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Title: A novel role for the RNA polymerase I transcription factor UBTF in maintaining genome stability through the regulation of highly transcribed RNA Pol II genes

Running Title: Non-nucleolar functions for UBTF

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Abstract

Mechanisms to coordinate programs of highly transcribed genes required for cellular homeostasis and growth are unclear. Upstream binding transcription factor 1 (UBTF, also called UBF) is thought to function exclusively in RNA polymerase I (Pol I)-specific transcription of the ribosomal genes. Here we report that, the two isoforms of UBTF (UBTF1/2) are also enriched at highly expressed Pol II-transcribed genes throughout the mouse genome. Further analysis of UBTF1/2 DNA binding in immortalised human epithelial cells and their isogenically matched transformed counterparts reveals an additional repertoire of UBTF1/2-bound genes involved in the regulation of cell cycle checkpoints and DNA damage response. As proof of a functional role for UBTF1/2 in regulating Pol II transcription, we demonstrate that UBTF1/2 is required for recruiting Pol II to the highly transcribed histone gene clusters and for their optimal expression. Intriguingly, lack of UBTF1/2 does not affect chromatin marks or nucleosome density at histone genes. Instead it results in increased accessibility of the histone promoters and transcribed regions to micrococcal nuclease, implicating UBTF1/2 in mediating DNA accessibility. Unexpectedly, UBTF2, which does not function in Pol I transcription, is sufficient to regulate histone gene expression in the absence of UBTF1. Moreover, depletion of UBTF1/2 and subsequent reduction in histone gene expression is associated with DNA damage and genomic instability independent of Pol I transcription. Thus, we have uncovered a novel role for UBTF1 and UBTF2 in maintaining genome stability through coordinating the expression of highly transcribed Pol I (UBTF1 activity) and Pol II genes (UBTF2 activity).

Introduction

Transcription of ribosomal RNA (rRNA) genes (rDNA) is mediated by RNA polymerase I (Pol I) and its associated transcription machinery to produce the 45S rRNA precursor of the 28S, 5.8S, and 18S rRNA components of the ribosome (McStay and Grummt 2008). In rapidly growing

mammalian cells, rRNA synthesis accounts for 35-60% of all RNA transcription (Moss and Stefanovsky 2002; Cavanaugh 2004). Accordingly, Pol I transcription rate is tightly coupled to cellular growth and proliferation rates (Hannan and Rothblum 1995; Moss and Stefanovsky 2002; McStay and Grummt 2008; Chan et al. 2011; Hein et al. 2012; Diesch et al. 2014). We have shown that the consistent upregulation of rRNA synthesis in cancer cells is necessary for malignant transformation in certain settings (Drygin et al. 2011; Bywater et al. 2012). While, small molecule inhibitors of Pol I transcription are currently in preclinical development and phase I trials for cancer therapy (Hein et al. 2013; Drygin et al. 2014; Poortinga et al. 2014). Despite this, the exact molecular mechanism by which Pol I transcription is coordinated with Pol II transcriptional programs to ensure orderly cellular homeostasis is not fully understood. In this report, we propose a functional role for the Pol I-specific transcription factor UBTF1/2 in coordinating Pol I-mediated rDNA transcription with expression of highly active Pol II transcribed genes.

UBTF1/2 belongs to the sequence non-specific class of HMG (high mobility group) proteins. It has 6 HMG-box homology domains and has been proposed to interact with DNA as a dimer and to induce six in-phase bends to generate a single 360° loop structure that resembles the nucleosome (Stefanovsky et al. 2001). UBTF1/2 binds across the transcribed and control regions of the rRNA genes (Supplementary Fig 1A)(O'Sullivan et al. 2002; Sanij and Hannan 2009), probably explaining its multiple roles in preinitiation complex (PIC) formation, transcription initiation and elongation (Reviewed in (Moss et al. 2007; McStay and Grummt 2008; Sanij and Hannan 2009). Indeed, total ablation of *Ubt1/2* eliminates all rRNA gene transcription and leads to changes in rDNA chromatin (Hamdane et al. 2014).

We have previously shown that UBTF1, but not the naturally occurring splice variant UBTF2, is essential in determining and maintaining the euchromatic state of active rDNA in mammalian cells (Sanij et al. 2008). Further, we have reported that depletion of *Ubt1/2* by RNA interference

(RNAi) silences active rRNA genes and is associated with histone H1-induced assembly of transcriptionally inactive rDNA chromatin (Sanij et al. 2008). Indeed, UBTF1/2 localizes with decondensed active rDNA while inactive rDNA appear as bright dense loci devoid of UBTF1/2 (Supplementary Fig 1B) that are enhanced in number by *Ubt1/2*-loss (Hamdane et al. 2014). This is in agreement with its reported function in establishing and maintaining the undercondensed structure of active nucleolar organizer regions (NORs), which is required for rDNA transcription and nucleolar assembly (Mais et al. 2005; Sanij et al. 2008; Sanij and Hannan 2009; Grob et al. 2014; Hamdane et al. 2014).

Intriguingly, UBTF2 was identified in a functional screen as an enhancer of the beta-catenin pathway (Grueneberg et al. 2003). Further, UBTF1/2 was noted to be putatively enriched at Pol II genes across the human genome (Zentner et al. 2011). However, the role of extra-nucleolar UBTF1/2 is unknown. Indeed, the prevailing consensus in the transcription field is still overwhelmingly that UBTF1/2 is exclusively a Pol I transcription factor (Grob et al. 2014). Here we demonstrate that in addition to the Pol I loci, UBTF1/2 is enriched at and regulates highly expressed Pol II-transcribed genes including histone gene clusters. Further, ChIP-seq analysis of UBTF1/2 binding in tumorigenic human epithelial cells compared to an isogenically matched primary cells reveals an additional repertoire of UBTF1/2 target genes involved in regulation of cell cycle checkpoints and DNA damage response. Depletion of *Ubt1/2* by RNAi leads to DNA damage and genomic instability independent of Pol I transcription. Together, our data demonstrate a novel and unexpected role for UBTF1 and UBTF2 in coordinating Pol I-mediated rDNA transcription with expression of distinct sets of highly active Pol II transcribed genes that control cell cycle progression and genome integrity.

Results

UBTF1/2 binds highly active Pol II transcribed genes. To identify species and cell type

independent core set of UBTF1/2 target genes, we performed ChIP-seq analysis in mouse NIH3T3 cells (crisis immortalized embryonic fibroblasts) and the human primary mammary epithelial cell line (HMEC) immortalised by expressing TERT, the catalytic subunit of telomerase (Elenbaas et al. 2001). We identified respectively, 3705 and 10726 genomic regions with significant UBTF1/2 enrichment across the mouse and human genomes of which respectively 46% and 52% overlapped any exon/intron of known RefSeq genes (Fig 1A&B). In both mouse and human cells ~40% of all UBTF1/2 binding overlapped with first exons and introns of annotated genes. Indeed, UBTF1/2 enrichment shows preference for binding near transcription start sites (TSS), whereas no significant binding preference was observed at the transcription termination sites (TTS) (Fig 1C). Gene ontology analysis using the Genomic Regions Enrichment of Annotation Tool (GREAT)(McLean et al. 2010) identified chromatin assembly and nucleosome organization and assembly as the biological processes most significantly enriched with UBTF1/2-bound genes and common between the mouse and human datasets (Supplementary Fig 2A,B). These categories include chromatin remodeling factors and significantly a large number of histone genes (Supplementary Tables 1&2), implicating a role for UBTF1/2 in their transcriptional regulation. To confirm the UBTF1/2 enrichments at these non-rDNA loci identified by ChIP-seq, we examined UBTF1/2 DNA binding manually by quantitative ChIP (qChIP) at genes encoding histone genes, chromatin remodeling factors (*Asf1a*, *Smarca5*, and *Dnmt3a*) and also potential UBTF1/2 target genes that did not appear within gene ontological categories, e.g., deoxyuridine triphosphatase (*Dut*), the oncogene *Myc* and the member of the structural maintenance of chromosome family (*Smc4*) (Fig 1D). The qChIP data showed significant enrichment in UBTF1/2 at these genes as compared to controls including the intergenic spacer (IGS) of rDNA that does not bind UBTF1/2 (Fig 1D).

We intersected the lists of annotated genes bound by UBTF1/2 within $\pm$ 2kb of TSSs in the NIH3T3 and HMEC data sets and identified 699 genes that were common between the mouse and

human analyses (Fig 1E, Supplementary Table 14). Gene ontology analysis of the intersected genes showed regulation of gene silencing, chromatin and nucleosome assembly categories to be the most significantly enriched with UBTF1/2 bound genes (Supplementary Fig 2C). The gene lists for these ontology terms consisted mainly of histone genes and chromatin remodeling factors (Supplementary Tables 3-5), suggesting a conserved function for UBTF1/2 in their transcriptional regulation. Given that histone genes are amongst the most highly expressed genes in the human genome (Anamika et al. 2012), we investigated whether UBTF1/2 binding correlates with transcriptional activity. Indeed, a correlation of UBTF1/2 DNA binding with global gene expression in NIH3T3 cells identified genes enriched with UBTF1/2 at their TSSs to be expressed at high levels compared to non-UBTF1/2 bound genes and all genes in the genome (Fig 1F).

We next examined the enrichments of a variety of posttranslational histone modifications, Pol II binding and DNase I hypersensitivity (HS) profiles at UBTF1/2 bound regions in NIH3T3 cells (Fig 2A). We observed little correlation between UBTF1/2 binding and the presence of the transcriptional repressive mark H3K9me3. However, almost 50% of the UBTF1/2 peaks overlapped with the activating H3K4me3, H4K9ac and H4 hyperacetylation marks and Pol II enrichment. Further, almost 70% of UBTF1/2 bound regions are defined as DNase I hypersensitive sites, indicative of open and accessible chromatin (Fig 2A). Thus, our data strongly suggest specific preference for UBTF1/2 binding to open chromatin structures associated with active promoters and gene bodies.

Interestingly, UBTF1/2 ChIP-seq peaks accounts for only 1.7% of all DNase I HS regions across the genome (Fig 2A). This suggests that UBTF1/2 preferentially binds a specific subclass of open highly transcribed chromatin. In order to confirm the specificity of UBTF1/2 binding to DNA, we performed ChIP-seq analysis of POLR1A, the largest subunit of the RNA Pol I complex as both Pol I and UBTF1/2 co-occupy the highly transcribed Pol I-dependent rDNA loci (Supplementary Fig 1A). We detected 1409 and 9296 Peaks of Pol I binding across the mouse and human

genomes some of which we validated manually by qChIP (Supplementary Fig 3A). However, a comparison of UBTF1/2 and POLR1A ChIP-seq analysis in NIH3T3 and HMEC cell lines revealed little overlap in binding (less than 8% and 3%) across the mouse and human genomes, respectively (Fig 2B). Thus the Pol I transcription apparatus does not simply colocalize indiscriminately at open chromatin and that the enrichment at classes of genes involved in chromatin assembly and nucleosome organization is specific to UBTF1/2. Further, the data strongly suggest that UBTF1/2 does not recruit Pol I to Pol II genes.

Importantly, gene ontology analysis on the intersected UBTF1/2 and Pol II bound regions revealed negative regulation of megakaryocyte differentiation, chromatin assembly and regulation of gene silencing, to be the biological processes most significantly enriched (Supplementary Fig 2D). The UBTF1/2-bound gene lists belonging to these ontology terms consisted mainly of chromatin remodeling factors and histone genes (Supplementary Tables 6-9). Thus, our data collectively along with the known role of UBTF1/2 in regulating rRNA gene transcription, suggest that UBTF1/2 is required for transcription of distinct classes of highly expressed genes involved in cellular proliferative growth including regulators of chromatin organization.

UBTF1/2 are enriched at a novel subset of Pol II genes in transformed human cells. To determine whether malignant transformation is associated with altered UBTF1/2 enrichment at Pol II genes, we compared UBTF1/2 enrichment in the HMECs (Fig 1B) to UBTF1/2 enrichment in the tumorigenic HMLER cells, an isogenic HMEC-derived cell line expressing the SV40 large-T, *TERT*, and an oncogenic allele of the *HRAS* gene (expressing *HRAS*^{V12G}) that forms tumors in nude mice (Elenbaas et al. 2001). We identified 11990 genomic regions with UBTF1/2 enrichment. We next intersected the UBTF1/2 ChIP-seq data in HMECs and HMLERs to identify common and unique regions of UBTF1/2 enrichment in HMLERs (Fig 2C & Supplementary Fig 2E&F) and identified 3687 UBTF1/2-bound genomic sequences common to both the primary and the tumorigenic cell lines, with genes encoding chromatin and nucleosome assembly being

among the most overrepresented (Supplementary Fig 2E, Supplementary Table 10). Thus the regulation of histone and chromatin remodeling genes is a fundamentally conserved function of UBTF1/2 in normal and transformed cells. Gene ontology analysis of the UBTF1/2-bound genomic regions unique to the tumorigenic HMLERs revealed biological processes involved in regulating cell cycle checkpoints, DNA damage checkpoints and DNA damage responses (Supplementary Fig 2F). Thus, in response to oncogenic transformation, UBTF1/2 targets an additional cohort of target genes whose products include mediators of ATR/ATM-regulated DNA damage response, signal transduction by TP53 and G1 to S transition of cell cycle (Supplementary Tables 11-13).

Identification of functional occupancy by UBTF1/2 at the histone gene clusters. To determine if UBTF1/2 binding to Pol II regulated genes has a functional effect on gene expression, we performed expression array analysis in control and *Ubt1/2* depleted NIH3T3 cells using two independent short interfering RNAs (siRNAs)(Supplementary Table 15). We intersected the ChIP-seq and microarray expression datasets to identify genes whose expression was significantly altered by *Ubt1/2* knockdown and were bound by UBTF1/2 within 500 bp of their TSSs. Gene ontology analysis, using the MetaCore pathways software (Thomson Reuters), of the biological processes that are enriched in the intersected list identified a significant overrepresentation of genes belonging to chromatin/nucleosome assembly and DNA packaging, including canonical histone genes and histone gene variants indicating that their transcription may be directly regulated by UBTF1/2 (Table 1). We then chose the histone gene clusters for further functional characterization in examining UBTF1/2's role in mediating Pol II transcription.

The five classes of genes encoding the canonical histone proteins (H2A, H2B, H3, H4 and H1) are arranged in repetitive units in one major and two smaller gene clusters (Marzluff et al. 2002) somewhat reminiscent of the rDNA repeats. Histone mRNAs are transcribed at a high rate as the cells enter S-phase in parallel with DNA replication (Marzluff et al. 2008). In addition, there are

replication-independent variant histone genes that are not located within the histone gene clusters and are constitutively transcribed throughout the cell cycle. These genes encode histone variants such as H1.0, H2A.Z, H2A.X, H3.3A and H3.3B, which are known to have distinct functions (Marzluff et al. 2008).

We detected robust peaks of UBTF1/2 enrichment at 33/45 canonical histone genes and 4/6 histone H1 genes within histone cluster 1, 4/10 genes within histone cluster 2 (Fig 3A&B) and also at the single histone H4 gene located on chromosome 12 in NIH3T3, but none at the small histone cluster 3. In addition, UBTF1/2 binding was detected at 6 histone gene variants, which are dispersed across the genome including *H2afy*, *H2afx*, *H2afz*, *H3f3a*, *H3f3b* and *H1f0* (Fig 3C). A similar enrichment of UBTF1/2 was observed at histone gene clusters and histone variants in HMEC (Supplementary Fig 3B&C and Supplementary Tables 16&17).

Using qChIP, we confirmed significant enrichment of UBTF1/2 binding at the canonical and histone variant genes compared to two regions that ChIP-seq data indicates are not bound by UBTF1/2 (the major satellite repeats and an amplicon specific to *Hist1h1a*, Fig 3D). These data suggest that histone genes are likely *bone fide* targets of UBTF1/2.

UBTF1/2 mediates histone gene expression. As *Ubt1/2* depleted NIH3T3 cells display a delay in G1-S progression (Supplementary Fig 3D), we examined the effect of *Ubt1/2* depletion on histone mRNA levels in sorted G1 and S populations from *siEgfp* control and *Ubt1/2* knockdown samples to control for any cell cycle bias. Histone H4 and *H2afx* mRNA levels were significantly reduced following *Ubt1/2* knockdown in the G0/G1 and in the S phase samples (Fig 4A), suggesting that the decrease in histone mRNA expression is a direct consequence of *Ubt1/2* knockdown rather than accumulation of cells in G1. Indeed, in the G0/G1 populations, mRNA levels of all canonical and histone variant mRNAs were significantly decreased following *Ubt1/2* depletion (Fig 4B). Thus, UBTF1/2 plays a permissive role in mediating mRNA expression of canonical and variants histone genes rather than functioning in the activation of canonical histone

gene expression in S-phase. Furthermore, consistent with UBTF1/2 regulating mRNA expression of histone genes, transient *Ubt1/2* knockdown significantly reduced the abundance of histone proteins as represented by H2A and H1 (Supplementary Fig 4A&B).

UBTF1/2 mediates Pol II recruitment and transcription of histone genes independent of chromatin modifications or nucleosome loading. To examine the mechanism underlying UBTF1/2-mediated regulation of histone gene expression, we performed sequential ChIP assays (ChIP-reChIP) experiments to investigate the overlap between UBTF1/2 and Pol II or Pol I at histone genes. The data showed that UBTF1/2 and Pol II simultaneously occupy histone genes (Supplementary Fig 4C). Consistent with the lack of overlap observed in UBTF1/2 and Pol I binding across the genome (Fig 2B), we observed no enrichment in Pol I occupancy at histone genes (Fig 4C, Supplementary Fig 4C). This eliminates the possibility of UBTF1/2 recruiting Pol I to histone gene loci.

To confirm that UBTF1/2 directly regulates transcription of histone genes via Pol II, we examined Pol II recruitment to histone genes in response to modulation of UBTF1/2 levels. To a large extent, the phosphorylation status of the C-terminal domain (CTD) of the largest subunit of Pol II defines the stage of transcription (Phatnani and Greenleaf 2006). Pol II is recruited into the PIC with a hypophosphorylated CTD, the CTD is phosphorylated on Serine 5 (Ser5) during initiation and promoter clearance and then on Serine 2 (Ser2) during elongation (Fuda et al. 2009). We observed significant reductions in total Pol II and Pol II (Ser5) loading at histone H2a, H4 and H1 genes in *Ubt1/2* depleted cells (Fig 4C), suggesting that UBTF1/2 is required for recruitment and initiation of Pol II transcription at histone genes. Interestingly, we also observed proportionally greater reduced occupancy of elongating Pol II (Ser2) at histone genes compared to the observed reduction in Pol II loading/ initiation following *Ubt1/2* knockdown. This suggests a role for UBTF1/2 in mediating Pol II elongation rates, consistent its ability to mediate

Pol I elongation (Stefanovsky et al. 2006).

Ubt1/2 knockdown did not correlate with any significant changes in chromatin modifications at histone H2a and H1 genes (Supplementary Fig 4D) indicating that UBTF1/2's role in regulating histone gene expression is downstream or independent of the establishment of the euchromatic environment within the histone gene clusters. Furthermore, we did not observed significant changes in the enrichment of histones H3, H4 or linker histone H1 loading at histone H2a and H1 genes following *Ubt1/2* knockdown (Supplementary Fig 4D). Thus, UBTF1/2 does not alter nucleosome loading at histone genes. To further examine the effect of UBTF1/2 binding on chromatin remodeling events at histone genes, we designed primers tiling the 340 bp region that spans the promoter, the TSS and the transcribed region of *Hist1h2ad* and performed qChIP (Fig 4D). UBTF1/2 binding at *Hist1h2ad* was approximately 10-15 fold higher at amplicons within the transcribed region compared to the promoter (Fig 4E). We then examined nucleosome positioning at *Hist1h2ad* by chromatin accessibility assay (CHART-PCR) (Rao et al. 2001). Micrococcal nuclease (MNase) accessibility measurements of control samples revealed the region spanning -194 to +15 bp of *Hist1h2ad* (amplicons P-1, P-2 and A) is highly sensitive to MNase digestion compared to the transcribed region (+32 to +144 bp, amplicons B and C) (Fig 4F), suggesting that it is depleted of a nucleosome as expected for a highly constitutively transcribed gene. Despite the fact that histone genes are present within an open chromatin context (Hovhannisyan et al. 2003; Medina et al. 2012), *Ubt1/2* knockdown led to a significant decrease in protection against MNase digestion at the promoter and also at the transcribed region (Fig 4F). This suggests that UBTF1/2 interacts directly with DNA as opposed to nucleosomes and its binding modulates DNA accessibility to promote Pol II binding independent of nucleosome occupancy. The data further suggest that UBTF1/2-wrapped chromatin (Stefanovsky et al. 2001) is a preferred template for Pol II than histone-wrapped chromatin.

UBTF2 is required for optimal histone gene expression. UBTF2 is inactive in regulating rDNA transcription compared with UBTF1 (Grummt 1999; Hannan et al. 1999; Stefanovsky and Moss 2008) and does not function in remodeling chromatin at the rDNA repeats in the absence of UBTF1 (Sanij et al. 2008). To address the relative contribution of UBTF1 and UBTF2 isoforms to Pol II histone gene expression, we performed replacement experiments with rat FLAG-*Ubt1* and FLAG-*Ubt2* cDNAs, which contain nucleic acid sequence differences in the region targeted by the murine-specific sir*Ubt1/2* (Supplementary Fig 5A) (Sanij et al. 2008) and performed qChIP assays using anti-FLAG antibodies. We found that both FLAG-rUBTF1 and FLAG-rUBTF2 can interact with histone genes independently of endogenous UBTF1 and UBTF2 (Supplementary Fig 5B). Next, we performed RNAi knockdown targeting *Ubt1* and *Ubt2* mRNAs or only *Ubt1* mRNA (we are unable to selectively target *Ubt2* by RNAi) (Fig 5A). While total UBTF1/2 enrichment was significantly reduced at histone genes in *Ubt1/2* depleted cells, *Ubt1* knockdown cells exhibited similar enrichment of total UBTF1/2 loading to control cells, indicating that UBTF2 is able to fully occupy histone genes independently of UBTF1 (Fig 5B). Moreover, while *Ubt1/2* knockdown significantly reduced histone H2a, H1 and H4 mRNA levels, *Ubt1* knockdown failed to reduce histone mRNA levels demonstrating that UBTF2 is sufficient to regulate histone gene expression in the absence of UBTF1 (Fig 5B). In direct contrast, at the rDNA loci, both *Ubt1/2* and *Ubt1* knockdown led to similar 50% reduction in 45S rRNA levels (Fig 5A). Thus, although UBTF2 is known to be inactive in Pol I transcription (Grummt 1999; Hannan et al. 1999; Stefanovsky and Moss 2008) and chromatin remodeling at rDNA (Sanij et al. 2008), these data demonstrate that UBTF2 functions to enhance Pol II gene expression.

***Ubt1/2* knockdown leads to DNA damage and genomic instability.** In eukaryotes, DNA damage and repair occurs in the context of chromatin and thus histones availability affects genome integrity and stability (Ye et al. 2003; Gunjan et al. 2005). Thus, the loss of histone gene

expression following *Ubt1/2* depletion might be associated with DNA damage and or genome instability. To investigate this possibility, we used the comet assay to detect DNA damage at the single cell level and found that *Ubt1/2* knockdown cells exhibited significantly longer comet tails than those of control cells, indicating that they accumulated DNA damage (Fig 6A).

The appearance of micronuclei is another characteristic marker of damaged chromosomes. Micronuclei are small nuclei separated from the main nucleus, containing chromosomes or chromosome fragments derived from mitotic spindle dysfunction (Fenech 2006). We performed a cytokinesis-block micronucleus (CBMN) assay to quantify micronuclei appearing after a single complete nuclear division. *Ubt1/2* knockdown cells displayed a significant increase in binucleated cells exhibiting micronuclei formation (Fig 6B&D). To examine whether the micronucleus phenotype observed following *Ubt1/2* depletion was simply caused by reductions in rDNA transcription and its subsequent consequences on nucleolar structure or function, independent of possible effects on Pol II transcription, we performed the CBMN assay following RNAi depletion of the Pol I-specific transcription factor *Rrn3* (Fig 6C&D). *RRN3* has no known functions outside nucleolar rDNA transcription. Knocking down *Ubt1/2*, *Ubt1* or *Rrn3* have similar consequences in reducing 45S rRNA levels (Fig 5A&6C). However, while knockdown of *Ubt1/2* led to a significant increase in micronuclei, *Rrn3* knockdown, and thus selective inhibition of rDNA transcription, failed to increase micronuclei numbers. Moreover, *Ubt1* knockdown only modestly increased the percentage of cells exhibiting micronuclei, consistent with the proposed model that UBTF2 plays a prominent role in regulating histone genes and other Pol II transcribed genes. These data reveal that the genomic instability following *Ubt1/2* knockdown is independent of its effects on rDNA transcription per se and is likely to be a direct consequence of reductions in Pol II gene expression, in particular histone genes. Further, immunofluorescence for UBTF1/2 combined with rDNA FISH analysis demonstrated that the micronuclei associated with *Ubt1/2* knockdown did not overlap with the rDNA signal indicating that genomic material in these micronuclei are not solely derived from rDNA-bearing

chromosomes (Supplementary Fig 6) and do not merely reflect genomic instability of the rDNA repeats but rather genome-wide chromosome instability.

Discussion:

UBTF1/2 couples the regulation of a subset of highly transcribed Pol I and Pol II genes. In this study we demonstrate a fundamental role for the Pol I-transcription factor UBTF1/2 in regulating highly transcribed Pol II-genes, including the histone gene clusters. Moreover, in transformed cells, we demonstrate that UBTF1/2 is enriched at an additional cohort of genes involved in DNA damage and repair including mediators of ATR/ATM-regulated DNA damage response, signal transduction by TP53 and G1 to S transition of cell cycle. Activation of the ATM/ATR-regulated DNA damage network following oncogenic stress is proposed to be a biological response to delay or protect against cancer (Bartkova et al. 2005) by pausing cells in S phase or G2 to allow appropriate repair of damaged DNA. It is tempting to speculate that this additional cohort of UBTF1/2 target genes represents a regulated response to oncogenic stress to couple the rate of rDNA transcription with DNA integrity and fidelity of chromatin assembly.

Thus collectively, the data indicates that UBTF1/2 binding is dynamic, context dependent and potentially associated with malignant transformation. While a number of Pol II transcription factors have been implicated in the regulation of Pol I, for example MYC, TP53 and RUNX2 (Hannan et al. 2013), our data provide the first specific *in vivo* example of a Pol I factor with dual functions in the regulation of Pol I and Pol II.

Interestingly, although ribosomal protein (RP) encoding genes, like histone genes, are also highly expressed genes (Castillo-Davis et al. 2002), they did not appear in the gene ontological analysis of genes bound by UBTF1/2 in NIH3T3 (Supplementary Fig 2A) nor did they change in mRNA levels upon *Ubt1/2* knockdown (Supplementary Table 15). In fact, UBTF1/2 ChIP-seq peaks in NIH3T3 cells were detected at only 5 RP genes. Thus, the data do not support a model in which

ribosome biogenesis is coordinated at the transcriptional level by UBTF1/2. Instead it is consistent with the prevailing view that in mammals, the major mode of coordinated regulation of ribosome biogenesis is achieved post-transcriptionally via specific conserved signaling networks including the PI3K/AKT/TOR and ERK pathways (White 2008; Chan et al. 2011; Hannan et al. 2011; Bywater et al. 2013; Hannan et al. 2013; Chauvin et al. 2014). This appears in contrast to lower organisms such as yeast where transcription of the rRNAs by Pol I and the RPs by Pol II is coordinated (Laferte et al. 2006; Berger et al. 2007). We note that in higher eukaryotes RPs have evolved extra-ribosomal functions, for example in regulating nucleolar stress and TP53 accumulation through the binding of certain RPs to MDM2 (Boulon et al. 2010; Deisenroth and Zhang 2010; Hein et al. 2013). The transcriptional uncoupling of rRNA and RPs synthesis in higher eukaryotes may allow for regulation of the extra-ribosomal functions of RPs by mechanisms and signaling pathways independent of those that modulate ribosome biogenesis.

Ubt1/2 are splice variants from a single transcript and their expression is dynamically regulated by over 90% during terminal differentiation and proliferation, which coincides with dramatic changes in rDNA transcription rate (Larson et al. 1993; Moss and Stefanovsky 2002; Sanij et al. 2008; Sanij and Hannan 2009; Poortinga et al. 2011; Bywater et al. 2012; Poortinga et al. 2014). Conversely, they are robustly upregulated under proliferative conditions (Moss and Stefanovsky 2002; Sanij and Hannan 2009; Bywater et al. 2012; Poortinga et al. 2014). Critically, our previous studies demonstrated that UBTF2 does not function in Pol I transcription raising the perplexing question about UBTF2 function *in vivo* (Sanij et al. 2008). In this study, we demonstrated that UBTF2 is sufficient in the absence of UBTF1 to modulate Pol II gene expression. Moreover, depletion of *Ubt1* alone had little effect on Pol II-dependent transcription of the histone genes but robustly repressed Pol I-dependent transcription of the rDNA (Fig 5). Together, these data suggest that UBTF1 functions predominantly in regulating Pol I while UBTF2 functions in regulating Pol II. Alternative splicing for the same gene to yield two distinct mRNA isoforms,

with specific roles in regulating Pol I (*Ubt1*) and Pol II (*Ubt2*) transcription, respectively, appears to be an ideal evolutionary mechanism to couple the regulation of highly transcribed genes required for fundamental process such as rRNA synthesis and chromatin assembly.

Recruitment of UBTF1/2 to open chromatin. The interphase genome is organized into self-interacting topological domains flanked by boundaries. These regions are remodeled by Pol II and topoisomerases activities creating supercoiling domains. Under-wound negatively supercoiled domains are transcriptionally active, enriched in “open” chromatin fibers, DNase I hypersensitive sites and TSSs (Naughton et al. 2013) reminiscent of chromatin structures bound by UBTF1/2 (Fig 1C&F, Fig 2A). Like other HMG proteins, UBTF1/2 has little intrinsic DNA binding specificity *in vitro*. One possibility is that UBTF1/2 may recognize specific topological features that are characteristic of specific DNA regions with very high constitutive transcriptional activity. Indeed, intersection of UBTF1/2 bound regions with DNA supercoiling data across 7 genomic loci that distinguish between negative supercoiled “under-wound” and positively supercoiled “over-wound” DNA (Naughton et al. 2013) demonstrates that ~83% of the overlapping regions represent under-wound DNA (data not shown). Thus UBTF1/2 may “mark” and stabilize regions of constitutively active “under-wound” DNA structures. In turn, this may facilitate rapid induction of mRNA synthesis during the cell cycle or in response to extracellular stimuli.

UBTF1/2 as a mediator of genomic stability. UBTF1/2 deficiency but not reductions in Pol I transcription *per se* resulted in genome wide instability in NIH3T3 cells (Fig 6). Almost certainly the role UBTF1/2 plays in maintaining high levels of canonical and non-canonical histones and other genes involved in chromatin assembly and DNA damage response contributes to the genomic instability upon *Ubt1/2* knockdown. This would be consistent with the observations that histone insufficiency during S-phase results in spontaneous DNA damage and genomic instability (Han et al. 1987; Myung et al. 2003; Ye et al. 2003; Prado and Aguilera 2005; Clemente-Ruiz and

Prado 2009) and that manipulation of core histone function or abundance causes defects in chromosome segregation (Meeks-Wagner and Hartwell 1986; Pinto and Winston 2000).

Thus, through its ability to coordinately regulate the expression of some of the most highly transcribed Pol I and Pol II genes in the genome including the histones clusters and rDNA repeats, our data demonstrate a previously unrecognised but fundamental role for the two isoforms of UBTF1/2 in coupling Pol I transcription and the cell's capacity to grow with the fidelity of chromatin assembly and DNA repair. Intriguingly, it has recently been demonstrated that inhibition of Pol I is an effective mechanism to treat hematological cancers with drugs targeting Pol I transcription now in clinical trials (Bywater et al. 2012; Bywater et al. 2013). We propose that the unique characteristics of UBTF1/2 to target a subset of Pol II genes critical for cell proliferation, in addition to Pol I, make it a highly attractive target for cancer therapy.

Methods

ChIP and ChIP-seq analysis

Standard ChIP was carried out as described (Poortinga et al. 2004; Sanij et al. 2008). Antibodies and primer sequences are listed in Supplementary Data. ChIP-reChIP experiments were carried out as described in (Mendoza-Parra 2012). Anti-UBTF1/2 sera were cross-linked to protein A beads using disuccinimidyl suberate. UBTF1/2 ChIP products were eluted in (1%SDS, 0.1 M NaHCO₃), diluted 1:10 and subjected to a second ChIP using antibodies directed against Pol I, Pol II or RS.

For ChIP-seq of UBTF1/2 ChIP DNA and input gDNA from NIH3T3, libraries from two biological replicates were prepared for the Illumina Genome Analyzer II platform at Peter MacCallum Cancer Centre. ChIP-seq libraries of histone modifications and Pol II in NIH3T3, libraries were also prepared for the Illumina Genome Analyzer II platform. Sequencing reads were mapped to the mouse genome database mm9 using Burrows-Wheeler Aligner (BWA)(Li

and Durbin 2009). ChIP-seq of UBTF1/2 ChIP, Pol I and gDNA samples in HMEC were performed using the Illumina Genome Analyzer II platform, while ChIP-seq of UBTF1/2 ChIP and gDNA samples in HMLER cells was performed using Illumina HiSeq 1000. Sequences were mapped to the human genome (hg19). MACS 1.4 (Model-based Analysis of ChIP-seq (Zhang et al. 2008)) was used to call peaks over input DNA and the fold enrichment for each peak was calculated using the R package (Gentleman et al. 2004). Only peak regions with a false discovery rate (FDR) below 10% and p-value below 0.00001 were selected and annotated with RefSeq annotation using the R package ChIPpeakAnno (Zhu et al. 2009). The distribution of UBTF1/2 peaks relative to RefSeq genes as well as the comparison of ChIP-seq datasets was performed using the Sole-Search software tool (Blahnik et al. 2010). The DNase I hypersensitivity profile in NIH3T3 cells were obtained from the ENCODE Broad UCSC Genome Browser (<https://www.encodeproject.org/>, Accession: ENCSR000CNS).

Gene expression analysis

We performed microarray analysis (Affymetrix, Mouse Exon ST 1.0 arrays) on three biological replicates of *Ubt1/2* knockdown samples using two independent siRNA oligos, a non-silencing *siEgfp* or Mock transfected NIH3T3 samples (Supplementary Table 18). The arrays were normalized using Robust Multi-Array Average expression measure (RMA) (Irizarry et al. 2003) and differential expression was then determined using a linear model and the Limma package (<http://bioinf.wehi.edu.au/limma/>) (Smyth 2005). Moderated t-statistics were generated and significance was assessed using log fold change and FDR adjusted P-value.

Correlation analysis of UBTF1/2 ChIP-seq and microarray expression data

Expression data of exponentially growing NIH3T3 was obtained using Affymetrix, Mouse Exon ST 1.0 arrays (Mock samples from previous section). Triplicates were normalized using the RMA method (Irizarry et al. 2003) and expression values for all genes, genes with significant UBTF1/2

peaks <2kb from their TSS, or genes with no UBTF1/2 binding at their TSS, were obtained. Statistical significance between the groups was assessed using t-tests.

Cell culture, siRNA knockdown and RNA expression analysis

NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C. HMECs and HMLER cells were cultured in HuMEC ready medium (12752010, life technologies). Dharmafect 2 reagent (Dharmacon) was used to transfect siRNA at 40 nM. The tetracycline-regulated rat *Ubt1* and *Ubt2* MEF-3T3 cell lines were established as reported in (Sanij et al. 2008). Cells were lysed and RNA was extracted and first-strand cDNA was synthesized using random hexamer primer and Superscript III (Invitrogen). Quantitative PCR (qPCR) was performed in duplicate using the FAST SYBR Green dye on the StepOnePlus™ real-time PCR system (Applied Biosystems). Primer sequences are listed in (Supplementary Table 20).

Western blotting and immunofluorescence

20-50 mg of whole cell lysates were resolved by SDS-PAGE, electrophoretically transferred onto PVDF membranes (Millipore) and analyzed using enhanced chemiluminescence (ECL) detection (GE Healthcare). For IF, cells were fixed in a 4% paraformaldehyde (10 min at room temperature), washed with PBS and blocked with (5% skim milk powder, 0.5% chicken serum in PBS and 0.3% Triton X-100) for 30 min. Cells were sequentially incubated with anti-UBTF1/2 sera and Alexa Fluor 594 anti-rabbit secondary antibody (Molecular Probes). Stained cells were fixed again with 4% formaldehyde, counterstained with DAPI in Vectashield mounting media (Vector Labs). Images were acquired on an Olympus BX-51 microscope equipped with a Spot RT camera (model 25.4), using the UPlanAPO 40X, NA 1.2 water immersion objective and the Spot Advanced software, version 4.6.4.3. Settings for adjusting the image after acquisition (i.e. gamma adjust and background subtract settings) were identical for all images. Detailed FISH

protocol is provided in Supplementary Methods.

COMET assay

Cells collected and processed as described in manufacture's protocol (Trevigen, Comet Assay 4250-050-K). Images were acquired on an Olympus BX-51 microscope using the Olympus UPlanAPO 20X, NA 1.2 water immersion objective as described above. Quantitation was performed using MetaMorph (Molecular Devices, Sunnydale, CA, USA).

CHART-PCR assay

Cells were collected and mononucleosome preparations were performed using EpiScope nucleosome preparation kit (#5333) (Takara). Briefly, nuclei were isolated from control and UBTF1/2 depleted cells and incubated at 37°C for 30 min with MNase. A control without MNase was included for each sample. gDNA was then isolated and subjected to qPCR using FAST SYBR Green dye on the StepOnePlus™ real-time PCR system (Applied Biosystems). The relative level of MNase-resistance was calculated after normalization to mock-digested DNA.

Data access

Raw ChIP-seq data is available through the NCBI Sequence Read Archive (SRA, <http://www.ncbi.nlm.nih.gov/sra/>) Study accession number (SRP039369). Processed ChIP-seq data and Microarray Expression Data have been submitted to the Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) Study accession number (GSE63255) and (GSE55461), respectively.

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Disclosure declaration

We have no conflict of interest to declare.

Figure legends

Fig 1. ChIP-seq analysis of UBTF1/2. Distribution of UBTF1/2 binding sites in NIH3T3 (**A**) and HMEC (**B**) with respect to RefSeq genes as determined by Sole-Search. (**C**) Average UBTF1/2 ChIP-seq enrichment profiles at all unique TSSs and TTSs in the mouse genome ± 5 kb. (**D**) qChIP analysis of UBTF1/2 binding in NIH3T3 cells to ChIP-seq genomic regions that resides within the indicated genes. The % of DNA immunoprecipitated with anti-UBTF1/2 or rabbit serum (RS) antibodies was calculated relative to the unprecipitated input control. ($n=3-7$) Ave $\pm$ s.e.m, * p -value < 0.05 , ** p -value < 0.01 compared to corresponding RS samples. Amplicons at the enhancer (ENH) and intergenic spacer (IGS) of rDNA were used as a positive and negative control for UBTF1/2 binding, respectively. Primers to core histone genes recognize multiple genes within each class (Supplementary Table 19). (**E**) Venn diagram indicating the overlap of UBTF1/2-bound genes between the NIH3T3 and HMEC cell lines. (**F**) Density histograms of gene expression levels in exponentially growing NIH3T3 cells for all genes, genes with significant UBTF1/2 ChIP-seq peaks < 2 kb from their TSS, or genes with no UBTF1/2 binding at their TSS. Statistical significance between groups was assessed using t-tests.

Fig 2. UBTF1/2 binding correlates with markers of open active chromatin. (**A**) Venn diagram indicating overlap of UBTF1/2 binding sites with various chromatin marks, DNase I HS and Pol

II binding in NIH3T3. (C) Venn diagram indicating the overlap of UBTF1/2 and Pol I (POLR1A) binding sites in NIH3T3 and HMEC. (E) Venn diagram indicating the overlap of UBTF1/2 binding sites between HMEC and HMLER cell lines.

Fig 3. UBTF1/2 binds histone genes. (A) IGV (Integrated Genome Viewer) screenshots of mapped reads from UBTF1/2 ChIP and input gDNA at mouse histone gene cluster 1, cluster 2 (B) and histone variant genes *H2afx* and *H3f3a* (C) in NIH3T3 cells. (D) qChIP analysis of UBTF1/2 binding to canonical and variant histone genes in NIH3T3 cells. qChIPs were performed as described in Fig 1D. ($n=4$) Ave $\pm$ s.e.m, * p -value < 0.05 , ** p -value < 0.01 compared to corresponding RS samples. Amplicons at *Hist1h1a* and major satellite repeats were used as a negative control for UBTF1/2 binding.

Fig 4. UBTF1/2 regulates histone gene expression by mediating Pol II recruitment. (A) NIH3T3 cells were transfected with *sirEgfp* or *sirUbt1/2#1* for 48h then collected and incubated with 5mM Vybrant DyeCycle™ Violet stain (Life Technologies). Cells were then analysed using BD FACSAria and G1 and S phases populations were sorted based on DNA content. Total RNA was extracted and *Ubt1/2*, Histone H4 and *H2afx* mRNA levels were determined by reverse transcription qPCR. mRNA levels were normalized to beta-2-microglobulin (*B2m*) mRNA and expressed as fold change relative to *sirEgfp*/G0/G1, ($n=4$). (B) Total RNA samples from G1 population of NIH3T3 cells transfected with *sirEgfp* or *sirUbt1/2#1* as in A were analysed by reverse transcription qPCR for mRNA expression of various canonical and variant histone genes as indicated, ($n=4$). (C) UBTF1/2 mediates Pol II recruitment, initiation and elongation at histone genes. qChIP analysis of the histone H2a, H4 and H1 genes in *sirEgfp* or *sirUbt1/2#1* transfected NIH3T3 cells using antibodies against Pol I ($n=2$), total Pol II ($n=7$), phospho Pol II-Ser5 ($n=4$) or phospho Pol II-Ser2 ($n=4$). The % of DNA immunoprecipitated with the indicated antibodies or RS was calculated relative to the unprecipitated input control. % of DNA of RS controls were

subtracted. **(D)** *Ubt1/2* knockdown leads to increased DNA accessibility at *Hist1h2ad*. Screenshots of IGV with the mapped reads from UBTF1/2 ChIP and input gDNA in NIH3T3 to *Hist1h2ad*. Primers used for qPCR are indicated (Supplementary Table 21). **(E)** qChIP analysis of UBTF1/2 binding at *Hist1h2ad* in *sirEgfp* or *sirUbt1/2#1* transfected NIH3T3 cells. qChIPs were performed as described in Fig 4C, ($n=3$). **(F)** Chromatin remodeling of the *Hist1h2ad* by UBTF1/2. Nuclei from NIH3T3 cells transfected with either *sirEgfp* or *sirUbt1/2#1* for 48h were incubated with or without MNase. Extracted gDNA was subjected to qPCR using primers outlined in D. MNase accessibility was expressed as a percentage of undigested gDNA sample, ($n=3$). In all graphs **(A-F)**, error bars represent Ave $\pm$ s.e.m, * p -value < 0.05 , ** p -value < 0.01 , *** p -value < 0.001 compared to corresponding *sirEgfp* samples.

Fig 5. The UBTF2 isoform mediates histone gene expression. **(A)** Total protein lysates from NIH3T3 cells transfected with *sirEgfp*, *sirUbt1/2#1*, *sirUbt1/2#2* or *sirUbt1* for 48h were analysed by western blotting (top panel). RNA was also extracted and 45S rRNA precursor levels were determined by reverse transcription qPCR using primers to the 5' external transcribed region (ETS) (Supplementary Table 20). 45S rRNA levels were normalized to *Gapdh* mRNA and expressed as fold change relative to *sirEgfp* control ($n=3$) Ave $\pm$ s.e.m. * p -value < 0.05 (bottom panel). **(B)** Top panel, qChIP analysis of UBTF1/2 binding to histone H2a, H1 and H4 genes in NIH3T3 cells transfected with either *sirEgfp*, *sirUbt1/2#1* or *sirUbt1* for 48h ($n=3$) Ave $\pm$ s.e.m, * p -value < 0.05 , compared to *sirEgfp* sample. qChIPs were performed as described in Fig 4C. Bottom panel, G1 populations of NIH3T3 cells transfected as above were sorted as described in Fig 4A and total RNA was extracted. Histone H2a, H1 and H4 mRNA levels were determined by reverse transcription qPCR. mRNA levels were normalized to *B2m* mRNA and expressed as fold change relative to *sirEgfp* control, ($n=4$) Ave $\pm$ s.e.m, * p -value < 0.05 relative to corresponding *sirEgfp* samples.

Fig 6. *Ubt1/2* knockdown leads to DNA damage and genomic instability. **(A)** NIH3T3 cells transfected with *siEgfp* or *siUbt1/2#1* for 48h and DNA damage was measured by comet assay. Representative images of SYBR Green stained DNA of *siEgfp* control cells, showing undamaged and supercoiled DNA remaining within the nuclear membrane while in *siUbt1/2#1* cells denatured DNA fragments migrate out from the nucleus in a comet tail. The tail length for approximately 50 nuclei for each sample from two independent experiments was measured using metamorph software. The graph on the right panel represents Ave $\pm$ s.e.m, **p*-value < 0.05 compared to *siEgfp* control. **(B)** *Ubt1/2* knockdown leads to abnormal mitosis as measured by the CBMN assay. NIH3T3 cells transfected with *siEgfp* or *siUbt1/2#1* were incubated for 24h then Cytochalasin B, an inhibitor cytokinesis was added at 3 μ g/ml for further 24h. DAPI staining was then performed and percentages of binucleated cells exhibiting micronuclei were scored (yellow arrows) (*n*=3) Ave $\pm$ s.e.m, **p* < 0.05. **(C)** 48hr after transfecting NIH3T3 cells with *siEgfp*, *siUbt1/2#1* or *siRrn3*, total RNA was extracted and *Ubt1/2* mRNA, *Rrn3* mRNA and 45S rRNA precursor levels were determined by reverse transcription qPCR. mRNA levels were normalized to *B2m* mRNA and expressed as fold change relative to *siEgfp* control (*n*=3) Ave $\pm$ s.e.m. *** *p*-value < 0.001, ***p*-value < 0.01, **p*-value < 0.05 compared to corresponding *siEgfp* controls. **(D)** NIH3T3 cells were transfected with siRNA oligos as indicated and the CBMN assay was performed as described in B. (*n*=3) Approximately 100 cells were counted in each experiment and percentages of binucleated cells exhibiting micronuclei were scored, Ave $\pm$ s.e.m. **p*-value < 0.05 compared to *siEgfp* control.

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Figure 1

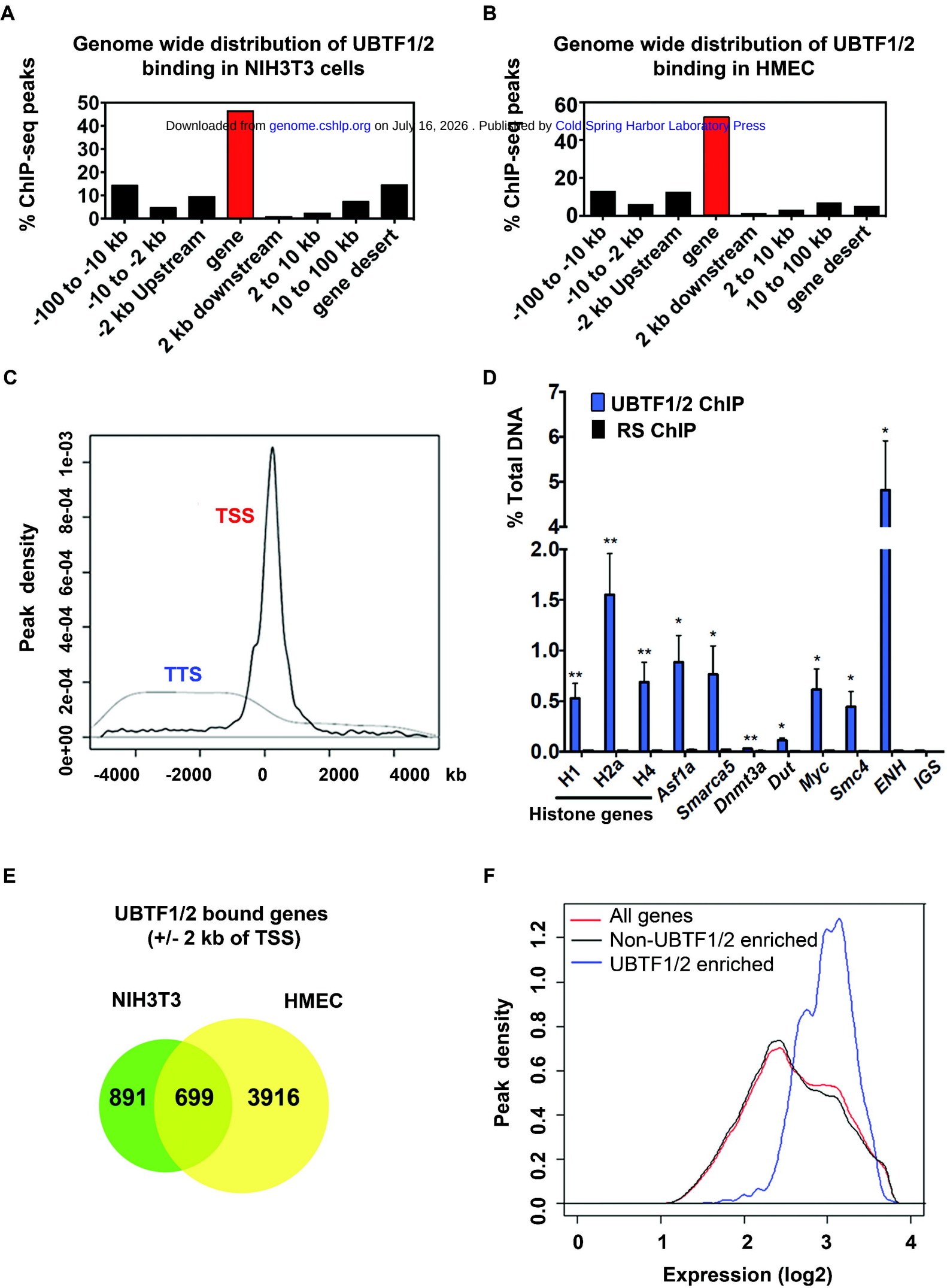
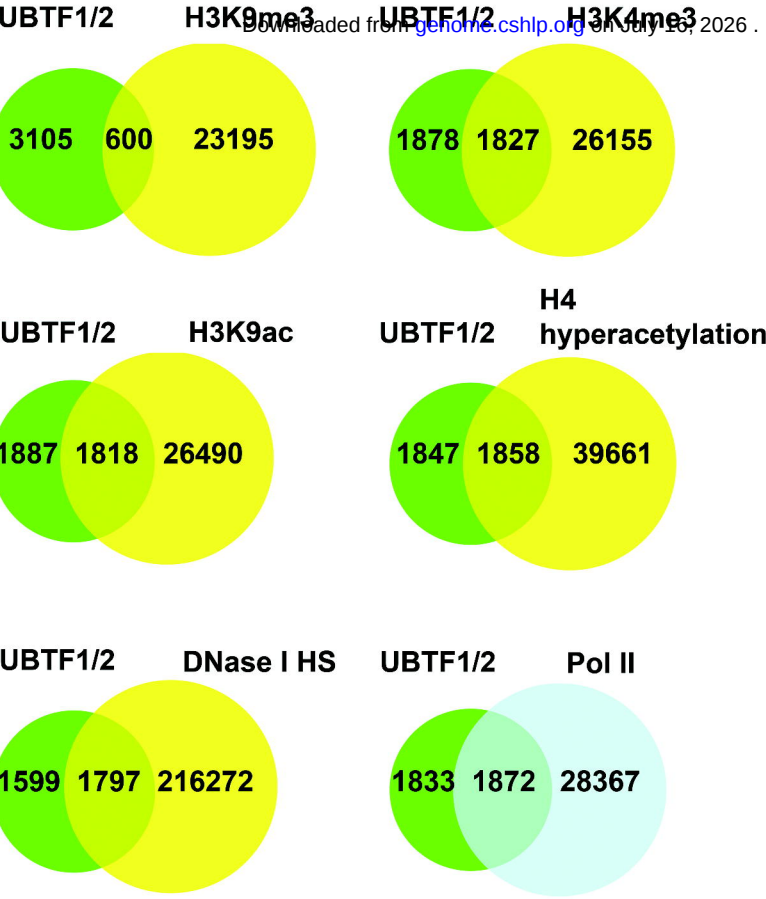
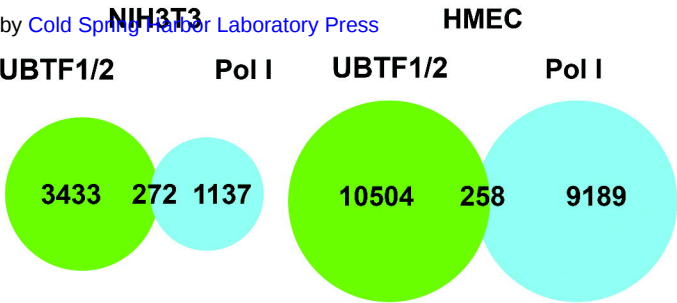


Figure 2

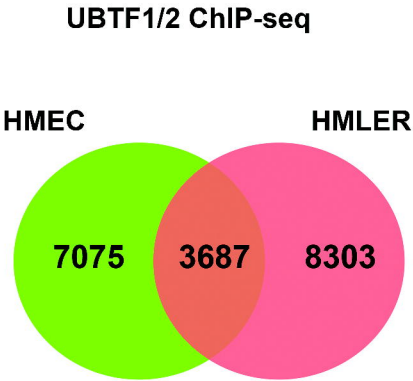
A



B



C



A

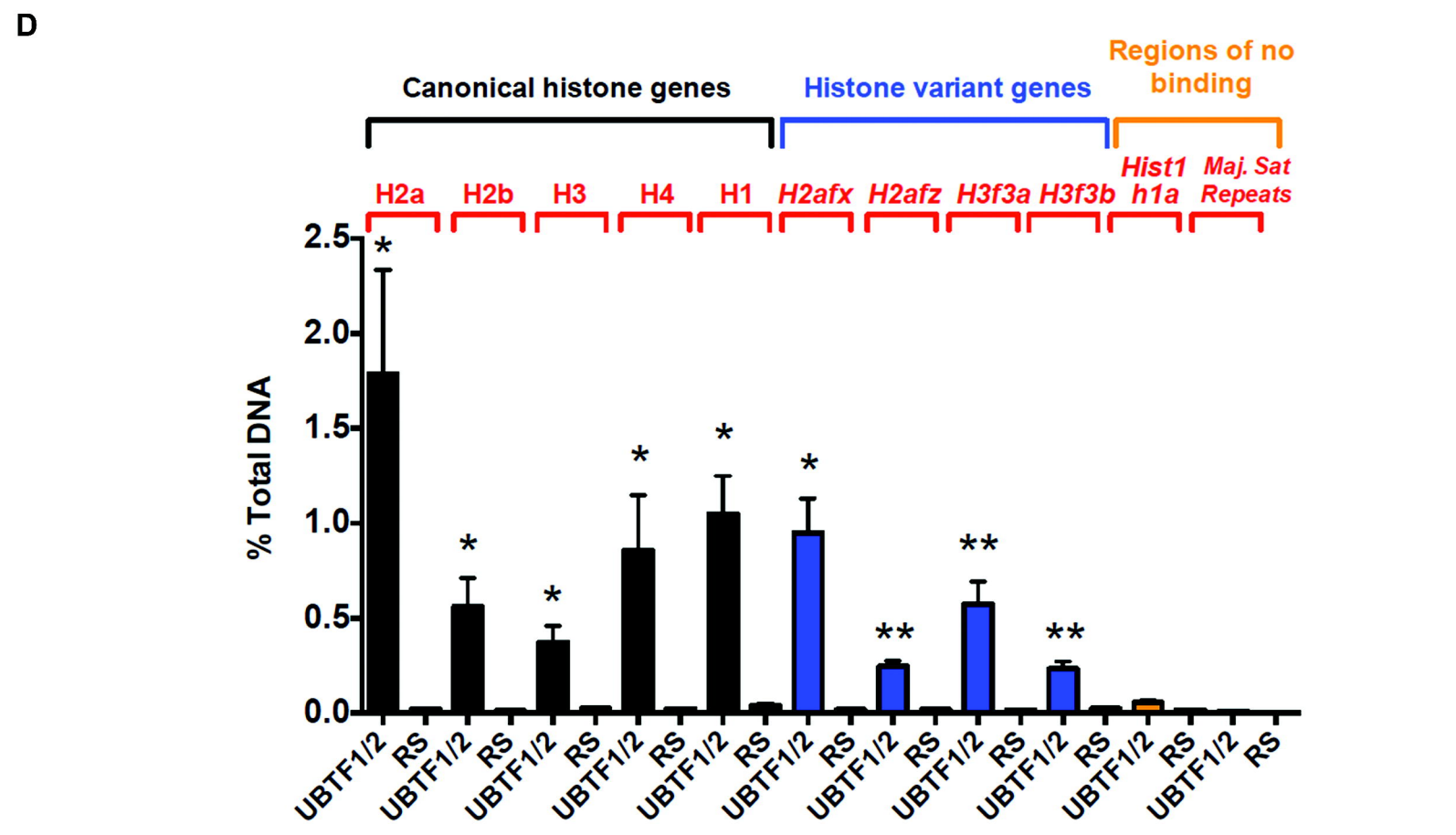


Figure 4

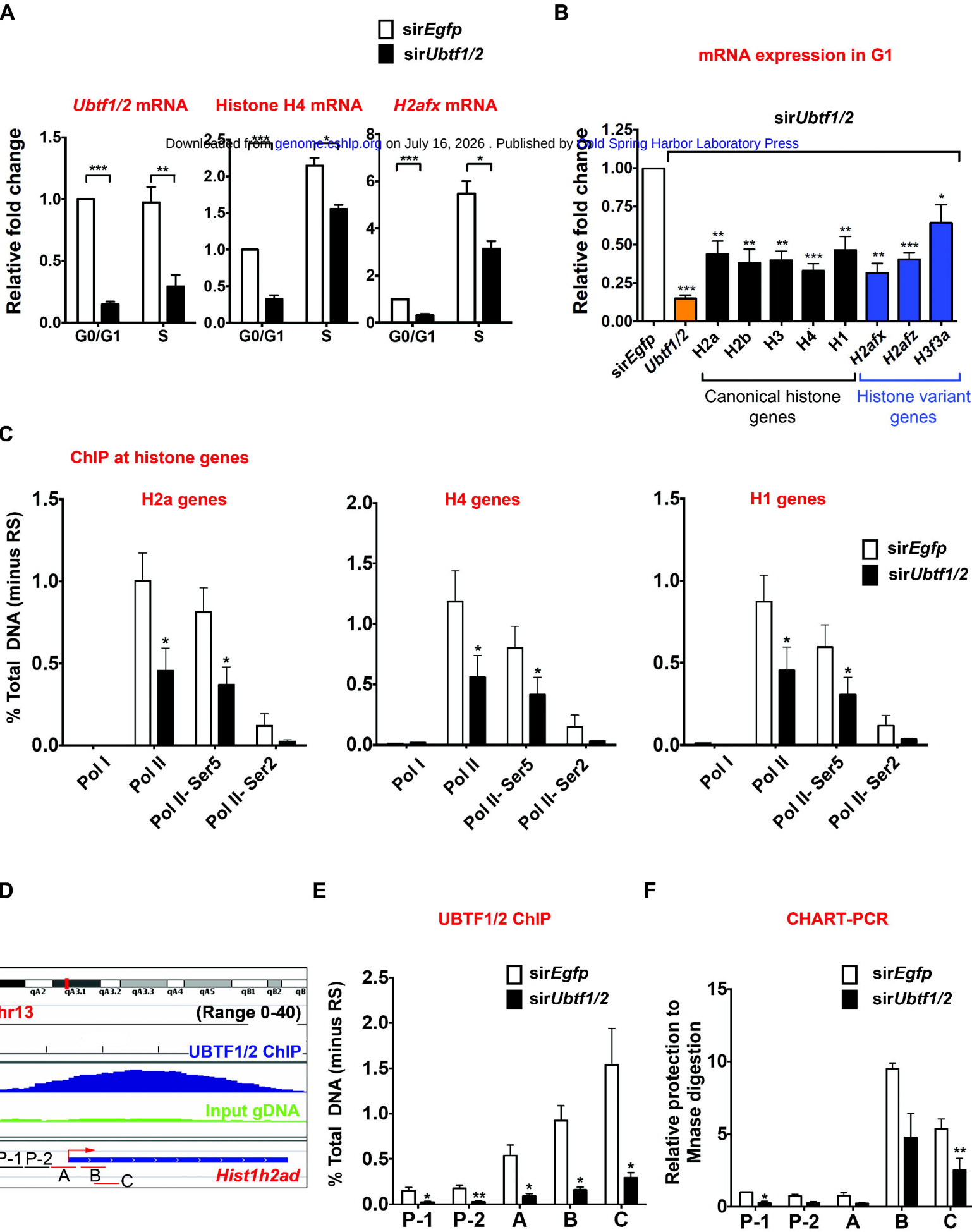


Figure 5

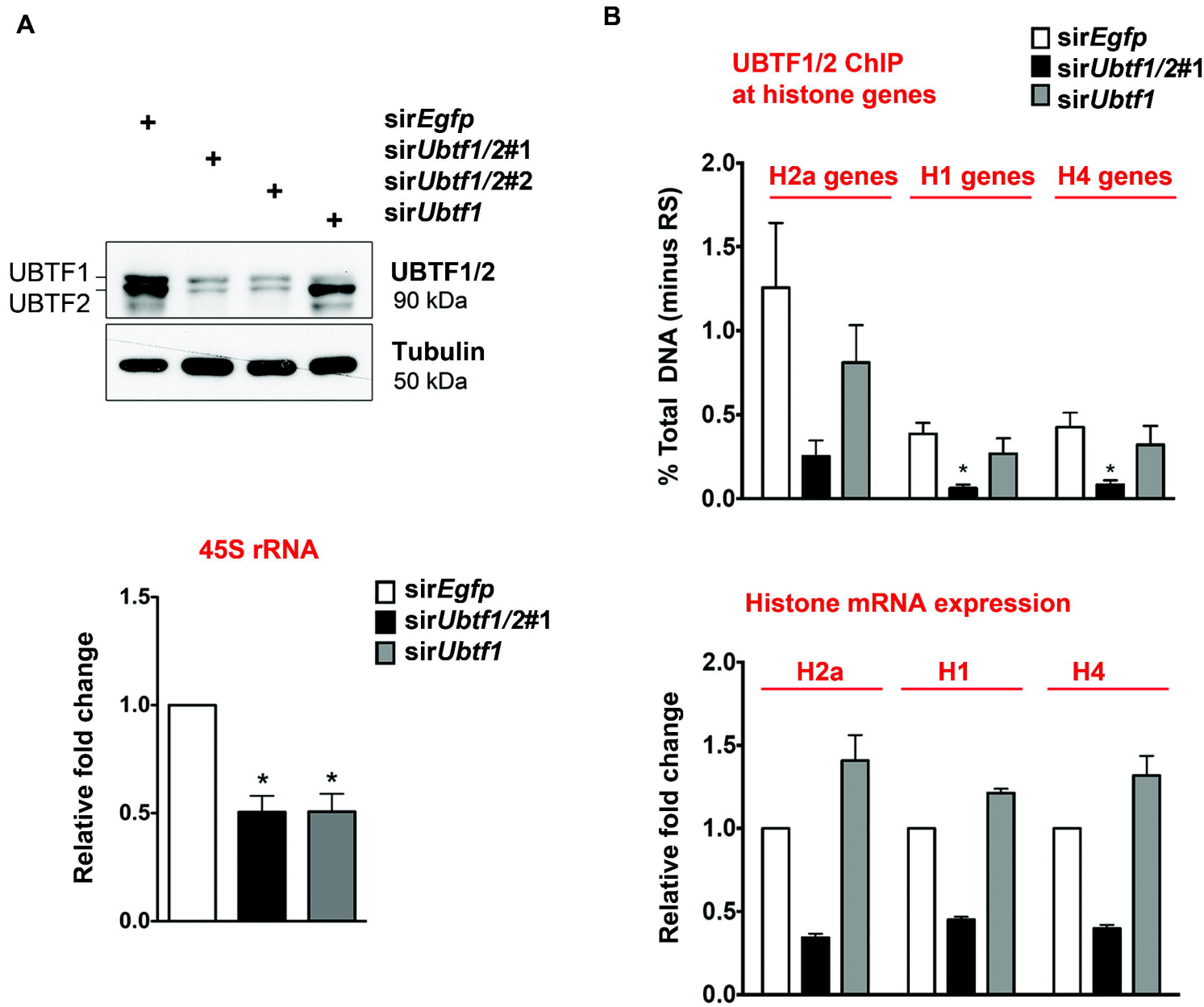


Figure 6

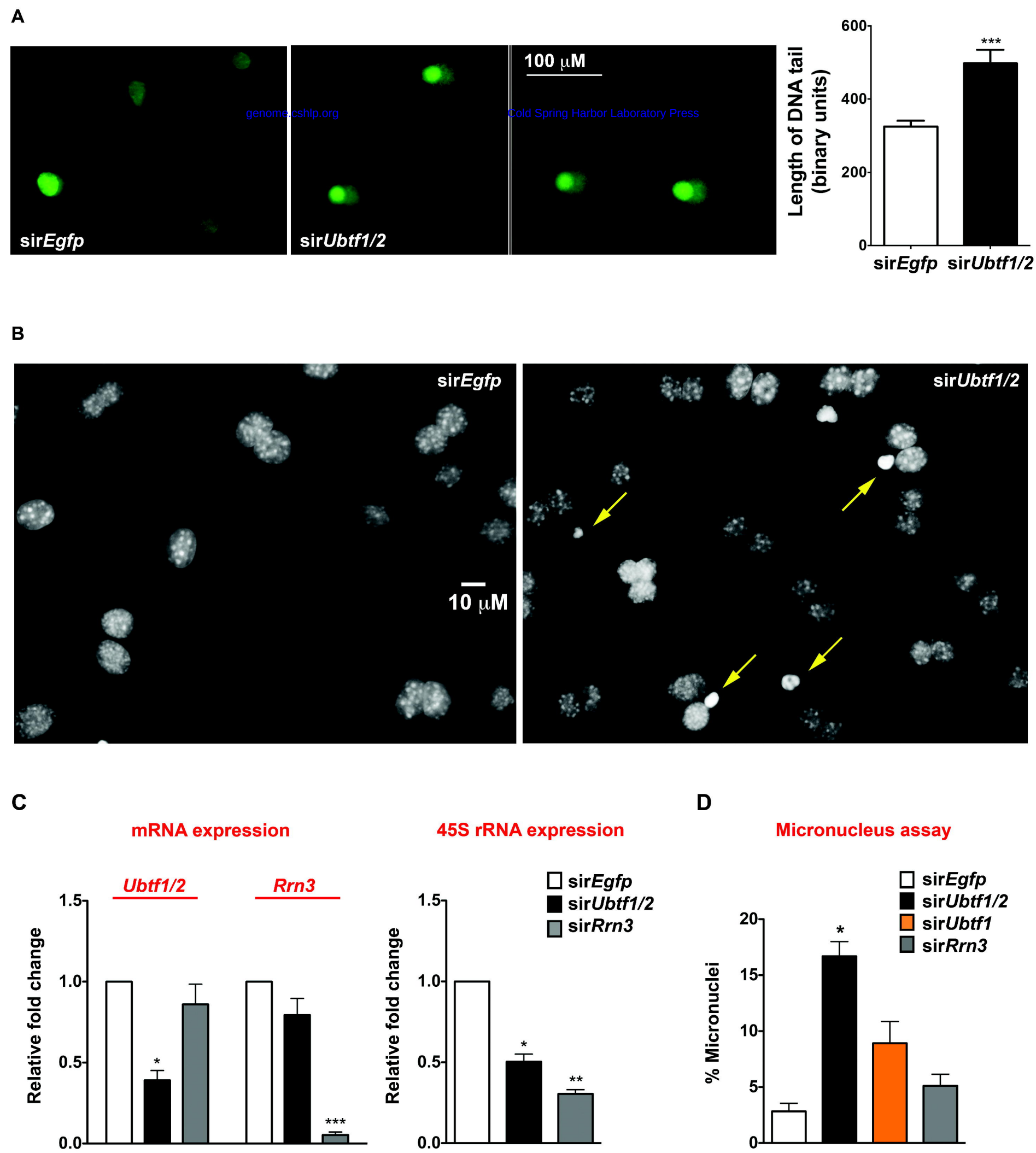


Table 1: MetaCore ontology analysis of UBTF1/2-bound genes within 500 bp of their TSSs that are differentially expressed following *Ubt1/2* knockdown in NIH3T3 cells. Only the most significantly enriched gene ontologies are listed. p-value denotes the significance of the number of genes from the intersected list (In Data) when compared to the total number of genes in the gene ontology classification (Total).

Enrichment analysis report			Common	
Enrichment by GO processes				
#	Processes	Total	p-value	In Data
1	Chromatin assembly	130	3.185E-13	14
2	Enzyme linked receptor protein signaling pathway	820	3.217E-13	29
3	DNA packaging	176	1.397E-12	15
4	Chromatin assembly or disassembly	146	1.599E-12	14
5	Protein-DNA complex assembly	146	1.599E-12	14
6	Nucleosome assembly	124	3.266E-12	13
7	Protein-DNA complex subunit organization	154	3.332E-12	14
8	System development	3997	4.532E-12	64
9	Multicellular organismal development	4704	7.630E-12	70
10	DNA conformation changes	205	1.270E-11	15