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Research article

SUBCELLULAR LOCALIZATION OF FULL-LENGTH HUMAN MYELOID LEUKEMIA FACTOR 1 (MLF1) IS INDEPENDENT OF 14-3-3 PROTEINS

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Abstract: Myeloid leukemia factor 1 (MLF1) is associated with the development of leukemic diseases such as acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). However, information on the physiological function of MLF1 is limited and mostly derived from studies identifying MLF1 interaction partners like CSN3, MLF1IP, MADM, Manp and the 14-3-3 proteins. The 14-3-3-binding site surrounding S34 is one of the only known functional features of the MLF1 sequence, along with one nuclear export sequence (NES) and two nuclear localization sequences (NLS). It was recently shown that the subcellular localization of mouse MLF1 is dependent on 14-3-3 proteins. Based on these findings, we investigated whether the subcellular localization of human MLF1 was also directly 14-3-3-dependent. Live cell imaging with GFP-fused human MLF1 was used to study the effects of mutations and deletions on its subcellular localization. Surprisingly, we found that the subcellular localization of full-length human MLF1 is 14-3-3-independent, and is probably regulated by other as-yet-unknown proteins.

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Abbreviations used: AML – acute myeloid leukemia; CMV – cytomegalovirus; CSN3 – subunit 3 of the COP9 signalosome; DMEM – Dulbecco's modified Eagle's medium; GFP – green fluorescent protein; MADM – MLF1 adaptor molecule; Manp – MLF1-associated nuclear protein; MDS – myelodysplastic syndrome; MLF1 – myeloid leukemia factor 1, MLF1IP – MLF1-interacting protein; NES – nuclear export sequence; NLS – nuclear localization signal; NPM – nucleophosmin

Key words: MLF1, 14-3-3, Binding site, Protein-protein interactions, Subcellular localization, Acute myeloid leukemia, Myelodysplastic syndrome, Site-directed mutagenesis, Confocal microscopy, Live cell imaging

INTRODUCTION

Myeloid leukemia factor 1 (MLF1) is a 30-kDa protein that became of interest because of its association with the development of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). For example, it has been reported to be expressed in AML and MDS patients that show an enhanced malignant phenotype [1]. In addition, MLF1 has been found to be expressed in lung squamous cell carcinoma [2], while the *MLF1* gene is amplified in oesophageal cancers [3]. Further indications of the potential oncogenic activity of MLF1 are its inhibition of erythropoietin-induced differentiation and $p27^{Kip1}$ accumulation [4] and the observation that a disturbance of MLF1 shuttling affects p53 stability and susceptibility to transformation [5]. In addition, recent results suggest that MLF1 is critical for normal and pathological blood cell development [6, 7].

MLF1 was originally described in a translocation between *MLF1* on chromosome 3 and nucleophosmin (*NPM*) on chromosome 5, which yields the oncogene *NPM-MLF1* [8]. The resulting fusion protein NPM-MLF1 consists of the 175 N−terminal amino acids of NPM and almost the entire MLF1 protein (17-269) and is localized mainly in the nucleus [8]. This localization is very likely caused by the NPM portion of the NPM-MLF1 fusion, since NPM is a nucleolar phosphoprotein [9]. The nuclear localization suggested that the transforming activity of MLF1 in AML was due to its ectopic expression, which may cause an enhanced interaction with nuclear protein partners [10]. Interestingly, although MLF1 localizes to the cytoplasm in non-hemopoietic cells [8], it preferentially resides in the nucleus of hemopoietic cells [8-11]. Beyond these reports, information on the physiological function of MLF1 is limited and is derived mostly from studies identifying MLF1 interaction partners, such as CSN3 [12], MLF1IP [13, 14], MADM [15], Manp, and the 14-3-3 proteins [11, 5-17].

14-3-3 proteins are eukaryotic proteins of 25 to 30 kDa that influence a variety of physiological processes [18]. Lacking intrinsic enzymatic activity, they exhibit their biological function by binding to target proteins via short phosphorylated sequences [19, 20]. Thereby, they modify the target proteins' ability to interact with other proteins, enzymatic activity or subcellular localization [21]. For example, 14-3-3 proteins regulate the activity of the cell-cycle phosphatase Cdc25 [22, 23], the protein kinase C-RAF [24, 25] and the transcriptional modulator YAP [26, 27], and stabilize the tumour suppressor p53 [28, 29]. 14-3-3 proteins have been associated with various cancers [30], the virulence of human pathogenic organisms [31, 32], and the development of neurodegenerative diseases [33].

In this study, we investigate the influence of 14-3-3 proteins on the subcellular localization of human MLF1 in human cells. In contrast to the previous research on the mouse homologue [11], we found that there is no direct influence of 14-3-3 proteins on the nucleo-cytoplasmic distribution of human MLF1 in HEK293T, Cos-7 and K-562 cells. These results indicate a more complex regulation mechanism of human MLF1 than the one suggested for the mouse homologue and imply that the subcellular localization of human MLF1 is not directly 14-3-3-dependent.

MATERIALS AND METHODS

DNA, PCR and cloning

Human MLF1 cDNA (GenBank Accession number: BC007045) was obtained from Open Biosystems and amplified by PCR using primers containing restriction sites for BglII, XhoI, EcoRI or PstI. GFP-fusion constructs were created by restriction and subsequent ligation of the MLF1 DNA into the pEGFP-C1 and pEGFP-N1 vectors (Clontech). Human 14-3-3ε was cloned into the pOPIN(C)-mCherry vector by the Dortmund Protein Facility. All of the constructs were confirmed by sequencing.

Site-directed mutagenesis of MLF1-constructs

Site-directed mutagenesis was performed using a QuikChange Kit (Stratagene). All of the mutated constructs were confirmed by sequencing.

Cell culture and transfection

HEK293T and Cos-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/l glucose, 584 mg/l L-glutamine and 110 mg/l Na-pyruvate (Life Technologies). For cultivation, 10% fetal bovine serum (GIBCO), 2 mM L-glutamine, 100 U penicillin and 0.1 mg/ml streptomycin (Sigma) were added and cells were incubated at 37ºC and 10% CO_2 in a humidified atmosphere. Experiments were performed with 5 to 20 cell passages. Cells were seeded on glass cover slips in 24-well plates and transiently transfected according to the manufacturer's instructions. HEK293T cells were transfected with 100 ng of plasmid DNA and the Mammalian Transfection Kit (Stratagene), while Cos-7 cells were transfected with 200 ng DNA and the GeneJammer Transfection Reagent (Stratagene).

Live cell imaging

Cells were examined 48 h after transfection using a Leica TCS SP2 confocal microscope. GFP and mCherry were excited using an argon ion laser at 488 nm and a GreNe ion laser at 543 nm, respectively. At least 14 cells from three independent experiments were used for quantification of fluorescence intensities with ImageJ version 1.43m.

RESULTS AND DISCUSSION

One of the most prominent functions of 14-3-3 proteins is governing the subcellular localizations of their binding partners. In the case of transcriptional regulators like Foxo [34], YAP [26] or TAZ [35], their influence on nucleo-cytoplasmic shuttling is well-established. Taking into account the findings reported for the mouse homologue of MLF1 [11], we hypothesized that the biological function of 14-3-3 binding to human MLF1 could be the control of its nuclear import and export.

Localization of GFP-fused human full-length MLF1 is 14-3-3-independent in HEK293T and Cos-7 cells

We transfected HEK293T cells with a GFP-MLF1 construct and analyzed its subcellular localization using confocal microscopy (Fig. 1). Wild-type MLF1 was localized almost exclusively in the cytoplasm (Fig. 1A) when GFP was fused to the N-terminus (GFP-MLF1). It was localized to a certain extent in the nucleus when GFP was fused to the C-terminus (MLF1-GFP). MLF1 contains one well-described 14-3-3-binding motif (amino acids 31-36) and a non-canonical 14-3-3-binding site (amino acids 145-150) that has not yet been confirmed experimentally [11]. Mutation of the S34 phosphorylation site in the well-described 14-3-3 interaction motif of MLF1 to alanine (S34A) had no influence on its subcellular localization pattern. As such findings can be strongly dependent on the cell line used for the experiments, we also studied this phenomenon in Cos-7 (Fig. 1C, D) and K-562 cells (data not shown) with no obviously different results.

Since MLF1 contains one nuclear export sequence (NES, 89-98) and two nuclear localization sequences (NLS, 168-174 and 232-236, Fig. 2B) [5], we validated the principal capability of our GFP-MLF1 constructs for nucleo-cytoplasmic shuttling by mutating the NES. Introduction of the amino acid substitutions L89E and L89A resulted in a strong predominantly nuclear localization, thereby validating the functionality of the MLF1 protein used in this study (Fig. 2A). These findings are well in line with the previous finding that the L89A mutant shows a predominant nuclear localization [5]. The less drastic L89V mutation led to a phenotype that resembled the wild type but showed a slight increase in the nuclear population of MLF1. However, the additional introduction of the 14-3-3-binding mutation S34A, which yielded the MLF1 S34A-L89V double mutant, did not show a different phenotype compared to the single L89V mutant (Fig. 2A).

Localization of C-terminally shortened GFP-fused human MLF1 exhibits a weak 14-3-3 dependency in HEK293T cells

To analyze the subcellular localization relative to the 14-3-3-binding site, different deletion constructs of MLF1 were tested in HEK293T cells (Fig. 2B). N-terminal constructs comprising the first 38 ($MLF1_{38}\Delta C$), 47 ($MLF1_{47}\Delta C$) and 176 ($MLF1_{176}\Delta C$) amino acids fused to GFP were analyzed (Fig. 2C). $MLF1_{38}\Delta C$-GFP

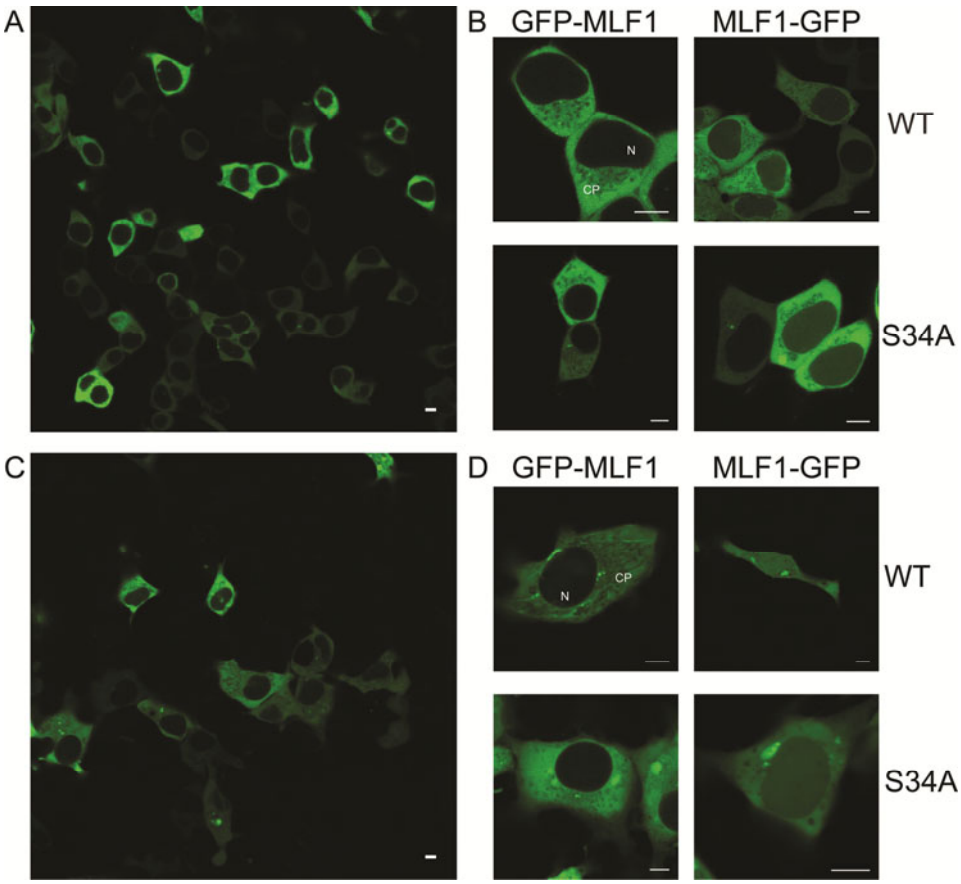

Fig. 1. Localization of human MLF1 in HEK293T and Cos-7 cells is influenced by the orientation of the GFP-fusion protein. A – HEK293T cells were transfected with the pEGFP-C1 vector containing MLF1 DNA. The GFP-MLF1 fusion protein can be detected in the cytoplasm (CP) by confocal fluorescence microscopy. B – Detailed images of the transfected cells reveal that, while N-terminally GFP-fused MLF1 is exclusively localized to the cytoplasm, C-terminally GFP-fused MLF1 is to a small extent also distributed in the nucleus (N), regardless of a functional 14-3-3-binding site. Indeed, 14-3-3 binding which is mediated via serine 34 (S34) does not influence the localization of human MLF1 in HEK293T cells. This is demonstrated by the use of an MLF1 S34A mutant, which shows the same cellular distribution as the wild-type protein. C and D – The same results were obtained using Cos-7 cells. Here, the effect of the C-terminally fused GFP is even more distinct. This becomes clear by comparing the exclusive cytoplasmic localization of N-terminal fused MLF1 (C and upper left panel in D) with the more or less even distribution of C-terminally GFP-fused MLF1 throughout the nucleus and the cytoplasm. Scale bar = 10 μm.

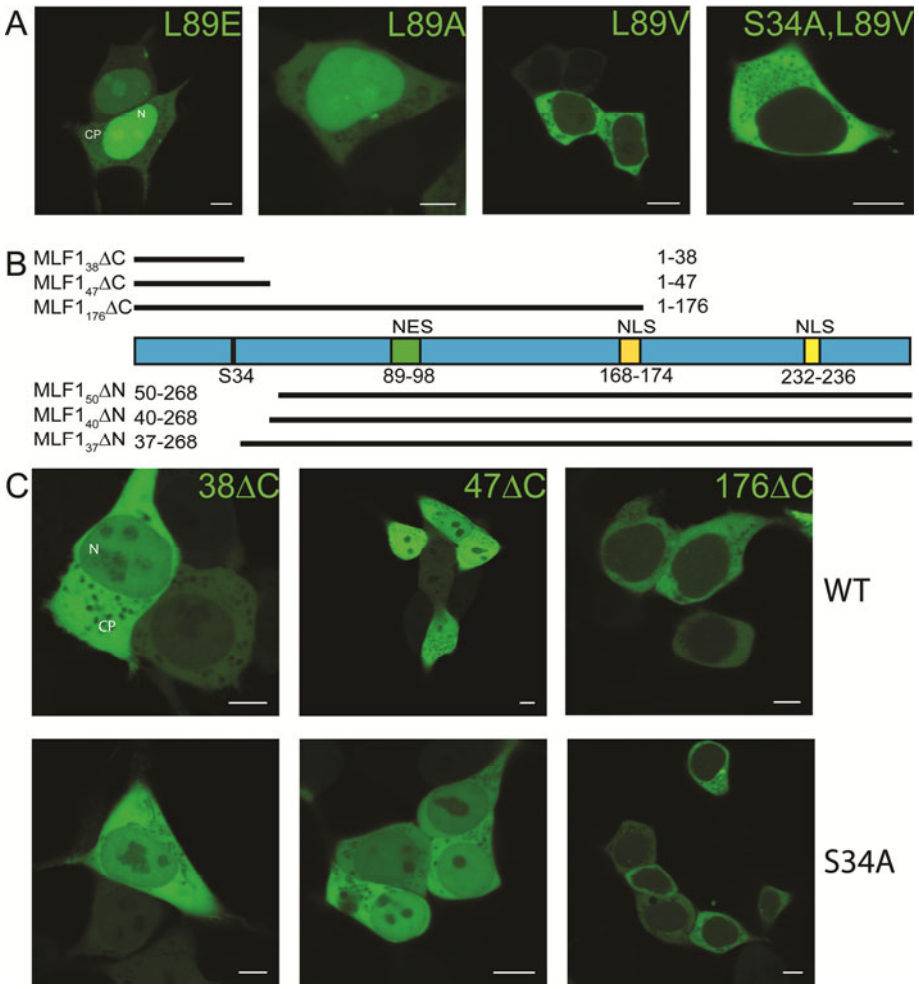

Fig. 2. Assessing the functionality of the MLF1 protein by mutating the NES and using various truncated MLF1 proteins in HEK293T cells. A – The NES of MLF1 consisting of amino acids 89-98 was mutated by exchanging L89 for the amino acids glutamate (L89E), alanine (L89A) or valine (L89V). Both L89E and L89A mutants could be detected in the nucleus (N), implying that the MLF1 protein is functional and may translocate from the cytoplasm (CP) to the nucleus under the right conditions. However, the L89V mutant, which closely resembles native NES, was detected in the cytoplasm. The L89V mutant containing the 14-3-3-binding mutation S34A was also visible in the cytoplasm, suggesting a more complex and not directly 14-3-3-dependent regulation of the MLF1 protein. B – N- and C-terminal-truncated MLF1 proteins were created. C – HEK293T cells were transfected with C-terminal-truncated MLF1-GFP fusion proteins. While the proteins that neither contain an NES nor a NLS (38ΔC and 47ΔC) are distributed evenly throughout the cytoplasm and the nucleus, the protein that contains the NES and a single NLS is mainly located in the cytoplasm (176ΔC, upper panel). This cellular localization is not influenced by introducing the 14-3-3-binding mutation S34A (lower panel). Scale bar = 10 μm.

and MLF1$_{47}$ΔC-GFP were found evenly distributed in the cytoplasm and the nucleus (Fig. 2C, upper panel). Again, a mutation rendering the 14-3-3-binding motif non-phosphorylatable and therefore dysfunctional (S34A) did not influence this distribution (Fig. 2C, lower panel). The MLF1 construct harbouring the NES and one NLS (MLF1$_{176}$ΔC-GFP) was found predominantly in the cytoplasm. Here also, the S34A mutation did not change the localization of GFP-MLF1 (Fig. 2C). The same results were obtained when the GFP-tag was placed at the N-terminus of the shortened MLF1 proteins (data not shown).

Although 14-3-3 proteins are expressed ubiquitously in eukaryotic cells [36], it is conceivable that the amount of endogenous 14-3-3 proteins might be insufficient to show an influence on the subcellular localization of the MLF1 protein, which is expressed under the control of the strong CMV promoter. The seven isoforms of human 14-3-3 proteins are highly conserved, with the conserved regions mainly localized in the amphipathic groove, which is responsible for the interaction with phosphorylated 14-3-3-binding motifs [18]. Therefore, as a representative of the 14-3-3 protein family, the ε isoform was co-expressed as a 14-3-3-mCherry fusion protein with MLF1-GFP in HEK293T cells. This protein is mainly localized in the cytoplasm and only to a small extent in the nucleus of HEK293T cells (Fig. 3). Interestingly, the C-terminally shortened MLF1 proteins show a different subcellular localization dependent on 14-3-3ε-mCherry co-expression.

While the 14-3-3-binding S34A mutants of the MLF1$_{38}$ΔC- and MLF1$_{47}$ΔC-GFP proteins are evenly distributed throughout the cytoplasm and the nucleus in the presence of 14-3-3ε-mCherry, the corresponding wild-type proteins show an increased localization in the cytoplasm (Fig. 3A). Therefore, the background-corrected GFP fluorescence intensities of the cytoplasm (CP) and nucleus (N) were quantified for at least 14 different cells from three individual experiments with ImageJ software. The median and the corresponding median absolute deviation (MAD) of the CP/N ratios were calculated and compared for the wild-type and S34A mutants and for the single transfected cells and the co-expression with 14-3-3ε-mCherry (Fig. 3B). For the 14-3-3-binding S34A mutants, no difference between the CP/N ratio for single and co-transfected cells can be observed. However, the wild-type proteins show an increased CP/N ratio in the presence of 14-3-3ε-mCherry, indicating a possible interaction of these proteins in the cytoplasm.

Localization of N-terminally shortened GFP-fused human MLF1 is 14-3-3-independent in HEK293T cells

To further analyze a possible role of the N-terminal 14-3-3-binding region on MLF1 localization, a series of N-terminal deletions of MLF1 was tested in HEK293T cells (Fig 2B). This would also exclude the possibility that residual binding affinity to 14-3-3 located around S34 accounted for the fact that the observed effect of the S34A mutants was only weak. The N-terminal deletions GFP-MLF1$_{37}$ΔN, GFP-MLF1$_{40}$ΔN and GFP-MLF1$_{50}$ΔN (all lacking the functional

14-3-3-binding site surrounding S34) displayed an exclusive cytoplasmic localization (Fig. 4). For the GFP-MLF1$_{50}$ΔN protein, an exclusive cytoplasmic localization has been reported before [5]. This further strengthens the finding that the 14-3-3 interaction motif does not directly influence the subcellular localization of human MLF1.

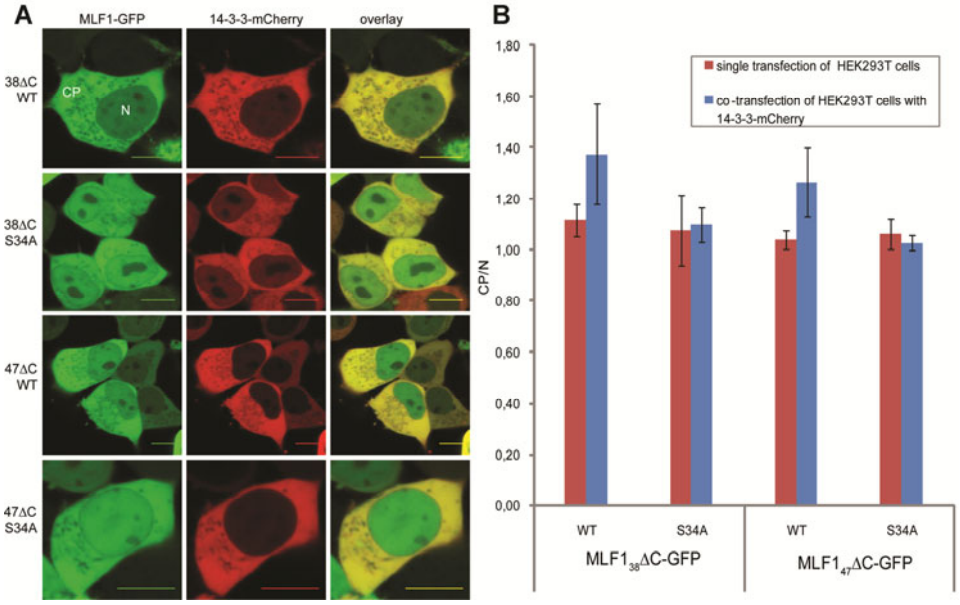

Fig. 3. Co-transfection of HEK293T cells with C-terminally shortened MLF1-GFP proteins as wild type (WT) or 14-3-3-binding mutant (S34A) and 14-3-3ε-mCherry. A – Co-expression of 14-3-3ε-mCherry has a weak influence on the subcellular localization of C-terminally shortened MLF1, as the WT and S34A proteins show a different localization in the presence of 14-3-3ε-mCherry. While the WT proteins are localized more to the cytoplasm (CP) in the presence of 14-3-3ε-mCherry, the 14-3-3-binding mutants (S34A) are distributed evenly throughout the cytoplasm and the nucleus (N). B – The ratio of GFP fluorescence CP/N was calculated for the different proteins. The median of 14 individual cells was calculated and the error bars represent the corresponding median absolute deviation (MAD). Scale bar = 10 μm.

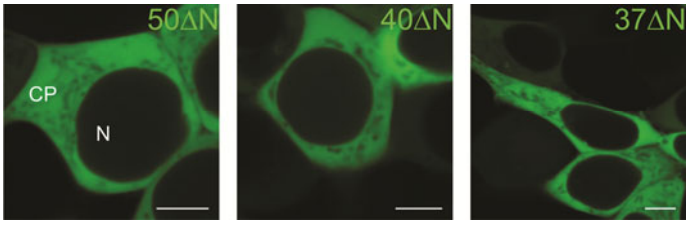

Fig. 4. Localization of N-terminally shortened GFP-MLF1 in HEK193T cells. To investigate to what extent 14-3-3 binding plays a role in cellular localization of MLF1, N-terminally truncated MLF1 proteins lacking the entire 14-3-3-binding site were created. These GFP-MLF1 fusion proteins can only be detected in the cytoplasm (CP). N = nucleus, scale bar = 10 μm.

Implications for the regulation of the subcellular localization of human MLF1

The 14-3-3-binding site surrounding S34 is one of the only known functional features of the MLF1 sequence, along with one NES and two NLS [5, 11]. Direct physical association of MLF1 and 14-3-3 proteins has been demonstrated [15] with the functional consequence of this interaction remaining largely unknown. Recently, we reported on the crystal structure of the primary 14-3-3-binding motif of MLF1 in a complex with 14-3-3ε, revealing mostly conserved but also unique features of this protein-protein interface [17]. However, the physiological function of the MLF1/14-3-3 protein interaction remains elusive.

In this study, we demonstrate that the subcellular localization of the human full-length MLF1 is 14-3-3-independent. This is rather unexpected because for the mouse homologue, a direct 14-3-3 dependency on the subcellular localization of MLF1 has been shown [11]. Employing N- and C-terminally fused GFP fusion proteins, we aimed to reveal possible artificial effects caused by the GFP-tag. Furthermore, with the use of different NES mutants we could show that the tested MLF1-GFP fusions are functional and able to translocate to the nucleus. Only for the C-terminally shortened MLF1 proteins could we observe a very weak effect of 14-3-3 on the subcellular localization when 14-3-3ε-mCherry was co-expressed in HEK293T cells. The use of N-terminally shortened MLF1 proteins indicated that the subcellular localization of the C-terminal part of MLF1 is 14-3-3-independent. A sequence alignment of the mouse and human MLF1 proteins revealed that even though the proteins share a sequence homology of 80%, the C-terminus (amino acids 208-268) is less well conserved, with only 65% sequence homology (Fig. 5). This may explain the differences that have been observed for the full-length mouse and human MLF1 proteins.

The subcellular localization of human MLF1 may be regulated by other as-yet-unknown proteins that presumably interact with the C-terminal part of this protein. Their role may be subject to future studies that will also help to evaluate the role of 14-3-3 proteins in MLF1 regulation more clearly.

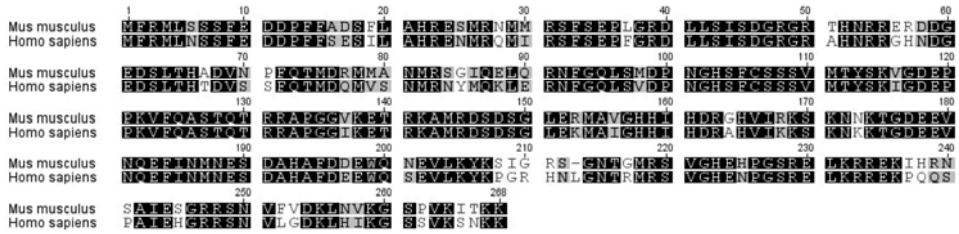

Fig. 5. Sequence alignment of human and mouse MLF1. Black = 100% homologue, dark grey = 80-100% homologue, light grey = 60-80% homologue, white = less than 60% homologue. The sequence alignment was performed with the Geneious Pro 4.8.3 Software (Biomatters) using the matrix Blosum62.

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