CRISPRi is not strand-specific at all loci and redefines the transcriptional landscape

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ABSTRACT

CRISPRi, an adapted CRISPR-Cas9 system, is proposed to act as a strand-specific roadblock to repress transcription in eukaryotic cells using guide RNAs (sgRNAs) to target catalytically inactive Cas9 (dCas9) and offers an alternative to genetic interventions for studying pervasive antisense transcription. Here we successfully use click chemistry to construct DNA templates for sgRNA expression and show, rather than acting simply as a roadblock, sgRNA/dCas9 binding creates an environment that is permissive for transcription initiation/termination, thus generating novel sense and antisense transcripts. At HMS2 in Saccharomyces cerevisiae, sgRNA/dCas9 targeting to the non-template strand for antisense transcription results in antisense transcription termination, premature termination of a proportion of sense transcripts and initiation of a novel antisense transcript downstream of the sgRNA/dCas9 binding site. This redefinition of the transcriptional landscape by
CRISPRi demonstrates that it is not strand-specific and highlights the controls and locus understanding required to properly interpret results from CRISPRi interventions.

INTRODUCTION

Eukaryotic genomes are pervasively transcribed but the effect of this inter- and intragenic transcription is not fully understood (Mellor, Woloszczuk, & Howe, 2016). Of particular interest is the function of antisense transcription, which alters the chromatin in the vicinity of sense promoters (Lavender et al., 2016; Murray et al., 2015; Pelechano & Steinmetz, 2013), and is associated with repression, activation or no change in levels of the corresponding sense transcript (Murray et al., 2015). Discerning the mechanism by which antisense transcription functions in gene regulation is confounded by the difficulty in ablating antisense transcription without direct genetic intervention (Bassett et al., 2014). A new approach, CRISPR interference (CRISPRi) (Qi et al. 2013), can circumvent these issues by avoiding the need to manipulate endogenous DNA sequences. An endonucleolytically dead version of Cas9 (dCas9) is recruited by single base-pairing guide RNAs (sgRNAs) targeting the non-template (NT) DNA strand, where it blocks transcription strand-specifically at the loci tested (Lenstra, Coulon, Chow, & Larson, 2015; Qi et al., 2013). In a further adaptation, dCas9 fusion with transcriptional repressors or activators allows both negative and positive regulation of transcription respectively (Gilbert et al., 2013, 2014), but the strand-specificity is lost and thus is not suitable for strand-specific repression of antisense transcription. The sgRNA component of the CRISPRi system consists of two regions: a constant region (82 nt) that binds to dCas9 and a variable region (20 nt) that is responsible for targeting. The modular nature of the sgRNA lends itself to production from DNA templates constructed using synthetic copper(I)-catalysed alkyne-azide cycloaddition (CuAAC) click chemistry (El-Sagheer & Brown, 2010). The stable artificial triazole DNA linker generated is biocompatible, being read and accurately copied by DNA and RNA polymerases (Birts et al., 2014; El-Sagheer & Brown, 2011; El-Sagheer, Sanzone, Gao,
Tavassoli, & Brown, 2011). We show that click chemistry is an efficient method for sgRNA template construction and that when combined with dCas9, these sgRNAs are as effective as sgRNAs produced from chemically synthesised full-length DNA templates at reducing levels of transcripts in vivo. However, it is still not completely understood how the CRISPRi system functions strand-specifically or why sgRNAs must target the non-template strand.

Here we use the CRISPRi system to study the effect of blocking antisense transcription at loci with well characterised sense:antisense transcript pairs. We have previously used a promoter deletion of the antisense transcript SUT650 at the HMS2 locus to show that SUT650 represses HMS2 sense transcription (Nguyen et al., 2014). Now we use CRISPRi to examine the effects of blocking SUT650 antisense transcription to ask (i) whether SUT650 represses HMS2 sense transcription without a genetic intervention and (ii) whether CRISPRi is strand-specific using, in addition to HMS2, an engineered GAL1 gene with a well characterised antisense transcript (Murray et al., 2012, 2015).

The main conclusion from this study is that CRISPRi at the HMS2 locus is not fully strand-specific and results in (i) premature termination of the sense transcript and (ii) initiation of a new unstable antisense transcript in the vicinity of the sgRNA binding site. As transcription from this new antisense initiation site extends in to the HMS2 promoter, there is no net change in HMS2 sense transcript levels. Thus CRISPRi redefines the transcriptional landscape at HMS2. This suggests that routine use of CRISPRi for gene expression analysis will require rigorous analysis of transcript integrity and function before conclusions can be drawn.

RESULTS & DISCUSSION

DNA templates for sgRNA production can be made using click chemistry

CRISPRi-mediated transcriptional repression requires co-expression of a mature sgRNA and dCas9 (Fig. 1A). A small library of single-stranded DNAs, comprised of templates for sgRNA variable regions, were joined to the constant region using click chemistry (Fig. 1B) and used successfully as templates
for PCR, with no significantly different efficiencies when compared to control full-length synthesised oligonucleotides (Fig. 1C). The PCR products were inserted in place of a *URA3* selection cassette in the endogenous *snR52* locus for expression of a transcript that is then processed to form the mature nuclear-retained sgRNA (Fig. 1A). Levels of dCas9 protein were uniform between strains (Fig. 1 – Figure Supplement 1). Neither insertion of *URA3* into *snR52* nor dCas9 expression in the control strains affected growth rate, although strains expressing some sgRNAs grew more slowly indicating a physiological effect (Fig. 1D).

**CRISPRi represses the production of antisense transcripts at *HMS2* and *GAL1**

CRISPRi represses transcription when sgRNAs/dCas9 are targeted to the non-template (NT) strand next to a protospacer adjacent motif (PAM) (Qi et al., 2013) (Fig. 2A). Firstly, we used an engineered version of *GAL1* that has a stable antisense transcript (*GAL1 AS*) initiating within an *ADH1* terminator inserted into the *GAL1* coding region (Murray et al., 2012) (Fig. 2B). *GAL1 AS* is present in cells grown in glucose-containing media when the *GAL1* gene is repressed and is reduced as cells are switched into galactose-containing media and *GAL1* sense is induced (Fig. 2C lanes 1-3). We designed sgRNAs adjacent to two PAM sequences on the non-template strand near the antisense transcription start site (TSS) (AS+28NT and AS+112NT) and a third strand-specificity control sgRNA on the template strand in this region (AS+93T) (Fig. 2B). Only sgRNA AS+112NT/dCas9 caused significant (p=0.004) reduction in *GAL1* antisense transcript levels, as assessed by Northern blotting (Fig. 2C,D).

Next, we examined the *HMS2* locus which has a stable antisense transcript, *SUT650*, initiating within the 3’ coding region of *HMS2* (Nguyen et al., 2014; Pelechano, Wei, & Steinmetz, 2013). We designed three *SUT650*-targeting sgRNAs, located 59, 243 and 276 nucleotides downstream of the major *SUT650* TSS on the non-template strand (sgRNAs AS+59NT, AS+243NT, AS+276NT respectively) (Fig. 2E). Note that there is considerable heterogeneity in the *SUT650* TSS (Fig. 2 – Figure Supplement 1) and thus in some cells, sgRNA AS+59NT may be binding upstream of the *SUT650* TSS. AS+243NT/dCas9 significantly (p=0.011) reduced *SUT650* (Fig. 2F lanes 1&3, 2G) and
this repression was comparable for the clicked and control full-length synthetic constructs (Fig. 2G).

However, there was no obvious effect of the other two sgRNAs/dCas9 on SUT650 levels (Fig. 2F lanes 1, 4&5, 2G). Consistently, we could only detect dCas9 binding to the site of sgRNA AS+243NT binding and not to the other two SUT650-targeting sgRNA binding sites tested (Fig. 2 – Figure Supplement 2).

CRISPR efficiency has been linked to nucleosome occupancy (Horlbeck, Witkowsky, et al., 2016; Isaac et al., 2016) but we found no correlation between the level of repression and the nucleosome occupancy (Knight et al., 2014) at each of the SUT650 sgRNA target regions (Fig. 2 – Figure Supplement 3). However, other factors such as sequence determinants can also influence repression (Horlbeck, Gilbert, & Villalta, 2016). These results at GAL1 and HMS2 confirm CRISPRi is suitable for reducing levels of antisense transcripts in yeast but controls are needed for each sgRNA designed to ensure that repression has been achieved.

**CRISPRi repression of SUT650 induces a new shorter antisense transcript at HMS2**

At HMS2, sgRNA AS+243NT/dCas9 was able to reduce SUT650 transcript levels significantly (Fig. 2F,G). Since SUT650 is a substrate for the major cytoplasmic 5’-3’ exonuclease Xrn1 (Fig. 2F, lanes 1&2), we investigated whether the CRISPRi-induced SUT650 reduction was due to direct repression of SUT650 transcription and/or a reduction in SUT650 stability. Whilst SUT650 was not stabilised upon XRN1 deletion in the strain expressing AS+243NT/dCas9, supporting previous studies that CRISPRi operates via a transcriptional block (Qi et al., 2013) (Fig. 2F, lanes 3&6, transcript B), we detected a shorter antisense transcript (Fig. 2F, lane 6, transcript B'). To map transcript B', we used a series of Northern blotting probes across the locus (Fig. 2H). With antisense-specific probes H1 and H2 in the HMS2 gene promoter and 5’ coding region respectively, we could detect antisense transcript B', indicating that it extends into the HMS2 sense promoter. However, probe H4 at the 3’ end of the HMS2 gene was unable to hybridise to B', indicating that B' initiates upstream of this site. So whilst CRISPRi successfully blocked the original SUT650, a new Xrn1-sensitive unstable antisense transcript (B') was created. This is potentially due to changes in the chromatin structure caused by...
AS+243NT/dCas9 binding to this region (Horlbeck, Witkowsky, et al., 2016; Isaac et al., 2016) or by blocking SUT650, creating an environment that is permissive for transcription initiation (Murray et al., 2012). We note that using probe H4 we were also unable to detect SUT650 initiating from its WT start site but terminating at the AS+243NT binding site, due to its small size (~243 nt).

**CRISPRi-induced GAL1 AS repression does not affect the GAL1 sense transcript**

We tested whether GAL1 AS repression by AS+112NT/dCas9 affected the GAL1 sense transcript. Previously we mutated a TATA-like sequence to ablate antisense transcription but observed no difference in GAL1 sense transcript levels in the population under repressive or activating conditions (Murray et al., 2015). Using CRISPRi, we also observed no significant change in GAL1 sense transcript levels or size (Fig. 3A,B). Additionally, we observed no leaky expression of the GAL1 sense transcript under repressive conditions (glucose) when the GAL1 AS transcript was blocked (Fig. 3A, t=0). To support a strand-specific transcriptional block of antisense transcription, GAL1 sense transcript polyA site usage was unaffected by sgRNA AS+112NT/dCas9 (Fig. 3C). Furthermore, XRN1 deletion in this strain also did not affect polyA site usage, ruling out a partial double-stranded transcriptional block and subsequent destabilisation of the resulting truncated sense transcript (Fig. 3C).

**CRISPRi-induced SUT650 repression truncates the HMS2 sense transcript**

Previous work shows that reducing SUT650 transcription increases HMS2 sense transcript levels (Nguyen et al., 2014). However, blocking SUT650 by AS+243NT/dCas9 did not similarly increase HMS2 sense levels (Fig. 3D, lanes 1&3). To our surprise, in addition to the full-length HMS2 sense transcript (A), we detected a considerably shortened HMS2 sense transcript (A'). Since the combined levels of the truncated and full-length transcripts are similar to those in the strain not expressing an sgRNA, independent of the presence of XRN1 (Fig. 3D, lanes 1&3 or 2&6), we hypothesised that the AS+243NT/dCas9 complex bound within the HMS2 coding region may be causing partial premature sense transcription termination, leading to transcript A'. Thus, we mapped the 3' end of HMS2 S by Northern blotting using a series of strand-specific probes across the locus (Fig. 3E). Both transcripts A...
and A1 could clearly be detected using the probes upstream of the AS+243NT/dCas9 binding site (probes H1, H2, H3) but the truncated transcript was undetectable using probe H4 downstream of this site. Thus the transcriptional block caused by AS+243NT/dCas9 is not strand-specific and redefines the transcriptional landscape at the HMS2 locus by creating a chromatin environment that is suitable for both transcription initiation and termination. To see if we could more directly target and block HMS2 sense transcripts, we designed three sgRNAs to bind the non-template strand adjacent to PAM sites near the HMS2 sense TSS (S+77NT, S+106NT, S+179NT) and monitored their effects on both HMS2 sense and antisense transcripts (Fig. 3 – Figure Supplement 1). However, these sgRNAs gave no CRISPRi repression as indicated by the unchanged transcript levels and integrity.

CRISPRi-induced antisense repression at HMS2 and GAL1 is not as efficient as previous methods

We compared the repression efficiency of CRISPRi with our previously used genetic interventions. At GAL1, mutation of a TATA-like sequence within the ADH1 terminator greatly reduced the level of the GAL1 antisense transcript (Fig. 4A,B) (Murray et al 2015). Lowered GAL1 AS transcript levels could result from either reduced transcription or transcript destabilisation (or a combination of the two). To differentiate between these possibilities, we deleted the major cytoplasmic 5’-3’ exonuclease XRN1. Since XRN1 deletion in the TATA mutant strain only slightly increased GAL1 AS levels, this suggests that most repression was at the level of transcription rather than altering transcript stability (Fig. 4B,C). By contrast, XRN1 deletion in the strain expressing AS+112NT/dCas9 did result in some upregulation of GAL1 AS (Fig. 4B,C), indicating that antisense transcript repression was not as great in this strain. Unlike at HMS2 (Fig. 2F,G), this stabilised GAL1 AS transcript was the same size as in the control and so likely represents stabilisation of the transcripts escaping repression rather than novel transcripts.

Next we compared the efficacy of AS+243NT/dCas9 with previous experiments to ablate SUT650, where we replaced the entire HMS2 coding region, including the antisense TSS with the URA3 coding region (HMS2:URA3) (Nguyen et al., 2014). This successful block of SUT650 transcription increased
levels of the \textit{HMS2} sense transcript, the di-cistronic \textit{HMS2-BAT2} transcript and consequently decreased levels of the downstream gene, \textit{BAT2}, due to transcriptional interference (Fig. 4D-F).

Whilst CRISPRi resulted in a similar decrease in \textit{SUT650} as \textit{HMS2:URA3}, neither the \textit{HMS2}, \textit{BAT2} nor \textit{HMS2-BAT2} di-cistronic transcripts were altered (Fig. 4F). The discovery that whilst \textit{AS+243NT/dCas9} represses \textit{SUT650}, a new unstable \textit{HMS2} antisense transcript is induced and the sense transcript is prematurely terminated could explain why blocking \textit{SUT650} using CRISPRi and the \textit{URA3} gene body replacement strategies did not give the same results (Fig. 4F). Thus CRISPRi is not as effective as a genetic mutation in reducing levels of either the \textit{GAL1} or \textit{HMS2} AS transcripts.

\textbf{Concluding remarks}

Although CRISPRi has been used to strand-specifically repress antisense transcription at \textit{GAL10} (Lenstra et al., 2015) and \textit{GAL1} (this work), here we demonstrate that this is not true at \textit{HMS2}. This may reflect the vastly different transcription levels for the galactose inducible genes (high) compared to \textit{HMS2} (low). Furthermore, a study in human K562 cells found that CRISPRi-induced repression did not correlate with which strand was targeted at 49 genes, although the mechanism behind this observation was not investigated (Gilbert et al., 2014).

This work extends our previous hypothesis (Nguyen et al., 2014), allowing us to propose that, rather than acting as a roadblock, the binding of the sgRNA/dCas9 complex at \textit{HMS2} creates a new chromatin environment, either directly or indirectly via the blocking of antisense transcription, which is permissible for transcription initiation and termination. Thus, new transcription units are generated that result in novel sense and antisense transcripts of varying stabilities that are therefore not always detectable. This redefinition of the transcriptional landscape highlights the levels of controls and locus understanding needed before results from using CRISPRi can be interpreted.

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### Key Resources Table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|------------------------------------|-------------|---------------------|-------------|------------------------|
| gene *(Saccharomyces cerevisiae)*   | HMS2        |                     | YJR147W; SGD ID S000003908; RRID:SCR_003093; SGD, RRID:SCR_004694 |            |
| gene *(S. cerevisiae)*             | GAL1        |                     | YBR020W; SGD ID S000000224; RRID:SCR_003093; SGD, RRID:SCR_004694 |            |
| gene *(S. cerevisiae)*             | BAT2        |                     | YJR148W; SGD ID S000003909 RRID:SCR_003093; SGD, RRID:SCR_004694 |            |
| gene *(S. cerevisiae)*             | snR52       |                     | SGD ID S000006443; RRID:SCR_003093; SGD, RRID:SCR_004694 |            |
| strain, strain background *(S. cerevisiae)* | see Supplementary File 1C | see Supplementary File 1C | *Saccharomyces cerevisiae*, NCBI-Taxon:1247190; SGD, RRID:SCR_004694 | see Supplementary File 1C |
| genetic reagent *(S. cerevisiae)* | see Supplementary File 1C | see Supplementary File 1C | see Supplementary File 1C | see Supplementary File 1C |
| antibody                           | Tubulin (rat monoclonal YL1/2) | Abcam | Abcam ab6160 RRID:AB_305328 | (1:3333) |
| antibody                           | Cas9 (rabbit polyclonal) | Diagenode | C15310258 RRID:AB_2715516 | (1:5000 Western blot); 5 µl/ChIP |
| recombinant DNA reagent            | see Supplementary | Eurofins Genomics | see Supplementary | see Supplementary |
sgRNA design

PAM sequences within 300 bp of the TSS in question were identified and the 20 bp immediately adjacent to these were used to design the variable regions of the sgRNAs. These sequences were run through an off-spotter algorithm (https://cm.jefferson.edu/Off-Spotter/) to minimise any off-target effects (Pliatsika & Rigoutsos, 2015). The sequence for the engineered constant region and SUP4 terminator were taken from (Dicarlo et al., 2013; Mali et al., 2013). The HMS2 sgRNA templates were synthesised as two DNA oligonucleotides to be joined by click chemistry, with the majority of the constant region on one oligonucleotide that could be used for all reactions. To increase the efficiency of the click reaction, the click linkage was designed between a dCpT dinucleotide within the Cas9 handle region. The sequences for the HMS2 and GAL1 sgRNA templates are shown in
Supplementary File 1A. Full-length sgRNA templates were also synthesised for the GAL1 sgRNAs and as a control for the HMS2 click chemistry constructs (Eurofins Genomics).

**Click chemistry**

Single-stranded DNA oligonucleotides complementary to the constant and variable sgRNA regions were synthesised by standard phosphoramidite oligonucleotide synthesis with 3’-alkyne or 5’-azido modifications respectively (El-Sagheer et al., 2011) (Supplementary File 1A). The click chemistry reaction was performed with a Cu¹ catalyst and 24-30 nt splint DNAs (El-Sagheer et al., 2011). PCR was performed for second strand synthesis, amplification and incorporation of sequences homologous to the site of insertion in *S. cerevisiae*. PCR efficiencies were obtained using qPCR performed three times on a Corbett 6000 Rotorgene with four serial 10-fold dilutions in triplicate. Analysis was performed using Rotor-Gene Q Series Software (Qiagen).

**Strain construction**

dCas9, without fusion to a transcriptional repressor domain, was expressed from a plasmid (# 46920 Addgene (Qi et al., 2013)) under the control of the TDH3 promoter. The DNA templates for sgRNA production were integrated in place of an exogenous URAS cassette immediately downstream of the splice site in the endogenous snR52 locus using homologous recombination followed by 5-FOA selection. Correct insertion was confirmed by genomic DNA Sanger sequencing. SNR52 is a Pol III-transcribed C/D box small nucleolar RNA (snoRNA) gene and so does not undergo extensive post-transcriptional processing such as capping and polyadenylation, and transcripts from this locus are retained in the nucleus. Additionally, the locus contains a self-splicing site that produces a mature transcript without additional machinery, allowing precise production of a mature sgRNA without any unwanted extensions. Primer sequences for strain construction are listed in Supplementary File 1B.

**Yeast growth**

The strains used in this study are listed in Supplementary File 1C. Strains were grown to mid-log at 30°C in complete synthetic media lacking leucine (for dCas9 plasmid selection). For experiments studying the GAL1 locus, yeast were grown to mid-log in rich media (YP 2% D/YP 2% Gal) so that the GAL1 induction kinetics were similar to what we had observed previously (Murray et al., 2012, 2015). dCas9 expression was unaffected by the temporary absence of plasmid selection (Fig. 1 – Figure Supplement 1).

**Yeast growth controls**
Assessment of growth in liquid culture (CSM-leucine) was achieved using a Bioscreen 240 spectrophotometer that automatically measures the optical density of cultures at 30°C every 20 min for 24 h. Doubling times were extracted from the gradient of the curves during logarithmic growth.

**Northern blotting**

Northern blotting was performed as before (Murray et al., 2012; Nguyen et al., 2014) using asymmetric PCR or *in vitro* transcription with T7 RNA polymerase to generate the radioactive strand-specific probes for *GAL1* and *HMS2* respectively. Primers for these reactions are listed in Supplementary File 1D. Northern blots were quantified using Fiji/ImageJ (Schindelin et al., 2012) and images acquired using a FLA 7000 phosphorimager (GE Healthcare). Images in the figures are scans of exposures to X-ray film.

**Western blotting**

dCas9 expression was confirmed after each experiment using an anti-Cas9 antibody (Diagenode C15310258) at 1:5000 dilution and anti-Tubulin (Abcam ab6160) at 1:3333.

**Chromatin Immunoprecipitation**

Yeast cells were fixed with 1 % formaldehyde (30 min, 22°C) followed by the addition of glycine to 125 mM for 5 min. Cell pellets were collected by centrifugation (3000 rpm, 5 min) before washing twice with 10 ml cold PBS. Cells were re-suspended in cold FA-150 buffer (10 mM HEPES pH 7.9, 150 mM NaCl, 0.1 % SDS, 0.1 % sodium deoxycholate, 1 % Triton X-100) and broken using 1 ml glass beads on a MagnaLyser (Roche) at (2 x 1 min, 6000 rpm, 4°C).

Fixed chromatin was sheared by sonication using a biorupter (Diagenode, 30 min, 30 s on, 30 s off, high setting), cleared by centrifugation (10 000 rpm, 15 min, 4°C) and incubated with 5 µl anti-Cas9 antibody (Diagenode C15310258) and 30 µl protein A-dynabeads pre-blocked with 200 µg/ml yeast tRNA, 200 µg/ml BSA and 200 µg/ml glycogen (15-20 h, 4°C).

Beads and attached chromatin were collected using a magnetic rack and washed with TSE-150 buffer (20 mM Tris-Cl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1 % SDS, 1 % Triton X-100) for 3 min, TSE-500 buffer (20 mM Tris-Cl pH 8.0, 500 mM NaCl, 2 mM EDTA, 0.1 % SDS, 1 % Triton X-100) for 3 min, LiCl buffer (0.25 M LiCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA, 1 % dioxycholate, 1 % NP-40) for 15 min and twice with TE, all at 22°C. After washing, chromatin was eluted from the beads for 30 min at 65°C with elution buffer (0.1 M NaHCO3, 1 % SDS).

Addition of 350 mM NaCl and incubation for 3 h at 65°C reversed the cross-links before treatment of samples with RNase A for 1 h at 37°C and proteinase K overnight at 65°C. DNA
was purified using a PCR-purification kit (Qiagen) and eluted in 400 µl 1 mM Tris-Cl pH 8.0.

Input DNA was diluted accordingly. Real-time quantitative PCR (qPCR) was carried out using a Corbett 6000 Rotorgene and Sybr green qPCR master mix (Bioline). Data ([IP - no antibody control]/input) were expressed as a percentage of the input (relative to sgRNA+276NT).

Real-time qPCR primers are listed in Supplementary File 1E.

**3' RACE mapping**

Mapping of the 3' end of the GAL1 sense transcript was performed as (Nguyen et al., 2014). Primers are listed in Supplementary File 1F.

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**FIGURE LEGENDS**

**Figure 1. Experimental strategy for sgRNA template synthesis and incorporation into yeast.**

A. An outline of the experiment. DNA templates for sgRNA production were generated using a click chemistry reaction between a single-stranded DNA oligonucleotide with a 3’ alkyne group encoding the constant region (dark blue) and a number of single-stranded DNA oligonucleotides with 5’ azido groups encoding the different variable regions (green). The resulting single-stranded DNA oligonucleotide was purified, amplified by PCR and transformed into yeast to replace the *URA3* selection cassette that had previously been introduced into the endogenous snR52 locus. Correct insertion was confirmed by Sanger sequencing. The expression of the sgRNA is driven from the endogenous RNA polymerase III snR52 promoter and processed to produce a mature sgRNA. The sgRNA couples with enzymatically dead Cas9 (dCas9, red), expressed under the control of the *TDH3* promoter off a plasmid, to block transcription. See Methods for more detail. B. A polyacrylamide gel visualised by UV shadowing reveals a high efficiency of the click reaction for all the HMS2 constructs (see Figures 2E and Figure 3 – Figure Supplement 1 for positions of the sgRNA binding sites). The sizes of the variable region, DNA splint, constant region and clicked product are indicated. C. PCR efficiencies of the clicked and full-length synthetic DNA oligonucleotides as measured by qPCR with a serial dilution series. N=3, errors are standard error of the mean (SEM), all differences are not significant p>0.05. See Source Data File 1, tab 2. D. Doubling times (min) of the indicated yeast strains grown in complete synthetic media minus leucine. C, strains constructed using clicked oligonucleotides; FLS, strains constructed using full-length synthetic oligonucleotides. N=10, errors are SEM, * p<0.05. See Source Data File 1, tabs 3-4.

**Figure 1 - Figure Supplement 1. Levels of dCas9 protein in the experimental strains.**

Western blots showing levels of dCas9 produced in the strains expressing the indicated sgRNAs against HMS2 (left panel) and GAL1 (right panel). HMS2 strains constructed using the clicked and control full-length synthetic DNA oligos are shown. Tubulin levels are used as a loading control. Images are taken from the same gels with the same exposure times but with some lanes spliced out (indicated with vertical lines).
**Figure 2. CRISPRi reduces antisense transcripts at GAL1 and HMS2.**

A. A schematic demonstrating the previously reported requirement of non-template strand targeting by the sgRNA/dCas9 complex for strand-specific transcriptional repression. Arrows indicate the direction of transcription. NT, non-template; T, template; red, dCas9; PAM, protospacer adjacent motif; green/blue, sgRNA. B. A schematic of the engineered GAL1 locus showing the site of insertion of the ADH1 terminator (T, red box), the transcription start site for the stable GAL1 antisense transcript and the positions of the designed sgRNAs (green vertical lines) targeting the non-template (AS+28NT, AS+112NT) and template (AS+93NT) strands with respect to antisense transcription. The position of the Northern blotting probe detecting the GAL1 AS transcript is shown in purple. C. A Northern blot showing the reduction in GAL1 antisense transcript (black arrowhead) in the strain expressing sgRNA AS+112NT/dCas9 relative to the no sgRNA control (GAL1:ADH1 snR52::URA3 with dCas9). sgRNAs AS+28NT and AS+93T do not alter GAL1 AS levels. Samples were taken from cells grown in glucose (t=0) and at the indicated times after transfer to galactose-containing media (min). Positions of the rRNA are indicated by the short horizontal lines. Ethidium bromide-stained rRNA is used as loading control. D. Quantification of Northern blotting for the GAL1 AS transcript at t=0 in the control no sgRNA strain and the strain with sgRNA AS+112NT. N=6, errors are SEM, * p=0.004. See Source Data File 1, tabs 10-12. E. A map of the HMS2 locus showing the HMS2 AS transcript, SUT650 (black arrow, transcript B) and positions of the three sgRNAs targeting SUT650 (green vertical lines). The position of the Northern blotting probe to detect SUT650 (H1 AS) is shown by the purple line. F. A Northern blot probed with HMS2 antisense probe H1 (see schematic in (E)) showing the level of SUT650 (black arrowhead, transcript B) in the no sgRNA control (snR52::URA3 with dCas9) strain and strains expressing the indicated antisense-targeting sgRNAs. Deletion of XRN1 allows detection of a shorter antisense transcript (B') in the strain expressing AS+243NT. Positions of the rRNA are indicated by the short horizontal lines. *represents cross-hybridisation with the 25S rRNA. A blot probed for the 18S rRNA is used as loading control. G. Quantification of the level of SUT650 (transcript B) reduction in the strains expressing each of the 3 antisense sgRNAs relative to the control no sgRNA strain. N=4-8, errors represent SEM, *p<0.05. Click-linked and full-length synthetic sgRNA AS+243NT templates behave similarly. See Source Data File 1, tabs 5-9. H. Northern blots with a series of HMS2 antisense-specific probes. A new shorter antisense transcript (B') can be detected upon XRN1 deletion in the strains expressing sgRNA AS+243NT/dCas9. Two clones of the CRISPRi strains produced using clicked (C) or full-length synthetic (FLS) DNA oligos for strain construction are shown. Positions of the antisense-specific probes (purple) and the site of sgRNA AS+243NT/dCas9 binding (green vertical line) are shown in the schematic. Positions of the rRNA on
the Northern blot are indicated by the short horizontal lines. *represents cross-hybridisation with the 25S rRNA. Ethidium bromide-stained rRNA is used as loading control.

**Figure 2 - Figure Supplement 1. TIF-seq data showing the heterogeneity in SUT650 transcript start site.**

Transcript start and end sites on the Crick strand of Chromosome X for SUT650 as mapped by Transcript Isoform Sequencing (TIF-seq) (Pelechano et al., 2013). The main SUT650 transcript start site at position 705,244 bp was used in the positioning of the HMS2 AS-targeting sgRNAs shown in the schematic below.

**Figure 2 - Figure Supplement 2. dCas9 is only detected at the HMS2 AS+243NT sgRNA binding site.**

Chromatin immunoprecipitation for dCas9 in the control strain with no sgRNA (snRS2::URA3 with dCas9) and in the strains with the indicated SUT650-targeting sgRNAs at the predicted sites of SUT650-targeting sgRNA binding in HMS2 (sites A and B). Primer positions for qPCR are shown in purple on the locus map. Robust dCas9 signal is only detected at the binding site of the repressive sgRNA AS+243NT. Signal normalised to input and sgRNA AS+276NT. N=3, error are SEM. See Source Data File 1, tabs 13-17.

**Figure 2 - Figure Supplement 3. Nucleosome occupancy over the HMS2 sgRNA target sites does not anti-correlate with level of repression.**

Nucleosome occupancy map for HMS2 as measured by sequencing of a sample that has been under- and over-digested with MNase (Knight et al., 2014). The schematic underneath shows the positions of the sgRNA targeting sites. Relative nucleosome occupancy at each sgRNA binding site is shown in the table for the under- and over-digested samples.

**Figure 3. HMS2 and GAL1 sense transcripts are differently affected by blocking of their respective antisense transcripts.**

A. A Northern blot probed for the GAL1 sense transcript (black arrow) in the control strain with no sgRNA (GAL1 ADH1t snRS2::URA3 with dCas9) and in the strain expressing sgRNA AS+112NT/dCas9. Samples were taken from cells grown in glucose (t=0) and at the indicated times after transfer to galactose-containing media (min). Ethidium bromide-stained rRNA is used as a loading control. B. Quantification of the Northern blot shown in (A) at t=90. N=4, error bars represent SEM, change is statistically non-significant (n.s.) p=0.062. See Source Data File 1, tabs 10-12. C. A schematic showing the results of GAL1 sense transcript 3’ end mapping by RACE in the strains indicated. All distances are shown relative to the sense TSS. Transcript cleavage and polyadenylation occurs beyond the
position of the GAL1 AS-blocking AS+112NT sgRNA shown (green vertical line). D. A Northern blot probed with HMS2 sense probe H1 (purple) showing the level of HMS2 sense (black arrowhead, transcript A) in the no sgRNA control (snR52::URA3 with dCas9) strain and strains expressing the indicated antisense-targeting sgRNAs. A truncated sense transcript (A') was also detected in the strain expressing AS+243NT. C and C' are read-through HMS2-BAT2 transcripts (see Nguyen et al., 2014). Positions of the rRNA are indicated by the short horizontal lines. Ethidium bromide-stained rRNA is used as loading control.

E. Northern blots with a series of HMS2 sense-specific probes detecting the regions indicated in the schematic. The position of the sgRNA AS+243NT/dCas9 binding site is shown (green vertical line). Positions of the rRNA are indicated by the short horizontal lines. Ethidium bromide-stained rRNA is used as loading control.

Figure 3 - Figure Supplement 1. HMS2 sense-targeting sgRNAs/dCas9 are not effective at repressing HMS2 sense and do not alter antisense transcript levels.

A. A Northern blot probed with HMS2 sense probe H1 (purple) showing the level of HMS2 sense (black arrowhead, transcript A) in the no sgRNA control (snR52::URA3 with dCas9) strain and strains expressing the indicated sense-targeting sgRNAs in the presence or absence of XRN1. Positions of the rRNA are indicated by the short horizontal lines. The 18S rRNA is used as loading control. *represents cross-hybridisation with the 25S rRNA. B. Quantification of the Northern blot in (A). N=2, errors represent the SEM. See Source Data File 1, tab 6. C. A Northern blot as (A) but probed with HMS2 antisense-specific probe H1. Ethidium bromide-stained rRNA is used as loading control. D. Quantification of the Northern blot in (C). N=2, errors represent the SEM. See Source Data File 1, tab 6.

Figure 4. CRISPRi block of SUT650 and GAL1 AS is not as efficient as previous genetic interventions.

A. A schematic showing the previously used genetic intervention to repress the GAL1 AS transcript (Murray et al., 2015). In the TATA mutant construct, a TATA box within the engineered ADH1 terminator (T) is scrambled and the level of GAL1 antisense transcript is reduced. B. A Northern blot probed for the GAL1 antisense transcript (black arrowhead) comparing the two methods of reducing antisense at this locus in the presence (top blot) or absence (bottom blot) of XRN1. All samples were run on the same gel and the blots were exposed to film for the same time. Samples were taken from cells grown in glucose (t=0) and at the indicated times after transfer to galactose-containing media (min). Positions of the rRNA are indicated by the short horizontal lines. Ethidium bromide-stained rRNA is used as loading control. C. Quantification of the Northern blot in (B) at t=0. N=2, errors represent the SEM, * p =0.026, n.s. non-significant. See Source Data File 1, tab 12. D. A schematic of the HMS2-BAT2 locus, showing the HMS2 sense and antisense transcripts (A and B), the HMS2-BAT2
478  di-cistronic transcripts (C and C’) and the BAT2 sense transcript (D). Cells switch between sense- and
479  antisense-dominant states in which the indicated transcripts are produced (Nguyen et al., 2014). E.
480  The HMS2-BAT2 locus and associated transcripts in the wildtype (WT) strain and strains that repress
481  the SUT650 transcript. In the HMS2:URA3 strain, the entire coding region of HMS2 is replaced by
482  that of URA3. F. Northern blots comparing the efficiency of SUT650 reduction by replacement of the
483  HMS2 coding region with that of URA3 versus CRISPRi with sgRNA AS+243NT and the relative effects
484  of these two strategies on the HMS2 sense and BAT2 transcripts shown in (E). The WT (BY4741) and
485  HMS2:URA3 strains have been transformed with plasmid pRS315 to allow for growth on complete
486  synthetic media lacking leucine and thus comparisons with the CRISPRi strains. Positions of the rRNA
487  are indicated by the short horizontal lines. *represents cross-hybridisation with the 25S rRNA.
488  Ethidium bromide-stained rRNA is used as loading control.

491  Supplementary File 1. Supplementary Tables (referenced in the Materials and Methods)
492  Supplementary File 1A. Templates for sgRNAs
493  Supplementary File 1B. Primers for strain construction
494  Supplementary File 1C. Saccharomyces cerevisiae strains used in this study
495  Supplementary File 1D. Primers for construction of Northern blot probe templates
496  Supplementary File 1E. Primers for real-time qPCR at HMS2
497  Supplementary File 1F. Primers for 3’ RACE mapping of the GAL1 sense transcript
498  Source Data File 1. Numerical data for all graphs (referenced in Figure Legends)
499  Tab 1. Notes about yeast strain names
500  Tab 2. Data supporting Fig. 1C
501  Tab 3. Data supporting Fig. 1D
502  Tab 4. Analysis of data in Tab 3 for Fig. 1D
503  Tab 5. Quantitation of all Northern blots for CRISPRi of HMS2 AS transcripts supporting Figs 2-4
504  Tab 6. Quantitation of all Northern blots for CRISPRi of HMS2 S transcripts experiments 1 and 2
505  supporting Fig. 3 – Figure Supplement 1.
506  Tab 7. Quantitation of all northern blots for CRISPRi of HMS2 S transcripts experiments 3 and 4
507  supporting Fig. 3 – Figure Supplement 1.
508  Tab 8. Data analysis of comparison of CRISPRi versus genetic intervention (URA3) for HMS2
509  supporting Fig. 4D-F
Tab 9. HMS2 sliding probes supporting Figs. 3E and 4F
Tab 10. GAL1 AS CRISPRi data supporting Fig. 3A-B
Tab 11. All data for GAL1 CRISPRi supporting Figs. 2-4
Tab 12. Data analysis of comparison of CRISPRi versus genetic intervention (TATA mutant) for GAL1 supporting Fig. 4B-C
Tab 13. Normalised dCas9 ChIP data at HMS2 supporting Fig. 2 – Figure supplement 2.
Tab 14. Combined dCas9 ChIP data at HMS2 for replicates 1-3 supporting Fig. 2 – Figure supplement 2.
Tab 15. Raw Ct data for dCas9 ChIP at HMS2 replicate 1 supporting Fig. 2 – Figure supplement 2.
Tab 16. Raw Ct data for dCas9 ChIP at HMS2 replicate 2 supporting Fig. 2 – Figure supplement 2.
Tab 17. Raw Ct data for dCas9 ChIP at HMS2 replicate 3 supporting Fig. 2 – Figure supplement 2.

All raw data can be found at Mendeley Data: http://dx.doi.org/10.17632/p2652vcdz8.1
A

Constant region
Library of variable regions

PCR

Transform

Yeast cell

CRISPRi

dCas9

sgRNA

B

Post-click reaction

Clicked product (119 nt)

Constant region (93 nt)

Splint (30 nt)

Variable region (26 nt)

C

qPCR efficiency

0.0

0.2

0.4

0.6

0.8

1.0

1.2

1.4

1.6

S+77NT
S+106NT
S+179NT
AS+243NT
AS+276NT
AS+59NT

Full-length synthetic

Clicked

D

Doubling time (min)

0

20

40

60

80

100

120

140

160

snR52

WT ::URA3 ::URA3 ::HMS2 AS+243NT

dCas9

- - + + +

- - + + +
Figure S1:

**HMS2 strains:**

- **dCas9**
  - No sgRNA
  - S+77NT
  - S+106NT
  - S+179NT
  - AS+243NT
  - AS+276NT
  - AS+59NT

- **Tubulin**
  - Full-length
  - synthetic
  - Clicked

**GAL1 strains:**

- **dCas9**
  - No sgRNA
  - S+122NT
  - AS+99NT

- **Tubulin**
  - Full-length
  - synthetic
  - Clicked

| kDa | No sgRNA | S+77NT | S+106NT | S+179NT | AS+243NT | AS+276NT | AS+59NT |
|-----|----------|--------|---------|---------|----------|----------|---------|
| 190 |          |        |         |         |          |          |         |
| 135 |          |        |         |         |          |          |         |
| 58  |          |        |         |         |          |          |         |
| 46  |          |        |         |         |          |          |         |
Figure 2

A. Target NT strand: transcriptional block

B. GAL1 T strand:

C. No sgRNA | AS+28NT | AS+112NT | AS+93T

D. Relative to no sgRNA

E. HMS2

F. xrn1Δ

G. Relative to no sgRNA

H. No sgRNA | H1 AS | H2 AS | H4 AS | HMS2 | AS+243NT | No sgRNA | H1 AS | H2 AS | H4 AS | HMS2 | AS+243NT

xrn1Δ

xrn1Δ

xrn1Δ

rRNA
Figure 2 Supplement 2

- dCas9 (relative to AS+276NT)
- No sgRNA
- AS+243NT
- AS+276NT
- AS+59NT

HMS2

SUT650
MNase-seq (under-digested)

(over-digested)

| sgRNA     | Relative nucleosome occupancy |
|-----------|------------------------------|
|           | Under | Over |
| S+77NT    | 4.295 | 14.17 |
| S+106NT   | 16.04 | 33.99 |
| S+179NT   | 9.391 | 11.51 |
| AS+243NT  | 16.53 | 19.36 |
| AS+276NT  | 11.97 | 15.09 |
| AS+59NT   | 22.7  | 28.27 |
Figure 3

A. **GAL1**

B. Bar graph showing RNA levels relative to no sgRNA t=90.

C. DNA sequence

3' RACE

No sgRNA

AS+112NT

AS+112NT xrn1Δ

Major sense polyA site

GAL1

D. **C&C' HMS2-BAT2 di-cistron**

E. **C&C' HMS2-BAT2 di-cistron**

HMS2

HMS2

HMS2

xrn1Δ
**Figure 3 Supplement 1**

**A**  
No sgRNA  
No sgRNA xrn1Δ  
- xrn1Δ  

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| No sgRNA | No sgRNA | xrn1Δ | S+77NT | S+106NT | S+179NT | S+77NT | S+179NT |
| S+18NT | S+106NT | S+179NT | S+18NT | S+106NT | S+179NT | S+18NT | S+106NT | S+179NT |

**B**  
HMS2 S (A)  
relative to no sgRNA  

**C**  
HMS2-BAT2 di-cistron  

**D**  
HMS2 AS (B)  
relative to no sgRNA  

**Notes:**  
- S+77NT, S+106NT, S+179NT denote specific RNA modifications.  
- No sgRNA indicates samples without short guide RNA.  
- xrn1Δ indicates samples with RNA helicase xrn1 deleted.
