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The Substrate of Greatwall Kinase, Arpp19, Controls Mitosis by Inhibiting Protein Phosphatase 2A

Aicha Gharbi-Ayachi, *et al.*

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Ensa are dephosphorylated, presumably by protein phosphatase(s) other than PP2A-B55 δ , such as PPI (29, 30). The threshold for activation of Gwl and entry into mitosis depends on the balance of activities of these as-yet unidentified phosphatases and that of MPF.

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19. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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31. We thank H. Mahubani, J. Kirk, and L. Egbuniwe for care of frogs and other members of the laboratory for advice and reagents, especially J. Gannon for the antibody to doubly phosphorylated Thr¹⁴-Tyr¹⁵ of Cdk1 (CP 3.2). S.M. was supported by a fellowship from the Japan Society for the Promotion of Science.

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Methods

Figs. S1 to S4

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The Substrate of Greatwall Kinase, Arpp19, Controls Mitosis by Inhibiting Protein Phosphatase 2A

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Initiation and maintenance of mitosis require the activation of protein kinase cyclin B–Cdc2 and the inhibition of protein phosphatase 2A (PP2A), which, respectively, phosphorylate and dephosphorylate mitotic substrates. The protein kinase Greatwall (Gwl) is required to maintain mitosis through PP2A inhibition. We describe how Gwl activation results in PP2A inhibition. We identified cyclic adenosine monophosphate–regulated phosphoprotein 19 (Arpp19) and α -Endosulfine as two substrates of Gwl that, when phosphorylated by this kinase, associate with and inhibit PP2A, thus promoting mitotic entry. Conversely, in the absence of Gwl activity, Arpp19 and α -Endosulfine are dephosphorylated and lose their capacity to bind and inhibit PP2A. Although both proteins can inhibit PP2A, endogenous Arpp19, but not α -Endosulfine, is responsible for PP2A inhibition at mitotic entry in *Xenopus* egg extracts.

Mitotic entry and exit are controlled by activation and inactivation of the cyclin B–Cdc2 protein kinase (1). However, maintained phosphorylation of mitotic proteins also requires inhibition of the protein phosphatase 2A (PP2A), the main phosphatase that dephosphorylates mitotic substrates (although a role of protein phosphatase 1 has also been reported in *Xenopus* egg extracts) (2–5).

The protein kinase Greatwall regulates PP2A activity during mitosis (2, 4, 6). Depletion of Gwl

from mitotic egg extracts induces mitotic exit, whereas depletion of Gwl from interphase egg extracts prevents mitotic entry (2–4). These phenotypes, as well as the phenotypes observed in Gwl knockdown human cells (6), are mediated by PP2A. Although Gwl and PP2A do interact, direct phosphorylation of PP2A by this kinase has not been observed (2, 4), suggesting that Gwl may inhibit PP2A through an intermediary protein. We used biochemical fractionation of cytosolic factor (CSF) meiotic arrested *Xenopus* egg extracts and in vitro phosphorylation by Gwl to identify substrates of this kinase.

We fractionated proteins from interphase extracts on a heparin column. Eluted proteins were precipitated with ammonium sulfate (50 to 70%), resuspended, and fractionated by gel filtration. Samples from the fractions were phosphorylated in vitro with a hyperactive form of human Gwl (K72M mutant) (7), and the phosphoryl-

ated bands were analyzed by mass spectrometry. Maximal phosphorylation was observed in the gel filtration fractions corresponding to molecular sizes of 30 to 70 kD (Fig. 1A, fraction 3) and 20 to 50 kD (Fig. 1A, fraction 4). These two fractions increased phosphorylation of a band of 20 kD. Forty different proteins were detected in this band by mass spectrometry, among them the cyclic adenosine monophosphate–regulated phosphoprotein 19 (Arpp19), a protein that strongly resembles the small protein α -Endosulfine. The functions of Arpp19 and α -Endosulfine are unclear (8, 9); however, oocytes from *Drosophila* with mutant α -Endosulfine have a prolonged prophase and fail to progress to metaphase (10). Cdc2 isolated from these oocytes has normal kinase activity, but the oocytes show a reduced amount of in vivo phosphorylation of mitotic substrates, a phenotype reminiscent of the one observed in Gwl-depleted *Xenopus* egg extracts (4). Although by mass spectrometry we identified peptides specific to Arpp19 but not for α -Endosulfine (fig. S1), their sequence similarity indicates that both may be Gwl substrates. We therefore tested glutathione S-transferase fusion proteins made from Arpp19 and α -Endosulfine, as possible substrates of Gwl. Both proteins were phosphorylated in vitro by Gwl (Fig. 1B). The endogenous proteins were present in small amounts in *Xenopus* egg extracts, particularly Arpp19, which was very difficult to detect (Fig. 1C). The analysis of the protein sequences of Arpp19 and α -Endosulfine revealed the presence of seven potential serine or threonine phosphorylation sites conserved in both proteins (Fig. 1D). We made individual mutants of Arpp19 in which serine (S) or threonine (T) was mutated to alanine (A) and tested whether they were phosphorylated in vitro by Gwl. All mutants were phosphorylated by Gwl except mutant S62A. Similarly, mutation of this conserved serine of human α -Endosulfine to alanine (S67A) also prevented the phosphorylation of this protein by Gwl (Fig.

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ID). These results indicate that this serine is the Gwl phosphorylation site in both Arpp19 and α -Endosulfine.

We analyzed the effects of Arpp19 and α -Endosulfine added to interphase *Xenopus* egg extracts. Either of these two proteins, if first phosphorylated in vitro by Gwl, promoted rapid mitotic entry as shown by the dephosphorylation of tyrosine 15 of Cdc2 and the phosphorylation of the mitotic substrates, followed by a subsequent exit from mitosis as indicated by the degradation of cyclin A and cyclin B2 (Fig. 1E). In a second experiment, to easily visualize mitotic entry, we prevented cyclin degradation by depleting the anaphase-promoting complex constituent Cdc27. In these extracts, the addition of phosphorylated Arpp19 or α -Endosulfine pro-

moted a rapid entry into mitosis and the maintenance of the mitotic state. Nonphosphorylated protein or the Arpp19 S62A or the α -Endosulfine S67A phosphorylation mutants had no effect (Fig. 2A).

Gwl mediates entry into mitosis through the inhibition of PP2A, and so we suspected that Arpp19 and α -Endosulfine could mediate mitotic entry by directly inhibiting PP2A. However, we could not exclude the possibility that these two proteins also regulate cyclin B-Cdc2 activity to promote entry into mitosis. To determine whether Arpp19 or α -Endosulfine could induce mitotic entry through the regulation of cyclin B-Cdc2 activity, we depleted Cdc25 in interphase egg extracts and analyzed whether the two proteins still induced mitotic entry (II). We also de-

pleted Cdc27 to prevent cyclin degradation and to easily visualize mitotic entry in these extracts. Either phospho-Arpp19 or phospho- α -Endosulfine induced entry into mitosis in these extracts where only cyclin A-Cdc2 appeared to be active (Fig. 2B). Thus, Arpp19 and α -Endosulfine are substrates of Gwl that promote mitotic entry, likely through the regulation of PP2A but not through the regulation of cyclin B-Cdc2.

Accordingly, when Gwl was depleted from interphase egg extracts, the addition of α -Endosulfine and Arpp19 still induced mitotic entry, further indicating that they act downstream of Gwl (Fig. 2C). These two proteins promoted entry into mitosis in Gwl-depleted interphase extracts only when they were thio-phosphorylated, indicating that when Gwl is present, dephosphorylation

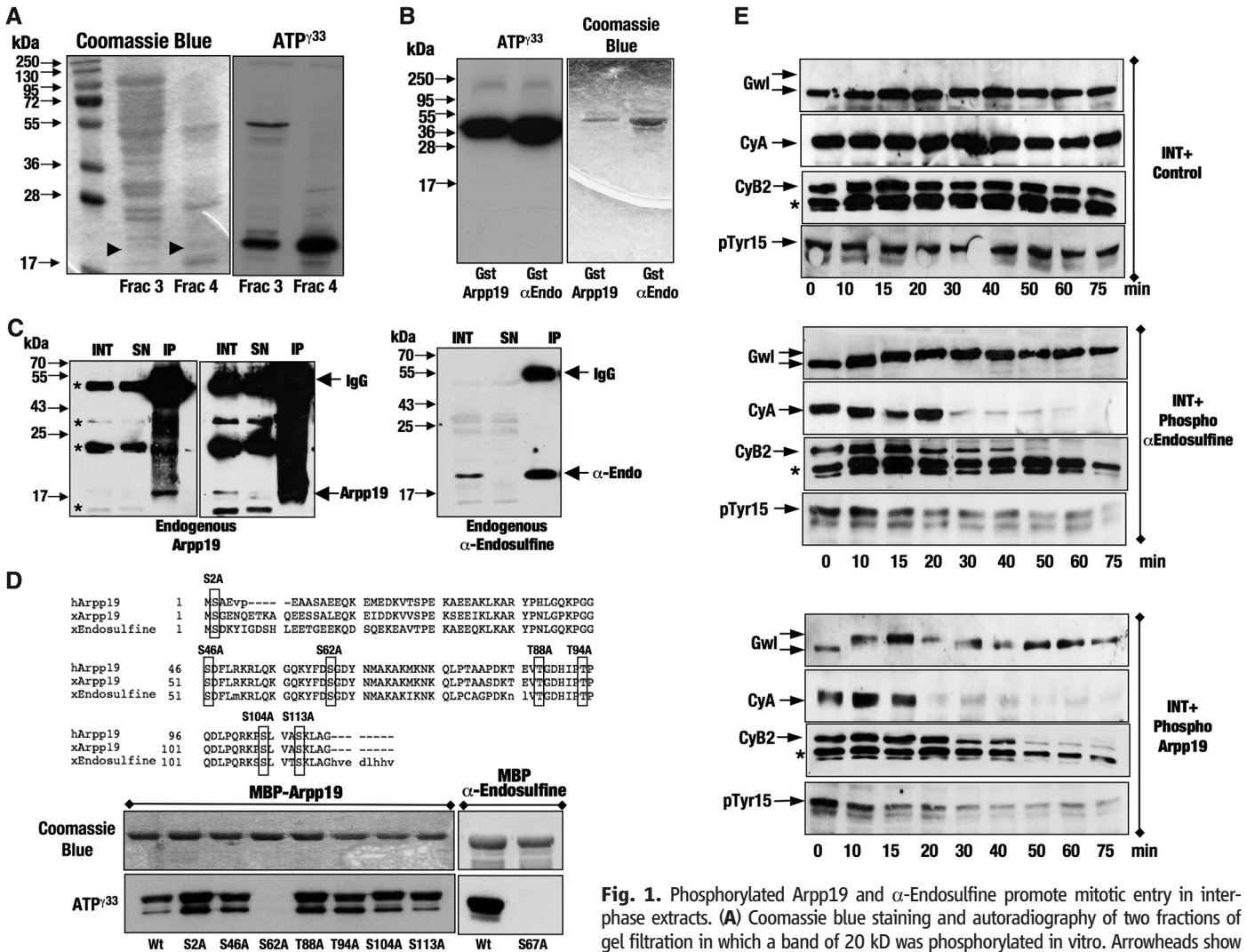


Fig. 1. Phosphorylated Arpp19 and α -Endosulfine promote mitotic entry in interphase extracts. (A) Coomassie blue staining and autoradiography of two fractions of gel filtration in which a band of 20 kD was phosphorylated in vitro. Arrowheads show bands analyzed by mass spectrometry. (B) In vitro phosphorylation of GST-Arpp19 and GST- α -Endosulfine by Gwl. (C) Western blot performed with antibody to full-length human Arpp19 (two exposure times) and to C-terminal *Xenopus* α -Endosulfine showing equal amounts of interphase extract and supernatant [10 μ l in Arpp19 immunoprecipitation (IP) and 2 μ l in α -Endosulfine IP] and an IP corresponding to 20 μ l of interphase extract. To improve detection of endogenous Arpp19, we boiled extract and supernatant before adding Laemmli buffer. (D) Protein sequence alignment of human and *Xenopus* Arpp19 and *Xenopus* α -Endosulfine. The conserved S/T sites that were mutated to A are indicated. (E) Interphase extracts (INT) were supplemented with phospho- α -Endosulfine or phospho-Arpp19 at a final concentration of 170 ng/ μ l, or were left untreated (Control). We determined mitotic entry by analyzing the phosphorylation of Gwl, the dephosphorylation of tyrosine 15 on Cdc2, and the abundance of cyclin A and cyclin B2. Nonspecific bands are indicated with an asterisk. Abbreviations for the amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

ation of these two substrates is counterbalanced by endogenous Gwl, whereas when Gwl is absent, these two proteins are rapidly dephosphorylated and do not promote or maintain the mitotic state.

Moreover, the addition of thio-phosphorylated Arpp19 or α -Endosulfine to Gwl-depleted CSF extracts also prevented mitotic exit (Fig. 2D), demonstrating that Arpp19 and α -Endosulfine are phosphorylated by Gwl and act downstream of this kinase to promote mitotic entry, possibly by directly inhibiting PP2A. Accordingly, phosphorylation of the Gwl-specific site on Arpp19

and α -Endosulfine (S62/S67) decreased simultaneously with dephosphorylation of Gwl at mitotic exit (Fig. 2E), and it increased again concomitantly with the phosphorylation of this kinase at mitotic entry (Fig. 2F).

To determine whether Arpp19 or α -Endosulfine could bind and inhibit PP2A, we incubated glutathione *S*-transferase (GST)-Arpp19 and GST- α -Endosulfine with interphase or with mitotic egg extracts and tested for association of PP2A with the fusion proteins. GST- α -Endosulfine (moderately) and GST-Arpp19 (very weakly) bound PP2A in interphase egg extracts, in which Gwl is

inactive. However, the binding increased (17 times for GST-Arpp19 and twice for GST- α -Endosulfine) in mitotic egg extracts, in which Gwl is fully active, suggesting that the association of these two proteins with the A and C subunits of PP2A is increased by their Gwl-dependent phosphorylation. In support of this idea, the mutation to alanine of the Gwl phosphorylation site S62 in Arpp19 and S67 in α -Endosulfine prevented the binding of either protein to PP2A. However, this association appears not to be exclusively regulated by Gwl-dependent phosphorylation because the α -Endosulfine mutant D66A did not bind

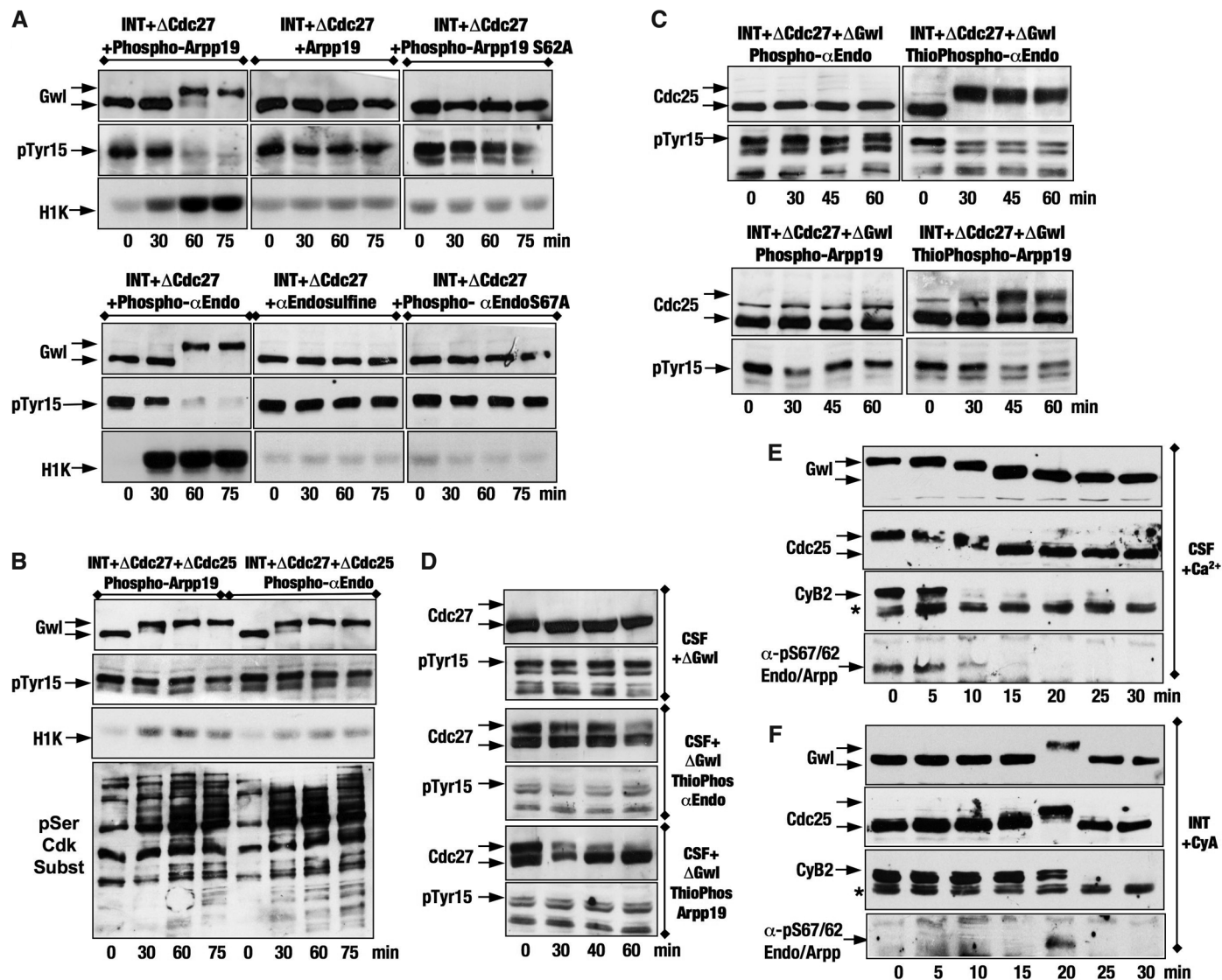


Fig. 2. Addition of thio-phosphorylated Arpp19 and α -Endosulfine rescues the phenotype induced by Gwl depletion in interphase and mitotic egg extracts. (A) Interphase extracts were depleted of Cdc27 and supplemented at a final concentration of 170 ng/ μ l with the proteins GST-Arpp19 and GST- α -Endosulfine phosphorylated or not in vitro by Gwl. The Gwl-phosphorylation mutants Arpp19-S62A and α -Endosulfine-S67A were also used. (B) Mitotic entry was analyzed in interphase extracts that were depleted of Cdc27 and Cdc25 and supplemented with phospho-Arpp19 and phospho- α -Endosulfine (final concentration of 170 ng/ μ l). The phosphorylation of cyclin B-Cdc2 substrates was analyzed with an antibody recognizing the phospho-Serine

Cdk consensus motif. (C) Interphase extracts were depleted of Cdc27 and Gwl and supplemented with phosphorylated or thio-phosphorylated Arpp19 and α -Endosulfine. (D) Mitotic extracts (CSF) were supplemented with thio-phosphorylated Arpp19 or α -Endosulfine and depleted of Gwl. (E) Mitotic exit was induced in CSF extracts by the addition of Ca^{2+} , and the amounts of cyclin B2 and phosphorylation of Cdc25, Gwl, and the Gwl-specific sites of Arpp19 and α -Endosulfine were analyzed. (F) Interphase extracts were supplemented with cyclin A (final concentration of 60 nM), and the kinetics of the phosphorylation of the indicated proteins as well as the levels of cyclin B2 were analyzed.

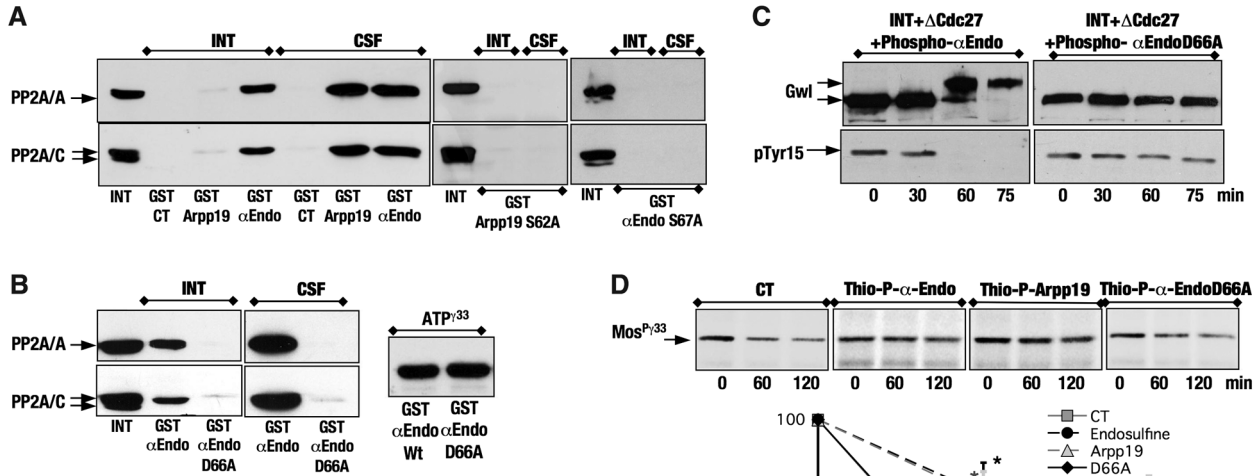
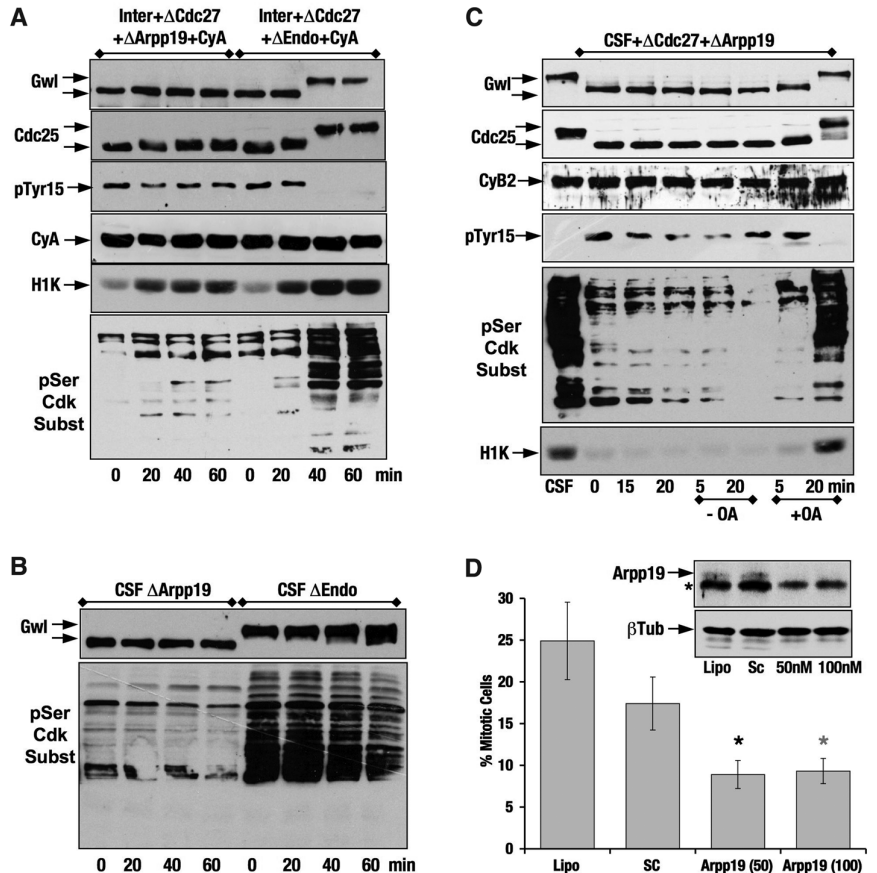


Fig. 3. Inhibition of PP2A by phosphorylated Arpp19 and α -Endosulfine. (A) Interphase or mitotic extracts were supplemented with GST-Sepharose, GST-Arpp19-Sepharose, GST- α -Endosulfine-Sepharose, the GST-Arpp19 (S62A)-Sepharose, or GST- α -Endosulfine (S67A)-Sepharose, and the binding of PP2A subunits A and C was analyzed by GST precipitation and Western blot. (B) Similar to (A) except for the addition of GST- α -Endosulfine-Sepharose and GST- α -Endosulfine (D66A)-Sepharose. Lower panels show the *in vitro* phosphorylation of the α -Endosulfine (D66A) mutant by Gwl. (C) Effect of the phospho- α -Endosulfine (D66A) mutant on mitotic entry in interphase extracts. (D) PP2A complex obtained by immunoprecipitation from interphase extracts was left untreated (CT) or was incubated for 10 min with 5.3 μ g of either thio-phospho-GST-Arpp19, thio-phospho-GST- α -Endosulfine, or thio-phospho-GST- α -Endosulfine (D66A) and then mixed with phosphoradiolabeled myelin basic protein-c-Mos fusion protein. At the indicated times, 15 μ l of supernatant was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Gels were scanned by a Typhoon Scanner and quantified with Image software. Statistical analysis of the results obtained from two different independent experiments was performed with an unpaired Student's *t* test. The percentage of phosphorylated c-Mos present at each time was expressed as the mean \pm SD. Statistical differences between control versus either Arpp19 or α -Endosulfine at the two time points are indicated. **P* < 0.03; #*P* < 0.04

Fig. 4. Endogenous Arpp19 but not α -Endosulfine is required to promote mitotic entry. (A) Interphase extracts were depleted of Cdc27 and either Arpp19 or α -Endosulfine and then supplemented with cyclin A. (B) Mitotic extracts were depleted of Arpp19 or α -Endosulfine, and the mitotic state was analyzed. (C) Mitotic extracts were depleted of Cdc27 and Arpp19 and 20 min later were (+OA) or were not (-OA) supplemented with okadaic acid (final concentration of 0.7 μ M), and the mitotic state was analyzed. (D) HeLa cells were transfected or not (Lipo) with scrambled small interfering RNA (siRNA) (SC) or 50 or 100 nM of Arpp19 siRNA for 24 hours, then synchronized by thymidine and released into nocodazole (50 ng/ml) for 10 hours. The percentage of mitotic cells was measured by a two-dimensional fluorescence-activated cell sorting (propidium iodide-antibody against phosphoserine Cdk) and expressed as the mean \pm SD. Statistical differences between scrambled and either 50 or 100 nM Arpp19 siRNA are indicated (**P* < 8.03 $\times 10^{-6}$ and **P* < 1.45 $\times 10^{-6}$). The cellular levels of endogenous Arpp19 at each condition are shown.



PP2A, although it was normally phosphorylated by Gwl at residue S67 (Fig. 3B). Moreover, the addition of this mutant previously phosphorylated by Gwl into interphase extracts did not induce mitotic entry (Fig. 3C), consistent with the idea that Gwl may inhibit PP2A by promoting the binding of its inhibitors, Arpp19 and α -Endosulfine.

We tested directly the effect of Arpp19 and α -Endosulfine on PP2A activity obtained from immunoprecipitated CSF extracts by assessing dephosphorylation of the cyclin B–Cdc2 substrate c-Mos in vitro in the presence or in the absence of thio-phosphorylated-Arpp19, thio-phosphorylated- α -Endosulfine, and thio-phosphorylated- α -Endosulfine D66A. Thio-phosphorylated-Arpp19 and thio-phosphorylated- α -Endosulfine significantly decreased dephosphorylation of c-Mos by PP2A. Thio-phosphorylated- α -Endosulfine mutant D66A, which does not bind PP2A, had no effect (Fig. 3D).

To test the physiological role of Arpp19 and α -Endosulfine, we depleted interphase extracts of Cdc27 and then depleted them with specific antibodies against Arpp19 or α -Endosulfine (fig. S2), and finally supplemented them with cyclin A. Depletion of Arpp19, but not of α -Endosulfine (Fig. 1C), completely inhibited entry into mitosis (Fig. 4A)—a phenotype that was rescued by adding back this thio-phosphorylated protein (fig. S3). Similarly, only depletion of Arpp19 from CSF ex-

tracts caused rapid exit of mitosis (Fig. 4B). This exit appeared to be mediated by a reactivation of PP2A because the inhibition of PP2A with okadaic acid caused these extracts to reenter mitosis (Fig. 4C). Thus, despite the inhibitory effects of both Arpp19 and α -Endosulfine on PP2A, only Arpp19 appears to regulate mitotic entry and exit in *Xenopus* egg extracts. Consistent with this, only Arpp19 is phosphorylated during mitosis (fig. S2) and, despite the presence of larger amounts of endogenous α -Endosulfine than endogenous Arpp19 in *Xenopus* egg extracts, only Arpp19 was identified in our biochemical analysis.

To investigate whether this mechanism was also conserved in human cells, we depleted Arpp19 from human cervical cancer (HeLa) cells using two different sequences of small interfering RNA (siRNA) (fig. S4). Depletion of Arpp19 reduced the number of mitotic cells by 50% compared to that in cells treated with a scramble siRNA, suggesting that Arpp19 also promotes mitotic entry in human cells (Fig. 4D and fig. S4).

Our results demonstrate an essential role of Arpp19 in regulating mitosis and provide a mechanism by which Gwl can influence cell cycle control through regulation of PP2A. Whether Arpp19 might be dephosphorylated at mitotic exit remains to be elucidated. Perhaps other physiological pathways might be regulated by α -Endosulfine-dependent inhibition of PP2A.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S4
References

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Cholinergic Interneurons Control Local Circuit Activity and Cocaine Conditioning

Ilana B. Witten,^{1*} Shih-Chun Lin,^{1,2*} Matthew Brodsky,^{1*} Rohit Prakash,^{1*} Ilka Diester,¹ Polina Anikeeva,¹ Viviana Gradinaru,¹ Charu Ramakrishnan,¹ Karl Deisseroth^{1,3,4,5†}

Cholinergic neurons are widespread, and pharmacological modulation of acetylcholine receptors affects numerous brain processes, but such modulation entails side effects due to limitations in specificity for receptor type and target cell. As a result, causal roles of cholinergic neurons in circuits have been unclear. We integrated optogenetics, freely moving mammalian behavior, in vivo electrophysiology, and slice physiology to probe the cholinergic interneurons of the nucleus accumbens by direct excitation or inhibition. Despite representing less than 1% of local neurons, these cholinergic cells have dominant control roles, exerting powerful modulation of circuit activity. Furthermore, these neurons could be activated by cocaine, and silencing this drug-induced activity during cocaine exposure (despite the fact that the manipulation of the cholinergic interneurons was not aversive by itself) blocked cocaine conditioning in freely moving mammals.

Acetylcholine is an important and widely studied neurotransmitter, which acts on a variety of receptors and target cells (1–5). Pharmacological and genetic studies have elucidated the complex and often opposing influences of the individual subtypes of muscarinic and nicotinic acetylcholine receptors on numerous biological processes, but no study has yet resolved the question of the causal role of cholinergic neurons themselves within a central nervous system

tissue (6–11). Addressing such a question would require a novel paradigm for selective and temporally precise control (activation and inhibition) of cholinergic neurons within living mammalian tissues, because previous investigations have resulted in contradictory findings linked to challenges with specificity and temporal resolution. For example, elegant in vivo pharmacological approaches have shown (12–14) that cholinergic transmission in the nucleus accumbens (NAc) [a

structure involved in natural reward-related behaviors and responses to drugs such as cocaine (15–19)] is required for reward learning, but novel studies of molecular ablation of cholinergic interneurons within the NAc instead have reported enhanced reward learning (20). Cholinergic interneurons within the NAc are particularly intriguing because they constitute less than 1% of the local neural population (21), yet they project throughout the NAc and provide its only known cholinergic input (22). Relevant cholinergic receptors are expressed locally, and nicotinic and muscarinic pharmacological agonists can exert complex influences on medium spiny neurons (MSNs, which represent >95% of the local neuronal population and constitute the output of the NAc) (23–25). However, the net effect (if any) of the cholinergic interneurons on any aspect of NAc physiology or behavior is unknown.

We undertook an optogenetic approach to resolve this question by selectively driving or blocking action potential firing in these cells. To

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