# **Supporting Information**

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#### **SI Materials and Methods**

Protein Expression and Purification. Cultures were grown in Luria-Bertani (LB) media to an  $OD_{600}$  of 1.0, at which point protein expression was induced by addition of isopropyl-beta-Dthiogalactopyranoside (IPTG) to a final concentration of 150  $\mu$ M. Culture growth was continued overnight at 23 °C, after which cells were harvested and resuspended in GST pulldown buffer (50 mM Tris, pH 7.5, 300 mM KCl, 5 mM DTT). The cell suspension was lysed using an EmulsiFlex homogenizer (Avestin) and cleared by centrifugation for 45 min at  $28,000 \times g$ . The fusion protein was purified from the cleared lysate by passage over Uniflow glutathione-agarose resin (Clontech), and the GST tag was removed from the eluted protein by thrombin digestion. Final purification was conducted by anion exchange (MonoQ; GE Healthcare) and gel filtration chromatography (S200; GE Healthcare), leaving the protein in a final buffer of 10 mM Tris, pH 7.5, 100 mM KCl, and 2 mM DTT at a concentration, as measured by UV absorbance, of 10 mg/mL. Selenomethionesubstituted Cog4-(525-785) (SeCog4) was purified in the same way, except using E. coli strain B834, and with 10 mM DTT added to all buffers.

**Crystallization Structure Determination.** The native crystals used for data collection were grown by hanging drop vapor diffusion in drops containing a 1:1 ratio of protein stock to well buffer (5% PEG-4000, 10 mM ammonium nitrate, 2.5% glycerol, 10 mM DTT). SeCog4-(525–785) crystals were grown by vapor diffusion in hanging drops containing a 1:1 ratio of protein stock to well buffer (4.5% PEG-8000, 8 mM ammonium nitrate, 3% glycerol, 5 mM DTT). Native Cog4-(525–785) crystals were cryoprotected using mother liquor supplemented with 20% (wt/vol) PEG-4000 and 30% (vol/vol) glycerol, while SeCog4-(525–785) crystals were transferred stepwise through intermediate buffers of 2:1 and 1:2 mother liquor:cryoprotectant to minimize osmotic stress on the crystal.

**Quantitative Co-Immunoprecipitation.** Stable *COG4* KD HeLa cells expressing different Cog4-myc mutant constructs for 72 h were lysed on ice in 1 mL DPBST (DPBS, 1% Triton X-100, and

protease inhibitor mixture; Roche Applied Science). Lysates were incubated with 30  $\mu$ L Protein A-Sepharose CL-4B resin, mixed by gentle inversion for 1 h on ice, and centrifuged 10 min at 20,000× g. Supernatants were transferred to new Eppendorf tubes containing 3  $\mu$ g affinity-purified antibodies against human Cog6 and incubated overnight on ice. After centrifuging as above, 60  $\mu$ L Protein G-Agarose resin (50% slurry) were added to each tube and incubated for 2 h with gentle inversion at 4 °C. The beads were then washed 4 times with TBST, transferred to new Eppendorf tubes, resuspended in 60  $\mu$ L 2× SDS/PAGE sample buffer, and heated 5 min at 95 °C to elute bound proteins. Subsequent immunoblotting was performed with anti-Myc and -Cog6 antibodies.

Analysis of Glycosylation Defects. For analysis of glycosylation defects, transfected cells were grown in DMEM/F-12 medium supplemented with 15 mM HEPES, 2.5 mM L-glutamine, 10% FBS, and 1  $\mu$ g/mL puromycin for 70 h, after which plasma membranes were stained with Alexa-647-labeled GNL for 30 min on ice (to prevent endocytosis), fixed with 4% paraformal-dehyde, and imaged using a Zeiss LSM510 confocal microscope. The resulting images were quantified by manual counting of the percentage of cells stained with GNL (n > 100).

**CPY Secretion Assay.** Triplicate 3-day-old single colonies of yeast strains were grown overnight in YPD, back-diluted to an OD<sub>600</sub> of 0.1 in fresh YPD, and grown for 6 h at room temperature. The cells were removed by centrifugation and lysed in 0.2 M NaOH, and the remaining media was concentrated 10-fold by trichlo-roacetate precipitation. The equivalent of 100  $\mu$ L media and 10  $\mu$ L cells were analyzed for CPY by western blotting; band densities were quantified using ImageJ.

**Antibodies.** Antibodies used for western blotting were generated by us or purchased from commercial sources. Antibodies (and their dilutions) were as follows: Rabbit affinity-purified anti-hCog4 (0.8  $\mu$ g/mL; R.S. and V.V.L.), anti-hCog6 (0.5  $\mu$ g/mL; R.S. and V.V.L.), anti-hCog6 (0.5  $\mu$ g/mL; R.S. and V.V.L.), anti-myc (1:10,000; Bethyl Laboratories), mouse monoclonal anti- $\beta$ -actin (1:10,000; Sigma), and anti-CPY (0.5  $\mu$ g/mL; Molecular Probes).



Fig. S1. Comparison of the two Cog4-(525–785) monomers composing each crystallographic asymmetric unit. Trp 721, which lies in a crystal contact, is highlighted.

DNAS

Structure		H1 H2 H2	
H. sapiens	526	OGKFDTKGIESTDEAKMSFLVTL <mark>NN</mark> VEV <mark>SENISTL</mark> KKTLESDCTKLFSOGIGGEOAOAKFDSCLSDLAAVSNKFRDLLOEGLTELNSTAIKP	618
B. taurus	526	OGKFDTKGIESTDEAKLSFLVTLNNVEVCSENISTLKKTLESDCTKLFSOGIGGEOAOAKFDSCLSDLAAVSGKFRDLLOEGLTELNSTAIKP	618
D. rerio	518	OGKITNLTOTLGIESOEOAKSAYLVTINNVEVCSENISTLKKNLESDCAKLFSOGASSDHAKEKIDSCLSDLVNTSSKFKDLLOEGLOELNNTAIRP	614
D. melanogaster	r520	OGKLHSSDADRGRANFLVOLNNADISTEYIETLCOTMEOEIAGTFPOTTOVEROMLDSCLTELKAVRDALKATVDFGMOOLRSSVIKP	607
C. albicans	615	GAIVGSLOTTPNNTKLISFIIYINTVAMAOEYFTKVFONINKDSYLOSYYPFGKDKSKISNILOODFLDPFTSVSNKIISESLINLYNOSIKN	707
S. cerevisiae	586	OAVVAEDEDSILALHHYLIYLNYLYLSKYYVHRLLSIEILEDDSORILRDNFPFDNDAAOLONLIIN-SEKLVLEOTDKLSKWAVKYLFONILON	679
Structure		H3 H4 H5 H6 H6 -	
H. sapiens	619	OVOPWINSFFSVSHNIE	694
B. taurus	619	ovopwintflsvshnie	694
D. rerio	615	OVKPWISSFLSVSHNIE	690
D. melanogaster	r 608	RINPWINOFLNYSHNIN	683
C. albicans	708	KLLLLVNEFFTDIPTANNENNYVIYSSNNINDPTILIKFTSNWOSLMKPYLOTLHKT-LWSKLLRLVVVNLTNLLPKKLFMILNKL	792
S. cerevisiae	680	RVRNLLGTVFVNSASSNSSTSNQKNVSRDYSAGSNQKNYIT <mark>S</mark> IEDFEDLSQINS <mark>F</mark> NSKWNQLIIPYKNILHNE-AYAELLSVIVDY <mark>IV</mark> TTL <mark>C</mark> QRIWTL	776
Structure			
H. sapiens	695	TENRLCCLOFDKELRSLIAYLTTVATWITCOKBARLSOMATILNDERVIE ILDYWGPNSGPLTWRLTPAEVROVLALEIDFRSEDIKRLRL	785
B. taurus	695	TENELCELOFDERELESLIAYLTTV#TWTIEDERAFLSOMATILNL®RVT®ILDYWGANSGPLTWELTPASVROVLALRIDFRS®DIKELFL	785
D. rerio	691	TESRLCCLOFDKELRALVAYLSSVESWTIRDKEARLTOMATILNLERVSEILDYWGPNSGPLTWRLTPASVROVLALRVDFRSEDIKRLRL	781
D. melanogaster	r 684	SENELCCLVLDOPVRALCSYLTGARSWSVRDKVTRISOTATLINDKITELSEYWNPENNKEMSSWHLTPNEVRTFLTRNDFRIEDIKRLOL	776
C. albicans	793	KINELCATKIEKDVSYLINEICRDNYIREKOVRITOIVLLVCMDEEWEESNOPVTKARETEEDGGEGRDDFDEIGCINWVLTPOERIOIRKYRI	889
S. cerevisiae	777	EFNELCYTKUDRELSLFIGMMCGLNYNLREKALKITOIVLLLGUDDDNADLTTGDIKDDENGTADWVINSOERIKASMMKIDRT	860

Fig. S2. Multiple sequence alignment of Cog4 homologs. The top 83 PSI-BLAST results from a search using *H. sapiens* Cog4 were aligned using ClustalW; a selection of aligned sequences is shown. Squares below the sequence indicate residues that mediate interdomain contacts; triangles represent residues mutated in Figs. 2 and 3.

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WT	vp	s1	N60 T60	7A 8A	<u>N60</u>	07A	T60	<u>88</u>	
ML	Μ	L	Μ	L	Μ	L	Μ	L	
			A ST	-	111	-	1.11	•	Unprocessed Processed Underglycosylated

**Fig. S3.** Mutational analysis of Cog4p function in *S. cerevisiae*. (*A*) Plasmids encoding truncated *S. cerevisiae* Cog4p (represented as in Fig. 2*A*) were tested for their ability to support growth as the sole source of *COG4* after plating on 5FOA. Color coding is as in Figs. 1 and 2. (*B*) Cog4p missense mutants were tested for growth defects on YPD at 23 ° C and 37 °C. (C) Mislocalization/secretion of CPY was measured by comparing intracellular (cell lysate: L) and extracellular (10-fold concentrated cell-free media: M) pooled triplicate samples by western blotting.



Fig. S4. Comparison of the Cog4-(525–785) dimerization interface to the Sec6p structure. (A) End-on view of the Cog4-(525–785) dimer. (B) As in panel A, but with the aligned Sec6p (2FJI, residues 411–805 out of 805) in place of the second (gray) Cog4-(525–785) monomer.



Fig. S5. Interdomain interaction mediated by N551 (human)/T608 (yeast).

**v** 



Fig. S6. Western blot analysis of Cog4 levels in stable shCOG4 transfected cells. Proteins from total lysates of parental HeLa T2-GFP cells and no. 39 stable shCOG4 cells were analyzed by western blotting using antibodies against Cog4 and actin.

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## Table S1. Phasing and refinement statistics

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		SeMet MAD				
	Native	λ1 (peak)	$\lambda 2$ (inflection)	$\lambda$ 3 (remote)		
Resolution, Å	50–1.9 (1.97–1.90)	50–2.2 (2.28–2.20)	50–2.2 (2.28–2.20)	50–2.2 (2.28–2.20)		
Wavelength, Å	1.0809	0.9792	0.9794	0.9611		
Completeness, %	99.3 (98.2)	99.7 (98.7)	99.7 (97.4)	99.2 (93.5)		
R <sub>sym</sub> , %	7.7 (35.7)	6.5 (51.4)	6.6 (54.8)	6.8 (65.6)		
Redundancy	5.4 (5.1)	5.3 (3.7)	5.3 (3.4)	5.2 (2.9)		
Total reflections*	406,090	491,197	492,460	479,448		
Unique reflections*	74,854	92,779	92,872	92,524		
<i>Ι/σ(I)</i>	26.5 (3.8)	25.7 (2.4)	25.2 (2.1)	22.9 (1.6)		
Space group	<i>P</i> 3 <sub>2</sub> 21					
Unit cell, Å						
a = b	87.1	86.7	86.7	86.7		
с	214.8	217.7	217.7	217.7		
MAD phasing						
Figure of merit			0.52			
Number of Se sites			10			
Refinement						
R <sub>work</sub> (R <sub>free</sub> ), %	19.4 (22.2)					
Reflections in work (free) set	71,032 (3,753)					
% reflections set aside for R <sub>free</sub>	5					
Number of atoms						
All atoms	4,375					
Protein	3,990					
Waters	393					
Average <i>B</i> factor, Å <sup>2</sup>						
All atoms	28.9					
rmsd						
Bond length, Å	0.013					
Bond angle, °	1.217					
B factor across main chain bonds, Å <sup>2</sup>	2.387					
Ramachandran statistics						
Most favored, %	96.5					
Additional allowed, %	3.2					
Generously allowed, %	0.2					

\*The number of reflections is lower in the higher-resolution native dataset due to the treatment of I+ and I- as distinct reflections in the MAD datasets only.

#### Table S2. Yeast strains used in this work

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Strain	Genotype	Source
MS3128	MAT a ura3–52 leu2–3,112 his3∆200 trp1∆1 vps1::LEU2	M. Rose, Princeton University
LY314	MAT $\alpha$ ura3 leu2 his3 cog4 $\Delta$ ::Gm pNB167 (2 $\mu$ , YPT1, URA3)	Lupashin lab (10)
RG25512	his $3\Delta1$ /his $3\Delta1$ leu $2\Delta0$ /leu $2\Delta0$ lys $2\Delta0$ /LYS2 MET15/met15 $\Delta0$ ura $3\Delta0$ /ura $3\Delta0$	Invitrogen
	COG4/cog4∆::KanMX	
BCR13	his $3\Delta 1 \ \text{leu} 2\Delta 0 \ \text{lys} 2\Delta 0 \ \text{ura} 3\Delta 0 \ \text{cog} 4\Delta$ ::KanMX pBCR114 (CEN URA3 COG4)	This study
BCR17	his3∆1 leu2∆0 lys2∆0 ura3∆0 cog4∆::KanMX pBCR95 (CEN LEU2 COG4)	This study
BCR19–51	As pBCR17, with mutant cog4 plasmids as indicated in Table 3.	This study

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## Table S3. Summary of phenotypes

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		Yeast	Yeast	Human GNL
Human mutant	Yeast mutant	strain	growth	staining
ΔQ525–785	ΔP564–861		Lethal	+
ΔP618–785	∆N679–861		Lethal	+
∆R741–785	∆D822–861	BCR19	WT	+
N549A	N607A	BCR51	WT	
N550A	T608A	BCR50	WT	
N549A N550A	N607A T608A	BCR29	Ts	-
I558D	V616D	BCR37	WT	
V596D	V657D	BCR38	WT	
K664A	K746A	BCR35	WT	
K664E	K746E	BCR36	WT	
E688A				-
K706E	R788E	BCR33	WT	
D705A K706A	D787A R788A	BCR32	WT	
A713D T716D	G795D C798D	BCR39	WT	
R724E	R805E	BCR31	WT	
R724A D725A	R805A E806A	BCR30	WT	
R729A	K810A	BCR21	WT	+
R729W	K810W	BCR41	WT	+
\$731D	T812D	BCR40	WT	
E740A	D821A	BCR24	WT	
E764A	E848A	BCR23	WT	++
E764R	E848R	BCR25	WT	
R729A E764A	K810A E848A	BCR26	WT	
D774A	D858A	BCR20	WT	
D774R	D858R	BCR22	WT	
E740A D774A	D821A D858A	BCR28	WT	
E740R D774R	D821R D858R	BCR27	WT	
D705A K706A A713D T716D R724A D725A S731D	D787A R788A G795D C798D R805A E806A T812D	BCR43	Ts	
A713D T716D R724A D725A S731D	G795D C798D R805A E806A T812D	BCR48	WT	
D705A K706A R724A D725A \$731D	D787A R788A R805A E806A T812D	BCR46	WT	
D705A K706A A713D T716D \$731D	D787A R788A G795D C798D T812D	BCR42	WT	
D705A K706A A713D T716D R724A D725A	D787A R788A G795D C798D R805A E806A	BCR47	Ts	-
A713D T716D R724A D725A	G795D C798D R805A E806A	BCR44	WT	
R724A D725A \$731D	R805A E806A T812D	BCR45	WT	
D705A K706A R724A D725A	D787A R788A R805A E806A	BCR49	WT	