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RACK1 release from the ribosome couples translational regulation with starving signaling and possibly depends on phosphorylation of key serine and threonine residues

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ABSTRACT

The balance between protein anabolism and catabolism sets the foundations on which cells build their homeostasis. RACK1 is a ribosome-associated scaffold protein involved in signal transduction. On the ribosome, RACK1 enhances specific translation. Conversely, upon growth factor/nutrient starvation, RACK1 is present in a ribosome-free form and inhibits protein synthesis. However, the precise role of RACK1 when not bound to the ribosome still requires elucidation. Here, we show that extra-ribosomal RACK1 increases LC3-II accumulation, thereby mimicking an autophagy-like phenotype. Next, based on the ribosome-bound structure of RACK1, we suggest a possible mechanism for RACK1 release from the ribosome which relies on phosphorylation of precise amino acid residues, namely Thr39, Ser63, Thr86, Ser276, Thr277, Ser278, Ser279. Specifically, by performing an unbiased *in silico* screening using phospho-kinase prediction tools, we propose that, upon starving, AMPK1/2, ULK1/2 and PKR are the strongest candidate protein kinases to phosphorylate RACK1. This may be relevant in the context of caloric restriction and cancer therapy, where repressing translation of specific mRNAs would open important therapeutic avenues. Overall, our work provides novel insight into RACK1 function(s) by connecting its ribosomal and extra-ribosomal activities with translation and signaling.

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Introduction

mRNA Translation, or protein synthesis, is a complex multi-step process that is required for cell survival. Ribosomes are a fundamental part of the translational machinery. Each ribosome is composed of a 40S and a 60S subunit. With the assistance of specific task-oriented translation factors, the 40S ribosomal subunits scan the mRNA for translation start codons. Once a suitable codon is found, the 60S subunit joins with the 40S to form a translational competent 80S ribosome, which proceeds to decode the mRNA by matching each codon with the complementary anti-codon presented by amino acid-loaded tRNAs. It is the 60S subunit duty to catalyze peptide bond formation between the amino acids which are being added to the nascent polypeptide chain.

Along with translation factors, several ribosomal proteins have been shown to have roles in modulating the translation of specific mRNA classes. Among those proteins, the eukaryotic scaffold protein RACK1 has been extensively studied. RACK1 has an impressive variety of interactors (1,2). Originally identified as a recruiter for activated PKCs (3), RACK1 most consistent partner is the 40S ribosomal subunit (4). 40S-bound RACK1 is located in close proximity to the mRNA exit channel(5) and has thus been shown to act as a hub connecting specific signaling pathways with the ribosomal machinery.

Deletion of Asc1, the homolog of RACK1 in the yeast *S. cerevisiae*, is not lethal (6). Moreover, RACK1-depleted mouse embryos survive until late gastrulation (7), demonstrating that RACK1 is not *per se,* fundamental for eukaryotic translation at the single-cell level. This said, several works in different model organisms, from yeast to human, have shown that RACK1 specifically modulates mRNA quality control or protein synthesis (7–13).

While RACK1 presence on the ribosome is well established, studies on its cytoplasmic counterpart, unbound from the ribosome, are scanty (13–15). To date, it remains largely unclear how many of the numerous RACK1 interactors are exclusive for its ribosome-bound form. Notably, our previous work showed that only ribosome-docked RACK1 efficiently recruits the translation initiation factor eIF4E to promote cap-dependent translation *in vitro* (13). In that same study, an excess of ribosome-free RACK1 was shown to be inhibitory for translation *in vivo* but not *in vitro* and such an effect was likely due to the activation of extra-ribosomal signaling with negative feedback on translation.

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In this context, it is very relevant to understand how RACK1 binding/unbinding occurs and how it may be regulated. By structural analysis on yeast ribosomes, RACK1 ortholog Asc1 has been shown to present several positively charged amino acids which interact with the negatively charged backbone of the 18S rRNA (16). Mutating those specific amino acids, from positively to negatively charged, reduced protein affinity for the ribosome. The same stands true for homologous mutations in human RACK1 (12,13,15), pointing to a conserved RACK1-ribosome interaction mechanism.

Thus, it is plausible that amino acid post-translational modifications, like phosphorylation, can be elicited to achieve RACK1 release from the ribosome. Indeed, RACK1 is found phosphorylated upon nutrient-starving conditions (17).

Here, by closely looking at RACK1 structure, we determined which specific residues of RACK1 may be involved in a dynamical balance of association/dissociation with the 40S ribosomal subunit. We propose RACK1 release from the ribosome as a way to repress specific translation (i.e., cap-dependent) when it is not useful for cells.

Materials and Methods

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Simone Gallo (gallo.simone16@gmail.com).

Cell lines

HEK293 cells were cultured in DMEM (Lonza) supplemented with 10% FBS (Euroclone) and 1% PSG (Euroclone) at 37°C with 5% CO_2.

Fluorescent staining and cellular imaging

HEK293 cells for intracellular staining were seeded on glasses previously coated with poly-lysin (Sigma-Aldrich). Cells were fixed with 3% paraformaldehyde, 2% sucrose in PBS for 20 minutes at room temperature. After washing in PBS, the cells were permeabilized with PBS-Triton 0.1% for 10 minutes at room temperature. PBS with 2% BSA was used for blocking for 2 hours at room temperature. Primary antibodies were incubated overnight at 4°C, and after three washes with PBS, secondary antibodies were incubated for 3 hours at room temperature in the dark as described in (18). After three washes with PBS, cells were mounted on slides with Prolong Gold (Life Technologies). All the antibodies were diluted in a blocking solution. We used mouse anti-RACK1 and rabbit anti-rpS6 as primary antibodies. As secondary antibodies, we employed donkey anti-mouse and donkey anti-rabbit (Alexa Fluor® secondary antibodies, Molecular Probes 1:500). Nuclei were stained with DAPI (Molecular Probes NucBlue® Live ReadyProbes®). The cells were examined by confocal microscopy (Leica SP5) at magnification 252X. Immunofluorescence experiments were performed at least three times in triplicate.

Western blot

Equal amounts of proteins (20 μg per sample) from clarified lysates were loaded onto SDS-PAGE gels. After separation, the proteins were transferred by using eBlot™ Protein Transfers (GenScript) on PVDF membranes. The membranes were blocked in 5% BSA (Calbiochem) in PBS 0.1% Tween-20 for 1 hour before overnight incubating with primary anti- LC3B antibodies (1:100 Cell Signaling; cat. No 2775) as described in (19). After washes in PBS 0.1 % Tween-20, the membranes were incubated with appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology). The signal was then developed with Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare) and the images were acquired from LAS-3000 Luminescent Image Analyzer (FujiFilm).

Structural modeling

The software RasWin (20,21), available at http://www.openrasmol.org/, was used for structural modelling of RACK1 on the ribosome. The source for the structural data was a published work showing the human 80S ribosome by cryo-electron microscopy ((22); ID at https://www.rcsb.org/: 4ug0).

Sequence alignment

The alignment of RACK1 ortholog sequences were done with T-Coffee (23), available at http://tcoffee.crg.cat/apps/tcoffee/index.html. The file was processed with the software BOXSHADE (https://embnet.vital-it.ch/software/BOX_form.html).

In silico kinase screening

The software GPS5(24), available at http://gps.biocuckoo.cn/, was employed to perform a whole sequence analysis for Ser/Thr kinases to potentially phosphorylate RACK1.

Results and discussion

The extra-ribosomal pool of Asc1, the *S. cerevisiae* homolog of RACK1, has been shown to increase in non-growing compared to exponentially-growing cells (14). This suggests that ribosome-free Asc1 might have a specific role in resting conditions which is distinct from its role in regulating translation when bound to the 40S ribosomal subunit. We wanted to test whether this function could be evolutionary conserved and also work for human RACK1. To do so, we performed immunofluorescence experiments both in exponentially growing and serum-deprived HEK293 cells. We stained for both endogenous RACK1 and rpS6, a protein that localizes on the 40S and which is widely used as a ribosomal marker. While RACK1 and rpS6 mostly co-localized in growing cells, after 2 hours of serum deprivation, their distribution was dramatically altered to the point of becoming mutually exclusive (Fig. 1A), indicating drastic relocalization of RACK1 away from the ribosome upon starving conditions also in mammalian cells.

We then went to determine if the forced expression of ribosomal-free RACK1 could mimic a nutrient deprivation phenotype also in exponentially growing cells. For this purpose, we employed the R36D K38E RACK1 mutant, a previously characterized RACK1 variant with low affinity for the ribosome (13). Since autophagy is one of the major intracellular pathways activated downstream of nutrient deprivation signaling, we determined if an excess of extra-ribosomal RACK1 could upregulate autophagic fluxes. Intriguingly, HEK293 cells transiently expressing the R36D K38E RACK1 mutant showed a significant ac-

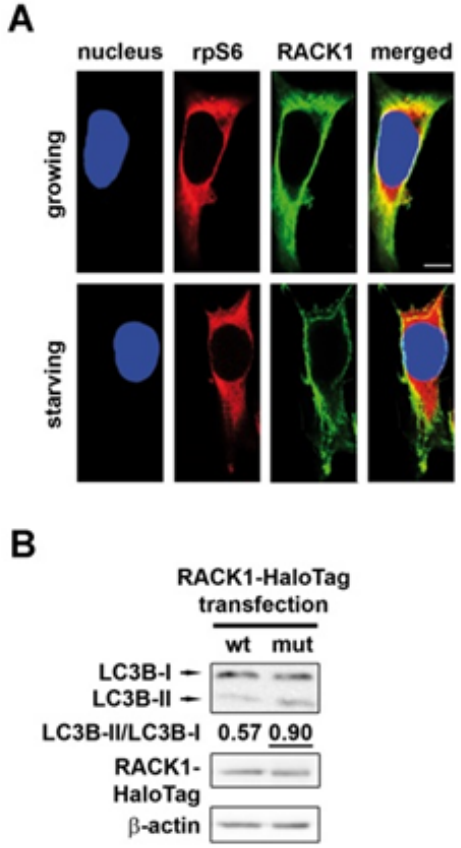


Figure 1. Growth factor/nutrient starvation in human cells promotes RACK1 release from ribosomes. (A) RACK1 co-localization with the 40S ribosomal subunit as assessed by immunofluorescence staining for endogenous RACK1 and rpS6. Scale bar: 5 μm. (B) Amounts of total and cleaved LC3B assessed through western blotting after wild-type and R36D K38E mutant RACK1 transient transfection.

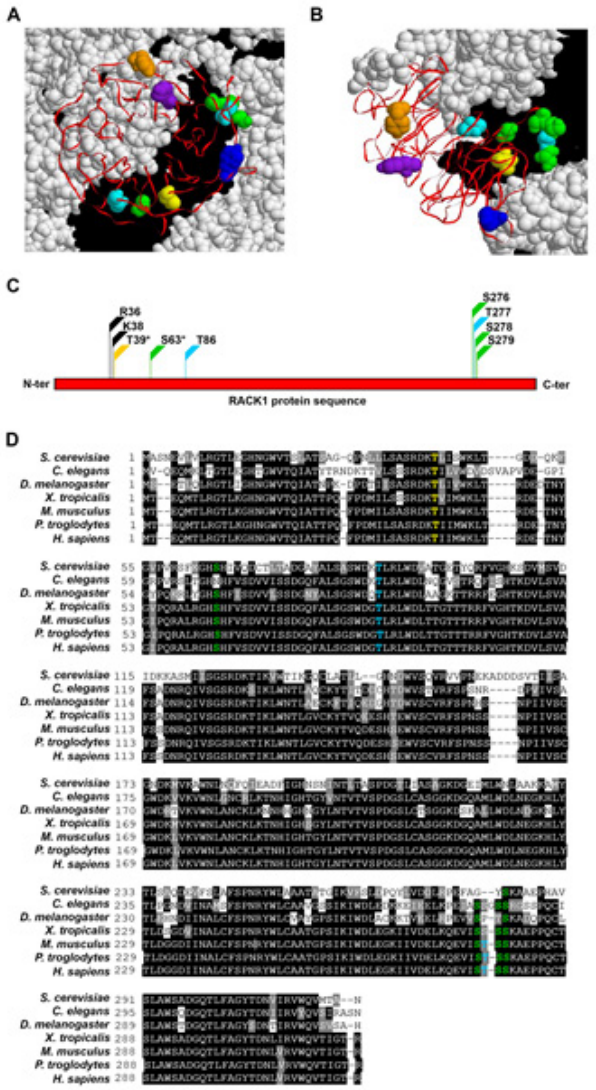


Figure 2. Ser/Thr phosphorylation may mediate RACK1 release from the ribosome. (A) Top and (B) side views of RACK1 (red) on human 40S (ribosomal proteins, light grey; rRNA, black). Specific residues are highlighted by different colors. Thr39: yellow; Thr86 and Thr277: cyan; Ser63, Ser276, Ser278, Ser279: green; Tyr52: blue. Tyr228: orange; Tyr246: purple. (C) The residues of interest in RACK1 amino acid sequence are listed. Residues phosphorylated in conditions of nutrient starvation are marked (*). (D) Alignments of RACK1 ortholog sequences from yeast (*Saccharomyces cerevisiae*), nematode (*Caenorhabditis elegans*), insect (*Drosophila melanogaster*), frog (*Xenopus tropicalis)* mammals (mouse, *Mus musculus*; chimpanzee, *Pan troglodytes*; human, *Homo sapiens*). In black: conserved residues; in grey: conservative mutations; in white: divergences.

cumulation of the lipidated form of LC3B (LC3B-II) (Fig. 1B), an indicator of increased autophagic flux, in accordance to previous studies (25). Taken together, these data show that an increase in ribosome-free RACK1 correlates with a starving-like phenotype independent of media nutrient conditions.

Next, we wanted to speculate on the mechanism responsible for RACK1 release from the ribosome during starvation. The rapid shift we observed in RACK1 distribution is compatible with an active removal of the protein from the ribosomal machinery. A molecular mechanism that would account for such a kind of regulation is phosphorylation. Hence, we focused on the phosphorylatability of key residues of RACK1. We speculated that to diminish RACK1 affinity for the ribosome *in vivo*, the protein might be phosphorylated on residues that are: i) exposed on the surface of the protein and ii) directly interacting with the backbone of the 18S rRNA. We used these two criteria for exploring RACK1 phosphorylatable amino acids. By modeling the structure of human ribosomes(22) and that of RACK1, we identified seven candidate phosphorylatable serine and threonine residues: Thr39, Ser63, Thr86, Ser276, Thr277, Ser278, Ser279 (Figure 2).

Interestingly, two of RACK1 most characterized phosphorylated residues, Tyr52 and Tyr246, which are involved in FAK and Src signaling, respectively (26,27), did not fulfill our requirements, as they localize on the side of the protein which does not interact with the ribosome, and were not considered in our final list (Figure 2B). Among our seven candidate residues, we predicted Thr39, Ser63 and Thr86 to be less exposed to other protein interactions when RACK1 is properly bound to the ribosome, while Ser276, Thr277, Ser278 and Ser279, which are part of a loop not covered by the rest of the protein, to be more available and more easily accessible also when RACK1 is properly ribosome-bound (Figure 2A-B). To analyze if our candidate phosphorylatable residues were conserved, we performed multiple alignments between the sequence of human RACK1 and that of its orthologues in various model organisms (Fig. 2D). RACK1 is a very evolution-

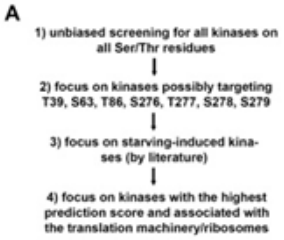
ary-conserved protein and, in fact, all of the seven residues we pinpointed are consistently maintained through most of the organisms analyzed. In particular, Thr39, Thr86 and Ser279 are conserved from the yeast *S. cerevisiae* to humans.

Then, we looked for potential kinases with the best consensus to phosphorylate the Ser/Thr residues we selected (Fig. 3A). The prediction analysis was carried out by feeding the human RACK1 protein sequence (https://www.ncbi.nlm.nih.gov/protein/NP_006089.1) into the GPS5 software (24,28). At first, to test the efficacy of the tool, we performed an unbiased screening, including all kinase families and all Ser/Thr residues. The software indicated Tyr52 to be phosphorylatable by Focal Adhesion Kinase PTK2B, and Tyr228 and Tyr246 to be phosphorylatable by Src (Figure 3B), as confirmed by the literature (26,27), indicating that the tool is efficient and reliable. Then, we focused on the putative phosphorylation of the seven Ser/Thr residues we determined as potentially relevant in RACK1 regulation. To further restrict the range of candidate protein kinases, we considered only those which are known to be present in the cytoplasm and to act upon nutrient/growth factor deprivation. We considered only the kinases with the top prediction scores and gave particular attention to the ones already known to be associated with the translational machinery and/or to interact with ribosomes. Considering all these parameters, AMPK1/2, PKR and ULK1 emerged as the strongest candidate kinases to phosphorylate RACK1 (Fig. 3C). Other notable players included: Jnk/SAPK1, a stress-related kinase known to interact with RACK1 in the context of translation regulation (12), and GCN2, an eIF2α kinase activated upon amino acids deprivation (29).

Altogether, the evidence we presented highlights RACK1 as a node where the translational machinery interfaces with nutrient-starving signaling and suggests that RACK1 release from the ribosome might be regulated by phosphorylation of key serine and threonine residues by kinases involved in growth factor signaling.

RACK1 is reported to interact with a surprisingly wide array of players, including ribosomal proteins and other components of the translational machinery and extra-ribosomal partners. On these bases, several functions have been proposed for RACK1, both in translation-related and unrelated contexts. However, a dichotomy emerges since most works look at RACK1 ribosomal and extra-ribosomal functions as two entirely separate fields, which are now quite puzzling to reconcile. In this work, we address RACK1 release from the ribosome as: i) mediated by phosphorylation on specific residues and ii) linking translation regulation with nutrient-starving signaling.

RACK1 phosphorylation on key amino acids which are in contact with the rRNA backbone disrupts RACK1 interaction with the ribosome, as shown in yeast by phospho-mimetic *asc1* mutants (16). RACK1 position on the ribosome suggests that only part of the candidate residues we propose can be phosphorylated at any given time. It is noteworthy that among the amino acids hidden under the RACK1 protein structure, Thr39 and Ser63 have been found to be phosphorylated upon nutrient/growth factor starving conditions (17). This suggests that RACK1 interaction with the ribosome can be modulated in order to expose those residues and allow for their phosphorylation. Ser276, Thr277, Ser278 and Ser279 could play a crucial



B

residue	prediction score	UniPROT ID	kinase
Tyr52	578.695	Q14289	PTK2B/ FAK2
Tyr228	18.896	P12931	SRC
Tyr246	14.706		

C

residue	prediction score	UniPROT ID	kinase	known involvement in translation?
Thr39	60.886	Q13131	AMPK α1	yes, direct mTORC1 inhibitor
Ser279	54.568	P54646	AMPK α2	yes, direct mTORC1 inhibitor
Ser276	16.868	Q96RR4	CaMKK2	no
Ser63	11.077	P53355	DAP kinase 1	no
Thr39	2.068	Q9UIK4	DAP kinase 2	no
Ser279	2.068			
Ser276	3.175	Q9P2K8	GCN2	yes, eIF2α kinase
Ser279	3.317			
Ser276	2.913	P45983	Jnk/ SAPK1	yes, RACK1 interactor
Thr39	70.079	P19525	PKR	yes, eIF2α kinase
Thr277	72.830			
Ser63	39.511	Q13464	ROCK1	no
Thr277	21.599	Q15831	STK11	no
Ser63	39.053	O75385	ULK1	yes, direct mTORC1 inhibitor
Ser63	3.130	Q8iYT8	ULK2	yes, direct mTORC1 inhibitor
Thr86	3.493			

Figure 3. *In silico* analyses pinpoint the putative kinases phosphorylating RACK1 upon starving. (A) Scheme representing the steps of our approach to screen for candidate kinase which phosphorylate RACK1. (B) Software prediction revealing that PTK2B, FAK and Src are putative kinases phosphorylating RACK1 on Tyr52, Tyr228 and Tyr246, as confirmed in the literature. (C) Software prediction of starving-activated kinases phosphorylating RACK1 on T39, S63, T86, S276, T277, S278 and S279. Kinases known to be directly involved in translation regulation are annotated, reflecting step 4 of the scheme in panel (A).

role by being phosphorylated first and mediating a partial detachment of RACK1 from the 40S ribosomal subunit. Notably, Thr39 is next to Lys38, which is one of the targeted residues in the R36D K38E RACK1 mutant. Moreover, Thr39 and the nearby residues are evolutionarily conserved from yeast to mammals, pinpointing this region of the protein as fundamental in establishing or disrupting the interaction between RACK1 and the ribosome.

We show on one side that RACK1 release from the ribosome is promoted by growth factor/nutrient starving, while, on the other, that an excess of ribosome-free RACK1 can activate an autophagy-like response even without actual starving conditions. Hence, RACK1 appears to be part of a more complex chain, which links the sensing of growth factors and nutrient availability to the appropriate physiological response. Indeed, in growing cells RACK1 resides on the ribosome where it enhances specific translation (9,11,13); conversely, extra-ribosomal RACK1 inhibits translation (13) and concomitantly activates autophagic fluxes. This supports the hypothesis that RACK1 release from the ribosome acts as a regulatory mechanism to repress specific translation (i.e., cap-dependent) when it is not useful for cells.

Deprivation of growth factors and/or nutrients is known to activate specific signaling pathways and repress translation through different means. Among all the kinases that could possibly phosphorylate RACK1, we found GCN2,

PKR and PERK, which act via eIF2α phosphorylation, and AMPK1/2 and ULK1/2, which are part of a common pathway that negatively regulates mTORC1 activity. Hypothetically, by triggering RACK1 release from the ribosome, such kinases would counteract the formation of active ribosomes and be even more efficient in their specific modulation of cellular metabolism.

When pathological conditions arise, for example when metabolism is altered, RACK1 could indeed become a valuable therapeutic target to be regulated. Such approach may also be exploited in a context of caloric restriction and would have particular relevance in cancer biology since RACK1 is highly expressed in cancer cells and its levels correlate with resistance to chemotherapy (30). In such a scenario, starvation coupled to treatment with anti-tumoral drugs targeting translation may develop a triple synergy against tumoral cells (as summarized in Figure 4).

In conclusion, we propose that RACK1 can be actively released from the ribosome by means of phosphorylation. In so doing, RACK1 would modulate both translation and starving signaling. These results represent an important advancement to further integrate the ribosomal and extra-ribosomal facets of RACK1 activity in cells.

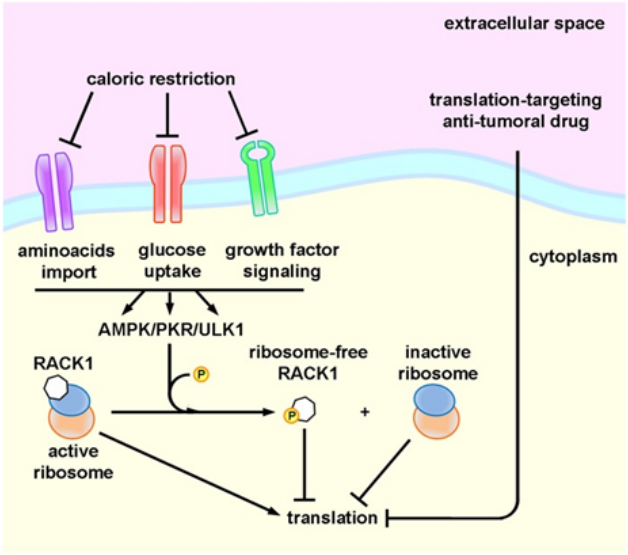


Figure 4. Scheme describing how RACK1 acts as a node linking starving responses and signaling pathways to translation regulation and how this could be exploited for cancer therapy. RACK1 release from the ribosome under starving conditions results in translationally inactive ribosomes and in ribosome-free RACK1 which acts, *per se*, as a translation repressor via signaling. In such a scenario, translation targeting by anti-tumoral drugs will generate a triple synergy of translation blockade.

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Interest conflict

We declare no competing financial interests or conflict of interest.

Consent for publication

The author read and proved the final manuscript for publication.

Author's contributions

SG designed and performed the experiments, analyzed data, and wrote the manuscript. NM performed the experiments, analyzed data, and wrote the manuscript. MK, GL, PC and MM performed the experiments. MI and all authors commented on and critically revised the manuscript.

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