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NF1-RAC1 axis regulates migration of the melanocytic lineage

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ABSTRACT

Metastases's spreading is the main cause of mortality for advanced stage cancer patients, including melanoma. The formation of metastases is favored by enhanced migratory and invasive capacities of tumor cells. Tumor suppressor gene *NF1* is a negative regulator of RAS and its deregulation plays an important role in several aspects of melanoma transformation and progression. However, very little is described about the role of NF1 in cellular migration and invasion. In this study, our results show on the one hand, that the loss of NF1 expression delays migration of human melanoblasts *via* a RAC1-dependent mechanism. On the other hand, our data indicate that NF1 loss in melanoma cells is enhancing migration, intravasation and metastases formation *in vivo*. Moreover, not only this phenotype is associated with an up-regulation of PREX1 but also patient-derived melanoma samples with low NF1 expression present increased levels of PREX1. In sum, our study brings new elements on the mechanism controlling cellular migration in the context of *NF1* loss. These data are of prime interest to improve treatment strategies against all *NF1*-mutated tumors, including this subtype of melanoma.

Introduction

Deregulation of tumor cells' migration and invasion strongly contributes to the dissemination of cancer metastases. The quick spreading of cutaneous melanoma metastases is considered as one of the main cause for its high mortality rate (2 and 1.3 per 100,000 cases in Western Europe for male and female respectively) [1]. In addition to invading the dermis, late stage melanoma cells can migrate along vascular structures (angiotropism) and reach other parts of the body such as the lungs, the liver or the brain. Interestingly, the phenomenon of extravascular migration is also observed in early melanocyte progenitors during embryonic development [2–4].

Promotion of cellular migration and invasion is described to be driven by essential signalings such as PI3K and Rho/ROCK [5–8]. In particular, Rho GTPases family member RAC1 can control cell migration through the regulation of lamellopodia formation and actin polymerization. Moreover, RAC1-guanine exchange factor (GEF) PREX1 is necessary for melanoblast and melanoma migration [9]. Indeed this study showed on the one hand, that *Prex1*-deficient mice present unpigmented belly spots suggesting impaired melanoblast migration during development, and on the other hand, that metastasis formation is significantly reduced in a *Prex1*-deficient melanoma model triggered by co-mutations in *Nras* and *Ink4a* specifically in the melanocyte lineage.

In addition to RAC1, Neurofibromin 1 (NF1) is also suggested to be involved in cell migration *via* regulation of actin and microtubule network dynamics [10,11]. In the same line, NF1 directly interacts with focal adhesion kinase (FAK), a protein which plays a crucial role in cellular motility and spreading processes [12]. Moreover, NF1 is described to negatively regulate RAC1 in a RAS-independent manner [13] which contributes to a decreased migratory phenotype. To the contrary, another study indicated that NF1 participates in the inhibition of LIMK2, a downstream effector of the Rho-ROCK pathway, resulting in a highly motile phenotype [14]. NF1 may also impact migration in a RAS-dependent manner, as it negatively regulates RAS [15]. In melanoma, mutations in tumor suppressor gene *NF1* are the third most common after *BRAF* and *NRAS*, and cover about 10% to 15% of the patients [16]. *NF1* was described not only to play a role in the melanocyte lineage during early development but also during melanoma formation and progression to drug resistance [17,18].

Because no therapeutic strategy targeting the migration of melanoma cells has been approved so far and because we know very little on how NF1 is involved in this particular process, we aimed here at investigating the role of NF1 in the migration of melanoma cell lines and melanoblasts,

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by exploring its molecular link to known migratory pathways such as PREX1/RAC1.

Materials and methods

Cell lines

Melanoma cell lines SK-mel-23 (gift from Cornelia Mauch, University of Köln), Mel 501 (gift from AG Pawelec, University of Tübingen), SK-mel-103 (gift from CNIO, Madrid, Spain) and A-375 (purchased from ATCC) were cultured in DMEM Medium (High Glucose, GlutaMax, Gibco™, 31966047) supplemented with 10% fetal bovine serum (FBS), MEM Non-essential Amino Acid Solution (Sigma-Aldrich®, M7145), 1% ß-Mercaptoethanol, 1% Penicillin (100 units/mL) and Streptomycin (100 mg/mL). Human iPSC-derived melanoblasts were derived in our laboratory at the DKFZ and cultured in the same medium as for melanoma cell lines, as described previously [19]. Use of patient-derived material was approved by the ethics committee approval no. 2009-350N-MA at the University Medical Center Mannheim, Germany. All cells were maintained at 37 ˚C in a humid incubator with 5% CO2.

Immunohistochemistry and TMA

Tissue representative of three independent experiments was stained on 5 μm paraffin sections using routine method. Primary antibodies used for IHC were rabbit anti-NF1 (Santa Cruz) and rabbit anti-Ki67 (abcam).

The tissue microarray (TMA) used in this study was described in detail before [20]. It was generated at the core facility of the National Center for Tumor Diseases (NCT), Department of Pathology, University of Heidelberg. Briefly, after deparaffinization and antigen retrieval, the slides were washed and incubated with blocking serum (0.1% bovine serum albumin in PBS plus 5% normal rabbit serum) for 1 h followed by avidin-biotin blocking reagent. Incubation with antibodies against human NF1 (Santa Cruz), PREX1 (Sigma-Aldrich) and RAC1 (Cytoskeleton Inc.) in a dilution of 1:400. This was followed by incubation with corresponding secondary antibodies (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) and by incubation with ABC reagent (Vectastain ABC Kit, Vector Laboratories, CA). Finally, staining was completed utilizing DAB (DAB Peroxidase Substrate Kit, Vector Laboratories, Burlingame, CA). Staining reaction was stopped in distilled H2O, and the slide was counter-stained with hematoxylin (Thermo Scientific, Waltham, MA). Two blinded individuals performed scoring of tissue microarrays applying a quantity/intensity-based IHC scoring system. Primary or metastatic samples were analysed for the percentage of positive cells in the whole tumor section and were ranked according to an overall IHC score ranging from 0 (no staining) to 12 (highest staining).

Western blot and qPCR

Whole cell extracts representative of three independent experiments were prepared from hiPSC-derived melanoblasts and melanoma cell lines and the extracted proteins were analysed. Primary antibodies used for western blot included: rabbit anti-NF1 (Santa Cruz), mouse anti-alpha-ACTININ (Santa Cruz), rabbit anti-phospho-ERK, anti-phospho-AKT, anti-total-ERK and anti-total-AKT (Cell Signaling Technology).

Pulldown of GTP-RAC was performed with glutathione affinity beads using GST-fused PAK-PBD. The amount of activated RAC1 was determined by western blot using a RAC1 specific antibody (Cytoskeleton inc. Cat. # BK035).

RNA from the same samples was extracted using RNeasy kit (Qiagen). The RNA was treated with RNase-free DNase I and reverse-transcribed with oligo(dT) primer using the First-Strand Synthesis Kit (Fermentas). Quantitative Real-time PCR (qPCR) was performed using the SYBR Green Supermix (Applied Biosystems) on a 7500 Real-Time PCR system (Applied Biosystems). All values were normalized to housekeeping gene 18S. Primers for qPCR were as follow: 18S, 5′-gaggatgaggtggaacgtgt-3′ (fwd) and 5′-tcttcagtcgctccaggtct-3′ (rev); NF1, 5′-acgagtgtctcatgggcagat-3′ (fwd) and 5′-actgttgtaagtgtcaggtccttttaag-3′ (rev); PREX1, 5′-ggcattcctgcatc-3′ (fwd) and 5′-cgggtgtaaacaatccaagg-3′ (rev) (Eurofins Genomics).

siRNAs and shRNAs

Human *NF1* siRNA clones were purchased from Qiagen and transfected with Lipofectamine® RNAiMAX (Life Technologies). Hs_NF1_6 FlexiTube siRNA - SI02664438 (CAGGTGGCTTGGGATCAATAA); Hs_NF1_11 FlexiTube siRNA - SI04949924 (TACGAATGGCACCGAGTCTTA). Human *NF1* shRNA was purchased from GeneCopoeia Inc. (CCAACTCCTACTTTAGAAC). Human *PREX* siRNA from Qiagen was used (PREX1.6 SI03246383 – CAGGGTCAGCCCACCCTTCAA).

Scratch assay

To analyze their migratory potential, cells were plated in a 2-well-culture-insert (Ibidi, Planegg, Germany, 80209) with culture medium. After attachment to the plate, cells were incubated overnight with FBS-free culture medium. The day after, inserts were removed, cells were washed with PBS and incubated with 10% FBS culture medium with Hydroxyurea (1 mM). Cell migration was monitored at the indicated time points. TScratch Software was used for quantitative analysis of the closing gap.

RAC1 activation assay

The Rac1 G-LISA® kit contains a Rac-GTP-binding protein linked to the wells of a 96 well plate. The bound active Rac1 is detected with a Rac1 specific antibody. Secondary antibody conjugated to horseradish peroxidase (HRP) allows for detection. Absorbance was read at 490 nm. Data are background subtracted (Cytoskeleton inc. Cat. # BK128). RAC1 activator (Cytoskeleton inc. CN04) or RAC1 inhibitor (Tocris Bioscience NSC 23766) were included in some of the experiments. Rac1 activity is represented as a fold change of stimulated compared to non-stimulated condition.

Chicken chorioallantoic membrane (CAM) metastasis assay

This assay can be used to evaluate intravasation, distant metastasis as well as *in vivo* tumor growth and utilises a real time PCR method to identify human specific Alu sequences on a background of chicken DNA in the estimation of distant micro-metastasis. Fertilized special pathogen free (SPF) eggs were incubated for 10 days in a Marsh incubator (Lyon Electric, USA) at 37 ˚C with automated intermittent rotation. On the 10th day of incubation, the eggs were visualised in dark room under a light source to evaluate the development of the egg, as well as identify and mark a CAM surface with good vasculature on which the cells will be later inoculated. A pin size hole is then made in the area of the air sac, and another in the area of the marking and suction was then applied with a pipette suction bulb over the air sac, which caused the creation of an artificial air sac and lowering of the CAM membrane under the marked area. A miniaturized 1 cm window is then made on the shell over the displaced membrane with an electric hand saw (Dremel, Wisconsin, USA), after which 2 million cells transduced with shNF1 or control shRNA were carefully inoculated onto the upper CAM in 50 μl of serum free medium in the vicinity of a prominent blood vessel. The window was then covered with adhesive tape and the eggs incubated without rotation for a further 7 days in the incubator. On the 17th day of the experiment, the eggshells were cut open along the latitudinal axis in the midline with a pair of dissecting scissors, and the chicken embryos decapitated. The lungs and liver were harvested and placed immediately into 15 mL tubes containing tissue lysis buffer. The developing tumors on the upper CAM were also dissected out of the CAM membrane and weighed. Genomic DNA was isolated from the liver and lungs and the number of metastasized cells evaluated using human specific Alu-PCR [21].

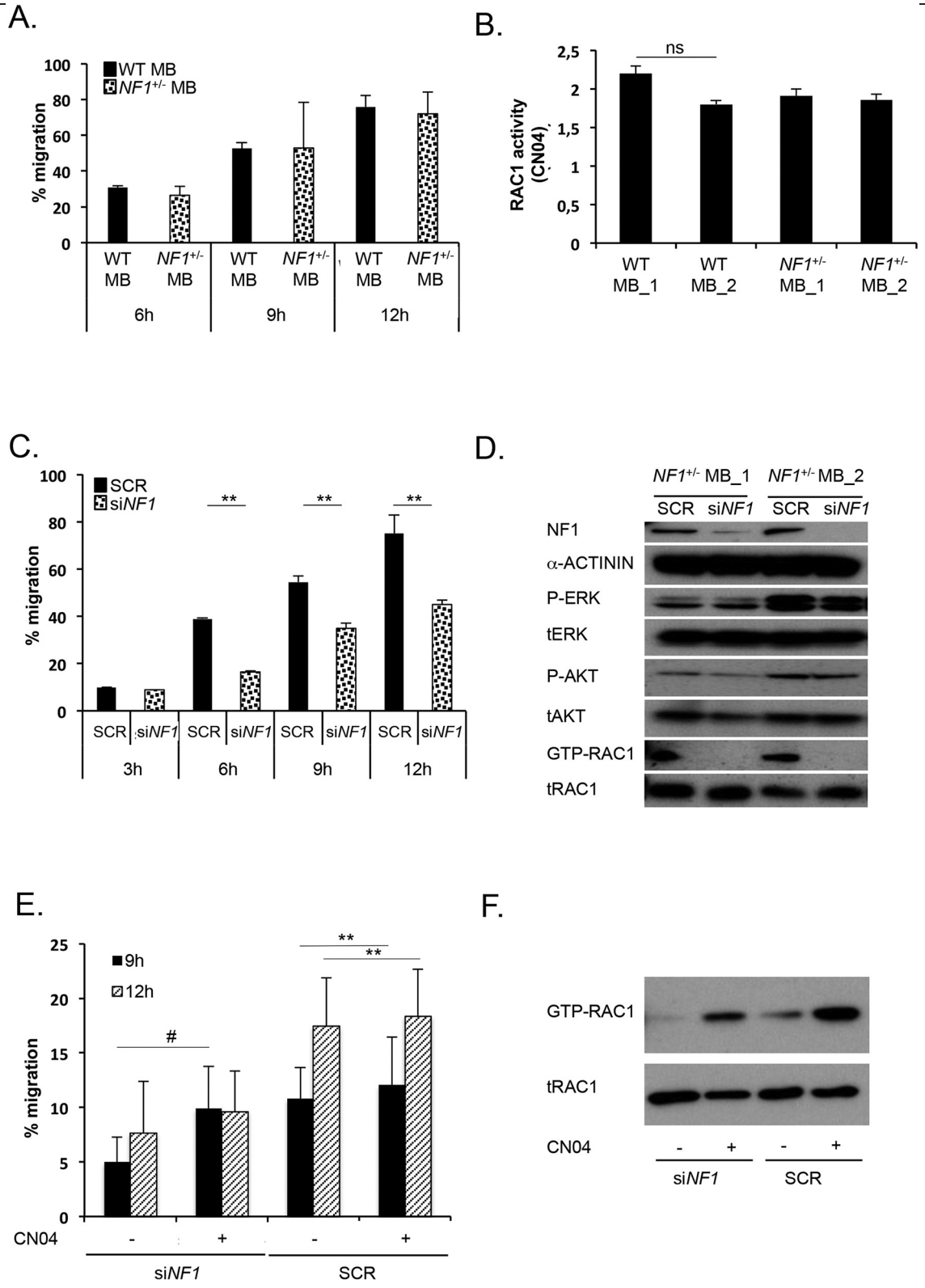

Fig. 1. Loss of *NF1* reduces RAC1-driven melanoblast migration. A. Scratch-like migration assay representing the percentage of cell coverage after 6 h, 9 h and 12 h using either WT or *NF1*$^{+/-}$ melanoblasts (MB) in the presence of a RAC1 activator (CN04). B. RAC1 activity was measured by G-lisa in WT and *NF1*$^{+/-}$ melanoblasts (MB). C. Scratch-like migration assay after 3 h, 6 h, 9 h and 12 h in *NF1*$^{+/-}$ melanoblasts 48 h-post transfection with either a scramble siRNA (SCR) or with an *NF1*-specific siRNA (si*NF1*). D. Expression status of NF1 and expression of phosphorylated and non-phosphorylated ERK and AKT in *NF1*$^{+/-}$ melanoblasts by western blot. α-actinin was used as a loading control. GTP-RAC1 pulldown and total lysates were blotted with α-RAC1 antibody. E. Scratch-like migration assay representing the percentage of cell coverage after 9 h and 12 h in *NF1*$^{+/-}$ melanoblasts 48 h-post transfection with either a scramble siRNA (SCR) or with an *NF1*-specific siRNA (si*NF1*) and in the presence or absence of a RAC1 activator (CN04). *: SCR *vs.* si*NF1*, #: -CN04 *vs.* + CN04. F. GTP-RAC1 pulldown and total lysates were blotted with α-RAC1 antibody in the presence or absence of a RAC1 activator (CN04). $^{**}P < 0.01$, $^{*}P < 0.05$, ns: not significant (unpaired Student's *t*-test). All error bars represent the SEM of at least three independent experiments.

Results

Loss of NF1 reduces RAC1-driven melanoblast migration

First, we used a human induced pluripotent stem cell (hiPSC)-derived melanoblast model to investigate the effect of *NF1* loss of expression on cell migration [22]. As we previously described, these hiPSCs were reprogrammed from skin fibroblasts of two patients with neurofibromatosis type 1 and therefore carried heterozygous *NF1*$^{+/-}$ germline mutations [23]. Although these *NF1* mutations led to >50% reduction of NF1 protein level in melanoblasts, they did not affect the migration capacity of these cells when compared to control *NF1*$^{+/+}$ melanoblasts (WT MB), which were generated from healthy donors' fibroblasts (Fig. 1A). We observed no significant difference in the activity of RAC1 between each *NF1*$^{+/+}$ melanoblast lines (WT MB_1 and WT MB_2) and this activity was not changed in *NF1*$^{+/-}$ melanoblast lines (*NF1*$^{+/-}$ MB_1 and *NF1*$^{+/-}$ MB_2) (Fig. 1B).

Curiously, after additional *NF1* silencing (si*NF1*) which reduced the remaining NF1 expression in *NF1*$^{+/-}$ melanoblast lines, the cell migration was significantly reduced compared to control cells transfected with scramble siRNA (SCR), from 6 h to 12 h time point (Fig. 1C). As observed by western blot, although NF1 expression was greatly impaired, it did not affect the activation status of ERK or AKT, two main downstream targets of NF1-regulated RAS. However, the activity of RAC1 was greatly impaired by the additional *NF1* silencing as observed after GTP-RAC1 pulldown and compared to total lysates (Fig. 1D).

Moreover, although the stimulation of *NF1*$^{+/-}$ melanoblasts transfected with scramble siRNA (SCR) with a RAC1 activator (CN04) did not provoke a change in cell migration when compared to unstimulated control condition, it partially rescued the reduction of cell migration observed in si*NF1*-transfected *NF1*$^{+/-}$ melanoblasts after 9 h and 12 h (Fig. 1E). Accordingly, RAC1 activity assessed by pulldown, decreased after *NF1* silencing but increased again under CN04 stimulation (Fig. 1F). These data show that a > 50% reduction of *NF1* expression leads to an impairment of migration and is involving the activation of RAC1 in human melanoblasts.

Loss of NF1 increases melanoma migration and is associated with increased PREX1 expression

Next, we silenced *NF1* in three melanoma cell lines (SK-mel-23, SK-mel-103 and Mel501) and assessed their migration capacity. Two different siRNAs (NF1.6 and NF1.11), led to an impairment of >50% *NF1* RNA expression compared to control conditions with scramble siRNA (SCR) in the three cell lines (Fig. 2A). As mentioned above, RAC1 guanine exchange factor PREX1 is important for melanoma cell migration and invasion since metastasis formation is impaired in a *Prex1*-deficient melanoma model [9]. In the same line, we observed a slight (up to 2.5 fold) but significant upregulation of *PREX1* in *NF1*-silenced melanoma cell lines (Fig. 2B). Of note, these results were also validated at the protein level (data not shown).

We then followed the migration rate of SK-mel-23, SK-mel-103 and Mel501 over a period of 24 h. *NF1* silencing led to an increased cell migration with both siRNAs compared with the scramble siRNA condition (SCR) in all three cell lines mostly at 6 h time point (Fig. 2C). At 6 h, the migration rate of SK-mel-23 and SK-mel-103 increased up to 2,5 folds and that of Mel501 increased up to 6 folds. At 12 h, the increase of the migration rate was less pronounced and after 24 h the effect was weak (SK-mel-23) or not observed (SK-mel-103 and mel501).

Interestingly, an additional silencing of *PREX1* had a strong delaying effect on the migration rate (Fig. 2D). In SK-mel-23, the migration showed up to 94% reduction at 12 h and up to 83% at 24 h. At 6 h however, the reduction was non-significant in the SCR and NF1.6 conditions (due to lower number of analysed samples) and reached 39% in the NF1.11 condition. In SK-mel-103, up to 52% reduction occurred at 6 h under *PREX* silencing when compared to scramble condition (scramble). At 12 h, this reduction only reached a maximum of 35% and at 24 h, only 18% reduction occurred in SCR condition but was non-significant in *NF1* silencing conditions. In Mel501 cell line, a maximal reduction of 92% occurred at 6 h and at 12 h under *PREX* silencing when compared to scramble condition (scramble) regardless of *NF1* silencing. Finally, at 24 h, a 62% reduction occurred in SCR condition but only reached 19% in *NF1* silencing conditions. Of note, *PREX* silencing was validated and led to a maximal reduction of 80% RNA expression (Supplemental Fig. 1). These data suggest that PREX1 is a strong regulator of migration even in the absence of NF1 expression.

In the next experiment, we tested the involvement of RAC1 in the NF1-mediated migration phenotype (Fig. 2E). In the three cell lines, a RAC1 inhibitor had a strong delaying effect on the migration rate after 6 h and 12 h, whether *NF1* was silenced or not. SK-mel-23 showed a reduction of 40% to 74% compared to control condition (control) at 6 h and about 90% reduction at 12 h. SK-mel-103 showed a reduction up to 96% at 6 h and up to 80% at 12 h. Mel501 showed an average reduction of 97% at 6 h and of 93% at 12 h. However, the reduction of migration observed at 24 h was lower although still significant in comparison with the control condition (control) (SK-mel-23: 32% to 54%; SK-mel-103: 11% to 19%; Mel501: 26% to 42%) (Fig. 2E). This result could be explained by the exhaustion of RAC1 inhibitor after 24 h.

All together, these results reveal that *NF1* silencing leads to an increased migration of melanoma cell lines which may involve the presence of PREX1 and the activity of RAC1.

Loss of NF1 increases melanoma metastasis formation and intravasation in vivo

In order to investigate the role of NF1 on cellular migration and invasion *in vivo*, we performed a chicken chorioallantoic membrane (CAM) Assay with *NF1*-silenced melanoma cell lines SK-mel-103, Mel501 and A-375.

Tumor cells were inoculated on top of an artificial air sac in chicken eggs 10 days after fertilization. Seven days later, the grown tumors in the upper CAM were collected and analysed. Histological stainings of these tumors generated from the three cell lines showed a decreased NF1 expression in *NF1*-silenced cells compared to control cells transfected with scramble shRNA (SCR), confirming the silencing was still efficient after the graft. However, cell proliferation based on Ki67 staining did not significantly changed between *NF1*-silenced cells and control cells (Fig. 3A and Supplemental Fig. 2). Similarly, the size and weight of the collected tumors (primary tumors) were measured but did not show a significant difference between *NF1*-silenced conditions and scramble conditions (Fig. 3B). Tumor size ranged between 0.8 cm and 1.7 cm and tumor weight ranged from 0.08 g to 0.5 g.

Importantly, cell intravasation, measured by the evaluation of tumor cell number in the lower CAM 54 h post-inoculation (see Material and Methods section) systematically increased in *NF1*-silenced cells compared to control cells in the three cell lines (Fig. 3C). Moreover, the number of lungs and liver metastases were significantly increased with *NF1*-silenced cells compared to control cells transfected with scramble shRNA (Fig. 3D).

These results indicate that *NF1* silencing in melanoma cells *in vivo* does not impact the tumor growth but rather promotes the dissemination of metastases as well as the intravasation capacity of the cells. Moreover, based on the Ki67 staining of the obtained tumors, no significant difference was observed between both tested groups, suggesting the observed metastasis formation is proliferation-independent.

PREX is upregulated in low NF1 expressing melanoma metastases

Tissue microarray covering primary and metastatic patient-derived melanoma samples was stained to evaluate NF1, PREX and RAC1 protein expression (Fig. 4A). The staining was performed and analysed as previously described [24]. Primary tumors group and metastatic tumors group were processed separately and subgroups were selected based on the overall NF1 staining in the whole tumor section. After selection of samples with an NF1's overall score below 6 (NF1 low), we could not observe any significant correlation with PREX1 or RAC1 expression in primary tumors. However, metastatic samples with NF1 low expression showed a discrete but significant correlation with high PREX1 expression. This tendency

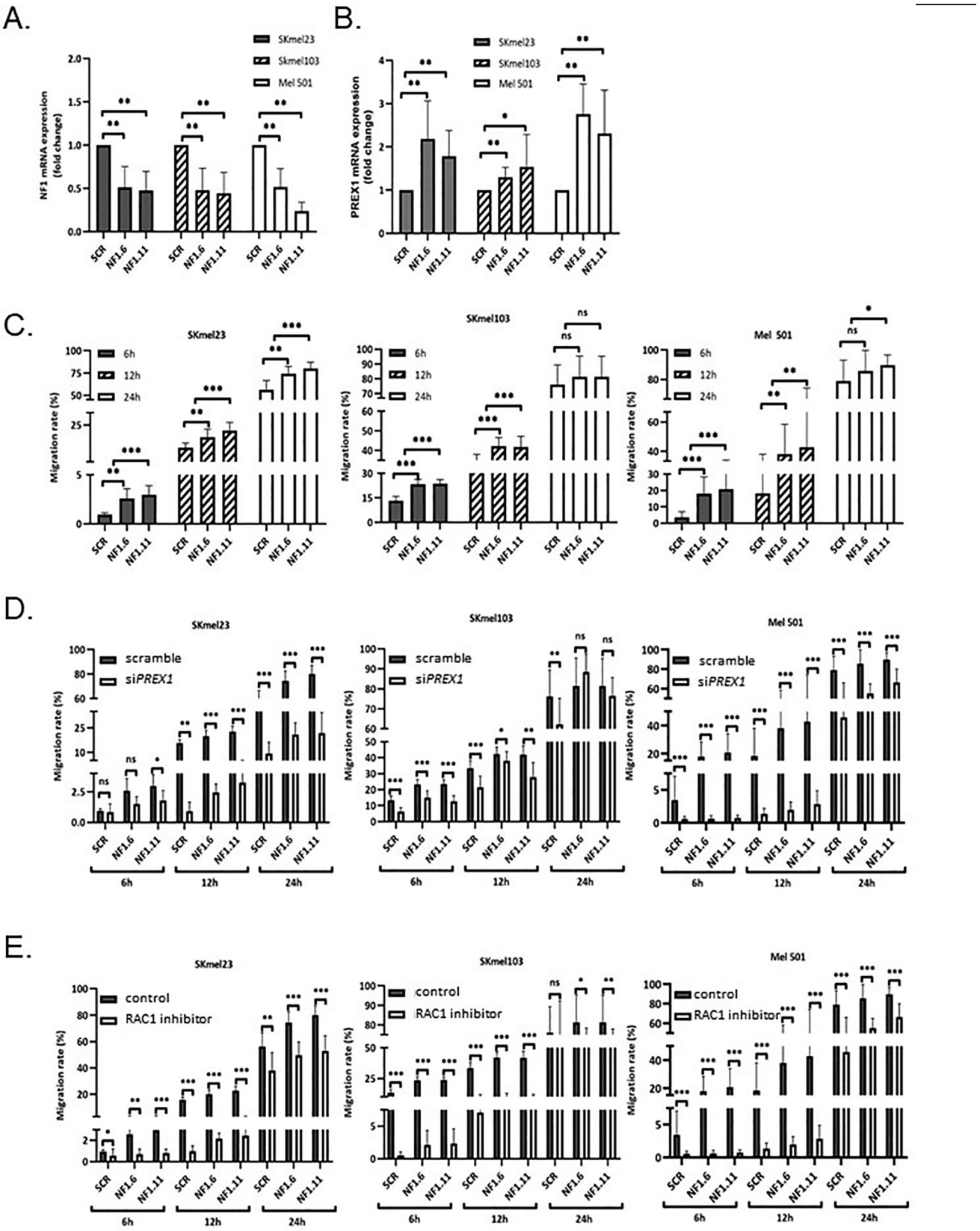

Fig. 2. Loss of *NF1* increases melanoma migration and is associated with increased *PREX1* expression. A. *NF1* mRNA expression under *NF1* silencing with two siRNAs (NF1.6 and NF1.11) in SK-mel-23, Mel501, and SK-mel-103 melanoma cell lines. B. *PREX1* mRNA expression under *NF1* silencing with two siRNAs in SK-mel-23, Mel501, and SK-mel-103 cell lines. C. Scratch-like migration assay representing the percentage of cell coverage after 6 h, 12 h and 24 h under *NF1* silencing in SK-mel-23, Mel501, and SK-mel-103 cell lines. D. Scratch-like migration assay as in C, after additional transfection with siRNA control (scramble) or with *PREX1* siRNA (si*PREX1*). E. Scratch-like migration assay as in C. in the absence (control) or presence (RAC1 inhibitor) of a RAC1 inhibitor. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ (unpaired Student's *t*-test). All error bars represent the SEM of at least three independent experiments.

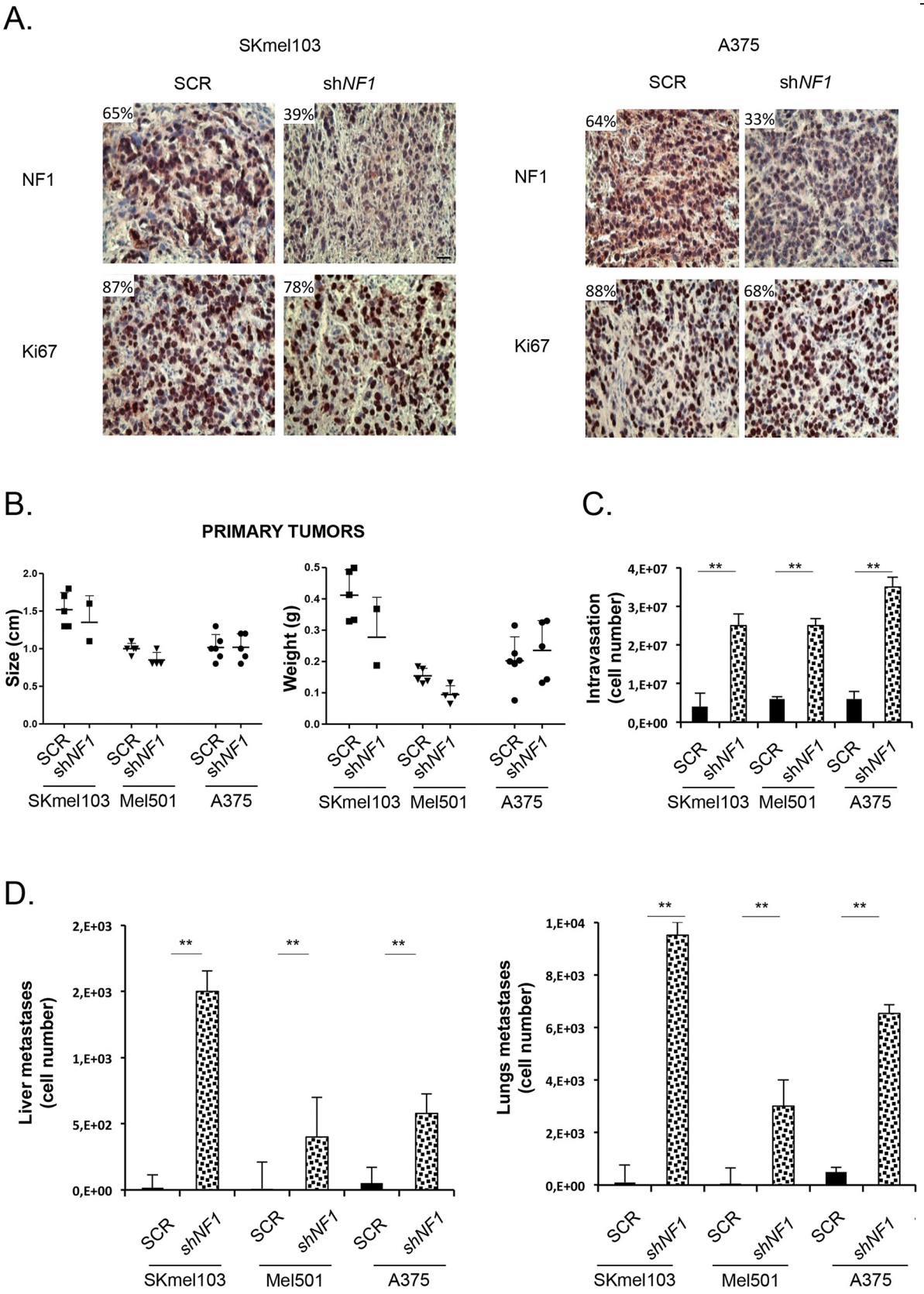

Fig. 3. Loss of *NF1* increases melanoma metastasis formation and intravasation *in vivo.* A. Chicken Chorioallantoic Membrane (CAM) Assay. Tumors grown from the respective cell lines were stained for NF1 and Ki67. Bar, 20 μm. Percentages represent staining's quantification (related to Supplemental Fig. 2). B. Graphs represent the tumors' size and weight seven days after injection of *NF1*-silenced SK-mel-103, Mel501 and A-375 cell lines. C. Graph represents the intravasation rate of *NF1*-silenced SK-mel-103, Mel501 and A-375 cell lines compared to their respective controls 54 h post-inoculation. D. Graph represents the number of lungs and liver metastases, extrapolated from human DNA quantification by qPCR for *NF1*-silenced SK-mel-103, Mel501 and A-375 cell lines seven days post-inoculation. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ (unpaired Student's *t*-test). All error bars represent the SEM of at least three independent experiments.

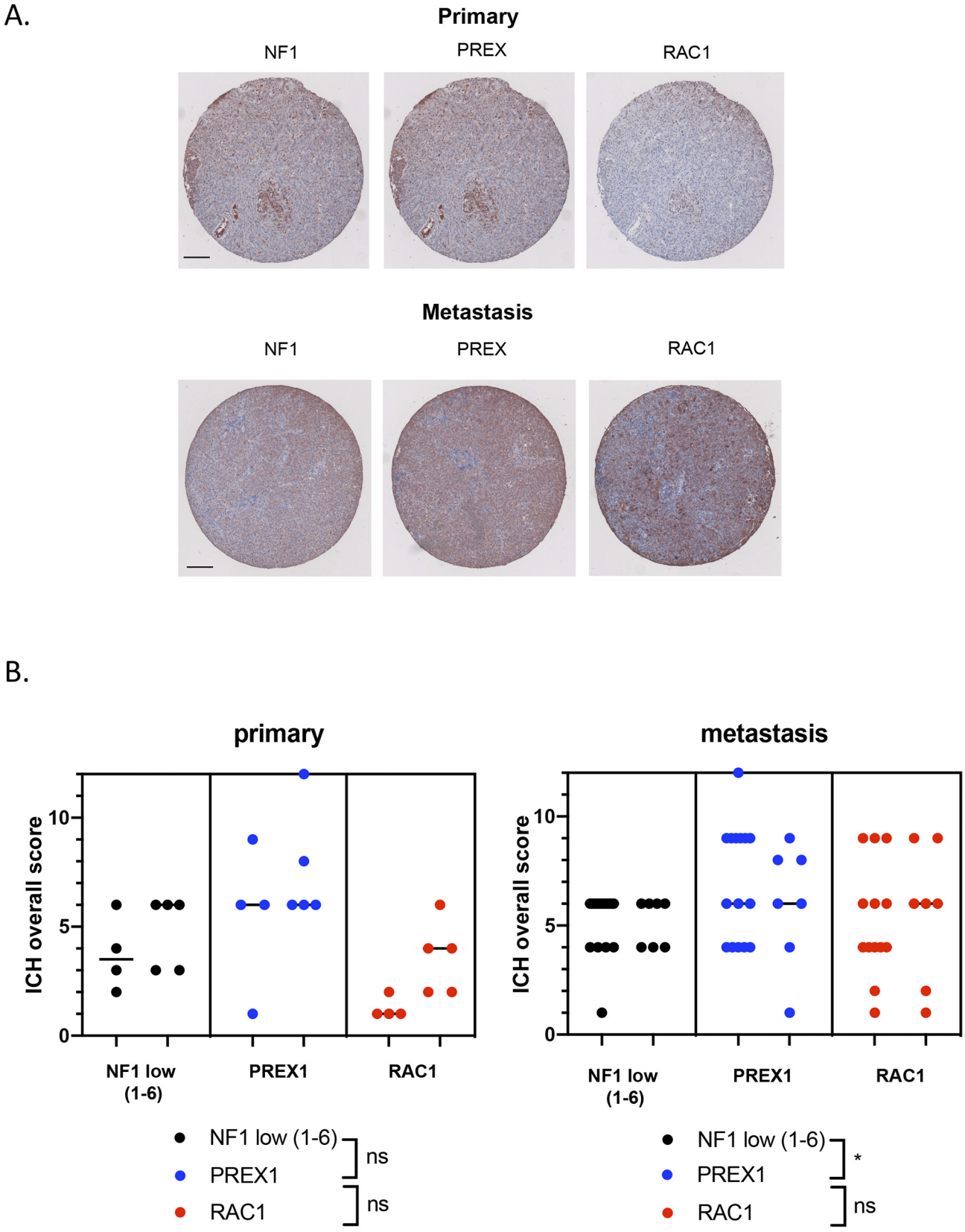

Fig. 4. PREX is upregulated in low NF1 expressing melanoma metastases. A. Representative microphotographs of Tissue Microarray (TMA) containing primary and metastatic melanoma samples analysed by immunohistochemistry using a specific antibody against NF1, RAC1 and PREX1. Bar, 100 µm. B. Scoring of the immunohistochemistry staining was performed according to our previously described protocol [24]. Duplicates of valid punch samples are represented for each condition. Significance was tested using two-tailed *t*-test with $*P < 0.05$ and ns: not significant.

was not observed with RAC1 expression, suggesting that NF1-mediated regulation of PREX may lead to a change in RAC1 activity instead of a change in protein expression (Fig. 4B).

Discussion

NF1$^{+/-}$ hiPSC-derived melanoblasts did not show any migration defect or alteration in RAC1 activity compared to *NF1*$^{+/+}$ control conditions. Only an additional silencing of the remaining *NF1* expression led to a migration delay. This result suggests an *NF1* dosage dependent effect on migration as it has already been described on pigmentation during developement [30]. Indeed, *Nf1* haploinsufficency specifically in melanocytes (*via Mitf*-cre) was not enough to cause skin pigmentation defect, however, the homozygous *Nf1* knockout did induce darker skin.

The migration delay we observed under *NF1* loss in *NF1*$^{+/-}$ melanoblasts is in line with data obtained in an *Nf1* mouse model. This report showed that loss of *Nf1* during brain development impaired cerebellar proper evolution, partially due to a defect of granule neuron progenitors' migration [15]. An ERK inhibitor could reverse this phenotype, involving the Mapk signaling in this mechanism. In our experiments however, we could not detect an increased activation of ERK after *NF1* silencing in *NF1*$^{+/-}$ melanoblasts, suggesting no obvious RAS activation in this context. This is most likely due to an already maximal activation of the RAS signaling by the *NF1* mutation itself. This result argues in favor of a migration regulation by a RAS/MAPK-independent mechanism. This result also provides rationale for the rescue of *cKit*- or *Mitf*-mutated mice's unpigmented bellyspots *via* Mapk activation when they are crossed with *Nf1*$^{+/-}$ mice [31].

We observed a defect in RAC1 activity under *NF1* silencing in *NF1*$^{+/-}$ melanoblasts, and we could partially reverse the migration delay with a RAC1 activator. These results strongly suggest a role of RAC1 in the migration mechanism regulated by NF1 in the context of human melanoblasts. Indeed, a RAS-independent regulatory mechanism of migration was described to link NF1 to RAC1 and LIM Kinases [13,14].

Interestingly, silencing of *NF1* in melanoma cell lines led to a global migration increase associated with an increase of *PREX1* expression. In line with our results, NF1-associated tumors such as malignant peripheral nerve sheath tumors (MPNSTs) or neurofibromas are described to be invasive and their migratory phenotype dependent on the activation of several signalings including MEK, AKT, mTORC1/2, STAT3 and ROCK [32,38]. Moreover, RHO-regulated genes have been shown as drivers of malignancy in plexiform neurofibromas [36,37].

In order to understand the apparent contradiction of the migration phenotype between melanoma cell lines and our melanoblast model, we should hypothesize that PREX1 is differently involved. The observed RAC1 defect after *NF1* loss in melanoblasts suggests a potential decrease of PREX1 expression or activity. We should also not exclude the possibility of RAC1-associated GAPs involvement in the regulation by NF1, which could therefore lead to a decrease of RAC1 activity.

Since we observed an upregulation of PREX after *NF1* silencing in melanoma cell lines and since PREX1 plays a crucial role in metastasis spreading in a melanoma mouse model [9], we decided to alter *PREX1* expression on top of *NF1* silencing. Additional *PREX1* silencing strongly decreased migration compared to *NF1* silencing alone. Yet, since migration was already impaired in cells only silenced for *PREX1*, a causal relationship between *NF1*-dependent *PREX1* upregulation and enhanced migration cannot be verified with certainty.

Our xenograft experiments in the chicken embryo showed that *NF1* loss does not impact tumor formation at the inoculation site but rather promotes metastases development in the liver and the lungs, as well as it increases the melanoma cells' intravasation capacities. The notion that tumor growth regulation at distant sites varies from the primary site has been known for a while. It was shown for example that a particular set of genes, so-called metastasis suppressor genes, could suppress metastasis formation *in vivo* without inhibiting primary tumor growth [25]. However, the role of *NF1* gene is particularly well documented in melanoma and several recent articles reported the particular phenotype of *NF1*-mutated melanoma tumors in comparison to those with wild-type *NF1* and their generally worse outcome [26–28]. These data highlight the important role of NF1 in melanoma advanced stages as well as in the primary situation. In addition, *NF1* loss was described to participate to melanoma formation by suppressing *BRAF*-induced senescence in melanocytes [29]. In our CAM model, the injection site imperfectly reproduces a "primary melanoma" as it is missing the tumor microenvironment of the skin (fibroblasts and keratinocytes) which greatly participate to tumor growth *via* the paracrine secretion of protumorigenic factors. This is one probable reason why *NF1* silencing had no effect on the tumor growth. Nevertheless our data clearly demonstrate that *NF1* loss is accelerating the formation of melanoma intravasation and metastasis. At this moment, additional experiments are needed to assess the involvement of PREX1/RAC1 in the formation of metastases *in vivo*.

Importantly, we observed an upregulation of PREX1 in patient-derived metastatic tumors with low NF1 expression, but this was not the case in patients with primary tumors. These data suggest a role of PREX1 specifically during tumor progression, and it would be interesting to correlate PREX1 upregulation with the aggressiveness of these tumors or with the rapidity of metastases formation.

We have also observed a strong migration delay during early time points in the presence of a RAC1 inhibitor, confirming the role of RAC1 in tumor cell migration. Nevertheless, it could be important to verify RAC1 activity in patient samples with low *NF1* expression, as we would expect an increased activity due to PREX1 upregulation. Indeed, between 5% and 10% patients with melanoma carry a mutation in the switch domain of *RAC1*, and this mutation is associated with tumor thickness, increased proliferation and presence of lymph node metastases [34,35].

Conclusion

In summary, we have shown in this study that tumor suppressor gene *NF1* not only regulates migration of human melanoblasts but also that of melanoma cells. This mechanism most likely involves an interaction with PREX1/RAC1, which are key controllers of cellular migration. The precise interaction mechanism remains however to be explored. Interestingly, it was described that PREX1/RAC1 are mediators of the response to HER/ErbB receptors and G-Protein Coupled Receptor CXCR4 in breast cancer [33]. Since melanoma tumors express these two receptors, it could be suggested to test their activation status in samples with low NF1 expression. Moreover, FDA-approved inhibitors for HER2 are classically used for breast cancer carrying HER2 mutations and could be tested in this context. Finally, regulators of migration and invasion in melanoma such as WNT and TFGβ signalings are also involved in the control of melanocyte lineage commitment during early development and converge to melanocyte-specific transcription factor MITF [39]. Based on the known genetic link between *NF1* and *MITF*, it could be expected that *NF1* loss-induced migration phenotype involves an interaction between NF1 and one of these two pathways.

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Declaration of competing interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2020.100858.

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