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Myotubularin-related protein 7 activates peroxisome proliferator-activated receptor-gamma

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Abstract

Peroxisome proliferator-activated receptor-gamma (PPAR γ) is a transcription factor drugable by agonists approved for treatment of type 2 diabetes, but also inhibits carcinogenesis and cell proliferation in vivo. Activating mutations in the Kirsten rat sarcoma viral oncogene homologue (*KRAS*) gene mitigate these beneficial effects by promoting a negative feedback-loop comprising extracellular signal-regulated kinase 1/2 (ERK1/2) and mitogen-activated kinase kinase 1/2 (MEK1/2)-dependent inactivation of PPAR γ . To overcome this inhibitory mechanism, we searched for novel post-translational regulators of PPAR γ . Phosphoinositide phosphatase *Myotubularin-Related-Protein-7* (MTMR7) was identified as cytosolic interaction partner of PPAR γ . Synthetic peptides were designed resembling the regulatory coiled-coil (CC) domain of MTMR7, and their activities studied in human cancer cell lines and C57BL6/J mice. MTMR7 formed a complex with PPAR γ and increased its transcriptional activity by inhibiting ERK1/2-dependent phosphorylation of PPAR γ . MTMR7-CC peptides mimicked PPAR γ -activation in vitro and in vivo due to LXXLL motifs in the CC domain. Molecular dynamics simulations and docking predicted that peptides interact with the steroid receptor coactivator 1 (SRC1)-binding site of PPAR γ . Thus, MTMR7 is a positive regulator of PPAR γ , and its mimicry by synthetic peptides overcomes inhibitory mechanisms active in cancer cells possibly contributing to the failure of clinical studies targeting PPAR γ .

Introduction

The nuclear transcription factor PPAR γ has been established as a target in type 2 diabetes for many years. In addition to its lipid lowering and insulin sensitizing properties, pharmacological activation of PPAR γ shows benefits in malignant and inflammatory human diseases^{1–3}, and inhibition of RAS-ERK1/2 signalling was observed in (pre)clinical models^{4–6}. It is the latter characteristic that renders the nuclear receptor a promising target in gastrointestinal tumours with frequent, activating mutations in the RAS-ERK1/2 signalling cascade, e.g.

in colorectal cancer (CRC)⁷. In this setting, mutations in *RAS* genes are a major obstacle for effective treatment in advanced disease⁸, and new drugable targets which inhibit RAS-ERK1/2 signalling are needed⁹. However, serious adverse effects limit the long-term monotherapy with PPAR γ -ligands in metabolic diseases¹⁰. Nonetheless, combination with chemo- or biological therapies may offer novel strategies against cancer^{6,11,12}. Clinical trials investigating the use of PPAR γ -agonists have yet failed to show sufficient efficacy^{13,14}.

One reason for this discrepancy between preclinical and clinical studies may rely on the complex regulation of PPAR γ by the RAS-ERK1/2 signalling cascade, which has not been taken into account in any of the before mentioned trials: we^{15,16} and others^{17,18} demonstrated that downstream effectors of RAS inhibit PPAR γ , e.g. by ERK1/2-dependent phosphorylation as well as by nuclear

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export and cytosolic sequestration through MEK1. In addition to this regulatory mechanism, off-target side effects of the first generations of PPAR γ -agonists even resulted in an increased proliferation rate of tumour and vascular cells, as they involve PPAR γ -receptor independent (“non-genomic”) activation of RAS¹⁹ and phosphoinositide 3-kinase (PI3K)²⁰ signalling, especially at higher dosages.

We therefore hypothesized that the resulting decrease in nuclear transcriptional activity of PPAR γ , due to its cytosolic sequestration in the presence of an active RAS cascade promotes its targeting to so far unknown cytosolic effectors. Therefore, unravelling novel effectors or modulators of PPAR γ could be a promising approach to overcome this obstacle, especially concerning tumours harbouring activating mutations of RAS genes, which are primarily unresponsive to PPAR γ activation.

In this context, we identified 76 kDa myotubularin-related protein 7 (MTMR7), a member of the myotubularin (MTM) family of lipid phosphatases, as a novel interaction partner of PPAR γ . MTMs consist of N-terminal plextrin homology (PH), central protein tyrosine phosphatase (PTP), SET-interaction (SID) and C-terminal coiled-coil (CC) domains^{21,22}. Homo- and heterodimerization between a catalytically active member of the family with an enzymatically inactive one, e.g. MTMR6/7/8 with MTMR9, is mediated via the CC domain resulting in an increased enzymatic activity²³. For murine MTMR7, a truncated 54 kDa isoform has been described lacking this domain²⁴. The active enzyme then dephosphorylates phosphatidyl-inositol-3-monophosphate (PI(3)P) and -3,5-bisphosphate (PI(3,5)P₂). MTMs are membrane-bound and localize to endosomes, with the exception of MTMR7, being present in a soluble form in the cytoplasm²⁴ using free inositol-1,3-bisphosphate (Ins (1,3)P₂) as a substrate.

In addition to the previously reported expression of MTMR7 in brain, muscle, liver and kidney²⁴, we detected MTMR7 in the gastrointestinal tract²⁵. In contrast to other MTMs, characterized as “survival phosphatases”^{21,22}, we demonstrated that MTMR7 reduces proliferation of CRC cells in vitro, even in the presence of activating mutations of KRAS and active insulin signalling, due to inhibition of both RAS-ERK1/2 and PI3K-AKT-mTOR signalling²⁵.

In the present study, we describe a novel regulatory mechanism of PPAR γ which augments its transcriptional activity via its interaction with MTMR7. In addition, we offer new insights into the subcellular distribution of MTMR7 in response to external stimuli and identified the CC domain of MTMR7, by designing and modifying a peptide resembling this domain, as a potential novel pharmacological activator of PPAR γ in vitro and in vivo.

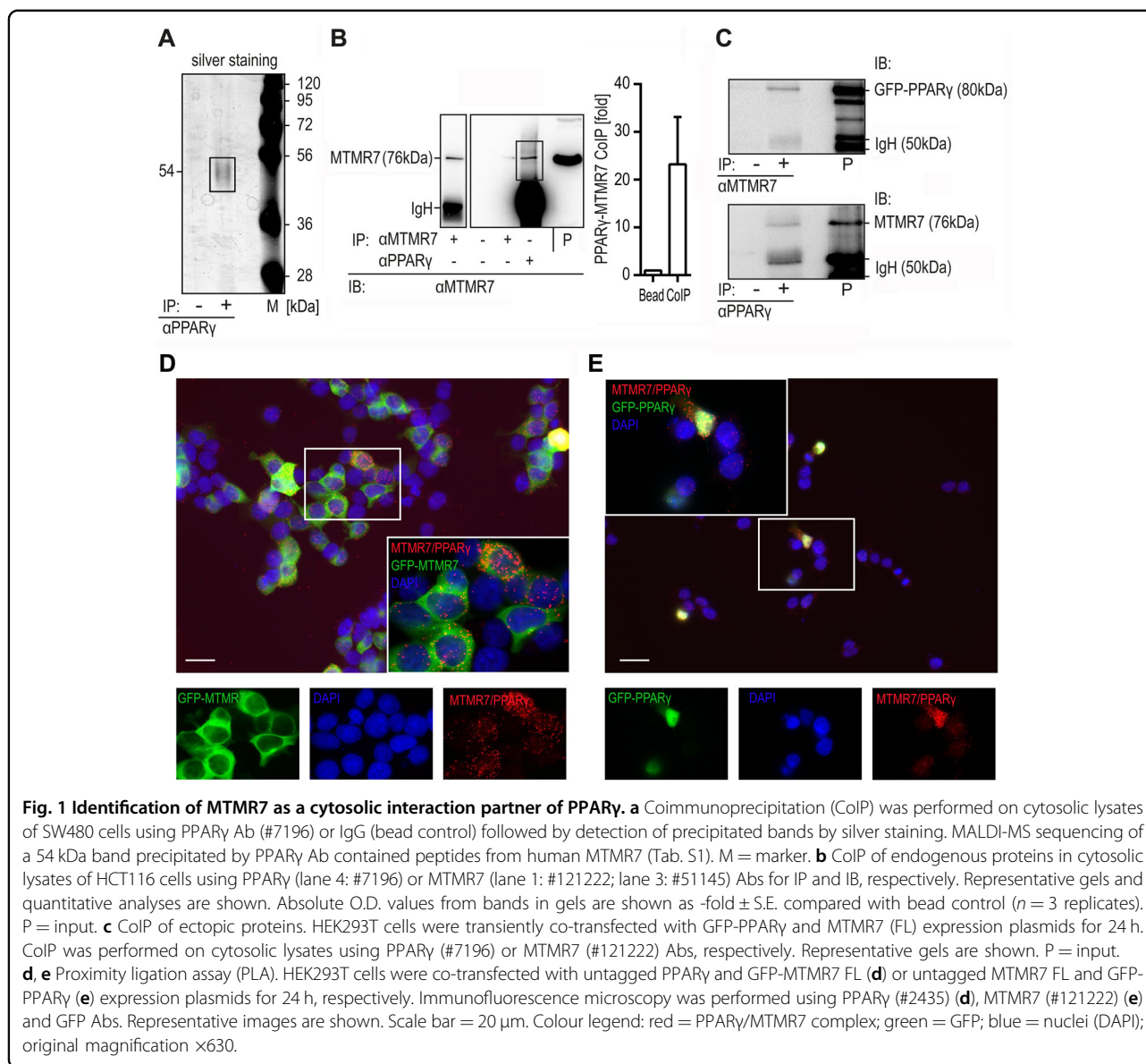
Results

MTMR7 is a cytosolic binding partner of PPAR γ

In cancer cells with constitutive activation of RAS-ERK1/2 signalling, PPAR γ can be translocated from the nucleus to the cytosol by a previously described MEK1-dependent export mechanism^{15,26}. However, the function of cytosolic PPAR γ is unknown. To identify novel binding partners which may act as regulators or effectors for cytosolic PPAR γ , a matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) protein interaction screen was performed in the aneuploid human CRC cell line SW480, which has mutated alleles of the KRASG12V gene²⁷ and a high amount of extra-nuclear PPAR γ . SW480 cells were disrupted by hypotonic lysis without detergents, a procedure which extracts cytosolic proteins. Coimmunoprecipitation (CoIP) was then performed with PPAR γ antibody (Ab) or control IgG followed by detection of precipitated bands by silver staining. Peptides within a band of 54 kDa were coprecipitated by the PPAR γ Ab and identified to correspond to human MTMR7 by MALDI-MS sequencing (Fig. 1a; Tab. S1). Most of the peptides covered the internal and C-terminal part of the enzyme, including the catalytic phosphatase, SID and CC domains.

For detection of the cytosolic complex between endogenous MTMR7 and PPAR γ , we resorted to HCT116, a human KRASG13D mutated CRC cell line which expresses high amounts of full-length (FL) MTMR7 (76 kDa) protein²⁵. Subcellular fractionation (SCF) of normal cycling HCT116 cells was performed, and cytosolic lysates were immunoprecipitated with MTMR7 or PPAR γ Ab-conjugated or unconjugated beads. Immunoblotting (IB) was performed with MTMR7 Ab (Fig. 1b). MTMR7 Abs were directed against the C-terminal region of the enzyme and pulled down a 76 kDa band corresponding to the FL protein.

For detection of ectopic proteins, we employed HEK293T cells which have low amounts of endogenous FL MTMR7 (76 kDa) protein²⁵. Cells were transiently co-transfected with expression plasmids encoding for GFP-PPAR γ and MTMR7 (FL) protein for 24 h. After SCF, CoIP from cytosolic lysates was performed using MTMR7 or PPAR γ -Ab-conjugated beads. IB against MTMR7 detected an 80 kDa band, representing GFP-PPAR γ after MTMR7 pulldown (Fig. 1c upper panel). For CoIP performed with PPAR γ Ab-conjugated beads, a 76 kDa overexpressed band was detected by the MTMR7 Ab (Fig. 1c lower panel). To corroborate the findings on this interaction, proximity ligation assay (PLA) was conducted in HEK293T cells after a 24 h transfection with GFP-MTMR7 (FL) (Fig. 1d) or GFP-PPAR γ (Fig. 1e) expression plasmids, respectively. Thereafter, cells were subjected to immunofluorescence imaging, and colocalization of MTMR7 and PPAR γ proteins was visualized as pink dots.



These data indicated that MTMR7 forms a complex with PPAR γ .

Nuclear translocation of MTMR7 in response to growth factors and PPAR γ -agonist

To interrogate the subcellular distribution of MTMR7 and PPAR γ , immunofluorescence microscopy was performed on normal cycling HCT116 cells expressing high levels of endogenous MTMR7 protein (Fig. 2a)²⁵. In this setting, predominant cytosolic localization could be detected ($p = 0.057$ nuclear “N” vs. cytosolic “C”, Mann–Whitney test, $n = 4$ replicates). In addition, we evaluated formalin-fixed paraffin-embedded (FFPE) tissue specimens from CRC patients ($n = 67$ cases) with regard to MTMR7 expression and subcellular localization by

means of immunohistochemistry (IHC). Of these patients, 41 (61%) showed a positive MTMR7 staining. In 19 (46%) of these MTMR7 positive cases, a nuclear staining was detected (Fig. 2b).

For 25 samples, an additional staining for PPAR γ was observed, 16 (64%) of which exhibited a positive nuclear staining for PPAR γ (Fig. 2c). Nuclear PPAR γ staining was associated with nuclear localization of MTMR7 ($p = 0.04$, Fisher’s exact test, $n = 67$ patients).

To further study stimulation-dependent nuclear translocation of MTMR7, immunofluorescence microscopy was performed in HCT116 cells expressing high endogenous MTMR7 FL protein. Cells were serum-deprived for 16 h, followed by incubation with PPAR γ -agonist rosiglitazone (rosi) (1 μ M, R1), epidermal growth factor

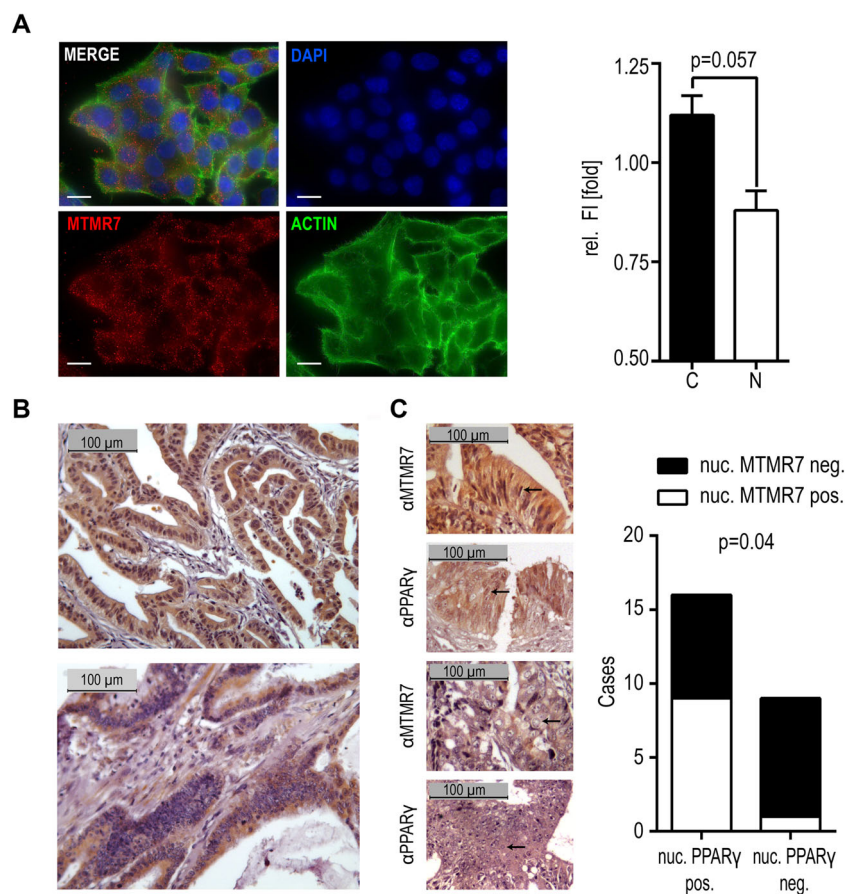


Fig. 2 Subcellular distribution of MTMR7 protein variants. **a** Localisation of MTMR7. Immunofluorescence microscopy was performed using MTMR7 Ab (#121222) in HCT116 cells, expressing endogenous MTMR7 protein. Left: Representative images. Right: Quantification of nuclear (“N”) and cytoplasmatic (“C”) fluorescence intensity (FI) revealed a predominant cytoplasmatic localization of MTMR7. Data are -fold FI \pm S.E. compared with control ($p = 0.057$ N vs. C, Mann–Whitney test, $n = 4$ replicates). Scale bar = 10 μ m. Colour legend: red = MTMR7, green = actin (phalloidin), blue = nuclei (DAPI); original magnification $\times 630$. **b** Localisation of MTMR7 in human CRC patients’ tissues ($n = 67$ cases). Immunohistochemistry (IHC) using MTMR7 Ab (#121222) revealed a subset of patients with positive nuclear MTMR7 staining (upper panel), whereas it was detectable mainly in the cytosol of other cases (lower panel). Representative images are shown; original magnification $\times 200$. **c** Reduced nuclear localization of PPAR γ is associated with increased cytosolic localization of MTMR7. IHC staining with MTMR7 (#121222) and PPAR γ (#7196) Abs was performed on matched human CRC patients’ tissue specimens. Left: Representative images of cases with (upper two panels) or without (lower two panels) nuclear MTMR7 and PPAR γ staining are shown; representative nuclei are marked by arrows. Right: Quantitative analyses evinced that lower numbers of PPAR γ ⁺ nuclei correlated with reduced MTMR7⁺ nuclei ($p = 0.04$, Fisher’s exact test, $n = 67$ cases). Reduction of nuclear PPAR γ staining was defined as <30% positive nuclei.

(EGF) (50 ng/ml) or foetal calf serum (FCS) (20% v/v) for 1 h (Fig. 3a, b). Upon stimulation with either FCS or rosi, an increase in the nuclear fluorescence intensity (FI) was detected (FCS $p = 0.0173$, R1 $p = 0.0431$), while there was a trend for a predominant cytoplasmic localization in starved cells ($p = 0.11$) (N vs. C, two-way ANOVA, Sidak’s multiple comparisons test, $n = 3$ replicates). Thus, endogenous MTMR7 translocates from the cytosol to the nucleus in response to serum or PPAR γ -activation.

However, this method did not discriminate between FL and small isoforms of MTMR7^{24,25}. We therefore studied subcellular localization in HEK293T cells which had low endogenous MTMR7 FL protein. Cells were starved for

16 h and then stimulated with serum or PPAR γ -agonist, followed by SCF and IB against MTMR7 (Fig. 3c). Upon incubation with 20% (v/v) FCS or rosi (1 μ M, R1), the endogenous, truncated MTMR7 isoform (54 kDa) accumulated in the nuclear fraction (N vs. C: FCS $p = 0.0440$, R1 $p = 0.0008$; two-way ANOVA, Sidak’s multiple comparisons test, $n = 3$ replicates). All Abs used in the present study recognized the C-terminal region of the MTMR7 FL protein (Tab. S2), indicative of a stimulus-dependent mobility of both isoforms.

To characterize the subcellular localization of MTMR7 in situ, we cultivated patient-derived organoids (PDOs) of four patients harbouring activating *KRAS* mutations and

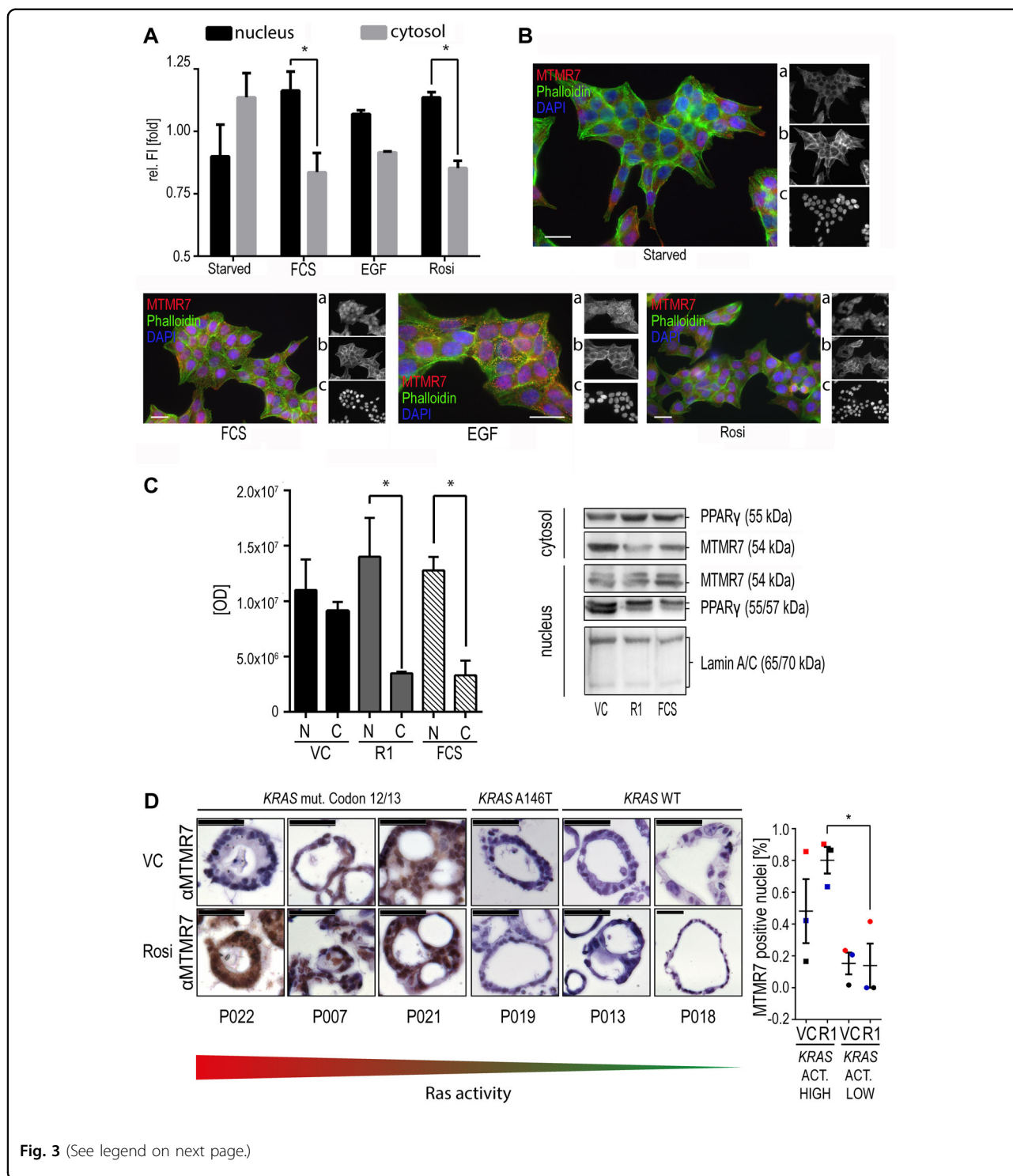


Fig. 3 (See legend on next page.)

two patients with wild-type (WT) *KRAS*. The PDOs were incubated for 48 h in medium containing 1 μ M rosi or vehicle control (VC, DMSO) (Fig. 3d): On baseline, 4 of the 6 PDOs showed a positive MTMR7 staining, 3 of which had an activating *KRAS* codon 12 or 13 mutation.

Remarkably, the rosi-stimulated PDOs showed an increase in nuclear MTMR7. This effect was more pronounced in the PDO lines with the activating *KRAS* mutations of codon 12 or 13 as compared with *KRAS* WT PDO lines or the line with the A146T mutation ($p = 0.016$

(see figure on previous page)

Fig. 3 Nuclear translocation of MTMR7 in response to growth factors and PPAR γ -agonist. **a** Immunofluorescence microscopy. HCT116 cells were serum-deprived for 16 h ("starved") and subsequently stimulated with serum (20% v/v FCS), EGF (50 ng/ml) or rosi (1 μ M) for 1 h, followed by fixation and staining of endogenous MTMR7 (Ab: #121222). FI signals from the nuclear and cytosolic compartments were normalized to the overall FI. Data are -fold FI \pm S.E. (* p < 0.05 N vs. C, two-way ANOVA, Sidak's multiple comparisons test, n = 3 replicates). While there was no difference in the distribution of endogenous MTMR7 in starved cells, 1 h stimulation with serum, EGF or rosi increased nuclear MTMR7 FI. **b** Representative images from **(a)** using Abs against MTMR7 (red, a), actin (phalloidin) (green, b), nuclei (DAPI) (blue, c). Scale bars = 20 μ m. Original magnification: \times 630. **c** Endogenous MTMR7 translocates into the nucleus in response to growth factors and PPAR γ -agonist. HEK293T cells were serum-deprived for 16 h followed by incubation with vehicle control (VC; DMSO), rosi (R; 1 μ M) or 20% (v/v) FCS for 1 h. Thereafter, cells were subjected to SCF and IB for MTMR7 (Ab: #150458). Representative gels and quantitative analyses. Mean O.D. values \pm S.E. from bands in gels are shown (* p < 0.05 N vs. C, two-way ANOVA, Sidak's multiple comparisons test, n = 3 replicates). C = cytoplasm; N = nucleus. **d** MTMR7 translocates into the nucleus in patient-derived organoids (PDOs). Cell lines from six different CRC patients were cultivated for 48 h in medium containing VC or 1 μ M rosi (R1). Left panel: IHC against MTMR7 (Ab: #9406043) was performed, and 4 of 6 PDO lines showed a positive MTMR7 staining at baseline which increased upon rosi stimulation. In 2 of 3 PDO lines with activating *KRAS* mutations, MTMR7 staining intensity increased after rosi stimulation. Each PDO is labelled by its patient ID. The *KRAS*-mutation type is given above the representative images. Right panel: The percentage of MTMR7⁺ nuclei after rosi stimulation was higher in PDO lines with activating codon 12 and 13 mutations of *KRAS* (p = 0.016 high vs. low *KRAS* activity, two-way ANOVA, Holm–Sidak's multiple comparisons test, n = 6 cases). Pre- and post-stimulation pairs are coded in the same colour.

high vs. low *KRAS* activity, two-way ANOVA, Holm–Sidak's multiple comparisons test, n = 6 cases). Thus, PPAR γ -agonist facilitates nuclear accumulation of MTMR7 in vitro and ex vivo in patient-derived cancer stem cells.

MTMR7 increases nuclear transcriptional activity of PPAR γ

To assess, whether MTMR7 alters the transcriptional activity of PPAR γ , we performed reporter gene assays using a luciferase plasmid with a PPAR γ -responsive enhancer element (PPRE) in front of a basal promoter. HCT116, SW480 and HEK293T cells were transfected with empty vector (EV) or MTMR7 (FL) expression plasmid followed by stimulation with rosi (0.1–10 μ M) for 48 h (Fig. 4a–c). MTMR7 overexpression increased basal (HCT116 & SW480: p < 0.0001) and ligand-dependent (HCT116 & SW480: p < 0.0001; HEK293T: 1 μ M: p = 0.0295, 10 μ M: p = 0.0425) reporter gene activity compared with EV control (replicates: HCT116: n = 12; SW480: n = 6; HEK293T: n = 12; two-way ANOVA, Sidak's multiple comparisons test, EV vs. MTMR7).

To explore whether these results correlate with increased PPAR γ -target gene expression, in silico analysis using the cBioportal database of cancer genomics was conducted²⁸. MTMR7 mRNA expression \leq 1 S.D. of the mean positively correlated with a reduced expression of a broad set of PPAR γ -target genes in the CRC dataset provided by *TCGA Nature 2012* (Fig. 4d). This finding was reproduced in the dataset provided by the *Pancancer Atlas* (not shown).

To elucidate a possible mechanism underlying the observed increased PPAR γ -activity in presence of MTMR7, we performed SCF followed by IB for phosphorylated PPAR γ , using an Ab specific for the bona fide ERK1/2-phosphorylation site at serine 82/84. To this end, HEK293T cells were transfected with EV or MTMR7 (FL) and treated with vehicle control (VC), 20% (v/v) FCS or

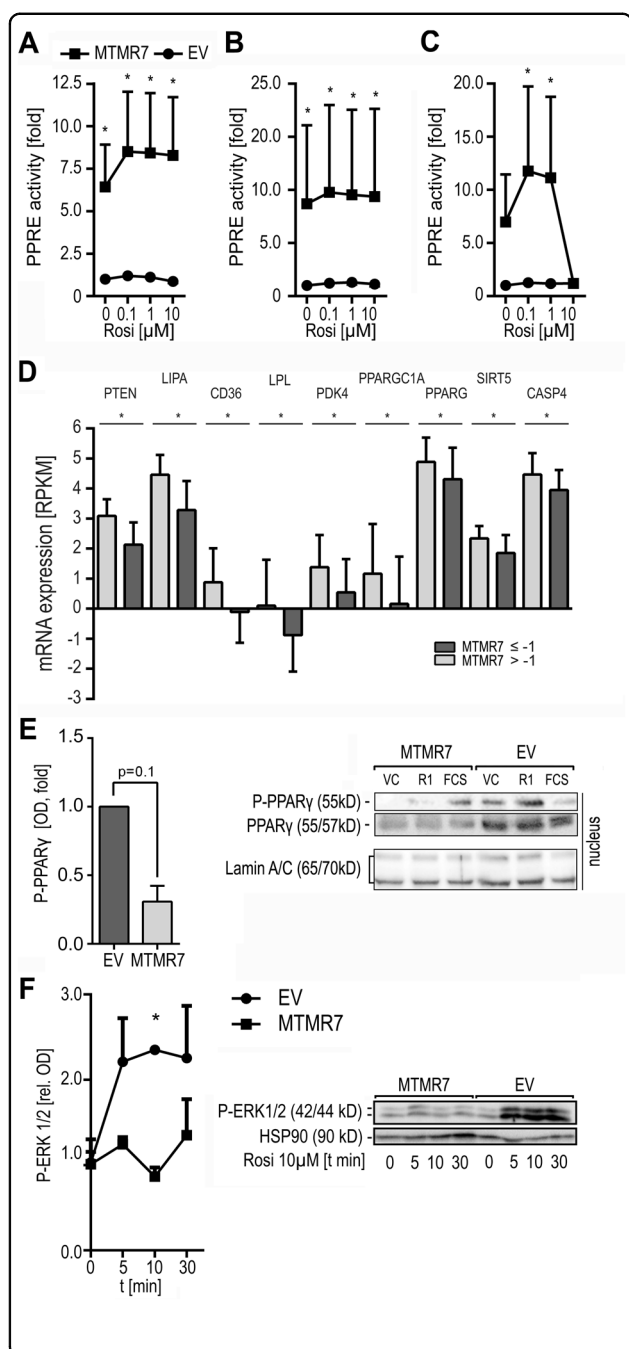
1 μ M rosi (R1) for 24 h, respectively (Fig. 4e). After MTMR7 overexpression, a trend for reduced amount of nuclear, phosphorylated (i.e. inactivated) PPAR γ was detectable. Hence, MTMR7 seems to increase PPAR γ -activity indirectly by reducing its inhibitory phosphorylation through the RAS-MEK1/2-ERK1/2 pathway¹⁶.

MTMR7 inhibits PPAR γ -agonist-mediated ERK1/2 activation

Since at least some of the off-target side effects of rosi (and related glitazones) may be mediated by the aberrant activation of MEK1/2-ERK1/2 signalling^{29,30}, we investigated the effect of MTMR7 overexpression on rosi-mediated ERK1/2 phosphorylation. To this end, HCT116 cells were transiently transfected with EV or MTMR7 (FL), serum-deprived for 16 h and subsequently incubated with 10 μ M rosi, a concentration higher than its IC₅₀ and shown to stimulate ERK1/2³⁰. This effect was abrogated by overexpression of MTMR7, leading to reduced amounts of phosphorylated ERK1/2 (p = 0.0449, Kruskal–Wallis test), particularly after 10 min of rosi stimulation (p = 0.0392 EV vs. MTMR7, Dunn's multiple comparisons test) (n = 3 replicates) (Fig. 4f). A similar result was shown for SW480 cells (Supplementary Fig. 1A).

Design of MTMR7-CC mimicry peptides

The main regulatory mechanism of catalytically active MTMs is the formation of homo- or heterodimers with partner MTMs and effector proteins by means of the C-terminal coiled-coil (CC) domain³¹. We therefore hypothesized that the CC domain of MTMR7 is the region of the protein responsible for the interaction with PPAR γ . To test this idea, we designed a synthetic peptide to substitute for the MTMR7 FL protein. There is no crystal structure of the MTMR7's CC domain or any other MTM available. However, Kim et al. identified and



characterized the CC domain of MTMR2³². Performing multiple alignment analysis of the amino acid (aa) sequences of MTMR7, MTMR2, MTMR6, MTMR8 and MTMR9, we identified a stretch of 30 amino acids at the C-terminus of MTMR7 (Fig. 5a1).

This leucine-rich region showed a high coil-forming capacity using NCOILS version 1.0 (ExPASy; Supplementary Fig. 1B). Therefore, we used this region as a basis for the design of a mimicry peptide (PEP) of the MTMR7-CC. Pepwheel, provided by EMBOSS, predicted the

Fig. 4 MTMR7 increases the transcriptional activity of PPAR γ .

a–c MTMR7 promotes PPAR γ -dependent reporter gene expression. HCT116 (**a**), SW480 (**b**) and HEK293T (**c**) cells were transfected with empty vector (EV) or MTMR7 FL expression plasmid, respectively, together with PPAR γ -reporter plasmid (PPRE-luc) for 24 h before stimulation with rosi (0–1 μ M) for additional 24 h. Luciferase activity was measured in total cell lysates, normalised to protein content and calculated as -fold \pm S.E. compared with control (* p < 0.05 EV vs. MTMR7, two-way ANOVA Sidak's multiple comparisons test; replicates: HCT116 n = 12; SW480 n = 6; HEK293T n = 12). **d** Downregulation of MTMR7 mRNA is associated with decreased expression of PPAR γ target genes. In silico analysis using the Colorectal Adenocarcinoma (TCGA, Nature 2012) dataset provided by the cBioPortal database for Cancer Genomics revealed a downregulation \geq 1 S.D. in 38 of 182 CRC patients analysed. This downregulation was associated with reduced "Reads per kilo base per million mapped reads" (RPKM) counts of PPAR γ target genes. Data are means \pm S.D. and significances (* p < 0.05 high vs. low MTMR7 mRNA) as disclosed by the provider. **e** MTMR7 reduces inhibitory phosphorylation on serine 82/84 on PPAR γ 1. HEK293T cells were transfected with MTMR7 (FL) for 6 h, followed by serum-deprivation for 16 h and subsequent stimulation with vehicle (VC, DMSO), rosi (1 μ M, R1) or 20 % (v/v) FCS for 60 min, followed by SCF. IB of unphosphorylated and phosphorylated PPAR γ was performed. Data are absolute O.D. values calculated as -fold \pm S.E. compared with EV control (p = 0.1 EV vs. MTRM7, Mann–Whitney test, n = 3 replicates). **f**, MTMR7 abrogates rosi-mediated activation of ERK1/2. HCT116 cells were transfected with MTMR7 FL plasmid for 6 h, followed by serum-deprivation for 16 h and stimulation with rosi (10 μ M) for the times indicated (min). IB on total cell lysates detected reduced ERK1/2 phosphorylation in presence of MTMR7. Data are absolute O.D. values normalized to HSP90 calculated as -fold \pm S.E. (p = 0.0449, Kruskal–Wallis test, subgroup analysis: 10 min, p = 0.0392 EV vs. MTMR7, Dunn's multiple comparisons test; n = 3 replicates).

selected sequence to form an amphipathic α -helix (Supplementary Fig. 1C). In addition, a modified peptide (MP), consisting of the same amino acid residues in a scrambled order, but containing a canonical LXXLL coactivator motif for nuclear receptors³³ (including PPAR γ) was designed (Fig. 5a2). Both peptides were myristoylated at the N-terminus and amidated at the C-terminus to enhance uptake into cells and minimize proteolysis.

MTMR7 mimicry peptides activate PPAR γ in vivo and in vitro

To determine the effect of the peptides on the transcriptional activity of PPAR γ , HEK293T cells were transfected with the PPRE-luciferase reporter plasmid for 24 h. Thereafter, treatment with vehicle control (VC), MP or PEP (both at 1 μ M) was performed in presence or absence of rosi (1 and 10 μ M). As for overexpression of MTMR7 FL protein, treatment with both peptides increased the transcriptional activity of PPAR γ (Fig. 5b, * p < 0.05 vs. VC, two-way ANOVA, Holm–Sidak's multiple comparisons test, n = 7 replicates). A similar effect was observed in HCT116 (n = 3) and SW480 (n = 5) replicates (* p < 0.05 vs. VC, one-sample t test). Treatment

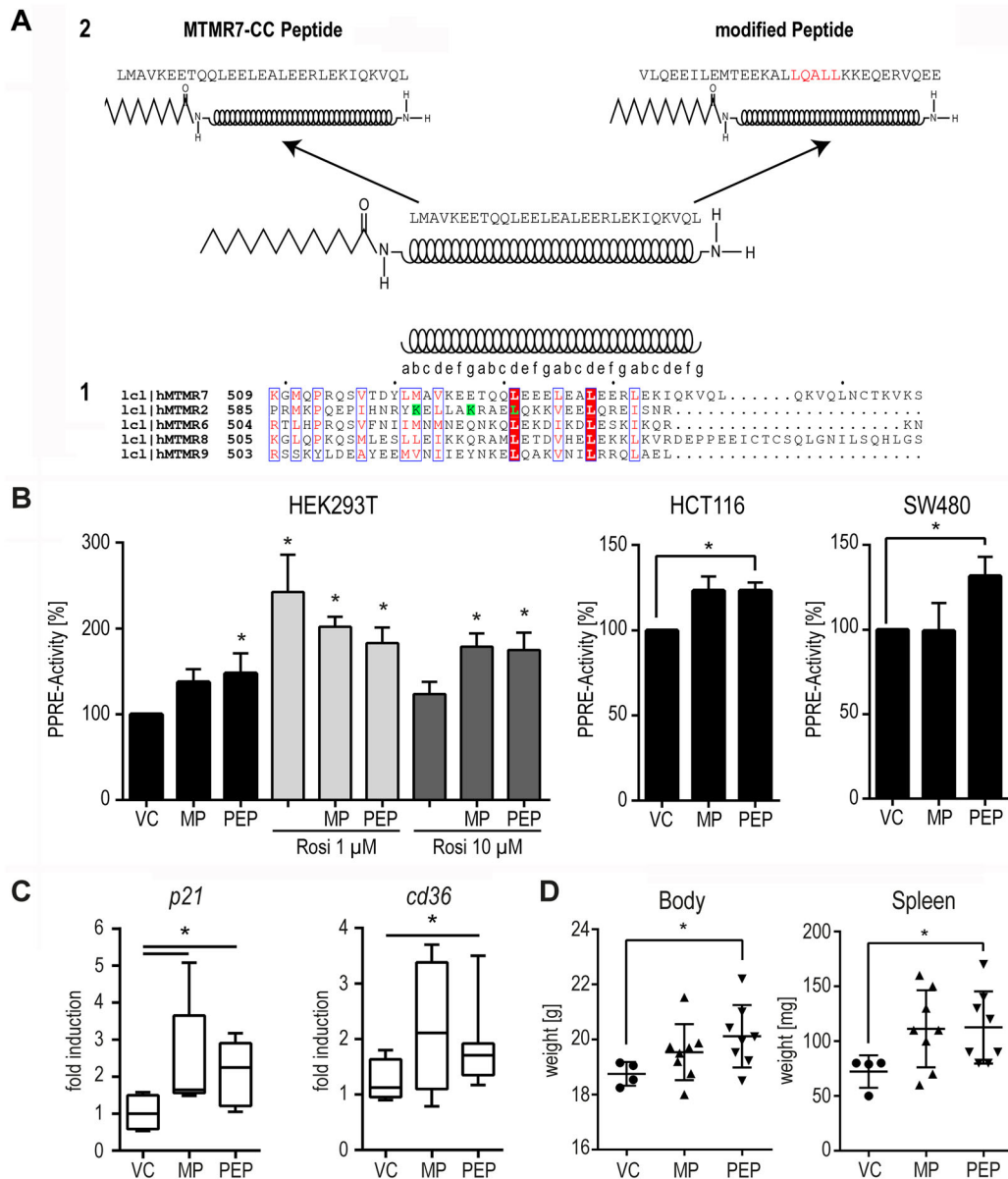
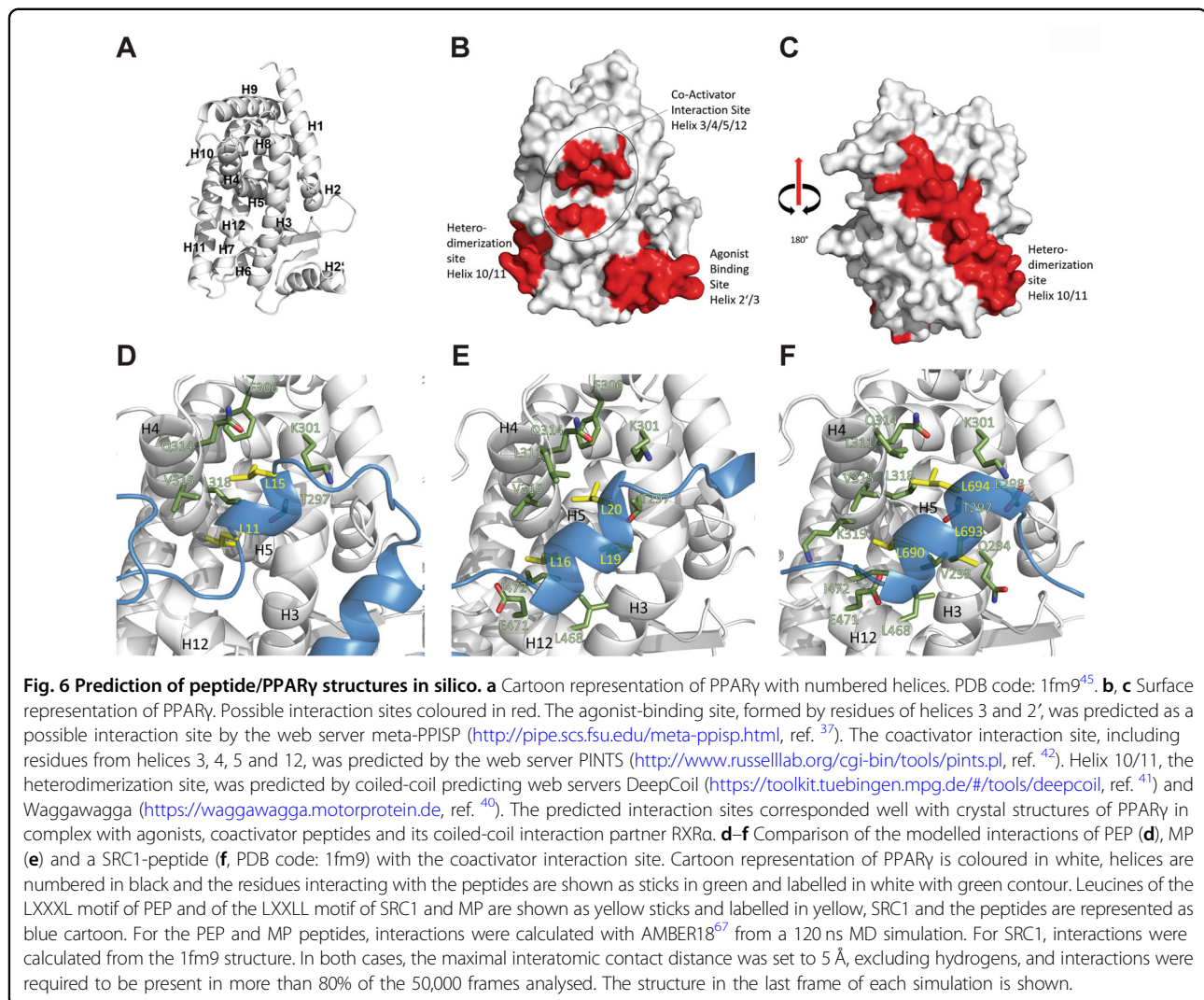


Fig. 5 Design of MTMR7-CC mimicry peptides which activate PPAR γ in vitro and in vivo. **a**, **1**: Alignment of the amino acid (aa) sequences of the coiled-coil (CC) domains of MTMR7, 2, 6, 8 and 9. The heptad repeat is denoted with the letters a–g, with the leucines of MTMR2 residing in the d position. Residues of MTMR2 required for heterodimerization are marked green³². This figure was drawn using ESPrnt (<http://esprnt.ibcp.fr>).

a, **2**: MTMR7 harbours a 30 aa sequence of MTMR7 with high coil-forming capacity. The N-terminus was modified by myristoylation, the C-terminus was amidated. A modified peptide consisting of the same aa sequence in a scrambled order, but with a PPAR γ coactivator LXXLL motif, was designed as control. **b** MTMR7-CC peptides promote transcriptional activity of PPAR γ . HEK293T, HCT116 and SW480 cells were transfected with PPAR γ -luciferase reporter plasmid (PPRE-luc) for 24 h before incubation with 1 μ M of a peptide mimicking the coiled-coil (CC) domain of MTMR7 (PEP) and a modified peptide (MP) with a scrambled α -helix composition but a preserved LXXLL coactivator motif in presence or absence of rosi (1 & 10 μ M) for additional 24 h. Luciferase activity normalised to protein content was calculated as % \pm S.E. compared with vehicle control (* p < 0.05 vs. VC, HEK293T: two-way ANOVA, Holm–Sidak’s multiple comparisons test, n = 7 replicates; HCT116: one-sample t test, n = 3 replicates, SW480: one-sample t test; n = 5 replicates). **c** PEP and MP activate PPAR γ -target genes in vivo. C57BL6/J mice (pCEA-SV40-Tag) were treated for 14 days with MP, PEP (both at 30 mg/kg*d, four times a week) or vehicle control (VC, DMSO) by intraperitoneal injection, respectively. RT-qPCRs detecting PPAR γ -target genes were performed on RNA extracted from snap-frozen whole tissue samples derived from the distal colon. MP and PEP treatment increased $P21^{(Cip1/Waf1)}$ and $Cd36$ mRNA expression. CT-values were normalized to $B2m$ and calculated as -fold \pm S.E. and compared with controls (* p < 0.05 vs. VC, Mann–Whitney test, VC: n = 4, MP: n = 8, PEP: n = 8 mice per group). **d** PEP and MP increase spleen weight and induce body weight gain, thereby mimicking a rosi-like phenotype⁶⁰: pCEA-SV40-Tag mice were treated as above. Body and spleen weights were measured after 14 days of therapy. Data are means \pm S.E. compared with controls (* p < 0.05 vs. VC, Mann–Whitney test, VC: n = 4, MP: n = 8, PEP: n = 8 mice per group).



with concentrations of rosi higher than its IC_{50} ($>1 \mu M$) reduced PPAR γ activity, presumably due to a “non-genomic” activation of the MEK1/2-ERK1/2-pathway and subsequent phosphorylation and inactivation of PPAR γ ¹⁶. Of note, exposure of cells to MP and PEP reversed this effect: In the presence of both peptides, increased PPAR γ activity was detectable even after treatment with 10 μM rosi (Fig. 5b, $*p < 0.05$ vs. VC, two-way ANOVA, Holm–Sidak’s multiple comparisons test, replicates: HEK293T: $n = 7$, HCT116: $n = 3$, SW480: $n = 5$). To address the question whether this in vitro efficacy could be translated into the in vivo situation, C57BL6/J mice (pCEA-SV40-Tag)³⁴ were treated for 14 days with MP, PEP (both at 30 mg/kg*d) or vehicle control (VC) by intraperitoneal injection (four times per week), respectively. RT-qPCRs (Tab. S2) detecting exemplary PPAR γ -target genes were performed on total RNA extracted from snap-frozen whole tissue samples derived from the distal colon (Fig. 5c). *P21*^(Cip1/Waf1) and *Cd36* mRNAs were

increased in animals treated with either of the peptides (VC: $n = 4$ mice, MP: $n = 8$ mice, PEP: $n = 8$ mice, $*p < 0.05$ vs. VC, Mann–Whitney test). This effect was accompanied by an increase in body weight (VC vs. PEP: $p = 0.0112$; VC vs. MP: $p = 0.1$, Mann–Whitney test) and spleen weight (VC vs. PEP: $p = 0.0182$; VC vs. MP: $p = 0.0639$, Mann–Whitney test) (Fig. 5d). Taken together, the MTMR7-CC mimicry peptides activated PPAR γ in vitro and in vivo.

Prediction of peptide/PPAR γ structures in silico

To gain a better understanding of the potential molecular mechanism of the mimicry peptide mediated activation of PPAR γ , a computational approach was employed to generate plausible models of the peptide/PPAR γ complexes. Both peptides (PEP and MP) were predicted to form α -helical secondary structures by the web servers PredictProtein³⁵ and Agadir³⁶ (Supplementary Figs. 2, 3). Therefore, they were both modelled as

α -helices, but the terminal myristoylation and the amide caps were omitted.

We then used three different web servers to identify putative peptide binding sites on the 12-helices bundle of the ligand-binding domain (LBD) of PPAR γ (Fig. 6a). This analysis yielded three candidate regions for interaction (Fig. 6b). The web server meta-PPISP³⁷ predicted the residues surrounding the agonist-binding pocket of PPAR γ to be the most likely interaction site on the protein³⁸ (Fig. 6b), mainly formed by residues of helices 2' and 3 and the loop between them³⁹. The coiled-coil (CC) predictors, Wagga-wagga⁴⁰ and DeepCoil⁴¹, predicted only helix 10/11 as a possible site for coiled-coil interaction on PPAR γ . Helix 10 mediates heterodimerization between PPAR γ and RXR α and forms a coiled-coil with helix 10 of RXR α (Fig. 6c). The PINTS web server⁴² yielded several crystal structures of PPAR γ and related proteins with LXXLL motif-harboring coactivators or corepressors^{43–46}. The latter had been co-crystallized in an α -helical conformation at the coactivator interaction site³⁹ which is formed by helices 3, 4, 5 and 12 of PPAR γ (Fig. 6b).

As the heterodimerization and the coactivator-binding sites were already experimentally shown to be binding sites for α -helical peptides and protein regions, they were thought to be more likely interaction sites than the agonist-binding site. Docking was performed using two different web servers that employ different approaches: The web server GalaxyPepDock⁴⁷ docked the MP exclusively with the canonical LXXLL motif at the coactivator binding site. The PEP was also docked exclusively to this site either with its LXXXL or LXXL motifs. The second tool used was the web server ClusPro⁴⁸. The ClusPro docking resulted in complexes with the peptides docked to varying sites on PPAR γ , including helix 10/11 and the proximity of the coactivator interaction site.

To assess which of these complexes might be the most favourable ones, all-atom molecular dynamics (MD) simulations followed by calculations of binding free energies were performed on 23 complexes from the two web servers with MP and PEP bound to different sites on the nuclear receptor. Peptides were bound either to the heterodimerization site or the coactivator interaction site. The results of the free energy calculations indicated that the coactivator interaction site is a more favourable binding site than the heterodimerization site (Tab. S3). Interactions were calculated for the most energetically favourable complex between each of the peptides and PPAR γ , as well as for PPAR γ with the classical coactivator peptide from SRC1. As seen in the last frame of the MD simulation, PEP bound to the coactivator interaction site with its N-terminal LXXXL motif (Fig. 6d), while MP and SRC1 bound to the interaction site with their canonical LXXLL motifs, respectively (Fig. 6e, f). Comparing the calculated interactions to those described by Nolte et al.³⁹,

the binding mode of MP seemed to be similar to SRC1, involving residues on helices 3, 4 and 12 (Tab. S4), including K301 on helix 3 and E471 on helix 12 (Fig. 6e). These two residues have been described to form a charged clamp that is thought to position the coactivator motif on SRC1 in the correct orientation at the coactivator interaction site³⁹.

PEP was not found to interact with residues on helix 12 in more than 80% of the 120 ns MD simulation and, therefore, is unlikely to make use of the charged clamp for positioning (Fig. 6d). The leucine at position five in the LXXLL/LXXXL motifs engaged in the majority of the interactions by inserting into a hydrophobic cleft between helices 3, 4 and 5, as described for SRC1³⁹. For both peptides, this leucine was calculated to interact with F306 inside this cleft, an interaction residue not shown for SRC1.

The structures of the complete complexes of PPAR γ with PEP or MP at the last frame of the MD simulations are shown in Supplementary Fig. 4. Outside the coactivator interaction site, their conformations differ. PEP was found to drape its C-terminal end in a stable helix over the entrance of the agonist-binding pocket in proximity of helices 2' and 3, while its N-terminus remained unstructured and did not form stable interactions. The N-terminus of MP stayed close to helices 7 and 11, while its C-terminus was close to helix 1.

In conclusion, the *in silico* prediction of the structures of peptide/PPAR γ complexes indicates a coactivator-like binding mode that may explain the capacity of the peptides to stimulate the transcriptional activity of PPAR γ .

Discussion

In the present study, we describe a novel role for MTMR7 in human CRC, identifying it as a binding partner and positive regulator of PPAR γ . The transcriptional activity of PPAR γ is regulated by ligand binding, post-translational modifications and subcellular localisation⁴⁹. The here described MTMR7-PPAR γ interaction complex offers a novel mode of PPAR γ regulation by compartmentalization and a possible explanation for the pro- vs. anti-tumour effects of PPAR γ -agonists^{50–52}. Previously, we characterized MTMR7 as an inhibitor of ERK1/2 and AKT/mTOR signalling²⁵, thus acting as a dual blockage of two interconnected and compensatory oncogenic pathways downstream of receptor tyrosine kinases (e.g. the EGF receptor) and RAS^{53,54}. MTMR7 may thus enforce the classical nuclear function of PPAR γ by inhibiting the RAS-ERK1/2 cascade, which otherwise evokes inactivation of PPAR γ by ERK1/2-mediated phosphorylation and MEK1/2-dependent sequestration of PPAR γ in the cytosol¹⁶, where it interacts with molecules that restrain PPAR γ activity including caveolin-1²⁶ and heat shock proteins⁵⁵.

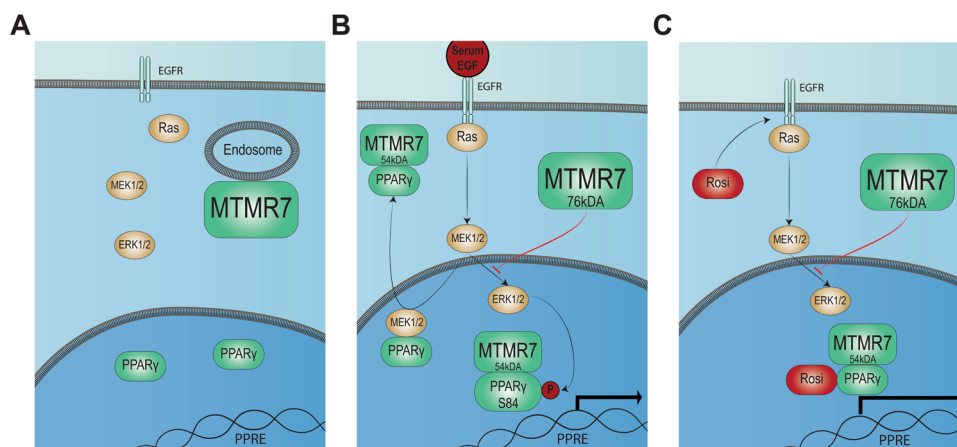


Fig. 7 MTMR7-PPAR γ signalling model. MTMR7 was identified as a novel PPAR γ interactor, which increased PPAR γ activity *in vivo* and *in vitro*. We propose a full-length (FL) isoform (76 kDa) in the cytosol, harbouring an N-terminal PH domain for binding to plasma and/or intracellular membranes (e.g. endosomes), and a mobile truncated isoform (54 kDa), presumably consisting of the C-terminal part of MTMR7 harbouring the coiled-coil (CC) domain, which was also found in the nucleus. **a** In resting cells, MTMR7 is bound to endosomes (or other vesicular structures), and ERK1/2 and MEK1/2 reside in the cytosol, whereas PPAR γ is localized in the nucleus. **b** Upon stimulation with EGF (or serum), the kinases translocate into the nucleus where ERK1/2 phosphorylate PPAR γ on Ser84, while MEK1/2 export PPAR γ to the cytosol, two events resulting in inactivation of PPAR γ . We suggest that MTMR7 counteracts the inhibitory effects of the two kinases: (1) MTMR7 FL stays in the cytosol and/or attached to membranes via its PH domain and inhibits MEK1/2-dependent ERK1/2 activation by an yet unknown mechanism, presumably involving altered PIP metabolism and EGFR traffic as shown for other MTMs. This event abrogates post-translational inactivation of PPAR γ by the above mentioned kinases (in **b**), resulting in increased transcriptional activity of PPAR γ . (2) Truncated MTMR7 translocates to the nucleus, directly binds to PPAR γ via its coiled-coil (CC) domain and acts as a nuclear receptor coactivator harbouring LXXLL-like motifs similar to SRC1. Thereby, the transcriptional activity of PPAR γ on target gene promoters is restored (e.g. *P21^{CIP1/WAF1}*, e.a.). **c** PPAR γ -ligand (exemplified by rosi) binds to PPAR γ and enhances its transcriptional activity in the nucleus. In addition, rosi triggers “non-genomic” trans-activation of plasma membrane receptors (e.g. EGFR, GPR40) and downstream kinases (in **b**). As for EGF/serum (in **b**), MTMR7 counteracts rosi-dependent “non-genomic” activation of MEKs/ERKs and promotes transcriptional activity of PPAR γ in the nucleus together with its mode of action as a SRC1-like coactivator.

In the current study, we provide evidence that MTMR7 also reduces the rapid “non-genomic activation” of ERK1/2 by the PPAR γ -agonist rosi^{56,57}. Through this mechanism, which has been shown to be mediated by EGF- or G-protein-coupled receptors (e.g. GPR40) in the plasma membrane, MTMR7 may prevent the adverse side effect of this class of PPAR γ -ligands on cell proliferation and organ damage^{49–52}. This finding is of particular significance in light of the plethora of negative clinical trials using PPAR γ agonists in the therapy of gastrointestinal malignancies^{14,58}: since the loss of MTMR7 is a common event in CRC, induced by stimuli including insulin or insulin-like growth factors²⁵, the MTMR7 expression status might be a predictive parameter when targeting PPAR γ in the setting of anti-proliferative therapies.

Moreover, we offer new insights into the subcellular distribution of MTMR7. While myotubularins in general are regarded to be cytosolic proteins^{21,22}, we collected data suggesting that MTMR7 shuttles between the cytosol and the nucleus in response to stimuli of the RAS signalling cascade or to PPAR γ agonist (here exemplary for rosi). In mice²⁴, full-length (FL) 76 kDa and truncated 54 kDa isoforms of MTMR7 are expressed. The latter lacks the C-terminal coiled-coil (CC) domain and may thus be incapable of oligomerization with other

MTMs²⁴. The peptides identified in SW480 cells by MALDI-MS also suggest the existence of a 54 kDa isoform of MTMR7 in human CRC cells, forming the complex with PPAR γ . Western blot analyses using four different Abs against the C-terminal domain of MTMR7, as well as previously published exon-selective RT-PCRs from several human CRC cell lines²⁵ confirmed the presence of truncated <54 kDa MTMR7 (Supplementary Fig. 5). We may hence conclude that the identified isoform is truncated at the N-terminus, containing the PH domain, responsible for membrane association. This truncation might therefore enable MTMR7 to shuttle into the nucleus, together with its binding partner PPAR γ (see model in Fig. 7), to enforce the transcriptional activity of the nuclear receptor.

This mode of action is further supported by the observed efficacy of the MTMR7-CC mimicry peptide, resembling the coiled-coil (CC) domain of the phosphatase, which was able to activate PPAR γ *in vitro* and *in vivo*: Notably, it induced transcription of PPAR γ target genes as well as body weight gain, a well-known adverse effect attributed to PPAR γ agonists of the thiazolidinedione class (e.g. rosi)^{49,59,60}.

Exploitation of different web servers for binding site prediction and docking, in combination with MD

simulations, pointed at the coactivator interaction site of PPAR γ being the most likely binding site for the peptides, similar to those of the bona fide coactivator SRC1, inserting the leucine at position 5 of its LXXLL motif into a hydrophobic cleft between helices 3, 4, 5 and 12³⁸. Complexes covering this interaction site were more stable and energetically favourable compared with any other candidate sites like the heterodimerization interface or the agonist-binding pocket. MP and PEP interacted with residues that were also reported for SRC1 by Nolte et al.³⁹. As such, MP made use of the charged clamp between E471 and K301 and interacted with several residues on helix 12. Both peptides were also found to interact with F306, implicating that the LXXLL/XXXL motifs might reach further into the cleft and contribute to the stable binding of the peptides to the coactivator interaction site. Notably, stretches of the peptides unfolded from their initial helical conformation assigned, whereas other parts, and especially the ones bound to the coactivator-binding site, stayed helical throughout the MD simulations. The stable helix formation of this peptide region agrees with reports on the behaviour of the LXXLL motif in SRC1 that is thought to be unstructured in its apo-form but to form a short amphiphatic α -helix upon binding to the coactivator-binding site^{61,62}.

While the computational methods favour the coactivator interaction site and resulted in a seemingly plausible interaction prediction, one important short-coming of this model for the PPAR γ /PEP complex is that PEP does not contain the canonical LXXLL motif that was deemed to be necessary for this kind of coactivator-like interaction in the past⁶². The fact that MP gave more favourable binding free energies than PEP supports the reported importance of all three leucines. Cell-free competition assays with both peptides and SRC1 on recombinant PPAR γ -LBD protein would be necessary to experimentally confirm our *in silico* predictions.

Preliminary findings revealed that the peptides were unable to displace rosi from the PPAR γ -LBD, thus unlikely to act as true agonists; instead, rosi increased MTMR7/PPAR γ complex formation and MTMR7 protein expression in CRC cells (not shown). These observations may indicate that, alike MTMR9, ligand-activated PPAR γ could stabilize the MTMR7 protein, and, vice versa, MTMR7 may lead to a quasi-allosteric activation of PPAR γ via the SRC1 coactivator site. However, future in depth nuclear magnetic resonance (NMR) or co-crystallization studies will be required to experimentally prove the predicted *in silico* models.

Taken together, our study identified a novel positive regulator of PPAR γ based on (i) inhibition of ERK1/2 signalling and (ii) direct interaction with the CC domain of MTMR7. Additional studies will be necessary to

characterize the pharmacological properties of the peptides to allow further development of this novel PPAR γ activator for future clinical applications.

Materials and methods

Reagents and plasmids

Chemicals were from Merck/Sigma (Darmstadt, Germany). Antibodies (Abs) used are listed in Table. S2. Untagged and GFP-tagged PPAR γ 1 and PPRE-luc plasmids were mentioned previously¹⁵. Human full-length (FL) MTMR7 cDNA (start codon MEHIRT, aa 1–660, 76 kDa, NM_004686.4) was in pTarget (pT) vector (Promega GmbH, Mannheim, Germany) with or without GFP-tag²⁵. Transient transfection and luciferase assays were performed as described⁶³.

Peptides

PEP corresponded to the original leucine-rich aa sequence of MTMR7 (aa 521–550; SwissProt ID: Q9Y216.3: LMAVKEETQQL~~EEEELEALEERLEKI~~QKVQL) (Tab. S1). MP was designed based on the PEP sequence using a random scrambling web tool (<https://web.expasy.org/randseq/>): VLQEEILEMTEEK~~ALLQALLKKEQ~~ERVQEE) generating a canonical LXXLL nuclear receptor coactivator motif. Both peptides were synthesized by automated, solid phase peptide synthesis on Rink amide resin and coupled at the N-terminus with myristidic acid (ETH Zürich, Switzerland). The resulting peptides were characterised by HPLC and MALDI-MS for purity and sequence confirmation and provided as lyophilized powders⁶⁴.

Cell culture

Human embryonic kidney (HEK293T) and colon adenocarcinoma cell lines (all from the American Type Culture Collection, Rockville, MD) were maintained as before²⁶. PDOs from CRC tissues were cultivated as published in refs. ^{65,66}. All cultures were routinely tested for contamination with Mycoplasma (InVivogen, Toulouse, France).

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Author contributions

All authors cooperated and contributed to, critically reviewed and approved the paper. E.B., M.E. and R.S. defined the research theme. E.B., L.H., M.S., P.W., T.G., T.S. and V.H. designed methods, carried out the experiments, analysed the data and interpreted the results. J.B. conducted sequencing and bioinformatics analyses. F.R., J.W.B. and V.P. synthesized the peptide. D.S. modelled 3D structures, performed and analysed simulations with the help and supervision of A.N.A. and R.W. C.R. provided clinical samples, performed and analysed immunohistochemical staining. D.S., E.B. and P.W. wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest. Parts of the cell line data have been deposited under <https://doi.org/10.11588/heidok.00025716>; <https://doi.org/10.11588/heidok.00023843>.

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