Enhancer Regulation of Transcriptional Bursting Parameters Revealed by Forced Chromatin Looping

Highlights
- β-globin transcriptional burst fraction and size increase in erythroid maturation
- Forcing enhancer-promoter contacts enables studies of enhancer function
- The β-globin enhancer predominantly determines burst fraction
- Dynamically alternating enhancer contacts account for promoter competition in cis

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In Brief
Transcription occurs in discontinuous bursts, but the impact of enhancers on burst parameters is poorly understood. Bartman et al. show that looped contacts between the β-globin enhancer and promoter stimulate burst fraction but not burst size. Rapidly alternating enhancer-promoter contacts underlie in cis competition of genes for enhancer activity.

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Enhancer Regulation of Transcriptional Bursting Parameters Revealed by Forced Chromatin Looping

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SUMMARY

Mammalian genes transcribe RNA not continuously, but in bursts. Transcriptional output can be modulated by altering burst fraction or burst size, but how regulatory elements control bursting parameters remains unclear. Single-molecule RNA FISH experiments revealed that the β-globin enhancer (LCR) predominantly augments transcriptional burst fraction of the β-globin gene with modest stimulation of burst size. To specifically measure the impact of long-range chromatin contacts on transcriptional bursting, we forced an LCR-β-globin promoter chromatin loop. We observed that raising contact frequencies increases burst fraction but not burst size. In cells in which two developmentally distinct LCR-regulated globin genes are cotranscribed in cis, burst sizes of both genes are comparable. However, allelic co-transcription of both genes is statistically disfavored, suggesting mutually exclusive LCR-gene contacts. These results are consistent with competition between the β-type globin genes for LCR contacts and suggest that LCR-promoter loops are formed and released with rapid kinetics.

INTRODUCTION

Transcription of most genes in mammalian cells occurs in bursts interspersed with refractory periods of varying lengths, both in cell lines and in tissues (Bahar Halpern et al., 2015; Cai et al., 2008; Chubb et al., 2006; Golding et al., 2005; Levsky et al., 2002; Molina et al., 2013; Raj et al., 2006; Raj and van Oudenaarden, 2009; Suter et al., 2011; Zenklusen et al., 2008). Transcription of a given gene can be increased through modulation of several different burst parameters. For instance, the gene could increase in burst fraction (the number of alleles transcribing per cell, which is related to how frequently bursts occur) or burst size (the number of RNA molecules produced per burst). One or both of these parameters can change depending on the system (Dar et al., 2012; Octavio et al., 2009; Raj et al., 2006; Zopf et al., 2013). For example, serum-induced transcription activation of β-actin augments both burst frequency and burst size, while serum induction of c-Fos leads to an increase in burst frequency without changing burst size (Kalo et al., 2015; Senecal et al., 2014). Recent evidence further suggests that maintenance of mRNA concentrations in response to changes in cell size are achieved through changes in burst size, while gene dosage compensation in the cell cycle is regulated by burst frequencies (Padovan-Merhar et al., 2015). However, the molecular regulation of transcriptional bursts remains unclear.

Enhancers are distal genetic elements that regulate transcription of their target genes by diverse modes, which include altering chromatin structure and histone modifications, recruiting basal transcription factors, or increasing transcriptional elongation (Bulger and Groudine, 2011; Spitz and Furlong, 2012). However, most studies on enhancer function are population based, leaving open how enhancers affect transcription dynamics at individual alleles.

The β-globin locus control region (LCR) is a powerful distal enhancer that is required for high-level transcription of all β-type globin genes (Bender et al., 2000; Epner et al., 1998; Reik et al., 1998). The LCR engages the embryonic (ε-globin), fetal (γ-globin), and adult-type (β-globin) globin genes through looped contacts in a developmentally appropriate manner (Carter et al., 2002; Palstra et al., 2003; Tolhuis et al., 2002). Proposed mechanisms by which the LCR activates transcription include recruiting tissue-specific and general transcription factors as well as RNA polymerase II, moving the locus toward the center of the nucleus and outside the chromosome territory, as well as promoting transcription elongation (Bender et al., 2012; Epner et al., 1998; Sawado et al., 2003). How these mechanisms impact transcriptional burst size versus fraction is unresolved. A related open question is how the LCR controls bursting in the context of multiple active or potentially active β-type globin genes within the locus.

A landmark study using RNA FISH demonstrated that at a fraction of alleles, γ-globin and β-globin located on the same chromosome can be transcribed during the same developmental stage (Wijgerde et al., 1995). This study further provided evidence that the appearance of allelic co-expression of γ-globin and β-globin results from rapidly alternating interactions of these genes with the LCR. Subsequent studies that included kinetic experiments provided additional support for the idea that LCR-promoter contacts are so dynamic that the appearance of allelic co-transcription is actually the result of very rapid transcriptional switches (Gribnau et al., 1998; Sankaran et al., 2009; Trimborn et al., 1999). A model in which only one promoter interacts with
Figure 1. β-Globin Transcriptional Burst Fraction and Size Increase during Erythroid Maturation
(A) Model of how bursts can be modulated during erythroid maturation to increase β-globin mRNA levels.
(B) Single-molecule RNA FISH of β-globin intron and exon to identify transcription sites in G1E-ER4 cells 4 or 24 hr after estradiol addition (arrows indicate transcription sites; intron and exon channels are set to same intensities across images; maximum merges [maximum signal from 45 stacks] shown).
(C) Representative experiment showing fluorescence intensities of β-globin transcription sites in G1E-ER4 cells at indicated time points after estradiol addition (ticks on x axis = medians for this experiment).
(D) Mean number of alleles transcribing β-globin per cell at time points after estradiol addition. n = 3 biological replicates.
(E) Median fluorescence intensities of β-globin transcription sites in G1E-ER4 cells following estradiol addition. n = 3 biological replicates.

(legend continued on next page)
the LCR at any given time would also explain competition among the genes. However, none of these studies excluded the possibility that the LCR acts on both genes simultaneously. Besides simultaneous LCR-gene contacts, an additional scenario compatible with allelic co-expression is that one gene could be transcribed dependently and the other independently of the LCR. Indeed, the α-type globin genes are transcribed in the absence of the LCR, albeit at much lower levels (Bender et al., 2000). The application of quantitative RNA FISH to examine transcriptional bursting parameters might allow discrimination between these models.

Here, we used quantitative single-molecule RNA FISH (Femino et al., 1998; Raj et al., 2008) to measure transcriptional burst size and burst fraction of the β-globin gene during erythroid maturation. We observed increases in both transcriptional burst fraction and size during this process. The enhancing effects of the LCR on β-globin transcription are predominantly explained by augmenting burst fraction with a modest, but significant, contribution to burst size, as revealed by LCR deletion experiments. To study the relationship between LCR-promoter contact frequencies on bursting parameters, we applied a forced chromatin looping system. Specifically, forced tethering of the nuclear factor Ldb1 via designer zinc fingers to a chosen β-type globin gene promoter leads to recruitment of the LCR and transcription activation (Deng et al., 2012; 2014). We found that in murine erythroblasts, engagement of the LCR increased β-globin burst frequency but not burst size. Similarly, in primary adult human erythroid cells, redirecting the LCR toward the γ-globin genes and away from β-globin genes increased γ-globin burst fraction while lowering β-globin burst fraction. A significant proportion of alleles co-transcribed β- and γ-globin, but allelic co-transcription of β- and γ-globin was statistically disfavored. Strikingly, the burst size of co-transcribed genes is not reduced compared to singly transcribing genes, further supporting the model that promoters compete for LCR activity and that LCR-promoter looping controls burst fraction. In concert, these results provide new insights into mechanisms of enhancer function and highlight the use of targeted alterations of chromatin architecture for functional studies of enhancers.

**RESULTS**

**β-Globin Transcriptional Burst Fraction and Burst Size Increase during Erythroid Maturation**

An increase in transcription at a given allele can occur through elevated burst fraction, burst size, or a combination of both (Figure 1A). We first measured β-globin transcriptional bursting parameters in the G1E-ER4 murine erythroblast cell line during the course of erythroid maturation, when β-globin transcription is strongly induced. Maturation of these cells is dependent on the hematopoietic transcription factor GATA1, which is expressed in these cells as an estrogen receptor fusion protein. Addition of estradiol triggers erythroid maturation and increases contacts between the LCR and the β-globin promoter, faithfully reproducing normal terminal erythroid maturation (Vakoc et al., 2005; Weiss et al., 1997; Welch et al., 2004). We measured transcription of the adult β-globin (Hbb-b1) gene at different time points during G1E-ER4 maturation using single-molecule RNA FISH (Femino et al., 1998; Raj et al., 2008, 2006). Transcription sites were identified by nuclear colocalization of spectrally distinguishable signals from probes specific for β-globin introns and exons (Figure 1B) (Levesque and Raj, 2013).

We verified that transcription sites faithfully reported recent transcriptional events by blocking transcription with actinomycin D and counting β-globin transcription sites in human erythroid cells (Figure S1A). This control showed that 90% of transcription sites were lost at 30 min post-drug treatment, and half disappeared by just 10 min, validating that our transcription site identification faithfully reported ongoing transcription.

Both burst fraction and average burst size increased during differentiation (Figures 1B–1E, Table S1), indicating that both parameters contribute to total β-globin production during cell maturation. Specifically, the increase in burst fraction from 4 to 24 hr of maturation was 3.4-fold, while the change in burst size from 4 to 24 hr was 1.8-fold (Figures 1D and 1E). The burst fraction increase corresponded to an increase in the proportion of cells transcribing 1, 2, or 3 active β-globin alleles (Figure S1C). The overall increase in transcriptional output was consistent with the profile of RNA polymerase II (Pol II) binding at the β-globin locus, as determined by chromatin immunoprecipitation (ChIP) (Figure 1F). Moreover, we observed an increase in the fraction of Pol II in the gene body relative to the promoter (Figure 1G), reflecting an increase in elongating Pol II, consistent with the observed increases in burst fraction and size. To address possible variabilities in fluorescence intensities between slides and experiments, we concurrently measured cyclin A2 mRNA intensities in the experiments shown in Figures 1B–1E (Figure S1B). We found that cyclin A2 mRNA intensities were consistent between experiments. The subtle downward trend in cyclin A2 signal during erythroid maturation is opposite to that of the β-globin transcription sites (Figure 1E), such that normalization of β-globin bursting size to cyclin A2 would result in slightly larger changes during maturation.

One question raised by this analysis is whether the variation in burst size might be strongly regulated by global transcriptional differences between cells (“extrinsic noise”) (Elowitz et al., 2002; Swain et al., 2002), as opposed to allele-level differences (“intrinsic noise”). If, for example, transcription factor concentrations between cells varied widely, and if this variation were to determine differences in burst size, then two alleles bursting in the same cell should tend to have similar burst sizes. To quantify the contribution of extrinsic noise to burst size, we measured whether the sizes of bursts were correlated in cells that displayed two transcriptional bursts at various stages of maturation. We

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(F) Mean anti-RNA polymerase II ChIP in G1E-ER4 cells at indicated times after estradiol addition in the globin locus normalized to input. Primer pairs targeting distinct regions of the gene are listed on the x axis. The silent CD4 locus served as a negative control. n = 3 biological replicates.

(G) Mean ratio of RNA polymerase II ChIP signal at β-globin exon 2 to β-globin promoter. n = 3 biological replicates. Error bars represent SEM. See also Figures S1 and S2 and Table S1.
found that the correlation was not strong (maximum $R^2 = 0.293$), and therefore differences between cells do not strongly determine individual burst sizes as measured by RNA FISH (Figures S1D–S1F). The correlation was slightly weaker at 9 hr of maturation compared to later time points, suggesting that cell-to-cell variability may play a slightly larger role in determining burst size variation later in differentiation. We note, however, that staggered initiation timing of bursts, as well as technical noise, can contribute to apparent intrinsic noise in this system.

To test whether global changes during erythroid maturation might nonspecifically alter the measurements of bursting parameters, we examined transcription of $Gata2$, a gene that is active in erythroid precursors but silenced during maturation (Weiss et al., 1994). We found that the burst size and burst fraction of $Gata2$ are both reduced during maturation, suggesting that both parameters are regulated in a gene-specific manner (Figure S2).

In sum, $\beta$-globin burst fraction and burst size increase during erythroid maturation. This finding raises the question of how these bursting parameters are governed and whether burst fraction and burst size are governed independently of each other.

Enhancer Deletion by Genome Editing Decreases $\beta$-Globin Burst Fraction and Size

Relatively little is known about the molecular control of bursting parameters. In particular, it is unknown how distal regulatory elements influence burst fraction and burst size. Previous studies indicate that the LCR influences both burst fraction and size (Bender et al., 2012). To quantify the contributions of the LCR to these parameters via single-molecule RNA FISH, we generated two distinct G1E-ER4 cell lines with homozygous deletions of the LCR using different guide RNA pairs (Figures 2A and S3A–S3G). Cells were induced with estradiol for 24 hr to examine the effect of the LCR in its most active state. The $\beta$-globin burst fraction of LCR-deleted cells is greatly reduced (311-fold and 194-fold in line 1 and line 2, Figures 2B–2C, Table S2) when compared to control cells. For measurements of burst size we had to examine a large number of cells (8,216 cells total between all conditions and replicates) given the strong reduction in burst frequencies in cells lacking the LCR. Moreover, we pooled across biological replicates and both clones in order to gain the number of transcription sites necessary to make a statistical comparison of burst size. Burst size was reduced approximately 3-fold in cells lacking the LCR, compared to parental G1E-ER4 cells (3-fold for each clone, Figures 2B and 2D). We note that we measured an equal number of transcription sites from each clone, and
the means of both populations were similar, justifying the pooling of the two clones in the final analysis. These results suggest that the LCR predominantly controls burst fraction with a lesser, but significant, impact on burst size.

**Forced Enhancer-Promoter Contacts Increase β-Globin Transcriptional Burst Fraction but Not Burst Size**

The LCR forms looped contacts with the promoters it regulates. Since the LCR influences both burst fraction and size, we set out to test whether these two effects are both controlled by LCR-promoter contact frequencies or whether they are mechanistically separable. We used a recently developed strategy to force LCR-promoter contacts that involves tethering of the self-association domain (SA) of nuclear factor Ldb1 by a designer zinc finger (ZF) to the β-globin gene promoter, which leads to recruitment of the LCR and transcription activation (Figure 3A) (Deng et al., 2014, 2012). The unique advantage of this system is that it allows targeted formation of enhancer-promoter contacts without otherwise perturbing the locus or cell state. G1E cells lack GATA1 and as a result have few, if any, LCR-promoter contacts.

G1E cells expressing a ZF-SA fusion protein specific for the murine β-globin promoter (mZF-SA) exhibit LCR-promoter contact frequencies approximating those of GATA1 replete cells (Deng et al., 2012). We infected G1E cells with a control retroviral vector or a derivative expressing mZF-SA as part of an IRES-GFP construct, followed by fluorescence-activated cell sorting (FACS) to enrich for infected cell populations. Increasing LCR-promoter contacts by mZF-SA significantly increased β-globin burst fraction compared to controls (Figure 3B, Table S3). Surprisingly, burst size was unchanged by mZF-SA expression (Figures 3C–3G).

β-globin transcription was confirmed to be increased by qPCR (Figure S4A). Together,
these results suggest that enhancer-promoter contacts augment the likelihood for a burst to occur but do not determine burst size. The use of the mZF-SA reagent thus enabled the uncoupling of enhancer looping from other potential enhancer functions that might impact on the β-globin locus.

**β-Globin Transcriptional Burst Fraction and Size Increase during Human Erythroid Maturation**

In order to investigate whether the regulation of β-globin transcriptional bursting during erythroid maturation is conserved between mice and humans, we examined primary human erythroblasts. Human CD34+ hematopoietic precursors from peripheral blood were expanded and differentiated toward the erythroid lineage using a previously described two-phase liquid culture system (San-karan et al., 2008). We measured adult type β-globin (HBB) burst size and fraction before and after 3 days of erythroid differentiation induction. During this process we observed an increase in both burst size and fraction of the β-globin gene, similar to our observations in maturing murine erythroid cells (Figures 4A and 4B). The increases in burst fraction (1.7-fold) and average burst size (1.6-fold) are comparable in magnitude and thus are both likely to contribute to increased β-globin production during erythroid maturation.

**Forced Switching of Enhancer Contacts between Two Promoters Reciprocally Alters Burst Fraction**

To examine whether the human LCR regulates bursting in the same manner as the murine LCR, we employed a forced looping strategy (Figure 4C) (Deng et al., 2014). The human β-globin locus spans several globin genes, including the adult type β-globin and a fetal form called γ-globin (HBG). We reported previously that expression of a ZF-SA construct targeting the γ-globin promoter (hZF-SA) in human primary erythroid cells increased LCR contacts with the γ-globin gene at the expense of the β-globin gene with corresponding changes in transcription, consistent with a mechanism in which these genes compete for LCR function (Deng et al., 2014). An advantageous feature of the human system is that perturbations of LCR looping leave unaltered
endogenous nuclear factors, the LCR per se, and the maturation state of the cells.

We infected primary human cultures with lentiviral vectors expressing either GFP (control) or hZF-SA, purified infected cells by FACS, and quantified bursting parameters by RNA FISH. We observed that erythroblasts from different donors expressing control vector produced between 5% and 35% γ-globin and 65%–95% β-globin. The variation between samples is presumably due to differences among donors or the way blood samples were obtained and processed. Forced enhancer-promoter looping between the LCR and γ-globin promoter increased the burst fraction of γ-globin transcription sites per cell and decreased the burst fraction and size of β-globin sites slightly (Figures 4D–4H, Table S4), suggesting that the two genes compete for LCR activity. Furthermore, formation of γ-globin-LCR contacts increased γ-globin burst fraction without affecting β-globin burst size, consistent with the results from the murine cell line (Figures 4E–4H). Changes in burst fraction corresponded to changes in mRNA levels in the same samples: γ-globin mRNA increases with hZF-SA, while β-globin trends downward (Figures S4B and S4C). The human LCR thus governs burst fraction of target genes via enhancer-promoter contact while governing burst size through another independent mechanism.

Allelic, but Not Cellular, Co-transcription Is Disfavored in Primary Human Erythroblasts

We observed co-transcription of β- and γ-globin on the same chromosome in a fraction of cells, as shown by colocalized γ-globin and β-globin intron RNA FISH signals, suggesting that competition between different globin genes might be not absolute (Figure 5A). Similar co-transcription of globin genes had been previously observed in murine systems (Gribnau et al., 1998; Sankaran et al., 2009; Trimborn et al., 1999; Wijgerde et al., 1995). Allelic co-transcription could mean that (1) the LCR switches rapidly between the β-globin and γ-globin genes to alternately trigger bursts, (2) the LCR acts on both genes simultaneously, or (3) occasional transcription of one or both genes occurs in an enhancer-independent manner. If the LCR truly switched rapidly (model 1), co-transcriptional events should be less frequent than expected than if the two genes were transcribing independently.

To test whether co-transcription is indeed statistically disfavored, we first examined whether γ- and β-globin are transcribed in the same cell, regardless of whether or not the two genes are transcribed from the same chromosome. We
quantified cells transcribing β-globin, γ-globin, both, or neither and then performed a Fisher exact test for independence of their transcription (Figures S5A–S5C). The null hypothesis is that a cell transcribing β-globin has the same likelihood of transcribing γ-globin as a cell NOT transcribing β-globin. This is represented by an odds ratio of one. Alternatively, an odds ratio less than one would show negative correlation between transcription of the two genes in a single cell, potentially suggesting some trans-regulation mediating competition between the genes. We found that the odds ratio was not consistently greater or less than 1 in our analysis of the cells of four donors (Figure S5C). This suggests that on a cellular level, transcription of β- and γ-globin is independent.

Next, we investigated whether co-transcription on a single allele copy of γ- and β-globin was independent, treating all alleles as a pool irrespective of which cell they were in. This analysis required estimating the number of non-transcribing alleles in the population, which is not measurable by RNA FISH. We thus performed cell-cycle analysis of cultured human cells using RNA FISH: cells with histone 4 mRNA are in S-phase, cells without histone 4 mRNA with a small nucleus are in G1, while cells without histone RNA with a larger nucleus are in G2 (Padovan-Merhar et al., 2015). Using this method, we found that 40% of cells were in G1, 31% in S, and 29% in G2 (Figure S5D). Since the β-globin locus is early replicating in erythroid cells (Goren et al., 2008), we conservatively assumed that each S and G2 cell had 4 copies, resulting in an average of 3.2 copies per cell (40% of 2 alleles + 60% of 4 alleles = 3.2 alleles). However, we also carried out calculations with different possible numbers of alleles: 2.6 alleles (which assumes that globin is replicated at the end of S phase) and 3 alleles (if globin is replicated near the middle of S) (Figures S5E and S5F).

Using this estimate for allele number, we quantified allelic transcription in cells from four human donors. Co-transcription in cis represented 1.1%–3.0% of total globin locus copies depending on the donor (example shown in Figure 5C). We then measured the relationship between transcription of β- and γ-globin in cis using the Fisher exact test (Figures 5B–5D, Table S5). The results suggest that allelic co-transcription of β- and γ-globin is mutually inhibitory, with odds ratios ranging from 0.39 to 0.76 depending on the donor (Figures 5B–5D). Note that this was the most conservative measurement of the odds ratio of allelic β- and γ-globin co-transcription. If the globin locus were not replicated at the very beginning of S phase in all cells, then the average number of alleles per cell would be less than 3.2. Estimating either 3.0 or 2.6 globin alleles per cell on average reduces the odds ratios for each donor and decreases all p values below p = 0.05 (Figures S5E and S5F). This analysis therefore supports the model that the LCR rapidly switches between promoters to allow co-transcriptional events to occur. In contrast, if the LCR could act on both genes at once, or if one gene could transcribe without LCR contact (models 2 and 3), we would expect to see co-transcription occurring at a higher rate.

Strikingly, we found that the average burst sizes of genes that are co-transcribing are not significantly different from those genes that are not, consistent with the model of dynamic enhancer switching (model 1) and the finding above that changing enhancer-promoter contacts does not alter burst size (Figure 5E).

If the LCR switches dynamically between genes (model 1), a further prediction would be that γ-globin burst sizes would not be correlated with β-globin burst sizes because bursts detected by FISH appear largest in the middle of the bursting interval and diminish during the beginning and end of the burst. In contrast, model 2 (simultaneous contacts of the LCR with both globin genes) would predict a positive correlation in the burst sizes since LCR activity on a target gene increases burst size. We observed a lack of correlation between allelic β- and β-globin burst sizes, consistent with transcription of the two genes occurring in a staggered fashion and lending further support for model 1 (Figure 5F).

In sum, our results agree with a model in which the LCR interactions with the β-globin gene disfavor those with the γ-globin gene and vice versa, accounting for competition between the two genes. Definitive evidence for this model would require direct imaging of LCR-promoter contacts in live cells, which is not yet possible.

DISCUSSION

We employed single-molecule RNA FISH to quantify transcriptional bursting parameters at the murine and human β-globin loci during cellular differentiation and following targeted changes to LCR-promoter contacts. During erythroid maturation, both β-globin burst fraction and burst size increase. Deletion of the LCR dramatically reduces burst fraction with comparatively modest effects on burst size. In order to selectively interrogate the influence of LCR-promoter contacts on bursting parameters, looped contacts were forged via targeted tethering of the self-association domain of Ldb1. Forced enhancer-promoter contacts increased burst fraction without affecting burst size. In concert, these findings suggest a connection between enhancer-promoter contact frequencies and burst fractions. The γ-globin and β-globin genes positioned in cis can be co-expressed but compete for LCR contacts and tend not to be initiated simultaneously, consistent with rapidly alternating and highly flexible looped enhancer promoter contacts.

The finding that the LCR influences increase in both the burst fraction and size is consistent with a previous report (Bender et al., 2012). However, single molecule RNA FISH experiments in the present study enabled a more quantitative assessment of burst parameters, revealing that augmenting burst fraction is the predominant mechanism by which the LCR enhances β-globin transcription. To bypass potential secondary effects of LCR deletion and to examine the consequences specifically of LCR-promoter contact frequency on bursting parameters, we employed a forced LCR-promoter looping approach. Notably, the looping-inducing construct ZF-SA triggered increased burst frequency to the same extent as did GATA1-induced maturation, but without modulating burst size. We saw essentially the same behavior in primary human cells under conditions in which the LCR-associated protein complexes and cell maturation stage are presumed to be largely unperturbed. This confirms that the major regulatory function of the LCR is to increase the likelihood of the β-globin genes to be actively transcribed. These conclusions are in line with observations made with live-cell imaging, which showed that increasing levels of
transcription factor binding to a reporter construct predominantly increased burst fraction with little effect on burst size or duration (Larson et al., 2013).

These data beg the question of how exactly enhancer looping governs burst occurrence. Is enhancer-promoter contact required only to initiate a transcriptional burst, or does it have to persist for the duration of the burst? Solving this issue definitively would require both live imaging of transcription as well as measuring the duration of enhancer-promoter contacts in live cells, the latter of which is technically difficult. The observation that co-transcriptional spots of γ-globin and β-globin only persist for a few minutes after cessation of transcription suggests that enhancer-promoter contacts and the resulting transcriptional burst are indeed very dynamic on a scale of minutes in the case of this locus. We acknowledge that temporal inferences on LCR promoter-contact oscillations and burst lengths are limited by the half-life of the intron used to image transcription. Therefore, it remains possible that the contact durations and/or the burst lengths are even shorter than our measurements allow.

It remains unclear how regulatory elements such as the LCR contribute to burst size during cell maturation, although our data suggests that the mechanism is independent of enhancer-promoter contact. One possibility is that increases in transcription factor occupancy at the locus or progressive changes in histone modifications that accompany maturation promote RNA polymerase density or processivity (Forsberg et al., 2000; Johnson et al., 2003; Letting et al., 2003). LCR deletion may block such maturation-induced changes: LCR deletion in G1E-ER4 cells led to reduced occupancy at the promoter by GATA1 (S.C.H. and G.A.B., unpublished data). However, loss of the LCR in whole animals lost chromatin accessibility, histone acetylation, and transcription factor occupancy at the promoters largely intact (Epner et al., 1998; Reik et al., 1998; Song et al., 2010; Vakoc et al., 2005). The LCR may also contribute to burst size by stimulating transcription elongation via physical contacts with the gene body (Kolovos et al., 2012; Larkin et al., 2012; Lee et al., 2015). Our forced looping approach predominantly facilitates enhancer contacts with the promoter while increasing gene body contacts less than GATA1-induced maturation does (Deng et al., 2012). An increase in elongation rate could allow more polymerase molecules to traverse the gene body in quick succession, thus increasing burst size. Therefore, decreased GATA1 occupancy, reduced LCR-gene-body contacts, or related consequences of LCR deletion likely account for the reduction in transcriptional burst size. However, we also acknowledge inherent technical limitations to the ability to distinguish burst size and fractions under conditions in which transcription is extremely rare, such as in the LCR-deficient cells.

Co-expression in cis of γ- and β-globin genes could result from simultaneous contacts of the LCR with both genes or from rapidly alternating contacts of the LCR to the two genes such that one interaction disfavors the other. Our results clearly support the latter model since active transcription of one gene lowers the probability of transcription of the other gene. Additional support for alternating LCR-gene contacts derives from the lack of correlation in burst sizes between γ- and β-globin genes co-transcribed in cis. Prior experiments involving pharmacologic transcription elongation blockage followed by release into productive elongation showed that co-transcriptional FISH signals for globin genes in cis re-appeared slower than signals from individually transcribing genes (Gribnau et al., 1998). Together with experiments showing that the LCR promotes phosphorylation of RNA polymerase II and elongation (Sawado et al., 2003; Song et al., 2007), the simplest model accommodating prior and current findings is that the LCR forms looped contacts with the promoters to increase the probability of paused RNA polymerase to convert into the actively elongating form.

If two genes in cis compete for LCR function in a mutually exclusive manner, no co-transcription should be detected, yet co-transcription appears at approximately 1.1%–3.0% of alleles. However, RNA FISH experiments are limited in their temporal resolution by the half-life of the introns under investigation. In our experiments, duration of RNA FISH signals was measured to average ~10 min following transcription inhibition. Thus, any fluctuations in LCR-gene contacts at a timescale below 10 min would appear as co-transcription even if LCR-promoter contacts are entirely mutually exclusive. However, we cannot not rule out rare simultaneous LCR contacts or occasional enhancer-independent firing that might lead to truly concurrent transcription. Addressing such possibilities might be feasible once live imaging techniques have reached the required spatial and temporal resolution.

Future studies will examine the generality of enhancer effects on transcriptional bursting. The LCR is a well-studied example, but distinct modalities of enhancer actions are a likely possibility. Moreover, different drug treatments or transcription factor perturbations may alter bursting in other ways that may help understand the molecular underpinnings of transcriptional control.

**EXPERIMENTAL PROCEDURES**

**Murine Cell Culture, Infection, and Sorting**

G1E cells and G1E-ER4 cells were cultured and differentiated as described (Weiss et al., 1997).

Cells were infected with the MIGR-1 retrovirus expressing only GFP or mZF-SA followed by an IRES element and GFP (Deng et al., 2012). Infections were performed as described (Tripic et al., 2009). Cells were expanded for 2 days and sorted using a BD FacsAria to purify GFP+ infected cells from control mZF-SA samples. Finally, estradiol was added for 9 hr and transcription was measured by FISH or qPCR.

**Human Primary Cell Culture, Infection, and Sorting**

Human peripheral blood mononuclear cells were obtained from de-identified healthy blood donors after informed consent by the University of Pennsylvania Stem Cell Core. CD34+ hematopoietic precursor cells were isolated from peripheral blood mononuclear cells using CD34+ magnetic bead positive selection. Cells were expanded in SFEM media (StemCell) with 10 μM hydrocortisone (Sigma Aldrich), 100 ng/ml SCF, 5 ng/ml IL-3, 100 ng/ml erythropoietin (Peprotech); protocol adapted from Guda et al. (2015), Neidez-Nguen et al. (2002), and Sankaran et al. (2008). For FISH studies measuring co-transcription, cells were examined after 9–12 days of expansion.

For HZF-SA experiments, cells were infected after 8–11 days of expansion. HZF-SA was expressed in a lentivirus driven by the ankyrin promoter and coupled to IRES-GFP. The control vector expressed only GFP (Deng et al., 2014). Infections were carried out as described (Deng et al., 2014). The expansion phase was extended for 7 more days, and then cells were sorted and examined.

For human erythroid maturation experiments, cells were expanded for 11 days after isolation, then cells were differentiated for 3 days in SFEM with...
3 U/ml erythropoietin without other cytokines such that d0 and d3 (as in Figures 4A and 4B) correspond to day 11 and day 14 of total culture, respectively.

Actinomycin D treatment was performed by adding 1 μg/ml of actinomycin D to human cells for the stated period of time, after 8–11 days of expansion of cells.

**Generation of Enhancer-Deleted G1E-ER4 Cells**

Cas9 and guide RNA plasmids were transiently co-transfected into G1E-ER4 cells using an Amaxa Nucleofector 2b (Lonza, program G-016, reagent kit R). Transfected single cells were sorted into a 96-well plate using a FACS Aria II (BD Biosciences). Single-cell clones were expanded and screened by PCR, followed by DNA sequencing.

**Chromatin Immunoprecipitation**

We performed ChIP as previously described (Letting et al., 2004), using the N-20 Pol II antibody (Santa Cruz sc899). ChiP-qPCR was performed with Power SYBR Green (Invitrogen).

**qRT-PCR**

We isolated RNA using TRIzol (Life Technologies). Reverse transcription was performed using Power SYBR Green (Invitrogen).

**Single-Molecule RNA FISH Imaging**

We performed single-molecule RNA FISH as described previously (Femino et al., 1998; Raj et al., 2008, 2009). Briefly, we fixed cells in 1.85% formaldehyde for 10 min at room temperature and stored them in 70% ethanol at 4°C until imaging. FISH probes consisted of oligonucleotides conjugated to fluorescent dyes. We hybridized pools of FISH probes to samples, followed by DAPI staining and wash steps performed in suspension. Samples were cytospun onto slides for imaging on a Nikon Ti-E inverted fluorescence microscope.

**Image Analysis**

We manually segmented boundaries of cells from bright-field images and localized RNA spots using custom software written in MATLAB (Raj et al., 2010), with subsequent analyses performed in R. Transcription sites for mouse β-globin, Gata2, and human γ-globin are identified by co-localization of spots in the intron and exon channels for a given mRNA and for human β-globin by bright nuclear intron spots. Alleles co-transcribing human γ-globin and β-globin are identified by colocalization of transcription sites. Fluorescence intensities of transcription sites were determined by 2D Gaussian fitting on processed image data.

**Plotting and Graphics**

We used R packages dplyr and ggplot2 to produce nearly all figures, followed by DAPI staining and wash steps performed in suspension. Samples were cytospun onto slides for imaging on a Nikon Ti-E inverted fluorescence microscope.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2016.03.007.

**AUTHOR CONTRIBUTIONS**

C.R.B., A.R., and G.A.B. conceived and designed the study; C.R.B., S.C.H., and C.C.-S.H. carried out experiments; and C.R.B., A.R., and G.A.B. wrote the manuscript with input from all authors.

**ACKNOWLEDGMENTS**

The authors thank members of the Blobel and Raj labs for careful reading and suggestions. This work was supported by an NIH GRFP fellowship to C.R.B.; NIH New Innovator Award 1DP2OD008514 to A.R.; NIH R03 1R33EB019767 to A.R., an NSF CAREER Grant Number 1350601 to A.R., 1R01 HL119479 to G.A.B., and NIH 1U01HL129998 to A.R. and G.A.B.

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**REFERENCES**


Supplemental Information

Enhancer Regulation of Transcriptional Bursting
Parameters Revealed by Forced Chromatin Looping

Caroline R. Bartman, Sarah C. Hsu, Chris C.-S. Hsiung, Arjun Raj, and Gerd A. Blobel
Figure S1, related to Figure 1. A) Effect of actinomycin D treatment on transcription site persistence, n=3 human donors, cell numbers for 0min, 10min, 30min and 60min samples were 105, 59, 64, 120; 118, 72, 84, 96; and 154, 111, 112, 150 for each replicate respectively. B) Fluorescence intensity of cyclin A2 mRNA from the same experiments shown in 1D and 1E, n=3 biological replicates, number of cells for 4, 9, 24 and 48h time points were 168, 180, 105, 98; 234, 151, 116, 99; and 433, 154, 130 and 103 for the 3 replicates respectively. C) Proportion of cells with different numbers of alleles transcribing β-globin for one replicate from 1D and 1E. Cell numbers were 234, 151, 116, 99 for 4hr, 9hr, 24hr and 48hr time points, representative of 3 biological replicates. D) Correlation of fluorescence intensities in cells with two transcription sites (pooled data from 3 experiments, 9h maturation time point as shown in Figure 1D-1E), number of cells with two transcription sites were 25, 16, and 47 for each replicate. E) Correlation of fluorescence intensities in cells with two transcription sites (pooled data from 3 experiments, 24h maturation time point as shown in Figure 1D-1E), number of cells with two transcription sites were 13, 16, and 43 for each replicate. F) Correlation of fluorescence intensities in cells with two transcription sites (pooled data from 3 experiments, 48h maturation time point as shown in Figure 1D-1E), number of cells with two transcription sites were 7, 7, and 26 for each replicate. All error bars represent SEM.
Figure S2, related to Figure 1. A) Representative images of Gata2 transcription sites in G1E-ER4 cells after 0 or 13 hours estradiol addition. Red: Gata2 intron probes; green: Gata2 exon probes. C) Fluorescence intensity of Gata2 transcription sites in G1E-ER4 cells at 0 or 13 after estradiol addition in a representative experiment (number of transcription sites per time point: 0hr 142, 13hr 43; number of cells per time point: 0hr 99, 13hr 131). D) Mean number of Gata2 transcription sites per cell at 0 or 13 hours after estradiol addition. N=3 biological replicates, number of cells in 0 hours condition was 99, 101 and 156, number of cells in 13 hours condition was 131, 120, 119. E) Fluorescence intensity of Gata2 transcription sites in G1E-ER4 cells at 0 or 13 hours after estradiol addition. N=3 biological replicates, number of cells in 0 hours condition was 99, 101 and 156, number of cells in 13 hours condition was 131, 120, 119; number of transcription sites in 0 hours condition was 142, 151, 204, number of transcription sites in 13 hours condition was 43, 21, 29. All error bars represent SEM.
Figure S3, related to Figure 2. A) Schematic of Cas9 gRNA target sites for LCR deletion and PCR amplicon selection for screening of clones. B) PCR screening of WT G1E-ER4 cells and LCR KO clones 1 and 2, confirming deletion of the LCR and formation of a novel junction amplicon. C) Sanger sequencing of LCR region of LCR KO clone 1, showing sequence of deletion. D) Sanger sequencing of LCR region of LCR KO clone 2, showing sequence of deletion. E) qPCR of β-globin mRNA in wild-type and LCR KO clones matured for 24h with estradiol, N=3 biological replicates. F) qPCR of Alas2 mRNA in wild-type and LCR KO clones matured for 24h with estradiol, N=3 biological replicates. G) qPCR of α-globin mRNA in wild-type and LCR KO clones matured for 24h with estradiol, N=3 biological replicates.
Figure S4, related to Figures 3 and 4. A) β-globin nascent RNA measured by RT-qPCR in G1E-ER4 cells induced for 9 hours with estradiol, infected with control vector or mZF-SA expressing vector, normalized to GAPDH mRNA and control (n=3). B) γ-globin mRNA measured by RT-qPCR in primary human erythroid cells infected with control vector or hZF-SA expressing vector, normalized to GAPDH mRNA and control (n=5 donors). C) β-globin mRNA measured by RT-qPCR in primary human erythroid cells infected with control vector or or hZF-SA expressing vector, normalized to GAPDH mRNA and control. Paired t-test, p=0.13. (n=5 donors). Error bars represent SEM.
Figure S5, related to Figure 5. A) Model demonstrating how cellular β- and γ-globin transcription was quantitated in primary human cells using RNA FISH data. B) Example of cellular β- and γ-globin transcription from a single donor (636 total cells). Fisher exact test odds ratio=1.48, p-value=0.052. C) Fisher exact test odds ratio and 95% confidence interval for 4 human samples. (Cells per sample: A 423, B 179, C 636, D 1066). D) Cell cycle phase identification of human primary cells, and calculation of predicted average number of globin alleles per cell. (Prediction is required given that RNA FISH does not measure non-transcribing alleles, but this number is needed to calculate statistical independence of co-transcription). E) Fisher exact test odds ratio and 95% confidence interval for 4 human samples for competition between cis-competition of globin alleles, estimating 3.0 alleles per cell. (Cells per sample: A 423, B 179, C 636, D 1066). F) Fisher exact test odds ratio and 95% confidence interval for 4 human samples for cis-competition of γ- and β-globin alleles, estimating 2.6 alleles per cell. (Cells per sample: A 423, B 179, C 636, D 1066).
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Table S3, related to Figure 3.
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*Table S4, related to Figure 4.*
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Table S5, related to Figure 5
Supplemental Experimental Procedures

Murine cell culture, infection and sorting
G1E cells were previously derived through deletion of GATA1 in mouse embryonic stem cells, followed by in vitro differentiation (Weiss et al., 1997). We cultured a subline of G1E cells, G1E-ER4, in which GATA-1-ER was transduced by retrovirus (Weiss et al., 1997). G1E-ER4 cells were induced to mature by the addition of 100nM estradiol to culture media.

Cells were infected with the MIGR-1 retrovirus expressing only GFP or mZF-SA followed by an IRES element and GFP (Deng et al., 2012).

In order to produce retrovirus, HEK293T cells were plated and grown to 90% confluence in a 10ml plate. Media was removed and replaced with 4ml warm media. Transfection buffer consisted of 0.5ml 0.5M HEPES pH 7.1, 0.45ml 2M NaCl, 10ul of 100mM Na2HPO4, and 4.05ml water. DNA mixture consisted of 62.5 microliters of 2M CaCl2, 50 microliters of NTE buffer (8.66g NaCl, 10ml 1M Tris-HCl pH 7.4, 2ml 0.5M EDTA pH 8.0, remainder of 100ml H2O), 15 microliters MIGR1 plasmid (1 microgram/microliter), 15 microliters pCL-Eco packaging plasmid (1 microgram/microliter), and 357.5 microliters H2O. 500 microliters of the transfection buffer was then added to DNA mixture in a dropwise fashion, then slowly mixed by pipet 12 times. This mixture was added dropwise to cells in 10 ml dish (now in 4 ml media). Cells were replaced in incubator. Media was changed at 6 hours post infection. Media was harvested at 48 hours post infection.

For retroviral infection, two million G1E or G1E-ER4 cells were plated in a 12-well plate in 1.5 ml media, to which 500 microliters of 48 hour transfected supernatant (as above) was added, along with 8 microgram/microliter polybrene and 10mM HEPES. Cells were spun at 500xg for 2 hours at room temperature, then media was changed and cells were replaced in incubator.

Cells were expanded for two days and sorted using a BD FACS Aria II to purify GFP+ infected cells from control and mZF-SA samples. Finally, 100nM estradiol was added for 9 hours and transcription was measured by FISH or qPCR.

Human primary cell culture, infection and sorting
Human peripheral blood mononuclear cells were obtained from de-identified healthy blood donors after informed consent by the University of Pennsylvania Stem Cell Core. CD34+ hematopoietic precursor cells were isolated from peripheral blood mononuclear cells using CD34+ magnetic bead positive selection (CD34+ MicroBead Kit, Miltenyi Biotec, #130-046-702; MS magnetic columns, Miltenyi Biotec, #130-042-201). Cells were expanded in SFEM media (StemCell) with 10^6M hydrocortisone (Sigma Aldrich), 100 ng/ml SCF, 5ng/ml IL-3, 100ng/ml erythropoietin (Peprotech), protocol adapted from (Guda et al., 2015; Neildex-Nguyen et al., 2002; Sankaran et al., 2008).

For FISH studies measuring co-transcription, cells were examined after 9-12 days of expansion.

For hZF-SA experiments, cells were infected after 8-11 days of expansion. hZF-SA was expressed in a lentivirus driven by the ankyrin promoter, and coupled to IRES-GFP (Deng et al., 2014). The control vector expressed only GFP. Infections were carried out as described above (Deng et al., 2014). The expansion phase was extended for 7 more days, and then cells were sorted and examined.

For human erythroid maturation experiments, cells were expanded for 11 days after isolation, then cells were differentiated for 3 days in SFEM with 3U/ml erythropoietin without other cytokines such that d0 and d3 (as in Figure 4A-B) correspond to day 11 and d14 of total culture respectively.

Actinomycin D treatment was performed by adding 1 microgram/ml of actinomycin D to human cells for the stated period of time, after 8-11 days of expansion of cells.

Generation of enhancer-deleted G1E-ER4 cells
Cas9 guide RNA design was performed using crispr.mit.edu. Cas9 and guide RNA plasmids were transiently co-transfected into G1E-ER4 cells using an Amaxa Nucleofector 2b (Lonza, program G-016, reagent kit R). Transfected single cells were sorted into a 96 well plate using a FACS Aria II (BD Biosciences). Single cell clones were expanded and screened by PCR, followed by DNA sequencing. Guide RNA sequences are as follows: gRNA Clone 1 ACTGGGAATGACCTTGTCAA, gRNA Clone 2:GAGATGTGAGTTTACAGTTC gRNA Shared: GGAAGAATATGTTTGGCTA. PCR primers for screening were: -2.5kb F: TCAAGGAAACTGCAATGGAA, -2.5kb R: TTTGCTGCTTGGGAACACT+,+3kb F: TAGCCAGAAACCCACTGAG,+3kb R: ATAAGCAGTCCCTGCCCCTT, +10kb F: AAAGGAACCTGGAATTTACTGG,+10kb R: AACCACACTCCCAATGTCCCCA,+18kb F: ATGAGGCTCCTCACTCTCC,+18kb R: GCATTAGCCTTCACCTCCAC, Scr F: CCAATCTGCTGGTGTTCTT,Scr R: CTTTCCCAACCTTTTTCCA.
Chromatin Immunoprecipitation

ChIP-seq of total Pol II was performed using N-20 (Santa Cruz, cat.# sc899). ChIP was performed as follows on approximately 10 million cells for each sample. Protease inhibitor (P8340, Sigma) was added to the following buffers right before use: Cell Lysis Buffer (10mM Tris pH 8, 10mM NaCl, 0.2% NP-40/Igepal), Nuclear Lysis Buffer (50mM Tris pH 8, 10mM EDTA, 1% SDS), and IP Dilution Buffer (20mM Tris pH 8, 2mM EDTA, 150mM NaCl, 1% Triton X-100, 0.01% SDS).

Agarose beads slurry was prepared by mixing Protein A (Invitrogen 15918014) and Protein G (Invitrogen 15920010) agarose beads at 1:1 ratio, washed with PBS 3 times, and the mixed beads resuspended in 1:1 volume in PBS (volumes indicated below are for the slurry in PBS, but PBS is removed by centrifugation and aspiration prior to bead use). For use in "pre-clearing" step, 50 micrograms rabbit IgG was mixed with 50 microliters agarose beads slurry. For use for immunoprecipitation step, 70 microliter bead slurry was mixed with 10 micrograms antibody for each immunoprecipitation and incubated overnight at 4 degrees C to allow binding ("pre-bound").

Additional buffers were prepared as follows: IP Wash Buffer 1 (20mM Tris pH 8, 2mM EDTA, 50mM NaCl, 1% Triton X-100, 0.1% SDS), High Salt Buffer (20mM Tris pH 8, 2mM EDTA, 500mM NaCl, 1% Triton X-100, 0.01% SDS), IP Wash Buffer 2 (10mM Tris pH 8, 1mM EDTA, 0.25mM LiCl, 1% NP-40/Igepal, 1% Nonidet P-40, 1% SDS), Elution Buffer (100mM NaHCO3, 1% SDS).

All steps performed on ice or at 4 degrees C unless otherwise noted. Cells were fixed in 1% formaldehyde in PBS for 10 minutes with agitation, then quenched for 5 minutes with 0.125 M glycine. Fixed cells were washed with PBS, then resuspended in 1ml Cell Lysis Buffer and incubated for 20min on ice, then resuspended in 1ml Nuclear Lysis Buffer and incubated for 20min on ice, then diluted with 0.6 ml of IP Dilution Buffer. Samples sonicated at 4 degrees C for 45 minutes using either EpiShper (Active Motif), then centrifuged at 21130g for 10 min to remove cellular debris. Supernatant was transferred to new tube and mixed with "pre-clear" beads and rotated at 4 degrees C overnight, then centrifuged at 821g to pellet beads. Two hundred microliters of supernatant was saved as "input." The remaining supernatant was mixed with beads prebound with antibodies and rotated overnight at 4 degrees C. Beads were washed sequentially once with IP Wash Buffer 1, twice with High Salt Buffer 1, once with IP Wash Buffer 2, then twice with Tris-EDTA pH 8.0 (BP2473-1, Fisher Scientific) with centrifugation at 5283g for 2min and aspiration of supernatant before resuspension with each subsequent buffer). Then, beads were pelleted by centrifugation and supernatant aspirated.

All steps from this point on performed at room temperature unless otherwise noted. Beads were resuspended in 100 microliters Elution Buffer twice sequentially and the supernatant from both steps combined for a final eluate volume of 200 microliters. Twelve microliters of 5M NaCl and 2 microliters of 10mg/ml RNase A (10109169001, BMB) were added to the 200 microliter eluted samples and 200 microliter input samples and incubated overnight at 65 degrees C. Then, 3 microliters of 20mg/ml Proteinase K (3115879, BMB) was added and incubated at 65 degrees C for an additional 2h. Ten microliters of 3M sodium acetate pH 5 was added to each sample and DNA purified per the instructions of the QIAquick PCR Purification Kit (cat.# 28106, Qiagen). Inputs were eluted in 133.4 microliters water and immunoprecipitated samples in 60 microliters water. DNA was stored at -20 degrees C until further processing.

ChIP-qPCR was performed with Power SYBR Green (Invitrogen). Standard curves were constructed using the ChIP input to allow normalization of sample Ct values as follows: each input was diluted 1:10, then three further serial dilutions at 1:4 were performed. ChIP qPCR primers were as follows: mouse β-globin promoter F: CAGGGAGAAATATGCTTGTCATCA; mouse β-globin promoter R: GTGAGCAGATTGGGCCCCTACC; mouse β-globin exon 2 F: AACCAGTGCCCTGAATCCTTGG; mouse β-globin exon 2 R: AGCCCTGAAGTTCTCAGGATCC; mouse β-globin intron 2 F: CTTCCTCTCTTCTCTTCTCTTTCTTAAAT; mouse β-globin intron 2 R: AATGAAACTGAGGAAAGGAAAGG; mouse β-globin 3'UTR F: GCCCTGGCTCAAGAATCCA; mouse β-globin 3'UTR R: TTCACAGGCAGCAGGAA; mouse CD4 F: CCAGAACATTCGGCAGCATT; mouse CD4 R: GGTAAGAGGAGGTGTGTCACCTTT.

RT-qPCR

We isolated RNA using TRIzol (Life Technologies) and the RNEasy mini kit (Qiagen). Reverse transcription was performed with iScript (Bio-Rad). qPCR was performed with Power SYBR Green (Invitrogen). Transcript levels were quantified using the delta-delta-CT method and normalized to GAPDH. RT-qPCR primers were as follows: mouse β-globin nascent RNA F: GCCCTGAGATCTGGTATTTTCT; mouse β-globin nascent RNA R: TGGAAATCTTGCCGCCAGGT; mouse Alas2 mRNA F: TATGTGCAGGCCATCAACTACCCA; mouse Alas2 mRNA R: TTTCCATCATCTGAGGCTGGT; mouse α-globin mRNA F: CACCACAAAGACCTAATTCC; mouse α-globin mRNA R: CAGTGCGTCAGAGGCTTTA; mouse GAPDH F:
AGGTGTGTCCCTGCGACTTCA; mouse GAPDH R: CCAGGAAATGAGCTTGACAAAG; human β-globin mRNA F: TGGGCAACCCTAAGGTGAAG; human β-globin mRNA R: GTGAGCCAGGCCATCACTAAA; human γ-globin mRNA F: TGGCAAGAAGGTGCTGACTTC; human γ-globin mRNA R: GCAAAGGTGCCCTTGAAGATC; human GAPDH F: ACCACAGTCCATGCCATC; human GAPDH R: CCATCACGCCACAGTTC.

Single-molecule RNA FISH imaging
We performed single-molecule RNA FISH as described previously (Femino et al., 1998; Raj et al., 2006; 2008). Briefly, we fixed cells in 1.85% formaldehyde for 10 min at room temperature, and stored them in 70% ethanol at 4 degrees C until imaging. FISH probes consisted of oligonucleotides conjugated to fluorescent dyes as follows: human β-globin to Alexa594 or Cy3, human γ-globin intron to Alexa594 or Cy3, human γ-globin exon to ATTO 647N, mouse β-globin intron to Alexa594, mouse β-globin exon to Cy5, human HIST1H4E to ATTO 647N, Gata2 introns to Alexa594, Gata2 exons to Cy3. We hybridized pools of FISH probes to samples, followed by DAPI staining and wash steps performed in suspension. Samples were cytospun onto slides for imaging on a Nikon Ti-E inverted fluorescence microscope using a 100x Plan-Apo objective (numerical aperture of 1.43), a cooled CCD camera (Pixis 1024B from Princeton Instruments), and filter sets SP102v1 (Chroma), SP104v2 (Chroma), and 31000v2 (Chroma) for Cy3, Cy5/ATTO 647N, and DAPI, respectively. Custom filter (Omega) was used for Alexa594. We took optical z-sections (typically 45) at intervals of 0.35 microns, spanning the vertical extent of cells, with 1s exposure time for Cy3, Cy5, ATTO 647N, and Alexa594, and 100ms for DAPI.

Image Analysis
We manually segmented boundaries of cells from bright field images and localized RNA spots using custom software written in MATLAB (Raj et al., 2010), with subsequent analyses performed in R. Transcription sites for mouse β-globin, Gata2, and human γ-globin are identified by co-localization of spots in the intron and exon channels for a given mRNA, and for human β-globin by bright nuclear intron spots. Alleles co-transcribing human γ-globin and β-globin are identified by colocalization of transcription sites. Fluorescence intensities of transcription sites were determined by 2D Gaussian fitting on processed image data.

Plotting and graphics
We used R packages plyr and ggplot2 to produce nearly all figures, followed by cosmetic adjustments in Adobe Illustrator. Several figures were produced using Graphpad Prism.