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INVESTIGATION

Evidence for an Integrated Gene Repression Mechanism Based on mRNA Isoform Toggling in Human Cells

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ABSTRACT We recently described an unconventional mode of gene regulation in budding yeast by which transcriptional and translational interference collaborate to down-regulate protein expression. Developmentally timed transcriptional interference inhibited production of a well translated mRNA isoform and resulted in the production of an mRNA isoform containing inhibitory upstream open reading frames (uORFs) that prevented translation of the main ORF. Transcriptional interference and uORF-based translational repression are established mechanisms outside of yeast, but whether this type of *integrated* regulation was conserved was unknown. Here we find that, indeed, a similar type of regulation occurs at the locus for the human oncogene *MDM2*. We observe evidence of transcriptional interference between the two *MDM2* promoters, which produce a poorly translated distal promoter-derived uORF-containing mRNA isoform and a well-translated proximal promoter-derived transcript. Down-regulation of distal promoter activity markedly *up-regulates* proximal promoter-driven expression and results in local reduction of histone H3K36 trimethylation. Moreover, we observe that this transcript toggling between the two *MDM2* isoforms naturally occurs during human embryonic stem cell differentiation programs.

KEYWORDS
gene expression regulation
MDM2
LUTI
transcript isoform
uORF translation

Gene expression regulation enables differential decoding of identical genetic material. It has generally been studied as a set of sequential processes, with transcription setting the core pattern of expression and translational and post-translational regulation modulating the final output. The concept of an exclusively linear model for the regulation of genetic information decoding is partly the result of the largely isolated discovery and subsequent study of each regulatory step. This approach has been necessary for and successful in providing a deep understanding of the biochemical mechanisms that mediate gene regulation, as well as for defining the types of regulatory mechanisms that exist at each level. Such regulatory mechanisms include, for example, transcriptional interference, in which transcription from one promoter locally represses transcription from another (Hausler and Somerville 1979; Adhya and Gottesman 1982; Cullen *et al.* 1984; Emerman and Temin 1984; Proudfoot 1986; Hirschman *et al.* 1988; Corbin and Maniatis 1989; Boussadia *et al.* 1997; Greger *et al.* 2000; Martens *et al.* 2004; Kim *et al.* 2012; Van Werven *et al.* 2012; Kim *et al.* 2016). Transcriptional repression by this mechanism has been associated with *cis*-enrichment of inhibitory chromatin marks, typically by production of a noncoding transcript (Carrozza *et al.* 2005; Kim *et al.* 2012; Van Werven *et al.* 2012; Kim *et al.* 2016). An example of a similarly established translational repression mechanism is based on translation of upstream ORFs (uORFs) in the 5′ leader region of some mRNAs at the expense of ORF translation [reviewed in (Wethmar *et al.* 2010; Barbosa *et al.* 2013; Wethmar 2014; Hinnebusch *et al.* 2016)]. Typically, uORF-mediated

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translational repression is viewed as a switch-like mechanism, where the uORFs prevent translation of the downstream ORF under certain conditions, but this repression can be bypassed under other conditions.

Recently, we described a form of gene regulation that relies on the obligate coupling of transcriptional interference and uORF-mediated translational repression to downregulate protein expression (Figure 1A; Chen *et al.* 2017; Chia *et al.* 2017). During budding yeast meiosis, the amount of protein for the conserved kinetochore protein Ndc80 is determined by toggling between two functionally distinct *NDC80* mRNA isoforms. The mRNA isoform produced from a distal promoter cannot be efficiently translated due to uORF translation that prevents ribosomes from reaching and translating the *NDC80* ORF, and the transcription of this isoform interferes with the proximal *NDC80* promoter activity in *cis*. In this manner, production of a 5′-extended mRNA isoform inhibits Ndc80 protein production (Chen *et al.* 2017; Chia *et al.* 2017). In the case of *NDC80*, the uORF-mediated repression appears to be constitutive, conditional only on the existence of the 5′-extended transcript, rather than condition-specific like the best studied regulatory uORF cases [(Mueller and Hinnebusch 1986; Vattem and Wek 2004), for example]. Thus, translational down-regulation by uORFs is simply a consequence of the choice of an upstream transcription start site. Re-expression of Ndc80 protein is achieved by a developmentally induced switch in promoter usage, from distal to proximal, during meiotic progression.

Improved methodology for genome-wide gene expression measurements have resulted in a more complete characterization of the set of transcripts expressed and regions translated than was previously possible. These studies have provided evidence for translation of thousands of uORFs and widespread existence of alternate transcript isoforms [(Ingolia *et al.* 2009; Ingolia *et al.* 2011; Brar *et al.* 2012; Sterne-Weiler *et al.* 2013; Dieudonné *et al.* 2015; Floor and Doudna 2016; Wang *et al.* 2016), for example], including during the yeast meiotic program. Despite their prevalence, the biological impact of both alternative transcript production and uORF translation to gene expression output in most of these newly identified cases has been unclear. Using analyses of parallel global mRNA, translation, and protein datasets, we found that many of the uORFs and alternate transcripts seen during the yeast meiotic program were indicative of the mode of coordinate regulation seen for *NDC80*, with at least 379 other genes showing protein levels that are driven by this type of integrated transcriptional and translational control over time through meiotic development (Cheng *et al.* 2018). It was also recently found that this mode of regulation functions to mediate down-regulation of proteins involved in aerobic respiration as a core part of the unfolded protein response [UPR; (Van Dalfsen *et al.*, 2018)].

We noted that some of the hallmarks of the integrated mode of gene repression that were seen for *NDC80* regulation, and thus used to annotate new cases in yeast, are also known to be common in mammals. For example, almost half of human genes show evidence of alternative promoter usage, resulting in transcript isoforms that differ in their 5′ leader (Wang *et al.* 2016). Additionally, transcripts with extended 5′ leaders that contain uORFs result, in some cases, in a poorly translated transcript compared to isoforms with shorter 5′ leaders (Law *et al.* 2005; Floor and Doudna 2016). Alternative uORF-containing transcripts were also previously defined for several individual mammalian genes, including Mouse double-minute 2 homolog (*MDM2*), an oncogene and repressor of the tumor suppressor p53 (Arrick *et al.* 1994; Barak *et al.* 1994; Brown *et al.* 1999;

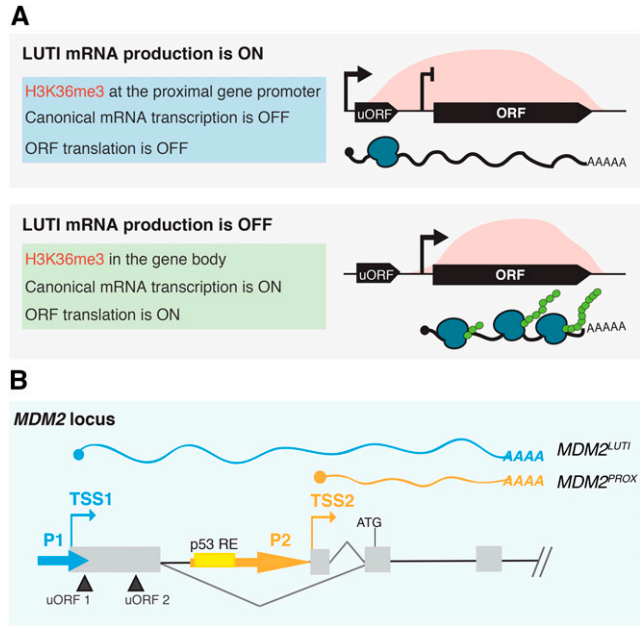

Figure 1 Illustrations of LUTI-based gene repression and *MDM2* gene locus, A. Model for LUTI-based gene repression. Top panel: LUTI mRNA production causes an increase in the co-transcriptional H3K36me3 marks at the proximal gene promoter and transcriptional repression of the canonical mRNA isoform. Because LUTI mRNA is not well translated due to uORFs in its extended 5′ leader and because the well-translated canonical mRNA is repressed, the net effect of LUTI mRNA production is the downregulation of translation from the LUTI target gene locus. Bottom panel: In the absence of LUTI expression, transcription from the canonical gene promoter occurs, leading to translation. B. Illustration of the *MDM2* gene structure. *MDM2* is transcribed from two different transcription start sites (TSS1 and TSS2) regulated by two different promoters (P1 and P2). Transcription from the distal TSS1 produces a 5′-extended, uORF-containing transcript, which is poorly translated. Hereafter, the P1 promoter-driven transcript isoform is referred to as $MDM2^{LUTI}$, while the P2-driven isoform, transcribed from the proximal TSS2 is referred to as $MDM2^{PROX}$. p53 RE refers to the location of the p53 response element within the P2 promoter.

Hughes and Brady 2005). The *MDM2* transcript isoform produced from the distal P1 promoter contains a longer 5′ leader than the one produced from the proximal P2 promoter (Figure 1B) and this P1-derived *MDM2* isoform specifically is poorly translated due to the presence of two uORFs in its extended 5′ leader, as established by polysome analyses and reporter assays (Landers *et al.* 1997; Brown *et al.* 1999; Jin *et al.* 2003). These assays had established differential translation of the two individual *MDM2* isoforms but had not investigated whether a relationship existed between them.

The integrated mode of gene repression seen for *NDC80* in yeast relies on three key features [Figure 1A, (Chen *et al.* 2017; Chia *et al.* 2017)]. First, a developmentally regulated switch between alternative promoters for the same gene leads to the usage of different transcription start sites (TSSs). Second, due to upstream open reading frame (uORF)-mediated translational repression, the distal promoter-generated transcript is inefficiently translated. Third, transcription from the distal promoter represses the expression of the canonical mRNA isoform through transcriptional interference associated with co-transcriptional histone modifications. When all three features exist together, and only then, the activation of

NDC80 transcription can result in a *decrease* in translation from this locus. We termed the distal promoter-generated transcript "*NDC80*^{LUTI}" for long undecoded transcript isoform, because, despite containing the entire *NDC80* ORF, *NDC80*^{LUTI} is not efficiently translated into protein (Chen *et al.* 2017; Chia *et al.* 2017). We noted that the regulation at the *MDM2* locus shared key features with this LUTI-based mechanism, with the important exception that it was unknown whether there was transcriptional interference between the two *MDM2* promoters.

It is well established that *MDM2* P2 can be activated by p53 (Wu *et al.* 1993; Barak *et al.* 1994; Honda *et al.* 1997), but little is known about the factors that activate P1 and whether transcription from the P1 promoter affects P2 activity had not been explored. To our knowledge, this was true of all other individually and globally defined examples of alternative transcripts of differential translatability, as transcriptional interference and uORF-based translational control have been topics studied independently, and typically by different labs. Transcriptional interference is an essential feature of the LUTI mechanism, because it causes a toggle between the two transcript isoforms, which is necessary for effective gene expression repression. Transcriptional interference also enables efficient developmental regulation in the case of *NDC80*. Evidence of transcriptional interference from nearby transcription has previously been established in human cells (reviewed in (Shearwin *et al.* 2005; Palmer *et al.* 2011)), but these cases have not involved the production of ORF-encoding mRNAs. Nevertheless, we hypothesized that the type of integrated regulation that is common in yeast might occur at the *MDM2* locus. Here, we report evidence that this is indeed the case, based on observed transcriptional interference between the two promoters at this locus. We also observe developmental regulation of the two *MDM2* transcript isoforms and conclude that the type of integrated transcriptional and translational regulation that we described as a developmental gene regulatory strategy in yeast is also seen in human cells. These findings suggest value in considering translational and transcriptional regulation not only as independent steps, but rather, as potential collaborators in gene expression outcomes.

MATERIALS AND METHODS

Cell lines

MCF-7-dCas9 and -dCas9-KRAB cells were cultivated at 37° with 5% CO_2 in high glucose Dulbecco's modified Eagle media (GlutaMax, Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin. K562-dCas9 cell lines were cultivated at 37° with 5% CO_2 in RPMI1640 media (Gibco) supplemented with 10% FBS and 10mM HEPES. hESCs (WIBR3 NIH#0079) were maintained in culture as described in (Lengner *et al.* 2010). The differentiation into definitive endoderm was performed using the STEMdiff Definitive Endoderm Kit (Stem Cell Technologies) following the manufacturer's instructions. MCF-7-dCas9 and -dCas9-KRAB cells were kindly provided by Howard Y. Chang (Stanford University). K562-dCas9 cells were kindly provided by Jonathan Weissman (University of California, San Francisco). Cell lines were authenticated by STR profiling and were tested to be negative for mycoplasma (MycoAlert *Mycoplasma* Detection Kit, Lonza).

RNA isolation, cDNA synthesis and quantitative polymerase chain reaction

Total RNA from hESCs differentiating into definitive endoderm and hESCs differentiating into neurons, MCF-7-dCas9, MCF-7-dCas9-KRAB and K562-dCas9 cells was isolated using Trizol (Life Technologies) according to the manufacturer's instructions. Equal amounts of RNA were primed with random hexamers and reverse transcribed using SuperScript II Reverse Transcriptase (ThermoFisher) according to the manufacturer's instructions. RNA levels were quantified using SYBR Green/Rox (ThermoFisher) and the StepOnePlus Real-time PCR system (ThermoFisher). Primers used for RT-qPCR are in Table 1.

Samples for total RNA isolation from hESCs differentiating into neurons (hESC, NPC, neurons D14, neurons D50) were a gift from Helen Bateup [University of California, Berkeley].

CRISPRi knockdowns

sgRNAs targeting *MDM2*^{LUTI} were designed and cloned into the lentiviral pU6-sgRNA EF1Alpha-puro-T2A-BFP vector. Lentivirus was packaged by co-transfecting sgRNA-expression plasmids and the packaging vectors pCMV-dR8.91 and pMD2.G into 293T cells using the TransIT-LT1 Transfection Reagent (Mirus). Cells were treated with ViralBoost (Alstem) to allow for efficient lentivirus production and lentivirus was harvested 72 h post-transfection. CRISPRi-directed gene knockdown was achieved by transducing MCF-7-dCas9, -dCas9-KRAB and K562-dCas9 cell lines with sgRNA-containing lentivirus in the presence of 8μg/ml polybrene (Millipore Sigma). Successfully transduced cells were puromycin-selected (ThermoFisher; 1.3μg/ml for MCF-7 and 3μg/ml for K562 cells) and harvested 7 days post-infection. The pU6-sgRNA EF1Alpha-puro-T2A-BFP vector was a gift from Jonathan Weissman (Addgene plasmid # 60955).

H3K36me3 Chromatin immunoprecipitation (ChIP)

K562-dCas9 cells ($5 \times 15cm^2$ plates per sample) were treated with 1% formaldehyde (16%, methanol free, Ultra Pure, Polysciences) for 10 min at room temperature to crosslink DNA and protein. The crosslinking reaction was stopped by adding 0.125M PBS-glycine and cells were harvested by centrifugation. Cells were subsequently resuspended in ice-cold PBS containing 0.25mM PMSF and 10ug/ml aprotinin (Millipore) and pelleted by centrifugation. Chromatin immunoprecipitation of these pellets was performed as previously described (Testa *et al.* 2005) with minor modifications. Chromatin was sonicated 50 × 30 sec ON/30 sec OFF with a Bioruptor Pico (Diagenode) to obtain fragment sizes of ~200 bp. The sheared samples were incubated in RIPA buffer II (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate, 140 mM NaCl) containing protease inhibitors and PMSF, with Dynabeads Protein A (Invitrogen) for 2 h at 4° on rotation. After removal of Dynabeads Protein A, precleared lysates were immunoprecipitated overnight with 4 ug of rabbit anti-mouse IgG (Ab46540, Abcam) or anti-Histone H3 tri methyl lysine 36 (Ab9050, Abcam). Immunoprecipitates were recovered by incubation for 2 h at 4° with previously blocked Protein A Dynabeads in RIPA buffer II (1 μg/μl bovine serum albumin, protease inhibitors, and PMSF). Reverse crosslinked input DNA and immunoprecipitated DNA fragments were amplified with SYBR Green/Rox (ThermoFisher) and quantified with the StepOnePlus Real-time PCR system (ThermoFisher).

Data availability

All the reagents generated in this study are available upon request. Supplemental material available at Figshare: https://doi.org/10.25387/g3.7670699.

Table 1 Primers used in this study

Target gene	Primer	5′-3′ sequence
MDM2^{PROX}	*MDM2*^{PROX} forward	GTGGCGATTGGAGGGTAGAC
	MDM2^{PROX} reverse	TTGTGCACCAACAGACTTTA
MDM2^{LUTI}	*MDM2*^{LUTI} forward	AAACTGGGGAGTCTTGAGGG
	MDM2^{LUTI} reverse	CAGACATGTTGGTATTGCACAT
GAPDH	*GAPDH* forward	AATCCCATCACCATCTTCCA
	GAPDH reverse	TGGACTCCACGACGTACTCA
NANOG	*NANOG* forward	CCAACATCCTGAACCTCAGCTAC
	NANOG reverse	GCCTTCTGCGTCACACCATT
SOX2	*SOX2* forward	CACACTGCCCCTCTCACACAT
	SOX2 reverse	CATTTCCCTCGTTTTTCTTTGAA
OCT4	*OCT4* forward	TCGAGAACCGAGTGAGAGGC
	OCT4 reverse	CACACTCGGACCACATCCTTC
CXCR4	*CXCR4* forward	AGTGAGGCAGATGACAGATA
	CXCR4 reverse	ACAATACCAGGCAGGATAAG
SOX17	*SOX17* forward	GCCGAGTTGAGCAAGATG
	SOX17 reverse	GGCCGGTACTTGTAGTTG
MDM2 (ChIP)	*MDM2 A* forward	GAGTGGAATGATCCCCGAGG
	MDM2 A reverse	GGTTTTCGCGCTTGGAGTC
	MDM2 B forward	CAGACACGTTCCGAAACTGC
	MDM2 B reverse	CCAATCGCCACTGAACACAG
	MDM2 C forward	CACAGATTCCAGCTTCGGAAC
	MDM2 C reverse	GCCATGCTACAATTGAGGTATACG
	MDM2 D forward	TGGCCAGTATATTATGACTAAACGA
	MDM2 D reverse	CACGCCAAACAAATCTCCTA

RESULTS

A key prediction, if *MDM2* were regulated by a LUTI-based mechanism, would be an inverse relationship between the two *MDM2* transcript isoforms, such that reduction in transcription from P1 should lead to increased transcription from P2. We were able to reliably assay the levels of the P1- and P2-derived transcripts by reverse transcription followed by quantitative PCR (RT-qPCR) using isoform-specific primers, based on the differential splicing of the two transcripts that results in unique 5′ sequences (Figure S1). To directly test if P1-dependent transcription interferes with P2-dependent transcription, we inhibited transcription from P1 by using CRISPRi (Gilbert *et al.* 2013; Qi *et al.* 2013). We first examined MCF-7 breast cancer cells stably encoding the catalytically dead Cas9 (dCas9), which is thought to interfere with PolII elongation when targeted near transcription start sites (Larson *et al.* 2013; Qi *et al.* 2013). Expressing each of four different single guide RNAs (sgRNAs) targeting the P1 promoter region led to modest but significant increases, of up to twofold, in the P2-derived *MDM2*^{PROX} transcript levels, which was associated with the expected reduction of transcription from P1 (Figure 2A and Figure S2A). This result was notable, given that the maximal knockdown of P1 activity was only 40% relative to control cells in these lines (Figure 2A). We tried to enhance the P1 transcriptional knockdown by using CRISPRi in MCF-7 cells that carry a version of dCas9 fused to the Krüppel-associated box (KRAB) transcriptional repression domain (Gilbert *et al.* 2013). However, targeting of dCas9-KRAB to the P1 promoter led to repression of both the P1 and P2 promoters (Figure S3). This finding is consistent with the long-range effect of the KRAB domain up to 1Kb (Gilbert *et al.* 2014), beyond the 845 bp distance between the P1 and P2 regulated transcription start sites. Therefore, we performed all subsequent experiments using cell lines that stably expressed dCas9 without the KRAB domain, as this first-generation version of CRISPRi allowed us to achieve promoter-specific repression.

We further probed the relationship between P1 and P2 by knockdown of the gene encoding p53 (*TP53*) in MCF-7 cells using CRISPRi. Given that p53 is a well-characterized transcriptional activator for P2, it was not surprising that *TP53* knockdown resulted in a significant, 43% reduction of the P2-derived *MDM2*^{PROX} transcript (Figure 2B, left panel). However, additional CRISPRi knockdown of the P1-derived transcript, hereon referred to as *MDM2*^{LUTI}, still resulted in the transcriptional activation of P2, as evidenced by the 2- to threefold increase in *MDM2*^{PROX} levels in this background compared to the *TP53* knockdown alone (Figure 2B, right panel; Figure S2B; Figure S4). The observation that *MDM2*^{LUTI} repression leads to an increase in *MDM2*^{PROX} expression, even in cells with reduced p53 levels, suggests that transcription from P1 actively represses P2 activity and that relief of this repression alone can lead to increased expression of *MDM2*^{PROX} independent of p53, consistent with transcriptional interference at this locus. To test whether transcriptional interference based on *MDM2*^{LUTI} occurs in a different cell type, we performed similar experiments in K562, a *TP53*$^{-/-}$ myeloid leukemia cell line that routinely shows robust CRISPRi-based repression (Gilbert *et al.* 2014). Inhibition of *MDM2*^{LUTI} transcription in these cells resulted in a dramatic increase (up to 10-fold) in *MDM2*^{PROX} expression (Figure 3A and Figure S2C). A range of *MDM2*^{LUTI} knockdown efficiencies were achieved in this cell line. Consequently, the degree of P1 down-regulation generally correlated with the degree of P2 activation (Figure 3A), suggesting tunability of the transcriptional interference at this locus.

H3K36me3 is a co-transcriptionally established modification that occurs in regions downstream of active promoters (Xiao *et al.* 2003; Bannister *et al.* 2005; Mikkelsen *et al.* 2007), and in budding yeast is associated with a decrease in spurious transcription initiation from within transcribed genes (Li *et al.* 2003; Xiao *et al.* 2003; Carrozza *et al.* 2005; Keogh *et al.* 2005; Kim *et al.* 2016) and in noncoding RNA transcription-dependent repression of gene promoters (Kim *et al.* 2012; Van Werven *et al.* 2012). H3K36me3 is enriched at the proximal *NDC80* promoter as a result of *NDC80*^{LUTI} transcription and is involved in the transcriptional interference seen at the *NDC80* locus (Chia *et al.* 2017). In mammalian cells, H3K36me3 has been

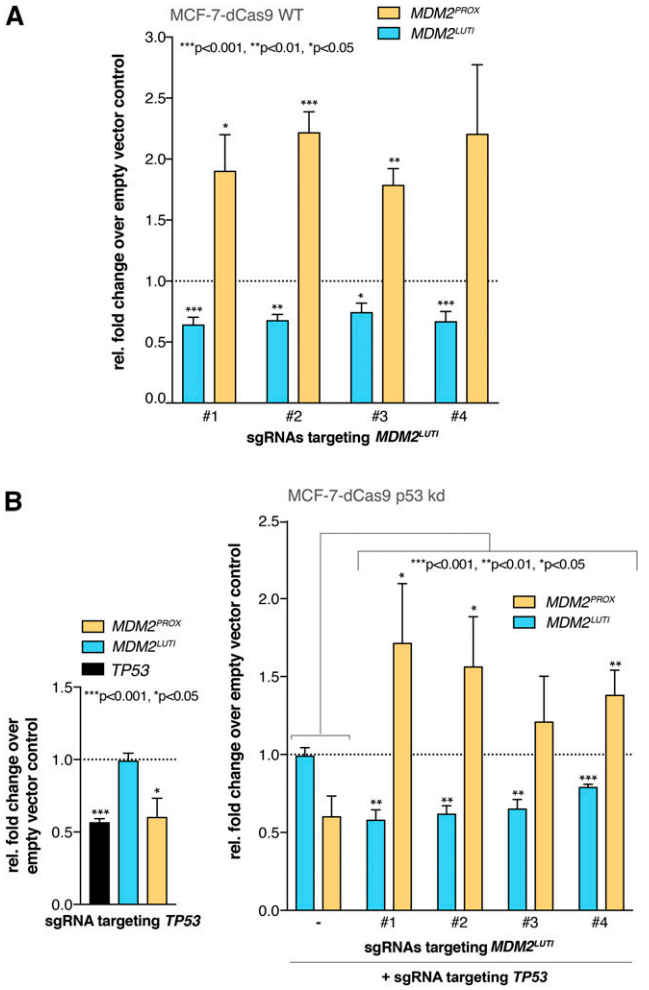

Figure 2 Downregulation of *MDM2*^{LUTI} leads to an increase in the expression of *MDM2*^{PROX} in MCF-7 cells, independent of p53 expression. A. RT-qPCR data displaying the changes of *MDM2*^{LUTI} and *MDM2*^{PROX} mRNA expression in MCF-7-dCas9 stable cells. The transcription of *MDM2*^{LUTI} was inhibited by CRISPRi using four different sgRNAs (#1-4). Data were normalized to *GAPDH*, and the fold change relative to the expression of *MDM2*^{LUTI} and *MDM2*^{PROX} in the cells transfected with an empty vector was calculated. Data points represent the mean of at least 3 independent biological replicates. Error bars represent standard error of the mean (SEM). Two-tailed Student's *t*-test was used to calculate the *P*-values in this figure and all of the subsequent figures. B. RT-qPCR data showing the change in the expression level of *TP53*, *MDM2*^{LUTI} and *MDM2*^{PROX} in MCF-7-dCas9 cells after CRISPRi-mediated *TP53* knockdown (left) or CRISPRi-mediated TP53- and *MDM2*^{LUTI}-double knockdown (right, sgRNA #1 through #4), relative to the cells transfected with an empty vector. Data were normalized to *GAPDH*. Data points represent the mean of four biological replicates. Error bars represent SEM.

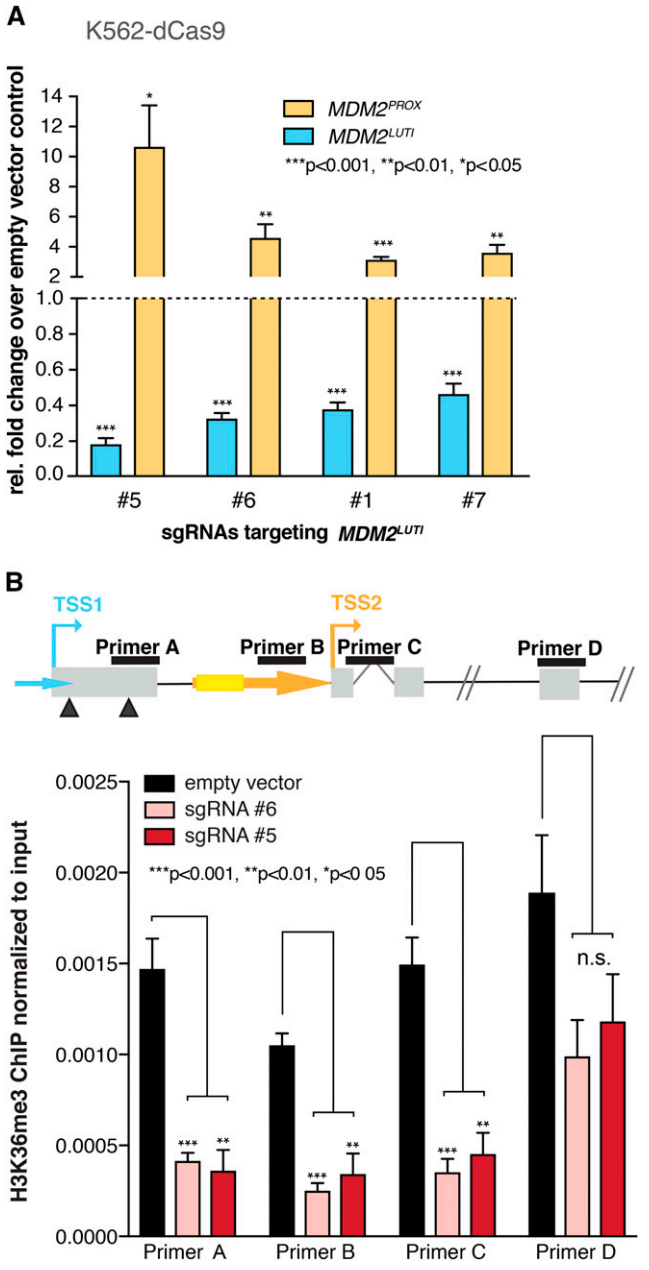

Figure 3 Downregulation of *MDM2*^{LUTI} reduces repression-associated histone marks at the P2 promoter. A. RT-qPCR data displaying the changes in *MDM2*^{LUTI} and *MDM2*^{PROX} expression levels in a stable K562-dCas9 cell line in which the transcription of *MDM2*^{LUTI} had been inhibited by CRISPRi using four different sgRNAs (#5, #6, #1, and #7). Data were normalized to *GAPDH*, and the fold change relative to cells transfected with the empty vector was calculated. Data points represent the mean of at least 3 independent biological replicates. Error bars represent SEM. B. Chromatin immunoprecipitation (ChIP) data displaying H3K36 trimethylation (H3K36me3) enrichment around the proximal TSS (TSS2) in K562-dCas9 cells after CRISPRi-mediated inhibition of *MDM2*^{LUTI} expression. Location of the four different primer pairs (A, B, C, and D) are shown in the schematic above the graph. Arrowheads indicate the location of uORFs in exon 1 and the yellow box indicates the p53 response element. Data points represent the mean of 4 independent biological replicates. Error bars represent SEM. n.s. = not significant.

implicated in silencing, including the repression of spurious intragenic transcription (Dhayalan *et al.* 2010; Xie *et al.* 2011; Wagner and Carpenter 2012; Baubec *et al.* 2015; Suzuki *et al.* 2017), but its involvement in promoter repression has been less clear. We found that down-regulation of *MDM2*^{LUTI} expression resulted in a greater than threefold decrease in the H3K36me3 signal over the P2 promoter (Figure 3B, Figure S5). In contrast, H3K36me3 signal remained high within the *MDM2* gene body, likely due to increased *MDM2*^{PROX} transcription under these conditions. These data are consistent with a

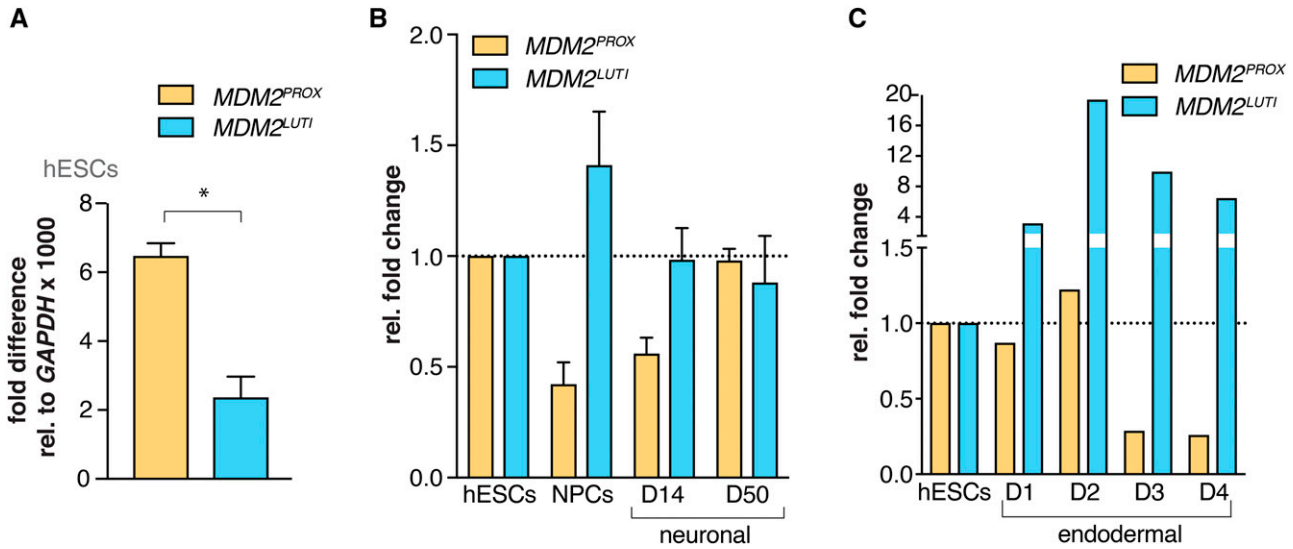

Figure 4 P1- and P2-driven *MDM2* transcript isoform toggling can be seen during human embryonic stem cell differentiation. A. RT-qPCR data showing the fold differences of $MDM2^{LUTI}$ and $MDM2^{PROX}$ transcript levels relative to *GAPDH* expression levels x1000 in human embryonic stem cells (hESCs). Error bars represent the range measured for two biological replicates. B. RT-qPCR data showing the relative expression of $MDM2^{LUTI}$ and $MDM2^{PROX}$ transcripts in human embryonic stem cells (hESCs), neural progenitor cells (NPCs), Day 14 and Day 50 neurons (D14 and D50). Data were normalized relative to $MDM2^{LUTI}$ or $MDM2^{PROX}$ transcript abundance in hESCs. Error bars refer to the range measured for two biological replicates. C. RT-qPCR data showing the changes in the expression of $MDM2^{PROX}$ and $MDM2^{LUTI}$ in hESCs differentiating into endoderm. D1-D4 refers to days after transfer of the hESCs to endoderm differentiation medium. Data were normalized relative to $MDM2^{LUTI}$ or $MDM2^{PROX}$ transcript abundance in hESCs.

mechanism whereby $MDM2^{LUTI}$ expression represses transcription from the P2 promoter through co-transcriptional histone modifications, and provide further support for a model in which the proximal *MDM2* promoter is controlled by a similar mechanism to that defined in yeast.

In budding yeast, developmentally controlled switching between the LUTI and canonical mRNA isoforms occurs pervasively during meiotic differentiation (Cheng *et al.* 2018). To begin to test whether such transcript toggling naturally occurs in human cells, we used two different human Embryonic Stem Cell (hESC) differentiation models. In human hESCs, both *MDM2* transcript isoforms were expressed (Figure 4A). When these cells were induced to undergo neuronal differentiation, a transient switch in transcript isoform expression from $MDM2^{PROX}$ to $MDM2^{LUTI}$ was evident between hESCs and neuronal precursors (NPCs) (Figure 4B). We also observed an anti-correlation between $MDM2^{PROX}$ and $MDM2^{LUTI}$ expression as hESCs differentiated into an endodermal fate, as determined by endoderm-specific markers (Figure 4C, Figure S6). This inverse pattern of proximal and distal promoter usage seen during hESC differentiation suggests that the LUTI-based mechanism regulates *MDM2* expression during normal cellular differentiation.

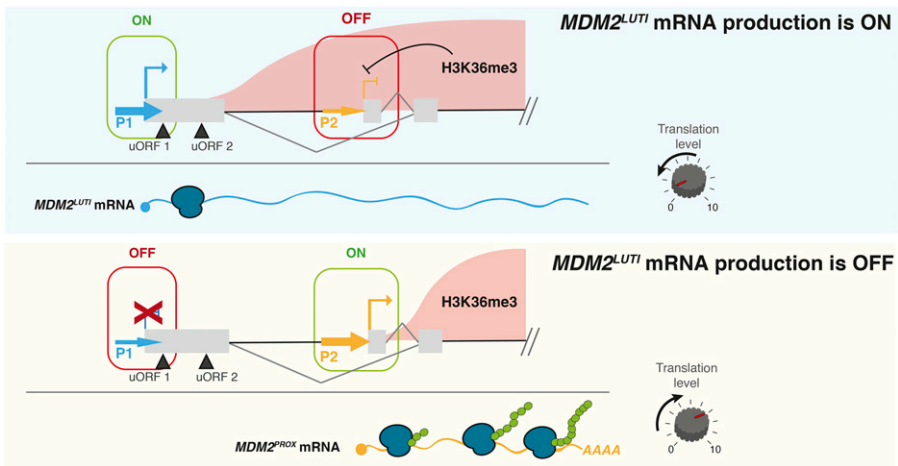

Figure 5 Model of the LUTI mRNA based mechanism of the *MDM2* gene. The *MDM2* gene has two promoters, P1 and P2. $MDM2^{PROX}$ is regulated by P2 whereas $MDM2^{LUTI}$ is regulated by P1. In comparison to $MDM2^{PROX}$, $MDM2^{LUTI}$ is poorly translated because of the existence of two upstream open reading frames (uORFs) in its extended 5′-leader. Top panel: When P1 promoter is active ("ON"), $MDM2^{LUTI}$ transcription establishes H3K36 trimethylation at the downstream P2 promoter and causes repression of P2 ("OFF"). As a result, $MDM2^{LUTI}$ becomes the predominant transcript product from the *MDM2* locus. Bottom panel: When P1 promoter is "OFF", transcriptional repression of the downstream P2 promoter is relieved, culminating in the expression of $MDM2^{PROX}$. $MDM2^{PROX}$ is efficiently translated, resulting in higher MDM2 translation.

DISCUSSION

We report here that an integrated transcriptional and translational regulatory strategy that relies on obligate coupling of transcriptional interference and uORF-based translational repression occurs in human cells. Based on the ubiquitous use of alternative promoters and uORF translation in humans (Ingolia *et al.* 2011; Floor and Doudna 2016; Wang *et al.* 2016; Tresenrider and Unal 2018), this type of regulation may control the expression of many mammalian genes. Both the type of transcriptional control described here and the uORF-based translational regulation already established for the distal promoter-derived *MDM2* transcript are known modes of gene regulatory control. However, our study shows that transcriptional interference can be combined with the production of alternate transcripts of differential translatability in mammals, in a manner that resembles the LUTI-based regulation seen in yeast. These findings suggest value in revisiting the sequential model of gene expression control that dominates the interpretation of past and current data in favor of a more holistic view of different levels of gene expression control.

Canonical models to explain the prevalence of mammalian alternative promoter usage suggest that one promoter might serve as a "back-up" or that the use of two promoters could simply allow activation by different transcription factors that are present in different cell types (Davuluri *et al.* 2008). However, in the case of *MDM2*, we argue that its two promoters are fundamentally different in function. The P1 promoter produces a poorly translated $MDM2^{LUTI}$ transcript and the production of $MDM2^{LUTI}$ from this promoter interferes with P2 activity in *cis*, reducing the transcription of the well-translated $MDM2^{PROX}$ isoform. Therefore, P1-driven $MDM2^{LUTI}$ mRNA production serves to downregulate *MDM2* expression. Further, this repression occurs in a tunable manner (Figure 5). A similar trend was also observed for the *Adh* gene in fruit fly, which is expressed from two distinct promoters (Jorgensen *et al.* 2018). Both findings are consistent with the notion that transcriptional interference can be used to tune gene expression, rather than acting as an on-off switch (Chia *et al.* 2017).

Contrary to traditional gene regulatory models, mRNA and protein abundances generally show poor correlations over developmental programs in genome-scale yeast and vertebrate studies (Peshkin *et al.* 2015; Cheng *et al.* 2018). In yeast, hundreds of such cases can be explained by LUTI-based regulation, whereby developmentally regulated transcript distal and proximal promoter usage drives final protein output levels (Cheng *et al.* 2018). In these conditions, we found that a single developmentally regulated transcription factor could drive distinct sets of canonical and LUTI targets, which resulted in coordinate up- and down-regulation of protein levels for the two target sets (Cheng *et al.* 2018). We propose that this mechanism may be advantageous for developmental programs, which are typically characterized by multiple transcription-factor driven switches in cell stage, because of the ability to temporally coordinate up- and down-regulation of gene sets at each transition. In principle, whether a gene is in one set or another could be defined simply by the position of the binding site for a given transcription factor relative to the ORF and uORF sequences (Chia *et al.* 2017; Cheng *et al.* 2018; Otto and Brar 2018; Tresenrider and Unal 2018). The natural toggling between *MDM2* isoforms during differentiation shown here suggests that the broad use of this type of regulation for developmental modulation of gene expression may be conserved.

MDM2 levels are elevated in a variety of cancers (reviewed in (Rayburn *et al.* 2005)) and this elevation has been attributed in some cases to an increase in translation of the pool of *MDM2* transcripts, based on increased transcription from the P2 (Landers *et al.* 1997; Capoulade *et al.* 1998; Brown *et al.* 1999). Much research has focused on identifying alternate transcription factors that can activate P2—as it is clear that transcription can occur from this promoter in the absence of p53—and several have been found (Phelps *et al.* 2003; Zhang *et al.* 2012), but relatively little is known about P1 regulation. Our study argues that MDM2 expression levels could be modulated by changes in activation of P1 alone, suggesting a promising new general area for the development of gene regulatory tools that modulate P1 activity, and the activity of other yet-to-be-identified LUTI mRNA promoters, as a means to fine tune gene expression.

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AUTHOR CONTRIBUTIONS

IH, AT, XD, RT, LYC, EÜ, and GAB designed experimental strategies and methods and performed data analysis; IH, JCB, VJ, AT, CDD, and LYC executed experiments; IH, EÜ, and GAB wrote the manuscript.

LITERATURE CITED

Adhya, S., and M. Gottesman, 1982 Promoter occlusion: transcription through a promoter may inhibit its activity. Cell 29: 939–944. https://doi.org/10.1016/0092-8674(82)90456-1

Arrick, B. A., R. L. Grendell, and L. A. Griffin, 1994 Enhanced translational efficiency of a novel transforming growth factor beta 3 mRNA in human breast cancer cells. Mol. Cell. Biol. 14: 619–628. https://doi.org/10.1128/MCB.14.1.619

Bannister, A. J., R. Schneider, F. A. Myers, A. W. Thorne, C. Crane-Robinson *et al.*, 2005 Spatial distribution of di- and tri-methyl lysine 36 of histone H3 at active genes. J. Biol. Chem. 280: 17732–17736. https://doi.org/10.1074/jbc.M500796200

Barak, Y., E. Gottlieb, T. Juven-Gershon, and M. Oren, 1994 Regulation of mdm2 expression by p53: alternative promoters produce transcripts with nonidentical translation potential. Genes Dev. 8: 1739–1749. https://doi.org/10.1101/gad.8.15.1739

Barbosa, C., I. Peixeiro, and L. Romao, 2013 Gene expression regulation by upstream open reading frames and human disease. PLoS Genet. 9: e1003529. https://doi.org/10.1371/journal.pgen.1003529

Baubec, T., D. F. Colombo, C. Wirbelauer, J. Schmidt, L. Burger *et al.*, 2015 Genomic profiling of DNA methyltransferases reveals a role for DNMT3B in genic methylation. Nature 520: 243–247. https://doi.org/10.1038/nature14176

Boussadia, O., F. Amiot, S. Cases, G. Triqueneaux, H. Jacquemin-Sablon *et al.*, 1997 Transcription of unr (upstream of N-ras) down-modulates N-ras expression in vivo. FEBS Lett. 420: 20–24. https://doi.org/10.1016/S0014-5793(97)01479-8

Brar, G. A., M. Yassour, N. Friedman, A. Regev, N. T. Ingolia *et al.*, 2012 High-resolution view of the yeast meiotic program revealed by ribosome profiling. Science 335: 552–557. https://doi.org/10.1126/science.1215110

Brown, C. Y., G. J. Mize, M. Pineda, D. L. George, and D. R. Morris, 1999 Role of two upstream open reading frames in the translational control of oncogene mdm2. Oncogene 18: 5631–5637. https://doi.org/10.1038/sj.onc.1202949

Capoulade, C., B. Bressac-de Paillerets, I. Lefrere, M. Ronsin, J. Feunteun *et al.*, 1998 Overexpression of MDM2, due to enhanced translation, results in inactivation of wild-type p53 in Burkitt's lymphoma cells. Oncogene 16: 1603–1610. https://doi.org/10.1038/sj.onc.1201702

Carrozza, M. J., B. Li, L. Florens, T. Suganuma, S. K. Swanson *et al.*, 2005 Histone H3 methylation by Set2 directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. Cell 123: 581–592. https://doi.org/10.1016/j.cell.2005.10.023

Chen, J., A. Tresenrider, M. Chia, D. T. McSwiggen, G. Spedale *et al.*, 2017 Kinetochore inactivation by expression of a repressive mRNA. eLife 6: e27417. https://doi.org/10.7554/eLife.27417

Cheng, Z., G. M. Otto, E. N. Powers, A. Keskin, P. Mertins *et al.*, 2018 Pervasive, Coordinated Protein-Level Changes Driven by Transcript Isoform Switching during Meiosis. Cell 172: 910–923 e916. https://doi.org/10.1016/j.cell.2018.01.035

Chia, M., A. Tresenrider, J. Chen, G. Spedale, V. Jorgensen *et al.*, 2017 Transcription of a 5′ extended mRNA isoform directs dynamic chromatin changes and interference of a downstream promoter. eLife 6: e27420. https://doi.org/10.7554/eLife.27420

Corbin, V., and T. Maniatis, 1989 Role of transcriptional interference in the Drosophila melanogaster Adh promoter switch. Nature 337: 279–282. https://doi.org/10.1038/337279a0

Cullen, B. R., P. T. Lomedico, and G. Ju, 1984 Transcriptional interference in avian retroviruses–implications for the promoter insertion model of leukaemogenesis. Nature 307: 241–245. https://doi.org/10.1038/307241a0

Davuluri, R. V., Y. Suzuki, S. Sugano, C. Plass, and T. H. Huang, 2008 The functional consequences of alternative promoter use in mammalian genomes. Trends Genet. 24: 167–177. https://doi.org/10.1016/j.tig.2008.01.008

Dhayalan, A., A. Rajavelu, P. Rathert, R. Tamas, R. Z. Jurkowska *et al.*, 2010 The Dnmt3a PWWP domain reads histone 3 lysine 36 trimethylation and guides DNA methylation. J. Biol. Chem. 285: 26114–26120. https://doi.org/10.1074/jbc.M109.089433

Dieudonné, F. X., P. B. O'Connor, P. Gubler-Jaquier, H. Yasrebi, B. Conne *et al.*, 2015 The effect of heterogeneous Transcription Start Sites (TSS) on the translatome: implications for the mammalian cellular phenotype. BMC Genomics 16: 986. https://doi.org/10.1186/s12864-015-2179-8

Emerman, M., and H. M. Temin, 1984 Genes with promoters in retrovirus vectors can be independently suppressed by an epigenetic mechanism. Cell 39: 449–467. https://doi.org/10.1016/0092-8674(84)90453-7

Floor, S. N., and J. A. Doudna, 2016 Tunable protein synthesis by transcript isoforms in human cells. eLife 5: e10921. https://doi.org/10.7554/eLife.10921

Gilbert, L. A., M. A. Horlbeck, B. Adamson, J. E. Villalta, Y. Chen *et al.*, 2014 Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. Cell 159: 647–661. https://doi.org/10.1016/j.cell.2014.09.029

Gilbert, L. A., M. H. Larson, L. Morsut, Z. Liu, G. A. Brar *et al.*, 2013 CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 154: 442–451. https://doi.org/10.1016/j.cell.2013.06.044

Greger, I. H., A. Aranda, and N. Proudfoot, 2000 Balancing transcriptional interference and initiation on the GAL7 promoter of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 97: 8415–8420. https://doi.org/10.1073/pnas.140217697

Hausler, B., and R. L. Somerville, 1979 Interaction in vivo between strong closely spaced constitutive promoters. J. Mol. Biol. 127: 353–356. https://doi.org/10.1016/0022-2836(79)90335-8

Hinnebusch, A. G., I. P. Ivanov, and N. Sonenberg, 2016 Translational control by 5′-untranslated regions of eukaryotic mRNAs. Science 352: 1413–1416. https://doi.org/10.1126/science.aad9868

Hirschman, J. E., K. J. Durbin, and F. Winston, 1988 Genetic evidence for promoter competition in Saccharomyces cerevisiae. Mol. Cell. Biol. 8: 4608–4615. https://doi.org/10.1128/MCB.8.11.4608

Honda, R., H. Tanaka, and H. Yasuda, 1997 Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett. 420: 25–27. https://doi.org/10.1016/S0014-5793(97)01480-4

Hughes, T. A., and H. J. Brady, 2005 Cross-talk between pRb/E2F and Wnt/beta-catenin pathways: E2F1 induces axin2 leading to repression of Wnt signalling and to increased cell death. Exp. Cell Res. 303: 32–46. https://doi.org/10.1016/j.yexcr.2004.09.014

Ingolia, N. T., S. Ghaemmaghami, J. R. Newman, and J. S. Weissman, 2009 Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. Science 324: 218–223. https://doi.org/10.1126/science.1168978

Ingolia, N. T., L. F. Lareau, and J. S. Weissman, 2011 Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. Cell 147: 789–802. https://doi.org/10.1016/j.cell.2011.10.002

Jin, X., E. Turcott, S. Englehardt, G. J. Mize, and D. R. Morris, 2003 The two upstream open reading frames of oncogene mdm2 have different translational regulatory properties. J. Biol. Chem. 278: 25716–25721. https://doi.org/10.1074/jbc.M300316200

Jorgensen, V., J. Chen, H. Vander Wende, D. Harris, S. W. Wong-Deyrup *et al.*, 2018 Tunable Transcriptional Interference at the Endogenous Alcohol Dehydrogenase Gene Locus in Drosophila melanogaster. bioRxiv. https://doi.org/10.1101/452649

Keogh, M. C., S. K. Kurdistani, S. A. Morris, S. H. Ahn, V. Podolny *et al.*, 2005 Cotranscriptional set2 methylation of histone H3 lysine 36 recruits a repressive Rpd3 complex. Cell 123: 593–605. https://doi.org/10.1016/j.cell.2005.10.025

Kim, J. H., B. B. Lee, Y. M. Oh, C. Zhu, L. M. Steinmetz *et al.*, 2016 Modulation of mRNA and lncRNA expression dynamics by the Set2-Rpd3S pathway. Nat. Commun. 7: 13534. https://doi.org/10.1038/ncomms13534

Kim, T., Z. Xu, S. Clauder-Munster, L. M. Steinmetz, and S. Buratowski, 2012 Set3 HDAC mediates effects of overlapping noncoding transcription on gene induction kinetics. Cell 150: 1158–1169. https://doi.org/10.1016/j.cell.2012.08.016

Landers, J. E., S. L. Cassel, and D. L. George, 1997 Translational enhancement of mdm2 oncogene expression in human tumor cells containing a stabilized wild-type p53 protein. Cancer Res. 57: 3562–3568.

Larson, M. H., L. A. Gilbert, X. Wang, W. A. Lim, J. S. Weissman *et al.*, 2013 CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nat. Protoc. 8: 2180–2196. https://doi.org/10.1038/nprot.2013.132

Law, G. L., K. S. Bickel, V. L. MacKay, and D. R. Morris, 2005 The undertranslated transcriptome reveals widespread translational silencing by alternative 5′ transcript leaders. Genome Biol. 6: R111. https://doi.org/10.1186/gb-2005-6-13-r111

Lengner, C. J., A. A. Gimelbrant, J. A. Erwin, A. W. Cheng, M. G. Guenther *et al.*, 2010 Derivation of pre-X inactivation human embryonic stem cells under physiological oxygen concentrations. Cell 141: 872–883. https://doi.org/10.1016/j.cell.2010.04.010

Li, B., L. Howe, S. Anderson, J. R. Yates, 3rd, and J. L. Workman, 2003 The Set2 histone methyltransferase functions through the phosphorylated carboxyl-terminal domain of RNA polymerase II. J. Biol. Chem. 278: 8897–8903. https://doi.org/10.1074/jbc.M212134200

Martens, J. A., L. Laprade, and F. Winston, 2004 Intergenic transcription is required to repress the Saccharomyces cerevisiae SER3 gene. Nature 429: 571–574. https://doi.org/10.1038/nature02538

Mikkelsen, T. S., M. Ku, D. B. Jaffe, B. Issac, E. Lieberman *et al.*, 2007 Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 448: 553–560. https://doi.org/10.1038/nature06008

Mueller, P. P., and A. G. Hinnebusch, 1986 Multiple upstream AUG codons mediate translational control of GCN4. Cell 45: 201–207. https://doi.org/10.1016/0092-8674(86)90384-3

Otto, G. M., and G. A. Brar, 2018 Seq-ing answers: uncovering the unexpected in global gene regulation. Curr. Genet. 64: 1183–1188. https://doi.org/10.1007/s00294-018-0839-3

Palmer, A. C., J. B. Egan, and K. E. Shearwin, 2011 Transcriptional interference by RNA polymerase pausing and dislodgement of transcription factors. Transcription 2: 9–14. https://doi.org/10.4161/trns.2.1.13511

Peshkin, L., M. Wuhr, E. Pearl, W. Haas, R. M. Freeman, Jr. *et al.*, 2015 On the Relationship of Protein and mRNA Dynamics in Vertebrate Embryonic Development. Dev. Cell 35: 383–394. https://doi.org/10.1016/j.devcel.2015.10.010

Phelps, M., M. Darley, J. N. Primrose, and J. P. Blaydes, 2003 p53-independent activation of the hdm2–P2 promoter through multiple transcription factor response elements results in elevated hdm2 expression in estrogen receptor alpha-positive breast cancer cells. Cancer Res. 63: 2616–2623.

Proudfoot, N. J., 1986 Transcriptional interference and termination between duplicated alpha-globin gene constructs suggests a novel mechanism for gene regulation. Nature 322: 562–565. https://doi.org/10.1038/322562a0

Qi, L. S., M. H. Larson, L. A. Gilbert, J. A. Doudna, J. S. Weissman *et al.*, 2013 Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152: 1173–1183. https://doi.org/10.1016/j.cell.2013.02.022

Rayburn, E., R. Zhang, J. He, and H. Wang, 2005 MDM2 and human malignancies: expression, clinical pathology, prognostic markers, and implications for chemotherapy. Curr. Cancer Drug Targets 5: 27–41. https://doi.org/10.2174/1568009053332636

Shearwin, K. E., B. P. Callen, and J. B. Egan, 2005 Transcriptional interference–a crash course. Trends Genet. 21: 339–345. https://doi.org/10.1016/j.tig.2005.04.009

Sterne-Weiler, T., R. T. Martinez-Nunez, J. M. Howard, I. Cvitovik, S. Katzman *et al.*, 2013 Frac-seq reveals isoform-specific recruitment to polyribosomes. Genome Res. 23: 1615–1623. https://doi.org/10.1101/gr.148585.112

Suzuki, S., Y. Murakami, and S. Takahata, 2017 H3K36 methylation state and associated silencing mechanisms. Transcription 8: 26–31. https://doi.org/10.1080/21541264.2016.1246076

Testa, A., G. Donati, P. Yan, F. Romani, T. H. Huang *et al.*, 2005 Chromatin immunoprecipitation (ChIP) on chip experiments uncover a widespread distribution of NF-Y binding CCAAT sites outside of core promoters. J. Biol. Chem. 280: 13606–13615. https://doi.org/10.1074/jbc.M414039200

Tresenrider, A., and E. Unal, 2018 One-two punch mechanism of gene repression: a fresh perspective on gene regulation. Curr. Genet. 64: 581–588. https://doi.org/10.1007/s00294-017-0793-5

van Dalfsen, K.M, S. Hodapp, A. Keskin, G.M. Otto, C.A. Berdan *et al.*, 2018 Global proteome remodeling during ER stress involves Hac1-driven expression of long undecoded transcript isoforms. Dev Cell. 46(2): 219–235.e8. https://doi.org/10.1016/j.devcel.2018.06.016

van Werven, F. J., G. Neuert, N. Hendrick, A. Lardenois, S. Buratowski *et al.*, 2012 Transcription of two long noncoding RNAs mediates mating-type control of gametogenesis in budding yeast. Cell 150: 1170–1181. https://doi.org/10.1016/j.cell.2012.06.049

Vattem, K. M., and R. C. Wek, 2004 Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. Proc. Natl. Acad. Sci. USA 101: 11269–11274. https://doi.org/10.1073/pnas.0400541101

Wagner, E. J., and P. B. Carpenter, 2012 Understanding the language of Lys36 methylation at histone H3. Nat. Rev. Mol. Cell Biol. 13: 115–126. https://doi.org/10.1038/nrm3274

Wang, X., J. Hou, C. Quedenau, and W. Chen, 2016 Pervasive isoform-specific translational regulation via alternative transcription start sites in mammals. Mol. Syst. Biol. 12: 875. https://doi.org/10.15252/msb.20166941

Wethmar, K., 2014 The regulatory potential of upstream open reading frames in eukaryotic gene expression. Wiley Interdiscip. Rev. RNA 5: 765–778. https://doi.org/10.1002/wrna.1245

Wethmar, K., J. J. Smink, and A. Leutz, 2010 Upstream open reading frames: molecular switches in (patho)physiology. BioEssays 32: 885–893. https://doi.org/10.1002/bies.201000037

Wu, X., J. H. Bayle, D. Olson, and A. J. Levine, 1993 The p53-mdm-2 autoregulatory feedback loop. Genes Dev. 7: 1126–1132. https://doi.org/10.1101/gad.7.7a.1126

Xiao, T., H. Hall, K. O. Kizer, Y. Shibata, M. C. Hall *et al.*, 2003 Phosphorylation of RNA polymerase II CTD regulates H3 methylation in yeast. Genes Dev. 17: 654–663. https://doi.org/10.1101/gad.1055503

Xie, L., C. Pelz, W. Wang, A. Bashar, O. Varlamova *et al.*, 2011 KDM5B regulates embryonic stem cell self-renewal and represses cryptic intragenic transcription. EMBO J. 30: 1473–1484. https://doi.org/10.1038/emboj.2011.91

Zhang, X., Z. Zhang, J. Cheng, M. Li, W. Wang *et al.*, 2012 Transcription factor NFAT1 activates the mdm2 oncogene independent of p53. J. Biol. Chem. 287: 30468–30476. https://doi.org/10.1074/jbc.M112.373738

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