RACK1 cooperates with NRAS<sup>Q61K</sup> to promote melanoma in vivo

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**ABSTRACT**

Melanoma is the deadliest skin cancer. RACK1 (Receptor for activated protein kinase C) protein was proposed as a biological marker of melanoma in human and domestic animal species harboring spontaneous melanomas. As a scaffold protein, RACK1 is able to coordinate the interaction of key signaling molecules implicated in both physiological cellular functions and tumorigenesis. A role for RACK1 in rewiring ERK and JNK signaling pathways in melanoma cell lines had been proposed. Here, we used a genetic approach to test this hypothesis in vivo in the mouse. We show that Rack1 knock-down in the mouse melanoma cell line B16 reduces invasiveness and induces cell differentiation. We have developed the first mouse model for RACK1 gain of function, Tyr::Rack1-HA transgenic mice, targeting RACK1 to melanocytes in vivo. RACK1 overexpression was not sufficient to initiate melanomas despite activated ERK and AKT. However, in a context of melanoma predisposition, RACK1 overexpression reduced latency and increased incidence and metastatic rate. In primary melanoma cells from Tyr::Rack1-HA, Tyr::NRas<sup>Q61K</sup> mice, activated JNK (c-Jun N-terminal kinase) and activated STAT3 (signal transducer and activator of transcription 3) acted as RACK1 oncogenic partners in tumoral progression. A sequential and coordinated activation of ERK, JNK and STAT3 with RACK1 is shown to accelerate aggressive melanoma development in vivo.

1. Introduction

Cutaneous melanoma is the deadliest skin cancer. Melanoma has a high metastatic capacity. Despite recent clinical breakthroughs, the majority of metastatic melanoma patients do not survive [1]. The study of a minipig melanoma model revealed an overexpression of RACK1 (Receptor for activated protein kinase C) mRNA in melanoma cells [2]. RACK1 protein is strongly expressed in melanoma cells of primary tumors and metastases in different mammalian species: patients [2], horses [3] and dogs [4]. In sharp contrast, RACK1 is not detected in normal skin melanocytes or in navi by immunofluorescence [2–4]. Interestingly, RACK1 increased the survival of human melanoma MeWo cells following UV induced-apoptosis. Moreover, inhibition of RACK1 expression using RNA interference was shown to reduce the tumorigenicity of MeWo cells in a xenograft model [5].
RACK1 is a ubiquitous and abundant protein [6]. It is a scaffold containing seven WD40 repeats considered protein-protein interaction platforms. Through its ability to coordinate the interaction of key signaling molecules, RACK1 is thought to integrate various pathways involved in both physiological and tumorigenic cellular functions making it a signaling hub [7]. Yet, the extent to which the multiple binding partners of RACK1 are coordinated has not been much tested in vivo. In an attempt to alter RACK1 levels in mammals, the group of S. Biffo obtained one mouse line with a hypomorphic Rack1 allele. While homozygosity for that hypomorphic Rack1 allele resulted in a lethal phenotype, heterozygous adult mice showed no major phenotype except for a belly spot and pigmented tail and paws [8], typical features of a developmental defect in melanoblast migration.

A role for RACK1 in the crosstalk between ERK (Extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinase) signaling in melanoma was proposed to set up a feed forward mechanism triggering tumor progression [9]. In the light of these in vitro data, we hypothesized that gain of function of RACK1 targeted to melanocytes in the context of NRas constitutive activation would accelerate melanogenesis by strengthening converging tumor signaling.

As for other solid cancers, cutaneous melanoma development is considered as a multistep process. Melanogenesis requires a combination of gain of function mutations in oncogenes and loss of function mutations in tumor suppressor genes [10]. The first spontaneous metastasizing melanoma model harbored the NRasG12K mutation in a deleted Cdkn2a background [11]. To test whether an overexpression of RACK1 was sufficient to trigger melanoma, we created Tyr::Rack1-HA transgenic mice in which a hemaglutinin (HA) epitope-tagged-RACK1 is expressed off the Tyrosinase promoter. We show here that RACK1 overexpression is not sufficient to trigger nevi or melanomas despite ERK and AKT activation. Yet, in a context of melanoma predisposition, RACK1 melanocytic overexpression reduced latency and increased incidence and metastatic rate. We found activated JNK and STAT3 as partners of RACK1 in melanogenesis.

2. Materials and methods

2.1. Mice and genotyping

Mouse Rack1 cDNA was tagged with HA by PCR before insertion in a pBSK-UPT::Tyr-SV40 plasmid [12]. Micro-injection of the linearized vector was made in B6CBAF1/J fertilized oocytes. Tyr::Rack1-HA transgenic founders were characterized by Southern blot analysis and PCR genotyping. Data come from the 7th backcross onwards on C57BL/6J background. The Pax3GFP and -Cdkn2a alleles and Tyr::NRasG12K transgene have been backcrossed onto the C57BL/6J background for > 15 generations [13]. Animal care and use for this study were approved by the ethical board of Allfort Veterinary School in accordance with European Union Standards (agreement number 16, notice 14/02/12-4). To identify the Tyr::Rack1-HA transgene, the following primers were used: forward: 5’-ggcagatgcagacgacctg-3’ and reverse 5’-taatagttggcgcctgcegccctggtaccatatg-3’. PCR conditions were 30 s at 94 °C, 30 s at 65 °C, 30 s at 72 °C for 30 cycles and a final extension step at 72 °C for 10 min. The other genotyping conditions were as described [13].

2.2. Histologic analysis and immunofluorescence in mouse samples

Complete necropsy and systematic pathological analysis were performed on all mice as described [14]. Immunofluorescence was performed with mouse monoclonal anti-RACK1 (Transduction Laboratories, dilution 1:150, BD Biosciences, Le Pont de Clai, France), chicken polyclonal anti-GFP (Abcam, 1:600, Paris, France), mouse monoclonal anti-HA (Covance, 1:600, Rueill-Malmaison, France), rabbit anti-cytokeratin5 (Thermo Scientific, 1:100, Fisher Scientific, Illkirch, France) and rabbit polyclonal anti-pERK (Thr202/Tyr204, 1:200) and anti-pAKT (Ser473, 1:50) (Cell Signaling, Oxyme, St Quentin, France) and rabbit anti-ERK 1:100, goat anti-Ki67 1:100, anti-STAT3, anti-JNK (D-2) (Santa Cruz, Heidelberg Germany) antibodies. Nuclear counter-staining was achieved with 4’,6-diamidino-2-phenylindole (DAPI) (1:1000, Invitrogen). Sections were examined with a Zeiss Axio Observer Z1M ApoTome microscope (Carl Zeiss S.A.S., Le Pecq, France). Controls without the first antibodies showed no unspecific labeling. Images were processed with the AxioVision computer program version 4.6 (Carl Zeiss). Figures are representative of the skin samples evaluated (n > 8 for each mouse line). All images shown are individual sections of z series stack. Final figures were assembled with Adobe Photoshop CS3 (Adobe Systems; USA). Quantification of Ki67/GFP positive nuclei was performed on images obtained at 40 x in regions positive for Ki67, counting at least 30 GFP+ cells per field, 2 fields per mouse, 6 mice per genotype.

2.3. Fluorescent activated cell sorting (FACS), cell culture, soft agar assays and immunofluorescence

Skin melanocytes and melanoma cells from primary tumors (n = 6) or metastases (lymph nodes n = 6; lung n = 4; liver n = 1; brain n = 1) were isolated, FACS-sorted and cultured as previously described [13]. B16 melanoma cell line was grown in DMEM medium with 10% fetal calf serum and penicillin/streptomycin. All cells were grown at 37 °C under 5% CO2 at pH 7.0–7.1. ERK inhibitor U0126, 5 μM and 10 μM, and JNK inhibitor SP600126 20 μM (Sigma-Aldrich, Saint-Quentin Fallavier, France) were incubated for 24 or 48 h. Human cells UACC903 were cultured as previously described [9]. Soft agar tests were made in 96-well plates as described [13].

For immunofluorescence on cells plated onto coverslips, fixation lasted 15 min in 2% PFA and permeabilization with ice-cold methanol, 10 min. Immunolabeling on cells or agar slices was performed like in tissue section with the omission of the antigen retrieval step. Antibodies used were mouse monoclonal anti-Ki67 (1:100, Novoceastra, Newcastle upon Tyne, UK), rabbit polyclonal anti-pPKCa/Thr (Thr638/641, 1:100), anti-pJNK (pSAPK Thr183/Tyr185, 1:25), anti-pSTAT3 (Tyr705, 1:100) (Cell Signaling) and as above.

2.4. RNA interference and transduction

Mouse Rack1 shRNA sequence (ID# 61854) corresponding to a sequence inside exon 2, was obtained from Ambion (Invitrogen): GGT-CACCTCCCATTTGTTATT and the scramble sequence used was GT-CACCTACCTTTGTTATT [15]. Lentiviral vectors with GFP reporter of infections were produced as previously described [16]. Three Stat3 shRNA (ID# 424803, 424802, 641819) were obtained from Open Biosystem (Thermo Fisher Scientific) as lentiviral vectors. Transduction was performed with at 0.45 ng/μl of lentiviral titer in presence of polybrene. RNA was collected on the third day.

2.5. RNA extraction and quantitative RT-PCR

RNA extractions were performed on 20,000 FACS-sorted cells following RNA XS kit manufacturer instructions (Macherey Nagel, Germany) as described [13]. RNA sequencing (RNA-Seq) on shScramble and shRack1-treated melanocytes, primary melanoma from Tyr::NRas+; Pax3GFP+/- cells with or without Tyr::Rack1-HA was performed on technical triplicates of viral infection. Libraries were prepared by select- ing polyadenylated mRNA using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA). When performed on plated infected cells, RNA was prepared from 106 cells in 6 well plates. qPCR assays on cDNA from primary cells infected with shRack1 were performed using TATAA Granscript cDNA Supermix for reverse transcription and TATAA SYBR
GranMaster mix on a Light Cycler480 qPCR instrument (Roche) (TATAA Biocenter, Czech Republic). Aicb, Gapdh, Tubb5 and Rnp2 were used as reference genes. Experiments were carried out at least twice in triplicates.

2.6. Protein extractions, immunoprecipitation, Western-blot analyses and JNK kinase assay

Experiments were performed as described [13], at least twice. Antibodies used were anti-tubulin (Cell Signaling), anti-STAT3, anti-JNK (D-2) (Santa Cruz, Heidelberg Germany) and the same as above. JNK immunokinase assays were performed with endogenous JNK as previously described [5].

2.7. Statistical methods

Error bars in the figures represent standard errors of the mean. The two-tailed Student’s t-test or nonparametric Mann-Whitney U test were used to assess differences between groups. A P-value < 0.05 was considered as statistically significant (*** P < 0.001, ** P < 0.01).

Other details regarding transgenesis construct, histological analysis, immunofluorescence in skin samples and cells, RNA interference and transduction, quantitative RT-PCR, RNA seq or Western blot are available in the supplementary section.

3. Results

3.1. Effect of RACK1 knock-down on metastatic melanoma cell clonogenicity and differentiation

To test the importance of RACK1 in melanoma development we developed a RACK1 shRNA lentivirus by inserting a previously validated sequence [15] into a backbone allowing visual control of infection [16] (Fig. 1a). The murine melan-a cell line, which is a nontransformed immortalized melanocytic line, and the highly metastatic B16 melanoma line were used to test RACK1 knock-down. With a transduction efficacy around 90%, RACK1 protein was efficiently reduced by shRack1 as evaluated by immunofluorescence and Western blot analyses without affecting unrelated proteins like Tubulin, three days post transduction of metastatic B16 cells (Fig. 1a). However, B16-shRack1 cells were pro-

![Image](image-url)

Fig. 1. RACK1 knock-down reduced B16 melanoma cells invasiveness and led to cell differentiation.a: i Lentiviral vector backbone for shRNA expression with GFP control of transduction; ii Western-blot analysis for RACK1 and Tubulin in shRack1 and shScramble B16 cells 3 days post-transduction (3dpt); iii Fluorescent microscopy analysis of direct GFP signal (green) and immunolabeling for RACK1 (red) on 3 day-transduced cells. b: Aspect of shRack1 and shScramble-treated B16 cultures 14 days after transfection. Note the differentiation and loss of GFP+ cells in shRack1-treated B16 cells. c: Soft agar assay of shRack1 and shScramble-treated B16 cells 3dpt. Remarks for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
gressively lost from day 10 to day 20 post transduction, being replaced by cells not transduced, hence not expressing the shRNA (Fig. 1b). Remaining fluorescent B16-shRACK1 cells showed a differentiated phenotype. They switched from the typical rounded B16 shape to a melanocyte-like shape with dendritic extensions and higher melanin content (Fig. 1b), as did melan-a cells (Fig. S1).

We assessed the effect of RACK1 silencing on anchorage-independent cell growth of 3 day transduced cells. Only 40% of B16-shRACK1 cells formed clones in soft agar compared to 86 and 88% in B16-shScramble and B16-mock cells, respectively ($\chi^2$ test; $P < 0.0005$) (Fig. 1c). Furthermore, the B16-shRACK1 clones were smaller than the controls (Fig. 1c). This reduced size of B16-shRACK1 clones relates to a sharp reduction of Ki67 and pSTAT3 staining (Fig. 1d).

### 3.2. Overexpressed RACK1 in melanocytic cells from the Tyr::NRasG61K melanoma model

We next determined RACK1 expression in the Tyr::NRasG61K; Cdkn2a−/− mouse melanoma model, referred to as Tyr::NRas thereafter. These mice carry a melanocyte-targeted NRasG61K transgene which leads to constitutive ERK activation [11]. Tyr::NRas mice develop early dermal melanocytic proliferation responsible for skin hyperpigmentation, which can eventually progress to a malignant lesion [14]. We have shown that introduction of the Pax3GFP allele allows the identification of fluorescent melanocytic cells in the skin without affecting melanoma development [13,17]. Immunofluorescence on cutaneous melanomas showed a cytoplasmic RACK1 signal in GFP⁺ cells (Fig. 2a). RACK1 was immunodetected in all lesions from Tyr::NRas; Pax3GFP⁺ and Tyr::NRas mice.

We also tested melanoma-free skin. In control Pax3GFP⁺; Cdkn2a−/− mice, RACK1 protein was highly expressed in the cytoplasm of keratinocytes, here considered as positive controls. In contrast, melanocytes identified as GFP⁺ cells were negative for RACK1. We excluded the possibility that melanocytes displayed membrane RACK1 signal with triple immunostaining against RACK1, GFP and cytokeratin 5 (CK5), a marker of basal keratinocytes. Instead, in Tyr::NRas; Pax3GFP⁺ mice, skin sections displayed a specific cytoplasmic RACK1 signal in GFP⁺, CK5⁺ melanocytes (Fig. 2b). Follicular, interfollicular and dermal melanocytes displayed this specific melanocytic RACK1 signal (Fig. S2). To test whether RACK1 overexpression occurred at the transcript level, GFP⁺ melanocytes were sorted by FACS from neonatal skins. Higher RACK1 mRNA levels were observed in Tyr::NRas; Pax3GFP⁺ melanocytes isolated from neonatal skin compared to Cdkn2a−/−; Pax3GFP⁺ control pup littermates (Fig. 2c). Thus, ERK activation together with RACK1 overexpression is associated with melanoma and melanoma predisposition in this model.

To study signaling associated with the progression stages, we established primary cultures from neonatal skin, primary tumor, locoregional metastasis, and distant metastasis which, as expected, were pERK⁺ according to NRasG61K expression when assayed by immunofluorescence and Western blot (Fig. 2d) [13]. In these cells, overexpression of RACK1 mRNA (Fig. 2e) and high RACK1 protein level were identified (Fig. 2f). Interestingly, a 60 kDa band in addition to the predicted 36 kDa band was revealed in tumor cells compared to melanocytes (Fig. 2f). Denaturing conditions did not support dimerization of RACK1 (not shown). Inhibition of MEK1 with U0126 did not alter RACK1 reactivity in melanoma cells (Fig. S3).

### 3.3. Activated ERK and transiently increased skin melanocytes in pups with RACK1 gain of function in melanocytes

To address a causative role of RACK1 in melanoma development, we generated Tyr::Rack1-HA transgenic mice. We used the 6.1 kb promoter sequence of the mouse Tyrosinase gene in combination with the 3.6 kb distal control region [12] to target the expression of the mouse Rack1 gene (MGI:101849) that encodes RACK1, tagged by HA, to the melanocytic lineage (Fig. 3a). RACK1-HA expression from the Tyr::Rack1-HA transgene was detected when transfected into B16 cells in vitro (Fig. 3a).

Five Tyr::Rack1-HA transgenic founders were obtained using classical transgenesis (Fig. 3a iii). All founders were viable, fertile and reached adulthood without displaying any overt phenotype. Three lines were established with offspring from three distinct founders and analysed in detail. In order to easily identify melanocytes, the three lines were crossed with Pax3GFP/+ mice. Melanocytes were FACS-sorted on GFP from back skin of 3 day-old pups for each transgenic line and quantitative RT-PCR assays were performed. As expected, Rack1 mRNA levels in melanocytes were higher in Tyr::Rack1-HA; Pax3GFP/+ mice than in Pax3GFP/+ littermates in each transgenic line (Fig. 3b). Rack1/Actb ratios were comparable to ratios measured in the Tyr::NRas mice.

Protein expression of RACK1 in melanocytes in furry and glabrous skin was assessed by immunofluorescence (Fig. 3c, Fig. S4). In Tyr::Rack1-HA; Pax3GFP/+ melanocytes, GFP and HA protein signals were colocalized (Fig. 3c, third line). Moreover, a specific cytoplasmic RACK1 signal was detected in GFP and HA-positive melanocytes (Fig. 3c). Triple immunostaining against RACK1, GFP and CK5 confirmed the melanocytic origin of the RACK1 signal (Fig. 3c, fourth line). Thus, in our three Tyr::Rack1-HA transgenic mouse lines, Rack1 mRNA was over-expressed and RACK1 protein was detected in melanocytes. This excluded a role of the integration site of the transgene.

RACK1 was reported to associate with the core kinases of the ERK pathway and RACK1 reduction resulted in lower ERK activity while RACK1 overexpression produced an increased ERK activation [18,19]. We tested ERK activation in melanocytes of transgenic Tyr::Rack1-HA; Pax3GFP/+ and control mice by immunostaining (Fig. 3d). In control Pax3GFP/+ melanocytes, pERK signal was hardly detected. Instead, in Tyr::Rack1-HA; Pax3GFP/+ melanocytes, nuclear GFP and pERK signals were co-localized. Besides, pERK signal was also identified in keratinocytes. Noteworthy, total ERK expression was equivalent in both sample types (Fig. S5). We checked whether the PI3K/akt pathway was also activated in the skin of Tyr::Rack1-HA transgenics. Nuclear pAKT signal was detected as well in Tyr::NRas', Pax3GFP/+ skins, as opposed to Pax3GFP/+ skin (not shown). These data suggest that RACK1 overexpression associated with ERK and AKT activation. We studied whether this pERK immunodetection corresponded to a proliferative signal translating efficient ERK activation. Tyr::Rack1-HA; Pax3GFP/+ skin biopsies presented 26% more GFP⁺ cells than Pax3GFP/+ skins (Fig. 3e). Yet, no coat or skin hyperpigmentation was visible in any of the mouse lines. Over 17 months of follow-up, no melanocytic lesions were detected in any of the three transgenic lines (n > 15 mice/lane). In addition, the morphology of the skin was normal indicating that RACK1 overexpression alone was not sufficient to drive melanoma development (Fig. S6).

### 3.4. Accelerated melanoma appearance with RACK1-HA expression in a context of Tyr::NRas⁺ melanoma predisposition

Melanoma penetrance is not complete in the Tyr::NRas model [11]. In our colony, 33% of mice develop melanoma [14]. To assess the effects of RACK1 expression in a genetic background predisposing to melanoma, we produced Tyr::Rack1-HA; Tyr::NRas; Pax3GFP/+ mice for the three Tyr::Rack1-HA independent transgenic lines. These mice developed melanocytic lesions ranging from benign to malignant tumors [14] (Fig. 4a). No differences in latency and incidence were seen between the three transgenic lines. Nevertheless, 78% of the Tyr::Rack1-HA; Tyr::NRas; Pax3GFP/+ mice in each line showed reduced latency within about 10 months, mean 7.5 months (Mann-Whitney test; P < 0.002) (Fig. 4b). Besides, 55% of mice developed
melanomas, indicating a clear increase in incidence of primary cutaneous malignant lesions (n = 22/31, y2 test; P < 0.05) (Fig. 4a).

Moreover, distant metastases were more frequently identified in Tyr::Rack1-HA; Tyr::NRas+; Pax3GFP/+ mice (55%, n = 12/22) compared to Tyr::NRas+; Pax3GFP/+ mice (36%, n = 9/25) (Fig. 4c). Histological analysis of the lesions detected no differences between Tyr::Rack1-HA lesions and those of control Tyr::NRas+; Pax3GFP/+ littermates (Fig. 4a). However, when the proliferation status of melanocytic cells was analysed using Ki67 labeling, Tyr::Rack1-HA bearing melanomas presented a higher index than controls (Fig. 4d) (32.9 ± 17.8 versus 22.3 ± 8.1 respectively, Student’s t-test; P < 0.05).

The higher incidence, lower latency and higher frequency of mitosis provide the first in vivo evidence of a contribution of RACK1 to melanoma development. Histological data point to an acceleration of the proliferative status of the lesions.

3.5. JNK and STAT3 as oncogenic partners of RACK1 in melanoma development

To investigate the clinical advantage conferred by the overexpression of RACK1 in the Tyr::NRas+ model, we explored candidate proteins related to metastasis. STAT3 activation has been shown to promote metastasis in melanoma [20]. To test whether PKC and JNK activation were involved in melanomagenesis induced by activated NRAS as modeled in vitro [9], we isolated primary melanocytic cells from neonatal skin and at different stages of tumoral progression from Tyr::NRas+; Pax3GFP/+ mice. pPKCa/βγ pJNK and pSTAT3 were analysed by immunofluorescence. Phosphorylation of PKCa/βγ was detected both in melanocytes and in tumoral cells, independently of their tumoral progression stage. pJNK, instead, started to be observed in
melanoma cells from primary lesions and was stronger in cells isolated from metastases (Fig. 5a). While no signal was visible on neonatal melanocytes and only a small number of primary cutaneous melanoma cells showed a signal, a high proportion of metastatic cells were found positive for pSTAT3 (Fig. 5a). RACK1 cytoplasmic immunoreactivity instead is detected in all cells (Fig. 5a).

Next, we analysed whether JNK and RACK1 interacted in Tyr::NRas+ melanoma cells. Positive interaction was shown between pJNK and RACK1 by Western blot analysis after RACK1 immunoprecipitation in primary melanoma and metastases. RACK1–pJNK interaction occurred at basal levels, without specific induction suggesting constitutive activation of JNK in these cells (Fig. 5b). We also validated the activation of STAT3 in melanoma cells by Western blot as well as its binding to RACK1, after RACK1 immunoprecipitation. Despite the activation of STAT3 in primary melanoma cells RACK1–pSTAT3 interac-

Fig. 3. Targeted RACK1 overexpression in melanocytes is not sufficient to initiate melanoma.

i. Scheme of the Tyr::Rack1-HA construct and genotyping strategy (orange arrows). ii. Western blot analysis for HA after RACK1 immunoprecipitation in B16 cells transfected with the above construct and the human RACK1-HA. iii. Phenotype of the transgenic mice. iv. Detection of the transgene by PCR. b. Reverse transcription-qPCR for Rack1 RNA in melanocytes isolated by FACS from Tyr::Rack1-HA Pax3(+/−) mice from each of the three transgenic lines (dotted bar) compared to littermates (green bar). Results shown are Rack1 expression normalized to Actb from triplicates. c. ApoTome microscopy analysis of triple labeling for GFP (red), RACK1 (green) and CK5 (magenta) or GFP (red), HA (cyan) and RACK1 (green) in control Pax3(+/−) mice and in Tyr::Rack1-HA; Pax3(+/−) mice of transgenic line 1. Arrows point to melanocytes overexpressing RACK1. Bar: 10 μm. d. ApoTome microscopy analysis of double labeling for GFP (red) and phospho-ERK (green) in control Pax3(+/−) mice and in Tyr::Rack1-HA; Pax3(+/−) mice of transgenic line 1. Arrowheads point to melanocytes overexpressing pERK. Nuclear counterstaining in blue. Barc 10 μm. e. Percentages of GFP cells detected in the dorsal skin of 6 day-old pups from Pax3(+/−) (green), Tyr::Rack1-HA; Pax3(+/−) (dotted) and Tyr::NRas+; Pax3(+/−) (hatched) lines. **P < 0.01, ***P < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 4. Targeted RACK1 overexpression reduces latency and increases incidence of melanoma development in Tyr::N Ras+ mice. a, Histological features (haematoxylin-eosin-saffron staining) of melanocytic nevi, primary cutaneous melanoma and lung metastases in Tyr::N Ras+; Cdkn2a+/−; Pax3GFP+ mice and Tyr::RACK1-HA; Tyr::N Ras+; Cdkn2a+/−; Pax3GFP+ mice. Note that lesions are histologically indistinguishable among groups. b, Survival (Kaplan-Meier plot) for Tyr::RACK1-HA; Tyr::N Ras+; Pax3GFP+ mice (n = 22, pink curve) compared to Tyr::N Ras+; Pax3GFP− control mice (n = 25, grey curve). Mice from the three transgenic lines were pooled in the Kaplan-Meier graph. c, Percentages of lung metastases in Tyr::RACK1-HA; Tyr::N Ras+; Pax3GFP+ mice (pink bar) compared to control Tyr::N Ras+; Pax3GFP− (grey bar). Gross feature of a Tyr::RACK1-HA; Tyr::N Ras+; Pax3GFP+ lung with multiple melanoma metastases. d, ApoTome microscopy analysis of double labeling for Ki67 (red), GFP (green) and RACK1 (cyan) in control Tyr::N Ras+; Pax3GFP− mice and in Tyr::RACK1-HA; Tyr::N Ras+; Pax3GFP+ mice. Nuclear counterstaining in blue. Bars: 10 μm. Arrowheads point to Ki67+, GFP+, RACK1+ melanocytic cells. Arrow points to a mitosis, RACK1−. In the lower row, intense RACK1+ cells are keratinocytes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 5. JNK and STAT3 are oncogenic partners of RACK1 in ERK melanoma development. a, Fluorescent microscopy analysis of immunolabeling for pPKCα/β, pJNK, pSTAT3 and RACK1 in Tyr::NRas; Pax3GFP/+ cells from each stage of melanoma progression. Bars: 10 μm. pPKCα/β was detected at all stages, pJNK was negative in nontransformed melanocytes and pSTAT3 appeared in metastases. b, Western blot analysis for pJNK after RACK1 immunoprecipitation in melanoma and lymph node metastases cells. Western blot for pJNK on the 10% inputs used for immunoprecipitation. Below: control immunoprecipitation without RACK1 antibody. Both cell types showed constitutive pJNK-RACK1 interaction. c, Western blot analysis for pSTAT3 after RACK1 immunoprecipitation in lung metastasis cells. Protein interaction between RACK1 and pSTAT3 appears only in metastatic melanoma cells. Western blot analysis for pSTAT3 and STAT3 in primary melanoma and metastasis cells. Tubulin was used as loading control.
tion was only detected in metastases (Fig. 5c). These data suggest that JNK and STAT3 are activated prior or during the metastatic process.

3.6. Coordinated expression of RACK1, JNK and STAT3 regulates invasive potential

To investigate RACK1, JNK and STAT3 coordination, we verified whether RACK1 levels were under c-Jun transcriptional control treating cells with a JNK inhibitor, SP600125. SP600125 reduced the levels of both 36 kDa and 60 kDa forms of RACK1 in all melanoma cell types (Fig. 6a). A reduction of pJNK was also found (Fig. 6a). When we compared FACS-sorted cells derived from primary melanomas of Tyr::RACK1-HA; Tyr::NRas; Pdx2GFP/+ mice with the ones from Tyr::NRas; Pdx2GFP/+ mice, we observed pJNK immunoreactivity in Tyr::Rack1-HA melanoma cells not only in the nucleus but also in the cytosol (Fig. 6b). We reasoned that if RACK1 overexpression accelerated the appearance of tumorigenic properties, a correlation with JNK activity would be found in Tyr::Rack1-HA melanoma cells. To test this hypothesis we carried out clonogenic and JNK activity assays. In soft agar assay, cells overexpressing Rack1 started forming clones earlier than control colonies (Fig. 6c). SP600125 treatment of Tyr::RACK1-HA melanoma cells prevented colony formation in agar over a 6 days period (Fig. 6c). When immunokinese assays using GST-c-Jun1-86 as substrate were performed on primary cells of melanomas, an increased JNK activity was revealed in Tyr::Rack1-HA melanoma cells compared to controls Tyr::NRas+ (Fig. 6d). These data point to a stronger JNK activity in RACK1 overexpressing melanomas.

Out of 5 enhancer regions described by ENCODE and identified on human RACK1 gene code (www.genecards.org), three (GH05E181172, GH05E181215, GH05E181240) present binding sites for STAT3. To test whether STAT3 transcriptionally regulates RACK1 in Tyr::NRas+ cells, we transduced interfering lentiviral shStat3 vectors. STAT3 knock-down in lung metastasis cells led to Rack1 mRNA (Fig. 6e) and RACK1 protein reduction on both the 36 kDa and the 60 kDa bands (Fig. 6eii). STAT3 knock-down effect on RACK1 mRNA and protein levels was also effective in human UACC903 melanoma cell lines as illustrated in Fig. 6f. As the JNK pathway was shown to activate STAT3 in epithelial cells [21], we tested whether JNK was regulating STAT3 in melanoma cells. In agreement with above findings, pSTAT3 signal was reduced in SP600125-treated mouse cells (Fig. 6a). Inhibition of JNK activation did also reduce STAT3 activation in human melanoma cell lines (Fig. 6g).

In order to further dissect the mechanism engaged by RACK1 overexpression to allow melanoma cells to metastasize, we examined the transcriptome of melanoma cells from primary lesions in Tyr::NRas+ and Tyr::Rack1-HA, Tyr::NRas+ by RNA-seq. Ingenuity Pathway Analysis on numerically differentially expressed genes resulted in the enrichment of tissue morphology, cellular movement, growth and proliferation pathways. Stat3 appeared increased in Tyr::Rack1-HA, Tyr::NRas+ with a fold change of 1.3. We intended to analyse the effect of shRack1. In these heterogeneous primary cultures, estimation of the efficacy of lentiviral vector transduction was around 50% for both shrRack1 and shScramble (Fig. 6h). By immunohistochemistry, RACK1 protein downregulation was detected in the transduced cells (Fig 6h) but no reduction of Rack1 mRNA was noticeable for any of the 6 transcripts of Rack1, from which only 2 (ENSMUST0000002640 and ENSMUST00000125166) can be translated. Among the genes that exhibited increased expression in Tyr::Rack1-HA, Tyr::NRas+ cells and decreased expression following shRack1 treatment, Agrp1 encoding the adhesion G protein-coupled receptor G1, has been shown to dually regulate angiogenesis during melanoma progression [22]. Quantitative RT-PCR on Rack1 exon 2, targeted by the interfering sequence, showed a slight reduction upon shRack1 treatment (Fig. 6i). Reduction of RACK1 was related to a clear decrease in Agrp1 expression in melanoma cells from Tyr::Rack1-HA, Tyr::NRas+ and Tyr::NRas+ mice. The Agrp1 reduction was stronger on Tyr::Rack1-HA, Tyr::NRas+ cells, in which Stat3 reduction was observed, in contrast to Tyr::NRas+ cells (Fig. 6i). In agreement with these results, RACK1 knock-down in B16 cell agar colonies led to pSTAT3 reduction (Fig. 1dii). To test whether angiogenesis could be responsible for the clinical outcome of Tyr::Rack1-HA, Tyr::NRas+ melanomas, we performed a smooth muscle actin (aSMa) immunofluorescence on the mouse primary melanoma lesions of Tyr::Rack1-HA, Tyr::NRas+ and Tyr::NRas+ models. aSMa is a marker of myofibroblasts and pericytes used to identify capillaries and vascular structures. Fig. 6j shows the higher density of aSMa+ and vascularisation in heavily pigmented lesions in the Tyr::Rack1-HA genotype.

4. Discussion

RACK1 overexpression has been described in different solid cancers. Its detection in situ was proposed as a diagnostic marker in melanoma [2] and several carcinomas [23–25]. Many studies have shown that RACK1 is involved in multiple aspects of cancer progression in vitro (reviewed by [7]). As cell monolayers are far from in vivo complexity, we wanted to test whether the model, in which NRASG12D triggers ERK/c-Jun accumulation with subsequent RACK1 increase and JNK activation, was valid in the mouse. In this study, we generated Tyr::Rack1-HA transgenic mice to test a RACK1 role in melanoma initiation and progression in vivo. Our results support the model of RACK1 as a major player in melanomagenesis.

We showed that transgenic Tyr::Rack1-HA mice overexpressed Rack1 in melanocytes with concomitant detection of pERK and pAKT. Nevertheless, Tyr::Rack1-HA mice did not develop either hyperpigmentation nor melanoma suggesting that additional alterations are required for both events. In Tyr::NRas+ transgenic mice, NRASG12D triggers MAPK and PI3K/AKT pathways activation [10]. This mutation leads to hyperproliferation of skin melanocytes and spontaneous melanoma development [11]. We showed here that these mice also overexpress RACK1 in melanocytes. Thus, the difference in phenotypes could be ascribed to a differential compartmentalization of the proteins in the two melanocyte-types engaging then distinct genetic programs [26]. Alternatively, it could hint at the existence of additional pathways triggered by the NRASG12D mutation, independent of ERK, JNK, AKT, STAT3 pathways and scaffold RACK1.

Breeding Tyr::Rack1-HA mice with Tyr::NRas+ mice enhanced melanoma initiation and progression, with reduced latency and increased incidence as well as distant metastases in three independent mouse lines. We provided a first evidence of a role for RACK1 in melanomagenesis in vivo.

RACK1 serves as a signaling hub that has been implicated in the regulation of several pathways. To identify proteins mediating a role for RACK1 in melanomagenesis, we determined the activation status of PKCα/β, STAT3 and JNK in primary cultures. pPKCα/β was detected during the early stages as described [2], whereas STAT3 and JNK proteins were found activated in all lymph node metastatic cells which are representative of the first metastatic stage. Clinically, improved therapy for melanoma will benefit from understanding the metastatic process at a molecular level. In our primary culture cells, JNK activation marked progression to lymph-node metastases. Fittingly, a pathology study showed JNK activation associated with poor prognosis [27]. However, an oncogenic role of the JNK/c-Jun pathway in melanoma development is still under debate [28]. The tumor suppressor p16/Cdkn2a was reported to exert its inhibitory role on tumor cells by suppressing JNK activity [29]. In our system, Cdkn2a deletion was not sufficient alone to trigger JNK activation. Lopez-Bergami et al. described that RACK1 mediates JNK activation by PKC in vitro [3]. Moreover, in human melanoma cell lines, constitutively active ERK provides signals to increase the activity of JNK via a rewired signaling [9]. In the pri-
**Fig. 6.** RACK1 regulation and angiogenesis in melanoma.

**a** Western blot analysis of pc-Jun and RACK1 after SP600125 inhibition of JNK on protein lysates from primary melanoma and metastases from Tyr::NRas*; Pax3GFP/+ mice and primary melanoma cells in Tyr::Rack1-HA; Tyr::NRas*; Pax3GFP/+. SP600125 20 μM reduced RACK1 levels after 48 h treatment. pc-Jun reduction is shown.

Western blot analysis of RACK1, pSTAT3, STAT3 and pJNK, in Tyr::NRas*; Pax3GFP/+ melanoma lung metastatic cells after treatment with 20 μM SP600125 for 48 h and quantification. 

**b** pJNK immunoreactivity on cells from primary melanomas of Tyr::Rack1-HA; Tyr::NRas*; Pax3GFP/+ mice compared to the ones from Tyr::NRas*; Pax3GFP/+ mice. Bars: 10 μm. Note a higher pJNK signal on Rack1 overexpressing cells.

**c** Soft agar assay over 6 days with melanoma cells overexpressing RACK1-HA and controls without or with SP600125 (20 μM). RACK1-HA cells started forming clones earlier, and SP600125 prevented it. Bars: 10 μm. In vitro JNK kinase assay. Cell lysates from Tyr::NRas*; Pax3GFP/+ and Tyr::Rack1-HA; Tyr::NRas*; Pax3GFP/+ melanomas and Tyr::NRas*; Pax3GFP/+ melanocytes were subjected to immunoprecipitation using JNK antibody followed by kinase assay using GST-c-Jun as substrate. i, ii Quantitative RT-PCR for Stat3 (dark blue bars) and RACK1 (hatched light bars) mRNAs on lung melanoma metastasis cells after treatment with lentiviral vectors coding for shStat3 (02, 03, 19) or control. Results shown are Stat3 or RACK1 expression normalized to Actb from triplicates. * P < 0.05, *** P < 0.001.

**d** Western blot for RACK1, STAT3 and Actin after STAT3 knock-down.
mary melanoma cells from Tyr::Rack1-HA; Tyr::NRas; Pax3\superscript{3GFP}\superscript{a} mice, pJNK was detected both in the nucleus and cytoplasm. JNK activity was increased as determined in the kinase assay. Although we have not explored the underlying mechanism of JNK activation, our data support the previous in vitro model where JNK activation is being driven by PKC, ERK and Rack1. Data are consistent with the positive correlation observed between cytoplasmic pJNK and pERK in human melanomas [27], where Rack1 is overexpressed [2]. Phospho-STAT3 on Ser705 was mainly found in lymph-node metastatic cells, which is consistent with progressive activation found in human melanoma cell lines [20] [30] and in tumor samples. In melanoma cells, activated STAT3 was reported to sustain expression of the anti-apoptotic genes, Bcl-xL (Bcl2-like 1) and Mcl-1 (Myeloid cell leukemia sequence 1) [31]. Constitutive STAT3 activation might be a crucial event in metastasis development. We identified STAT3 as a direct Rack1 partner in melanoma development. Confirming the functional interaction between STAT3 and Rack1 we found that Rack1 was regulated at the mRNA level by STAT3. Noteworthy, STAT3-mediated maintenance of NF-xB activity occurs in human A2058 melanoma cell line [32]. In turn, NF-xB is known to upregulate the transcription of Rack1 through direct interaction with its promoter thus contributing to cell survival in PC12 cells [33]. Besides, inhibition of JNK activity led to decreased activation of STAT3. This was reported in colon cancer cells after treatment with AS601245, another JNK inhibitor [34].

B16 mouse melanoma cells harbor both STAT3 activation and JNK activation (data not shown). Rack1 silencing in B16 cell line led to reduced invasive capacities and cell differentiation and loss. The differentiated state with dendritic extensions closely resembles the in situ status of melanocytes in normal skin where no Rack1 is detected [2,3]. Yet, our results seem to be in apparent contradiction with the recent publication of Marubashi et al. which showed that Rack1 knock-down in melan-a cells decreased their dendrivity [35]. In fact, this specific phenotype corresponded to forskolin treated cells, and it might depend upon the extent of RNA silencing and the time before observation. Previous observations showed that mouse fibroblast cells with silenced Rack1 contained more and longer focal adhesions [18,19].

We observed an additional 60 kDa band revealed by Rack1 antibody only in transformed cells, which does not appear in the literature. This band decreased in the presence of the JNK inhibitor or STAT3 knock-down suggesting it could reflect a form of Rack1 related to malignancy. Further investigation must be carried out to understand the significance of this isoform.

RNA-seq data were produced in order to determine the targets of signaling pathways induced by Rack1 overexpression that cause the phenotype changes between normal and tumoral melanocytes with different potential. Tyr::Rack1-HA; Tyr::NRas; Pax3\superscript{3GFP}\superscript{a} primary melanoma cells expressed a higher basal level of Adgrg1 mRNA. Interestingly, Adgrg1 has been related to angiogenesis [22], and Rack1 overexpressing melanomas did appear to contain more dSMA\superscript{a} cells, a marker of angiogenesis. Adgrg1 activation was recently shown to promote melanoma migration [37]. Melanomas have a high angiogenic potential. These observations are in agreement with early findings on Rack1 expression during angiogenesis [38]. Biological replicates of tumors should be used to formulate new hypotheses on transcriptional targets of Rack1-involving pathways. The wide implication of Rack1 in physiological processes precludes the possibility to directly act on this protein [7]. Identification of Rack1-protein interactions holds the opportunity to develop new therapeutics by the design of peptides interfering with specific binding partners of Rack1 [39,40].

In summary, we demonstrated that Rack1 is implicated in melanomagenesis. Rack1 enhanced melanoma initiation and progression in vivo. We propose that activated STAT3 adds to JNK as Rack1 partners in the metastatic process. Specific interference between Rack1 and its partners must be further analysed as a potential therapeutic improvement in melanoma treatment.

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**Authors contributions**

JJP, GEM designed the study, CC performed all studies on the mouse and mouse cell assays, MEP and PLB on human cell lines and the JNK assay, SL, PS performed mouse transgenesis, FB and ERG scored histological samples, JEz performed immunofluorescence, PHC carried out FACS, SP, UM provided lentiviral vectors expressing shRack1, GAH advised with constructs, DE, performed RNA-seq, JB bioinformatic analysis, EB converted gene names. CC and GEM wrote the manuscript.

**Uncited reference**

[36]

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