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1 **Myeloid lncRNA *LOUP* Mediates Opposing Regulatory Effects of RUNX1 and RUNX1-ETO in**
2 **t(8;21) AML**

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35 KEY POINTS

- 36 • lncRNA *LOUP* coordinates with RUNX1 to induces *PU.1* long-range transcription, conferring
37 myeloid differentiation and inhibiting cell growth.
- 38 • RUNX1-ETO limits chromatin accessibility at the *LOUP* locus, causing inhibition of *LOUP* and
39 *PU.1* expression in t(8;21) AML.

41 KEYWORDS

42 Chromatin structure; enhancer; promoter; lncRNA; long-range transcription; single-cell sequencing; cell
43 type-specific gene induction; transcription factor fusion; t(8;21) AML; myeloid differentiation

45 ABSTRACT

46 The mechanism underlying cell type-specific gene induction conferred by ubiquitous transcription
47 factors as well as disruptions caused by their chimeric derivatives in leukemia is not well understood.
48 Here we investigate whether RNAs coordinate with transcription factors to drive myeloid gene
49 transcription. In an integrated genome-wide approach surveying for gene loci exhibiting concurrent
50 RNA- and DNA-interactions with the broadly expressed transcription factor RUNX1, we identified the
51 long noncoding RNA *LOUP*. This myeloid-specific and polyadenylated lncRNA induces myeloid
52 differentiation and inhibits cell growth, acting as a transcriptional inducer of the myeloid master
53 regulator *PU.1*. Mechanistically, *LOUP* recruits RUNX1 to both the *PU.1* enhancer and the promoter,
54 leading to the formation of an active chromatin loop. In t(8;21) acute myeloid leukemia, wherein RUNX1
55 is fused to ETO, the resulting oncogenic fusion protein RUNX1-ETO limits chromatin accessibility at the
56 *LOUP* locus, causing inhibition of *LOUP* and *PU.1* expression. These findings highlight the important
57 role of the interplay between cell type-specific RNAs and transcription factors as well as their oncogenic
58 derivatives in modulating lineage-gene activation and raise the possibility that RNA regulators of
59 transcription factors represent alternative targets for therapeutic development.

61 INTRODUCTION

62 Lineage-control genes that dictate cellular identities are often expressed in dynamic and
63 hierarchical patterns.¹⁻³ Disturbance of these established normal patterns results in anomalies.⁴ In the
64 blood system, the ETS-family transcription factor PU.1 (also known as Spi-1) is essential for myeloid
65 differentiation. *PU.1* is silent in most tissues and cell types but expressed at highest levels in myeloid
66 cells including granulocytes and monocytes.⁵ Downregulation of *PU.1* impairs myeloid cell
67 differentiation leading to acute myeloid leukemia (AML).^{6,7} *PU.1* is a major downstream transcriptional

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68 target of Runt-related transcription factor 1 (RUNX1) that is expressed in many different cell types and
69 plays diverse biological roles in hematopoiesis, development of neurons, hair follicles, and skin.⁸⁻¹² In
70 AML with t(8;21) chromosomal translocation, a portion of *RUNX1* containing the Runt DNA binding
71 domain is fused to *ETO*, giving rise to the oncogenic transcription factor fusion RUNX1-ETO.^{13,14}
72 Previously, we have reported that RUNX1-ETO inhibits *PU.1* expression¹⁵ but the mechanism
73 underlying this transcriptional inhibition remains to be determined. In general, how broadly expressed
74 transcription factors, such as RUNX1, modulate cell type- and gene-specific induction and how their
75 chimeric derivatives disrupt this normal regulation in leukemia are poorly understood.

76
77 Transcription of many cell type-specific genes are induced by enhancer elements, which are
78 located at variable distances from gene targets.^{16,17} For instance, *PU.1* transcription is induced by the
79 formation of a specific chromatin loop resulting from the interaction between the upstream regulatory
80 element (URE) (-17 kb in human and -14 kb in mouse) and the proximal promoter region (PrPr).¹⁸⁻²⁰
81 Interestingly, abrogation of RUNX1-binding motifs at the URE reduces URE-PrPr interaction, resulting
82 in decreased *PU.1* expression in myeloid cells.^{8,15} Because RUNX1 is broadly expressed, it remains
83 unclear how this transcription factor modulates chromatin structure in such a gene- and cell type-
84 specific manner.

85
86 With advances in whole transcriptome sequencing over the last decade, thousands of noncoding
87 RNAs (ncRNA) have been unveiled.²¹ Arbitrarily defined as ncRNAs having at least 200 nucleotides in
88 length, long noncoding RNAs (lncRNA) are implicated to display tissue-specific expression patterns^{22,23}
89 and might undergo post-transcriptional processing such as splicing and polyadenylation.²⁴ Through
90 interactions with DNAs, proteins and other RNAs, lncRNAs regulate fundamental cellular processes
91 including transcription, RNA stability, and DNA methylation.²⁴⁻²⁶ To date, only a few lncRNAs have been
92 precisely mapped and functionally defined,²³ leaving most lncRNAs poorly annotated and largely
93 unexplored.

94
95 In this study, we identified a myeloid-specific lncRNA termed “Long noncoding RNA Originating
96 from the URE of P*U.1*”, or *LOUP*, from an integrated genome-wide approach aimed at screening for
97 gene loci exhibiting concurrent RNA- and DNA-interactions with RUNX1. We demonstrated that *LOUP*
98 induces *PU.1* expression, conferring myeloid differentiation, and inhibiting cell growth. *LOUP* serves as
99 a central hub in opposing regulation by RUNX1 and its derived oncogenic fusion, RUNX1-ETO. Our
100 findings provide a model explaining how a lineage gene is activated in normal myeloid development
101 and dysregulated in leukemia.

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103 **METHODS**

104 **Cell lines and Cell Culture**

105 U937, HL-60, K562, HEK293T, RAW 264.7, NB4, Jurkat, Kasumi-1 and THP-1 cells were obtained
106 from the American Type Culture Collection (ATCC). U937, HL-60, NB4, Jurkat, and K562 cells were
107 cultured in full RPMI-1640 medium (supplemented with 10% (vol/vol) fetal bovine serum (FBS; Cellgro)
108 and 1% penicillin-streptomycin). Kasumi-1 cells were cultured in the same medium plus 20% (vol/vol)
109 FBS. THP-1 cells were cultured in full RPMI-1640 medium supplemented with 2-mercaptoethanol to a
110 final concentration of 0.05 mM. HEK293T and RAW 264.7 cells were cultured in DMEM supplemented
111 with 10% (vol/vol) FBS and 1% penicillin-streptomycin. All cells were grown at 37°C in 5% (vol/vol) CO₂
112 and humidified incubators.

113

114 **AML patient sample collection**

115 Bone Marrow (BM) samples were obtained from newly diagnosed AML patients at the Tor Vergata
116 University Hospital, Rome with informed consent. Diagnoses were performed according to “The 2016
117 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia”.²⁷
118 Bone marrow mononuclear cells (BM-MNCs) were isolated by Ficoll gradient centrifugation using
119 Lympholyte-H (Cedarlane), according to the manufacturer’s instructions.

120

121 Methods for assaying interactions of RNA, DNA, and protein with chromatin, chromatin structure and
122 gene expression manipulation as well as bioinformatic analyses are in supplemental methods.

123

124 **RESULTS**

125 **Identification of RUNX1-interacting RNAs at myeloid gene loci**

126 We started out by performing a transcriptome-wide survey for RUNX1-interacting RNAs in the
127 monocytic cell line THP-1 using formaldehyde RNA immunoprecipitation sequencing (RIP-seq).^{28,29}
128 RUNX1-interacting RNAs were captured by anti-RUNX1 antibody (Figures S1A-C) and sequenced by
129 paired-end massively parallel sequencing. By annotating 14,067 high-confident RUNX1-RIP peaks to
130 the GRCh38.p12 gene catalog,³⁰ we identified 5,774 gene loci carrying at least one of these peaks
131 (Figure S1D, left). Most of the peaks were detected within transcript bodies and promoters (Figure
132 S1E). To identify genes exhibiting concurrent RUNX1-RNA and RUNX1-DNA interactions, we
133 annotated 24,132 high-confident RUNX1-ChIP peaks to the same gene catalog and identified 13,272
134 corresponding gene loci (Figure S1D, right). The majority of these peaks were found at intronic,
135 promoter, and intergenic regions (Figure S1F). Because most of peaks identified by both RUNX1-RIP
136 and RUNX1-ChIP peaks were distributed at coding gene loci (Figures 1A-B), we focused our analyses

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137 on this gene group. By intersecting these genes with a list of 78 myeloid genes defined by their known
138 roles in myeloid development, or myeloid molecular markers (Table S1), we obtained 15 myeloid gene
139 loci displaying both RUNX1-RIP and RUNX1-ChIP peaks (Figure 1C). *PU.1*, a master regulator of
140 myeloid development and a well-known transcriptional target of RUNX1,⁸ was among these genes.
141 Intriguingly, we observed RNA peaks at the upstream region of *PU.1* (Figure 1D). We further validated
142 this observation by RUNX1 RIP-qPCR (Figure 1E). Additional myeloid genes showing RUNX1-RIP
143 peaks and RUNX1-ChIP peaks are presented in Figure S1G. The presence of previously
144 uncharacterized RNAs, arising from the upstream region of the *PU.1* locus, and able to interact with
145 RUNX1, suggests their potential role in controlling *PU.1* expression through RUNX1-mediated
146 transcriptional regulation.

147

148 **Characterization of the RUNX1-interacting lncRNA *LOUP***

149 To map the RUNX1-interacting transcript(s), we inspected the RNA expression and epigenetic
150 landscape at the upstream region of the *PU.1* locus (Figure 2A). Remarkably, the RNA-seq track view
151 revealed two distinct RNA peaks. A narrow peak was observed at the URE, which corresponded to an
152 area of open chromatin in myeloid cells as indicated by strong DNase I hypersensitivity signals (Figure
153 2A, DNase-seq). This element was also enriched with histone post-translational modifications such as
154 H3K27ac, H3K4me1, and H3K4me3 (Figure 2A, ChIP-seq), which are typical features of active
155 enhancers.^{31,32} A broad peak was proximal to the promoter region. Notably, these peaks were present
156 in myeloid cell lines (THP-1 and HL-60) and primary monocytes, but not in the lymphoid cell line Jurkat,
157 which does not express *PU.1* mRNA, indicating a cell-type specific expression pattern. RT-PCR and
158 Sanger sequencing analysis identified exon junctions connecting these two peaks in both human and
159 murine cell lines (Figure S2A). Strand-specific RT-PCR analysis confirmed that the transcript is sense
160 with respect to the *PU.1* gene (Figure 2B). To locate the 5' end, we inspected Cap analysis gene
161 expression sequencing (CAGE-seq) tracks from the FANTOM5 project,³³ and identified a strong CAGE-
162 seq peak, located within the URE and in the sense genomic orientation (Figure 2A, CAGE-seq),
163 suggesting the presence of the 5' end of a transcript. Using the P5-linker ligation method outlined in
164 Figure S2B, we identified the 5' end including a transcription start site (TSS) of the RNA within the
165 homology region 1 (H1) of the URE¹⁸ (Figure S2C). Although a splicing event was detected within the
166 second exon, intron retention was dominant as shown by the presence of a ~2.3 Kb major transcript
167 and a ~1.0 Kb minor transcript (Figures 2C and S2D). The transcripts were detectable in the myeloid
168 cell line U937, but not in the lymphoid cell line Jurkat, further indicating their cell-type specificity (Figure
169 2C). Notably, the RNA exhibited very low coding potential similar to that of other known lncRNAs
170 (Figure S2E) as assessed by PhyloCSF software.³⁴ Additionally, no known protein domains were found
171 (data not shown) using PFAM software.³⁵ Thus, we named the RNA transcript “long noncoding RNA

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172 originating from the URE of *PU.1*, or *LOUP*. qRT-PCR analyses of subcellular fractionations revealed

173 that *LOUP* resides in both the cytoplasm and the nucleoplasm compartments, and was particularly

174 enriched in the chromatin fraction (Figure S2F). The lncRNA is polyadenylated, being detected from

175 oligo(dT)-primed cDNAs (Figure 2B) and enriched in the polyA⁺ RNA fraction (Figures 2C-D and S2G).

176 *LOUP* is low abundant lncRNA; the spliced form is expressed as ~40, 14, and 5 copies per cells in

177 U937, HL-60, and NB4, respectively (Figure 2E). The lncRNA was barely detectable as its premature

178 (non-spliced) form in total RNA as well as in the nuclear RNA fraction (Figures S2H-I). Altogether, these

179 findings established *LOUP* as a polyadenylated lncRNA that emanates from the URE and extends

180 toward the PrPr.

181

182 ***LOUP* is myeloid-specific lncRNA that is co-expressed with myeloid lineage gene *PU.1***

183 We sought to explore *LOUP* expression in normal tissues and cell types. By examining the *LOUP*

184 transcript profile in different human tissue types from the Illumina Body Map dataset, we noticed that

185 this lncRNA was barely detectable in most tissues but elevated in leukocytes (Figure 3A). Remarkably,

186 comparing with two of its closest neighbor genes, *PU.1* and *SLC39A13* (Figure S2D), the *LOUP*

187 expression pattern was similar to that of *PU.1* mRNA (Figures 3A-B) but not of *SLC39A13* (Figure

188 S3A). Additionally, *LOUP* transcript levels were not correlated with that of its interacting partner,

189 *RUNX1* (Figure S3B). To further delineate the relationship between *LOUP* and *PU.1* transcript levels

190 and lineage identity in individual blood cells, we employed single-cell RNA-seq analyses (scRNA-seq).

191 scRNA-seq data of human mononuclear cells isolated from peripheral blood (PBMC) and bone marrow

192 (BMMC) were retrieved from the 10x Genomic Project³⁶ and pooled together to maximize coverage of

193 hematopoietic cell lineages (Figure S3C). Notably, *LOUP* and *PU.1* were both enriched in the myeloid

194 cells, comprising of monocytes, macrophages and granulocytes (Figures S3D-E). Expectedly, *RUNX1*

195 was broadly expressed in myeloid cells as well as lymphoid cells (T, B, and Natural Killer (NK)) (Figure

196 S3F). By stratifying the mononuclear cell population into *LOUP*^{high}/*PU.1*^{high} and *LOUP*^{low}/*PU.1*^{low} groups

197 based on *LOUP* and *PU.1* expression levels (see methods for details), we noted that *LOUP*^{low}/*PU.1*^{low}

198 cells were associated with T, B, and NK cells. Remarkably, 99.3% of *LOUP*^{high}/*PU.1*^{high} cells were linked

199 to the myeloid identity (Figure 3C). Consistent with this observation, top biological processes

200 associated with expression of *LOUP* and *PU.1* were mono/macrophage and granulocyte functions

201 (Figure S3G and Table S2). We further examined expression patterns of *LOUP* and *PU.1* during

202 myeloid differentiation. qRT-PCR analyses of purified murine hematopoietic cell populations showed

203 low *Loup* levels in long-term hematopoietic stem cells (LT-HSC), short-term hematopoietic stem cells

204 (ST-HSC), common myeloid progenitors (CMP), and megakaryocyte-erythroid progenitors (MEP).

205 Remarkably, *Loup* expression was elevated in myeloid progenitor cells (granulocyte-macrophage

206 progenitors, GMP) and was highest in definitive myeloid cells (Figure 3D). A similar expression pattern

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207 was seen with *PU.1* (Figures 3E). Taken together, our data indicated that *LOUP* and *PU.1* transcript
208 levels were associated with the myeloid identity, warranting further investigation regarding molecular
209 relationship between *LOUP* and *PU.1* in myeloid cells.

210

211 ***LOUP* induces *PU.1* expression, promotes myeloid differentiation and inhibits cell growth**

212 To test our hypothesis that *LOUP* induces *PU.1* expression, we investigated the impact of loss-of-
213 function of *LOUP* on *PU.1* expression. In order to deplete *LOUP*, we employed CRISPR/Cas9 genome-
214 editing platform which introduces small insertion and deletion (indel) mutations in the *LOUP* gene via
215 the non-homologous end-joining (NHEJ) DNA repair mechanism.^{37,38} The macrophage cell line U937
216 that expresses high levels of *LOUP* (Figure 2E) was stably transduced with lentiviruses carrying Cas9
217 and *LOUP*-targeting or non-targeting sgRNAs. Double-positive mCherry (CAS9) and eGFP (sgRNA)
218 cells were selected by fluorescence-activated cell sorting (FACS) (Figures 4A and S4A), and derived
219 cell clones were analyzed by Sanger DNA sequencing and Inference of CRISPR edits (ICE) analysis.³⁹
220 Cell clones having indels at *LOUP*-targeting genomic locations (Figures S4B-D) displayed >80%
221 depletion of *LOUP* RNA levels (Figure 4B, left panel). This depletion was paralleled by a significant
222 reduction in *PU.1* mRNA levels (Figure 4B, right panel). In gain-of-function experiments, transient *in*
223 *trans*-overexpression of *LOUP* in K562 cells resulted in significant induction of *PU.1* (Figure 4C).
224 Remarkably, *in cis* locus-specific induction of endogenous *LOUP* via the CRISPR/dCas9-VP64
225 activation system yielded a comparable increase in *PU.1* expression, despite producing lower *LOUP*
226 transcripts than the ectopic *in trans*-expression (Figures 4D-E). Consistent with the important role of
227 *PU.1* in myeloid differentiation,^{6,7,40,41} *LOUP* depletion was associated with a reduction in expression of
228 the myeloid marker CD11b (Figure 4F). Furthermore, *LOUP* depletion increases cell proliferation
229 whereas enforced *LOUP* reduced cell number suggesting that *LOUP* inhibits cell growth (Figures 4G-
230 H). Together, these results demonstrate that *LOUP* promotes myeloid differentiation and inhibits cell
231 growth and that this lncRNA regulator exerts its inducing effect on *PU.1* expression primarily in *cis*.

232

233 ***LOUP* induces enhancer-promoter communication by interacting with chromatin at the *PU.1*** 234 **locus**

235 We have previously reported that the formation of a chromatin loop mediated by URE-PrPr
236 interaction is crucial for *PU.1* induction.^{18,19} Because *LOUP* arises from the URE and extends toward
237 the PrPr, we reasoned that *LOUP* drives long-range transcription of *PU.1* by promoting URE-PrPr
238 interaction. To elucidate this, we quantified interaction strengths of the URE with the PrPr and the
239 surrounding area by chromosome conformation capture and Taqman qPCR (3C-qPCR) (Figure 5A).
240 Consistent with previous reports,^{18,19} we detected strong interaction between the URE and the PrPr, but
241 not between the URE and other genomic regions, including the upstream *PU.1* promoter, intergenic

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242 sequences, and the *MYBPC3* gene body downstream of the *PU.1* locus. Interestingly, *LOUP* depletion
243 caused a significant reduction in URE-PrPr communication (Figures 5B). To provide evidence
244 supporting our prediction that *LOUP* recruits the URE to the PrPr by physically interacting with the two
245 elements, we employed the Chromatin Isolation by RNA Purification (ChIRP) assay.⁴² Biotinylated
246 *LOUP*-tiling oligos were able to capture endogenous *LOUP* RNA in U937 cells (Figure 5C). Enrichment
247 of the URE and the PrPr co-captured with *LOUP* RNA was observed in ChIRPed samples with *LOUP*-
248 tiling probes but not LacZ-tiling controls, suggesting that *LOUP* occupies both the URE and the PrPr
249 (Figure 5D). Taken together, our data indicate that by interacting and bringing to close proximity two
250 regulatory elements, the URE and the PrPr, *LOUP* promotes the formation of a functional chromatin
251 loop within the *PU.1* locus that is critical in inducing *PU.1* expression.

252

253 ***LOUP* binds the Runt domain of RUNX1 and coordinates recruitment of RUNX1 to the enhancer**
254 **and the promoter**

255 We next sought to gain a deeper mechanistic understanding of how *LOUP* modulates the chromatin
256 structure in a gene specific manner. Point mutations abolishing the RUNX binding sites in the URE are
257 known to disrupt chromosomal interactions between the URE and the PrPr.¹⁵ Additionally, we
258 demonstrated that *LOUP* interacts with RUNX1 at the *PU.1* locus (Figure 1). Therefore, we asked
259 whether *LOUP* mediates the URE-PrPr interaction by cooperating with RUNX1. In line with the previous
260 finding in murine cells,¹⁵ we observed RUNX1 occupancy at the URE in primary CD34⁺ cells isolated
261 from healthy donors and patients with AML. Importantly, we noticed a peak at the PrPr, indicating that
262 RUNX1 also occupies the PrPr (Figure 6A). We further inspected the genomic region surrounding the
263 PrPr and found a RUNX-DNA binding consensus motif at -220 bp relative to the *PU.1* mRNA
264 transcription start site. To determine if this motif is functional, we performed biotinylated DNA pull-down
265 (DNAP) assays. Wild-type probes, containing the RUNX consensus motifs embedded in the URE and
266 the PrPr, efficiently captured endogenous RUNX1 from U937 nuclear extracts. In contrast, probes
267 mutating the RUNX1 binding sequence, displayed drastic reductions in RUNX1 occupancy (Figures 6B
268 and S5A). These results suggest that RUNX1 binds its DNA consensus motif at both the URE and the
269 PrPr. RUNX1 is known to form homodimers to modulate transcription.^{43,44} Thus, we reasoned that
270 *LOUP* promotes looping formation by conferring occupancy of RUNX1 dimers concurrently at their
271 binding motifs within the URE and the PrPr. Indeed, *LOUP* depletion reduced RUNX1 occupancy at
272 both the URE and the PrPr (Figure 6C), indicating that *LOUP* promotes placement of RUNX1 dimers at
273 the URE and the PrPr. By aligning *LOUP* sequence with itself using the Basic Local Alignment Search
274 Tool (BLAST), we unexpectedly uncovered a highly repetitive region (RR) of 670 bp near the 3' end of
275 *LOUP* (Figure S5B). Interestingly, by performing RNA pull-down assays (RNAP) assay, we noted that
276 biotinylated *LOUP* RR was able to capture endogenous RUNX1 proteins in U937 nuclear extracts at a

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277 level that was comparable to biotinylated full-length *LOUP*, indicating that the RR contains RUNX1-
278 binding region (Figure 6D). To further locate the binding region, we first computed potential interaction
279 strength of putative elements within the RR to RUNX1 protein by using the catRAPID algorithm.⁴⁵ By
280 doing so, we identified two ~100 bp candidate regions, termed region 1 (R1) and region 2 (R2) within,
281 with high interaction scores (Figures S5C and 6E). RNAP analysis confirmed that R1 and R2 bind
282 recombinant RUNX1 (Figure 6F). Additionally, recombinant Runt domain of RUNX1 was able to bind
283 R1 and R2 (Figure 6G), suggesting that the Runt domain is responsible for *LOUP* binding. These data,
284 together, suggests that *LOUP* binds RUNX1 and coordinates deposition of RUNX1 dimers to the URE
285 and the PrPr.

286

287 **RUNX1-ETO down-regulates *LOUP* in t(8;21) AML by inhibiting histone H3 acetylation and**
288 **reducing promoter accessibility**

289 We further examined how the oncogenic fusion protein RUNX1-ETO, derived from t(8;21)
290 chromosomal translocation, affects the regulatory function of *LOUP*. By examining *LOUP* transcript
291 profiles in an AML RNA-seq dataset downloaded from The Cancer Genome Atlas (TCGA), we noticed
292 that *LOUP* RNA levels were significantly lower in t(8;21) AML patients as compared to AML patients
293 with normal karyotype (Figure 7A, left panel). Consistent with our data demonstrating the *PU.1* is a
294 downstream target of *LOUP*, *PU.1* levels were also lower in t(8;21) AML patients (Figure 7A, right
295 panel). These finding were further confirmed by qRT-PCR using patient samples (Figure 7B). Thus, we
296 reasoned that *LOUP* may act as an inhibitory target of RUNX1-ETO in t(8;21) AML. Indeed, depletion of
297 RUNX1-ETO in t(8;21) AML cells Kasumi-1, resulted in a robust increase in *LOUP* transcript levels
298 which was accompanied by a significant induction in *PU.1* mRNA (Figure 7C). RUNX1-ETO is capable
299 of recruiting Nuclear Receptor Corepressor Histone Deacetylase Complex and associates with histone
300 deacetylase activity.⁴⁶⁻⁴⁸ To examine whether RUNX1-ETO inhibits *LOUP* transcription by affecting local
301 histone acetylation, we analyzed histone acetylation and chromatin accessibility at the URE, where
302 *LOUP* transcription is initiated, upon depletion of RUNX1-ETO.⁴⁹ As expected, knockdown of RUNX1-
303 ETO reduces RUNX1-ETO occupancy at the URE (Figure 7D, top panel). Interestingly, depletion of
304 RUNX1-ETO resulted in robust induction of the H3K9Ac histone acetylation mark that is associated
305 with active promoters and Dnase I accessibility at the URE (Figure 7D, middle and bottom panels),
306 indicating that RUNX1-ETO inhibits *LOUP* transcription by deacetylating and limiting its promoter
307 accessibility.

308

309 In summary, we established *LOUP* as a myeloid-specific lncRNA that promotes myeloid
310 differentiation and inhibits cell growth via cooperating with RUNX1 to induce *PU.1* expression, and that
311 RUNX1-ETO disrupts the action of *LOUP* in t(8;21) AML. Thus, lncRNA *LOUP* acts as a regulatory hub

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312 delivering opposing effects from a broadly expressed transcription factor and its oncogenic derivative
313 on long-range transcription of an important lineage gene (Figure 7E).

314

315 **DISCUSSION**

316 In this study, we discovered that RUNX1, which is expressed and exerts its regulatory roles in
317 diverse cell types,^{50,51} cooperates with a myeloid-specific lncRNA *LOUP* to induce long-range
318 transcription of *PU.1*, and that RUNX1-ETO impairs *LOUP*-mediated *PU.1* induction by inhibiting *LOUP*
319 expression in t(8;21) AML. Our study reported several important mechanistic findings. We reveal *LOUP*
320 as a cellular RNA-interacting partner of RUNX1. We also demonstrate that *LOUP* recruits RUNX1 to
321 respective RUNX1-binding motifs at both the URE and the PrPr, thereby promoting formation of the
322 URE-PrPr chromatin loop at the *PU.1* locus. Additionally, we identify a repetitive region serving as the
323 RUNX1-binding platform for *LOUP*. Furthermore, we show that *LOUP* is a inhibitory target of RUNX1-
324 ETO, in t(8;21) AML. These findings provide important insight into how long-range transcription is
325 induced in a gene-specific manner by ubiquitous transcription factors and how their chimeric derivative
326 disrupt normal gene induction in leukemia.

327

328 Our findings that RUNX1, known to be crucial for the URE-PrPr interaction, occupies both the URE
329 and the PrPr of the *PU.1* locus, provides a molecular understanding of locus-specific activation. We
330 propose that, once the URE and the PrPr are brought into close proximity, RUNX1 molecules that are
331 parts of separate URE- and PrPr-bound complexes might interact, resulting in the formation of the
332 URE-PrPr (enhancer-promoter) transcriptional activation complex. In supporting of this mechanism,
333 RUNX1 sites at enhancers and promoters have been shown to be critical for induction of *CSF2*
334 (encoding GM-CSF), *CD34*, and *CEBPA* (encoding C/EBP α),^{43,52-54} suggesting that RUNX1 could also
335 contribute to specific enhancer-promoter docking at these gene loci. In line with this notion, locus-
336 specific enhancer-promoter interaction could be induced by artificially tethering transcription factor to
337 promoter.⁵⁵ Our findings, therefore, support a model in which specific and on-target enhancer-promoter
338 interactions are achieved by transcription factors, bound to specific motifs both at the enhancer and the
339 target promoter, that are able to dimerize or multimerize, thereby helping to fuse enhancer and
340 promoter transcriptional complexes together.

341

342 How chromatin-bound protein complexes at enhancers and target promoters are brought together in
343 a highly specific manner is still poorly understood. Our findings offer several exciting avenues that
344 might explain how locus-specific induction is accomplished. First, we demonstrated that *LOUP*
345 modulates recruitment of RUNX1 to its binding motifs at both the URE and the PrPr, suggesting that
346 *LOUP* might serve as an “RNA bridge”, bringing the separate RUNX1-containing-URE and -PrPr

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347 transcriptional complexes into proximity which finally fused into an URE-PrPr complex via RUNX1
348 dimerization. Second, locus specificity might also be enhanced based on our finding that *LOUP* arises
349 from the URE and acts *in cis* to modulate chromatin looping at the nearby *PU.1* locus. Accordingly,
350 even when a small number of transcripts are being produced, local molecular concentration of *LOUP*
351 could be enriched enough to profoundly influence rapid *PU.1* mRNA induction. Indeed, we found that
352 *LOUP* is a low-abundance lncRNA but is enriched in the chromatin fraction. Third, we revealed that
353 *LOUP* is expressed exclusively in myeloid cells. This could explain why RUNX1, which is expressed in
354 diverse cell types, induces URE-PrPr interaction and *PU.1* expression specifically in myeloid cells.
355 These findings, together, provide mechanistic understanding of gene-specific enhancer-promoter
356 interaction and cell type-specific gene induction.

357
358 Our findings also contribute to the growing body of knowledge with regard to molecular functions of
359 lncRNAs. Indeed, among thousands of lncRNAs that are implicated to arise throughout the genome,
360 only a few have been precisely mapped and molecularly characterized.²³ The herein described lncRNA
361 *LOUP*, presenting as spliced and polyadenylated transcripts, binds the Runt domain of RUNX1 via a
362 repetitive region. To our knowledge, *LOUP* is the first cellular RNA-interacting partner of RUNX1 being
363 reported. Remarkably, we also discovered that *LOUP* is down-regulated by RUNX1-ETO. It also worth
364 mentioning that a normal allele of *RUNX1* is retained alongside RUNX1-ETO fusion gene in t(8;21)
365 AML cells⁵⁶ and that RUNX1-ETO is implicated to exert opposing effect by competing with RUNX1 for
366 binding to protein partners and the same chromatin locations.^{49,57,58} Collectively, our findings uncover a
367 heretofore-unknown cross-regulation and molecular interactions of lncRNAs with transcription factors
368 and their oncogenic derivatives, providing mechanistic understanding underlying their molecular
369 functions.

370
371 In summary, we identified lncRNA *LOUP* with several important molecular features, including cell-
372 type specific expression and harboring a RUNX1-binding platform enabling *LOUP* to coordinate with
373 RUNX1 to drive long-range transcription of *PU.1* in myeloid cells. *LOUP*, a downstream inhibited target
374 of the oncogenic fusion protein RUNX1-ETO, is capable of inducing myeloid differentiation and
375 inhibiting cell growth. Our finding raises the possibility that RNA regulators of transcription factor
376 represent alternative targets for therapeutic development and provide a molecular mechanism
377 explaining, at least in part, how ubiquitous transcription factors contribute to enhancer-promoter
378 communication in both cell-type and gene-specific manner and how their chimeric derivatives disrupt
379 this normal regulation in leukemia.

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394

395 **AUTHORSHIP CONTRIBUTIONS**

396 DGT and BQT designed the study with contribution from AKE, PBS, MTV, PPP, and ADR. BQT,
397 SU, AKE, SC, PZ, HZ, EL, FM, EK, EF, CG and ART performed experiments. BQT, VEA and GH
398 analyzed ChIP-seq data; BQT and MB analyzed RIP-seq and scRNA-seq data; BQT and TMN
399 analyzed bulk RNA-seq data and performed CRISPRa; RC and DET designed and performed RIP-seq
400 experiment; LY, HY and BQT analyzed TCGA data, CW and BQT performed PhyloCSF analysis; BQT
401 and SU drew schematics; BQT and DGT wrote the manuscript with input from authors, especially, AKE,
402 EL, MB, TMN, PBS, MTV, PPP, and ADR. DGT supervised the project.

403

404 **DISCLOSURE OF CONFLICTS OF INTERESTS**

405 The authors declare no competing interests.

406

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537

538 **FIGURE LEGENDS**

539 **Figure 1. Screening of gene loci exhibiting concurrent RUNX1 RNA and DNA interactions in**
540 **THP-1 cells**

541 (A and B) Pie charts showing proportions of RUNX1 RIP-seq peaks and RUNX1 ChIP-seq peaks in
542 coding and noncoding gene families. ChIP-seq data was from published source ⁵⁹ under the Gene
543 Expression Omnibus (GEO) accession number: GSE79899.

544 (C) Venn diagram intersecting RUNX1 RIP-seq, RUNX1 ChIP-seq gene lists and the myeloid gene list.

545 (D) Gene track view of the *PU.1* locus including the upstream region (highlighted in blue). Shown are
546 RIP-seq tracks (Input, IgG and RUNX1) and RUNX1 ChIP-seq tracks (GSM2108052). Data was
547 integrated in the UCSC genome browser.

548 (E) RUNX1 RIP-qPCR confirmation. Left panel: Location of three PCR amplicons (#1, #2, #3). Right
549 panel: Enrichment of RNAs captured by anti-RUNX1 antibody and IgG control at three amplicons
550 relative to input. Error bars indicate SD (n=3).

551 See also Figure S1 and Table S1.

552

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554 **Figure 2. Characterization of long noncoding RNA *LOUP***

555 (A) Gene track view of the genomic region encompassing the *PU.1* locus. RNA-seq tracks include THP-
556 1, HL60, primary monocytes, and Jurkat. DNase-seq and ChIP-seq are overlay tracks of monocyte and
557 myeloid cell lines. These data were processed from published data in GEO (see methods for details).
558 CAGE-seq track was imported from the FANTOM5 project. #1, #2 and arrows point to locations of the
559 RNA peaks.

560 (B) RT-PCR analysis of *LOUP*'s transcript features. First-strand cDNAs were generated from HL-60
561 total RNA using a primer that does not anneal to the *PU.1* locus (Unrelated), Random hexamers,
562 Oligo(dT), and strand-specific primers (Anti-sense and Sense).

563 (C) Northern blot analysis of *LOUP*. polyA⁻ and polyA⁺ RNA fractions were isolated from U937 and
564 Jurkat cells. Top panel: schematic of the probe location spanning exon junction (E1 and E2a; see
565 Figure S2D). Middle panel: Northern blot detection of *LOUP*'s major and minor transcripts. Lower panel:
566 RNA gel demonstrating relative migration between 28S and 18S rRNAs stained with ethidium bromide.

567 (D) qRT-PCR analysis of *LOUP* levels in polyA⁻ and polyA⁺ RNA fractions isolated from HL-60 cells.
568 Error bars indicate SD (n=3). ***p < 0.001.

569 (E) Calculation of *LOUP* transcript per cell by qRT-PCR. *LOUP* RNA standard curve was generated by
570 *in vitro* transcription. Error bars indicate SD (n=3).

571 See also Figure S2.

572

573 **Figure 3. Expression profiles of *LOUP* and *PU.1* in normal tissues and cell lineages**

574 (A and B) Transcript profiles of *LOUP* and *PU.1* in human tissues. Shown are transcript counts from the
575 Illumina Body Map RNA-seq data dataset (AEArrayExpress: E-MTAB-513). Error bars indicate SD
576 (n=2).

577 (C) Proportion of cell lineages corresponding to *LOUP* and *PU.1* transcript levels. Myeloid: includes
578 monocytes, macrophages and granulocytes; T_{CD4+}: T helper cell; T_{CD8+}: Cytotoxic T cell; T_{reg}: Regulatory
579 T cell; B: B lymphocyte; Plas: Plasma cell; NK: Natural killer cell; DC: Dendritic cell; Ery: Erythrocyte;
580 Meg: Megakaryocyte.

581 (D and E) qRT-PCR analysis of *Loup* RNA and *PU.1* mRNA levels in murine hematopoietic stem,
582 progenitor, and mature (myeloid) cell populations. LT-HSC: long-term hematopoietic stem cells;
583 ST-HSC: short-term hematopoietic stem cells; CMP: common myeloid progenitors, MEP:
584 megakaryocyte-erythroid progenitors; LMPP: lymphoid-primed multipotent progenitors;
585 GMP: granulocyte-macrophage progenitors, myeloid cells (Mac1⁺Gr1⁺). Data are shown relative to LT-
586 HSC. Error bars indicate SD (n=2).

587 See also Figure S3 and Table S2.

588

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589 **Figure 4. The effect of *LOUP* on *PU.1* expression, myeloid differentiation and cell growth**

590 (A) Schematic diagram of the upstream genomic region of the *PU.1* locus. Shown are sgRNA-binding
591 sites (#D1 and #D2) for *LOUP* depletion using CRISPR/Cas9 technology.
592 (B) qRT-PCR expression analysis for *LOUP* (left panel) and *PU.1* (right panel) in non-targeting (N) and
593 *LOUP*-targeting (L) U937 cell clones. Data are shown relative to N1 control.
594 (C) qRT-PCR expression analysis of *LOUP* RNA (left panel) and *PU.1* mRNA (right panel) in K562 cells
595 transfected with *LOUP* cDNA or empty vector (EV) by electroporation.
596 (D) Schematic diagram of the *LOUP* promoter region showing sgRNA-binding sites (#A1 and #A2) for
597 *LOUP* induction. Distance from the TSS of *LOUP* is indicated in bp
598 (E) qRT-PCR expression analysis of *LOUP* (left panel) and of *PU.1* (right panel) in K562 dCas9-VP64-
599 stable cells infected with *LOUP*-targeting (#A1 and #A2) or non-targeting (control) sgRNAs.
600 (F) FACS analysis of CD11b myeloid marker in U937 cell clones with *LOUP* homozygous indels (L2a
601 and L2b) and controls (N1 and N2) using PACBLUE-conjugated CD11b antibody.
602 (G) Edu incorporation was measured by flow cytometry for cell proliferation.
603 (H) Trypan blue exclusion and manual cell counts for kinetics of cell growth (shControl v.s. shLOUP
604 (#A1 and #A2).
605 Error bars indicate SD (n=3). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$, n.s: not significant.
606 See also Figure S4.

607

608 **Figure 5. 3C and ChIRP assays measuring the effect of *LOUP* on chromatin looping**

609 (A) Schematic diagram illustrating potential 3C interactions between the URE and genomic viewpoints
610 surrounding the *PU.1* locus. Included are restriction recognition sites of A_{po}I used in the assay. -8Kb,
611 and -4Kb: distances from the PrPr in kilo bases. Int: intergenic.
612 (B) 3C-qPCR TaqMan probe-based assay comparing crosslinking frequencies at chromatin viewpoints.
613 The U937 cell clone L2a, carrying a *LOUP* homozygous indel that does not alter the recognition pattern
614 of A_{po}I (Figure S4D), was used to compare with non-targeting control (sgControl, N1). n.d.: not
615 detectable.
616 (C) qRT-PCR assay evaluating levels of *LOUP* RNA and control *GAPDH* captured by biotinylated
617 *LOUP*-tiling and LacZ-tiling probes using ChIRP.
618 (D) ChIRP assay assessing *LOUP* occupancies at the URE, the PrPr, and *ACTB* promoter. *LOUP*-tiling
619 oligos were used to capture endogenous *LOUP* in U937 cells. LacZ-tiling oligos were used as negative
620 control.
621 Error bars indicate SD (n=3); * $p < 0.05$; **** $p < 0.0001$, n.s: not significant.

622

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623 **Figure 6. *LOUP* cooperates with RUNX1 to facilitate URE-PrPr interaction**

624 (A) Gene track view of the ~26 kb region encompassing the URE and the PrPr. Shown are RUNX1
625 ChIP-seq tracks derived from CD34⁺ cells from healthy donors (GSM1097884), an AML patient with
626 FLT3-ITD AML (GSM1581788), and a non-t(8;21) AML patient (GSM722708) (top panel). The bottom
627 panel is a schematic showing the corresponding genomic locations of *LOUP* and the 5' region of *PU.1*.
628 (B) DNA pull-down assay showing binding of RUNX1 to the RUNX1-binding motifs at the URE and the
629 PrPr. Proteins captured by biotinylated DNA oligos (wt: wildtype oligo containing RUNX1-binding motif,
630 mt: oligo with mutated RUNX1-binding motif) in U937 nuclear lysate were detected by immunoblot.
631 (C) ChIP-qPCR analysis of RUNX1 occupancy at the URE and the PrPr. *LOUP*-depleted U937
632 (sg*LOUP*, L2a) and control (sgControl, N1) clones were used. PCR amplicons include the URE
633 (contains known RUNX1-binding motif at the URE), PrPr (contains putative RUNX1-binding motif in the
634 PrPr) and GENE DESERT (a genome region that is devoid of protein-coding genes). Error bars indicate
635 SD (n=3).
636 (D) RNA pull-down analysis of the RUNX1-*LOUP* interaction. Upper panel: Schematic diagram of
637 *LOUP* showing relative position of the repetitive region RR. Arrows underneath the diagram illustrate
638 direction and relative lengths of *in vitro*-transcribed and biotin-labeled *LOUP* fragments (Bead: no RNA
639 control; EGFP: *EGFP* mRNA control; AS: full-length antisense control; S: full-length sense, and RR:
640 repetitive region). Lower panel: *LOUP* fragments were incubated with U937 nuclear lysate. Retrieved
641 proteins were identified by immunoblot.
642 (E) Schematic diagram of the repetitive region RR showing predicted binding regions R1 and R2.
643 (F and G) RNAP binding analysis of R1 and R2 with recombinant full-length and Runt domain of
644 RUNX1. *In vitro*-transcribed and biotin-labeled RNAs include R1-AS (R1 antisense control); R1-S (R1
645 sense); and R2-S (R2 sense). The vertical line demarcates where an unrelated lane was removed from
646 the figure.

647 See also Figure S5.

648

649 **Figure 7. Effects of RUNX1-ETO on regulatory function of *LOUP***

650 (A) Transcript count for *LOUP* levels in AML patient samples (RNA-seq data was retrieved from TCGA
651 portal. normal: normal karyotype n=87, t(8;21) n=7); Mann-Whitney U test: **p<0.01, ****p<0.0001.
652 (B) RT-qPCR analysis of AML patient samples. normal: normal karyotype (n=14), t(8;21) (n=7).
653 Mann-Whitney U test: ***p<0.001.
654 (C) qRT-PCR expression analysis of *RUNX1-ETO* (left panel), *LOUP* RNA (middle panel) and *PU.1*
655 mRNA (right panel) in Kasumi-1 cells transfected with Renilla-targeting shRNA (shControl) and
656 RUNX1-ETO targeting shRNA (shRUNX1-ETO). Error bars indicate SD (n=4), *p<0.05, **p<0.01,
657 ***p<0.001.

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658 (D) Gene track view at the *LOUP* locus including the URE where *LOUP* transcription initiation is
659 located. Shown are RUNX1-ETO CHIP-seq tracks (top panels), H3K9Ac CHIP-seq tracks (middle
660 panels) and DNase-seq tracks of Kasumi-1 cells upon depletion of RUNX1-ETO. Cells were transfected
661 with either nontargeting siRNA (siControl) or RUNX1-ETO-targeting siRNA. Data was processed from
662 published dataset (GEO: GSE29222) and integrated in the UCSC genome browser.

663 (E) Model of how *LOUP* coordinates with RUNX1 to modulate chromatin looping resulting in PU.1
664 induction, myeloid differentiation, and cell growth, and how RUNX1-ETO interferes with *LOUP*-
665 mediated molecular functions.

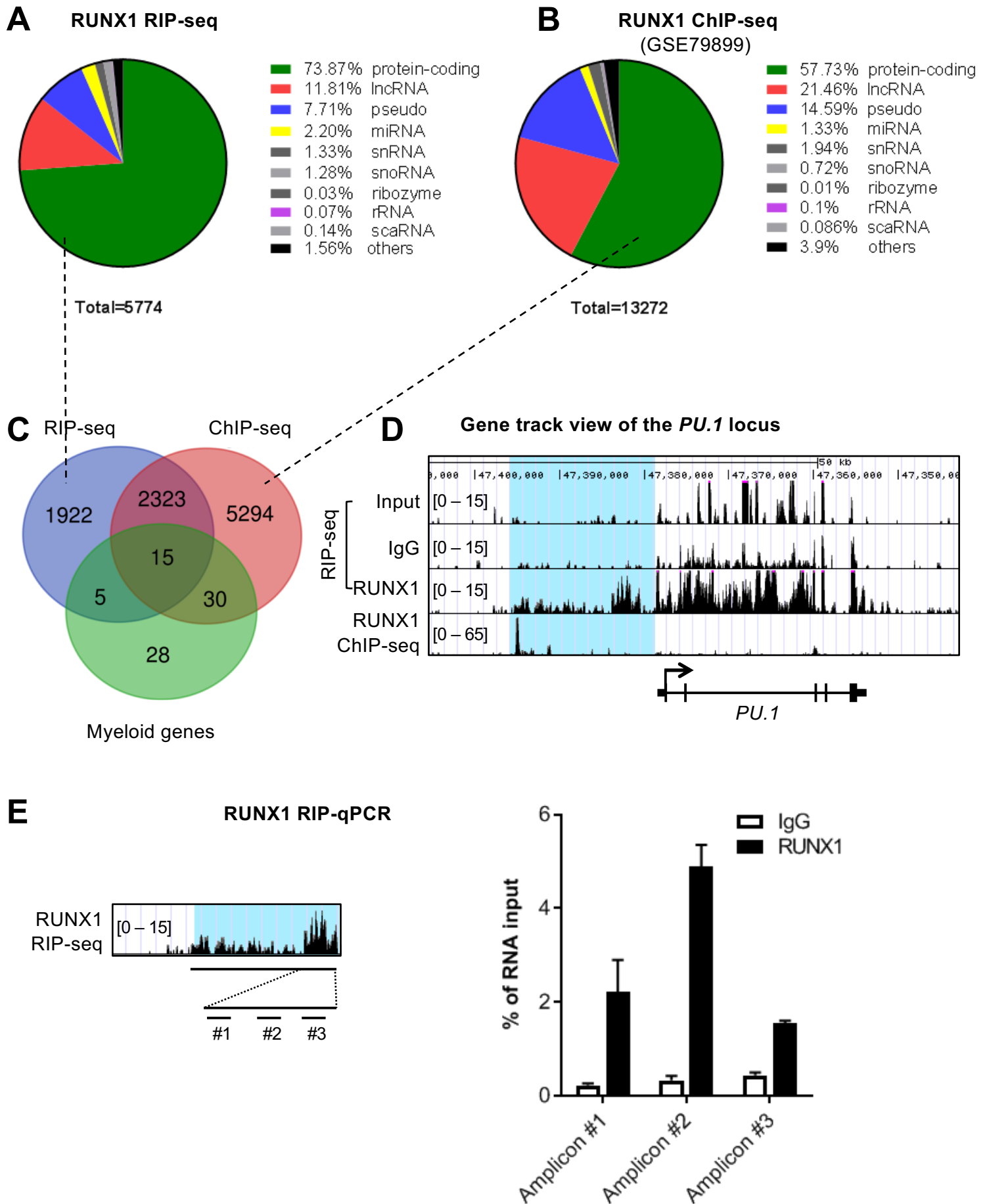


Fig1

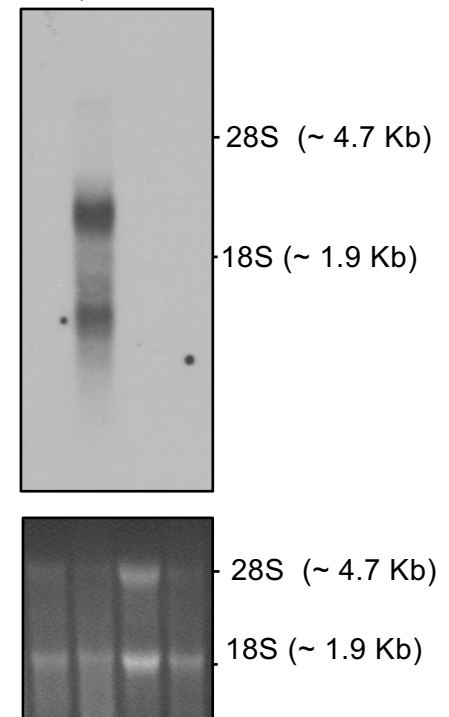
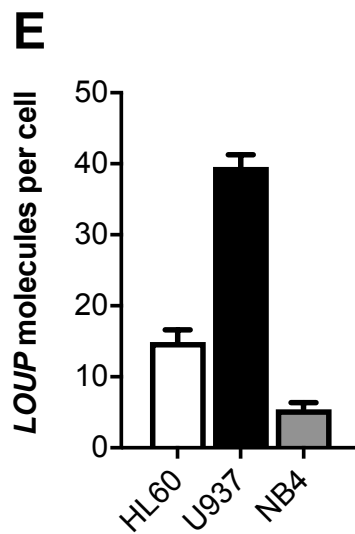
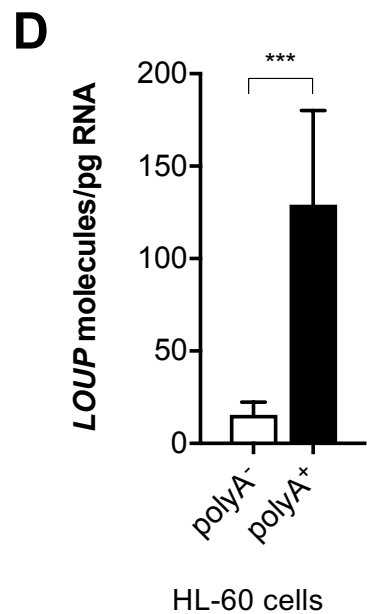
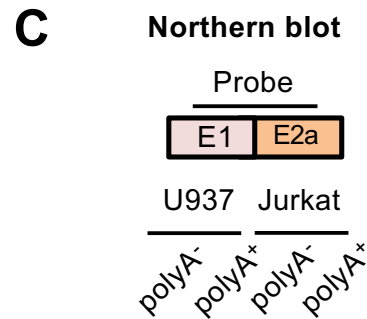
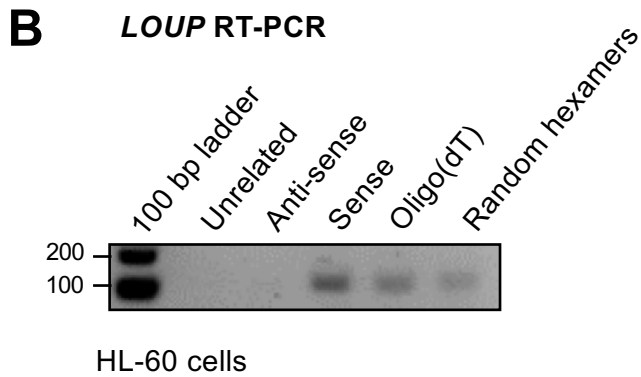
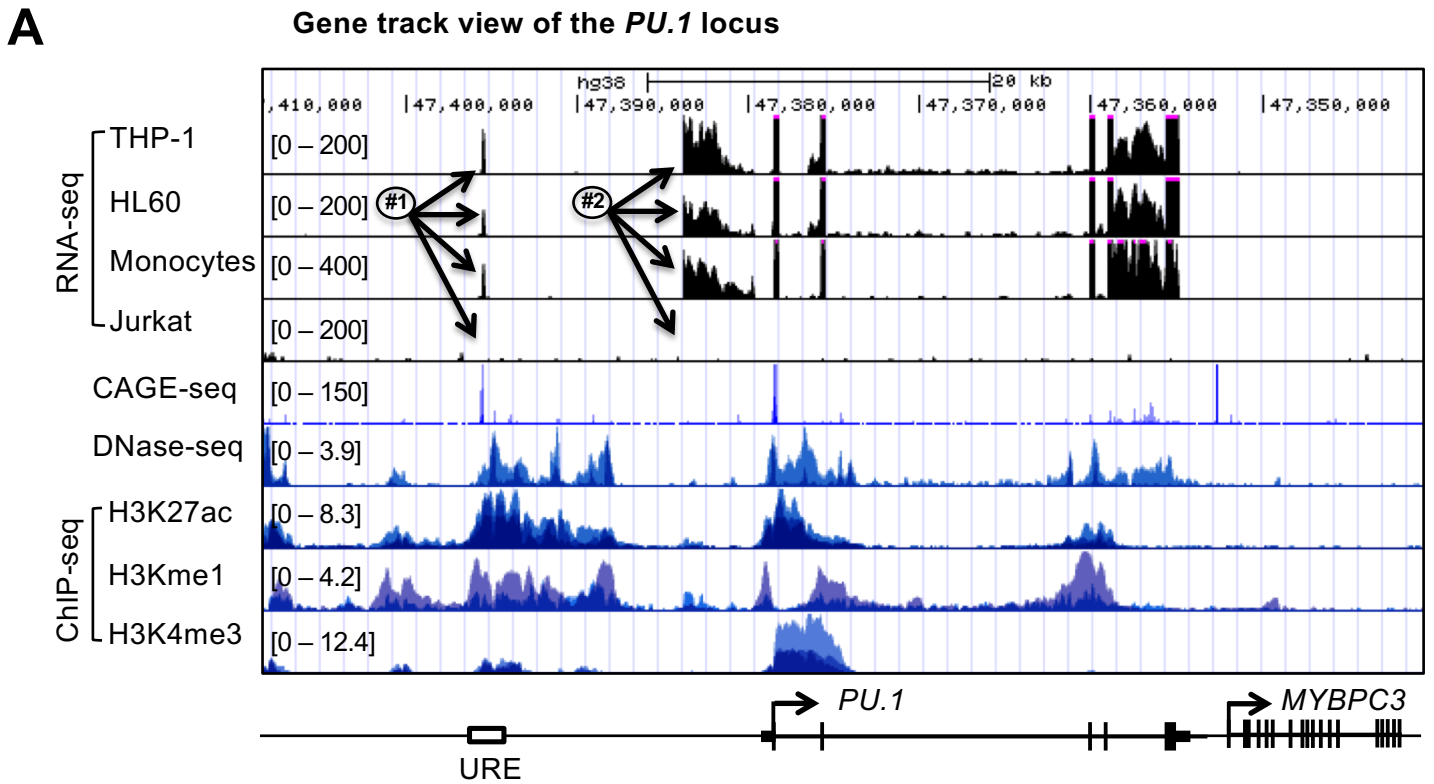


Fig2

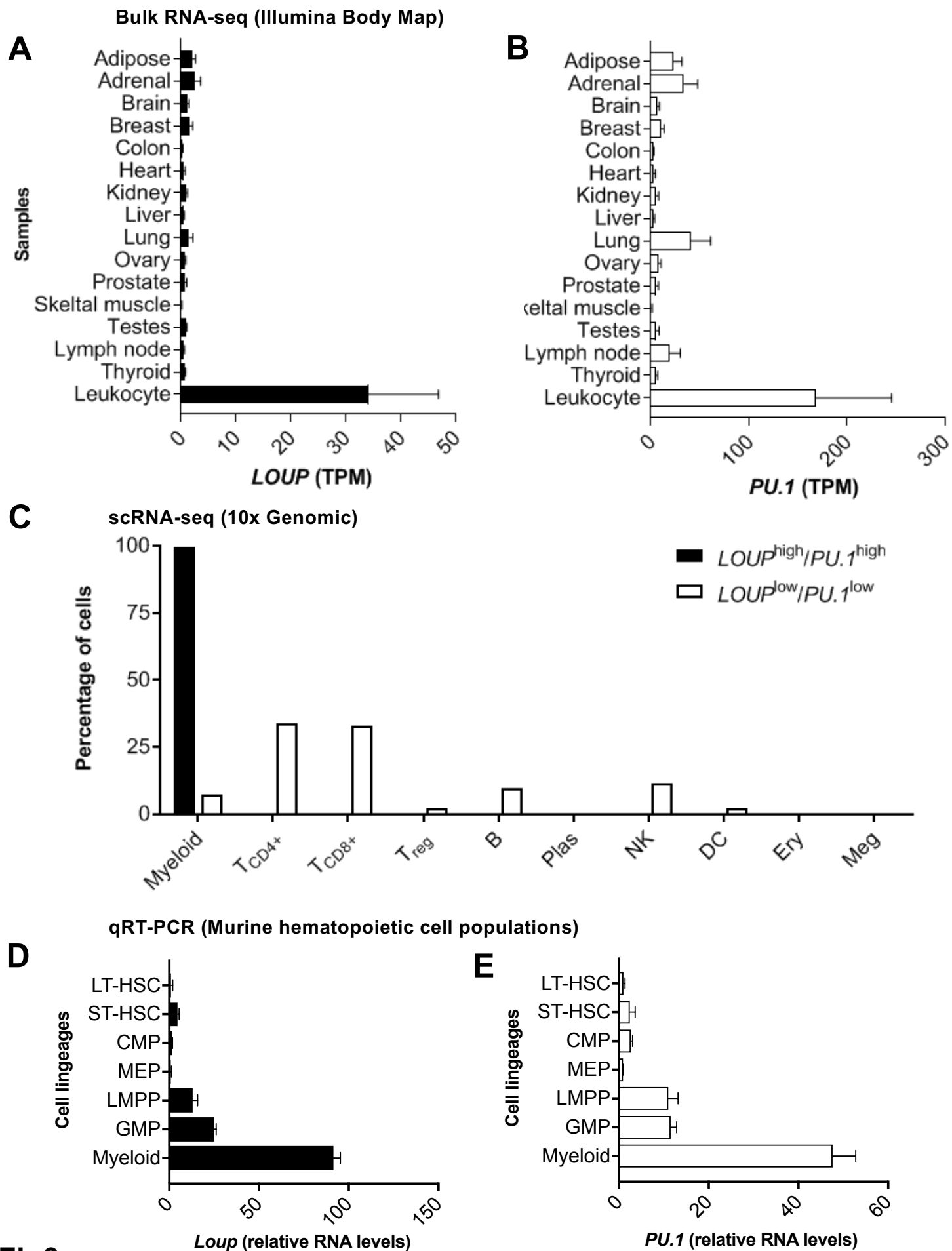


Fig3

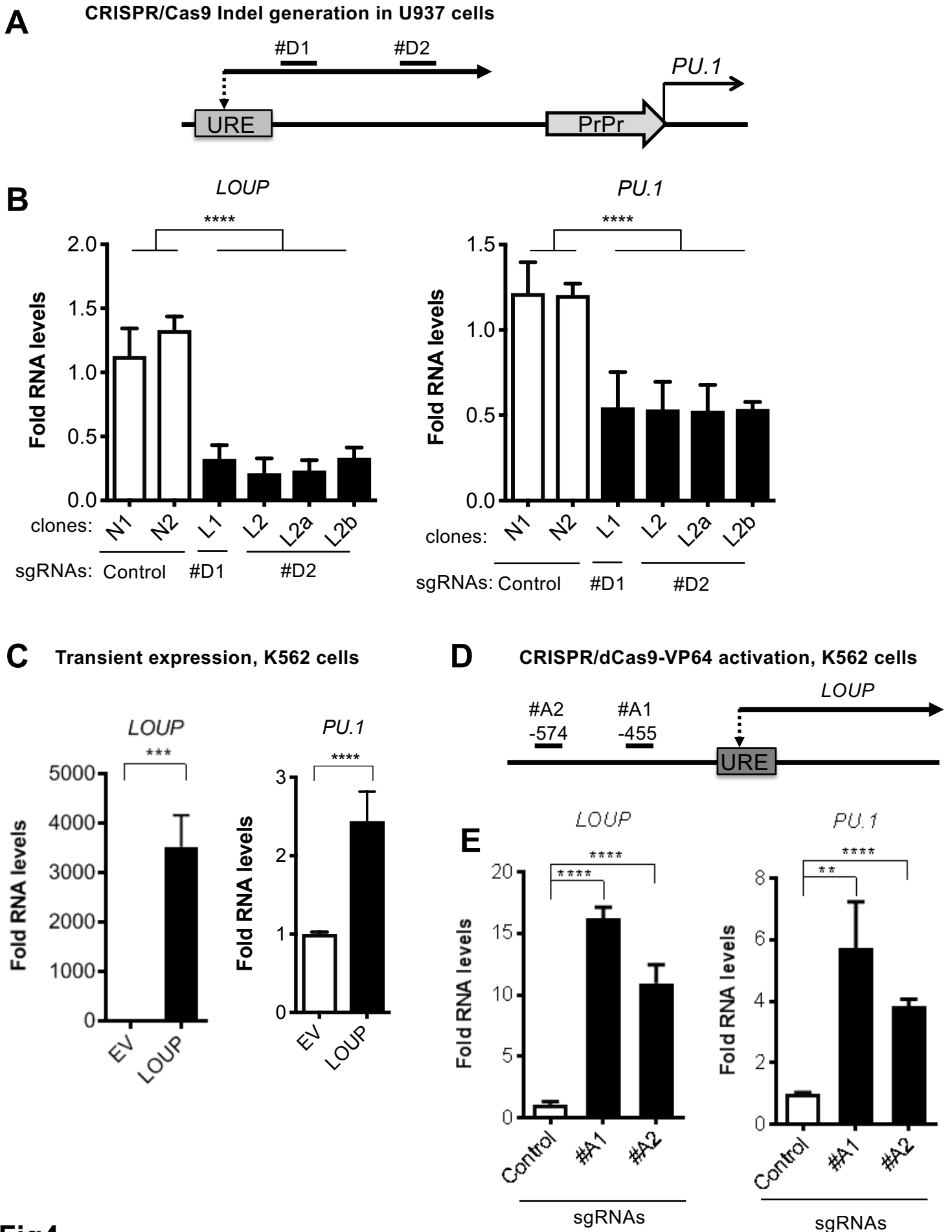
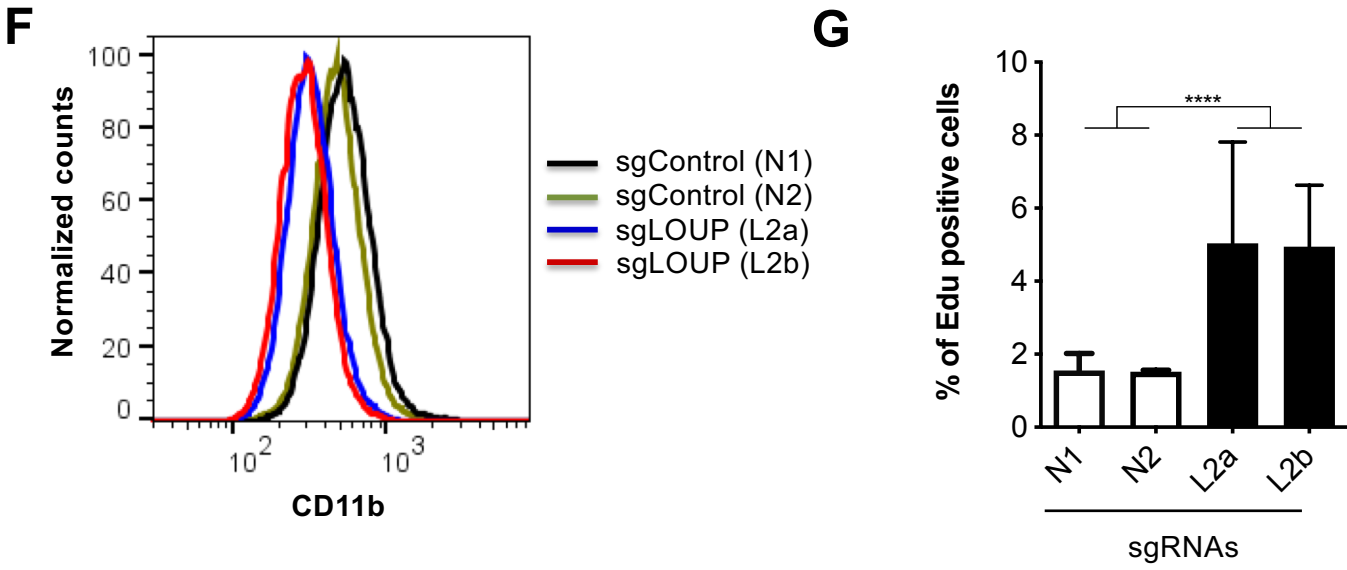


Fig4

CRISPR/Cas9 Indel generation in U937 cells



H CRISPR/dCas9-VP64 activation, K562 cells

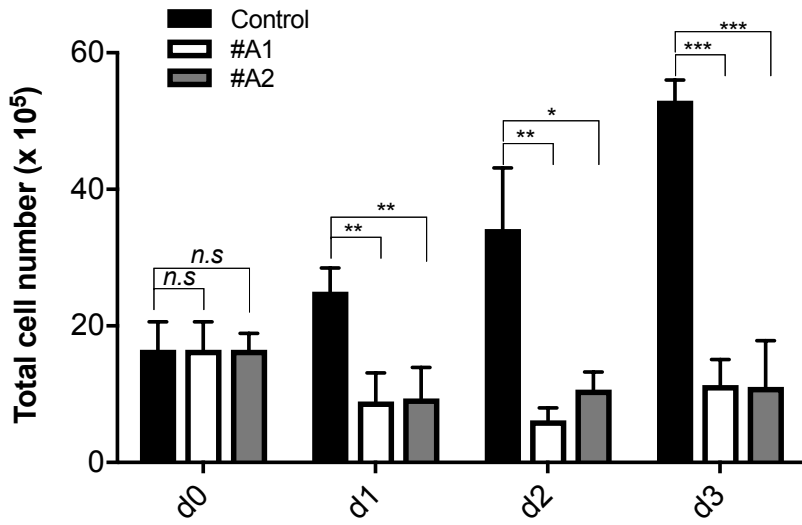
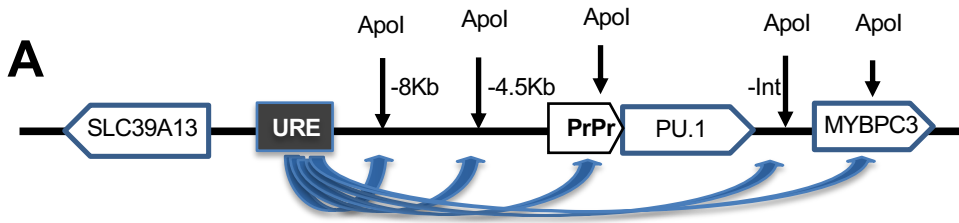
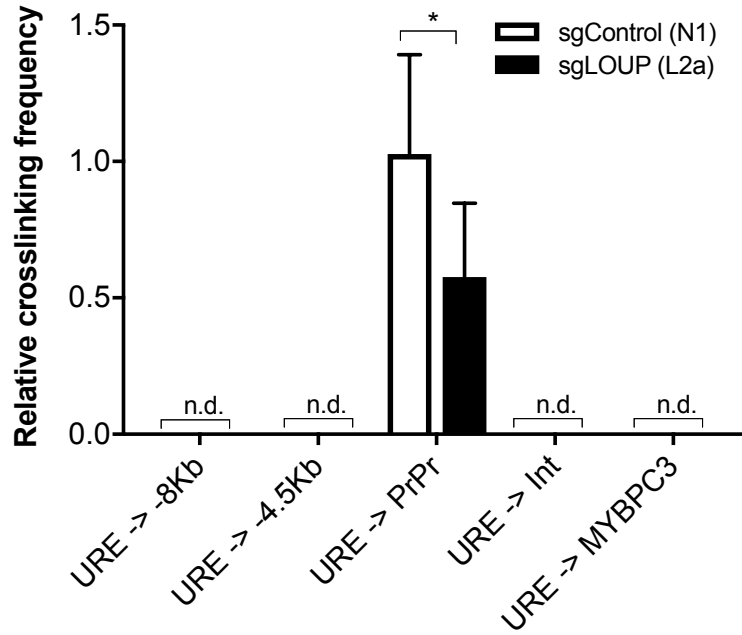


Fig4



B 3C-qPCR TaqMan probe-based assay measuring URE interactions



ChIRP assay of LOUP interactions with chromatin elements

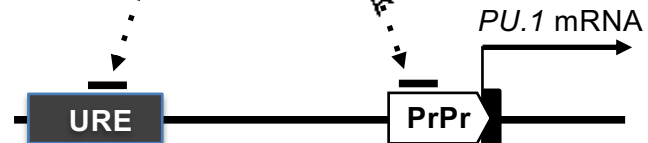
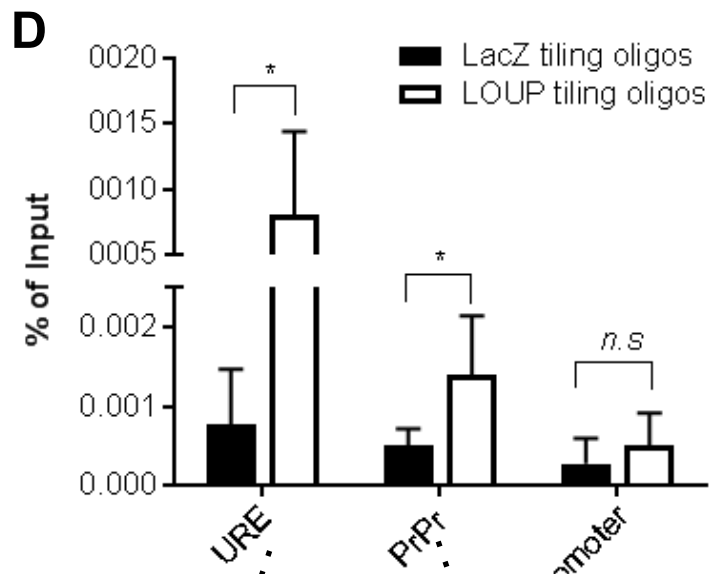
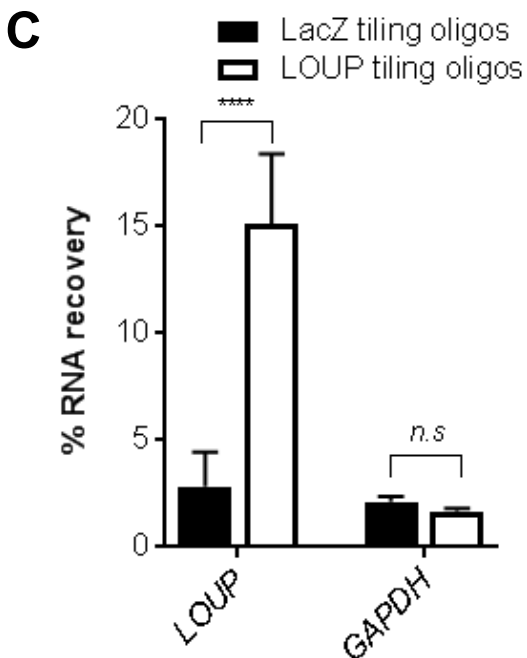
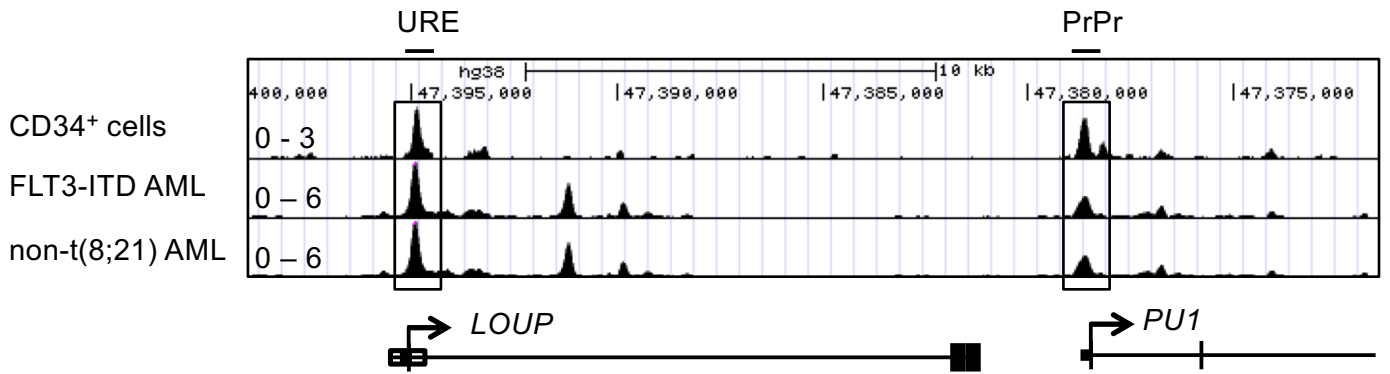
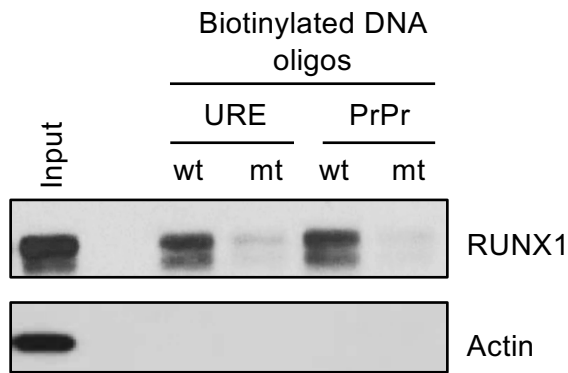


Fig5

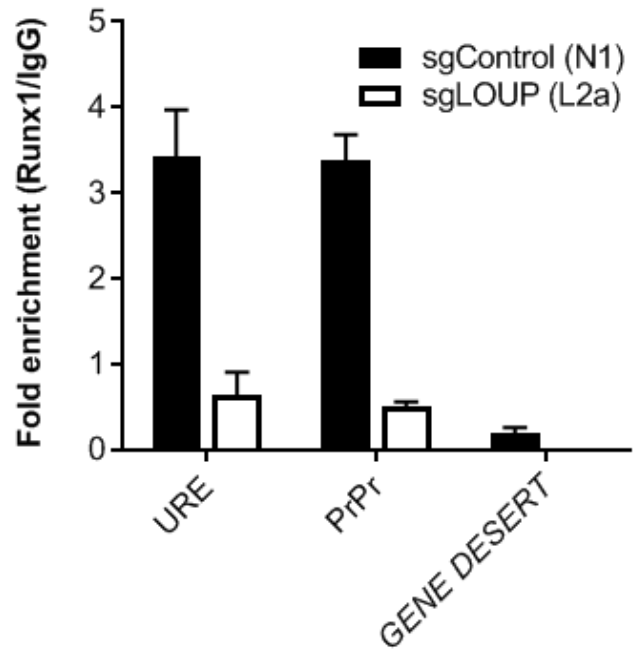
A Gene track view of RUNX1 ChIP-seq at *PU.1* locus



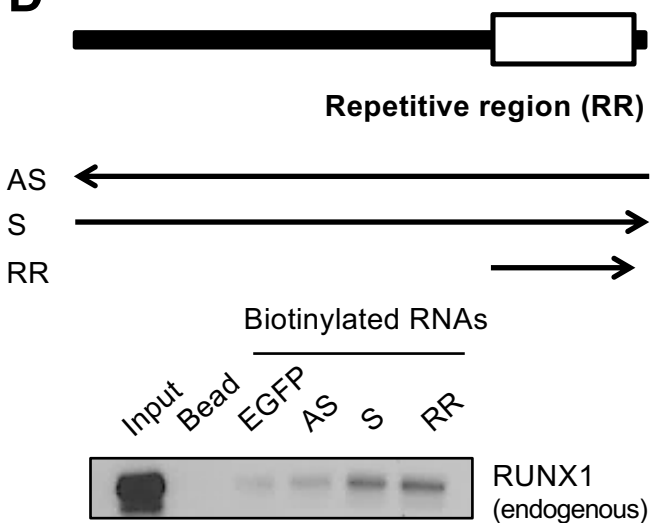
B RUNX1-DNA binding (DNAP)



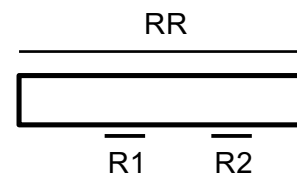
C RUNX1 chromatin occupancy (ChIP-qPCR)



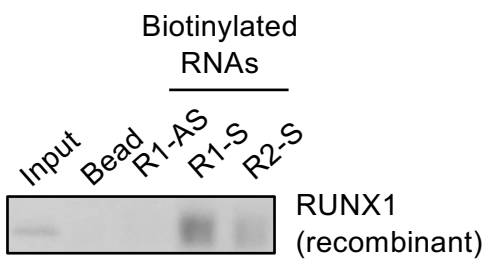
D LOUP-RUNX1 binding (RNAP)



E



F



G

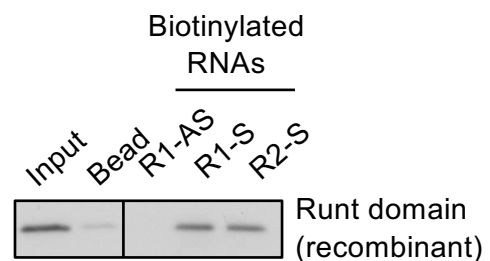


Fig6

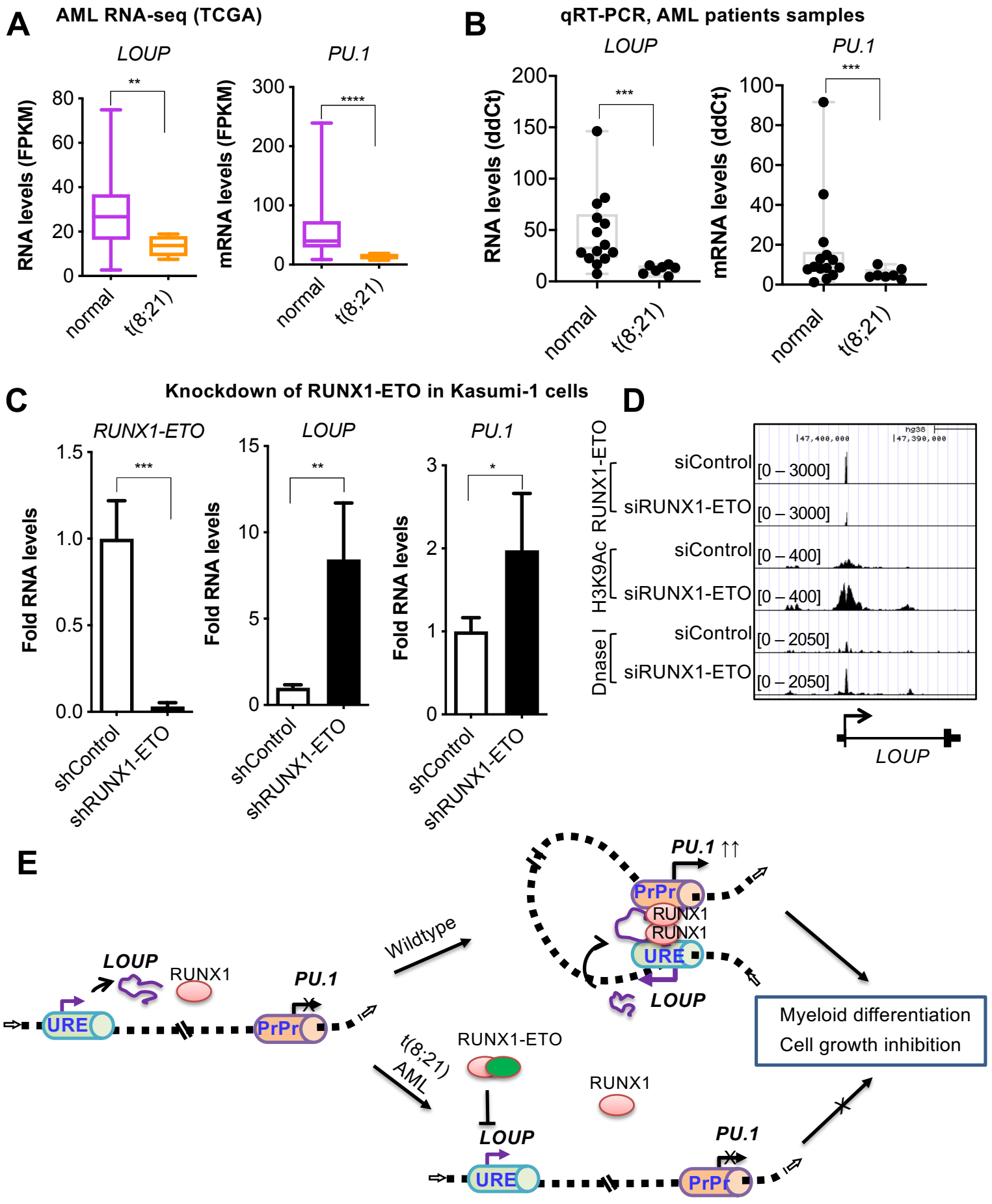


Fig7