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The COP9/signalosome complex is conserved in fission yeast and has a role in S phase

Kirsten E. Mundt*, Joanne Porte*, Johanne M. Murray†, Constantinos Brikos*†, Per U. Christensen*, Thomas Caspari*, Iain M. Hagan‡, Jonathan B.A. Millar§, Viesturs Simanis¶, Kay Hofmann¥ and Anthony M. Carr*

The COP9/signalosome complex is conserved from plant to mammalian cells. In *Arabidopsis*, it regulates the nuclear abundance of COP1, a transcriptional repressor of photomorphogenic development [1,2]. All COP (constitutive photomorphogenesis) mutants inappropriately express genes that are normally repressed in the dark. Eight subunits (Sgn1–Sgn8) of the homologous mammalian complex have been purified [3,4]. Several of these have been previously identified through genetic or protein interaction screens. No coherent model for COP9/signalosome function has yet emerged, but a relationship with cell-cycle progression by transcriptional regulation, protein localisation or protein stability is possible. Interestingly, the COP9/signalosome subunits possess domain homology to subunits of the proteasome regulatory lid complex [5,6]. Database searches indicate that only Sgn5/JAB1 is present in *Saccharomyces cerevisiae*, precluding genetic analysis of the complex in cell-cycle regulation. Here we identify a subunit of the signalosome in the fission yeast *Schizosaccharomyces pombe* through an analysis of the DNA-integrity checkpoint. We provide evidence for the conservation of the COP9/signalosome complex in fission yeast and demonstrate that it functions during S-phase progression.

Addresses: *MRC Cell Mutation Unit, The University of Sussex, Brighton BN1 9RR, UK. †School of Biological Sciences, The University of Sussex, Brighton BN1 9QG, UK. ‡University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, UK. §Division of Yeast Genetics, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK. ¶Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges, Switzerland. ¥Bioinformatics Group, MEMOREC Stoffel GmbH, Stöckheimer Weg 1, D-50829 Köln, Germany.

Correspondence: Anthony M. Carr
E-mail: a.m.carr@sussex.ac.uk

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Results and discussion

Identification of an *S. pombe* signalosome subunit

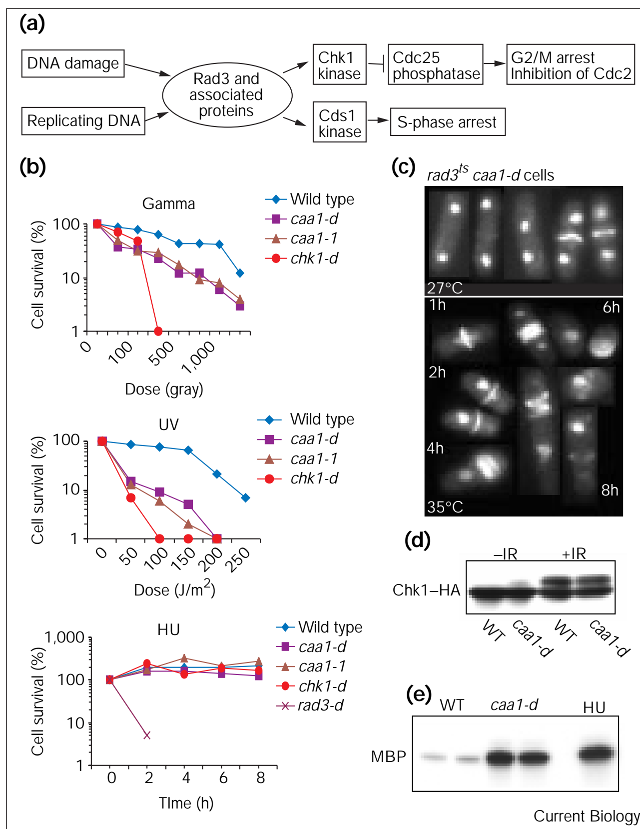
All eukaryotic cells respond to DNA damage and problems arising during DNA replication by activating DNA-integrity checkpoints, which arrest cell-cycle progression (Figure 1a) [7]. DNA damage induces a Chk1-dependent mitotic delay, and ectopic expression of Chk1 (Chk1^{oe}) mimics checkpoint activation [8]. We screened *S. pombe* for chromosomal mutants able to overcome cell-cycle arrest induced by *chk1^{oe}*. The gene corresponding to one such mutant, *caa1-1* (Chk1 arrest attenuator), encodes a homologue of Sgn1 (Gps1) [9] and COP11 [10]. Cells deleted for *caa1* (*caa1-d*) were viable, albeit slow growing and heterogeneous in cell length at division (14.4–20.4 µm, mean 17.9 µm) compared with *caa1$^+$* cells (14.4–15.9 µm, mean 15 µm). Comparisons of *caa1-1* and *caa1-d* cells suggested that *caa1-1* is a loss-of-function mutant. *Caa1-d* and *caa1-1* cells were sensitive to gamma and UV radiation, but not to hydroxyurea (HU) treatment, which inhibits replication (Figure 1b).

We analysed genetic interactions between *caa1-d* and DNA-integrity-checkpoint mutants. *Caa1-d* was synthetically lethal with *rad3-d*, *chk1-d*, *cds1-d* and *cdc2.3w* (an allele of *cdc2* defective in the S/M checkpoint [11]), suggesting that *caa1-d* cells require checkpoint functions for viability. Using the temperature-sensitive *rad3^{ts}* mutation [12], we analysed the terminal phenotype of *caa1-d rad3^{ts}* cells. Unlike the two single mutants, mitosis was aberrant following loss of checkpoint function (Figure 1c). The interaction of *caa1-d* with both the *chk1-d* and *cds1-d* mutations is intriguing as Chk1 function is thought to be G2-specific, whereas Cds1 is S-phase-specific [13,14]. We therefore examined Cds1 kinase activity [13] and Chk1 phosphorylation [14] in *caa1-d* cells. Chk1 was phosphorylated after irradiation in *caa1-d* cells, but was also phosphorylated at low levels in the absence of exogenous DNA damage (Figure 1d), suggesting that *caa1-d* cells suffer intrinsic DNA damage. In *caa1-d* cells, Cds1 kinase appeared to be as highly activated as in a HU-treated wild-type control (Figure 1e), although protein levels were not significantly increased.

Caa1-d mutants have problems in S phase

In fission yeast, S phase is initiated soon after mitosis and completed before cytokinesis is finished. Fluorescence-activated-cell-sorting (FACS) analysis of the DNA content of wild-type *S. pombe* cells therefore shows largely a 2N profile, with a small population of replicating cells displaying a

Figure 1



Characterisation of *caa1* mutants. **(a)** The DNA-integrity structure checkpoint. A diagram showing the signal transduction pathway that leads to cell-cycle arrest in response to activation of the DNA-integrity checkpoints. DNA damage and perturbed DNA replication are detected by different sensors that feed through a common transduction system to activate either Cds1 (in response to S-phase perturbations) or Chk1 (in response to DNA damage). The aim of the checkpoint is to maintain the inhibitory phosphorylation on Tyr15 of the mitotic inducer Cdc2. The elements of this pathway are conserved in metazoan cells. **(b)** *Caa1* mutants are sensitive to radiation. Survival of *caa1-1* and *caa1-d* cells compared with *caa1*$^+$ (wild type) and *chk1-d* cells after exposure to gamma or UV irradiation at different doses, and to 10 mM hydroxyurea (HU) for the indicated times. The effect of HU treatment on *rad3-d* cells is also shown. **(c)** Checkpoint activity is required for the viability of *caa1-d* cells. Staining of DNA and septal material (with DAPI and calcufluor, respectively) in *rad3*^{ts} *caa1-d* cells at the permissive temperature (27°C) and at the indicated times after shift to the restrictive temperature (35°C). **(d)** Chk1 activation in *caa1-d* cells. Western blot of endogenous Chk1–HA protein in *caa1*$^+$ (WT) cells and in *caa1-d* cells before and after irradiation with 500 Gy (± IR). Note the presence of phosphorylated Chk1 in the absence of irradiation in *caa1-d* cells. **(e)** Cds1 kinase activity is high in *caa1-d* cells. Cds1 kinase assays were essentially performed as described in [14]; Cds1 was immunoprecipitated from two different *caa1*$^+$ (WT) and two different *caa1-d* cell extracts. Cds1 kinase activity was subsequently measured using MBP as a substrate. For a positive control of Cds1 activity, HU was added to a *caa1*$^+$ culture for 3 h to activate the replication checkpoint.

DNA content between 2N and 4N. We examined the DNA content of exponential *caa1-d* cells. Compared with *caa1*$^+$, *caa1-d* populations showed a broad peak that was biased towards a < 2N DNA content, suggesting a delay in the completion of replication while cells had divided. Wild-type cells accumulated as a 1N population 3 hours after the addition of HU. In contrast, *caa1-d* cells produced a broader G1/S peak, consistent with an increased proportion of *caa1-d* cells being trapped in S phase when nucleotide precursors are depleted (Figure 2a).

As a further measure of the duration of S phase, we examined levels of Mik1 protein, which phosphorylates and inactivates p34^{Cdc2}. Mik1 is only present in the nucleus of S-phase cells and is stabilised in response to activation of the replication checkpoint (P.U.C., personal communication) [15]. Mik1 protein levels in exponential *caa1-d* cells were 2–3 times higher than those seen in *caa1*$^+$ cells (Figure 2b). Furthermore, in *caa1*$^+$ populations, S phase normally coincided with septation and only 10% of single cells showed nuclear Mik1. In a *caa1-d* population, nuclear Mik1 staining was evident in 26% of single cells, consistent with S phase being extended so that septation and cytokinesis are completed before replication (Figure 2c). Interestingly, *caa1-d* and *mik1-d* showed a strong genetic interaction and double mutants formed microcolonies and exhibited severe morphological phenotypes in liquid culture (Figure 2d). Consistent with the observed increase in Mik1 levels, we found that *caa1-d* partially suppressed the mitotic advancement seen upon loss of Wee1 function (see Supplementary material). Taken together, these data demonstrate that *caa1-d* cells experience significant problems in passing through S phase. However, we found no strong genetic interactions with cell division control (*cdc*) mutations in genes encoding elements of the replication machinery. This suggests that the S-phase delay is not caused by a specific effect on one of the known aspects of replication.

Constitutive DNA damage is often a consequence of incomplete or perturbed replication, and results in genomic instability [16] that can be monitored by mini-chromosome stability [17]. Mini-chromosome[16] was lost from *caa1-d* cells approximately 175-fold more frequently per division than in *caa1*$^+$ cells (frequencies: 6.9% compared with 0.04%).

Nuclear import and export are not globally affected by *caa1* deletion

A role for the signalosome in regulating nuclear import and export has been suggested [2]. The nuclear localisation of Cdc25 — the activating phosphatase for mitotic p34^{Cdc2} — is a target of the Chk1-dependent DNA-damage response. Cdc25 is constitutively imported into the nucleus, but during G1 and S phases (and in response to DNA damage) it is rapidly exported [18]. Nuclear Cdc25 levels in unperturbed *caa1-d* cells were strikingly reduced compared with levels in *caa1*$^+$ cells, but total levels of Cdc25 were unaffected (Figure 2e). Low levels of nuclear Cdc25 probably reflect the extended S phase of *caa1-d* cells. Addition of leptomycin B, which targets the nuclear export signal

(NES) receptor Crm1 [19,20] and inhibits export, led to a rapid nuclear accumulation of Cdc25 in *caa1-d* cells (Figure 2f). This demonstrates that nuclear import *per se* is not dramatically reduced in the *caa1-d* mutant. Consequently, as the reduction of nuclear Cdc25 staining in the *caa1-d* cells is apparent, Crm1-dependent nuclear export must be functional in *caa1-d* cells.

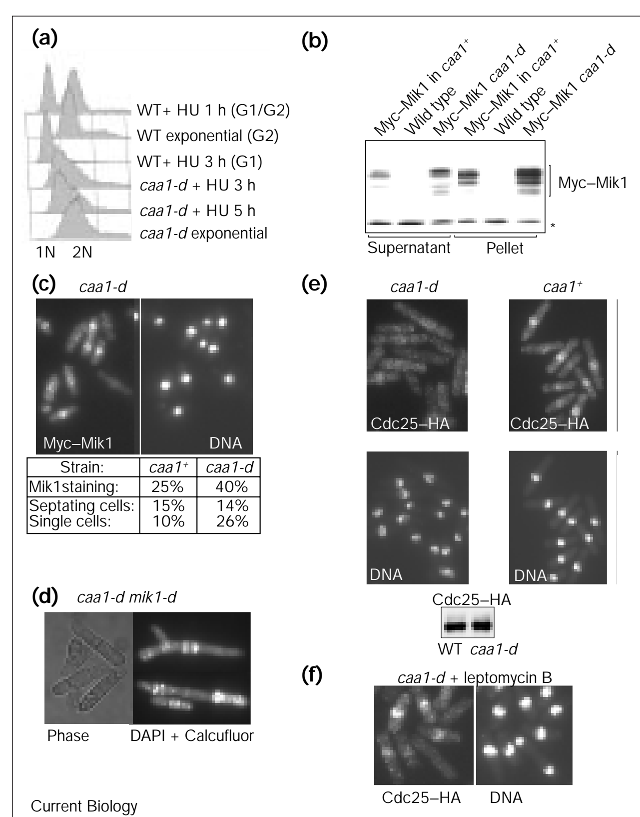
Conservation of the COP9/signalosome complex in fission yeast

To determine whether other COP9/signalosome subunits are conserved in *S. pombe*, we used a two-hybrid interaction assay to screen *caa1* as bait against an *S. pombe* cDNA library. Of twenty positive colonies, fifteen contained a plasmid encoding an *S. pombe* homologue of human Sgn2 ($Sgn2^{Hs}$). Caa1 and *S. pombe* Sgn2 ($Sgn2^{Sp}$) co-immunoprecipitated (Figure 3a) and an *sgn2-d* mutant was phenotypically indistinguishable from *caa1-d*. Epistasis analysis showed no additional sensitivity in the *caa1-d sgn2-d* double mutant compared with the single mutants (Figure 3b). In size-exclusion gel filtration, Caa1 eluted in size fractions around 500 kDa, a size similar to the human signalosome [3] and consistent with the conservation of a multi-subunit complex (Figure 3c). Caa1 was predominantly nuclear throughout the cell cycle, with no changes observed after irradiation (Figure 3d). Finally, two further putative *S. pombe* signalosome subunits, $Sgn4^{Sp}$ and $Sgn5^{Sp}$, exist in the genome database. Taken together, these data strongly predict the existence of a COP9/signalosome-like complex in fission yeast.

The existence of a Cop9/signalosome-like complex in fission yeast is surprising as it is absent in budding yeast and has been suggested to play a role in developmental processes in multicellular organisms. Mitotic control and checkpoint regulation are both structurally and functionally more conserved between *S. pombe* and metazoans than between *S. cerevisiae* and metazoans. If, as has been suggested, the signalosome has a role in cell-cycle regulation, it is possible that its conservation in *S. pombe* reflects similarities in the organisation of the cell cycle. Our data provide evidence that the Cop9/signalosome complex in *S. pombe* is required for the co-ordination of S phase. The molecular targets are not yet identified and are probably numerous. In this context, the homology to the regulatory lid complex of the proteasome, which confers specificity to ubiquitinated proteins, is provocative [5,6]. Perhaps the signalosome targets proteins, possibly modified by ubiquitin-like molecules, to structures within the nucleus that organise chromatin-associated transcription, replication and/or repair factors.

We have been unable to obtain data that explain the identification of *caa1-1* as a suppressor of Chk1 overexpression. This may be a direct effect, or it could be an indirect consequence of disturbed cell-cycle progression and constitutive activation of the DNA-integrity checkpoint pathways.

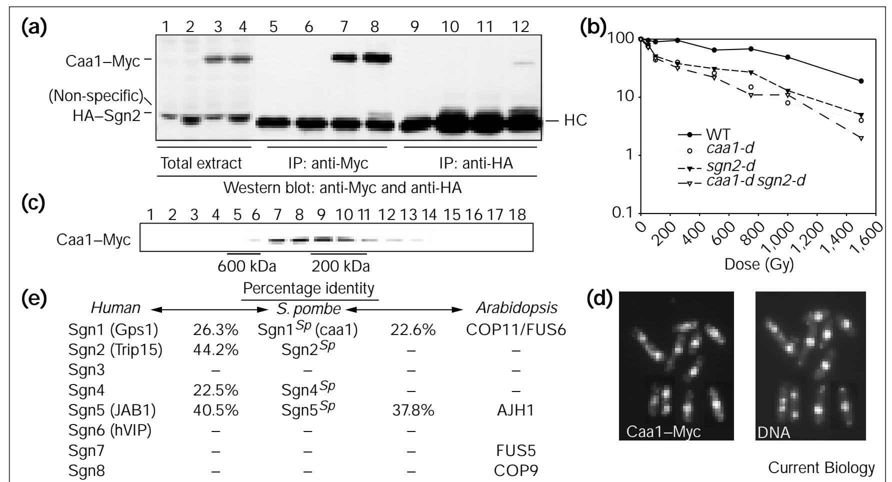
Figure 2



S-phase phenotypes of *caa1-d* cells. **(a)** FACS analysis of the DNA content of *caa1-d* and *caa1+* (WT) cells after staining with propidium iodide. The profiles are, from top to bottom: WT cells arrested for 1 h with hydroxyurea (HU) to create a 1N DNA peak; a predominant 2N peak in an exponentially growing WT culture; WT cells arrested for 3 h with HU to block cells in G1; *caa1-d* cells arrested with HU for 3 h; *caa1-d* cells arrested with HU for 5 h; exponentially growing *caa1-d* cells. **(b)** Mik1 protein levels are elevated in *caa1-d* cells. Mik1 protein levels were examined using a strain carrying genomically Myc-tagged Mik1. Protein extracts from exponentially growing *Myc–mik1 caa1+*, untagged wild-type, and *Myc–mik1 caa1-d* cells were separated into a high-speed supernatant and a pellet fraction. Equal amounts of total protein from each fraction were analysed (the asterisk indicates a non-specific crossreacting band). **(c)** Mik1 protein persists in *caa1-d* cells after the completion of cell division. Immunolocalisation of Myc–Mik1 using anti-Myc antibody and DAPI staining of DNA. Nuclear Myc–Mik1 staining in *caa1-d* and *caa1+* septating and single cells was scored in over 300 cells for each strain (lower panel). **(d)** Phenotype of the *caa1-d mik1-d* double mutant. *Caa1-d mik1-d* double mutant cells in liquid medium are shown in phase and stained with DAP I and calcufluor for DNA and septum material, respectively. **(e)** Localization of haemagglutinin-tagged Cdc25 (Cdc25–HA) in *caa1+* and *caa1-d* cells. Immunolocalisation of Cdc25–HA using anti-HA antibody and DAPI staining of DNA shows a drastic decrease in the nuclear staining of Cdc25–HA in *caa1-d* cells. Anti-HA western blotting demonstrates that total protein levels of Cdc25–HA are equal in *caa1-d* and *caa1+* (WT) cells. **(f)** Cdc25 is imported into the nucleus in *caa1-d* cells. Immunolocalisation of Cdc25–HA after blocking nuclear export with leptomycin B for 40 min demonstrates that Cdc25–HA is imported into the nucleus.

Future studies combining genetic and biochemical analysis should clarify this and help to establish the elusive function of the signalosome.

Figure 3



Human		S. pombe		Arabidopsis
Sgn1 (Gps1)	26.3%	$Sgn1^{Sp}$ (caa1)	22.6%	COP11/FUS6
Sgn2 (Trip15)	44.2%	$Sgn2^{Sp}$	–	–
Sgn3	–	–	–	–
Sgn4	22.5%	$Sgn4^{Sp}$	–	–
Sgn5 (JAB1)	40.5%	$Sgn5^{Sp}$	37.8%	AJH1
Sgn6 (hVIP)	–	–	–	
Sgn7	–	–	–	FUS5
Sgn8	–	–	–	COP9

Conservation of the COP9/signalosome complex. **(a)** $Sgn2^{Sp}$ and $Caa1^{Sp}$ interact *in vivo*. Cells carrying a genomically (13Myc)-epitope-tagged *caa1* and non-tagged wild-type (WT) cells were transformed with either pREP41 vector or *HA–Sgn2* in pREP41. Cultures were grown under non-inducing conditions to allow low expression of HA–Sgn2 from the thiamine-repressible *nmt1* promoter. Western blot of total extracts from: lane 1, WT cells + vector; lane 2, WT cells + HA–Sgn2; lane 3, Caa1–Myc cells + vector; lane 4, Caa1–Myc cells + HA–Sgn2. The total cell extracts used in immunoprecipitations (IPs) for lanes 5–8 and 9–12 were the same as those described for lanes 1–4. Caa1–Myc and HA–Sgn2 were immunoprecipitated using anti-Myc antibody 9E10 or anti-HA antibody 12CA5, respectively. Co-immunoprecipitating protein was revealed by immunoblotting with both anti-Myc and anti-HA antibodies. HC, IgG heavy chain. **(b)** *Caa1-d* and *sgn2-d* are epistatic. Survival of *caa1-d, sgn2-d* and *caa1-d sgn2-d* mutant cells were compared with wild-type (WT) cells after exposure to gamma irradiation at different doses. **(c)** Caa1 is associated with a macromolecular complex. After gel filtration analysis of Caa1–Myc, fractions 1–18 were immunoblotted with anti-Myc antibody 9E10. The elution pattern of size markers is indicated below the panel. **(d)** Caa1–Myc localises to the nucleus. Immunofluorescence staining of genomically tagged Caa1–Myc in exponentially growing cells. **(e)** Conservation of the COP9/signalosome complex between humans, fission yeast and plants.

Supplementary material

Supplementary material including figures showing alignments of Sgn2 and Caa1 with their homologs, genetic interactions between *caa1* and *wee1/cdc25*, control experiments showing that the moderate phosphorylation of Chk1 in *caa1-d* cells is not the cause of the lack of Cdc25 in the nucleus, a table showing double mutants of *caa1-d*, and additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

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Supplementary material

The COP9/signalosome complex is conserved in fission yeast and has a role in S phase

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Figure S1

Caa1 alignment. Caa1 is homologous to Cop11 and Gps1. Amino-acid alignments of human Sgn1 (Gps1), *Arabidopsis* Cop11 (Fus6) and *S. pombe* Caa1. Identical residues are shaded in black; similar residues are shaded in grey.

```
HS Sgn1     1  MEVDGTPRRGGCKMPLPVQVFNLQGAVEPMQIDVDPQEDPQNAPDVNYVVENPSLDLEQYAASYSGLMRIERQLFIADHCP...TLRVEA
AT Cop11    1  MERDEEAGG.......PMMEMCTNGGEETSN..............RRPIISGEPLDIEAYAALYKGRTKIMRLLFIANHCGGNHALQFDA
SP Caa1     1  ..........................................MSLNLLVNQEELKRYLDEYGVWSKIFRALFVARNSKP..LRSFCV

HS Sgn1    88  LKMALSFVQRTFNVDMYEEIHRKLSEATRELQNAPDAIPESGVEPPALDTAWVEATRKKALLKLEKLDTDLKNYKGNSIKESIRRGHDDL
AT Cop11   70  LRMAYDEIKKGENTQLFREVVNKIGNRL..............GEKYGMDLAWCEAVDRRAEQKKVKLENELSSYRTNLIKESIRMGYNDF
SP Caa1    44  HYAIKELKEKTYNLELYQSLFEEFQDC..............FENEQLDVEWVESVTFHRKQNLEQLRRELKAYKNNLIRESIRAAQLDL

HS Sgn1   178  GDHYLDCGDLSNALKCYSRARDYCTSAKHVINMCLNVIKVSVYLQNWSHVLSYVSKAESTPEIAEQRGERDSQTQAILTKLKCAAGLAEL
AT Cop11  146  GDFYYACGMLGDAFKNYIRTRDYCTTTKHIIHMCMNAILVSIEMGQFTHVTSYVNKAEQNPETLEP.........MVNAKLRCASGLAHL
SP Caa1   119  ASFFADVGQFDSALRSYAKVREYCTNAGQIAHLSLELMRISIWIGNYSHVLAFGSRAKSTVSAAME..........LTSPIYAYCGLANF

HS Sgn1   268  AARKYKQAAKCLLLASFDHCD.FPELLSPSNVAIYGGLCALATFDRQELQRNVISSSSFKLFLELEPQVRDIIFKFYESKYASCLKMLDE
AT Cop11  227  ELKKYKLAARKFLDVNPELGNSYNEVIAPQDIATYGGLCALASFDRSELKQKVIDNINFRNFLELVPDVRELINDFYSSRYASCLEYLAS
SP Caa1   199  CLGDYEEALAHFLKVETDTSD...GIITKTDISVYISLCALACWDHKRLIQELERNEEFNALSDLDPSLRRCLQAKCNRKYSLLLDTLQQ

HS Sgn1   357  MKDNLLLDMYLAPHVRTLYTQIRNRALIQYFSPYVSADMHRMAAAFNTTVAALEDELTQLILEGLISARVDSHSKILYARDVDQRSTTFE
AT Cop11  317  LKSNLLLDIHLHDHVDTLYDQIRKKALIQYTLPFVSVDLSRMADAFKTSVSGLEKELEALITDNQIQARIDSHNKILYARHADQRNATFQ
SP Caa1   286  NAQDYSLDMYLAPQLTNLFSLIRERSLLDYLIPYSALPFSKIAVDFHIDENFIEKNLLEIIEAKKLNGKVDSLQKRVYIEPSSEP.ENFE

HS Sgn1   447  KSLLMGKEFQRRAKAMMLRAAVLRNQIHVKSPPREGSQGELTPANSQSRMSTNM
AT Cop11  407  KVLQMGNEFDRDVRAMLLRANLLKHEYHARSARKL...................
SP Caa1   375  DIKNIAQTSLLYSKALYLQTLAAMNTENTDDEAPVIAQTNITAEDSQN......
```

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Figure S2

Sgn2 alignment. The Caa1-interacting protein Sgn2^{Sp} is homologous to Sgn2^{Hs}. Amino-acid alignments of human Sgn2 (HS Sgn2) and *S. pombe* Sgn2 (SP Sgn2). Identical residues are shaded in black, similar residues are shaded in grey.

```
SP Sgn2     1  MSRFTNDFMLEDDENYDFEFEDDDDDMIEPYVDVENCYYNSKSLKEENPESALTSFYSIVEKCGRQQNEWAFKALKQITKINFQLKKYDD
HS Sgn2     1  MSDMEDDFMCDDEEDYDLEYSEDSNS..EPNVDLENQYYNSKALKEDDPKAALSSFQKVLELEG.EKGEWGFKALKQMIKINFKLTNFPE

SP Sgn2    91  MLQSYQRLLGYTNWLSITKNYSEKSIYNIVEYASSCENTEFLEKFYDVTTKALQNLNNERLMLKVLMHVARFLLTQKNYHKFKYLLRQMH
HS Sgn2    88  MMNRYKQLLTYIR.SAVTRNYSEKSINSILDYISTSKQMDLLQEFYETTLEALKDAKNDRLWFKTNTKLGKLYLEREEYGKLQKILRQLH

SP Sgn2   181  GAFVRTKIILLQIQNRGTHLLELYSLEIQMYSDIEDNKRLKELYQSSLRVKTAIPHPRIMGIIRECGGKMHMQENQWSEAQTNFFESFKS
HS Sgn2   177  QS.CQTDDGEDDLK.KGTQLLEIYALEIQMYTAQKNNKKLKALYEQSLHIKSAIPHPLIMGVIRECGGKMHLREGEFEKAHTDFFEAFKN

SP Sgn2   271  YDEAGSSDRIRVLKYLVLANMLSESEINPFDSPETQPYKDNPHIIAMTKLVEAYQIRDITAVERYTNLSH.DILDDDFIRQYVDXILYSI
HS Sgn2   265  YDESGSPRRTTCLKYLVLANMLMKSGINPFDSQEAKPYKNDPEILAMTNLVSAYQNNDITEFEKILKTNHSNIMDDPFIREHIEELLRNI

SP Sgn2   360  RSQVLIELVKPYTSVKLSLLAKKLGVSISIIEQALVGLIIDERVNGKIDMVNEVFTISQPKNTIHNQLVEDVQKLWNTATK........
HS Sgn2   355  RTQVLIKLIKPYTRIHIPFISKELNIDVADVESLLVQCILDNTIHGRIDQVNQLLELDHQKRGGARYTALDKWTNQLNSLNQAVVSKLA
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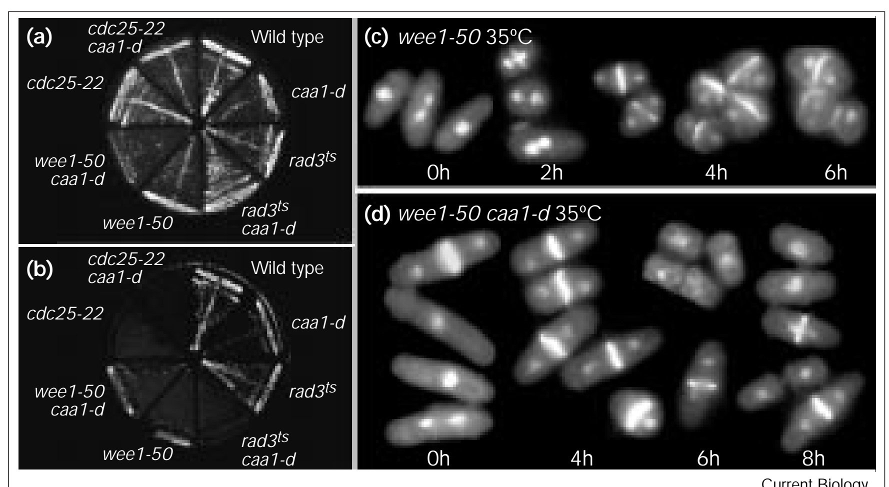
Current Biology

Figure S3



Genetic interactions between *caa1* and *wee1/cdc25*. Growth of temperature-sensitive mutants in a *caa1-d* background. **(a,b)** Growth on YEA plates at (a) 27°C or (b) 35°C of wild-type, *caa1-d, rad3^{ts}, rad3^{ts} caa1-d, wee1-50, wee1-50 caa1-d, cdc25-22,* and *cdc25-22 caa1-d* cells. **(c,d)** Fluorescence microscopy of (c) *wee1-50* and (d) *wee1-50 caa1-d* cells after a temperature shift from 27°C to 35°C. DNA and septal material were stained with DAPI and calcufluor, respectively.

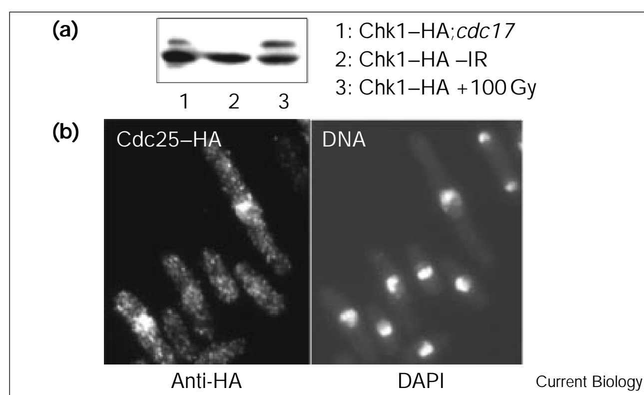
Table S1

Double mutants of *caa1-d* with genes mutant in replication, regulation of mitotic entry or checkpoint function.

Double mutants with *caa1-d*	Phenotype at 27°C	Phenotype at 35°C
Cdc1-P13 (polδ small subunit)	Caa1-d	Cdc
Cdc6-23 (polδ catalytic subunit)	More elongated than *cdc6*	Cdc
Cdc10 M17 (transcription factor)	Caa1-d	Cdc
Cdc10 V50 (transcription factor)	Caa1-d	Cdc
Cdc17-K42 (DNA ligase)	Caa1-d	Cdc
Cdc18-K46 (replication initiator)	Caa1-d	Cdc
Cdc21-M68 (MCM protein)	Caa1-d	Cdc
Cdc24-M34 (novel)	More elongated than *cdc24*	Cdc
Polα^{ts} (primase)	Slightly more elongated than *polα^{ts}*	Cdc
Cdc25-22 (mitotic inducer, *cdc2* PPAse)	Caa1-d	Cdc
Wee1-50 (mitotic inhibitor, *cdc2* kinase)	More elongated than *wee1-50*	Supression of *wee1-50* phenotype
Cdc2.33 (mitotic inducer)	Caa1-d	Cdc
Cdc2.3w (mitotic inducer)	Synthetic lethal	
Mik1-d (Wee1-like kinase)	Low viability	
Rad3-d (ATM homologue)	Synthetic lethal	
Chk1-d (G2 checkpoint kinase)	Synthetic lethal	
Cds1-d (replication checkpoint kinase)	Synthetic lethal	

Cdc, cell cycle control.

Figure S4



Controls for Cdc25 localisation. Nuclear localisation of Cdc25–HA in *cdc17* cells. **(a)** Chk1-HA;*cdc17-K42* cells were grown at the permissive temperature (27°C) and the level of Chk1–HA phosphorylation (lane 1) was compared to that in *cdc17*$^+$ cells before (–IR; lane 2) and after (lane 3) irradiation with 100 Gy. **(b)** Localisation of Cdc25–HA in Cdc25–HA;*cdc17-K42* cells at 27°C. The left-hand panel shows staining with anti-HA antibody 12CA5; the right-hand panel shows staining with DAPI. These data indicate that it is unlikely that the weak G2 checkpoint activation (as judged by Chk1 phosphorylation) is responsible for the absence of nuclear localisation of Cdc25 in *caa1-d* cells. Cdc25 localises in the nucleus of *cdc17-K42* cells in G2 at 27°C, despite higher levels of intrinsic Chk1 phosphorylation.

Supplementary materials and methods

Isolation and genetics of caa1 *and* sgn2

50,000 cells were mutagenised using UV radiation and Chk1 cDNA expression from the *nmt1* promoter in REP1 induced. The radiation-sensitivity of 500 surviving colonies was tested in the absence of *Chk1^{oe}*. One radiation-sensitive mutant, *caa1-1*, was analysed further. Transformation with a genomic library and selection for radiation-resistance identified a plasmid encoding a 1266 bp open-reading frame (with three predicted introns removed). Introns and 3′ end were confirmed by RACE-PCR and cDNA sequencing. Caa1 has 422 amino acids and a molecular mass (M_r) of 48.6 kDa. Full-length *caa1* in pAS was screened against an *S. pombe* cDNA library in pACT2D (deleted for the HA tag) [S1]. The predicted Sgn2 protein has 440 amino acids and a M_r of 51.8 kDa. The coding sequences (amino acids 6–383 of *caa1* and 25–413 of *Sgn2*) were replaced by *ura4*$^+$ or *LEU2*$^+$, respectively [S2]. Integrations were confirmed by Southern blot analysis. Checkpoint measurements and survival experiments were as described [S3,S4]. Genomic tagging of *caa1* was performed using published methods [S5]. The *caa1–13Myc:kan*$^+$ cells were phenotypically wild-type. The locus was verified by backcrossing against *caa1::ura4*$^+$.

Cell extracts, gel filtration and immunolocalisation

Extracts were prepared and analysed by Superdex 200 (HR10/30 Pharmacia) chromatography as described [S6]. For immunofluorescence microscopy, cells were fixed for 10 min in 3% paraformaldehyde and processed as described [S7]. Primary antibody dilutions were: 12CA5 anti-HA (Babco), 1:250; and 9E10 anti-Myc (Pharmagen), 1:50. Secondary antibody dilutions were: Cy3-conjugated anti-mouse (Jackson labs), 1:250; and FITC-conjugated anti-mouse (DAKO), 1:150. To block nuclear export, 5 ng leptomycin B per ml of culture was added for different intervals. Nuclear Cdc25–HA accumulated within 20 min.

Supplementary references

S1. Durfee T, Becherer K, Chen P, Yeh S, Yang Y, Kilburn A, *et al.*: **The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit.** *Genes Dev* 1993, **7:**555-569.

S2. Barbet NC, Muriel W, Carr A: **Versatile shuttle vectors and genomic libraries for use with *Schizosaccharomyces pombe*.** *Gene* 1992, **114:**59-66.

S3. Edwards R, Carr A: **Analysis of radiation-sensitive mutants of fission yeast.** *Methods Enzymol* 1997, **283:**471-494.

S4. al-Khodairy F, Fotou E, Sheldrick K, Griffiths D, Lehmann A, Carr A: **Identification and characterization of new elements involved in checkpoint and feedback controls in fission yeast.** *Mol Biol Cell* 1994, **5:**147-160.

S5. Bahler J, Wu J, Longtine M, Shah N, McKenzie A, Steever A, *et al*: **Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*.** *Yeast* 1998, **14:**943-951.

S6. Niwa O, Matsumoto T, Yanagida M: **Construction of a minichromosome by deletion and its mitotic and meiotic behaviour in fission yeast.** *Mol Gen Genet* 1986, **203:**397-405.

S7. Hagan I, Hyams J: **The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe*.** *J Cell Sci* 1988, **89:**343-357.