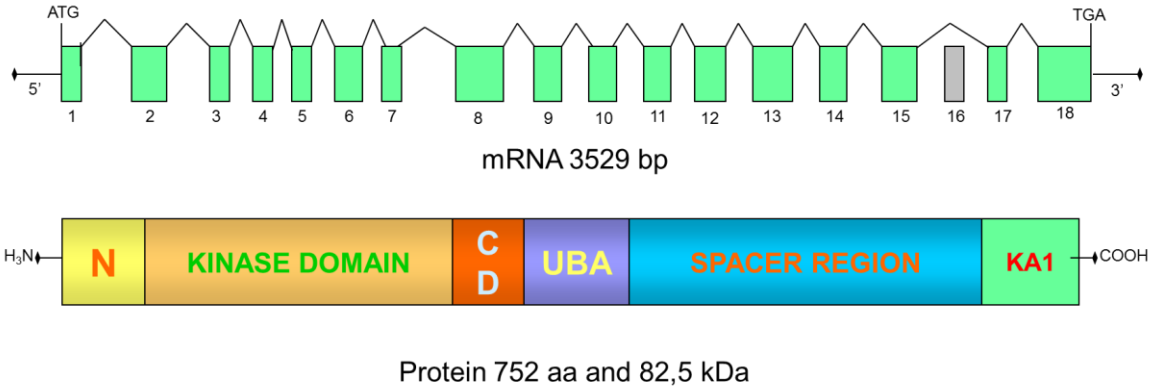


Figure 5. Schematic representation of MARK4L mRNA and protein. In MARK4L, the exon 16 is skipped, by alternative splicing causing a shift of the reading frame that changes the STOP codon generating the MARK4L protein, which possesses all the classical MARK domains.

The *MARK4* gene is ubiquitously expressed, with the highest levels in brain and testis (Beghini *et al.*, 2003; Trinczek *et al.*, 2004). The MARK4 sequence shows 55% homology with the other MARK proteins, with the highest homology with MARK3. Both MARK4 isoforms present the characteristic domain and protein structure of the MARK proteins, with the amino-terminal header, the catalytic domain, the UBA domain, the spacer region and the tail domain. The catalytic domain is the more conserved region among MARK proteins, with a 90% of sequence homology. This domain contains the three loops that are involved in the regulation and function of the protein, and in particular the T-loop that has the conserved sequence LDTFCGSP, with the two regulatory phosphorylation sites (T214 and S218). The spacer region is the less conserved sequence between the MARKs.

As a consequence of alternative splicing, the two MARK4 isoforms differ in the C-terminal domain: MARK4L contains the KA1 as the other AMPK-related kinases, while MARK4S has a domain with no homology to any other known structure. MARK4 presents the lowest sequence homology in the tail region, as compared to MARK1-3 proteins. Nevertheless the prediction of secondary structure shows that this domain folds up in a conformation similar to those of MARK1, 2 and 3, suggesting an analogous autoinhibitory function (Marx *et al*., 2010).

3.2 MARK4 interactors

By means of Tandem Purification Affinity and immunoprecipitation experiments 20 MARK4 interacting proteins have been identified (Brajenovic *et al.*, 2004). Among these, Cdc42 and PKCλ, that are components of the PAR-6 complex, have been involved in cell polarity. In addition MARK4 interacts with the transforming growth factor-β-induced anti-apoptotic factor, an ortholog

of Miranda, a centrosomal protein implicated in neuroblast asymmetric division in *Drosophila* (Mollinari *et al., 2002).*

Many different 14-3-3 proteins have been identified to interact with MARK4 and in particular the 14-3-3η. As previously mentioned, 14-3-3 proteins are involved in the regulation of several cellular process by binding phosphorylated proteins and has been proposed they could directly regulate MARK4 or act as bridging factors between several proteins acting in different pathways. Other MARK4 interactors appear to be associated with cytoskeleton, such as ARHGEF2, a microtubule-associated exchange factor for Rho and Rac GTPases, and phosphatase 2A which has been involved in the regulation of tau (Sontag *et al.,* 1999). In addition MARK4 co-precipitates in complex with α, β and γ tubulin, non-muscle myosin and actin (Trinczek *et al.,* 2004).

As previously reported MARK4 interacts and is regulated by LKB1 and aPKC kinases and the deubiquitinating enzyme USP9X (Brajenovic *et al.* 2004).

3.3 Regulation of MARK4

Given that the protein structure is conserved between MARK proteins, regulation of MARK4 is analogous to that of the other MARK members, and involves phosphorylation, ubiquitination and proteins interaction.

MARK4 is activated by LKB1 and MARKK-induced phosphorylation of the conserved threonine residue (T214) in the activation loop (Brajenovic *et al.,* 2004), whereas is inhibited by GSK3β phosphorylation of the S218 residue.

Moreover it has been demonstrated that MARK4 is polyubiquitinated *in vivo*, and interacts and is deubiquitinated by the USP9X enzyme (Al-Hakim *et al.,* 2008). The interaction between MARK4 and USP9X was previously shown by tandem affinity purification and immunoprecipitation (Brajenovic *et al.,* 2004). In the latter study, the role of the interplay between these two enzymes has been elucidated. In particular the USP9X silencing increases the polyubiquitination of MARK4, while the overexpression of USP9X prevents ubiquitination. The addition of ubiquitin chains does not control MARK4 stability, but rather may block the phosphorylation and activation by LKB1. MARK4 mutants that do not bind USP9X appear hyper-ubiquitinated and not phosphorylated at T214 in the T-loop. The proposed model suggests that when MARK4 is ubiquitinated, the ubiquitin chains might compete with the kinase domain for the UBA domain binding (that is necessary for the activation). Disruption of the interaction between the UBA and the Kinase domain destabilises the catalytic domain and thus blocks the phosphorylation by LKB1 (Figure 6) (Al-Hakim *et al.,* 2008).

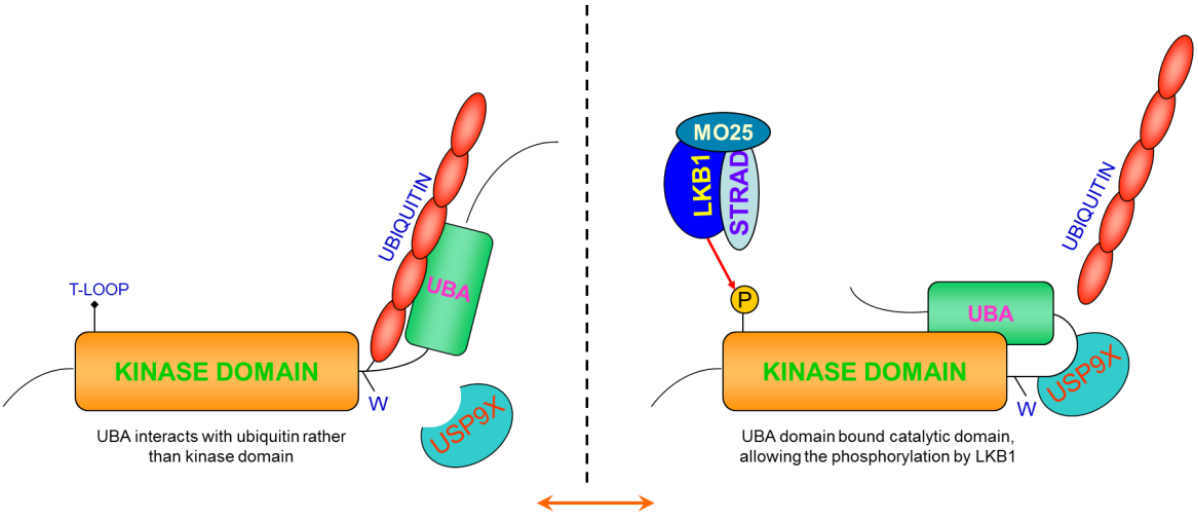


Figure 6. Model of the regulation of MARK4 phosphorylation by USP9X and ubiquitin (modified from Al-Hakim *et al.*, 2008).

Otherwise polyubiquitin chains may cover sterically the T214 present in the T-loop or could cause conformational changes favouring the dephosphorylation by protein phosphatases (Al-Hakim *et al.*, 2008).

It has also been demonstrated that MARK4 interacts with the aPKC, and as reported for MARK2 and MARK3, might be phosphorylated and inhibited by this kinase (Brajenovic *et al.*, 2004).

3.4 MARK4 subcellular localisation

It has been reported that exogenous GFP-conjugated MARK4 localises in normal and aberrant interphase centrosomes in CHO (Chinese Hamster Ovary) and neuroblastoma cell lines, a finding which is in contrast to the cytoplasmic localisation of the other members of the MARK family. It was also hypothesised that inactive MARK4 is located close to the nucleus, probably associated with the endoplasmic reticulum, and following activation it co-localises in centrosome to exert its functions (Trinczek *et al.*, 2004). These results have been confirmed and expanded by our data obtained by immunofluorescence experiments with MARK4L specific antibody in glioma cell lines (Magnani *et al.*, 2009). In particular it has been shown that endogenous MARK4L localises at normal and aberrant interphase centrosomes and maintains this association also in all the mitotic phases, co-localising with γ-tubulin throughout the entire cell cycle. The centrosome association was not abolished by nocodazole treatment, that induces microtubule depolymerisation, suggesting that MARK4L is a core component of centrosomes. Furthermore two novel MARK4L localisation sites have been identified, at the midbody during cytokinesis and in the nucleolus. All these results

were validated by immunoblotting experiments performed on centrosome, midbody and nucleolus fractions (Magnani *et al.*, 2009).

Recently we have demonstrated, by immunofluorescence experiments, that also the MARK4S isoform, localises at centrosomes and midbody, in glioma cell lines (Magnani *et al.*, 2011). However, unlike MARK4L, the short isoform is not detectable in the nucleolus. Moreover in the same study we highlighted that the two isoforms are not present in the nucleolus of normal cell lines, including human normal neural progenitors, fibroblasts and myoblasts, but both are associated with the centrosomes and midbody, as observed in glioma cell lines. We also found that MARK4L is detectable in the nucleolus of glioblastoma-derived cancer stem cells, but not in normal neural stem cells. Therefore the nucleolar localisation appears to be a specific feature of MARK4L in transformed cells (Magnani *et al.*, 2011).

3.5 MARK4 in the central nervous system: potential role in gliomagenesis

MARK4 is ubiquitously expressed, but elevated levels have been found in the brain, suggesting that MARK4 kinase may play important functions in this organ (Trinczek *et al.*, 2004; Moroni *et al.*, 2006). The two isoforms are differentially expressed in the cells of the central nervous system (CNS), and their expression is regulated during neuron differentiation processes. In particular MARK4S is the prevalent isoform in human and mouse brain (Moroni *et al*., 2006), is not detectable in undifferentiated neural progenitors and neuroblastoma NT2 (NTera2) cells, but is up-regulated during neuronal differentiation of both cell systems (Moroni *et al.*, 2006). The short isoform was thus proposed to be a neuron-specific marker in the CNS. Conversely, MARK4L is present in undifferentiated progenitors and NT2 cells, and its expression is maintained at the same levels in differentiated neurons (Moroni *et al.*, 2006). Indeed both MARK4 isoforms were found expressed in neurons by immunohistochemistry experiments, indicating that both proteins may play a role in neurons (Moroni *et al.* 2006).

MARK4 gene maps at 19q13.2, a region frequently altered in glioma, the most common tumour of the CNS. By characterising the chromosomal rearrangements involving the 19q13 band in glioma, it has been found that *MARK4* gene is duplicated in the Mi-4 glioblastoma (IV grade glioma) cell line (Beghini *et al.*, 2003). Array-CGH analysis of 25 primary glioma cell lines confirmed the duplication in the Mi-4 cell line and revealed that *MARK4* is included in a "gain" region in a few of the tested cell lines. In addition this experiment showed that *MARK4* is never deleted in the analysed samples (Roversi *et al.*, 2006), a finding intriguing as *MARK4* maps close and marks the centromeric boundary of the LOH area observed in glioma (Hartmann *et al.*, 2002).

In order to deepen the role of MARK4 in glioma, its expression has been extensively investigated using different approaches. By means of semi-quantitative PCR, MARK4L was found up-regulated in all the glioma samples analysed (8 tissue samples and 26 cell lines), with a direct correlation between MARK4L expression levels and malignancy grade. In addition MARK4L expression was found to be restricted to undifferentiated neural progenitor cells, while its expression was down-regulated during glial differentiation. On the contrary MARK4S was found highly expressed in whole normal brain (showing low levels of MARK4L) and hardly detectable in glioma tissues and undifferentiated human neural progenitors. These results led to hypothesise that MARK4L might be a mitogen protein, important for proliferation and consequently highly enriched in proliferating or undifferentiated cells (Beghini *et al.*, 2003).

Recently we have demonstrated by parallel real-time PCR and immunoblotting experiments that both MARK4 isoforms are concurrently expressed in a set of 21 glioma cell lines and 36 glioma tissues of different malignancy grade. MARK4L is the predominant isoform, while MARK4S levels appear significantly decreased in comparison and have an inverse correlation with malignancy grade. MARK4S reduction attests a switch towards the expression of MARK4L and an associated loss of cell differentiation. Moreover also in glioblastoma derived cancer stem cells (GBM-CSC) and normal neural stem cells (NSC), MARK4L is the predominant isoform, whereas MARK4S is hardly detectable. Accordingly, immunohistochemistry experiments on both human and rodent tissues from adult and embryonic brain, showed that only MARK4L is expressed in the embryonic ventricular zone and adult sub-ventricular zone which are known sites of neural stem cells (Magnani *et al.*, 2011). The subverted MARK4L/MARK4S ratio observed in GBM and GBM-CSC recapitulates the MARK4 expression profiling of NSC, with prevalence of MARK4L. The overall body of data suggested that the expression of the two MARK4 isoforms is tightly regulated during the proliferation/differentiation of neural stem cells and that changes in their expression levels may be a molecular marker of tumour transformation (Magnani *et al.*, 2011).

3.6 Potential role of MARK4 in Alzheimer's disease

Alzheimer's Disease (AD), a multifactorial common disease with a small mendelian counterpart is one of the most investigated neurodegenerative disorders.

One of the pathological hallmarks of AD is the hyperphosphorylation of *tau*, a microtubule associated protein, mainly expressed in the central nervous system. It has been demonstrated that hyperphosphorylated tau accumulates in the somato-dendritic compartments of neurons aggregating abnormally in paired helical filaments that form insoluble neurofibrillary tangles. As mentioned above, tau can be phosphorylated at Ser262 by MARK proteins, causing a reduction of the

microtubule-binding affinity and the consequent detachment from MTs. The unbounded tau can be subsequently phosphorylated by other kinases, including CDK5 and GSK3, leading to its altered localisation and cleavage (Chin *et al.,* 2000; Gamblin *et al.,* 2003; Drewes *et al.,* 2004). In addition AD is characterised by other hallmarks including amyloid plaques, synapses defects and loss of neurons. Other studies have revalued MT alterations in AD, suggesting that phosphorylation of tau is not the initial event of the disease (Chatterjee *et al.,* 2009) but a consequence of β-amyloid aggregation. It has been suggested that the abnormal β-amyloid might prime transport defects and improper distribution of tau into the somatodendritic compartments (Zempel *et al.,* 2010) or alternatively that tau might be the major mediator of Aβ toxicity (Lewis *et al.,* 2001; Gotz *et al.,* 2001; Rapport *et al.,* 2002; Oddo *et al.,* 2003). Many studies confirmed the involvement of MARK proteins in the development of Alzheimer's disease and the central role of MARK4 in this pathology has been highlighted by three recent works.

A genome-wide association study suggested a probable link between MARK4 and late onset Alzheimer's disease (Seshadri *et al.,* 2010). In addition it has recently been demonstrated that MARK4 has a more pronounced role, than other MARK proteins, in the pathological phosphorylation of tau in Alzheimer's disease. It has been found that MARK4 expression and MARK4-tau interaction are more elevated in AD cases compared to control samples (Gu *et al.,* 2013). A merging evidence derives from another study which showed that MARK4 is a mediator of β-amyloid toxicity on synapses and dendritic spines. This study demonstrated that MARK4 overexpression in rat hippocampal neurons led to tau hyperphosphorylation, reduced expression of synaptic markers, and loss of dendritic spines and synapses. The same alterations were obtained by treatment of neurons with β-amyloid oligomers. Interestingly the Aβ toxic effects could be abrogated by the inhibition of the endogenous MARK proteins, suggesting that these kinases might be the link between β-amyloid and tau pathology (Yu *et al.,* 2012).

3.7 Functions of MARK4

MARK4 is the less characterised and studied member among MARK proteins, and more is known about its features that are universal to all members of the MARK family than features that are unique to MARK4, most of which remain to be elucidated. In particular many functions were ascribed to MARK4 based on its specific subcellular localisation and on the differential expression of its two isoforms in human tissues. Only recently, several works have demonstrated that this kinase is involved in the regulation of different processes, including spermatogenesis (Tang *et al.,* 2012), cell growth (Li & Guan 2012), ciliogenesis (Kuhns *et al.,* 2013) and cell cycle (Rovina *et al.,* submitted).

MARK4 and spermatogenesis. It has been reported that MARK4 is highly expressed in testis (Beghini *et al.*, 2003; Trinczek *et al.*, 2004), however its role in this tissue is still unknown. Insights on MARK4 localisation and possible functions in spermatogenesis came out from a recent study (Tang *et al.*, 2012). Employing a rat testis model, it has been shown that MARK4 is expressed in both Sertoli and germ cells and is localised at the apical and basal ectoplasmic specialisation (ES) a testis-specific adherens junction, involved in the maintenance of cell polarity and adhesion (Tang *et al.*, 2012). It has also been found that MARK4 expression is stage-specific during the epithelial cycle and strongly associated with the integrity of the apical ES. The authors hypothesised that MARK4 may regulate microtubules during spermatogenesis and given its localisation in ES close to actin filament bundles, may mediate the cross talk between microfilaments and microtubules (Tang *et al.*, 2012).

mTORC1 regulation. The mammalian target of rapamicin (mTOR) is a conserved kinase that is involved in the regulation of several cellular processes. This protein forms two distinct complexes, namely mTORC1 and mTORC2, the former implicated in cell growth, by inhibiting autophagy and stimulating protein synthesis (Wullschleger *et al.*, 2006). Deregulation of mTORC1 complex contributes to many human diseases including cardiovascular, metabolic and autoimmunity disorders and cancer. Many extra and intra cellular signals, among which nutrients, energy levels, growth factors and stress conditions have been identified to regulate mTORC1. A recent study provided evidence on the role of MARK4 as negative regulator of mTORC1 based on the findings that MARK4 knockdown increased mTORC1 activity whereas MARK4 overexpression significantly blocked its activity (Li & Guan 2012). Interestingly, MARK4 was found to inhibit the activation of mTORC1 acting on Rag GTPases, which are implicated in amino acid signalling, but not to block the effect of Rheb, that directly interacts to activate mTORC1. It has been found that MARK4 also phosphorylates Raptor, a key component of the mTORC1 complex, and this phosphorylation interferes with the interaction between Raptor and Rag, which is important for the activation of mTORC1 (Li & Guan 2012).

Ciliogenesis. The primary cilium is a microtubule-based structure that is involved in the coordination of important signalling pathways in both embryonic and adult tissues. The cilium is constituted by a microtubule core structure, the axoneme, surrounded by the ciliary membrane. The microtubules that form the axoneme are nucleated by the basal body, a structure derived from the mother centriole of the centrosome. The assembly of primary cilium starts at the G0/G1 phase of the cell cycle and then follows an ordered sequence of steps, that are regulated by different proteins. Involvement of MARK4 in ciliogenesis has been highlighted by a recent study demonstrating that

MARK4, which localises to the basal body of the primary cilium, is a positive regulator of the early steps of the process (Khuns *et al.*, 2013). Indeed MARK4 knockdown leads to loss of primary cilia formation, impairing the initiation of axoneme elongation. It is also shown that MARK4 is involved in the regulation of the centrosomal localisation of ODF2 (outer dense fiber protein 2), a protein involved in ciliogenesis, as MARK4 depletion reduces centrosomal levels of ODF2, while overexpression prompted ODF2 accumulation in this compartment. Based on MARK4 interaction with ODF2 *in vivo* and MARK4 ability to phosphorylate ODF2 *in vitro* the authors conclude that MARK4 contributes to ciliogenesis acting on ODF2 and triggering its localisation in centrosomes (Khuns *et al.*, 2013).

The regulation of centrosome and cell cycle. It has been suggested that MARK4 could play a role in cell cycle progression, since localises at centrosome and midbody (Trinczek *et al.*, 2004; Magnani *et al.*, 2009; Magnani *et al.*, 2011).

By knockdown experiments on fibroblasts and glioma cell lines, we have recently demonstrated that MARK4 is implicated in the maintenance of cell morphology shaped by the cytoskeleton and is involved in the regulation of cell cycle, driven by ordered centrosome dynamics (Rovina *et al.*, submitted).

We have shown that silencing of MARK4S affects the morphology of both fibroblasts and glioma cells (G32). In particular fibroblasts lose their typical spindle-shaped morphology, appearing shorter and polygonal, while G32 cells lose their cell-cell interactions and appear rounded or abnormally stretched. Furthermore, proliferation of fibroblasts and G32 cells is severely reduced following MARK4S depletion, as indicated by the growth curves of interfered cells. By cytofluorimetric analysis we have demonstrated that MARK4S silenced cells are blocked in G1 phase, as the percentages of cells in S and G2/M phases are reduced and the percentage of cells in G1 phase is increased. In agreement with these observations we noticed that silenced cells display duplicated centrosomes, apical to the nucleus, a feature typical of the G/S phase transition, indicating that the centrosome cycle is altered and the cells are arrested in G1 phase. We thus hypothesised that MARK4 might be a crucial regulator of the G1/S checkpoint, and that critical levels of MARK4 are crucial for the proper functions of centrosome during cell cycle (Rovina *et al.*, submitted).

4. Cytoskeleton, centrosomes, midbody and cell division

4.1 Cytoskeleton

The cytoplasm of eukaryotic cell is spatially organised by a network of protein filaments, which constitute the cytoskeleton. Cytoskeleton arrays build up complex structures that change during the cell cycle and between different cell types. This protein network providing the cell with shape, support and movement consists of three main structural components: microtubules, intermediate filaments and microfilaments (Table 2).

Properties	***Microtubules***	***Intermediate filaments***	***Microfilaments***
Prototype proteins	Tubulins	Many, tissue-specific	Actin
Diameter (nm)	25	10-12	5-8
Structure	Conserved	Diverse (with conserved subdomains)	Conserved
Expression	Eukaryotes	Absent from yeast	Eukaryotes
Solubility	High	Low	High
Polarity	Yes	No	Yes
Cellular location	Cytoplasm	Nucleus (laminin), cytoplasm (other)	Cytoplasm
Binding proteins	Many	Few	Many
Phosphorylation	Limited	Extensive	Limited

Table 2. Characteristics of the three major cytoskeletal structures (Modified from Omary *et al*., 2006).

4.1.1 Microtubules

Microtubules are crucial components of the cytoskeleton that play a main role in several cellular functions like cell migration and polarisation, intracellular transport, organisation of the intracellular space, chromosome segregation and cell division.

Microtubules are complex hollow tubular structures, that are formed by the reversible association of heterodimers of α and β tubulin, arranged in a pseudo-helix of thirteen laterally associated linear protofilaments with a longitudinal seam (Lawson & Carazo Salas 2013). In most animal cells, MTs are nucleated at the centrosome, that acts as a MTOC (MT organizing Centre; see centrosome paragraph). Many different proteins are involved in their nucleation and organisation, among them the γ-tubulin associated with other proteins, forms a ring complex (γTuRC), onto which α and β tubulin dimers are added to build a microtubule. Hence MT appear to be polarised with a minus-end capped and anchored at the MTOC and a plus-end usually localised at the cell periphery (Forges *et al.,* 2012). Microtubules stochastically alternate between phases of growth (polymerisation), pause and shrinkage (depolymerisation) separated by rescue (transition from shrinkage to growth phase) or catastrophe (transition between growth phase and shrinkage) events (Figure 7). This dynamic

behaviour, called "dynamic instability", permits sampling of the three-dimensional space, consenting microtubules to efficiently capture cellular targets, like mitotic kinetochores (Mitchison & Kirschner 1984; Hayles & Nurse 2001). Dynamic instability is regulated by binding, hydrolysis and exchange of GTP by β-tubulin. During MTs polymerisation heterodimers of GTP-bound tubulin are added at the plus-end. A slight delay between polymerisation and GTP hydrolysis by β-tubulin forms a GTP-tubulin cap. Loss of this cap induces a quick depolymerisation (Drechsel & Kirschner 1994).

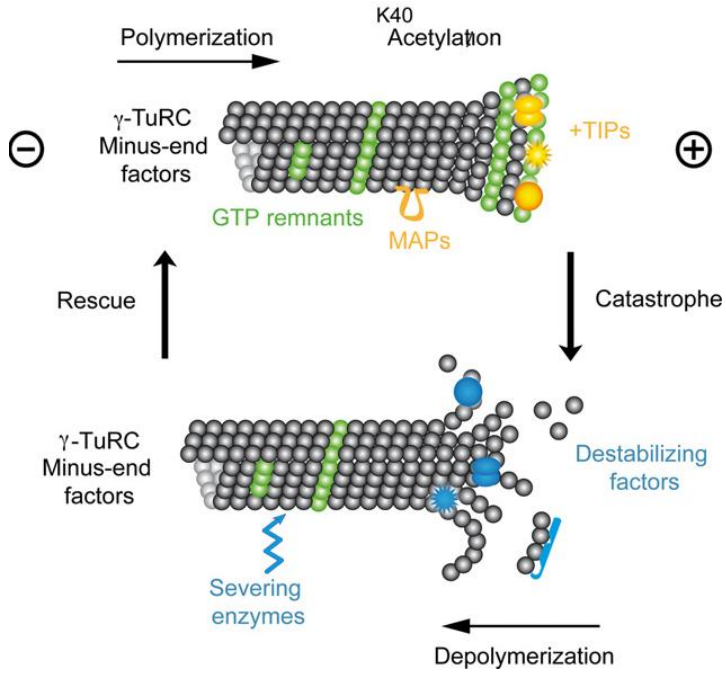


Figure 7. Schematic view of microtubule dynamics and regulation. Microtubules stochastically alternate between phases of growth, pause and shrinkage (Modified from Forges *et al.* 2012).

This dynamic behaviour is fundamental for the important functions played by MTs and is highly regulated by multiple factors. In particular microtubule dynamics are controlled by a balance between the activity of different microtubule-stabilising and destabilising factors, that work by altering microtubule integrity, especially at the plus-end, thereby regulating indirectly microtubule length.

Among stabilising proteins two classes have been identified: the classical microtubule associated proteins (MAPs), that permeate the microtubules lattice and the plus-end tracking proteins (+TIPs), that act through specific interactions with the plus end (Amos & Schlieper 2005). The MAPs are proteins that bind to and stabilise microtubules. Different parts of these proteins interact with the luminal and external tubule face, therefore they probably co-assemble during polymerisation and act from within. The MAP family includes tau and MAP2 that are strongly expressed in neurons and MAP4 which is present in all non-neuronal cells. All these proteins have a conserved protein

structure with an N-terminal projection domain, which extends from the microtubule surface and may affect MT spacing and cytoplasmic transport, and a C-terminal microtubule binding domain that contains a proline-rich sequence and the KGXS motif which is phosphorylated by MARK proteins (Drewes *et al.*, 1998).

+TIPs normally accumulate at polymerising plus-end and undergo repeated cycles of association and dissociation, and dynamically track the growing plus-end. In addition to the regulation of MT dynamics, they are involved in the interactions of microtubules with chromosomes during mitosis and with the cellular cortex.

Many different proteins have been identified to have destabilising effect on MTs, either by cutting microtubules (for example katanin and spastin) or by inducing depolymerisation (Lawson & Carazo Salas 2013).

As post-translational modifications of tubulin are also linked to the regulation of MT dynamics, these modifications seem to establish a readable code for MAPs and motors (Janke & Kneussel 2010). Microtubule modifications such as glutamylation, detyrosination, acetylation and glycylation are almost all carried out on polymerised tubulin, and they are mostly linked to MT stability.

All these regulation systems are necessary for the microtubule functions in the cells. Indeed microtubules play a crucial role in different cell processes including cell architecture and motility, they give shape to cells, organise the intracellular space, serve as intracellular transport tracks, and are key component in important cellular structures like axonemes and mitotic spindle.

4.1.2 Intermediate filaments

Intermediate filaments are the least studied and understood of the three cytoskeletal systems that are present in all vertebrate cells. They are constituted by subunits that form 10-12 nm filaments, of intermediate size compared with the two other cytoskeletal components (microfilaments 5-8 nm, microtubules 25 nm). Despite the structure of these filaments is maintained, the protein compositions is not conserved, as they are encoded by ~75 different genes (Goldman *et al.*, 2012). In addition the transcription of these genes is regulated during development, and they are expressed in cell-, tissue- and differentiation-specific fashions. Based on domain and sequence homology, intermediate filament proteins have been classified into five groups (Table 3).

Intermediate filament (name)	Type	Size (kDa)	Cell or tissue distribution
Cytoplasmic			
Keratins (K9-K20)	I	40-64	Epithelia (hair Ha 1-8)
Keratins (K1-K8)	II	52-68	Epithelia (hair Hb 1-6)
Vimentin	III	55	Mesenchymal
Desmin	III	53	All muscle
GFAP	III	52	Astrocytes
Peripherin	III	54	Peripheral neurons
Syncoilin	III	54	Muscle (mainly skeletal/cardiac)
Neurofilament-L	IV	61	Central and peripheral neurons
Neurofilament-M	IV	90	Central and peripheral neurons
Neurofilament-H	IV	110	Central and peripheral neurons
α-Internexin	IV	61	Central neurons
Nestin	IV	240	Neuroepithelia
Synemin	IV	180 (α), 150 (β)	All muscle
Nuclear			
Lamins A/C	V	62-78	Nuclear lamina
Lamins B1, B2	V	62-78	Nuclear lamina
Other			
Phakinin (CP49)	Orphan	46	Lens fibre cells
Filensin	Orphan	83	Lens fibre cells

Table 3. Features of intermediate filaments proteins.

All intermediate filament proteins show a similar structure as they consist of a central coil-coil α-helical road domain, that is flanked by a non-α-helical N-terminal head and a C-terminal domain of different lengths (Figure 8). The road domain is subdivided into coil 1 (subdomains 1A and 1B) and coil 2 (subdomains 2A and 2B), which are interrupted by a non-α-helical linker. Intermediate filaments assemble from a coiled-coiled dimer, and the responsible region is the highly conserved, α-helix central road domain. These dimers associate in a hierarchical fashion to produce intermediate filaments. Some intermediate filament proteins form homopolymeric filaments (for example vimentin and desmin), whereas others are obligate non-covalent heteropolymers (e.g. keratins and neurofilaments) (Toivola *et al.*, 2005).

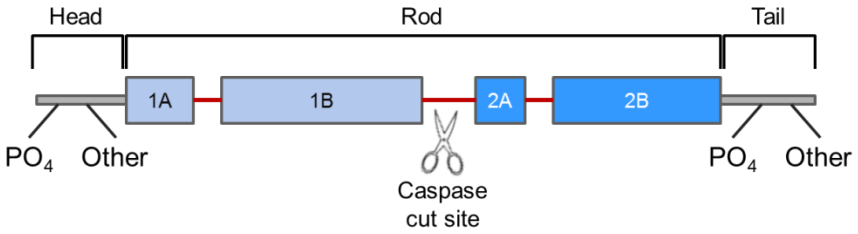


Figure 8. Schematic illustration of intermediate filaments protein domain. All members of the intermediate filament family present a similar structure with a central coil-coil α-helical road domain, flanked by a non-α-helical N-terminal head and a C-terminal domain.

The regulation of intermediate filaments mainly involves post-translational modifications and binding proteins. The principal post-translational modifications include phosphorylation, glycosylation (within head/tail domain) and transglutamination. Phosphorylation, the best studied of the post-translational modifications, takes a central role for their regulation; by contrast the regulation of microtubules and microfilaments occurs primary via their associated proteins. The phosphorylation typically involves multiple Ser/Thr residues, preferentially within the head and tail domains. Phosphorylation and dephosphorylation are crucial for the regulation of intermediate filament dynamics primarily by modifying their intrinsic properties including solubility, conformation and filament organisation, and, additionally, by regulating other post-translational modifications (Omary *et al.* 2006).

In addition to post-translational modifications, caspase-mediated proteolysis is another mechanism involved in the regulation of intermediate filaments. In particular, caspase-cleavage primarily takes place at a conserved motif within the linker region.

Finally many different intermediate filament-associated proteins have been identified, including kinases, phosphatases, cytoskeletal linker proteins, adaptor proteins and junctional proteins. These proteins can regulate or be regulated by intermediate filaments.

Intermediate filaments exhibit many different mechanical and non-mechanical functions. The principal role of intermediate filaments is structural, as they are involved in the maintenance of the shape and in the protection of cells from mechanical and non-mechanical stresses (for example oxidative injury). More recent studies have demonstrated that intermediate filaments are also expressed in the leading edge of cells and are essential for cell polarity and migration. In addition to these well-established functions, intermediate filaments play a role in the regulation of the protein targeting and organelle functions, contributing to subcellular organisation, organelle shape and mechanical stability and have been also associated to the modulation of protein (Toivola *et al.* 2005)

Among the big protein family of intermediate filaments, vimentin is one of the most familiar members, as it is the main intermediate filament protein in mesenchymal cells and is a developmental marker of cells and tissues.

Vimentin interacts, directly or indirectly, with many different cell structures including plasma membrane, lipid components, Golgi, nucleus, lysosomes, microtubules and microfilaments, supporting its involvement in multiple cellular processes. For example vimentin plays an important role in cell adhesion, by regulating integrin functions. On the other hand it has a key role in the lymphocyte attachment to vascular endothelium and in the transcellular migration through endothelial cells (Nieminen *et al.* 2006).

Several studies have demonstrated the link between vimentin and signal transduction and, due to the high number of vimentin interactors, it has been proposed that vimentin, acting as a binding platform and scaffold for signalling elements working in the same regulatory pathway, could modulate the transduction of the signal (Pallari & Eriksson 2006).

4.1.3 Microfilaments

Microfilaments are the smallest component of the cytoskeleton, and are constituted by actin filaments and associated proteins. Actin is one of the most abundant and conserved proteins present in eukaryotic cells and can be subdivided in three broad classes: α-, β- and γ-actins.

Actin is mainly present in the cytoplasm, and can be found in both monomeric (G-actin, globular-actin) and polymerised (F-actin, filamentous-actin) form. The G-actin molecule is constituted by a single polypeptide chain of 375 amino acid residues (~ 42kDa), that folds into two equally sized domains divided by a deep cleft that harbours a binding pocket for small molecules like nucleotides (ATP) (Michelot & Drubin 2011).

Actin undergoes cycles of polymerisation and disassembly between the globular and the filamentous form, that is the prevalent form in the eukaryotic cytoplasm. The polymerisation process can be essentially divided into three main phases: 1) a slow initial association to a dimer that is more prone to quickly dissociate into monomers than to assemble; 2) the formation of a stable trimer that is the nucleus of polymerisation; and 3) the elongation phase in which actin monomers are rapidly assembled (Dos Remedios *et al.* 2003). As the initial polymerisation is kinetically unfavourable, cells need additional factors, namely actin filament nucleators (for example Arp2/3 complex), that stabilise dimer formation. Actin filaments assemble as a right-handed, double-stranded helices, and appear very dynamic (Schoenenberger *et al.* 2011). Two components are essential for the dynamic behaviour of these filaments: the intrinsic asymmetry of the G-actin subunit and the interaction with actin binding proteins. The structural polarity of the subunits and the rapid polymerisation of ATP-charged monomers combined with the slow ATPase activity of actin, lead to filaments with a fast growing end (called barbed end) and a slow growing end (namely pointed end). Consumption of ATP-actin at the barbed end results in a critical level of subunits where extra ATP-actin addition is balanced by the loss of ADP-actin at the pointed end. This sequence of events leads to filaments of constant length but with a dynamic nature, as the subunits travel from the fast growing to the slow growing end, a process called “treadmilling” (Wegner 1976). In cells treadmilling is strongly controlled by different protein ligands, that can interact with either end of the filament, and can facilitate or block the elongation and stability of actin filaments (Schoenenberger *et al.* 2011).

The intrinsic asymmetry of actin and its interaction with different ligand proteins explain the dynamic of actin filaments, that are involved in many different vital processes. Actin filaments, in cells, are not found as disorganised meshwork, but as organised assemblies localised in specific regions of the cytoplasm where they can execute their functions. In particular they are involved in the regulation of fundamental processes including cell shape (filamentous actin forms a cortical web that underlines plasma membrane), cell motility and migration (lamellipodia and filopodia are built of actin filaments), vesicular movement, phagocytosis, cytokinesis and molecular transport between the plasma membrane and the nucleus.

4.2 Centrosomes

The centrosome is a small non-membranous organelle, normally positioned centrally in the cell, close to the nucleus (Fukasawa 2002). It is considered the primary microtubule-organising centre (MTOC) in animal cells, as it can regulate nucleation and spatial organisation of microtubules (Bettencourt-Dias & Glover 2007).

Centrosomes consist of two distinct domains (Figure 9):

- the centriolar domain that includes the two centrioles, namely mother and daughter, which are cylindrical organelles consisting of nine microtubule triplet structures. The mother centriole presents subdistal and distal appendages, that dock cytoplasmic microtubules;
- the pericentriolar domain, an electron-dense matrix called pericentriolar material (PMC), consisting of many fibres and proteins that surround the centrioles. The PMC is a crucial structure that anchors and nucleates cytoplasmic microtubules during interphase and mitosis, by associating α and β tubulin dimers from a γ-tubulin ring.

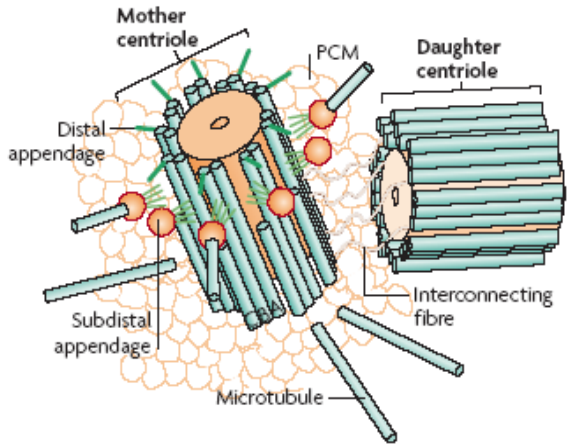


Figure 9. Structure of the centrosome. Centrosomes are constituted of two centrioles enclosed by an electron-dense matrix, the pericentriolar material (Modified from Bettencourt-Dias & Glover 2007).

The centrosomes have many different functions during interphases and mitosis. As above mentioned, centrosome controls the nucleation and organisation of the microtubule network, and so it is involved in the regulation of several processes including cell adhesion, polarity and motility in interphase, and spindle pole organisation and chromosome segregation during mitosis. The ability of the centrosome to organise the microtubule network depends on its capacity to nucleate, anchor and release microtubules. The PCM is a key structure, containing many different regulatory components, such as γ-tubulin. The changes in the microtubule-nucleating capacity during the cell cycle, seem to be directed by a balance of factors that limit or promote the recruitment of MT-organising molecules.

It has been proposed that centrosomes can also act as signalling platforms because they host different regulatory complexes, including checkpoint and tumour suppressor proteins (Doxsey *et al.,* 2005a). In addition centrosomes might play a role in cell cycle regulation, and in particular they may regulate the G1/S transition and the cytokinesis (Doxsey *et al.,* 2005b).

The number of centrosomes is tightly regulated and, similarly to the genome, is intimately coupled to the cell cycle. Centrosome duplicates only once in the cell cycle, during G1/S transition and in S phase, to ensure the formation of a bipolar spindle in mitosis.

Centrosomes that are aberrant in size, shape, composition (also with improper phosphorylation or expression of centrosomal proteins) or number are frequently observed in many tumours (Yamamoto *et al.*, 2004; Kastsetos *et al.*, 2006). A surplus of centrosomes can lead to the formation of aberrant multipolar mitotic spindles, which can be responsible for chromosomes mis-segregation and, consequently, for the chromosomal instability (CIN) often found in tumours (Ganem *et al.*, 2009).

4.3 Cytokinesis and Midbody

Cytokinesis occurs at the very end of mitosis and is the process that leads to physical division of a single cell (mother cell) into two daughter cells. This event implicates the accurate coordination of independent pathways involved in the regulation of cell cycle, microtubules, actin, membrane and organelle dynamics.

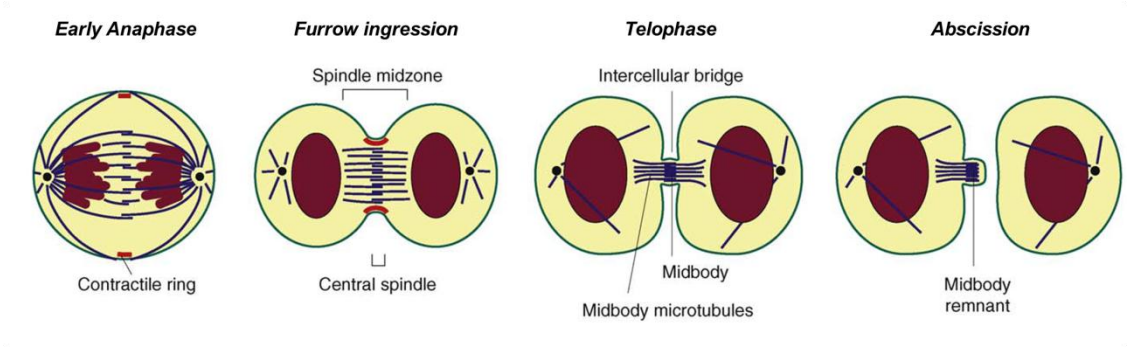


Figure 10. Schematic representation of the progression through cytokinesis (Modified from Steigemann & Gerlich 2009)

Cell division follows a well-ordered progression of steps, in which mitosis is the first (Figure 10). During mitosis the mother cell forms the mitotic spindle, consisting of two microtubule asters located at the cell poles, that allows the equal segregation of chromosomes into the daughter cells.
During anaphase, after sister chromosomes have been separated, the residual non-kinetochore overlapping microtubules, form a structure called spindle midzone. Together with the spindle asters, the central spindle, present in the midzone establishes the position of the actomyosin ring, that initiates to assemble and contract. At this stage, the post-mitotic sister cells remain connected by an intracellular bridge, and the remnant of the spindle midzone persists as a structure called midbody (Mullins & Biesele 1977; Mullins & McIntosh 1982). This structure is constituted by overlapping antiparallel bundles of microtubules surrounded by an electron-dense matrix of proteins. The microtubules present in the midbody are acetylated (a post-translational modification associated with stable microtubules) and resistant to different perturbations, including depolymerisation drugs. However, it has been demonstrated a permanent growth of microtubules both inwards and outwards from the midbody, indicating that midbody MTs are less stationary than initially thought (Steigemann & Gerlich 2009).
The midbody provides an anchor for the cleavage furrow, which will divide the mother cell inside the midzone, in a point which is equidistant from the two asters. In this furrow, the contractile acto-myosin ring grows up and shrinks, producing the "stricture" of the cell, until the two opposing membrane surfaces come in contact and merge, closing and delimiting the two daughter cells. Coinciding with abscission, the midbody MT-bundles adjacent to one side of the midbody brusquely disassemble. Notwithstanding midbody MT-bundles lateral to the midbody disassemble during abscission, they remain stable at their overlap region, which can persist as remnant throughout multiple cell cycles in some cell types (Gromley *et al.*, 2005), while in others it is degraded by autophagy (Pohl & Jentsch 2009).

It has been suggested that the midbody might play an important role in the cytokinesis and in particular it might be involved in preserving a bipolar spindle and in correctly separating the cytoplasm between the daughter cells.

Materials and Methods

1. Cell Cultures

1.1 Primary Glioma Cell Lines

The oligoastrocytoma (G157) and glioblastoma (G32) cell lines used in the present study were selected from a panel of 21 human primary glioma cell lines, that were obtained from post-surgery specimens and characterised as described elsewhere (Magnani *et al.,* 1994; Perego *et al.,* 1994; Beghini *et al.,* 2003; Roversi *et al.,* 2006). In Table 1 are reported the features of the two selected cell lines.

Cell line	***Sex/Age at surgery***	***Histological diagnosis***	***WHO grade***	***Karyotype***
G157	M / 31	Oligoastrocytoma	II	not evaluated
G32	M / 63	Glioblastoma	IV	64, XXY, +1, +7, +7, +8, +8, +10, +13, +14, +14, +18, +19, +21, +22, +3mar

Table 1. Clinic and karyotypic features of the gliomas from which the cell lines derive.

Both glioma cell lines were grown in RPMI supplemented with 5% foetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin at 37°C and 5% CO_2. Before reaching confluence, during exponential growth phase cells were detached using trypsin-EDTA (0.05%) and split. To verify the absence of mycoplasma contamination cells were analysed by immunofluorescence with DAPI.

1.2 Normal Human Fibroblasts

Normal human adult fibroblasts were grown at 37°C in a 5% CO_2 atmosphere in RPMI supplemented with 10% FBS and 100 U/ml Penicillin-Streptomycin. Fibroblasts were obtained from "Cell Line and DNA Biobank from Patients Affected by Genetic Diseases" (G. Gaslini Institute, Genoa).

1.3 Human Embryonic Kidney 293T cell line

Human Embryonic Kidney 293T (HEK293T) cells were grown in DMEM high glucose, supplemented with 10% FBS and 100 U/ml Penicillin-Streptomycin, at 37°C in a 5% CO_2 atmosphere. The absence of mycoplasma was confirmed by immunofluorescence with DAPI.

2. Flow Cytometry analysis

Flow cytometry is a technology that allows to measure and then analyse multiple physical properties of single particles, generally cells, as they flow in a fluid stream through a ray of light. The characteristics that can be analysed include particle's relative size, relative granularity or internal complexity and relative fluorescence intensity. These properties are determined by an optical-to-electronic coupling system that registers how the cell scatters incident light and emits fluorescence.

A flow cytometer is composed of three main systems (Figure 1):

- the **fluidics** system, that transports particles in a fluid stream to the laser beam for interrogation;
- the **optics** system, that consists of lasers to illuminate the particles and optical filters to direct the arising light signals to the detectors;
- the **electronics** system, that converts detected light signals into electronic signals, which can be processed by computer.

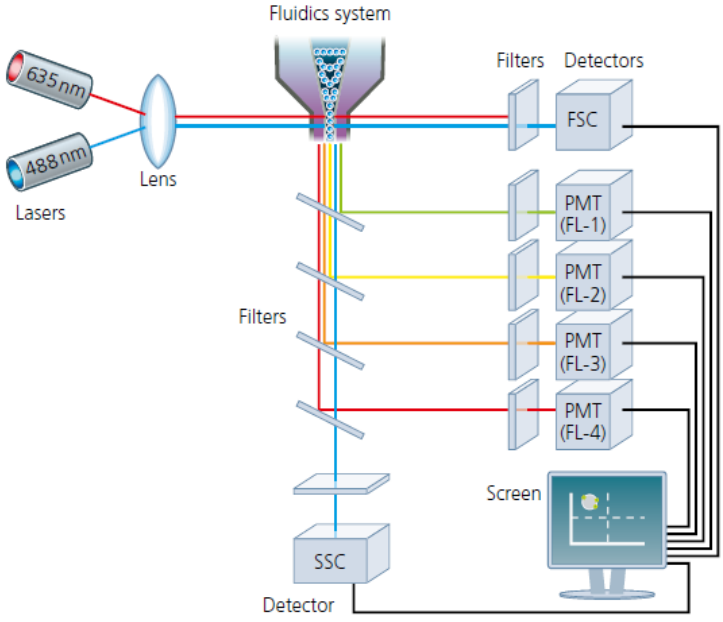


Figure 1. Schematic representation of a typical flow cytometer setup (Modified from Rahman M – Introduction to Flow Cytometry)

When a sample is injected into flow cytometer, the particles are causally distributed in three-dimensional space. To be properly interrogated by the detection system, the sample must be ordered, by the fluidics system, into a stream of single particles: a central channel, through which the sample is injected, is surrounded by an outer sheath of faster flowing fluid that accelerates the

particles and confines them to the centre of the sample core. This process, known as hydrodynamic focusing, creates a single file of particles which can be analysed by passing through one or more beams of light.

When particles pass through a laser beam they deflect the incident light causing its scattering. The extent to which this occurs depends on the physical properties of the particle. Forward-scattered light (FSC) is proportional to cell-surface area and is a measurement of the mostly diffracted light that is detected just off the axis of the incident light beam in the forward direction by a photodiode.

Side-scattered light (SSC) is a measurement of mostly refracted and reflected light and is collected at 90° to the laser beam. SSC is related to the internal complexity and cell granularity.

Both FSC and SSC are unique for each particle and a correlated measurement of the two can be used to distinguish different cell types in a heterogeneous sample.

Fluorescence measurements can provide qualitative and quantitative information about fluorochrome-labeled cell molecules. In particular different molecules including cell surface receptor or specific intracellular proteins, can be highlighted using fluorescent ligand or polyclonal/monoclonal antibodies labelled with fluorochromes. Other substances such as DNA and RNA, can be stained with fluorochromes that bind to them in a stoichiometric manner.

Flow cytometers use distinct fluorescence channels to detect light emitted, and the number of detectors will vary according to the instrument and its manufacturer. Once a cell flows through the laser light, the emitted SSC and fluorescence are diverted to photomultiplier tubes while a photodiode collects the FSC. All of the signals are focused to their specific detectors via a system of optical filters and mirrors. The filters block light by absorption and can be classified in three major types:

- **long pass filters**, that transmit wavelengths equal to or longer than a definite wavelength;
- **short pass filters**, that transmit light wavelengths equal to or shorter than a specified wavelength;
- **band pass filters**, that transmit light within a specified narrow range of wavelengths.

Dichroic mirrors are devices that direct light of different wavelengths in diverse directions. In particular they allow the passage of the specified wavelengths in the forward direction and deflect at 90° angle the blocked light.

When light signals hit a side of photodetector, they are converted in a proportional number of electrons that are amplified to create a greater electrical current that is then converted to a voltage pulse. The measurement from every detectors is referred to as a “parameter” (for example FSC, SSC or fluorescence), and the data acquired in each parameter are known as “events” and represent the number of cells showing the physical feature or marker of interest.

Flow cytometers are paired with a computer for data acquisition and analyses, with the possibility to measure several parameters simultaneously and a graphical output known as "cytogram". The operator can establish a threshold value and define an "electronic gate", to exclude irrelevant events (for example debris) or to select the cell population of interest.
The flow cytometry has many different application in cell biology including: absolute cell count, recognition of particular cell population, viability and apoptosis, differentiation and cell cycle distribution (Rahman M – Introduction to Flow Cytometry; Introduction to Flow Cytometry: A Learning Guide, BD Biosciences).

2.1 Evaluation of MARK4L and MARK4S expression during cell cycle

The evaluation of MARK4 isoforms expression during cell cycle phases was performed using flow cytometry, on cells stained with specific MARK4 antibodies and propidium iodide (PI) for DNA counterstaining.

Sample preparations

Cells were harvested using trypsin/EDTA 0.05%, counted and washed with cold PBS. The pellet was then accurately resuspended in 1 ml of Saline GM solution on ice using 21G needle and syringe. To fix cells preventing aggregation, 3 ml of cold 96% methanol were added dropwise while the suspension was vortexed.

Saline GM solution	
Glucose	1.1 g/l
NaCl	8.0 g/l
KCl	0.4 g/l
$Na_2HPO_4 \bullet 2H_2O$	0.2 g/l
KH_2PO_4	0.15 g/l
EDTA	0.2 g/l

MARK4L and MARK4S – DNA staining

To evaluate MARK4L and MARK4S expression during the cell cycle, fixed samples were washed three times with PBS and permeabilised with PBS containing 0.1% Triton X-100 for 7 min on ice. After three washes with PBS, cells were incubated with PBS containing 5% BSA and 0.1% Tween 20 for 15 min on ice, to block non-specific binding. After one wash with PBS cells were incubated

with anti-MARK4L (GenScript Corporation, Piscataway, NJ, USA) or anti-MARK4S (ab5262; Abcam, Cambridge, UK) antibodies diluted 1:100 in 400 µl PBS containing 0.1% Tween 20 overnight at 4°C. Cells were washed with PBS and then incubated with FITC-conjugated anti-rabbit (Sigma, Saint Louis, MI, USA) or Alexa488-conjugated anti-goat (Invitrogen, Paisley, UK) antibodies diluted 1:200 in PBS containing 0.1% Tween 20 for 1 hour at room temperature. Cells were washed with PBS, and then incubated with 1 ml of PBS containing propidium iodide (PI) 2.5 µg/ml and 25 µl RNAse A (1mg/ml) overnight at 4°C before the analysis with cytofluorimeter. The fluorescence intensity of cells stained with PI correlates with the DNA amount: given that DNA content duplicates during S phase, it is possible to distinguish G0/G1, S and G2/M.

Cytofluorimetric analysis was performed using FACS-Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and fluorescence pulses were detected using a laser beam at 488 nm and a band pass filter at 530 ± 30 nm for anti-MARK4L or MARK4S green fluorescence and 620 ± 35 nm for DNA content analysis. For each samples 10000 events were acquired.

3. RNAi

To knockdown MARK4S in fibroblast cells, we performed RNAi using Stealth RNAi™ siRNA duplexes (Invitrogen), transfected by lipid-mediated transfection with Lipofectamine™ RNAiMAX (Invitrogen). Stealth siRNA used in silencing experiments is customised siRNA, designed to specifically target exon 16, skipped in MARK4L, and thus able to determine specific MARK4S silencing. The sequence of MARK4S siRNA is reported in Table 2.

siRNA	***Sense***	***Antisense***	***Target Exon***
Stealth siRNA #10	CGAUCCCUCUAAACGGCAGAACUCU	AGAGUUCUGCCGUUUAGAGGGAUCG	16

Table 2. Sequence of MARK4S siRNA

siRNA negative control (siRNA#1, Ambion, Paisley, UK), not targeting any specific gene product (because designed to have no similarity to human transcript) was used to distinguish non-specific (NS) effects on siRNA treated cells.

Based on previous experiments, we performed knockdown with the following time table:

- Day 0: Reverse transfection;
- Day 2: Forward transfection;
- Day 3: analysis of siRNA effects.

In reverse protocol, transfection mix is prepared and then dispensed in each plate and only after, cells and medium are dispensed. In forward transfection the mix is generally prepared and added the day after the plating of cells.

RNAi duplex-Lipofectamine RNAiMAX complex were prepared as follow. RNAi duplexes were diluted in RPMI Medium without serum and antibiotics to attain the optimal final concentration. After mixing, Lipofectamine RNAiMAX was added to the mix, incubated for 20 minutes at room temperature and then dispensed in each plate. Cells, diluted in RPMI with 10% FBS without antibiotics were added to the plates and then incubated at 37°C 5% CO_2 for 48 hours, before the forward transfection.

In forward transfection the mix was prepared and incubated as above described for reverse protocol. The same plates used for reverse transfection were treated after 48 hours as follows. Culture medium was removed and correspondent amount of transfection mix (Table 3) was added to each

plate. RPMI with 10% FBS without antibiotics was added to each plate, then incubated at 37°C 5% CO_2 until ready to assay for gene knockdown.

Culture Vessel	***Transfection mix Volume***	***siRNA concentration***	***Lipofectamine RNAiMAX***	***Reverse transfection***		***Forward transfection***
				Cell Number	***Cell suspension***	***Culture medium***
35 mm	500 µl	60 nM	5 µl	100,000	2 ml	2 ml
100 mm	1,000 µl	60 nM	10 µl	600,000	8 ml	8 ml

Table 3. Cell number and concentration of reagents used for reverse and forward transfection.

4. Overexpression studies

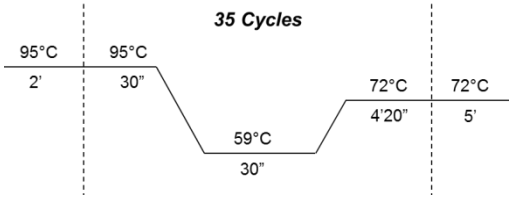
MARK4L and MARK4S overexpression studies were carried out in normal human fibroblasts and in the oligoastrocytoma G157 cell line, using different plasmids containing wild type MARK4L/MARK4S or the kinase dead (KD) mutants. In addition we used plasmid with only GFP as negative control to discriminate the non-specific effects due to transfection and overexpression.

4.1 Plasmid construction

Total mRNA from the ReNcellCX cell line was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with random examers, according to the manufacturer's protocol.

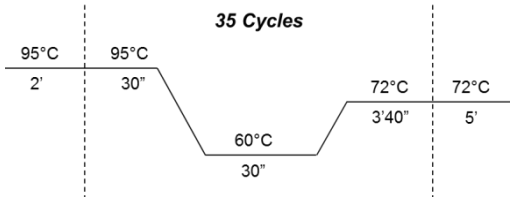
Full-length MARK4L open reading frame was amplified by PCR from this cDNA using *Pfu* DNA polymerase (Promega, Madison,WI, USA) and MARK4L specific primers also harbouring BamHI and EcoRI restriction site (Forward primer 5'-GTACCTAGGATCCTGTCTTCGCGG-3' and reverse primer 5'-GATGATGAATTCGGTGGCTCAGAG-3'). The following conditions were used:

10x Buffer	5	µl
dNTP mix (10 mM)	1,2	µl
Forward primer (10 µM)	1	µl
Reverse primer (10 µM)	1	µl
Pfu polymerase	1	µl
cDNA	2	µl
H_2O	to 50	µl



Full-length MARK4S cDNA was PCR-amplified from the cDNA clone OCABo5050C0222D (Source BioScience, Cambridge, UK) by means of *Pfu* DNA polymerase (Promega) and MARK4S specific primer also harbouring BglII and EcoRI restriction site (Forward primer 5'-TACCTAAGATCTTGTCTTCGCGG-3' and reverse primer 5'-GTTGATGAATTCGCCCTACACTCC-3'). We used the following conditions:

10x Buffer	5	µl
dNTP mix (10 mM)	1,2	µl
Forward primer (10 µM)	1	µl
Reverse primer (10 µM)	1	µl
Pfu polymerase	1	µl
cDNA	2	µl
H_2O	to 50	µl



MARK4L and MARK4S cDNA were then digested, purified and cloned into the BamHI/EcoRI site of the mammalian plasmid pcDNA4/HisMax (Invitrogen), a vector that includes a cleavable N-terminal Xpress™ tag for the detection of the recombinant protein with mouse anti-Xpress™ antibody (Invitrogen).

MARK4 cDNA and 500 ng of vector were digested in parallel, to make compatible ends, with BamHI/EcoRI or BglII/EcoRI restriction enzymes for MARK4L and MARK4S respectively. Following purification the insert was joined into plasmid using T4 DNA ligase (Promega). *E. coli* DH5α cells were chemically transformed with the recombinant DNA construct and then plated onto LB agar plate containing 100 µg/ml of ampicillin, in order to select the transformed bacterial cells. MARK4L and MARK4S cDNAs were also sub-cloned into pAcGFP1-C2 (Clontech, Mountain View, California USA) to obtain GFP-MARK4L and GFP-MARK4S constructs, respectively, using the protocol above described.

4.2 Mutagenesis of MARK4L and MARK4S vectors

The Thr214 and Ser218 are phosphorylation sites that are important for the activation and regulation of the MARK4 protein isoforms. To obtain catalytically inactive forms of MARK4L and MARK4S we mutated both the Thr214 and Ser218 into alanine, using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies Inc., Santa Clara, CA, USA) according to manufacturer's instruction. This technique is performed using a high-fidelity DNA polymerase for mutagenic primer-directed replication of both plasmid strands to obtain mutated vectors. The procedure can be divided in three steps:

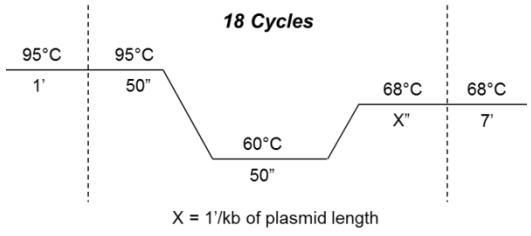
1. Extension of oligonucleotide primers (each complementary to opposite strands of the plasmid) during temperature cycling to produce the mutated vectors.
2. Digestion of the parental template using a specific endonuclease that recognises hemimethylated and methylated DNA.
3. Bacterial transformation with the mutated plasmid.

For MARK4L and MARK4S mutation we mutagenized the pAcGFP1-C2 MARK4L/MARK4S using the following primers:

- Forward primer: 5'-TCGAAGCTGGACGCCTTCTGCGGGGCCCCCCCTTATGCCGCCCCG-3'
- Reverse primer: 5'-CGGGGCGGCATAAGGGGGGGCCCCGCAGAAGGCGTCCAGCTTCGA-3'

The reaction mix was prepared and then cycled according to this protocol:

10x reaction buffer	5	µl
dsDNA template	10	ng
Forward primer	125	ng
Reverse primer	125	ng
dNTP mix	1	µl
QuikSolution	3	µl
PfuUltra HF DNA polymerase	1	µl
H_2O	to 50	µl



We then added 1µl of *Dpn* I restriction enzyme directly to each amplification reaction, mixed by pipetting the solution up and down and then incubated at 37°C for 1 hour to digest parental DNA. Following this step, XL10-Gold ultracompetent cells were chemically transformed and then were plated onto LB agar supplemented with 50 µg/ml of Kanamycin, in order to select the bacteria that incorporated the mutagenized vector.

4.3 Plasmid extraction and sequence analysis.

Transformed bacteria, containing the plasmid, were grown overnight at 37°C in 3 ml or 50 ml of LB supplemented with the appropriate antibiotic (Ampicillin or Kanamycin). Plasmid were then extracted using PureYield™ Plasmid Miniprep System or PureYield™ Plasmid Midiprep System (Promega) according to the manufacturer's instructions.

Plasmid quantity and quality were determined by measuring absorbance at 230, 260 and 280 nm with the ND-1000 Spectrophotometer (NanoDrop products, Waltham, MA, USA, by Thermo Fisher Scientific, Inc.).

To confirm that MARK4L or MARK4S cDNA was correctly oriented in frame with the fusion tag and without sequence variations, all the generated plasmids were sequenced.

4.4 Transfection of Fibroblasts

Fibroblasts were transfected by lipid-mediated transfection with Lipofectamine™ LTX & plus reagents (Invitrogen), according to manufacturer's instructions. Briefly, one day before transfection, cell were plated at the appropriated concentration in RPMI with 10% FBS without antibiotics. The day of transfection we prepared plasmid-Lipofectamine™ LTX complex as follows: plasmid DNA was diluted into Opti-MEM® I Reduced Serum medium (Invitrogen), without serum and antibiotics, to attain the optimal final concentration. After gentle mixing, the optimised volume of PLUS Reagent was added to the diluted DNA and then incubated at room temperature for 5 min. Subsequently Lipofectamine LTX was added to the mix. Following 30 minutes of incubation at room temperature, the DNA-Lipid complexes were added dropwise to the plate containing cells. Fibroblasts were incubated at 37°C, 5% CO_2 until ready to testing for transgene expression.

Transfection conditions, including cell number, plasmid and Lipofectamine concentration, used for these experiments are reported in Table 4.

Culture Vessel	*Cell Number*	*Volume plating medium*	*Volume dilution medium*	*DNA*	*Plus Reagent*	*Lipofectamine LTX*
35 mm	80,000	2 ml	500 µl	3 µg	3 µl	7.0 µl
100 mm	600,000	10 ml	1000 µl	22 µg	22 µl	52.9 µg

Table 4. Fibroblasts transfection conditions.

4.5 Transfection of G157 and HEK293T cells

G157 and HEK293T cells were transfected by lipid-mediated transfection with X-treameGENE HP DNA Transfection Reagent (Roche, Roche Diagnostic, Manheim, Germany), according to manufacturer's instructions. Briefly, one day before transfection, cell were plated at the appropriated concentration in culture medium with FBS and without antibiotics. The day of transfection we prepared plasmid-lipid complex as follows: plasmid DNA was diluted into serum-free medium, to reach the optimal final concentration. After mixing, the optimised volume of X-treameGENE HP DNA Transfection Reagent was added to the diluted DNA, mixed and then incubated at room temperature for 20 min. Subsequently the transfection complexes were added dropwise to the plate containing cells, and then incubated at 37°C, 5% CO_2 until ready to testing for transgene expression. Transfection conditions, including cell number, plasmid and Lipofectamine concentration, used for these experiments are reported in Table 5.

Cells	*Medium*	*Culture Vessel*	*Cell Number*	*Volume plating medium*	*Volume dilution medium*	*DNA*	*X-treameGENE HP DNA*
G157	RPMI 5% FBS	35 mm	200,000	2 ml	400 µl	3 µg	9 µl
		100 mm	1,500,000	10 ml	1000 µl	22 µg	66 µl
HEK	DMEM 10% FBS	60 mm	1,000,000	5 ml	500 µl	6 µg	18 µl
		100 mm	2,000,000	10 ml	1000 µl	22 µg	66 µl

Table 5. G157 and HEK cell lines transfection conditions

5. Immunofluorescence

Immunofluorescence permits the visualisation of the subcellular localisation of a specific protein in cultured cells.

For immunofluorescence experiments cells were seeded onto glass chamber slides and processed at around 70% confluence or at the end of transfection period (24 h).

For better visualisation of centrosomes and microtubules, cells were washed in microtubule-stabilising buffer for 4 min, fixed with methanol at -20°C for 12 min, and then permeabilized in PBS 0.1% Triton X-100 for 4 min.

Microtubule-stabilising buffer	80 mM PIPES pH 6,9, 1 mM $MgCl_2$, 1 mM EGTA, 4% (w/v) polyethylene glycol 6,000

Non-specific binding was blocked by incubating the fixed cells with 5% bovine serum albumin (BSA) in PBS for 20 min in a humidified chamber, before the incubation with primary antibodies.

Primary antibodies and dilutions	
• Rabbit anti-**MARK4 1+2+3+4** (phospho Thr 215) (ab111437, Abcam);	1:50
• Mouse anti-**γ-tubulin** (clone GTU-88; Sigma);	1:200
• Mouse anti-**β-tubulin** (clone TUB 2.1, Sigma);	1:100
• Mouse anti-**vimentin** (sc-6260, Santa Cruz Biotechnology, Santa Cruz, CA, USA);	1:100
• Mouse anti-**Xpress** (Invitrogen).	1:100

Antibodies were diluted in PBS 0.1% Tween 20, 5% goat serum (the serum of the animal where the secondary antibodies are raised, goat, was used to reduce non-specific binding), and incubated overnight at 4°C.

After three washes in PBS (10 min), the secondary antibodies, diluted in PBS 0.1% Tween 20 - 5% goat serum, were incubated for 1 hour at room temperature in dark humidified chamber.

Primary antibodies and dilutions	
• Goat anti-rabbit IgG-FITC (Sigma);	1:200
• Goat anti-mouse IgG-TRITC (Sigma).	1:200

Following three washes of 8 minutes in PBS, the slides were mounted with DAPI antifade (Vector Labs, Burlingame, CA, USA) and examined using an Olympus IX51 inverted fluorescence

microscope, equipped with an Olympus DP71 super high-resolution colour digital camera and U-MNIBA2 excitation 460/490 (FITC), U-MWIG3 excitation 530/550 (TRITC) and U-MNU2 (DAPI) filters. Images were acquired and processed using the F-View II-Bund-cell F software (Olympus, Tokyo, Japan).

6. Protein Analyses

6.1 Protein extraction

Harvested cells were washed with cold PBS, counted and resuspended in lysis buffer (100 µl / 2,000,000 cells).

Lysis Buffer	150 mM NaCl, 50 mM Tris pH 7.5, 1% NP-40, 0.25% deoxycholic acid, protease inhibitor cocktail (Complete EDTA-free, Roche) and phosphatase inhibitor (Halt™ Phosphatase Inhibitor Cocktail, Thermo Scientific).

The suspension was incubated on ice for 30 minutes with occasional inversion to obtain complete lysis. The lysate was centrifuged at 14,000 rpm for 25 minutes, at 4°C to prevent protein degradation. The supernatant (whole cell lysate) was stored at -20°C.
Protein concentration was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA), according to the manufacturer's protocol, with the ND-1000 Spectrophotometer.

6.2 Co-immunoprecipitation

Anti-vimentin antibody (sc-6260, Santa Cruz Biotechnology) was incubated with PureProteome™ Protein G Magnetic Beads (Millipore) for 1 hour at room temperature with continuous mixing. After three washes of the immobilised antibody in PBS containing 0.1% Tween 20, 1 mg of lysate was added and incubated at 4°C for 2h with agitation. Beads were extensively washed with PBS containing 0.1% Tween 20 and the immunoprecipitated proteins were eluted in non-reducing SDS loading buffer (Blue loading buffer pack, Cell Signaling Technology Inc., Beverly, MA, USA) by denaturating at 90°C for 10 minutes and subjected to SDS-PAGE, together with the corresponding whole cell lysates.

6.3 Immunoblotting

The immunoblotting procedure allows to separate proteins, according exclusively to their molecular weight, and to identify using specific antibodies, the presence or absence of a specific protein, its size and its relative expression.
For each sample equal amounts of the extracted proteins (20 µg) were supplemented with reducing SDS loading buffer (Blue loading buffer pack, Cell Signaling Technology) and denatured 3 minutes at 99°C.

Proteins were then separated by 4% stacking (100V) and 10% resolving (130V) SDS PolyAcrylamide Gel Electrophoresis (SDS-PAGE). The separated proteins were transferred by semi-dry electroblotting (10V, 30 minutes) to a PVDF membrane (Roche). The molecular weight standard used were Biotinylated protein ladder (Cell Signaling Technology) and ColorBurst electrophoresis marker (Sigma).

After electroblotting membranes were washed twice (10 min each) in PBS-T (0.3% Tween, Sigma) and non-specific binding was blocked by incubating the membrane in 5% skimmed milk, PBS-T for 1 hours at RT, in agitation.

PBS	100mM NaCl, 80mM Na_2HPO_4, 20mM NaH_2PO_4.

Membranes were then incubated with appropriate primary antibodies in PBS-T at 4°C overnight in agitation. For protein quantification, the membranes were cut horizontally immediately after the blocking step and incubated with the appropriate antibodies.

Primary antibodies and dilutions	
• Rabbit anti-**MARK4L** (GenScript corporation);	1:5,000
• Goat anti-**MARK4S** (ab5262; Abcam);	1:1,250
• Rabbit anti-**MARK4 1+2+3+4** (phospho Thr 215) (ab111437, Abcam);	1:1,000
• Mouse anti-**vimentin** (sc-6260, Santa Cruz Biotecnology);	1:1,000
• Mouse anti-**GAPDH** (ab8245; Abcam).	1:10,000

Following four washes in PBS-T membrane were incubated with secondary antibodies (in PBS-T) for 1 hour at room temperature (RT).

Secondary antibodies and dilutions	
• Goat anti-rabbit IgG-HRP (sc-2004; Santa Cruz Biotechnology);	1:10,000
• Donkey anti-goat IgG-HRP (sc-2020, Santa Cruz Biotechonlogy)	1:25,000
• Anti-biotin, HRP-linked antibody (Cell Signaling).	1: 2,500
The secondary antibodies are conjugated to HRP (Horse Radish Peroxidase), the anti-biotin antibody allows detecting the biotinylated protein ladder.	

After four washes in PBS-T and two in PBS, bounded antibodies were detected by covering the membranes with peroxidase/enhancer solution (Westar ηC Cyanagene Bologna, Italy) for 5 minutes and Blot images were acquired with Gbox Chemi XT4 system (Syngene, Cambridge, UK). Semi-quantitative analysis of MARK4L and MARK4S protein levels was performed using the Gene Tools Gel Analysis software (Syngene). MARK4L and MARK4S protein levels were normalised against GAPDH protein levels.

For immunoblot with the anti-MARK 1+2+3+4 antibody (phospho Thr215), both primary and secondary antibodies were diluted in TBS-T (50 mM Tris, 150 mM NaCl, 0.3% Tween 20), membranes were washed in TBS-T/TBS, and TBS-T containing 5% bovine serum albumin (BSA) was used to block non-specific binding.

Results

1. MARK4L and MARK4S expression during cell cycle phases

To elucidate the function of MARK4 during cell cycle we first determined the expression profile of MARK4L and MARK4S during each specific cell cycle phase. To this purpose we performed bi-parametric flow cytometry experiments on the glioblastoma G32 cell line, using PI to stain DNA content and specific antibodies to detect MARK4L or MARK4S protein expression. As negative control we used cells stained only with FITC-conjugated secondary antibody. Results of this analysis showed that MARK4L is not expressed at (a) particular cell cycle phase(s), but is continuously expressed throughout the cell cycle (Figure 1a).

Similar results were obtained for MARK4S, which appeared to be expressed across all the cell cycle phases too (Figure 1b). Analogous results were achieved in fibroblasts (data not shown), allowing to generalise the main result obtained on glioma cells to normal cells.

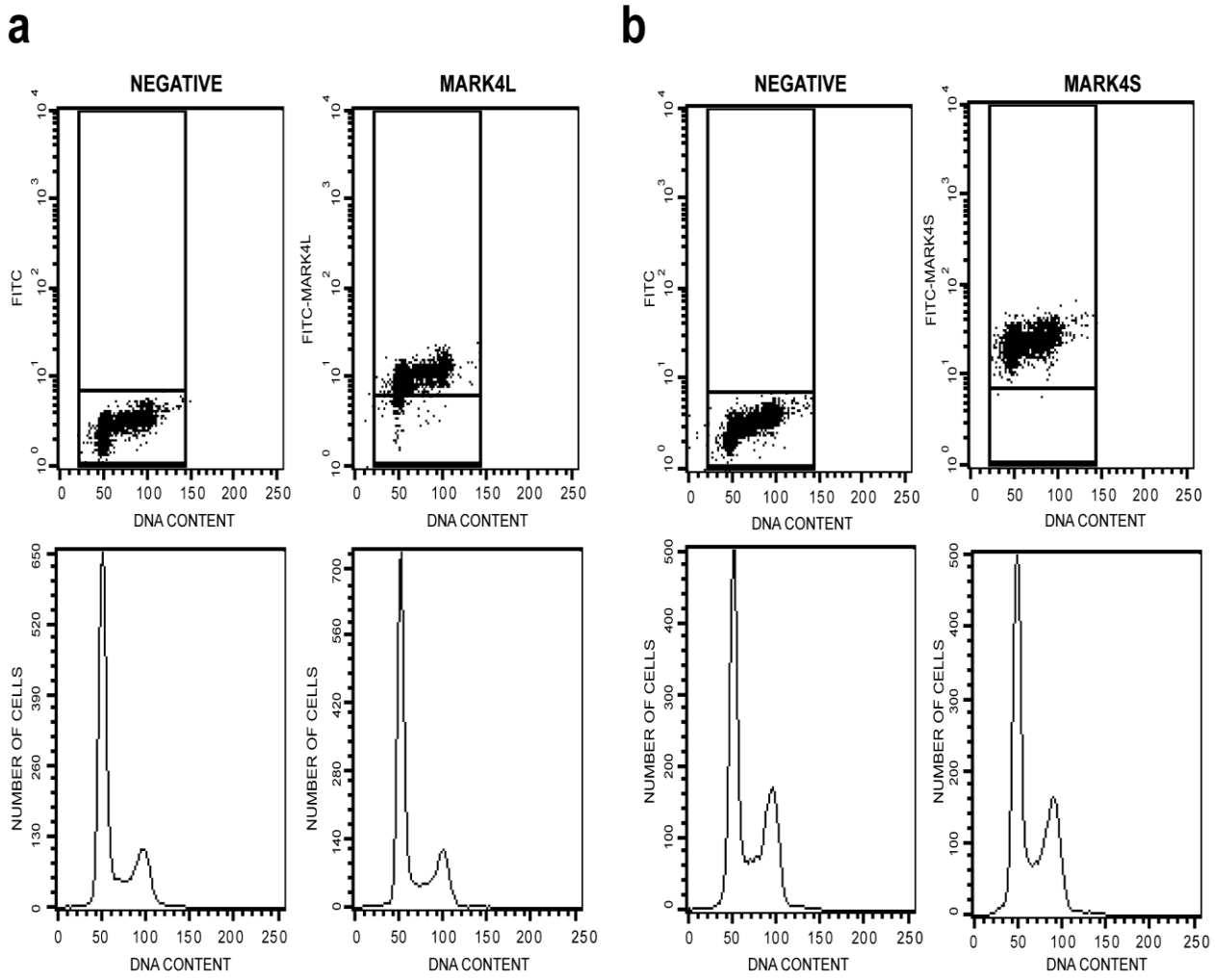


Figure 1. Cytofluorimetric analysis of MARK4L and MARK4S expression throughout the cell cycle of G32 glioblastoma cells. Both MARK4L (a) and MARK4S (b) are expressed in all cell cycle phases. Cells stained with only FITC-conjugated secondary antibody were used as negative control.

2. Activation status of MARK4 during cell cycle

As described in the introduction section, MARK4 activation requires the phosphorylation of the conserved Thr214 located in the activation loop. To asses phosphorylated-active MARK4 in human fibroblasts and glioma cells (G157), we performed immunofluorescence experiments using an antibody against all phosphorylated MARK proteins. The antibody was produced against the synthetic phosphopentapeptide L-D-T^{P}-F-C, derived from human MARK 1+2+3+4 centred around the phosphorylation site of the threonine in the T-loop. This sequence is identical in all MARK proteins so its choice does not allow to raise antibodies specific for a single MARK protein. To overcome the limit given by the recognition of all phospho-MARKs we focused our attention on MARK4 subcellular localisation sites, centrosomes and midbody (Magnani *et al.*, 2009; Magnani *et al.*, 2011), which are not shared by the other MARK proteins and hence kept into account only signals in the above mentioned positions.

MARK4 appeared phosphorylated throughout the cell cycle, with prevalence of the activated form in mitosis. In interphase, only a fraction of fibroblasts (about 29%) (Figure 2a) and G157 cells (nearly 31%) (Figure 2b) exhibited fluorescence corresponding to phosphorylated MARK4 at single or duplicated centrosomes.

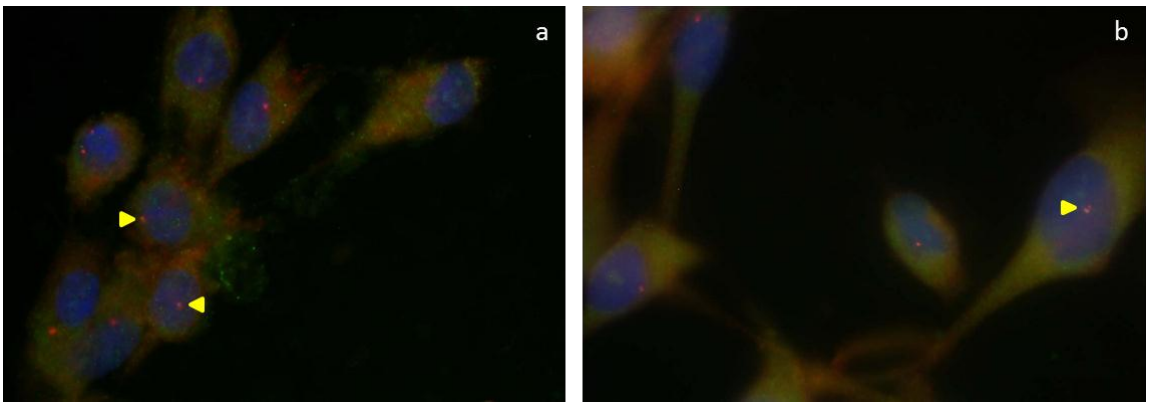


Figure 2. Immunofluorescence analysis of active MARK4 using an anti-phosphorylated MARK antibody. Representative images of phosphorylated-MARK4 (green) and γ-tubulin (red) immunostaining in interphase fibroblasts (a) and G157 cells (b). Centrosomes are labeled with phosphorylated-MARK4 (yellow arrowheads) only in a few cells.

By contrast, MARK4 is phosphorylated during all phases of mitosis and cytokinesis. Fluorescence corresponding to phosphorylated MARK4 was always detected at centrosome during all mitotic stages (Figure 3d-f) and interestingly, also in the pre-mitotic phase during centrosome separation e migration (Figure 3a-c).

Active MARK4 is present also in the midbody during cytokinesis, especially at the borders of the midbody at the lateral constriction zone (Figure 3g-h).

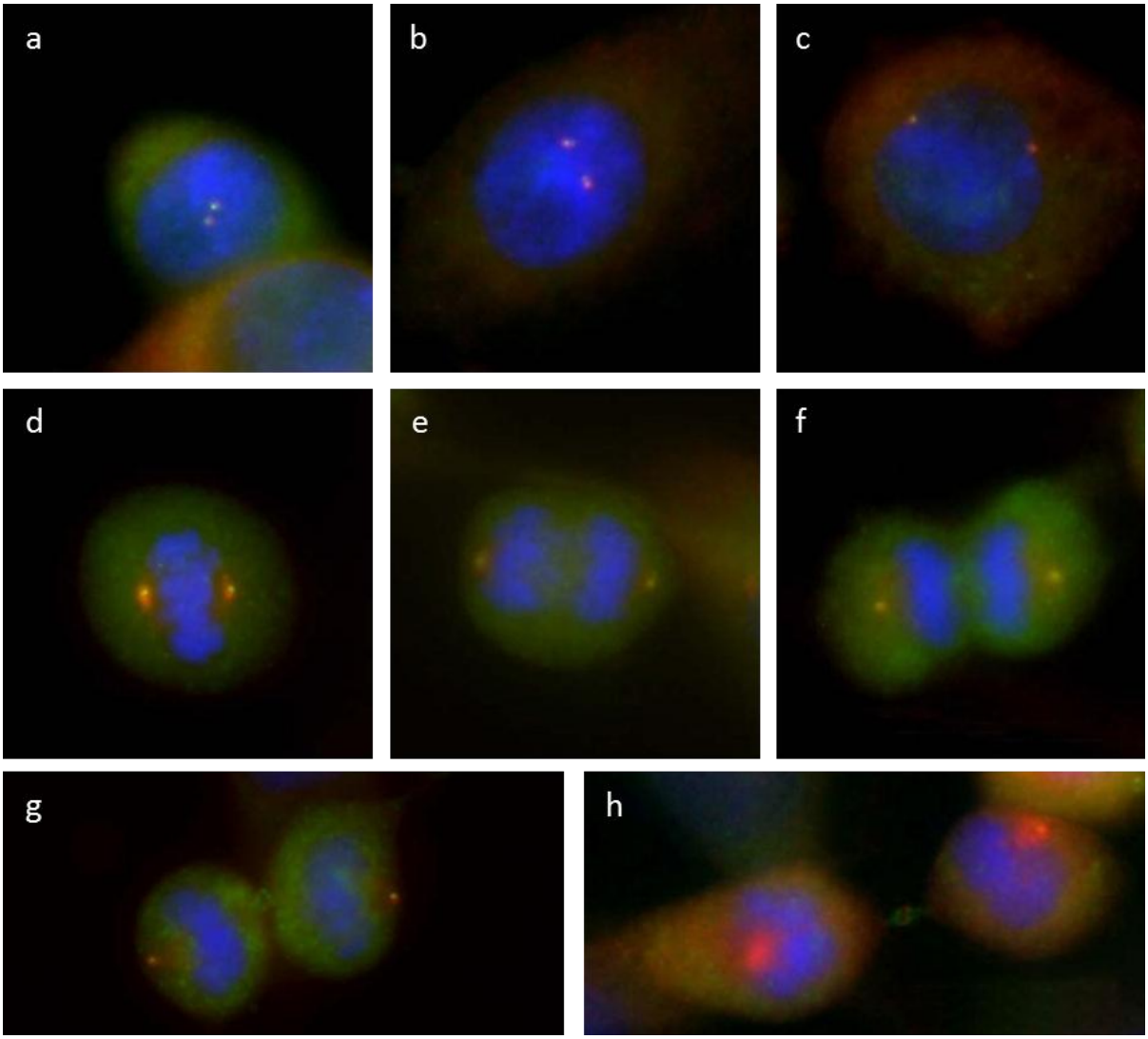


Figure 3. Immunofluorescence experiments of active MARK4 using an anti-phosphorylated MARK antibody. Representative images of G157 cells showing co-localisation between phosphorylated-MARK4 (green) and γ-tubulin (red) immunostaining at centrosomes during centrosome separation (a-c) and across all phases of the mitosis (d-f). Positive signals are also visible at the midbody during cytokinesis (g and h).

To verify that the signals detected by the phospho-MARK antibody at the centrosome are due to MARK4, we performed immunofluorescence experiments on MARK4S depleted fibroblasts.

Following treatment with MARK4S or non-specific siRNA for 72 h, we verified the depletion of endogenous MARK4S by Western blot (Figure 4a) and performed immunofluorescence with the phospho-MARK antibody. In MARK4S siRNA treated cells a significant reduction of positive phospho-MARK4 signals as compared to non-specific control, can be observed at the interphase

centrosomes (10% vs 30%) (Figures 4b and c). These data suggest that the centrosome signals observed with the anti-phospho-MARK antibody are imputable to MARK4.

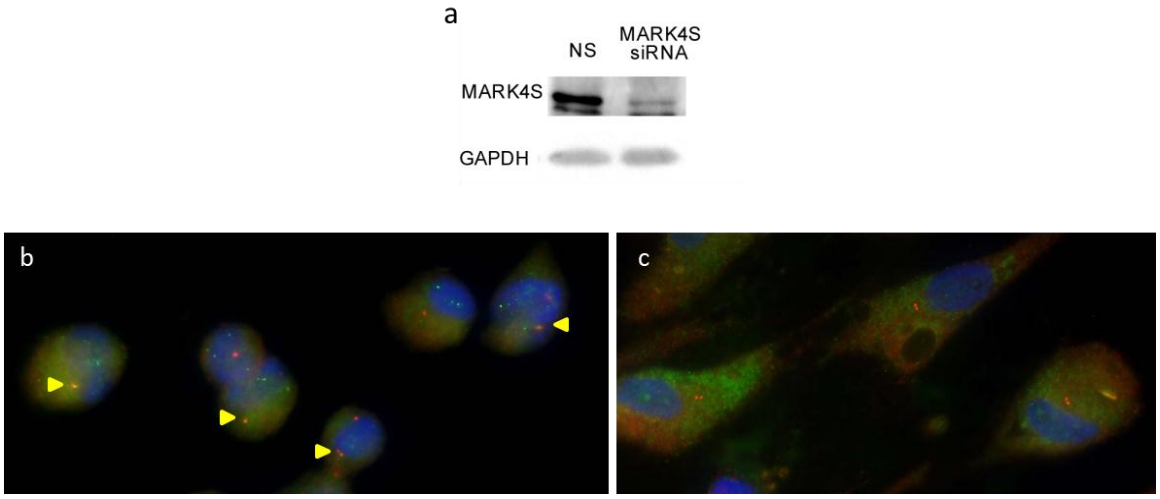


Figure 4. Analysis of phospho-MARK4 in fibroblasts after MARK4S siRNA treatment for 72. (a) Western blot analysis of MARK4S protein levels, in MARK4S siRNA-treated human fibroblasts compared to levels in non-specific siRNA-treated control cells, show a decrease of MARK4S protein. The protein levels of MARK4S were normalised against the protein level of the housekeeping gene GAPDH. Representative images of phosphorylated-MARK4 (green) and γ-tubulin (red) immunostaining in non-specific siRNA-treated control (b) and MARK4S siRNA-treated (c) fibroblasts. In MARK4S depleted cells a significant reduction of positive phospho-MARK4 signals as compared to non-specific control, can be observed.

3.Overexpression experiments

3.1 Evaluation of MARK4L and MARK4S expression

Before investigating the effects of MARK4 overexpression we verified that the GFP- and Xpress-MARK4L/MARK4S fusion proteins were correctly expressed. We transfected HEK293T cells with the MARK4L or MARK4S vectors (both GFP and Xpress) and then analysed the presence of the recombinant proteins by Western blot using specific MARK4 antibodies. As shown in Figure 5, MARK4L and MARK4S are well expressed and recognised by their specific antibodies. Endogenous MARK4L is also detected by the anti-MARK4L antibody (Figure 5), at difference of MARK4S which is only weakly expressed by HEK cells.

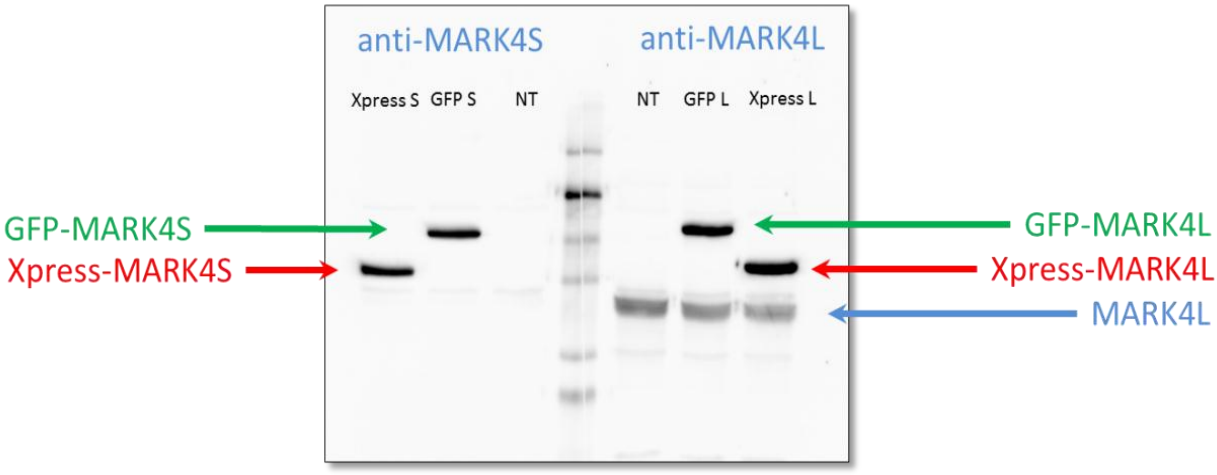


Figure 5. Immunoblot experiments of HEK293T cells overexpressing MARK4L or MARK4S. Both GFP- and Xpress-tagged proteins are expressed and recognised by the anti-MARK4L or anti-MARK4S antibodies. Endogenous MARK4L is also detected by the MARK4L antibody. NT, non-transfected; GFP L, overexpressed GFP-MARK4L; Xpress L, overexpressed Xpress-MARK4L; GFP S, overexpressed GFP-MARK4S; Xpress S, overexpressed Xpress-MARK4S.

We observed that a high expression level of MARK4L or MARK4S is cytotoxic. Therefore, in order to plan the timing of overexpression experiments, we decided to analyse the expression levels of the recombinant proteins at different post transfection times (4, 8, 24 and 48 h) in HEK293T cells.

As shown in Figure 6 at 4h post-transfection both exogenous MARK4 isoforms were not yet detected by Western blot, indicating that monitoring should be delayed. Both MARK4L and MARK4S begin to be detectable 8 h after transfection, and their levels increase as expected at 24h.

At 48h the levels appear not increased in comparison to those at 24h, probably due to the death of a fraction of overexpressing cells (Figure 6).

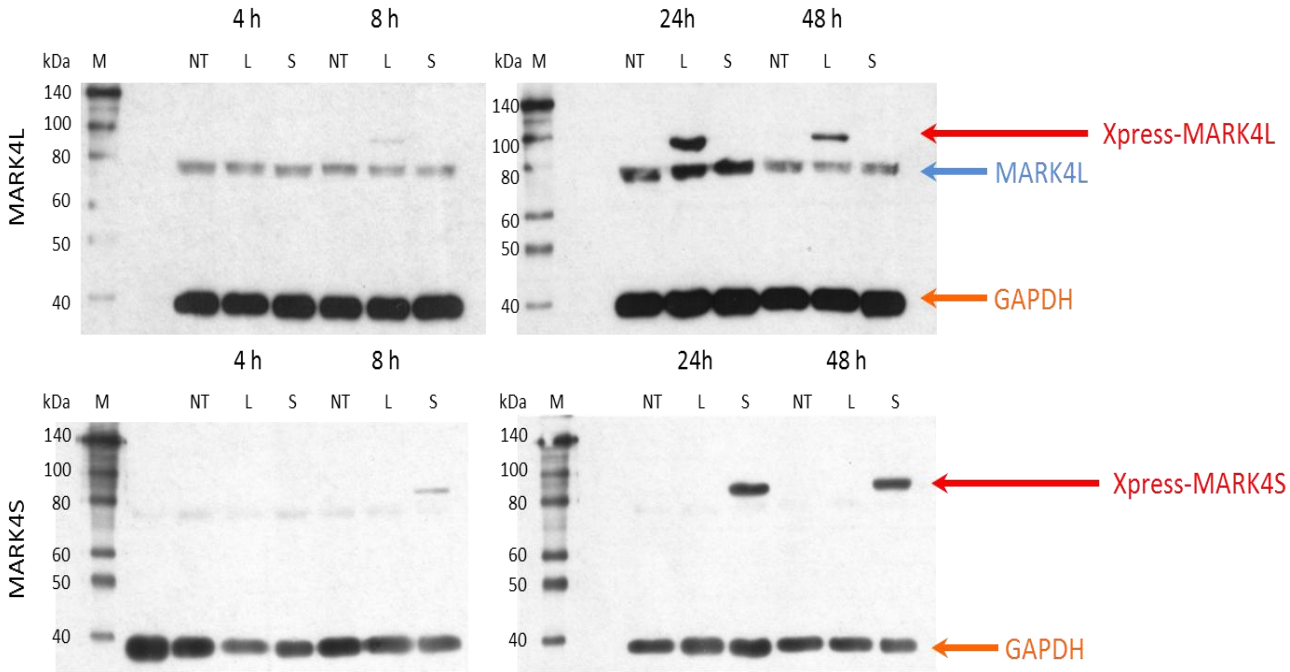


Figure 6. Western blot analysis of cells overexpressing MARK4L or MARK4S at 4h, 8h, 24h and 48h post-transfection. Immunoblot analysis of HEK293T cells with ant-MARK4L or anti-MARK4S antibody shows that both MARK4 isoforms begin to be detectable 8 h after transfection. The expression levels increase at 24h whereas remain stable at 48h. GAPDH was used as control protein. The membrane was cut after the blocking step and incubated with the appropriate antibodies. The MARK4L antibody also detects the endogenous MARK4L protein. NT, non-transfected; L, overexpressed MARK4L; S, overexpressed MARK4S.

We then verified that the catalytically inactive MARK4 mutant (Kinase Dead, KD), in which Thr214 and Ser218 are substituted by alanine were correctly expressed in both fibroblasts and the G157 cell line. At 24 h post transfection both wild type and mutant MARK4L and MARK4S are well detectable by Western blot with specific MARK4 antibodies (Figure 7a). In addition the activation status of both overexpressed MARK4L and MARK4S was controlled by Western blot with the anti-phosphorylated MARK antibody. As proof of evidence for the correct working of the system wild-type MARK4L and MARK4S were found phosphorylated and thus active, whereas KD MARK4L and MARK4S were not (Figure 7b).

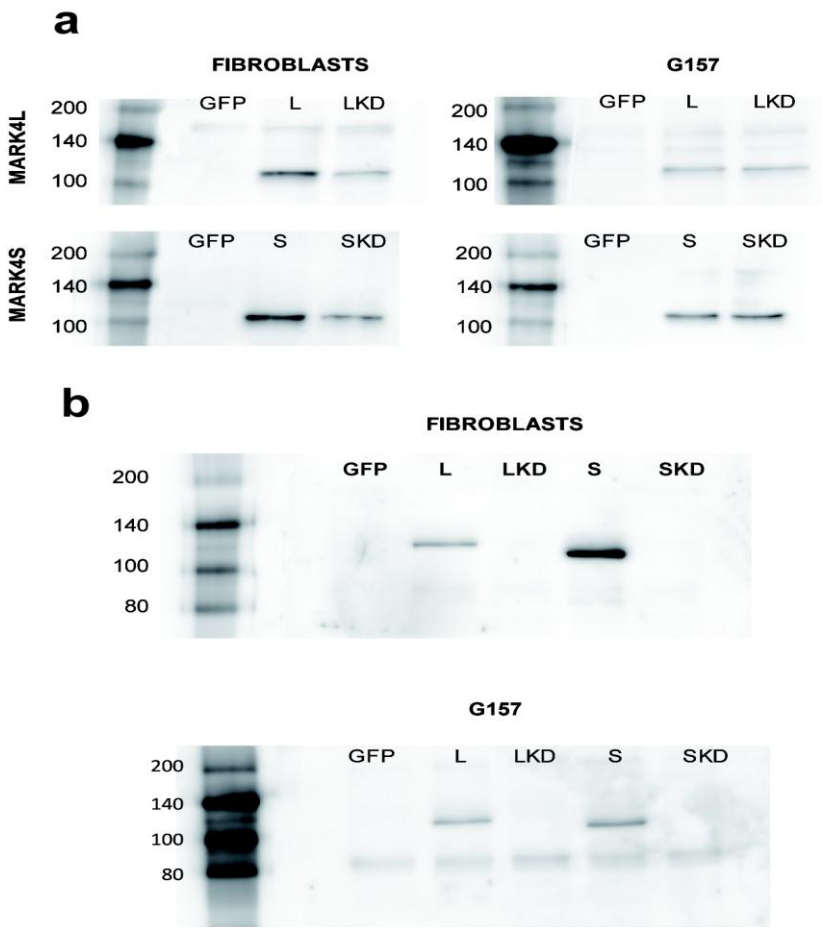


Figure 7. Western blot analysis of cells overexpressing MARK4L or MARK4S. (a) Immunoblot analysis of fibroblasts and G157 cells confirming the overexpression of wild-type MARK4L or MARK4S, and their KD mutants. (b) The anti-phosphorylated MARK antibody recognises overexpressed wild-type MARK4L and MARK4S but does not detect KD MARK4L or MARK4S (mutagenized at Thr214). GFP, GFP alone; L, overexpressed MARK4L; LKD, overexpressed KD MARK4L; S, overexpressed MARK4S; SKD, overexpressed KD MARK4S.

3.2 Overexpression of MARK4L or MARK4S reduces the density of the microtubule network

As underlined in the introduction the main biological function of MARK proteins is to phosphorylate MAPs, leading to the destabilisation of microtubules. To investigate the effects of MARK4L and MARK4S on the MT network, we performed immunofluorescence experiments on our system of fibroblasts and G157 glioma cells transiently transfected with GFP-tagged wild-type MARK4L/MARK4S or the respective KD mutants.

At 48 h after transfection, most cells overexpressing MARK4L or MARK4S had detached from chamber slides and died, confirming the cytotoxicity observed at this time and thus precluding monitoring the effect of MARK4L/MARK4S overexpression. Therefore, we selected for the analysis the 24 h post-transfection time, when the cells were still viable and exogenous MARK4L and MARK4S could be detected by Western blot (Figure 7).

Immunofluorescence staining with an anti-β-tubulin antibody revealed that the MT network was altered in a large number of cells overexpressing MARK4L (Figures 8 and 9). In particular, the density of the MT network was lower in MARK4L overexpressing cells than in non-transfected cells or GFP-transfected cells (Figures 8 and 9).

The effect of MARK4S overexpression was similar, in both the cell systems. Most of the transfected cells showed, as observed for MARK4L, an MT array less dense as compared to non-transfected or GFP-transfected cells (Figures 8 and 9).

To verify whether these alteration of MTs were caused by the kinase activity of the two MARK4 isoforms, or by non-specific effects of overexpression, we transfected cell with catalytically inactive MARK4L or MARK4S mutants, in which Thr214 and Ser218 were mutated to alanine. In this case the recipient cells had a normal aspect and did not show any alteration of the microtubule network (Figures 8 and 9), demonstrating that the effects on microtubules can be ascribed to the phosphorylation activity of MARK4.

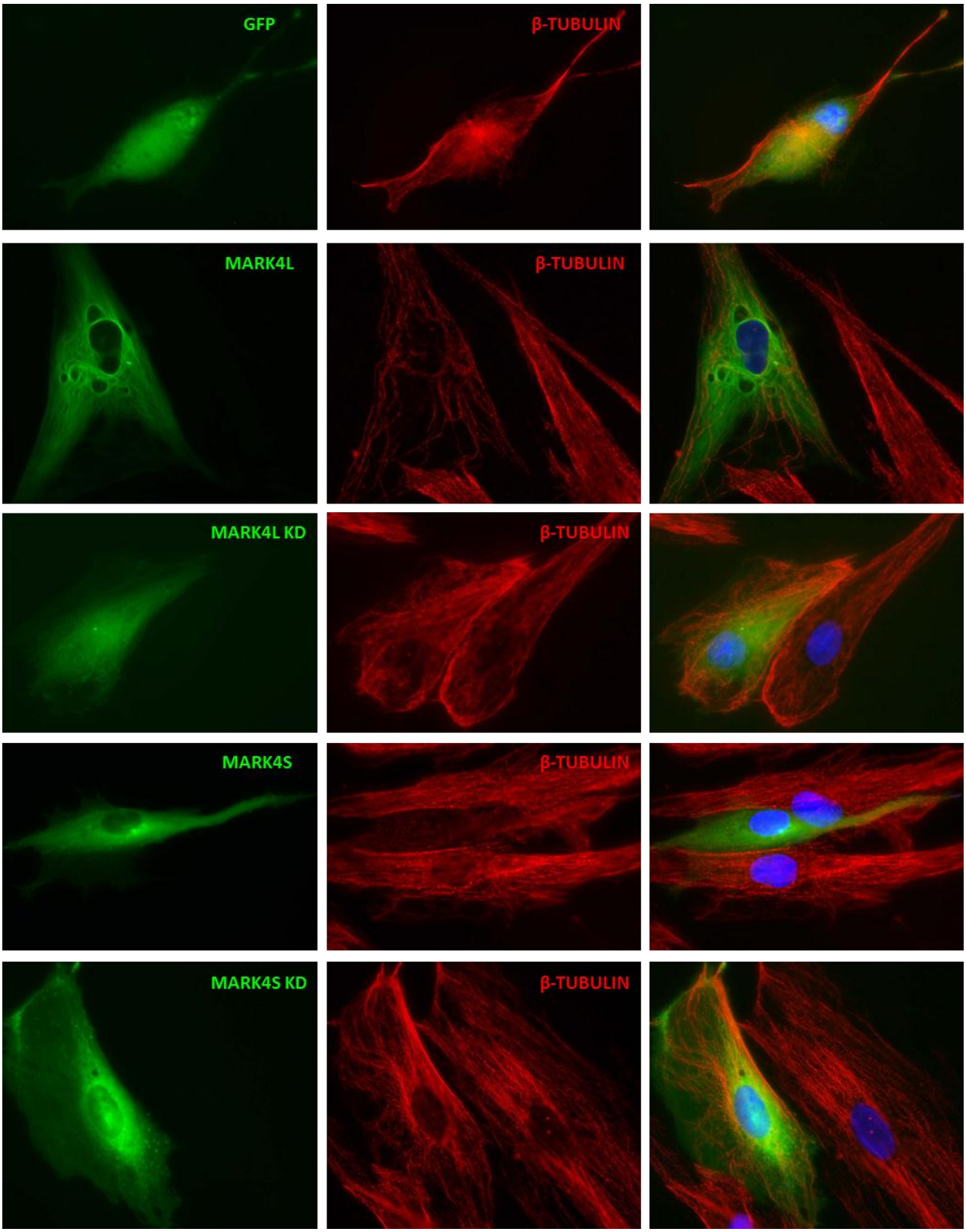


Figure 8. Effect of MARK4 overexpression on the MT network in fibroblasts. Immunofluorescence labeling with an anti-β-tubulin antibody (red) shows a reduced density of the MT network in fibroblasts overexpressing wild-type MARK4L or MARK4S (green) in comparison to surrounding non-transfected or GFP-transfected cells. Overexpression of KD MARK4L or MARK4S does not disrupt or weaken the MT network.

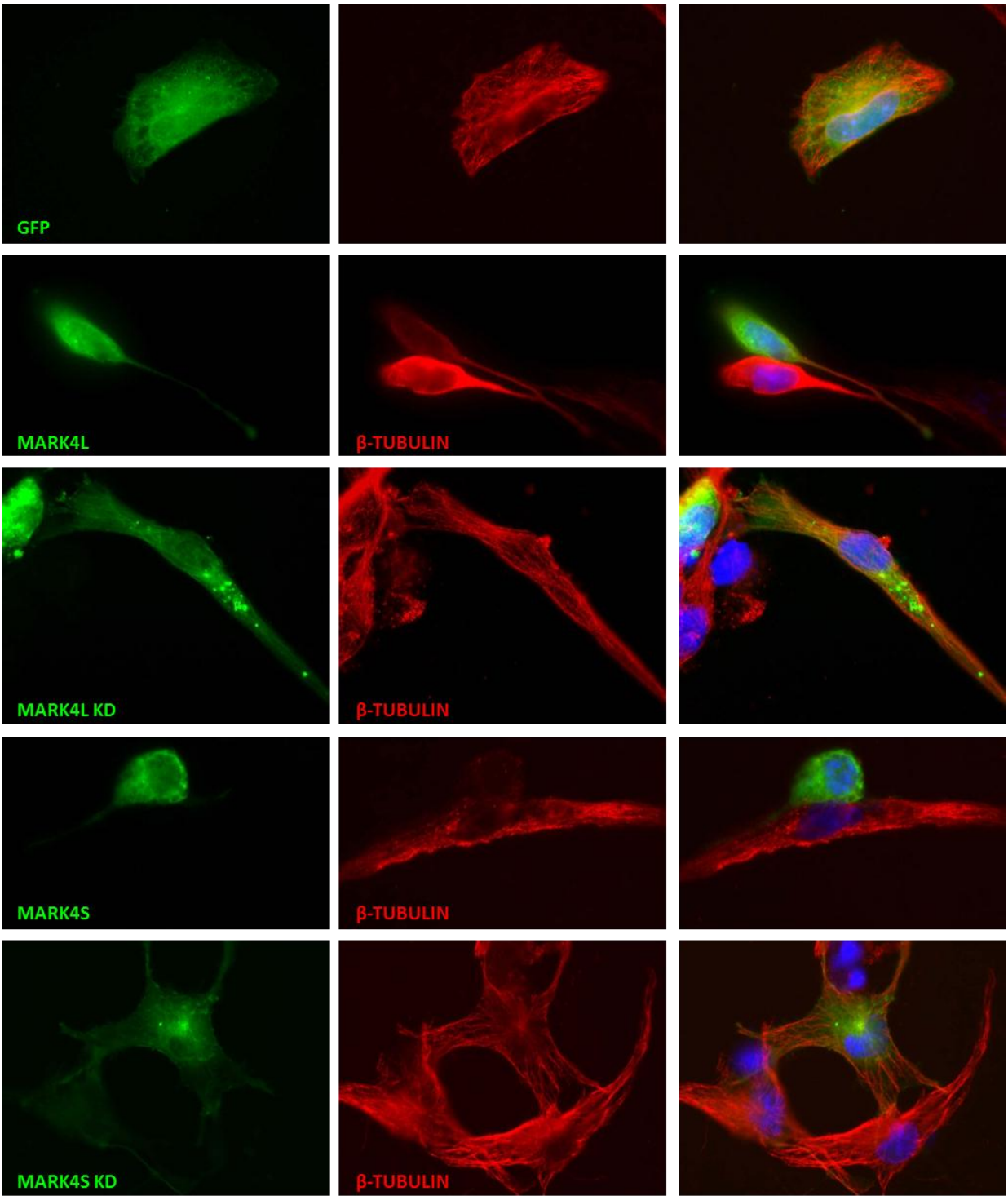


Figure 9. Effect of MARK4 overexpression on the MT network in G157 cells. Immunofluorescence labeling with an anti-β-tubulin antibody (red) shows a reduced density of the MT network in G157 cells overexpressing wild-type MARK4L or MARK4S (green) in comparison to surrounding non-transfected or GFP-transfected cells. Overexpression of KD MARK4L or MARK4S does not disrupt or weaken the MT network.

3.3 MARK4L co-localises with the intermediate filament protein vimentin in fibroblasts

Microscopic observations of fibroblasts overexpressing MARK4L showed that many cells (more than 50% of transfected cells) displayed GFP fluorescence in a filamentous pattern that resembled the intermediate filament network, suggesting that MARK4 might interact with other cytoskeletal structures in addition to microtubules. In order to explore this hypothesis, we first excluded by immunofluorescence experiments with an anti-β-tubulin antibody, that the observed bundles contain microtubules. As shown in Figure 10 the anti-β-tubulin antibody did not label the filamentous pattern of overexpressed MARK4L, indicating that tubulin was not present in these filaments.

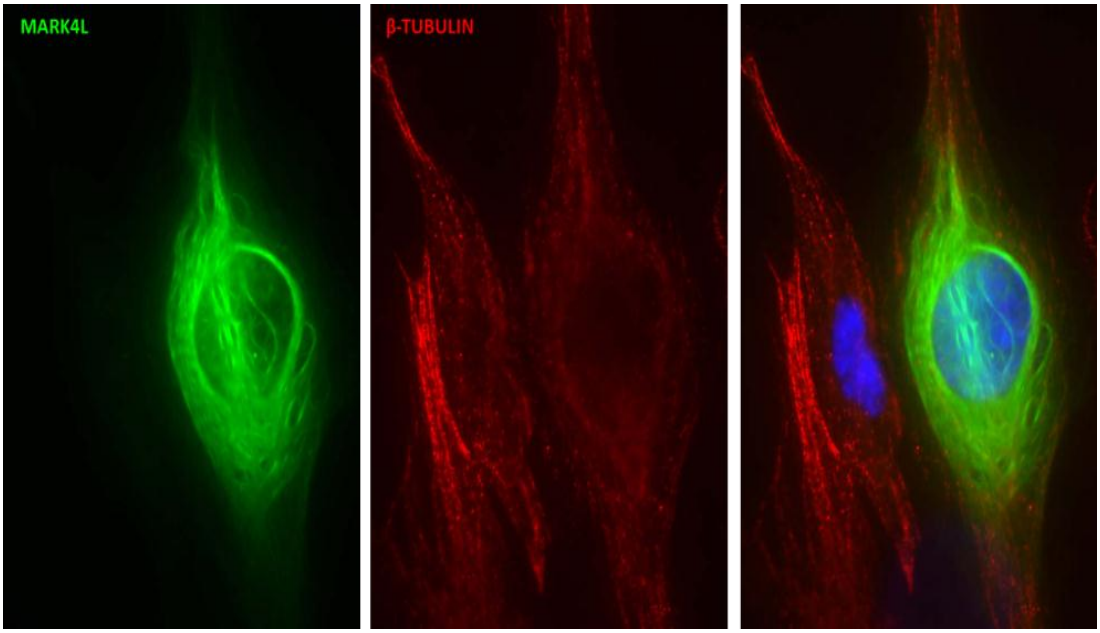


Figure 10. Overexpressed MARK4L shows a filamentous pattern in fibroblasts. Immunofluorescence staining with an anti-β-tubulin antibody (red) highlights that the bundles observed in fibroblasts overexpressing MARK4L do not contain microtubules.

Next we performed immunofluorescence experiments using an antibody against vimentin, the main component of intermediate filaments in fibroblasts. Overexpressed MARK4L appeared to localise to bundle structures that were also labelled with the anti-vimentin antibody (Figure 11). In contrast overexpressed MARK4S localised to these bundle structures to a lesser extent and only co-localised with the anti-vimentin antibody labelling in a few cells (less than 5%) (Figure 11).

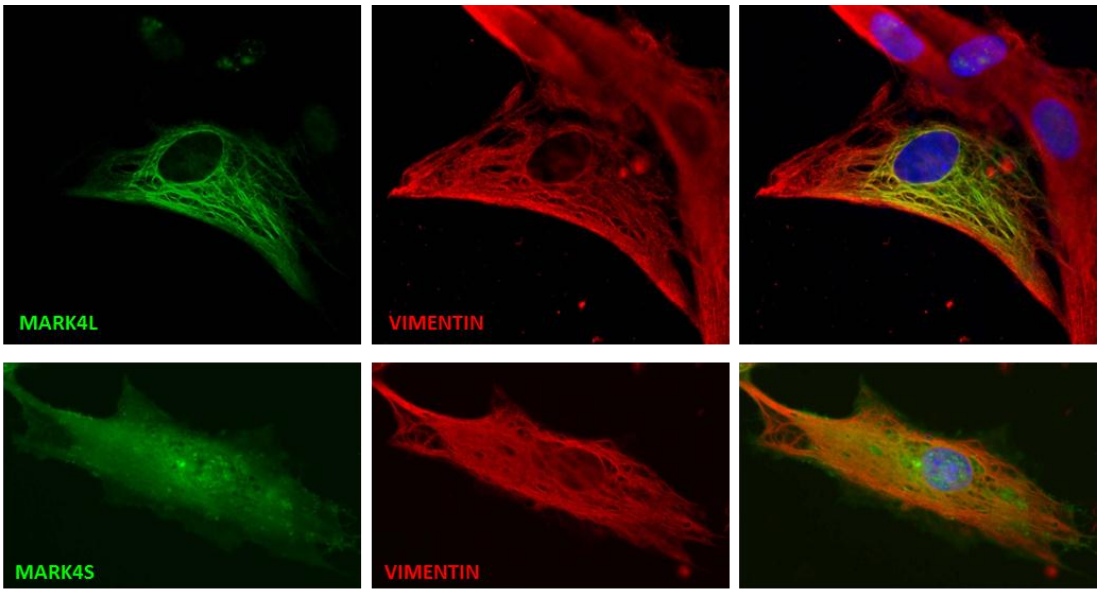


Figure 11. Co-localisation of overexpressed MARK4L with intermediate filaments in fibroblasts. Overexpressed MARK4L (green) exhibits a filamentous pattern that co-localises with anti-vimentin antibody labelling (red). Overexpressed MARK4S shows a less evident co-localisation with vimentin.

The co-localisation between MARK4L and vimentin is particularly evident in the perinuclear zone and in addition, in some overexpressing cells, vimentin filaments appeared re-structured as compared to those in GFP-transfected cells, and showed the formation of bundling (Figure 12).

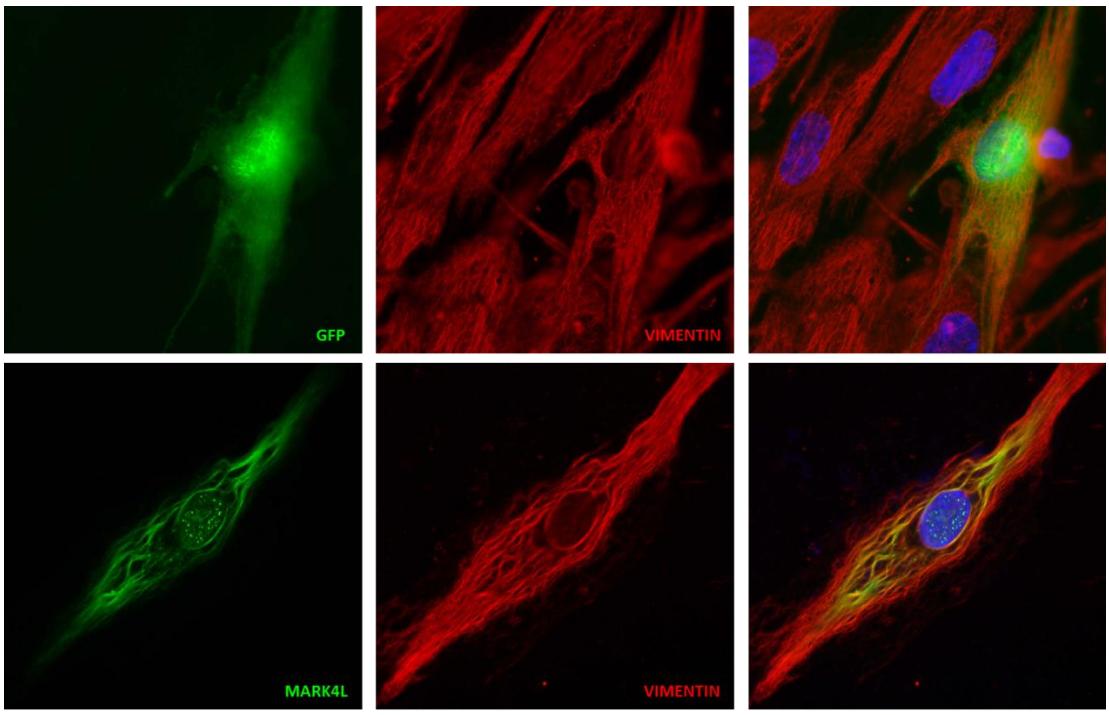


Figure 12. Comparison of intermediate filaments in GFP or MARK4L overexpressing fibroblasts. Immunofluorescence staining with an anti-vimentin antibody (red) shows that the intermediate filaments in MARK4L overexpressing fibroblasts are reorganised, forming bundle structures. In contrast overexpression of GFP alone does not alter their structure.

We next asked whether MARK4L activity is involved in the co-localisation with vimentin and in the reorganisation of these cytoskeletal filaments. We thus performed immunofluorescence experiments on fibroblasts transfected with kinase dead MARK4L. As shown in Figure 13, MARK4L KD in contrast to the wild type, does not alter the structure of intermediate filaments and co-localised to these fibres to a lesser extent (Figure 13).

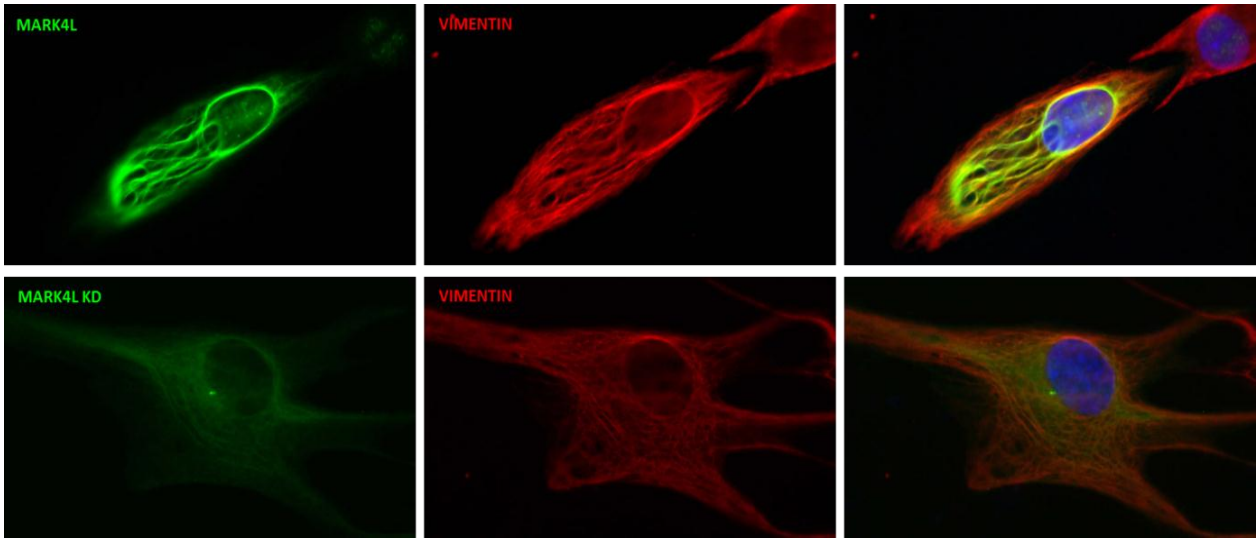


Figure 13. Effect of MARK4L wild type or kinase dead on vimentin filaments in fibroblasts. Overexpressed MARK4L (green) co-localises and alters the vimentin cytoskeleton (red). In contrast the kinase dead mutant (green) co-localises only partially with anti-vimentin antibody (red) and does not form bundle structures.

Finally we asked whether MARK4L present in these structures is active and could exert its kinase functions and if the tag protein could influence this localisation. We thus transfected fibroblasts with pcDNA4/HisMax, a vector in which MARK4L is fused with the N-terminal-Xpreess™ tag, and then performed double immunofluorescence experiments with the anti-phspho-MARK1+2+3+4 and with anti-Xpress antibodies. The overexpressed MARK4L showed the same filamentous pattern observed after transfection of GFP-MARK4L (Figure 14), demonstrating that the co-localisation with vimentin is not influenced by the tag. In addition the phospho-MARK antibody, co-localised with the recombinant MARK4L indicating that the protein is active (Figure 14).

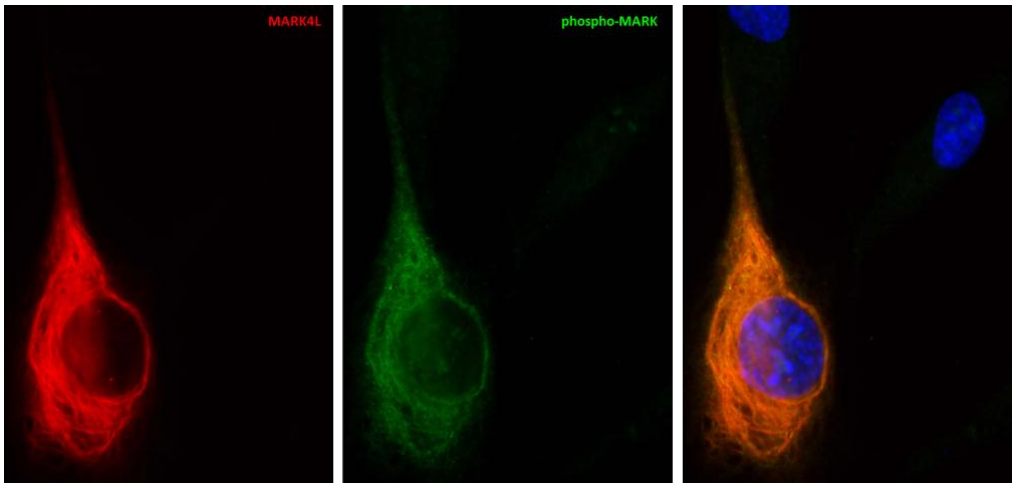


Figure 14. Co-localisation between anti-Xpress and anti-phospho-MARK antibody signals. Overexpressed MARK4L fused with Xpress-tag (red) shows a filamentous pattern similar to that observed for GFP-MARK4L and co-localises with the anti-phosphoMARK antibody labelling (green), indicating the phosphorylation of the exogenous protein.

In order to better interpret the results of MARK4L overexpression we carried on co-immunoprecipitation experiments in non-transfected fibroblasts to verify whether the endogenous MARK4L interacts directly with vimentin. Preliminary results using the anti-vimentin antibody, showed that vimentin interacts with MARK4L, since a band corresponding to MARK4L is detected in the vimentin immunoprecipitate sample (Figure 15).

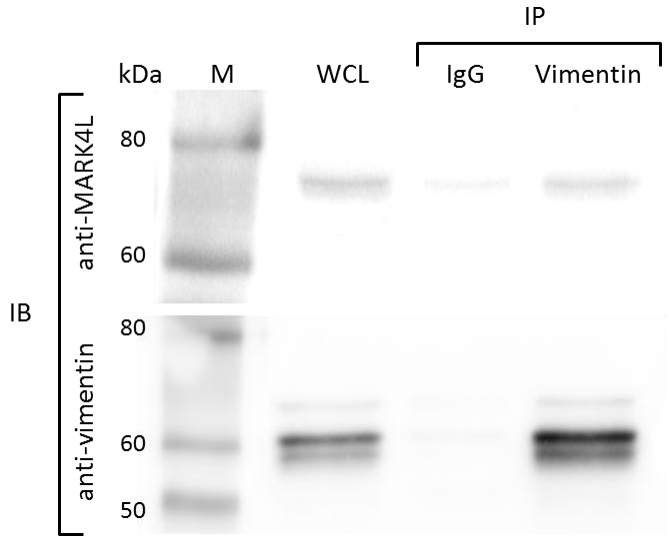


Figure 15. Co-immunoprecipitation of MARK4L and vimentin in normal fibroblasts. Whole cell lysate (WCL) of normal fibroblasts and immunoprecipitations (IP) with anti-vimentin or control mouse IgG (IgG) antibodies were analysed by immunoblotting with the indicated antibodies. A band corresponding to MARK4L is clearly visible in the vimentin IP sample. M: standard molecular masses.

Discussion

Substantial evidence has been acquired on the important roles played by MARK4, a member of the MARK family of AMPK-related kinases, in the establishment and maintenance of cell polarity, in cell cycle control and in intracellular signal transduction (Drewes *et al.,* 1997; Ebneth *et al.,* 1999; Marx *et al.,* 2010; Li & Guan 2013). For many years MARK4 remained the less characterised member of the MARK family and the functions ascribed to it were based on its homology to the other MARK proteins. Only recently an increasing number of studies focused on MARK4 functions have been published, delineating this protein as an extremely versatile and multifaceted kinase.

As the least phylogenetically conserved of the MARK proteins, MARK4 has several unique features: the expression in two different isoforms, MARK4L and MARK4S, and a distinctive localisation at the centrosome and at the midbody during cytokinesis (Trinczek *et al.,* 2004; Magnani *et al.,* 2009; Magnani *et al.,* 2011). The localisations of MARK4 at structures that are fundamental to cell division highlight its link with microtubules dynamics in cycling cells. Recent studies also revealed that MARK4 is a crucial regulator of MT-based structures, such as primary cilium, whose assembly is initiated at G0/G1 phase (Khuns *et al.,* 2013), and ectoplasmic specialisations of rat testis spermatids and Sertoli cells, which are dependent on the tubulin cytoskeleton (Tang *et al.,* 2012).

Despite the increasing attention in literature about MARK4 function, the dual nature, represented by its two MARK4L and MARK4S isoforms, was scarcely addressed. In particular among the recent published studies, only one has focused on the role played by a single isoform, namely MARK4L (Khuns *et al.* 2013); notwithstanding the possible involvement of the second one was not evaluated.

As already mentioned, the two MARK4 isoforms differ in the C-terminal region, suggesting different functions that could be directly mediated by the protein structure or depend on the specific proteins interacting with their differential end domain. In addition, the involvement of MARK4L and MARK4S in distinct processes is proposed by their different expression profiling in the central nervous system in both physiologic conditions during normal terminal differentiation and gliomagenesis (Beghini *et al.,* 2003; Moroni *et al.,* 2006; Magnani *et al.,* 2011).

In both these contexts deepening the possible differences between the short and long isoform in processes like cell cycle and cytoskeleton regulation appears to be of particular interest. Continuing our previous studies on MARK4, we wanted to further elucidate the role played by MARK4 in the cell cycle progression and in the regulation of the cytoskeleton in both normal (fibroblasts) and transformed cells (primary glioma cell lines). In particular we planned to face the intriguing problem of MARK4 dual nature, in order to highlight possible isoform-specific functions.

To achieve this aim we first investigated the expression and activation status of MARK4 throughout the cell cycle, since protein kinases involved in cell cycle control are thinly regulated in their spatio-

temporal expression and their activity is often acquired by post-translational modifications and protein-protein interactions (Bayliss *et al.,* 2012). Accordingly, cytofluorimetric analysis showed that both MARK4 isoforms are not expressed during a particular cell cycle phase(s) but are continuously expressed throughout the cell cycle in both G32 glioblastoma cells and fibroblasts.

We next performed immunofluorescence experiments to assess the activation of MARK4 in fibroblasts and glioma cells during cell cycle and established that MARK4 in its functionally active form prevails during mitosis and cytokinesis. As the antibody marking phospho-MARK4 is directed against an epitope shared by all MARK proteins and MARK4 can thus be only recognized through its peculiar centrosome localisation, shared by both MARK4 isoforms, this approach could not discriminate between MARK4S and MARK4L isoforms. In particular we highlighted that phospho-MARK4 is present in the centrosome at all the mitotic phases and in the midbody during cytokinesis. In addition active MARK4 was also detected in centrosomes during their separation and migration to the cell poles. Interestingly, we have previously demonstrated that MARK4S depletion alters centrosome cycle and in particular depleted cells showed duplicated centrosomes positioned apically to the nucleus, that are unable to migrate (Rovina *et al.,* submitted). Taken together these data support the involvement of MARK4 in the regulation of centrosome separation and migration. It has been demonstrated that the separation and migration of centrosomes involve microtubules and motor proteins, including both kinesin and dynein (Tanenbaum & Medema 2012). Interestingly MARK proteins have been reported to be involved in the regulation of microtubules and MT-based transport (Drewes *et al.,* 1997; Mandelkow *et al.,* 2004). The overall observations let us to hypothesize that MARK4 might regulate centrosome separation and migration through its action on microtubules and MT-transport.

Overall, expression and activation data suggest that MARK4 is required throughout the cell cycle and phosphorylation of MARK4, which is required for its activation, mainly occurs during the dynamic phases of cell cycle including centrosome migration, mitosis and cytokinesis.

Consistent evidences on MARK4 involvement in the regulation of cytoskeleton and cytoskeleton-driving processes came from our overexpression experiments in both fibroblasts and glioma cell line (G157). These experiments allowed us to discriminate the effects of each single MARK4 isoform making feasible to highlight possible different functions between them.

Overexpression of MARK4L or MARK4S led to a sharp decrease in microtubule density in both fibroblasts and G157 cells, as monitored by immunofluorescence. By contrast, overexpression of KD MARK4L or MARK4S, in which the Thr214 and Ser218 residues of the phosphate acceptor site were mutated, did not affect the microtubule network, indicating that these effects on microtubules are dependent on the kinase activity of MARK4.

Our experiments highlight that both MARK4 isoforms have similar effects on the microtubules array in both normal and glioma cells. This datum is novel in two ways. First the two isoforms have been assayed for the first time in parallel; second their effect is monitored both in normal and glioma cells, allowing to generalise this function to physiological and pathological conditions. Indeed the activity exerted by both MARK4 isoforms on microtubules can be considered the main biological function of MARKs, since is shared by all the members of the family and can involve these kinases in the regulation of many different processes. However the distinctive subcellular localisation of MARK4, in addition to its different activation during cell cycle suggests that the microtubule depolymerisation activity could be directed to more specific processes namely mitosis and cytokinesis as compared to the other MARK proteins.

The regulation of microtubules dynamics during mitosis and cytokinesis is tightly controlled by several proteins that act for the proper progression of these processes. Many studies have demonstrated that overstabilisation but also a decrease in the stability of MTs during mitosis result in an increased tendency to monopolar spindle formation (Tanenbaum & Medema 2012). Since we have shown that MARK4 regulates microtubules and is always active during mitosis, we can hypothesize that MARK4 plays an important role in bipolar spindle assembling, by regulating MTs dynamics. In addition the localisation of active MARK4 in the midbody, especially at the borders of the midbody in the lateral constriction zone, also suggests that MARK4 isoforms might be involved in the regulation of microtubule disassembly that starts just in the lateral midbody zone during abscission (Steigemann & Gerlich 2009).

Taken together, our data on phosphorylated-MARK4 and overexpression show that active MARK4 plays a key role in dynamic assembly/disassembly of the MT network during cell cycle and in particular in the organisation of the mitotic spindle from prophase to anaphase and the midbody at cytokinesis.

The roles of MARK4 likely extend to other fundamental cytoskeleton structures, such as intermediate filaments, similar to other centrosomal kinases. For example, Aurora B not only controls centrosome separation, spindle formation and mitotic progression, but also phosphorylates vimentin during cytokinesis and thereby controls the assembly of intermediate filaments (Li & Li 2006).

Interestingly, in fibroblasts overexpressed MARK4L co-localises with vimentin in filament structures. Furthermore, this co-localisation is particularly evident in the perinuclear zone, and in some overexpressing cells, vimentin filaments appear more unorganised as compared to those in GFP-transfected cells, and show the formation of bundling structures (Figure 11-12). These alterations are clearly imputable to the kinase activity of MARK4L since overexpression of kinase

dead mutant did not remodel the intermediate filaments. In agreement with this data the overexpressed MARK4L present in these structures is demonstrated to be phosphorylated and thus able to exert its kinase function.

In contrast to MARK4L, overexpressed MARK4S co-localises with vimentin to a lesser extent and only in few cells (about 5% of overexpressing fibroblasts). Interestingly, despite the two isoforms are strictly intermingled, as inferred by their shared subcellular localisation and by the common activity on MTs, their different co-localisation with vimentin structures might support a different role for them in the regulation of other cytoskeletal structures. A similar behaviour has been observed for the two isoforms of Oxysterol-binding-protein (OSPB)-related protein 4 (ORP4). Indeed ORP4-S co-localises with vimentin altering the structure of intermediate filaments, whereas ORP4-L displays a diffuse staining pattern (Wang *et al.,* 2002). The strikingly different behaviour of MARK4L and MARK4S as regards vimentin co-localisation deserves further biochemical studies to be confirmed and to be interpreted.

The three major components of the cytoskeleton interact with each other and their connections are important for many different functions including cell polarisation and motility. In particular the interaction between intermediate filaments and microtubules has been the focus of many studies highlighting the relevance of MTs for the correct organisation of intermediate filaments (Liem 2013). Several proteins have been implicated in the connection between intermediate filaments and microtubules, and among them the role played by APC (Adenomatous polyposis coli) has been recently disclosed (Sakamoto *et al.,* 2013). In particular it has been demonstrated that APC is required for microtubule interaction with intermediate filaments and for microtubule-dependent rearrangements of intermediate filaments during migration processes (Sakamoto *et al.,* 2013). Our data suggest that MARK4L might be considered another bridging protein between the microtubules and the intermediate filament networks. In particular the long isoform could regulate the re-organisation of these cytoskeleton structures that occurs during mitosis and cytokinesis. Phosphorylation is the main mechanism that regulates intermediate filaments functions (Omary *et al.* 2006) and it has been demonstrated that during mitosis intermediate filaments are phosphorylated in a spatio-temporal manner by distinct protein kinases (Sihag *et al.,* 2007). In particular it has been reported that vimentin is phosphorylated by Cdk1 from prometaphase to metaphase (Tsujimura *et al.,* 1994) and by Aurora-B (Goto *et al,.* 2003) and Rho-kinase (Goto *et al.,* 1998) from anaphase to the end of mitosis. These phosphorylations regulate the structure and the separation of the vimentin filaments.

Based on our data we are incline to hypothesize that MARK4L may act at this level, by phosphorylating vimentin and thus re-modelling the filament structure. As currently we do not have

any direct evidence of MARK4L-dependent phosphorylation, we are keeping into account as alternative mechanism that the re-organisation of intermediate filaments may be effected, through the regulation of MT-dependent transport, being thus connected to MARK4L indirectly. Interestingly bundle-like structures have been observed following overexpression of MAPs, and they have been thought to result from the stabilisation of intermediate filaments and their accumulation at the perinuclear zone (Trinczek *et al.,* 1999). MAP overexpression inhibits the binding of motor proteins to MTs and thus prevents MT-based motility of intermediate filaments (Trinczek *et al.,* 1999). Joining this evidence to that herein acquired on MTs destabilization, likely through increased MAPs phosphorylation, in MARK4 overexpressing cells, a reshaping of intermediate filaments, as described by Trinczek, might be envisaged. In our system MARK4 overexpression by disrupting the MT scaffold might inhibit the movement of intermediate filaments along MTs thereby affect the dynamics of intermediate filaments.

In conclusion, overexpression experiments demonstrate the important role played by MARK4 isoforms in the regulation of cytoskeleton structures. A prevalence of active MARK4 is required during the dynamic phases of cell division. Moreover, the finding that MARK4L co-localises with intermediate filaments highlights the role of this protein in connecting different types of cytoskeletal filaments, as it has been reported for other centrosomal serine-threonine kinases. The overall data merge in demonstrating the dynamic involvement of MARK4L and MARK4S in structures like centrosomes, midbody and cytoskeleton, that are crucial for mitosis and cytokinesis. The emerging multifaceted role of MARK4 kinase in cell cycle paves the way for future studies aimed at establishing its interactions with the multiple proteins residing at the different cytoskeleton, centrosome and centrosome-related structures and their dynamic change during the cell cycle.

Perspectives

We plan to develop this project on the two points of MARK4 phosphorylation and its co-localisation with vimentin which we consider the most novel results.

As concerns MARK4 switch to the active form, further advances will be focused to the following aim:

- deepen the phosphorylation of MARK4 during interphase by co-staining with other cell cycle markers (PCNA, CENP-F for example) to delineate when phospho signal is first detected.

In regards to the relationship of MARK4 with vimentin, experiments are in progress to:

- confirm the interaction between MARK4L and vimentin by co-immunoprecipitation and *in vitro* binding assays;
- verify the possible phosphorylation of vimentin by MARK4L;
- analyse the soluble/insoluble ratio of vimentin following overexpression of MARK4.

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