

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, $\Delta ira1\Delta ira2$ cells bypassed mitotic arrest in response to spindle damage, indicating that Ira1 and Ira2 may be involved in the spindle assembly checkpoint (SAC). However, $\Delta ira1\Delta ira2\Delta mad2$ cells are more sensitive to spindle damage than $\Delta mad2$ or $\Delta ira1\Delta ira2$ cells are, suggesting that Ira1/2 and Mad2 function in different pathways. Overexpression of CTD but not CSRD/RasGAP partially rescued the hypersensitivity of $\Delta ira1\Delta ira2\Delta mad2$ 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 protein 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

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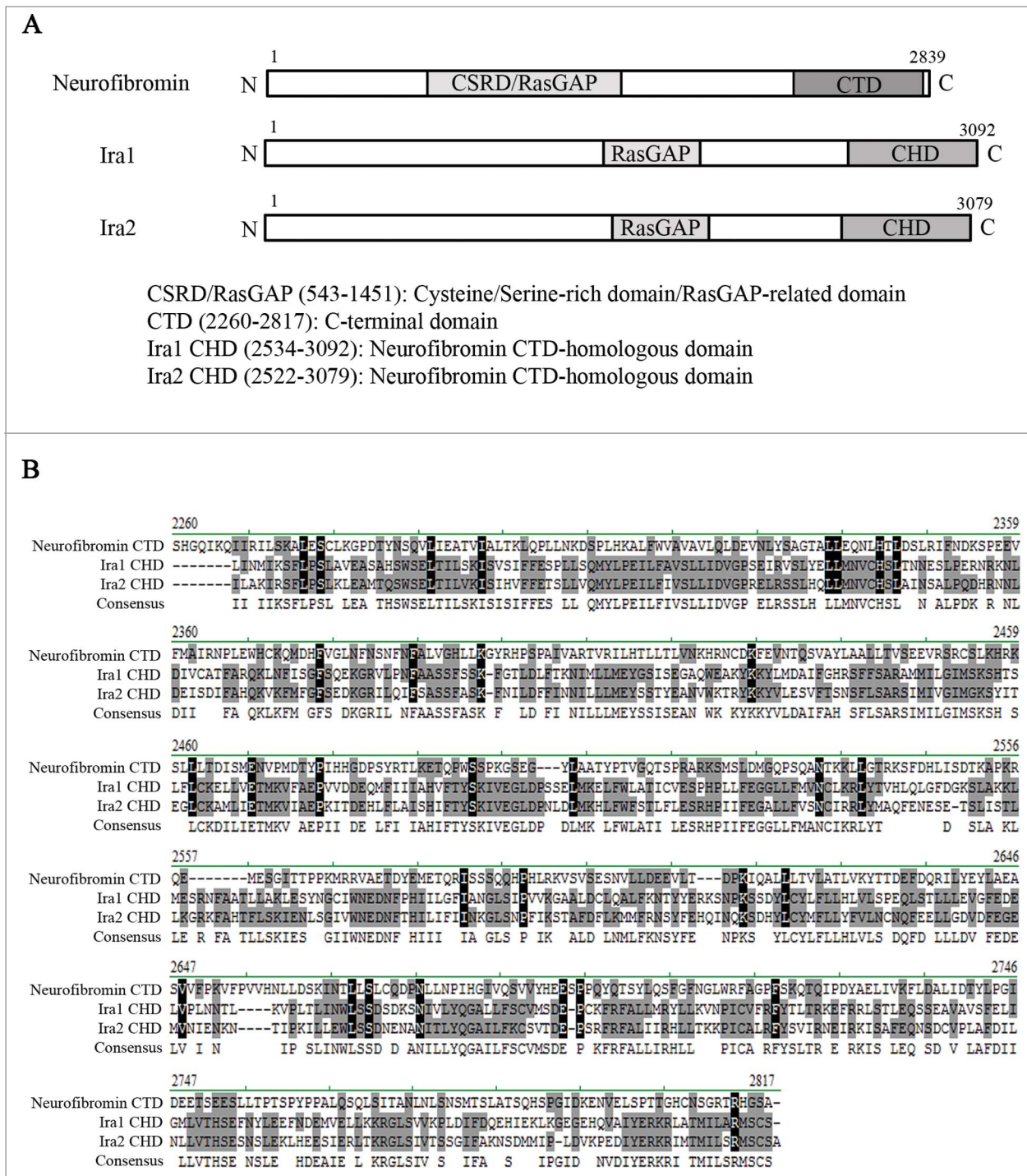


Figure 1. Neurofibromin and its yeast homologs, Ira1 and Ira2. **(A)** Schematic diagram of the full-length neurofibromin, Ira1, and Ira2. The cysteine and serine-rich domain/Ras-GTPase activating protein domain (CSRD/RasGAP) and C-terminal domain (CTD) of neurofibromin are conserved in its yeast homologs Ira1 and Ira2. CSRD/RasGAP is shown in light gray, and the neurofibromin CTD and Ira 1/2 neurofibromin CTD-homologous domain (CHD) in dark gray. **(B)** Alignment of the CTD and CHD amino acid sequences using Vector NTI software (Invitrogen). Identical amino acids are shown in black, conserved amino acids in gray.

chromosomes in the presence of spindle damage in all eukaryotes.¹⁶ 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.¹⁷⁻¹⁹ 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).⁵ 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. $\Delta ira1$ and $\Delta ira2$ 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 *GALI* promoter could rescue the heat-sensitive phenotypes of $\Delta ira1$ and $\Delta ira2$. As shown in Fig. 2B, the induced expression of CSRD/RasGAP by galactose rescued the heat shock

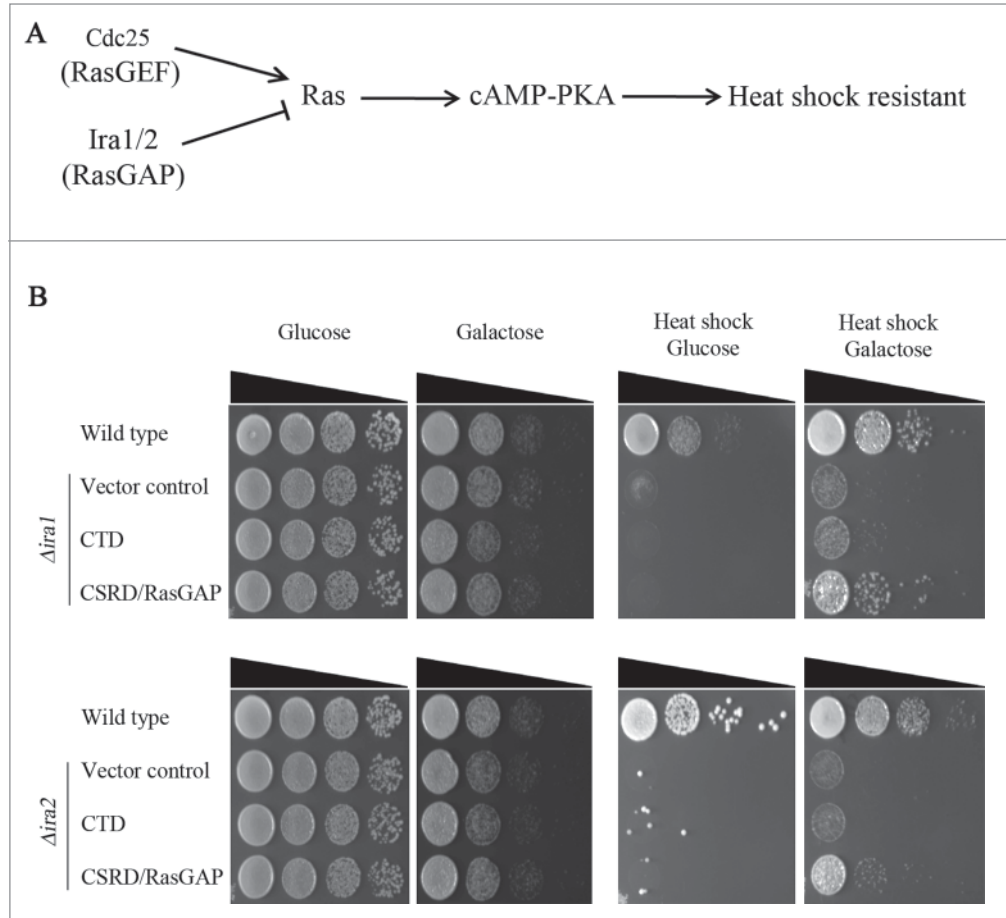


Figure 2. The neurofibromin cysteine and serine-rich domain/Ras-GTPase activating protein domain (CSRD/RasGAP) but not the C-terminal domain (CTD) rescues heat sensitivity in $\Delta ira1$ and $\Delta ira2$ budding yeast cells. (A) The heat shock-sensitive Ras signaling pathway in budding yeast. (B) A complementation assay for heat sensitivity using the neurofibromin CSRD/RasGAP domain and CTD. *pCEN-P_{GALI}-3HA-NF1-CTD*, *pCEN-P_{GALI}-3HA-NF1-CSRD/RasGAP*, and *pCEN-P_{GALI}-3HA* were respectively transformed into $\Delta ira1$ (strain YSK2620) and $\Delta ira2$ (strain YSK2622) cells. *pCEN-P_{GALI}-3HA* was transformed into wild-type (W303a) cells and used as a control. Cells were serially diluted, spotted on plates containing either glucose or galactose, and then incubated at 30°C for 2 days. For heat shock (right panel), cells were incubated at 55°C for 30 min prior to the transfer to 30°C.

sensitivity of $\Delta ira1$ and $\Delta ira2$ cells. In contrast, $\Delta ira1$ and $\Delta ira2$ 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

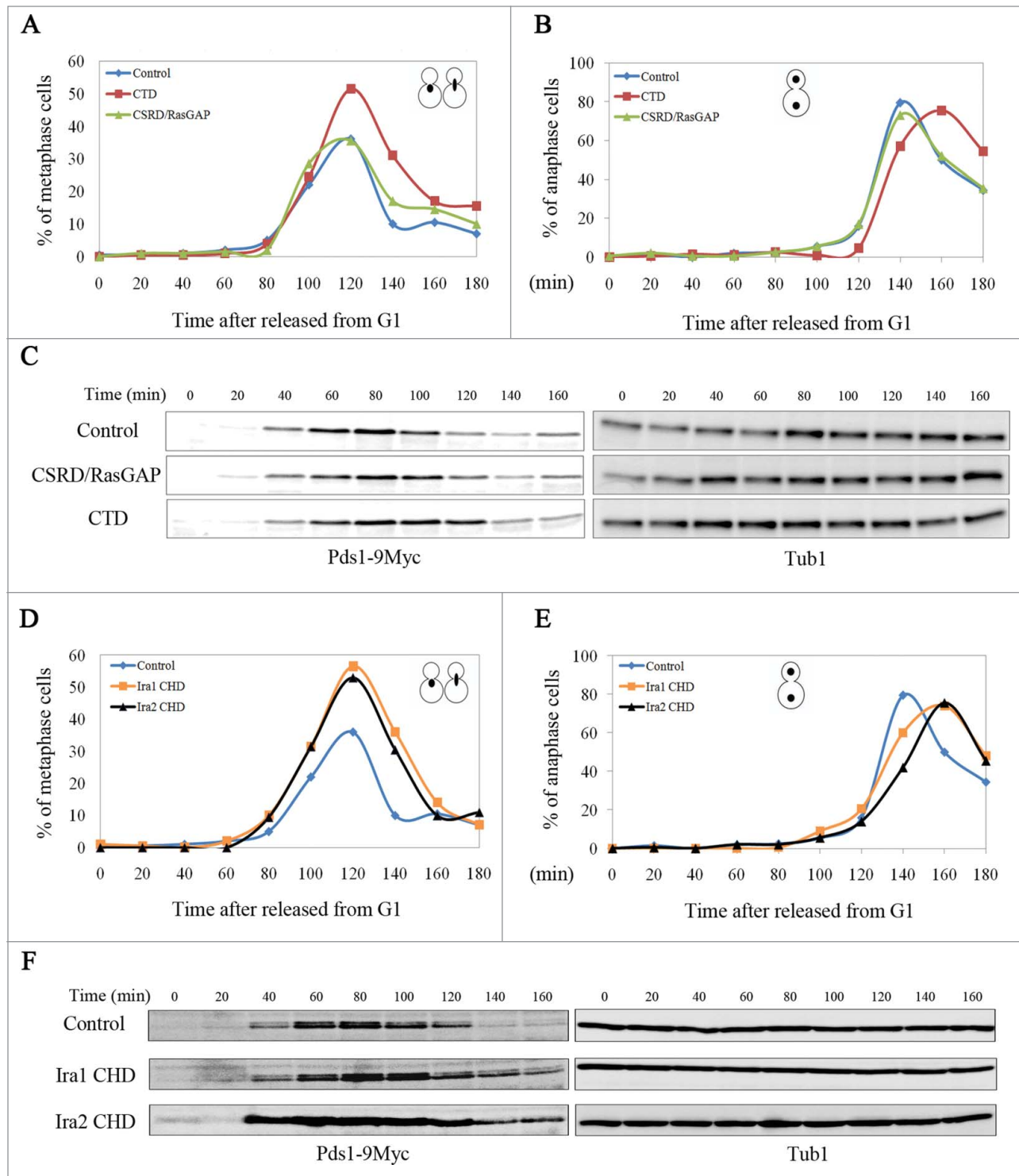


Figure 3. The neurofibromin C-terminal domain (CTD) and the Ira1/2 neurofibromin CTD-homologous domain (CHD) regulate the metaphase to anaphase transition in budding yeast. *pCEN-P_{GAL1}-3HA-NF1-CTD*, *pCEN-P_{GAL1}-3HA-NF1-CSR/D/RasGAP*, *pCEN-P_{GAL1}-3HA-IRA1-CHD*, *pCEN-P_{GAL1}-3HA-IRA2-CHD*, or *pCEN-P_{GAL1}-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 (CSR/D/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, CSR/D/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.^{20,21} 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.¹⁷ Consistent with this report, we found that the viability of $\Delta mad2$ 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 $\Delta ira1$ and $\Delta ira2$ cells was partially decreased in response to benomyl, but not to the same extent as in ($\Delta mad2$ cells. As this result

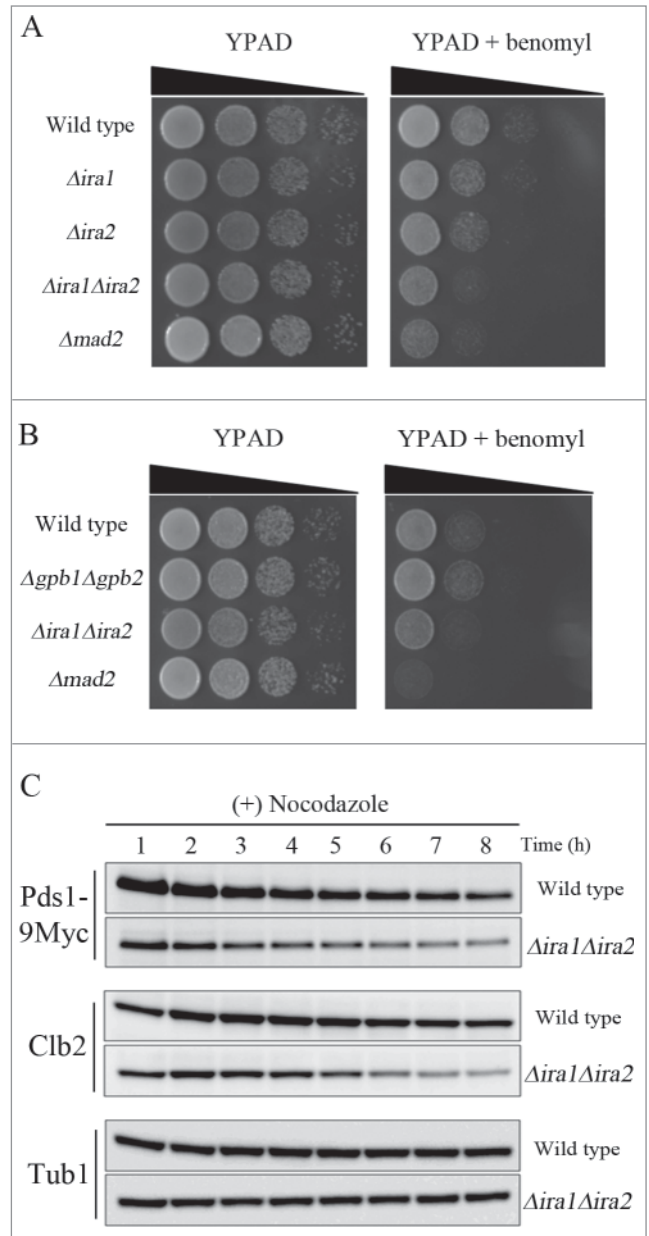


Figure 4. Ira1 and Ira2 are involved in the spindle assembly checkpoint (SAC) pathway. **(A)** $\Delta ira1\Delta ira2$ cells are sensitive to spindle damage. Wild-type, $\Delta ira1$ (strain YSK2620), $\Delta ira2$ (strain YSK2622), $\Delta ira1\Delta ira2$ (strain YSK2866), and $\Delta mad2$ (strain 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 μ g/mL benomyl, and incubated at 25°C. **(B)** $\Delta gpb1\Delta gpb2$ cells are proficient for SAC. Wild-type, $\Delta gpb1\Delta gpb2$ (strain YSK2929), $\Delta ira1\Delta ira2$ (strain YSK2866), and $\Delta mad2$ (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 μ g/mL benomyl and incubated at 25°C. **(C)** $\Delta ira1\Delta ira2$ cells bypassed mitotic arrest in response to spindle damage. Wild-type and $\Delta ira1\Delta ira2$ cells expressing Myc-tagged Pds1 (strains YSK2992 and YSK3001, respectively) were grown to early log phase at 25°C and then treated with 15 μ g/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 $\Delta mad2$ mutant cells, $\Delta ira1\Delta ira2$ 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 $\Delta gpb1\Delta gpb2$ cells in the presence of benomyl (Fig. 4B). The $\Delta gpb1\Delta gpb2$ mutant cells were not as sensitive to benomyl as the $\Delta ira1\Delta ira2$ and $\Delta mad2$ 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 $\Delta ira1\Delta ira2$ 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 $\Delta ira1\Delta ira2$ 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 $\Delta ira1\Delta ira2$ 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 $\Delta ira1\Delta ira2\Delta mad2$ and compared its sensitivity to benomyl with that of the $\Delta ira1\Delta ira2$ and $\Delta mad2$ mutants. Interestingly, $\Delta ira1\Delta ira2\Delta mad2$ cells were more sensitive to benomyl at either 25°C or 30°C than $\Delta ira1\Delta ira2$ and $\Delta mad2$ 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, $\Delta ira1\Delta ira2$, $\Delta mad2$, and $\Delta ira1\Delta ira2\Delta mad2$ cells in response to nocodazole. As shown in Fig. 5B, $\Delta mad2$ cells showed a drop in Pds1 levels at approximately 100–110 min after nocodazole treatment. Importantly, Pds1 degradation was exacerbated in $\Delta ira1\Delta ira2\Delta mad2$ 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 $\Delta ira1\Delta ira2$ cells only begins to degrade approximately 3–4 h after nocodazole treatment (Fig. 4C), the level of Pds1 expression in $\Delta ira1\Delta ira2$ 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

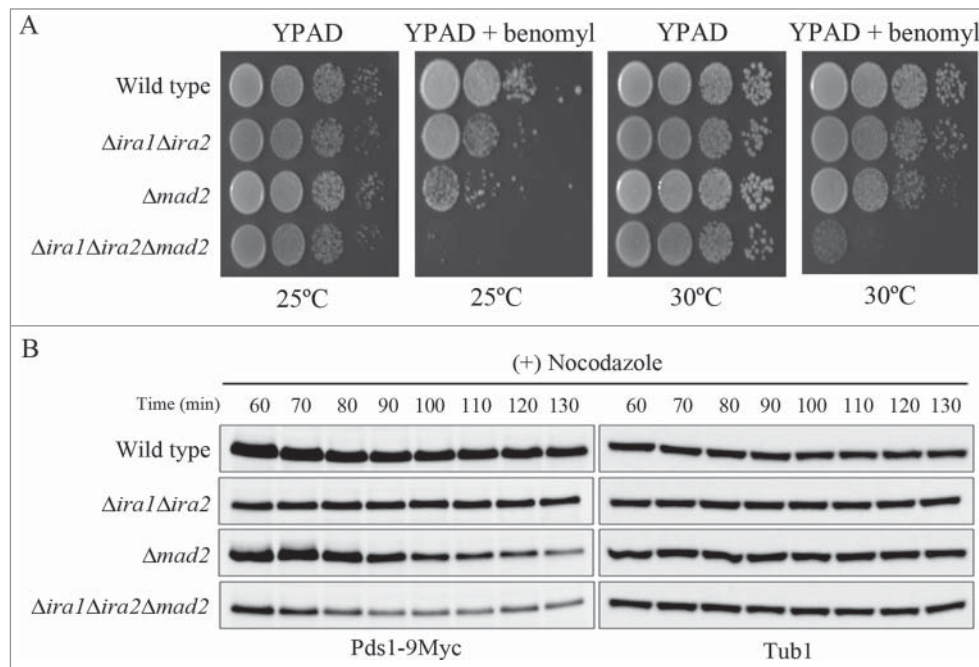


Figure 5. Ira1 and Ira2 function independently of Mad2 in the spindle assembly checkpoint (SAC) pathway. **(A)** $\Delta ira1\Delta ira2\Delta mad2$ was highly sensitive to spindle damage. Wild-type, $\Delta ira1\Delta ira2$ (strain YSK2866), $\Delta mad2$ (strain YSK2668), and $\Delta ira1\Delta ira2\Delta mad2$ (strain YSK2925) cells were grown to mid-log phase, serially diluted 10-fold, spotted onto either YPAD plates or YPAD plates containing 10 μ g/mL benomyl and then incubated at either 25°C or 30°C. **(B)** $\Delta ira1\Delta ira2\Delta mad2$ cells are more sensitive to nocodazole than $\Delta ira1\Delta ira2$ or $\Delta mad2$ cells. Wild-type, $\Delta ira1\Delta ira2$, $\Delta mad2$, and $\Delta ira1\Delta ira2\Delta mad2$ cells expressing Myc-tagged Pds1 (strains YSK2992, YSK3001, YSK2984, and YSK3003, respectively) were grown to early log phase at 25°C and then treated with 15 μ g/mL nocodazole for 130 min. Cells were collected every 10 min from 60 min, and then Pds1 expression (Pds1-9Myc) was detected by western blotting using an anti-Myc antibody. α -Tubulin (Tub1) served as a loading control.

SAC-defective phenotype of $\Delta ira1\Delta ira2\Delta mad2$ 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 $\Delta ira1\Delta ira2\Delta mad2$ 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 $\Delta ira1\Delta ira2\Delta mad2$ 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 $34.8 \pm 6.7\%$ of the CTD-expressing cells, as compared to $43.8 \pm 5.3\%$ of cells expressing the control vector and $41.5 \pm 6.4\%$ of CSRD/RasGAP-expressing cells (Fig. 6B). These results show that CTD functions independently of RasGAP in the metaphase to anaphase transition.

Discussion

NFI is a tumor suppressor, and mutations found in *NFI* 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.

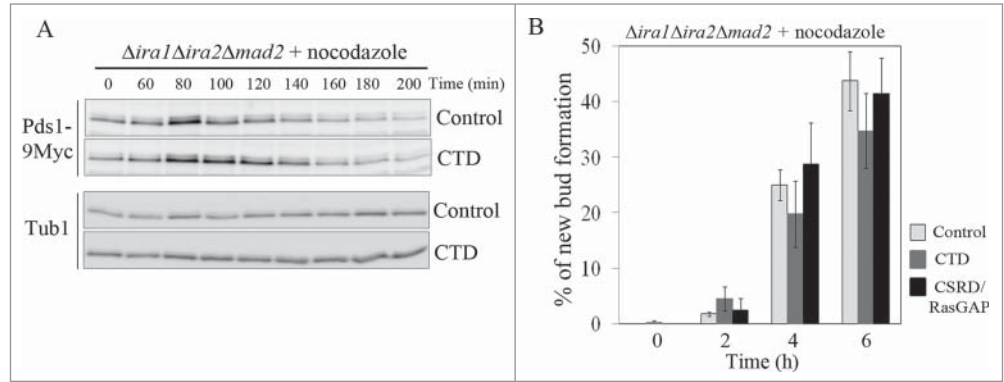


Figure 6. The neurofibromin C-terminal domain (CTD) functions in the spindle assembly checkpoint (SAC) pathway. (A, B) CTD expression partially rescued the SAC-defective phenotype. *pCEN-P_{GAL1}-3HA-NF1-CTD*, *pCEN-P_{GAL1}-3HA-NF1-CSRD/RasGAP*, and *pCEN-P_{GAL1}-3HA* were transformed into $\Delta ira1\Delta ira2\Delta mad2$ cells expressing Myc-tagged Pds1 to produce strain YSK3003. (A) Cells were synchronized at G1 with α -factor (5 μ g/mL), the expression of CTD under the *GAL1* promoter was induced for 1 h, and then the cells were released into galactose medium containing 15 μ g/mL nocodazole at 25°C. After 60 min, cells were collected every 20 min for 200 min. Then, Pds1 expression (Pds1-9Myc) was detected by western blotting using an anti-Myc antibody. α -Tubulin (Tub1) served as a loading control. (B) Cells expressing CTD and the neurofibromin cysteine and serine-rich domain/Ras-GTPase activating protein domain (CSRD/RasGAP) were released from G1 arrest into galactose medium containing 15 μ g/mL nocodazole and collected every 2 h for 6 h. New bud formation was scored at each time point (n = 400). Three independent experiments were performed, and the average was plotted with standard deviations.

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^{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 S52AS88A mutant should have facilitated Pds1 degradation in $\Delta mad2$ cells in response to spindle damage. However, when we overexpressed the phosphorylation-defective Cdc20 S52AS88A mutant in $\Delta mad2$ 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^{Cdc20} in response to DNA damage,^{29,32} we also examined the possibility that Ira1 and Ira2 may promote

Table 1. Yeast strains used in this study

Name	Genotype	Source*
W303a (Y300)	<i>MATa ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-100</i>	Elledge SJ
Σ1278b	<i>MATa ura3-52</i>	Lorenz et al., 1997
YSK2866	Y300 except as <i>Δira1::URA3 Δira2::URA3</i>	Sanchez Y
YSK2202	W303a except as <i>bar1 Δbfa1::HIS3 PDS1-9Myc:hphNT1 SIC1-3HA:Kan^R [pRS304-BFA1-GFP]</i>	This study
YSK2620	W303a except as <i>Δira1::KAN^R</i>	This study
YSK2622	W303a except as <i>Δira2::KAN^R</i>	This study
YSK2668	W303a except as <i>Δmad2::URA3</i>	This study
YSK2929	W303a except as <i>Δgpb1::KAN^RΔgpb2::HIS3</i>	This study
YSK2992	W303a except as <i>PDS1-9Myc:hphNT1</i>	This study
YSK3001	Y300 except as <i>Δira1::URA3 Δira2::URA3 PDS1-9Myc:hphNT1</i>	This study
YSK2925	Y300 except as <i>Δira1::URA3 Δira2::URA3 Δmad2::KAN^R</i>	This study
YSK2984	W303a except as <i>Δmad2::URA3PDS1-9Myc:hphNT1</i>	This study
YSK3003	Y300 except as <i>Δira1::URA3 Δira2::URA3 Δmad2::KAN^R PDS1-9Myc:hphNT1</i>	This study
YSK2949	Σ1278b except as <i>Δleu2::hisG IRA1-3HA:G418</i>	Heitman J
YSK3103	Σ1278b except as <i>Δleu2::hisG IRA1-3HA:G418 PDS1-9Myc:hphNT1</i>	This study

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^{Cdc20}-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 CSRD/RasGAP, CHD, Cdc20, and Cdc20 S52AS88A were subcloned into pCEN-*P_{GAL1}-3HA*. The expression of CSRD/RasGAP, 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 AxioVision 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 MgSO₄, 50 mM NaF, 100 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM dithiothreitol,

Table 2. Plasmids used in this study

Plasmids	Source*
pCR2.1-TOPO carrying CSRD/GRD	Mangoura D
pEGFP-C1 carrying CTD	Mangoura D
pFA6a-His3MX6- <i>P_{GAL1}-3HA</i>	Pringle JR
pRS315- <i>P_{GAL1}-3HA</i>	This study
pRS315- <i>P_{GAL1}-3HA-NF1 CTD</i>	This study
pRS315- <i>P_{GAL1}-3HA-NF1 CSRD/RasGAP</i>	This study
pRS315- <i>P_{GAL1}-3HA-IRA1 CHD</i>	This study
pRS315- <i>P_{GAL1}-3HA-IRA2 CHD</i>	This study
pMW034	Sanchez Y
pMW036	Sanchez Y
pRS315- <i>P_{GAL1}-3HA-CDC20</i>	This study
pRS315- <i>P_{GAL1}-3HA-CDC20 S52A S88A</i>	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 A agarose (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-Clb2 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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