Supporting Information

Title: It takes two (Las1 HEPN endoribonuclease domains) to cut RNA correctly

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Running Title: Las1 requires dual HEPN nuclease domains

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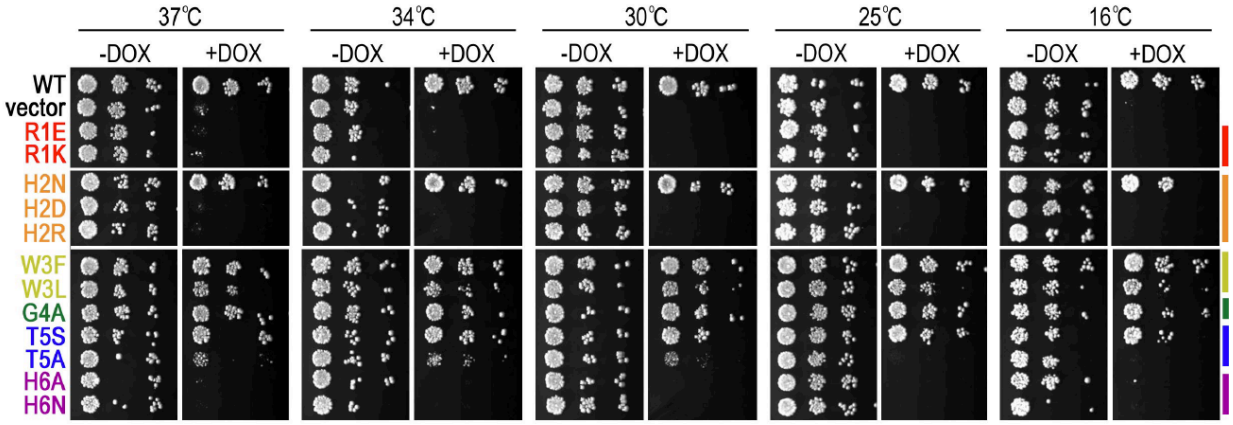

Fig. S1. *In vivo* assay for complementation of *S. cerevisiae LAS1* over a temperature range. The *S. cerevisiae tet-LAS1/Myc-GRC3* strain was transformed with plasmids encoding Flag-Las1 RHxhTH variants (see Table S2). Serial dilutions were spotted on YPD in the absence (-DOX) and presence (+DOX) of 40 μg/ml doxycycline and incubated at 37°C, 34°C, 30°C, 25°C and 16°C for 2-6 days.

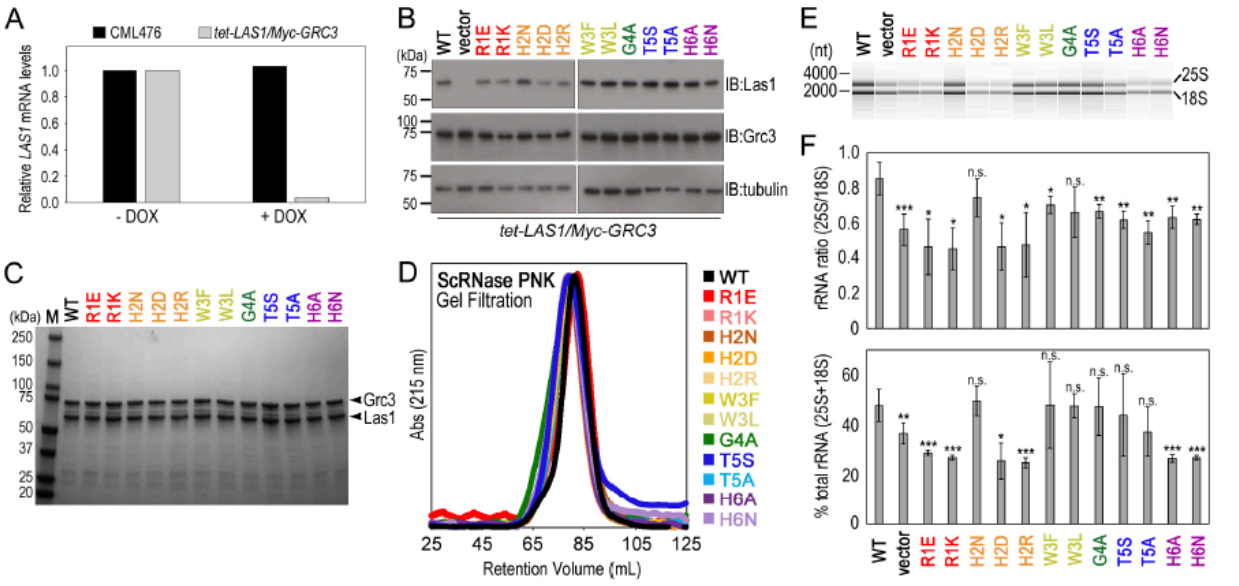

Fig. S2. Las1 RHxhTH variants retain their association to Grc3. (*A*) RT-PCR of the parent (CML476) and tet (*tet-LAS1/Myc-GRC3*) yeast strains with primers specific for *LAS1*. (*B*) Transformed *S. cerevisiae tet-LAS1/Myc-GRC3* strains were grown to mid-log phase at 30°C in the presence of 40 μg/ml doxycycline. Cells were lysed and equal amounts of whole cell lysate were separated by SDS-PAGE and analyzed by western blotting with anti-Flag (Las1), anti-Myc (Grc3) and anti-tubulin (loading control). IB is an abbreviation for immunoblotting. (*C*) SDS-PAGE analysis of purified recombinant ScRNase PNK complex comprised of full length Grc3 and Las1 RHxhTH variants. (*D*) Gel filtration of ScRNase PNK variants containing Las1 RHxhTH missense mutants. Wild-type RNase PNK (black) was previously shown to form a hetero-tetramer (23) and has the same retention volume as the recombinant ScRNase PNK variants. This suggests that the ScRNase PNK variants maintain higher-order assembly. (*E*) Extracted total RNA (250 ng) from transformed *tet-LAS1/Myc-GRC3* strains were analyzed using a bioanalyzer to monitor the levels of mature 25S and 18S rRNA. (*F*) Quantification of mature rRNA shown in panel *E* reveals a reduced ratio in mature 25S/18S rRNA (top) and total mature rRNA (25S + 18S) (bottom). The average and standard deviation were calculated from three technical replicates. n.s. not significant, $*P < 0.05$, $**P < 0.007$, and $***P < 0.0007$ were calculated by two-tailed Student's *t* tests.

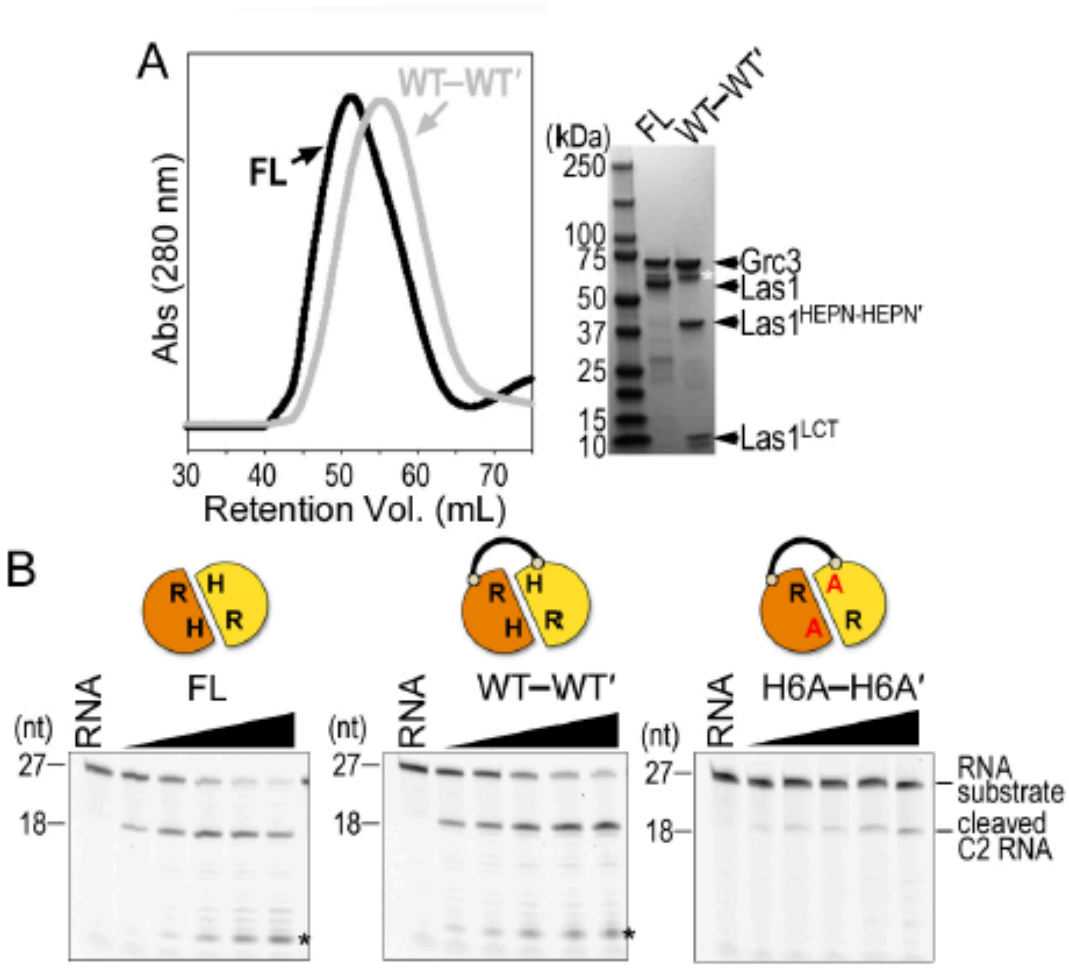

Fig. S3. Functional *S. cerevisiae* RNase PNK chimera for *in vitro* assays. (*A*) Gel filtration and SDS-PAGE analysis of full length ScRNase PNK (FL; black), composed of Las1 and Grc3, and chimeric ScRNase PNK (WT-WT′; gray), comprised of Las1 HEPN-HEPN′, Las1 LCT and Grc3. White asterisk marks a Grc3 degradation product. Chimeric ScRNase PNK has a similar retention volume to the full length complex suggesting the chimera maintains its higher-order assembly despite lacking its CC domain. (*B*) Denaturing urea gels of reactions containing chimeric ScRNase PNK variants (0-3.2 μM) encoding missense mutations to the Las1 RHxhTH motif. Protein variants were incubated with C2 RNA substrate (50 nM) for 1 hour at 37°C. RNA defines reactions set in the absence of protein and black asterisks marks the accumulation of a non-specific band that is dependent on the presence of active RNase PNK complex and a 3′-fluorescent label.

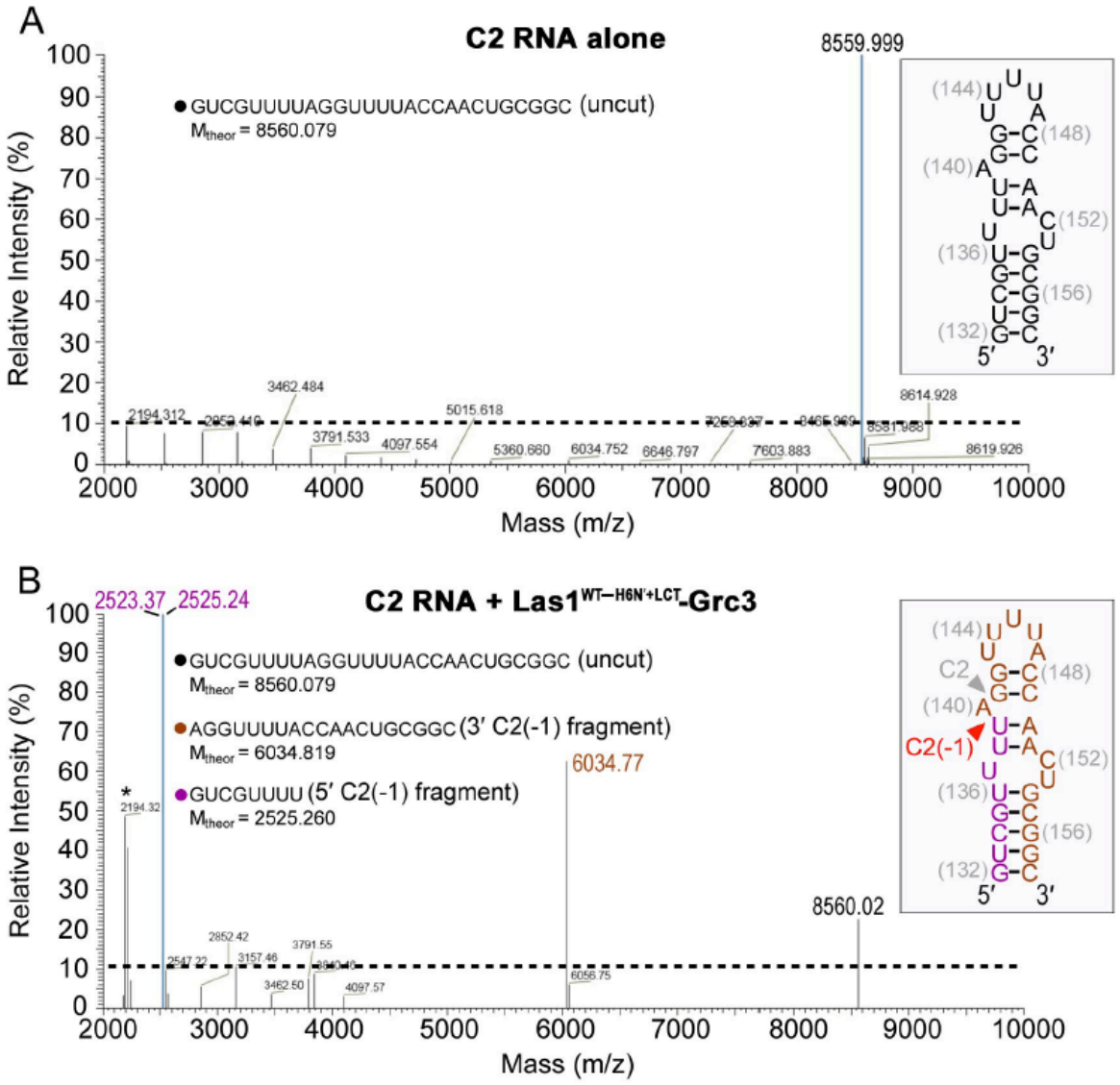

Fig. S4. Identification of off-target RNA products using mass spectrometry. (*A*-*B*) Spectra by LC-ESI-MS of C2 RNA substrate incubated in the (*A*) absence and (*B*) presence of chimeric RNase PNK containing Las1$^{WT\text{-}H6N'}$. RNA (10 μM) was incubated in the absence and presence of protein (13 μM) for 30 minutes at 37°C. Observed ions of *m/z* 8560 is indicative of the uncut C2 RNA substrate. Whereas ions of *m/z* 2525.3 and 6034.8 correspond to 5′- and 3′-RNA fragments, respectively, produced following cleavage at an off-target site (C2(-1); red arrowhead) between U139 and A140 of the *S. cerevisiae* ITS2. The 8-nucleotide 5′-fragment (magenta) harbors a 2′,3′-cyclic phosphate and the 19-nucleotide 3′-fragment (brown) contains a 5′-hydroxyl end confirming the ScLas1$^{WT\text{-}H6N'}$ variant is responsible for the off-target RNA hydrolysis.

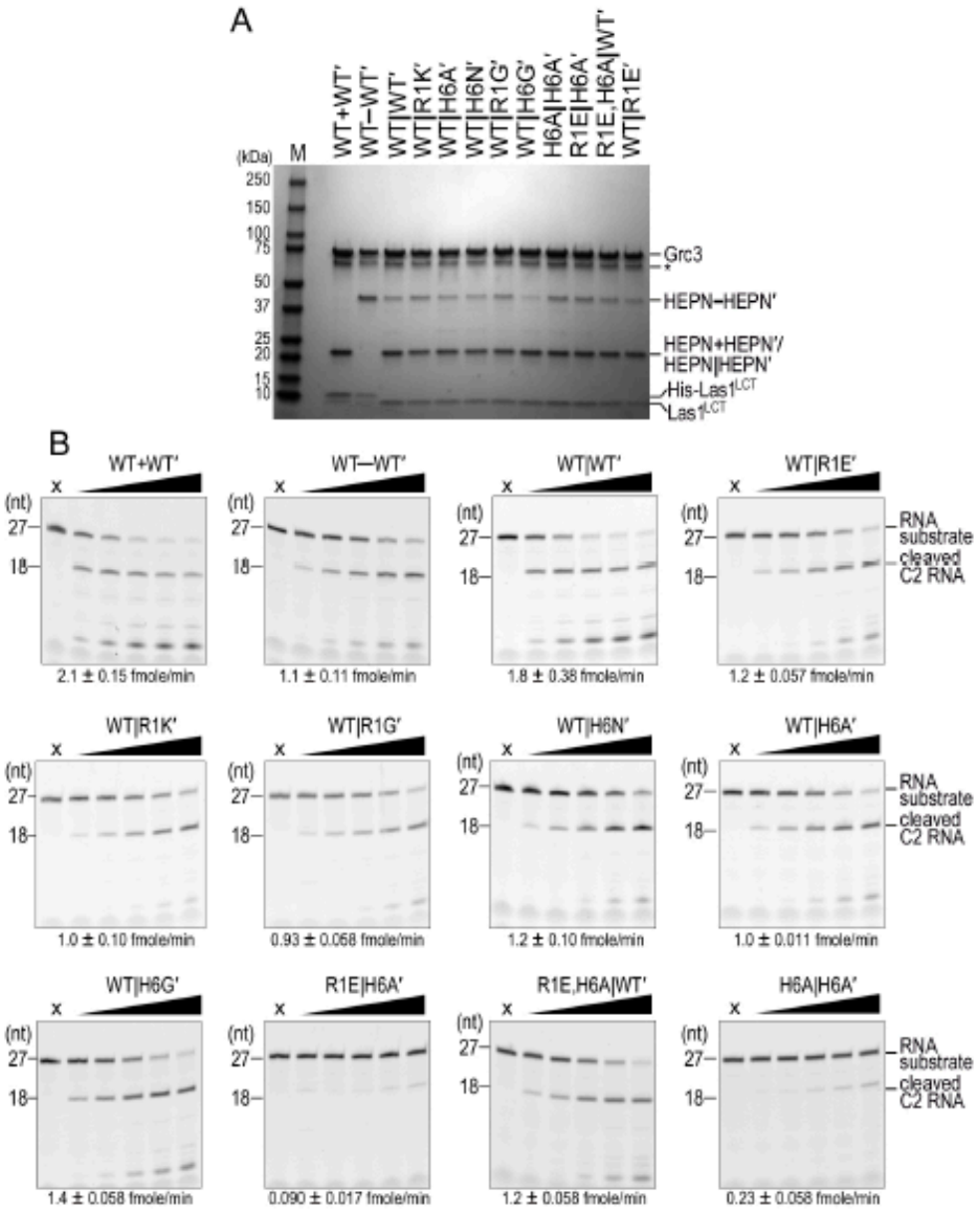

Fig. S5. TEV cleaved RNase PNK chimeras. (*A*) SDS-PAGE analysis of purified ScRNase PNK (WT+WT′), composed of Las1 (1-179 aa; 468-502 aa) and Grc3, chimeric ScRNase PNK (WT-WT′), comprised of Las1 HEPN-HEPN′, Las1 LCT and Grc3, and TEV cleaved ScRNase PNK variants (WT|WT′). (B) Representative denaturing urea gels of reactions containing chimeric ScRNase PNK variants (0-3.2 μM) encoding missense mutations to the Las1 RHxhTH motif. Protein variants were incubated with C2 RNA substrate (50 nM) for 1 hour at 37°C. The specific activity calculated from three biological replicates is shown below each gel.

Table S1. Yeast plasmids used in this study

Plasmid	Variant	*LAS1*	Vector	Source
pMP580	ScLas1	WT; residues 1-502, 3x N-terminal FLAG Tag	YCplac33	(26)
pMP600	ScLas1^{R1E}	R129E; residues 1-502, 3x N-terminal FLAG Tag	YCplac33	This study
pMP648	ScLas1^{R1K}	R129K; residues 1-502, 3x N-terminal FLAG Tag	YCplac33	This study
pMP632	ScLas1^{H2N}	H130N; residues 1-502, 3x N-terminal FLAG Tag	YCplac33	This study
pMP631	ScLas1^{H2D}	H130D; residues 1-502, 3x N-terminal FLAG Tag	YCplac33	This study
pMP654	ScLas1^{H2R}	H130R; residues 1-502, 3x N-terminal FLAG Tag	YCplac33	This study
pMP601	ScLas1^{W3F}	W131F; residues 1-502, 3x N-terminal FLAG Tag	YCplac33	This study
pMP602	ScLas1^{W3L}	W131L; residues 1-502, 3x N-terminal FLAG Tag	YCplac33	This study
pMP603	ScLas1^{G4A}	G132A; residues 1-502, 3x N-terminal FLAG Tag	YCplac33	This study
pMP590	ScLas1^{T5S}	T133S; residues 1-502, 3x N-terminal FLAG Tag	YCplac33	This study
pMP656	ScLas1^{T5A}	T133A; residues 1-502, 3x N-terminal FLAG Tag	YCplac33	This study
pMP591	ScLas1^{H6A}	H134A; residues 1-502, 3x N-terminal FLAG Tag	YCplac33	This study
pMP649	ScLas1^{H6N}	H134N; residues 1-502, 3x N-terminal FLAG Tag	YCplac33	This study

Table S2. Yeast strains used and constructed in this study

Strain	Genotype	Source
CML476	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa	EUROSCARF
tet-LAS1	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$:*LAS1*	This Study
tet-LAS1 + pMP580	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$:*LAS1*; *pMP 580 (3xFlag-LAS1; WT)*	This Study
tet-LAS1 + ycplac33	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$:*LAS1*; *ycplac33 (empty vector)*	This Study
tet-LAS1/Myc-GRC3 (yMP125)	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$:*LAS1*; trp1:3xMyc:*GRC3*	(26)
tet-LAS1/Myc-GRC3 + pMP580	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3xMyc:*GRC3*; *pMP 580 (3xFlag-LAS1; WT)*	(26)
tet-LAS1/Myc-GRC3 + ycplac33	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3xMyc:*GRC3*; *ycplac33 (empty vector)*	(26)
tet-LAS1/Myc-GRC3 + pMP600	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3xMyc:*GRC3*; *pMP 600 (3xFlag-LAS1; R129E)*	This Study
tet-LAS1/Myc-GRC3 + pMP648	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3xMyc:*GRC3*; *pMP 648 (3xFlag-LAS1;* R129K*)*	This Study
tet-LAS1/Myc-GRC3 + pMP632	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3xMyc:*GRC3*; *pMP 632 (3xFlag-LAS1;* H130N*)*	This Study
tet-LAS1/Myc-GRC3 + pMP631	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3xMyc:*GRC3*; *pMP 631 (3xFlag-LAS1;* H130D*)*	This Study
tet-LAS1/Myc-GRC3 + pMP654	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3xMyc:*GRC3*; *pMP 654 (3xFlag-LAS1;* H130R*)*	This Study
tet-LAS1/Myc-GRC3 + pMP601	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3xMyc:*GRC3*; *pMP 601 (3xFlag-LAS1;* W131F*)*	This Study
tet-LAS1/Myc-GRC3 + pMP602	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3xMyc:*GRC3*; *pMP 602 (3xFlag-LAS1;* W131L*)*	This Study
tet-LAS1/Myc-GRC3 + pMP603	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3xMyc:*GRC3*; *pMP 603 (3xFlag-LAS1; G132A)*	This Study

tet-LAS1/Myc-GRC3 + pMP590	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3xMyc:*GRC3*; *pMP 590 (3xFlag-LAS1; T133S)*	This Study
tet-LAS1/Myc-GRC3 + pMP656	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3xMyc:*GRC3*; *pMP 656 (3xFlag-LAS1; T133A)*	This Study
tet-LAS1/Myc-GRC3 + pMP591	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3xMyc:*GRC3*; *pMP 591 (3xFlag-LAS1; H*134A*)*	This Study
tet-LAS1/Myc-GRC3 + pMP649	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3xMyc:*GRC3*; *pMP 649 (3xFlag-LAS1; H134N)*	This Study

Table S3. *E. coli* expression plasmids used and constructed in this study

Plasmid	Variant	*GRC3*	*LAS1*	Vector	Source
pMP001	ScLas1-Grc3	1-632 aa	1-502 aa	pST39	(23)
pMP538	ScLas1^{R1E}-Grc3	1-632 aa	1-502 aa; R129E	pST39	This Study
pMP593	ScLas1^{R1K}-Grc3	1-632 aa	1-502 aa; R129K	pST39	This Study
pMP531	ScLas1^{H2N}-Grc3	1-632 aa	1-502 aa; H130N	pST39	This Study
pMP530	ScLas1^{H2R}-Grc3	1-632 aa	1-502 aa; H130R	pST39	This Study
pMP594	ScLas1^{H2D}-Grc3	1-632 aa	1-502 aa; H130D	pST39	This Study
pMP629	ScLas1^{W3F}-Grc3	1-632 aa	1-502 aa; W131F	pST39	This Study
pMP628	ScLas1^{W3L}-Grc3	1-632 aa	1-502 aa; W131L	pST39	This Study
pMP541	ScLas1^{G4A}-Grc3	1-632 aa	1-502 aa; G132A	pST39	This Study
pMP517	ScLas1^{T5S}-Grc3	1-632 aa	1-502 aa; T133S	pST39	This Study
pMP595	ScLas1^{T5A}-Grc3	1-632 aa	1-502 aa; T133A	pST39	This Study
pMP539	ScLas1^{H6A}-Grc3	1-632 aa	1-502 aa; H134A	pST39	This Study
pMP592	ScLas1^{H6N}-Grc3	1-632 aa	1-502 aa; H134N	pST39	This Study
pMP673	ScLas1$^{WT\text{-}WT'+LCT}$-Grc3	1-632 aa	HEPN-HEPN′: 1-179 aa, 2x GGGGS linker, 1-185 aa + LCT: 469-502 aa	pST39	This Study
pMP681	ScLas1$^{WT\text{-}H6A'+LCT}$-Grc3	1-632 aa	HEPN-HEPN′: 1-179 aa, 2x GGGGS linker, 1-185 aa (H134A) + LCT: 469-502 aa	pST39	This Study
pMP682	ScLas1$^{WT\text{-}H6N'+LCT}$-Grc3	1-632 aa	HEPN-HEPN′: 1-179 aa, 2x GGGGS linker, 1-185 aa (H134N) + LCT: 469-502 aa	pST39	This Study
pMP683	ScLas1$^{WT\text{-}R1E'+LCT}$-Grc3	1-632 aa	HEPN-HEPN′: 1-179 aa, 2x GGGGS linker, 1-185 aa (R129E) + LCT: 469-502 aa	pST39	This Study
pMP684	ScLas1$^{WT\text{-}R1K'+LCT}$-Grc3	1-632 aa	HEPN-HEPN′: 1-179 aa, 2x GGGGS linker, 1-185 aa (R129K) + LCT: 469-502 aa	pST39	This Study
pMP691	ScLas1$^{R1E\text{-}H6A'+LCT}$-Grc3	1-632 aa	HEPN-HEPN′: 1-179 aa (R129E), 2x GGGGS linker, 1-185 aa (H134A) + LCT: 469-502 aa	pST39	This Study
pMP697	ScLas1$^{R1E,H6A\text{-}WT'+LCT}$-Grc3	1-632 aa	HEPN-HEPN′: 1-179 aa (R129E, H134A), 2x GGGGS	pST39	This Study

			linker, 1-185 aa + LCT: 469-502 aa		
pMP695	ScLas1$^{H6A\text{-}H64A'+LCT}$-Grc3	1-632 aa	HEPN-HEPN′: 1-179 aa (H134A), 2x GGGGS linker, 1-185 aa (H134A) + LCT: 469-502 aa	pST39	This Study
pMP301	ScLas1-Grc3	1-632 aa	1-179 aa; 469-502 aa	pST39	(23)
pMP773	ScLas1$^{WT\text{-}WT'+LCT}$-Grc3	1-632 aa	HEPN-HEPN′: 1-179 aa, GGGGS-TEV-GGGS linker, 1-185 aa + LCT: 469-502 aa	pST39	This Study
pMP774	ScLas1$^{WT\text{-}R1K'+LCT}$-Grc3	1-632 aa	HEPN-HEPN(R1K)′: 1-179 aa, GGGGS-TEV-GGGS linker, 1-185 aa + LCT: 469-502 aa	pST39	This Study
pMP775	ScLas1$^{WT\text{-}H6A'+LCT}$-Grc3	1-632 aa	HEPN-HEPN(H6A)′: 1-179 aa, GGGGS-TEV-GGGS linker, 1-185 aa + LCT: 469-502 aa	pST39	This Study
pMP776	ScLas1$^{WT\text{-}H6N'+LCT}$-Grc3	1-632 aa	HEPN-HEPN(H6N)′: 1-179 aa, GGGGS-TEV-GGGS linker, 1-185 aa + LCT: 469-502 aa	pST39	This Study
pMP777	ScLas1$^{WT\text{-}R1G'+LCT}$-Grc3	1-632 aa	HEPN-HEPN(R1G)′: 1-179 aa, GGGGS-TEV-GGGS linker, 1-185 aa + LCT: 469-502 aa	pST39	This Study
pMP778	ScLas1$^{WT\text{-}H6G'+LCT}$-Grc3	1-632 aa	HEPN-HEPN(H6G)′: 1-179 aa, GGGGS-TEV-GGGS linker, 1-185 aa + LCT: 469-502 aa	pST39	This Study
pMP779	ScLas1$^{H6A\text{-}H6A'+LCT}$-Grc3	1-632 aa	HEPN(H6A)-HEPN(H6A)′: 1-179 aa, GGGGS-TEV-GGGS linker, 1-185 aa + LCT: 469-502 aa	pST39	This Study
pMP780	ScLas1$^{R1E\text{-}H6A'+LCT}$-Grc3	1-632 aa	HEPN(R1E)-HEPN(H6A)′: 1-179 aa, GGGGS-TEV-GGGS linker, 1-185 aa + LCT: 469-502 aa	pST39	This Study
pMP781	ScLas1$^{R1E\text{-}H6A'+LCT}$-Grc3	1-632 aa	HEPN(R1E H6A)-HEPN′: 1-179 aa, GGGGS-TEV-GGGS linker, 1-185 aa + LCT: 469-502 aa	pST39	This Study
pMP782	ScLas1$^{R1E\text{-}WT'+LCT}$-Grc3	1-632 aa	HEPN(R1E)-HEPN′: 1-179 aa, GGGGS-TEV-GGGS linker, 1-185 aa + LCT: 469-502 aa	pST39	This Study

Table S4. RT-PCR Primers

Primer	Sequence
Las1RT1Fwd	5′ TGGAAGCGACTTAGAAACGAG 3′
Las1RTT1Rev	5′ CTTGCGTGTGAACTTTTCCTC 3′
Tfc1RTFwd	5′ GCTGGCACTCATATCTTATCGTTTCACAATGG 3′
Tfc1RTRev	5′ GAACCTGCTGTCAATACCGCCTGGAG 3′