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Skeletal muscle Rac1 mediates exercise training adaptations towards muscle glycogen resynthesis and protein synthesis☆

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ABSTRACT

Long-term exercise training elicits tremendous health benefits; however, the molecular understanding is incomplete and identifying therapeutic targets has been challenging. Rho GTPases are among the most regulated groups of proteins after exercise in human skeletal muscle, yet, unexplored candidates for mediating the effects of exercise training.

We found that the Rho GTPase Rac1 was activated acutely after multiple exercise modalities in human skeletal muscle. Loss of Rac1 specifically in muscle attenuated contraction-induced muscle protein synthesis, diminished improvements in running capacity, and prevented muscle hypertrophy after exercise training in mice. Additionally, *Ncf1** mice revealed that Rac1 regulated glycogen resynthesis via a NOX2-dependent mechanism. Molecularly, Rac1 was required for contraction-induced p38MAPK signaling towards HSP27, MNK1, and CREB phosphorylation. In vivo muscle-targeted overexpression of a hyperactive Rac1-mutant elevated reactive oxidant species production during exercise but did not affect muscle mass. Using mass spectrometry-based proteomics, we found that loss or gain of Rac1 muscle protein affected pathways related to cytoskeleton organization, muscle adaptation, and large ribosomal subunits. Thus, skeletal muscle Rac1 mediates both molecular and functional adaptation to exercise training.

1. Introduction

Long-term exercise training offers substantial health benefits, but its molecular mechanisms are poorly understood, making therapeutic target identification challenging. The Rho-family GTPases are a group of proteins vital for the cell's ability to integrate signaling networks and adapt in response to external cues [1]. Recent advances in mass spectrometry-based proteomics and phosphoproteomics have revealed that Rho GTPases are amongst the most regulated proteins in response to exercise [2,3]. In skeletal muscle, exercise and muscle contraction activate the Rho GTPase Rac1 [4], which controls intramyocellular glucose uptake [5,6] and NOX2-dependent reactive oxygen species (ROS) production [7,8].

Regular exercise training leads to multiple health benefits and can prevent and/or treat lifestyle- and age-related diseases [9]. The beneficial effect of regular exercise is mediated through signaling networks

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that result in molecular adaptations, particularly within skeletal muscle, leading to increased muscle mass and function [10–12]. Where acute exercise can lead to the posttranslational regulation of >4000 phosphosites [2], long-term exercise training leads to a marked change in protein content in skeletal muscle [13,14]. Targeting the mechanisms underlying exercise training adaptations could potentially improve health [15,16]. However, the advancement of therapeutic potential is limited by the incomplete understanding of the mechanisms that improve muscle health through exercise [2]. Rho GTPases, like Rac1, are key for integrating signaling networks, adapting to external cues, and regulating metabolism, making them promising candidates for the benefits of physical activity. Whether Rac1 drives long-term exercise adaptations, and thus could be a therapeutic target, remains unknown.

Here, we hypothesized that skeletal muscle Rac1 mediates exercise-induced adaptations in muscle. Using gain- and loss-of-function mouse models in combination with human muscle biopsies, we find that Rac1 was necessary for the improvement in exercise performance and muscle hypertrophy. This was likely explained by the necessity of Rac1 to regulate post-exercise protein synthesis, glycogen resynthesis and intramyocellular signaling. Here, we describe a novel Rac1-dependent regulation of a p38MAPK-HSP27-CREB signaling cascade. Ultimately, skeletal muscle Rac1 was required for optimal adaptations to exercise training.

2. Methods

2.1. Human experiments

Acute exercise study: These samples were acquired from a previous study. Please see Ref. [2] for further information.

One-legged kicking training study: These samples were acquired from a previous study. Please see Ref. [17] for further information.

2.2. Animals

Inducible muscle-specific Rac1 knockout mice (Rac1 imKO): These mice have been described elsewhere [5,18]. Female, age-matched, 12–16 weeks (License: 2016-15-0201-01043). Briefly, the KO was introduced by adding doxycycline to the water (1g/1L) for 3 consecutive weeks. All mice received doxycycline, including control mice. Following the doxycycline treatment, a 3-week washout period was applied before the start of the intervention to avoid potential off-target effect of the treatment. For the exercise training study (12 weeks), all mice were single-housed and received two weeks of doxycycline after six weeks of voluntary wheel running to avoid potential re-expression of endogenous Rac1 expression. *For NOX2 studies*: Female B10.Q control and B10.Q. p47phox mutated (*Ncf1**) mice contain a point mutation in exon 8, describedin a previous study [19]. Age-matched non-littermate control and *Ncf1** mice between 12 and 16 weeks of age were used for experiments (license: 2017-15-0201-01311). *For rAAV-studies:* 8 weeks old female C57BL/6JBomTac mice were used (Taconic, Denmark). All animals were maintained on a 12:12-h light-dark cycle at 22 °C ± 2 °C and received a standard rodent diet (Altromin, no. 1324; Chr. Pedersen, Ringsted, Denmark) and water *ad libitum*. All experiments were approved by the Danish Animal Experimental Inspectorate.

2.2.1. Recombinant adeno associated virus (rAAV) – the expression of a constitutive activated Rac1

Recombinant adeno-associated serotype-6 pseudotyped viral vectors (rAAV6) was created as previously described [20] and constructed to express a constitutively activated H-Rac1 mutant (Rac1:G12V). An intramuscular injection of the rAAV Rac1:G12V was injected in gastrocnemius (1E+10^{10}vg) and tibialis anterior (1E+10^{10}vg) muscle in one of the legs two weeks (acute exercise) or eight weeks (for long-term overexpression) prior to the terminal experiment. The contralateral leg was injected with an empty vector (rAAV:MCS), creating a study-design where the mice functioned as their own control (also described elsewhere [20]).

Body composition: Total fat and lean body mass were measured by nuclear magnetic resonance using an EchoMRI™ (USA).

Treadmill exercise protocols: Running capacity test: All mice were familiarized to the treadmill in the weeks prior to any running protocols. This consisted of 10 min of running at 0.16 m/s (0° incline) on three separate days. On a 15° incline, the running capacity test started with a 5 min warm-up at 0.16 m/s before gradually increasing the speed by 0.02 m/s per minute until exhaustion.

In situ muscle contraction protocol: This protocol was developed and described elsewhere [21]. In short, mice were fasted 2 h prior to *in situ* contraction (nine sets of contraction bouts of 1 min in duration (3 s of 10 V stimulations of pulses with a duration of 0.1 ms at a frequency of 100 Hz, repeated every 10 s), with a 30 s break between bouts). 0.2 mm acupuncture needles (TAI-CHI; B.C. Medical, Nykoebing SJ, Denmark) were inserted into the proximal and distal parts of the m. quadriceps femoris muscle. The mice were kept anaesthetized by inhalation of 2 % isoflurane during the entire procedure. For the acute contraction experiment (Fig. 2A), the mice were sacrificed immediately after the cessation of the contractions. Four hours after the contraction, the mice were anaesthetized using 2 % isoflurane and given a retro-orbital injection of 21.75 mg kg−1 body weight puromycin (Calbiochem, San Diego, CA, USA) in saline to measure muscle protein synthesis [22].

Glycogen measurements: Skeletal muscle glycogen was measured using the *in vitro* hexokinase method as previously described [5].

Muscle preparation and immunoblotting: Muscle tissue was pulverized in liquid nitrogen before being homogenized in a modified GSK3-buffer (10 % glycerol, 1 % NP-40, 20 mM sodium pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 20 mM β-glycerophosphate, 10 mM NaF, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 8.0), 2 mM Na3VO4, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 3 mM benzamidine) as described previously [5]. The homogenization was performed using a Tissue-Lyser II with stainless steel grinding balls (2 × 30s at 30 Hz) (Qiagen, USA). After 30 min of end-over-end at 5 °C, the samples were centrifuged at 9.500 *g* for 20 min at 4 °C. The supernatant was collected discarding the remaining pellet.

Lysate protein concentration was determined using the bicinchoninic acid method. Bovine serum albumin (BSA) was used as a standard (Pierce). Immunoblotting of relevant phosphorylation-sites of proteins as well as total proteins was performed by standard immunoblotting techniques using commercially available equipment (BioRad Laboratories, USA). After the transfer of protein to polyvinylidene difluoride membranes, these were subsequently blocked for 5 min in TBS-Tween 20 containing either 2 % skim milk or 3 % BSA at room temperature. The membranes were incubated overnight with primary antibodies at 4 °C. The primary antibodies used can be seen in Table 1. On the following day, the primary antibody was removed, and horseradish peroxidase-conjugated secondary antibody was applied and incubated for 45 min at room temperature. Imaging and visualization of bands were performed using Bio-Rad ChemiDocTM MP Imaging System and enhanced chemiluminescence (ECL^{+}; Amersham Biosciences). For the effect of voluntary wheel running exercise training on Rac1 protein content in skeletal muscle (Fig. 5B), the control mice from the Rac1 imKO exercise training cohort (12 weeks intervention, Fig EV 3A), and a separate cohort (female, 6 weeks intervention) of WT mice were combined. The different cohorts are indicated with a change in color of the individual values.

RNA extraction and quantitative real-time PCR: Total RNA was extracted from 15 to 20 mg quadriceps using TRIzol (Qiagen, USA). One mL TRIzol was added to all samples and homogenized with stainless steel grinding balls for 2 min at 30 Hz using a TissueLyser II bead mill (Qiagen, USA). Then, 200 μL chloroform (Sigma, USA) was added to each sample and tubes were inverted 5 times and incubated at room temperature (RT) for 2–3 min. Following incubation, the samples were centrifuged at 8.000 RPM for 20 min at 4 °C. The aqueous phase

Table 1
Overview of antibodies and dilutions.

Antibody	Company	Number #	Dilution
Rac1	BD biosciences	#610651	1:1000 in 2 % skim milk
FLAG	Sigma-Aldrich	F1804	1:1000 in 2 % skim milk
PAK1	Cell Signaling Technology	#2602	1:1000 in 2 % skim milk
PAK2	Cell Signaling Technology	#2608	1:1000 in 2 % skim milk
phospho(p)PAK1 thr(T) 423/pPAK2 T402	Cell Signaling Technology	#2601	1:1000 in 3 % BSA
RhoGDIa	Cell Signaling Technology	#2564	1:1000 in 2 % skim milk
RhoA	Cell Signaling Technology	#2117	1:1000 in 2 % skim milk
CDC42	Cell Signaling Technology	#2462	1:1000 in 3 % BSA
p-P38 MAPK T180/Y182	Cell Signaling Technology	#9211	1:1000 in 3 % BSA
P38 MAPK	Cell Signaling Technology	#9212	1:1000 in 2 % skim milk
p-ERK1/2 MAPK T202/Y204	Cell Signaling Technology	#9101	1:1000 in 2 % skim milk
ERK1/2 MAPK	Cell Signaling Technology	#9102	1:1000 in 2 % skim milk
pHSP27 S82	Cell Signaling Technology	#2406	1:1000 in 2 % skim milk
pMNK1 T197/202	Cell Signaling Technology	#2111	1:1000 in 2 % skim milk
MNK1	Cell Signaling Technology	#2195	1:1000 in 2 % skim milk
pCREB S133	Cell Signaling Technology	#9198	1:1000 in 3 % BSA
Puromycin	Sigma-Aldrich	MABE343	1:1000 in 2 % skim milk
p-p70S6K T389	Cell Signaling Technology	#9205	1:1000 in 2 % skim milk
p70S6K	Cell Signaling Technology	#2708	1:1000 in 2 % skim milk
p-rS6 S235/236	Cell Signaling Technology	#2211	1:1000 in 2 % skim milk
rS6	Cell Signaling Technology	#2217	1:1000 in 2 % skim milk
p-4EBP1 T37/46	Cell Signaling Technology	#9459	1:1000 in 3 % BSA
4EBP1	Cell Signaling Technology	#9644	1:1000 in 2 % skim milk
$gp91^{phox}$	BD Biosciences	611414	1:1000 in 2 % skim milk

(400–500 μL) was added to 300 μL of pre-cooled isopropanol/70 % ethanol (Sigma, USA), and immediately mixed via pipetting up and down 7–8 times. All samples were treated with 80 μL DNase I Incubation Mix (Qiagen, USA) and incubated at RT for 15 min. RNA was then further purified following the manufacturer's protocol (Qiagen, USA). Finally, RNA was eluted by adding 30 μL RNase-free water and centrifuged at 8000 rpm for 2 min at 4 °C. RNA extractions were kept on ice and their concentrations and purity were determined using a NanodropTM 2000/2000c spectrophotometer (Thermo Fisher Scientific).

Complementary (c)DNA was generated by retro-transcription reaction using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems) and diluted to 10 ng/μL with nuclease-free H_2O. mRNA content was determined using real time quantitative PCR (qPCR). Two technical replicates were analyzed per sample. qPCR was performed using the QuantStudio 6 and 7 Flex Real-Time PCR System (Applied Biosystems). The setting of the cycles used was the default for comparative Ct studies. All measurements were normalized to house-keeping mRNAs: *β-actin, sdha* or *Gapdh*. All primer sequences are listed in Table 2.

Total oxidant production: The staining of oxidants was performed with the commercially available 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (#C6827, Thermo Fisher Scientific, USA). Cryo-sections (10 μm) of the embedded TA muscles were cut at −10 °C and applied to microscope glasses. The samples were rinsed with PBS for 5 min before applying H_2DCFDA (concentration). This compound is light-sensitive, and the stainings were performed with minimum light. The samples were incubated overnight at room temperature in complete darkness. The next day, any remaining H_2DCFDA was removed with a pipette before being rinsed 3 × 5 minutes in PBS. The samples were subsequently covered with mounting medium (H-1000, Vector Laboratories) enabling the use of laser microscopy. Confocal laser microscopy (LSM 780 confocal microscope, Zeiss) was used to visualize the oxidation of H_2DCFDA as indication of total oxidants. Laser microscopy was performed blinded for interventions. The fluorescence-intensity was calculated from the average intensity of several different fibers from at least three independent pictures using ImageJ/Fiji [23].

2.2.2. Skeletal muscle proteomics

2.2.2.1. Sample preparation for mass spectrometry. Skeletal muscle samples were lysed using 4 % sodium dodecyl sulfate in 100 mM Tris HCl, pH 8.5. Homogenization was performed with pre-chilled magnetic beads on the BeatBox tissue homogenizer (PreOmics). Homogenate was boiled at 95° on a shaking incubator for 5 min and tip-probe sonicated for 30 s, with 1 s on/off at an amplitude of 50 % (Bioruptor). Following centrifugation at 20.000*g* for 10 min, supernatant was stored in fresh tubes at −20°. Protein content was measured using a DC assay (Thermo Fisher) and an aliquot of 40 μg protein was reduced and alkylated with 40 mM Chloroacetamide (CAA) and 10 mM pH-neutral Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), at 45 °C for 5 min.

Proteins were digested according to Protein aggregation capture protocol [24] on the KingFisher Flex robot (Thermo Fisher Scientific). Briefly, protein-to-bead ratio of 1:4 (MagReSyn Hydroxyl Beads, Resyn

Table 2
Overview of primers.

Gene	Species	Company	Forward sequence	Reverse sequence
HkII	Mouse	TAG Copenhagen	TGATCGCCTGCTTATTCACGG	AACCGCCTAGAAATCTCCAGA
Pdk4	Mouse	TAG Copenhagen	AGGGAGGTCGAGCTGTTCTC	GGAGTGTTCACTAAGCGGTCA
Akt1	Mouse	TAG Copenhagen	ATGAACGACGTAGCCATTGTG	TTGTAGCCAATAAAGGTGCCAT
Angptl4	Mouse	TAG Copenhagen	CATCCTGGGACGAGATGAACT	TGACAAGCGTTACCACAGGC
Tead3	Mouse	TAG Copenhagen	CAACCAGCACAATAGCGTCCA	CTGAAAGCTCTGCTCGATGTC
Tead4	Mouse	TAG Copenhagen	ATGTCGTCTGCACAGATCGTC	TTGCCAAAACCCTGAGATTGC
Smad1	Mouse	TAG Copenhagen	GCGGGTGCAGCTTGAAAATC	GCACTGCTCACCTTCACGA
Nr4a3	Mouse	TAG Copenhagen	AGGATTCACTGATCTCCCCAA	GATGCAGGACAAGTCCATTGC
Sik1	Mouse	TAG Copenhagen	TCATGTCGGAGTTCAGTGCG	ACCTGCGTTTTGGTGACTCG
Ppargc1α	Mouse	TAG Copenhagen	TATGGAGTGACATAGAGTGTGCT	CCACTTCAATCCACCCAGAAAG
Tfam	Mouse	TAG Copenhagen	ATTCCGAAGTGTTTTTCCAGCA	TCTGAAAGTTTTGCATCTGGGT
β-actin	Mouse	TAG Copenhagen	GGTCATCACTATTGGCAACGA	GTCAGCAATGCCTGGGTACA
Sdha	Mouse	TAG Copenhagen	GGAACACTCCAAAAACAGACCT	CCACCACTGGGTATTGAGTAGAA
Gapdh	Mouse	TAG Copenhagen	AGGCCGGTGCTGAGTATGTC	TGCCTGCTTCACCACCTTCT

Biosciences) was prepared in 70 % Acetonitrile. Washing steps were conducted twice with 100 % Acetonitrile and once with 70 % ethanol. Overnight protein digestion was performed with Trypsin and LysC in 100 mM Tris (1:100 and 1:500 enzyme:protein ratio, respectively). The next morning, enzyme activity was quenched with 1 % TFA in two steps.

Digested samples were cleared by centrifugation at 20.000*g* for 10 min and de-salted using Sep-Pak C18 96-well plates (Waters). Peptides were eluted in two steps with 40 % and 60 % Acetonitrile and vacuum-dried. Following re-suspension, peptide concentrations were measured with NanoDrop (Thermo Fisher Scientific) and 200 ng of peptides were loaded on equilibrated Evotips, according to manufacturer's instructions (Evosep).

2.2.2.2. Mass spectrometry. Peptide separation was performed with the Evosep ONE HPLC system on a 15 cm column with a diameter of 150 μM filled with 1.5 μm C18 beads (Pepsep). Chromatographic peptide separation was performed using the default 30 samples per day method. Peptides were injected into a timsTOF Pro 2 (Bruker) via CaptiveSpray source with a 20um emitter. Mass spectrometric data was collected in diaPASEF mode over a *m/z* range of 100–1700. The cycling time was set to 1.8 s and covered an ion mobility range of 1.6–0.6 $1/K_0$. For ion mobility calibration, three Agilent ESI-L Tuning Mix ions were used (622.0289, 922.0097 and 1221.9906 *m/z*). A long gradient method with 16 diaPASEF scans was used, which incorporated two 25 Da windows per ramp and a mass range of 400.0–1201.0 Da with a mobility range of 1.43–0.60 $1/K_0$. The linear decrease in collision energy was set from 59 eV at $1/K_0 = 1.3$–20 eV at $1/K_0 = 0.85$ Vs cm^{-2} and both accumulation time as well as PASEF ramp time were 100 ms.

2.2.2.3. Data processing and bioinformatics. Raw MS files were quantified in Spectronaut Version 18.6 in directDIA + mode with default settings against the reviewed Mouse FASTA obtained from UniProt (January 2024, 17137 entries). Data processing and downstream analysis was performed in R studio (version 4.3.1). Log2 transformed data was median scaled and proteins were filtered for 70 % valid values per group, resulting in 3727 proteins for the Rac1 imKO ET study and 3622 proteins for the rAAV study.

Rac1 imKO study: Differentially expressed proteins were identified by applying a linear model with eBayes smoothening (limma package) including the main effects of genotype (Wildtype, KO) and condition (Exercise trained, sedentary) and their interaction term. In addition, contrasts between the four groups were computed, allowing individual comparisons.

rAAV study: Paired samples t tests were performed.

To assess statistical significance, the Xiao significance score was applied, which combines the fold change and the p-value [13,25]. A Xiao significance score below 0.05 was considered statistically significant.

Gene set enrichment analysis (GSEA) of gene-ontology biological processes (GO-BP), molecular function (GO-MF), and cellular compartments (GO-CC) was performed using the clusterProfiler package. P-values were adjusted with the Benjamini-Hochberg method with the false discovery rate (FDR) set to 5 % and GO terms with more than 70 % similarity were filtered out using the simplify function.

ClueGo: The ClueGo Enrichment analyses [26] were performed as right-sided hypergeometric tests with a Bonferroni FDR <0.05 for the proteins regulated >0.2 fold change, functionally related terms are grouped, and color coded based on overlapping proteins. The most significantly regulated terms in each group are labeled.

Graphics: The graphics shown in the current study were made in ©BioRender (Toronto, Canada) or ©Inkscape.

Statistical analyses: Results are shown as mean +SEM incl. individual variables when applicable. For the human and rAAV-studies, parried students t-test's or two-way repeated measures ANOVA was applied when suitable. For Rac1 mKO studies, two-way or repeated measures ANOVA was applied as indicated by connecting lines. A Sidak post hoc analysis was performed if main effects during ANOVA analysis occurred. A Mann Whitney test was performed instead of a *t*-test, if the samples did not reach normality (Shapiro-Wilk test) after log-transformation. All statistical analyses were performed using GraphPad Prism, version 10 (GraphPad Software, Inc., USA).

3. Results

3.1. Rac1 is activated upon muscle contraction and regulates protein synthesis, glycogen resynthesis, and p38 MAPK signaling post-contraction

To investigate the involvement of Rac1 in the adaptations to exercise training, we first validated that Rac1 signaling is increased acutely by a resistance-type muscle contraction in mouse skeletal muscle. Rac1 activation was inferred by measuring the phosphorylation (p) of PAK1 threonine(T)423, which is a direct downstream target of Rac1 (Fig. 1A) [27–31]. As expected, Rac1 signaling increased acutely after *in situ* contraction, indicated by a 45 % increase in pPAK1 T423 (Fig. 1A). To test the dependency of Rac1 in the adaptations after exercise, we used this *in situ* muscle contraction protocol in inducible skeletal muscle-specific Rac1 KO mice (Rac1 imKO) and littermate control mice, a protocol known to increase intramyocellular signaling toward muscle protein synthesis (puromycin incorporation) in the hours after muscle contraction [21] (Fig. 1B).

The protein synthesis in recovery was lower in Rac1 imKO mice compared to controls, evidenced by decreased puromycin incorporation in the Rac1 imKO muscles 4 h after contraction (Fig. 1C). Additionally, the replenishment of muscle glycogen stores [32] also failed to increase during the recovery from contraction in Rac1 imKO. Specifically, contraction led to 40 % increased muscle glycogen content 4 h after contraction in the control, but not the Rac1 imKO mice (Fig. 1D). These findings indicate an important role of Rac1 in protein and glycogen synthesis in recovery from muscle contraction.

3.2. HSP27, MNK1, and CREB are novel Rac1-dependent targets in contracting muscle

Molecularly, the increase in muscle protein synthesis after contraction is mediated by mTORC1-dependent and independent mechanisms (Fig. 1E) [33–36]. We observed that contraction increased mTORC1 signaling 4 h after contraction towards p70S6K (T389)/rS6 (S235–S236) and 4EBP1 (T37-46) independent of Rac1 (Fig. 1F), suggesting that Rac1 is dispensable for the activation of mTORC1 in the recovery from muscle contraction. Mitogen-activated protein kinases (MAPKs) are another group of proteins that have been implicated in contraction-induced muscle signaling [37]. ERK1/2 MAPK phosphorylation was not affected by Rac1 imKO (Fig. 1G). The phosphorylation of p-p38MAPK T180/Y182 robustly increased 4 h into recovery from *in situ* contraction, irrespective of genotype (Fig. 1H). Yet, we noted that Rac1 mKO mice had 50 % increased total p38MAPK protein content, thus, Rac1 mKO mouse muscle exhibited 50 % reduced p-p38 MAPK T180/Y182 in relation to total p38 MAPK protein content compared to control littermates (Fig. 1H).

To understand the downstream consequences of lower relative contraction-stimulated p38 MAPK phosphorylation, we next investigated suggested p38 MAPK targets. These include HSP27 (Heat-shock protein 27, also known as HSPB1) [38], MNK1 (MAP kinase-interacting serine/threonine-protein kinase 1) [39,40], and CREB (cAMP response element-binding protein) [41]. These proteins are known to be involved in vital cellular processes, including gene transcription, protein translation, and functioning as chaperones. Contraction increased the phosphorylation of HSP27, MNK1, and CREB in the recovery from contraction in control mice (Fig. 1I). In Rac1 imKO muscle, pHSP27 S82 was abolished in recovery from contraction (Fig. 1I). Also, both pMNK1 T197/202 and pCREB S133 were lower in Rac1 imKO compared to

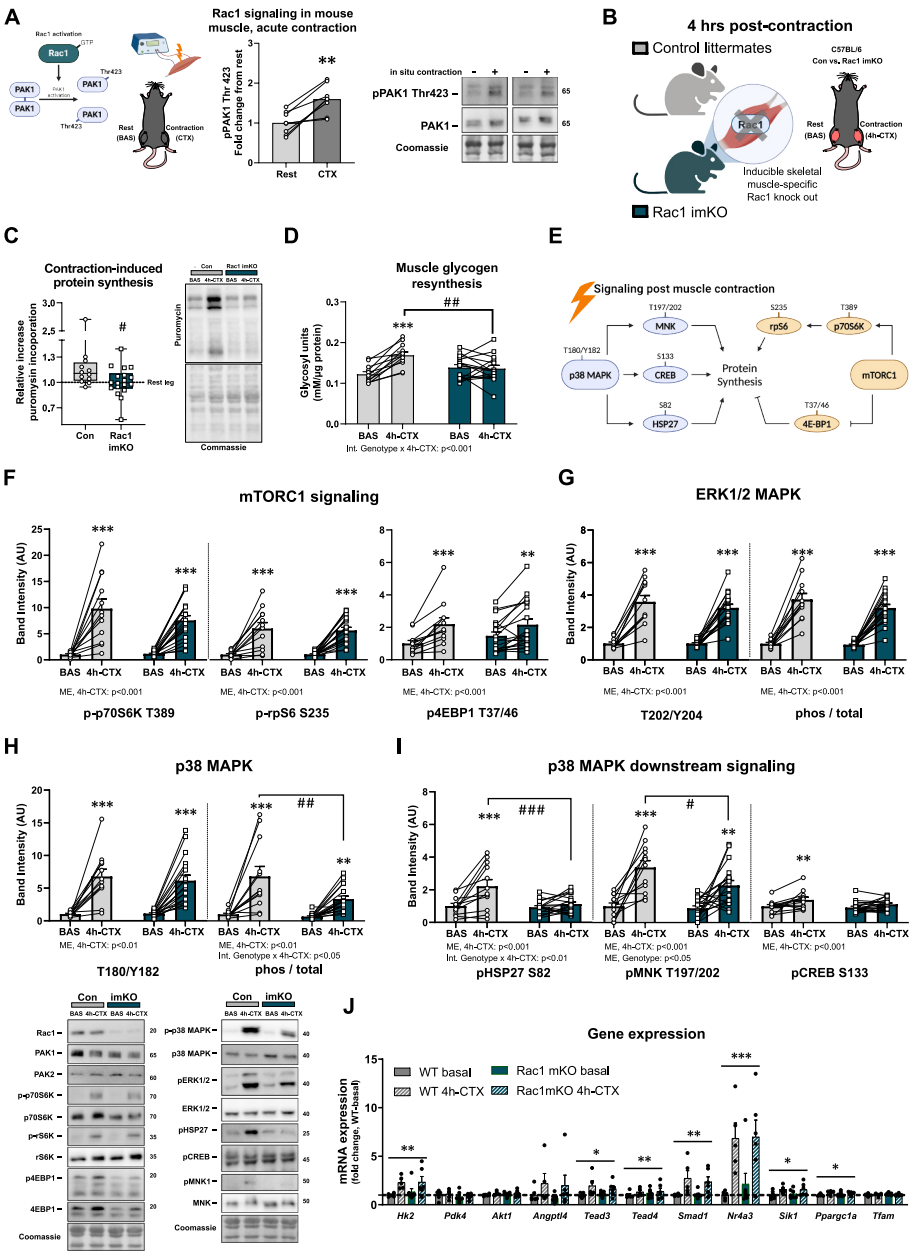

Fig. 1. Post-contraction protein synthesis and glycogen resynthesis require Rac1, which regulates p38 MAPK singling. (A) Schematic illustration of Rac1-PAK1 signaling at rest and *in situ* contracted (CTX) mouse quadriceps muscle. (B) Experimental setup of the investigation of post-contraction (4 h, 4h-CTX) intramyocellular signaling in littermate control (Con) and inducible muscle-specific Rac1 knockout (Rac1 imKO) mice. (C) Relative increase in protein synthesis (puromycin incorporation measured by Western blot) and (D) glycogen synthesis 4 h after contraction in quadriceps muscle. (E) Signaling events leading to protein synthesis in muscle. The effect of *in situ* contraction on: (F) mTORC1 signaling, (G) ERK MAPK phosphorylation, (H) p38 MAPK phosphorylation, and (I) p38 MAPK signaling. Representative blots are shown in the bottom right corner. Acute contraction in mice (A): n = 8. Post-contraction in mice (B–G), Control mice: n = 12, Rac1 imKO mice: n = 18. Significant differences basal leg vs. contraction are indicated; */**/*** = $p < 0.05/p < 0.01/p < 0.001$. Significant differences Rac1 imKO vs. control are indicated as; #/# #/# # # = $p < 0.05/p < 0.01/p < 0.001$. Data are presented as boxplots or bar plots, mean + SEM incl. individual values.

control mice after contraction (Fig. 1I). Surprisingly and in contrast to the above described results, at the transcriptional level, Rac1 mKO had no detectable effect 4 h post-contraction, as both control and Rac1 mKO mice exhibited comparable increases in established contraction-responsive mRNAs (Fig. 1J).

Collectively, these results provide evidence that HSP27, MNK1, and CREB are Rac1-dependent targets in skeletal muscle, which may be implicated in the decreased protein synthesis and glycogen resynthesis observed in Rac1 imKO mice in the recovery from contraction.

3.3. *Rac1 regulates glycogen resynthesis, but not protein synthesis, via the NOX2 complex*

Rac1 is necessary for the assembly and activation of the NOX2 complex to produce ROS during acute exercise [7]. Rac1's involvement in NOX2 activation is required for Angiotensin-II induced cardiac hypertrophy [42], as well as exercise-stimulated glucose uptake in muscle [7]. Thus, we hypothesized that Rac1 may affect protein synthesis and glycogen metabolism in the recovery from contraction via its key role in exercise-induced NOX2 activation.

To test this, we used a mouse model carrying a loss-of-function mutation in the *Ncf1* gene (**Ncf11* KO mice). This mutation leads to

an impaired function of the regulatory NOX2 subunit, p47^{phox} [43,44] and blocks NOX2-dependent production of ROS during acute exercise in muscle [7] (Fig. 2A, (*Ncf1* mice)). Thus, the *Ncf1* mouse can be used to determine the role of NOX2-induced ROS production in the regulation of post-contraction metabolism. Four hours into recovery from contraction (Fig. 2B), we observed equally increased protein synthesis (puromycin incorporation) in *Ncf1* mice compared to control mice (Fig. 2C). Intriguingly, the increase in muscle glycogen in recovery was abolished

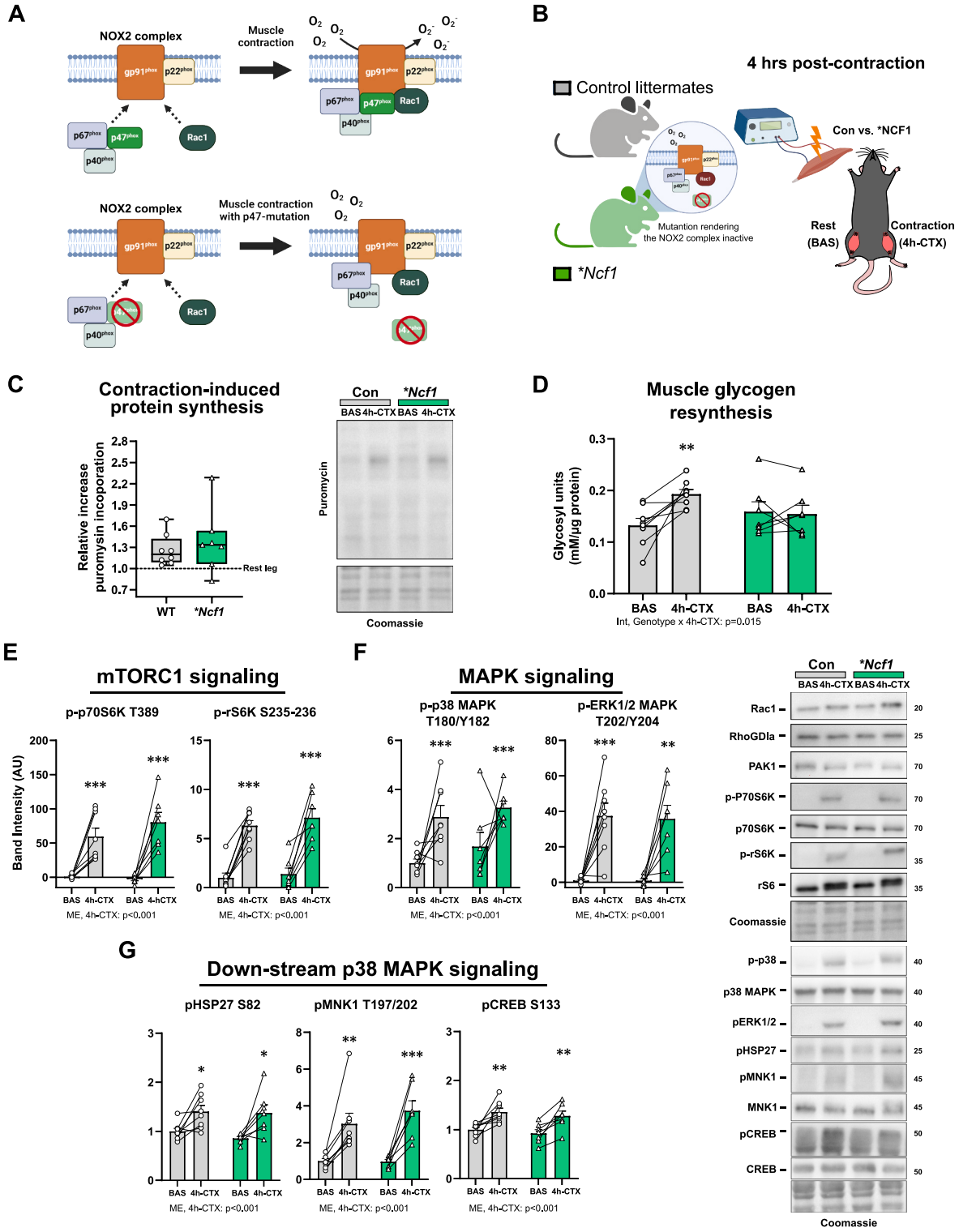

Fig. 2. Rac1 affects post-contraction protein synthesis independent of NOX2 activity. (A) Illustration of the activation of the NOX2 complex and subsequent O_2^--production with and without p47phox mutation (*Ncf1*). (B) Contraction protocol in *Ncf1*mice. (C) Relative increase in protein synthesis (puromycin incorporation measured by Western blot) in quadriceps muscle from control mice and mice carrying a mutation in the *Ncf1* gene (p47^{phox}). (D) Muscle glycogen after contraction. (E) Phosphorylation of mTORC1 substrates, (F) p38 MAPK and ERK1/2 MAPK, and (G) p38 MAPK substrates. Representative blots are shown in the bottom right corner. Control: n = 8, *Ncf1*: n = 7. Significant differences from basal vs. contraction are indicated; */**/*** = $p < 0.05/p < 0.01/p < 0.001$. Data are presented as boxplots or mean + SEM incl. individual values.

in muscle lacking NOX2 activity (Fig. 2D). mTORC1 signaling (Fig. 2E), p38 MAPK-signaling (Fig. 2F), and downstream targets of p38 MAPK all increased independently of the NOX2 complex in recovery from contraction (Fig. 2G).

Together, these data suggest that Rac1 mediates p38 MAPK signaling and protein synthesis in recovery of contraction independent of NOX2-mediated ROS production. In contrast, post-contraction muscle glycogen resynthesis was regulated via the Rac1-dependent NOX2 complex, which is also involved in exercise-stimulated glucose uptake [5,7].

3.4. Endurance, sprint, and resistance exercise activate Rac1 signaling in human skeletal muscle

Different exercise modalities activate distinct molecular signals in muscle, leading to varied functional outcomes. Recent omics studies suggest Rho GTPases are highly activated during exercise [2]; and to investigate the translatability of the observed signaling events in Rac1 imKO mice, we assessed Rac1 activity and p38 MAPK down-stream signaling in human skeletal muscle before, immediately after, and 3 h post-recovery from endurance, sprint, and resistance exercise (Fig. 3A) [2]. In human skeletal muscle, pPAK1 T423 levels increased by 50–80 % immediately after the cessation of endurance, sprint, and resistance exercise (Fig. 3B). Interestingly, pPAK1 T423 remained 30 % elevated 3 h into recovery after resistance exercise, while the phosphorylation returned to pre-exercise levels after recovery from endurance and sprint exercise (Fig. 3B). Translational importance of p38 MAPK signaling events was confirmed in human exercised muscle, where all exercise modalities induced phosphorylation of HSP27 and MNK1 (Fig. 3C–D). Interestingly, pHSP27 S82 responded similar to Rac1 signaling and remained elevated 3 h after resistance exercise (Fig. 3C), resembling the pattern observed in Rac1 imKO mouse muscle after contraction. Expectedly, total Rac1 and PAK1 protein content were not changed by acute exercise in human skeletal muscle (Fig. 3E).

3.5. Muscle Rac1 protein content increases after long-term exercise training

Having established that Rac1 regulates signaling events and protein synthesis in the hours after contraction, we next investigated whether Rac1 is necessary for long-term exercise training adaptations. We analyzed whether total Rac1 protein content is regulated by training. In muscle biopsy samples from lean healthy men before and after a single-leg knee-extensor exercise training intervention [17], three weeks of exercise training led to a 30 % increase in Rac1 protein content in the trained muscle (Fig. 4A). Similarly, Rac1 protein content also increased in mouse gastrocnemius muscle following voluntary wheel running exercise training (Fig. 4B). Thus, Rac1 protein is modestly training-sensitive in both human and mouse skeletal muscle.

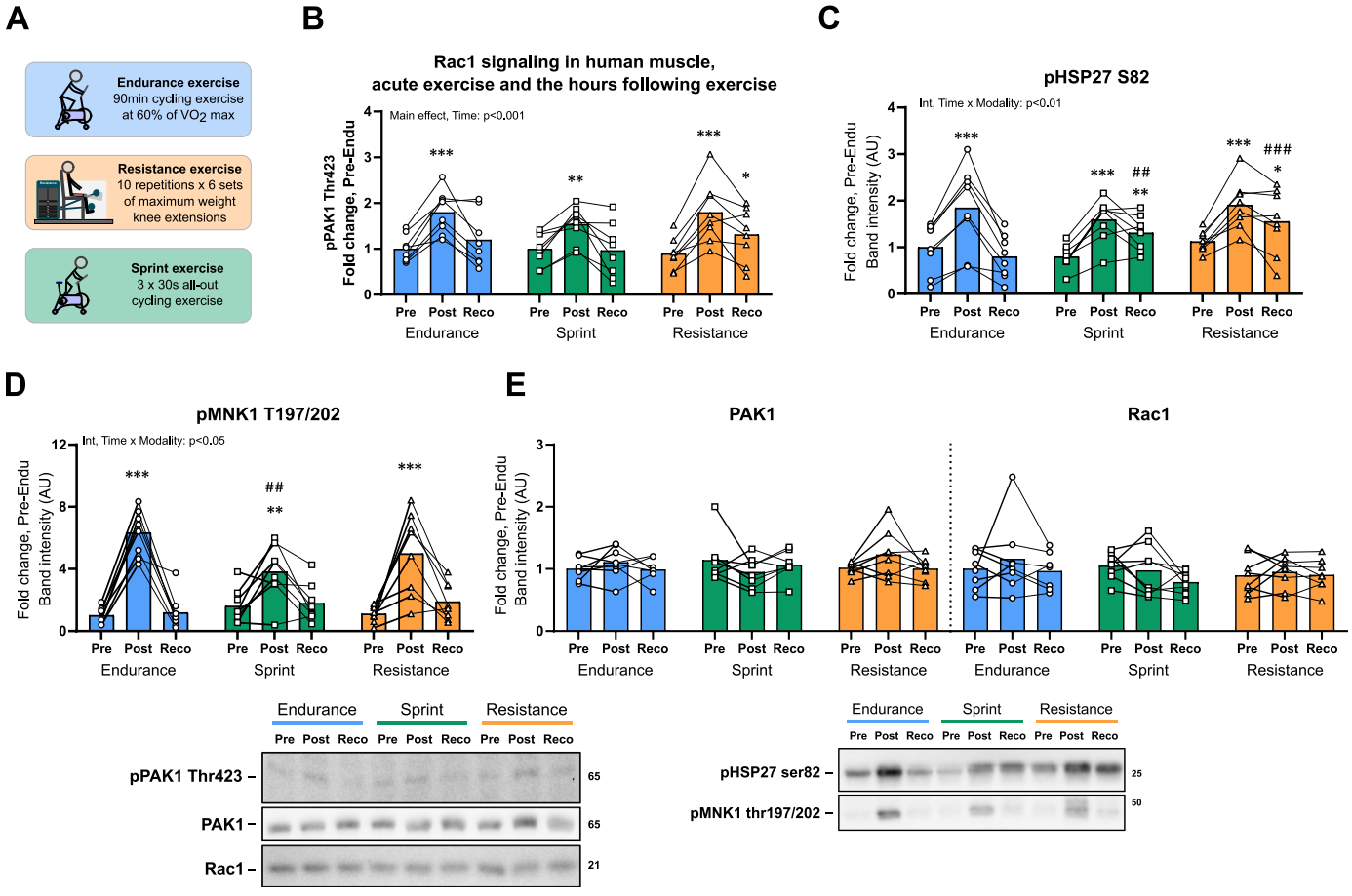

Fig. 3. Rac1 signaling is increased by several exercise modalities in skeletal muscle of young healthy men. (A) Overview of the exercise interventions. (B) Rac1 signaling (pPAK1 T423) in vastus lateralis skeletal muscle of young healthy men before (Pre), immediately after (Post), and 3 h into the recovery (Reco) from endurance, sprint, or resistance exercise. (C) pHSP27 S82. (D) pMNK1 T197/202. (E) PAK1 and Rac1 protein content. n = 8. Representative blots are presented at the bottom of the figure. Significant differences between pre and post/reco are indicated as; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Significant differences between modalities vs. endurance are indicated as; # # = $p < 0.01$, # # # = $p < 0.001$.

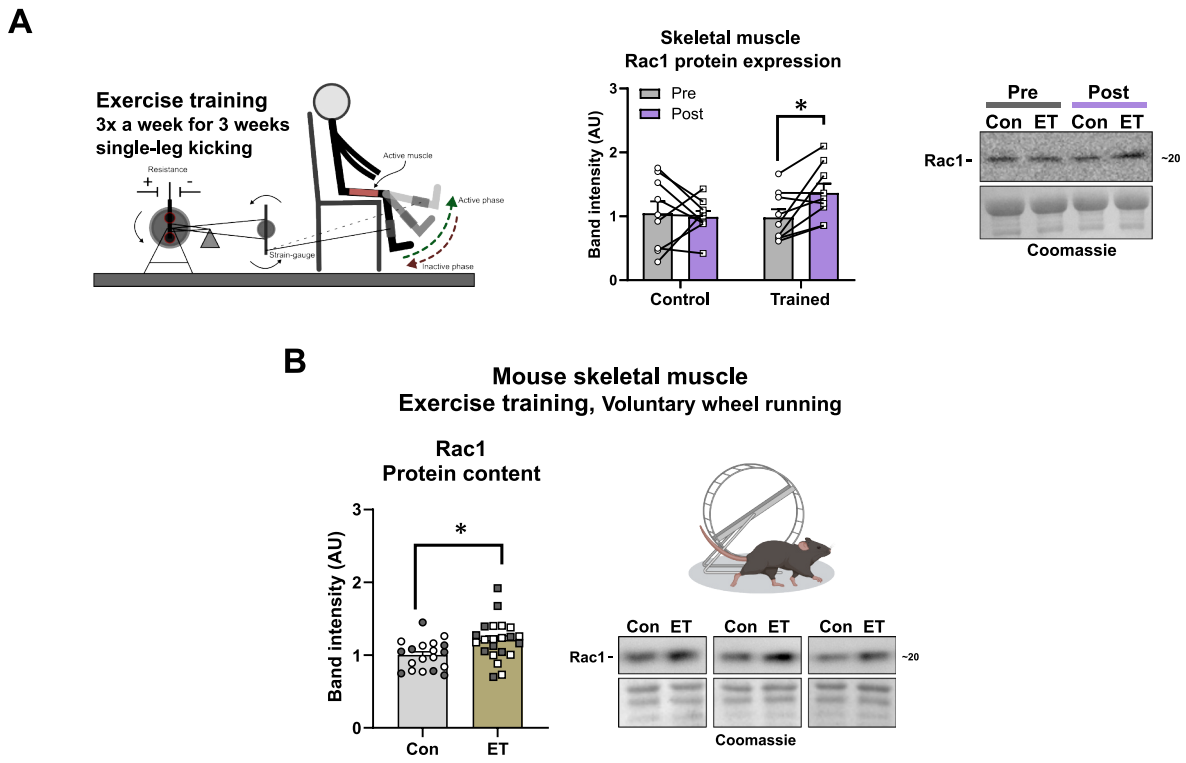

Fig. 4. Exercise training increases Rac1 protein content in human and mouse skeletal muscle. (A) Effect of single-leg exercise training (ET) on Rac1 protein content in human vastus lateralis skeletal muscle (n = 9). (B) Effect of voluntary wheel running (ET) on gastrocnemius Rac1 protein content in mice. Mouse study: n = 19–22, color of dots indicate two separate studies. Significant differences after training intervention are indicated; * = $p < 0.05$. Data are presented as mean + SEM incl. individual values, connected when applicable.

3.6. Rac1 mediates improved running capacity and muscle mass gain after exercise training

To determine the necessity and mechanistic role of Rac1 in exercise training adaptations, we next performed a 12-week voluntary wheel running exercise training intervention in our Rac1 imKO mice (Fig. 5A). This exercise modality robustly induces adaptations in skeletal muscle of mice [14,45–47]. During the intervention, Rac1 imKO and control littermate mice covered the same daily and total running distance (Fig. 5B). The exercise training intervention expectedly [14,46] increased running capacity by 45 % in control mice. In contrast, the Rac1 imKO mice did not significantly improve their running capacity compared to untrained sedentary Rac1 imKO mice (Fig. 5C). Thus, exercise-trained Rac1 imKO mice displayed significantly reduced running capacity compared to control exercise trained mice (Fig. 5D). Rac1 imKO mice had similar running capacity compared to littermate controls before the ET intervention (not shown), in line with our previous observations [5]. While Rac1 was necessary for the acute resynthesis of glycogen after electrically-induced contraction, the elevation of muscle glycogen after long term exercise training was similar between control and Rac1 imKO mice (Fig. 5E). Intriguingly, only the control mice increased their body weight (+10 %) in response to the training intervention (Fig. 5F). This difference was caused by training-induced increases in lean body mass (+10 %, Fig. 5F), which was also observed for muscle weight of gastrocnemius (+20 %) and tibialis anterior (+15 %) in the control, but not the Rac1 imKO mice (Fig. 5G). Exercise training reduced body fat by 20 % in both genotypes (Fig. 5F), revealing muscle-selective differences in adaptations between control and Rac1 imKO mice.

To determine the molecular adaptations to exercise training, we next measured the content of proteins involved in Rac1-related signaling. Interestingly, the protein content of the two other Rho GTPases, CDC42 and RhoA ($p = 0.07$), was increased after exercise training, independent of Rac1 (Fig. 5H). Moreover, CDC42 protein was elevated in Rac1 imKO mice, suggesting a possible compensatory response to Rac1 deficiency (Fig. 5H). In gastrocnemius muscle, the kinases down-stream of Rac1, PAK1 and PAK2, were both increased in Rac1 imKO muscles, where PAK2 protein content also increased (50 %) with exercise training in control mice (Fig. 5H). In contrast, the protein content of the Rac1 inhibitor, Rho guanine dissociation inhibitor α (RhoGDIα) [48], was neither affected by Rac1 imKO nor exercise training (Fig. 5H). Collectively, these data show that Rac1 is activated in response to multiple exercise modalities in human skeletal muscle and that Rac1 is necessary for improved exercise performance and muscle hypertrophy in response to exercise training in mice.

3.7. Lack of Rac1 alters the proteomic adaptations to exercise training

The adaptation to exercise training is a highly dynamic process, where hundreds of proteins are increased and decreased in response to training in skeletal muscle [13,14]. To get a broader understanding of the role of Rac1 in skeletal muscle adaptations to exercise training, we next determined the whole muscle proteome in response to exercise training in Rac1 imKO and control muscle using LC-MS/MS mass spectrometry. We identified a total of 3727 proteins (Fig. 5I) and exercise training led to an increase of 111 proteins, while 30 proteins decreased independent of genotype (Fig. 5J). Gene Set Enrichment Analyses (GSEA) showed that exercise training in general led to increased expression of proteins involved in oxidative phosphorylation, carboxylic acid catabolic processes, and respiratory chain complexes, while decreasing proteins involved in proteasome complexes (data not shown).

In the trained muscles, Rac1 imKO mice only showed minor differences on a protein level compared to trained control mice (Fig. 5K, increased: 5, decreased: 14). ClueGO enrichment of the muscle proteome [26] showed clusters involved in musculoskeletal movement,

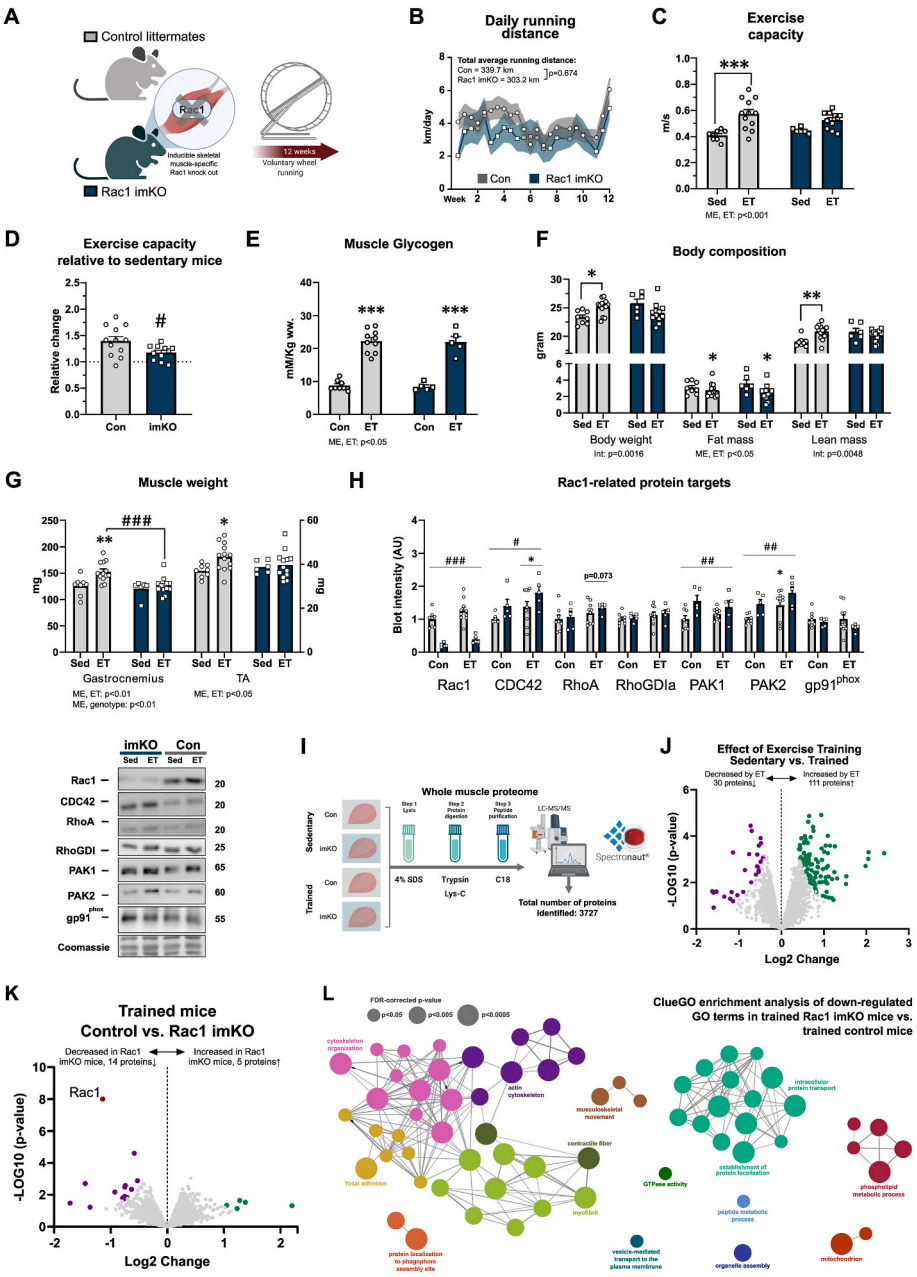

Fig. 5. Rac1 mediates critical adaptations to exercise training. (A) Lack of skeletal muscle Rac1 during exercise training (ET) was investigated using inducible skeletal muscle-specific Rac1 knockout mice (Rac1 imKO, compared to control littermate mice (Con). (B) Running distance during the intervention. Effect of exercise training on (C) exercise capacity. (D) change in exercise capacity relative to sedentary (Sed) mice, (E) muscle glycogen, (F) body mass and composition. (G) muscle weight. (H) Effect of ET on protein content of proteins involved in muscle metabolism including representative blots. For control mice: n = 8–13. For Rac1 imKO mice: n = 5–13. Significant effects of training are indicated; */**/*** = $p < 0.05/p < 0.01/p < 0.001$. Significant effects of Rac1 imKO compared to controls are indicated as; # = $p < 0.05$, # # = $p < 0.01$, # # # = $p < 0.001$. Data are presented as mean +SEM incl. individual values. (I) Schematic illustration of whole proteomic analyses of mouse muscle from control (Con) and skeletal muscle-specific Rac1 knockout mice (Rac1 imKO). (J) Volcano plot of the main effect of exercise training (ET) in mouse muscle. (K) Volcano plot and (L) ClueGo enrichment analysis of muscle from trained mice. For the ClueGo analyses: Right-sided hypergeometric test, Bonferroni FDR <0.05 for the proteins regulated >0.2 fold change, functionally related terms are grouped, and color coded based on overlapping proteins. The most significantly regulated terms in each group are labeled. Sedentary mice: n = 5, trained mice: n = 4–5.

cytoskeleton organization, myofibrils, intracellular protein transport, and phospholipid metabolic processes were all downregulated in muscle from trained Rac1 imKO mice compared to trained muscle from control mice (Fig. 5L). Thus, the muscle proteome showed a global dysfunction of proteins involved in cytoskeleton organization and muscle adaptation when lacking Rac1 in skeletal muscle during exercise training.

3.8. Rac1 protein content is increased in trained muscle and elevates proteins involved in the large ribosomal subunits and mitochondria

Having found that Rac1 was required for various molecular and physiological adaptations to exercise, we next investigated if elevated Rac1 activity in skeletal muscle would result in exercise-like adaptations. To this end, we used recombinant adeno-associated viral vector (rAAV6) with muscle tropism [49] to overexpress a hyperactive (H)-Rac1 mutant (Rac1:G12V). Rac1:G12V is a naturally occurring

mutation located at the P-loop (phosphate-binding structure) of Rac1 and impairs Rac1-dependent hydrolysis of GTP, making this Rac1 mutant constitutively active [50–52] (Fig. 6A). When overexpressed in muscle, H-Rac1 increased pPAK1 T423 (+30 %) and pPAK2 T402 (+35 %) at rest and during exercise despite reduced PAK1 protein content (Fig. 6B and C). In line with Rac1's crucial role in NOX2-dependent exercise-induced ROS production, H-Rac1 overexpression led to a 50 % increase in oxidant production (DCFH fluorescence) in skeletal muscle during treadmill running, but not at rest (Fig. 6D). This was likely also affected by an elevated (+170 %) protein content of the catalytic subunit of the NOX2 complex, gp91^{phox}, by H-Rac1 (Fig. 6B and C).

Having validated our Rac1 mutant, we next overexpressed Rac1: G12V for 8 weeks to investigate whether active Rac1 would suffice to induce a trained muscle phenotype towards muscle growth (Fig. 6E). After 8 weeks, Rac1 increased by 100 %, while CDC42 and RhoA protein content were unaffected by Rac1:G12V overexpression (Fig. 6E). Next, we took an unbiased approach to investigate the whole muscle proteome (Fig. 6F). Quantifying a total of 3480 proteins (Fig. 6F), we observed 35 proteins that were decreased while 16 proteins were increased in Rac1: G12V muscles compared to MCS control (Fig. 6F). Performing ClueGo analyses, an expected clustering of proteins involved in GTP binding and cytoskeleton organization were increased by Rac1:G12V. Interestingly, overexpression of H-Rac1 also led to an increase in two clusters involved in the large ribosomal subunits and mitochondria (Fig. 6G). Despite these molecular changes, Rac1:G12V overexpression did not affect muscle size (Fig. 6H). These data indicate that while Rac1 activity and protein levels increase in response to exercise, constitutively activating Rac1 in skeletal muscle is insufficient to induce training-like adaptations towards muscle growth.

4. Discussion

Skeletal muscle Rho GTPases are highly regulated proteins in response to exercise, yet their role in exercise-mediated adaptations remained elusive. Here we demonstrate that the Rho GTPase, Rac1 is activated during various exercise modalities in human skeletal muscle. This activation is likely critical for exercise adaptations, as mice lacking muscle Rac1 showed no improvements in running capacity or muscle growth following exercise training. Molecularly, Rac1 was necessary for increased protein and glycogen synthesis in recovery from contraction. Here, glycogen resynthesis depended on Rac1-dependent NOX2 activation. Additionally, we identify HSP27, MNK1, and CREB as Rac1-dependent contraction-responsive targets in skeletal muscle. Finally, we demonstrate that exercise training increased Rac1 protein levels in both human and mouse muscle. While overexpressing a hyperactive Rac1 mutant modestly altered the muscle proteome, it did not produce exercise-like adaptations towards muscle growth. Together, these findings suggest that Rac1 orchestrates molecular and functional exercise adaptations of skeletal muscle.

The finding that Rac1 was activated in response to multiple exercise modalities in human skeletal muscle was exciting because all these modalities are known to lead plethora of long-term health benefits, including increased muscle growth and function [15]. For example, resistance exercise, known to promote muscle growth by increasing muscle protein synthesis [53], activated Rac1 signaling. Our results indicate a role for Rac1 in these exercise-mediated adaptations, since mice lacking muscle Rac1 showed no exercise training-induced muscle growth or improvements in running capacity. These findings corroborate Rac1's critical role in regulating cardiac hypertrophy [54–56]. Our findings also align with a reduced muscle mass [57] and late-onset myopathy [58] in double PAK1 and PAK2 knockout mice. Lastly, the **Ncf1* mice have previously shown lack improvements in exercise capacity [59] and have diminished muscle hypertrophy when subjected to a high-fat diet [60] following exercise training. The lack of muscle hypertrophy in Rac1 imKO mice was associated with the reduction in protein synthesis in the recovery from muscle contraction, identifying a new signaling mechanism for exercise-induced muscle growth and function.

Mechanistically, Rac1-induced contraction signaling was independent of mTORC1 [61]. Although Rac1 has been shown to regulate mTORC1 signaling in non-muscle cells, [62], our results show that this Rac1–mTORC1 axis does not appear to operate in mature skeletal muscle in response to contraction, although it is possible that different time points may yield distinct signaling profiles. Instead, we identified the p38 MAPK targets HSP27, MNK1, and CREB as Rac1-dependent contraction-responsive proteins. Notably, HSP27 and CREB are of interest, as whole-body HSP27 KO mice have smaller muscle fibers [63], while CREB coactivator overexpression increases muscle mass [64], though their roles in exercise adaptation is not tested. Additionally, p38MAPK and CREB have previously been shown to induce *Pgc1α* gene expression following muscle contraction [65,66]. While the precise mechanism by which Rac1 regulates p38 MAPK remains unclear, it may involve Rac1's role in stretch-induced mechanotransduction during contraction [67]. These findings further support that mTORC1-independent pathways regulate muscle mass regulation [33].

In addition to its role in protein synthesis, Rac1 was required for glycogen resynthesis after contraction. This process also depended on NOX2, which requires Rac1 for activation in response to muscle contraction and exercise [7]. Rac1-dependent NOX2 activation is required to stimulate glucose uptake during exercise [4–7]. While glucose uptake was not directly measured in this study, a reduction in glucose uptake during or possibly in the hours following the contraction could explain the lower glycogen resynthesis observed in both Rac1 imKO and **Ncf1* mouse muscle.

We also found that Rac1 protein content increased in response to long-term exercise training in both human and mouse skeletal muscle. Although supraphysiological Rac1 hyperactivation enriched proteins related to the large ribosomal subunit, mitochondria, and cytoskeletal reorganization, it did not mimic an exercise-training phenotype. Consistent with this, exercise training had a more pronounced effect on the muscle proteome than Rac1 activation alone, as shown by our findings and previous studies [14,68,69]. These findings uncover new Rac1-regulated pathways and highlight that activating Rac1 as a single exercise-sensitive protein is insufficient to drive the extensive changes observed in muscle following exercise training.

In conclusion, our findings establish Rac1 as a critical regulator of exercise-induced molecular and functional adaptations in skeletal muscle, highlighting its pivotal role in promoting running capacity, muscle growth, and recovery, with potential implications for optimizing therapeutic strategies for muscle-related conditions.

CRediT authorship contribution statement

Steffen H. Raun: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Carlos Henriquez-Olguín:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Emma Frank:** Writing – review & editing, Investigation. **Farina Schlabs:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Nanna Just Hahn:** Writing – review & editing, Investigation. **Jonas Roland Knudsen:** Writing – review & editing, Methodology, Investigation. **Mona S. Ali:** Writing – review & editing, Investigation. **Nicoline R. Andersen:** Writing – review & editing, Investigation. **Lisbeth L.V. Møller:** Writing – review & editing, Investigation. **Jonathan Davey:** Methodology. **Hongwei Qian:** Methodology. **Ana Coelho:** Writing – review & editing, Investigation. **Christian S. Carl:** Writing – review & editing, Resources. **Christian T. Voldstedlund:** Writing – review & editing, Resources. **Bente Kiens:** Writing – review & editing, Resources. **Rikard Holmdahl:** Writing – review & editing, Resources, Investigation. **Paul Gregorevic:** Writing – review & editing, Resources, Methodology. **Thomas E. Jensen:** Writing – review & editing, Investigation. **Atul S. Deshmukh:**

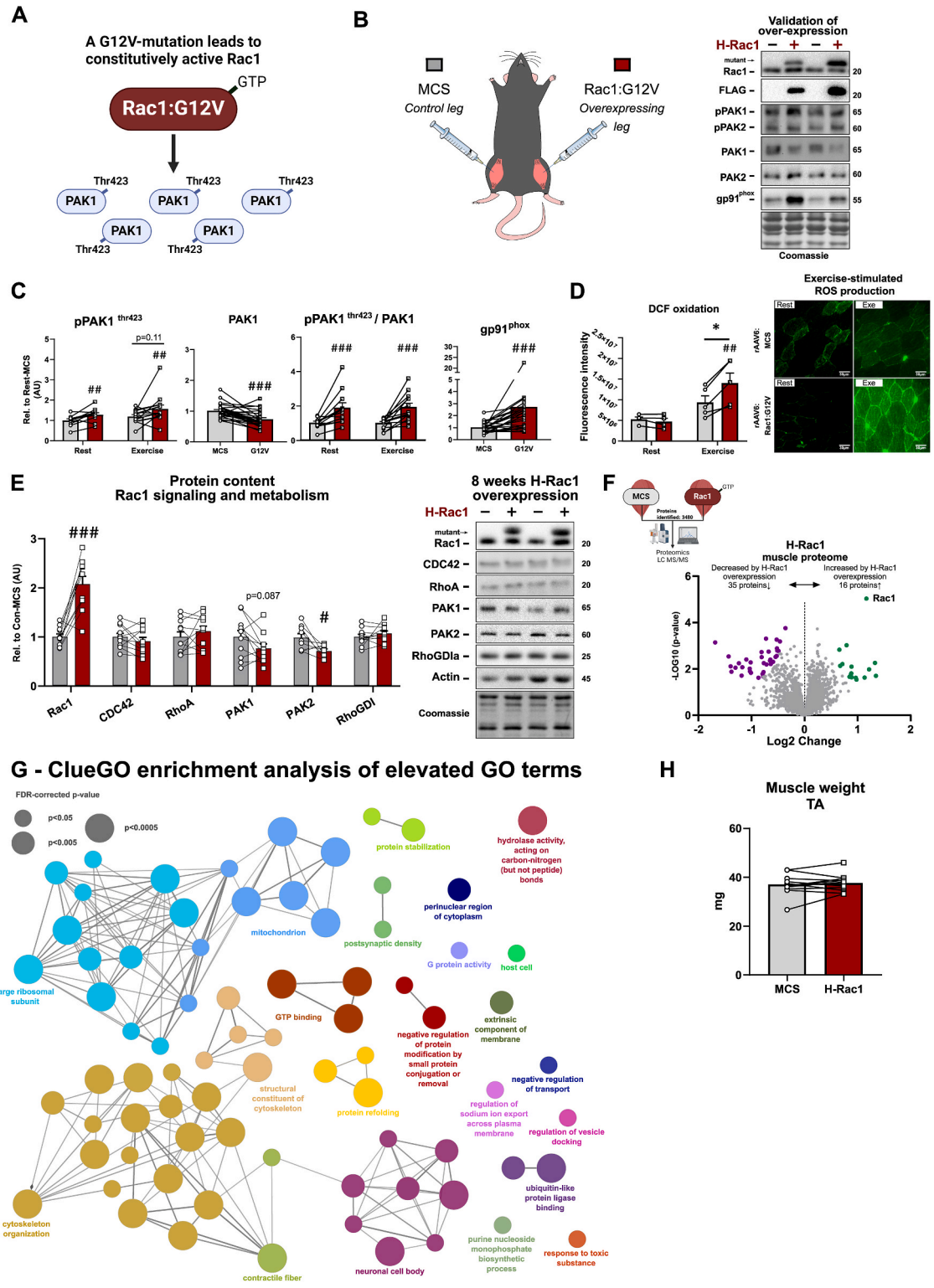

Fig. 6. H-Rac1 increases exercise-induced ROS production and enriches the proteome in proteins related to the cytoskeleton organization, large ribosomal subunits, and mitochondria. (A) Constitutively active G12V Rac1 (H-Rac1) was overexpressed in muscle using recombinant adeno-associated virus. (B) Experimental design. (C) immunoblotting validation of H-Rac1 overexpression. (D) DCF-oxidation (oxidant production) at rest and during exercise in tibialis anterior muscle. (E) Effect of 8 weeks of H-Rac1 overexpression on muscle protein content measured by immunoblotting in gastrocnemius muscle. (F) Schematic presentation of proteomic analyses (n = 6). Volcano plot of the muscle proteome in muscle overexpressing H-Rac1. (G) ClueGo enrichment analysis of GO terms of the proteins elevated >0.2 Fold Change by H-Rac1. Right-sided hypergeometric test, Bonferroni FDR <0.05, functionally related terms are grouped, and color coded based on overlapping proteins. The most significantly regulated terms in each group are labeled. (H) Effect of H-Rac1 on muscle weight. For H-Rac1 studies: n = 4–10. Significant differences of acute exercise are indicated as; * = $p < 0.05$. Significant differences of H-Rac1 overexpression are indicated as; # = $p < 0.05$, # # = $p < 0.01$, # # # = $p < 0.001$. Data are presented as mean +SEM incl. individual values, paired samples connected with lines when applicable. LC-MS/MS, liquid chromatography–tandem mass spectrometry.

Writing – review & editing, Resources, Methodology. **Erik A. Richter:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Lykke Sylow:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper. Additional data related to this paper may be requested from the corresponding authors (LS/SHR) upon reasonable request.

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Declaration of competing interest

The authors of the current manuscript have no conflict of interest associated with the current manuscript.

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