Supplementary Table I. **Yeast strains used in this work**

Name	Genotype	Source or reference
CTY5	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 RDI1-3HA-klTRP1*	This study
CTY35	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 RDI1-3myc-His3MX6 LEU2-3HA-RHO1*	This study
CTY36	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 RDI1-3myc-His3MX6 LEU2-3HA-RHO4*	This study
CTY38	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO4 Δrdi1::klTRP1*	This study
CTY37	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO1 His3MX6-GAL1-3HA-RDI1*	This study
CTY40	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO4 His3MX6-GAL1-CDC20*	This study
CTY42	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO4 Δerg6::klTRP1*	This study
CTY43	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-CDC42*	This study
CTY44	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 RDI1-3myc-His3MX6 LEU2-3HA-CDC42*	This study
CTY45	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-CDC42 His3MX6-GAL1-3HA-RDI1*	This study
CTY54	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 RDI1-3myc-His3MX6 LEU2-3HA-RHO2*	This study
CTY55	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 RDI1-3myc-His3MX6 LEU2-3HA-RHO3*	This study
CTY56	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 RDI1-3myc-His3MX6 LEU2-3HA-RHO5*	This study
CTY60	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO1 Δrdi1::klTRP1*	This study
CTY64	*MAT***a** *his3::hisG leu2::hisG trp1::hisG ura3-52 Δrdi1::KanMX6*	This study
CTY65	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO4 Δpep4::KanMX6*	This study
CTY67	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 His3MX6-GAL1-GFP-RHO4*	This study
CTY68	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-CDC42 Δrdi1::His3MX6*	This study
CTY71	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO4 rsp5-1*	This study
CTY74	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO4 Δcla4::His3MX6*	This study
CTY86	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-CDC42 Δrdi1::klTRP1 Δcla4::His3MX6*	This study
CTY87	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO1 Δrdi1::klTRP1 Δcla4::His3MX6*	This study
CTY88	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-CDC42^{G12V}*	This study
CTY89	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-CDC42^{D118A}*	This study
CTY90	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO1^{G19V}*	This study
CTY91	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO1^{D125A}*	This study
CTY95	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δpep4::His3MX6 LEU2-3HA-RHO4^{G81V}*	This study
CTY96	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δpep4::His3MX6 LEU2-3HA-RHO4^{D197A}*	This study
CTY99	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-CDC42 Δcla4::His3MX6*	This study
CTY100	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO1 Δcla4::His3MX6*	This study
ESM1193	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δlte1::KanMX6*	Höfken and Schiebel (2002)
MBY37	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO1*	This study
MBY38	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO2*	This study
MBY39	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO3*	This study

MBY40	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO4*	This study
MBY41	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-3HA-RHO5*	This study
PC344	*MAT***a**/*MAT*α *ura3-52/ura3-52*	Paul Cullen
PPY966	*MAT***a** *his3*::*hisG leu2*::*hisG trp1*::*hisG ura3-52*	Tiedje et al. (2007)
THY77	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δlte1::KanMX6 Δrga2::klTRP1*	This study
THY78	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δlte1::KanMX6 Δbem3::klTRP1*	This study
THY99	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δlte1::KanMX6 Δrga1::klTRP1*	This study
THY497	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 His3MX6-GAL1-3HA-RDI1*	This study
THY521	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δrdi1::klTRP1*	This study
THY524	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δlte1::KanMX6 Δrdi1::klTRP1*	This study
THY529	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 RDI1-9myc-klTRP1*	This study
THY530	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δbem3::klTRP1*	This study
THY541	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δrga2::klTRP1*	This study
THY567	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 RDI1-9myc-klTRP1 KanMX6-GAL1-3HA-STE20*	This study
THY568	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 RDI1-9myc-klTRP1 KanMX6-GAL1-3HA-CLA4*	This study
THY651	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 RDI1-3myc-His3MX6*	This study
THY686	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δlte1::KanMX6 Δrga2::klTRP1 LEU2-CDC14-GFP*	This study
THY687	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δlte1::KanMX6 Δbem3::klTRP1 LEU2-CDC14-GFP*	This study
THY688	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δlte1::KanMX6 Δrdi1::klTRP1 LEU2-CDC14-GFP*	This study
THY689	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 LEU2-CDC14-GFP*	This study
THY690	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δlte1::KanMX6 Δrga1::klTRP1 LEU2-CDC14-GFP*	This study
THY691	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δlte1::KanMX6 LEU2-CDC14-GFP*	This study
THY692	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δrga1::klTRP1*	This study
THY697	*MAT***a** *his3*::*hisG leu2*::*hisG trp1*::*hisG ura3-52 Δste20::hphNT1*	This study
THY705	*MAT***a**/*MAT*α *ura3-52/ura3-52 Δrdi1::hphNT1/Δrdi1::KanMX6*	This study
THY706	*MAT***a**/*MAT*α *ura3-52/ura3-52 Δste20::hphNT1/Δste20::KanMX6*	This study
THY718	*MAT***a**/*MAT*α *ura3-52/ura3-52 URA3-3HA-CDC42*^{G12V}	This study
THY719	*MAT***a**/*MAT*α *ura3-52/ura3-52 URA3-3HA-CDC42*	This study
YPH499	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*	Sikorski and Hieter (1989)

klTRP1 encodes the *Kluyveromyces lactis TRP1* gene.
hphNT1 encodes the *E.coli hph* gene.

Supplementary Table II. **Plasmids used in this work**

Name	**Construction**	**Source or reference**
pEG(KT)	2μm, *URA3, pGAL1-GST*	Mitchell et al. (1993)
pKT10-GAL	2μm, *URA3*, *GAL1*	Masuda et al. (1994)
pKT10-GAL-*RDI1*	pKT10-GAL carrying *RDI1*	Masuda et al. (1994)
pCT22	pEG(KT) carrying *YGK3*	This study
pIS3	pEG(KT) carrying *Rho4(G81V)*	This study
pMB5	pEG(KT) carrying *RHO1*	This study
pMB6	pEG(KT) carrying *RHO2*	This study
pMB7	pEG(KT) carrying *RHO3*	This study
pMB8	pEG(KT) carrying *RHO4*	This study
pMB9	pEG(KT) carrying *RHO5*	This study
pMB10	pEG(KT) carrying *CDC42*	This study
pML2	pEG(KT) carrying *SKM1*	This study
pMVB112	*CEN*, *URA3, GAL1-myc-CLA4(K594A)*	Versele and Thorner (2004)
pMVB113	*CEN*, *URA3, GAL1-myc-CLA4*	Versele and Thorner (2004)
pRS316	*CEN*, *URA3*	Sikorski and Hieter (1989)
pRS425	2μm, *LEU2*	Christianson et al. (1992)
pRS426	2μm, *URA3*	Christianson et al. (1992)
pTH79	pRS426 carrying *CDC42*	This study
pTH246	pRS425 carrying *RDI1*	This study

References

Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H., and Hieter, P. (1992). Multifunctional yeast high-copy-number shuttle vectors. Gene *110*, 119-122.

Mitchell, D.A., Marshall, T.K., and Deschenes, R.J. (1993). Vectors for the inducible overexpression of glutathione S-transferase fusion proteins in yeast. Yeast *9*, 715-722.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics *122*, 19-27.

Tiedje, C., Holland, D.G., Just, U., and Höfken, T. (2007). Proteins involved in sterol synthesis interact with Ste20 and regulate cell polarity. J. Cell Sci. *120*, 3613-3624.

Versele, M., and Thorner, J. (2004). Septin collar formation in budding yeast requires GTP binding and direct phosphorylation by the PAK, Cla4. J. Cell Biol. *164*, 701-715.

Supplemental figure legends

Figure S1. Strong overexpression of *RDI1* is lethal.

(A) Overexpression of *RDI1* from a multi-copy plasmid under control of the endogenous promoter does not affect cell growth. Serial dilutions (1:10) of the indicated strains were spotted on YPD and SC-Leu plates and grown for 2 days at 30°C.

(B) Overexpression of *RDI1* placed under control of the *GAL1* promoter has no effect on cell growth. Serial dilutions of the indicated strains were spotted on a YPD and a YP plate containing 3% raffinose and 2% galactose and were grown for 2 days at 30°C.

(C) Strong overexpression of *RDI1* is lethal. Serial dilutions of cells carrying either *GAL1-RDI1* on a 2μm plasmid (pKT10-GAL-*RDI1*) or the empty plasmid (pKT10-GAL) were spotted on either YPD or SC-Ura plates containing 3% raffinose and 2% galactose and were grown for 2 days at 30°C.

(D) Cells overexpressing *RDI1* do not form a bud. Wild type cells carrying either *GAL1-RDI1* on a 2μm plasmid (pKT10-GAL-*RDI1*) or the empty plasmid (pKT10-GAL) were grown in SC-Ura medium with 3% raffinose. Galactose was added to induce expression of *RDI1*. At the indicated time points cells were fixed with formaldehyde and the number of unbudded cells was determined. At least 100 cells were analyzed at each time point, and the data represent the average of three independent experiments.

(E) Strong overexpression of *RDI1* results in the depolarization of the actin cytoskeleton. Cells were treated as in (D). 6 hours after induction with galactose, cells were fixed with formaldehyde and stained with rhodamine-phalloidin to visualize the actin cytoskeleton.

Figure S2. Rdi1 binds to Cdc42, Rho1 and Rho4.

Co-precipitation of Rdi1 with Cdc42, Rho1 and Rho4. Rdi1-3HA cells carrying constructs encoding *GST-RHO* gene fusions placed under control of the *GAL1* promoter were grown in selective medium in the presence of 3% raffinose. Overexpression of *RHO* GTPases was induced for 3 hours by addition of 2% galactose. Subsequently cells were lysed and precipitated with glutathione sepharose beads. Eluted proteins were analyzed by immunoblotting with anti-GST and anti-HA antibodies.

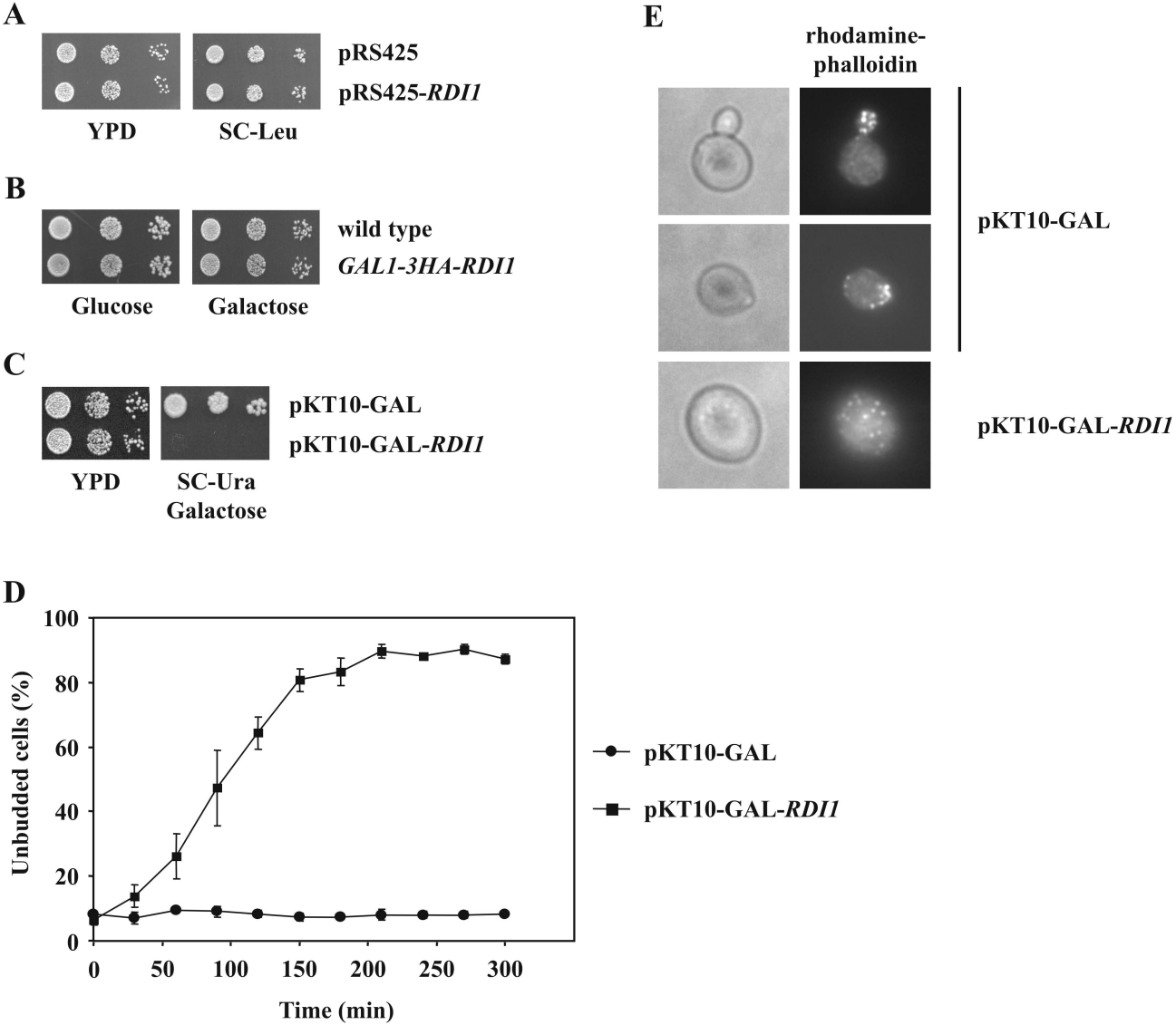
Figure S3. Fractionation of Rho GTPases.

Cells expressing 3HA-tagged Rho proteins were lysed and separated by centrifugation at 100,000 × g to obtain the soluble supernatant and membrane pellet fractions. To compare both

fractions with each other, the pellets were resuspended in the same volume as the supernatants and same volumes were analyzed by Western blotting with antibodies against the HA epitope. The results shown in this figure are representative of two independent experiments.

Figure S4. *CLA4* overexpression and deletion, respectively, do not affect the amount of cytosolic Rho4.
(A) Cells expressing *3HA-RHO4* carrying either *GAL1-myc-CLA4* on a plasmid or the empty plasmid, respectively, were induced for 120 minutes. Cells were lysed and equal amounts of protein extract were separated by centrifugation at 100,000 × g. 3HA-Rho4 was detected by immunoblotting using antibodies against the HA epitope.
(B) Exponentially growing *3HA-RHO4* cells of the wild type and Δ*cla4* background were lysed, fractionated by centrifugation at 100,000 × g and analyzed by immunoblotting.

Figure S5. Rho4 degradation does not depend on the ubiquitin ligase Rsp5.
Cells expressing *3HA-RHO4* in the wild type and *rsp5-1* background, respectively, carrying either *GAL1-RDI1* on a plasmid (pKT10-GAL-*RDI1*) or the empty plasmid (pKT10-GAL) were grown at the permissive temperature of 23°C. Cells were then either kept at 23°C or shifted to 37°C for 2 hours. At the same time galactose was added to induce *RDI1* expression. Equal amounts of protein were analyzed by SDS-PAGE followed by immunoblotting. Note, that at both, 23°C and 37°C, Rho4 degradation is less efficient than at 30°C (e.g. Fig. 5A).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pRS425-RDI1
YPD
SC-Leu
B
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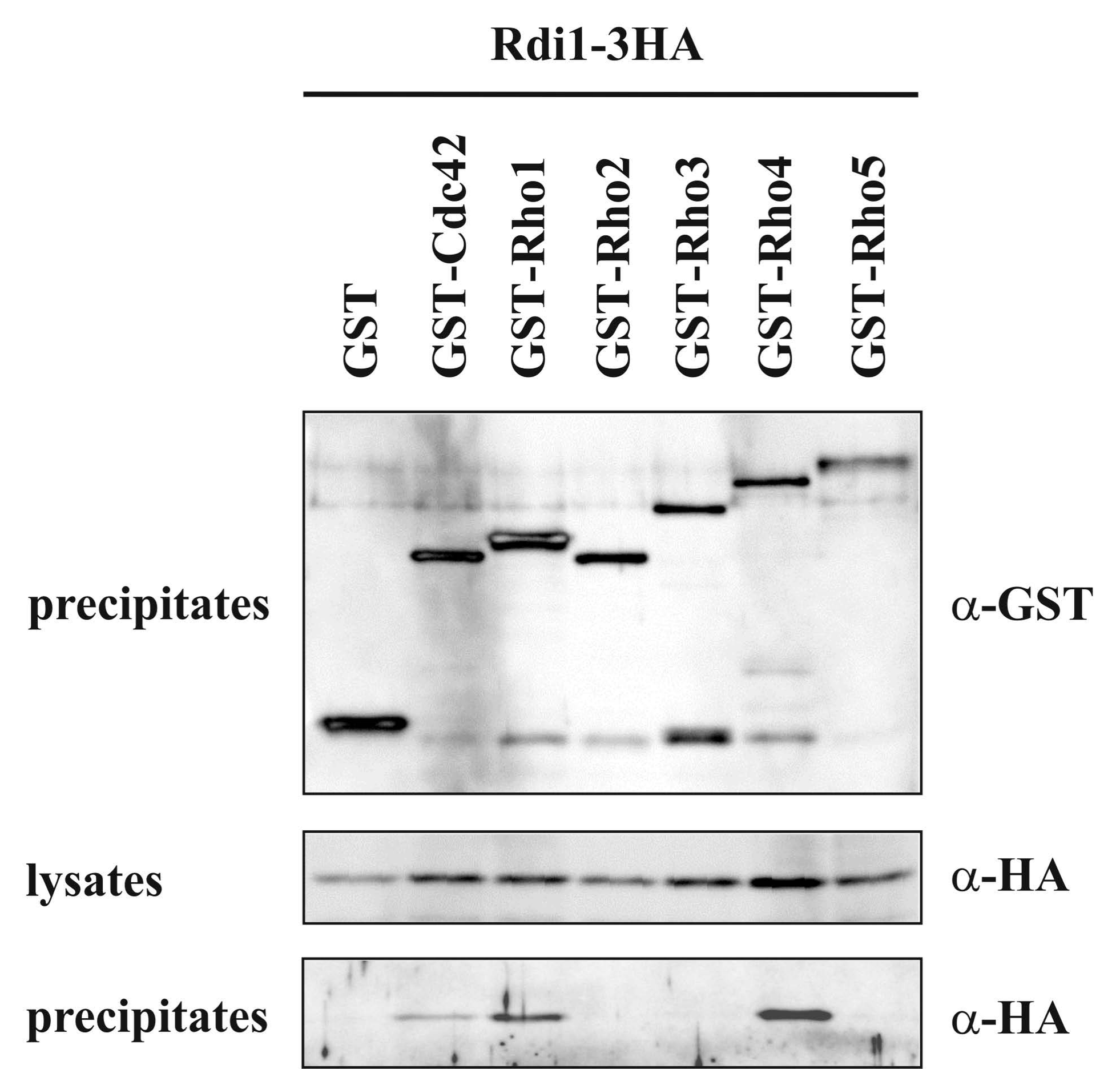
GAL1-3HA-RDI1
Glucose
Galactose
C
pKT10-GAL
pKT10-GAL-RDI1
YPD
SC-Ura
Galactose
E
rhodamine-
phalloidin
pKT10-GAL
pKT10-GAL-RDI1
D
Unbudded cells (%)
100
80
60
40
20
0
0
50
100
150
200
250
300
Time (min)
pKT10-GAL
pKT10-GAL-RDI1


Rdi1-3HA
GST
GST-Cdc42
GST-Rho1
GST-Rho2
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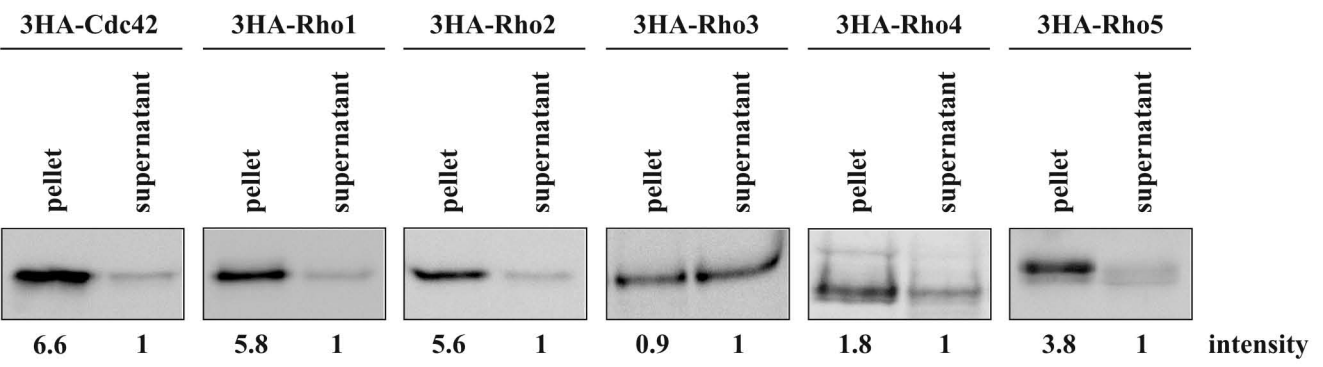
GST-Rho4
GST-Rho5
precipitates
α-GST
lysates
α-HA
precipitates
α-HA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3HA-Rho1
3HA-Rho2
3HA-Rho3
3HA-Rho4
3HA-Rho5
pellet
supernatant
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pellet
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pellet
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pellet
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1
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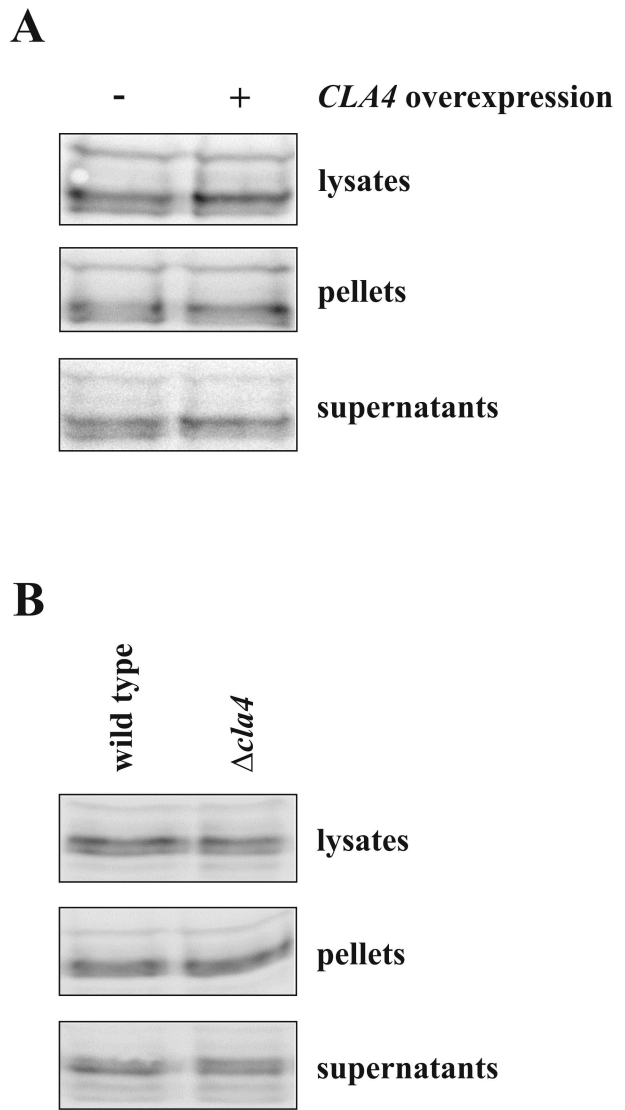
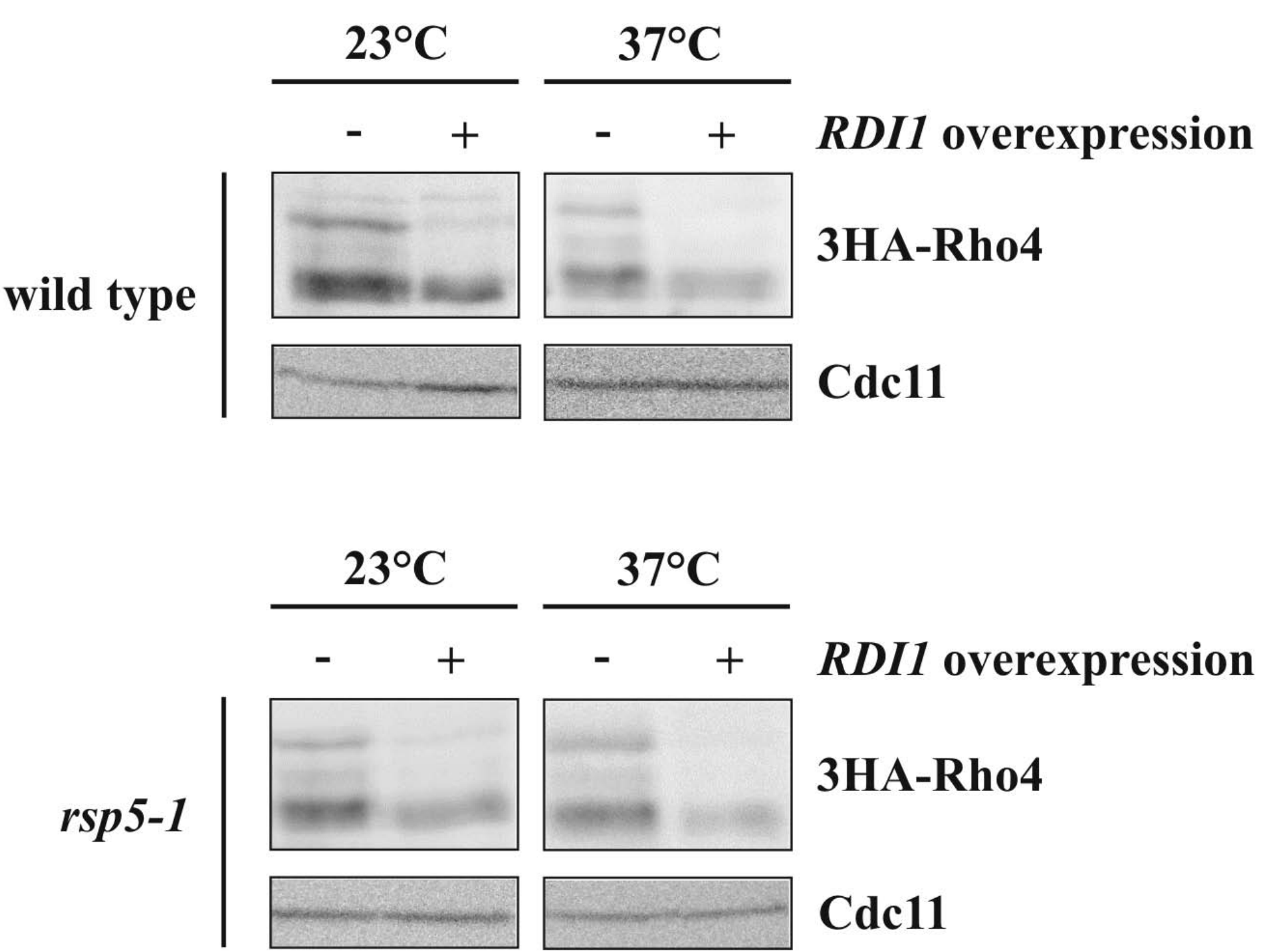
1
3.8
1
intensity


A
-
+
CLA4 overexpression
lysates
pellets
supernatants
B
wild type
Δ*cla4*
lysates
pellets
supernatants


23°C
37°C
- + - +
RDI1 overexpression
wild type
3HA-Rho4
Cdc11
23°C
37°C
- + - +
RDI1 overexpression
rsp5-1
3HA-Rho4
Cdc11