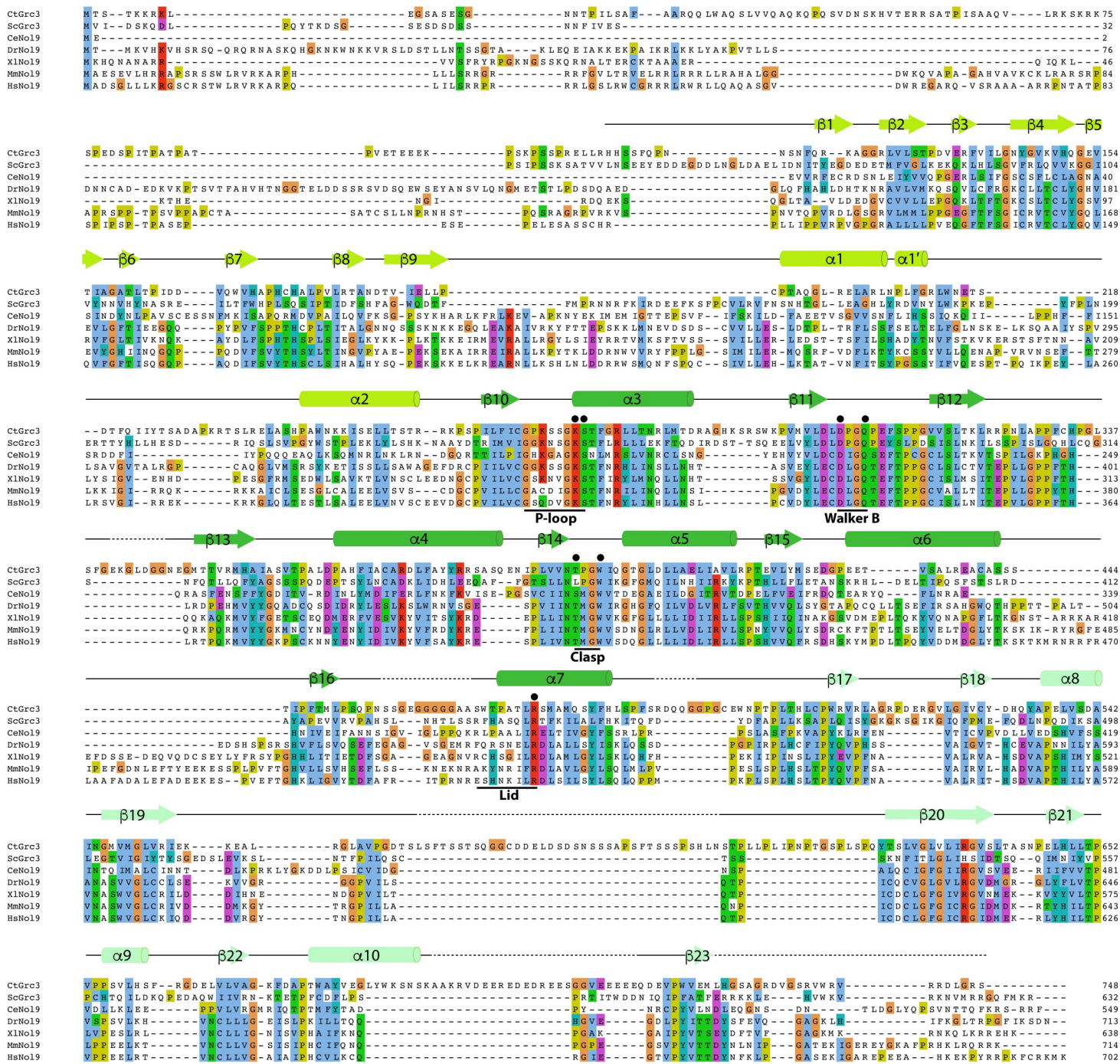**Supplementary Note 1| Las1 multiple amino acid sequence alignment.**

a, Schematic of Las1 domain boundaries. Black bars define the Las1 regions used to generate sequence alignments. The Las1 coiled-coil (CC) domain alignment is not displayed due to low sequence conservation. **b,** Sequence alignments for the Las1 HEPN nuclease domain. Alignments were performed with PROMALS3D (Pei, J. et al., *Nucleic Acids Res.* 36, 2295-2300, 2008) and illustrated in JalView (Waterhouse, A.M. et al., *Bioinformatics.* 25, 1189-1191, 2009). The secondary structure is shown above. The black bracket defines the N-terminus of Las1 that forms an interface with Grc3. The red bracket indicates the HEPN core and the green bracket in α4 marks the HEPN dimer interface. Residues targeted for mutagenesis are marked with a black dot. Abbreviations are as follows: *Chaetomium thermophilum* (Ct), *Saccharomyces cerevisiae* (Sc), *Caenorhabditis elegans* (Ce), *Danio rerio* (Dr), *Xenopus laevis* (Xl), *Mus Musculus* (Mm), and *Homo sapiens* (Hs). **c,** Sequence alignments for the Las1 C-terminal tail (LCT). Alignments were performed with PROMALS3D and illustrated in JalView as seen in panel B. Las1 LCT hydrophobic residues engaged with the Grc3 C-terminal domain are marked by arrowheads.

Secondary structure labels: β1 β2 β3 β4 β5 β6 β7 β8 β9 α1 α1′ α2 β10 α3 β11 β12 β13 α4 β14 α5 β15 α6 β16 α7 β17 β18 α8 β19 β20 β21 α9 β22 α10 β23

Block 1

```
CtGrc3  MTS--TKKRKL------------------------------EGSASESG-----------NNTPILSAF---AARQQLWAQSLVVQAQKQPQSVDNSKHVTERRSATPISAAQV----LRKSKRK 75
ScGrc3  MVI--DSKQDL----------------PQYTKDSG--------SESDSDSS----------NNFIVES-------------------------------------------------------- 32
CeNol9  ME----------------------------------------------------------------------------------------------------------------------- 2
DrNol9  MT---MKVHKVHSRSQ-QRQRNASKQHGKNKWNKKVRSLDSTLLNTSSGTA----KLEQEIAKKEKPAIKRLKKLYAKPVTLLS--------------------------------------- 76
XlNol9  MKHQNANARR-----------------VVSFRYRPGKNGSSKQRNALTERCKTAAAER-----------------------------------------------------QIQKL--- 46
MmNol9  MAESEVLHRRAPSRSSWLRVRKARPH-----------LLLSRRGR----RRFGVLTRVELRRLRRRLLRAHALGG----------------DWKQVAPA-GAHVAVKCKLRARSRP 84
HsNol9  MADSGLLLKRGSCRSTWLRVRKARPQ----------LILSRRPR----RRLGSLRWCGRRRLRWRLLQAQASGV------------------DWREGARQ-VSRAAA-ARRPNTATP 83
```

Block 2 (β1–β5)

```
CtGrc3  SPEDSPITPATPAT-------------------PVETEEEK----------PSKPSSPRELLRHHSSFQPN-------------NSNFQR--KAGGRLVLSTPDVERFVILGNYGVKVHQGEV 154
ScGrc3  --------------------------------PSIPESKSATVVLNSEEYEDDEGDDLNGLDAELIDNITYEGDEDETMFVGLKEKQKLHLSGVFRLQVVKGGI 104
CeNol9  --------------------------------------------------------------EVVRFECRDSNLEIYVVQPGERLEIFGSCSFLCLAGNA 40
DrNol9  DNNCAD-EDKVKPTSVTFAHVHTNGTELDDSSRSVDSQEWSEYANSVLQNGMETSTLPDSDQAED----------------GLQPHAHLDHTKNRAVLVMKQSQVLCFRGKCLLTCLYGHV 181
XlNol9  I--------KTHE------------NGI---------------------RDQEKS----------QGLTA--VLDEDGVCVVLLEPGQKLFTGKCSLTCLYGSV 97
MmNol9  APRSPP-TPSVPPAPCTA--------SATCSLLNPRNHST------PQSRAGRFVRKVS------PNVTQPVRDLGSGRVLMMLPPGEGFTFSGICRVTCVYGQL 168
HsNol9  SPIPSP-TPASEP-------------ESE--------YELESASSCHR--------PLLIPPVRPVGPGRALLLLPVEQGFTFSGICRVTCVYGQV 149
```

Block 3 (β6–β9, α1, α1′)

```
CtGrc3  TIAGATLTPIDD----VQWVHAPHCHALPVLRTANDTV-IELLP--------------------------CPTAQGL-RELARLNPLFGRLWNETS---------------------------- 218
ScGrc3  VYNNVHYNASRE----ILTFWHPLSQSIPTIDFSHFAG-WQDIF---------FMPRNNRFKIRDEEFKSFPCVLRVFNSNHTGL-LEAGHLYRDVNVLWKPKEP------YFPLN 199
CeNol9  SINDYNLPAVSCESSNPMKISAPQRMDVPAILQVFKSG-PSYKHARLKFRLKEV-APKNYEKIMEMIGTTEPSVF--IPSKILD-FAEETVSGVVSNFLIHSSIQKQII-----LPPHF--FISI 151
DrNol9  EVLGFTIEEGQQ----PYPVFSPPTHCPLTITALGNNQSSSKNKKEGQLEAKAIVRKYFTTEPSKKLMNEVDSDS--CVVLLES-LDTPL-TRFLSSFSELTELPGLNSKE-LKSQAAIYSPV 295
XlNol9  RVFGLTIVKNQK----AYDLFSPHTHSPLSIEGLKYKK-PLKTKKEIRMEVRALLRGYLSIEYRRTVMKSFTVSS-SVILLER-LEDST-TSFILSHADYTNVFSTKVKERSTSFTNN--AV 209
MmNol9  EVYGHIINQGQP----PQDVFSVYTHSYLTINGVFYAE-PEKSGEKAIRREIKALLKPYTKLDDRNWVVRYFPPLG--SIMILER-MQSRF-VDFLKTYKCGSSYVLLQENAP-VRVNSEF--TT 279
HsNol9  QVFGPTISQGQP----AQDIFSVYTHSCLSIHALHYSQ-PEKSKKELKRARNLLKSHLNLDDRRWSMQNFSPQC--SIVLLEH-LKTAT-VNFITSYPGSSYIPVQESPT-PQIKPEY--LA 260
```

Block 4 (α2, β10, α3, β11, β12 — P-loop, Walker B)

```
CtGrc3  --DTFQIIYTSADAPKRTSLRELASHPAWNKKISELLTSTR--RKPSPILFICGPKSSGKSTFGRLLTNRLMTDRAGHKSRSWKPVMVLDLDPGQPEFSPPGVVSLTKLRRPNLAPPFCHPGL 337
ScGrc3  ERTTYHLLHESD------RIQSLSVPGYWSTPLEKLYLSHK-NAAYDTRIMVIGGKNSGKSTFLRLLLEKFTQDIRDST-TSQEELVYLDLDPGQPEYSLPDSISLNKILSSPISLGQHLCQG 314
CeNol9  SRDDFI---------IYPQQQEAQLKSQMNRLNKLRN-DQQRTTILPIGHKGAGKSNLMRSLVNRCLSNG---------YEHVVVLDCDIGQSEFTPCGCLSLTKVTSPILGKPHGH---249
DrNol9  LSAVGVTALRGP------CAQGLVMSRSYKETISSLLSAWAGEFDRCPIILVCGGKSSGKSTFNRHLINSLLNHT--------ASVEYLECDLGQTEFTPPGCLSLCTVTEPLLGPPFTH---401
XlNol9  LYSIGV--ENHD------PESGFRMSEDWLSLAVKTLVNSCLEEDNGCPVILVCGSKNVGKSTFIRYLMNQLLNHT--------SSVGYLDCDLGQTEFTPPGCISLNSITEPVLGPPFTH---313
MmNol9  LKKIGI--RRQK------RKKAICLSESGLCALEELVSVS---CDGCPVILLCGACDIGKSTFNRILINQLLNSI--------PGVDYLECDLGQTEFTPPGCVALLTITEPLLGPPYTH---380
HsNol9  LRSVGI--RREK------KRKGLQLTESTLSALEELVNVSCEEVDGCPVILVCGSQDIGKSTFNRYLINHLLNSL--------PCVDYLECDLGQTEFTPPGCISLLNITEPVLGPPFTH---364
```
**P-loop** (under CtGrc3 GKSTFGR region), **Walker B** (under LDLDPGQP region)

Block 5 (β13, α4, β14, α5, β15, α6 — Clasp)

```
CtGrc3  SFGEKGLDGGNEGMTTVRMHAIASVTPALDPAHFIACAKDLFAYYRRSASQENIPLVVNTPWIQGTGLDLLAELIAVLRPTEVLYMSEDGPEET------VSALREACASSS-----------444
ScGrc3  S-----------NFQTLTLQFYAGSSSPQDEPTSYLNCADKLIDHLEEQAF--FGTSLLNLPGWIKGFGMQILNHIIRKYKPTHLLFLETANSKRHL--DELTIPQSFSTSLRD----------412
CeNol9  ----------QRASFENSFFYGDIIQV-RDINLYMDIFERLFNKFKVISE-PGSVCIINSMGWVTDEGAEILDGITRVTDPELFVEIFRDQTEARYQ----FLNRAE-------------------339
DrNol9  ---------LRDPEHMVYYGQADCQSDIDRYLESLKSLWRNVSGE------SPVIINTMGWVRGHGFQILVDLVRLPSVTHVVQLSYGTAPQCQLLTSEFIRSAHGWQTHPETT-PALT--SLD 504
XlNol9  ---------QQKAQKMVYFGETSCEQDMERFVESVKYVITSYKRD-----EPLINTMGWVRKGFGLLLLIDIIRLLSPSHIIQINAKGSVDMEPLTQKVYQNAPGFLTKGNST-ARRKAR 418
MmNol9  ---------QRKPQRMVYYGKMNCYNDYENYIDIVKYVFRDYKRE-----FPLIVNTMGWVSDNGLRLLVDLIRVLSPNYVVQLYSDRCKFTPTLTSEYVELTDGLYTKSKIK-RYRGFE 485
HsNol9  ---------LRTPQKMVYYGKPSCKNNYENYIDIVKYVFSAYKRE-----FPLIVNTMGWVSDQGLLLLIDLIRLLSPSHVVQFRSDHSKYMPDLTPQYVDDMDGLYTKSKTKMRNRRFR 470
```
**Clasp** (under PWIQGTGL region)

Block 6 (β16, α7, β17, β18, α8 — Lid)

```
CtGrc3  ----------------TIPFFTMLPSQPNSSGEGGGGGAASWTPATLRSMAMQSYFHLSPFSRDQQGGPGCEWNPTPLTHLCPWRVRLAGRPDERGVLGIVCY-DHQYAPELVSDA 542
ScGrc3  ----------------AYAPEVRVRPAHSL--NHTLSSRFHASQLRFTFKKILALFHKITQFD--------YDFAPLLKSAPLQISYGKGKSGIKGTQFPME-FQDLNPQDIKSA 498
CeNol9  ----------------HNIVEIFANNSIGV-IGLPPQKRLPAALIRELTIVGYFSSRLPR-----------PSLASFPKVAPIKLEFTKFEN----------VTICVPVDLLVEDSHVFSS 419
DrNol9  ----------------EDSHSPSRSHVFLSVQSEFEGAG--VSGEMRFQRSNELRDLALLSYISKLQSSD----------PGPIRPLHCFIPYQVPHSS--------VAIGVT-HCEVAPNNILYA 593
XlNol9  EFDSSE-DEQVQDCSEYLFRSYPHLIETDFSGA----GEAGNVRCDLRDLAMLGYLSLQHFH----------PEKIIPINSLIPYEVPFNA--------VALRVA-HSDVAPSHIMYS 521
MmNol9  IPEFGDNLEFTYEEKESSPLPVFTGHVLLSVHSEFLSS---KNEKNRAKYNRIFRDLAVLGYLSQLMLPV---------PESLSPLHSLTPYQVPFSA--------VAIRVL-HADVAPTHILYA 589
HsNol9  LAAFADALEFADEEKES--PVEFTGHKLIGVYTDFAFR---ITPRNRESHNKLRDLSILSYLSQLQPPM--------PKPLSPLHSLTPYQVPFNA--------VALRIT-HSDVAPTHILYA 572
```
**Lid** (under RESHNKLRDL region)

Block 7 (β19, β20, β21)

```
CtGrc3  INGMVMGLVRIEK----KEAL------RGLAVPGDTSLSFTSSTSQGGCDDELDSDSNSSSAPSFTSSSPSHLNSTPLLFLIPNPTGSPLSPQYTSLVGLVLIRGVSLTASNPELHLLTP 652
ScGrc3  LEGTTVIGIYTYSGEDSLEVKSL---------NTFPILQSC------------------TSS----------SKNFITLGLIHSIDTSQ--QIMNIYVP 557
CeNol9  INTQIMALCINHT----DLKPRKLYGKDDLPSICVIDG--------------NSP--------ALQCIGFGIIRGVSVEE-RIIFVVTP 481
DrNol9  ANASVVGLCCLSE----KVVGR----------GGPVILS-----------------QTP-------ICQCVGLGVLRGVDMGR-GLYFLVTP 646
XlNol9  VNASWVGLCRILD----DIHNE----------NDGPVILT-----------------QTP-------ICDCLGFGIVRGVNMEK--KVYYVLTP 575
MmNol9  VNASWVGLCRIVD----DMKGY----------TRGPILLA-----------------QNP-------ICDCLGFGICRGIDMDK--RTYHILFP 643
HsNol9  VNASWVGLCKIQD----DVRGY----------TNGPILLA-----------------QTP-------ICDCLGFGICRGIDMEK--RLYHILTP 626
```

Block 8 (α9, β22, α10, β23)

```
CtGrc3  VPPSVLHSF--RGDELVLVAG-KFDAPTWAYVEGLYWKSNSKAAKRVDEEREDEDREESGGVEEEEEQDEVPWVEMLHGSAGRDVGSRVWRV---------RRDLGRS 748
ScGrc3  PCHTQILDKQPEDAQWIIVRN-KTEPPFCDFLPS---------------------PRTITWDDNIQIPFATFERRKLK----HVWKV--------RKNVMRRGQYMRF 632
CeNol9  VDLLKLEE---------PPVLVRGMRIQPTMFYTAD---------PY------NRCPVVLNDLEQGNS--DN------TLDGLYQPSVNTTQFKRS-RRF---- 549
DrNol9  VSPSVLKH---------VNCLLLG-EISLPKILLTQQ------------HGVF------GDLPYITTDYSFEVQ--GAGKLH---------IFKGLTRPGFIKSDN- 713
XlNol9  LVPESLRL-------VNCLLIG-NISIPHKFKNQ----------PGAK------GAIPYVTSEYDFTVF--GAGKMK-------------VALRKRREHK------ 638
MmNol9  LPPEELKT--------VNCLLVG-SISIPHCIFQNQ---------PGPE------GSVPVYTTDYNLNIP--GATEKIGEREYGKAFPRHKLRQRRK-------- 714
HsNol9  VPPEELRT--------VNCLLVG-AIAIPHCVLKCQ---------RGIE------GTVPYVTTDYNFKLP---GASEKIGAREPEEA---HKEKPYRRPKFCRKMK 702
```

Supplementary Note 2| Grc3 (Nol9) multiple amino acid sequence alignment.
Alignments of Grc3 and its mammalian homologs Nol9 were performed with PROMALS3D (Pei, J. et al., *Nucleic Acids Res.* 36, 2295-2300,2008) and illustrated in JalView (Waterhouse, A.M. et al., *Bioinformatics.* 25, 1189-1191, 2009). The secondary structure is shown above, with the NTD colored chartreuse, the PNK colored dark green, and the CTD colored light green. Residues previously shown to be important for Grc3 polynucleotide kinase activity are indicated with a dot (Pillon, M.C. et al., *RNA.* 24, 721-738, 2018). Abbreviations are as follows: Chaetomium thermophilum (Ct), Saccharomyces cerevisiae (Sc), Caenorhabditis elegans (Ce),Danio rerio (Dr), Xenopus laevis (Xl), Mus Musculus (Mm), and Homo sapiens (Hs).

Supplementary Table 1 | RNA oligos used in this study

RNA	Sequence	Source
SS-RNA	5'-ACGUACGCGGAAUACUUCGAA-3'	IDT
SC-ITS2-3-RNA	5′-GUCGUUUUA-3′	IDT
SC-ITS2-5-RNA	5′-GGUUUUACCAACUGCGGC-3′	IDT
SC-ITS2-cleaved-RNA	SC-ITS2-3-RNA annealed to SC-ITS2-5-RNA	IDT
SC-ITS2-RNA	5′-GUCGUUUUAGGUUUUACCAACUGCGGC/36-FAM/-3′	IDT
CT-ITS2-RNA	5′-UGUGUUGGGGdeoxyACCCGCGGCUGCUCG CGGGCCCUGAAAAGCA-3′	IDT

Supplementary Table 2 | 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
tetO$_7$-*LAS1* (yMP 125)	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$:*LAS1*; trp1:3Myc:*GRC3*	This Study
tetO$_7$-*LAS1* + *pMP 580*	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3Myc:*GRC3*; *pMP 580 (3Flag-LAS1; WT)*	This Study
tetO$_7$-*LAS1* + *pMP 581*	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3Myc:*GRC3*; *pMP 655 (3Flag-LAS1; R129E, H134A)*	This Study
tetO$_7$-*LAS1* + *pMP 655*	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3Myc:*GRC3*; *pMP 655 (3Flag-LAS1; H130A)*	This Study
tetO$_7$-*LAS1* + *YCplac33*	*MATα;* ura3-52; leu2Δ1; his3Δ200; GAL2; CMVp(tetR'-SSN6)::LEU2; trp1::tTa; kanMX:tetO$_7$: *LAS1*; trp1:3Myc:*GRC3*; *YCplac33 (empty vector)*	This Study