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Phosphate (P\textsubscript{i})-regulated heterodimerization of the high-affinity sodium-dependent P\textsubscript{i} transporters PiT1/Slc20a1 and PiT2/Slc20a2 underlies extracellular P\textsubscript{i} sensing independently of P\textsubscript{i} uptake

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Extracellular phosphate (P\textsubscript{i}) can act as a signaling molecule that directly alters gene expression and cellular physiology. The ability of cells or organisms to detect changes in extracellular P\textsubscript{i} levels implies the existence of a P\textsubscript{i}-sensing mechanism that signals to the body or individual cell. However, unlike in prokaryotes, yeasts, and plants, the molecular players involved in P\textsubscript{i} sensing in mammals remain unknown. In this study, we investigated the involvement of the high-affinity, sodium-dependent P\textsubscript{i} transporters PiT1 and PiT2 in mediating P\textsubscript{i} signaling in skeletal cells. We found that deletion of PiT1 or PiT2 blunted the P\textsubscript{i}-dependent ERK1/2-mediated phosphorylation and subsequent gene up-regulation of the mineralization inhibitors matrix Gla protein and osteopontin. This result suggested that both PiTs are necessary for P\textsubscript{i} signaling. Moreover, the ERK1/2 phosphorylation could be rescued by overexpressing PiT2 transport-deficient PiT mutants. Using cross-linking and bioluminescence resonance energy transfer approaches, we found that PiT1 and PiT2 form high-abundance homodimers and P\textsubscript{i}-regulated low-abundance heterodimers. Interestingly, in the absence of sodium-dependent P\textsubscript{i} transport activity, the PiT1-PiT2 heterodimerization was still regulated by extracellular P\textsubscript{i} levels. Of note, when two putative P\textsubscript{i}-binding residues, Ser-128 (in PiT1) and Ser-113 (in PiT2), were substituted with alanine, the PiT1-PiT2 heterodimerization was no longer regulated by extracellular P\textsubscript{i}. These observations suggested that P\textsubscript{i} binding rather than P\textsubscript{i} uptake may be the key factor in mediating P\textsubscript{i} signaling through the PiT proteins. Taken together, these results demonstrate that P\textsubscript{i}-regulated PiT1-PiT2 heterodimerization mediates P\textsubscript{i} sensing independently of P\textsubscript{i} uptake.

Phosphorus is the sixth most abundant element in the human body, constituting ~1% of total body weight (1). About 85% of total phosphate can be found in the skeleton, where it is a major constituent of hydroxyapatite crystals deposited on the extracellular organic matrix during the mineralization process. The remaining 15% is found mainly in cells from soft tissues and in extracellular volume, where it represents <1% of total phosphate (2–4). In plasma, ~16% of circulating phosphate is found as organic phosphate bound to proteins and lipids, whereas the major part (84%) is orthophosphate, or free inorganic phosphate (Pi),\textsuperscript{5} that can be filtered by the kidney (1). At physiological pH, the monovalent H\textsubscript{2}PO\textsubscript{4} is and the divalent HPO\textsubscript{4} forms are present at a 1:4 molar ratio (5). Although this plasma Pi represents a small fraction of total body phosphorus, it serves as an exchange pool between the various Pi-containing and -regulating organs, and disturbances in Pi homeostasis can affect almost all organ systems.

In addition to the widespread structural and metabolic functions of Pi, it has become increasingly apparent during the past 15 years that extracellular Pi can act as a signaling molecule directly altering gene expression and cell phenotype (6–9). The abundance of Pi in the skeleton has led to early studies describing the effects of extracellular Pi in this organ. Exposing cultured chondrocytes to a high level of extracellular Pi leads to their terminal maturation and subsequent matrix mineralization (10–13). Similarly, the apoptosis of terminally differentiated hypertrophic chondrocytes was shown to be dependent upon the circulating plasma Pi levels in vivo (14). In both of these in vitro and in vivo approaches, the Pi-mediated apoptosis of chondrocytes is dependent upon the activation of the MAPK ERK1/2 pathway (15–17), but not of other mitogen-

\textsuperscript{5}The abbreviations used are: Pi, inorganic phosphate; iLoop, large intracellular loop; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; eYFP, enhanced yellow fluorescent protein; BRET, bioluminescence resonance energy transfer; qPCR, quantitative PCR; F1–F3, fractions 1–3, respectively.

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This article contains Figs. S1–S5.

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activated protein kinases, such as p38 or c-Jun N-terminal kinase. Interestingly, the Pi-dependent activation of the ERK1/2 pathway up-regulated the gene expression of the mineralization inhibitors matrix Gla protein (Mgp) and osteopontin (Opn), most likely setting off a feedback mechanism to control Pi-induced mineralization (7, 16, 18, 19). The elevated extracellular Pi level was also shown to be important in osteoblast proliferation and differentiation (16, 20–24), cementoblast formation (25), odontoblast differentiation (26, 27), and osteoclast differentiation (28–30).

The Pi-mediated signaling underlies the notion that cells must possess a Pi-sensing mechanism on the surface of or within the cell that is able to detect and respond to the variation of extracellular Pi levels. The ability of organisms to detect changes in extracellular levels of other metabolites (such as Ca$^{2+}$, glucose, or amino acids) has already been described (31–33), and emerging evidence suggests that similar events are at work to mediate the cellular effects of Pi (8, 34–36). Although the identity of the molecules involved in these mechanisms is still unknown in mammals, Pi-sensing machineries have been characterized in prokaryotic and eukaryotic unicellular organisms (37). In *Escherichia coli*, the phosphate transporter PstS and other periplasmic proteins (PstC, Pst, and PstB/PhoU) detect the variation of external Pi concentrations. In case of a low extracellular Pi level, this system increases the efficiency of Pi retention in the bacteria (38). In *Saccharomyces cerevisiae*, a low extracellular Pi level resulted in induction of the Pi transporter Pho84, now identified as the essential component of the Pi-sensing system (38, 39). Interestingly, following Pi restriction, it was demonstrated that Pho84 could trigger the rapid activation of protein kinase A without transporting Pi (40).

In mammals, the Slc20a1/PiT1 and Slc20a2/PiT2 proteins are expressed at the plasma membrane and have been described as mediating the intracellular uptake of Pi with a high affinity (41–43). PiT1 and PiT2 have a wide tissue distribution, being the only Pi transporters expressed in bone (44, 45). Interestingly, their expression can be modulated by extracellular Pi (21, 43, 44, 46, 47), and previous studies have suggested that they can mediate downstream effects of extracellular Pi. In bone, the elegant study of Kimata et al. (48) suggests that the chondrocyte response to extracellular Pi is mediated by a PiT1-dependent up-regulation of cyclin D1 through ERK1/2 pathway activation. The authors hypothesize that PiT1-driven conformational changes of PiT1 could be involved in the Pi-sensing mechanism. In parathyroid cells, PiT1 was suggested to act as a Pi sensor to modulate the secretion of the phosphaturic parathyroid hormone (49). On the other hand, based on its property of oligomerizing upon extracellular Pi variation, PiT2 was also proposed to serve as a Pi sensor (50). Although these data support a possible role for PiT1 or PiT2 as Pi sensors, little is known about the underlying mechanisms. Because PiT1 and PiT2 have very close Pi transport characteristics (51), they may also share Pi-sensing properties and thus have interconnected roles in Pi sensing. Moreover, because PiT-independent functions have been highlighted recently for PiT1 (52–56), the involvement of Pi transport in the Pi sensing by PiT1 or PiT2 remains to be investigated.

In this report, we investigated the role of PiT1 and PiT2 as Pi sensors in osteoblastic and chondrocytic cell lines. We show that both PiT1 and PiT2 are required for mediating Pi-dependent signaling. We demonstrate that PiT1 and PiT2 could interact together and that extracellular Pi modulates this interaction. Finally, we show that cellular Pi uptake is not required to mediate Pi signaling through the PiT proteins.

Results

Requirement of both PiT1 and PiT2 for Pi-mediated signaling

We first investigated whether PiT1 and/or PiT2 were involved in the Pi-dependent up-regulation of Mgp and Opn expression. To this aim, using RNA interference, we established stably transfected osteoblastic MC3T3-E1 clones in which PiT1 or PiT2 expression was knocked down. In MC3T3-E1 shPiT1 clones, PiT1 gene expression showed a 63% reduction, together with a significant up-regulation of PiT2 (Fig. 1A). Similarly, the MC3T3-E1 shPiT2 clones displayed a 62% decrease in PiT2 mRNA level, together with a significant up-regulation of PiT1 (Fig. 1A). Comparable results were observed when cells were incubated with 10 mM extracellular Pi concentration (Fig. S1A). Interestingly, the sodium-dependent Pi uptake was similar in MC3T3-E1 shPiT1 and shPiT2 clones and control MC3T3-E1 cells (Fig. 1B), suggesting that a depletion of either PiT may be compensated by the remaining PiT, as was previously suggested (56). Consistent with this possibility, MC3T3-E1 clones stably transfected with both shPiT1 and shPiT2 resulted in a 52% reduction of both PiTs (Fig. 1A), resulting in a similar decrease in sodium-dependent Pi uptake (Fig. 1B). In contrast to wild-type differentiated MC3T3-E1 cells in which Mgp and Opn expression was up-regulated following stimulation with 10 mM extracellular Pi for 24 h, the up-regulation of Mgp and Opn expression in PiT-depleted MC3T3-E1 clones was blunted (Fig. 1C). The defect in PiT-dependent Mgp and Opn up-regulation arose despite a normal Pi transport in the shPiT1 or shPiT2 MC3T3-E1 clones, suggesting that a variation in intracellular Pi content is unlikely to account for defects in PiT-dependent signaling in the absence of either PiTs. Because the ERK1/2 signaling pathway was shown to be required for PiT-dependent regulation of Mgp and Opn expression (16, 19), we investigated the PiT-dependent ERK1/2 activation in differentiated PiT-depleted MC3T3-E1 clones. We showed that following a 30-min (Fig. S1B) or 24-h (Fig. 1D) stimulation with 10 mM extracellular Pi, the activation of ERK1/2 pathway was blunted in shPiT1, shPiT2, or shPiT1/shPiT2 clones, as compared with untransfected and shScramble-transfected cells. Similar data were obtained in three separate PiT-depleted MC3T3-E1 clones (Fig. S1, B–D) and in transiently transfected MC3T3-E1 cells (Fig. S2). Interestingly, the effect of PiT depletion on the activation of ERK1/2 pathway following stimulation with 10% fetal bovine serum (FBS) was not as pronounced as PiT stimulation, arguing for a specificity of PiT1 and PiT2 in the PiT-dependent activation of the ERK1/2 pathway.

Moreover, we performed similar experiments in the MC615 chondrogenic cell line. We used a transient transfection approach leading to a 50 and 56% deletion of PiT1 and PiT2 mRNA levels, respectively (Fig. 2A). A similar extent of PiT
deletion was obtained at the protein level, as shown by immuno- 
fluorescence (Fig. S3). MC615 cells were also used to rescue 
PiT deletion by overexpressing human PiT1 and PiT2 in PiT1- 
and PiT2-depleted cells, respectively (Fig. 2B). Similar to what 
was observed in MC3T3-E1 cells, depletion of either PiT1 or 
PiT2 in MC615 cells blunted the activation of the ERK1/2 path-
way by 10 mM extracellular Pi, despite the up-regulation of the 
remaining PiT (Fig. 2C). When human PiT2 was overexpressed 
in PiT1-depleted MC615 cells, we could rescue the PiT-depen-
dent ERK1/2 phosphorylation (Fig. 2C). Similar results were 
obtained when human PiT2 was overexpressed in PiT2-de-
pleted MC615 cells or when both human PiT1 and PiT2 were 
overexpressed in PiT1–PiT2–depleted MC615 cells (Fig. 2C). 
This further illustrated the requirement of both PiT1 and PiT2 
for the PiT-dependent ERK signaling in cell lines of skeletal 
origin.

PiT1 and PiT2 form hetero-oligomers upon variation of 
external Pi concentrations

The requirement of both PiT1 and PiT2 for Pi-dependent 
ERK1/2 signaling may indicate the existence of a functional 
protein complex comprising both PiTs. This possibility is also 
reinforced by the presence in PiT1 and PiT2 protein sequences 
of a conserved and highly hydrophobic 127-amino acid domain 
that was suggested to be important for determining the quater-
nary structure of the protein (57). To investigate the formation of hetero- and homo-oligomers, we used HEK293T cells that 
are easy to transfect, allow a robust expression of PiTs at the 
plasmamembrane, and have been shown to have similar Pi-med-
iated ERK1/2-specific activation (58). In addition, because PiT 
proteins are often difficult to detect using total cell extracts in 
Western blot experiments, we used a crude cell fractionation 

Figure 1. Pi-dependent Mgp and Opn gene regulation and ERK1/2 signaling require both PiT1 and PiT2 in MC3T3-E1 cells. A, RT-qPCR analysis of PiT1 (white bars) and PiT2 (black bars) mRNA levels in untransfected (UT) or stably transfected MC3T3-E1 cells, as indicated. Data are means ± S.E. (*, versus shScramb, 

p < 0.05, n = 3). B, sodium-dependent Pi uptake was measured in untransfected or stably transfected MC3T3-E1 cells, as indicated. Data are means ± S.E. (n = 3). C, RT-qPCR analysis of Mgp and Opn mRNA levels in untransfected or stably transfected MC3T3-E1 cells, as indicated. Cells were incubated in low-serum (0.5%) medium for 24 h and stimulated with 1 mM (white bars) or 10 mM (black bars) extracellular Pi concentration for 24 h. Data are means ± S.E. (error bars) (#, 
p < 0.05; ##, p < 0.01 versus 1 mM Pi control; and *, p < 0.05; **, p < 0.01 versus shScramb; n = 3). D, Western blot analysis of ERK1/2 phosphorylation (P-ERK 1/2) in untransfected or stably transfected MC3T3-E1 cells, as indicated. Cells were incubated in low-serum (0.5%) medium for 24 h and stimulated for another 24 h with 1 mM or 10 mM extracellular Pi concentration or with 10% FBS used as a positive control for ERK1/2 phosphorylation. Total ERK1/2 proteins were used as a loading control.
approach to analyze an enriched plasma membrane protein fraction revealed by the specific expression of the Na/K-AT\(^{+}\)-Pase, as shown in Fig. 3A. When analyzing the enriched plasma membrane fraction from PiT1- and/or PiT2-transfected cells by Western blotting, we could observe protein complexes after cellsurface cross-linking using BS\(^{3}\), a membrane-impermeable cross-linker (Fig. 3B). The apparent molecular mass of 142–165 kDa that we detected from cells transfected with hPiT2 alone recapitulated the results obtained by Salau\"en et al. (50) demonstrating the formation of PiT2 homodimers. Similarly, the 151–188 kDa apparent molecular mass band detected from cells transfected with hPiT1 is consistent with the association of two PiT1 molecules. When cells were co-transfected with both hPiT1 and hPiT2, no change was observed in the band profile, apart from a less intense signal due to transfection with 50% fewer hPiT1 or hPiT2 plasmids. In this condition, we could not detect a distinct band at the theoretical PiT1-PiT2 heterodimer molecular mass, most likely due to the expected similar molecular weights of PiT homo- and heterodimers.

To study further the PiT oligomerization, we then used a BRET approach. To this aim, we constructed hPiT1 and hPiT2 chimeric proteins expressing the eYFP acceptor or Rluc donor. Because structure-function studies have excluded a role of the large intracellular loop (iLoop) in Pi transport and retrovirus binding (59–62) and showed no overlapping between iLoop and the highly hydrophobic domain (57), we substituted the iLoop with eYFP and Rluc sequences (Fig. 3C). When expressed in HEK293T, the chimeric hPiT1-eYFP or -Rluc and hPiT2-eYFP or -Rluc proteins could be visualized at the plasma membrane, as shown by confocal microscopy (Fig. 3D), enabling us to study their role in detecting the variation of extracellular Pi levels. We performed saturation BRET experiments in living cells to investigate their potential hetero- and homo-oligomerization. As shown in Fig. 3E, we obtained typical BRET-saturable curves when using hPiT1-eYFP and hPiT2-Rluc proteins, together with a high BRET ratio. In contrast, when the BTN3A2 protein was used instead of either PiT, no saturation could be achieved, together with a weak BRET ratio (Fig. S4, A and B). These data support the notion that PiTs can form hetero- and homo-oligomers specifically. We confirmed the specificity of the hetero-oligomers by a competition assay (Fig. S4C) whereby overexpression of untagged PiT1 or PiT2 reduces the BRET ratio, whereas unrelated BTN3A2 expression does not. We next determined whether the interaction of PiTs could be modulated by the variation of extracellular Pi concentration. We therefore performed the same saturation BRET experiments after a 10-min stimulation with 1, 3, or 10 mM extracellular Pi concentration. Results reported in Fig. 3 (F and G) and Fig. S5A showed that saturation curves were different upon extracellular Pi concentration only for hPiT1-hPiT2 hetero-oligomers. Indeed, calculating the BRET 50 values from these curves, we showed a significant decrease at 10 mM extracellular Pi, suggesting a stronger interaction between PiT1 and PiT2 in this condition, whereas no variation was observed for homo-oligomers. In all conditions, the BRET max values did not vary significantly (Fig. S5B).

Figure 2. Pi\(^{-}\)-dependent ERK1/2 signaling requires both PiT1 and PiT2 in MC615 cells. A, RT-qPCR analysis of mPiT1 (white bars) and mPiT2 (black bars) mRNA levels in transiently transfected MC615 cells, as indicated. Data are means \(\pm\) S.E. (error bars) (**, \(p < 0.01\); ***, \(p < 0.001\) versus shScramb; \(n = 3\)). B, RT-qPCR analysis of hPiT1 (white bars) and hPiT2 (black bars) mRNA levels in transiently transfected MC615 cells, as indicated. The endogenous murine PiT1 or PiT2 genes were used as reference genes to evaluate the overexpression of the transfected human PiT genes. Data are expressed as mean \(\pm\) S.E. (***, \(p < 0.001\) versus shScramb + pcDNA, \(n = 3\)). N/A, not applicable. C, Western blot analysis of ERK1/2 phosphorylation (P-ERK 1/2) in transiently transfected MC615 cells, as indicated. Cells were incubated in low-serum (0.5%) medium for 24 h and stimulated for 30 min with 1 mM or 10 mM extracellular Pi concentration as indicated. Total ERK1/2 proteins were used as a loading control.
Pivotal role of PiT1 and PiT2 in bone phosphate sensing

A

|      | Ctrl  | hPIT1+hPIT2 |
|------|-------|-------------|
| F1   |       |             |
| F2   |       |             |
| F3   |       |             |

Na/K-ATPase: 87 kDa
PiT1: 79 kDa
PiT2: 75 kDa

B

|      | NPT1 | NPT2 | NPT1+NPT2 | UT |
|------|------|------|-----------|----|
| F1   |      |      |           |    |
| F2   |      |      |           |    |
| F3   |      |      |           |    |

IB PiT1: 151-188 kDa
IB PiT2: 142-165 kDa

C

hPIT1 or hPIT2

hLoop1, hLoop2

D

hPIT1-eYFP / hPIT2-Rluc

hPIT1-eYFP / hPIT1-Rluc

hPIT2-eYFP / hPIT2-Rluc

E

BRET ratio vs. eYFP/Rluc

F

BRET ratio vs. eYFP/Rluc

10 mM Pi
3 mM Pi
1 mM Pi

G

BRET 50 vs. [Pi] mM

1 mM Pi
3 mM Pi
10 mM Pi
Pivotal role of PiT1 and PiT2 in bone phosphate sensing

Our results illustrated that both PiT proteins are important for P<sub>I</sub>-dependent ERK signaling and that Pi<sub>I</sub> can modulate PiT hetero-oligomerization. To elucidate whether the Pi<sub>I</sub> transport function of PiTs was important to mediate Pi<sub>I</sub>-dependent ERK1/2 signaling, we used the previously reported hPiT<sub>1</sub>S<sup>128A</sup> and hPiT<sub>2</sub>S<sup>113A</sup> Pi<sub>I</sub>-transport−deficient mutants (56, 63, 64). When hPiT<sub>1</sub>S<sup>128A</sup> was overexpressed in PiT1-depleted cells in which Pi<sub>I</sub>-dependent ERK1/2 activation was lost, we could rescue the activation of ERK1/2 signaling (Fig. 4A). Similarly, we could rescue the Pi<sub>I</sub>-dependent ERK1/2 activation in PiT2-depleted cells by overexpressing hPiT<sub>2</sub>S<sup>113A</sup> mutant. This was also true when cells depleted from both PiTs were transfected by both hPiT<sub>1</sub>S<sup>128A</sup> and hPiT<sub>2</sub>S<sup>113A</sup> (Fig. 4A). These data demonstrated that the Pi<sub>I</sub> transport function of PiTs was dispensable for the Pi<sub>I</sub>-dependent ERK1/2 activation. To further study whether the sodium-dependent Pi<sub>I</sub> transport function of PiTs was important to mediate Pi<sub>I</sub>-dependent PiT hetero-oligomerization, we performed BRET experiments in the absence of Na<sup>+</sup>. In this condition where Pi<sub>I</sub> transport was blunted, the variation of extracellular Pi<sub>I</sub> concentrations was still able to modulate PiT interaction (Fig. 4B and C and Fig. S5C). Because Pi<sub>I</sub> was able to modulate PiT interaction without being transported, this suggests that the binding of Pi<sub>I</sub> to PiT proteins rather than its actual uptake into the cell may be involved in the modulation of PiT interaction. We next generated mutated versions of the chimeric hPiT<sub>1</sub>-eYFP and hPiT<sub>2</sub>-Rluc proteins in which Ser<sup>128</sup> or Ser<sup>113</sup> was replaced by an alanine residue. As expected, overexpression of chimeric hPiT<sub>1</sub>S<sup>128A</sup>-eYFP and hPiT<sub>2</sub>S<sup>113A</sup>-Rluc resulted in decreased sodium-dependent Pi<sub>I</sub> transport compared with transporting chimeric PiT proteins (Fig. 4D). Interestingly, using a BRET approach, we showed that although hPiT<sub>1</sub>S<sup>128A</sup>-eYFP and hPiT<sub>2</sub>S<sup>113A</sup>-Rluc were still able to interact together, this interaction was not modulated anymore by extracellular Pi<sub>I</sub> variations (Fig. 4E (left) and Fig. S5C), consistent with a role for Ser<sup>128</sup> or Ser<sup>113</sup> in Pi<sub>I</sub> binding. When BRET experiments were performed with a transport-deficient mutant (hPiT<sub>1</sub>S<sup>128A</sup>-eYFP or hPiT<sub>2</sub>S<sup>113A</sup>-Rluc) and a transporting chimeric PiT protein (hPiT<sub>1</sub>-eYFP and hPiT<sub>2</sub>-Rluc), we could recover the modulation of PiT interaction by Pi<sub>I</sub> (Fig. 4E (middle and left) and Fig. S5C), further supporting a role of Ser<sup>128</sup> or Ser<sup>113</sup> in the Pi<sub>I</sub>-dependent interaction of PiTs.

**Discussion**

The ability of a cell to detect changes in extracellular Pi<sub>I</sub> levels is paramount for its adequate response to environmental fluctuations and critical for the appropriate modulation of Pi<sub>I</sub> homeostasis and skeletal mineralization. In this work, we provided mechanistic insights into the molecular events leading to the detection of changes in extracellular Pi<sub>I</sub> concentrations using skeletal cell lines as a model.

Skeletal cells are constantly exposed to high local extracellular Pi<sub>I</sub> levels, mainly due to the continual resorption and formation of the mineralized extracellular matrix of bone and the need for tremendous quantities of Pi<sub>I</sub> for mineralization purposes (65). This has made bone a model of choice to study Pi<sub>I</sub> signaling, where it has been shown in early studies to regulate the programmed cell death of hypertrophic chondrocytes (10, 11, 13). In subsequent studies, we and others have shown that Pi<sub>I</sub> could regulate skeletal mineralization by controlling the expression of the mineralization inhibitors Mgp and Opn through the activation of the ERK1/2 pathway (7, 15, 16, 18, 19). Our present study brings evidence for a role of PiT1 and PiT2 in transmitting the Pi<sub>I</sub> signal to the cell by showing that the Pi<sub>I</sub>-dependent up-regulation of Mgp and Opn and ERK1/2 phosphorylation were blunted in PiT1- or PiT2-depleted cells. A role for PiT1 in mediating ERK1/2 signaling has been reported earlier (48, 55); however, a similar role for PiT2 has never been illustrated before.

A critical aspect in studying the molecular events involved in P<sub>I</sub>-signaling is whether or not P<sub>I</sub> needs to be transported within the cell to fulfill its role. This question is particularly relevant in view of the role of PiT1 and PiT2 as mediators of P<sub>I</sub> signaling because these two proteins have well-described P<sub>I</sub> transport functions (41–43). By using Pi<sub>I</sub>-transport−deficient mutants of PiT proteins, we could rescue the ERK1/2 signaling, demonstrating that PiT1 and PiT2 can mediate a P<sub>I</sub> signal without transporting the ion. Furthermore, the loss of ERK1/2 signaling in the absence of PiT1 or PiT2 was not associated with a change in cellular Pi<sub>I</sub> uptake, further indicating that the transport of Pi<sub>I</sub> into the cell is not necessary for Pi<sub>I</sub> signaling.

These data have major implications for the understanding of the P<sub>I</sub>-signaling mechanism. The absence of functional compensation by PiT1 in PiT2-depleted cells, and vice versa, together with the requirement of both PiTs for Pi<sub>I</sub> signaling, suggested that they could interact functionally and/or physically to mediate the P<sub>I</sub> signal. Consistent with this hypothesis, using a BRET approach, we showed that PiT proteins could

**Figure 3. Specific interaction between PiT1 and PiT2 varies upon extracellular Pi<sub>I</sub> concentration.** A, Western blotting analysis of Na<sup>+</sup>/K<sup>+</sup>-ATPase, hPiT1, and hPiT2 expression in pmax-GFP-transfected (Ctrl) or hPiT1 and hPiT2 co-transfected HEK293T cells after crude cellular fractionation, as indicated (see “Experimental procedures” for details). Overexpression of hPiT1 and PiT2 allowed a better signal than Ctrl. B. Western blotting analysis (UB) of hPiT1 (left) and hPiT2 (right) expression in HEK293T cells untransfected (UT) or transfected with hPiT1, hPiT2, or both after cell surface cross-linking using bis(sulfosuccinimidyl)suberate and cellular fractionation. Enriched plasma membrane fraction (F<sub>P</sub>) was analyzed. C, schematic representation of chimeric hPiT1-eYFP or -Rluc and hPiT2-eYFP or -Rluc proteins used for BRET assays. The DNA region encoding for the large internal loop of PiT1 and PiT2 (iLoop1 and iLoop2, respectively) was analyzed.

A critical aspect in studying the molecular events involved in P<sub>I</sub>-signaling is whether or not P<sub>I</sub> needs to be transported within the cell to fulfill its role. This question is particularly relevant in view of the role of PiT1 and PiT2 as mediators of P<sub>I</sub> signaling because these two proteins have well-described P<sub>I</sub> transport functions (41–43). By using Pi<sub>I</sub>-transport−deficient mutants of PiT proteins, we could rescue the ERK1/2 signaling, demonstrating that PiT1 and PiT2 can mediate a P<sub>I</sub> signal without transporting the ion. Furthermore, the loss of ERK1/2 signaling in the absence of PiT1 or PiT2 was not associated with a change in cellular Pi<sub>I</sub> uptake, further indicating that the transport of Pi<sub>I</sub> into the cell is not necessary for Pi<sub>I</sub> signaling.

These data have major implications for the understanding of the P<sub>I</sub>-signaling mechanism. The absence of functional compensation by PiT1 in PiT2-depleted cells, and vice versa, together with the requirement of both PiTs for Pi<sub>I</sub> signaling, suggested that they could interact functionally and/or physically to mediate the P<sub>I</sub> signal. Consistent with this hypothesis, using a BRET approach, we showed that PiT proteins could
form homo- and heterodimers. More strikingly, we could illustrate that the formation of PiT1-PiT2 heterodimers only was affected by the variation of extracellular Pi levels. We could not quantify the relative importance of hetero- versus homodimers, but the cross-linking data supported the idea that the Pi-sensitive PiT1-PiT2 heterodimers were present at much lower quantities. It is possible that a low-abundance Pi-sensitive PiT1-PiT2 heterodimer may be more effectively tunable than a highly abundant Pi sensor. This abundance may also be consistent with the on/off Pi effect on ERK1/2 signaling that we have observed when deleting the PiT proteins.

Although a Pi-sensitive PiT1-PiT2 heterodimer is likely to represent an important component of the Pi-sensing machinery, deciphering the detailed functioning of such a sensor requires additional work. Nevertheless, our data provide several important mechanistic insights that may give clues to the understanding of the Pi-signaling cascade. We showed that in the absence of Na⁺, which blunts the Pi transport activity of PiT proteins, the formation of PiT1-PiT2 heterodimers was still sensitive to extracellular Pi variations. This is consistent with the idea that Pi transport is not a prerequisite step for PiT transport (51). In line with this possibility, when PiT1 and PiT2 were deleted, PiT1-PiT2 heterodimer was not responsive anymore at extracellular Pi variations. The substitution of only one serine, however, rescued Pi sensitivity of the PiT1-PiT2 heteroduplex, illustrating the complex relationship between the structural arrangement

**Figure 4.** Pi-dependent ERK1/2 signaling and PiT1-PiT2 hetero-oligomerization are independent of Pi transport. A, Western blot analysis of ERK1/2 phosphorylation (P-ERK 1/2) in transiently transfected MC615 cells, as indicated. Overexpression of Pi transport–deficient hPiT1 (PiT$^{S128A}$) and/or hPiT2 (PiT$^{S113A}$) was performed in PiT1–, PiT2–, or PiT1-PiT2–depleted cells, respectively. Cells were incubated in low-serum (0.5%) medium for 24 h and stimulated for 30 min with 1 or 10 mM extracellular Pi concentration. Total ERK1/2 proteins were used as a loading control. B, sodium-dependent and -independent Pi uptake was measured in HEK293T cells transfected as indicated. Data are means ± S.E. (error bars) (*, p < 0.05 versus sodium-dependent, n = 4). C, BRET 50 index was measured from BRET saturation curves obtained after a 10-min stimulation with 1, 3, or 10 mM extracellular Pi concentration from HEK293T co-transfected with hPiT1-eYFP and hPiT2-Rluc in an Na⁺free medium. Data are means ± S.E. (*, p < 0.05 versus 1 mM Pi condition, n = 4). D, sodium-dependent Pi uptake was measured in HEK293T cells transfected with pcDNA6A plasmid (Ctrl) or with plasmids containing normal (hPiT1 and hPiT2) or Pi transport–deficient mutants (hPiT$^{S128A}$ and hPiT$^{S113A}$) with or without BRET chimeric acceptor or donor, as indicated. Data are means ± S.E. (*, p < 0.05 versus Pi-transporting PiTs, n = 4). E, BRET 50 index was measured from BRET saturation curves obtained after 1, 3, or 10 mM extracellular Pi concentration stimulation for 10 min from HEK293T co-transfected with the indicated plasmids. Data are means ± S.E. (*, p < 0.05 versus 1 mM Pi condition, n = 4–5).
of the $P_i$ binding site and $P_i$ sensitivity. A determination of the crystal structure of PiT proteins is therefore necessary to help determine the identity of the $P_i$ binding site and its role in $P_i$ transport and sensing.

An interesting consequence of our work is that PiT proteins should not be considered anymore as $P_i$ transporters solely but also as $P_i$ receptors able to mediate $P_i$ signaling by activating specific downstream pathways. Such a hybrid transporter-receptor behavior may indicate that the PiT1-PiT2 heteroduplex could behave as a $P_i$ transport, whereby conformational changes during the transport cycle (including the $P_i$-binding step) affect a signal transduction component that triggers a downstream signaling pathway (66). The difference between a transceptor and a pure receptor is that the transport can also transport the ligand into the cell. In recent years, evidence for transporters functioning as transceptors has been obtained in several eukaryotic systems (67). Interestingly, in yeast, the Pho84 phosphate carrier that is considered as an essential component of the $P_i$-sensing system was characterized as a $P_i$ transporter (40). In prokaryotic or lower eukaryotic organisms, true transceptors could be functionally characterized by specific mutants that either lack the signaling capacity and retain normal transport or have lost transport but retain signaling. In the case of PiT proteins, the signaling capacity is lost when either PiT1 or PiT2 is deleted, indicating that the possible transceptor function may only be revealed by heterodimerization and may not be associated with structural changes of either PiT. The gain of function provided through PiT heterodimerization implies that a unique protein complex mediates $P_i$ signaling. This underlies the idea that specific PiT1 and PiT2 protein partners may be involved in this process. If this is the case, the identification of PiT-specific partners in the future may provide unique targets to modulate $P_i$ signaling in specific organs or specific physiological conditions.

In summary, this study provided mechanistic insights into the $P_i$-signaling cascade in skeletal cell line models by unraveling PiT1-PiT2 heterodimers as essential components of the $P_i$-sensing machinery. Although in vivo studies are required to strengthen the physiological relevance of these findings, our work may help in deciphering the mechanisms underlying the ability of the organism to respond to the serum $P_i$ level variations, which is the necessary first step in the $P_i$ homeostasis-regulating cascade.

**Experimental procedures**

**Cells and culture conditions**

Murine preosteoblastic MC3T3-E1 cells were seeded at $10^4$ cells/cm$^2$ and cultured for 10 days in $\alpha$-minimum essential medium GlutaMAX™ (catalogue no. 32751, Thermo Fisher Scientific, Saint-Aubin, France) supplemented with 10% FBS and 1% penicillin/streptomycin. Murine chondrogenic MC615 cells were seeded at $10^4$ cells/cm$^2$ and maintained in a medium consisting of Dulbecco’s modified Eagle’s medium (DMEM) high glucose GlutaMAX™/Ham’s F-12 (1:1) (catalogue no. 31966 and 31765, respectively, Thermo Fisher Scientific) supplemented with 10% FBS and 1% penicillin/streptomycin. HEK293T cells were maintained in DMEM with high glucose GlutaMAX™ supplemented with 10% FBS, 10 mM HEPES, and 50 $\mu$g/ml gentamicin. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO$_2$ in air, and media were renewed every 2–3 days. When indicated, cells were incubated in low-serum (0.5%) medium for 24 h and stimulated with various concentrations of $P_i$ for 30 min or 24 h. $P_i$ was added as a mixture of NaH$_2$PO$_4$ and Na$_2$HPO$_4$ (pH 7.4), as described previously (10).

**RNA interference**

Inactivation of PiT1 or PiT2 were performed by cloning an shPiT1 (56) or shPiT2 (see sequences in Table 1) into pSUPER vector (68) expressing a puromycin resistance gene. A scramble sequence cloned into pSUPER was used as a negative control (56). Stable knockdown of PiT1 or PiT2 was obtained by transfecting MC3T3-E1 cells with 5 $\mu$g of the pSUPER-shRNAs using the T-20 program of the Amaxa nucleofector system (Cell Line Nucleofector™ Kit V VCA-1003, Lonza, Bâle, Switzerland). Cells were plated at limiting density, and puromycin-resistant clones were picked, expanded, and tested for PiT expression. Experiments were performed with three independent stable transfectants, and the data presented illustrate a representative clone. Transient inactivation of PiT was also performed in MC3T3-E1 and MC615 cells using transfection as described above. These transient experiments were stopped 72 h after transfection.

**Table 1**

| Applications | Target genes | Primer sequences (5’ to 3’) | Accession Number |
|--------------|--------------|-----------------------------|------------------|
| RNA interfere | mPiT2 | F: ACTAGATCCCGCGGCCGAGTTAACAAAGGAACATTCATTTACAAAGCACTG | N/A |
| Site-directed mutagenesis | hPiT2/FS-| F: ATGTGGGCGCTGTCGCTGTCGACACCCCTC | N/A |
| mPiT2/FS-66His | R: AGCTAGATTTCCTGCTCTTATGAGAGAGTACCAAT | |
| hPiT2/FS-66His | R: ATGCATTCTCATGAAAGTCGACACCCCTC | N/A |
| hPiT2/FS- | R: TCCTGATTTCCTGCTCTTATGAGAGAGTACCAAT | N/A |
| hPiT2/FS-66His | R: ATGCATTCTCATGAAAGTCGACACCCCTC | N/A |
| mPiT1, mPiT2 | hPiT1 | F: TGGTGGGCGCTGTCGCTGTCGACACCCCTC | N/A |
| shPiT2 | R: ATGCATTCTCATGAAAGTCGACACCCCTC | N/A |
| mPiT1 | shPiT2 | F: TGGTGGGCGCTGTCGCTGTCGACACCCCTC | N/A |
| shPiT1 | R: ATGCATTCTCATGAAAGTCGACACCCCTC | N/A |
| shPiT2 | F: TGGTGGGCGCTGTCGCTGTCGACACCCCTC | N/A |

* siRNA sequences are underlined. # desired mutation is underlined.
Pivotal role of PiT1 and PiT2 in bone phosphate sensing

Gene expression analysis

Total RNA was isolated from cells using the NucleoSpin RNA II kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer’s instructions. RNA was reverse transcribed using Affinity Script (Agilent Genomics, Santa Clara, CA) as per the manufacturer’s recommendations. Real-time PCR was performed on a Mx3000P system (Stratagene, San Diego, CA) using Brilliant III Ultra-Fast SYBR QPCR Master Mix (Agilent Genomics). The following temperature profile was used: denaturation at 95 °C for 3 min, amplification during 40 cycles of 5 s at 95 °C and 20 s at 60 °C, followed by a step at 95 °C for 1 min and 65 °C for 30 s. Expression of target genes were normalized to GAPDH expression levels and were calculated as described previously (69). Primer sequences are listed in Table 1.

Immunofluorescence and confocal microscopy

One day after transient transfection, MC615 cells were fixed/permeabilized in methanol at −20 °C for 5 min and blocked in 1% bovine serum albumin for 1 h at room temperature. Immunodetection of PiT1 and PiT2 was performed using rabbit anti-mouse PiT1 or PiT2 antibody, respectively (generously provided by Dr. G. Friedlander), at a 1:200 dilution overnight at 4 °C, and goat anti-rabbit Alexa488 secondary antibody (catalogue no. A11008, Thermo Fisher Scientific) at a 1:1,000 dilution for 1 h at room temperature. The nuclei were counterstained using 1 μg/ml TO-PRO 3 iodide solution (Thermo Fisher Scientific). The stained cells were mounted in Prolong Gold antifade mounting medium (Thermo Fisher Scientific). Images were acquired with a Nikon Eclipse TE2000E confocal microscope (Nikon, Minato-ku, Tokyo, Japan) equipped with an ×60 oil immersion objective. The averaged intensity for PiT1 and PiT2 staining was determined using Metamorph version 7.5 software.

HEK293T cells were seeded at 3 × 10⁴ cells/cm² in μ-slide 8-well ibiTreat (catalogue no. 80826, Ibidi, Madison, WI) precoated with poly-l-lysine (Sigma-Aldrich). Cells were transfected with 0.125 μg/well of plasmid using JetPrime (Polyplus transfection, Illkirch, France) according to the manufacturer’s instructions. Twenty-four hours post-transfection, HEK293T cells were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and blocked in 1% bovine serum albumin for 1 h at room temperature. Immunodetection of hPiT1-Rluc, hPiT2-Rluc, and BTN3A2-Rluc was performed using anti-Rluc antibody (catalogue no. GTX47953, GeneTex, Irvine, CA) at a 1:100 dilution as a loading control. Anti-rabbit (catalogue no. 7074, Cell Signaling) and anti-mouse (catalogue no. A11008, Thermo Fisher Scientific) at a 1:1,000 dilution for 1 h at room temperature. Blots were probed with primary antibodies for 1 h at room temperature. Proteins were transferred to PVDF membrane, and blocking was performed with 5% nonfat dry milk/TBST overnight at 4°C, followed by secondary antibodies for 1 h at room temperature. Anti-β-actin clone AC-74 (Sigma-Aldrich) was used at a 1:5,000 dilution. hPiT1 (catalogue no. 12423-1-AP) and anti-phospho-ERK1/2 (catalogue no. 9102) were from Cell Signaling (Danvers, MA) and used at a 1:2,000 dilution. Anti-PiT1 (catalogue no. 12820-1-AP) and anti-phospho-PiT2 (catalogue no. 12820-1-AP) were from Proteintech (Rosemont, IL) and used at a 1:1,000 dilution. Anti-Na/K-ATPase (catalogue no. ab7671) was from Abcam (Cambridge, UK) and used at a 1:5,000 dilution. Monoclonal anti-β-actin clone AC-74 (Sigma-Aldrich) was used at a 1:5,000 dilution as a loading control. Anti-rabbit (catalogue no. 7074, Cell Signaling) and anti-mouse (catalogue no. A9917, Sigma-Aldrich) secondary antibodies were used at 1:2,000 and 1:80,000 dilutions, respectively. Signal detection was performed using ECL Western blotting detection reagent and ECL hyperfilm (GE Healthcare) or ChemiDoc Imaging System (Bio-Rad).

Crude cellular fractionation and cross-linking

HEK293T cells were seeded at 10⁵ cells/cm² in 6-well plates and transfected with 1 μg/well each of hPiT1 and hPiT2 plasmids using JetPrime (Polyplus transfection) according to the manufacturer’s instructions. P₁ uptake in HEK293T cells was measured 24 h after transfection. To perform uptake experiments in MC3T3-E1 cells, PiT-depleted MC3T3-E1 clones were seeded at 1.5 × 10⁵ cells/cm², and uptake was performed 24 h later. P₁ uptake was performed as described previously (70). Briefly, after three washing steps, cells were incubated in an uptake medium containing 100 μM [³²P]KH₂PO₄ (0.5 μCi/ml) in the presence of 137 mM NaCl (total P transport) or N-methyl-D-glucamine (sodium-independent transport) at 37 °C for 10 min. sodium-dependent P₁ transport was calculated as the difference between total and sodium-independent P₁ transports. Cells were washed three times and lysed with 0.1 ml NaOH solution, and aliquots of cell lysates were taken for the determination of protein content (Pierce BCA protein assay kit, Thermo Fisher Scientific) and the radioactivity by liquid scintillation counting (Ultima Gold LLT, PerkinElmer Life Sciences) in a Hitachi 300 SL β counter.
Table 2

| Primer sequences used for FastCloning |
|--------------------------------------|
| Chimeric constructs \* | Primer for eYFP in | Primer sequences (5’ to 3’) |
| eYFP insert in hPiT1 plasmid | pcDNA6A-hPiT1 | F: GACGAGAAGAAGAAGTAAGGC<br> R: AGGACATCACTTACCCCTCCG |
| Insert | pEYFP-N1 | F: AATAAGTGTAACCTGTTGGAAGCGAACGGCAG<br> R: ACTTCCGCTGTGCTGCTGCTGCTG |
| Rluc insert in hPiT1 plasmid | pcDNA6A-hPiT1-Rluc | F: GACGAGAAGAAGAAGTAAGGC<br> R: AGGACATCACTTACCCCTCCG |
| Insert | pEYFP-N1 | F: AATAAGTGTAACCTGTTGGAAGCGAACGGCAG<br> R: ACTTCCGCTGTGCTGCTGCTGCTG |
| eYFP insert in hPiT2 plasmid | pcDNA6A-hPiT2 | F: GGGGCGGCGGGGCGGGA<br> R: CTATCCGCTGATAAGACCTTCC |
| Insert | pEYFP-N1 | F: AATAAGTGTAACCTGTTGGAAGCGAACGGCAG<br> R: ACTTCCGCTGTGCTGCTGCTGCTG |
| Rluc insert in hPiT2 plasmid | pcDNA6A-hPiT2-Rluc | F: GGGGCGGCGGGGCGGGA<br> R: CTATCCGCTGATAAGACCTTCC |
| Insert | pEYFP-N1 | F: AATAAGTGTAACCTGTTGGAAGCGAACGGCAG<br> R: ACTTCCGCTGTGCTGCTGCTGCTG |
| BTN3A2 in hRlucC1 plasmid | pcDNA6A-hRlucC1 | F: ATGACAACCAGAACGGTGAC<br> R: GGGCCTTACCAGGAGATCG |
| Insert | pEYFP-N1 | F: AATAAGTGTAACCTGTTGGAAGCGAACGGCAG<br> R: ACTTCCGCTGTGCTGCTGCTGCTG |
| BTN3A2 in EYPPh1 plasmid | pcDNA6A-EYPPh1 | F: ATGTGGAGACAGGGGCGGGA<br> R: ATGCGAGCCTCGATGCGATCT |
| Insert | pEYFP-N1 | F: AATAAGTGTAACCTGTTGGAAGCGAACGGCAG<br> R: ACTTCCGCTGTGCTGCTGCTGCTG |

\* overlap sequences underlined.

**Construction of chimeric and transport-deficient PiT plasmids**

The hPiT1 and hPiT2 sequences were previously cloned into pcDNA6A plasmid (56). The V5-His\(_6\) fusion tag sequence present in pcDNA6A was excluded by introducing a stop codon at the end of the hPiT coding sequence using site-directed mutagenesis (QuickChange, Agilent Genomics) (see primers in Table 1). The iLoop of hPiT1 and hPiT2 was then replaced by eYFP or RLuc proteins using FastCloning (71). Briefly, using Phusion\(^\text{®}\) high-fidelity DNA polymerase (New England Biolabs) and overlapping specific primers (Table 2), we PCR-amplified overlapping sequences were reassembled by E. coli-mediated recombination-ligation following transformation in high-efficiency NEB \(^\text{®}\) 10-β competent cells (New England Biolabs, Ipswich, MA). In the final hPiT1-eYFP or -RLuc chimeric constructs, the eYFP or RLuc coding sequences were inserted in place of iLoop1 (amino acids 268–492). Similarly, the eYFP or RLuc coding sequence was inserted in place of iLoop2 (amino acids 256–450) in the hPiT2-eYFP or -RLuc constructs. The integrity of the constructs was verified by sequencing. To serve as a control for BRET experiments, the BTN3A2 DNA sequence (generously provided by Dr. Scotet, INSERM UMR1232, Centre de Recherche en Cancérologie et Immunologie, Nantes-Angers, France), encoding a small cell surface-expressed protein, was fused to the N terminus of eYFP or RLuc coding sequences using the same strategy (primers used are reported in Table 2).

**BRET saturation assays**

HEK293T cells were seeded at 5 × 10\(^4\) cells/cm\(^2\) in 12-well plates. The next day, cells were co-transfected using JetPrime (Polyplus transfection) with a fixed amount of hPiT1-RLuc (50 ng/well), hPiT2-RLuc (100 ng/well), or BTN3A2-RLuc (10 ng/well) plasmids (encoding BRET donors) and variable amounts of hPiT1-eYFP or hPiT2-eYFP (from 12.5 to 1,500 ng/well) or BTN3A2-eYFP (from 1.56 to 50 ng/well) plasmids (encoding BRET acceptors). The pcDNA6A empty vector was used to compensate for the variable amounts of transfected DNA and to ensure equivalent transfection conditions in each well. Twenty-four hours later, transfected cells were detached using 0.5 mM EDTA solution and seeded at 1.5 × 10\(^5\) cells/cm\(^2\) in white flat-bottom 96-well plates in duplicate. BRET experiments were performed 48 h post-transfection. Cells were washed once with 0.9% NaCl solution and stimulated with various concentrations of Pi for 10 min. Pi was added as a mixture of Na\(_2\)HPO\(_4\) and Na\(_2\)HPO\(_4\) (pH 7.4). When indicated, cells were previously starved of Pi by incubating cells overnight with DMEM high glucose no phosphates (catalogue no. 11971, Thermo Fisher Scientific) supplemented with 10% FBS, 10 mM HEPES, and 50 μg/ml gentamicin before Pi stimulation. The coelenterazine h substrate (UPR3078, Interchim Uptima, Montluçon, France) was added at a final concentration of 5 μM by automated injection in the Mithras LB940 plate reader (Berthold Technologies, Versailles, France), and 485- and 530-nm light emissions were measured consecutively several times. The BRET ratio was calculated as the ratio of light emitted by the acceptor fusion protein at 530 nm over the light emitted by the donor fusion protein at 485 nm. Values were
Pivotal role of PiT1 and PiT2 in bone phosphate sensing
corrected with the background signal calculated from a well without donor fusion protein. The BRET 50 was calculated as the eYFP/Rluc value at which the BRET ratio is half of the maximum BRET ratio achieved at saturating substrate concentration.

Statistical analysis
Data are expressed as mean ± S.E. GraphPad version 5.0 software was used to perform Mann–Whitney tests. A p value of <0.05 was considered statistically significant. Unless otherwise stated, experiments were repeated at least three times.

Author contributions—N.B., G.C., and A.B. conducted most of the experiments, from conception and design to acquisition of data or analysis and interpretation of data. S.S. provided technical assistance. L.B., J.G., and S.B.C conceived the idea and supported the coordination of the project. N.B. and L.B. wrote the paper. N.B., G.C., A.B., S.B.C, J.G., and L.B. made adjustments to the final paper version. All authors reviewed the results and approved the final version of the manuscript.

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