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# The *Drosophila* mitotic inhibitor Frühstart specifically binds to the hydrophobic patch of cyclins

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**The hydrophobic patch of cyclins interacts with cyclin-dependent kinase (Cdk) substrates and p27-type Cdk inhibitors. Although this interaction is assumed to contribute to the specificity of different Cdk–Cyclin complexes, its role in specific steps of the cell cycle has not been demonstrated. Here, we show that in *Drosophila* the mitotic inhibitor Frühstart (Frs) binds specifically and with high affinity to the hydrophobic patch of cyclins. In contrast to p27-type Cdk inhibitors, Frs does not form a stable interaction with the catalytic centre of Cdk and allows phosphorylation of generic model substrates, such as histone H1. Consistent with a 2.5 times stronger binding to CycA than to CycE *in vitro*, ectopic expression of *frs* induces endocycles, in a manner similar to that reported previously for down-regulation of *CycA* or *Cdk1*. We propose that binding of Frs to cyclins blocks the hydrophobic patch to interfere with Cdk1 substrate recognition.**

Keywords: *Drosophila*; cell-cycle inhibitor; hydrophobic patch; cyclin; blastoderm

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## INTRODUCTION

The activity of cyclin-dependent kinase (Cdk) is controlled by several mechanisms. Inhibitors of the p14 family bind to Cdk6, leading to disruption of the Cdk6–cyclin (Cyc)D complex, whereas inhibitors of the p27 family form a stable association with the G1/S-phase-specific Cdk2–Cyclin complex by binding to the hydrophobic patch of cyclin and also to the catalytic centre of the kinase subunit (Pavletich, 1999). Common to G1/S- and G2/M-specific cyclins is the hydrophobic patch that acts as a binding site for some Cdk substrates and also for p27-type inhibitors (Zhu *et al*, 1995; Adams *et al*, 1996; Chen *et al*, 1996; Brown *et al*, 1999). The K/RxL motif (called KxL in the following) of substrates and p27-type inhibitors binds to a hydrophobic patch on the cyclin surface. Although this interaction has been studied in great detail (Adams *et al*, 1999; Lowe *et al*, 2002; Lacy *et al*, 2004), the role of the hydrophobic patch in substrate specificity of various Cdk–Cyclin complexes, and to which degree it is required for substrate phosphorylation (Wittenberg, 2005), remains to be established. It is also unknown whether the hydrophobic patch is essential for specific steps of the cell cycle.

The problem of specificity is best exemplified in budding yeast, which has only one Cdk, CDC28. By association with cyclins specific for G1 or G2, differential groups of substrates are assumed to be phosphorylated. By analysing the phosphorylation of a library of Cdk substrates *in vitro*, Loog & Morgan (2005) found that the G2/M-specific Cdk1–Clb2 (Clb2 for Cyclin B-like 2) phosphorylated all substrates equally well, whereas the S-phase-specific Cdk1–Clb5 showed preference for a small subset of substrates, but poorly phosphorylated most substrates. Efficient phosphorylation of these S-phase specific substrates depended on the hydrophobic patch of Clb5; therefore, it was proposed that the hydrophobic patch would contribute to the correct selection of S-phase-specific substrates.

The new protein Frühstart (Frs) and the pseudo-kinase Tribbles (Trbl) are specific cell-cycle regulators that are active in certain cell types and developmental stages (Lee & Orr-Weaver, 2003). Frs delays entry into mitosis in the mesoderm anlage during invagination and is involved in turning off the rapid nuclear division cycles after 13 rounds during the blastoderm stage (Seher & Leptin, 2000; Großhans & Wieschaus, 2000; Großhans *et al*, 2003). As no obvious motifs are present in the Frs primary structure, the molecular mechanism of cell-cycle inhibition by Frs is currently unknown.

Here, we show that *frs* inhibits mitotic entry but not G1/S phase. Consistent with the physiological function, preferential binding of Frs to CycA compared with CycE is shown. Complex formation requires the KxL motif of Frs and the hydrophobic patch of cyclins. But, in contrast to p27-type inhibitors, Frs does not associate with the kinase subunit.

## RESULTS

### *frs* inhibits entry into mitosis but not S phase

The precocious mitosis during ventral furrow formation in *frs* mutant embryos clearly shows the function of *frs* in the G2/M transition (Großhans & Wieschaus, 2000; Seher & Leptin, 2000).

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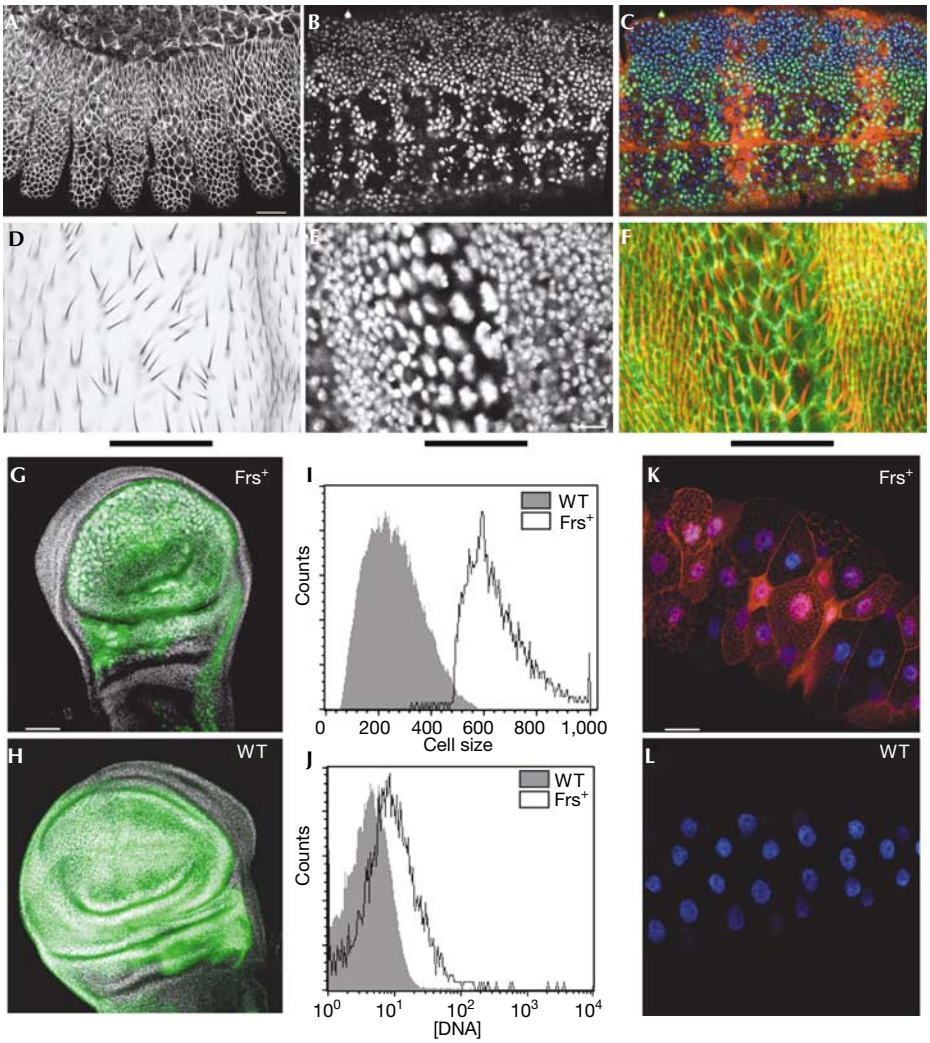


**Fig 1** | *frs* inhibits M phase but not S phase. (**A–C**) Genotype: pairedGal4/UASfrs[H]. (**A**) Cell number in the epidermal segments with *frs* expression was half that of neighbouring segments (38 ± 3 compared with 20 ± 3 cells per 34 μm$^2$, $n = 10$, stained for discs-large (Dlg). Scale bar, 20 μm. (**B,C**) Progression through S phase was marked by BrdU incorporation (25 min pulse). (**B**) BrdU, white. (**C**) BrdU, green; Frs, red; DNA, blue. (**D–F**) *frs* expressed in a stripe (indicated by the black bar) in pupal wing imaginal discs. Genotype: patchedGal4/UASfrs[J]. (**D**) Section of a differentiated wing. (**E,F**) Pupal discs (phalloidin, green; cell borders, red; higher optical section showing the trichomes and DAPI, white). Scale bar, 20 μm. (**G–J**) *frs* expressed in wing discs. Genotypes: (**G**) MS1096Gal4/+; UASfrs[C]/UASGFP, and (**H**) MS1096Gal4/+; UASGFP/+. The expression domain is indicated by GFP expression (green) and DNA (white). Scale bar, 50 μm. Cells were sorted by GFP expression and cell size, and analysed according to DNA content. (**I**) Cells from wild-type discs and size-selected population of large cells from the *frs*-expressing discs. (**J**) DNA content of the cell populations shown in (**I**). (**K**) *frs* expressed in third instar salivary glands in comparison with wild-type glands (**L**; DNA, white; Frs, red). Genotypes: (**K**) patchedGal4/UASfrs[C], (**L**) patchedGal4/UASGFP. Scale bar, 50 μm. BrdU, 5-bromodeoxyuridine; DAPI, 4′,6′-diamidino-2-phenylindol; Frs, Frühstart; GFP, green fluorescent protein; UAS, upstream activating sequence.

To test whether *frs* also inhibits G1-specific Cdk, we expressed *frs* in a striped pattern during mid-embryogenesis. This ectopic expression inhibited the last zygotic cell cycle, cycle 16, as shown by the halved cell density (Fig 1A). The reduced number of cells was not due to inhibition of S phase, as 5-bromodeoxyuridine (BrdU) was incorporated in S phase 16 in those embryos (Fig 1B,C). In contrast to *frs* expression, similar expression of *dacapo*, the p27 homologue as reported by Lane *et al* (1996), inhibits S phase, as measured by BrdU incorporation. These experiments show that *frs* affects cell-cycle progression, but not by inhibiting the G1/S transition.

To assess the activity of *frs* in cells with a full cell cycle of G1 and G2 phases, we used imaginal discs of *Drosophila* larvae. Expression of *frs* in the wing imaginal discs induced morphological defects in pupal discs and adult wings (Fig 1D–F), which are consistent with inhibition of the mitotic Cdk1 (Sauer *et al*, 1995; Hayashi, 1996; Weigmann *et al*, 1997; Vidwans *et al*, 2002). The cells of wing imaginal discs were much larger and had bigger

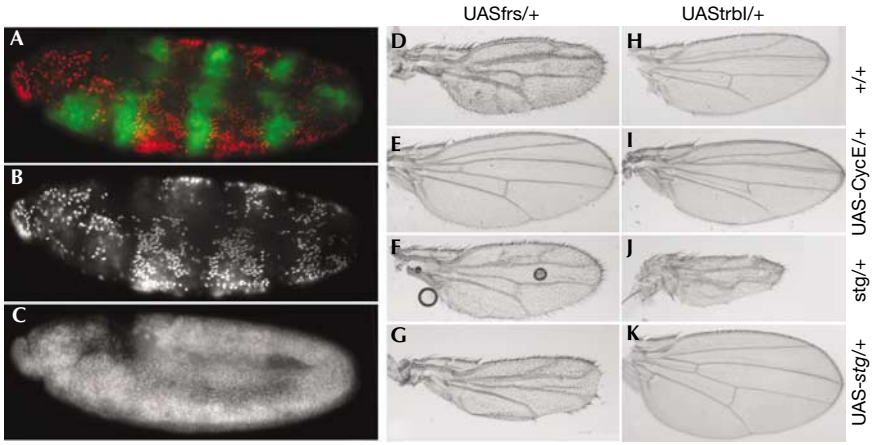


**Fig 2** | Frs inhibits the constitutively active *Cdk1-AF* allele and does not genetically interact with *string*. (**A–C**) Embryo expressing Cdk1-14A15F (uniformly by heat shock) and *frs* (UASfrs[H], in stripes with the pairedGal4) were stained for Frs (**A**, green), mitotic nuclei (pH3-S10; (**A**) red, (**B**) white and DNA (**C**, white). Expression of Cdk1-AF induces premature mitoses that are inhibited in the regions with Frs expression. (**D–K**) Wings of flies in which *frs* (**D–G**), *trbl* (**H–K**), CycE (**E,I**) or *string* (stg; **G,K**) was expressed with a Gal4 line specific for the wing blade (MS1096) or that was heterozygotes for *string* (**F,J**). Genetic interactions can be observed by the improved or worsened morphology. Cdk, cyclin-dependent kinase; CycE, cyclin E; Frs, Frühstart; trbl, tribbles; UAS, upstream activating sequence.

nuclei with strong DNA staining (Fig 1E–H), as confirmed by fluorescence-activated cell sorting analysis (Fig 1I,J). This phenotype produced groups of wing hairs with disturbed polarity in the differentiated wings of adults (Fig 1D,F). The higher DNA content suggests that *frs* induced endocycles. Conversely, expression of *frs* during endocycles in the larval salivary glands (Fig 1K,L) did not prevent the growth of the tissue, suggesting that ectopic expression of *frs* at the level applied did not stop normally occurring endocycles; however we, do not exclude a weak interference with Cdk2-controlled endocycles. Endocycles and similar morphological defects are also induced by downregulation of *Cdk1* or *CycA* in *Drosophila* and mitotic Cdk in yeast (Moreno & Nurse, 1994; Sauer *et al*, 1995; Hayashi, 1996; Weigmann *et al*, 1997; Vidwans *et al*, 2002). The *frs* loss-of-function and gain-of-function phenotypes show preferential inhibition of the G2/M transition and thus specificity for the mitotic Cdk1 complexes.

Entry into mitosis during the zygotically controlled cell cycles is triggered by *string* (*Drosophila melanogaster* CDC25). To test whether *frs* interferes with the activation of Cdk1 by *string*, we coexpressed an allele of Cdk1 (Cdk1AF, ubiquitously induced by heat shock) that does not require dephosphorylation by *string* to be active with *frs* (in stripes with pairedGal4; Fig 2A–C). Almost no mitotic chromatin was detected in regions of the embryo with *frs* expression, whereas between the stripes of *frs* expression many mitotic chromosomes, which synchronously entered mitosis, were observed. Control embryos of the same stage with no Cdk1AF expression, showed fewer mitotic nuclei. Furthermore, we tested genetic interactions of *frs* and *string* in wings. As described before, ectopic expression of *frs*, as well as *trbl*, induces endocycles in wing imaginal discs. The resulting phenotype is not significantly altered by increasing or decreasing the expression of *string*, whereas overexpression of *CycE* suppresses the phenotype. By contrast, the phenotype caused by expression of *trbl* is modified by *string* but not by *CycE*. These experiments indicate that *frs* inhibits Cdk1 by a method other than interfering with activation by *string*.

## Frs associates with cyclins

To investigate the molecular mechanism of how Frs inhibits mitotic Cdk1 complexes, we identified interactors of Frs both biochemically and using a yeast two-hybrid screen. We isolated components of the nuclear transport machinery and the nuclear pore (supplementary Fig S1 online), which suggested that Frs shuttles between the nucleus and the cytoplasm. Furthermore, we isolated two independent clones of CycE with the yeast two-hybrid system (supplementary Fig S1 online). We confirmed the formation of Frs–Cyclin complexes *in vitro*. Beads loaded with GSTfrs (GST for glutathione-*S*-transferase) specifically associated with $^{35}$S-labelled CycA, CycB, CycB3 and CycE (Fig 3A). By contrast, we did not detect any binding of GSTfrs to Cdk1 (Fig 3A). Next, we concentrated on CycA as it is required—in contrast to CycB and CycB3—for mitotic entry in *Drosophila* (Lee & Orr-Weaver, 2003). The Frs–CycA–Cdk1 complex could be immunoprecipitated with CycA and Cdk1 antibodies from embryos (Fig 3B). By contrast, CycE immunoprecipitates did not contain detectable amounts of Frs, which suggests that Frs specifically associates with mitotic Cdk complexes *in vivo*.

To quantify complex formation and to assess the extent to which Frs binds preferentially to mitotic cyclins, we measured the kinetic parameters of complex formation with amino-terminally truncated cyclins by surface plasmon resonance (SPR; Fig 3C; Table 1; supplementary Fig S2 online). We detected binding of CycAΔN170 to immobilized Frs with a nanomolar binding constant ($K_D = 38$ nM; Fig 3C; Table 1), whereas the interaction of human (h) Cdk2 and Frs could not be measured ($K_D > 1$ μM; Fig 3C). In comparison, as determined by isothermic calorimetry, the cyclin-dependent kinase inhibitor p27 bound to both hCycA and hCdk2 with nanomolar binding constants ($K_D$(hCycA) = 25 nM and $K_D$(hCdk2) = 70 nM; Lacy *et al*, 2004). These and our data show that Frs binds as tightly as p27 to CycA, indicating that Frs is able to block the binding site of cyclins. In contrast to p27, Frs does not stably bind to the kinase subunit. Furthermore, binding of Frs to the Cdk–CycAΔN170 complex was no stronger

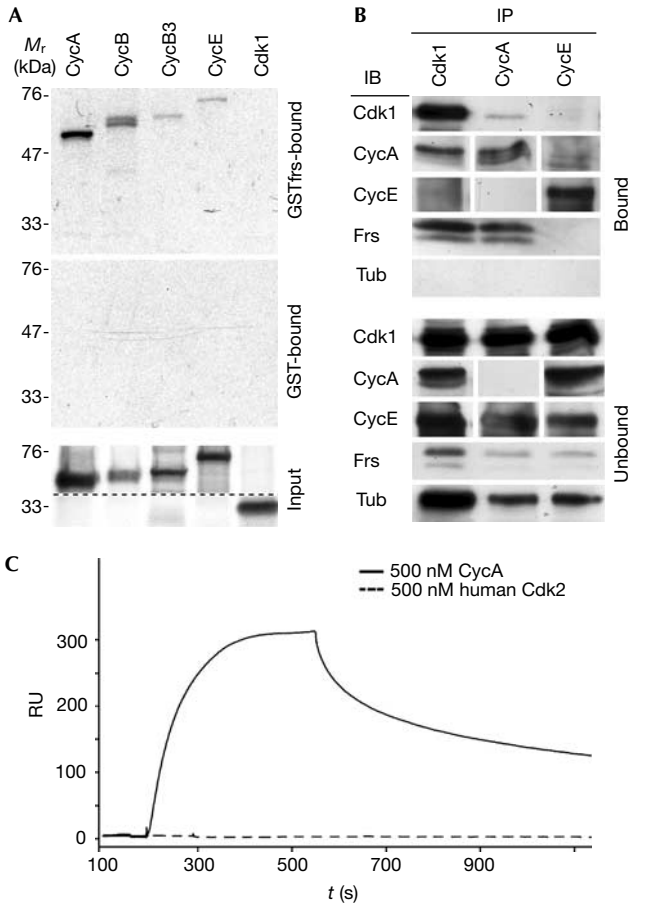


**Fig 3** | Frs binds to cyclins with high affinity. (**A**) Frs binds cyclins but not Cdk1 *in vitro*. $^{35}$S-labelled cyclins or Cdk1 were incubated with beads loaded with GSTfrs or GST. Bound (50%) and unbound (100%) fractions were analysed by SDS–PAGE and autoradiography. (**B**) Frs associates with the CycA–Cdk1 complex *in vivo*. Cdk1, CycA or CycE immunoprecipitates obtained from lysed embryos (stage 5) were analysed by western blotting with the indicated antibodies. To achieve higher loading for CycA and CycE, a second immunoprecipitation was performed with the unbound fraction with CycA and CycE antibodies. Loading per slot in embryo equivalents (bound/unbound): Cdk1 (50/50), CycA (2,000/2,000), CycE (2,000/2,000), Frs (2,000/50), tubulin (10/10). (**C**) Quantitative analysis of complex formation with immobilized Frs ($H_{10}$ZZ-frs) by surface plasmon resonance with human Cdk2 (500 nM) and CycAΔN170 (500 nM). Cdk, cyclin-dependent kinase; Cyc, cyclin; Frs, Frühstart; GST, glutathione-*S*-transferase; IB, immunoblottting; IP, immunoprecipitation; RU, response units; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

than its binding to isolated CycAΔN170 (Table 1; supplementary Fig S2 online). To address the cell-cycle specificity of Frs, we determined the binding constant of Frs to the G1/S cyclin CycEΔN300. We measured a binding constant of $K_D = 96$ nM (Fig 3C; Table 1), showing that Frs preferentially binds to CycA compared with CycE, and thus showing a molecular specificity of Frs; however, this is not absolute. Both binding constants are in the range of the estimated concentration of Frs ($c \approx 100$ nM; supplementary Fig S2 online) during mid-cellularization, when *frs* expression reaches its highest values (Großhans *et al*, 2003).

## The KxL motif of Frs is required for mitotic inhibiton

To improve analysis of the interaction, we mapped the important residues of Frs and cyclin that are required for the interaction. In an unbiased approach, we screened a library of random *frs* mutants for clones that had lost the ability to bind to CycE but not to Nup50 (Nuclear pore protein 50), in the yeast two-hybrid system (Fig 4A). The isolated clones either encoded carboxy-terminal truncations or contained mutations in one of the last six codons or the stop codon (Fig 4A), indicating that the C-terminal part of Frs is required for binding to CycE. Close examination of the C-terminal sequence showed a KxL motif including a phenylalanyl residue at position +2 and a basic residue at position −3 (Fig 4B; supplementary Fig S2 online). This motif has been found in the cyclin-dependent kinase inhibitor and Cdk substrates and has been thoroughly analysed (Fig 4B; Moreno & Nurse, 1994; Zhu *et al*, 1995; Adams *et al*, 1996; Chen *et al*, 1996; Sanchez-Diaz *et al*, 1998; Brown *et al*, 1999; Foley *et al*, 1999; Lowe *et al*, 2002). Substituting the basic and hydrophobic residues of the KxL motif with alanine (Frs86ASA) severely affected the Frs–Cyclin complex formation in the two-hybrid assay (Fig 4C), as well as in the *in vitro* binding assay (Fig 4D). Furthermore, a peptide comprising the C-terminal residues of Frs with the KxL motif interfered with Frs–CycA association, although competition was effective only at high concentrations (Fig 4C). To confirm that Frs binds to the hydrophobic patch, three hydrophobic amino-acid residues of CycA were mutated to alanine (Schulman *et al*, 1998). This mutation did not interfere with complex formation of CycA with hCdk2 but compromised binding to Frs (Fig 4D). In conclusion, we were able to identify a KxL motif at the C terminus of Frs that is required for binding to the hydrophobic patch of CycA.

To test whether binding to cyclin is essential for *frs* function *in vivo*, we introduced the mutated KxL motif into a genomic rescue construct and tested its ability to complement the ventral furrow phenotype of *frs* (Großhans *et al*, 2003). We found that mutations in the putative nuclear export signal (frs11AxxA) did not affect *frs* function, whereas Frs with a mutated KxL motif was not active (Table 2; supplementary Fig S3 online). These data indicate that *frs* requires binding to the hydrophobic patch of cyclins *in vivo*.

## Frs inhibits Cdk kinase activity *in vitro*

Frs does not stably bind to the kinase subunit; therefore, we investigated whether Frs would interfere with Cdk kinase activity. In an *in vitro* kinase assay, we compared the effect of Frs on the phosphorylation of model substrates (histone H1 (calf), Rb (human) and LaminDmO (*Drosophila*)) by Cdk1 obtained by immunoprecipitation from embryonic lysates (Fig 4E; supplementary Fig S4 online). We found that the Cdk1 and Cdk2 kinase activities were inhibited by increasing concentrations of GSTfrs. However, significant inhibition was observed only at concentrations of Frs that were more than 10 times higher than the binding constant of Frs to CycA. As Frs is itself a Cdk1 substrate, inhibition might be caused by substrate competition (supplementary Figs S4,S5 online). We tested whether phosphorylation of Frs is important for its function. In the cyclin binding assay, we did not observe a differential behaviour of pre-phosphorylated Frs

**Table 1** | Frühstart binds with high affinity to cyclins

| Protein | $k_{off}$ ($\times 10^{-3}$ $s^{-1}$) | s.d. | $k_{on}$ ($\times 10^{4}$ $M^{-1}$ $s^{-1}$) | s.d. | $K_d$ (nM) | s.d. |
|---|---|---|---|---|---|---|
| CycAΔN170 | 2.80 | 0.01 | 7.30 | 0.03 | 38 | 0.2 |
| CycEΔN300 | 6.78 | 0.05 | 7.04 | 0.09 | 96 | 1.4 |
| human Cdk2-CycAΔN170 | 4.39 | 0.04 | 4.00 | 0.03 | 100 | 1.0 |

s.d. standard deviation.

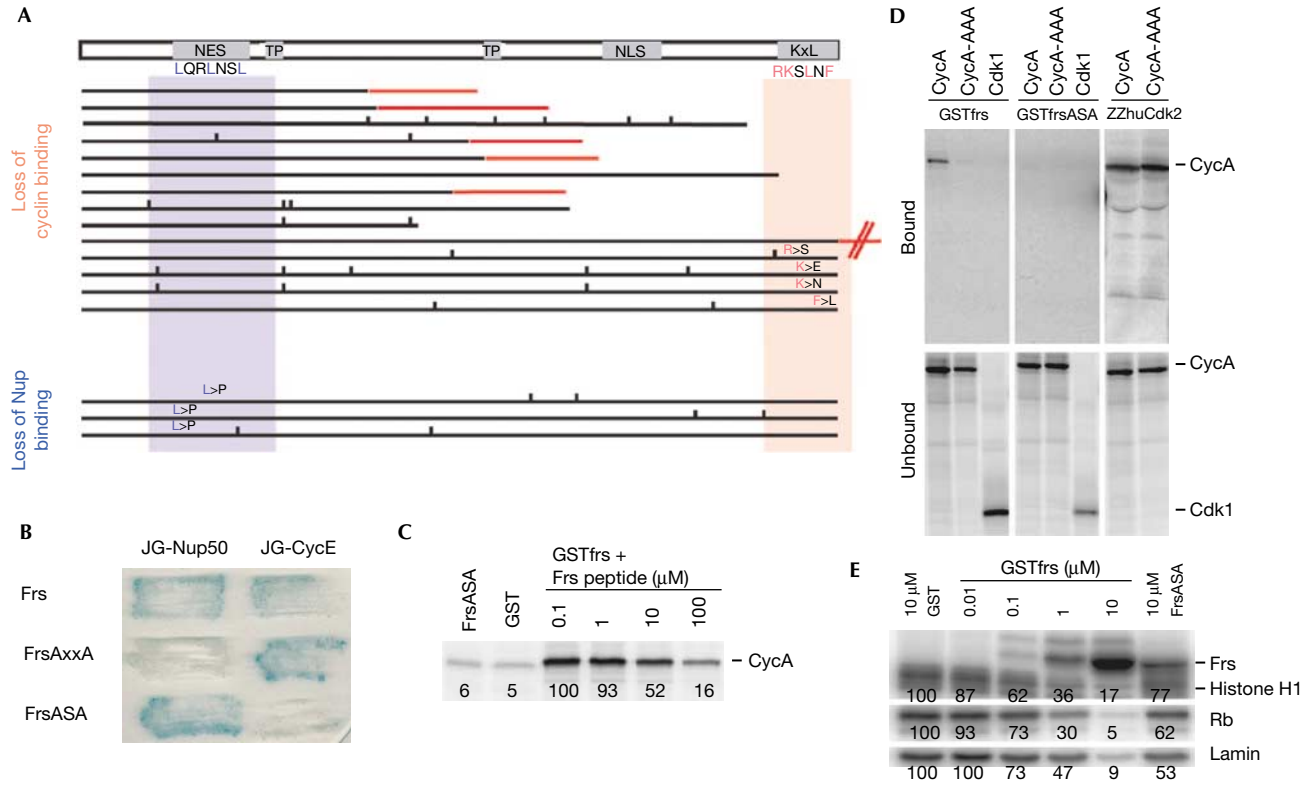


**Fig 4** | The carboxy-terminal KxL motif of Frs binds to the hydrophobic patch of Cyclin A. (**A**) Interaction mutants of Frs. Schematic structure of Frs showing the position of the predicted nuclear export signal (NES), the Cdk1 phosphorylation sites (TP), the predicted nuclear localization signal (NLS) and the cyclin binding site (KxL). The positions of multiple mutations in clones defective in Nup50 or CycE interaction are shown by black tick marks. New C-terminal sequences caused by frameshift mutations are shown in red. Regions with clustered mutations are marked in red and blue text. (**B**) Alanyl substitutions in the KxL motif and putative NES do not interact with CycE or Nup50, as assayed by the two-hybrid system in yeast. Blue staining of the lacZ reporter indicates interaction. (**C**) A peptide with the 15 C-terminal residues of Frs interferes with binding of $^{35}$S-labelled CycA to GSTfrs. Quantification of the autoradiograph is indicated. Note that the peptide concentrations are much higher than the binding constant of full-length Frs. Residues of Frs outside the C-terminal portion might contribute to complex formation. (**D**) The hydrophobic patch of CycA is required for Frs binding. $^{35}$S-labelled CycA, Cdk1 or CycA-AAA (three residues of the hydrophobic patch mutated to alanine) were incubated with beads loaded with GSTfrs, GSTfrs86ASA or $H_{10}$ZZhuCdk2. Bound (50%) and unbound (100%) fractions were analysed by SDS–PAGE and autoradiography. (**E**) Frs inhibits CDK1 phosphorylation. Cdk immunoprecipitates from embryonic lysates were incubated with 3 μM of the indicated substrate (lamin, histone H1 and Rb) and the indicated concentrations of GST, GSTfrs or GSTfrs86ASA in kinase buffer containing [$^{32}$P]ATP. The reaction products were analysed by SDS–PAGE and autoradiography. Relative quantification of [$^{32}$P] incorporation is indicated below the respective bands. $K_m$ values are approximately 1 μM (GSTfrs), 10 μM (GSTfrsASA), 2 μM (histone H1) and 49 μM (lamin; Stuurman, 1997). Cdk, cyclin-dependent kinase; Cyc, cyclin; Frs, Frühstart; GST, glutathione-*S*-transferase; Nup50, Nuclear pore protein 50; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

(supplementary Fig S5 online). Furthermore, a transgene of *frs*, with mutated phosphorylation sites, at least in part complemented the *frs* mutant phenotype (Table 2; supplementary Fig S3 online), indicating that phosphorylation of Frs by Cdk1 does not have an important role.

## DISCUSSION

In summary, Frs binds specifically and tightly to the hydrophobic patch of cyclins. Although Frs does not inhibit model substrate phosphorylation by Cdk complexes at nanomolar concentrations *in vitro*, it specifically inhibits mitotic Cdk1–Cyclin function

**Table 2**|The KxL motif is required for *frs* function

| Genotype of male | Normal ventral furrow | Open ventral furrow | Penetrance*(%) |
|---|---|---|---|
| Df *frs*/TM3 | 100 | 78 | 88 |
| frs$^{+}$; Df *frs*/TM3 | 120 | 20 | 28 |
| frs86ASA; Df *frs*/TM3 | 59 | 53 | 94 |
| frs11AxxA; Df *frs*/TM3 | 121 | 19 | 27 |
| frs22A48A; Df *frs*/TM3 | 55 | 25 | 62 |

*As 50% of the embryos were marked with the reporter gene in a cross with compound females, only 50% of the unmarked embryos that were scored have the expected genotype; the penetrance number takes this into account; Df, deficiency chromosome; Frs, Frühstart; TM, third multiple 3 balancer chromosome.

*in vivo*. The question is how Frs functions. Cdk complexes can shuttle between the cytoplasm and the nucleus during interphase and remain in the nucleus early in prophase. In principle, blocking of the hydrophobic patch might affect the nuclear accumulation of Cdk during prophase or might affect the interaction of Cdk1 with other regulators, such as String/Twine or Wee1/Myt1 (Myt for membrane-associated tyrosine and threonine specific cdc2 inhibitory protein kinase). Frs might interfere with Cdk1 activation by the phosphatase String; however, this model is less likely as *frs* inhibits the *Cdk1-AF* allele that cannot be inhibited by phosphorylation.

Alternatively, the hydrophobic patch might be important for substrate specificity of mitotic Cdks. The hydrophobic patch would not be essential for the recognition of our model substrates, but only for the—yet unknown—subset of mitotic Cdk1 substrates that triggers mitosis. Such a model is consistent with the situation in yeast, in which the phosphorylation of many substrates might not depend on the hydrophobic patch of the cyclins Clb2 and Clb5. Only a subset of the Clb5 substrates requires the hydrophobic patch for efficient phosphorylation (Loog & Morgan, 2005). In contrast to *Drosophila*, in which blocking of the hydrophobic patch by Frs inhibits M phase, the hydrophobic patch in yeast seems to be essential for efficient selection of S-phase-specific substrates. Although the mitotic inhibitors substrate inhibitor of Cdk (Sic1), replication uncoupled from mitosis 1 (Rum1) and roughex (Rux) are not structural homologues of Frs (Moreno & Nurse, 1994; Sanchez-Diaz *et al*, 1998; Foley *et al*, 1999), they might share the mechanism of Cdk inhibition, in that they might also block the hydrophobic patch of mitotic cyclins to prevent efficient phosphorylation of some Cdk substrates.

Independent of the actual mechanism of Cdk1 inhibition, our study suggests that in contrast to yeast, in which the hydrophobic patch seems to be essential for efficient selection of S-phase-specific but not M-phase-specific substrates, in *Drosophila*, the hydrophobic patch might be important for controlling entry into mitosis. Identification of the Cdk1 substrates by proteomic studies will allow a more systematic investigation of the role of the hydrophobic patch in cyclin-specific substrate selection.

## METHODS

The experimental procedures and materials are described in detail in the supplementary information online.

The experiments with flies were performed by using standard protocols. The *frs* complementation test is described by Großhans *et al* (2003). Frs-interacting clones were isolated from a ovarian complementary DNA library with lex-frs, LEU2 and lacZ reporter genes (Großhans *et al*, 1999). Frs interaction mutants were isolated from a library produced by error-prone PCR by screening with Nup50 and CycE preys.

Purified GST proteins were cleaved using thrombin and further purified by gel filtration. The $H_{10}$ZZhuCdk2 protein was expressed from a bicistronic plasmid with Cdk activating protein kinase (CAK) kinase. Binding tests were performed with $^{35}$S-labelled proteins and GST fusion proteins on glutathione–Sepharose, and analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and phospho-imaging. The peptide corresponding to the C-terminal residues of Frs was YEADKNFIKARKSLNF. Immunoprecipitates with protein A–Sepharose were subjected to western blotting or kinase assays. Kinase assays were performed with equivalents of 50 embryos for 10 min at 25 °C, if not otherwise noted. The sample was analysed by SDS–PAGE, and signal was detected and quantified by a phospho-imager.

For SPR, $H_{10}$ZZfrs was immobilized on the flow cells of a CM5 sensor chip (Biacore, Freiburg, Germany). The rate constants were calculated by fitting to 1:1 Langmuir binding model.

**Supplementary information** is available at *EMBO reports* online (http://www.emboreports.org).

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## Supplemental data:

**Detailed description of methods and materials:**

*Genetics, embryology, histology*

Fly stocks and crosses were kept at 25°C if not otherwise indicated (Roberts 1998). Following specific stocks were used UASCdk2myc$_6$ (Meyer et al, 2000), UASfrs (Großhans et al, 2003), HS-Cdk1-T14A-Y15F (Sprenger et al, 1997), MS1096-Gal4, pairedGal4, patchedGal4, Df(3L)BK10, TM3-hb-lacZ (Fly stock centre, Bloomington, USA). Deletions of the *frs* locus were produced by mobilisation of the transposon KG(3)0224. *white* revertants were selected for lethality over Df(3L)BK10 and breakpoints mapped by PCR. The transheterozygotic combination of two excisions (KG7/KG19) delete *frs* and the two proximal genes *gdl* and *Eip71CD*. Embryos, larval and pupal discs were fixed with 4% formaldehyde and stained by standard procedures with following antibodies: Frs (1 $\mu$g/ml, ref. 10), pHistone3-S10 (0.5 $\mu$g/ml, rabbit, Upstate), pHistone3-S10 (0.2 $\mu$g/ml, mouse, clone 3H10, Upstate), BrdU (4 $\mu$g/ml, Roche), ß-galactosidase (0.2 $\mu$g/ml, Roche), Dlg (monoclonal 4F3, 0.5 $\mu$g/ml), secondary antibodies labelled with Alexa dyes (4 $\mu$g/ml), DAPI (0,2 $\mu$g/ml), Phalloidin coupled with Alexa dyes (6 nM, Molecular Probes). Samples were mounted in Aquapolymount (Polyscience) and analysed with a confocal microscope (63x, 40x oil lenses, Leica). Adult wings were washed in ethanol, mounted in Hoyers/lactic acid (Roberts, 1998) and photographed under bright-field illumination with a 4x objective (Zeiss).

*Expression of Cdk1-AF by heatshock*

Embryos from a cross of prdGal4/TM3 females with UASfrs[H]/+; HS-Cdk1-AF males were were heatshocked on the apple juice plate for 30 minutes in a 37°C water bath. After 10 min recovery in a water bath at room temperature, the embryos were dechorionated, fixed with 4% formaldehyde/PBS and stained for Frs, mitotic nuclei. and DNA. Photographs of the three colour channels were taken separetely with a fluorescence microscope (Zeiss) with a 20x objective and a digital camera (ProgRes C10, Jenoptik).

*Rescue assay with frs transgenes*

Embryos from crosses of C(3) females with males of indicated genotypes were stained with p-histone3 (mitotic marker) and ß-galactosidase antibodies and scored for ventral furrow formation. Only embryos without ß-galactosidase staining were scored. In this class the expected proportion of Df *frs* hemizygous embryos is 50%. Penetrance refers to this expected number. Df *frs* was Df(3L)BK10 and was balanced with a TM3 carrying a hunchback-lacZ reporter gene.

*Molecular genetics:*

DNA was amplified by vent DNA polymerase (NEB biolabs) with primers introducing suitable restriction sites. The purified and digested PCR products were cloned into specified plasmids and sequenced. Site-directed mutations were produced by inverse PCR with appropriate oligonucleotides and Pfu DNA polymerase (Stratagene) and selected by DpnI (Roche) digest. The region of interest of the mutagenised plasmids was sequenced and recloned into a new plasmid.Preparation and construction of plasmids is described in detail in the table SI.

*Protein expression:*

Proteins were stored in 10% gycerol at -70°C. Protein concentrations were determined by the Bradford assay. GST and GSTfrs expression were induced for 4 h, 0.1 mM IPTG, 37°C. GST proteins were isolated by chromatography of the cleared lysate (PBS, 150 mM NaCl and 1 mM DTT) with 1 ml GSH Sepharose colums (Amersham) and eluted with 50 mM Tris/HCl [pH 8], 50 mM NaCl, 1 mM DTT, 10 mM Gluthathion. GSTRb expression was induced for 6 h at 18°C and purified as the other GST proteins. GSTCycAΔN170, GSTCycEΔN300 proteins were expressed for 6 h at

18°C, 0.1 mM IPTG. The cleared lysate (PBS, 150 mM NaCl and 1 mM DTT) was incubated with GSH sepharose beads for 1 h. Following washing, the beads (50 µl) were incubated with 10 U of thrombin (Serva) in 50 mM Tris [pH8], 200 mM NaCl, 3 mM $CaCl_2$, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 0.005% Tween20 for 4 h at 4°C. The cleaved proteins from the supernatant were further purified by gel filtration (Superdex200 10/300, Amersham) and concentrated by Vivaspin colums (Satorius). His-tagged proteins were expressed by 0.1 mM IPTG for 4 h, 37°C, purified from the cleared lysate (20 mM Na-phosphate [pH 8], 500 mM NaCl, 20 mM imidazol) by chromatography with Ni-NTA sepharose (Amersham) and eluded with 20 mM Na-phosphate [pH 8], 500 mM NaCl, 250 mM imidazol. The $H_{10}$ZZhuCdk2 protein was expressed from a bicistronic plasmid with CAK kinase for 6 h at 18°C, 0.1 mM IPTG.

*Binding tests*

Proteins were labelled with [35S]-methionin in a coupled transcription-translation reaction (TNT kit, Promega). [35S]-labelled proteins were incubated with GST, GST- or ZZ-fusion proteins (approx. 2 µg) immobilized on gluthathion or IgG sepharose (10 µl) in 1 ml of 10 mM Na-phosphate [pH 7.4], 600 mM NaCl, 0.1% Tween for 2 h, 4°C. Following five-times washing, the bound fraction (50%) as well as the unbound (100%) fraction were analysed by SDS-PAGE and autoradiography with a phosphimager. The peptide corresponding to the C-terminal residues of Frs (YEADKNFIKARKSLNF, synthesised by Peptide Speciality Lab., Heidelberg) was dissolved in PBS to 5 mM. The concentration was determined by absorption at l=280 nm.

*Immunoprecipitation*

Staged embyos were dechorionated, frozen in liquid nitrogen and stored at -70°C. Embryos were lysed in 1 ml 50mM Hepes [pH 7.5], 150 mM NaCl, 0,5% TritonX-100, 10% Glycerin with a glass homogeniser. Following centrifugation and lipid extraction with triclorotrifluoroethan, antibodies (Cdk1 (5 µl serum), CycA (2 µl serum, Sprenger et al, 1997), CycB (5 µg, monoclonal #F2F4), CycE (2 µl serum, Knoblich et al, 1994), myc (2 µg, Roche)) were added to the lysate. After 2 h incubation the immunocomplexes were isolated with proteinA sepharose (10 µl beads, 1 h, 4°C). The washed beads served as source of kinase activity. For Co-immunoprecipitation experiments bound fraction and the supernatant were analysed by SDS-PAGE and western blotting (semi-dry transfer to nitrocellulose membrane) with following antibodies CycA (monoclonal A12, 0.5 µg/ml), Cdk1 (PSTAIR, Sigma, 0.1 µg/ml), Frs (0.2 µg/ml), a-tubulin (B512, Sigma, 1:50000). To detect CycA and CycE in the unbound fraction, CycA and CycB were immunoprecipitated from the supernatant. The blots were developed with peroxidase coupled antibodies (Sigma, 0.1 µg/ml) and chemiluminescence (ECL, Amersham).

*Kinase assay*

Immunocomplexes were isolated from 0-12 h wild-type embryos or embryos from a cross of pairedGal4 with UASCdk2myc$_6$ (Meyer et al, 2000). Kinase assays were performed with 10 µl beads (corresponding to 50 embryo equivalents) in 16 µl in kinase buffer (80 mM, ß-glycerophosphat [pH 7.2], 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 50 µM ATP) and 2 µCi [32P] ATP per reaction, 3 µM of substrate and indicated amounts of Frs for 10 min, 25°C. Substrates were expressed in *E. coli* and affinity purified (GSTRb(aa379-928, Adams et al, 1996), H6LaminDmO(aa1-179, Stuurman 1997) or purchased (histone H1, Calbiochem). The kinase reaction was stopped by addition of SDS-PAGE sample buffer and analysed by SDS-PAGE and autoradiography with a phospho-imager. For quantification of [32P] incorporation the original picture was analysed and background was subtracted. The polyclonal Cdk1 antibody was raised against a peptide corresponding to the C-terminal residues of Cdk1 (CILEHPYFNGFQSGLVRN, synthesised by Peptide Speciality Lab., Heidelberg) according to Knoblich et al (1994).

*Surface plasmon resonance (SPR)*

$H_{10}$ZZfrs (400 response units) were immobilised with N-hydroxysuccinimide and N-ethyl-N'-(3-diethylaminopropyl)carbodiimide on the flow cells of a CM5 sensor chip (Biacore). A reference flow cell was generated in parallel but without Frs protein. For the measurements 120 $\mu$l of the sample ($H_{10}$ZZhuCdk2 and CycAΔN170 in 10 mM Hepes [pH 7.4], 150 mM NaCl, 50 $\mu$M EDTA, 0.005% Tween20. CycEΔN299 in 50 mM Tris [pH 8], 200 mM NaCl, 3 mM $CaCl_2$, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 0.005% Tween20) were applied to the chip at 20 $\mu$l/min at 25°C. The rate constants were calculated from each sensogram by fitting to 1:1 Langmuir binding model using a global fitting method from the BIAevaluation 4.0.1 software.

*Two hybrid assay*

Frs interacting clones were isolated from a ovarian cDNA library with lex-frs and LEU2 and lacZ reporter genes (Großhans et al, 1999) and sequenced following PCR amplification from the yeast DNA. Interaction mutants were isolated by a mating scheme from a library of *frs* mutants generated by PCR mutagenesis. The coding sequence of *frs* was amplified by error-prone PCR (10 mM Tris-HCl, [pH 8.3], 50 mM KCl, 7 mM $MgCl_2$, 0,3 mM $MnCl_2$, 10% DMSO, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dCTP, 1 mM dTTP) with Taq polymerase and cloned into pEG202, resulting in more than one mutation per 500 bp. Yeast clones (approx. 1000, strain EGY48 with a chromosomal LEU2 reporter) with library plasmids (pJG-frs*) were crossed in parallel with yeast of the other mating type (RFY206/pSH18-34 lacZ reporter) harbouring pJG-CycEΔN319 or pJG-Nup50 (full-length) plasmids. Diploid clones were tested for LEU2 and lacZ reporter gene activation. The *frs* coding sequence of isolated clones was isolated by PCR and sequenced.

*Flow cytometry.*

Wing imaginal discs from third instar larvae (15 per experiment) were dissected in PBS and digested in 500 $\mu$l trypsin (0.5 g/l) with EDTA (0.2 g/l, Gibco) for 4 h. Following staining with Hoechst (0.5 $\mu$g/ml), cells were sorted according to the GFP signal and analysed according to cell size and DNA staining (Becton Dickinson FACS AriaTM, Cell Quest program.

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**Table SI:** Plasmids used in this study

| Name of plasmid | Oligonucleotides for PCR | Description |
|---|---|---|
| pCS-frs | JG70(EcoRI) JG71(XhoI) | PCR with P1 DS08110, cloned into pCS2 EcoRI/XhoI |
| pCS-frs86ASA | JG185 JG186 | Mutagenesis of pCS-frs |
| pCS-frs11AxxA | JG196 JG197 | Mutagenesis of pCS-frs |
| pCS-frs48A | JG211 JG212 | Mutagenesis of pCS-frs |
| pLex-frs | JG95(EcoRI) JG71(XhoI) | PCR with pCS-frs cloned into pEG202 EcoRI/XhoI |
| pLex-frs86ASA | JG95(EcoRI) JG71(XhoI) | PCR with pCS-frs86ASA cloned into pEG202 EcoRI/XhoI |
| pLex-frs11AxxA | JG95(EcoRI) JG71(XhoI) | PCR with pCS-frs11AxxA cloned into pEG202 EcoRI/XhoI |
| pQE-ZZ-tev-frs-H6 | JG116(NcoI+ATG G) JG117(BamHI) | PCR with pCS-frs cloned into pQE80-ZZ-tev-H6 NcoI/BamHI |
| pQE-frs | JG96(BamHI) JG71(XhoI) | PCR from pCS-frs as BamHI/XhoI cloned into pQE30 BamHI/SalI |
| pQE-H10-ZZ-tev-frs | - | frs BamHI/HindIII cleaved from pQE30-frs and cloned into pQE80-H10-ZZ BamHI/HindIII |
| pGST-frs | JG95(EcoRI) JG71(XhoI) | PCR with pCS-frs cloned into pGEX4T-1 EcoRI/XhoI |
| pGST-frsASA | JG95(EcoRI) JG71(XhoI) | PCR with pCS-frsASA cloned into pGEX4T-1 EcoRI/XhoI |
| pGST-frs11AxxA | JG95(EcoRI) JG71(XhoI) | PCR with pCS-frs11AxxA cloned into pGEX4T-1 EcoRI/XhoI |
| pGST-frs48A | JG95(EcoRI) JG71(XhoI) | PCR with pCS-frs48A cloned into pGEX4T-1 EcoRI/XhoI |
| pGST-frs22A48A | PG57 PG58 | Site directed mutagenesis of pGEX-frs48A |
| pQE-CycAΔN170 | JG213(NcoI) JG214 (BglII) | PCR with pSP64 HA-CycA cloned into pQE80N60 NcoI/BglII |
| pGST-CycAΔN170 | - | CycAΔN170 NcoI/BglII from pQE-CycAΔN170 cloned into pGEX4T-1-thrombin-tev NcoI/BamHI |
| pGST-CycEΔN300 | PG55(EcoRI) PG56(XhoI) | PCR with pSP64- CycE cloned into pGEX4T-1-thrombin-tev EcoRI/XhoI |
| pSP-CycA-AAA | JG209 JG210 | Mutagenesis of pSP64-CycA, M235L239W242 to AAA |
| pBKS-frsΔCDS | JG141(BglII) JG147(BglII,NheI) | Inverse PCR with pBKS-frs-3'HSP70. Genomic fragment of frs without the coding sequence |
| pBKS-frsΔCDS+frs | JG182(BglII) JG183(XbaI) | PCR with pCS-frs cloned as BglII/XbaI into pBKS-frsΔCDS BglII/NheI |
| pBKS-frsΔCDS+frs86ASA | JG107 JG182(BglII) | PCR with pCS-frs86ASA XbaI/BglII and cloned into pBKS-frsΔCDS BglII/NheI |
| pBKS-frsΔCDS+frs11AxxA | JG182(BglII) JG183(XbaI) | PCR with pCS-frs11AxxA BglII/XbaI and cloned into pBKS-frsΔCDS BglII/NheI |
| pCasper-HSfrs | - | frs (cDNA) as EcoRI/XhoI from pCS-frs into pCasper-HS EcoRI/XhoI |
| pCasper-frs | - | Cloned from pBKS-frsΔCDS+frs XhoI/XbaI into pCasper4 XhoI/XbaI |
| pCasper-frs86ASA | - | Cloned from pBKS-ΔCDS-frs86ASA XhoI/XbaI into pCasper4 XhoI/XhoI |
| pCasper-frs11AxxA | - | Cloned from pBKS-ΔCDS-frs11AxxA XbaI/XhoI into pCasper4 XhoI/XbaI |

| | |
|---|---|
| pBKS-frs-3'HSP70 | Großhans et al (2003) Dev Cell 5: 285-294 |
| pCS-frs | Großhans et al (2003) Dev Cell 5: 285-294 |
| DmO lamin His-L(1-179) | Stuurman N (1997) FEBS Lett. 401: 171-174 |
| pQE-H10-ZZ-huCdk2 CAK | D. Görlich |
| pSP64 HA-CycA | Foley E, Sprenger F (2001) Curr Biol 11: 151-160 |
| pSP64 HA-CycB | Foley E, Sprenger F (2001) Curr Biol 11: 151-160 |
| pNB40 CycB3 | C. Lehner |
| pSP-CycE | F. Sprenger |
| pSP64 Cdk1 | F. Sprenger |
| pGEX-huRb(379-928) | Kaelin et al (1991) Cell 64: 521-532. |

**Supplementary figure 1: Interacting proteins of Frs**

**A**, Interacting proteins isolated in a two-hybrid screen. Frs interacting clones were isolated with lexA fused to full-length Frs from an ovarian cDNA library with lacZ and LEU2 reporter genes. Specificity of the interaction was confirmed with a series of other lexA fusion proteins. Isolated clones were partially sequenced. The truncations are shown for selected examples. **B**, Biochemical interactors of Frs. ZZfrsH6 (50 $\mu$g) bound to IgG sepharose was incubated with embryonic extract (0-12 h, 50 mM Hepes [pH 7.5], 10 mM $MgCl_2$, 100 mM NaCl, 10% Glycerol) at 4°C overnight. Bound proteins were eluded with 1.5 M $MgCl_2$ and analysed by SDS-PAGE and silver staining. Other basic proteins (ribosomal protein S7, 23a) served as controls. To create nuclear conditions 5 $\mu$M RanQ63LDC180 was added to the extract. The identity of bands was determined by mass-spectrometry.

**Supplementary figure 2: Frs binds to Cyclins with high affinity.**

Quantitative analysis of complex formation with immobilised Frs ($H_{10}$ZZ-frs) by surface plasmon resonance. **A**, with CycAΔN170 (concentrations as indicated), **B,** CycEΔN300 (concentrations as indicated), **C**, huCdk2-CycAΔN170 complex (concentrations as indicated). **D**, 2 $\mu$g of the proteins used in the SPR experiment were analysed by SDS-PAGE and staining with Coomassie. human Cdk2 (huCdk2) was used, since it is difficult to isolate soluable Cdk1 from *E. coli*. **E**, Indicated amounts of recombinant $H_{10}$ZZfrs, total extracts from precisely staged embryos (mid-cellularisation) and total extract of embryos deficient for *frs* (ΔKG9/ΔKG17) were analysed by SDS-PAGE and western blot with Frs antibody that was raised against a GSTfrs fusion protein. We estimate that during mid-cellularisation one embryo contains approximately $10^{-11}$ g Frs. Given a volume of $10^{-8}$ l per embryo, the concentration would be $10^{-7}$ M. **F**, Sequence comparison of K/RxL motifs in mitotic inhibitors (Frs, Rux, SIC1, RUM1), p27 and Cdk2 substrates (Rb, p107). Marked residues are assumed to interact with the hydrophobic patch and a neighbouring acidic residue.

**Supplementary figure 3: Expression of *frs* alleles from a transgene.**

Embryos from stocks carrying a deficiency of the *frs* region (Df(3L)BK10), indicated $frs^+$ transgenes and a balancer chromosome with a lacZ reporter gene (TM3, hb-lacZ) were fixed and stained with Frs (white) and ß-galactosidase (red) antibodies. Embryos homozygous for the *frs* deficiency were recognised by the absense of ß-galactosidase staining.Genotypes: **A**, **C**, **E**, **G**, homo- or heterozygous for the balancer, **B**, **D**, **F**, **H**, homozygous for the *frs* deficiency. **A**, **B** frs86ASA, **C**, **D** frs11AxxA, **E**, **F** frs22A48A, **G**,**H** no $frs^+$ transgene.

**Supplementary figure 4: Inhibition of Cdk activity by Frs.**

**A**, Specificity of kinase activity. Kinase activity (3 $\mu$M Lamin, 10 min) associated with Nup50 and Cdk1 immuno-complexes from embryonic lysates. **B**, Cdk2-myc immuno-complexes from embryos expressing Cdk2-myc were analysed for presence of Cdk2-myc and Cdk1 by western blotting (loading each 10 embryo equivalents). **C**, Frs inhibits substrate phosphorylation by Cdk1 and Cdk2. In dicated Cdk and cyclin immuno-complexes (50 embryo equivalents per reaction) from embryonic lysates were incubated with 3 $\mu$M substrate (histon H1, Rb, Lamin) and indicated concentrations of GST, GSTfrs or GSTfrs86ASA in kinase buffer containing [$^{32}$P]-ATP. The reaction mix was analysed by SDS-PAGE and autoradiography. Relative quantification of [$^{32}$P] incorporation is indicated below the respective bands. **D**, Comparable inhibition of Cdk1

and Cdk2 complexes. Kinase activity of Cdk1 and Cdk2-myc immuno-complexes in the presence of 3 $\mu$M Lamin and increasing concentrations of GSTfrs (measured in triplicates). [$^{32}$P] incorporation was quantified following autoradiography. Error bars are standard deviations. **E**, A Frs peptide comprising the C-terminal 15 residues does not inhibit Cdk1. Kinase activity (3 $\mu$M Lamin) of Cdk1 immuno-complexes in the presence of increasing concentrations of the Frs peptide, GST or GSTfrs. **F**, Cdk1 phosphorylation sites in Frs. GST fusion proteins (10 $\mu$M) of indicated Frs alleles were incubated with Cdk1 immuno-complexes for 6 min on ice. Relative degree of phosphorylation was quantified following SDS-PAGE and autoradiography. **G**, Inhibition of Cdk1 by Frs alleles. Kinase activity (3 $\mu$M Lamin, 10 min on ice) of Cdk1 immuno-complexes in the presence of increasing concentrations of GST fusions proteins of indicated Frs alleles. Relative degree of phosphorylation was quantified following SDS-PAGE and autoradiography. **H**, Protein preparations used in the kinase assay. 2 $\mu$g of indicated proteins were analysed by SDS-PAGE and staining with Coomassie.

**Supplementary figure 5: Frs is a Cdk1 substrate.**

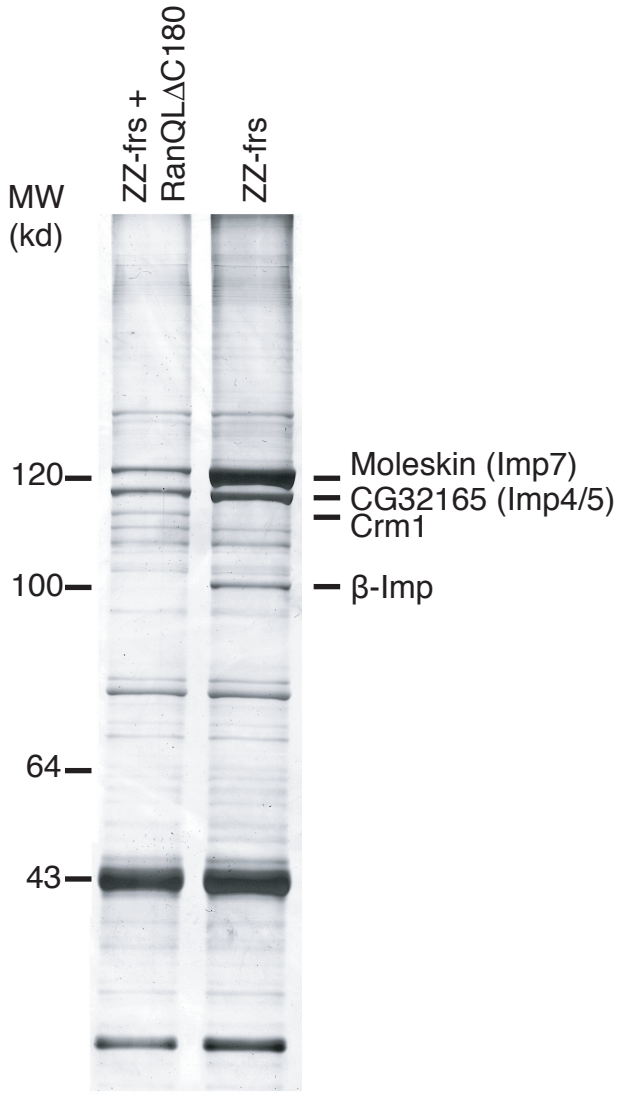
**A**, **B**, Frs is phosphorylated at T22 and T48. GSTfrs was incubated with Cdk1 immuno-complexes isolated from embryonic lysates for 10 min on ice. Following SDS-PAGE protein bands were digested with trypsin. Peptides were separated by HPLC and analysed by mass-spectroscopy. Spectra of the (A) T48 and (B) T22 peptides show twin peaks of fragments with and without
phosphate group (indicated by arrow). **C,** Phosphorylated and unphosphorylated Frs equally bind to CycA. GSTFrs and GSTFrs86ASA was incubated with or without Cdk1 immunocomplexes isolated from embryonic lysates for 10 min on ice prior to binding to GSH beads and the binding test with [$^{35}$S] labelled CycA. Fractions bound and unbound to the GSH beads were analysed by SDS-PAGE and autoradiography. Phosphorylation of the GST fusions proteins was controlled by addition of [$^{32}$P] ATP to an aliquot of the reaction.

# Sup. Fig. S1

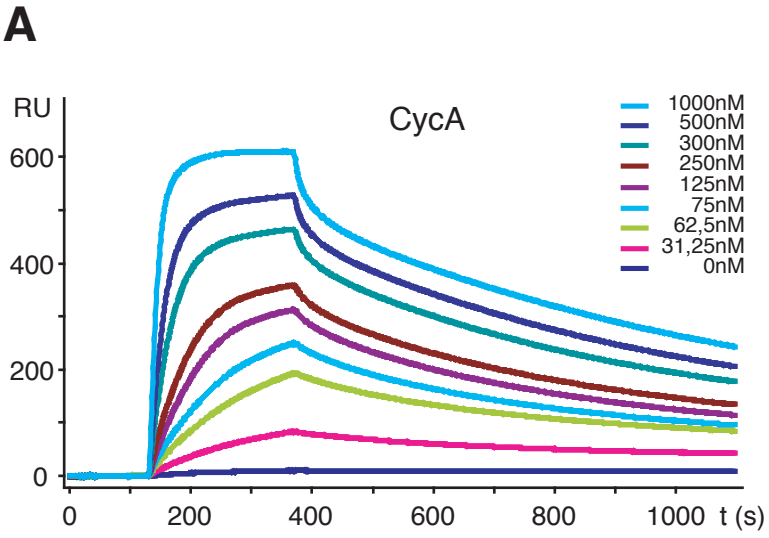
A

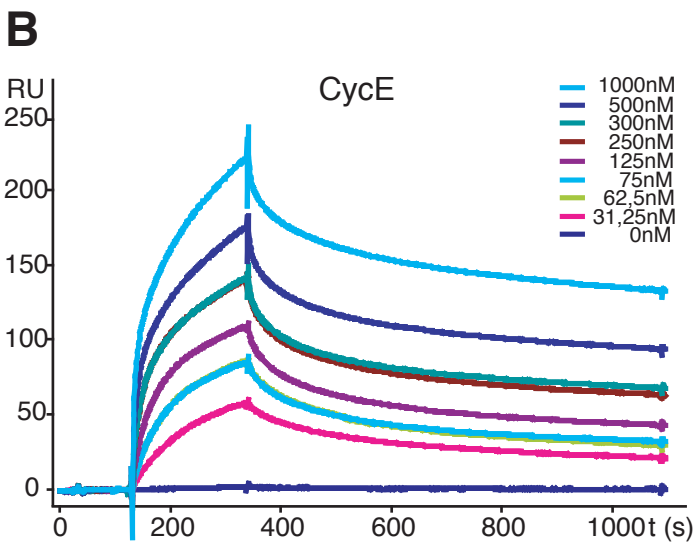
| gene | number of clones | clone size |
|---|---|---|
| Nup50/CG2158 | 31 | full length, ΔN562, ΔN249. |
| Nup214 | 6 | ΔN528, ΔN468, ΔN331. |
| Nup54/CG8831 | 5 | ΔN587, ΔN577, ΔN570. |
| Nup358 | 5 | ΔN573, ΔN519, ΔN502. |
| CycE | 2 | ΔN244, ΔN319. |

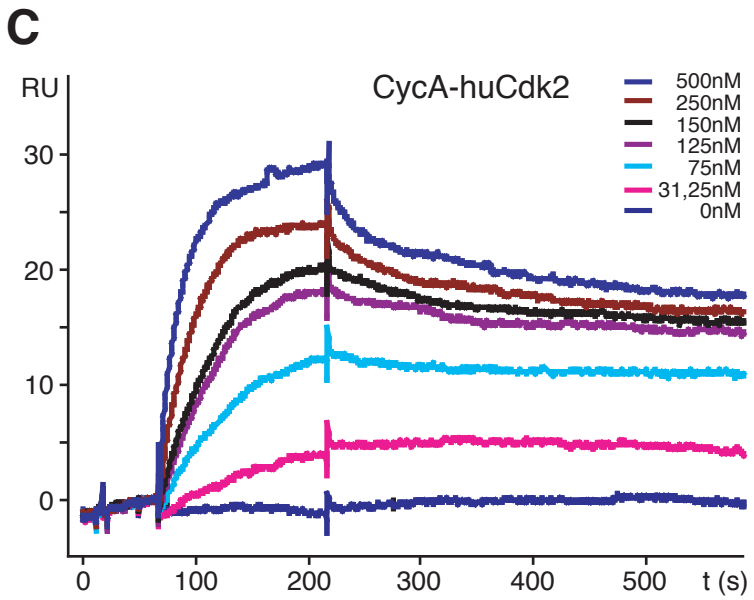
B

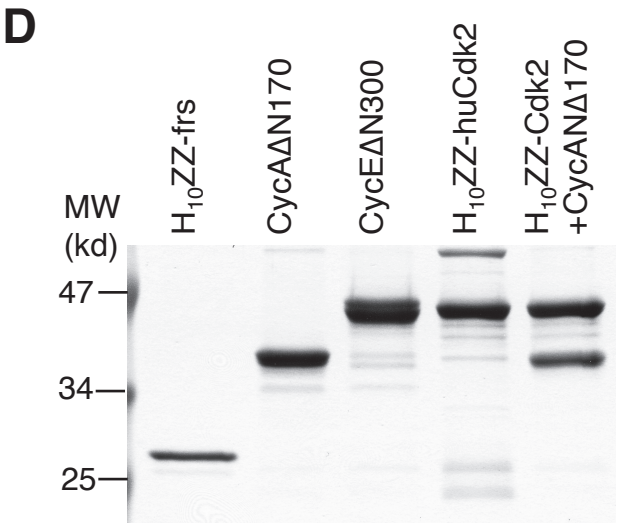


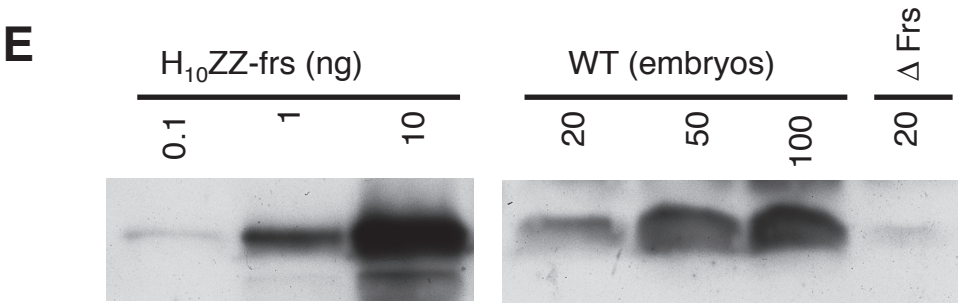
# Sup. Fig. S2



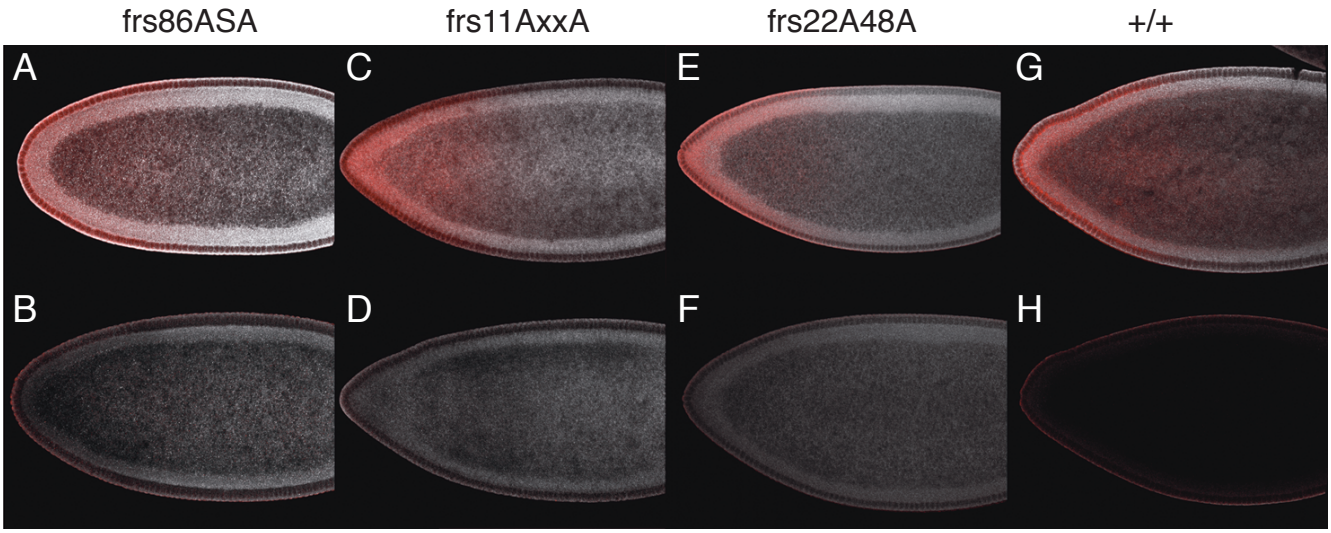



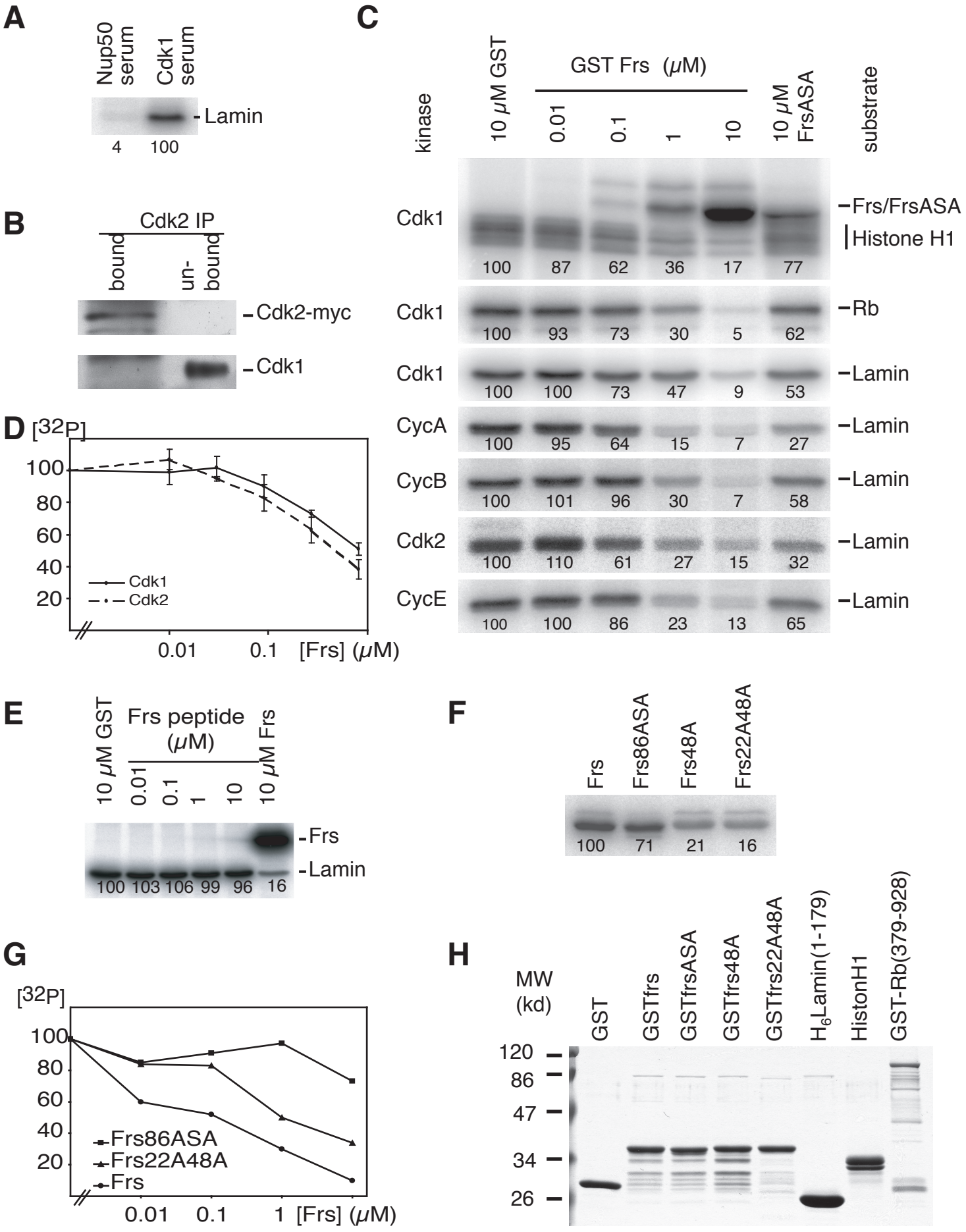

F

| | - 6 | - 5 | - 4 | - 3 | - 2 | - 1 | 0 | + 1 | + 2 | aa residues |
|---|---|---|---|---|---|---|---|---|---|---|
| Dmfrs | I | K | A | R | K | S | L | N | F | 82-90 |
| DmRux | R | C | V | R | R | T | L | F | T | 244-252 |
| SIC1 | R | F | K | P | K | A | L | F | Q | 270-278 |
| RUM1 | T | F | K | P | K | L | L | F | A | 138-146 |
| Rb | P | K | P | L | K | K | L | R | F | 869-877 |
| p27 | P | S | A | C | R | N | L | F | G | 26-34 |
| p107 | G | S | A | K | R | R | L | F | G | 654-662 |

## Sup. Fig. S3

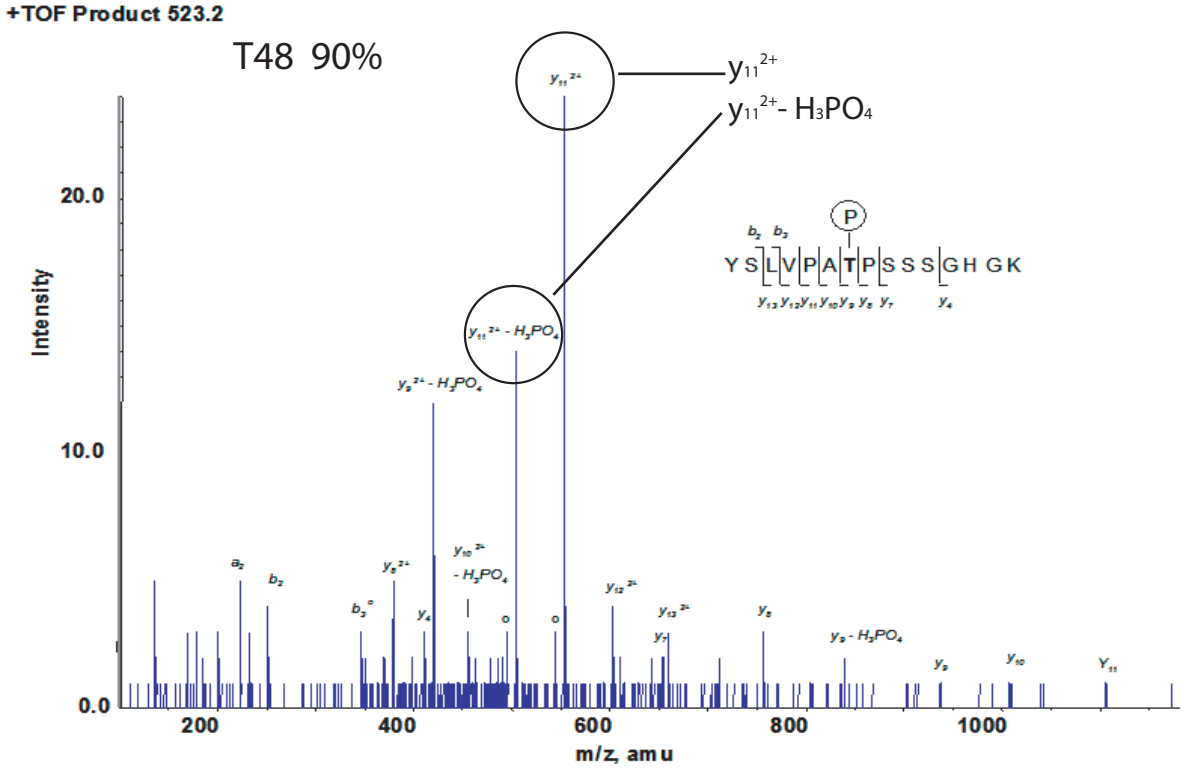


# Sup. Fig. S4

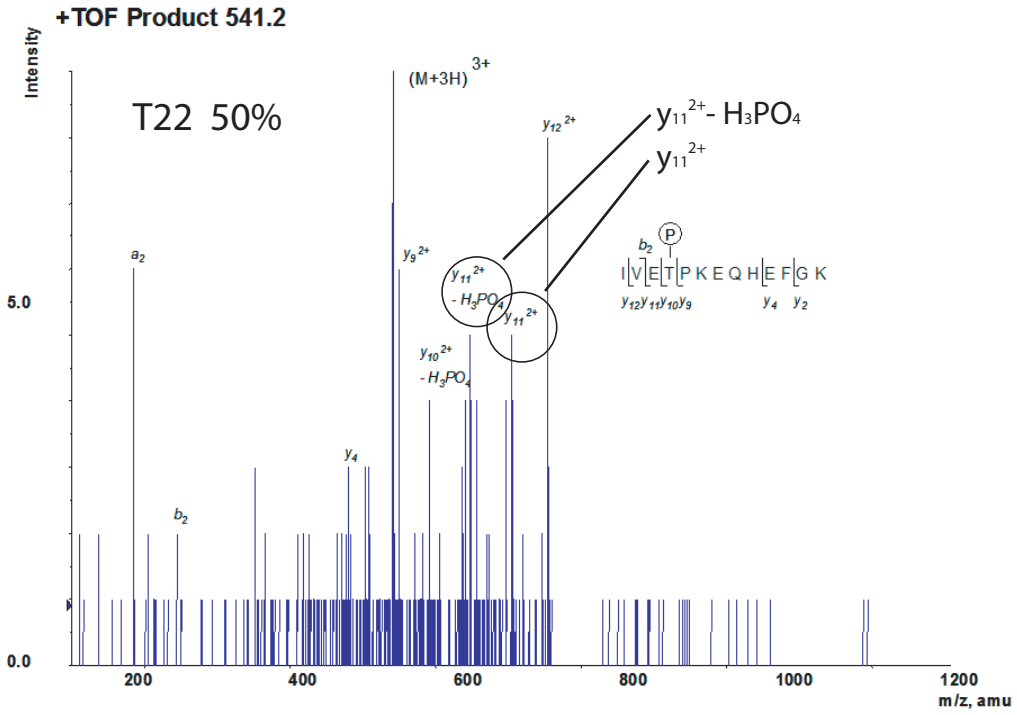

A
Nup50 serum
Cdk1 serum
Lamin
4
100
B
Cdk2 IP
bound
un-bound
Cdk2-myc
Cdk1
C
kinase
10 μM GST
GST Frs (μM)
0.01
0.1
1
10
10 μM FrsASA
substrate
Cdk1
Frs/FrsASA
Histone H1
100 87 62 36 17 77
Cdk1
Rb
100 93 73 30 5 62
Cdk1
Lamin
100 100 73 47 9 53
CycA
Lamin
100 95 64 15 7 27
CycB
Lamin
100 101 96 30 7 58
Cdk2
Lamin
100 110 61 27 15 32
CycE
Lamin
100 100 86 23 13 65
D
[32P]
100
80
60
40
20
Cdk1
Cdk2
0.01
0.1
[Frs] (μM)
E
10 μM GST
Frs peptide (μM)
0.01
0.1
1
10
10 μM Frs
Frs
Lamin
100 103 106 99 96 16
F
Frs
Frs86ASA
Frs48A
Frs22A48A
100 71 21 16
G
[32P]
100
80
60
40
20
Frs86ASA
Frs22A48A
Frs
0.01
0.1
1
[Frs] (μM)
H
MW (kd)
GST
GSTfrs
GSTfrsASA
GSTfrs48A
GSTfrs22A48A
H6Lamin(1-179)
HistonH1
GST-Rb(379-928)
120
86
47
34
26

# Sup. Fig. S5

A




B




C