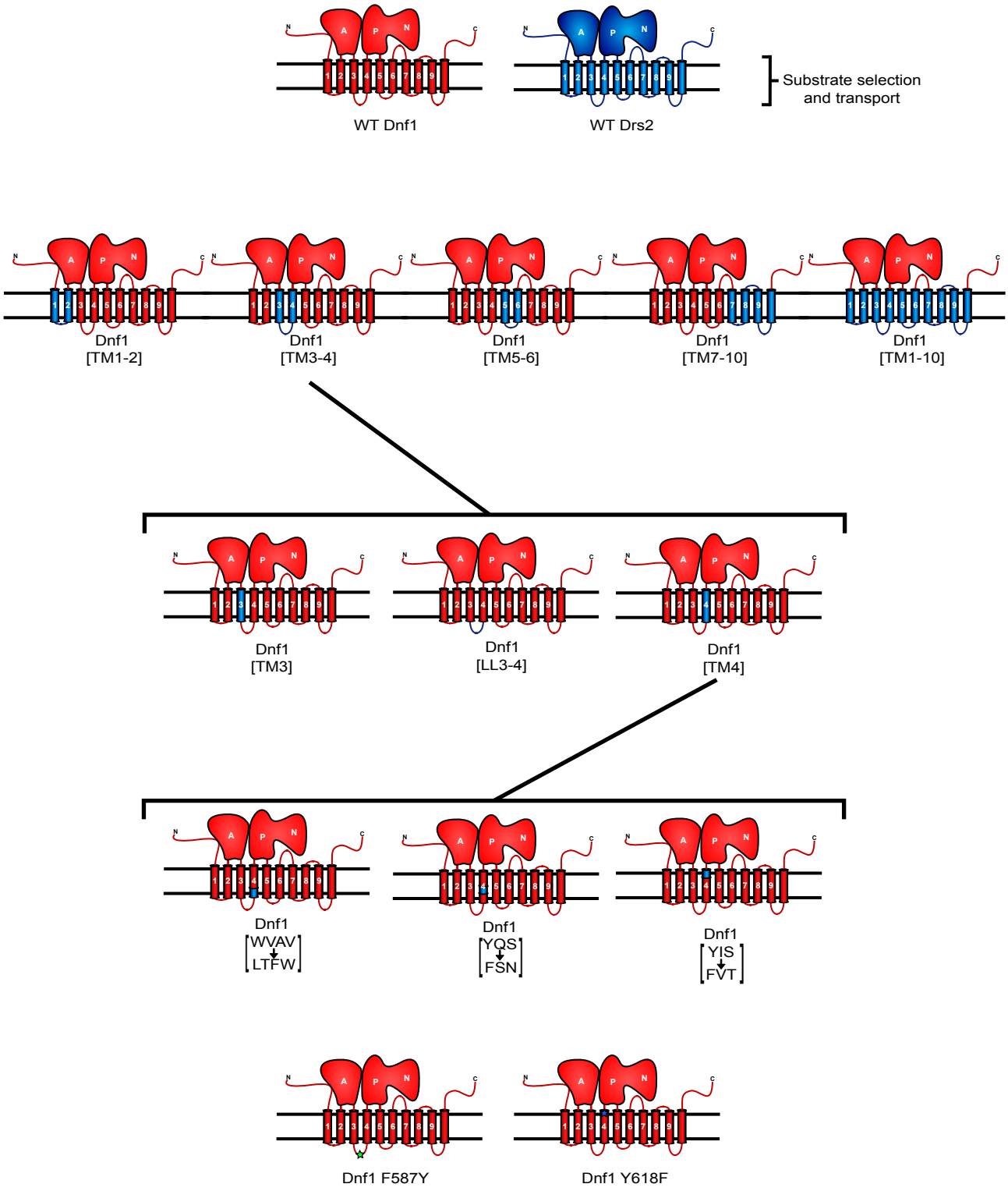

Figure S1. Topological diagrams of WT Dnf1 (red), WT Drs2 (blue) and most of the chimeras reported in this study. Black lines are membrane boundaries. A is the actuator domain, N is the nucleotide binding domain and P is the phosphorylation domain.

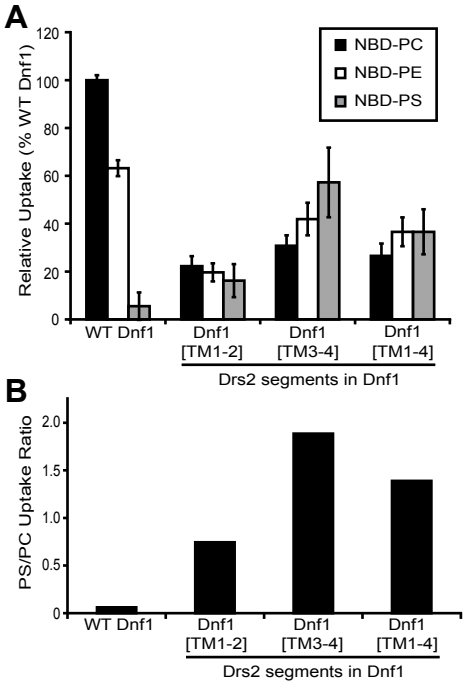

Figure S2. Activity and specificity of Dnf1[TM1-4]. (A) NBD-PC and NBD-PE uptake by Dnf1[TM1-4] is similar to Dnf1[TM3-4], but NBD-PS activity is slightly reduced PS. (B) The specificity of Dnf1[TM1-4] for PS is also slightly reduced relative to Dnf1[TM3-4], suggesting TM3-4 has the greatest impact on PS specificity.

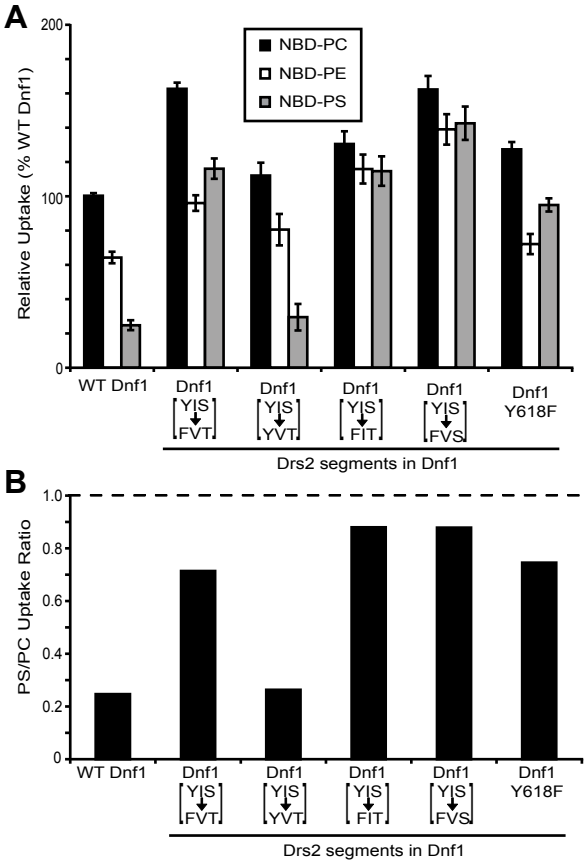

Figure S3. The enhanced uptake for all three substrates noted for Dnf1[YIS->FVT] was also observed for Dnf1[YI->FV] (A) Exchanging Y618F, I619V, and S620T in pairs reveals the enhanced activity seen in Dnf1[YIS->FVT] is dependent on both Y618F and I619V. (B) Each pairwise combination of changes containing Y618F results in a specificity for PS comparable to the Y618F point mutation.

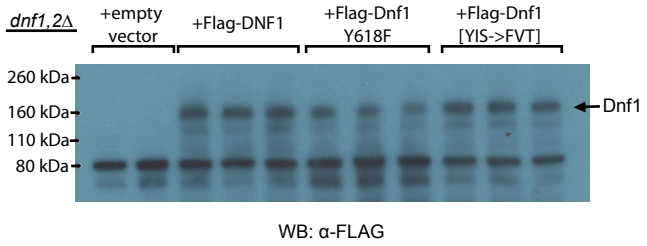

Figure S4. Expression levels of Flag-Dnf1, Flag-Dnf1[YIS->FVT] and Flag-Dnf1 Y618F are similar.

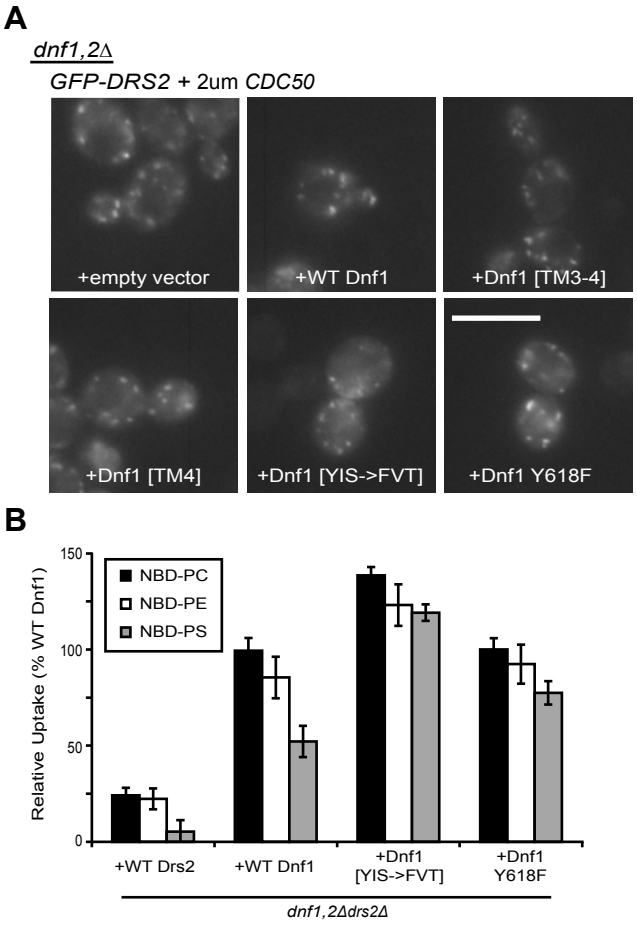

Figure S5. The PS uptake observed in Dnf1[Drs2] chimeras is not attributable to Drs2 (A) Localization of N-terminal GFP fused Drs2 does not change in the presence Dnf1[Drs2] chimeras in *S. cerevisiae*. The scale bar is 10μm. (B) NBD-PL uptake observed by Dnf1[Drs2] chimeras in a *dnf1,2Δdrs2Δ* strain demonstrated the uptake activity was independent of Drs2.

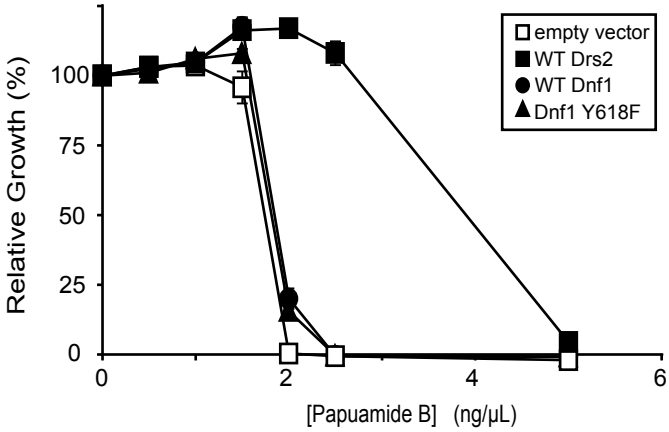

Figure S6. Dnf1 Y618F is unable to suppress the PapB hypersensitivity of the *drs2Δ* strain.

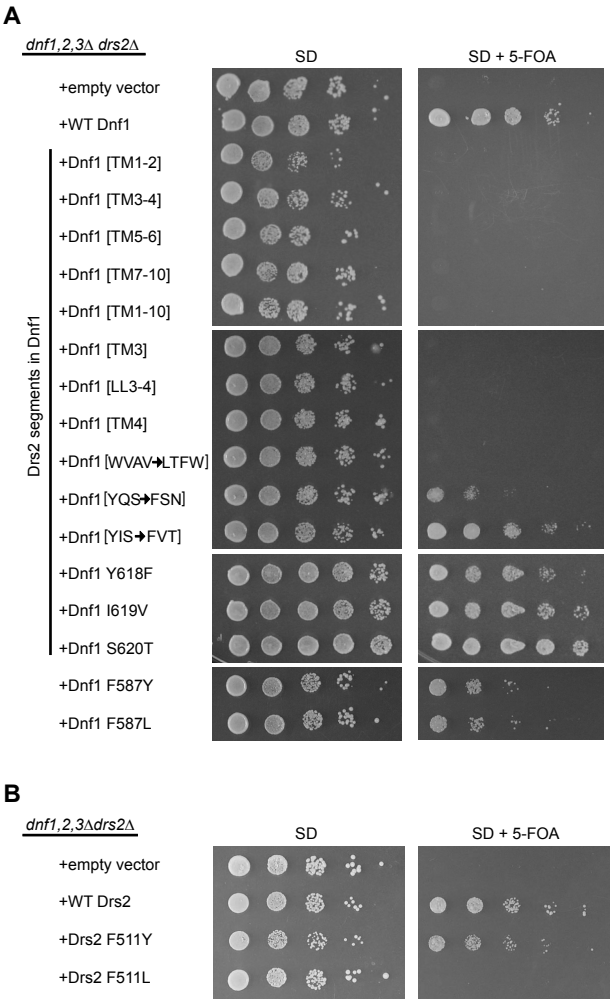

Figure S7. Complementation of *dnf1,2,3Δdrs2Δ* by *DNF1[DRS2]* chimeric genes. (A) Serial dilutions of strains harboring *DNF1[DRS2]* chimeric genes were plated on synthetic medium (left) or synthetic medium containing 5-FOA (right), which is lethal to cells expressing *URA3*. Growth on 5-FOA indicates the Dnf1[Drs2] chimera is capable of complementing the growth defect of the *dnf1,2,3Δdrs2Δ* strain in the absence of the covering *URA3-DRS2* plasmid. (A) Serial dilutions of strains harboring *DRS2[DNF1]* chimeric genes

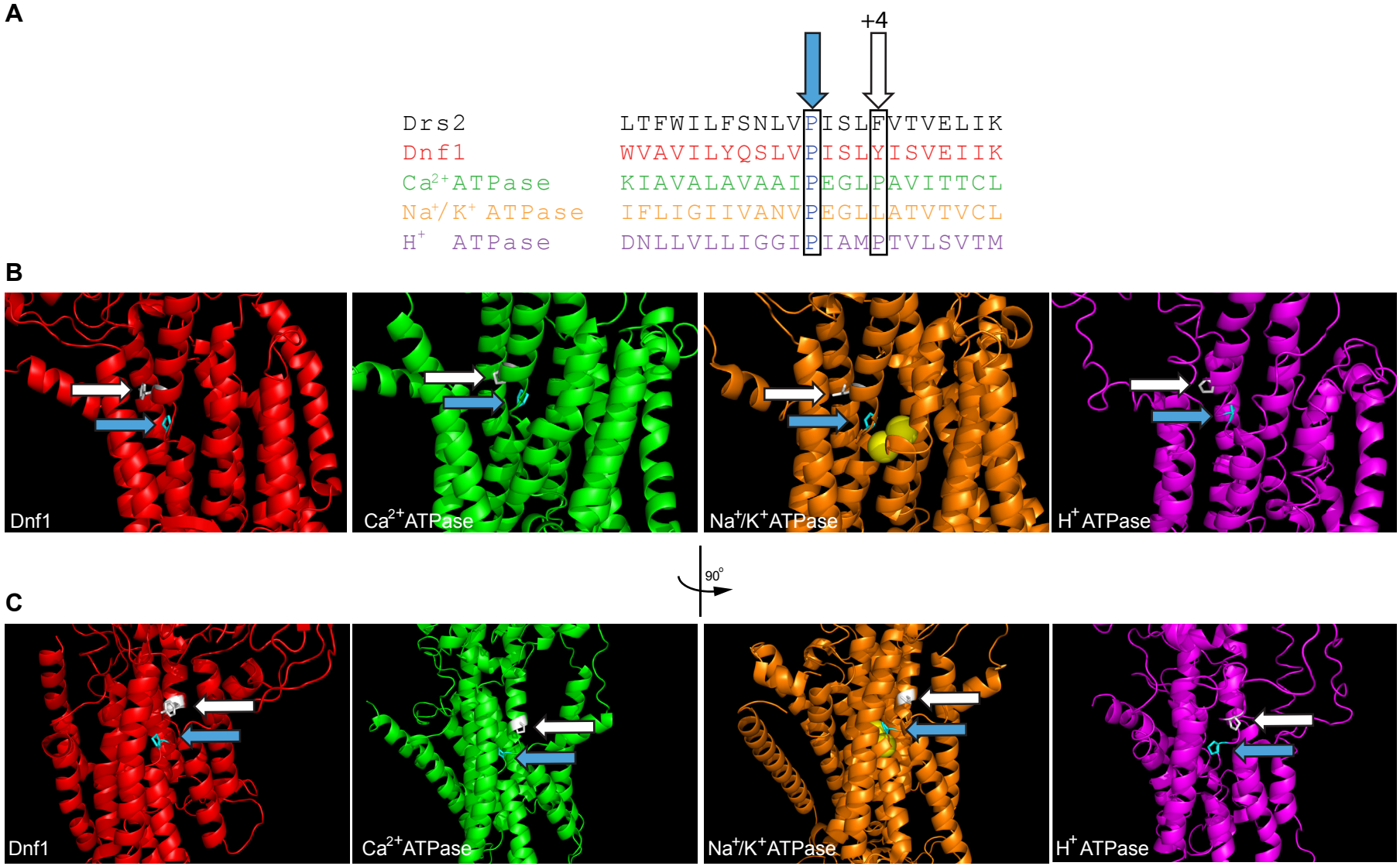

Figure S8. Structural conservation of the P-type ATPase TM domain. (A) Sequence alignment of TM4 of P4-ATPases Dnf1 and Drs2 with crystalized P-type ATPases. (B, C) Comparison of the conserved proline (blue arrow), and "proline plus 4" position (white arrow) between each crystal structure and the structural model of Dnf1. The crystal structures used in this image are: SERCA PBD 2AGV, Na+/K+ ATPase PBD 2ZXE, H+ ATPase PBD 3B8C.

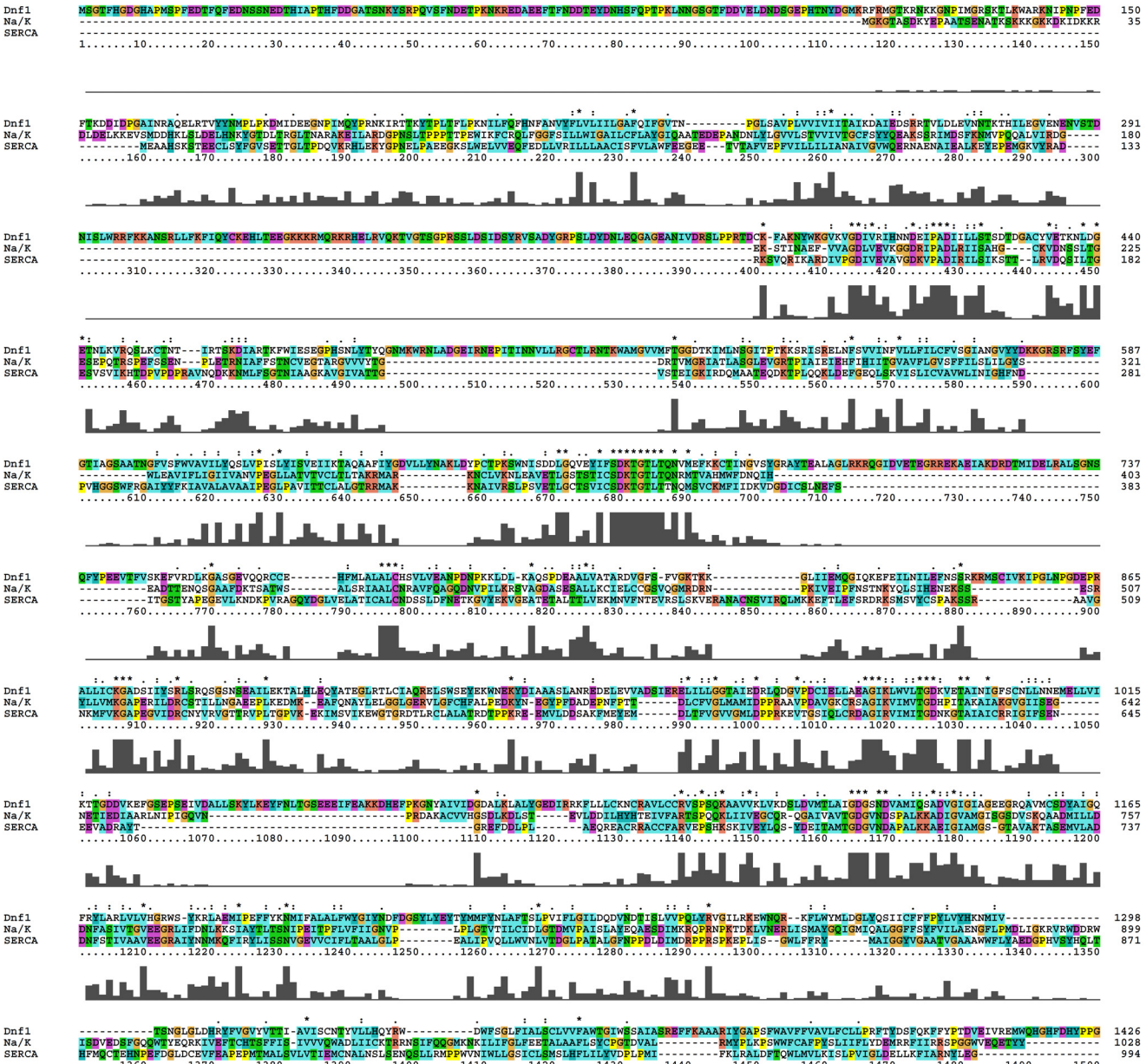

Figure S9. Alignment of Dnf1, Na+/K+ ATPase and SERCA used to generate the structural model. Image generated with Clustal X v2.0 (1).

1. Larkin MA, et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* 23:2947-2948.

Table S1. Yeast strains used in this study

Strain	Genotype	Plasmid	Source
BY4741	MATa *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*		Invitrogen
BY4742	MATα *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*		Invitrogen
PFY3275F	MATa *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 dnf1Δ dnf2Δ*		(1)
SCY119	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lem3Δ*		This study
ZHY615M2D	MATα *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 drs2Δ*		(1)
ZHY709	MATa *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 dnf1Δ dnf2Δ drs2Δ::LEU2*		(1)
ZHY704	MATα *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 dnf1Δ dnf2Δ dnf3Δ drs2Δ::LEU2 pRS416-DRS2*	pRS416-DRS2	(1)
XZY0035	BY4742 *CDC50::KanMX4-GPD atp2 Δ::URA3*		This study
RBY201	ZHY704	pRS313	This study
RBY204	ZHY704	pRS313-DNF1	This study
RBY240	ZHY704	pRS313-Dnf1[TM1-2]	This study
RBY255	ZHY704	pRS313-Dnf1[TM3-4]	This study
RBY2100	ZHY704	pRS313-Dnf1[TM5-6]	This study
RBY2121	ZHY704	pRS313-Dnf1[TM7-10]	This study
RBY2130	ZHY704	pRS313-Dnf1[TM1-10]	This study
RBY264	ZHY704	pRS313-Dnf1[TM3]	This study
RBY267	ZHY704	pRS313-Dnf1[LL3-4]	This study
RBY261	ZHY704	pRS313-Dnf1[TM4]	This study
RBY282	ZHY704	pRS313-Dnf1[WVAV->LTFW]	This study
RBY285	ZHY704	pRS313-Dnf1[YQS->FSN]	This study
RBY288	ZHY704	pRS313-Dnf1[YIS->FVT]	This study
RBY2177	ZHY704	pRS313-Dnf1 Y618F	This study
RBY2180	ZHY704	pRS313-Dnf1 I619V	This study
RBY2183	ZHY704	pRS313-Dnf1 S620T	This study
RBY2308	ZHY704	pRS313-Dnf1 F587Y	This study
RBY2311	ZHY704	pRS313-Dnf1 F587L	This study
RBY2200	ZHY704	pRS313-DRS2	This study
RBY2203	ZHY704	pRS313-Drs2 F511Y	This study
RBY2209	ZHY704	pRS313-Drs2 F511L	This study
RBY2810	SCY119	pRS416-GFP-DNF1 +pRS425-CDC50	This study
RBY2812	SCY119	pRS416-GFP-DNF1 +pRS425-LEM3	This study
RBY2814	SCY119	pRS416-GFP-Dnf1[TM1-2] +pRS425-CDC50	This study
RBY2816	SCY119	pRS416-GFP-Dnf1[TM1-2] +pRS425-LEM3	This study
RBY2818	SCY119	pRS416-GFP-Dnf1[TM3-4] +pRS425-CDC50	This study
RBY2820	SCY119	pRS416-GFP-Dnf1[TM3-4] +pRS425-LEM3	This study
RBY2826	SCY119	pRS416-GFP-Dnf1[TM4] +pRS425-CDC50	This study
RBY2828	SCY119	pRS416-GFP-Dnf1[TM4] +pRS425-LEM3	This study
RBY2838	SCY119	pRS416-GFP-Dnf1[YIS->FVT] +pRS425-CDC50	This study
RBY2840	SCY119	pRS416-GFP-Dnf1[YIS->FVT] +pRS425-LEM3	This study
RBY2842	SCY119	pRS416-GFP-Dnf1 Y618F +pRS425-CDC50	This study
RBY2844	SCY119	pRS416-GFP-Dnf1 Y618F +pRS425-LEM3	This study
RBY3210	XZY0035	pRS313-GPD-TAP2-Drs2	This study
RBY3213	XZY0035	pRS313-GPD-TAP2-Drs2 F511Y	This study
RBY3216	XZY0035	pRS313-GPD-TAP2-Drs2 F511L	This study
RBY3506	ZHY615M2D	pRS313-DNF1	This study
RBY3527	ZHY615M2D	pRS313-Dnf1 Y618F	This study
RBY3901	ZHY615M2D	pRS313	This study
RBY3904	ZHY615M2D	pRS313-DRS2	This study
RBY3907	ZHY615M2D	pRS313-Drs2 F511Y	This study
RBY3913	ZHY615M2D	pRS313-Drs2 F511L	This study
RBY4503	ZHY709	pRS313	This study
RBY4506	ZHY709	pRS313-DRS2	This study
RBY4509	ZHY709	pRS313-DNF1	This study
RBY4518	ZHY709	pRS313-Dnf1[YIS->FVT]	This study
RBY4521	ZHY709	pRS313-Dnf1 Y618F	This study

Table S1. Cont.

Strain	Genotype	Plasmid	Source
RBY501	PFY3275F	pRS416-GFP-DNF1 + pRS425-LEM3	This study
RBY537	PFY3275F	pRS416-GFP-Dnf1[TM1-2] +pRS425-LEM3	This study
RBY565	PFY3275F	pRS416-GFP-Dnf1[TM3-4] +pRS425-LEM3	This study
RBY571	PFY3275F	pRS416-GFP-Dnf1[TM5-6] +pRS425-LEM3	This study
RBY574	PFY3275F	pRS416-GFP-Dnf1[TM7-10] +pRS425-LEM3	This study
RBY577	PFY3275F	pRS416-GFP-Dnf1[TM1-10] +pRS425-LEM3	This study
RBY559	PFY3275F	pRS416-GFP-Dnf1[TM3] +pRS425-LEM3	This study
RBY562	PFY3275F	pRS416-GFP-Dnf1[LL3-4] +pRS425-LEM3	This study
RBY556	PFY3275F	pRS416-GFP-Dnf1[TM4] +pRS425-LEM3	This study
RBY601	PFY3275F	pRS313	This study
RBY604	PFY3275F	pRS313-DNF1	This study
RBY640	PFY3275F	pRS313-Dnf1[TM1-2]	This study
RBY667	PFY3275F	pRS313-Dnf1[TM3-4]	This study
RBY6100	PFY3275F	pRS313-Dnf1[TM5-6]	This study
RBY6121	PFY3275F	pRS313-Dnf1[TM7-10]	This study
RBY6130	PFY3275F	pRS313-Dnf1[TM1-10]	This study
RBY676	PFY3275F	pRS313-Dnf1[TM3]	This study
RBY679	PFY3275F	pRS313-Dnf1[LL3-4]	This study
RBY673	PFY3275F	pRS313-Dnf1[TM4]	This study
RBY682	PFY3275F	pRS313-Dnf1[WVAV->LTFW]	This study
RBY685	PFY3275F	pRS313-Dnf1[YQS->FSN]	This study
RBY688	PFY3275F	pRS313-Dnf1[YIS->FVT]	This study
RBY6151	PFY3275F	pRS313-Dnf1 Y618F	This study
RBY6154	PFY3275F	pRS313-Dnf1 I619V	This study
RBY6157	PFY3275F	pRS313-Dnf1 S620T	This study
RBY6160	PFY3275F	pRS313-Dnf1 Y618L	This study
RBY6200	PFY3275F	pRS313-Dnf1 F587Y	This study
RBY6203	PFY3275F	pRS313-Dnf1 F587L	This study
RBY658	PFY3275F	pRS313-Dnf1[TM1-4]	This study
RBY6172	PFY3275F	pRS313-Dnf1[YIS->YVT]	This study
RBY6175	PFY3275F	pRS313-Dnf1[YIS->FIT]	This study
RBY6178	PFY3275F	pRS313-Dnf1[YIS->FVS]	This study
RBY6503	PFY3275F	pRS313-Flag3-DNF1	This study
RBY6506	PFY3275F	pRS313-Flag3-Dnf1 Y618F	This study
RBY6509	PFY3275F	pRS313-Flag3-Dnf1[YIS->FVT]	This study
RBY9604	PFY3275F	+pRS416-GFP-Drs2 +pRS425-Cdc50 +pRS313	This study
RBY9607	PFY3275F	+pRS416-GFP-Drs2 +pRS425-Cdc50 +pRS313-Dnf1	This study
RBY9610	PFY3275F	+pRS416-GFP-Drs2 +pRS425-Cdc50 +pRS313-Dnf1[TM3-4]	This study
RBY9613	PFY3275F	+pRS416-GFP-Drs2 +pRS425-Cdc50 +pRS313-Dnf1[TM4]	This study
RBY9616	PFY3275F	+pRS416-GFP-Drs2 +pRS425-Cdc50 +pRS313-Dnf1[YIS->FVT]	This study
RBY9619	PFY3275F	+pRS416-GFP-Drs2 +pRS425-Cdc50 +pRS313-Dnf1 Y618F	This study
RBY9701	PFY3275F *lem3Δ::natNT2*		This study
RBY9707	RBY9701	pRS313	This study
RBY9710	RBY9701	pRS313-DNF1	This study
RBY9716	RBY9701	pRS313-Dnf1[TM4]	This study
RBY9719	RBY9701	pRS313-Dnf1[YIS->FVT]	This study
RBY9722	RBY9701	pRS313-Dnf1 Y618F	This study
RBY9750	PFY3275F *cdc50Δ::natNT2*		This study
RBY9756	RBY9750	pRS313	This study
RBY9759	RBY9750	pRS313-DNF1	This study
RBY9765	RBY9750	pRS313-Dnf1[TM4]	This study
RBY9768	RBY9750	pRS313-Dnf1[YIS->FVT]	This study
RBY9771	RBY9750	pRS313-Dnf1 Y618F	This study

1. Hua Z, Fatheddin P, Graham TR (2002) An essential subfamily of Drs2p-related P-type ATPases is required for protein trafficking between Golgi complex and endosomal/vacuolar system. *Mol Biol Cell* 13:3162-3177.

Table S2. Plasmids used in this study

Plasmid	Notes	Source
pRS313		(1)
pRS313-DNF1		(2)
pRS313-Dnf1[TM1-2]	N210-I260 replaced with F217-I267 (from Drs2)	This study
pRS313-Dnf1[TM3-4]	I545-S620 replaced with V440-T513 (from Drs2)	This study
pRS313-Dnf1[TM5-6]	L1184-L1240 replaced with I1008-F1064 (from Drs2)	This study
pRS313-Dnf1[TM7-10]	L1271-R1393 replaced with W1095-D1220 (from Drs2)	This study
pRS313-Dnf1[TM1-10]	N210-I260, I545-S620, L1184-L1240, L1271-R1393 replaced with F217-I267, V440-T513, I1008-F1064, W1095-D1220 (from Drs2)	This study
pRS313-Dnf1[TM3]	I545-Y574 replaced with V440-M469 (from Drs2)	This study
pRS313-Dnf1[LL3-4]	Y575-F602 replaced with S470-F495 (from Drs2)	This study
pRS313-Dnf1[TM4]	W603-S620 replaced with L496-T513 (from Drs2)	This study
pRS313-Dnf1[WVAV->LTFW]	W603L, V604T, A605F, V606W	This study
pRS313-Dnf1[YQS->FSN]	Y609F, Q610S, S611N	This study
pRS313-Dnf1[YIS->FVT]	Y618F, I619V, S620T	This study
pRS313-Dnf1 Y618F	Y618F	This study
pRS313-Dnf1 I619V	I619V	This study
pRS313-Dnf1 S620T	S620T	This study
pRS313-Dnf1 Y618L	Y618L	This study
pRS313-Dnf1 F587Y	F587Y	This study
pRS313-Dnf1 F587L	F587L	This study
pRS313-Dnf1[TM1-4]	N210-I260, I545-S620 replaced with F217-I267, V440-T513 (from Drs2)	This study
pRS313-Dnf1[YIS->YVT]	I619V, S620T	This study
pRS313-Dnf1[YIS->FIT]	Y618F, S620T	This study
pRS313-Dnf1[YIS->FVS]	Y618F, I619V	This study
pRS313-DRS2		(3)
pRS313-Drs2 F511Y	F511Y	This study
pRS313-Drs2 F511L	F511L	This study
pRS313-Flag3-DNF1	N-terminal 3xFlag fusion, *DNF1* promoter	This study
pRS313-Flag3-Dnf1[YIS->FVT]	Y618F, I619V, S620T	This study
pRS313-Flag3-Dnf1 Y618F	Y618F	This study
pRS416-DRS2		(4)
pRS416-GFP-DNF1	N-terminal GFP fusion, *PRC1* promoter	This study
pRS416-GFP-Dnf1[TM1-2]	N210-I260 replaced with F217-I267 (from Drs2)	This study
pRS416-GFP-Dnf1[TM3-4]	I545-S620 replaced with V440-T513 (from Drs2)	This study
pRS416-GFP-Dnf1[TM5-6]	L1184-L1240 replaced with I1008-F1064 (from Drs2)	This study
pRS416-GFP-Dnf1[TM7-10]	L1271-R1393 replaced with W1095-D1220 (from Drs2)	This study
pRS416-GFP-Dnf1[TM1-10]	N210-I260, I545-S620, L1184-L1240, L1271-R1393 replaced with F217-I267, V440-T513, I1008-F1064, W1095-D1220 (from Drs2)	This study
pRS416-GFP-Dnf1[TM3]	I545-Y574 replaced with V440-M469 (from Drs2)	This study
pRS416-GFP-Dnf1[LL3-4]	Y575-F602 replaced with S470-F495 (from Drs2)	This study
pRS416-GFP-Dnf1[TM4]	W603-S620 replaced with L496-T513 (from Drs2)	This study
pRS416-GFP-Dnf1[YIS->FVT]	Y618F, I619V, S620T	This study
pRS416-GFP-Dnf1 Y618F	Y618F	This study
pRS425-CDC50		(5)
pRS425-LEM3		This study

1. Sikorski RS, 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.
2. Liu K, Hua Z, Nepute JA, Graham TR (2007) Yeast P4-ATPases Drs2p and Dnf1p are essential cargos of the NPFXD/Sla1p endocytic pathway. *Mol Biol Cell* 18:487-500.
3. Natarajan P, Wang J, Hua Z, Graham TR (2004) Drs2p-coupled aminophospholipid translocase activity in yeast Golgi membranes and relationship to in vivo function. *Proc Natl Acad Sci USA* 101:10614-10619.
4. Hua Z, Fatheddin P, Graham TR (2002) An essential subfamily of Drs2p-related P-type ATPases is required for protein trafficking between Golgi complex and endosomal/vacuolar system. *Mol Biol Cell* 13:3162-3177.
5. Chen S, et al. (2006) Roles for the Drs2p-Cdc50p complex in protein transport and phosphatidylserine asymmetry of the yeast plasma membrane. *Traffic* 7:1503-1517.

Table S3. Primers used in this study

Primer	Sequence (5'->3')	For creation of constructs
Dnf1(rev)SOE1	AAATTCTTGGAACAAAAATTTTGGTAAGAATGTTAACGGGG	Dnf1[TM1-2]
Dnf1(forw)SOE3	GCCATGAAGGAATGTATCGAAGACTCAAGAAGAACCGTCCTAG	Dnf1[TM1-2]
Dnf1(rev)SOE6	GTTGATAATTTTCTCAACCCTGGACTTCTTGGTGGGTG	Dnf1[TM3-4], Dnf1[TM3]
Dnf1(rev)SOE8	TAGAATCCAAAATGTTAAAAATGAGACGAAACCGTTTGTC	Dnf1[TM4]
Dnf1 (forw) SOE8	ggcttattcttcaaagacTTTTGGGTTGCTGTTATTCTTTACC	Dnf1[LL3-4]
Dnf1(forw)SOE9	ATTTCTCTATTTGTCACCGTGGAGATCATCAAAACTGCACA	Dnf1[TM3-4], Dnf1[TM4]
Dnf1 (rev) SOE10	tttggcatctgcagtagaATATACACCATTTGCAATACCAGAAAC	Dnf1[LL3-4]
Dnf1 (forw) SOE10	attggtaatgttattatgTACGACAAAAAAGGCAGATCAC	Dnf1[TM3]
Dnf1 (rev) SOE11	CAAAATTGCGACAGAAATTCTCTTATAAGACCACCTACCGTGAAC	Dnf1[TM5-6
Dnf1 (forw) SOE13	TTTGTCATTGGTGTATTTGACCAGGACGTGAATGACACA	Dnf1[TM5-6]
Dnf1 (rev) SOE18	ATTAATAATCCATCCCCAGAACTTTCTTTGGTTCCATTCTTTC	Dnf1[TM7-10]
Dnf1 (forw) SOE20	TTTGCACTGGTAAGAGATTTCACATATGACAGTTTTCAAAAATTTTTC	Dnf1[TM7-10]
Dnf1(forw) WVAVmut	AACGGTTTCGTCTCATTTttaacattttggATTCTTTACCAATCTTTA	Dnf1[WVAV->LTFW]
Dnf1(rev) WVAVmut	TAAAGATTGGTAAAGAATccaaaatgttaaAAATGAGACGAAACCGTT	Dnf1[WVAV->LTFW]
Dnf1 (forw) YQSmut	TGGGTTGCTGTTATTCTTTtttcgaaTTTAGTCCCAATCTCTTTG	Dnf1[YQS->FSN]
Dnf1 (rev) YQSmut	CAAAGAGATTGGGACTAAAttcgaaaAAAGAATAACAGCAACCCA	Dnf1[YQS->FSN]
Dnf1 (forw) YISmut	CTTTAGTCCCAATCTCTTTGtttgtcaccGTGGAGATCATCAAAACTGC	Dnf1[YIS->FVT]
Dnf1 (rev) YISmut	GCAGTTTTGATGATCTCCACggtgacaaaCAAAGAGATTGGGACTAAAG	Dnf1[YIS->FVT]
Dnf1(forw)Y618F	CTTTAGTCCCAATCTCTTTGTttATTTCTGTGGAGATCATCAAAAC	Dnf1 Y618F
Dnf1(rev)Y618F	GTTTTGATGATCTCCACAGAAATaaACAAAGAGATTGGGACTAAAG	Dnf1 Y618F
Dnf1(forw)I619V	TAGTCCCAATCTCTTTGTACgtcTCTGTGGAGATCATCAAAAC	Dnf1 I619V
Dnf1(rev)I619V	GTTTTGATGATCTCCACAGAgacGTACAAAGAGATTGGGACTA	Dnf1 I619V
Dnf1(forw)S620T	GTCCCAATCTCTTTGTACATTaccGTGGAGATCATCAAAACTGC	Dnf1 S620T
Dnf1(rev)S620T	GCAGTTTTGATGATCTCCACggtAATGTACAAAGAGATTGGGAC	Dnf1 S620T
Dnf1(forw)Y618L	CTTTAGTCCCAATCTCTTTGTtgATTTCTGTGGAGATCATCAAAAC	Dnf1 Y618L
Dnf1(rev)Y618L	GTTTTGATGATCTCCACAGAAATcaACAAAGAGATTGGGACTAAAG	Dnf1 Y618L
Dnf1(forw)YIS->YVT	CTTTAGTCCCAATCTCTTTGTACgtcaccGTGGAGATCATCAAAACTGC	Dnf1[YIS->YVT]
Dnf1(rev)YIS->YVT	GCAGTTTTGATGATCTCCACggtgacGTACAAAGAGATTGGGACTAAAG	Dnf1[YIS->YVT]
Dnf1(forw)YIS->FIT	CTTTAGTCCCAATCTCTTTGtttATTaccGTGGAGATCATCAAAACTGC	Dnf1[YIS->FIT]
Dnf1(rev)YIS->FIT	GCAGTTTTGATGATCTCCACggtAATaaaCAAAGAGATTGGGACTAAAG	Dnf1[YIS->FIT]
Dnf1(forw)YIS->FVS	CTTTAGTCCCAATCTCTTTGtttgtcTCTGTGGAGATCATCAAAACTGC	Dnf1[YIS->FVS]
Dnf1(rev)YIS->FVS	GCAGTTTTGATGATCTCCACAGAgacaaaCAAAGAGATTGGGACTAAAG	Dnf1[YIS->FVS]
Dnf1(forw) +KpnI	GACGACGGTACCTCTGGAACTTTTCATGGCG	GFP-Dnf1
Dnf1(rev) +KpnI	GACGACGGTACCTATTAATTGTTCTGTTGTGTTCCGA	GFP-Dnf1
Drs2(forw)SOE1	TTAACATTCTTACCAAAATTTTTGTTCCAAGAATTTTCCAAA	Dnf1[TM1-2]
Drs2(rev)SOE3	GGTTCTTCTTGAGTCTTCGATACATTCCTTCATGGCAGAAAC	Dnf1[TM1-2]
Drs2(forw)SOE6	CCCACCAAGAAGTCCAGGGTTGAGAAAATTATCAACAGACAGATTATTC	Dnf1[TM3-4], Dnf1[TM3]
Drs2(forw)SOE8	AACGGTTTCGTCTCATTTTTAACATTTTGGATTCTATTTTCGAATC	Dnf1[TM4]
Drs2 (rev) SOE8	aataacagcaacccaaaaGTCTTTGAAGAATAAGCCAGCCTT	Dnf1[LL3-4]
Drs2(rev)SOE9	AGTTTTGATGATCTCCACGGTGACAAATAGAGAAATAGGAACTAGATTC	Dnf1[TM3-4], Dnf1[TM4]
Drs2 (forw) SOE10	attgcaaatggtgtatatTCTACTGCAGATGCCAAACATTT	Dnf1[LL3-4]
Drs2 (rev) SOE10	tctgccttttttgtcgtaCATAATAACATTACCAATTGAAGAAATTAAAA	Dnf1[TM3]
Drs2 (forw) SOE11	AGGTGGTCTTATAAGAGAATTTCTGTCGCAATTTTGTACTCTTT	Dnf1[TM5-6
Drs2 (rev) SOE13	GTCATTCACGTCCTGGTCAAATACACCAATGACAAAAGGGG	Dnf1[TM5-6]
Drs2 (forw) SOE18	TGGAACCAAAGAAAGTTCTGGGGATGGATTATTAATGGC	Dnf1[TM7-10]
Drs2 (rev) SOE20	AAAACTGTCATATGTGAAATCTCTTACCAGTGCAAAAATTGG	Dnf1[TM7-10]
Drs2 (forw) F511Y	CGAATCTAGTTCCTATTTCTCTATACGTCACCGTTGAATTAATC	Drs2[F511Y]
Drs2 (rev) F511Y	GATTAATTCAACGGTGACGTATAGAGAAATAGGAACTAGATTCG	Drs2[F511Y]
Drs2 (forw) F511L	CGAATCTAGTTCCTATTTCTCTATTgGTCACCGTTGAATTAATC	Drs2[F511L]
Drs2 (rev) F511L	GATTAATTCAACGGTGACcAATAGAGAAATAGGAACTAGATTCG	Drs2[F511L]
Dnf1(forw)@1500	TACTATTAATAACGTTCTGCTTCGTGG	
Dnf1(rev)@2297	CATCTTTGTTGTACTTCACCACTAGC	

SI Material and Methods

Cloning. GFP-Dnf1 was an N-terminal fusion generated by standard cloning of *DNF1* into the KpnI site in pRS416-GFP. KpnI sites were added to the 5' and 3' ends of *DNF1* by amplifying pRS313-*DNF1* with Dnf1 primers (Dnf1(forw)+KpnI and Dnf1(rev)+KpnI) to amplify a 4.7kb PCR fragment. The GFP-Dnf1 chimeras were generated by replacing the WT Dnf1 sequence through standard cloning using SphI and BseRI restriction sites from the pRS313 expression vectors (1).

Plasmid Shuffling Assay. A *dnf1,2,3*Δ*drs2*Δ strain harboring WT *DRS2* on a *URA3* plasmid was transformed with a second plasmid containing the *DNF1[DRS2]* chimera. When grown in the presence of 5-fluoroorotic acid (5FOA), any strain that is unable to lose the *URA3-DRS2* plasmid will die. Strains containing a Dnf1[Drs2] chimera capable of supporting growth of the *dnf1,2,3*Δ*drs2*Δ strain will be able to lose the *DRS2-URA3* plasmid and grow in the presence of 5FOA.

Cell Extracts and Western Blotting. Yeast containing N-terminal 3xFLAG tagged Dnf1 were grown in YPD to mid-log phase and harvested in SDS/urea sample buffer (40 mM Tris–HCl (pH 6.8), 8M urea, 0.1mM ethylenediaminetetraacetic acid, 1% 2-mercaptoethanol, 5% SDS and 0.25% bromophenol blue). 0.2OD cell lysate was separated by SDS-PAGE, transferred to PVDF membrane, and blotted with anti-FLAG M2 antibody (Sigma-Aldrich).

1. Sikorski RS, 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.