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RESEARCH ARTICLE

RACK1 depletion in a mouse model causes lethality, pigmentation deficits and reduction in protein synthesis efficiency

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Abstract The receptor for activated C-kinase 1 (RACK1) is a conserved structural protein of 40S ribosomes. Strikingly, deletion of RACK1 in yeast homolog Asc1 is not lethal. Mammalian RACK1 also interacts with many non-ribosomal proteins, hinting at several extraribosomal functions. A knockout mouse for RACK1 has not previously been described. We produced the first RACK1 mutant mouse, in which both alleles of RACK1 gene are defective in RACK1 expression (ΔF/ΔF), in a pure C57 Black/6 background. In a sample of 287 pups, we observed no ΔF/ΔF mice (72 expected). Dissection and genotyping of embryos at various stages showed that lethality occurs at gastrulation. Heterozygotes (ΔF/+) have skin pigmentation defects with a white belly spot and hypopigmented tail and paws. ΔF/+ have a transient growth deficit (shown by measuring pup size at P11). The pigmentation deficit is partly reverted by p53 deletion, whereas the lethality is not. ΔF/+ livers have mild accumulation of inactive 80S ribosomal subunits by polysomal profile analysis. In ΔF/+ fibroblasts, protein synthesis response to extracellular and pharmacological stimuli is reduced. These results highlight the role of RACK1 as a ribosomal protein converging signaling to the translational apparatus.

Keywords Initiation · eIF6 · PKC beta · 40S · Asc1p

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Introduction

Recent evidence has shown that the control of gene expression can occur at the level of translation. Several mechanisms have been proposed including a complex interplay between initiation factors (IF) and signaling pathways, *cis*-sequences in mRNA, and more recently miRNAs [1]. However, the most striking finding is the recent evidence that defects in ubiquitous ribosomal proteins can lead to tissue-specific alterations in gene expression, thus suggesting that even the ribosomal machinery itself can contribute to the regulation of translation [2]. One question to be addressed is whether each ribosomal protein contributes in a unique way, or alternatively alterations in multiple ribosomal proteins, belonging to either the large or the small ribosomal subunit, lead to overlapping phenotypes. Unfortunately, such analysis is strongly impaired by the nature of ribosomal proteins that are generally strictly necessary for all tissues, and difficult to manipulate. An alternative possibility is that ribosomal proteins, due to their intrinsic abundance, may perform ribosome-independent functions.

The receptor for activated C-kinase 1 (RACK1) was first characterized as an intracellular receptor for active PKC [3]. RACK1 binds with nanomolar affinity to the activated form of PKC beta II, leading to its stabilization and translocation to specific substrates [4]. Other forms of PKC can bind RACK1 at lower affinity [5]. Studies in

mammalian cells have complicated the issue by identifying other RACK1 partners. More than 100 interactions have been reported, raising the issue of their significance, or the puzzling question as to how RACK1 can bind so many partners [6]. Significant and extensively confirmed interactors in vitro include kinases such as src [7, 8] and JNK [9, 10], neuronal membrane-bound receptors [11, 12], RNA-binding proteins [13], adhesion molecules such as integrins [5, 14, 15], and enzymes such as PDE4D5 [16–18]. Accordingly, RACK1 has been reported to mediate many processes, including cell division [8, 19], apoptosis [20], adhesion and motility [5, 14, 15], development [21], stress responses [22, 23], neuronal activity [24], circadian rhythms [25], and energy metabolism status [26]. It should be stressed that RACK1 is a very abundant protein, thus indirectly supporting the possibility that it might participate in multiple processes. The question is how separate pools of RACK1 protein may be sorted to specific interactors in vivo, and which functions of RACK1 are necessary.

RACK1 is evolutionarily conserved. Homologs are described in budding yeasts (Asc1) and fission yeasts (cpc2). In the unicellular context, some puzzling properties of RACK1 have been described. First, RACK1 deletion in yeast cells is not lethal, and cells retain normal vitality and proper function unless challenged with various stresses, mostly related to nutrient sensing [27–31]. Early studies showed that in yeast RACK1 is associated with the small ribosomal subunit, suggesting that it may function as a non-essential ribosomal protein [28]. This finding has been spectacularly confirmed by independent biochemical and structural studies, which converged in showing that RACK1 is a component of the small ribosomal subunit 40S [29, 32], where it localizes at the back of the 40S head region, close to the mRNA exit channel [33, 34]. In the context of translation, RACK1 function has been mainly investigated in lower eukaryotes, where it modulates miRNA-mediated translational repression [35] and translational arrest [36]. Taken together, evidence in unicellular organisms and structural studies indicate that RACK1 can behave as a structural ribosomal protein in addition to cytoplasmic ribosomes. Notably, the ribosomal association of RACK1 is evolutionarily conserved [29, 37]. However, in some conditions, RACK1 does not behave as a core component of ribosomes: in stationary phase yeast cells a fraction of RACK1 is free in the cytosol, unbound to the small subunit [27].

In order to explain the ribosomal localization of RACK1 and the existence of multiple interactors, two main hypotheses, not necessarily mutually exclusive, can be devised. First, the role of RACK1 may be different between yeast and mammals, because many interactors of mammalian RACK1 are absent in yeast. Alternatively, RACK1 is a ribosomal protein in mammalian cells too, which may have acquired the additional function of translocating specific molecules to the ribosomal machinery [38]. In the past, we reconciled the ribosomal and signaling functions of RACK1 by suggesting that it acts as a PKC signaling platform on the ribosome; specifically, we have shown that most RACK1 resides on ribosomes, where it contributes to PKC-stimulated translation and regulates the function of eukaryotic IF 6 [37, 39]. The idea that RACK1 may act as a scaffold for PKC on ribosomes has been strengthened by the observation that, coupled to PKCβII, ribosomal RACK1 promotes the translation of specific mRNAs in ribosomes of cancer cells [38]. However, to elucidate the double role of RACK1 in higher eukaryotes, it is compelling to establish a mammalian model in which RACK1 is genetically depleted at the tissue level. Here, we describe for the first time a mouse defective in RACK1 expression.

Materials and methods

Reagents

All antibodies were from Cell Signaling (Boston, MA, USA) except RACK1 antibody that was from BD Bioscience (San José, CA, USA). β-Actin was from Sigma (St Louis, MO); eIF6 antibodies were as previously described [40]. Secondary antibody for fluorescence was Alexa Fluor 488 goat anti-mouse IgM. [^{35}S]Methionine, [^{32}P]γATP, and [^{32}P]α-dCTP were purchased from Perkin Elmer (Waltham, MA, USA). The cell culture media (DMEM) was purchased from Lonza (Basel, Switzerland).

Generation of knockout mice

Knockout mice were generated at EMBL (Monterotondo, Italy). Frt-flanked neomycin cassette was introduced in the region between exon 3 and exon 4 of the RACK1 gene. LoxP sites were situated before exon 3 and after the Frt-Neo-Frt sequence (see Fig. 1a). The construct was electroporated in C57 Black/6 embryonic stem cells (ES), and injected into foster mothers of the same background. All experiments were approved by the Ethics Committee of San Raffaele and complied with E.C. regulations (IACUC authorization SK398). For genotyping, the following primers were used for multiplex PCR: 5′-CCGAGACAAGACCATAAAGT (forward sequence in exon 3), 5′-GCAGCGCATCGCCTTCTATC (forward sequence in the Neo cassette), and 5′-ACGATGATAGGGTTGCTGCT (reverse sequence in exon 4) on the RACK1 gene. For p53, the following primers were used: 5′-ACAGCGTGGTGGTACCTTAT (forward sequence in p53 wild-type gene), 5′-CTATCAGGACATAGCGTTGG (forward sequence in

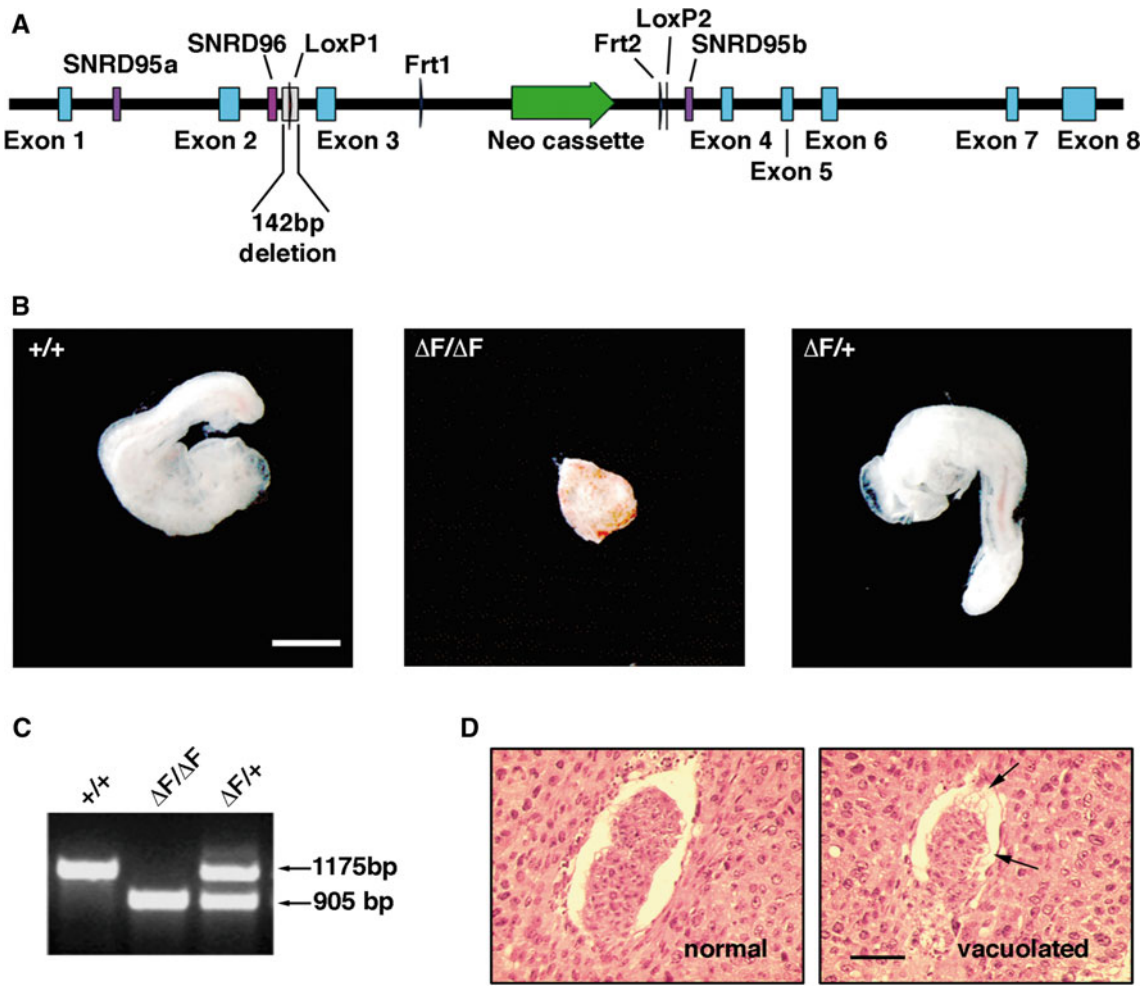

Fig. 1 RACK1 is essential for gastrulation. **a** Reconstruction of targeted RACK1 gene. The 142-bp deletion is at the level of the first loxP site (*grey square*) (scheme produced by MacVector software). **b**, **c** Resorbed knockout embryos at E9.5 (**b**, *scale bar* 1 mm) and their genotype identification (**c**). **d** Histological analysis of implantation sites showed the absence of the early egg cylinder and the presence of vacuolated areas (*arrows*) in one out of four E6.5 embryos (*scale bar* 80 μm)

p53 mutant gene) and 5′-TATACTCAGAGCCGGCCT (reverse sequence in p53 wild-type gene). The PCR for the presence of Flpe recombinase was performed using 5′-CTAGGCCACAGAATTGAAAGATCT (wild-type forward), 5′-GTAGGTGGAAATTCTAGCATCATCC (wild-type reverse), 5′-CACTGATATTGTAAGTAGTTTGC (Flpe forward), and 5′-CTAGTGCGAAGTAGTGATCAGG (Flpe reverse).

Northern blot analysis

For pre-rRNA processing analysis, total RNA was isolated from the livers of 16-day-old mice using TRI Reagent (Sigma, St Louis, MO, USA) following the manufacturer's protocol. RNA (5 μg) was separated on 1 % agarose-formaldehyde gel and analyzed by Northern blot hybridization using standard techniques. The oligonucleotide ITS2-2 complementary to nucleotides (nt) 239–271 of the ITS2 region was used as the probe for hybridization (5′-ACTGGTGAGGCAGCGGTCCGGGAGGCGCCGACG-3′), as described previously [41]. The probe was 5′-labeled using [^{32}P]γ-ATP and T4 polynucleotide kinase.

For snoRNA primer design, sequences for murine snoRNA U95 and U96 were designed using http://www.ensembl.org (SNORD95a: ENSMUSE00000521724; SNORD95b: ENSMUSE00000522004; SNORD96: ENSMUSE00000521949). Two primers were designed with antisense orientation to a portion of the snoRNA gene: 5′-GCCTCTGGATTTCAGCAAAG-3′ (for both SNORD95 transcripts), an 5′-GATTGGTTGACAATGTCGTC-3′ (for SNORD96). Total RNA was isolated from heads of E13.5 embryos by miRNeasy (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA (10 μg) was loaded on a 17 % TBE–polyacrylamide gel with 7 M

urea and run in 0.5× TBE (prepared from 10× TBE: 54 g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA, pH 8, per 0.5 l). Samples to load were prepared as follows. Two volumes of formamide were added to 1 volume of 5× loading dye (recipe for 10 ml: 5.75 ml 87 % glycerol, 0.5 ml 1 M Tris, pH 7.7, 0.1 ml 0.5 M EDTA, pH 8, 3.65 ml H_2O, bromophenol blue). To each sample was added 1.5 volumes of the resulting dye and the sample was boiled at 95 °C for 4 min. Polyacrylamide gels were run at 180 V at 4 °C in 0.5× TBE, stained with ethidium bromide in 0.5× TBE, and destained in 0.5× TBE. The gels were transferred at 4 °C at 90 mA on Hybond-N^+ nylon membrane and the membrane was UV crosslinked. Primer (10 pg) was end-labeled by incubation with 1 ml polynucleotide kinase T4 (NEBiolabs, Boston, MA) in the presence of 50 µCi of [^{32}P]α-ATP (3,000 Ci/mmol; Perkin-Elmer) for 1 h at 37 °C. The denatured labeled primer was added to the membrane, preincubated with UltraHyb buffer (Ambion, Carlsbad, CA) according to the manufacturer's instructions. The membrane was incubated overnight at 38 °C and washed at 50 °C with 2× SSC/0.2 % SDS (two washes 20 min each). The membrane was exposed to film at −80 °C.

Western blot analysis

Mouse tissues, mouse embryonic fibroblasts (MEFs) and HEK293 cells were analyzed by Western blotting. Mouse tissues and HEK293 cells were lysed in RIPA lysis buffer and mechanically homogenized. MEFs were lysed in lysis buffer (20 mM HEPES, pH 7.5, 100 mM KCl, 50 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 0.5 mM $NaVO_4$, 1 % Triton X-100, protease inhibitor cocktail) or RIPA buffer. The proteins of clarified and quantified extracts were separated by SDS-PAGE and then transferred onto PVDF membranes. The membranes were incubated with primary and secondary antibodies for protein detection.

Quantitative PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After treatment of total RNA with RQ1 RNase-free DNase (Promega. Fitchburg, WI, USA), reverse transcription was performed with M-MLV reverse transcriptase enzyme (Promega) according to the manufacturer's instructions. Reverse-transcribed complementary DNA (100 ng) was amplified with the probe for Gnb2l1 (TaqMan gene expression assay Mm00515010_m1; Applied Biosystems, Foster City, CA, USA) in an ABI PRISM 7900HT sequence detection system. GAPDH was the internal control for gene expression (TaqMan gene expression assay 4352932E; Applied Biosystems). Each sample was analyzed at least in triplicate.

Mouse embryonic fibroblast preparation and protein synthesis

MEFs were isolated from E13.5 embryos as previously described [42] and kept in DMEM complemented with 10 % fetal bovine serum, 2 mM glutamine and 1 % penicillin/streptomycin at 37 °C in an atmosphere containing 9 % CO_2.

MEFs were seeded at subconfluency into six-well plates starved for 4 h. The cells were stimulated with either insulin or phorbol 12-myristate 13-acetate (PMA) at, respectively, 100 and 50 nM, added 5 and 30 min before radioactive pulsing. The cells were metabolically labeled by pulsing for 30 min as previously described [43]. The two-tailed *t* test was used to calculate *p* values (**$p < 0.01$, *$p < 0.05$).

Immunostaining of embryos

Timed pregnancies were obtained as described previously [44]. Cryostat-cut serial sections of thickness 10 µm were analyzed by immunofluorescence as previously described [45] utilizing an anti-mouse IgM. Serial sections from all embryos derived from a pregnant mouse were evaluated by RACK1 immunostaining and Hoechst counterstaining.

Polysomal profiles

Whole livers from 16-day-old mice were homogenized by five strokes in a Dounce tissue grinder containing 500 µl of 50 mM Tris-HCl, pH 7.4, 240 mM KCl, 10 mM $MgSO_4$, 5 mM DTT, 250 mM sucrose, 2 % Triton X-100, 90 mg/ml cycloheximide, 30 U/ml RNasin (Promega, Fitchburg, WI) and protease inhibitor cocktail (Sigma, St Louis, MO). After clarification, 7.5 U heparin was added and the equivalent of 7 absorbance units at 254 nm were layered onto a 15–50 % sucrose gradient (gradient buffer: 25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$ and 200 U heparin). Gradients were centrifuged at 4 °C in a SW41Ti Beckman rotor for 3.5 h at 187,813 *g* as described previously [46]. The absorbance at 254 nm was recorded using BioLogic LP Software (Bio-Rad, Hercules, CA). Fractions were collected and further analyzed.

RNAi in HEK293 cells

Hek293 cells were transfected at 40 % confluence as previously described [39], and lysed after 48 h for western blot analysis.

Results

RACK1 locus integrity is necessary for early stages of mouse embryonic development

Originally, we sought to conditionally target the RACK1 locus, but targeted ES were never viable to germline transmission. After multiple attempts, we obtained two viable ES lines with a 142-nt deletion of the first loxP site in intron 2 and a floxed Neo cassette in intron 3 of RACK1 (Fig. 1a). This line resulted in a hypomorphic RACK1 allele (see below). We derived mice from these lines and bred them to heterozygosity. We named the heterozygous mice ΔF/+. Intercrosses of ΔF/+ did not lead to ΔF/ΔF pups after birth (287 pups, observed ΔF/ΔF none, expected 72; $p \leq 0.0001$). ΔF/+ and +/+ mice were born with the expected mendelian frequency. To understand at which stage development of ΔF/ΔF was defective, embryos were analyzed after implantation. At E9.5 we observed one out of four embryos resorbed, all of them being ΔF/ΔF mice (Fig. 1b, c). To examine the general appearance of earlier embryos, E6.5 implantation sites were analyzed by histology (Fig. 1d). At this age ΔF/ΔF embryos had disorganized implanted blastocysts with vacuolar areas (Figs. 1d, right; 2b, right), whereas all wild-type mice and ΔF/+ mice had compact trophectoderm and embryonic ectoderm (Figs. 1d, left; 2a, left). Levels of RACK1 in the vacuolated embryos were very low, as shown by immunofluorescence, compared to normal embryos (Fig. 2). We conclude that the ΔF/ΔF phenotype is lethal at gastrulation. Deletion of the neomycin cassette by Flpe recombinase rescued the phenotype (data not shown), suggesting that lethality is caused by interference of the neomycin cassette on RACK1 expression, as was demonstrated (see below).

ΔF/+ mice have a "belly spot" phenotype and a transient growth deficit

ΔF/+ mice showed a penetrant phenotype consistently as a white belly spot, and hypopigmented tail and paws (Fig. 3a). Remarkably, this phenotype is identical to the one of the natural mutant for the ribosomal proteins rpL24 (*Bst*) [47]. In contrast to the *Bst* mutant, we did not observe mild skeletal abnormalities [47]. Adult ΔF/+ mice, with the exception of the pigmentation deficit, were normal and had normal weights (Fig. 3b). Analysis of gene expression in adult mice showed no alteration in RACK1 protein levels (Fig. 3c).

The presence of a phenotype related to melanocyte migration, which occurs developmentally, without gross alterations in RACK1 levels of adult mice, prompted us to study other development-related changes. No gross alterations in embryonic development of ΔF/+ mice were found. However, we found a transient difference in the weight and length of young females, which was not seen in male littermates (Fig. 4a, b). We used this developmental window to analyze the expression of the RACK1 locus, at both the protein and mRNA levels in different tissues. We found a reduction in RACK1 protein in both the brain and in liver (Fig. 4c). Consistently, a downregulation of RACK1 mRNA was observed in the brain (Fig. 4d) and liver (Fig. 4e), but not in other tissues (not shown). None of

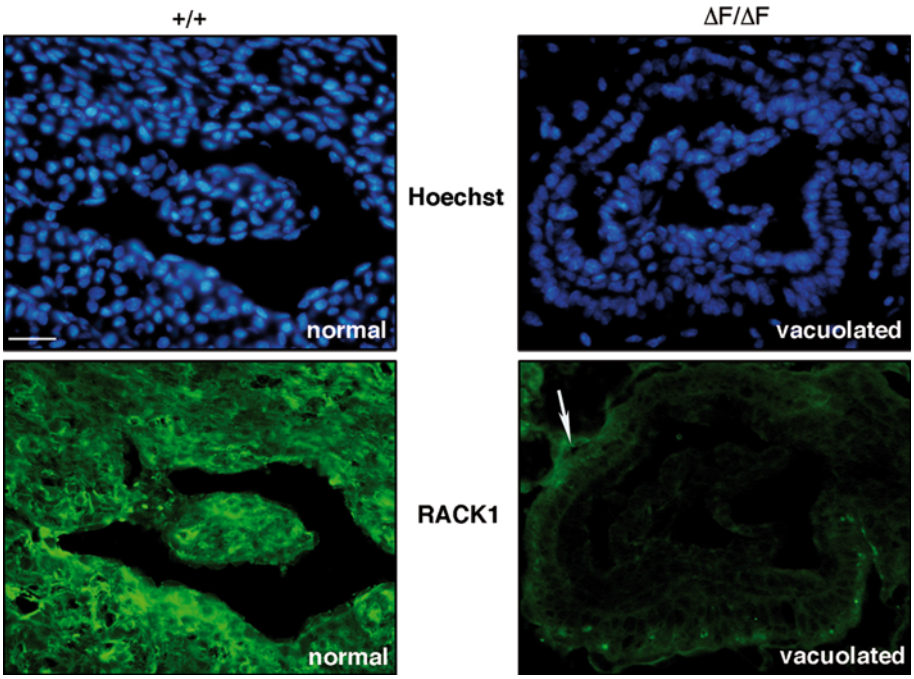

Fig. 2 RACK1 is reduced in the presumptive ΔF/ΔF mouse embryo. Immunofluorescence analysis of E6.5 embryos, showing nuclear staining (Hoechst, *blue*) and RACK1 staining [13]. RACK1 immunohistochemical staining was virtually absent in 25 % of E6.5 embryos (*bottom right*). The residual fluorescence (*arrow*) is due to autofluorescence, visible also in the red channel (not shown and [64]). *Scale bar* 50 μm

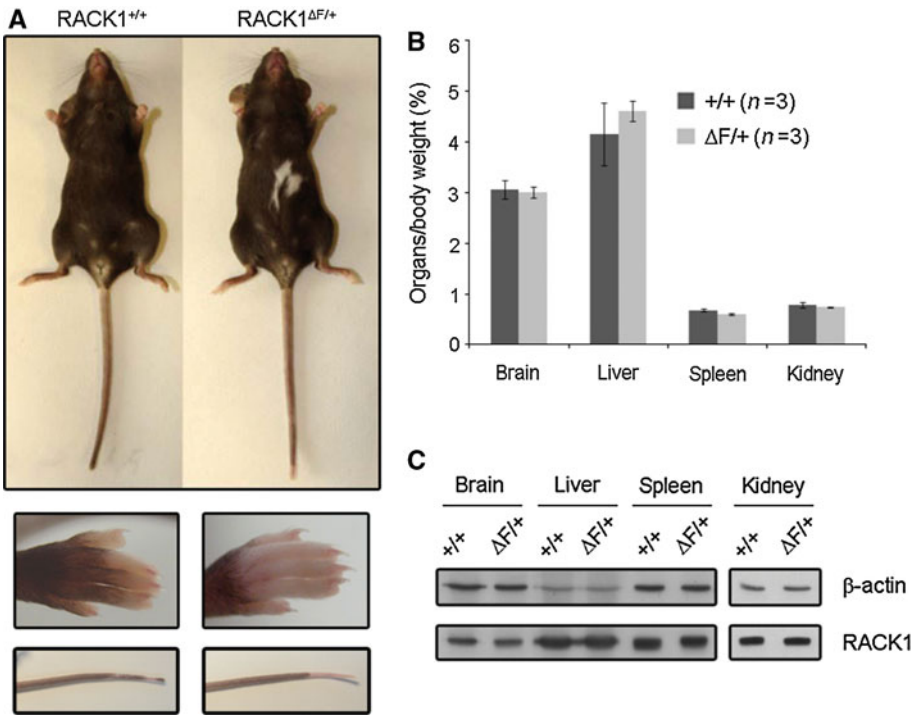

Fig. 3 Belly spot phenotype in RACK1 mutant mice. **a** White belly spot and hypopigmented paws and tail tips in ΔF/+ adult mice. **b** Organ size is normal in ΔF/+ adults. **c** Representative RACK1 protein expression in brain, liver, spleen and kidney

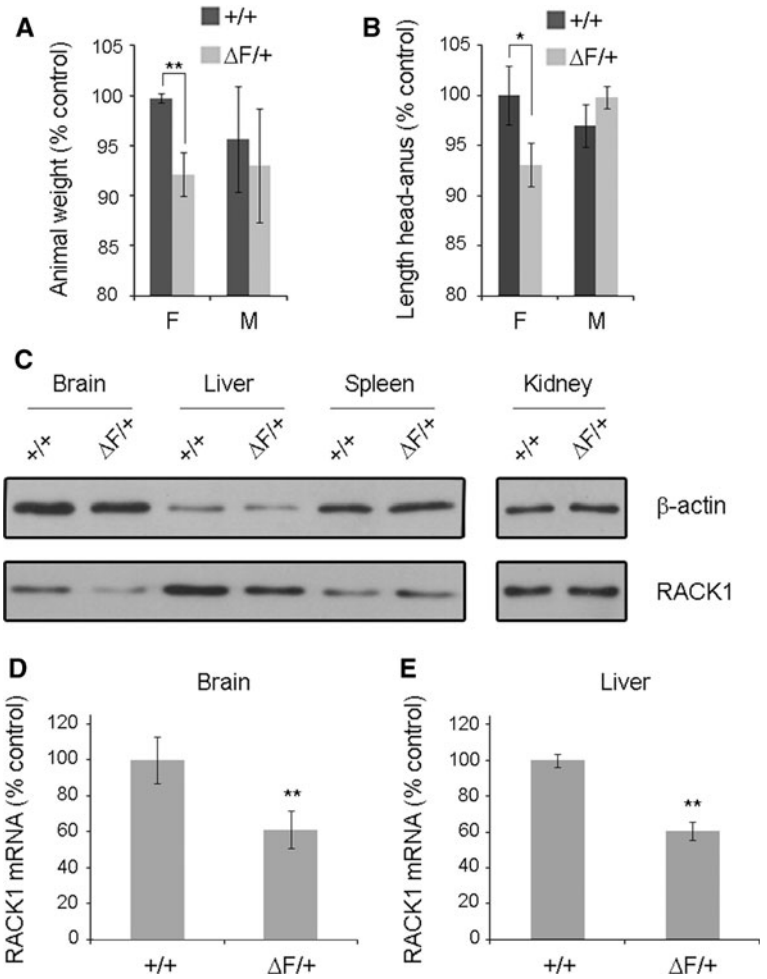

Fig. 4 RACK1 mutant young females have delayed growth and reduced RACK1 protein levels. **a**, **b** Weight and head-to-anus length of 11-day-old pups (*F* females, *M* males; $n = 17$ per genotype; $*p \leq 0.05$, $**p \leq 0.01$). **c** Representative protein expression in brain, liver, spleen and kidney. **d**, **e** Quantitative PCR for RACK1 mRNA levels in brain and liver of littermates ($n = 5$ per genotype)

the flanking genes in the same locus showed altered expression, indicating that the phenotype is due to hypomorphism of the RACK1 gene. These findings establish that in mammalian cells, in contrast to unicellular organisms, proper expression of RACK1 is necessary for tissue growth. The RACK1 gene contains three snoRNAs, U95a, U95b and U96 (see Fig. 1a), which are evolutionarily conserved and necessary for the modification of 28S rRNA of the 60S ribosomal subunit. We analyzed snoRNA expression and pre-rRNA processing, both essential for the correct production of ribosomal subunits. In ΔF/+ cells we did not detect differences in the levels of snoRNA U95 and U96 (Fig. 5a) compared to wild-type cells. Ribosomal maturation, 60S and 40S, also appeared normal (Fig. 5b).

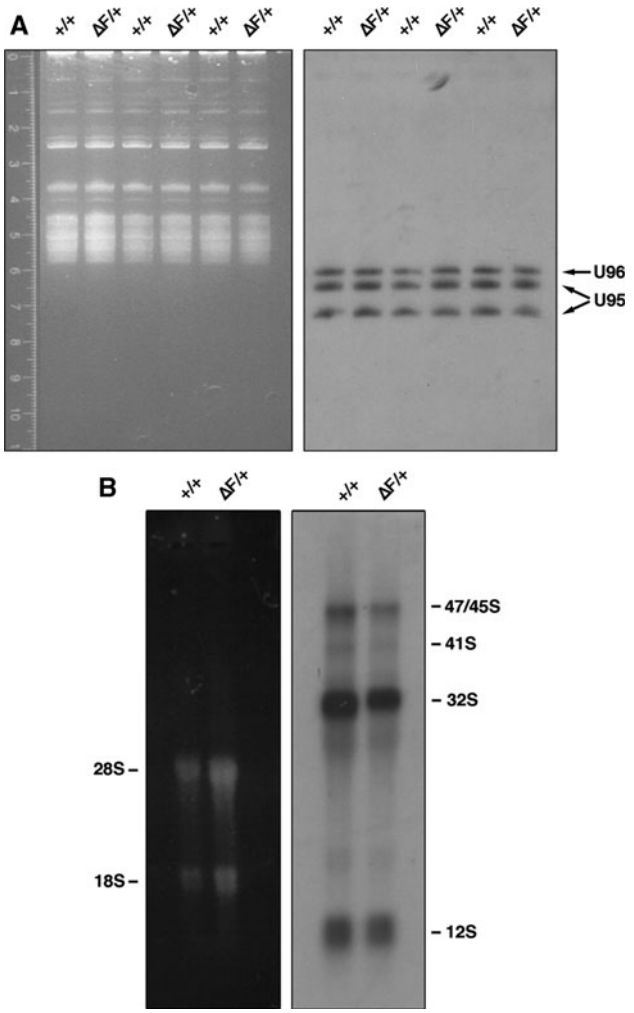

Fig. 5 snoRNA and rRNA production are not affected in mutant mice. **a** Northern blot analysis of snoRNAs in RNA samples from littermates, comparing wild-type and RACK1 ΔF/+ embryos (*left* ethidium bromide staining, *right* hybridization). The probes used are described in Materials and methods. **b** Representative Northern blot analysis for rRNA precursors (*left* ethidium bromide, *right* hybridization)

Protein synthesis is reduced in RACK1-deficient mice

RACK1 is a ribosomal protein. It has been previously demonstrated that RACK1 interacts with eIF6 in vitro, and transduces signaling to the translational machinery by PKC recruitment [39, 48]. We examined protein synthesis status in our model by methionine incorporation and polysomal profiles. Differences between wild-type and ΔF/+ cells were observed both in tissue-specific and stimulation-specific situations. Methionine incorporation was measured in MEFs derived from ΔF/+ mice, and compared to that in matched control. We found that ΔF/+ cells did not respond to stimulation with either insulin or the PKC agonist PMA (Fig. 6a, b, respectively). MEFs derived from ΔF/+ mice, compared to matched controls, had less RACK1 mRNA and protein (Fig. 6c, d). Livers from ΔF/+ mice had a higher 80S/polysome ratio (Fig. 7a, b) than those from wild-type littermates. Blotting of fractions with anti-eIF6 did not show consistent changes in its distribution. Consistent with our previous findings showing that modulation of translation by RACK1 is independent by the mTOR pathway [39], analysis of mTORC1/2 signaling ruled out its involvement in the altered status of ΔF/+ protein synthesis (Fig. 7c).

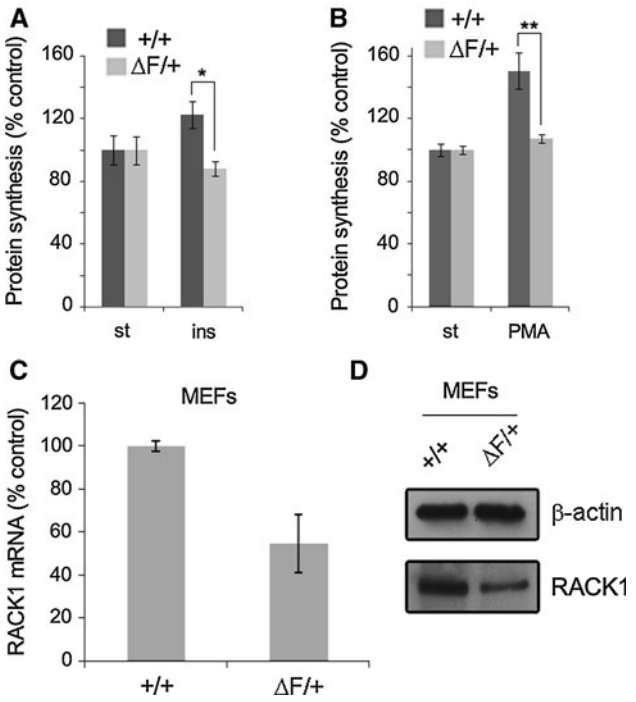

Fig. 6 RACK1 mutant MEFs have reduced PKC-stimulated translation. Global protein synthesis was measured by [^{35}S]methionine incorporation. Starved (*st*) MEFs were treated with (**a**) insulin (*ins*) and (**b**) PMA as described in Materials and methods ($n \geq 3$ per genotype; $*p \leq 0.05$, $**p \leq 0.01$). **c**, **d** RACK1 mRNA and protein levels of the analyzed MEFs

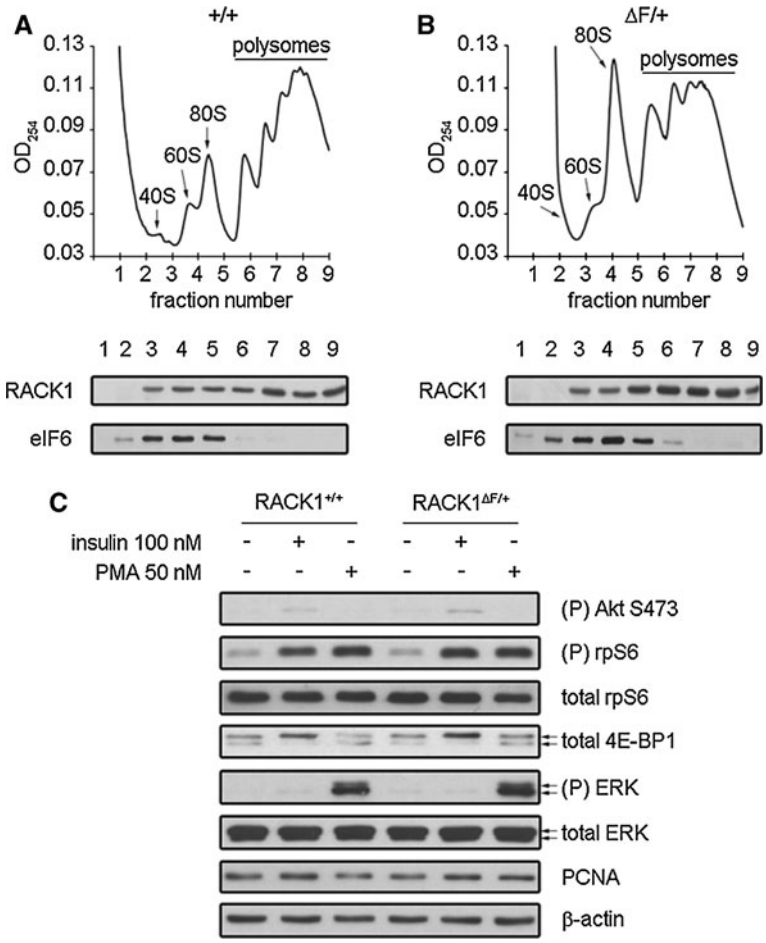

Fig. 7 RACK1 mutant tissues show mild 80S accumulation. **a**, **b** Polysome profiles in 16-day-old females (+/+ versus ΔF/+ littermates). **c** Western blot analysis of mTORC1/2 pathway under the same conditions as in **a** and **b**

p53 depletion partly rescues the phenotype of RACK1-deficient mice

Ribosomal depletion is associated with induction of p53, and consistently the phenotype of most ribosomal deficiencies is rescued by p53 depletion [49]. We could not detect p53 induction in ΔF/+ mice, likely because RACK1 reduction is too small to raise p53 levels in a detectable extent (not shown). However, induction of p53 was observed by siRNA-mediated RACK1 downregulation in 293 cells (Fig. 8a), and p53 depletion reduced the belly spot phenotype of ΔF/+, even though it did not abrogate it (Fig. 8b). However, p53 depletion was not sufficient to rescue the lethality of the ΔF/ΔF genotype. Finally, the survival of $p53^{-/-}$ mice was not significantly affected by the ΔF/+ genotype (Fig. 8c). We conclude that RACK1 depletion induces ribosomal stress, and that rescue of ribosomal stress may slightly ameliorate but not abolish the effects of RACK1 deficiency.

Discussion

This is the first description of a mammalian model in which RACK1 has been depleted. It allows a set of solid conclusions on the function of RACK1 in mammals, and the comprehension of some aspects of ribosomal biology. It is useful to summarize the main phenotype of RACK1 depletion: lethality at gastrulation; a white belly spot at heterozygosis; induction of p53 and amelioration, but not elimination, of the deficit due to RACK1 depletion; and reduction in PMA- and insulin-stimulated translation. Taken together the findings demonstrate that a prominent function of RACK1 is to act as an adaptor converging signaling to the ribosomal machinery, but retaining some features common among ribosomal proteins. We discuss here some technical caveats and draw attention to some aspects deserving future work.

The RACK1 locus has never been targeted in mammals. In spite of this, knockouts exist in unicellular organisms and many studies have been performed using a RNAi-based approach. Conventional knockouts of RACK1 have not been feasible, due to the reduced ability of ES to go germline (unpublished observations). In addition, all our efforts to obtain a conditional mouse have been impaired by recombination at the locus. Furthermore, the presence of snoRNAs genes in the RACK1 locus limits the possibility of selectively knocking down the RACK1 gene. These difficulties coupled to a limit in our current technology may

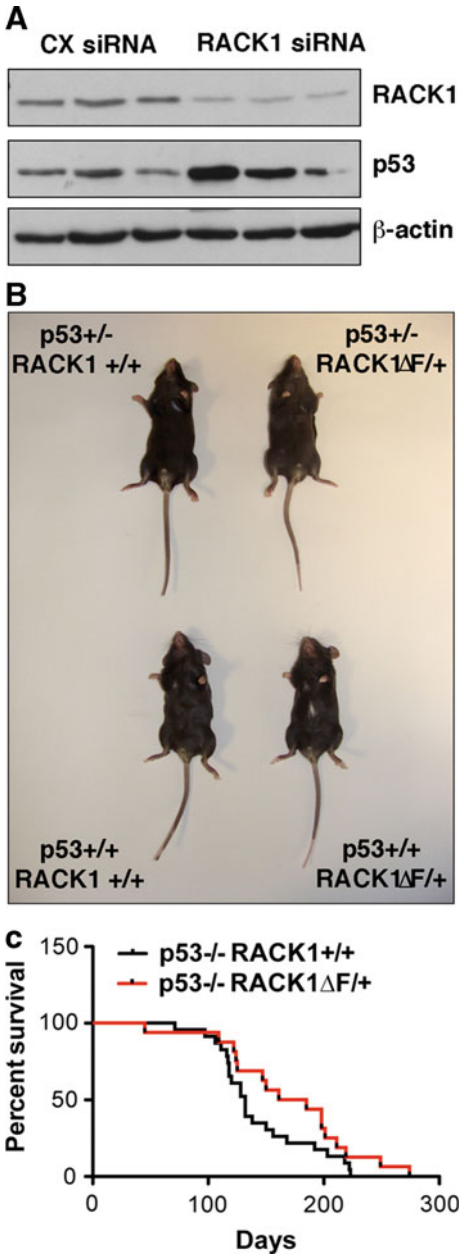

Fig. 8 p53 depletion slightly ameliorates the belly spot phenotype. **a** Induction of p53 stress by RACK1 depletion. 293 cells were transfected with siRNA in triplicate and p53 levels assayed by western blotting after 48 h. **b** Reduction in the pigmentation defect by p53 ablation. **c** Survival (Kaplan-Meier plot) of $p53^{-/-}$ mice in RACK1 wild-type versus mutant background ($n = 16$ $p53^{-/-}$ $RACK1^{+/+}$ males; $n = 23$ $p53^{-/-}$ $RACK1^{\Delta F/+}$ males)

explain our failure. An alternative is that ribosomal proteins, and in particular RACK1, are highly expressed, and the intronic manipulation we attempted in the locus was disfavored because of its negative effect on gene expression. In this perspective, obtaining a hypomorphic RACK1 mouse has been useful for understanding RACK1 function, and may provide a genetic basis for reconstitution experiments employing RACK1 mutants.

The phenotype of ΔF/+ mice could be possibly due to either RACK1 depletion or snoRNA depletion or both. Two lines of evidence suggest that the first interpretation is the more likely. First, we did not find evidence of snoRNA depletion in several tissues and ages; although this fact is superficially surprising, it should be noted that the rate of conversion of a RNA precursor to a mature snoRNA under normal conditions is totally unknown. Several (tens) snoRNA genes are processed by the same essential machinery and thus it is simply likely that a little depletion of the RNA precursor is not rate-limiting for the production of a mature snoRNA [50]. Second, at least in yeast cells, the mild phenotype of the RACK1 ortholog mutation is totally due to the depletion of the RACK1 protein and not to the hosted snoRNA [28]. Obviously, the situation may be totally different in the ΔF/ΔF background where lethality may be due to a combination of both RACK1 depletion and the loss of snoRNAs; reconstitution experiments on this background will allow the definition of the problem.

RACK1 literature is complex (for a recent review see [51]). Studies on unicellular models have shown that RACK1 orthologs are involved in ribosomal biology, and structural reconstructions of the 40S subunit locate RACK1 precisely on the small subunit surface. The phenotypic result of RACK1 homolog depletion in unicellular organisms is normal growth under rich conditions, accompanied by a variety of effects under stress [31, 50, 52–55]. Thus, in spite of being classified as a ribosomal protein by both structural and biochemical work [56], ribosomal RACK1 is not strictly essential. In apparent contrast, the literature on mammalian RACK1 deals mainly with its function as a scaffold protein for signaling molecules without taking into account its ribosomal localization. Importantly, studies on RACK1 in mammals have been greatly limited by the analysis of simple immortalized cell lines. In mammals, lethality is observed at gastrulation. In general, this result is compatible with a non-essential function of RACK1 in the context of ribosomal biology; scanty data exist, but null mutants for ribosomal proteins are lethal before implantation when maternal proteins are rapidly depleted upon dilution by proliferation [57]. Lethality at gastrulation is therefore compatible with a regulatory role of RACK1 on ribosomes, rather than structural.

Taken together our findings indicate that the primary function of RACK1 is related to the efficient use of ribosomes. This said, the effect of RACK1 depletion on metabolic labeling is evident upon stimulation, where it phenocopies eIF6 depletion [42], but seems very limited by analysis of polysomal profiles, which shows a modest increase in 80S formation. This is not surprising because 80S formation is the most sensitive index of impaired initiation, independent of the underlying causes, in vivo. The

partial overlap with eIF6 phenotype is not unexpected, considering also the role of eIF6 in lymphomagenesis in PKC beta-rich tissues [43] and the recently described role of RACK1 as a PKC adaptor for other IFs involved in chemoresistance and tumorigenesis [38]. Thus, some RACK1 ribosomal defects may be due to impaired stimulation of the translational machinery. Alternatively, depletion of a ribosomal protein generates a rapid induction of p53 through the ribosomal stress pathway, an event that is considered pivotal in ribopathies [58]. Indeed, we observe that RACK1 depletion in cultured cells generates an increase in p53 levels, but in vivo we did not observe induction of p53, likely due to its scattered induction in haploinsufficient cells and their possible elimination; more importantly, however, we showed that in vivo p53 depletion ameliorates the melanocyte-specific RACK1 phenotype. Another intriguing line of evidence on the primary role of RACK1 in ribosomal function, is that the phenotype of RACK1-depleted mouse closely resembles rpL24 hypomorphic mutant. rpL24 is a protein belonging to the large ribosomal subunit located near the eIF6 binding site. RACK1 binds eIF6 in a curiously similar manner to rpL24 hypomorphic mutants. RACK1 hypomorphic mice have a white belly spot that can be reverted by p53 depletion [59]. Taken together these findings show that RACK1 function is associated with ribosomes, and indicate intriguingly that alterations in ribosomal efficiency hit specifically the development of neural crest-derived melanocytes.

Referring to the melanocyte phenotype, it is intriguing to note that a role for RACK1 in PKC activation and migration of melanoma cells was predicted by in vitro studies [9, 10, 60]. Another interesting possibility is that disruption of RACK1-PDE4D5 (the cAMP-degrading phosphodiesterase) complex on the ribosome could affect the migration or melanin production of melanocytes. Inhibiting the cAMP-activated PKA signaling pathway decreases melanogenic gene expression [61]. It is possible that increased free PDE4D5 in RACK1-deficient melanocytes allows this phosphodiesterase to penetrate to other sites in the cell where it lowers cAMP levels so as to reduce PKA activity and decrease melanogenic gene expression. Indeed, it has been shown that upon RACK1 downregulation by RNAi the amount of PDE4D5 associated with the signaling scaffold beta-arrestin increases [62]. Since the melanocortin 1 receptor (MC1R), which constitutes a key regulator of melanism, is coupled to cAMP synthesis, this would provide a potential route whereby increased PDE4D5 association with MC1R-sequestered beta-arrestin would lower cAMP levels, PKA activation and melanin production (see for example reference [63]). It will be exciting to verify if melanoma cells show other particular features of the translational machinery and if PDE4D5 is involved in these processes.

Last, the delayed lethality of RACK1 mutants suggests that RACK1 may act on the efficiency of the translational apparatus and its integration with extracellular stimuli rather than with ribosome production. Consistent with this, we did not see anomalies in ribosomal production. However, we observed a reduction in PKC-stimulated translation. This effect phenocopies the outcome of eIF6 haploinsufficiency [42], confirming that the two molecules are involved in the same process on the translation machinery. However, their action is not linked to mTORC1/2 pathways, as we found here and in previous studies [39]. Recent work on this line has suggested a more general role of the RACK1 platform in the regulation of initiation of translation, always depending on PKC activation [38]. On the basis of our findings, we think that several of the effects observed in previous studies on the role of extraribosomal RACK1 should be reevaluated in the context of translational efficiency [6].

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