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FHOD1 formin is upregulated in melanomas and modifies proliferation and tumor growth

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\section{Introduction}

Cells undergo substantial functional alterations in order to obtain proliferative, invasive and metastatic properties which are associated with malignancy. Alterations that involve cell shape, signalling, adhesion, and migration depend on efficient remodelling of the actin cytoskeleton. Actin filaments not only determine shape but also form a scaffold for motor proteins that provide the contractile force for invasive movement and serve as regulators of intracellular signalling cascades. Dynamic actin filament remodelling is controlled by a multitude of actin-associated molecules [1]. Several classes of actin nucleating proteins assist the assembly of actin monomers into filamentous structures. Among them, the formin family is the largest.

Formins are large multi-domain proteins, which are defined by the presence of a highly conserved formin homology 2 (FH2) domain. This domain mediates nucleation or elongation of actin filaments. Other domains, present in most formins, regulate activation/inactivation and subcellular filament targeting. The biochemical properties of the individual formins are distinct and sometimes even counteract each other: once activated, they may nucleate, elongate, bundle, cap and even sever actin filaments. On the cellular level, individual formins have been shown to participate in the formation of diverse cellular protrusions, adhesions, and influence serum response factor-mediated transcription [2–4].

FHOD1 (formin homology 2 domain containing protein 1) is an efficient capping and bundling protein of actin filaments. In contrast to the majority of formin proteins, it does not elongate actin filaments \textit{in vivo} [5]. The participation of FHOD1 in cytoskeletal remodelling and cell migration has been established in fibroblasts, melanoma cells and breast cancer cells [6,7]. FHOD1 is one of formins regulating transcription from the serum response element (SRE) [7,8]. This occurs through changes in the pool of monomeric actin. With increased actin filament polymerization, the pool of monomeric actin is reduced, which allows the release of megakaryoblastic leukemia 1 (MKL-1, a.k.a. MAL or myocardin related transcription factor A, MRTF-A) which translocates/accumulates in nuclei [9]. MKL-1 binds to the SRE,
inducing transcription from many cytoskeletal and cell cycle progression associated genes [10,11]. FHOD1 has further been found to be essential for nuclear movement and centrosome orientation in fibroblasts polarizing for migration [12,13].

There are very few studies on formin expression and significance in clinical cancer. FMNL1 (Formin-like protein 1) is over-expressed in non-Hodgkin lymphoma [14], and high FMNL2 (Formin-like protein 2) expression is associated with metastasis in colorectal cancer [15]. Recently, we found that high expression of FMNL2 in localized cutaneous melanoma is associated with increased risk of recurrence during follow-up. In cell lines, FMNL2 expression was dependent on ERK MAPK signalling, a pathway commonly over activated melanoma [16]. In another study, we found that FHOD1 expression increased with cancer-associated EMT, a process that mediates invasion [17]. Intriguingly, FHOD1 has been shown to directly interact with components of the ERK MAPK pathway [18], which suggests that FHOD1 could, if expressed in melanoma, be a link between altered signalling and cellular behaviour. With this background, we wanted to investigate FHOD1 expression in melanocytic neoplasms and explore its roles in melanoma cell lines.

In this study, we found that FHOD1 is expressed in most melanomas. In order to study the functional role of FHOD1 in melanoma, we stably silenced FHOD1 expression in the WM164 melanoma cell line using shRNA constructs. Studying the effects of FHOD1 depletion, we found changes in cell morphology, migration, colony formation, cell-spreading assays and cell cycle analysis in vitro. Importantly, we found that tumor growth is reduced after FHOD1 depletion in a mouse melanoma xenograft model. Taken together, all these results suggest a role for FHOD1 in melanoma tumorigenesis.

2. Material and methods

2.1. Tumor histology and immunohistochemistry

The mouse melanoma xenografts were paraffine embedded, sectioned and haematoxylin and eosin stained. For immunohistochemistry, slides were sectioned at 3 μm, stained for Ki-67 and cleaved caspase 3 using a Ventana Discovery XT autostainer device (Ventana Medical Systems, Tucson, AZ). After a standard pretreatment with Cell Conditioning Solution CCI (Ventana), the slides were incubated with a Ki-67 polyclonal antibody (1:1000, Chemicon International, Billerica, MA) for 36 mins or Cleaved Caspase 3(D3E9) 1:100, Cell Signalling Technology, Danvers, MA) for 40 min, respectively. OminMap anti-Rb HRP using ChromoMap DAB, both from Ventana, were used for detection. FHOD1 immunohistochemistry was performed using a Labvision Autostainer device with a BrightVision Poly-HRP-anti-Rabbit IgG detection kit according to the manufacturer’s protocol (Immunologic, Duiven, the Netherlands). The slides were pressure cooked for 2 min for antigen retrieval. The primary antibody against FHOD1 (1:150, Sigma-Aldrich, St Louis, MI) was incubated for 1 h, the secondary antibody for 30 min. This antibody has been extensively characterized previously [17]. Diaminobenzene was used as chromogene.

The tumor histology was evaluated by microscopy of HE-stainings. For analysis of cell diameter and of Ki-67 staining, four photomicrographs were taken at 400x magnification from different tumors in each treatment group. The diameter of at least 50 cells was measured from each micrograph.

For the study of FHOD1 expression in nevi and melanoma, 8 benign nevi and 10 melanoma +3 paired metastatic melanoma FFPE samples were sectioned and immunohistochemically stained as described above for mouse xenografts. The samples were collected from the tissue archive of the Department of Pathology at Turku University Hospital with the approval of the Joint Committee on Ethics of the University of Turku and Turku University Hospital.

2.2. Cell lines and culture conditions

Metastatic melanoma cell lines WM164 and WM239 (BRAF V600E and BRAF V600D, respectively) were cultured in RPMI 1640 medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Biowest), 5 mM Ultraglutamine I (Lonza, Basel, Switzerland), 1 x non-essential amino acids (NEAA) (Lonza), and 100 U/ml penicillin-streptomycin (Gibco). Cutaneous Bowes melanoma cell line (BRAF WT) and the metastatic SK-Mel-28 cells (BRAF V600E mutated) were cultured in EMEM (Gibco) and MEM (Gibco), respectively, supplemented with 10% FBS, 5 mM Ultraglutamine I, and 100 U/ml penicillin-streptomycin. Malme-3 M (BRAF V600E mutated) metastatic cells were maintained in DMEM (Lonza) containing all the mentioned supplements.

2.3. Western blotting

Cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with inhibitors (1xComplete Mini -protease inhibitor cocktail [Roche] and 1XPhosStop a phosphatase inhibitor cocktail [Roche]). Insoluble cell debris was removed by centrifugation (21,000g for 30 min at 4°C). The protein concentration of lysates was determined by the Bradford method (BioRad) after 5x Laemml to samples. Equal amounts of total protein were separated by SDS-PAGE and transferred to nitrocellulose membrane (Whatman PROTRAN, PerkinElmer). The membranes were blocked with 5% dry milk in TBST (TBS, 0.1% Tween) and immunoblotted with different antibodies diluted in 5% bovine serum albumin (BSA) in TBST.

The rabbit anti-human FHOD1 antibody (Sigma-Aldrich) was used at 1:1000. The mouse anti-α-tubulin antibody (Sigma-Aldrich) was used 1:10,000 as a control for protein loading. The secondary antibodies were HRP-conjugated swine anti-rabbit and HRP-conjugated rabbit anti-mouse immunoglobulins (1:2500, Dako) diluted in block solution. Membranes were washed three times with TBST between the different steps.

2.4. Generation of stable FHOD1 knockdown cell lines

For establishment of stable FHOD1 knockdown cell lines, 300×10^3 WM164 cells were transfected in a 24 well plate using 2 μl of Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) and 50 ng of pRFP-C-RS HuSH shRNA RFP vectors (Origene, Rockville, MD, USA) containing either the scrambled shRNA cassette TR30015 (scramble control), human FHOD1 targeting shRNAs FI351919 (CGCTGTGCCAAGGTGGACTTTGAACAGCT; shRNA 1) or FI351920 (CCCGAGACAGAGATTCTCAGGTGGCT; shRNA 2). The transfection medium was changed after 24 h and the cells were kept in selection medium containing 1.0 μg/ml puromycin (Sigma) for 2 weeks. Next, the cells were transferred to 10 cm dish and colonies expressing RFP (red fluorescence protein) were isolated, amplified and analysed for FHOD1 expression by western blotting.

The FHOD1 shRNA clones were stable in culture and maintained a low FHOD1 level for at least 15 passages in medium which contained 0.5 μg/ml puromycin. Similar passages of the 3 clones were used in all the following studies.

2.5. Cell Immunofluorescence stainings and microscopy

Cells were plated on gelatin (Sigma-Aldrich) precoated coverslips (13 mm) and grown in complete medium for 24 h. Next, the cells were fixed with 4% parformaldehyde for 10 min at room temperature. The coverslips were washed with PBS and blocked with 3% BSA, 5% dry milk, 0.5% triton X-100 in PBS for 45 min. The rabbit anti-FHOD1 (1:200, Millipore) antibody was incubated 1 h at room temperature. Next, the coverslips were incubated with Alexa Fluor 568 goat anti-

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rabbit IgG (1:500, Invitrogen) or Alexa Fluor 488 goat anti-mouse IgG (1:500, Invitrogen).

Alexa Fluor 488 or 546-conjugated phalloidin (1:100, Invitrogen) were incubated together with the secondary antibodies in order to visualize filamentous actin. The mounting media contained DAPI for staining nuclei (ProLong Gold Antifade Mountant with DAPI, Life Technologies). After each staining step, the cells were washed three times with PBS. Images were taken with an Olympus BX60 fluorescence microscope. The images (1360×1024 px) were analysed with ImageJ 1.49b software (http://rsweb.nih.gov/ij/) for quantification of changes in morphology. Cell circularity was measured using ImageJ (Measure and Analyze Particles commands; circularity=4π(area/perimeter^2)). A circularity value of 1.0 indicates a perfect circle. For actin filament quantification, mean intensity of fluorescent phallodin in cell cytoplasm was calculated for 100 cells per cell line in 8 bit pictures using "IntDen" (Integrated Density; the product of Area and Mean Gray Value).

2.6. Migration and invasion assay

50×10^3 cells were grown overnight in 96-well Essen BioScience ImageLock microplates coated with 100 µg/ml of Matrigel (Corning, Lowell, MA) in complete medium. Wounds were precisely made by the 96-pin Wound-Maker provided with the IncuCyte FLR (Essen Bioscience, Ann Arbor, MI). After washing thoroughly with PBS to remove the detached cells, wounds were covered with 50 µl of Matrigel (for invasion) or 50 µl of medium (for migration). After 30 min in the incubator, 100 µl of completed medium was added and the cells were placed in the IncuCyte FLR. The wound images were automatically acquired by IncuCyte FLR from the incubator at 2 h intervals for 48 h. The kinetics of the relative wound density was analysed by IncuCyte software.

2.7. Transwell cell migration analysis

50×10^3 cells in 150 µl of medium without FBS were loaded in Boyden chambers (Millipore) placed in 24 well plates containing 900 µl of complete medium with 10% FBS. Cells were allowed to migrate through porous (8 µm) membranes for 48 h. After medium removal the chambers and wells were washed once with PBS. Non-migrating cells were removed from the upper chamber with a cotton swab, whereas migrating cells adherent to the underside of the filter were fixed and stained with Crystal Violet solution (0.05% Crystal Violet, 1% Formaldehyde, 1% methanol, PBS) for 10 min. The inserts were washed with water and allowed to dry overnight. The migrated cells were photographed in 4 random sites by light microscopy at a magnification of 100X. To quantify the amount of migrated cells in circularity=4π(area/perimeter^2). Cells were allowed to attach for 60 min, then fixed and stained with Crystal Violet for 10 min at room temperature and washed 5 times with distilled water. Representative pictures were taken from each well using 100X objective of a light microscope (Olympus IX70). Images (1280×1024 pixels) were analysed using ImageJ 1.49b software. The experiment was repeated four times.

2.8. Cell-spreading assay

For the cell-spreading assay 25×10^3 cells were seeded in 96 well plate (4 wells/cell line/experiment) precoated with fibronectin (25 µg/ml, Sigma-Aldrich). Cells were allowed to attach for 60 min, then fixed and stained with Crystal Violet for 10 min at room temperature and washed 5 times with distilled water. Representative pictures were taken from each well using 100X objective of a light microscope (Olympus IX70). Images (1280×1024 pixels) were analysed using ImageJ 1.49b software. The experiment was repeated four times.

2.9. Focal adhesion analysis

Cells were grown on coverslips previously precoated with fibronectin (25 µg/ml). The cells were allowed to attach for 60 min, then fixed and stained as previously described. Focal adhesions were visualized with Anti-Paxillin antibody (1:100, Transduction Laboratories) and images were taken using 10X objective with an Olympus BX60 fluorescence microscope. The images (2040×1536 px) were analysed with ImageJ 1.49b software for quantification of changes on size and number of focal adhesions. The average area and number of focal adhesions per cell were quantified from binary images using the Analyze Particle tool in ImageJ. Particles larger than 0.2 µm^2 were counted as focal adhesions.

2.10. Colony formation assay

2×10^3 cells were mixed in 500 µl complete medium containing 0.3% agarose and plated in 12 well plates (4 wells/cell line/experiment) previously precoated with 500 µl of 0.5% agar in the same medium. Cells were allowed to grow for 2 weeks. Eight random pictures (1280×1024 pixels) were taken from each well using 4X objective of a light microscope (Olympus IX70). The percentage of colonies (more than 30 cells) with diameter greater than 100 µm was calculated using ImageJ 1.49b software.

2.11. Proliferation assay

5×10^3 cells were plated in 96 well plates and allowed to grow for 72 h. Phase-contrast images were automatically acquired by IncuCyte FLR (Essen Bioscience) from the incubator at 2 h intervals. Proliferation was monitored by analyzing the occupied area (% confluence) of cell images over time by IncuCyte software (Essen Bioscience). The experiment was repeated four times.

2.12. Flow cytometric analysis

For cell cycle analysis 15×10^5 cells in 6 well plates were kept with serum free medium for 48 h and then trypsinised and resuspended in PBS. Analysis of cell viability was performed using Propidium Iodide (Molecular Probes, Eugene OR, USA) according to manufacturer’s instructions followed by fluorescence activated cell sorting with FACS Fortessa, (BD Biosciences, NJ, USA). Data obtained from the cell cycle distributions were analysed with FlowJo flow cytometry analysis software (Ashland, OR, USA).

2.13. Mouse model/inoculations

All animal experiments and handling were performed in accordance with the Finnish Animal Experiment Board and the institutional animal care policies of Turku University, Central Animal Laboratory, which fully meet the requirements as defined in the U.S. National Institutes of Health guidelines on animal experimentation. Adult female athymic nude Foxn1nu mice (Harlan laboratories, The Netherlands) were housed with free access to irradiated food (SDS RM3 (E) Soya free, irr. 25 kGy, Special Diet Service) and sterilized water under controlled conditions of light (12 h light/dark cycle) and temperature.

Three mice per cell line were subcutaneously inoculated in both flanks with 1×10^6 cells in 75 µl of RPMI media mixed with 75 µl of Matrigel (BD Biosciences). Mice were weighed weekly and tumor growth was measured with calipers to get the volume (π[length x width^2]/6) and by optical imaging (IVIS Spectrum, Perkin Elmer). The RFP fluorescence images were acquired using 570 nm excitation and 620 nm emission filters. When the tumor size at least in one mouse/group reached the level of predetermined maximal tumor size (17 mm length as determined in the animal experiment license), all mice in the
study group were euthanized under isoflurane anesthesia by blood collection through heart puncture and neck dislocation. Tumors and organs which showed fluorescence in IVIS imaging were fixed in 10% formalin for 24 h and paraffine-embedded (FFPE) for histological and immunohistochemical analysis.

2.14. MKL-1 nuclear translocation

WM164 cells lines were plated on gelatin coated coverslips and kept on incubator overnight with complete medium. Medium was replaced by starvation medium for 72 h before 20 min stimulation with medium containing 10% serum. Cells were washed with PBS and fixed using 4% PFA for 10 min in room temperature. MKL-1 staining was done as described above using a goat polyclonal anti MKL1 (MRTF-A) antibody (Santa Cruz) followayed by Alexa Fluor 488 Donkey anti-Goat IgG secondary antibody together with Alexa Fluor 546-conjugated phallolidin (1:100, Invitrogen) in order to visualize filamentous actin. The mounting media contained DAPI for staining nuclei (ProLong® Gold Antifade Mountant with DAPI, Life Technologies). After each staining step, the cells were washed three times with PBS. Images were taken with an Olympus BX60 fluorescence microscope. The images (2040×1536 px) were analysed with ImageJ 1.49b software (http://rsbweb.nih.gov/ij/) for quantification of MKL-1 positive nuclei of 1500 cells/cell line.

2.15. Statistical analysis

All the experiments were repeated three times, unless indicated. One-way ANOVA was performed using Tukey’s multiple comparison procedure to identify the differences between cell lines and error bars represent SEM.

3. Results

3.1. FHOD1 expression in melanocytic tumors and melanoma cell lines

First, we wanted to establish whether FHOD1 is expressed in human melanocytic tumors. For this purpose, 8 benign melanocytic nevi and 10 primary melanomas were immunohistochemically stained with a FHOD1 antibody. FHOD1 expression was found to be very low or absent in all benign nevi (Fig. 1A, upper panel). Clear upregulation could be seen in most primary melanoma and metastatic melanoma samples, as moderate or strong FHOD1 staining was seen in 8/10 cases (Fig. 1A, lower panel). The staining was similar in tumour centres and at tumor margins. In 2/10 melanoma samples, FHOD1 immunoreactivity was weak, comparable to the staining intensity seen in benign nevi.

Next we studied the expression of FHOD1 in several melanoma cell lines by western blotting. All studied cell lines expressed FHOD1, but the expression level varied. We found moderate FHOD1 expression in the Bowes cell line, high expression in SK-MEL-28, WM164 and WM239, while there was low expression Malme-3M (Fig. 1B). Immunofluorescence double staining with anti-FHOD1 and phallolidin showed FHOD1 to be localized in the cytoplasm of WM164 cells, mostly as small dots, but also along actin filaments (Fig. 1C).

We have earlier shown that FHOD1 expression is dependent of PI3K signalling, but not MAPK signalling in oral SCC cells [17] In line with the result in SCC cells, melanoma cell lines with active PI3K signalling lost FHOD1 expression with PI3K inhibition, while MAPK inhibition had negligible effect (not shown).

3.2. Phenotypic alterations in WM164 melanoma cells after FHOD1 knockdown

To investigate the functions of FHOD1 in the WM164 melanoma cell line, cells were transfected with shRNA plasmids either specifically targeting human FHOD1 mRNA or a scrambled control shRNA. Several stable clones with reduced FHOD1 were obtained and further analysed. Clones in which levels of FHOD1 protein were reduced 80% and 95% compared with parental or scrambled control cells were chosen for further experiments. These clones were named FHOD1 20% and FHOD1 < 5%, respectively. Scrambled control cells were named FHOD1 100% (Fig. 2A).

The reduction of FHOD1 was accompanied by a change in cellular morphology. The FHOD1 silenced melanoma cells contained less actin filaments, as demonstrated by fluorescent phallolidin staining (Fig. 2C, upper panel). Fluorescent actin filaments were quantified with ImageJ, by measuring mean intensity of fluorescence in cells. FHOD1 20% and FHOD1 < 5% had 25% and 10% less actin filaments compared to FHOD1 100% (not shown). The FHOD1 < 5% cells were less polarized, resulting in larger cells with a rounder shape. When quantified from the cell outline pictures (Fig. 2C, lower panel), the total area and circularity was significantly increased in FHOD1 < 5% (n=90) compared to both FHOD1 20% (n=91) and FHOD1 100% (n=101) (Fig. 2B).

3.3. Effects of FHOD1 silencing on melanoma cell migration, spreading and adhesion

To test whether the cells were able to migrate after FHOD1 knockdown, a wound healing migration assay was performed (Fig. 3A). The knockdown cells FHOD1 20% and FHOD1 < 5% showed significant healing delay in comparison to the control FHOD1 100% (P≤0.001 and P≤0.01 respectively, ANOVA). Next, the cells were challenged to invade into a Matrigel plug in the wound healing assay. Also invasion was reduced after FHOD1 depletion (Fig. 3B). The difference was highly significant in both FHOD1 depleted groups (P≤0.001). Transwell migration, using Boyden chambers and serum gradient, was used to challenge the cells to migrate as single cells (Fig. 3C and D). Knockdown cells lines FHOD1 20% and FHOD1 < 5% migrated significantly less than the control FHOD1 100% cell line (P≤0.01, ANOVA).

Cancer-associated EMT could account for increased migration and invasion. To check whether FHOD1 knockdown induces EMT, the expression of well-established EMT-markers vimentin,slug, snail, E-cadherin, N-cadherin, β Catenin, were investigated. No changes were found in western blotting, indicating that mesenchymal transcription factors were equally expressed in the knockdown and control cells (data not shown).

Cell spreading initiates adhesion in vitro. We asked whether the inferior migratory capacity of FHOD1 depleted cells might be related to adhesive properties. To test this, a cell-spread assay was performed. FHOD1 depleted and control cells were seeded on fibronectin-coated wells, fixed and stained after 60 min (Fig. 4A). The percentage of cells with spread morphology and cell areas were calculated (Fig. 4B and C). We found that a higher percentage of cells were spread in the FHOD1 depleted groups. The spread cells further had a greater cell area than the control cells. The difference was statistically significant in the FHOD1 < 5% cell line. The increased spreading of FHOD1 depleted cells suggested that adhesive structures might be altered. To visualize and evaluate focal adhesions, cells were stained for the focal adhesion protein paxillin (Fig. 4D). FHOD1 silenced cells contained significantly smaller focal adhesions (Fig. 4F), but the number of focal adhesions did not differ from control cells (Fig. 4E).

3.4. FHOD1 silencing reduces colony formation and proliferation

A colony-forming assay was performed by seeding cells in soft agar and quantifying colonies with a diameter greater than 100 µm after 2 weeks (Fig. 5A). The percentage of colonies with diameter greater than 100 µm was significantly higher in both FHOD1 100% and FHOD1 20% as compared to FHOD1 < 5%, while the number of FHOD1 20%
colonies did not significantly differ from the control (Fig. 5B).

Using IncuCyte™ live cell imaging, proliferation was monitored by analyzing the area occupied by cells (% confluence). The confluence of cells growing in normal conditions for 72 h showed that FHOD1 knockdown significantly reduces proliferation in WM164 melanoma cells (Fig. 5C).

To investigate the reduced proliferation further, cleaved caspase 3 expression was studied by western blotting. No increase of cleaved caspase was detected after FHOD1 knockdown (data not shown), suggesting that increased apoptosis does not account for reduced proliferation and colony formation. Cell cycle analysis, however, revealed a mechanistic explanation for the reduced proliferation. In FACS analysis, a significant arrest of FHOD1 depleted cells in G0+G1 was found (Fig. 5D).

3.5. FHOD1 knockdown reduces tumor growth in vivo

To study whether the effects of FHOD1 knockdown were relevant for tumor formation in vivo, FHOD1 depleted or control cells were injected subcutaneously in both flanks of athymic nude mice. Tumor growth was measured with calipers and optical imaging using IVIS Spectrum. Images of mice from different treatment groups at day 14 are presented in Fig. 6A. The cell lines regrettably had different levels of red fluorescence, making intergroup comparison by this method inappropriate. Therefore measurements with calipers were chosen as follow-up method. By palpation, the tumors grew at different rates between the groups; therefore mouse groups were sacrificed at diverse time points. Tumors derived from the FHOD1 100% cell line grew faster than FHOD1 depleted groups, resulting in sacrifice due to large...
tumor size at day 14 (Fig. 6B). At this time point a statistical difference in the tumor size was observed for the FHOD1 20% compared to control FHOD1 100% (P ≤ 0.01, ANOVA) (Fig. 6C). We could see statistical difference (P ≤ 0.01) also when FHOD1 20% and FHOD1 < 5% results were combined and then compared with the control using t-test (results not shown). In the FHOD1 depleted groups, the size limit was reached at 30 days for FHOD1 20%, and at 22 days for and FHOD1 < 5% (Fig. 6B). No fluorescence was detected in any of the collected organs thus no further analysis were done.

Histologically, mouse tumors formed by WM164 melanoma cells expressing different levels of FHOD1 were slightly different (Fig. 6D, top row). FHOD1 100% cells grew diffusely, whereas FHOD1 20% and FHOD1 5% formed a slightly lobular architecture, with strands of connective tissue seen within the tumor. In all three groups, the edges of the tumors were morphologically invasive. The size of the tumor cells was quantified, and found to be unaltered (results not shown).

FHOD1 immunohistochemistry revealed strong and diffusely cytoplasmic expression in FHOD1 100% tumors. In the knockdown tumors, a minor diffuse expression was seen in FHOD1 20% cells, and small islands of clearly FHOD1 positive cells were scattered in both FHOD1 20% and more frequently in FHOD1 < 5% groups (Fig. 6D, middle row). The clearer reduction of tumor growth in FHOD1 20% compared to FHOD1 < 5% may be explained by the groups of FHOD1 expressing cells within the FHOD1 < 5% tumors (Fig. 6D). The reduc-

Fig. 2. FHOD1 silenced WM164 melanoma cells are larger and more circular than control cells. A) FHOD1 expression in shRNA-expressing clones transfected with plasmids containing two shRNA sequences targeting human FHOD1 or a scrambled control sequence: FHOD1 100% (scramble control, transfected with shRNA TR30015), FHOD1 20% (transfected with shRNA FI351920), and FHOD1 < 5% (transfected with shRNA FI351919). Tubulin expression was used as a loading control. B) Morphological analysis of the cell lines expressing different levels of FHOD1. C) Representative images of FHOD1 stable knockdown cells stained with Alexa Fluor 488-conjugated phalloidin (upper panel) and the cell outline pictures (lower panel). Actin filaments in cells were quantified by measuring mean intensity of fluorescence in cells. FHOD1 20% and FHOD1 < 5% had 25% and 10% less actin filaments compared to control (not shown). ** P ≤ 0.01, ANOVA, Tukey. Scale bars: 50 µm.
tion of FHOD1 expression in the majority of cells corresponded to the levels previously estimated by western blotting (Fig. 2A). The tumors were stained for cleaved caspase 3, but no change was seen between groups, indicating that the effect of FHOD1 depletion on tumor growth is not due to increased apoptosis (results not shown). In Ki-67 staining, however, a clear reduction of proliferation was seen (Fig. 6D, bottom row; Fig. 6E) as a result of FHOD1 depletion. The reduced rate of proliferation most probably account for the slower growth of FHOD1 depleted tumors, reflecting the cell cycle arrest found in vitro.

Cell cycle arrest could possibly relate to the SRE, since it controls the expression genes involved in cell cycle progression. We wondered if FHOD1 knockdown could reduce the serum response that leads to transcription from the SRE. To address this issue, we measured the nuclear translocation of the SRE co-activator MKL-1 from the cytoplasm in FHOD1 control and knockdown cells after serum stimulation. The number of MKL-1 positive nuclei was significantly lower in FHOD1 depleted cells, suggesting that FHOD1 is affecting transcription from the SRE (Fig. 7A and B).

4. Discussion

While the understanding of melanoma biology and therapeutic alternatives are expanding rapidly, the mechanisms regulating melanoma cell growth and invasion are incompletely understood. Here we studied the association of FHOD1, a formin only recently associated with cancerous phenotypes, in melanoma using both tumor material and experimental approaches. We provide several types of evidence indicating an important role for FHOD1 in melanoma biology: First, we show that FHOD1 expression is increased in human melanomas, as compared to benign nevi. The expression in both nevi and melanomas is different from normal skin, where FHOD1 expression cannot be detected [17]. Second, we detect roles for FHOD1 in adhesion and migration as well as proliferation, all key processes that are dysregulated malignant cells. Finally, we find that FHOD1 participates in tumor growth in vivo. This is the first time FHOD1 expression has been studied in clinical benign and malignant melanocytic neoplasms, and the most comprehensive analysis of FHOD1 in melanoma biology to
The biochemical and cellular properties of individual formin proteins have been intensively studied. Many cell-specific functions have been described in detail. The expression and significance of formins in clinical cancer has not been as well characterized. In one study, expression of a formin closely related to FHOD1, FMNL2, was found to be increased in colorectal cancer and correlate with metastasis [19]. FMNL2 expression has also been found in melanoma (discussed further below). Another formin, FMNL1, is commonly overexpressed in basal breast cancer, a breast cancer type with poor prognosis [20,21]. Furthermore, FHOD1 expression has been shown to increase in malignancy: high expression is found in squamous cell carcinoma (SCC), while expression is not detected in normal oral squamous epithelium. In SCC cells, this upregulation is associated with EMT [20,21]. Finding FHOD1 in cancers of epithelial lineage is intriguing, since in normal tissues it is found mainly in cell types of mesenchymal lineage, such as endothelial cells and smooth muscle cells. The findings we describe here indicate that FHOD1 can be upregulated in malignancy of neuroectodermal lineage as well. Further studies will be needed to show whether FHOD1 upregulation is a common phenomenon in cancers that develop in mucosa or skin, irrespective of histological type.

In this study, we found FHOD1 protein expression to be moderate/high in a vast majority of malignant melanoma, as compared to benign melanocytic tumors, i.e. nevi. This profile is different from what we found when studying the expression of FMNL2 in clinical melanoma.
In that study, we found FMNL2 to be expressed in early melanomas, at levels that varied with similar frequency from lower to clearly higher than detected in surrounding benign squamous cells. In that study, we were encouraged to look for a prognostic role of FMNL2 expression, and indeed found that the FMNL2 expression level was a negative prognostic factor, when measured as recurrence-free survival or melanoma-specific survival [16]. The wide expression of FHOD1 in melanoma (and melanoma cell lines) suggested a role for FHOD1 in tumorigenesis, rather than prognosis. Therefore, we decided to explore the biological role of FHOD1 in melanoma. In our studies, we have found that silencing FMNL2 or FHOD1 does not influence the expression of the other (not shown).

We found that FHOD1 depletion was accompanied by clear morphological and functional alterations in cultured melanoma cells. Morphologically the cells were more rounded, had a greater area and less prominent actin filaments with the most efficient knockdown. In functional experiments, we found that the FHOD1 depletion brought a significant reduction of melanoma migration in wound healing, inva-
sion and even further reduction in Boyden chamber migration. These findings are in line with alterations seen in breast cancer and oral SCC lines earlier [7,17], suggesting that the maintenance of an elongated and actin fiber rich and migratory phenotype is a common function of FHOD1 regardless of cancer cell type. The reduction of migration in Boyden chambers was eye-catching. Experimental setups in wound healing and Boyden chambers are quite different, the first tests a more collective mode of migration and the second challenges cells to migrate individually through pores in a membrane. In the latter, adhesion and detachment of individual cells have essential roles. Studies from other groups have found FHOD1 involvement in adhesion: exogenously expressed FHOD1 accumulates into integrin clusters, which later mature to focal adhesions [22]. FHOD1 knockdown has been shown to impair cell spreading and focal adhesion formation in fibroblasts and osteosarcoma cells [22,23]. In line with this, we found that FHOD1 depleted melanoma cells contain clearly smaller focal adhesions than control cells. Focal adhesion size has been shown to predict cell migration. Increase in focal adhesion size enhances cell speed [24]. The smaller size of focal adhesions may contribute to reduction of melanoma cell migration, as well as more spread morphology after FHOD1 depletion, reflecting reduced mechanical tension with fewer actin filaments.

Fig. 6. FHOD1 knockdown decreases tumor growth. A) Melanoma cells from different FHOD1 groups were inoculated subcutaneously in both flanks of nude mice. Fluorescence images were acquired using IVIS Spectrum instrument using 570 nm excitation and 620 nm emission filters, to follow the growth of individual tumors. Overlay of light and fluorescent images 14 days after injections. The cell lines had different levels of RFP expression and images were taken with different exposure times (intergroup comparisons by fluorescence levels were therefore not possible). B) Curves of tumor growth and time of tumor removal. The tumors were followed up by caliper measurement. When the tumors reached a predetermined tumor volume, the mice were euthanized and tumors were removed. FHOD1 depleted cells formed tumors reaching predetermined size slower than FHOD1 100% cells (FHOD1 100%, 14 days; FHOD1 20%, 30 days, FHOD1 < 5%, 22 days). Mean ± SE of 5–8 tumors per group. C) Statistics of the tumor growth in each group and estimated tumor volume at day 14. D) H & E and immunohistochemistry of tumors. In the knockdown tumors, a minor diffuse FHOD1 immunoreactivity was seen in FHOD1 20% cells, and small islands of clearly FHOD1 positive cells were scattered in both 20% and 5% groups (middle row). Ki-67 staining is reduced in FHOD1 depleted cells (bottom row). Scale bars: 100 µm. E) Proliferation rates as determined by Ki-67 staining of different tumor groups. ** P < 0.01, ANOVA, Tukey.
An important and novel finding was inferior proliferation and cell cycle arrest in melanoma cells depleted of FHOD1. A possible mechanism behind this finding relates to transcriptional effects of FHOD1. FHOD1 is capable of activating the SRE through its effect on the actin cytoskeleton [7,8]. Testing this, we found that FHOD1 knockdown diminished the nuclear translocation of MKL-1 after serum stimulation, indicating that co-activation of the SRE was reduced. Taken together, our results suggest that FHOD1 depletion reduces transcription from the SRE, which sequentially causes cell cycle arrest and reduced proliferation. The effect on SRE also raises the possibility of FHOD1 influencing the transcription of focal adhesion genes in WM164, as another explanation for the smaller focal adhesions in knockdown cells. The SRE may control transcription of focal adhesion genes, as has been shown in NIH-3T3 fibroblasts [10].

In this study, the reduction of FHOD1 did not abrogate tumorigenesis in vivo. In mice, the FHOD1 silenced cells formed smaller tumors with reduced proliferation. The tumor margins were still histologically infiltrative. This suggests that a high level of FHOD1 is not indispensable for invasion of melanoma cells in vivo.

Taken together, our studies identify FHOD1 as a formin commonly upregulated in melanoma, and by studying its effects in the melanoma cell line WM164 we propose that its roles in melanoma cell biology are diverse, involving adhesion, migration and tumor growth. However, it may not be essential for maintenance of invasive capacity. Although the life expectancy in metastatic melanoma has increased with modern treatment including cytotoxic therapies, molecular targeted therapy and immunotherapy, advanced melanoma is still an incurable disease [25]. Targeting actin cytoskeleton modulating proteins could be a potential addition to the current treatment modalities. Future investigations should focus on the mechanisms of FHOD1 upregulation and activation in melanoma, to determine whether it could be utilized as a biomarker or drug target.

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Fig. 7. FHOD1 knockdown reduces MKL-1 nuclear translocation after serum stimulation. WM164 FHOD1 knockdown cell lines were starved for 72 h and then stimulated with 10% serum for 20 min. MKL-1 nuclear positivity was calculated to evaluate activation of the serum response factor. A) Representative merged fluorescence images of MKL-1 (green) localization in FHOD1 silenced cells. B) Histogram showing percentages of MKL-1 positive nuclei in control and FHOD1 depleted cells. Scale bar: 100 µm. White arrows indicate MKL-1 positive nuclei, while white arrowheads point out nuclei negative for MKL-1. *P<0.05, **P<0.01; ANOVA, Tukey.

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