



# Microtubule-associated protein/microtubule affinity-regulating kinase 4 (MARK4) plays a role in cell cycle progression and cytoskeletal dynamics



Davide Rovina<sup>a</sup>, Laura Fontana<sup>a</sup>, Laura Monti<sup>a</sup>, Chiara Novielli<sup>a</sup>, Nicolò Panini<sup>b</sup>, Silvia Maria Sirchia<sup>a</sup>, Eugenio Erba<sup>b</sup>, Ivana Magnani<sup>a,1</sup>, Lidia Larizza<sup>a,\*,1</sup>

<sup>a</sup> Medical Genetics, Department of Health Sciences, Università degli Studi di Milano, via A. di Rudinì 8, 20142 Milan, Italy

<sup>b</sup> Flow Cytometry Unit, IRCCS, Istituto di Ricerche Farmacologiche Mario Negri, Via La Masa 19, 20156 Milan, Italy

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## ABSTRACT

MARK4 is a serine-threonine kinase that phosphorylates MAP proteins, increasing microtubule dynamics. MARK4 differs from the other members of the MARK family for encoding two isoforms (MARK4L and MARK4S), differentially expressed in the nervous system, and for the peculiar localisation at the centrosome and the midbody. By cytofluorimetric analysis we showed that MARK4 is expressed throughout the cell cycle and preferentially activated during mitosis. Depletion of MARK4S affected the morphology and proliferation of fibroblasts and glioma cells, as the percentages of cells in S and G2/M phases were reduced and the percentage of cells in G1 was increased. In MARK4S silenced cells, centrosomes were duplicated and positioned apically to the nucleus, indicating that the centrosome cycle was altered and the cells arrested in G1 phase. Overexpression of MARK4L or MARK4S reduced the density of the microtubule network, confirming microtubules as the main target of MARK4, and revealed a novel co-localisation of MARK4 and vimentin. Taken together, our data confirm that MARK4 is a key component in the regulation of microtubule dynamics and highlight its major role in cell cycle progression, particularly at the G1/S transition. The co-localisation of vimentin and MARK4L suggests that MARK4 has a wide-ranging influence on cytoskeleton.

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## Introduction

Microtubule-associated protein/microtubule affinity-regulating kinase 4 (MARK4) is a member of a highly conserved protein kinase family that phosphorylates microtubule (MT)-associated proteins (MAPs) (including Tau, MAP2 and MAP4), which causes these proteins to detach from MTs and increases MT dynamics (Drewes et al., 1998; Trinczek et al., 2004).

The MARK4 gene encodes two alternatively spliced isoforms, MARK4S and MARK4L, which differ for the presence of exon 16 (Kato et al., 2001). The MARK4S mRNA contains all the 18 exons and encodes a 688 amino acid protein, which molecular weight is around 75 kiloDalton (kDa). The MARK4L mRNA arises from the skipping of exon 16, that causes the shift of the reading frame and changes the stop codon determining the synthesis of a longer

protein (752 amino acids), with a predicted molecular weight of 82.5 kDa. The two MARK4 proteins have an identical structure apart from the C-terminus, as MARK4L presents the MARK family characteristic kinase-associated 1 domain (KA1), while MARK4S has a unique domain with no homology with any other known structure (Kato et al., 2001; Moroni et al., 2006). In addition the two isoforms are differentially expressed in human tissues, particularly in the central nervous system (Beghini et al., 2003; Kato et al., 2001). Several studies report that MARK4S is expressed in normal brain tissue and neurons, suggesting that this isoform has a role in neuronal differentiation (Magnani et al., 2011; Moroni et al., 2006). Conversely, MARK4L is up-regulated in hepatocarcinoma cell lines (Kato et al., 2001), glioma cells and neural progenitor cells, pointing to a possible role of this isoform in cell proliferation (Beghini et al., 2003). Recently, we highlighted a subverted MARK4L/MARK4S ratio in glioblastoma and glioblastoma-derived cancer stem cells, that recapitulates the MARK4 expression profiling of neural stem cells with prevalence of MARK4L. This suggests that the expression of the two MARK4 isoforms is tightly regulated during the proliferation/differentiation of neural stem cells and that

\* Corresponding author. Tel.: +39 0250323206; fax: +39 0250323026.

E-mail address: [lidia.larizza@unimi.it](mailto:lidia.larizza@unimi.it) (L. Larizza).

<sup>1</sup> These authors contributed equally to this work.

changes in their expression levels may be a molecular marker of tumour transformation (Magnani et al., 2011).

The subcellular localisation of MARK4 also indicates it has a role in cell proliferation and cell cycle regulation. Trinczek et al. (2004) first described specific localisation of exogenous MARK4 with the centrosome and MTs, which is in contrast to the cytoplasmic localisation of the other members of the MARK family. We previously demonstrated that endogenous MARK4L and MARK4S associate with centrosomes and are also localised at the midbody in normal and glioma cells (Magnani et al., 2009, 2011). A role of MARK4 at the centrosome was also indicated by a study that investigated primary cilium formation, a process that occurs in G0/G1 phase and involves MARK4 via its interaction with centriolar proteins (Kuhns et al., 2013). Furthermore, overexpression of MARK4 in post-mitotic rat hippocampal neurons leads to tau hyperphosphorylation, which causes defects in synapses and dendritic spines that are characteristic of Alzheimer's Disease and other tauopathies. This indicates that MARK4 inhibitors are potential therapeutic tools (Yu et al., 2012). While the role of MARK4 in cycling and differentiated cells is being progressively elucidated, activation of MARK4 has not been extensively investigated. MARK4 activity, like that of all MARKs, is finely tuned by various post-translational modifications, including ubiquitination and phosphorylation, and by interactions with other proteins (Timm et al., 2008). In particular, MARK4 is activated by phosphorylation of the conserved threonine residue (Thr214) in the activation loop (T-loop) by LKB1 (Liver Kinase B1) and MARKK/TAO-1 (MARK Kinase/Thousand And One amino acids), whereas MARK4 is inhibited by phosphorylation of the Ser218 residue (Bright et al., 2009). Moreover, polyubiquitination does not control MARK4 stability. Rather, it inhibits phosphorylation and activation of MARK4 by LKB1 (Al-Hakim et al., 2008).

The involvement of MARK4 in cell proliferation and the MT network has already been reported (Trinczek et al., 2004), but it remains unknown whether the functions of MARK4 vary throughout the cell cycle. To investigate this issue we monitored the expression and the activation status of MARK4L and MARK4S during interphase and mitosis and performed functional experiments in which MARK4 was silenced or overexpressed in fibroblasts and glioma cells.

Taken together, our results show that MARK4 plays a role throughout cell division and in cytoskeletal dynamics of both non-cancerous and cancerous cells.

## Materials and methods

### Cell cultures

Human adult fibroblasts were grown as previously described (Magnani et al., 2011). The G32 glioblastoma and G157 oligoastrocytoma cell lines were selected from a panel of extensively characterised human primary glioma cell lines obtained from post-surgical specimens (Beghini et al., 2003; Magnani et al., 1994; Perego et al., 1994; Roversi et al., 2006).

Fibroblasts were obtained from "Cell Line and DNA Biobank from Patients Affected by Genetic Diseases" (G. Gaslini Institute, Genoa).

### RNA interference (RNAi)

RNAi was performed using Customised Stealth RNAi<sup>TM</sup> siRNA duplexes (Invitrogen, Paisley, UK), which were transfected by Lipofectamine<sup>TM</sup> RNAiMAX (Invitrogen) as instructed by the manufacturer. Transfection efficiency was assessed using a Cy3-luciferase siRNA (short interference RNA) (Upstate, Billerica, MA, USA), that is detectable by fluorescence microscopy. To define the optimal knockdown conditions, the knockdown efficiencies of

various concentrations of different siRNA duplexes were assessed by immunoblotting.

Due to the impossibility to obtain a MARK4L-specific siRNA, the Stealth RNAi<sup>TM</sup> siRNA #10 (MARK4S siRNA) (Invitrogen) was selected and used at a concentration of 60 nM to silence the sole MARK4S isoform. A siRNA negative control (siRNA#1 Ambion; Paisley, UK) was used, at the same concentration of the MARK4S siRNA, to determine the non-specific effects of siRNA-treatment on cells. To exclude off-target effects of siRNA treatment a second MARK4 siRNA (Silencer Validated siRNAs<sup>TM</sup>, Ambion), targeting a different sequence of MARK4 mRNA, was used. Cells were transfected by reverse protocol, and forward transfection was repeated 48 h after plating to extend RNAi till 72 h.

### Plasmids

The mammalian expression construct for MARK4L with an N-terminal Xpress<sup>TM</sup> tag was previously described (Magnani et al., 2011). Full-length MARK4S cDNA was PCR-amplified from the cDNA clone OCAB05050C0222D (Source BioScience, Cambridge, UK), and was then cloned into the pcDNA4/HisMax vector (Invitrogen). The pcDNA4/HisMax vector includes a cleavable N-terminal Xpress<sup>TM</sup> tag, of about 4 kDa, that can be detected using a specific mouse anti-Xpress<sup>TM</sup> antibody (Invitrogen).

MARK4L and MARK4S cDNAs were sub-cloned into pAcGFP1-C2 (Clontech, Mountain View, California, USA) to obtain GFP-MARK4L and GFP-MARK4S constructs, respectively. MARK4L and MARK4S kinase dead (KD) mutants were obtained by mutating the Thr214 and Ser218 residues to alanine using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. The generated vectors were sequenced to confirm that MARK4L or MARK4S cDNA was correctly orientated, in frame with the fusion tag and without sequence variations.

For transfection, fibroblasts were seeded onto chamber slides for 1 day to achieve 80% confluency and were then transfected with 2–3 µg plasmid DNA, using Lipofectamine LTX and Plus Reagent (Invitrogen), according to the manufacturer's instructions. Cells were subjected to immunofluorescence analysis 24 h after transfection.

### Immunoblotting

Proteins (15–25 µg) extracted using lysis buffer were resolved under reducing conditions and subjected to immunoblotting as previously described (Magnani et al., 2011).

Rabbit anti-MARK4L (GenScript Corporation, Piscataway, NJ, USA), goat anti-MARK4S (ab5262; Abcam, Cambridge, UK) and mouse anti-GAPDH (ab8245; Abcam) antibodies were used as previously described (Magnani et al., 2011). For Western blotting with the anti-MARK 1 + 2 + 3 + 4 antibody (phospho Thr215) (ab111437; Abcam), the antibody was diluted 1:1000 in TBS-T (50 mM Tris, 150 mM NaCl, 0.3% Tween 20), membranes were washed in TBS-T and TBS-T containing 5% bovine serum albumin (BSA) was used to block non-specific binding.

Blots were scanned using the Gbox Chemi XT4 system (Syngene, Cambridge, UK) and 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.

### Immunofluorescence

Immunofluorescence experiments were performed as previously described (Magnani et al., 2009, 2011). Rabbit anti-MARK4L 1:200 (GenScript Corporation), rabbit anti-MARK 1 + 2 + 3 + 4

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