Calmodulin interacts with Rab3D and modulates osteoclastic bone resorption

Sipin Zhu, Shek Man Chim, Taksum Cheng, Estabelle Ang, Benjamin Ng, Baysie Lim, Kai Chen, Heng Qiu, Jennifer Tickner, Huazi Xu, Nathan Pavlos & Jiake Xu

Calmodulin is a highly versatile protein that regulates intracellular calcium homeostasis and is involved in a variety of cellular functions including cardiac excitability, synaptic plasticity and signaling transduction. During osteoclastic bone resorption, calmodulin has been reported to concentrate at the ruffled border membrane of osteoclasts where it is thought to modulate bone resorption activity in response to calcium. Here we report an interaction between calmodulin and Rab3D, a small exocytic GTPase and established regulator osteoclastic bone resorption. Using yeast two-hybrid screening together with a series of protein-protein interaction studies, we show that calmodulin interacts with Rab3D in a calcium dependent manner. Consistently, expression of a calcium insensitive form of calmodulin (i.e. CaM1234) perturbs calmodulin-Rab3D interaction as monitored by bioluminescence resonance energy transfer (BRET) assays. In osteoclasts, calmodulin and Rab3D are constitutively co-expressed during RANKL-induced osteoclast differentiation, co-occupy plasma membrane fractions by differential gradient sedimentation assay and colocalise in the ruffled border as revealed by confocal microscopy. Further, functional blockade of calmodulin-Rab3D interaction by calmidazolium chloride coincides with an attenuation of osteoclastic bone resorption. Our data imply that calmodulin-Rab3D interaction is required for efficient bone resorption by osteoclasts in vitro.

Calmodulin is a versatile protein that regulates Ca(2+) homeostasis, synaptic plasticity, and cardiac excitability. It has been implicated in osteoclast differentiation, function, and survival. Calmodulin regulates Ca2+/calmodulin-dependent kinase II (CaMKII) and Ca2+/calmodulin-dependent protein phosphatase, calcineurin; both are critical for osteoclast differentiation. It also mediates osteoclast survival through a mechanism involving the binding of calmodulin with the death receptor Fas. In addition, calmodulin modulates acid transport and bone resorption. However, the underlining molecular events by which calmodulin regulates bone resorption remain to be elucidated.

Bone resorption is a highly regulated process that requires intense vesicular trafficking to sustain the structural and functional polarity of osteoclasts and enable efficient delivery of osteolytic cargo contents (i.e. Cathepsin K) and membrane exchange between the ruffled border and the opposing basolateral surface. Vesicular trafficking is modulated by sets of genetically conserved proteins among which members of the small Rab GTPase family are now firmly established regulators. To date, several Rab proteins have been implicated in bone resorption, although only Rab7 and Rab3D have been functionally characterized in osteoclasts. Rab7 is involved in the late endocytic pathway in the osteoclast polarization and bone resorption, whereas Rab3D modulates a post-TGN trafficking step that is required for maintenance of the osteoclastic ruffled border membrane and utilizes the dynin motor complex and microtubules via its direct interaction with Tctex-1 to facilitate vesicle delivery and/or retrieval during bone resorption.

Here, we identify calmodulin as a specific Rab3D interacting molecule by yeast two hybrid screening. We show that inhibition of calmodulin calcium binding perturbs this association in vivo by bioluminescence resonance energy transfer (BRET). Disruption of calmodulin-Rab3D interaction attenuated osteoclastic bone resorption in vitro.

1Department of Orthopaedics, The Second Affiliated Hospital, Wenzhou Medical University, Wenzhou, Zhejiang, 325027 China. 2School of Pathology and Laboratory Medicine, The University of Western Australia, Perth, WA 6009, Australia. 3Centre for Orthopaedic Research, School of Surgery, The University of Western Australia, Perth, WA 6009, Australia. 4School of Dentistry, Oral Biology Research Laboratory, The University of Western Australia, Perth, WA 6009, Australia. Correspondence and requests for materials should be addressed to H.X. (email: spine-xu@163.com) or N.P. (email: nathan.pavlos@uwa.edu.au) or J.X. (email: jiake.xu@uwa.edu.au)
We propose that calmodulin, via modulating calcium, imparts an additional layer of regulation on Rab3D trafficking during osteoclastic bone resorption.

Results

Calmodulin interacts with Rab3D. We have previously established a yeast two-hybrid approach to successfully uncover novel Rab3D interacting partners such as Tctex-1. Here we identify calmodulin as an additional binding partner of Rab3D. The interaction of calmodulin with Rab3D was verified by a yeast two hybrid assay, using a histidine-deficient plate (Fig. 1A). To further examine the interaction of calmodulin and Rab3D, we generated Rluc-calmodulin and EYFP-Rab3D fusion protein constructs and performed BRET protein-protein interaction assays. As shown in Fig. 1B, co-expression of Rluc and EYFP is shown as a negative control. Co-expression of Rluc and EYFP-Rab3D is shown as a negative control. (C) Flag-Rab3D proteins expressed in COS cells interact with calmodulin-saposin in the presence of 2 mM calcium. *Indicates p Value < 0.001 when compared with EYFP and Rluc. (D) Calmodulin calcium-insensitive mutant perturbs its interaction with Rab3D. Generation of a Rluc-calmodulin construct in which four aspartic acid residues at position 23, 59, 96, 132 were substituted with alanine, mimicking a calcium insensitive form of calmodulin. (E) BRET assays showing that the calcium insensitive form of calmodulin failed to interact with Rab3D. 1:1, 1:2 and 1:3 indicate that transfected plasmid ratio of EYFP-Rab3D/Rluc-calmodulin or EYFP-Rab3D/Rluc-calmodulin mutant 1234. Symbol *indicates p Value < 0.001 when compared with EYFP and Rluc-calmodulin control. Symbol # indicates p Value < 0.001 when compared Rluc-calmodulin with Rluc-calmodulin mutant 1234.

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calcium insensitive form of calmodulin18 (Fig. 1D). BRET assay results showed that the calcium insensitive form of camodulin attenuated the interaction with Rab3D (Fig. 1E).

The preferential interaction between Calmodulin and Rab3D in its GTP-bound conformation. Rab GTPases embed in organelle membranes via C-terminal prenylation motifs where they function as molecular switches that oscillate between GTP “active” and GDP “inactive” conformations. In their active state, Rabs recruit GTP-dependent effector proteins through which they elicit their biological function at various stages of vesicular transport. Therefore, we next asked whether the interaction between Calmodulin and Rab3D was dependent on the nucleotide and/or prenylation status of Rab3D. To access this, we employed several well characterised Rab3D variants16, which selectively disrupt the GDP/GTP exchange i.e. GTP-bound Rab3D (Rab3DQ81L), nucleotide empty RAB3D (Rab3DN135I) and prenylation motif deletion of Rab3D (Rab3ΔCXC) compared to wildtype Rab3D (Fig. 2A,B). These constructs were successfully expressed as EYFP fusion proteins in transfected COS cells as confirmed by western blot analyses (Fig. 2C). As with other bona fide Rab effector protein, calmodulin exhibited an enhanced association with a GTP-bound Rab3D (Rab3DQ81L) when compared to wild-type Rab3D, nucleotide empty Rab3D (Rab3DN135I) and prenylation motif deletion of Rab3D (Rab3ΔCXC) in BRET assays. *Indicates p Value < 0.001 when compared with EYFP and Rluc. # indicates p Value < 0.05 when compared to wild-type Rab3D, nucleotide-empty (Rab3DN135I) and prenylation motif deletion of Rab3D (Rab3ΔCXC).

Calmodulin and Rab3D are co-expressed during osteoclast formation and co-sediment in membrane fractions of osteoclasts. To begin to probe the potential relevance of the calmodulin-Rab3D association in osteoclasts, we first compared the gene expression profile of calmodulin and Rab3D in osteoclasts and their precursor cells. Bone marrow macrophages (BMM) were cultured in the presence of macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) for a period of 0, 1, 3, 5 days and then fixed and stained for tartrate resistant acid phosphatase (TRACP) activity, showing the presence of
multinucleated TRACP positive osteoclast like cells (Fig. 3A). In a parallel experiment, semi-quantitative RT-PCR was performed. Calmodulin gene expression appears to be constitutive during osteoclastogenesis by RANKL at an expression kinetic similar to that of Rab3D (Fig. 3B). Osteoclast marker gene expression of TRACP, Cathepsin K, V-ATPase d2, and calcitonin receptor (CTR) were induced by RANKL as compared to 36B4 gene expression as an internal control (Fig. 3B). Further, Western blot analysis showed that Calmodulin protein is constitutively expressed during osteoclastogenesis similar to Rab3D protein expression (Fig. 3C).

Next, sucrose gradient sedimentation assays were performed to examine calmodulin and Rab3D co-fractionation. Rab3D is present in small vesicles (F9-10) and large plasma membrane fractions (P) (Fig. 3D). Interestingly, calmodulin co-fractionated with Rab3D but only in the large membrane fraction (P) (Fig. 3D). By comparison, V-ATPase (d2) was also present in both vesicle and membrane fractions (Fig. 3D). Moreover, an association between Rab3D and calmodulin was further confirmed in bone-resorbing osteoclasts by immunofluorescence confocal microscopy (Fig. 4). In this instance colocalisation (yellow colour) was observed upon overlay of individual fluorescent channels for Rab3D (green) and Calmodulin (red), which were detected using antibodies specific to Rab3D and calmodulin, as validated in the Western blot analysis above (i.e. Fig. 3C). Interestingly, a subset of Rab3D-calmodulin colocalisation was observed within the F-actin ring/sealing zone (blue) that typically denotes the ruffled border membrane (Fig. 4A, region circumscribed by red line). This colocalisation was confirmed by correlative linescan analysis (Fig. 4B) which revealed overlap between the fluorescent peaks of Rab3D and calmodulin within the ruffled border region (Fig. 4A, white dashed line).

**Functional blockade of the calmodulin-Rab3D interaction by calmidazolium chloride attenuates osteoclastic bone resorption.** Finally, we set out to define the impact of the interaction of calmodulin with Rab3D on osteoclast function. To this end, we first tested the effect of calmidazolium chloride on the interaction of calmodulin and Rab3D using BRET assays. Interestingly, calmidazolium chloride perturbs the association of calmodulin and Rab3D, Rab3DQ81L, Rab3DN135I and Rab3ΔCXC (Fig. 5A). Further, to examine the effect of calmidazolium on osteoclastic bone resorption, BMM derived osteoclasts were seeded into bone slices in the presence and absence of calmidazolium chloride for 24 hours. Treatment of osteoclasts with calmidazolium chloride inhibited osteoclastic bone resorption (Fig. 5B) but did not affect the total number (Fig. 5C) and morphology of TRACP positive osteoclastic like cells at 1 μM and 5 μM (Fig. 5D). Taken together, these data suggest that
treatment of calmidazolium chloride perturbs the interaction of calmodulin with Rab3D, an effect that coincides with the attenuation of osteoclastic bone resorption in vitro.

Discussion

Calmodulin has versatile roles in regulating intracellular calcium homeostasis and diverse cellular processes including osteoclastic bone resorption. In this study we document that calmodulin interacts with Rab3D in a calcium-dependent manner. Both calmodulin and Rab3D proteins co-occupy membranes factions by a sucrose gradient ultracentrifugation sedimentation assay and colocalise in the ruffled border by confocal microscopy. Functional blockade of calmodulin and Rab3D interaction by calmidazolium chloride resulted in an attenuation of osteoclastic bone resorption. Considering that calmodulin is concentrated on the ruffled border membrane in osteoclasts, and that Rab3D is a functional requirement for ruffled border maintenance, it is plausible that calmodulin, through its interaction with Rab3D facilitates the delivery and calcium-sensitively of Rab3D-bearing vesicles at the ruffled border membrane during bone resorption (Fig. 6).

Bone resorption by osteoclasts is a multi-step process which culminates in the removal of an inorganic mineral layer primarily composed of crystalline hydroxyapatite and subsequent degradation of the underlying organic phases. This process involves continual content delivery and membrane recycling through vesicle trafficking, governed largely small Rab GTPases. Rab proteins have been implicated in the regulation of distinct events in vesicle transport on the exocytotic, endocytotic and transcytotic pathways. Because of this, in recent years, there has been mounting interest surrounding the role of Rab3D in intracellular transport. Rab3D was shown to mediate exocytosis in mast cells, adipocytes, and acini cells. GTPase-deficient Rab3D decreased nutrient induced insulin release. Moreover, actin coating of secretory granules during regulated exocytosis has been shown to correlate with the release of Rab3D. In addition, Rab3D has been suggested to play a role in the regulation of apically directed transcytosis in rat hepatocytes and appears to be essential for apical transport in polarized epithelia. Our previous data indicates that Rab3D modulates a post-TGN trafficking step that is required for osteoclastic bone resorption. Furthermore, we have shown that Rab3D interacts with Tctex-1 which regulates microtubule-directed trafficking of Rab3D vesicles via cytoplasmic dynein. The present study further extends the role of Rab3D in bone resorption by binding to calmodulin; a molecule previously implicated in acid secretion and bone resorption.

During the formation of ruffled border membrane domains, the osteoclastic plasmalemma can be further divided into several specialised functional domains. These include the basolateral domain and a functional secretory domain. In fact, more recent evidence suggests that the ruffled border is not a continuous domain but rather segregated into an “uptake” and “secretory” domain reflecting its dynamic endo-exocytic intracellular trafficking routes. Therefore it is likely that Rab3D mediates bone resorption along the exocytic pathway through its

Figure 4. Colocalisation between Rab3D and calmodulin in bone-resorbing BMM-derived osteoclasts. (A) Representative confocal microscopy images of individual and overlay fluorescent channels of Rab3D (green), calmodulin (red), F-actin (blue) and nuclei (magenta). Colocalisation between Rab3D and calmodulin appears as yellow in overlay. Red line demarcates the ruffled border region within the sealing zone. White line corresponds to the correlational linescan analysis in (B). Bar = 10 μm.
direct interaction with calmodulin that is located in the ruffled border membranes. In other systems, calmodulin has been shown to stimulate GTP binding to Rab3A that is complexed with GDI, which leads to the formation of an active GTP-bound form of the Rab3A-Ca (2+) /Calmodulin complex in synaptic membranes of activated nerve termini. Similarly, interaction between Rab3B and calmodulin was Ca (2+) -dependent. These findings provide evidence that Rab3B is primarily localized with the particulate fraction and that Ca (2+)/calmodulin could regulate function of this GTPase in platelets. It remains to be elucidated whether other Rab proteins might also interact with calmodulin and facilitate these molecular pathways in osteoclasts and other cells.

Previously, structural analysis revealed four Ca (2+) -binding domains in calmodulin that are important for the function of calmodulin, together with hydrophobic regions represent the sites of interaction with pharmacological agents. The three-dimensional structure of calmodulin has been determined crystallographically at 3.0 Å resolution. The molecule consists of two globular lobes connected by a long exposed alpha-helix, and each lobe is able to bind two calcium ions through helix-loop-helix domains. The flexibility of the protein may explain the fact that calmodulin is able to bind many different targets. We have found that the interaction of calmodulin with Rab3D is calcium dependent. During osteoclastic bone resorption, free calcium is to be released in the resorption compartment, which could in turn further facilitate the interaction of calmodulin with Rab3D. It has been suggested that a Rab3-calmodulin complex generated by elevated Ca (2+) concentrations mediated at least some of the effects of the GTPase and limited the number of exocytic events that occurred in response to secretory stimuli. We propose that the recruitment of calmodulin by Rab3D is an important requirement for osteoclast-mediated bone resorption.

**Figure 5. Functional blockade of the interaction of Rab3D and calmodulin by calmidazolium chloride attenuates osteoclastic bone resorption.** (A) BRET assays showing the effect of calmidazolium chloride on the interaction of calmodulin and Rab3D, Rab3DQ81L, Rab3DN135I and Rab3DΔCXC; respectively. (B) Treatment of osteoclasts with calmidazolium chloride inhibits osteoclastic bone resorption with quantitative analysis of bone resorption areas. BMM derived osteoclasts were seeded into bone slices in the presence and absence of calmidazolium chloride for 24 hours. (C) Total number of TRACP positive osteoclastic like cells. (D) Representative images of bone resorption assays showing the effect of calmidazolium on TRACP positive osteoclast morphology (upper panel), and osteoclastic bone resorption SEM images (lower panel). Scale bars are shown. * and #Indicate p Value < 0.001, and < 0.05 respectively when compared to untreated control.
Identification of interacting partners to Rab proteins will be important for drug design, for instance, Plekhm1 was found to co-localize with Rab7 to late endosomal/lysosomal vesicles, a putative function in vesicular transport in the osteoclast. This has been implicated in the development of osteopetrosis. Interestingly, alendronate inactivates osteoclasts by mechanisms that impair their intracellular vesicle transport, with apoptosis being only a secondary phenomenon to this. The anti-resorptive activity of NE10790 is thus likely due to disruption of Rab-dependent intracellular membrane trafficking in osteoclasts. Given that Rab-dependent intracellular membrane trafficking in osteoclastic bone resorption has been proposed to be a target of nitrogen-containing bisphosphonate drug NE10790, defining Rab3D interacting partners might facilitate the design of the next generation of bisphosphonate drugs.

Materials and Methods

Two-hybrid screening. Mouse Rab3D cDNA was inserted into pBTM116 and used to screen a pVP16-based yeast two-hybrid cDNA library as previously described. Briefly, for cDNA library screening, the yeast reporter strain L40 was first transfected with baits pLexA-Rab3D and subsequently transfected with the pVP16 mouse embryo cDNA library using lithium acetate and polyethylene glycol. Library plasmids were grown in the presence or absence of histidine. Positive clones isolated from the cDNA library were further analysed by co-transfection with pLexA-Rab3D and DNA sequencing.

RT-PCR. To determine the calmodulin and Rab3D gene transcripts in osteoclasts, total RNA was isolated from bone marrow cells treated with RANKL at various time points according to the manufacturer’s instructions (Qiagen, Sydney). For RT-PCR, single-stranded cDNA was prepared from 2 μg of total RNA using reverse transcriptase with an oligo-dT primer. Two μl of each cDNA was subjected to 30 cycles of PCR (94°C, 40 sec; 55°C, 40 sec; and 72°C, 40 sec) using specific primers to mouse rab3d gene (forward: 5′-ATG GCA TCC GCT AGT GAG-3′; reverse: 5′-CTA ACA GCT GCA GCT GCT-3′) and calmodulin (forward: 5′-CCA TGG CTG ACC AGC TGA-3′; reverse: 5′-GCT TCA CTT TGC AGT CAT-3′). Osteoclast markers were also used; including cathepsin K (forward: 5′-CCA GTG GGA GCT ATG GAA GA-3′; reverse: 5′-AAG TGG TTC ATG GCC AGT TC-3′); calcitonin receptor (forward: 5′-GGG ACT TTG ACA CAG CAG AA-3′; reverse: 5′-CGG ACT TTG ACA CAG AA-3′); V-ATPase-d2 (forward: 5′-GTG AGA CCT TGG AAG ACC AGC TGA-3′; reverse: 5′-GAG AAA TGT GCT CAG GGG CT-3′). As an internal control, the single stranded cDNA was PCR-amplified for 25 cycles using 36 B4 primers (forward: 5′-CTA TTG TGG GAG CAG ACA-3′; reverse: 5′-TCC TCC GAC TCT TCC TTT-3′).

In vitro protein interaction. The full length of mouse Rab3D cDNA was subcloned into Flag-tagged expression vector with CMV promoter to generate a Flag-Rab3D plasmid. To express Rab3D protein, 5 x 10^5 COS-7 cells was transfected with 10 μg of Flag-Rab3D plasmid using electroporation reagents (Qiagen, Sydney). After incubation for 48 hours, transfected cells were washed twice with PBS and lysed for 30 mins on ice with 750 μl of lysis buffer (50 mM Tris.Cl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 M EDTA, 1 μg/ml pepstatin A). Following centrifugation at 12,000 RPM for 5 mins at 4°C, supernatant was collected and 750 μl of binding buffer (20 mM Tris. Cl, pH 7.5, 50 mM KCl, 100 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 5 mM DTT) was added to the supernatant and mixed. One half of the sample was added to 50 μl of calmodulin immobilized in sepharose beads (Sigma, Sydney) in the presence and absence of 2 mM of calcium. The mixture was incubated overnight at 4°C with a shaker. The beads were washed three times with 300 μl of buffer containing equal volumes of lysis buffer and binding buffer. The bound proteins were boiled with 1 x SDS-PAGE sample buffer and analysed by Western blot analysis using an anti-FLAG monoclonal antibody (Sigma, Sydney).

Figure 6. A working model illustrating that calmodulin is concentrated on the ruffled border membrane in osteoclasts and mediates bone resorption process. Rab3D interaction with calmodulin is implicated for a role in bone resorption when Rab3D-bearing vesicles reach the resorbing ruffled border compartment of an osteoclast.
Construction of EYFP-Rab3D and Rluc-calmodulin. EYFP-Rab3DWT, EYFP-Rab3DQ81L, EYFP-Rab3DN135I, and EYFP-Rab3DΔCXC fusion constructs were reported previously. To generate Rluc-calmodulin construct, calmodulin mRNA was PCR-amplified with primers (forward: 5′-AAG AAT TCG GAG CAT GCC TCT GCA GCT CTG-3′; reverse: 5′-GGA TGC TCT AGA CTT TGA CCT TGC AGT CAT-3′). The amplified fragment was cloned into a PCR 2.1 T/A-cloning vector as outlined by the manufacturers’ instructions. The BamHI and EcoRI fragment was then cloned into the BamHI and EcoRI sites of pcDNA3.1-Rluc (Invitrogen) to make pcDNA-Rluc as previously described. To generate a Rluc-calmodulin calcium insensitive mutant, pcDNA3.1 calcium insensitive calmodulin mutant, in which the first Asp of each EF-hand is changed to Ala, was obtained from Dr. Blaise Z. Peterson and then subcloned into the pcDNA3.1-Rluc. These constructs were sequenced to confirm. Bioluminescence resonance energy transfer (BRET) assay and cell transfections were performed in COS cells as previously described using the Mithras LB940 BRET plate reader (Berthold Technologies, Inc., Germany).

Generation and isolation of osteoclastic cells and bone resorption assay. OCs were generated from mouse BMMs treated with RANKL (100 ng/ml) and M-CSF (10 ng/ml) in vitro as previously described. Bone resorption assay was carried out using mouse BMM-derived mature osteoclasts as previously described.

Sucrose gradient ultracentrifugation. Sucrose gradient centrifugation was performed as described previously with minor modifications. Briefly, ~3 × 10^6 OCs were pelleted, 500 μl of the clarified supernatant was layered on top of 12 ml of 5–20% linear sucrose density gradient prepared in the lysis buffer without Triton X-100. After centrifugation at 150,000 g for 18 h in a SW40 rotor (Beckman Coulter), 1 ml fractions were collected and analyzed by immunoblotting using antibodies to GFP (Santa Cruz, CA, USA), Rab3D (SynapticSystems, Goettingen), V-ATPase d2, and calmodulin (Sigma, Sydney).

Immunofluorescence confocal microscopy. Immunofluorescence detection of Rab3D in osteoclasts by confocal microscopy was performed as previously described. Briefly BMM-derived osteoclasts cultured on bone for 7–10 days were fixed with 4% paraformaldehyde, permeabilised with Triton X-100, blocked in 0.2% BSA-PBS, and immunostained with primary antibodies against Rab3D (Rabbit polyclonal; SynapticSystems, Goettingen) or calmodulin (mouse monoclonal, Sigma, Sydney) and then corresponding secondary Alexa-Fluor conjugated antibodies against rabbit (Alexafluor-488) and mouse Alexafluor-555). F-actin rings were detected by staining with Alexfluor647-conjugated Phalloidin (Molecular Probes, USA) and nuclei were visualised by staining with DAPI (Sigma, USA).

Statistics. The results are representative of at least three independent experiments. Single comparison tests were performed by using paired Student’s t-test in Microsoft Excel. All data are presented as the mean ± standard error of the mean (SEM). Statistical significance was determined at P values < 0.05.

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Author Contributions

Sipin Zhu, Shek Man Chim, Taksum Cheng, Estabelle Ang, Kai Chen, Heng Qiu carried out experiments and data analysis. Benjamin Ng, Baysie Lim, Jennifer Tickner provided technical assistance. Sipin Zhu and Jiakexu participated in draft manuscript. Jennifer Tickner, and Nathan Pavlos revised manuscript. Hua-Zi Xu, Nathan Pavlos and Jiakexu supervised the project, experimental designs and approved the final manuscript.

Additional Information

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