Functional activities and cellular localization of the ezrin, radixin, moesin (ERM) and RING zinc finger domains in MIR

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Received 4 August 2003; revised 3 September 2003; accepted 3 September 2003

First published online 19 September 2003

Edited by Lev Kisselev

Abstract Myosin regulatory light chain interacting protein (MIR) belongs to the ezrin, radixin, moesin (ERM) family of proteins involved in membrane cytoskeleton interactions and cell dynamics. MIR contains, beside the ERM domain, a RING zinc finger region. Immunocytochemistry showed that full-length MIR and the subdomains localize differently in cells. Cell fractionation revealed a similar distribution of full-length MIR and the RING domain protein in the Triton X-100-insoluble fraction. The neurite outgrowth inhibitory activity of MIR was attributed to the RING domain. MIR levels were controlled in the cells depending on the intact RING domain and proteasome activity. The dynamic regulation of MIR contributes to its effects on neurite outgrowth and cell motility.

Key words: Myosin regulatory light chain interacting protein; Ezrin, radixin, moesin (ERM) proteins; Neurite outgrowth; Ubiquitination

1. Introduction

The ERM proteins, ezrin, radixin and moesin, link the actin cytoskeleton to membrane-bound proteins located at different membrane sites, i.e. microvilli, membrane ruffles and cell–cell contact points [1–3]. These proteins have an actin binding site in the C-terminal part, and a FERM domain in the NH-terminal part. The FERM domain has been found in different proteins and is thought to be mediate protein–protein and protein–membrane interactions [4]. The FERM proteins can influence cell adhesion and signal transduction events through phosphorylation and interaction with phosphoinositides and the Rho family of proteins [1–3]. The ERM subgroup of proteins is known to interact with other proteins, such as CD44 and ICAM-1, -2 and -3, which helps in establishing membrane specializations [5]. In keeping with their known functions in controlling membrane–cytoskeleton interactions, the ERM proteins are usually localized close to the cell membrane. However, the exact mechanisms by which the ERM proteins mediate cellular interactions and influence cell motility are not fully understood.

We have recently described a novel ERM family protein, myosin regulatory light chain interacting protein (MIR), which has an ERM domain at the N-terminus but which lacks an actin binding site. In contrast, MIR has a RING zinc finger domain in the C-terminal region [6]. Studying PC12 cells, we observed that MIR inhibited neurite outgrowth induced by nerve growth factor. This effect was ascribed to the interaction of MIR with the myosin regulatory light chain (MRLC). To learn more about the mechanism underlying the action of MIR, we studied here which part of MIR, the ERM or the RING domain, is mainly involved in neurite outgrowth. We also determined the distribution of the full-length MIR and C- or N-terminally truncated MIR proteins in cells, and compared them with that for the ERM protein moesin. The results show that the neurite outgrowth inhibitory activity of MIR resides in the RING domain. The RING domain and the full-length MIR were mainly codistributed in the Triton X-100-insoluble fraction, whereas the ERM domain was also found in the soluble fraction. The ERM and RING domains were also differentially localized in cells and apart from moesin. The levels of MIR were downregulated in cells in a process depending on the activity of the proteasome and an intact RING domain, which exhibited ubiquitination activity.

2. Materials and methods

2.1. Plasmids

pEGFP-ERM (encoding aa 1–295 of MIR), pEGFP-RING (encoding aa 296–445 of MIR) and pEGFP-MIR C387A were generated from pEGFP-MIR [6] by polymerase chain reaction and confirmed by sequencing analysis. pEGFP was used as control.

2.2. Transfection, neurite outgrowth and solubility assay

Neuroblastoma N2-A cells were transfected with the following plasmids using Fugene (Roche): green fluorescent protein (GFP), GFP-ERM, GFP-RING and GFP-MIR. After 40 h, the number of cells bearing neurites longer than twice the cell diameter was scored. In each experiments more than 100 cells were counted, and data are expressed as mean ± S.E.M. For statistical analyses Student's *t*-test was used.

For solubility assays, COS-7 cells were transfected with the different plasmids. After 40 h the plates were washed once with phosphate-buffered saline (PBS). Cells were scraped into lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 3 mM EGTA, 0.5% Triton X-100 and protease inhibitors (Roche)), and kept for 5 min on ice. 30 μl were saved, mixed with 10 μl 4×sodium dodecyl sulfate (SDS) loading buffer and sonicated (total cell lysate). The remaining cell lysate was centrifuged at 13 000×*g* for 10 min, and the supernatant was mixed with 4×SDS loading buffer (soluble fraction); the pellet was dissolved in 1×SDS loading buffer and sonicated (insoluble fraction). Equal amounts were then analyzed on 10% SDS–polyacryl-

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Abbreviations: MIR, myosin regulatory light chain interacting protein; XIAP, X-linked inhibitor of apoptosis protein; MRLC, myosin regulatory light chain

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)01010-X

amide gel electrophoresis (PAGE) and subsequent Western blotting using a monoclonal anti-GFP antibody (Roche) and horseradish peroxidase-coupled anti-mouse secondary antibodies (Jackson).

2.3. Ubiquitination assay

N2-A cells were transfected with expression vectors for His-tagged ubiquitin [7] and either GFP, GFP-MIR, GFP-ERM, GFP-RING or MIR mutated in the RING domain (Cys387Ala). The mutation of the cysteine residues has been shown to abolish the ubiquitin ligase activity of proteins containing RING zinc fingers [8]. After 24 h, 10 μM MG132 in serum-free medium was added for 6 h and cells were lysed in ice-cold RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS and protease inhibitors). Immunoprecipitation was carried out with polyclonal anti-GFP antibodies (1:250, Clontech) and protein G-Sepharose. The beads were washed three times, and the pellet was boiled in 1×SDS–PAGE buffer. Cell lysates and immunoprecipitates were analyzed by SDS–PAGE followed by Western blotting using anti-GFP (1:1000, Roche) or anti-ubiquitin antibodies (1:1000, Affinity Research).

2.4. Immunocytochemistry

COS-7 cells were seeded on coverslips and transfected using Fugene. Sixteen hours after transfection the cells were washed with PBS, fixed in 4% paraformaldehyde and permeabilized in PBS/5% normal goat serum/0.05% Triton X-100. The cells were stained with anti-moesin antibodies (1:200, Upstate) and Cy3-tagged goat anti-rabbit secondary antibodies (Jackson). Signals were visualized with a Zeiss Axiophot microscope and analyzed with deconvolution software (Zeiss).

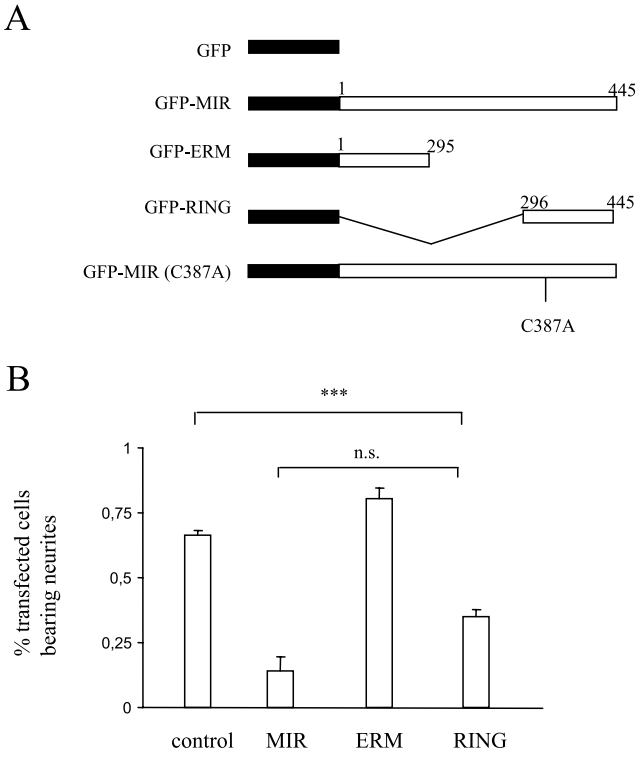

Fig. 1. Different MIR constructs and their effects on neurite outgrowth. A: The following MIR constructs were used in the present study. The numbers indicate the amino acid position in the protein. C387A shows mutated MIR protein with the cysteine residue replaced with alanine. B: N2-A neuroblastoma cells were transfected with the different constructs. Cells were incubated without serum for 16 h and the number of cells bearing neurites was determined. Experiments were done in triplicate, and the values represent mean ± S.E.M. ***$P \leq 0.01$ for control vs. the RING construct, n.s. not significant for control vs. the ERM construct.

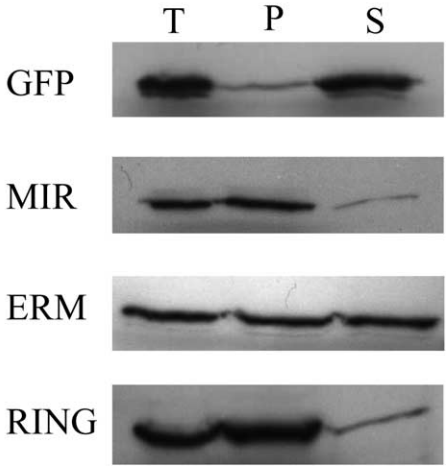

Fig. 2. Subcellular distribution of different full-length and truncated MIR proteins. COS-7 cells were transfected with the MIR constructs and subcellular compartments obtained as described in Section 2. Proteins were analyzed by Western blotting using an anti-GFP antibody.

3. Results and discussion

3.1. Activities of different MIR constructs in the control of neurite outgrowth

We have recently identified MIR, a novel member of the ERM protein family, as a potent inhibitor of neurite outgrowth expressed in brain [6,9]. In order to reveal which part of MIR is responsible for this inhibition, we generated deletion constructs fused to GFP and tested them in an assay for neurite outgrowth together with the full-length protein and GFP serving as control (Fig. 1). In accordance with earlier results, full-length MIR inhibits neurite outgrowth dramatically compared to the GFP control. The RING domain also exhibits neurite outgrowth inhibition, although somewhat attenuated compared to MIR. The difference between MIR and the RING domain only was not statistically significant ($P > 0.1$, $n = 300$). In contrast, the ERM domain did not decrease neurite outgrowth (Fig. 1). There was a slight increase in the number of cells bearing neurites expressing the ERM domain, but the difference was not statistically significant ($P > 0.1$, $n = 300$). Thus, the neurite outgrowth inhibitory activity in MIR is associated with the RING domain, whereas the ERM domain does not significantly contribute to this effect.

3.2. Subcellular fractionation of different MIR constructs

To study whether the different properties of the subdomains in MIR are related to their different localization in cells, we transfected COS-7 cells with the different constructs and analyzed the solubility of the proteins expressed. Fig. 2 shows that a large proportion of full-length MIR resides in the Triton-insoluble fraction, whereas only little protein was detected in the soluble fraction. This result was paralleled by the RING domain, which remained in the insoluble fraction with almost no protein in the soluble part. In contrast, the ERM domain of MIR was recovered about equally in the Triton-soluble and in the insoluble fractions (Fig. 2). These data correspond to those observed for the ERM protein ezrin.

Ezrin has a readily soluble N-terminus, i.e. the ERM domain, and a rather insoluble C-terminal part [10].

3.3. Subcellular localization of MIR constructs

To study the subcellular localization of the different subdomains of MIR cells in more detail, we performed immunocytochemical stainings of COS-7 cells after transfection of the constructs (Fig. 3). The data showed that full-length MIR and the ERM domain exhibited a similar distribution in the cells, with a punctuated staining pattern in the cells (Fig. 3d,g). The RING domain on the other hand was accumulated in subcellular structures in the cytoplasm (Fig. 3j). These resembled aggregates observed previously with other RING finger-containing proteins, such as the X-linked inhibitory of apoptosis protein (XIAP). In XIAP the occurrence of such intracellular aggregates of the protein was associated with proteasome activity and the presence of the RING domain [10]. To study whether the distribution of the RING domain in MIR is linked to its possible activity as ubiquitin ligase in cells, we set out to mutate one of the cysteine residues. These residues have been shown to be required for the intact function of the RING domain in different RING zinc finger-containing proteins [8]. The novel MIR construct, harboring a mutation in cysteine residue 387, C387A, in the RING domain (Fig. 1A), was then transfected into COS-7 cells. The results showed that the C387A mutant MIR protein showed a largely similar dis-

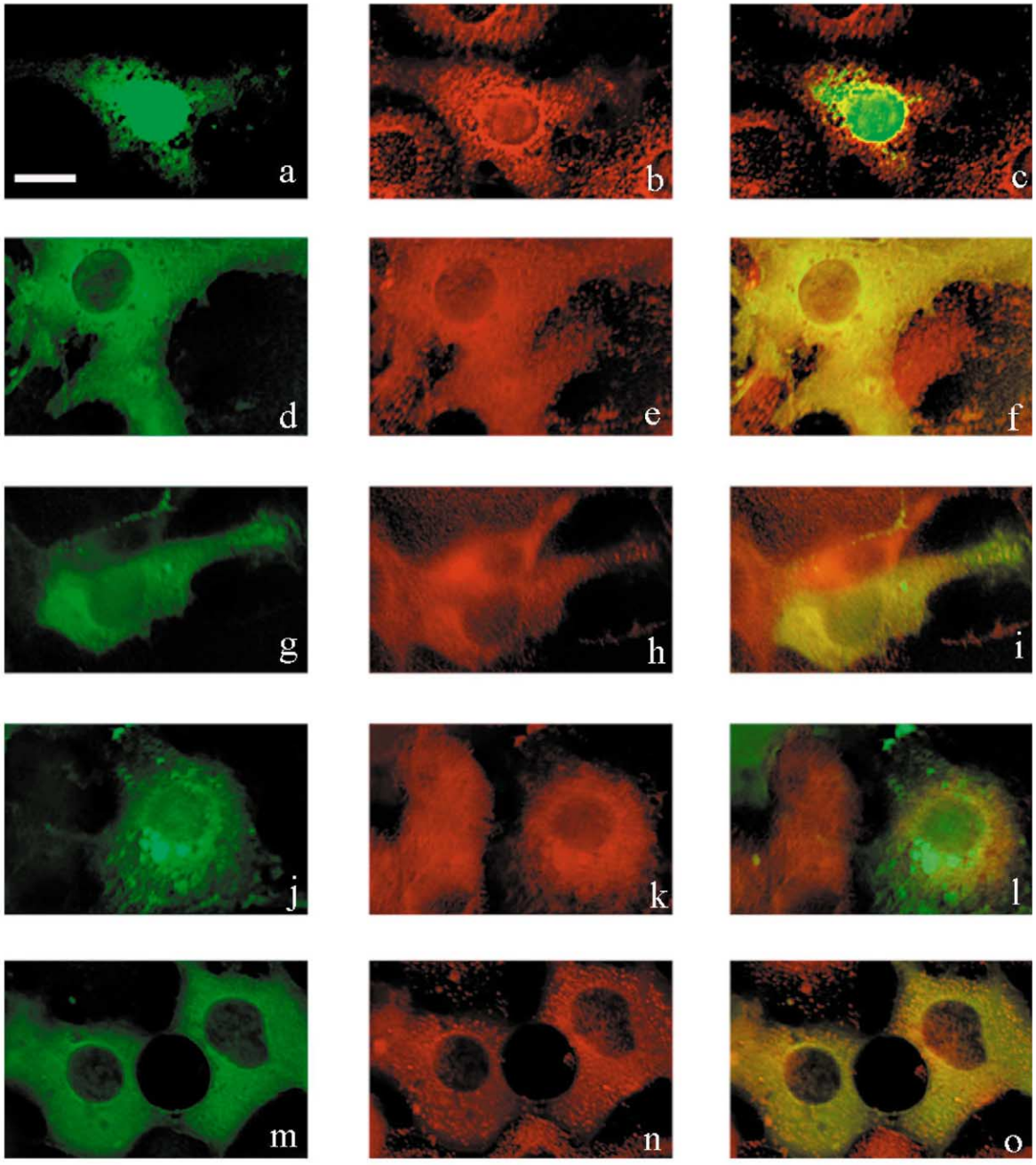

Fig. 3. Cellular localization of full-length and truncated MIR proteins. COS-7 cells were transfected and visualized using the following constructs: GFP (a–c), GFP-MIR (d–f), GFP-ERM (g–i), GFP-RING (j–l) and GFP-MIRC387A (m–o). Left panel, GFP fluorescence. Middle panel, an anti-moesin antibody was used for staining. Right panel, merged images of GFP fluorescence and moesin staining. Scale bar, 15 μm.

tribution to that of wild-type MIR (Fig. 3m), and different from the protein construct with intact RING domain only.

To further characterize the localization of the different MIR constructs, the cells were also stained for the ERM protein moesin. The results showed that moesin was distributed in a rather similar pattern to those of the ERM-containing and full-length MIR proteins (Fig. 3). This was also observed for the C387A mutant MIR protein (Fig. 3). However, the protein bearing an intact RING domain was not localized with moesin nor with any other of the proteins expressed (Fig. 3). To study whether moesin localizes in the same compartment as MIR, we analyzed the cells using the deconvolution method for protein detection. The results showed that there was no exact colocalization of moesin and of MIR and its subdomains in cells. The same result applied to double stainings using an anti-ezrin antibody (data not shown). This probably reflects the partly different functions of the two ERM proteins and MIR, with moesin and ezrin being involved in protein–protein interactions different from those of MIR.

Taken together the data show that the ERM domain alone is distributed in a similar fashion to that of full-length MIR in cells. In contrast, the RING domain was present in distinct subcellular structures in a dotlike fashion. The biochemical analyses for solubility, however, showed that full-length MIR is mainly insoluble similar to the RING domain, whereas the ERM domain is rather soluble. The results of these two types of studies are not necessarily in conflict and they reflect different aspects and behavior of the same protein and its subdomains. The results can be interpreted to indicate that the ERM domain in MIR mediates higher solubility of the protein, whereas the RING domain is involved in protein–protein interactions with a change in the cellular localization of the protein.

3.4. The RING domain in MIR mediates ubiquitination

The analysis of the expression of the different constructs revealed that the levels of the MIR and the RING domain proteins were downregulated in cells (Fig. 4A). In contrast the protein bearing the ERM domain was rather stable in the cells (Fig. 4A). Addition of the proteasome inhibitor MG132, however, blocked the observed decline in MIR and RING domain proteins. This indicates that MIR and the RING domain proteins are controlled by the activity of the proteasome and can be downregulated probably by ubiquitination. In keeping with this we observed that the C387A mutant of MIR lacking the intact functional RING domain was stable in the cells (Fig. 4A). The C387A mutant protein was also equally expressed in the absence and presence of MG132.

To study the role of ubiquitination in the function of the RING construct, we performed a ubiquitination assay for the protein. The results showed that under the conditions of protein decline, the construct containing the RING domain was readily ubiquitinated, as shown by the presence of ubiquitin chains in the protein (Fig. 4B). This process was dependent on the Cys387 residue in the RING domain as evident by data showing no ubiquitination using the mutant protein (Fig. 4B). These results show that the RING domain is ubiquitinated and downregulated by the proteasome. Similar data were also observed for the full-length MIR although the presence of ubiquitin chains was harder to detect due to the high molecular weight of the fusion EGFP-MIR protein (data not shown).

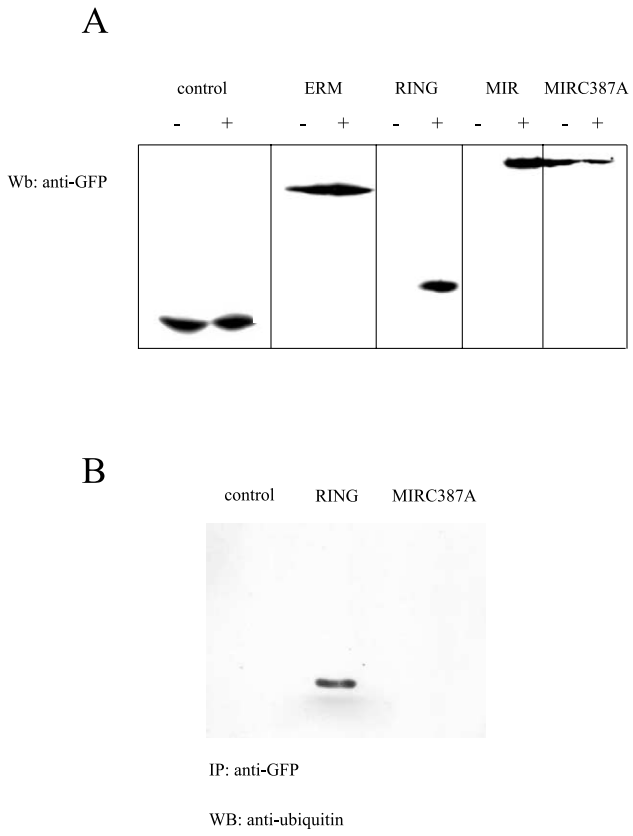

Fig. 4. Expression of full-length and truncated MIR proteins and their regulation by proteasome activity. A: N2-A cells were transfected with the different MIR constructs and incubated in the absence or presence of 10 μM MG132 to inhibit proteasomes. Proteins were detected by Western blotting using anti-GFP antibodies. Note the downregulation of full-length MIR and RING-containing proteins, which was inhibited by MG132. B: Transfected N2-A cells were incubated in the presence of 10 μM MG132. Immunoprecipitation was carried out using anti-GFP antibodies and protein G-Sepharose. Immunoprecipitates were analyzed by Western blotting with anti-ubiquitin antibodies to reveal ubiquitinated proteins. Note the ubiquitination of intact RING-containing protein but not of the C387A mutant.

3.5. Functional considerations regarding the MIR subdomains

Taken together we have shown that the two major protein motifs in MIR, the ERM and the RING finger domain, exhibited a differential functional activity in regulating neurite outgrowth in N2-A cells. The known neurite outgrowth inhibiting activity of MIR was associated with the function of the RING domain but not with that of the ERM region. The two truncated forms of MIR were also distributed in different compartments, as shown by cell fractionation and localization of the GFP fusion proteins in COS-7 cells. The ERM domain was almost evenly distributed within the cell, whilst the RING finger domain was found in the particular fraction. In keeping with these results, the ERM domain was soluble, whereas the RING domain was recovered in the Triton X-100-insoluble fraction, as shown also for full-length MIR. Functionally, the protein bearing the RING domain was found to be downregulated in cells, which could be attenuated by the presence of the proteasome inhibitor MG132. This was also observed

with the full-length MIR protein but not with the ERM domain nor with the C387A mutant protein, lacking ubiquitin ligase activity.

In this study we show that the expression of MIR can lead to its own degradation. We recently observed that MIR can also ubiquitinate the binding partner MRLC with effects on neurite outgrowth [11]. The present study shows that the ubiquitin ligase activity of the RING can also be directed towards the protein itself indicating that the degradation of MIR can be regulated by autoubiquitination. A similar dual mode of regulation has also been described for other RING finger-containing proteins such as the inhibitor of apoptosis proteins [12,13]. In this respect, XIAP was found to be able to ubiquitinate itself and also other substrates such as caspase-3 [13,14]. However, it is not fully understood how these two processes are interconnected in RING finger-containing proteins, and whether the different substrates can compete with each other for ubiquitination. We are currently studying which other proteins can be ubiquitinated by the RING domain in MIR, and whether these interactions affect the dynamics and function of MIR in the regulation of cell motility. The present results indicate a dynamic regulation of the levels and activity of MIR, which has a bearing on its cellular functions in neurite outgrowth and cell motility.

Acknowledgements: This study was supported by the Swedish Cancer Foundation (Cancerfonden), Barncancerfonden and Uppsala University.

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