The C-terminal domains of human neurofibromin and its budding yeast homologs Ira1 and Ira2 regulate the metaphase to anaphase transition

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Abbreviations: NF1, neurofibromatosis type 1; APC, anaphase promoting complex; CSRD/RasGAP, cysteine and serine-rich domain/Ras-GTPase activating protein; CTD, C-terminal domain; CHD, CTD-homologous domain; SAC, spindle assembly checkpoint.

The human tumor suppressor neurofibromin contains a cysteine and serine-rich domain/Ras-GTPase activating protein domain (CSRD/RasGAP) and a C-terminal domain (CTD). Domain studies of neurofibromin suggest it has other functions in addition to being a RasGAP, but the mechanisms underlying its tumor suppressor activity are not well understood. The budding yeast Saccharomyces cerevisiae is a good model system for studying neurofibromin function because it possesses Ira1 and Ira2, which are homologous to human neurofibromin in both sequence and function. We found that overexpression of CTD or a neurofibromin CTD-homologous domain (CHD) of Ira1/2 in budding yeast delayed degradation of the securin protein Pds1, whereas overexpression of CSRD/RasGAP did not affect Pds1 degradation. We also found that when CTD or CHD was overexpressed, the number of cells in metaphase was higher than in the control. These results demonstrate that CTD and CHD function in the metaphase to anaphase transition. In addition, Δira1Δira2 cells bypassed mitotic arrest in response to spindle damage, indicating that Ira1 and Ira2 may be involved in the spindle assembly checkpoint (SAC). However, Δira1Δira2Δmad2 cells are more sensitive to spindle damage than Δmad2 or Δira1Δira2 cells are, suggesting that Ira1/2 and Mad2 function in different pathways. Overexpression of CTD but not CSRD/RasGAP partially rescued the hypersensitivity of Δira1Δira2Δmad2 cells to microtubule-destabilizing drugs, indicating a role for CTD in the SAC pathway. Taken together, independently of RasGAP activity, the C-terminal domains of neurofibromin, Ira1, and Ira2 regulate the metaphase to anaphase transition in a Mad2-independent fashion.

Introduction

Neurofibromatosis type 1 (NF1) is a human genetic disorder affecting 1 in 3000–4000 individuals. It is caused by mutations in NF1 and increases the risk of tumor development in the central and peripheral nervous systems.1-3 NF1 encodes neurofibromin, a large protein (2,839 amino acids; 319 kDa) that contains several domains, including a cysteine and serine-rich domain/Ras-GTPase activating domain (CSRD/RasGAP) and a C-terminal domain (CTD).4-6 CSRD/RasGAP is thought to inhibit the GTPase Ras by accelerating hydrolysis of active Ras-GTP to inactive Ras-GDP.7-9 Li et al. suggested that loss-of-function mutations in CSRD/RasGAP might contribute to the development of tumors through aberrantly activating Ras signaling.10 However, analyses of NF1 patients found mutations not only in CSRD/RasGAP but also in CTD.11-13 Thus, CTD is strongly implicated in tumor suppression.

As NF1 is so large, it is difficult to clone and express. Thus, although NF1 was identified more than 25 years ago, the molecular function of neurofibromin in cell cycle progression has yet to be fully elucidated. However, the budding yeast Saccharomyces cerevisiae is a good model system for studying neurofibromin function, since it contains Ira1 and Ira2, which are homologous to human neurofibromin in both sequence and function (Fig. 1B).7,14,15

Transition from metaphase to anaphase is only activated if duplicated chromosomes are properly attached to the mitotic spindle.16 A surveillance mechanism called spindle assembly checkpoint (SAC) prevents segregation of duplicated...
chromosomes in the presence of spindle damage in all eukaryotes. Mad1, Mad2, Mad3, Bub1, Bub3 and Mps1 are key components in the SAC pathway, and each mutation of these genes bypasses the mitotic arrest in response to spindle damage in budding yeast. The SAC ensures the onset of anaphase via the securin Pds1. Pds1 must be degraded for the metaphase to anaphase transition, and this degradation is mediated through the ubiquitination of Pds1 by anaphase promoting complex.
(APC). APC-dependent ubiquitination of Pds1 is promoted by its direct interaction with Cdc20 in budding yeast.20-24 Mad2 lies the most downstream in SAC to regulate the degradation of Pds1.25 In response to spindle damage, the SAC stabilizes Pds1 by the Mad2-dependent sequestration of Cdc20 to delay the metaphase to anaphase transition in budding yeast.16,26

In this study, we found that the neurofibromin CTD can regulate the metaphase to anaphase transition in budding yeast independently of CSRD/RasGAP and that this function is conserved in CHD. Furthermore, Ira1 and Ira2 are involved in the SAC pathway in a Mad2-independent manner. These results suggest that neurofibromin CTD and Ira1/2 CHD regulate the metaphase to anaphase transition during mitosis independently of Mad2 in the SAC pathway.

Results

CSRD/RasGAP shows RasGAP activity in budding yeast, but its CTD functions independently of RasGAP activity

Neurofibromin is a huge protein composed of 2839 amino acids and contains several domains including the CSRD/RasGAP and the CTD (Fig. 1A).7 Ira1 and Ira2 are yeast homologues of human neurofibromin and their residues of both the RasGAP and the CTD domains are conserved (Fig. 1B).7,14,15 Due to its huge size, the full-length NF1 gene is difficult to be cloned and studied as a whole. In this study, we examined the function of distinct neurofibromin domains in the budding yeast cell cycle.

We first examined whether CSRD/RasGAP would have RasGAP activity in budding yeast. ∆ira1 and ∆ira2 cells are sensitive to heat shock stress since the lack of RasGAP hyperactivates Ras-PKA signaling (Fig. 2A), and this heat sensitivity is rescued by overexpressing the CSRD/RasGAP domain of neurofibromin (Fig. 2A).6,7 Therefore, we investigated whether overexpression of CSRD/RasGAP under the GAL1 promoter could rescue the heat-sensitive phenotypes of ∆ira1 and ∆ira2. As shown in Fig. 2B, the induced expression of CSRD/RasGAP by galactose rescued the heat shock sensitivity of ∆ira1 and ∆ira2 cells. In contrast, ∆ira1 and ∆ira2 cells did not proliferate following heat shock when CTD was overexpressed (Fig. 2B). These observations demonstrate that CSRD/RasGAP has RasGAP activity in budding yeast, but CTD does not.

CTD regulates the metaphase to anaphase transition in budding yeast

We next examined whether overexpression of CTD and CSRD/RasGAP affects cell cycle progression in budding yeast. The cells were first synchronized at G1 with α-factor, and then CTD or CSRD/RasGAP expression was induced for 1 h. The cells were then released from G1 arrest into galactose medium and collected every 20 min for 3 h to count the number of cells in metaphase (Fig. 3A) or in anaphase (Fig. 3B) after staining with 4',6-diamidino-2-phenylindole (DAPI). When either CTD or CSRD/RasGAP was overexpressed, metaphase cells began to accumulate approximately by 80 min after release, and by
Figure 3. The neurofibromin C-terminal domain (CTD) and the Ira1/2 neurofibromin CTD-homologous domain (CHD) regulate the metaphase to anaphase transition in budding yeast. pCEN-PGAL1-3HA-NF1-CTD, pCEN-PGAL1-3HA-NF1-CSRD/RasGAP, pCEN-PGAL1-3HA-IRA1-CHD, pCEN-PGAL1-3HA-IRA2-CHD, or pCEN-PGAL1-3HA were transformed into cells expressing Myc-tagged Pds1 (strain YSK2202). Cells transformed with these plasmids were synchronized at G1 with α-factor (50 ng/mL), and then the expression of CTD, the neurofibromin cysteine and serine-rich domain/Ras-GTPase activating protein domain (CSRD/RasGAP), and the Ira 1/2 CHD under the GAL1 promoter were induced for 1 h prior to release into galactose medium at 25°C. (A, B, D, and E) The cell cycle progression and (C and F) Pds1 expression level in cells expressing CTD, CSRD/RasGAP, or CHD. (A, B, D, and E) Cells were collected every 20 min and stained with 4',6-diamidino-2-phenylindole, and then metaphase and anaphase cells were counted (n = 200). (C and F) Cells were collected every 20 min, and then Pds1 expression was detected by western blotting using an anti-Myc antibody (Pds1-9Myc). α-Tubulin is shown as a loading control (Tub1).
120 min after release, the number of metaphase cells had greatly increased, particularly among cells expressing CTD. At 140 min after release, the number of metaphase cells expressing CSRD/RasGAP or the control vector sharply decreased, whereas the number of those expressing CTD remained relatively high. Similarly, the number of anaphase cells (large-budded cells with 2 divided nuclei) expressing CSRD/RasGAP or the control vector greatly increased until 140 min after release and sharply decreased thereafter, whereas the number of anaphase cells expressing CTD greatly increased until 160 min after release and then decreased thereafter. These observations suggest that CTD overexpression delays the metaphase to anaphase transition in budding yeast.

To verify the effects of CTD overexpression on the metaphase to anaphase transition in budding yeast, we examined the expression of yeast securin Pds1 by western blotting, as its degradation is required for the metaphase to anaphase transition. When cells synchronized at G1 were released with the induction of CTD or CSRD/RasGAP, as shown in Fig. 3C, Pds1 began to accumulate after 40 min, indicating that these strains have the same growth rate after release from G1 arrest. As expected from the results shown in Fig. 3A and B, at 120 min, Pds1 was maintained in CTD-expressing cells but was mainly degraded in cells expressing CSRD/RasGAP or the control vector (Fig. 3C). Taken together, these results demonstrate that CTD functions in the metaphase to anaphase transition in budding yeast by regulating Pds1 levels.

CHD also regulates the metaphase to anaphase transition

To test whether the CHD of Ira1/2 functions in the metaphase to anaphase transition, mitotic cell cycle progression during CHD overexpression was monitored. As with CTD overexpression, when CHD was overexpressed, the number of metaphase cells at 140 min was maintained at a relatively high level, and the number of anaphase cells was highest at 160 min. In contrast, the number of control cells in metaphase peaked at 120 min and declined sharply thereafter, and the number of control cells in anaphase peaked at 140 min (Fig. 3D, E). The expression of Pds1 was consistent with the nuclear morphology observed in the cells: Pds1 began to accumulate at 40 min and was completely degraded by 140–160 min in the control, whereas in CHD-expressing cells, Pds1 began to accumulate at 40 min but was maintained at 140–160 min (Fig. 3F). These results demonstrate that the function of CTD at the metaphase to anaphase transition is conserved in CHD.

Ira1 and Ira2 function in the SAC pathway in budding yeast

As Pds1 is a key target of the SAC in budding yeast, we next tested whether Ira1 and Ira2 were also involved in the SAC pathway. It had previously been reported that treatment of SAC mutants with benomyl, a microtubule-destabilizing compound, severely decreases cell viability. Consistent with this report, we found that the viability of Δmad2 cells was sharply reduced in response to benomyl treatment, because Mad2 is a key component of the SAC pathway (Fig. 4A). In contrast, the viability of Δira1 and Δira2 cells was partially decreased in response to benomyl, but not to the same extent as in Δmad2 cells. As this result

Figure 4. Ira1 and Ira2 are involved in the spindle assembly checkpoint (SAC) pathway. (A) Δira1/Δira2 cells are sensitive to spindle damage. Wild-type, Δira1 (strain YSK2620), Δira2 (strain YSK2622), Δira1/Δira2 (strain YSK2866), and Δmad2 (strain YSK2668) cells were grown to mid-log phase at 25°C, serially diluted 10-fold, spotted onto either YPAD plates or YPAD plates containing 10 μg/ml benomyl, and incubated at 25°C. (B) Δgpb1/Δgpb2 cells are proficient for SAC. Wild-type, Δgpb1/Δgpb2 (strain YSK2929), Δira1Δira2 (strain YSK2929), Δira1Δira2 (strain YSK2866), and Δmad2 (strain YSK2668) cells were grown to mid-log phase at 25°C, serially diluted 10-fold, spotted onto either YPAD plates or YPAD plates containing 10 μg/ml benomyl and incubated at 25°C. (C) Δira1Δira2 cells bypassed mitotic arrest in response to spindle damage. Wild-type and Δira1Δira2 cells expressing Myctagged Pds1 (strains YSK2992 and YSK3001, respectively) were grown to early log phase at 25°C and then treated with 15 μg/ml nocodazole. Cells were collected every 1 h for 8 h, and Pds1 and Clb2 expression (Pds1-9Myc and Clb2, respectively) was detected by western blotting using anti-Myc and anti-Clb2 antibodies, respectively. α-Tubulin (Tub1) served as a loading control.
might have been due to the functional redundancy of Ira1 and Ira2, we next examined the sensitivity of the Ira1 and Ira2 double-knockout strain YMW208 in response to benomyl. Like Δmad2 mutant cells, Δira1Δira2 mutant cells had significantly decreased cell viability in response to benomyl, suggesting that Ira1 and Ira2 function redundantly in the SAC pathway.

To determine whether the RasGAP activity of Ira1 and Ira2 is required for their function in the SAC pathway, we deleted both GPB1 and GPB2, which encode proteins that interact with Ira1 and Ira2 to maintain their RasGAP activity,15 and then examined the viability of Δgpb1Δgpb2 cells in the presence of benomyl (Fig. 4B). The Δgpb1Δgpb2 mutant cells were not as at all sensitive to benomyl as the Δira1Δira2 and Δmad2 mutants, indicating that the SAC function of Ira1 and Ira2 is independent of RasGAP activity.

To confirm that Ira1 and Ira2 function in the SAC pathway, we compared the expression of Pds1 and the mitotic cyclin Clb2 in Δira1Δira2 and wild-type cells in the presence of nocodazole, a microtubule-destabilizing compound. Since the metaphase to anaphase transition is delayed by microtubule defects, wild-type cells maintain expression levels of Pds1 and Clb2 when treated with microtubule-destabilizing drugs. However, Pds1 and Clb2 became degraded upon impairment of the SAC in budding yeast.26,27 When wild-type and Δira1Δira2 cells were grown to mid-log phase and treated with nocodazole, the wild-type cells maintained high levels of Pds1 and Clb2 expression for 8 h, whereas the Δira1Δira2 cells began to degrade Pds1 and Clb2 at 3–4 h and 5–6 h, respectively (Fig. 4C). This result confirms that Ira1 and Ira2 function in the SAC pathway to induce mitotic arrest in response to spindle damage.

Ira1 and Ira2 function independently of Mad2 in the budding yeast SAC pathway

Mad2 is a key downstream regulator of the SAC pathway that regulates the metaphase to anaphase transition in budding yeast; its deletion causes mitotic arrest in response to spindle damage to be bypassed.16,17,25,26 Thus, we asked whether Ira1 and Ira2 function in the Mad2 pathway in response to spindle damage. To address this question, we constructed the triple Ira1, Ira2, and Mad2 knockout mutant Δira1Δira2Δmad2 and compared its sensitivity to benomyl with that of the Δira1Δira2 and Δmad2 mutants. Interestingly, Δira1Δira2Δmad2 cells were more sensitive to benomyl at either 25°C or 30°C than Δira1Δira2 and Δmad2 cells (Fig. 5A). This result suggests that Ira1 and Ira2 might function independently of Mad2 in the SAC.

In order to verify that Ira1/2 and Mad2 delay the metaphase to anaphase transition in response to spindle damage via different pathways, we examined the kinetics of Pds1 degradation in wild-type, Δira1Δira2, Δmad2, and Δira1Δira2Δmad2 cells in response to nocodazole. As shown in Fig. 5B, Δmad2 cells showed a drop in Pds1 levels at approximately 100–110 min after nocodazole treatment. Importantly, Pds1 degradation was exacerbated in Δira1Δira2Δmad2 triple mutant cells: it began at 80–90 min after nocodazole treatment (Fig. 5B), suggesting that Ira1/2 and Mad2 respond to spindle damage via independent pathways.

Consistent with the previous observation that Pds1 in Δira1Δira2 cells only begins to degrade approximately 3–4 h after nocodazole treatment (Fig. 4C), the level of Pds1 expression in Δira1Δira2 cells was maintained for 130 min after nocodazole treatment (Fig. 5B). These results strongly suggest that Ira1 and Ira2 act in the budding yeast SAC pathway independently of Mad2.

CTD functions in the budding yeast SAC pathway while RasGAP does not

To determine whether CTD overexpression would rescue the
SAC-defective phenotype of Δira1Δira2Δmad2 cells, the cells were synchronized at G1 with α-factor, and the expression of CTD or CSRD/RasGAP was induced for 1 h. Then, the cells were released into galactose medium containing nocodazole, and cell cycle progression was monitored. As expected, overexpression of CTD in Δira1Δira2 Δmad2 cells partially restored their loss of mitotic arrest in response to spindle damage, whereas the expression of CSRD/RasGAP or vector control did not (Fig. 6; Fig. S3). As shown in Fig. 6A, Pds1 accumulation peaked at 80 min and sharply decreased by 100 min in cells expressing the control vector. In cells overexpressing CTD, Pds1 accumulation also peaked at 80 min, but at 100–120 min, a relatively high level of Pds1 expression was maintained (Fig. 6A). In contrast, the kinetics of Pds1 degradation in response to nocodazole in Δira1Δira2Δmad2 cells overexpressing CSRD/RasGAP was similar to that in cells expressing the control vector (Fig. S3). In addition, we counted the number of budded cells, a phenotype associated with bypass of mitotic arrest, in cells overexpressing CTD and CSRD/RasGAP. In this experiment, after the cells had been released from G1 arrest, they were collected every 2 h for 6 h and then examined for bud formation. Consistent with the results shown in Fig. 6A and Fig. S3, the loss of mitotic arrest was partially relieved in a time-dependent manner in cells expressing CTD: 6 h after release, new bud formation was observed in 34.8 ± 6.7% of the CTD-expressing cells, as compared to 43.8 ± 5.3% of cells expressing the control vector and 41.5 ± 6.4% of CSRD/RasGAP-expressing cells (Fig. 6B). These results show that CTD functions independently of RasGAP in the metaphase to anaphase transition.

Discussion

NF1 is a tumor suppressor, and mutations found in NF1 patients suggest a critical role for CTD as well as CSRD/RasGAP in cell cycle regulation.11-13 However, no definite mechanism of the CTD in the control of cell proliferation has been elucidated. In this study, we showed that CTD regulates the metaphase to anaphase transition independently of RasGAP activity during mitosis in budding yeast and this function is conserved in the CHD of the neurofibromin yeast homologs Ira1 and Ira2. Furthermore, our results suggested that both CTD and CHD are involved in the SAC pathway.

The highly conserved SAC is a surveillance mechanism that monitors improper spindle attachment to sister chromatids to maintain genomic stability prior to chromosome segregation in mitosis.28 SAC acts via delaying the degradation of the securin Pds1, which is an inhibitor of the metaphase to anaphase transition, through the Mad2-dependent sequestration of Cdc20 in response to spindle damage.16 Interestingly, we observed that Ira1, Ira2, and CTD function independently of Mad2 to delay Pds1 degradation in response to spindle damage. This raises a question of what could be a molecular mechanism of Ira1, Ira2 and the CTD of neurofibromin in regulating Pds1 in a Mad2-independent manner.

Previous studies have shown that in response to DNA damage in budding yeast, protein kinase A (PKA) phosphorylates Cdc20 on S52 and S88 to inhibit the interaction of Cdc20 and Pds1, which then delays the APC\(^{\text{Cdc20}}\)-mediated degradation of Pds1.29,30 In addition, it has been suggested that the Kelch proteins Gpb1 and Gpb2, which interact with Ira1/2 via CHD, inhibit PKA activity by promoting the interaction of the PKA regulatory and catalytic subunits.15,31 Thus, we hypothesized that CHD and CTD might activate PKA by binding Gpb1 and Gpb2 and thus block the interaction between Gpb1 and Gpb2 with PKA. If this were the case, the expression of a phosphorylation-defective Cdc20 S52A88A mutant should have facilitated Pds1 degradation in Δmad2 cells in response to spindle damage. However, when we overexpressed the phosphorylation-defective Cdc20 S52A88A mutant in Δmad2 cells, Pds1 degradation in response to nocodazole was not accelerated (Fig. S1). As the phosphorylation of Pds1 inhibits its degradation by blocking ubiquitination from APC\(^{\text{Cdc20}}\) in response to DNA damage,29,32 we also examined the possibility that Ira1 and Ira2 may promote...
the phosphorylation of Pds1 by an unknown pathway to induce the delay of Pds1 degradation in a Mad2-independent manner. However, we could not detect any Pds1 phosphorylation in response to spindle damage (data not shown), which is consistent with the previous report that Pds1 is phosphorylated by DNA damage but not by spindle damage.32 Therefore, we speculate that the phosphorylation of Pds1 is not directly correlated with the SAC function of Ira1 and Ira2.

In addition, we also hypothesized that Ira1 and Ira2 may bind directly to Pds1 to inhibit the interaction between Pds1 and Cdc20, which could then block the APC<sub>Cdc20</sub>-dependent ubiquitination of Pds1 in a Mad2-independent way. To test this idea, we first examined the physical interaction between Pds1 and Ira1 by co-precipitation in the presence or absence of nocodazole. However, as shown in Fig. S2A and B, Ira1 did not co-precipitate with Pds1. We also found that overexpressed CTD did not associate with Pds1 in budding yeast (Fig. S2C). Thus, the molecular mechanisms underlying the Mad2-independent regulation of Pds1 remain unclear and will be the subject of future studies. Nonetheless, this study reveals new insight into why mutations in the neurofibromin CTD can increase the risk for tumorigenesis in mammals.

**Materials and Methods**

**Yeast strains, culture, and plasmids**

The yeast strains and plasmids used in this study are described in Tables 1 and 2. All strains were constructed by PCR-based homologous recombination methods and verified by PCR and/or western blot analysis.33,34 Yeast cells were grown in YPAD medium (1% yeast extract, 2% bacto-peptone, 100 μg/mL adenine and 2% dextrose) or in synthetic complete drop-out medium prepared with yeast nitrogen base and necessary supplements.35 PCR-amplified CTD and CSRDRasGAP, CHD, Cdc20, and Cdc20 S52AS88A were subcloned into pCEN-P<sub>GAL1</sub>-3HA. The expression of CSRDRasGAP, CTD, CHD, Cdc20, and Cdc20 S52AS88A was induced using the GAL1 promoter as described by Kim et al.35 Spindle damage was induced by adding either benomyl (10 μg/mL) or nocodazole (15 μg/mL) to the culture at 25°C.

**Yeast spotting assay**

Cells were grown to mid-log phase and serially diluted 10-fold. The heat shock sensitivity assay was performed by incubating cells for 30 min at 55°C after being spotted on selective plates containing glucose or galactose.36 The spindle damage assay was performed by spotting cells grown to mid-log phase on YPAD plates containing 10 μg/mL benomyl.35

**Microscopy**

Harvested cells were fixed in 70% ethanol, washed once with distilled water, briefly sonicated, and stained with 1 μg/mL DAPI. Cells were observed by fluorescence microscopy using a 100× objective on an Axioplan2 (Zeiss), and the images were captured with an AxioCam CCD (Zeiss) camera using Axiowision software (Zeiss).35

**Co-immunoprecipitation**

For co-immunoprecipitation, cells were extracted in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% nonidet P-40, 2 mM EDTA, 5 mM MgSO<sub>4</sub>, 50 mM NaF, 100 mM β-glycerophosphate, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 1 mM dithiothreitol, 100 μg/mL benomyl]. The obtained pellets were extracted in lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% SDS, 100 mM NaF, 20 mM β-glycerophosphate, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 1 mM dithiothreitol, and 2 μg/mL benomyl. The precipitate was washed three times with distilled water, briefly sonicated, and stained with 1 μg/mL DAPI. Cells were observed by fluorescence microscopy using a 100× objective on an Axioplan2 (Zeiss), and the images were captured with an AxioCam CCD (Zeiss) camera using Axiowision software (Zeiss).35

| Name       | Genotype                          | Source*       |
|------------|-----------------------------------|---------------|
| W303a (Y300) | MATa ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-100 | Elledge SJ    |
| Σ1278b     | MATa ura3-52                      | Lorenz et al., 1997 |
| YSK2866    | Y300 except as Δira1::URA3 Δira2::URA3 | Sanchez Y     |
| YSK2202    | W303a except as bar1Δ63:His3 PDS1-9Myc:hphNT1 SIC1-3HA-Kan<sup>R</sup> [pRS304-BFA1-GFP] | This study    |
| YSK2620    | W303a except as Δira1::KAN<sup>R</sup> | This study    |
| YSK2622    | W303a except as Δira2::KAN<sup>R</sup> | This study    |
| YSK2668    | W303a except as Δmad2::URA3       | This study    |
| YSK2929    | W303a except as Δgpb1::KAN<sup>R</sup>Δgpb2::HIS3 | This study    |
| YSK2992    | W303a except as PDS1-9Myc:hphNT1   | This study    |
| YSK3001    | Y300 except as Δira1::URA3 Δira2::URA3 PDS1-9Myc:hphNT1 | This study    |
| YSK2925    | Y300 except as Δira1::URA3 Δira2::URA3 Δmad2::KAN<sup>R</sup> | This study    |
| YSK2984    | W303a except as Δmad2::URA3PDS1-9Myc:hphNT1 | This study    |
| YSK3003    | Y300 except as Δira1::URA3 Δira2::URA3 Δmad2::KAN<sup>R</sup> PDS1-9Myc:hphNT1 | This study    |
| YSK2949    | Σ1278b except as Δleu2::hisG IRA1-3HA:G418 | Heitman J     |
| YSK3103    | Σ1278b except as Δleu2::hisG IRA1-3HA:G418 PDS1-9Myc:hphNT1 | This study    |

**Table 2. Plasmids used in this study**

| Plasmids                          | Source*       |
|-----------------------------------|---------------|
| pCR2.1-TOPO carrying CSRDRasGAP   | Mangoura D    |
| pEFGP-C1 carrying CTD            | Mangoura D    |
| pFA6a-His3MX6-P<sub>GAL1</sub>-3HA | Pringle JR    |
| pRS315-P<sub>GAL1</sub>-3HA      | This study    |
| pRS315-P<sub>GAL1</sub>-3HA-NF1 CTD | This study    |
| pRS315-P<sub>GAL1</sub>-3HA-NF1 CSRDRasGAP | This study    |
| pRS315-P<sub>GAL1</sub>-3HA-IRA1 CHD | This study    |
| pRS315-P<sub>GAL1</sub>-3HA-IRA2 CHD | This study    |
| pMW034                           | Sanchez Y     |
| pMW036                           | Sanchez Y     |
| pRS315-P<sub>GAL1</sub>-3HA-CDC20 | This study    |
| pRS315-P<sub>GAL1</sub>-3HA-CDC20 S52A S88A | This study    |
1 mM phenylmethylsulphonyl fluoride, and protease inhibitor cocktail (Roche) by beadbeating (Biospec). To precipitate hemagglutinin (HA)-tagged Ira1, CTD, and CSRD/RasGAP, crude cell lysates (10 mg in lysis buffer) were incubated with an anti-HA antibody for 2 h at 4°C, followed by incubation with protein Aagarose (Invitrogen) for 2 h.

**Western blot analysis**

Cellular lysates were extracted in sodium dodecyl sulfate (SDS) sample buffer [60 mM Tris-HCl (pH 6.8), 25% glycerol, 10% SDS, 5% 2-mercaptoethanol, and 0.1% bromophenol blue] by boiling for 5 min at 90–100°C. Detection of Myc, Clb2, HA, and α-tubulin was performed using anti-Myc mouse monoclonal antibody (Cell Signaling, 1:5000 dilution), anti-Cla2 rat polyclonal antibody (generated in our lab, 1:5000 dilution), anti-HA mouse monoclonal antibody (Santa Cruz, 1:3000 dilution), and anti-α-tubulin mouse monoclonal antibody (Sigma Aldrich, 1:5000 dilution), respectively. An enhanced chemiluminescence system was used for blot analysis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Supplemental Materials**

Supplemental materials may be found here: http://dx.doi.org/10.4161/15384101.2015.945870

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