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RESEARCH ARTICLE

Figures and figure supplements

The proteasome biogenesis regulator Rpn4 cooperates with the unfolded protein response to promote ER stress resistance

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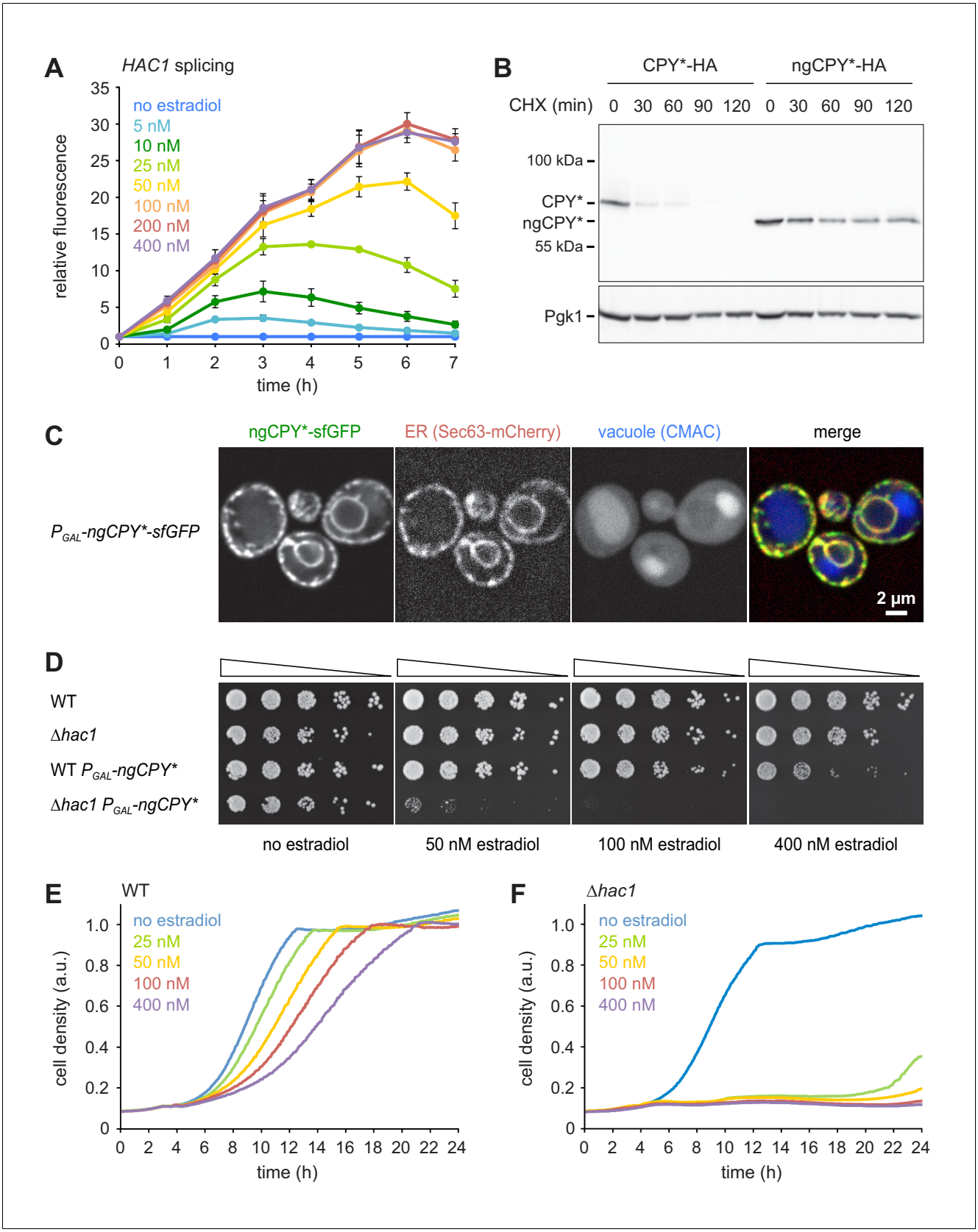

Figure 1. A titratable system for the induction of ER stress. (**A**) Flow cytometric measurement of GFP levels in cells harboring the *HAC1* splicing reporter and expressing ngCPY* under the control of the estradiol-inducible *GAL* promoter system. For each time point, data are normalized to

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Figure 1 continued

untreated cells. Mean ±SEM, n = 3. (**B**) Western blot of HA and Pgk1 from cells expressing CPY*-HA or ngCPY*-HA. Cells were treated with cycloheximide (CHX) for the times indicated. Pgk1 served as a loading control. (**C**) Images of cells expressing ngCPY*-sfGFP and the general ER marker Sec63-mCherry. Expression of ngCPY*-sfGFP was induced with 25 nM estradiol for 4 hr and cells were stained with the vacuole dye CMAC. (**D**) Growth assay on solid media of wild-type (WT) and Δ*hac1* cells expressing the estradiol-inducible artificial transcription factor GEM and, where indicated, ngCPY* under the control of the *GAL* promoter. For each strain, series represent fivefold dilution steps. (**E**) Growth assay in liquid media of WT cells expressing ngCPY* under the control of the estradiol-inducible *GAL* promoter system. a.u., arbitrary units. (**F**) As in panel E, but with Δ*hac1* cells.

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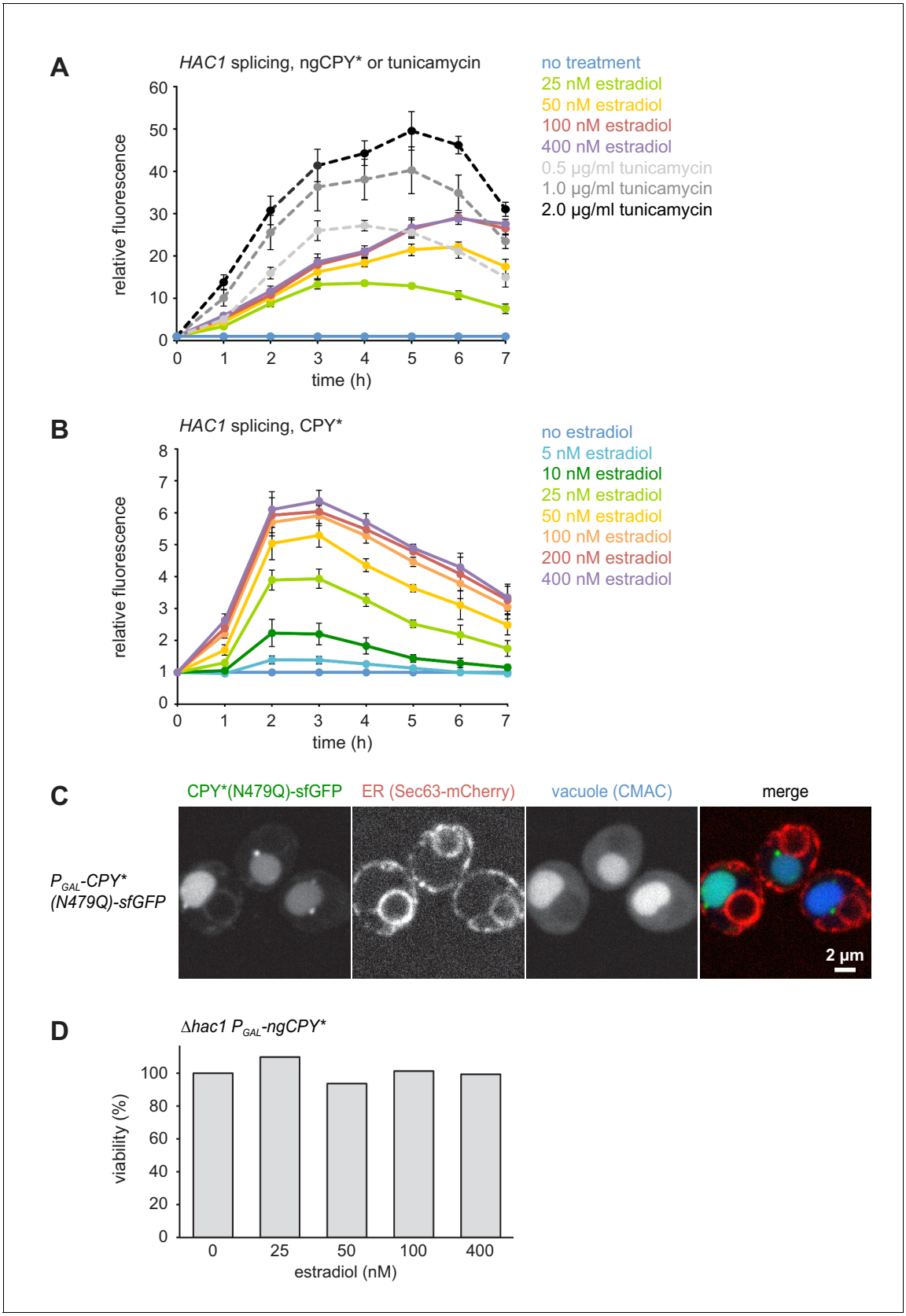

Figure 1—figure supplement 1. A titratable system for the induction of ER stress. (**A**) Flow cytometric measurement of GFP levels in cells harboring the *HAC1* splicing reporter and expressing ngCPY* under the control of the estradiol-inducible *GAL* promoter system. For each time point, data are

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Figure 1—figure supplement 1 continued

normalized to untreated cells. Mean ±SEM, n = 3. (**B**) As in panel A, but with cells expressing glycosylatable CPY*. (**C**) Images of cells expressing CPY* (N479Q)-sfGFP and the ER marker Sec63-mCherry. Expression of CPY*(N479Q)-sfGFP was induced with 25 nM estradiol for 4 hr and cells were stained with the vacuole dye CMAC. (**D**) Viability of Δ*hac1* cells after induction of ngCPY* expression with the indicated estradiol concentrations for 24 hr. Viability of untreated cells was set to 100%.

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Figure 2. Multicopy suppression of ngCPY* toxicity in Δ*hac1* cells. (**A**) Growth assay on solid media of wild-type (WT) and Δ*hac1* cells expressing ngCPY* under the control of the estradiol-inducible *GAL* promoter system and overexpressing *RPN4*, *SSZ1* or *PDR1* from extrachromosomal plasmids

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Figure 2 continued

where indicated. (**B**) Growth assay in liquid medium of WT and Δ*hac1* cells expressing ngCPY* under the control of the estradiol-inducible *GAL* promoter system. Δ*hac1* cells additionally overexpressed the indicated genes. Cells were grown without estradiol. a.u., arbitrary units. (**C**) As in panel B, but in the presence of 50 nM estradiol. (**D**) Quantification of growth assays as shown in panel B. Data are normalized to WT cells. Mean ±SEM, n = 4. (**E**) Quantification of growth assays as shown in panel C. Data are normalized to WT cells. Mean ±SEM, n = 4.

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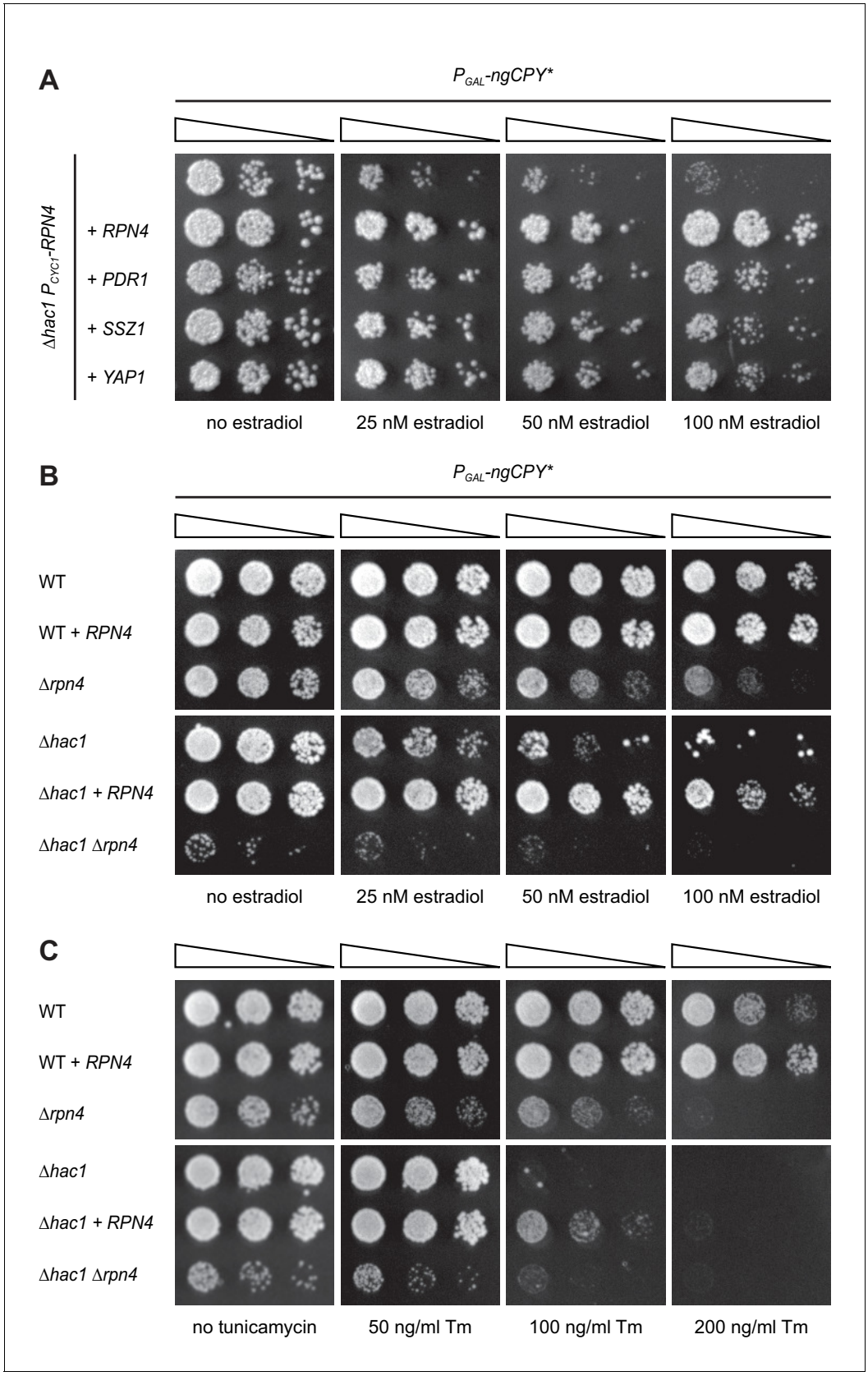

Figure 3. Rpn4 and the UPR are functionally linked. (**A**) Growth assay on solid media of Δ*hac1* cells containing the constitutive *CYC1* promoter in place of the endogenous *RPN4* promoter, expressing ngCPY* under the control of the estradiol-inducible *GAL* promoter system and overexpressing *RPN4*,

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SSZ1, *PDR1* or *YAP1* where indicated. (**B**) Growth assay on solid media of wild-type (WT), Δ*rpn4*, Δ*hac1* and Δ*hac1* Δ*rpn4* cells expressing ngCPY* under the control of the estradiol-inducible *GAL* promoter system and overexpressing *RPN4* where indicated. (**C**) As in panel B, but on media containing different concentrations of tunicamycin (Tm).

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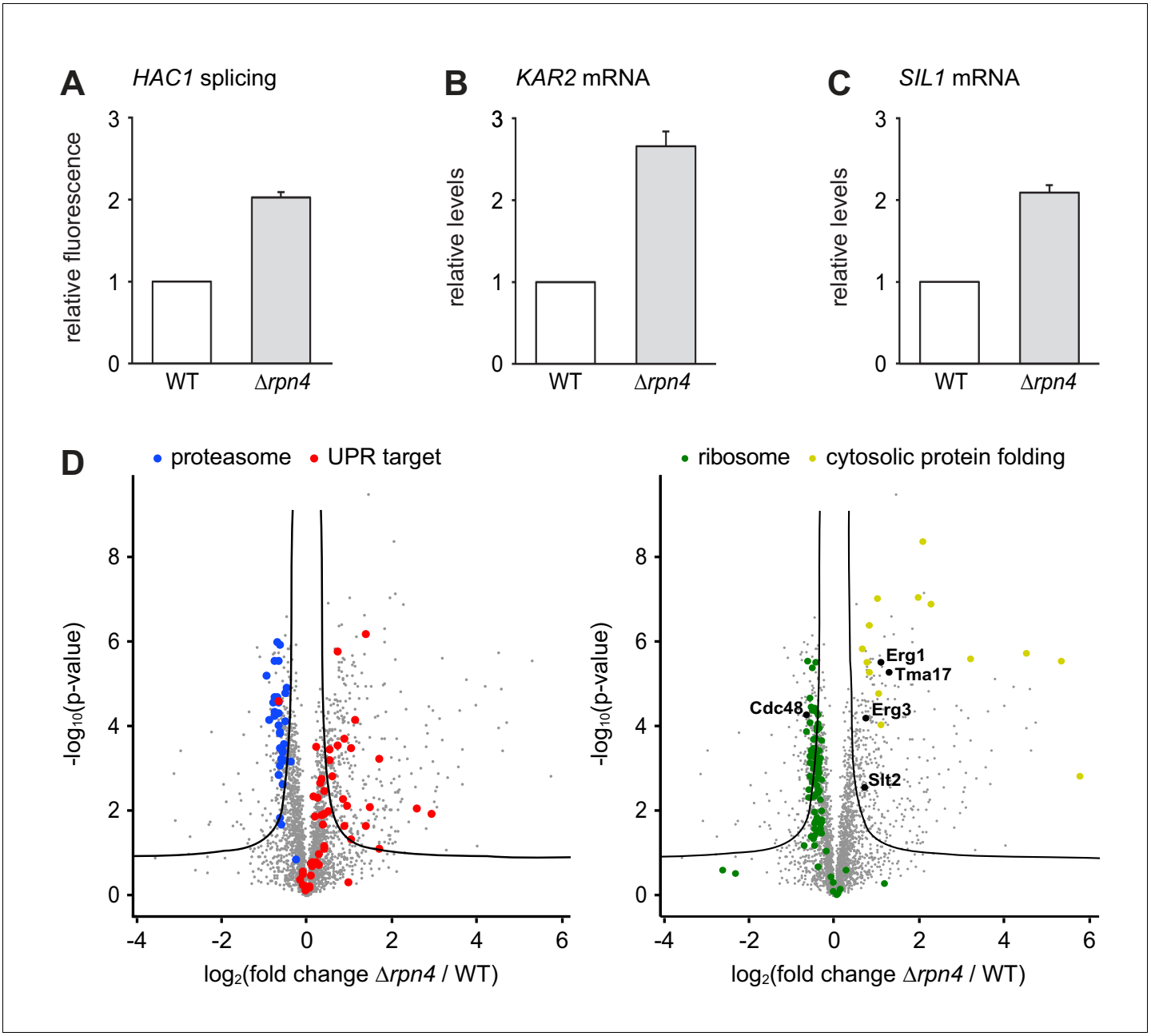

Figure 4. Loss of Rpn4 activates the UPR and triggers a broad adaptive response. (**A**) Flow cytometric measurement of GFP levels relative to cytosolic BFP in wild-type (WT) and Δ*rpn4* cells harboring the *HAC1* splicing reporter. Data are normalized to WT cells. Mean ±SEM, n = 3. (**B**) *KAR2* mRNA levels in WT and Δ*rpn4* cells as measured by quantitative real-time PCR. Data are normalized to WT cells. Mean ±SEM, n = 3. (**C**) As in panel B, but for *SIL1*. (**D**) Global effects of *RPN4* deletion on protein expression. For each protein, the x axis shows the average $\log_2$ fold change between WT and Δ*rpn4* cells (proteins increased in the Δ*rpn4* strain have positive values); the y axis shows the result of a t test for that difference (two-tailed; n = 4). The ''volcano'' lines indicate thresholds of significance. Proteins falling above the volcano lines are significantly changed. The left and right panels show the same plot but with different proteins highlighted. See ***Figure 4—source data 1*** for the data used to generate the plot. In Δ*rnp4* cells, proteasome subunits are downregulated (blue dots, $p=1.1\times10^{-17}$, n = 32), UPR targets are upregulated (red dots, $p=1.8\times10^{-9}$, n = 50), ribosomal proteins are downregulated (green dots, $p=7.5\times10^{-29}$, n = 89) and proteins involved in cytosolic protein folding are upregulated (yellow dots, $p=3.8\times10^{-9}$, n = 14).
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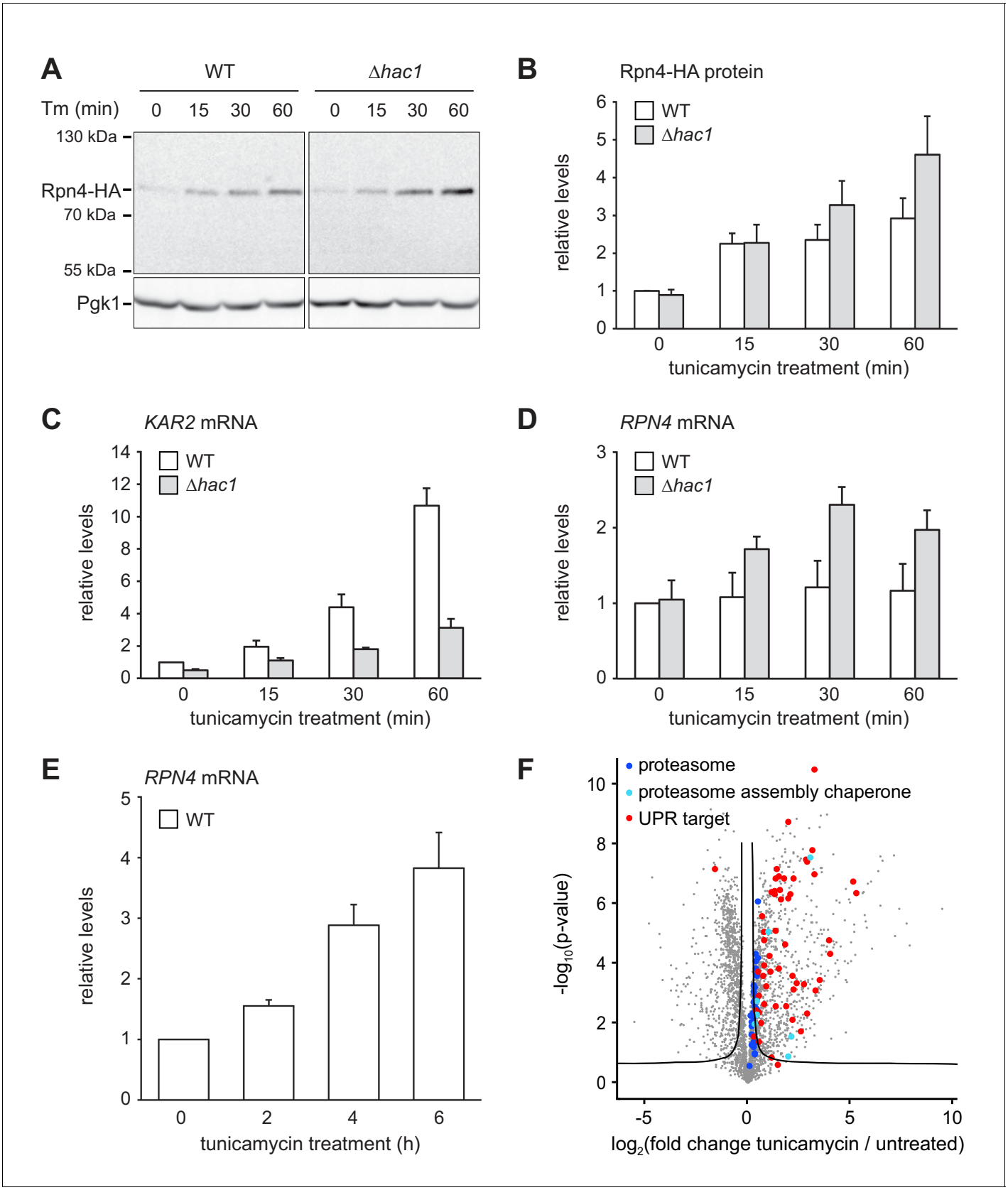

Figure 5. ER stress increases Rpn4 abundance, induces *RPN4* transcription and promotes proteasome biogenesis. (**A**) Western blot of HA and Pgk1 from wild-type (WT) and Δ*hac1* cells expressing Rpn4-HA and treated with 2 µg/ml tunicamycin (Tm) for the times indicated. (**B**) Quantification of Rpn4-

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HA protein levels relative to Pgk1 from western blots as shown in panel A. Data are normalized to WT cells at t = 0. Mean ±SEM, n = 5. (**C**) *KAR2* mRNA levels in WT and Δ*hac1* cells treated with 2 μg/ml tunicamycin for the times indicated. Data are normalized to WT cells at t = 0. Mean ±SEM, n = 3. (**D**) As in panel C, but for *RPN4*. (**E**) As in panel C, but for *RPN4* after treatment with 5 μg/ml tunicamycin for the times indicated. (**F**) Global effects of tunicamycin treatment on protein expression. For each protein, the x axis shows the average $\log_2$ fold change between untreated WT cells and WT cells treated with 5 μg/ml tunicamycin for 4 hr (proteins upregulated by the treatment have positive values); the y axis shows the result of a t test for that difference (two-tailed; n = 4). The ''volcano'' lines indicate thresholds of significance. Proteins falling above the volcano lines are significantly changed. See ***Figure 4—source data 1*** for the data used to generate the plot. Treatment with tunicamycin causes upregulation of proteasome subunits (dark blue dots, $p=7.6\times10^{-3}$, n = 32), proteasome assembly chaperones (light blue dots, $p=3.3\times10^{-3}$, n = 8), and UPR targets (red dots, $p=3.3\times10^{-22}$, n = 58).

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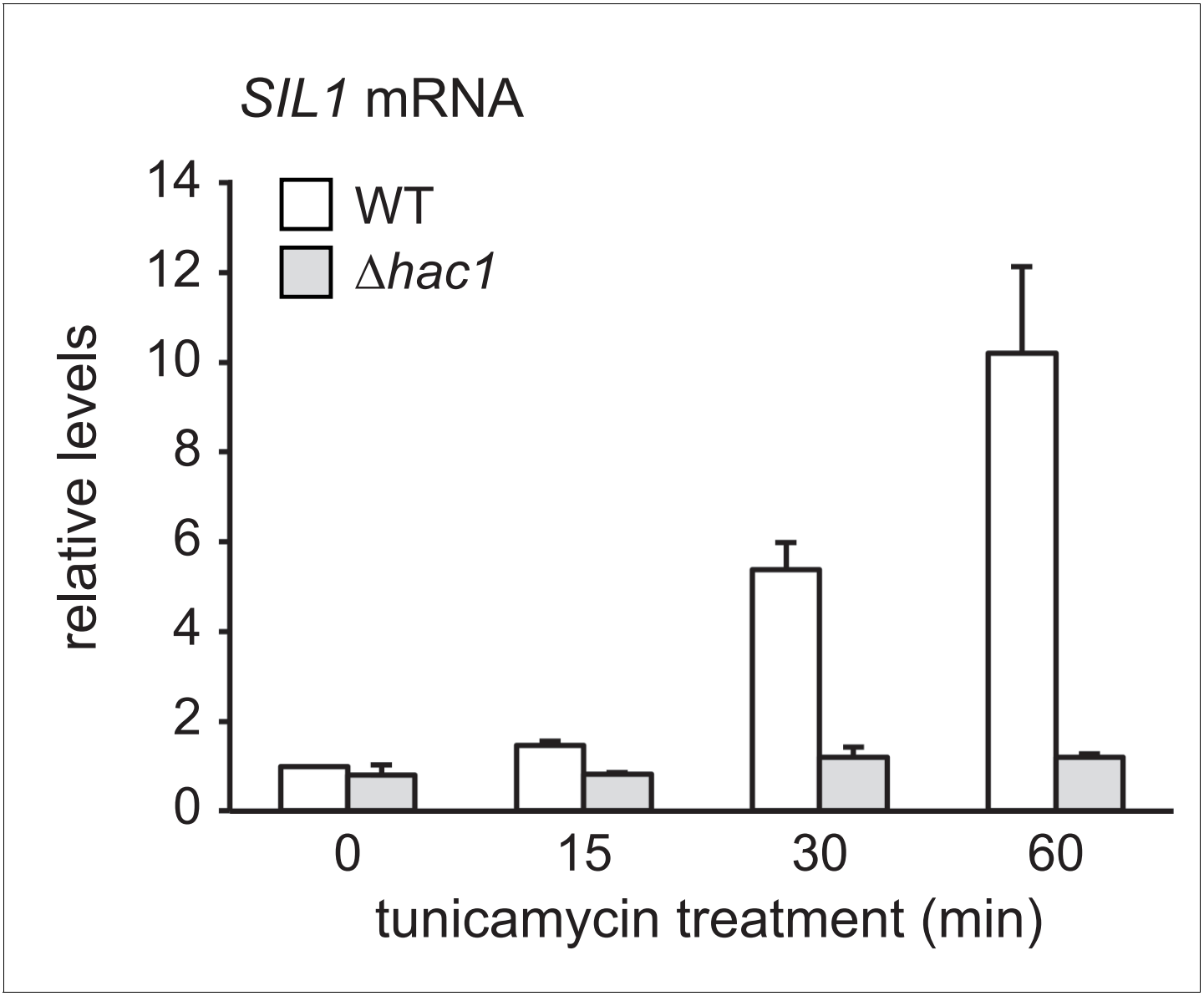

Figure 5—figure supplement 1. *SIL1* mRNA levels in wild-type and Δ*hac1* cells treated with tunicamycin. *SIL1* mRNA levels in wild-type (WT) and Δ*hac1* cells treated with 2 µg/ml tunicamycin for the times indicated. Data are normalized to WT cells at t = 0. Mean ±SEM, n = 3.

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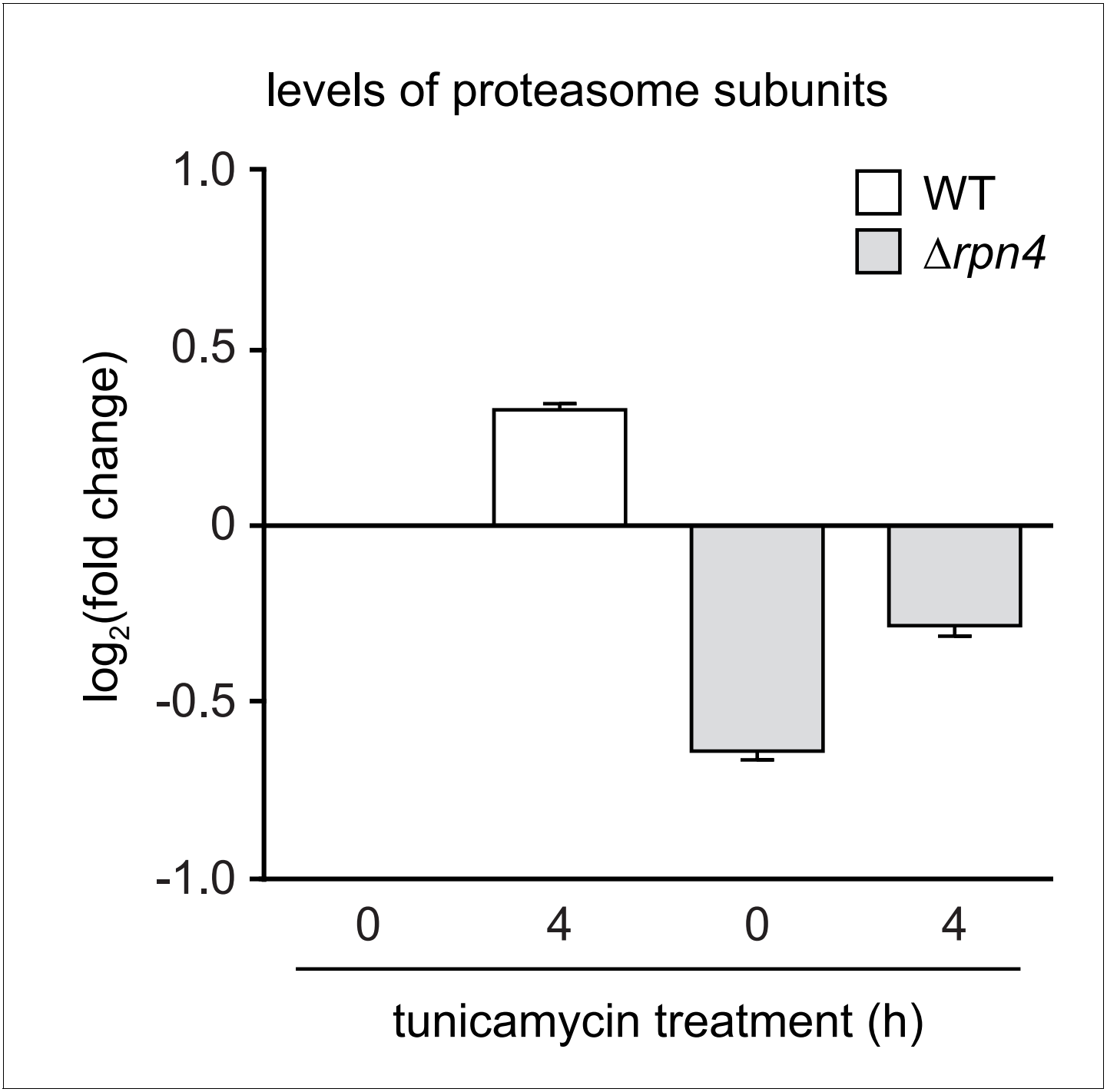

Figure 5—figure supplement 2. Effects of tunicamycin treatment on the levels of proteasome subunits in wild-type and Δ*rpn4* cells. Wild-type (WT) and Δ*rpn4* cells were left untreated (0 hr) or treated with 5 µg/ml tunicamycin for 4 hr. Proteasome subunit abundance was normalized to that in untreated WT cells (value = 0 in log2 space). Bars represent the mean log_2(fold change)±SEM, n = 32. See ***Figure 4—source data 1*** for the data used to generate the graph.

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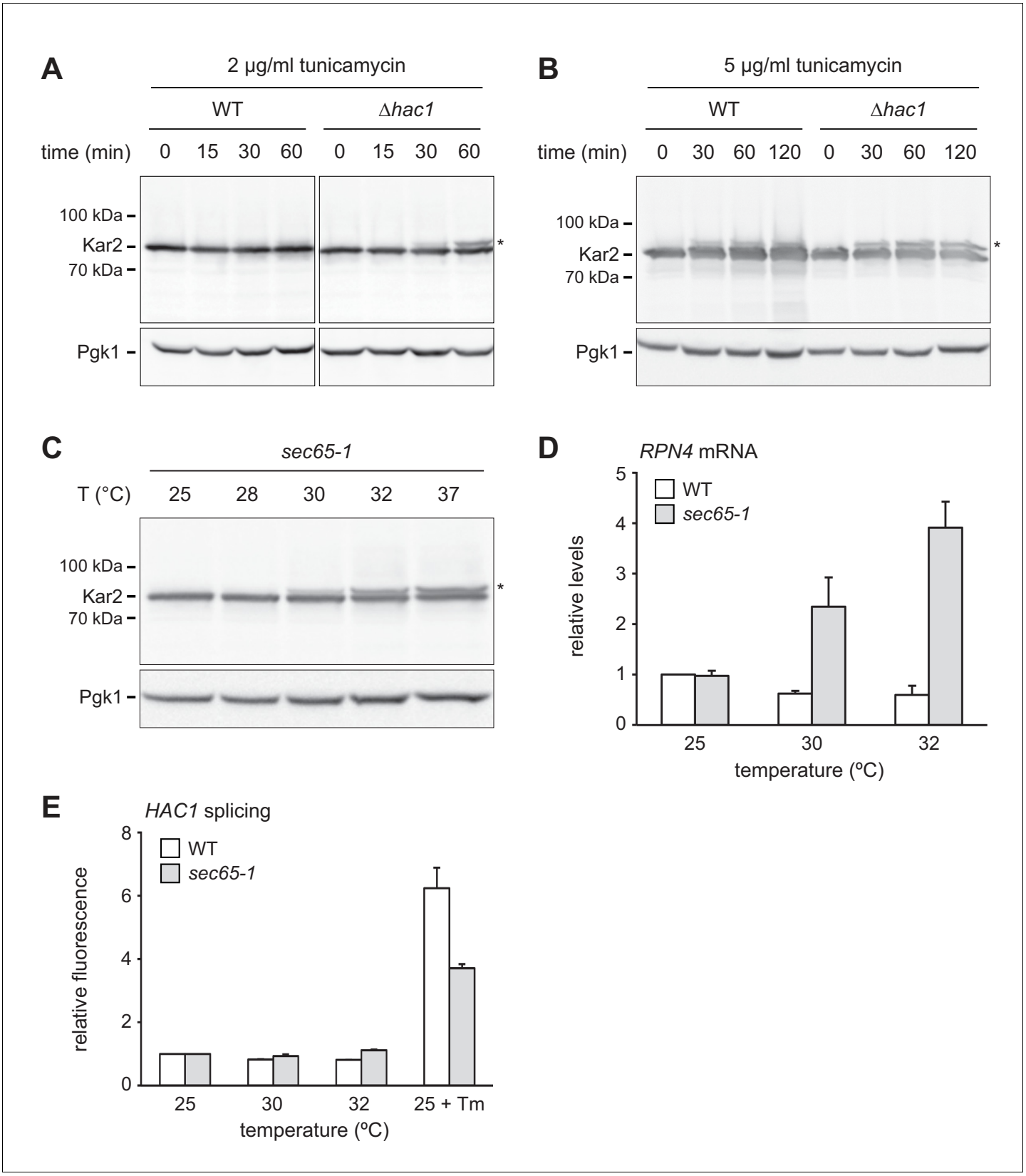

Figure 6. *RPN4* is upregulated by cytosolic mislocalization of secretory proteins. (**A**) Western blot of Kar2 and Pgk1 from wild-type (WT) and Δ*hac1* cells treated with 2 µg/ml tunicamycin for the times indicated. The asterisk indicates untranslocated ss-Kar2. (**B**) As in panel A, but with 5 µg/ml

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Figure 6 continued

tunicamycin. (**C**) Western blot of Kar2 and Pgk1 from *sec65-1* cells grown at 25˚C and shifted to the indicated temperatures for 90 min. The asterisk indicates untranslocated ss-Kar2. (**D**) *RPN4* mRNA levels in WT and *sec65-1* cells grown at 25˚C and shifted to the indicated temperatures for 90 min. Data are normalized to WT cells at 25˚C. Mean ±SEM, n = 3. (**E**) Flow cytometric measurement of GFP levels in WT and *sec65-1* cells harboring the *HAC1* splicing reporter. Cells grown at 25˚C were shifted to the indicated temperatures or treated with 2 µg/ml tunicamycin (Tm) for 90 min. For each strain, data are normalized to 25˚C. Mean ±SEM, n = 3.

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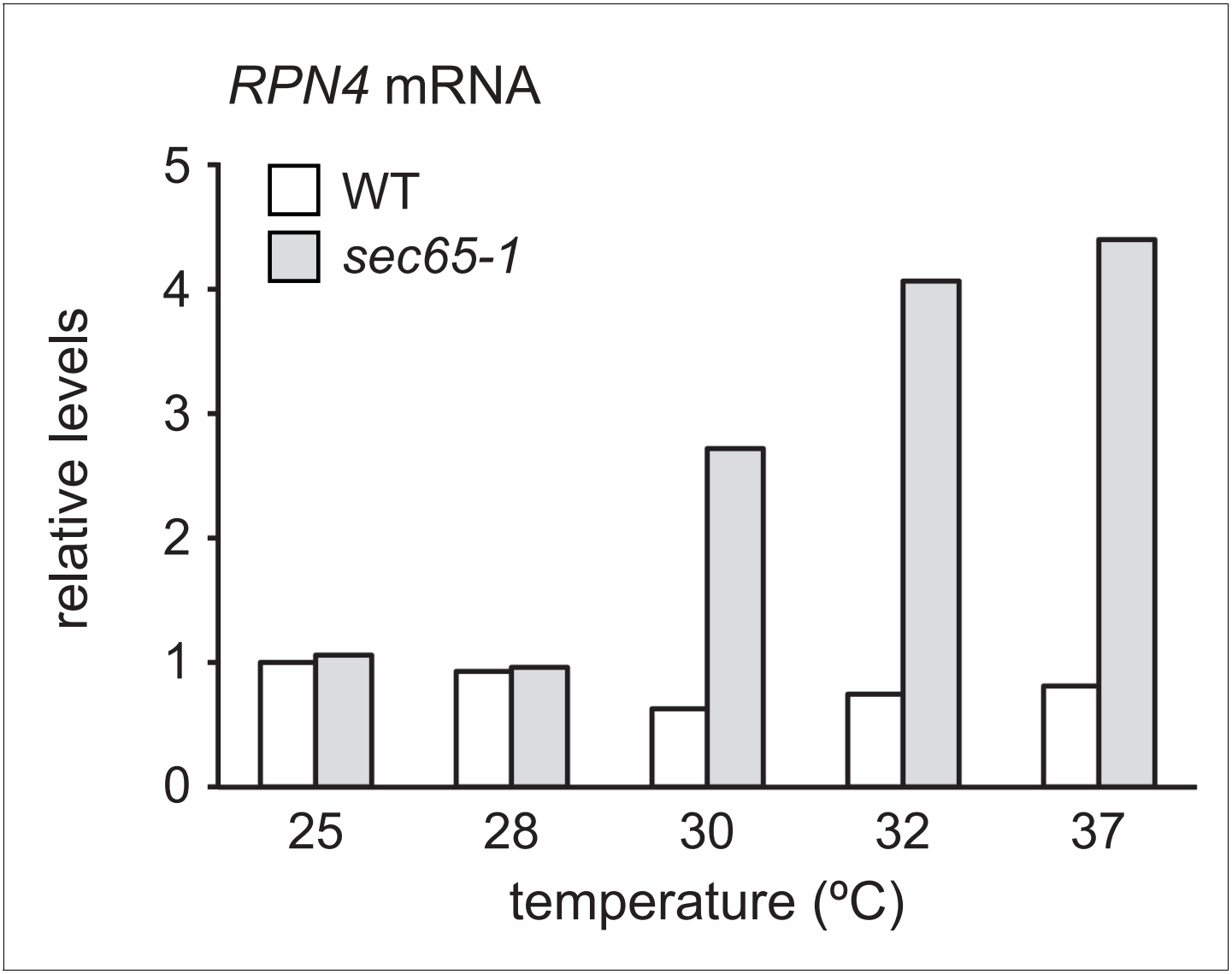

Figure 6—figure supplement 1. *RPN4* mRNA levels in wild-type and *sec65-1* cells at different temperatures. *RPN4* mRNA levels in wild-type (WT) and *sec65-1* cells grown at 25˚C and shifted to the indicated temperatures for 90 min. Data are normalized to WT cells at 25˚C.

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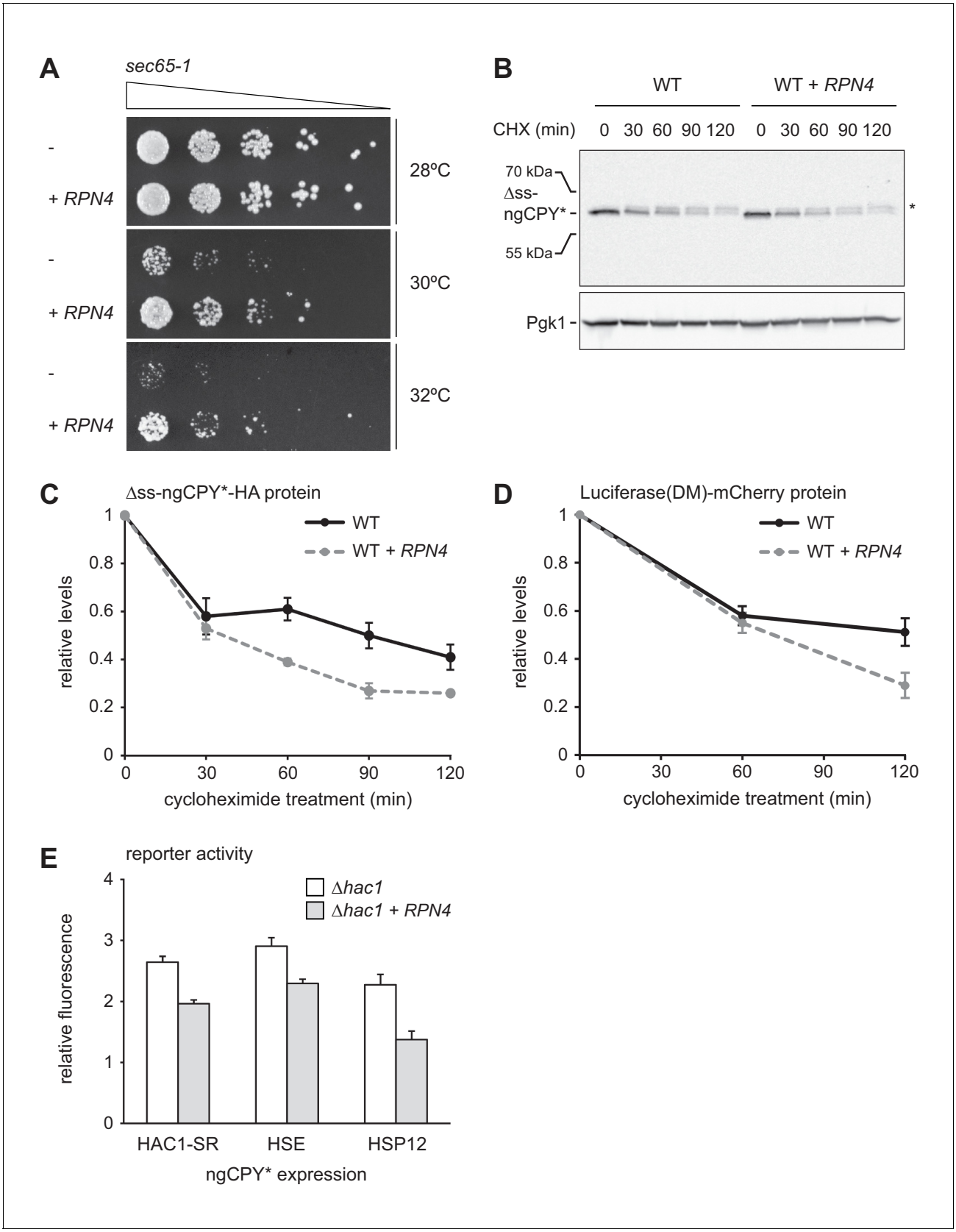

Figure 7. Rpn4 protects against cytosolic protein misfolding. (**A**) Growth assay on solid medium of *sec65-1* cells grown at different temperatures and overexpressing *RPN4* where indicated. (**B**) Western blot of HA and Pgk1 from cycloheximide-treated wild-type (WT) cells expressing Δss-ngCPY*-HA

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and additionally overexpressing *RPN4* where indicated. Expression of Δss-ngCPY*-HA was induced with 100 nM estradiol for 4 hr. The asterisk indicates a slower-migrating, post-translationally modified form of Δss-ngCPY*-HA. CHX, cycloheximide. (**C**) Quantification of Δss-ngCPY*-HA levels relative to Pgk1 from western blots as shown in panel B. For each strain, data are normalized to t = 0. Mean ±SEM, n = 3. (**D**) Luciferase(DM)-mCherry levels relative to Pgk1 and normalized to t = 0. Quantification is based on western blots of mCherry and Pgk1 from cycloheximide-treated cells expressing Luciferase(DM)-mCherry and additionally overexpressing *RPN4* where indicated. Mean ±SEM, n = 3. (**E**) Flow cytometric measurement of GFP levels in Δ*hac1* cells harboring the *HAC1* splicing reporter (HAC1-SR), HSE reporter or HSP12 reporter, expressing ngCPY* under the control of the estradiol-inducible *GAL* promoter system, and overexpressing *RPN4* where indicated. Expression of ngCPY* was induced with 100 nM estradiol for 5 hr. Data are normalized to cells not treated with estradiol. Mean ±SEM, n = 3.

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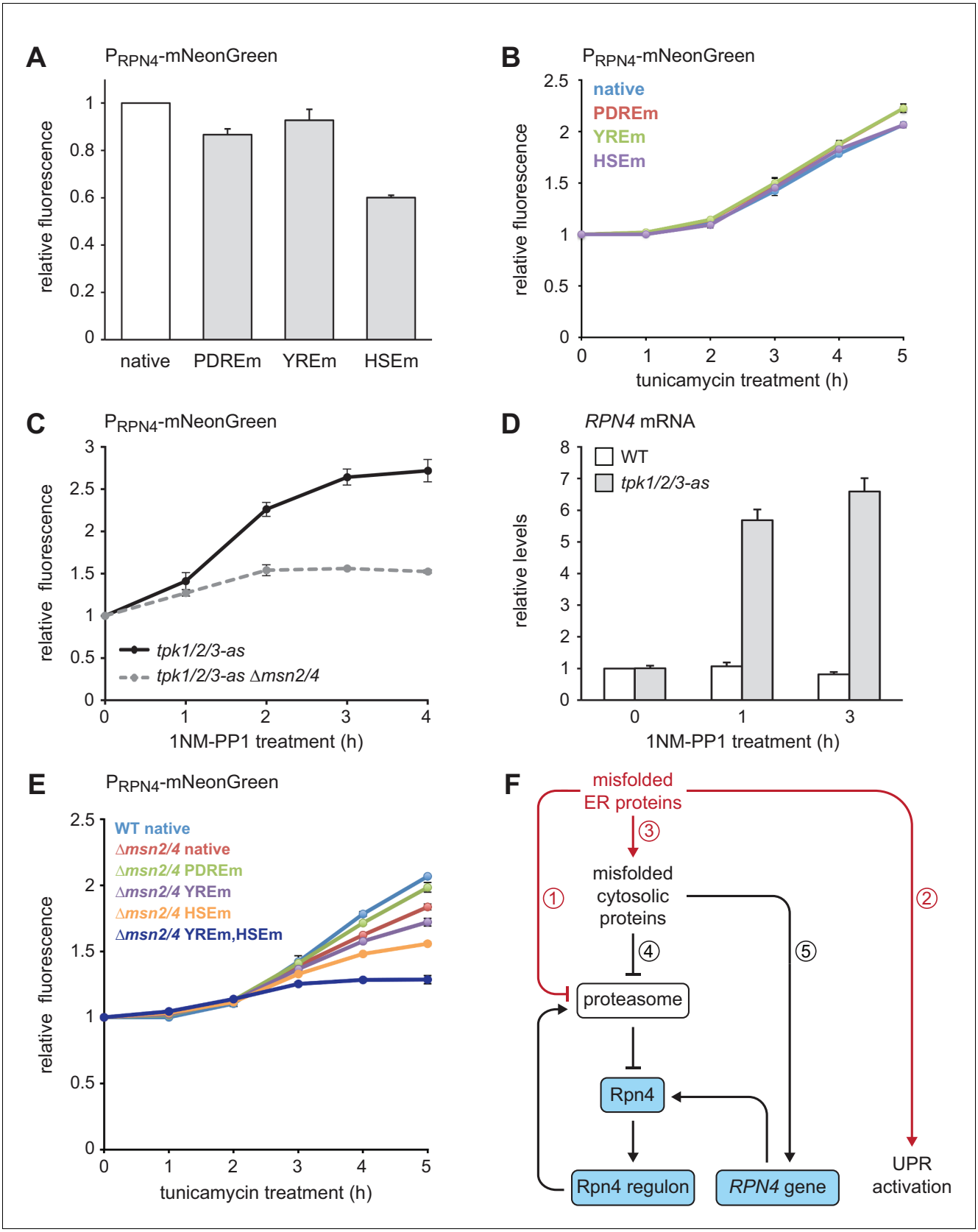

Figure 8. Multiple signaling pathways mediate *RPN4* induction by ER stress. (**A**) Flow cytometric measurement of the activity of *RPN4* reporter variants in untreated cells. Data are normalized to the reporter containing the native *RPN4* promoter. The other reporters contain mutations in the two Pdr1/3

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response elements (PDREm), the Yap1 response element (YREm) or the heat shock element (HSEm). Mean ±SEM, n = 4. (**B**) As in panel A, but after treatment with 5 µg/ml tunicamycin for the times indicated. Mean ±SEM, n = 3. (**C**) Flow cytometric measurement of the activity of the native *RPN4* reporter in *tpk1/2/3-as* and *tpk1/2/3* Δ*msn2/4* cells treated with the ATP analog 1NM-PP1 for the times indicated. The analog-sensitive *tpk1/2/3-as* alleles enable specific inhibition of protein kinase A with 1NM-PP1. Mean ±SEM, n = 3. (**D**) *RPN4* mRNA levels in wild-type (WT) and *tpk1/2/3-as* cells treated with 1NM-PP1 for the times indicated. Data are normalized to WT cells at t = 0. Mean ±SEM, n = 3. (**E**) Flow cytometric measurement of the activity of *RPN4* reporter variants in WT and Δ*msn2/4* cells treated with 5 µg/ml tunicamycin. Data are normalized to WT cells containing the reporter with the native *RPN4* promoter. Mean ±SEM, n = 3. (**F**) Model for the cooperation of Rpn4 and the UPR. ER protein misfolding causes increased flux through the ERAD pathway, which strains proteasome capacity and inhibits efficient protein degradation (1). In addition, the UPR is activated (2). Severe ER stress leads to translocation defects, causing mislocalization of secretory proteins to the cytosol, where they cannot fold properly (3). These cytosolic misfolded proteins further burden the proteasome (4). As a result, Rpn4 is stabilized, the Rpn4 regulon is activated and proteasome biogenesis is enhanced. If cytosolic misfolded proteins persist, the *RPN4* gene is induced (5), providing a second mechanism to increase Rpn4 abundance and augment proteasome biogenesis.

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