TABLE S1

Peptides pulled down by NHERF1

	Bio View Identified Proteins	Accession Number	Molecular Weight	Sequence Coverage
1	NHERF1_HUMAN Na^+/H^+ exchange regulatory cofactor NHE-RF1 OS=Homo sapiens GN=SLC9A3R1 PE=1 SV=4	O14745	39kDa	68%
2	TBB5_HUMAN Tubulin beta chain OS=Homo sapiens GN=TUBB PE=1 SV=2	P07437	50 kDa	59%
3	H3BRM5_HUMAN Cytochrome c oxidase subunit 5A, mitochondrial OS=Homo sapiens GN=COX5A PE=2 SV=1	H3BRM5 (+2)	8 kDa	57%
4	TCPB_HUMAN T-complex protein 1 subunit beta OS=Homo sapiens GN=CCT2 PE=1 SV=4	P78371	57 kDa	55%
5	SERB_HUMAN Phosphoserine phosphatase OS=Homo sapiens GN=PSPH PE=1 SV=2	P78330	25 kDa	55%
6	SDF2L_HUMAN Stromal cell-derived factor 2-like protein 1 OS=Homo sapiens GN=SDF2L1 PE=1 SV=2	Q9HCN8	24 kDa	52%
7	H0YFX9_HUMAN Histone H2A (Fragment) OS=Homo sapiens GN=H2AFJ PE=2 SV=1	H0YFX9 (+8)	10 kDa	52%
8	B4DEM7_HUMAN T-complex protein 1 subunit theta OS=Homo sapiens GN=CCT8 PE=2 SV=1	B4DEM7 (+1)	58 kDa	51%
9	PRDX4_HUMAN Peroxiredoxin-4 OS=Homo sapiens GN=PRDX4 PE=1 SV=1	Q13162	31 kDa	50%
10	HSP7C_HUMAN Heat shock cognate 71 kDa protein OS=Homo sapiens GN=HSPA8 PE=1 SV=1	P11142	71 kDa	49%
11	TBB4B_HUMAN Tubulin beta-4B chain OS=Homo sapiens GN=TUBB4B PE=1 SV=1	P68371	50 kDa	48%
12	CPSF5_HUMAN Cleavage and polyadenylation specificity factor subunit 5 OS=Homo sapiens GN=NUDT21 PE=1 SV=1	O43809	26 kDa	47%
13	SMU1_HUMAN WD40 repeat-containing protein SMU1 OS=Homo sapiens GN=SMU1 PE=1 SV=2	Q2TAY7	58 kDa	47%
14	MK01_HUMAN Mitogen-activated protein kinase 1 OS=Homo sapiens GN=MAPK1 PE=1 SV=3	P28482	41 kDa	46%
15	C1QBP_HUMAN Complement component 1 Q subcomponent-binding protein, mitochondrial OS=Homo sapiens GN=C1QBP PE=1 SV=1	Q07021	31 kDa	46%
16	DHE3_HUMAN Glutamate dehydrogenase 1, mitochondrial OS=Homo sapiens GN=GLUD1 PE=1 SV=2	P00367	61 kDa	45%
17	EFTU_HUMAN Elongation factor Tu, mitochondrial OS=Homo sapiens GN=TUFM PE=1 SV=2	P49411	50 kDa	45%
18	PP1α_HUMAN Ser/Thr protein phosphatase PP1-alpha catalytic subunit OS=Homo sapiens GN=PPP1CA PE=1 SV=1	P62136	38 kDa	45%
19	PA1B3_HUMAN Platelet-activating factor acetylhydrolase IB subunit gamma OS=Homo sapiens GN=PAFAH1B3 PE=1 SV=1	Q15102	26 kDa	45%
20	NPS3A_HUMAN Protein NipSnap homolog 3A OS=Homo sapiens GN=NIPSNAP3A PE=1 SV=2	Q9UFN0	28 kDa	45%

TABLE S2

PP1α peptides pulled down by NHERF1

Tryptic peptides	Charge	Mascot score
K.LNLDSIIGR.L	2	51.1
K.NVQLTENEIR.G	2	45.4
R.EIFLSQPILLELEAPLK.I	3	59.9
K.QSLETICLLLAYK.I	2	74.4
K.YPENFFLLR.G	2	47.1
K.TFTDCFNCLPIAAIVDEK.I	2	70.7
K.TFTDCFNCLPIAAIVDEK.I	3	45.0
K.IFCCHGGLSPDLQSMEQIR.R	3	59.3
R.IMRPTDVPDQGLLCDLLWSDPDKDVQGWGENDR.G	4	78.1
K.HDLDLICR.A	2	46.2
R.AHQVVEDGYEFFAK.R	2	44.9

Identified PP1α peptides pulled with NHERF1. Unique peptides along with their charge state and corresponding scores based on Mascot analysis were listed.

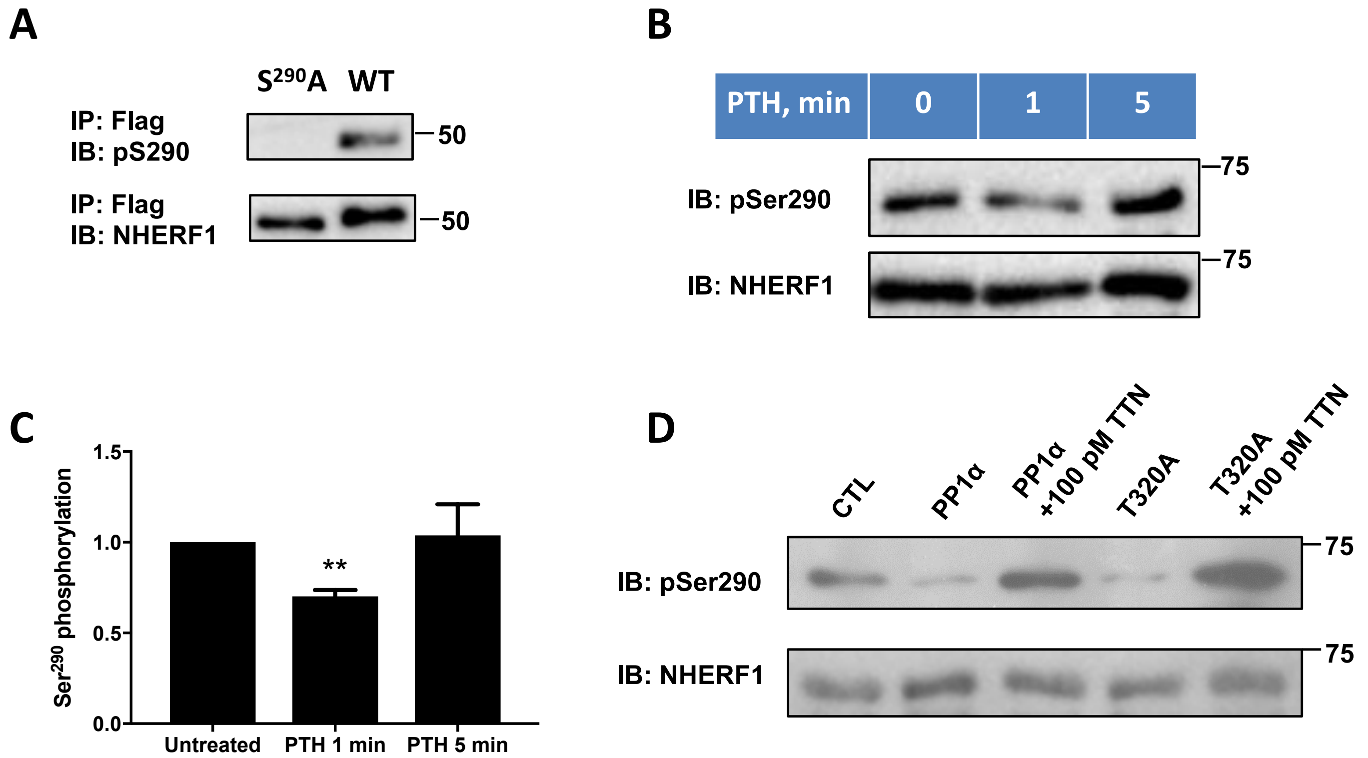

Fig. S1. A**nti-phospho-NHERF1 (pSer290) antibody detection of pSer290 dephosphorylation** . *A*, Alanine replacement at Ser^{290} virtually abolished Ser^{290} phosphorylation compared to wild-type NHERF1. Flag-WT- or -S290A-NHERF1 was transfected into GnTI- cells. 48 h later, cells were harvested and lysed, and the resulting supernatant after centrifugation was incubated with anti-Flag beads. Pull-downed proteins were analyzed by western blotting using a specific phospho-NHERF1 (pSer290) antibody. *B*, Representative blots from 3 independent assays show dynamic Ser^{290} phosphorylation upon PTH treatment. Double stable $GnTI^-$ cells expressing TAP-NHERF1 and Flag-PTHR were grown in 6-cm dishes for 48 h. Cells were serum-starved and subsequently treated with 100 nM PTH for the time indicated. Cells were harvested and lysed. The cleared supernatant after centrifugation was incubated with streptavidin beads. The resulting proteins were analyzed by western blotting. *C*, quantitative analysis of Fig. S1B showed a similar Ser^{290} dephosphorylation pattern as observed in Fig. 3, *A* and *B*. *D*, TTN blocks PP1α activity and prevents $pSer^{290}$ dephosphorylation. GFP-PP1α or GFP-PP1α-T320A was transfected into the double stable cells as described in Fig. S1*B*. 48 h later, cells were incubated with or without 100 pM TTN. NHERF1 protein was purified as noted in Fig. S1*B* and analyzed by immunoblotting.

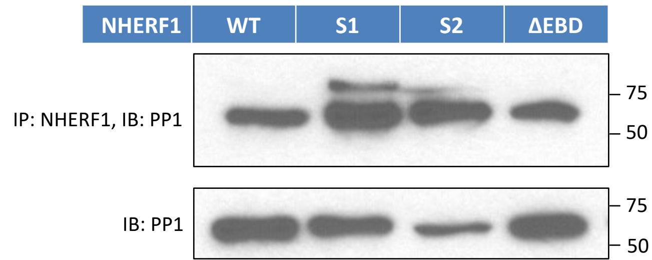

Fig. S2. **PP1α Does Not Bind PDZ1, PDZ2, or EBD of NHERF1**. HEK-293 cells were transfected with FLAG-NHERF1 (WT, S1, S2, △EBD) and GFP-PP1A. Two days later, NHERF1 was immunoprecipitated with anti-FLAG beads and PP1α was detected with a monoclonal anti-GFP antibody as detailed in Experimental Procedures.

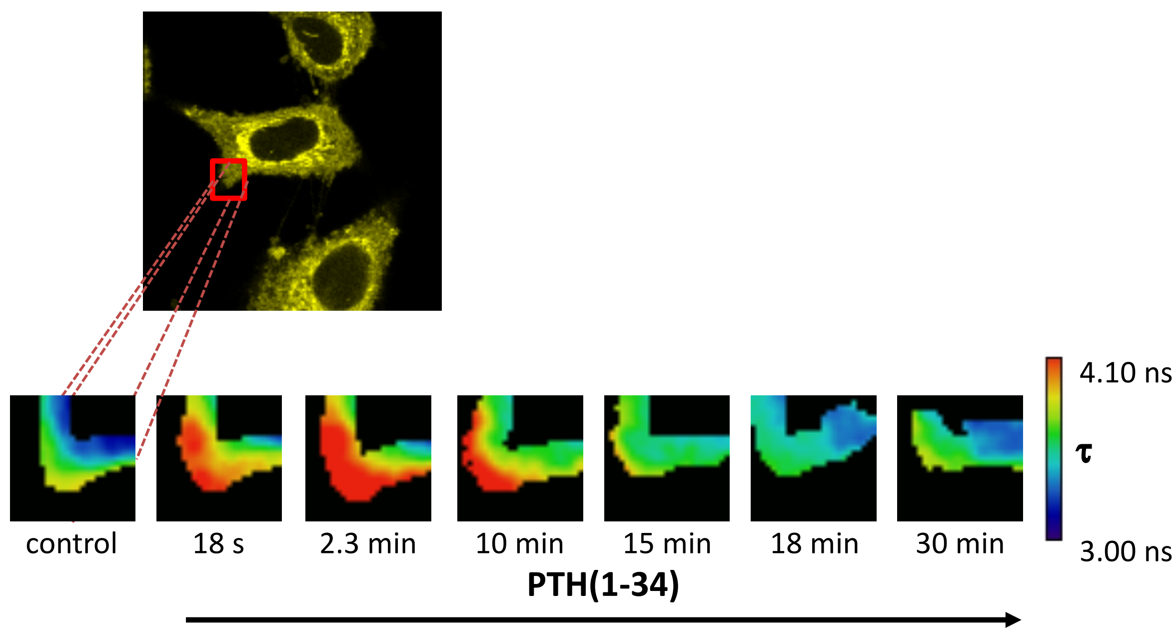

FIG. S3. **Representative FLIM images of 4-MeO-2-Me TM in MC3T3-E1 preosteoblasts after PTH treatment**. The region of interest near the cell membrane is selected for fast FLIM scanning. A time series of FLIM images are collected, obtaining the tau values of 4-MeO-2-Me TM in each pixel. This illustrative example shows a rapid increase in tau (τ), and a subsequent recovery to control levels. The changes in tau are related to changes in total phosphate concentration using the calibration in Fig. S4.

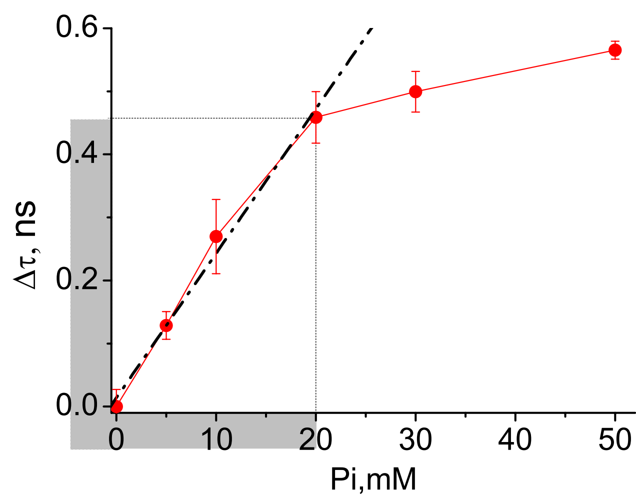

FIG. S4. **Intracellular calibration of changes in tau values of 4-MeO-2-Me TM in MC3T3-E1 preosteoblasts permeabilized with α-toxin**. Permeabilization with α-toxin assures equilibration of intra- and extracellular Pi concentrations. There is a good linear relation (0.99531) between the change in the tau (τ) value and the added total phosphate up to 20 mM. Intracellular phosphate concentrations were calculated from Δτ using the linear fit (black, dash-dotted line).

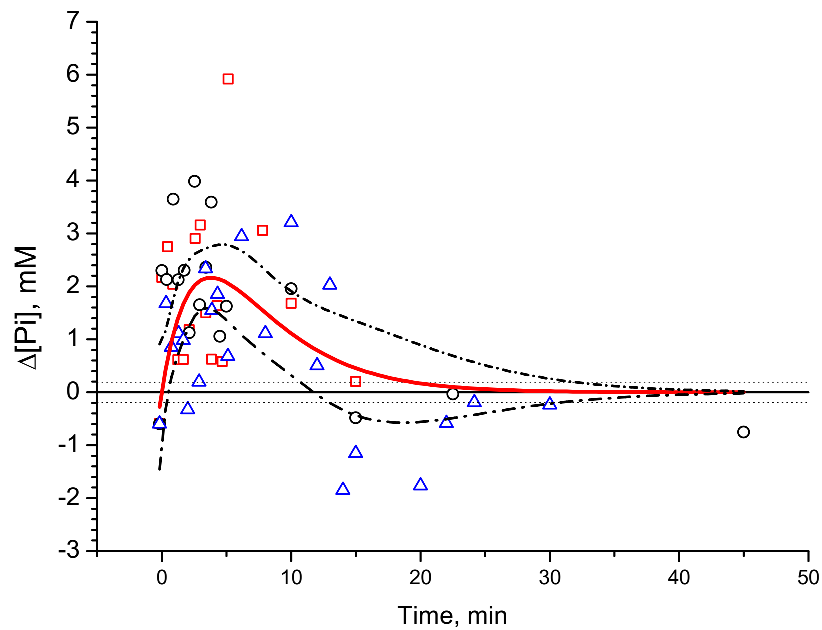

Fig. S5. **Intracellular phosphate in live cells**. PTH-induced changes of intracellular phosphate concentration (Δ[Pi], mM) were measured by FLIM as detailed in Experimental Procedures. MC3T3-E1 preosteoblasts were loaded with 2Me-4OMe-TM. Cells were treated with 100 nM PTH(1-34) in the presence of extracellular buffer containing 10 mM Pi. Different color symbols indicate repetitions of independent experiments supporting the reproducibility of the measurements. The red line represents a global fit of the 3 datasets to a double-exponential decay function, resulting in a rise time of 3.2 min, and a subsequent decay time of 4.5 min. Dashed-dotted lines indicate 95% confidence intervals of the fitting.

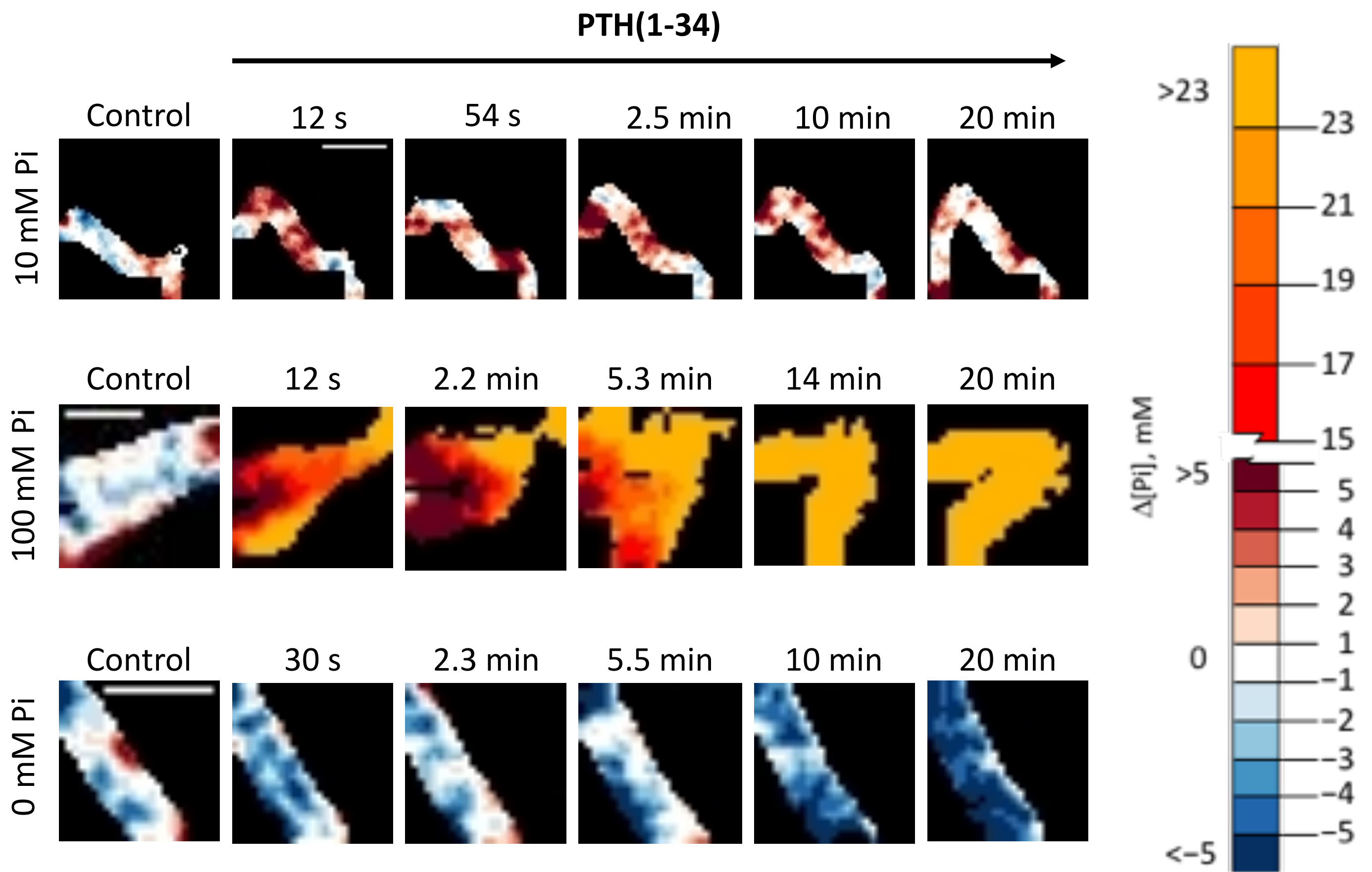

FIG. S6. **Real-time FLIM phosphate measurement in living cells**. Representative images of 4-MeO-2-Me TM in MC3T3-E1 preosteoblasts upon treatment with PTH(1-34) at different extracellular phosphate(Pi) concentrations. Extracellular buffer was 10 mM Pi (PBS, upper row), 100 mM Pi buffer (center), or 0 mM Pi (TMA + KCl, bottom). The color coding represents changes of the intracellular phosphate concentration. Scale bars represent 5 µm.

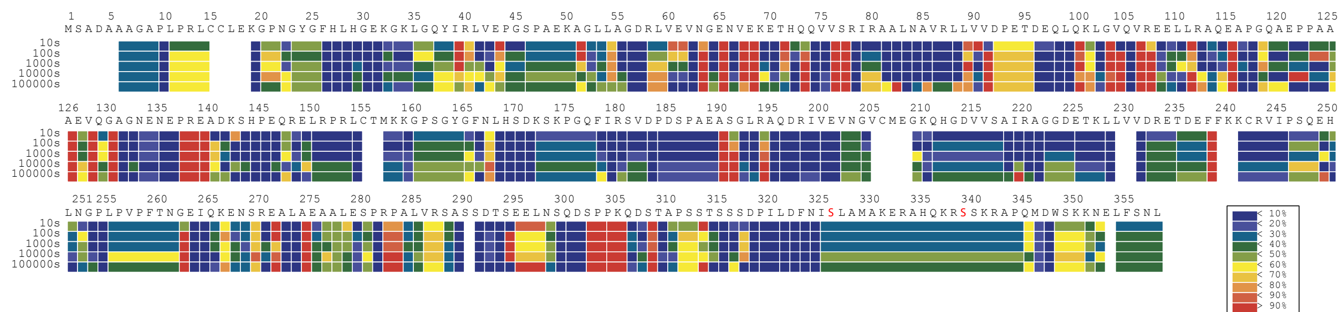

Fig. S7. **Time-dependent HDX heat map of wild-type NHERF1**. First-order analysis of NHERF1 uncorrected for deuterium back exchange. The color scale indicates percentage of deuterium incorporation. These findings are comparable to those reported (1).

1. Park, J. Y., Duc, N. M., Kim, D. K., Lee, S. Y., Li, S., Seo, M. D., Woods, V. L., and Chung, K. Y. (2015) Different conformational dynamics of PDZ1 and PDZ2 in full-length EBP50 analyzed by hydrogen/deuterium exchange mass spectrometry. Biochem. Cell Biol. **93**, 1-8