Intergenic Transcriptional Interference Is Blocked by RNA Polymerase III Transcription Factor TFIIIB in *Saccharomyces cerevisiae*

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ABSTRACT The major function of eukaryotic RNA polymerase III is to transcribe transfer RNA, 5S ribosomal RNA, and other small non-protein-coding RNA molecules. Assembly of the RNA polymerase III complex on chromosomal DNA requires the sequential binding of transcription factor complexes TFIIIC and TFIIIB. Recent evidence has suggested that in addition to producing RNA transcripts, chromatin-assembled RNA polymerase III complexes may mediate additional nuclear functions that include chromatin boundary, nucleosome phasing, and general genome organization activities. This study provides evidence of another such "extratranscriptional" activity of assembled RNA polymerase III complexes, which is the ability to block progression of intergenic RNA polymerase II transcription. We demonstrate that the RNA polymerase III complex bound to the tRNA gene upstream of the *Saccharomyces cerevisiae* *ATG31* gene protects the *ATG31* promoter against readthrough transcriptional interference from the upstream noncoding intergenic *SUT467* transcription unit. This protection is predominately mediated by binding of the TFIIIB complex. When TFIIIB binding to this tRNA gene is weakened, an extended *SUT467–ATG31* readthrough transcript is produced, resulting in compromised *ATG31* translation. Since the *ATG31* gene product is required for autophagy, strains expressing the readthrough transcript exhibit defective autophagy induction and reduced fitness under autophagy-inducing nitrogen starvation conditions. Given the recent discovery of widespread pervasive transcription in all forms of life, protection of neighboring genes from intergenic transcriptional interference may be a key extratranscriptional function of assembled RNA polymerase III complexes and possibly other DNA binding proteins.

IN eukaryotes, the process of transcription is divided among three RNA polymerases—RNA polymerase I, II, and III. In the yeast *Saccharomyces cerevisiae*, RNA polymerase III (Pol III) transcribes a variety of small RNAs, including transfer RNA (tRNA), 5S ribosomal RNA (5S rRNA), U6 spliceosomal RNA, snR52 small nucleolar RNA, 7SL RNA, and the RNA component of RNase P. Assembly of the transcription machinery on Pol III genes is mainly determined by the structure of the promoter. A unique feature of most Pol III promoters is the presence of internal control regions (ICRs) that are composed of conserved sequences separated by more variable regions. The most common promoter arrangement used by Pol III is the class II promoter, found mainly in the tRNA genes (tDNAs). Class II promoters consist of the conserved intragenic *A-box* and *B-box* sequences that are bound by the transcription factor complex TFIIIC (Pascali and Teichmann 2012; Acker *et al.* 2013).

In yeast, the entire Pol III transcription machinery bound to tDNAs consists of three multimeric protein complexes: the transcription factors TFIIIC (6 subunits) and TFIIIB (3 subunits), which are required for promoter recognition and preinitiation complex formation, and the 17 subunit Pol III enzyme (Geiduschek and Kassavetis 2001; Huang and Maraia 2001; Acker *et al.* 2013). The initial step in the transcription of tDNAs in yeast is the binding of the TFIIIC complex to the *A-* and the *B-box*es. The 6 subunits of TFIIIC are organized into two globular domains, τ_A (Tfc1p, Tfc4p, and Tfc7p) and τ_B (Tfc3p, Tfc6p, and Tfc8p). τ_B specifically binds to the *B-box* with high affinity and favors *A-box* binding by τ_A (Geiduschek and Kassavetis 2001). The most currently refined *B-box* consensus sequence, GWT*C*RANNC (Marck *et al.* 2006; Orioli

et al. 2012) contains a highly conserved cytosine residue (italicized), and mutation of this cytosine compromises TFIIIC binding (reviewed in Donze 2012). TFIIIC binding is required to recruit TFIIIB at most Pol III promoters. TFIIIB is composed of three proteins, *T*ATA-*b*inding *p*rotein (TBP), TFII*B*-*r*elated *f*actor (Brf1p) and B″ (*B*-*double prime*, Bdp1p). Binding of TFIIIB forms an exceptionally kinetically stable TFIIIB–DNA complex (Cloutier *et al.* 2001), which then recruits the Pol III enzymatic complex and helps maintain it for multiple transcription cycles in a process called facilitated recycling (Dieci and Sentenac 1996; Ferrari *et al.* 2004).

While Pol III and its transcription factors are generally thought to be dedicated to transcription of Pol III target genes, emerging studies have shown that either partial or complete DNA-bound Pol III transcription complexes can have effects on transcription, chromatin state, and genome organization of neighboring Pol II genes. These so-called "extratranscriptional" (Donze 2012) or "product independent" (Clelland and Schultz 2010) effects of Pol III complexes, mostly demonstrated in *S. cerevisiae*, include the following activities: targeting integration of Ty retroelements (Chalker and Sandmeyer 1990; Ji *et al.* 1993; Devine and Boeke 1996), displacement of nucleosomes (Morse *et al.* 1992), phasing of adjacent nucleosomes (Nagarajavel *et al.* 2013), position effect repression of adjacent Pol II promoters (Hull *et al.* 1994), chromatin boundary/insulator functions (Donze 2012), and pausing of replication forks (Deshpande and Newlon 1996; Sekedat *et al.* 2010). In some instances, the TFIIIC complex alone can mediate extratranscriptional functions, as *E*xtra *TFIIIC* (ETC) sites (Moqtaderi and Struhl 2004), chromosomal loci that bind only TFIIIC without recruiting TFIIIB or Pol III, can act as insulators (Simms *et al.* 2008), can directly regulate Pol II promoters (Kleinschmidt *et al.* 2011), and can tether chromosomal regions to the nuclear periphery (Hiraga *et al.* 2012).

Using the *S. cerevisiae* model system, we have previously described multiple types of extratranscriptional functions of the *TRT2* tDNA at the *STE6–CBT1* locus. In *MATα* cells, *TRT2* serves as a barrier to prevent repression of the neighboring Pol II-transcribed *CBT1* gene, whereas in *MAT*a cells *TRT2* exerts an apparent tRNA position effect, as deletion of *TRT2* results in an increase in *CBT1* gene transcription (Simms *et al.* 2004). This modest position effect (approximately threefold increase in *CBT1* mRNA levels) was shown to be due in part to the tDNA acting as an insulator, as it prevents inappropriate activation of the *CBT1* promoter by the Mcm1p transcription factor that binds to the nearby *STE6* upstream activation sequence (UAS) (Simms *et al.* 2008).

Manual inspection of the *S. cerevisiae* genome reveals that about one-quarter of all tDNAs lie between divergently transcribed genes in the yeast genome and could potentially show a similar insulator effect. Given the modest insulator effect observed at the *CBT1* locus, we investigated the *ATG31–tV(UAC)D–SES1* locus anticipating a more robust ef-

fect, as genome-wide expression data indicate that *SES1* is transcribed at considerably higher levels (∼70-fold) than is *ATG31* in rich media (Holstege *et al.* 1998; Xu *et al.* 2009). Our reasoning was that transcription factors responsible for the high level activation of *SES1* would more strongly activate *ATG31* upon deletion of the tDNA. Surprisingly, when we performed Northern blot analysis on RNA from wild-type and *tv(uac)d*Δ (referred to hereafter as *tdna*Δ) strains, we found that *ATG31* mRNA levels were not only increased, but that a *longer* transcript with an extended 5′-UTR (5′-untranslated region) replaced the normal transcript. We show here that this longer transcript is due to readthrough of the noncoding stable unannotated transcript *SUT467* (Xu *et al.* 2009), and mutations that inhibit TFIIIB complex assembly or stability allow readthrough. Progression of transcription from the upstream *SUT467* start site prevents normal *ATG31* transcriptional initiation, and the extended 5′-UTR inhibits translation of the *ATG31* coding sequence. Since Atg31p is required for autophagy, reduced translation results in compromised autophagy and fitness under nitrogen starvation conditions in strains exclusively expressing the extended transcript. This work identifies another novel extratranscriptional function of tDNAs, the ability to block progression of cryptic intergenic transcription, preventing subsequent deleterious transcriptional interference of an adjacent promoter.

Materials and Methods

Yeast cultures were grown in nutrient-rich YPD media (1% yeast extract, 2% peptone, and 2% dextrose) at 30° on a rotary shaker unless otherwise noted. For induction of autophagy, cells were grown to mid-log phase ($A_{600} = 0.7$) in YPD, collected by centrifugation (3000 rpm $\times$ 5 min), washed with water, then resuspended in nitrogen starvation media (1.7 g/liter yeast nitrogen base without amino acids and without ammonium sulfate, plus 2% dextrose). For Northern blot analysis of temperature-sensitive mutants, cultures were grown at 30° to an OD_{600} of 0.7 and then incubated at 37° for 1 hr before RNA extraction.

Plasmid pDD1232 was created by cloning a 1.35-kb *Xho*I–*Spe*I cut *ATG31–SES1* intergenic fragment (PCR amplified with oligos DDO1281/-1282, which added an artificial *Xho*I site) into Bluescript SK+ (all oligos used are listed in Supporting Information, Table S2). Two-step PCR mutagenesis was performed using pDD1232 as template and T7 and T3 primers with mutagenic primers (DDO184/-1284; DDO183/-1285, respectively) to amplify the fragment containing the *tdna* deletion; this fragment was then cloned into Bluescript SK+ as above to create pDD1233. Direct-site-directed mutagenesis was performed on pDD1232 to create pDD1248 (*tdna B-box*Δ), pDD1249 (*tdna B-box mutant*), pDD1261 (*tdna A-box mutant*), and pDD1260 (*tdna*Δ::*Eco*RI–*Bam*HI linker), using DDO1391/-1392, DDO1393/-1394, DDO1474/-1475, and DDO1466/-1467 primer sets, respectively. The *B-box* mutant had the invariant cytosine and following guanine bases changed to GC, and the *A-box* mutant scrambled the

entire consensus. Plasmids pDD1262 (flipped orientation of the tDNA) and pDD1272 (tdnaΔ::ETC9) were created by cloning EcoRI–BamHI-digested PCR-amplified fragments using DDO1468/-1469 (flip), or DDO1534/-1535 (ETC9), respectively, into EcoRI–BamHI-digested pDD1260. Yeast genomic DNA was used as PCR template. Plasmid pDD1263 (tdnaΔ::ETC4) was constructed by directly ligating complementary oligonucleotides (DDO1489/-1490) containing EcoRI–BamHI overhangs into pDD1260. All plasmids were confirmed by Sanger sequencing and are listed in Table S1.

Yeast strains were generated from wild-type S. cerevisiae W303-1a; genotypes of all strains used and generated in this study are given in Table 1. Parent tdnaΔ::URA3 (DDY4605–4607) strains were created by amplifying URA3 with primers DDO1279/-1280 containing homology to the flanking region of tV(UAC)D, and then this DNA was transformed into wild-type DDY3 followed by selection of Ura+ colonies and PCR identification of homologous recombinants. Linearized tdna mutant plasmids were digested with XhoI and SpeI, individually transformed into a tdnaΔ::URA3 strain, and 5-fluoroorotic acid (5-FOA) resistant colonies were isolated. Recombinants were identified by PCR and verified by DNA sequencing of the product. Yeast strains for chromatin immunoprecipitation (ChIP) were created by crossing existing BRF1–3X-FLAG (DDY1495) and TFC1–3X-FLAG (DDY3860) strains to DDY4607, and then FLAG-tagged Ura+ progeny were backcrossed to each tdna mutant.

The 9X-Myc epitope tag was amplified from a TRP1 marked cassette (Knop et al. 1999) using DDO1462/-1463 and transformed into wild-type and tdna mutants. ATG31–9X-myc Trp+ homologous recombinants were identified by PCR (DDO1419/-1464) and confirmed by Western blotting. ATG31 and ATG8 knockout strains were constructed by standard yeast homologous recombination. For autophagy induction alkaline phosphatase assays, pho13Δ pho8Δ60 strains were created by crossing a pho13Δ::URA3 pho8Δ60::HIS3 strain (kindly provided by Daniel Klionsky) to wild-type and tdna mutants.

RNA isolation and Northern blotting were performed as described (Simms et al. 2004). Most Northern results were verified with three (but at least two) independently isolated mutant strains; Table 1 lists only the specific strains shown in the figures. Primers used to amplify Northern probes are listed in Table S2. 5′-RACE analysis was performed on RNA isolated from the DDY420 brf1 II.6 mutant using the First Choice RLM-RACE kit (Ambion/Life Technologies, AM1700). Individual clones were sequenced by standard Sanger sequencing and mapped to the S. cerevisiae genome on the Saccharomyces Genome Database at http://www.yeastgenome.org (Cherry et al. 2012).

Quantitative RT–PCR was performed as follows: First-strand cDNA was synthesized from 0.5 μg of total RNA after DNase treatment (RQ1 DNase, Promega M6101). Synthesis was extended from long transcript specific primer DDO1284 using ProtoScript M-MuLV first-strand cDNA synthesis kit (NEB E6300S). Quantitative reverse transcription PCR (qRT–PCR) was performed on 1:4 diluted cDNA using primers DDO1606/-1555 and Sybr Green super mix (Bio-Rad 170-8882) with 60° annealing temperature. Results were normalized to amplicons from ACT1 control primers (DDO402+403). Reactions were run and analyzed using a Bio-Rad MyiQ as described (Kim et al. 2011) and examined by agarose gel electrophoresis to verify that only the predicted PCR products were amplified. The primers were designed to specifically amplify readthrough transcripts; the strategy is described and illustrated in Figure S2.

Chromatin immunoprecipitation was performed as described previously (Rusche et al. 2002). Anti-FLAG epitope antibody was purchased from Sigma (F1804). Primers DDO1527/-1555 or DDO1576/-1577 were used to amplify desired regions surrounding tV(UAC)D. For Western analysis, yeast minilysates were prepared by glass bead lysis of log-phase cultures directly in lysis buffer (50 mM Tris pH 7.5, 1% SDS, 5 mM EDTA, 14.3 mM β-mercaptoethanol, 1 mM PMSF, 2 μg/ml leupeptin and pepstatin). Pellets from 5 ml YPD culture at A600 1.0 were resuspended in 200 μl lysis buffer and then vortexed with glass beads at 4° for 10 min. Lysis buffer, 100 μl, was added, and the mixture was boiled for 3 min, cooled on ice, and centrifuged at 4° to remove cell debris. To 100 μl of clarified minilysate, an equal amount of 2× SDS–PAGE loading buffer was added, and after boiling, 15 μl was loaded on 12% acrylamide protein gel. Proteins were transferred to Millipore Immobilon membrane by semidry transfer and incubated in blotto (10× TBS/10% SDS/5% dry milk) for 1 hr. Primary Myc antibody (c-Myc 9E10, Santa Cruz Biotechnology), anti-mouse Ig-horseradish peroxidase secondary antibody (GE healthcare) were used for Western analysis. Immuno-star Western chemiluminescent kit (Bio-Rad) was used to detect the secondary antibody.

The alkaline phosphatase pho8Δ60 assay was performed as described (Klionsky 2007). The cell survival assay was adapted from Kabeya et al. (2007). Yeast strains were grown in YPD rich media to $A_{600} = 1.0$ (∼10^7 cells/ml). Cells were harvested by centrifugation, washed once with distilled water, and resuspended at 10^7 cells/ml in media lacking nitrogen. Cells were incubated for 6 days at 30° on rotatory shaker, and every other day 200 μl of culture dilutions was plated on YPD plates in triplicate. Plates were incubated at 30° for 48 hr and survival rate was obtained by counting resulting colonies.

Results

Mutation of the tDNA upstream of ATG31 results in readthrough of SUT467

Figure 1A depicts the ATG31–SES1 locus, showing the location of the tV(UAC)D tDNA, and the extent of transcripts normally produced from the region. To test the initial hypothesis that tV(UAC)D might act as an insulator by

Table 1 S. cerevisiae strains used and generated in this study

Name	Genotype	Source
DDY3	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1	J. Rine
DDY232	MATα his3-11,15 leu2-3,112 trp1-1 ura3-1 rpc31-236 hmrΔ	Donze lab
DDY246	MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 rpc160-Δ1::HIS3 p-rpc160-112	Donze lab
DDY261	MATα his3-11,15 leu2-3,112 trp1-1 ura3-1 tfc3-G349E hmrΔI	Donze lab
DDY416	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 hmrΔ brf1Δ::HIS3 p-brf1 II.9	Donze lab
DDY420	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 hmrΔ brf1Δ::HIS3 p-brf1 II.6	Donze lab
DDY947	MAT**a** his3-11,15 leu2-3,112 trp1-1 ura3-1 sas2Δ::TRP1	Donze lab
DDY1376	MAT**a** his3-11,15 leu2-3,112 trp1-1 ura3-1 nhp6a:URA3 nhp6b:HIS3	Donze lab
DDY1495	MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 BRF1:3XFLAG:KanMX nhp6b:HIS3	Donze lab
DDY1631	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 rsc2Δ::TRP1	Donze lab
DDY1676	MATα his3-11,15 leu2-3,112 trp1-1 ura3-1 rpd3::LEU2	Donze lab
DDY2058	MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 yta7Δ::TRP1	Donze lab
DDY2236	MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 HMR-ADE2 htz1Δ::KanMX	Donze lab
DDY2509	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 bdf1Δ::HIS3	Donze lab
DDY3860	MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 TFC1:3XFLAG:KanMX	Donze lab
DDY4300	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 tfc6 promoter mutant 3	Donze lab
DDY4607	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 tv(uac)dΔ::URA3	This study
DDY4624	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 tv(uac)dΔ	This study
DDY4625	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1	This study
DDY4652	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 tv(uac)dΔ	This study
DDY4653	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 tv(uac)dΔ	This study
DDY4764	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 atg31Δ::TRP1	This study
DDY4769	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 tv(uac)d B-boxΔ	This study
DDY4816	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 tV(UAC)D flip	This study
DDY4817	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 tv(uac)d A-box mutant	This study
DDY4819	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 tv(uac)dΔ::ETC4	This study
DDY4901	MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 tv(uac)dΔ::URA3 BRF1:3XFLAG:KanMX	This study
DDY4904	MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 tv(uac)dΔ::URA3 TFC1:3XFLAG:KanMX	This study
DDY4908	MATα his3-11,15 leu2-3,112 trp1-1 ura3-1 TFC1:3XFLAG:KanMX	This study
DDY4917	MATα his3-11,15 leu2-3,112 trp1-1 ura3-1 tv(uac)dΔ::ETC4 TFC1:3XFLAG:KanMX	This study
DDY4920	MATα his3-11,15 leu2-3,112 trp1-1 ura3-1 tv(uac)d B-box point mutant TFC1:3XFLAG:KanMX	This study
DDY4925	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 tv(uac)d B-box point mutant	This study
DDY4935	MAT**a** his3-11,15 leu2-3,112 trp1-1 ura3-1 tV(UAC)D flip BRF1:3XFLAG:KanMX	This study
DDY4938	MATα his3-11,15 leu2-3,112 trp1-1 ura3-1 BRF1:3XFLAG:KanMX	This study
DDY4943	MAT**a** his3-11,15 leu2-3,112 trp1-1 ura3-1 tv(uac)d A-box mutant BRF1:3XFLAG:KanMX	This study
DDY4946	MAT**a** his3-11,15 leu2-3,112 trp1-1 ura3-1 tv(uac)d B-box point mutant BRF1:3XFLAG:KanMX	This study
DDY4949	MAT**a** his3-11,15 leu2-3,112 trp1-1 ura3-1 tv(uac)dΔ::ETC4 BRF1:3XFLAG:KanMX	This study
DDY4970	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 tv(uac)dΔ::ETC9	This study
DDY5003	MATα his3-11,15 leu2-3,112 trp1-1 ura3-1 tv(uac)dΔ::ETC9 BRF1:3XFLAG:KanMX	This study
DDY5006	MATα his3-11,15 leu2-3,112 trp1-1 ura3-1 tv(uac)dΔ::ETC9 TFC1:3XFLAG:KanMX	This study
DDY5010	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ydl156Δ::KanMX	Research Genetics
DDY5012	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 ATG31-9X-myc::TRP1	This study
DDY5014	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 tv(uac)dΔ ATG31-9X-myc::TRP1	This study
DDY5018	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 tv(uac)d B-boxΔ ATG31-9X-myc::TRP1	This study
DDY5020	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 tv(uac)d B-box pt. mutant ATG31-9X-myc::TRP1	This study
DDY5044	MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 pho13::URA3 pho8Δ60::HIS3 atg8Δ::TRP	This study
DDY5046	MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 pho13::URA3 pho8Δ60::HIS3 atg31Δ::TRP	This study
DDY5051	MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 pho13::URA3 pho8Δ60::HIS3	This study
DDY5072	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 pho13::URA3 pho8Δ60::HIS3 tv(uac)dΔ	This study
DDY5074	MAT**a** his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 pho13::URA3 pho8Δ60::HIS3	This study
DDY5078	MATα his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 pho13::URA3 pho8Δ60::HIS3 tv(uac)d B-boxΔ	This study
DDY5081	MATα his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 pho13::URA3 pho8Δ60::HIS3 tv(uac)d B-box pt. mut.	This study
SG154.2	MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1Δ SCC2::scc2-D730V::HYG	J. Gerton
ROY1032	MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 HMRΔI smc1-2::LEU2 ts	R. Kamakaka
ROY1060	MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 HMRΔI smc3-1:: LEU2 ts	R. Kamakaka
ROY1063	MATα his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3-1 HMRΔI scc1-73::TRP1 ts	R. Kamakaka
ZFY155	MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 pho13::URA3 pho8Δ60::HIS3	D. Klionsky

All strains are isogenic to S. cerevisiae W303-1A except DDY5010, which is S288C.

preventing promiscuous activation of ATG31 by regulatory elements associated with the strong promoter of the neighboring divergent SES1 gene, we created tV(UAC)D deleted (tdnaΔ) mutant strains.

Northern blot analysis using a probe homologous to the ATG31 coding sequence (Figure 1B, left) showed not only an apparent slight increase in the level of ATG31 mRNA in the tdnaΔ strains (compared to ACT1 controls), but also an
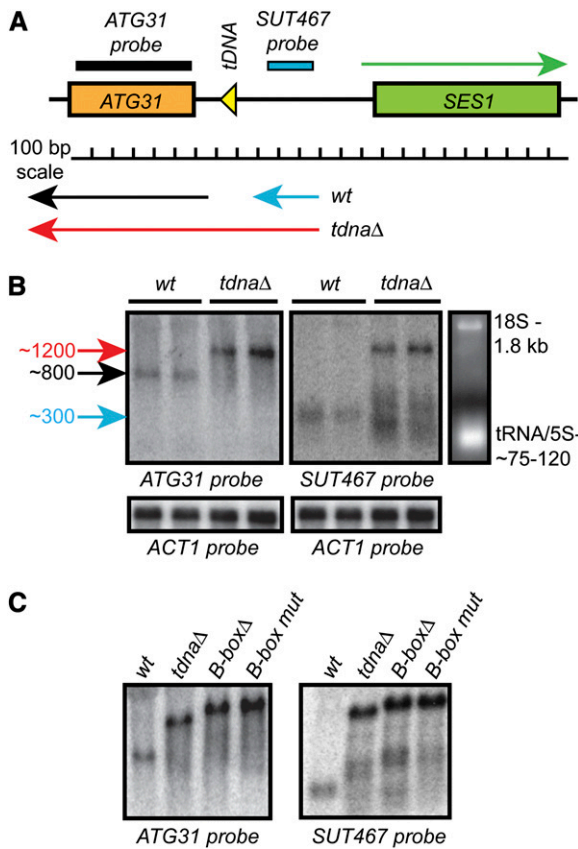
Figure 1 Mutation of the *tV(UAC)D* tRNA gene upstream of *ATG31* results in readthrough of the intergenic *SUT467* transcript. (A) Schematic of the *ATG31–SES1* locus on *S. cerevisiae* chromosome IV. Colored arrows indicate known annotated transcripts: *ATG31*, black; *SUT467*, blue. The overlapping red arrow represents the extended readthrough transcript. (B) Northern blot analysis of *ATG31* expression in wild-type and *tdna*Δ strains reveals the extended transcript. The *ATG31* coding sequence probe hybridized to RNA of ~800 bp in wild-type strains (black arrow) and to RNA of ~1200 bp after tDNA deletion (red arrow). The *SUT467* probe hybridized to the predicted ~300-bp transcript in wild-type cells (blue arrow) and to the same ~1200-bp extended transcript in *tdna*Δ strains. The normal *ATG31* transcript was absent in *tdna*Δ strains. Each pair of lanes contained total RNA from independent wild-type and mutant strains. (C) B-box deletion (*B-box*Δ) or mutation of the invariant cytosine in the B-box (*B-box mut*) also resulted in extended readthrough transcription. Strains used were: (B) DDY4625 and DDY3 (wild-type); DDY4653 and 4624 (*tdna*Δ); (C) DDY3 (wt); DDY4652 (*tdna*Δ); DDY4769 (*B-box*Δ); and DDY4925 (*B-box mut*).

increase in the length of the transcript by ~400 nucleotides (shifting from ~800 bases in wild-type strains to ~1200 bases), with apparent absence of the normal length mRNA. Recent tiling array and RNA-seq studies have identified widespread pervasive and intergenic transcription in eukaryotic cells, and in yeast this often appears to occur as bidirectional transcription from strong promoters (Neil *et al.* 2009; Xu *et al.* 2009). Inspection of data from these studies indicated that the *SUT467* intergenic transcript initiates upstream of the *SES1* promoter and terminates near the tDNA (Figure 1A); therefore, we hypothesized that the extended *ATG31* transcript in the *tdna*Δ strain was a readthrough

SUT467 transcript that interferes with the production of the normal *ATG31* transcript.

To confirm that the extended transcript in *tdna*Δ strains was due to readthrough of *SUT467* and not a 3′ extension, we repeated the Northern analysis using a probe specific for the transcribed *SUT467* RNA sequence (Figure 1B, right). The results showed that this probe hybridized to RNA of ~300 bases in wild-type strains, consistent with previous annotations of *SUT467*. The *tdna*Δ strains showed a longer ~1200-base transcript, the same length as when using the *ATG31* coding-sequence probe. We concluded that in the absence of the tDNA at this region, *SUT467* readthrough occurs and interferes with normal *ATG31* transcription initiation, producing only the observed extended RNA.

Since our gross deletion of the tDNA sequence removed 90 bp of chromosome IV, we confirmed this readthrough effect by creating strains that either had only the B-box sequence of the tDNA deleted or contained a mutation in the invariant cytosine residue in the B-box. Both of these mutations of the tDNA were expected to result in loss of TFIIIC binding and inhibition of Pol III complex assembly. Northern blot analysis with either probe shown in Figure 1C confirmed that in each of these mutant backgrounds, complete readthrough of *SUT467* occurred as in the *tdna*Δ strains. The slightly shorter transcript observed in the *tdna*Δ strain compared to the *B-box*Δ and point mutant strains is also consistent with the long transcript being a readthrough from upstream of the tDNA, as this reflects the 90-bp deletion. Also observed were shorter RNAs hybridizing to only the *SUT467* probe, which appear to terminate between the tDNA and *ATG31*.

TFIIIB binding is correlated with blocking of SUT467 readthrough

Our previous studies of heterochromatin barrier and insulator function of tDNAs have shown that assembled TFIIIC alone can block the spread of silencing from the *HMR* locus and can insulate a UAS from a promoter (Simms *et al.* 2008). To determine which components of the Pol III complex are required to block *SUT467* progression, we analyzed *ATG31* transcripts in various temperature-sensitive strains compromised for Pol III complex function and formation when pulsed at the nonpermissive temperature before RNA extraction. Figure 2A shows the results of Northern blot analysis of these strains. Temperature-sensitive mutations in RNA Polymerase III subunit genes *rpc31* and *rpc160* affect transcription initiation and elongation, respectively (Dieci *et al.* 1995; Thuillier *et al.* 1995). These mutants had relatively little effect on the ability of the tDNA to block progression of *SUT467* (lanes 2 and 3 compared to wild type in lane 1) as evidenced by a minimal alteration of the ratio of normal to extended transcripts. In contrast, mutations in the TFIIIB subunit encoding *BRF1* gene (*brf1*-II.6 and -II.9) that impair interactions of Brf1p with TBP (Andrau *et al.* 1999) showed a major shift to the longer extended transcript (lanes 4 and 5), with relatively little normal length
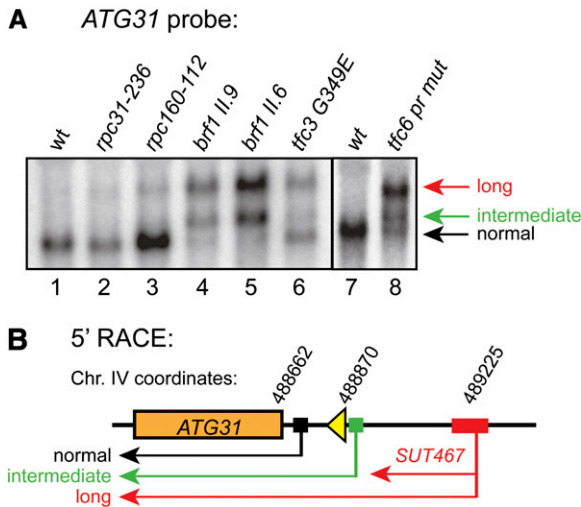
Figure 2 Pol III transcription factors are required to block *SUT467* readthrough transcription. (A) Northern analysis of temperature-sensitive mutants of the Pol III complex was performed as in Figure 1, except that each culture was shifted from 30° to 37° for 1 hr prior to RNA extraction. Extended *ATG31* transcripts are most prominent in TFIIIB and TFIIIC subunit mutants, which also express an intermediate length *ATG31* transcript. Strains used in lanes 1–8 were DDY3 (wt); DDY232 (*rpc31-236*); DDY246 (*rpc162–112*); DDY416 (*brf1 II.9*); DDY420 (*brf1 II.6*); DDY261 (*tfc3 G349E*); DDY3 (wt); and DDY4300 (*tfc6* promoter mutant). (B) 5′-RACE analysis of extended and intermediate *ATG31* transcripts. 5′-RACE was performed to map transcriptional start sites (TSS) for the various transcripts observed in the Northern blot analysis of the *brf1* II.6 mutant. Colored solid boxes represent the range of alternative TSS, which were observed in three distinct clusters. The exact *Saccharomyces* Genome Database coordinates of all mapped TSS are given in Figure S1.

RNA. Interestingly, this mutant also showed an intermediate length *ATG31* transcript that initiates just upstream of the tDNA coding sequence (see 5′-RACE analysis below).

Mutations involving TFIIIC also resulted in readthrough transcription. RNA isolated from the temperature-sensitive, DNA-binding defective *tfc3* G349E mutant strain (Lefebvre *et al.* 1994) showed apparent equal amounts of both normal and long *ATG31* transcripts, with a small relative amount of the intermediate transcript (Figure 2A, lane 6). A strain harboring a mutation in the *TFC6* promoter that results in reduced expression of Tfc6p and slow growth (Kleinschmidt *et al.* 2011) showed a similar pattern (lane 8), shifted a bit more to the long and intermediate transcripts. These results demonstrate that loss of TFIIIC function also results in readthrough *SUT467* transcription, but this could be due to loss of TFIIIB assembly in the absence of full TFIIIC activity.

To verify that the transcription start site of our readthrough transcript initiates in the region of the annotated *SUT467* transcriptional start site (TSS) and to map the TSS of the observed intermediate transcript, we performed 5′-RACE analysis on RNA isolated from the *brf1*-II.6 mutant, because it contains all three transcripts as detected by Northern blotting. As shown schematically in Figure 2B, 5′-RACE ends that correspond to the annotated *ATG31* mRNA, and within a 94-nucleotide range that overlaps the

annotated *SUT467* TSS, were mapped. The intermediate transcript was found to begin very close to the beginning of the tRNA coding sequence. The exact *Saccharomyces* Genome Database chromosome IV coordinates corresponding to each individually mapped 5′-RACE end are listed in Figure S1.

To further assess the mechanistic requirements of each Pol III transcription factor in preventing Pol II readthrough transcription, we constructed yeast strains specifically modified at the *ATG31* upstream tDNA locus and analyzed the long *vs.* short RNA phenotypes. Inverting the orientation of the tDNA had no effect, as no extended *ATG31* mRNA was detected (Figure 3A, lane 1). Mutation of the *A-box* within the tDNA or replacement of the tDNA with the *ETC4* site resulted in only the extended transcript being produced (Figure 3A, lanes 2 and 3). Each of these replacements was expected to bind TFIIIC, but not be able to efficiently recruit TFIIIB or Pol III. Interestingly, replacing the tDNA with the tDNA remnant upstream of the *TIM21* gene, recently referred to as *ETC9* (Nagarajavel *et al.* 2013), was sufficient to block readthrough transcription (Figure 3A, lane 4). This tDNA remnant has previously been shown to bind both TFIIIC and TFIIIB, but not the Pol III enzymatic complex (Guffanti *et al.* 2006).

These results suggest that recruitment of TFIIIB is the critical step that prevents readthrough transcription of *SUT467*, as binding of TFIIIC alone at the *ETC4* site is not sufficient to block Pol II progression. Mutation of the *A-box* has been demonstrated to impair TFIIIB assembly (Huibregtse and Engelke 1989), and this mutation also allows readthrough. These interpretations assume that each of these sequences used to replace the tDNA have the same *in vivo* binding characteristics at the *ATG31* locus as they do in their native chromosomal locations. To confirm such assumptions regarding the presence or absence of each transcription factor complex at these sequences when moved to the *ATG31* locus, we crossed a 3X-FLAG-epitope-tagged *BRF1* allele into each of these mutants. ChIP results using anti-FLAG antibody shown in Figure 3B demonstrate that the tDNA flip and *ETC9* alleles are strongly enriched for TFIIIB at levels comparable to wild-type tDNAs, while insertions unable to block readthrough transcription (*A-box* mut, *ETC4*, and *B-box* mut) had significantly reduced TFIIIB ChIP signals, comparable to background signals observed in the no antibody control panels. Primers amplifying a separate control tDNA on chromosome III showed similar enrichment in each of the samples, indicating that equivalent amounts of ChIP DNA were added to each PCR reaction.

We also created strains containing a 3X-FLAG-epitope-tagged *TFC1* allele to assess the binding of TFIIIC at modified *ATG31* loci. The results in Figure 3C show that TFIIIC but not TFIIIB is associated with the *ETC4* insertion, and both TFIIIC and TFIIIB are bound at the *ETC9* insertion and at the wild-type tDNA locus. The control tDNA again showed equivalent levels of enrichment in the ChIP samples. These results demonstrate an association of TFIIIB binding with
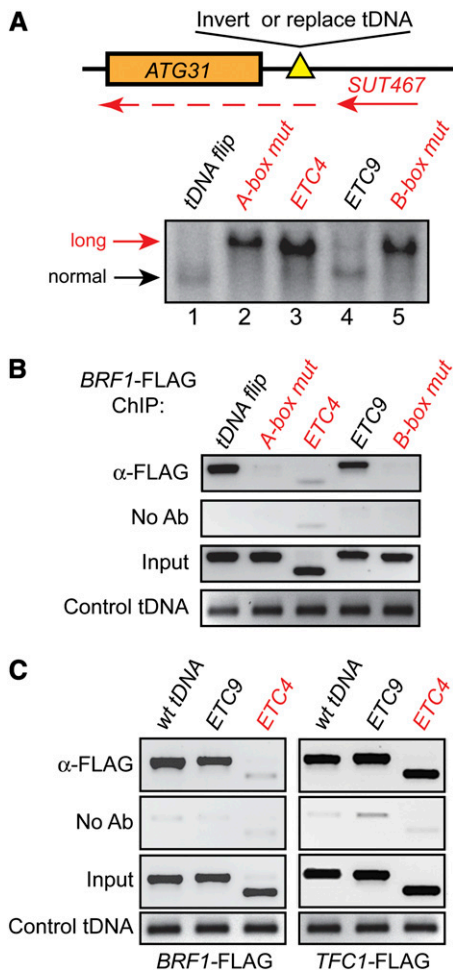
Figure 3 Binding of the TFIIIB complex is associated with blocking of *SUT467* readthrough transcription. Strains were constructed to recruit the entire Pol III complex, TFIIIB and TFIIIC, or TFIIIC alone to the *ATG31–SES1* intergenic region. Each construct was tested for the ability to block readthrough and for binding of Pol III transcription factor complexes to the ectopic locations. (A) Schematic of the modified *ATG31* loci and Northern blot of each strain using the *ATG31* probe. Lane 1, DDY4816 (tDNA flip); lane 2, DDY4817 (*A-box* mut); lane 3, DDY4819 (*ETC4* replacement); lane 4, DDY4970 (*ETC9* replacement); and lane 5, DDY4925 (*B-box* mut). Replacement of the tDNA by *ETC4*, or mutating the *A-box* or *B-box*, resulted in the presence of the extended transcript (red labels). However, inversion of the tDNA sequence or replacement with the *ETC9* sequence still blocked readthrough (black labels). (B and C) Confirmation of expected Pol III transcription factor binding in the above mutants by chromatin immunoprecipitation. Each tDNA mutant strain was crossed to strains containing either *BRF1–3X-FLAG* or *TFC1–3X-FLAG* alleles, and then subjected to ChIP analysis using anti-FLAG antibody. (B) The absence of TFIIIB upstream of *ATG31* in the *A-box* mutant, *ETC4* replacement, and *B-box* mutant correlates with the presence of the extended transcript, suggesting that TFIIIB binding is required to block readthrough. Strains used (left to right) were DDY4935, -4943, -4949, -5003, and -4946. (C) ChIP analysis of *BRF1–3XFLAG* and *TFC1–3XFLAG* strains demonstrates that TFIIIC but not TFIIIB is bound in *ETC4* replacement strains, indicating that TFIIIC binding alone cannot block readthrough transcription. Strains used (left to right) were DDY4938, -5003, -4949, -3860, -5006, and -4917.

blocking of *SUT467* transcription. Importantly, and contrary to results seen in our earlier tDNA heterochromatin blocking studies (Simms *et al.* 2008), TFIIIC binding alone to *ETC4* is not sufficient to block cryptic transcript readthrough.

Mutations in genes affecting tDNA heterochromatin barrier function have minimal impact on transcript blocking

Previous studies on the heterochromatin barrier activity of tDNAs revealed the involvement of other chromatin-associated proteins in this extratranscriptional function (Donze *et al.* 1999; Donze and Kamakaka 2001; Jambunathan *et al.* 2005; Braglia *et al.* 2007). To assess the potential role of these tDNA associated proteins in blocking readthrough transcription, we performed Northern blot analysis (using the *ATG31* coding sequence probe) on RNA isolated from a number of these mutants. The results in Figure 4A showed that each of these mutants contain mostly normal-length *ATG31* transcripts; however, low levels of readthrough are apparent in some strains, most obvious in *nhp6* (lane 3) and *smc3* (lane 12) mutants in the particular blot shown. However, the intensity of these signals was relatively weak and was often difficult to consistently distinguish from background in different blots.

To confirm these apparent low levels of readthrough, we used readthrough transcript-specific primers to develop an RT–PCR assay to measure differences in the relative levels of the long transcript compared to a wild-type strain. The inset in Figure 4B shows an inverted ethidium-stained gel image that verifies that the primers specifically amplified the readthrough cDNA, as the *B-box* mutant strain showed significantly higher levels of RT–PCR product than the wild-type strain. There also appears to be a low level of readthrough in the wild-type strain, which is consistent with a genome-wide transcriptome analysis that identified a single readthrough clone overlapping this locus (Miura *et al.* 2006). Quantitative RT–PCR was performed on the same RNA samples shown in the Northern blot in Figure 4A, and those that showed a significant increase in the long *ATG31* transcript relative to the wild-type parent are shown in Figure 4B. The *B-box* mutant strain measured ~80-fold more readthrough transcript than wild type in this assay, while other mutants were confirmed to have modest (ranging from ~2- to 15-fold) yet detectable increased levels of the long transcript as suggested by the Northern analysis.

The extended ATG31 transcript is not efficiently translated

Given the extended 5′-UTR present on the long *ATG31* transcript, we next asked to what extent translation of the Atg31 protein was affected by readthrough *SUT467* transcription. We created *ATG31*–9X-myc epitope-tagged strains in wild-type and tDNA mutant backgrounds and then analyzed Atg31 protein expression by Western blotting. In each strain producing the long transcript, we observed a drastic reduction in Atg31p levels (Figure 5A). Atg31p is required for
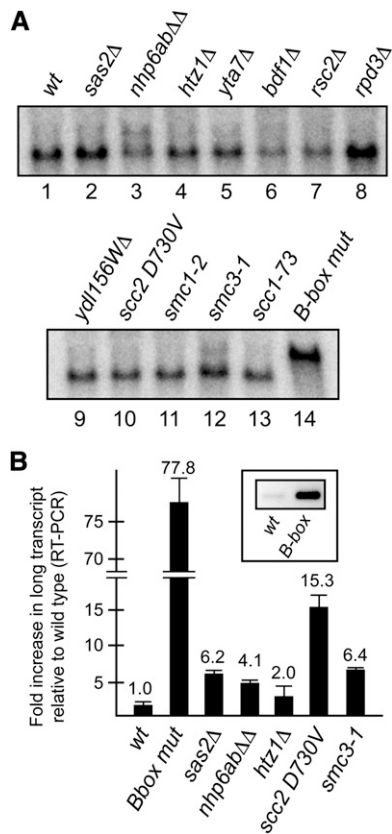
Figure 4 Genetic factors involved in tDNA chromatin boundary function have minimal effects on blocking of Pol II progression through *tV(UAC)D*. (A) RNA from strains containing mutations that weaken tDNA boundary function were analyzed by Northern blotting using the *ATG31* probe. Strains in lanes 1–9 were DDY3, -947, -1376, -2236, -2058, -2509, -1631, -1676, and -5010; lane 10, SG154.2; lanes 11–13, ROY1032, -1060, and -1063; lane 14, DDY4925. (B) RT–PCR analysis of readthrough transcription also shows only minimal effects.
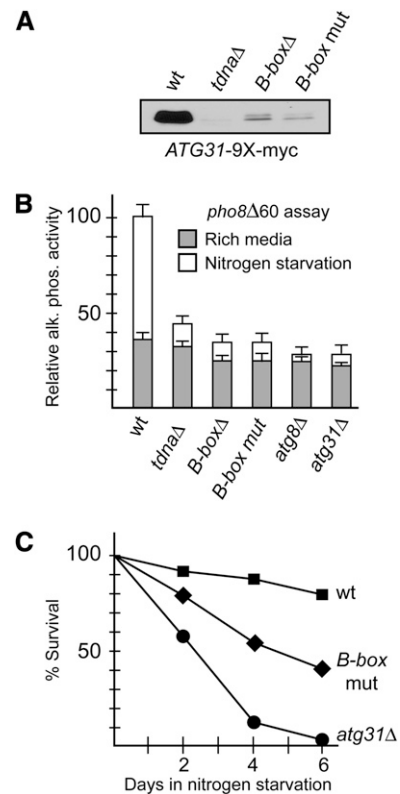

Figure 5 Mutations in *tV(UAC)D* inhibit expression and function of Atg31 protein. (A) Western blot analysis was performed on wild-type and tDNA mutants containing an *ATG31*–9X-myc tag allele to determine whether the extended transcript affects translation of Atg31p. Compared to wild type, tDNA mutants showed significantly reduced expression of Atg31p. No other bands were observed on the Western blot, demonstrating that the extended transcript does not lead to production of an extended polypeptide. Strains used were DDY5012 (wt), DDY5014 (*tdna*Δ), DDY5018 (*B-box*Δ), and DDY5020 (*B-box* mut). Extracts were prepared from cells grown in YPD media. (B) Inhibition of autophagy induction in yeast expressing the extended transcript as measured by the Pho8Δ60 alkaline phosphatase assay. Strains used were: DDY5051 (wt control), DDY5072 (*tdna*Δ), DDY5078 (*B-box*Δ), DDY5081 (*B-box* mut), DDY5044 (*atg8*Δ), and DDY5046 (*atg31*Δ). (C) Production of the extended transcript is associated with reduced survival under nitrogen starvation conditions. Strains used were: DDY5012 (wt), DDY5081 (*B-box* mut), and DDY4764 (*atg31*Δ).

autophagy in yeast. Autophagy is a conserved cellular response that recycles cellular components upon nutrient limitation and during normal regulated molecular turnover and involves the formation of autophagosome vesicles that capture and degrade macromolecules after fusion with other membrane bound vesicles (Reggiori and Klionsky 2013; Stanley *et al.* 2013).

Since Atg31p is required for the formation of autophagosomes upon nitrogen starvation (Kabeya *et al.* 2007), we tested the efficiency of this response using two well-characterized assays to measure autophagy induction. We first created a series of strains that produce the readthrough transcript and contain the *pho8*Δ60 and *pho13*Δ alleles. These mutations reduce background alkaline phosphatase levels, and the phosphatase activity of the precursor Pho8Δ60 protein can be activated only if it is proteolytically processed during autophagy (Klionsky 2007). Since *ATG31* is required for these events, we reasoned that the reduction in Atg31p levels due to the extended 5′-UTR would result in reduced processing of Pho8Δ60p upon induction of autophagy and therefore reduced levels of alkaline phosphatase activity

upon shifting cells to nitrogen starvation conditions. Figure 5B shows this to be the case, as strains producing the readthrough transcript showed significantly reduced induction of phosphatase activity compared to a *pho13*Δ *pho8*Δ60 strain producing only normal *ATG31* mRNA (wt in Figure 5B). Complete deletion of *ATG31* or *ATG8* in control strains severely reduced starvation-induced phosphatase activity as expected. These results confirm that induction of autophagy is compromised in strains that predominantly produce the long *ATG31* transcript.

As a second assay for the efficiency of autophagy induction, we tested the viability of yeast cells producing the long transcript when placed under autophagy-inducing conditions. Previous studies have shown that complete deletion of *ATG31* results in reduced survival of cells undergoing

nitrogen starvation due to inhibition of autophagy (Kabeya *et al.* 2007). When we tested a strain containing the *B-box* mutation in the tDNA (producing the long transcript), we found an intermediate level of survival compared with wild-type and *atg31Δ* strains (Figure 5C). This loss of survival is not due to effects on the neighboring *SES1* gene, as wild-type and *tdna* mutant strains show equivalent expression levels of *SES1* when analyzed by Northern blotting (Figure S3). This result suggests that readthrough of the cryptic transcript reduces Atg31p translation to a level that compromises fitness of the cells during nitrogen starvation.

Discussion

The results described in this study demonstrate that stable RNA polymerase III transcription factor complexes containing TFIIIB assembled at tDNAs have the capacity to block the progression of intergenic transcription by RNA polymerase II. High-throughput microarray and sequencing technologies have led to the identification of much more diversity in transcriptomes, from prokaryotes to humans, than was previously appreciated (Core *et al.* 2008; Dornenburg *et al.* 2010; Wei *et al.* 2011). In *S. cerevisiae*, such cryptic transcripts include the cryptic unstable transcripts (CUTs), stable unannotated transcripts (SUTs), Xrn1-sensitive unstable transcripts (XUTs), and meiotic unannotated transcripts (MUTs) (Wyers *et al.* 2005; Xu *et al.* 2009; Lardenois *et al.* 2011; van Dijk *et al.* 2011). Additionally, alterations in the prevalence of intergenic transcripts and transcript start and end sites have been observed under different growth and stress conditions (Xu *et al.* 2009; Waern and Snyder 2013). Since the vast majority of these cryptic transcripts and transcript isoforms have unknown functions, it has been speculated that they may represent inherent sloppiness of the transcriptional process, referred to as "transcriptional noise" (Struhl 2007).

Where functions of such pervasive transcription have been identified in *S. cerevisiae*, it appears that it is not necessarily the RNA produced but the act of transcription itself that leads to the observed function. The short noncoding *SRG1* transcript inhibits *SER3* expression by transcriptional interference and promoter occlusion mechanisms, as the path of the *SRG1* transcript overlaps transcription factor binding sites within the *SER3* promoter (Martens *et al.* 2004; Martens *et al.* 2005). In this case, the *SRG1* transcript terminates near the beginning of *SER3*, while mutation of the tDNA at *SUT467* results in uncontrolled readthrough all the way to the end of *ATG31*. Similar *cis*-linked mechanisms may be at work at other yeast loci where noncoding transcription appears to block initiation or elongation of *ADH1* (Bird *et al.* 2006), *IMD2* (Kuehner and Brow 2008), *URA2* (Thiebaut *et al.* 2008), *FLO11* (Bumgarner *et al.* 2009), *PHO84* (Camblong *et al.* 2007), and *IME4* (Hongay *et al.* 2006; Gelfand *et al.* 2011). There is evidence that there are also *trans*-effects of the noncoding RNA product regulating the *PHO84* locus (Camblong *et al.* 2009). Additionally,

full repression of yeast *GAL* genes (Houseley *et al.* 2008), *IME1* (van Werven *et al.* 2012), and again *PHO84* (Camblong *et al.* 2007) requires chromatin modifications associated with ongoing noncoding transcription. While more instances are likely yet to be identified, this handful of yeast genes has incorporated intergenic transcription into their regulatory programs and generally appears to use it as a means of repression. However, we show in this study that if left unchecked, progression of noncoding transcription can have negative consequences on neighboring gene expression, resulting in reduced fitness of cells. This result demonstrating a cryptic transcript-blocking activity of bound Pol III complexes can be added to the list of extratranscriptional effects of the RNA Polymerase III system.

Our results presented here demonstrate that the tDNA upstream of *ATG31* protects against such repressive transcriptional interference effects. Our data are consistent with a model in which TFIIIB, as part of the Pol III complex associated with the *tV(UAC)D* tDNA, serves as a physical impediment to elongating RNA Pol II initiating at the *SUT467* transcriptional start site. In the absence of TFIIIB, nearly complete readthrough by Pol II occurs to produce an extended *SUT467–ATG31* RNA transcript. This transcript is not efficiently, if at all, translated into Atg31 protein, as scanning ribosomes (Kozak 2005) attaching at the 5′ end of the extended transcript would encounter start and stop codons before reaching the *ATG31* start codon. This readthrough transcript appears to be both capped and polyadenylated, as the 5′-RACE protocol includes a phosphatase treatment before decapping and adaptor ligation, ensuring that only capped 5′ ends are mapped, and the long transcript is enriched in Northern analysis of poly(A)-purified RNA (A. Korde, unpublished data).

The small amount of Atg31p we detect in our Western blots likely results from low levels of normal *ATG31* transcripts that are undetectable in Northern blots from cells grown in rich media. Extracts from nitrogen-starved *tdna* mutants show a slight increase in protein levels, along with detectable normal *ATG31* transcripts in Northern blots of RNA isolated from the same cultures (A. Korde, unpublished data). This suggests that under conditions that induce *ATG31*, limited normal initiation is slightly enhanced, but protein levels are still lower than in wild-type cells.

Previous work from our lab and others has shown that certain extratranscriptional effects associated with tDNAs can be mediated by binding of the TFIIIC complex alone. Propagation of silencing at the *HMR* mating locus can be blocked by replacing the tDNA downstream of the *HMR*-I silencer with an *ETC* site, and insertion of an *ETC* site between UAS_G and *GAL10* insulates the promoter from Gal4p activation (Simms *et al.* 2008). Heterochromatin boundary activity of TFIIIC-only containing complexes is also observed in *Schizosaccharomyces pombe* (Noma *et al.* 2006; Scott *et al.* 2006). Additionally, the *ETC6* site within the *TFC6* promoter may modulate transcription by an insulator-like mechanism (Kleinschmidt *et al.* 2011). In this case

of preventing readthrough of intergenic transcription, the binding of TFIIIC alone is clearly not sufficient. While TFIIIC binds to *B-box* sequences *in vitro* with extremely high affinity (Lefebvre *et al.* 1994; Jourdain *et al.* 2003), this binding is somehow tempered by passage of the Pol III enzymatic complex during transcription of the internal control element regions.

On the other hand, after recruitment of TFIIIB by TFIIIC, the tightly bound TFIIIB complex appears to be fixed, as *in vitro* experiments have shown that TFIIIB–DNA complexes are resistant to high salt and heparin treatments (Kassavetis *et al.* 1990; Kassavetis *et al.* 1995). The fully assembled TFIIIB complex also is thought to be "kinetically trapped" (Cloutier *et al.* 2001), with a half-life on the order of a full yeast cell cycle, and fully assembled TFIIIB likely persists at tDNAs until regulated release during mitosis or stationary phase (Fairley *et al.* 2003; Roberts *et al.* 2003). Such characteristics of TFIIIB are consistent with our results that suggest that formation of this complex is the major impediment to cryptic transcript readthrough by *SUT467*. These results are also compatible with earlier *in vitro* studies that demonstrated the ability of Pol III to transcribe through assembled TFIIIC but not assembled TFIIIB (Bardeleben *et al.* 1994).

Our results suggest that TFIIIC yields to Pol II in a similar manner as it does to Pol III, since replacing the tDNA with *ETC4* allowed readthrough of Pol II even though chromatin immunoprecipitation analysis revealed that TFIIIC was bound to the ectopic *ETC4* site (Figure 3C). While we have not mapped the exact 3′ end of the *SUT467* transcript, the annotated end mapped by tiling array analysis (Xu *et al.* 2009) places it within ~20 bp of the 5′ extent of the expected TFIIIB footprint at this tDNA (schematically depicted in Figure S1). Estimation of this 5′ end of the TFIIIB footprint is based on earlier *in vitro* footprinting studies (Kassavetis *et al.* 1989) and a recent global "bootprinting" analysis of *in vivo* bound Pol III transcription factors (Nagarajavel *et al.* 2013). The location of the 3′ end of *SUT467* is consistent with TFIIIB being a transcriptional roadblock that is resistant to displacement by transcribing Pol II.

A curious sidelight to this study is the appearance of the intermediate length transcript in TFIIIB and TFIIIC mutants, but not in tDNA mutants. This is most likely initiated by Pol II, as in *brf1*, *tfc3*, and *tfc6* mutants, the tDNA terminator sequence is still present, so it is unlikely that this is a Pol III transcript. We speculate that in these mutants, TFIIIB binding still occurs, but is unstable, and perhaps dissociation of the Brf1p and Bdp1p subunits occurs before loss of TBP at the site. Such a lingering TBP might then recruit factors necessary to then subsequently recruit Pol II immediately upstream of the tDNA. Alternatively, the Pol III complex may mask a cryptic Pol II promoter, which is revealed in a subset of cells containing mutations in the Pol III transcription factors.

There is mounting evidence that a much larger fraction of genomes is transcribed than was previously appreciated. While RNA degradation pathways generally keep most of these transcripts at low levels (Wolin *et al.* 2012), it has become clear that the act of intergenic transcription can have significant effects on neighboring genes. Due to such observations, one must consider how mutation of a specific genomic locus may affect expression of nearby genes in addition to the targeted gene when assigning the actual cause of observed phenotypes (Wei *et al.* 2011). To assess the global nature of RNA Pol III extratranscriptional effects, we are conducting RNA-seq analysis of wild-type *vs.* Tfc6p underexpressing mutant strains. Previous studies have been conducted to determine the global effects of Pol III deficiencies (Conesa *et al.* 2005), but the RNA was analyzed by coding sequence microarray, which could not detect effects involving intergenic transcription. Inspection of preliminary RNA-seq results suggests that when the Pol III complex is globally compromised, several tDNA proximal genes may be affected as described here, and in other possibly unique ways (Q. Wang, A. Korde, and C. Nowak, unpublished results). This type of result also raises the question of how to interpret phenotypes due to mutations that may globally affect intergenic transcription (which may be relevant in mutants of other DNA and chromatin binding proteins), or as shown here for mutation at a specific locus, as unchecked cryptic transcription can lead to unexpected and even detrimental misexpression of downstream genes. While a subset of pervasive transcription products themselves may be noise, multiple mechanisms must exist to keep secondary effects of their production in check.

Acknowledgments

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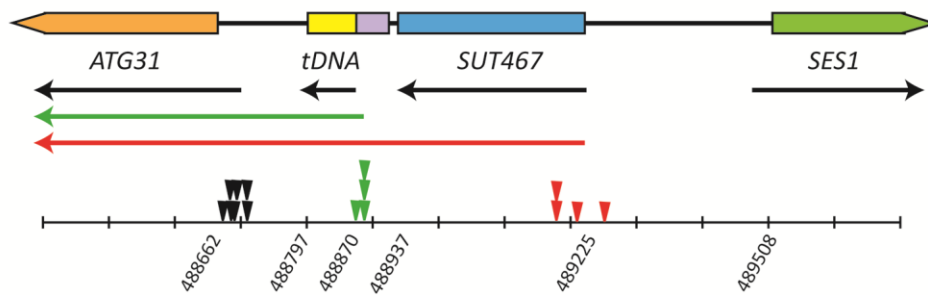
Communicating editor: M. Hampsey

GENETICS

Intergenic Transcriptional Interference Is Blocked by RNA Polymerase III Transcription Factor TFIIIB in *Saccharomyces cerevisiae*

Asawari Korde, Jessica M. Rosselot, and David Donze

Chromosome IV coordinate landmarks and mapped 5'-RACE Transcription Start Sites

Annotated landmarks:

ATG31 annotated coding sequence	488072 ← 488662
tDNA annotated coding sequence	488797 ← 488870
SUT467 annotated sequence	488937 ← 489225
SES1 annotated coding sequence	489508 → 490896
Predicted extent of TFIIIB protection	

5'-RACE mapped TSS (from brf1 mutant strain):

Normal *ATG31* TSS ▼ 488672, 488685 (x2) 488688, 488689, 488705 (x2)

Intermediate *ATG31* TSS ▼ 488872, 488881 (x3)

Long *ATG31* TSS ▼ 489165 (x2), 489209, 489259

Figure S1 *S. cerevisiae* Chromosome IV annotation and locations of 5' ends of normal, intermediate, and long *ATG31* transcripts. Annotations are as listed in the *Saccharomyces* Genome Database, and the predicted TFIIIB footprint was inferred from published data as described and referenced in the main text. Normal transcripts are shown as black arrows, with extended transcripts in green and red. Transcription start sites (TSS) were mapped by 5'-RACE as described in Materials and Methods. RNA was from the *brf1* mutant strain DDY420, as all three transcripts are present in this mutant. First strand cDNA synthesis was random primed and amplified by nested PCR using adaptor inner and outer primers (as described in Ambion First-Choice RLM RACE kit) with nested DDO-1541/1542 for the normal ATG31 mRNA, and DDO-1527/1364 for the intermediate and long transcripts. Since DDO-1364 lies in the normally untranscribed region between *ATG31* and the tDNA, only cDNA copied from extended transcripts are amplified. The coordinates are listed for each individual sequenced 5'-RACE clone, and their locations are marked on the map with arrowheads using the same color-coding scheme (coordinates as of October 11, 2013).
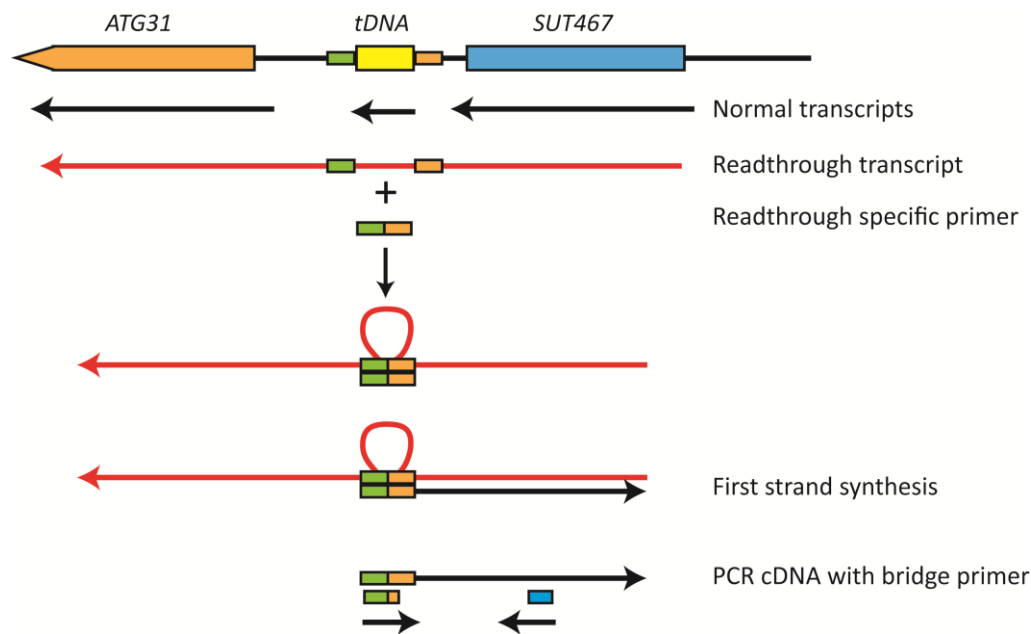
Figure S2 Strategy for RT-PCR quantitation of *SUT467-ATG31* extended transcripts. In order to quantify extended transcripts while minimizing amplification of trace amounts of chromosomal DNA remaining after DNase treatment, a bridge primer method was designed. First strand cDNA was synthesized with readthrough specific primer DDO-1284, which has homology to normally untranscribed regions on either side of the tDNA (green and orange boxes), but omitting the transcribed tDNA sequence. This oligo can only base pair with RNA that is transcribed from upstream through the tDNA, presumably looping out the tRNA sequence within the extended transcript. After first strand synthesis, qPCR was performed with the bridge primer DDO-1606, which is homologous to 21 base pairs of the green sequence, and only 5 base pairs within the orange sequence. By having only 5 contiguous base pairs of homology to the orange region, amplification of residual genomic DNA was minimized.
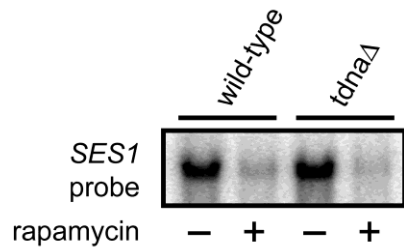
Figure S3 Mutation of tDNA does not affect *SES1* expression. Northern blot analysis of *SES1* in wild-type and *tdnaΔ* strains shown no significant difference in *SES1* mRNA levels. Treatment with rapamycin induces autophagy and results in decreased *SES1* mRNA levels, and this downregulation is also unaffected when the tDNA is deleted.

A. Korde, J. M. Rosselot, and D. Donze

Table S1 Plasmids

Name	Description	Source
pYM6	*9XMyc-klTRP1*	E. Schiebel[1]
pDD 1232	*ATG31-SES1* intergenic	This study
pDD 1233	*ATG31-SES1* intergenic *tv(uac)dΔ*	This study
pDD 1248	*ATG31-SES1* intergenic *tv(uac)d b-boxΔ*	This study
pDD 1249	*ATG31-SES1* intergenic *tv(uac)d b-box point mutant*	This study
pDD 1260	*ATG31-SES1* intergenic *tv(uac)dΔ::EcoRI-BamHI* linker	This study
pDD 1261	*ATG31-SES1* intergenic *tv(uac)d a-box mutant*	This study
pDD 1262	*ATG31-SES1* intergenic *tV(UAC)D* flip	This study
pDD 1263	*ATG31-SES1* intergenic *tv(uac)dΔ::ETC4*	This study
pDD 1272	*ATG31-SES1* intergenic *tv(uac)dΔ::ETC9*	This study

1 – Knop *et al.*, *Yeast* 15, 963-972 (1999)

Table S2   Oligonucleotides used in this study

Name	Sequence	Description
tV(UAC)D mutagenesis and replacements:		
DDO-183	AATTAACCCTCACTAAAGGG	T3 primer
DDO-184	GTAATACGACTCACTATAGGGC	T7 primer
DDO-1281	GACCTTATCTATTCCTGCTGTC	_ATG31_ intergenic _Spe_I
DDO-1282	CTTTTTTCTTCAAGTCCTCTCGAGCCTGTTGCTCCTTTTCAGTC	_SES1_ intergenic _Xho_I
DDO-1283	CAGATGGATGAACGATATTTCCTAC	_SES1_ CDS KO PCR check
DDO-1284	GATATGTACTTCAAATTATTATCTTTTACGAAACCCATTGGAAATGACTTAATCCC	tDNA SDM delete top
DDO-1285	GGGATTAAGTCATTTCCAATGGGTTTCGTAAAAGATAATAATTTGAAGTACATATC	tDNA SDM delete bottom
DDO-1325	GAGATCCCGATTCATCCAATTG	_ATG31_ CDS internal
DDO-1328	TATCTTGGATATTGACCACCCTTATATTATGGATATGTACTTCAAATTATTATCTTTT ACGCAGATTGTACTGAGAGTGC	_ATG31-SES1_ intergenic KO top
DDO-1329	GACAGAAGCACCACTCTTGTGGATTATCCTGAAAAAGTTATTTTACATTTAATATAG CGACTCCTTACGCATCTGTGCGG	_ATG31-SES1_ intergenic KO bottom
DDO-1330	CAAACATAATGTCCATCCTTATCAG	intergenic KO check
DDO-1391	CTTTACACGGCGAAGATCCCGTCGGTTGGATCAGAATCTTTTTTATC	_tv(uac)d B-box_ delete top
DDO-1392	GATAAAAAAGATTCTGATCCAACCGACGGGATCTTCGCCGTGTAAAG	_tv(uac)d B-box_ delete bottom
DDO-1393	CTTTACACGGCGAAGATCCCGAGTTGCAACCTCGGTTGGATCAGAATCTTTTTTATC	_tv(uac)d B-box_ mut. top
DDO-1394	GATAAAAAAGATTCTGATCCAACCGAGGTTGCAACTCGGGATCTTCGCCGTGTAAAG	_tv(uac)d B-box_ mut. bottom
DDO-1468	CAAATTATTATCGGATCCGATAAAAAAGATTCTGATCCAAC	_tV(UAC)D_ flip _Bam_HI end
DDO-1469	GTGGATTATCCTGAATTCGTTATTTTACATTTAATATAGCG	_tV(UAC)D_ flip _Eco_RI end
DDO-1474	CGCCGTGTAAAGGCGACGTCTTGAATCTAGATTGGACCGGAAACCCATTGGAA ATGAC	_tv(uac)d A-box_ mut. top
DDO-1475	GTCATTTCCAATGGGTTTCCGGTCCAATCTAGATTCAAGACGTCGCCTTTACACGGCG	_tv(uac)d A-box_ mut. bottom
DDO-1489	AATTCCACTCTCCCTTTTAATGCCTCCACGGAGGTTCGAATGGGTTATACTAGAA AGAAG	_ETC4 Eco_RI end
DDO-1490	GATCCTTCTTTCTAGTATAACCCATTCGAACCTCCGTGGAGGCATTAAAAGGGA GAGTGG	_ETC4 Bam_HI end
DDO-1534	AATATTTATAGCTCAGGATCCAAAAGACATGACGCATATAATGTAC	_ETC9_ PCR _Bam_HI end
DDO-1535	AATATTTATAGCTCAGAATTCAAGAAAAAAAACCGGATAATACCAGGTC	_ETC9_ PCR _Eco_RI end
Northern Analysis probes:		
DDO-1369	TAATACGACTCACTATAGTACGGAATTGGAGAGCATTTG	_ATG31_ CDS T7
DDO-1370	GAATGTTACAGTTACTGTTTATG	_ATG31_ CDS
DDO-1404	TAATACGACTCACTATAGCACAAGAGTGGTGCTTCTGTC	_SUT467_ T7
DDO-1366	CATGCTGCAGCTAATATCACC	_SUT467_
DDO-1371	TAATACGACTCACTATAGATTCATCATGACTGGAGCTTG	_SES1_ CDS T7
DDO-1372	GTTGGACATCAACCAATTTATC	_SES1_ CDS
ACT1 5'	ATGGATTCTGGTATGTTCTACCGC	_ACT1_ CDS
ACT1 3'	TAATACGACTCACTATAGGGCAACTCTCAATTCGTTGTAGAAGG	_ACT1_ CDS T7
5'-RACE, ChIP and qRT-PCR:		
DDO-59	CATACTCGAAGGGTAGTTGG	_tN(GUU)C_ upstream, Chr. III
DDO-60	GATTTTTCCATTCGCCATGC	_tN(GUU)C_ downstream, Chr. III
DDO-402	ATGGATTCTGAGGTTGCTGC	_ACT1_ ATG/intron qPCR
DDO-403	CAAAACGGCTTGGATGGAAAC	_ACT1_ internal qPCR
DDO-1284	GATATGTACTTCAAATTATTATCTTTTACGAAACCCATTGGAAATGACTTAATCCC	Extended transcript specific primer for qRT-PCR (same as _tDNA_Δ)
DDO-1364	GATATGTACTTCAAATTATTATCTTTTAC	Long transcript RACE inner
DDO-1527	GACGTTTATCTTGGATATTGAC	Long transcript RACE outer
DDO-1541	CATAAACAGTAACTGTAACATTC	_ATG31_ RACE inner
DDO-1542	TCTCTTCTAATCTGTACTTGAC	_ATG31_ RACE outer
DDO-1555	GTCCTTTGAATTGCAGGCATAAC	_ATG31_ intermediate
DDO-1576	CAATGTTTACCGGCTTAATAAG	_ATG31_ intergenic ChIP
DDO-1577	TTTCGACATATAATAGGCGAAC	_ATG31_ intergenic ChIP
DDO-1606	CTTCAAATTATTATCTTTTACGAAAC	qPCR bridge primer

Table S2 Oligonucleotides used in this study (cont.)

Name	Sequence	Description
Western Analysis, Phosphatase assay and Viability:		
DDO-1419	TTTGCGATCTGTCTCCTTTTC	*ATG31*-myc tag check
DDO-1451	CCAAGATAAACGTCGCATTCCCATTTTCTTATTAAGCCGGTAAACATTGCTGAAAT CTGCGAACAGGACTCCTTACGCATCTGTGCGG	*ATG31* delete top
DDO-1452	AATATAGAGAACATATACCTACATAAACAAGTTAAGAGAGTCTCATCCATGCGGC TTCATTTTTGCTTGCAGATTGTACTGAGAGTGC	*ATG31* delete bottom
DDO-1453	GATTTAGCAAAACTTAGAGAAATTG	*ATG31* delete PCR check
DDO-1462	GTTTGAATGACTTAATTAAACTCTACGTCACACAAAATGAACAATTACAAATGCTC TCCAATTCCGTAATCTCCGGTTCTGCTGCTAG	*ATG31*-9X-myc tag top
DDO-1463	AATATAGAGAACATATACCTACATAAACAAGTTAAGAGAGTCTCATCCATGCGGC TTCATTTTTGCTTGGCCAGAAGACTAAGAGGTG	*ATG31*-9X-myc tag bottom
DDO-1464	GCTATTCATCCAGCAGGCCTC	Myc tag/k. lactis *TRP1* ORF check
DDO-1540	GATTCAGAGTGGACTCGGAC	
DDO-1599	GGATTGATAAGAGAATCTAATAATTGTAAAGTTGAGAAAATCATAATAAAATAATT ACTAGAGACGCAGATTGTACTGAGAGTGC	*ATG8* delete top
DDO-1600	TGGCTAATGAGTCCCTATAATTTCGATTTTAGATGTTAACGCTTCATTTCTTTTCATA TAAAAGACTCCTTACGCATCTGTGCGG	*ATG8* delete bottom