**Correction**

**BIOCHEMISTRY**


The authors note that Figs. 1b, 1d, 1g, and 4e have been revised to include dividing lines between lanes to show where nonessential lanes were removed from a single original gel. Figs. 2c, 2d, and 3c have been revised to include boxes to indicate samples run on different gels, under the same conditions; these gels are aligned with respect to the indicated molecular weight markers. Figs. 4b and 4c have been revised to eliminate errors that occurred in the original version during file conversion. The changes were made to comply with the PNAS policy that requires dividing lines whenever entire nonessential lanes have been removed from a single original gel, and clear demarcation of samples run on separate gels. These changes do not affect the data presented nor the conclusions of the article. The corrected figures and their legends appear below.

**Fig. 1.** IP7-mediated protein phosphorylation requires prephosphorylation by protein kinases. (a) NSR1 and SRP40 purified from *E. coli* or *S. cerevisiae* were incubated with 5[β32P]IP7 and resolved by NuPAGE; immunoblotting with a tag-specific antibody (Left) and autoradiography to determine phosphorylation (Right). (b) GST or GST-tagged NSR1 fragment (amino acids 51–166) expressed in *E. coli* and immobilized on glutathione beads were preincubated with or without *S. cerevisiae* extract or ATP, washed with PBS, and then treated with 5[β32P]IP7 and resolved by NuPAGE; Coomassie brilliant blue R250 staining (Left) and autoradiography (Right). The extra phosphorylated protein bands in lanes 3–6 are yeast proteins that bound nonspecifically to glutathione beads. (c) GST-SRP40 purified from *E. coli* was preincubated with ATP and either native *S. cerevisiae* extract (N) or extract that had been boiled for 5 min (B) and then treated with 5[β32P]IP7; Coomassie brilliant blue R250 staining (Lower) and autoradiography (Upper). (d) GST or GST-tagged NSR1 fragment (amino acids 1–50) purified from *E. coli* was incubated with yeast extract and indicated nucleotides and then phosphorylated by 5[β32P]IP7; Coomassie brilliant blue R250 staining (Lower). (e) GST-SRP40 and GST or GST-tagged NSR1 fragment (amino acids 1–50) purified from *E. coli* with the catalytic A1 subunit of human CK2 (lane 3). Purified proteins were incubated with 5[β32P]IP7, as in b. (g) GST or GST-tagged NSR1 fragments (amino acids 27–50) purified from *S. cerevisiae* were preincubated in the absence or presence of λ-phosphatase and then phosphorylated by 5[β32P]IP7, as in b.
Fig. 2. IP7 phosphorylates a phosphoserine residue to generate pyrophosphoserine. (a) Extracts from S. cerevisiae expressing GST-tagged mouse Nopp140 fragment (amino acids 80–100) WT sequence and indicated point mutants were phosphorylated by 5[32P]IP7; autoradiography (Upper) and immunoblotting (Lower). (b) Sequences of individual peptides derived from Nopp140 (GenBank accession no. NP_941035). NOP, amino acids 76–100 of mouse Nopp140; NOP-2[pS], the same sequence as NOP except with phosphoserine at positions 88 and 91; NOP-pS 88 and NOP-pS 91, single phosphoserine residues at positions 88 and 91 respectively; NOP-2[MepS], methylphosphoserine at positions 88 and 91; NOP-2[ppS], pyrophosphoserine at positions 88 and 91. (c and d) The six synthetic peptides in b were incubated with 5[32P]IP7 (c) or with CK2 and γ[32P]ATP (d) and resolved by NuPAGE. Coomassie G250 staining (Left) and autoradiography (Right). In each peptide lane, there are multiple bands in the 3- to 9-kDa range, corresponding to peptide monomers and multimers, generated possibly because of electrostatic interactions between the positively charged lysines and negatively charged acidic and phosphate-containing residues (27). Peptide dimers were also observed by MALDI-TOF analysis (data not shown).

Fig. 3. The properties of pyrophosphoserine differ from those of phosphoserine. (a and b) NOP-2[pS] peptide was phosphorylated by CK2 and γ[32P]ATP (a) or by 5[32P]IP7 (pS), then treated with HCl and subjected to NuPAGE; Coomassie G250 staining (Left) and autoradiography (Right). (c) NOP-2[pS] peptide was phosphorylated by either CK2 and γ[32P]ATP or by 5[32P]IP7, as described in SI Materials and Methods. A fraction of the phosphorylated peptides was subjected to NuPAGE to ensure equivalent phosphorylation under both conditions; Coomassie G250 staining (Left) and autoradiography (Right). (d and e) The remaining CK2 phosphorylated peptide (d) and IP7-phosphorylated peptide (e) were hydrolyzed with 6 M HCl, and the resulting amino acids were resolved by HPLC. Data collected during the first 40 min of the HPLC run are presented; elution profiles of the first 3 aa (Upper) and corresponding radioactivity in each 1-ml fraction (Lower). Note that high levels of 32Pi in the IP7-phosphorylated sample compared with the CK2 and ATP phosphorylated sample are due to incomplete removal of 5[32P]IP7 from the peptide during desalting (see SI Materials and Methods).
Fig. 4.  λ-phosphatase does not dephosphorylate IP₇-phosphorylated proteins. (a) GST-tagged NSR1 fragment (amino acids 1–50) purified from S. cerevisiae was incubated without or with λ-phosphatase and then phosphorylated by 5β[³²P]IP₇ (lanes 1 and 2). Alternatively, the protein was first phosphorylated by 5β[³²P]IP₇ and then incubated without or with λ-phosphatase (lanes 3 and 4). The samples were resolved by NuPAGE and autoradiographed. (b) GST-tagged NSR1 fragment (amino acids 27–50) purified from S. cerevisiae was phosphorylated either by CK2 and γ[³²P]ATP (Left) or by 5β[³²P]IP₇ (Right) and then incubated with or without λ-phosphatase; Coomassie G250 staining (Left) and autoradiography (Right). (c) NOP-2[pS] peptide bound to streptavidin agarose beads was phosphorylated by either CK2 and γ[³²P]ATP (Left) or by 5β[³²P]IP₇ (Right) and then incubated with or without λ-phosphatase as in b. (d) S. cerevisiae expressing GST-tagged Nopp140 fragment (amino acids 80–100) (Left) or NSR1 fragment (amino acids 27–50) (Right) were labeled with inorganic orthophosphate [³²PO₄]. Purified radiolabeled proteins were incubated with or without λ-phosphatase; autoradiography (Upper) and immunoblotting (Lower). (e) NOP-2[pS] peptide (Left) or NOP-2[ppS] peptide (Right) were treated with or without λ-phosphatase, resolved by NuPAGE, and stained with either Coomassie G250 or Pro-Q Diamond phosphoprotein gel stain, as indicated.

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Protein pyrophosphorylation by inositol pyrophosphates is a posttranslational event

Rashna Bhandari1, Adolfo Saiardi1, Youssef Ahmadiben2, Adele M. Snowman*, Adam C. Resnick*, Troels Z. Kristiansen6, Henrik Molina6, Akhilesh Pandey6, J. Kent Werner, Jr.*, Krishna R. Juluri*, Yong Xu1, Glenn D. Prestwich1, Keykavous Parang1, and Solomon H. Snyder*.

In a previous study, we showed that the inositol pyrophosphate diphosphoinositol pentakisphosphate (IP5) physiologically phosphorylates mammalian and yeast proteins. We now report that this protein transfer reflects pyrophosphorylation. Thus, proteins must be prephosphorylated by ATP to prime them for IP7 phosphorylation. IP7 phosphorylates synthetic phosphopeptides but not if their phosphates have been masked by methylation or pyrophosphorylation. Moreover, IP7 phosphorylated peptides are more acid-labile and more resistant to phosphatases than ATP phosphorylated peptides, indicating a different type of phosphate bond. Pyrophosphorylation may represent a novel mode of signaling to proteins.

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1Present address: Division of Neurosurgery, Children’s Hospital of Philadelphia, Department of Neurosurgery, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

2To whom correspondence should be addressed at: 725 North Wolfe Street, WBSB 813, Baltimore, MD 21205. E-mail: ssnyder@jhmi.edu.

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APL6 and mammalian Nopp140, AP3β3A, and TCOF1 (data not shown).

The requirement for priming by prephosphorylation suggests that either (i) ATP phosphorylation at certain sites allosterically facilitates IP$_7$-mediated phosphorylation at other sites, or (ii) IP$_7$ directly phosphorylates amino acids that have been previously ATP-phosphorylated and hence provides a pyrophosphorylation or diphosphorylation modification. Earlier, we noted that IP$_7$ phosphorylation occurs primarily at stretches of serines flanked by acidic amino acids and concluded that phosphorylation occurs on the serine residues (18) (also see SI Fig. 6). In Nopp140, deletion of individual serine residues in such a stretch (amino acids 80–100) decreases IP$_7$ phosphorylation, implying that IP$_7$ can phosphorylate several of them (Fig. 2a). Substitution of the single threonine residue in this sequence has no effect on IP$_7$ phosphorylation. Because prephosphorylation by CK2 leads to IP$_7$-mediated phosphorylation of such sites, Nopp140 fragments containing phosphates at the CK2-preferred sites should be substrates for IP$_7$ phosphorylation in the absence of any other priming. To identify the preferred sites for CK2 phosphorylation, we phosphorylated an E. coli-expressed Nopp140 fragment comprising amino acids 80–100 and identified the major phosphorylation sites by mass spectrometry as serines 88 and 91 (data not shown). We synthesized a peptide with both of these serines prephosphorylated (Fig. 2b). This phosphopeptide is robustly phosphorylated by 5/H9252[32P]IP$_7$, whereas the corresponding unphosphorylated peptide is resistant to 5/H9252[32P]IP$_7$ phosphorylation (Fig. 2c). Using two peptides with phosphates at serines 88 and 91, respectively, we observe substantially greater phosphorylation at position 91 than 88 (Fig. 2c). This differential influence on discrete sites supports the specificity of IP$_7$-mediated phosphorylation. Both the unphosphorylated and phosphorylated peptides are phosphorylated by 5/H9253[32P]ATP and CK2 (Fig. 2d) on one or more of the available serine residues (data not shown). IP$_7$

![Fig. 1.](image-url)
phosphorylation of the prephosphorylated but not the unphosphorylated Nopp140 peptide is consistent with pyrophosphorylation. This possibility is further supported by mass spectrometric analysis showing no phosphorylation by IP7 of the unphosphorylated Nopp140 peptide but addition of one or two phosphates to the prephosphorylated peptides (SI Table 1). The maximum number of phosphates accepted by a peptide from IP7 equals the number of preexisting phosphate groups on the peptide; single phosphoserine-containing peptides accept one phosphate from IP7, whereas the peptide containing two phosphoserine residues accepts one or two phosphates.

The phosphorylated Nopp140 peptide might facilitate IP7 phosphorylation of nearby serines or might be pyrophosphorylated. To distinguish those alternatives, we synthesized the same phosphopeptide in which the two phosphates have been methylated to prevent pyrophosphorylation (Fig. 2b). The methylated phosphopeptide is not phosphorylated by 5β[32P]IP7 (Fig. 2c and SI Table 1), although it can be phosphorylated by γ[32P]ATP and CK2 (Fig. 2d).

The failure of the methylated phosphopeptide to be IP7-phosphorylated strongly implies that IP7 provides pyrophosphorylation. To further test this possibility, we synthesized the same Nopp140 peptide in which both serines 88 and 91 are phosphorylated. This pyrophosphorylated peptide resists IP7 phosphorylation, although it can be phosphorylated by CK2 (Fig. 2c and d and SI Table 1). This further supports the notion that IP7 pyrophosphorylates its targets.

If IP7 phosphorylates proteins on serines in the same fashion as ATP, then the properties of the phosphoserine bond should be the same with IP7 and ATP phosphorylation. By contrast, the chemical properties of a pyrophosphorylated serine differ from those of a conventional phosphoserine. We examined the acid sensitivity of the phospho-Nopp140 peptide after phosphorylation by γ[32P]ATP and CK2 or 5β[32P]IP7 (Fig. 3a and b). Phosphorylation by 5β[32P]IP7 is more labile to treatment with 3 M HCl compared with phosphorylation by γ[32P]ATP and CK2. Furthermore, we subjected the CK2 and ATP or IP7-phosphorylated peptides to hydrolysis in the presence of 6 M HCl to release single amino acids. Hydrolysis liberates a prominent peak of [32P]phosphoserine from the ATP-phosphorylated peptide but not from the IP7-phosphorylated peptide (Fig. 3c–e). Thus, the phosphate modification on serine is more labile to acid than the conventional phosphoserine elicited by ATP-mediated phosphorylation.

In contrast to the greater lability to acid treatment, the IP7-phosphorylated peptide is more stable to enzymatic dephosphorylation (Fig. 4a). As observed earlier (Fig. 1g), λ-phosphatase treatment before adding IP7 prevents IP7 phosphorylation of NSR1 (1–50). After IP7-phosphorylation, however, the protein is completely resistant to λ-phosphatase. λ-Phosphatase greatly reduces phosphorylation of CK2 and ATP phosphorylated NSR1 (27–50) or Nopp140 peptide but fails to affect the IP7-phosphorylated peptides (Fig. 4b and c). The same protein fragments are resistant to λ-phosphatase when purified from yeast labeled with inorganic orthophosphate [32PO4]i (Fig. 4d), implying that these proteins exist within yeast cells in a pyrophosphorylated form.

These experiments suggest that the pyrophosphoserine bond is resistant to λ-phosphatase. We directly tested this possibility using the Nopp140 peptide containing pyrophosphorylated serines at position 88 and 91 compared with the same peptide with phosphorylated serines at these positions. λ-Phosphatase abolishes phosphorylation of the phospho-Nopp140 peptide but does not affect the phosphorylated Nopp140 peptide (Fig. 4e and SI Table 2).

We also examined the sensitivity of the IP7-phosphorylated NSR1 (27–50) fragment to other protein phosphatases. The following protein phosphatases are completely inactive: calcineurin, protein phosphatase-1, and alkaline phosphatase (SI Fig. 7a). In addition, the pyrophosphatase enzymes, thermolabile inorganic pyrophosphatase and tobacco acid pyrophosphatase, fail to dephosphorylate the IP7-phosphorylated peptide (SI Fig. 7a). Because these enzymes hydrolyze inorganic pyrophosphate (Pi), it is likely that they do not recognize pyrophosphate linked to a serine residue. We also studied DIPP (diphosphoinositol polyphosphate phosphohydrolase), an enzyme that Shears and associates (20, 21) have shown to physiologically dephosphorylate IP7. As expected, pretreatment of the reaction mix containing IP7 lowers phosphorylation, whereas treatment with DIPP after IP7 phosphorylation has no effect (SI Fig. 7b).

In summary, we report that IP7 pyrophosphorylates proteins. Evidence includes the requirement of prephosphorylation by ATP to prime proteins for IP7 phosphorylation and the selective phosphorylation by IP7 of synthetic phosphopeptides but not of...
such peptides that are “blocked” by methylation or are already pyrophosphorylated. Moreover, the properties of IP7-phosphorylated peptides differ markedly from ATP-phosphorylated peptides in terms of acid lability, which is greater with IP7-phosphorylated peptides, and sensitivity to phosphatases, which is much less with IP7-phosphorylated peptides.

We wondered whether 4/6-PP-IP5, the novel isomer of IP7 identified by York and colleagues (15), and IP8 are able to phosphorylate proteins in a manner similar to 5-PP-IP5. Radio-labeled 4/6-PP-IP5 and IP8 phosphorylate proteins in extracts obtained from S. cerevisiae (SI Fig. 8), the pattern of phosphorylated proteins being identical to that obtained with 5-PP-IP5.

Fig. 3. The properties of pyrophosphoserine differ from those of phosphoserine. (a and b) NOP-2[pS] peptide was phosphorylated by CK2 and γ[32P]ATP (a) or by 5[32P]IP7 (b), then treated with HCl and subjected to NuPAGE; Coomassie G250 staining (Left) and autoradiography (Right). (c) NOP-2[pS] peptide was phosphorylated by either CK2 and γ[32P]ATP or by 5[32P]IP7, as described in SI Materials and Methods. A fraction of the phosphorylated peptides was subjected to NuPAGE to ensure equivalent phosphorylation under both conditions; Coomassie G250 staining (Left) and autoradiography (Right). (d and e) The remaining CK2 phosphorylated peptide (d) and IP7-phosphorylated peptide (e) were hydrolyzed with 6 M HCl, and the resulting amino acids were resolved by HPLC. Data collected during the first 40 min of the HPLC run are presented; elution profiles of the first 3 aa (Upper) and corresponding radioactivity in each 1-ml fraction (Lower). Note that high levels of [32P] in the IP7-phosphorylated sample compared with the CK2 and ATP phosphorylated sample are due to incomplete removal of 5[32P]IP7 from the peptide during desalting (see SI Materials and Methods).

Fig. 4. λ-phosphatase does not dephosphorylate IP7-phosphorylated proteins. (a) GST-tagged NSR1 fragment (amino acids 1–50) purified from S. cerevisiae was incubated without or with λ-phosphatase and then phosphorylated by 5[32P]IP7 (lanes 1 and 2). Alternatively, the protein was first phosphorylated by 5[32P]IP7 and then incubated without or with λ-phosphatase (lanes 3 and 4). The samples were resolved by NuPAGE and autoradiographed. (b) GST-tagged NSR1 fragment (amino acids 27–50) purified from S. cerevisiae was phosphorylated either by CK2 and γ[32P]ATP (Left) or by 5[32P]IP7 (Right) and then incubated with or without λ-phosphatase; Coomassie G250 staining (Left) and autoradiography (Right). (c) NOP-2[pS] peptide bound to streptavidin agarose beads was phosphorylated by either CK2 and γ[32P]ATP (Left) or by 5[32P]IP7 (Right) and then incubated with or without λ-phosphatase; Coomassie G250 staining (Left) and autoradiography (Right). (d) S. cerevisiae expressing GST-tagged Nopp140 fragment (amino acids 80–100) (Left) or NSR1 fragment (amino acids 27–50) (Right) were labeled with inorganic orthophosphate [32PO4]i. Purified radiolabeled proteins were incubated with or without λ-phosphatase; autoradiography (Upper) and immunoblotting (Lower). (e) NOP-2[pS] peptide (Left) or NOP-2[ppS] peptide (Right) were treated with or without λ-phosphatase, resolved by NuPAGE, and stained with either Coomassie G250 or Pro-Q Diamond phosphoprotein gel stain, as indicated.
Therefore, it is likely that the principal known inositol pyrophosphates are able to pyrophosphorylate proteins.

Nondenzyotic pyrophosphorylation is thermodynamically feasible. Semiempirical calculations show that, whereas the free enthalpy ($\Delta H$) of reaction for transfer of the $\beta$-phosphate from IP$_2$ to a simple primary alcohol is pH- and counter-ion-dependent, $\Delta H$ values at pH 6.8 can reach $-38.3$ kcal/mole (17). This strongly exothermic reaction equals or exceeds $\Delta H$ for phosphorylation by ATP. The high phosphorylation potential is attributable to the sterically and electronically crowded environment of the IP$_3$ pyrophosphate group. The phosphoserine to pyrophosphoserine conversion by IP$_7$ will require more advanced modeling, but two important clues emerge from the protein substrates for IP$_7$: first, that suggest how the 3D structures of the polypeptide may organize reagent and substrate to provide an entropic driving force to contribute to the overall free energy ($\Delta G$) for the reaction. First, the polypeptide chains must have Asp or Glu residues present for pyrophosphorylation to occur. Second, the pyrophosphorylation shows an absolute requirement for divalent cations, with Mg$^{2+}$ preferred (18). Both of these features are reminiscent of the prenyl diphosphate synthases, which feature DDXXD motifs that coordinate Mg$^{2+}$, which in turn activates the pyrophosphate as a leaving group (22). For serine pyrophosphorylation, one can imagine IP$_7$ phosphates organized by H-bonds within a polySer tract, whereas the IP$_3$ pyrophosphate could be activated and targeted to a particular phosphoserine phosphate with the assistance of an Asp/Glu-chelated Mg$^{2+}$.

What might be the physiologic role of pyrophosphorylation? Classic protein kinase-mediated phosphorylation modifies the conformation of proteins, enhancing or decreasing catalytic activity, determining protein localization, or altering protein–protein interactions. Presumably pyrophosphorylation also elicits such alterations in protein function. ATP-mediated phosphorylation is typically regulated by dephosphorylation, whereas pyrophosphorylated proteins are resistant to known protein phosphatases. This suggests that serine pyrophosphorylation may be a more stable modification within the cell, even though it is thermodynamically more unstable compared with phosphoserine. Such a conclusion is consistent with the fact that some of the best IP$_7$ targets, such as NSR1 and SRP40/Nopp140, exist endogenously as abundantly phosphorylated proteins (23–25).

### Materials and Methods

#### Synthesis of Inositol Pyrophosphate

The inositol pyrophosphate used throughout these studies was 5-PP-I(1,2,3,4,6)$_3$P$_3$, referred to as IP$_3$). The synthesis and purification of radiolabeled 5$[^{32}$P]IP$_3$ was carried out as described earlier (18). By using these procedures, 5$[^{32}$P]IP$_3$ was obtained at a specific activity of 60 Ci/mmol (1 Ci = 37 GBq) and used at 1–2 $\mu$Ci per reaction for protein phosphorylation. The synthesis of 5$[^{32}$P]-labeled 4/6-PP-IP$_3$ and IP$_5$ is described in SI Materials and Methods. Unlabeled 5-PP-I(1,2,3,4,6)$_3$P$_3$ was prepared by total synthesis by using modifications of an earlier method (26). Full experimental details are provided in SI Materials and Methods.

#### Preparation of Recombinant Proteins

The expression and purification of all recombinant proteins are described in SI Materials and Methods.

### Protein and Peptide Phosphorylation Assays

Cell lysates were obtained by resuspending yeast cells in ice-cold lysis buffer [20 mM Hepes (pH 6.8)/5 mM DTT/1 mM EGTA/1 mM EDTA/0.1% CHAPS, protease inhibitor mixture, and 200 mg/liter phenylmethylsulfonyl fluoride] and vortexing the sample in the presence of glass beads. The homogenates were centrifuged for 20 min at 15,000 × g, and supernatants were used in the assay. In experiments where lysates were used directly for phosphorylation, protein extracts (10–20 $\mu$g) were incubated in IP$_7$ phosphorylation buffer [25 mM Hepes (pH 7.4)/50 mM NaCl/6 mM MgCl$_2$/1 mM DTT] and 1 $\mu$Ci $[^{32}$P]IP$_3$ for 15 min at 37°C. The reactions were heated at 95°C for 5 min in sample buffer before separation by NuPAGE (Invitrogen, Carlsbad, CA). The gels were either stained and dried, or transferred to a PVDF membrane. Radiolabeled proteins were detected by autoradiography. Immunoblotting using anti-Xpress (Invitrogen) or anti-GST (Sigma, St. Louis, MO) antibodies, was performed according to standard procedures.

GST- or hexahistidine-tagged proteins purified from *S. cerevisiae* were used directly for phosphorylation by 5$[^{32}$P]IP$_3$. The protein (1–2 $\mu$g) was incubated in the presence of 5$[^{32}$P]IP$_3$, and the reaction was performed as described above. Where indicated, the purified proteins were preincubated with $\alpha$-phosphatase (400 units; New England Biolabs, Beverly, MA) at 30°C for 1 h, washed twice with PBS, and then used in a 5$[^{32}$P]IP$_3$ phosphorylation assay. GST fusion proteins purified from *E. coli*, were coupled to glutathione beads, and either incubated directly with 5$[^{32}$P]IP$_3$, as above or were first preincubated with 12 $\mu$g of extract prepared from *S. cerevisiae* (strain DDY1810) at 30°C for 1 h in IP$_3$ phosphorylation buffer with or without 1 mM ATP or other nucleotides. Where indicated, the *S. cerevisiae* extract was incubated in boiling water for 5 min, cooled, and then used for the preincubation with purified protein. After incubation with yeast extract, the glutathione beads were washed twice with PBS and once with IP$_3$ phosphorylation buffer and used for phosphorylation by 5$[^{32}$P]IP$_3$, as described above. In some cases, proteins purified from *E. coli* were first preincubated with 250 units of CK2 (New England Biolabs) in the supplied CK2 reaction buffer, along with 1 mM ATP at 30°C for 1 h, and then washed and used for 5$[^{32}$P]IP$_3$ phosphorylation. NSR1 amino acids 27–50 purified from *E. coli* cells coexpressing human CK2A1 was used directly in 5$[^{32}$P]IP$_3$ phosphorylation assays.

Peptides were synthesized as described in SI Materials and Methods. Synthetic peptides were used for both CK2 and ATP or IP$_3$-mediated phosphorylation assays. Peptide (3–6 $\mu$g) was incubated with 250 units of CK2 in CK2 phosphorylation buffer, 200 $\mu$M ATP, and 1 $\mu$Ci $[^{32}$P]ATP at 30°C for 15–30 min. For IP$_3$ phosphorylation, 3–6 $\mu$g of peptide was added to IP$_3$ phosphorylation buffer and 1–2 $\mu$Ci of 5$[^{32}$P]IP$_3$ at 37°C for 15 min, followed by incubation at 95°C for 5 min. The peptides were mixed with sample buffer and resolved on a 12% gel by using the NuPAGE system. The gels were stained by using Safe Stain (Invitrogen), dried, and used for autoradiography. Although peptide phosphorylation by IP$_3$ was routinely performed at 95°C to maximize the extent of phosphorylation, a time-dependent increase in phosphorylation is observed at 37°C (SI Fig. 9).

### Acid-Sensitivity Assay

To determine the sensitivity of CK2 and ATP or IP$_3$-phosphorylated NOP-2[S] peptide to treatment with acid, peptides were phosphorylated as described above and then incubated with either 1 M HCl at 50°C, with 3 M HCl at 25°C, or without any acid at 25°C, for 1 h. After incubation, the acid was neutralized with an appropriate volume of 10 M NaOH, and the reaction was mixed with sample buffer and subjected to NuPAGE. Phosphorylation was detected as described above. Peptide hydrolysis in the presence of 6 M HCl, followed by separation of amino acids by HPLC, was performed as described in SI Materials and Methods.

### $\alpha$-Phosphatase-Sensitivity Assay

Purified GST fusion proteins or NOP-2[S] peptide were phosphorylated by CK2 and $\gamma[^{32}$P]ATP or by 5$[^{32}$P]IP$_3$, as described above. After phosphorylation, the
biotinylated NOP-2[pS] peptide was bound to streptavidin agarose beads (Sigma) and washed with PBS. Protein or peptide was then incubated in the presence of λ-phosphatase (400 units; New England Biolabs) in supplied buffer according to the manufacturer’s instructions at 30°C, mixed with sample buffer, resolved by NuPAGE, and subjected to autoradiography. Expression and purification of 32[PO4]i-labeled GST fusion proteins and their treatment with phosphatase is described in SI Materials and Methods.

The Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen) was used to determine relative levels of phosphate on the NOP-2[pS] or NOP-2[ppS] peptides before or after treatment with λ-phosphatase. NOP-2[pS] peptide (6 μg) or NOP-2[ppS] peptide (24 μg) were incubated with 400 units of λ-phosphatase for 1 h at 30°C and then resolved by NuPAGE and stained with Pro-Q Diamond stain according to the manufacturer’s instructions.

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