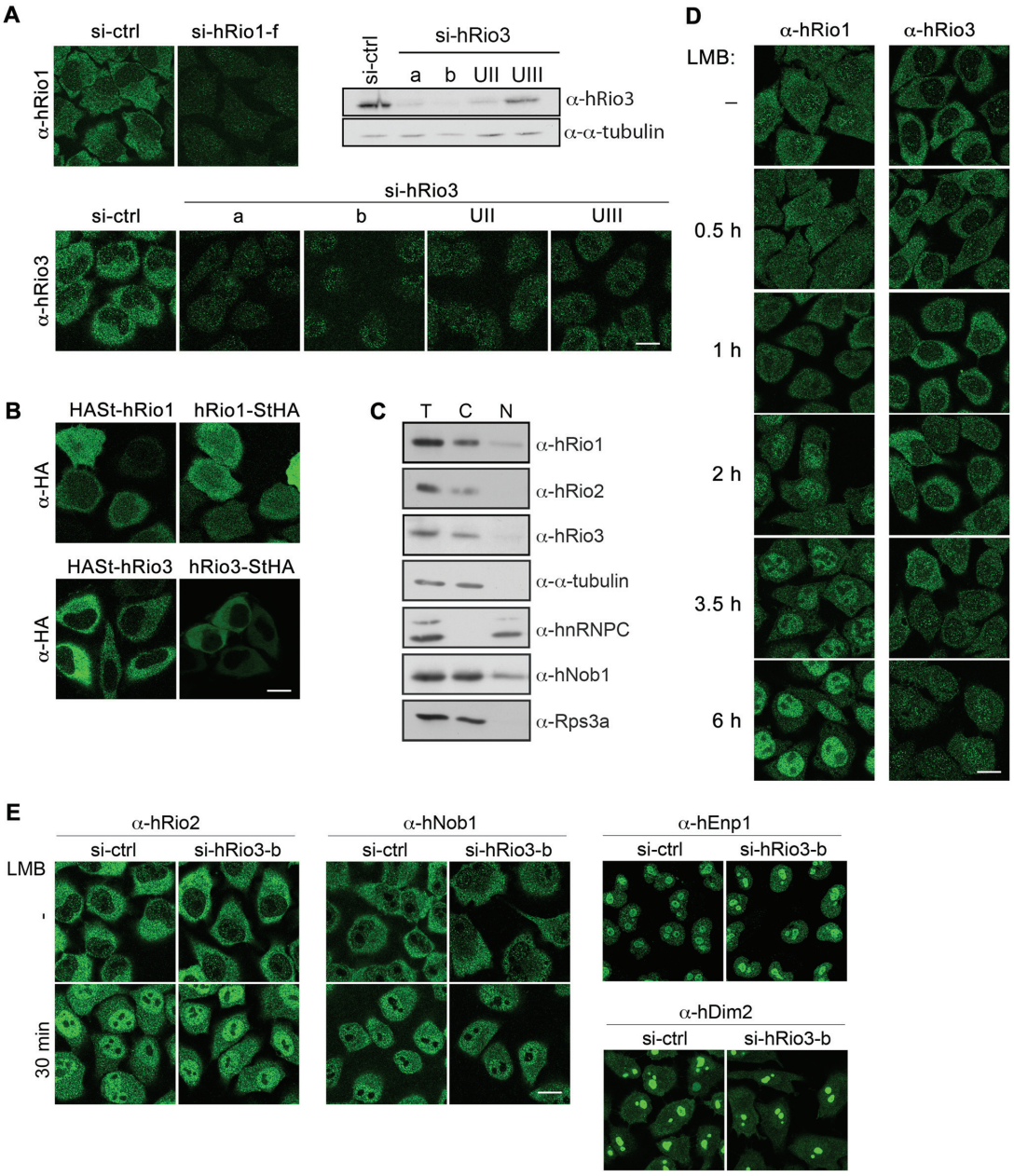

Figure S1. Localization of hRio1 and hRio3. Analysis of the effect of hRio3 depletion on the localization of 40S trans-acting factors.

(A) HeLa cells were depleted for hRio1 or hRio3 for 72 hrs. For hRio3, four different siRNAs targeting the open reading frame (a, b) or the 3'UTR (UII, UIII) were used. The efficiency of downregulation was analyzed by immunostaining and Western blotting. The siRNA hRio1-f (Figure 3A) was used to verify the specificity of the α–hRio1 antibody. Scale bar is 20 μm.
(B) HeLa cells were transiently transfected with HASt-hRio1, hRio1-StHA, HASt-hRio3 or hRio3-StHA. Cells were fixed after 24 hrs and the localization of the tagged hRio proteins analyzed by α–HA immunostaining. Scale bar is 20 μm.
(C) A hypotonic HeLa cell extract was separated into nuclear and cytoplasmic fractions. Total cell extract (T), cytoplasmic extract (C), and nuclear extract (N) were analyzed by Western blotting using the indicated antibodies. Whereas hRio2 and hRio3 are only found in the cytoplasmic fraction, a small amount of hRio1 is also found in the nuclear fraction. Tubulin and hnRNPC served as controls for fractionation.

(D) HeLa cells were treated with 20 nM LMB or solvent (ethanol, -) for the indicated times, followed by fixation and immunostaining with α–hRio1 or α–hRio3 antibodies. Upon CRM1 inhibition, hRio1 and hRio3 accumulate in the nucleoplasm after 2 hrs and 6 hrs, respectively. Scale bar is 20 μm.
(E) hRio3 was depleted from HeLa cells using si-hRio3-b for 72 hrs. Cells were fixed and the localization of the indicated 40S trans-acting factors was analyzed by immunostaining. Scale bar is 20 μm.

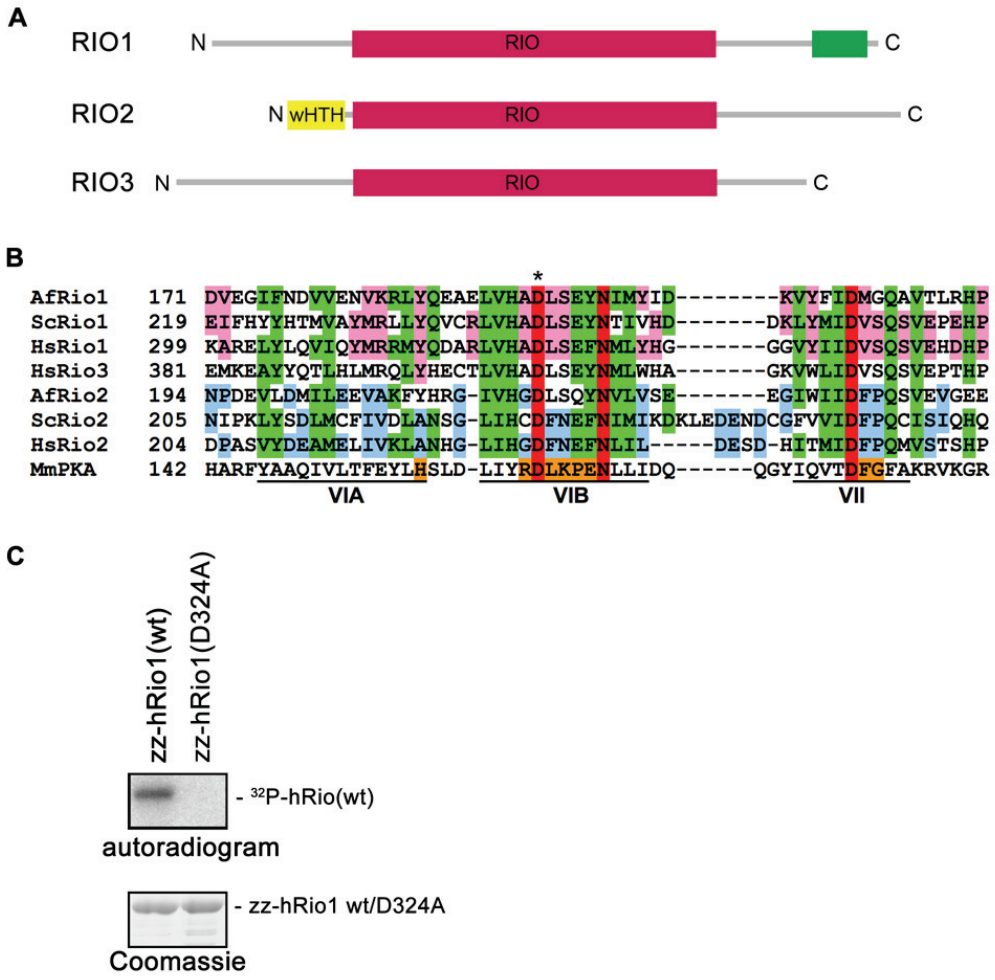

Figure S2. Asp324 is the catalytic base in hRio1.

(A) Schematic representation of the three subfamilies of RIO kinases. Magenta: RIO domain. Green: C-terminal domain of unknown function conserved in Rio1 kinases. Yellow: N-terminal winged helix-turn-helix (wHTH) domain of Rio2 proteins.
(B) Alignment of RIO kinases to PKA as a reference for eukaryotic protein kinases. Roman numerals mark the subdomains of the kinase domain. Color code of amino acids: Red: invariant in ePKs. Orange: highly conserved in ePKs. Green: conserved in RIO kinases. Rose: conserved in Rio1 subfamily. Light blue: conserved in Rio2 subfamily. Af *Archaeoglobus fulgidus*, Sc *Saccharomyces cerevisiae*, Hs *Homo sapiens*, Mm *Mus musculus*. The asterisk marks the conserved Asp mutated in hRio1(D324A).
(C) zz-tagged recombinant hRio1(wt) and hRio1(D324A) were tested for autophosphorylation activity in an in vitro kinase assay in the presence of ^{32}P-γ-ATP. Mutation of Asp324 to Ala in hRio1 results in the loss of its kinase activity.

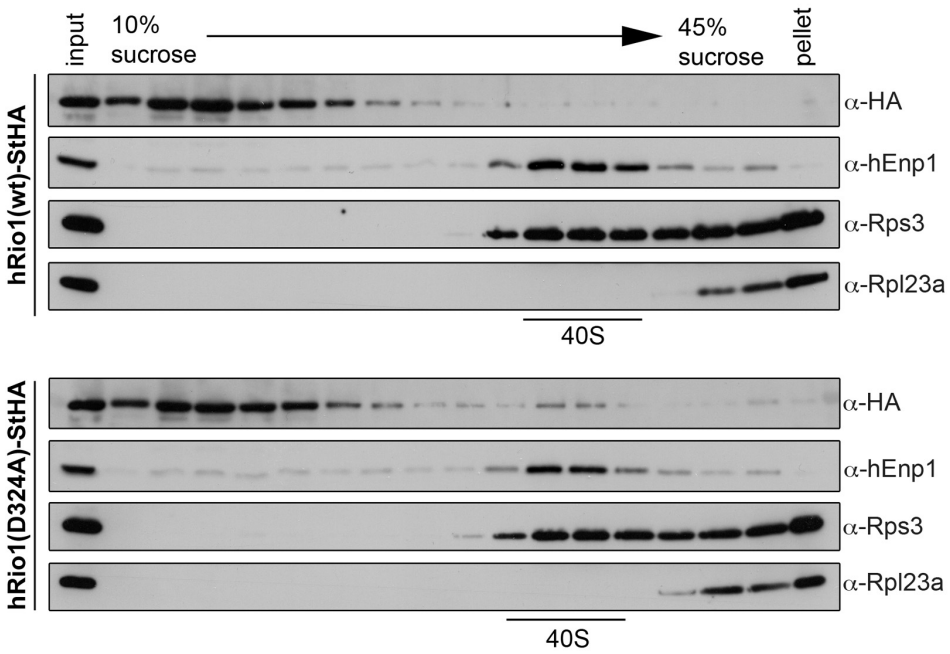

Figure S3. Sucrose gradient analysis of cell extracts from cells expressing hRio1(wt)-StHA or hRio1(D324A)-StHA.

Cell extracts of HEK293 cells expressing either hRio1(wt)-StHA (upper part) or the kinase inactive hRio1(D324A)-StHA mutant (lower part) were fractionated on TLS55 sucrose gradients (100 mM KCl) and analyzed by Western blotting using the indicated antibodies. Note that hRio1(D324A)-StHA partially cofractionates with (pre)-40S.

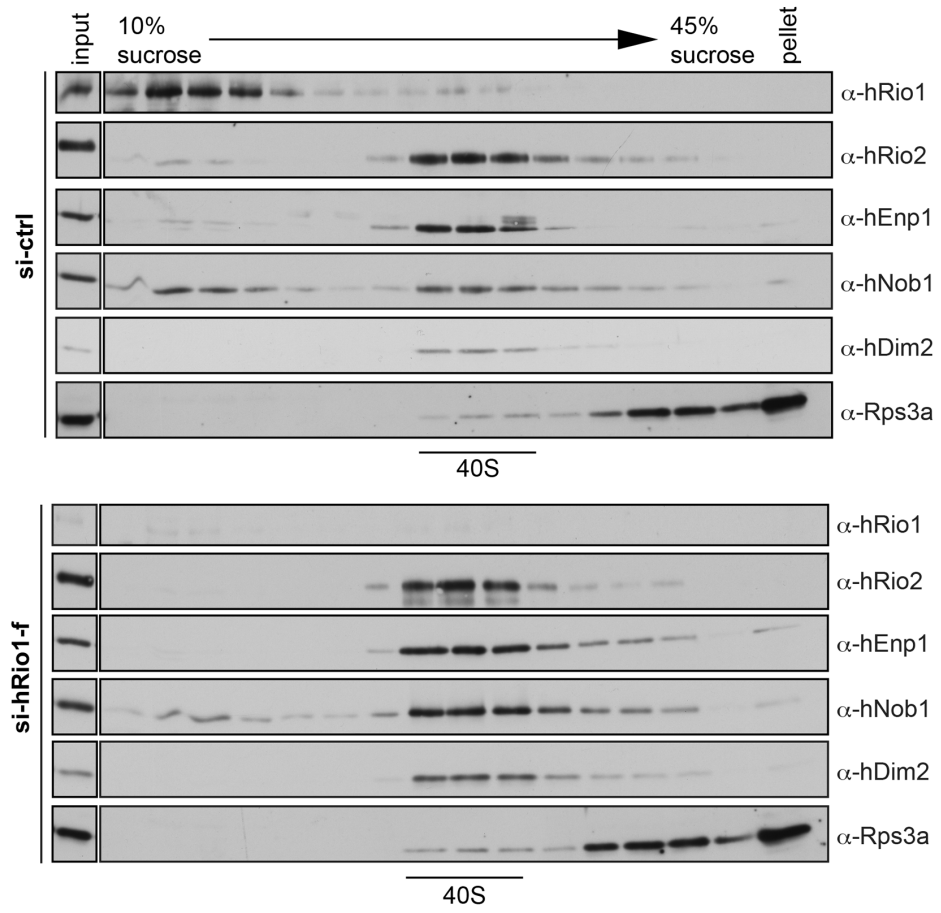

Figure S4. Sucrose gradient analysis of cell extracts after hRio1 depletion.

Hypotonic HeLa cell extracts of control cells (upper panel) and hRio1 knockdown cells (lower panel) were fractionated on TLS55 sucrose gradients and analyzed by Western blotting using the indicated antibodies. hEnp1, hDim2, hNob1 and hRio2, which are 40S-associated in control cells, still co-sediment with 40S after depletion of hRio1.

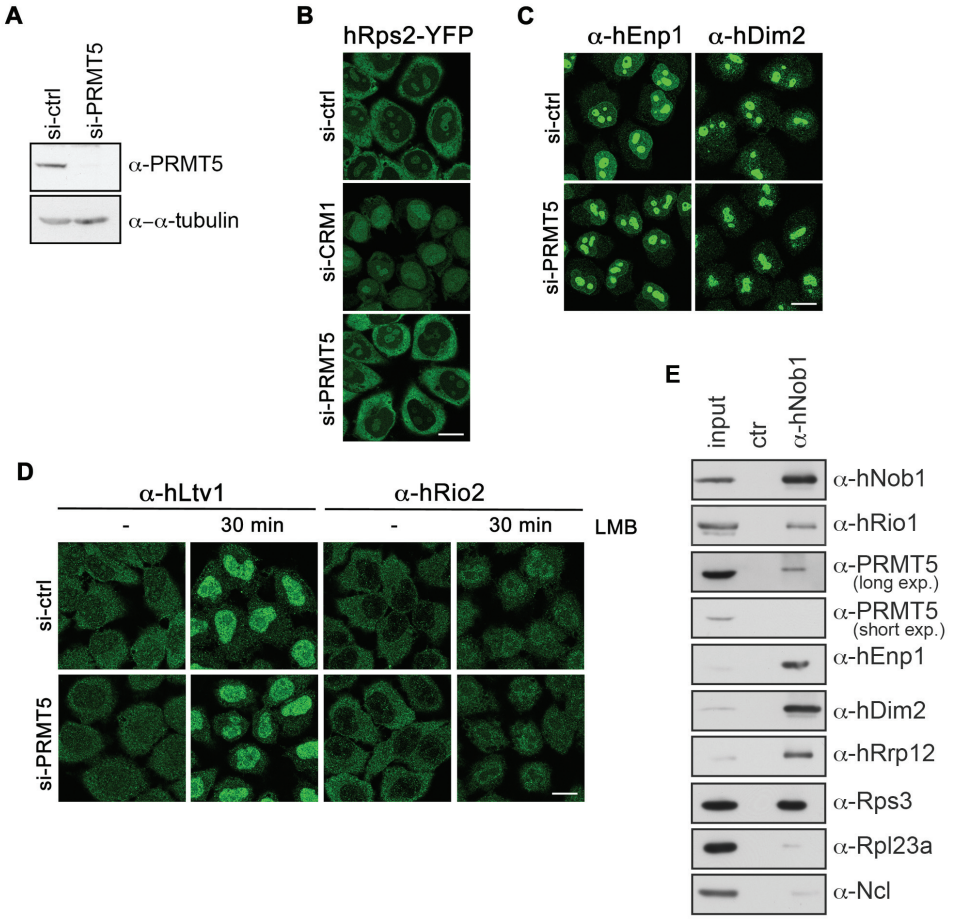

Figure S5. Depletion of PRMT5 by RNAi does not affect the localization of several reporter proteins of 40S biogenesis.

(A) Hela cells were treated with control or PRMT5 siRNAs for 72 hrs. Downregulation of PRMT5 was analyzed by Western blotting.
(B) hRps2-YFP HeLaK reporter cells were transfected with the indicated siRNAs. Expression of hRps2 was induced as described (Zemp *et al.*, 2009) after 52 hrs by addition of doxycycline for 16 hrs, followed by incubation in doxycycline-free medium for another 4 hrs. After altogether 72 hrs, cells were fixed and analyzed by confocal fluorescence microscopy.
(C) HeLa cells were treated as in (A). Cells were fixed and the localization of hEnp1 and hDim2 was analyzed by immunostaining, followed by confocal fluorescence microscopy.
(D) HeLa cells were treated as in (A). 30 min before fixation, cells were treated with 20 nM LMB or solvent (ethanol, -). The localization of hLtv1 and hRio2 was analyzed by immunostaining, followed by confocal fluorescence microscopy. Scale bars are 20 μm.
(E) hNob1 was immunoprecipitated as in Figure 1C (150 mM K acetate) from total HeLa cell extract using purified α-hNob1 antibodies coupled to beads. Empty beads served as control (ctr). Input extract and elutates were analyzed by immunoblotting using the indicated antibodies.

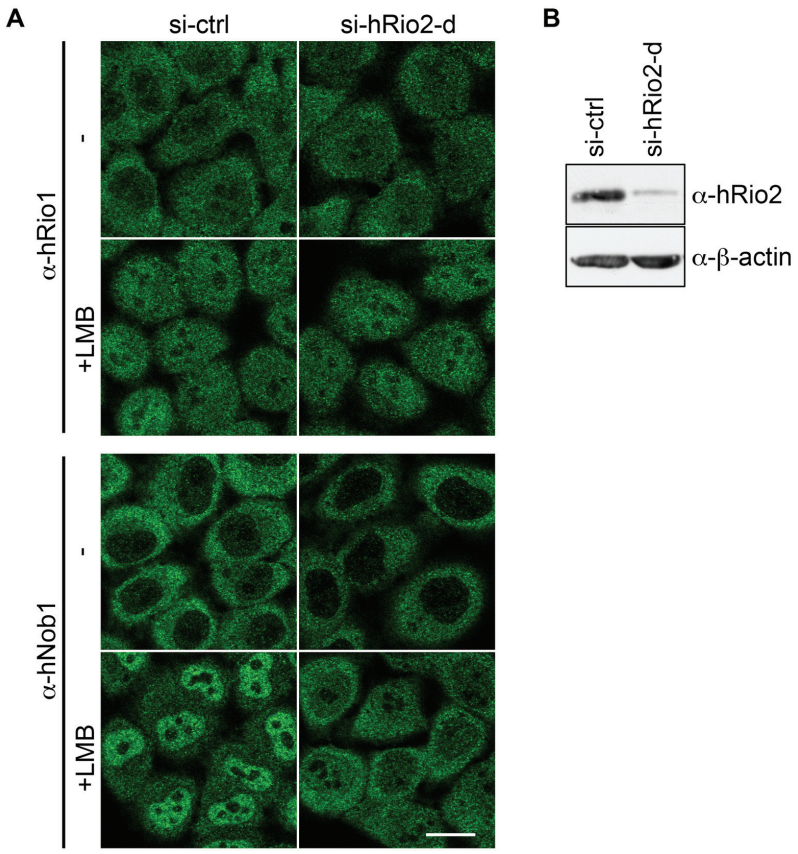

Figure S6. Depletion of hRio2 by RNAi does not affect the localization of hRio1.

(A) HeLa cells were treated with control or hRio2 siRNAs (9 nM) for 70 hrs. Then, cells were either treated with 20 nM LMB or solvent (ethanol, -) for 120 min. Cells were fixed and the localization of hRio1 analyzed by immunostaining, followed by confocal fluorescence microscopy. Localization of hNob1 was analyzed as a positive control, revealing delayed nuclear accumulation of hNob1 upon hRio2 depletion. Scale bar is 20 μm.
(B) Downregulation of hRio2 was analyzed by Western blotting. Immunoblotting of actin served as loading control.

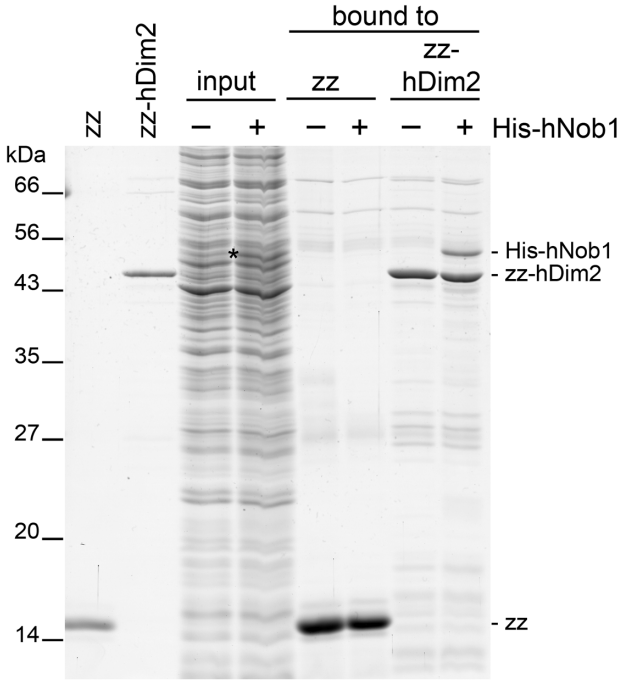

Figure S7. Direct binding of hNob1 to hDim2.

100 μl *E.coli* lysate or *E.coli* lysate supplemented with 4 μM purified, recombinant hNob1 (input,*) was supplemented with either zz or zz-hDim2 (each 2 μM), and incubated on ice for 2 hrs. Then, zz-proteins and bound material were retrieved on IgG sepharose beads. After washing three times with buffer (50 mM Tris (pH 7.6), 225 mM potassium acetate, 3 mM $MgCl_2$), bound proteins were eluted with SDS sample buffer and separated by SDS-PAGE followed by Coomassie staining.

Table S1: Number of unique peptides detected for proteins annotated in Figure 2.

hRio1(wt)-StHA	peptides
PRMT5	18
MEP50	8
CK2 a	12

hRio1(D324A)-StHA	peptides
hRio1:	32
PRMT5:	40
hNob1:	11
MEP50:	16
CK2 α:	19
CK2 α':	6
GNB2L1 (RACK1)	16
Rps2:	14
Rps3A:	16
hDim2 (PNO1):	10
Rps6:	8
Rps3:	27
Rps4:	23
Rps2:	11
Rps9:	21
Rps5:	10
Rps18:	18
Rps11:	17
Rps13:	9
Rps25:	6
Rps10:	7
Rps17:	8
Rps23:	6
Rps16:	12
Rps19:	10
Rpl23:	9
Rps14:	9
Rps15a:	9
Rps23:	6