

Ydj1 Protects Nascent Protein Kinases from Degradation and Controls the Rate of Their Maturation[∇]

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Ydj1 is a *Saccharomyces cerevisiae* Hsp40 molecular chaperone that functions with Hsp70 to promote polypeptide folding. We identified Ydj1 as being important for maintaining steady-state levels of protein kinases after screening several chaperones and cochaperones in gene deletion mutant strains. Pulse-chase analyses revealed that a portion of Tpk2 kinase was degraded shortly after synthesis in a *ydj1Δ* mutant, while the remainder was capable of maturing but with reduced kinetics compared to the wild type. Cdc28 maturation was also delayed in the *ydj1Δ* mutant strain. Ydj1 protects nascent kinases in different contexts, such as when Hsp90 is inhibited with geldanamycin or when *CDC37* is mutated. The protective function of Ydj1 is due partly to its intrinsic chaperone function, but this is minor compared to the protective effect resulting from its interaction with Hsp70. *SIS1*, a type II Hsp40, was unable to suppress defects in kinase accumulation in the *ydj1Δ* mutant, suggesting some specificity in Ydj1 chaperone action. However, analysis of chimeric proteins that contained the chaperone modules of Ydj1 or Sis1 indicated that Ydj1 promotes kinase accumulation independently of its client-binding specificity. Our results suggest that Ydj1 can both protect nascent chains against degradation and control the rate of kinase maturation.

Molecular chaperones prevent polypeptides from misfolding and promote acquisition of the folded state. In their absence, nascent polypeptides or proteins that become unfolded aggregate or become targeted for degradation via the ubiquitin proteasome pathway (22). Hsp70 is a molecular chaperone that functions early in protein folding process by interacting with nascent polypeptide chains cotranslationally or immediately after translation. Hsp70 functions in association with cochaperones that facilitate the cycle of nascent chain binding and release. Cochaperones that promote polypeptide loading are members of the Hsp40 family (12, 15). Those that stimulate polypeptide dissociation function in nucleotide exchange and belong to several structurally distinct protein families (1, 25, 28, 46).

Hsp40 chaperones comprise a large and diverse protein family (12, 15). Each member has a conserved “J” domain that functions in stimulating Hsp70’s ATPase. Members of type I and type II Hsp40s also interact with unfolded proteins and help to load them onto Hsp70. Once a polypeptide is bound to Hsp70, the Hsp40 J domain stimulates Hsp70’s ATPase, which helps stabilize the interaction by leading to closure of a helical lid structure over the polypeptide binding site. Binding of unfolded polypeptides to Hsp70 or Hsp40s is via hydrophobic interactions, and this prevents polypeptide aggregation (18). Polypeptide release is catalyzed by nucleotide exchange factors that facilitate release of ADP from Hsp70, allowing for ATP binding and opening of the lid retaining the polypeptide in the chaperone client-binding site. Cycles of binding and release,

catalyzed by Hsp40 and nucleotide exchange factors, can promote folding and prevent aggregation.

In addition to preventing aggregation, chaperones also function in the degradation of misfolded proteins by the proteasome. Inhibition of Hsp90, for example, leads to ubiquitination and degradation of many protein kinases and several nuclear receptors (52). Hsp90 is required in yeast for degradation of heterologously expressed proteins that are not folded by this chaperone, suggesting that it has distinct roles in both folding and degradation (38). In mammalian cells, chaperones promote degradation via interaction with the E3 ubiquitin ligase, Chip, which binds to both Hsp70 and Hsp90 via tetratricopeptide motifs (14, 39). Chip integrates the quality control process by regulating heat shock factor-dependent gene expression and also acting itself as a chaperone (16, 44).

The mechanisms underlying chaperone and cochaperone function in targeting polypeptides toward the proteasome are not well understood, although a common factor is Hsp70. Several genetic and biochemical studies have shown that Hsp70 is important for the degradation of misfolded cytosolic proteins (4, 38, 40). Hsp40s cooperate with Hsp70 in this process, based on the finding that *YDJ1* and *HLJ1* promote the degradation of cytosolic and membrane bound polypeptides (30, 40, 54). The results from biochemical studies support the view that Hsp40s promote degradation. For example, endoplasmic reticulum-associated degradation of misfolded membrane proteins is promoted by Hdj2, a Ydj1 ortholog (57). A more direct targeting function has been discovered for a neuronal Hsp40, Hsj1, which interacts directly with polyubiquitin chains via a ubiquitin interaction motif, and promotes ubiquitinylation of polypeptides in association with Chip (51).

Protein kinases represent a class of protein whose folding is dependent on the Hsp90 and Cdc37 in addition to the Hsp70/Hsp40 chaperone pair (9). Using in vitro chaperone-kinase

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assembly reactions, Arlander et al. demonstrated that chaperones bind to a misfolded kinase in a sequential manner, beginning with Hsp70/Hsp40 (2). The interaction of these chaperones with the misfolded kinase was required for subsequent Cdc37 binding, which led to Hsp90 recruitment. This final step is also promoted by the Hsp organizing protein called Hop, which acts as bridge between Hsp70 and Hsp90 (2, 31, 32). Cdc37 interacts primarily with the kinase N-lobe, while Hsp90 interacts with both N-lobe and C-lobe of a kinase catalytic subunit (42, 59). Complementary studies using yeast genetics identified similar chaperone components for kinase folding (17, 29, 31, 32) and showed that Cdc37 has a general role in kinome biogenesis in addition to protecting nascent kinase chains from degradation during or shortly after translation (36). In addition to the folding of nascent kinases, molecular chaperones also function to maintain the mature state of mutant kinases, such as oncogenic forms of Src or Lck (26, 53).

In the present study, we screened for chaperones that affected kinase steady-state levels in a similar manner to Cdc37. Ydj1, a yeast type I Hsp40 chaperone was identified as having such a role. We demonstrate here that Ydj1 protects a cohort of kinases similar to that protected by Cdc37. Our findings support the hypothesis that Ydj1 protects nascent chains during or immediately after synthesis and contrasts with other studies showing that this Hsp40 promotes degradation. In addition, we show that newly synthesized protein kinases that escape triage in *ydj1Δ* cells are largely protected from further degradation but fold slowly for a period of up to 2 h.

MATERIALS AND METHODS

Strains and plasmids. In the present study we used yeast strains from the TAP-tagged library (Open Biosystems, Huntsville, AL) based on strain S288C (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (24). The strain genotype was verified by PCR analysis using primers specific to the tag and kinase gene of interest. The yeast *ydj1Δ* and *erg6Δ* knockout strains were constructed by introducing KanMX4 module as described earlier (36). In the *ydj1* and *erg6* double mutant, *YDJI* was replaced by *URA3* and *ERG6* replaced by KanMX4.

The H34Q mutant was prepared from pRS315-Ydj1. Amino acid substitution was performed by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutation was confirmed by DNA sequencing. pGAL-Ydj1 was constructed by excising a 1.3-kb EcoRI fragment from pAV2 (8) and ligating into the low-copy-number vector pSEYc58GalP containing the GAL1-10 promoter. Plasmids expressing *YDJI*, *SIS1*, *YSY*, and *SYS* in pRS315 were kindly provided by Doug Cyr (University of North Carolina, Chapel Hill). pRS317-*ydj1ΔG/F* was a gift from E. Craig (University of Wisconsin, Madison). Ydj1ΔG/F was subsequently subcloned into pRS315 by using NotI and XmaI. The plasmid expressing *Escherichia coli* dnaJ was described previously (7).

Solubility assay. Cells expressing TAP-tagged kinases were grown at 30°C, harvested, washed once with sterile water, and resuspended in 500 μl of 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mg of pepstatin A/ml. Cells were lysed by bead beating and clarified by centrifugation at 16,000 × g for 10 min at 4°C. A portion (150 μl) of this supernatant was kept aside as total protein, and another 150 μl was spun in a Beckman TLA45 rotor at 100,000 × g for 30 min at 4°C. The supernatant was removed and designated as the soluble fraction. The pellet was washed once with the buffer described above and solubilized with 200 μl of 1× sodium dodecyl sulfate (SDS) sample buffer. Then, 50 μl of 4× SDS sample buffer was added to the total-protein and soluble-fraction samples. Equal amounts of each fraction were analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis with anti-TAP antibody (Open Biosystems).

Western blot analysis. Western blot analysis was performed on whole-cell extracts prepared in IPP150 (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% NP-40) or in 50 mM Tris (pH 7.5)–0.1 mM EDTA–1% SDS plus Complete protease inhibitor (Boehringer Mannheim) by glass bead lysis. The steady-state kinase level was quantified by Odyssey Li-Cor image processing software. Galactose induction experiments shown in Fig. 5 were conducted by growing cul-

tures in raffinose (2%) overnight. Cultures were diluted to an A_{600} of 0.2 and supplemented with either galactose or glucose (2% [vol/vol]), followed by incubation for 8 h with shaking at 30°C before extract preparation as described above.

Polyclonal antisera to Hsp90, Ydj1, and Sis1 were described previously (8, 35, 43). Hsp70 mouse monoclonal antisera was purchased from Stressgen (spa-822). Antisera to *E. coli* dnaJ was kindly provided by Ulrich Hartl. Anti-TAP was purchased from Open Biosystems.

Pulse-labeling. Pulse-labeling and immunoprecipitations were performed as described previously (5).

Kinase assays. Kinase assay of wild-type and *ydj1Δ* cells expressing TAP-tagged forms of Tpk2 and Rim11 were performed as described previously (36). Briefly, wild-type and *ydj1Δ* strains were grown overnight at 30°C in 100 ml of synthetic complete medium (minus histidine) to log phase. Cell extracts were prepared in IPP150 buffer, and the amount of protein was quantified by using Bradford reagent. Tpk2 was immunoprecipitated from 0.1 to 0.5 mg of extract from wild-type cells and from 3 mg of extracts from *ydj1Δ* mutant cells. The cell extracts were diluted to 0.45 ml in IPP150 and incubated with 50 μl of 50% (vol/vol) immunoglobulin G-Sepharose resin (GE) for 1 h at 4°C. The resin was washed and divided into two aliquots. One aliquot was used for the kinase assay, while the other was used for Western blot analysis with anti-TAP. The Western blots were quantified by using a Li-Cor Odyssey infrared detection system. Kinase assays for Tpk2 were performed by using a Pep-Tag nonradioactive protein kinase assay kit and quantified by spectrophotometry of agarose gel slices as described by the manufacturer (Promega), while Rim11 was assayed by measuring its ability to phosphorylate myelin basic protein (Upstate Biotechnology) and quantified by phosphorimaging.

RESULTS

Ydj1 influences the steady-state levels of protein kinases.

We searched for molecular chaperones and cochaperones besides Cdc37 that could affect the levels of yeast protein kinases, starting with C-terminal TAP tagged Tpk2, a homologue of mammalian cAMP-dependent protein kinase (PKA). The chaperones or cochaperones *AHA1*, *CPR6*, *CPR7*, *HCH1*, *SBA1*, *SSE1*, *STI1*, and *YDJI* were deleted from strains expressing a TAP-TPK2. These genes encode proteins that modulate Hsp90's ATPase activity (e.g., Aha1, Hch1, Sti1, and Sba1), act as chaperones (Cpr6, Cpr7, Sba1, Sse1 and Ydj1), and/or have peptidyl prolyl isomerase activity (Cpr6 and Cpr7 [37, 41]).

Tpk2 levels were assessed by Western blotting in comparison to a wild-type strain, using Pgk1 as a loading control. The results demonstrated that only upon deletion of the Hsp40 chaperone *YDJI* were Tpk2 levels reproducibly lowered compared to the wild type (Fig. 1A and data not shown). Reduction in kinase levels was associated with increased amounts of Hsp90 and Hsp70, although Pgk1 levels were unaffected by any of the chaperone deletions. Similar reductions in kinase levels and increases in Hsp90/Hsp70 were observed in a *ydj1Δ* strain expressing TAP-Rim11 (Fig. 1B). These increases suggest that deletion of *YDJI* promotes a stress response in these cells.

In a previous study we analyzed 65 different kinases for stability in a yeast strain mutated for *CDC37*, a chaperone essential for protein kinase folding (36). Among the 65 kinases tested, 51 had reduced levels compared to those present in the wild-type strain. We therefore tested a sampling of kinases that were unstable or stable in the *cdc37* mutant for a similar phenotype in the *ydj1Δ* mutant. For these experiments we analyzed Cdc28, Cmk1, Fus3, Pho85, and Slt2 as examples of kinases that were unstable in the *cdc37* mutant, and Cmk2 and Snf1 as kinases that were stable. Prs5, a component of ribosylphosphotransferase, was added to this analysis as an example of a kinase-related protein that was unaffected by mutation

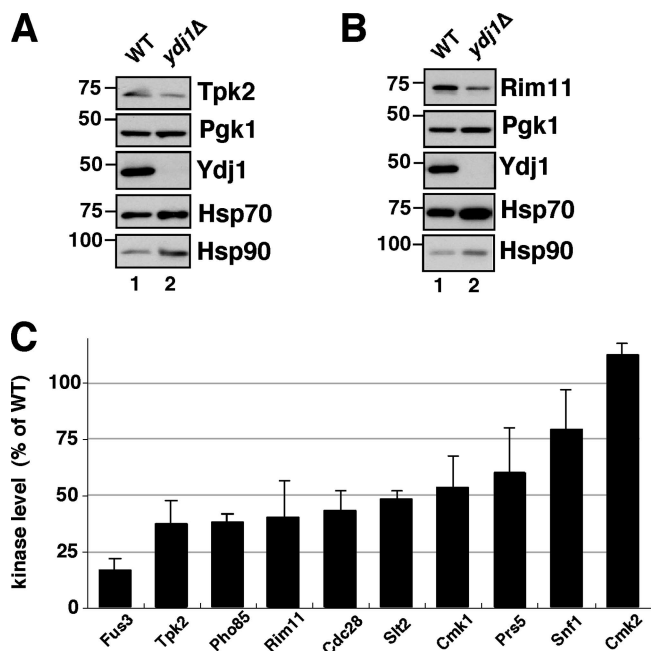


FIG. 1. Effect of *YDJ1* deletion on protein kinase levels. (A) Steady-state level of Tpk2 in wild-type and *ydj1* Δ yeast cells; (B) Rim11 in wild-type and *ydj1* Δ cells. Wild-type and *ydj1* Δ yeast cells were grown overnight at 30°C and kinases identified with anti-TAP. Western blots of the same samples were performed with anti-Pgk1, anti-Ydj1, anti-Hsp70, and anti-Hsp90. Pgk1 levels are shown as a loading control. (C) Steady-state levels of kinases in *ydj1* Δ strain. The results are expressed as the percentages of levels in wild-type cells in each case based on Western blot analysis. The bars indicate the standard deviation from three to four independent experiments. WT, wild type.

in *CDC37*. In accord with results from the *cdc37* mutant, we observed that Cdc28, Cmk1, Fus3, Pho85, and Slr2 had reduced levels in the *ydj1* Δ mutant (Fig. 1C). The mean reduction was \sim 2-fold, but Fus3 levels were reduced the most at 6-fold compared to the wild type. Strikingly, kinases that were stable in the *cdc37* mutant, Cmk2, and Snf1 displayed the least dependence on Ydj1 for their steady-state levels. Cmk2 steady-state levels were completely unaffected by the loss of Ydj1. Prs5, which was unaffected by mutation in *CDC37*, was reduced in the *ydj1* Δ mutant.

The fate of residual Tpk2 kinase that was synthesized and stable in a *ydj1* Δ mutant was addressed in two ways. First, we investigated whether the kinase accumulated in a soluble form or whether it aggregated. Lysates containing Tpk2 synthesized in the presence or absence of Ydj1 protein were centrifuged at $100,000 \times g$ for 1 h. A small enrichment of Tpk2 was observed in the pellet fraction in the *ydj1* Δ mutant, although the vast majority of the kinase was soluble. Next, we determined the specific activity of Tpk2 synthesized in the *ydj1* Δ mutant. Since there was less Tpk2 at steady state in the *ydj1* Δ mutant compared to the wild type, we first generated a standard curve of Tpk2 activity from the wild-type strain (Fig. 2B). The specific activity of a single concentration of Tpk2 isolated from the *ydj1* Δ mutant was then determined by comparison with the standard curve. As shown in Fig. 2B, the specific activity of Tpk2 in both strains was similar, although slightly reduced in

the *ydj1* Δ mutant. Therefore, Tpk2 is capable of acquiring the native state in the absence of Ydj1, but in reduced amounts. Similar findings were recorded for Rim11, which also has a very similar specific activity when derived from wild-type or *ydj1* Δ mutant strains, although its steady-state levels are reduced in the absence of Ydj1 (Fig. 1 and 2C).

Ydj1 affects the rate of protein kinase maturation. Previous studies showed that Cdc37 protects newly made protein kinases from degradation either during or shortly after translation (36). Since Ydj1 functions upstream of Cdc37 in in vitro kinase-chaperone assembly reactions (2), it seemed likely that Ydj1 might behave in similar manner, especially given that both chaperones interact with the same cohort of protein kinases.

We addressed the role of Ydj1 in biogenesis of newly translated Tpk2 and Cdc28 by pulse-chase analysis. For Tpk2, pulse-chase experiments revealed the presence of two distinct bands after denaturing gel electrophoresis (Fig. 3A). Based on previous studies with mammalian PKA, these represent immature (inactive, nonphosphorylated; faster migrating) and mature (active, autophosphorylated; slower migrating) conformers (56). This was verified with phosphatase treatment of ^{35}S -labeled wild-type cell extracts, which resulted in partial conversion of Tpk2 from the slow-migrating form to the fast-migrating form (PKA is relatively resistant to phosphatase treatment) (47). Mammalian PKA autophosphorylates in cells at two sites: Ser197 and Ser338 (27). The former is in the activation loop and is necessary for enzyme activity, while the latter facilitates stabilization of the kinase N-lobe (3, 56). It is likely, therefore, that the immature form of the kinase is an unstable conformer.

In the wild-type strain, the faster-migrating form of Tpk2 is a minor component after a 10-min pulse-labeling that disappears after 30 min of chase. In the *ydj1* Δ mutant, there is a lower level of Tpk2 after the pulse, a finding consistent with decreased levels found at steady state (Fig. 3A). Also, the faster-migrating band is more prominent at zero time of chase and appears to convert into the slower-migrating band over the 2-h chase time. This interpretation is based on the apparent decrease in faster-migrating band intensity and an increase in the amount of the slower-migrating band after 30 min of chase. The faster-migrating band then slowly disappears over the next 90 min (Fig. 3A and C). The relationship between the two forms of Tpk2 was investigated further in wild-type cells using short (2 min) pulse-labeling and chase reactions (Fig. 3B). Under these conditions, most Tpk2 was in the faster-migrating form after the short pulse that converts to the slower-migrating mature form completely within 10 min. In *ydj1* Δ cells this conversion takes 60 to 120 min (Fig. 3A and C).

When the pulse-chase reaction was performed in the *ydj1* Δ strain with the proteasome inhibitor MG132 (Fig. 3C), we noted increased Tpk2 levels after the pulse compared to the dimethyl sulfoxide-treated sample. This suggests that some nascent Tpk2 is targeted for degradation rapidly after translation in a manner similar to that of the quality control we observed in a *cdc37* mutant strain. In addition, the ratio of immature to mature Tpk2 was clearly altered in the two samples at 30 min of chase. In the MG132-treated sample, there were increased amounts of immature kinase, suggesting that some of the nascent Tpk2 was targeted for degradation in

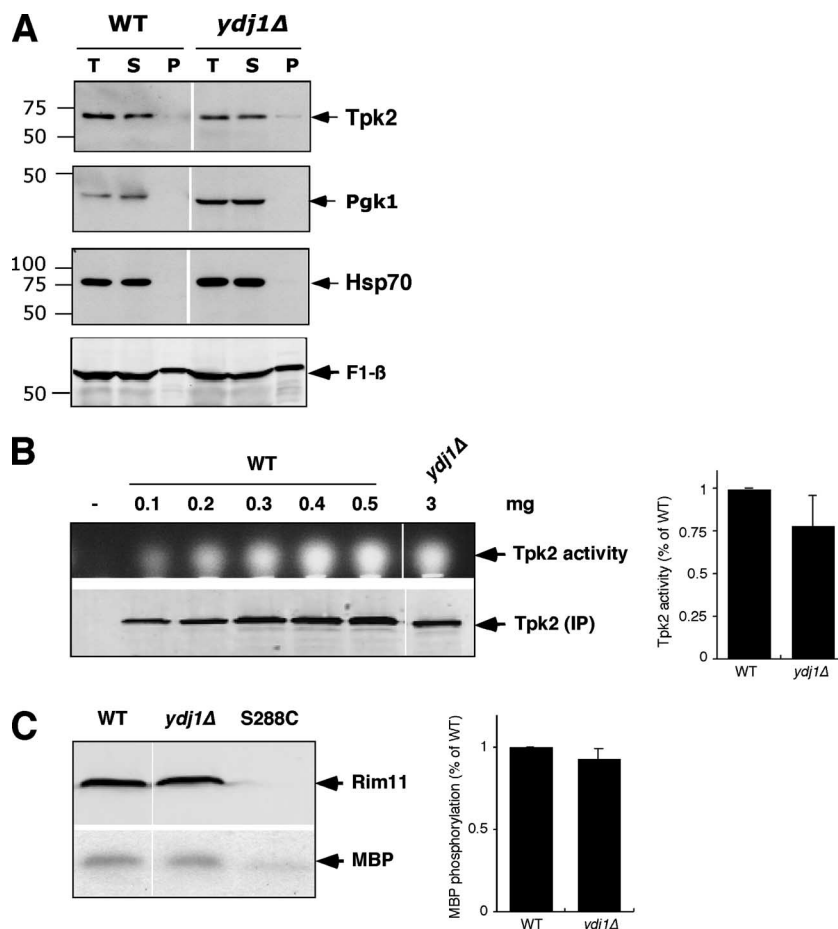


FIG. 2. Solubility and activity of protein kinases in *ydj1Δ* mutant strains. (A) Solubility assay of Tpk2 kinase. The solubility of Tpk2 was assayed in wild-type and *ydj1Δ* cells by comparing equal amounts of total lysate (T), supernatant (S), and pellet (P) fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. The same blot was analyzed with anti-Hsp70, anti-Pgk1, and anti-Atp2, which encodes the β subunit of the mitochondrial F1 ATPase. (B) Tpk2 activity in wild-type and *ydj1Δ* mutant strains. The upper panel shows the Tpk2 activity after immunoprecipitation of the kinase from various amounts of wild-type extract (expressed in milligrams) and from a 3-mg portion of extracts from *ydj1Δ* cells. The panel shows the fluorescence from a labeled peptide whose migration in an agarose gel depends on phosphorylation by immunoprecipitated Tpk2. The lower panel shows the amount of Tpk2 in each reaction as determined by Western blotting. The bar graph at the right shows the quantitation of Tpk2 activity after normalization to the amount of enzyme in the assay. Bars indicate the standard error of the mean values of three independent experiments. (C) Assay of Rim11 activity. The upper panel shows Western blot results of TAP-Rim11 immunoprecipitates and the amount of 32 P-labeled myelin basic protein (MBP) used in the assay. The panel on the right shows the MBP data quantified. Bars represent the standard error ($n = 3$). WT, wild type.

absence of Ydj1. However, it is also clear that a large proportion of the immature kinase managed to adopt the mature state after 120 min of chase, even in the presence of MG132. This shows that, in the absence of Ydj1, the nascent Tpk2 fate is in the balance, with some being susceptible to degradation while most can adopt the mature state.

A similar function for Ydj1 in affecting the rate of protein kinase maturation was characterized for Cdc28 (Fig. 4). In this case, there were similar levels of kinase produced after pulse-labeling in both wild-type and *ydj1Δ* mutant strains. However, Cdc28 in the *ydj1Δ* mutant had a slower migration at zero time of pulse and quantitatively converted into a faster migrating band after 30 min. In wild-type cells, Cdc28 always appears as the faster-migrating band, even after pulse-labeling. Based on previous studies with Cdc28 (20, 23) and mammalian Cdk (6), the slower-migrating band is the immature, nonphosphorylated form, whereas the faster-migrating band results from

phosphorylation with the Cdk activating kinase, Cak1. Note that the immature and mature forms of Cdc28 are the inverse of those observed for Tpk2. Importantly, Ydj1 does not appear to affect the levels of nascent Cdc28 after pulse-labeling as dramatically as does mutation in *CDC37* (compare Fig. 4A to Fig. 6A), only the rate with which it is converted from the immature form to the mature form. However, the kinase does appear to be degraded to some extent over the 2 h of chase, a finding consistent with the decrease we observed under steady-state conditions. These studies were conducted with nontagged Cdc28, but similar findings were made with TAP-Cdc28 (Fig. 4C). These combined studies show that Ydj1 functions to protect nascent kinases from degradation and also functions to promote efficient folding and/or maturation. The effect of Ydj1 on the rate of maturation is remarkable. In wild-type cells both Cdc28 and Tpk2 are almost quantitatively in the mature state within the 10-min pulse-labeling period. In the absence of

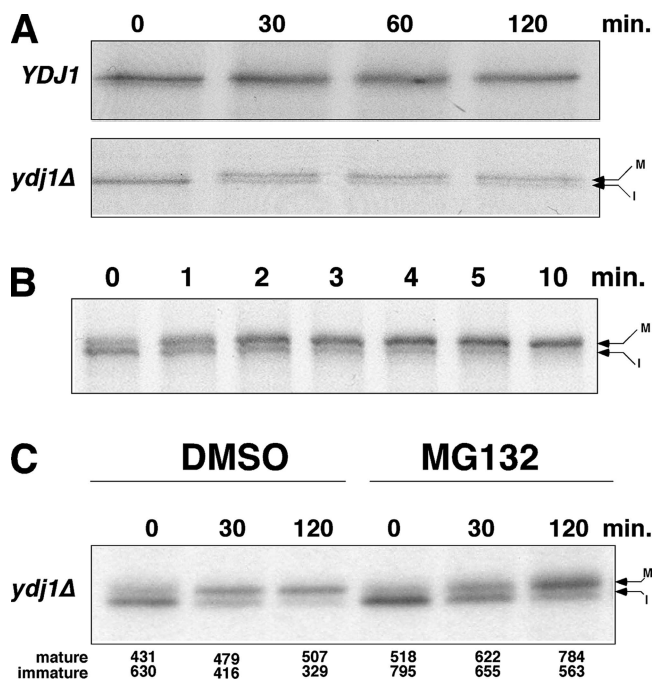


FIG. 3. Pulse-chase analysis of Tpk2 kinase in the wild type and the *ydj1*Δ mutant. (A) Pulse-chase analysis of TAP-tagged Tpk2 in wild-type (*YDJ1*) and *ydj1*Δ mutant cells. The chase times are indicated in minutes. (B) Pulse-chase analysis of wild-type cells after a 2-min pulse and chase from 0 to 10 min. (C) Pulse-chase analysis of TAP-Tpk2 in *ydj1*Δ cells. Cells were treated with dimethyl sulfoxide or 100 μM MG132 for 30 min before labeling. The strain background is *erg6*Δ, which improves MG132 permeability. The chase times are indicated in minutes. Mature (M, phosphorylated) and immature (I, nonphosphorylated) forms of Tpk2 are denoted by arrows. The relative amounts of mature and immature kinase in each lane were quantified, and the numbers appear below the panel.

Ydj1, it takes up to 2 h for Tpk2 to become fully mature, while for Cdc28 the fully mature state was generated within 30 min.

Ydj1 protects nascent kinases against degradation promoted by Hsp90 inhibition or mutation in *CDC37*. Previous analyses of Ydj1 suggested that it promotes polypeptide degradation (30, 40, 54), whereas in our case it appears to protect newly synthesized kinases from this same fate. We therefore addressed the extent of Ydj1's protective function in nascent protein kinase quality control using other models that enhance degradation. The first model involved use of the Hsp90 inhibitor, geldanamycin (GA), which promotes degradation of nascent kinases in yeast (9, 36). For Slt2 kinase, we observed that GA treatment resulted in a modest decline in kinase levels over a 2-h period (Fig. 5A and B). In contrast, Slt2 levels were reduced by ca. 80% in the presence of GA in *ydj1*Δ cells. These findings show that Ydj1 protects Slt2 from degradation when Hsp90 is inhibited. Tpk2, in contrast, is degraded in the presence of GA in wild-type cells or *ydj1*Δ mutant cells to a similar extent (compare Fig. 5C with the glucose panel in Fig. 5D).

Previous studies have noted differential degradation of kinases in GA-treated cells derived from human tumors and healthy tissues (11, 48, 58), but the underlying basis for this has never been fully established. One possibility is that chaperones controlling kinase degradation could be modulated. To test

this, we extended the findings presented above by analyzing whether *YDJ1* overexpression had any further capability to protect nascent kinases from degradation in the presence of GA. *YDJ1* was inducibly overexpressed from a galactose-regulated promoter in wild-type cells for these experiments (Fig. 5D and E). In the presence of glucose, which repressed *YDJ1* overexpression, GA promotes the degradation of Tpk2 over a 2-h period as described previously (36). However, this effect was suppressed by *YDJ1* overexpression (Fig. 5D, middle panel). This effect was not due to galactose replacing glucose in the medium since galactose by itself did not influence the degradation of Tpk2 in the presence of GA (Fig. 5D, lower panel). A similar finding was made for Slt2 kinase (Fig. 5F). Ydj1 is therefore not saturating for its capacity to protect nascent protein kinases from degradation in the presence of GA. For Tpk2, the protective action for Ydj1 in the presence of GA appears to be a gain of function since deletion of the chaperone did not influence GA-dependent kinase degradation (Fig. 5C and D).

The studies described above place Ydj1 in a similar role to Cdc37 as a protector of nascent kinases. We next explored whether these roles were redundant with each other. If this were so, then overexpression of *YDJ1* might suppress degradation occurring due to mutation in *CDC37*. Our model for these studies utilized degradation of Cdc28 in the *cdc37*^{S14A} mutant. As shown in Fig. 6A, Cdc28 is degraded rapidly in the *cdc37*^{S14A} mutant, as described previously (36). In contrast, *YDJ1* overexpression from a multicopy plasmid results in a reduced rate of degradation of Cdc28 in the *cdc37*^{S14A} mutant (Fig. 6A and the quantification shown in Fig. 6B). Importantly, a similar finding was observed when the *ydj1*^{H34Q} mutant was similarly overexpressed in the *cdc37*^{S14A} mutant (Fig. 6A and B). The *ydj1*^{H34Q} mutant contains a point mutation in the J domain that inhibits interaction of Ydj1 with Hsp70 (49). The ability of *ydj1*^{H34Q} to phenocopy wild-type Ydj1 suggests a direct role for Ydj1 in chaperoning nascent kinases that is independent of Hsp70. In contrast, overexpression of either the *YDJ1* or the *ydj1*^{H34Q} mutant in *cdc37*^{S14A} only delays the degradation of Cdc28, it does not completely prevent it. This is revealed by the failure of Cdc28 to achieve the mature state when *YDJ1* is overexpressed in *cdc37*^{S14A} in the pulse-chase analysis (Fig. 6A and data not shown). Furthermore, Western blot analysis of Cdc28 shows that its levels are only marginally higher in the *cdc37*^{S14A} mutant overexpressing *YDJ1* (Fig. 6C). These findings suggest that Ydj1 has overlapping functions with Cdc37 in stabilizing nascent kinases. The overlapping function is manifest in the way Ydj1 delays Cdc28 degradation in the *cdc37*^{S14A} mutant, although it clearly fails to promote maturation. This is probably related to Cdc37 chaperone function directly or indirectly in recruiting Hsp90.

Specificity of Ydj1 function in protein kinase maturation. The experiments described above suggest a model in which Ydj1 protects nascent kinases in association with Cdc37. In addition, Ydj1 contributes to the rate of kinase maturation. The results of experiments with *ydj1*^{H34Q} suggested a model in which the chaperone action of Ydj1 functioned to protect nascent kinases independently of Ydj1's interaction with Hsp70 (Fig. 6A and B). However, when Tpk2 levels were assayed in the *ydj1*Δ strain expressing *ydj1*^{H34Q} from a low-copy-number plasmid (Fig. 7A), they were similar to those

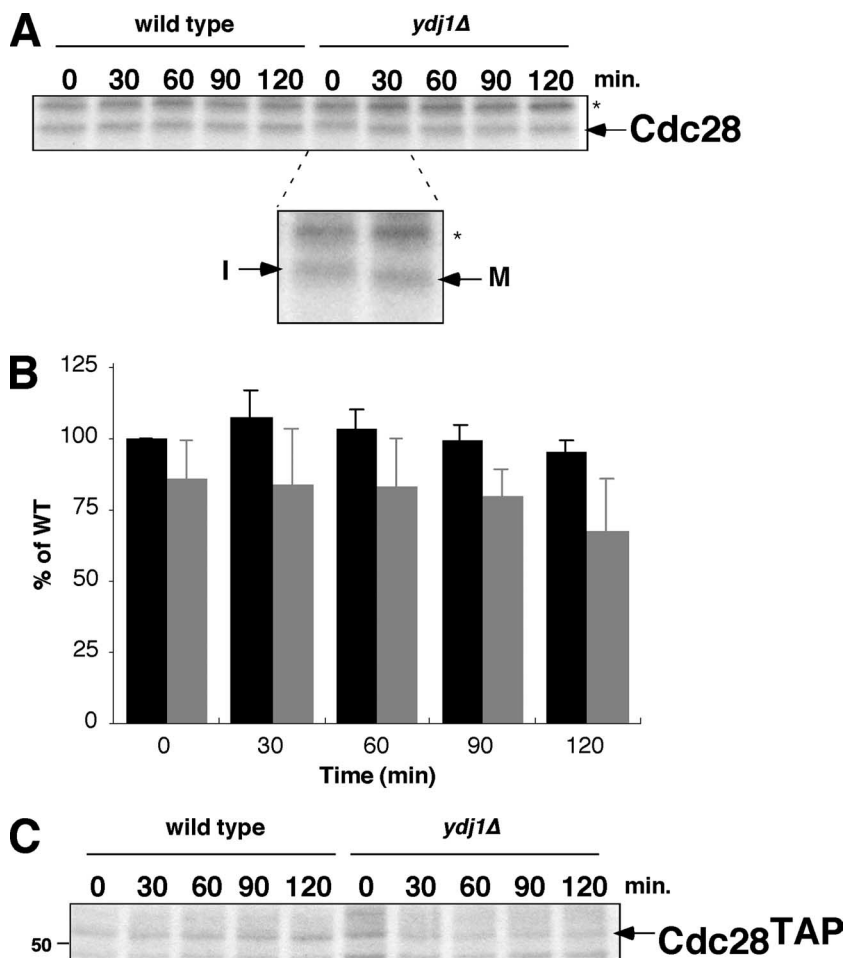


FIG. 4. Pulse-chase analysis of Cdc28. (A) Pulse-chase analysis of untagged Cdc28 immunoprecipitated with anti-PSTAIRE (which recognizes a peptide in the α -C helix) in wild-type and *ydj1Δ* mutant cells. Chase times are indicated in minutes. Cdc28 is denoted by arrow. The band labeled with an asterisk is nonspecific. The inset shows the difference in mobility of Cdc28 at 0 and 30 min of chase time. I, immature form of Cdc28; M, mature form. (B) Quantitation of Cdc28 levels in wild-type (WT) and *ydj1Δ* cells after phosphorimaging. The results are expressed as the percentages of the levels in wild-type cells at 0-min chase. Bars indicate the standard deviations of three independent experiments. Bars: ■, wild type; □, *ydj1Δ* mutant. (C) Pulse-chase analysis of TAP-tagged Cdc28 in wild-type and *ydj1Δ* cells as described above.

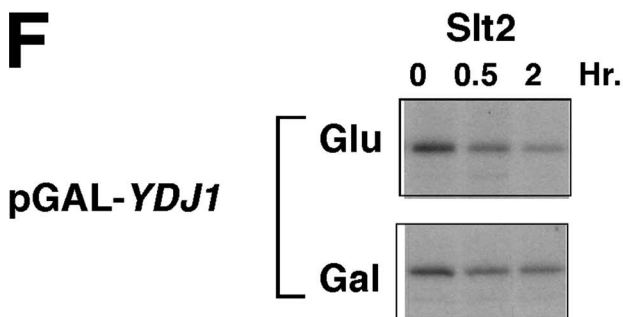
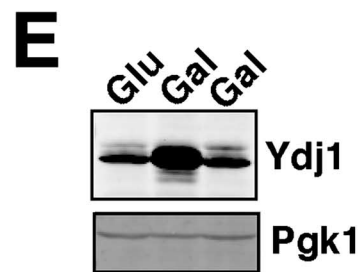
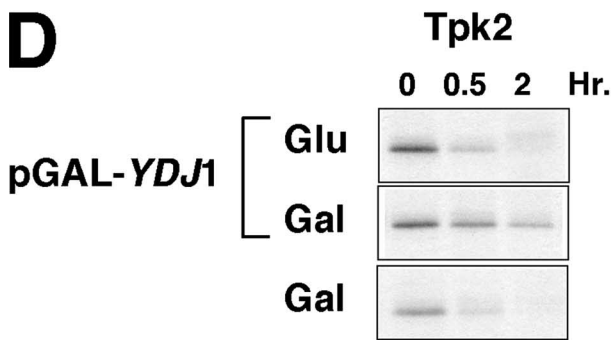
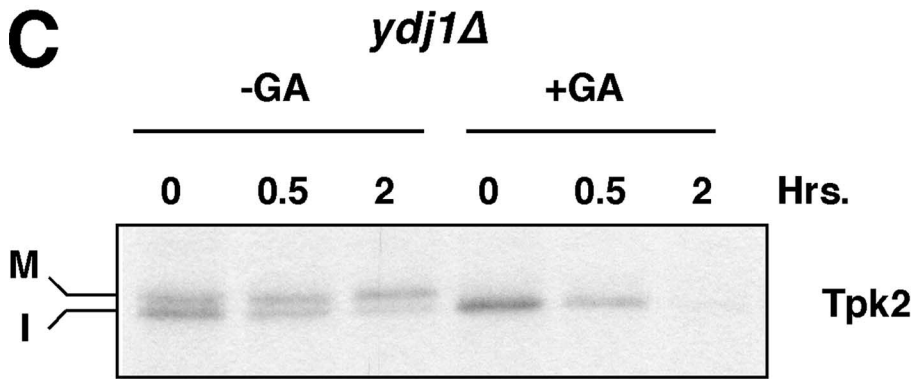
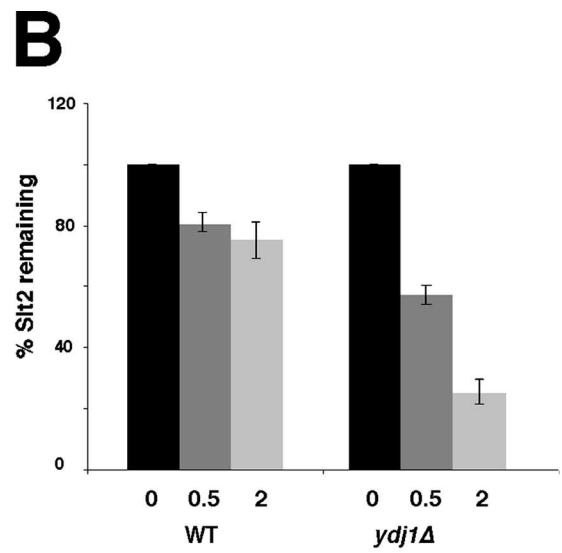
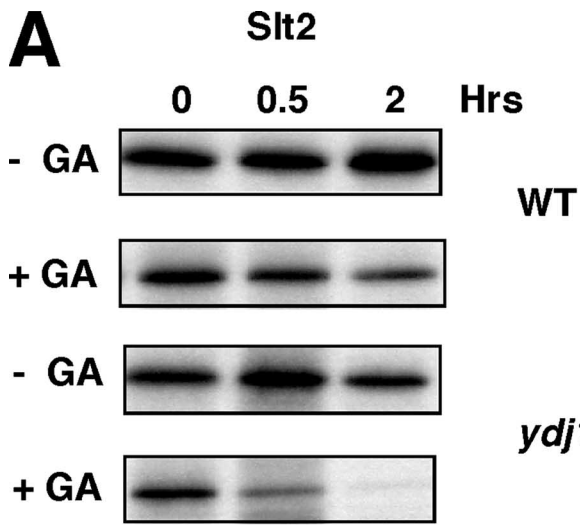
found in the *ydj1Δ* mutant alone. Similar findings were made when Tpk2 maturation was followed by pulse-chase analysis (data not shown). This indicates that under normal conditions, Ydj1 function in Tpk2 maturation involves Hsp70.

To further address the relationship between Ydj1 and Hsp70 in kinase maturation, we overexpressed the type II Hsp40, Sis1, in the *ydj1Δ* mutant. Previous studies showed that Sis1, a type II Hsp40, partially suppressed the slow-growth phenotype of a *ydj1Δ* mutant (8); accordingly, Sis1 may compensate for the loss of Ydj1 in kinase biogenesis. However, *SIS1* overexpression was largely unable to suppress reduced levels of Tpk2 in the *ydj1Δ* mutant strain (Fig. 7B). This finding suggested some specificity in the way in which Ydj1 and Hsp70 promote kinase maturation.

Both Ydj1 and Sis1 have distinct chaperone modules that are exchangeable and can specify Sis1 or Ydj1 action in a chimera (19). When we assayed these chimeras, however, those containing the Sis1 chaperone module (YSY; where Y represents Ydj1 domains, and S represents the Sis1 chaperone domain) were more effective at suppressing the loss of Tpk2 than

those containing the Ydj1 chaperone module (SYS; Fig. 7C). These findings with Sis1 and Sis1/Ydj1 chimeras suggest that the relationship between Ydj1 and Hsp70 for kinase folding is more complex than expected. They cannot be attributed just to J-domain regulation of Hsp70's ATPase or to specificity in polypeptide-binding by Ydj1. Similarly, deletion of the Ydj1 G/F domain, which separates the J domain from the chaperone-binding module, had little effect on Tpk2 maturation (Fig. 7D).

Finally, we investigated whether the specificity of Ydj1 could be retained by another type I dnaJ protein with the same domain organization. We analyzed *E. coli* dnaJ for this purpose, which is similar to Ydj1, although it is missing the far-nylation motif. The expression of dnaJ led to complete suppression of the *ydj1Δ* phenotype for Tpk2 and resulted in even greater levels of kinase in both wild-type and *ydj1Δ* strains, suggesting it is a more effective chaperone (Fig. 7E). These combined results provide insight into the importance of the C-terminal and perhaps dimerization domains of type I Hsp40s in protecting nascent chains from degradation, while the far-



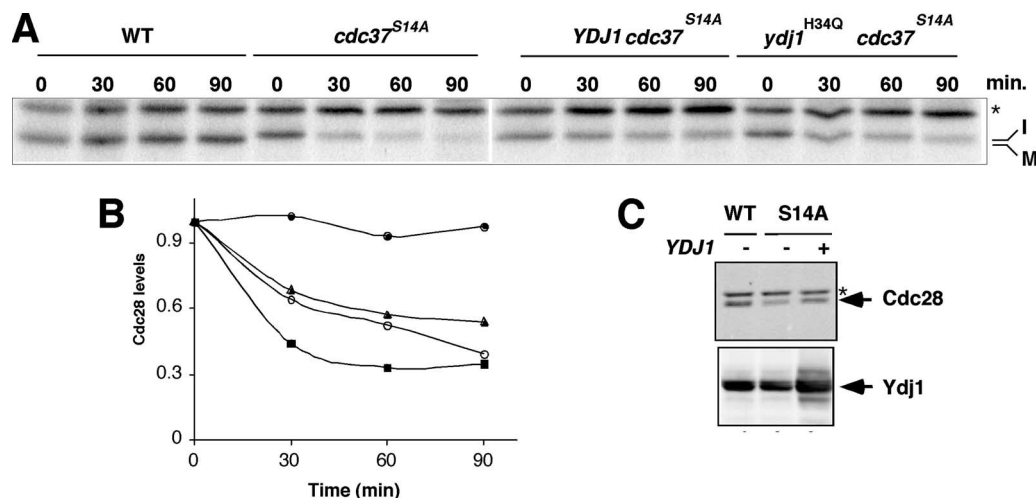


FIG. 6. Ydj1 protects against degradation of unstable Cdc28 in a *cdc37* mutant strain. (A) Pulse-chase analysis of Cdc28 in wild-type and *cdc37^{S14A}* mutant cells without or with overexpression of *YDJ1* or the mutant *ydj1^{H34Q}*. Chase times are given in minutes. Immature (I) and mature (M) forms of Cdc28 are indicated. The band labeled with an asterisk is nonspecific. (B) Quantitation of the data shown in panel A. Symbols: ●, wild type; ■, *cdc37^{S14A}* mutant; ▲, *cdc37^{S14A}* mutant with *YDJ1* overexpressed; ○, *cdc37^{S14A}* mutant with *ydj1^{H34Q}* overexpressed. (C) Western blot analysis of Cdc28 in wild-type and *cdc37^{S14A}* mutant cells (S14A) either with (+) or without (-) *YDJ1* overexpression. The lower panel shows the Ydj1 levels. The band labeled with an asterisk is nonspecific. WT, wild type.

nesylation of the Ydj1, which helps to anchor it to membranes (10), is not required for this function.

DISCUSSION

As polypeptide chains emerge from the ribosome, they interact with several chaperone machines. Among these are components of the RAC complex, which includes ribosome-binding Hsp70 and Hsp40 chaperones that promote efficient polypeptide translation (50). Other members of the Hsp70 and Hsp110 families also participate in nascent chain binding (55). More specific chaperones interact with nascent chains from distinct protein families, such as Cdc37, which protects nascent protein kinases from rapid degradation by the proteasome. In the present study, we characterized the type I Hsp40, Ydj1, as also having a protective role in protein kinase biogenesis at the earliest stages posttranslation. Ydj1 also has a role in kinase biogenesis that is distinct from Cdc37, by controlling the rate of kinase maturation as well as the yield of mature kinase.

Ydj1's ability to protect nascent protein kinases results from a direct effect and an indirect one via Hsp70. Of the two, it is the latter that has the greatest contribution to stabilizing protein kinases. In the *cdc37^{S14A}* mutant, overexpression of wild-type *YDJ1* or the mutant *ydj1^{H34Q}* led to a temporary stabilization of Cdc28. Since the *ydj1^{H34Q}* mutant cannot interact

with Hsp70, these findings suggest that the intrinsic chaperone function of Ydj1 promoted the stabilization, albeit weakly. Furthermore, the overall effects of expressing *ydj1^{H34Q}* in place of wild-type Ydj1 were negligible at steady state for Tpk2 (Fig. 7A), suggesting that Hsp70 binding is required for complete protection against degradation. As noted previously, Ste11 kinase synthesized in a *ydj1Δ* mutant had decreased amounts of bound Hsp70 (32), reinforcing the notion that Ydj1 interacts with nascent kinases in order to protect them, but this effect must be integrated with subsequent binding to Hsp70 to avoid degradation. Notably, Hsp90 and Cdc37 could still bind to Ste11 even in a *ydj1Δ* strain (32). Such binding likely represents a quality control step in kinase maturation that is at least partially distinct from that managed by Ydj1. This is because even in the absence of Ydj1 there is a strong effect of GA in promoting kinase degradation (Fig. 5C). These data therefore point to a role for both Hsp70 and Hsp90 chaperone machines in protecting newly synthesized kinases from degradation, as well as helping them to fold. Interestingly, both chaperones are upregulated in cells with *YDJ1* deleted (Fig. 1).

The notion that both Hsp70 and Hsp90 contribute to kinase stability fits with our finding that Tpk2 degradation is biphasic. The first phase is during the 10-min pulse-labeling period, in a manner similar to the way in which Tpk2 is degraded in the

FIG. 5. Effect of Ydj1 on the stability of nascent protein kinases under conditions that promote their degradation. (A) Effect of GA treatment on the stability of TAP-Slt2 kinase in wild-type and *ydj1Δ* cells, as indicated. Chase times are given in hours after pulse-labeling in the absence and presence of 50 μ M GA. (B) Quantitation of Slt2 based on the data shown in panel A and other data ($n = 3 \pm$ the standard error). (C) Pulse-chase analysis of TAP-Tpk2 in the absence and presence of 50 μ M GA in *ydj1Δ* cells. The chase times are indicated in hours. Mature (M) and immature (I) forms of Tpk2 are indicated. (D) Pulse-chase analysis of TAP-Tpk2 from cells containing pGAL-YDJ1 under noninducing condition (glucose; Glu) and inducing conditions (galactose, Gal). The lower panel shows pulse-chase results for Tpk2 in cells containing galactose but not the pGAL-YDJ1 plasmid. (E) Western blot analysis of Ydj1 protein before and after overexpression using the same cultures as in panel D. Pgl1 is shown as a loading control. (F) Effect of *YDJ1* overexpression on TAP-Slt2 stability in the presence of GA (50 μ M). Inducing and noninduced states are as described in panel D.

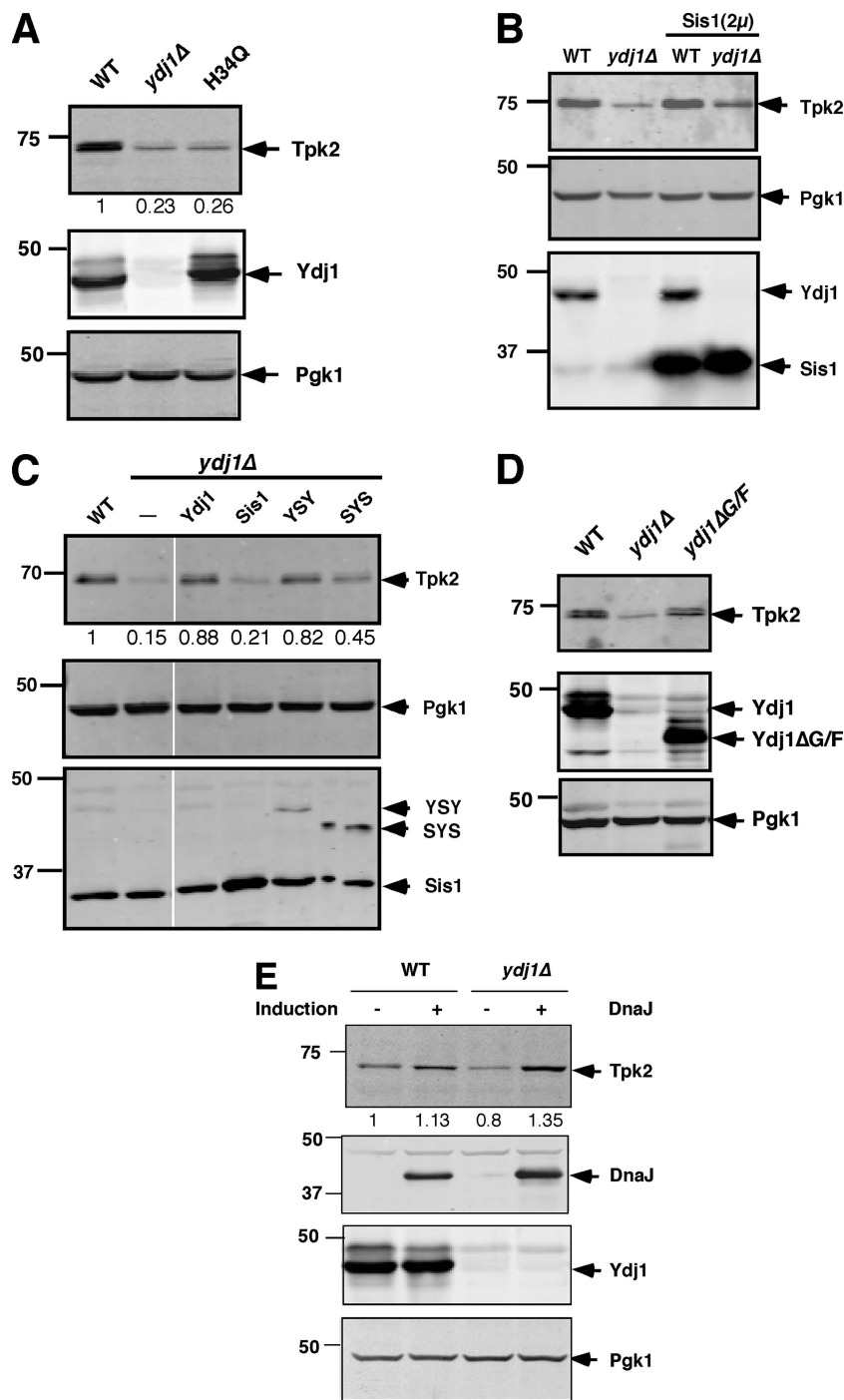


FIG. 7. Steady-state TAP-Tpk2 levels in *ydj1* mutants. (A) Western blot analysis of TAP-Tpk2 (Tpk2) in wild-type and *ydj1Δ* cells without or with the *ydj1^{H34Q}* mutant expressed from a low-copy-number plasmid. The middle panel shows Ydj1 levels, and the lower panel shows the levels of Pgk1 as a loading control. (B) Western blot analysis of wild-type and *ydj1Δ* mutants without or with overexpression of *SIS1*. The middle panel shows the levels of Pgk1 as a loading control, while the lower panel shows the levels of Ydj1 and Sis1. (C) Western blot of TAP-Tpk2 in wild-type and *ydj1Δ* cells with or without Sis1, YSY, and SYS chimeric chaperones as indicated. The middle panel shows Pgk1, and the lower panel shows the levels of Sis1 and Ydj1/Sis1 chimeras using anti-Sis1. (D) Effect of Ydj1's G/F region in kinase accumulation. Tpk2 levels were analyzed in wild-type and Ydj1ΔG/F-expressing cells in a *ydj1Δ* background. (E) Effect of bacterial DnaJ in kinase accumulation. The Tpk2 levels were measured after overexpression of DnaJ in wild-type and *ydj1Δ* yeast strains. DnaJ expression was carried out after induction with galactose for 8 h. Tpk2 was identified with anti-TAP antibody. The kinase levels were quantified and are shown as a percentage of the wild-type levels. Western blot analyses with the same samples were performed with anti-Pgk1, anti-DnaJ, and anti-Ydj1. Pgk1 levels served as a loading control. WT, wild type.

cdc37 mutant (36). Like Cdc37, therefore, Ydj1 protects nascent chains and, in its absence, the newly made kinase is largely directed toward the ubiquitin/proteasome system. Tpk2 that escapes initial triage either folds slowly or is subsequently degraded in a slower process. We did not observe the fast degradation of Cdc28 in a *ydj1Δ* strain, a finding consistent with a similar finding for the *cdc37* mutant (Fig. 6) (36). Instead, there is a delay in Cdc28 maturation, followed by a slow decrease in kinase levels. For both Cdc28 and Tpk2, however, the rate of kinase degradation is much more pronounced in the *cdc37* mutant than in the *ydj1Δ* strain. Despite this difference, Ydj1 and Cdc37 both contribute in a similar manner to the folding process; each stabilizes nascent kinases and helps promote binding to another chaperone, Hsp70 in the case of Ydj1 and Hsp90 in the case of Cdc37. Although Ydj1 is an abundant protein, it is still limiting in its capacity to protect newly synthesized kinases. When overexpressed, Ydj1 promotes greater stability of Tpk2 and Slr2 kinases in the presence of GA. Similarly, overexpressed Ydj1 or *ydj1^{H34Q}* delayed the degradation of unstable Cdc28 in *cdc37^{S14A}* mutant cells (Fig. 5 and Fig. 6).

Although our results provide strong support for a protective role for Ydj1 in kinase biogenesis, this is not a universal function. There have been several published reports showing that there is reduced degradation of unstable proteins in a mutant of *YDJ1* called *ydj1-151* (30, 40, 54). However, we found that the expression of *ydj1-151* largely suppressed the effect of complete *YDJ1* deletion and led to the accumulation of folded Tpk2 as measured by enzyme activity (data not shown). Our model, therefore, is that Ydj1 functions to promote kinase folding but is not involved in targeting misfolded kinases to the degradation machinery, at least under nonstress conditions.

Besides acting to protect nascent kinases, Ydj1 also affects the rate at which maturation takes place. This effect occurred in *ydj1Δ* and *ydj1^{H34Q}* mutants (Fig. 3 and data not shown), indicating that the effect depends more on the relationship between Ydj1 and Hsp70 than on the intrinsic chaperone action of Ydj1 itself. The basis for the reduced rate of kinase maturation in the *ydj1* mutations, however, remains obscure. One possibility relates to the finding that *ydj1* mutants grow slowly and thus have reduced rates of metabolic processes. However, pulse-chase analyses of Tpk2 maturation in other chaperone mutants that grow slowly (*cpr7Δ* and *sse1Δ*) suggested that this is not the case (not shown).

The ability of Ydj1 to protect nascent kinases has some specificity since *SIS1* overexpression was unable to suppress defects in kinase biogenesis in a *ydj1Δ* mutant. The lack of suppression occurs despite the finding that *SIS1* overexpression can improve growth of *ydj1Δ* cells (8). Furthermore, the growth of *ydj1Δ* cells can be improved by just increasing the abundance of the J domain, suggesting that impairment to Hsp70's functional cycle is the main factor that limits growth of *ydj1Δ* cells (45). Tests of which domains impart the functional specificity were largely negative but demonstrated that the chaperone modules specific to type I and type II Hsp40s were not responsible (Fig. 7). Further support for this view derives from the finding that mammalian Hdj2 (type I) and Hsp40 (type I) are interchangeable for their function in folding of progesterone receptor and Chk1 protein kinase (13, 21). Based on this, the specificity with which we observed Ydj1 function

may have more to do with how Sis1 is unable to interact with kinases or nuclear receptors in the presence of Hsp70. Previous studies showed that Ydj1 is capable of refolding luciferase in association with Hsp70 in vitro, whereas Sis1 does not function as efficiently in this capacity (34). The basis for their differential function is due in part to each having a distinct chaperone module, which can specify the function of Sis1 or Ydj1 in chimeric molecules (19). We note, however, that Sis1 also has a more stable interaction with the Hsp70 C terminus than does Ydj1 (33). The C-terminal region of Hsp70 interacts with the peptide-binding groove in the chaperone module of Sis1, suggesting that there could be competitive interactions. This might preclude Sis1 binding to nascent kinases in the presence of Hsp70. We speculate that such binding of Hsp70 to the Sis1 chaperone module is disrupted in the YSY chimera and that this is why it proves so effective compared to Sis1 alone (Fig. 7).

In conclusion, our combined findings suggest a model in which Ydj1 has two specific functions in protein kinase folding. The first involves protecting the newly synthesized kinase chain from degradation, and the second involves promoting efficient folding and maturation. Ydj1 by itself can protect nascent kinases, but not to the same extent that it can in association with Hsp70. The unexpected finding that Ydj1 can affect the rate of kinase maturation presents a new paradigm for its action in cellular quality control.

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