The role of calponin in the gene profile of metastatic cells: inhibition of metastatic cell motility by multiple calponin repeats

Thomas Lener, Gerald Burgstaller, Mario Gimona*

Institute of Molecular Biology, Department of Cell Biology, Austrian Academy of Sciences, Billrothstrasse 11, A-5020 Salzburg, Austria

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Abstract **Metastasis of diseased cells is the basic event leading to death in individuals with cancer. Establishment of metastasis requires that tumour cells migrate from the site of the primary tumour into the circulation system, escape from the vasculature and form secondary tumours at novel sites. These processes depend to a large degree on cytoskeletal remodeling. We show here that multiple copies of the short actin-binding module CLIK^{23} from human or *Caenorhabditis elegans* calponin proteins effectively inhibit cell motility on two dimensional matrices and suppress soft agar colony formation of metastatic melanoma and adenocarcinoma cells of murine and human origin. Ectopic expression of CLIK^{23} modules for 30 days results in the formation of multinucleated cells. The repeat displays true modular behaviour, resulting in increased cytoskeletal effects in direct correlation with the increase in number of modules. Our results demonstrate that the role of calponin in the signature profile of metastasising cells is that of a mechanical stabiliser of the actin cytoskeleton, which interferes with actin turnover by binding at a unique interface along the actin filament.**

Key words: Calponin repeat; Cell motility; Metastasis; Melanoma; Adenocarcinoma; Actin turnover

1. Introduction

Establishment of metastasis requires that tumour cells lose the ability to form contacts with their neighbouring cells, migrate from the site of the tumour into the circulation system, escape from the vasculature, invade novel sites and proliferate to form secondary tumours [1,2]. These processes require cytoskeletal remodeling and a number of cytoskeletal and regulatory proteins have been implicated in the progression of metastatic cell motility [3]. Current approaches towards understanding the mechanisms of metastatic disease have evolved beyond the descriptive or purely phenomenological stage. Modern research activities investigating malignancy caused by tumour cell metastasis employ complex model systems, direct genetic manipulations and, to an increasing extent, gene expression profiling [4].

A recently identified 17-gene signature profile [5] distinguishes primary from metastasising tumours. The profile, established initially for adenocarcinoma metastases, contains eight upregulated and nine downregulated genes. Strikingly, almost 50% of the downregulated genes affect components of the actin cytoskeleton, namely γ-actin, myosin heavy chain, myosin light chain kinase, and *h*1calponin (CaP). Expression levels of the smooth muscle *h*1CaP isoform are decreased in a number of tumours, including human fibrosarcoma [6], leiomyosarcoma [7] and osteosarcoma [8], and CaP expression is lost in malignant prostate tissue [9]. Reduced levels of CaP expression correlate with alterations in actin cytoskeleton stability in a number of soft-tissue tumours [7], and residual expression levels of *h*1CaP are associated with increased survival rates of osteosarcoma patients [8], while re-expression of CaP suppresses tumourigenicity in nude mice [6]. The molecular mechanism underlying the increased tumour forming potential of CaP-deficient cells remains, however, to be elucidated.

We show here that ectopic expression of multiple 23 amino acid residue calponin-like (CLIK^{23}) repeats reduces cell motility and colony formation in soft agar in cultured mouse melanoma and human adenocarcinoma cells. The results shed new light on the mechanisms underlying the cellular consequences of cytoskeletal remodeling during cellular transformation and plasticity, tissue invasion and metastatic progression of tumours, and arteriosclerosis.

2. Materials and methods

2.1. cDNA constructs

Green fluorescent protein (GFP)-tagged cDNA constructs were described before [10–13]. Fusion constructs ABD-*h*1CaP and CH-UNC-87 were generated by PCR of the respective isolated domains into GFP C1 and C3 vector (Clontech, Heidelberg, Germany), respectively.

2.2. Cell culture, transfection and immunofluorescence microscopy

Mouse B16F1 melanoma and human MDA-MB 231 or MCF-7 breast cancer cells (LGC Promochem, Wesel, Germany) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (PAA, Linz, Austria), penicillin/streptomycin (Gibco, Vienna, Austria) at 37°C and 5% CO_2. For transient expression, cells were grown in 60 mm plastic culture dishes and transfected using superfect or effectene (Qiagen, Hilden, Germany) at 70% confluence, essentially as described elsewhere [12]. Expression and stability of the constructs was assessed by Western blotting using a monoclonal antibody against GFP (Clontech, Heidelberg, Germany). Cells were replated onto 15 mm cover slips 16 h post-transfection and

*Corresponding author. Fax: (43)-6662-6396140.
E-mail address: mgimona@server1.imolbio.oeaw.ac.at (M. Gimona).

Abbreviations: CLIK^{23} repeat, 23 amino acid residue calponin-like repeat; F-actin, filamentous actin; GFP, green fluorescent protein; CaP, calponin; PFA, paraformaldehyde; h1, h2, genetic variants of CaP

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prepared for live or immunofluorescence microscopy. For immunofluorescence staining cells were washed three times in phosphate-buffered saline (PBS) (138 mM NaCl, 26 mM KCl, 84 mM Na_2HPO_4, 14 mM KH_2PO_4, pH 7.4), extracted in 3.7% paraformaldehyde (PFA; Merck, Darmstadt, Germany)/0.3% Triton X-100 in PBS for 5 min. and fixed in 3.7% PFA in PBS for 30 min. Fluorescence images were recorded on a Zeiss Axioscope equipped with an Axiocam driven by the manufacturer's software package (all from Zeiss, Vienna, Austria) using a 63× oil immersion lens.

2.3. Antibodies

Monoclonal and polyclonal antibodies to GFP were from Clontech (Heidelberg, Germany). Fluorescently labelled secondary antibodies and phalloidin labelled with Alexa 350 (blue), Alexa 488 (green) or Alexa 568 (red) were from Molecular Probes (Leiden, The Netherlands).

2.4. Soft agar colony formation

Colony formation on soft agar was performed as described [14] in 35 mm dishes. 1×10^4 cells were suspended in 2 ml of 0.4% bactoagar (Becton Dickinson Biosciences, Heidelberg, Germany) in DMEM w/o phenol red (Gibco, Vienna, Austria) and supplemented with 10% foetal bovine serum. Cells were transferred onto a base layer of 0.8% bactoagar. Plates were incubated at 37°C in a 5% CO_2 incubator for 3–6 weeks. The number of colonies was assessed by light microscopy at low magnification using a 10× lens at a minimum of six independent and random positions on the plate.

2.5. Live video microscopy and analysis

Cells were observed in an open, heated chamber (Warner Instruments, Hamden, CT, USA) at 37°C on a Zeiss Axiovert TV-135 inverted microscope equipped with epifluorescence, phase-contrast and DIC optics. The objectives 40×/NA 1.3 Plan-Neofluar and 100×/NA 1.4 Plan-Apochromat were used with or without 1.6 optovar intermediate magnification. 100 W tungsten lamps were used for fluorescence and phase contrast illumination. Data were acquired using a back-illuminated, cooled charge-coupled-device camera (Princeton Scientific Instruments, Monmouth, NJ, USA) driven by a 16 bit controller. The camera controller was driven by IPLabs software (Visitron Systems, Eichenau, Germany), and shutters were used on the

a

MODULE DESCRIPTION

		CLIK[23] module	CLIK[23] module	intervening sequences
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SM22	c	iglqmgsnrgasqagmtgygrpr	iglqmgsnrgasqagmtgygrprqiis	
*h*1CaP	c1	iglqmgtnkfasqqgmtaygtrr	iglqmgtnkfasqqgmtaygtrrhlydpklgtdqpldqat	
	c2	islqmgtnkgasqagmtapgtkr	islqmgtnkgasqagmtapgtkrqifepglgmehcdtln	
	c3	vslqmgsnkgasqrgmtvyglpr	vslqmgsnkgasqrgmtvyglprqvyd	
UNC-87	c1	ipsqagwnkgdsqklmtnfgtpr	ipsqagwnkgdsqklmtnfgtprntntrvksenlqeipedianrthge	
	c2	vrlqsgtnkycsqrgmtgfgsgr	vrlqsgtnkycsqrgmtgfgsgrdvcregvrvaqnpadlaelpeekirmsegi	
	c3	vrlqagtnkydsqkgmtgfgtgr	vrlqagtnkydsqkgmtgfgtgrrettkmvdskhpeydhekpdqse	
	c4	iplqsgtnkfasqkgmtgfgtar	iplqsgtnkfasqkgmtgfgtarrettkmvdsnhpdyshecsidqtt	
	c5	ipsqmgsnqyasqkgmtgfgqpr	ipsqmgsnqyasqkgmtgfgqprwevldpsiswqnrksqgm	
	c6	vrlqsgtnrfasqagmigfgtcr	vrlqsgtnrfasqagmigfgtcrnttfeaeggelpyeamkvseti	
	c7	ipsqagwnkgdsqkkmtsfgapr	ipsqagwnkgdsqkkmtsfgaprdvkgkhlkriweleypeeaeisldrl	
		Sequence Similarity	***Disorder Promoting Amino Acids***	

b

CONSTRUCT ANNOTATION

CH-*1-2-3*--	CaP (*h*1 or *h*2)
CH-*1-2-3*	CaPΔt (*h*1 or *h*2)
CH-CH-*1-2-3*--	ABD-CaP
CH-*1*	SM22α (C- or N-terminal fusion)
---*1-2-3-4-5-6-7*-	UNC-87 (1-7)
2-3-4-5-6-7-	UNC-87 (2-7)
3-4-5-6-7-	UNC-87 (3-7)
4-5-6-7-	UNC-87 (4-7)
5-6-7-	UNC-87 (5-7)
---*1-2-3*	UNC-87 (1-3)
CH-*1-2-3-4-5-6-7*-	CH-UNC
CH-CH-SP-SP-SP-SP-EF-EF-	α-actinin
CH-CH	ABD

Fig. 1. a: Sequence alignment of CLIK[23] repeats and adjacent intervening sequences from human SM22, human CaPs and *C. elegans* UNC-87. Left: only the 23 core residues of the module display sequence similarity. Right: CLIK[23] repeats and intervening regions contain primarily disorder promoting residues. b: GFP-tagged cDNA constructs of wild-type, fusion and deletion mutants carrying CLIK[23] modules (c) of varying number (1, 2, 3, ...) and origin (CaP, UNC-87 or SM22), or the ABD from non-muscle α-actinin. Δt: tail deletion mutants of CaP; CH-UNC and ABD-CaP: fusion mutants with one (*type 3*) or two (*type 1*/*type 2* tandem) CH domains, respectively, fused to the N-terminal end; SP and EF: spectrin repeats and EF hand motifs in α-actinin. c: Differential effects of ectopic GFP CLIK[23] module construct expression on cytoskeleton stability in B16F1 mouse melanoma cells. Stability of the actin filaments correlates to the number of CLIK[23] modules present in the mutant protein. Double immunofluorescence image using Alexa 568 phalloidin to visualise F-actin.

illumination ports to minimise photodamage [15]. The digital images were analysed on an Apple Power Macintosh G3, using IPLabs (Scanalytics, Fairfax, VA, USA) and Adobe Photoshop 2.5 and 5.5 software. A minimum of 10 independent experiments was performed for each construct tested.

3. Results and discussion

The actin-binding modules from CaP [16] or the *Caenorhabditis elegans* homologue UNC-87 [17], comprised of multiple CLIK^{23} repeats, are highly conserved at the amino acid level (Fig. 1a). Both the CLIK^{23} modules and intervening sequences consist of an excess of primarily disorder promoting amino acid residues [18,19], a hallmark of intrinsically unstructured proteins [20]. Overexpression of constructs containing multiple CLIK^{23} modules inhibits podosome formation in vascular smooth muscle cells [10], and causes protection of the actin cytoskeleton against remodeling by latrunculin and cytochalasin, or RhoKinase inhibition [11]. Thus, it was tempting to speculate that stabilisation of the actin cytoskeleton and interference with actin filament turnover rates in living cells will cause a general reduction in cell motility [21]. To test this hypothesis we here used 16 different GFP-tagged constructs (Fig. 1b). Transfection of GFP UNC-87(1–7) in B16F1 mouse melanoma cells induces the formation of stable actin stress fibres [12]. A similar induction was observed with deletion mutants 2–7, 3–7 and 4–7, which all bind and bundle actin in vitro [12] (not shown), whereas the constructs comprising the N-terminal (1–3) or the C-terminal (5–7) three CLIK^{23} repeats were not effective in maintaining a stable actin cytoskeleton in living cells (Fig. 1c).

Rapid remodeling of the actin cytoskeleton is a hallmark of motile cells [22]. In light of the phenotypic alterations induced by the CLIK^{23} modules we next investigated the influence on cell motility in living cells. For this purpose we first transiently transfected B16F1 cells with the various cDNA constructs and monitored their motile behaviour by live video microscopy in phase contrast and epifluorescence (Fig. 2a). Ectopic expression of GFP alone did not alter B16F1 motility on laminin-coated glass. Cells expressing either of the triple CLIK^{23} module-containing mutants (1–3) or (5–7), despite their low actin-binding capacity already displayed a significant reduction in motility compared to the non-transfected wild-type or GFP-transfected control cells. This reduction in motility further increased when the mutants containing four, five, six, or seven CLIK^{23} modules were used. The full-length UNC-87(1–7) protein reduced cell motility by about eight-fold. From these data we hypothesised that the inhibition of cell motility is directly related to the number of CLIK^{23} modules present in a given actin-binding protein. Indeed, the weak inhibitory activity measured with the UNC mutants (1–3) and (5–7) was comparable to that induced by the transient expression of *h*1CaP or *h*2CaP, which likewise contain three copies of the CLIK^{23} module. In further agreement with this hypothesis, GFP-tagged SM22 [23], which harbours a single CLIK^{23} module in its C-terminus, showed an even weaker, yet significant reduction of B16F1 motility. In contrast to UNC-87, CaP contains two negative regulatory domains at its C- and N-terminal ends. When the C-terminal tail sequences were deleted (CaPΔt mutants) to render the CLIK^{23} modules fully active, the inhibitory effect further increased compared to the full-length proteins. On the other hand, fusion of the *type3* CHD from CaP onto UNC-87(1–7) significantly reduced the motility inhibiting activity, suggesting that CaP activity in vivo may be regulated by the interactions of these two domains which, in concert, serve to modulate actin binding via the CLIK^{23} module interface.

Considering that the most effective UNC mutants (3–7), (2–7) and (1–7) not only bind, but also efficiently bundle filamentous actin (F-actin) in vitro and in vivo [12], it was plausible that the reduced motility was a direct effect of the bundling activity. To test this possibility we transiently transfected B16F1 cells with GFP-tagged non-muscle α-actinin, a prominent cellular cross-linker protein [24]. Strikingly, the expression of α-actinin caused only marginal reduction of B16 motility on laminin and the values measured were almost comparable to those obtained with the isolated actin-binding domain (ABD) of α-actinin which strongly binds to actin stress fibres but is incapable of bundling. Thus, cross-linking of the actin filaments cannot be the sole molecular basis for the reduction of cell motility. Actin-binding sites formed by either calponin homology (CH) domains [25] or CLIK^{23} modules occupy non-competing binding sites along the actin filament [12], suggesting that binding of CaP via the CLIK^{23} modules is independent of any putative interaction of the CH domain with the actin filament. Indeed, a mutant fusion protein of the ABD from α-actinin onto *h*1CaP, replacing the single *type 3* CH domain in CaP, was as effective in reducing B16 motility as was the wild-type CaP molecule. This indicates that the effects on the actin cytoskeleton are not additive, and that the CLIK^{23} modules functionally dominate over the CH domain module in this particular case. When we cultured transfected cells for a period of up to 30 days we observed a substantial increase in the number of multinucleated

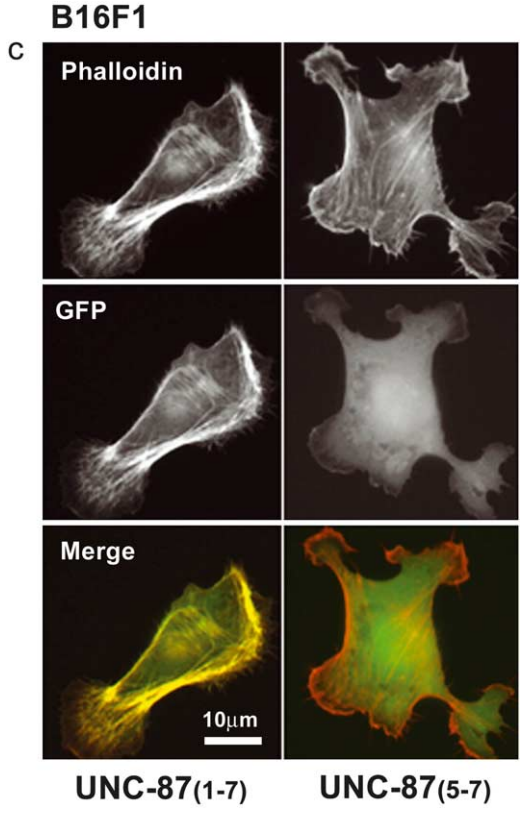

Fig. 1 (*Continued*).

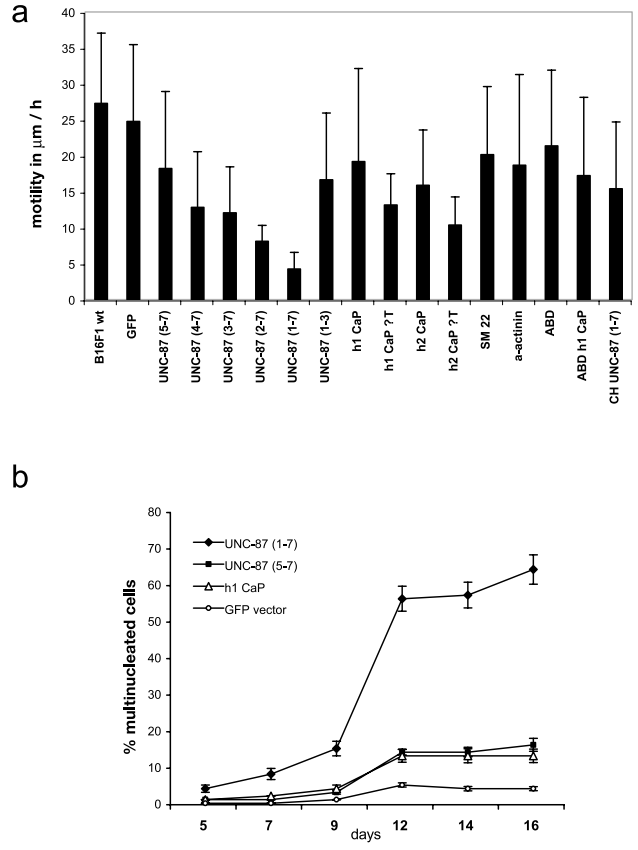

Fig. 2. a: Inhibition of metastatic cell motility in B16F1 cells transiently transfected with the indicated GFP constructs and monitored by live video microscopy in phase contrast and epifluorescence on laminin-coated glass. Values (given as S.D.) represent at least 15 individual experiments per construct tested. b: Ectopic expression of multiple CLIK^{23} module constructs increases the formation of multinucleated cells (>1 nucleus).

cells, again in direct correlation to the number of CLIK^{23} modules present in the constructs (Fig. 2b). This result argues for significant interference with cytokinesis as a consequence of the interference with actin filament turnover.

To this end we have demonstrated that binding of multiple CLIK^{23} modules significantly stabilises the actin cytoskeleton against rapid remodeling, likely by altering the balance in actin turnover, and impair cell motility on a two dimensional surface. However, cellular behaviour differs significantly in a two dimensional versus a three dimensional environment. To investigate the effect of the observed interference with actin cytoskeleton remodeling and turnover on the ability of B16F1 cells to form tumour-like colonies in vitro, we plated cells expressing any of the GFP-tagged constructs on soft agar and monitored colony formation over a period of up to 7 weeks. As shown in Fig. 3 the number of colonies was strongly reduced in UNC-87(1–7)-transfected cells. This reduction in colony number showed a similar dependence on the number of CLIK^{23} modules present on the constructs used for transfection as observed before in the motility assays. CLIK^{23} module-expressing cells not only formed fewer colonies (Fig. 3a), but these colonies also grew to a considerable size, compared to the microcolonies observed with wild-type or GFP-transfected B16F1 cells (Fig. 3b).

This finding indicated that proliferation was not impaired by the expression of the strongly inhibiting UNC-87(1–7) constructs. Indeed, when we measured the duplication time in cells expressing any of the above constructs the differences were marginal. However, when we attempted to select stable cell lines we observed a significant reduction in protein expression in clones expressing the most active UNC-87 mutants (1–7), (2–7), (3–7), but not for the triple CLIK^{23} mutants (1–3) and (5–7), or *h*1CaP. Since expression levels and transfection efficiency was almost identical for all constructs this result argues for a tolerance problem in the transfected cells upon prolonged expression. When we measured the content of GFP-fusion protein in adhesive cells versus cells that are released into the culture medium we observed that the levels in the adhesive cells were reduced while in the soluble fraction they remained almost constant. Closer inspection of the cells expressing UNC-87(1–7) demonstrated that with time the colonies also contained an increasing number of multinucleated cells (not shown), whereas GFP transfected cells did not. This result supports recent findings by Hossain et al. [26] and demonstrates that the unique actin-binding function of the CLIK^{23} module interface is sufficient for the inhibition of cytokinesis. One further interpretation of this result is that prolonged expression of the initial high levels of the multiple

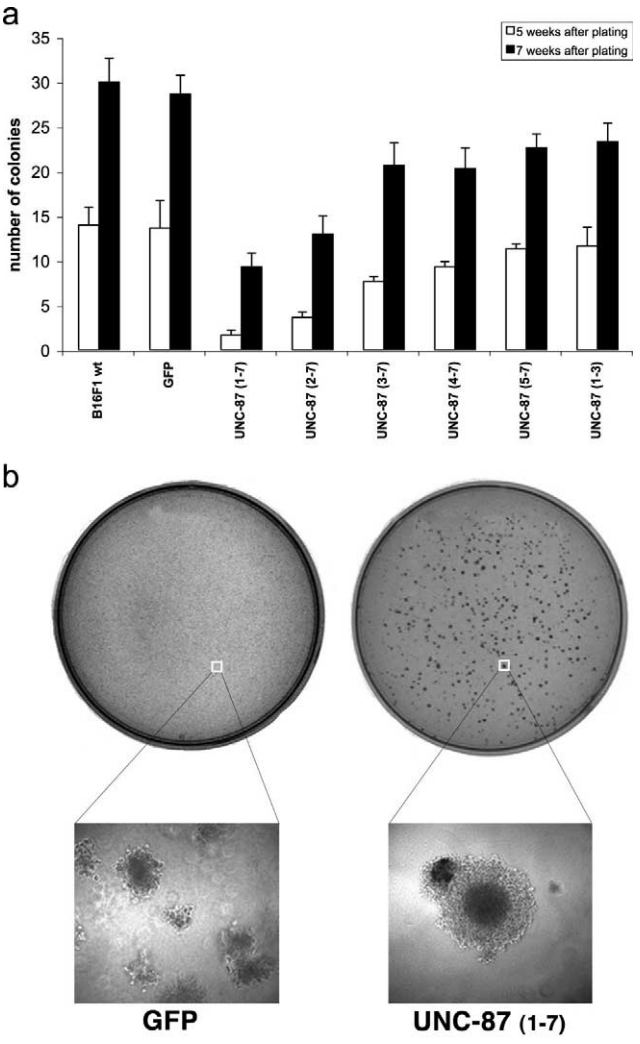

Fig. 3. a: Expression of CLIK^{23} modules reduces the number of colonies in soft agar. b: Selected colonies of B16F1 cells in soft agar expressing either GFP or GFP UNC-87(1–7).

CLIK[23] module constructs leads to cell death. Optical sectioning through colonies in soft agar indeed revealed a large number of dead cells in the centre of the colonies supporting this contention (not shown).

From these data we concluded that inhibition of cell motility together with the formation of large, compact colonies in a three dimensional matrix argue for a mechanism aiming at the inhibition of metastatic tumour progression. In comparison to normal tissues and benign melanocytic tumours, the expression of *h*1CaP is significantly suppressed in malignant melanomas [27], and suppression of CaP is coincident with the increased structural fragility of the vessel and the increased penetration by metastatic cells [28]. Together these findings suggest that high levels of *h*1CaP expression are important for the suppression of metastasis. To test if the hypothesis of regulation of actin turnover by CLIK[23] module binding may be of a more general value to metastatic carcinoma, we repeated the core experiments in a number of human cell lines focusing on the highly metastatic breast cancer cell lines MDA-MB 231 and MCF-7. The results, summarised in Fig. 4, demonstrate identical effects of the CLIK[23] modules in the human adenocarcinoma cell lines as observed for the mouse B16F1 system with respect to both subcellular localisation and induction of stress fibres, inhibition of cell migration, and colony formation in soft agar.

Considering that the clinical outcome of individuals can be

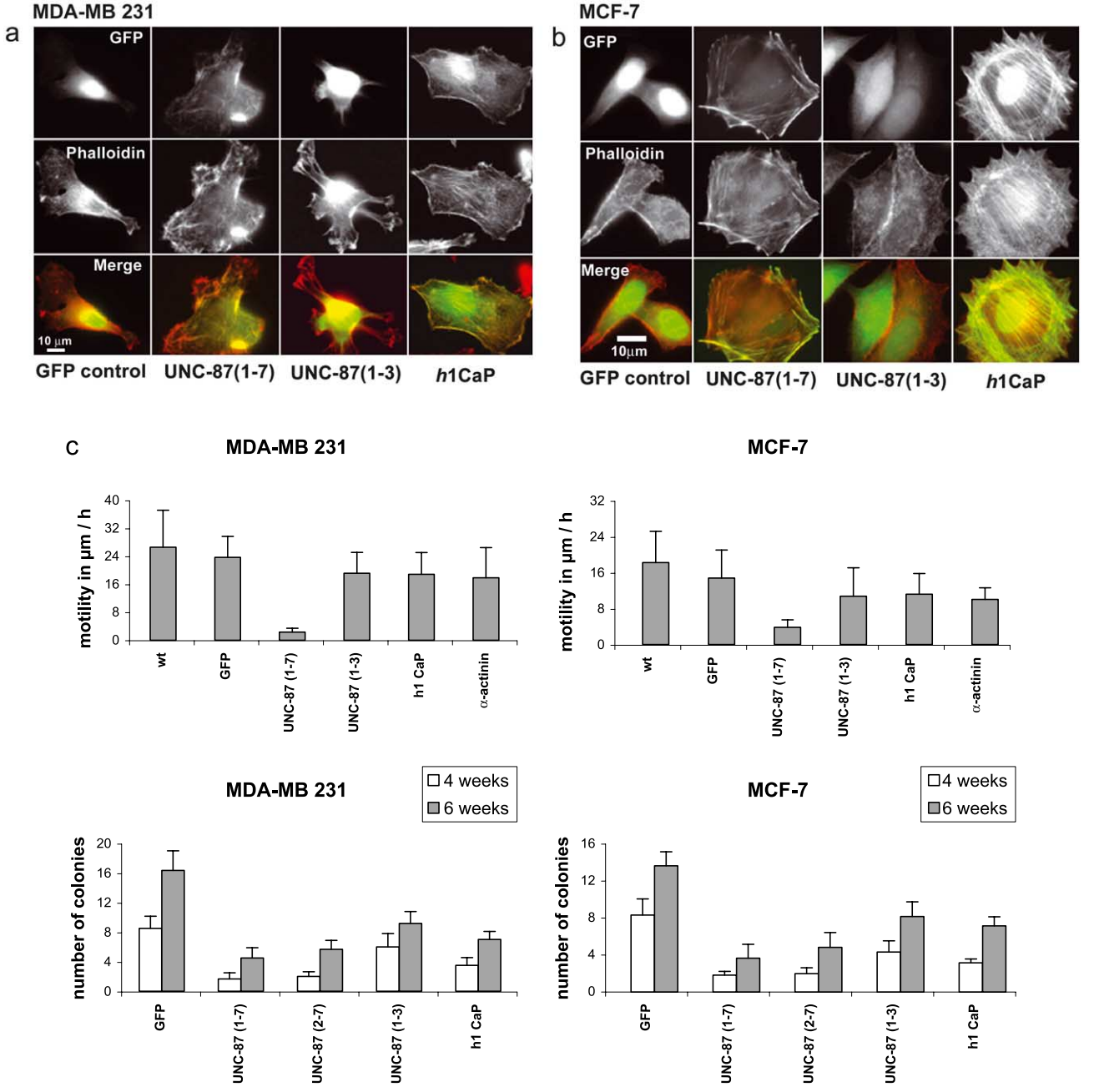

Fig. 4. a,b: Expression of multiple CLIK[23] modules stabilises the actin cytoskeleton in MDA-MB 231 (a) and MCF-7 (b) cells. Values represent at least 10 individual experiments per construct tested. c: Suppression of cell motility on matrix-coated glass and formation of colonies in soft agar in MDA-MB 231 and MCF-7 cells. Immunofluorescence as in Fig. 1c.

predicted using gene-expression profiles of primary tumours at diagnosis it is important to determine the factors, or *signature genes*, that are mechanistically important in the cascade of events leading to metastasis [4]. Our previous studies have highlighted CaP as a major stabilising component of actin filaments in living cells, and as a suppressor of actin cytoskeleton remodeling [10,11]. The conformational stability conferred to actin filaments by the binding of CaP reduces the rate of filament turnover by introducing subtle alterations in the filament's three dimensional structure [10,29]. Loss of CaP may alter the balance of the actin turnover cycle towards instability and increased polymerisation activity, an established hallmark of motile and invasive cells. These results are in excellent agreement with recent work from Hashimoto and colleagues [30] who have demonstrated that re-expression of *h*1CaP into cultured peritoneal cells from the CaP knockout mouse protects them from invasion by metastatic B16F10 melanoma cells. Our work extends these findings by demonstrating that the CLIK^{23} modules are sufficient for stabilising the actin cytoskeleton in metastatic melanoma and adenocarcinoma cell lines of mouse and human, irrespective of the species origin of the CLIK^{23} sequence. Similar processes are likely to be effective in other tissues. The preferential expression of acidic CaP in brain may point towards a role in the regulation of, e.g., glioblastoma motility in vitro [31].

Like CaP, the actin-binding protein SM22 (also known as transgelin) has been identified as a major downregulated component in a number of gene expression profiles aiming at the identification of tumour-related genes [32,33]. In addition, results from the Der lab [34] have revealed that SM22/transgelin is downregulated in epithelial cells upon activation of Ras. Moreover, this latter study demonstrated that reduced expression levels of SM22/transgelin are found in both breast carcinoma cell lines in vitro and benign human colon and breast tumours in vivo, suggesting that loss of SM22 expression contributes at an early stage to the development of cancers. In further agreement with our study the same group also showed that forced re-expression of SM22/transgelin cannot significantly revert the invasive properties of MDA-MB 231 cells. However, the study fell short of offering a potential mechanistic explanation for the observed importance of SM22/transgelin for the maintenance.

Albeit the establishment of signature gene profiles suffices to pin down the important factors for a given situation, the mere identification of the components does not automatically guarantee that there is sufficient functional data available for all the components in order to generate interaction maps and symptom–cause relationships. In this specific case we have only a vague idea about the physiological roles of CaP and SM22 in normal tissue, and even more so about the aberrant expression in tumour cells. In our ongoing studies we aim at defining the potential of vascular smooth muscle cells to adopt a motile phenotype in response to CaP and SM22/transgelin inactivation. Future work will aid in our understanding of the mechanisms underlying the cellular consequences of cytoskeletal remodeling by CLIK^{23} module-containing proteins during cellular transformation and plasticity.

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