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 $34 \mu\text{m}^2$, $n = 10$, stained for discs-large (Dlg). Scale bar, $20 \mu\text{m}$. (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, $20 \mu\text{m}$. (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, $50 \mu\text{m}$. 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, $50 \mu\text{m}$. 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 ³⁵S-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 *CycA*ΔN170 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 h*CycA* and h*Cdk2* with nanomolar binding constants (K_D (h*CycA*) = 25 nM and K_D (h*Cdk2*) = 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*–*CycA*ΔN170 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 CycA ΔN170 (500 nM). Cdk, cyclin-dependent kinase; Cyc, cyclin; Frs, Frühstart; GST, glutathione-S-transferase; IB, immunoblotting; IP, immunoprecipitation; RU, response units; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

than its binding to isolated CycA ΔN170 (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 CycE ΔN300 . We measured a binding constant of $K_D = 96 \text{ nM}$ (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 ($c \approx 100 \text{ nM}$;

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 inhibition

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} ($\times 10^{-3} \text{ s}^{-1}$)	s.d.	k_{on} ($\times 10^4 \text{ M}^{-1} \text{ s}^{-1}$)	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) Alanine 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 H₁₀ZZhuCdk2. 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 <i>frs</i> /TM3	100	78	88
<i>frs</i> ⁺ ; Df <i>frs</i> /TM3	120	20	28
<i>frs</i> 86ASA; Df <i>frs</i> /TM3	59	53	94
<i>frs</i> 11AxxA; Df <i>frs</i> /TM3	121	19	27
<i>frs</i> 22A48A; Df <i>frs</i> /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 H₁₀ZZhuCdk2 protein was expressed from a bicistronic plasmid with Cdk activating protein kinase (CAK) kinase. Binding tests were performed with ³⁵S-labelled proteins and GST fusion proteins on glutathione–Sephadex, and analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and phospho-imaging. The peptide corresponding to the C-terminal residues of Frs was YEADKNFIKARKSLNF. Immuno-precipitates with protein A–Sephadex 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, H₁₀ZZfrs 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.emboports.org>).

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