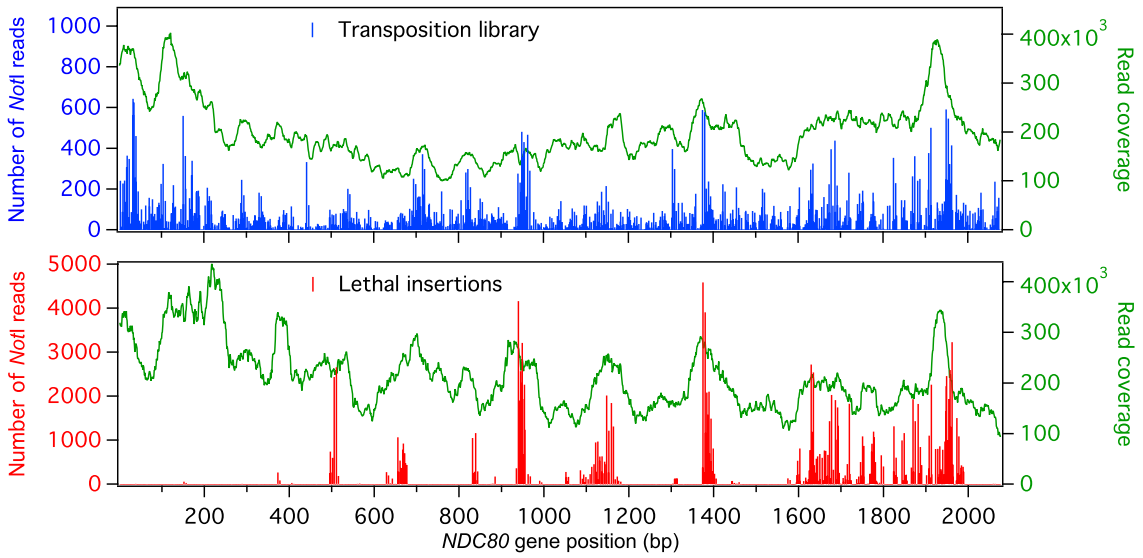

Figure S1 Positions of 15-bp insertions in the transposition library (blue lines) and from the non-sectoring red colonies (red lines) were determined by Illumina sequencing. Insertions were identified using the unique *Not*I recognition sequence and shown along the *NDC80* gene. The read coverage from the sequencing is shown in green.

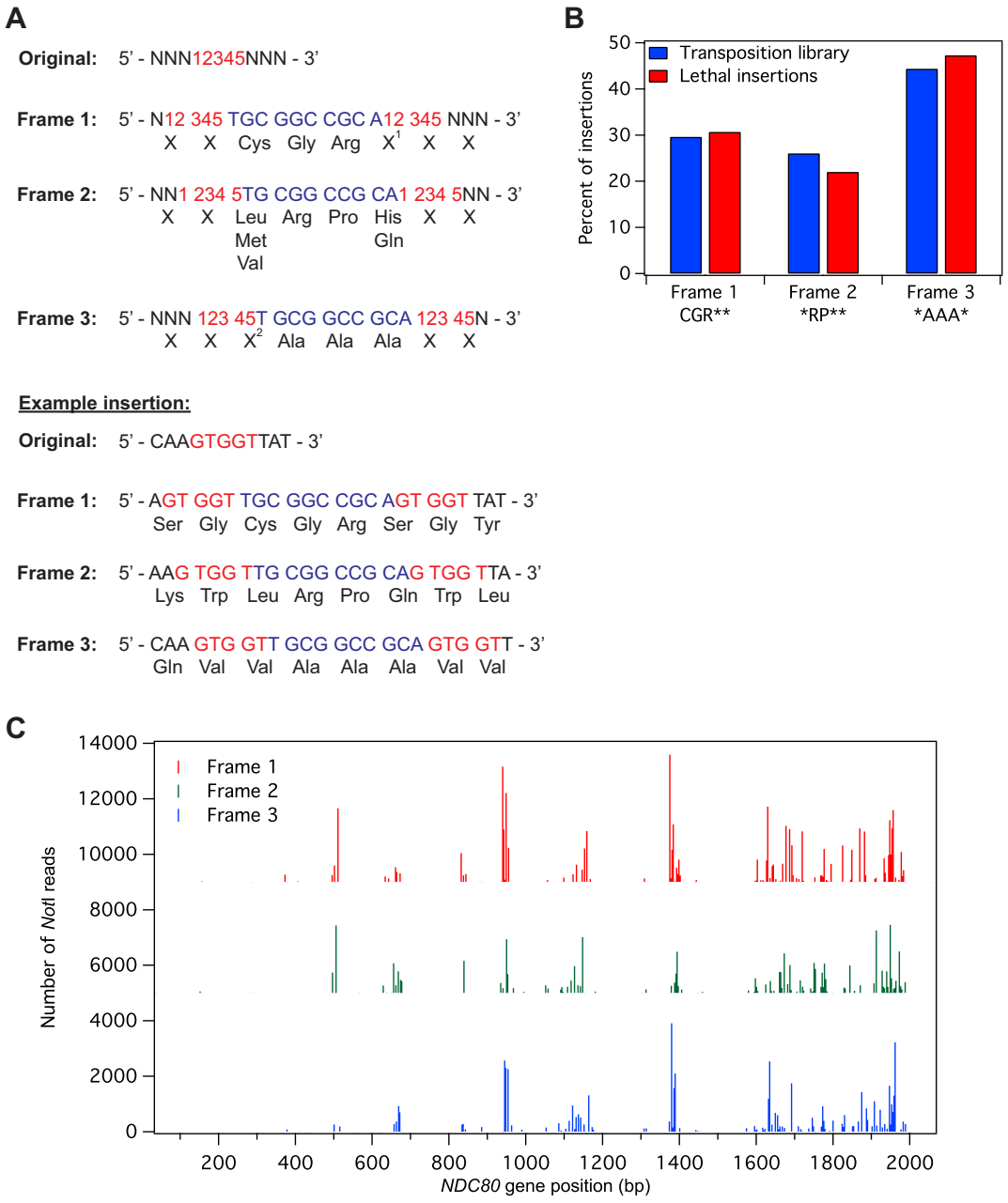

Figure S2 The reading frame targeted by transposition dictates the residues inserted. (A) The translation of the insertion depends on both the frame of insertion and the target sequence (adapted from Finnzymes Manual F-701). In the transposition library, each 15-bp insertion includes 5 bp of duplicated target sequence (red) and 10 additional nucleotides (blue). X is any amino acid; X^1 is Ile, Met, Thr, Asn, Lys, Ser, or Arg; X^2 is any amino acid except Gln, Glu, Lys, Met, and Trp. An example insertion is also shown. (B) The proportion of lethal insertions in each frame (red) is similar to that of the initial transposition library (blue). (C) The positions of lethal insertions (from Figure S1) are separated based on the frame of insertion. Traces are offset vertically for visual clarity.

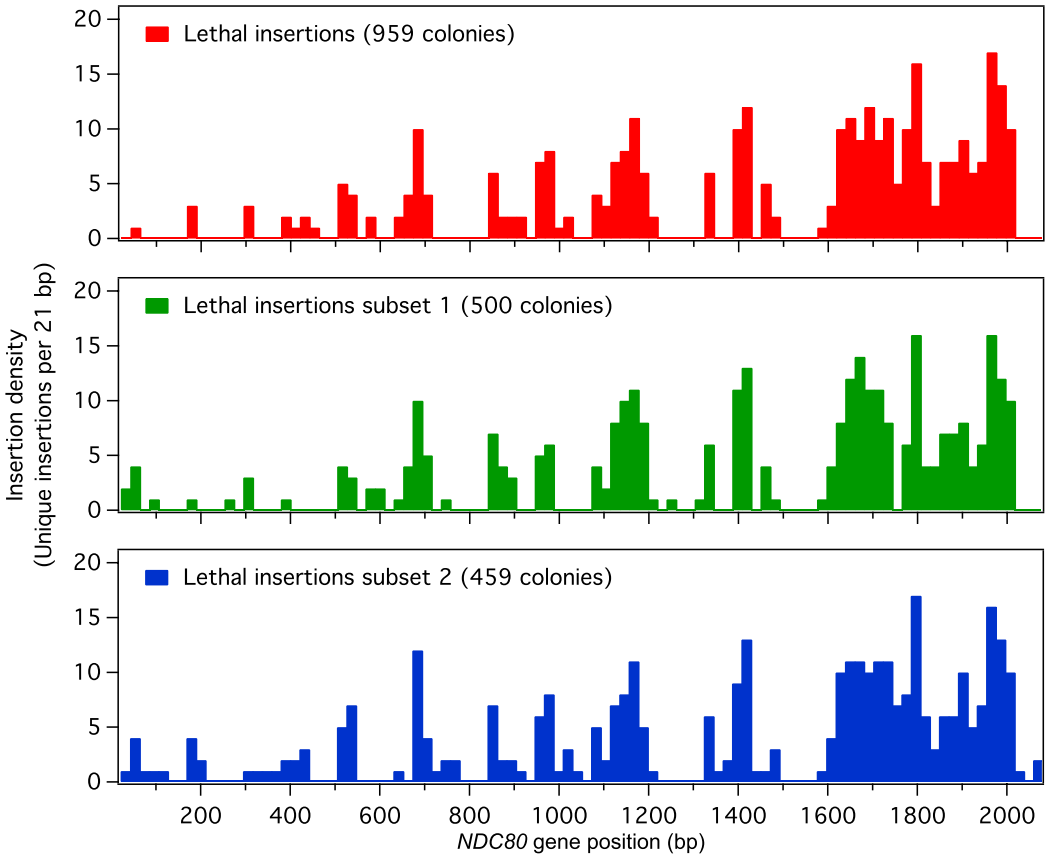

Figure S3 Validation of the lethal insertion clusters. Two subsets (green and blue bars) sequenced independently have the same lethal insertion clusters as the original 959 non-sectoring red colonies (red bars, from Figure 3D).

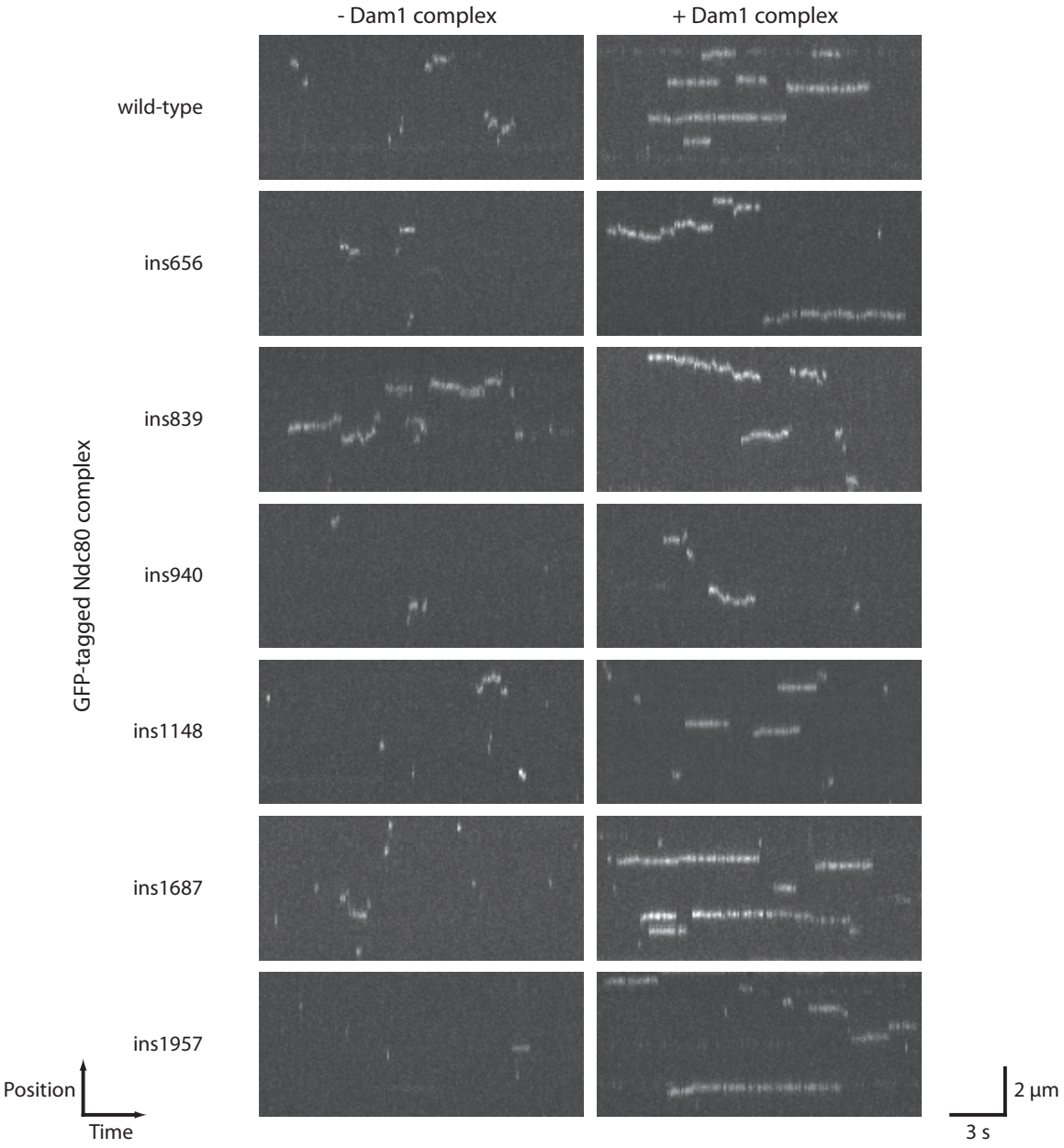

Figure S4 Lethal insertion mutants affect the ability of the Dam1 complex to enhance binding of the Ndc80 complex to microtubules. Using TIRF microscopy, single molecules of GFP-tagged Ndc80 complexes were visualized on taxol-stabilized microtubules. Representative kymographs show the binding and one-dimensional diffusion of 50 pM GFP-tagged Ndc80 complexes on microtubules in the presence or absence of 2.5 nM untagged Dam1 complex.

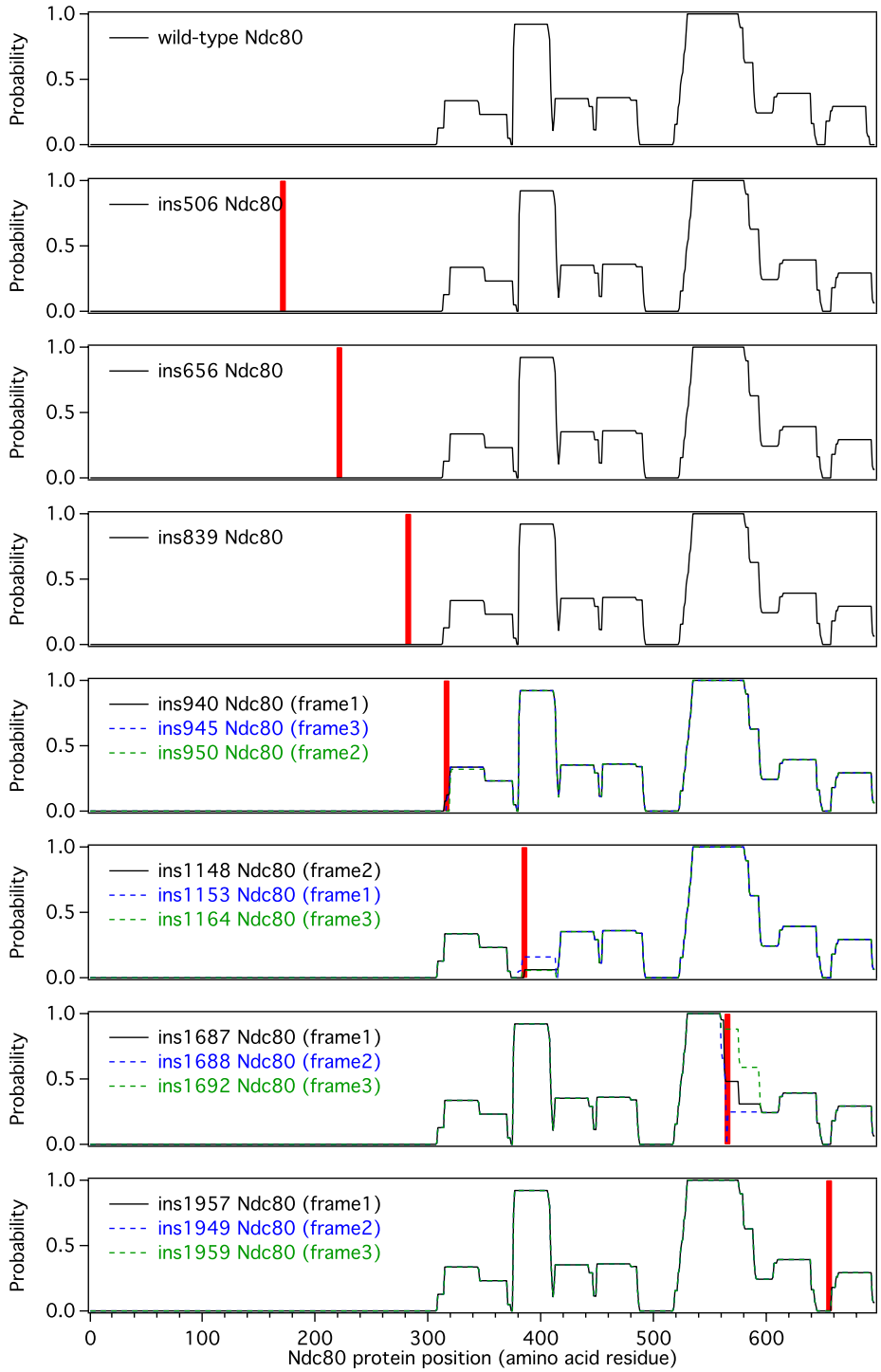

Figure S5 The effect of lethal insertions in Ndc80 on predicted coiled-coil formation. The probabilities of coiled-coil formation, as predicted by Paircoil2 (McDonnell *et al.* 2006), for Ndc80 containing the representative lethal insertions studied (black lines). Vertical red lines denote the positions of the representative insertions. The probabilities of coiled-coil formation for additional lethal insertions are also shown (blue and green dotted lines).

Table S1 Plasmids used in this study

Plasmid	Relevant markers	Reference
pRS316	*CEN6 ARSH4 URA3 Amp*^{r} f1 origin	SIKORSKI and HIETER 1989
pKG9	*NatMX*	GREENLAND *et al.* 2010
pJT12	*NDC80 ADE3 LYS2* in 2 µm vector	This study
pJT36	*NDC80* in pRS316	This study
pJT153	*GFP-NDC80* in pRS316	This study
pJT185	*ins506 GFP-ndc80* in pRS316	This study
pJT187	*ins656 GFP-ndc80* in pRS316	This study
pJT188	*ins839 GFP-ndc80* in pRS316	This study
pJT189	*ins940 GFP-ndc80* in pRS316	This study
pJT190	*ins1148 GFP-ndc80* in pRS316	This study
pJT154	*ins1687 GFP-ndc80* in pRS316	This study
pJT155	*ins1957 GFP-ndc80* in pRS316	This study
His_6-Spc24/Spc25 expression plasmid	*His_6-SPC24/SPC25* dicistron, *Kan*^{r}	WEI *et al.* 2005
Ndc80/Nuf2-GFP expression plasmid	*NDC80/NUF2-GFP* dicistron, *Amp*^{r}	POWERS *et al.* 2009
pJT138	*ins506 ndc80/NUF2-GFP* dicistron, *Amp*^{r}	This study
pJT139	*ins511 ndc80/NUF2-GFP* dicistron, *Amp*^{r}	This study
pJT140	*ins656 ndc80/NUF2-GFP* dicistron, *Amp*^{r}	This study
pJT141	*ins839 ndc80/NUF2-GFP* dicistron, *Amp*^{r}	This study
pJT142	*ins940 ndc80/NUF2-GFP* dicistron, *Amp*^{r}	This study
pJT143	*ins1148 ndc80/NUF2-GFP* dicistron, *Amp*^{r}	This study
pJT145	*ins1687 ndc80/NUF2-GFP* dicistron, *Amp*^{r}	This study
pJT146	*ins1957 ndc80/NUF2-GFP* dicistron, *Amp*^{r}	This study
Dam1 complex expression plasmid	*DAD4/DAD3/DAD2/SPC19/ASK1* and *DAD1/DUO1/SPC34-His_6/DAM1/HSK3* polycistrons, *Amp*^{r}	MIRANDA *et al.* 2005

Table S2 Yeast strains used in this study

Strain	Genotype	Reference
W303	*ade2-1oc can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	
JTY1	MAT**a**/α *ade2-1oc/ade2-1oc ade3Δ-100/ade3Δ-100 can1-100/can1-100 cyh2^r/CYH2^s his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/trp1-1 ura3-1/ura3-1*	This study
JTY4	MAT**a**/α *ade2-1oc/ade2-1oc ade3Δ-100/ade3Δ-100 can1-100/can1-100 cyh2^r/CYH2^s his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/trp1-1 ura3-1/ura3-1 NDC80/ndc80Δ::NatMX*	This study
JTY5-5C	MAT**a** *ade2-1oc ade3Δ-100 can1-100 cyh2^r his3-11,15 leu2-3,112 lys2Δ::HIS3 trp1-1 ura3-1 ndc80Δ::NatMX* [pJT12]	This study
JTY12-25A	MAT**a** *ade2-1oc ade3Δ-100 can1-100 cyh2^r his3-11,15 leu2-3,112 trp1-1 ura3-1 NDC80 NUF2-mCherry::hphMX*	This study
JTY29-1B	MATα *ade2-1oc ade3Δ-100 can1-100 cyh2^r his3-11,15 leu2-3,112 trp1-1 ura3-1 NUF2*	This study
JTY29-1C	MATα *ade2-1oc ade3Δ-100 can1-100 CYH2^s his3-11,15 leu2-3,112 trp1-1 ura3-1 NUF2-TAP::KanMX*	This study
JTY47-2A	MAT**a** *ade2-1oc ade3Δ-100 can1-100 CYH2^s his3-11,15 leu2-3,112 trp1-1 ura3-1 SPC24*	This study
JTY47-2B	MATα *ade2-1oc ade3Δ-100 can1-100 cyh2^r his3-11,15 leu2-3,112 trp1-1 ura3-1 SPC24-TAP::KanMX*	This study

Table S3 Oligonucleotides used for Illumina sequencing

Primer	Sequence
NDC80 forward PCR primer	TAAAGGGAACAAAAGCTGGG
NDC80 reverse PCR primer	ACGGCCAGTGAATTGTAA
Multiplex adapter 1	5' P-GATCGGAAGAGCACACGTCT
Multiplex adapter 2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
Multiplex forward PCR primer + index 1	AATGATACGGCGACCACCGCGATCTCGTGATACACTCTTTCCCTACACGACGCTCTTCCGATC
Multiplex forward PCR primer + index 2	AATGATACGGCGACCACCGCGATCTACATCGACACTCTTTCCCTACACGACGCTCTTCCGATC
Multiplex reverse PCR primer	CAAGCAGAAGACGGCATACGAGATACACTCTTTCCCTACACGACGCTCTTCCGATCT
Multiplex index sequencing primer	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
Multiplex read1 sequencing primer	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
TruSeq Indexed Adapter AD002	GATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATGTATCTCGTATGCCGTCTTCTGCTTG
TruSeq Indexed Adapter AD018	GATCGGAAGAGCACACGTCTGAACTCCAGTCACGTCCGCATCTCGTATGCCGTCTTCTGCTTG
TruSeq Indexed Adapter AD019	GATCGGAAGAGCACACGTCTGAACTCCAGTCACGTGAAAATCTCGTATGCCGTCTTCTGCTTG

Table S4 Illumina sequencing results

	Transposition library	Lethal insertions	Lethal insertion subsets	
			Subset 1	Subset 2
Number of sequencing runs	3	1	1	1
Number of 36-bp reads mapped to Chromosome IX	14,000,357	13,710,514	10,075,540	13,424,377
Number of Chromosome IX reads containing *Not*I	96,569	166,135	290,126	319,434
Number of Chromosome IX reads with *Not*I in *NDC80* + promoter/terminator	93,826	N/A	N/A	N/A
Number of Chromosome IX reads with *Not*I in *NDC80*	70,215	165,414	289,470	318,637
Number of unique *Not*I sites in *NDC80*	1,074	336	320	351
Coverage of *NDC80* gene (%)	52	16	15	17
Number of unique codons containing insertions	444	162	149	165
Coverage of Ndc80 protein (%)	64	23	22	24

N/A, not applicable

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