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Broadening of DNA replication origin usage during metazoan cell differentiation

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Abstract

We have examined whether replication of the chicken β-globin locus changes during differentiation of primary erythroid progenitors into erythrocytes. In undifferentiated progenitors, four major initiation sites and a replication fork pausing region (RFP) were observed. Forty-eight hours after induction of differentiation, the major sites were maintained, even within the activated β^A-globin gene, some minor sites were enhanced, three new sites appeared and the RFP disappeared. One of the activated origins showed increased histone H3 K9K14 diacetylation, but the others did not. These results demonstrate a broadening of DNA replication origin usage during differentiation of untransformed metazoan cells and suggest that neither histone H3 diacetylation, nor other histone modifications so far reported, nor transcription, are crucial determinants of origin selection in this system.

Introduction

Replication initiates at specific locations along the chromosomes of animal cells (for review (Gilbert, 2004). Origin types range from highly specific, efficient initiation sites (e.g.,(Kitsberg et al., 1993) to broad zones of multiple, inefficient sites (e.g; (Dijkwel et al., 2002). No clear consensus sequence has emerged from characterized origins. Origin specification is thought to involve recognition of structural and functional features of chromatin and not simply DNA sequences.

Origin usage is developmentally regulated. In Xenopus and Drosophila, initiation sites are distributed at random sequences in early embryos, but are confined to intergenic spacers at the onset of zygotic transcription, and are further rarefied during later development (Hyrien et al., 1995; Maric et al., 1999; Sasaki et al., 1999). Origin modulation by transcription was also reported for the initiation zone located downstream of the Chinese hamster dihydrofolate reductase (DHFR) gene. Promoter deletions that eliminate transcription allow the body of the DHFR gene to become a template for initiation (Saha et al., 2004). Conversely, invasion of the initiation zone by the transcription machinery due to deletions of the 3' end of the gene inactivate the origins (Mesner and Hamlin, 2005). Thus, potential origins appear to be widespread, but transcription can inactivate them. In the fly *Sciara*, transcriptional regulation has also been associated with the contraction of an initiation zone during DNA puff II/9A amplification. The initiation zone spans two quiescent transcription units in preamplification stages but contracts to a small area upstream of the genes, simultaneously with the appearance of RNA polymerase II at the upstream gene promoter, but before intense transcription (Lunyak et al., 2002). On the other hand, the binding of transcription factors to plasmid DNA can increase initiation at the factor binding site in Xenopus egg extracts (Danis et al., 2004), and the mapping of origins and RNA pol II along a Drosophila chromosome suggests that

transcription somehow facilitates ORC localization (MacAlpine et al., 2004). Thus, cellular differentiation might influence replication initiation by various mechanisms.

Developmental activation of cell-type specific origins was first reported in the slime mould Physarum polycephalum (Maric et al., 2003) and recently for the murine IgH locus (Norio et al., 2005). Due to the limited resolution (~5kb) with which the latter origins were mapped it remains unclear whether their activation correlates with specific changes in chromatin structure. Moreover, this report compared cell lines and did not document dynamic changes as they occur (Gilbert, 2005).

We report here for the first time dynamic metazoan origin activation using a homogeneous *ex-vivo* differentiation system. We have analysed how the replication of the chicken β−globin locus and surrounding sequences changes during differentiation into erythrocytes. The 55 kb region studied contains four independent chromatin domains : a folate receptor (FR) gene, a 16 kb stretch of condensed chromatin, the 30 kb β-globin gene cluster, and an olfactory receptor gene, separated from one another by three DNAse I-hypersensitive sites (HSA, 5'HS4 and 3'HS) (Figure 1B). Previously, we analysed the replication initiation pattern of the FR/β-globin region in the transformed 6C2 cell line (Prioleau et al., 2003). The 6C2 cell line mimics the CFU-E stage, which precedes the final differentiation in erythrocytes. We identified four replication origins located at the 5'HS4 insulator, 5' and 3' of the silent ρ-globin gene, and over a broader 1.5 kb region that covers the β^A-globin gene and its promoter. We showed that the four origins fire early in S phase, even though they harbor different epigenetic modifications. The 5'HS4 origin has unmethylated CpGs and hyperacetylated histones, whereas the other origins have methylated CpGs (if any) and hypoacetylated histones. Significant histone acetylation is only found over HSA and 5'HS4 in these cells. None of these epigenetic marks appears to be an obligatory feature of active origins. On the other hand, the overall condensed chromatin structure of the locus in 6C2 cells

may restrain initiation to specific sites by suppressing weak potential origins. To test this possibility, we have looked whether initiation becomes more dispersed during later differentiation, when the β-globin locus starts to decondense.

Results and Discussion

In vitro differentiation of chicken erythroid progenitors and replication mapping strategy

We used recently developed methods to obtain large amounts of normal chicken erythrocytic progenitors and induce their differentiation into erythrocytes *ex vivo* (Gandrillon et al., 1999). In this system, immature eythrocytic progenitors purified from chick bone marrow are maintained in culture with TGF-α, TGF-β and dexamethasone at an early immature stage (late BFU-E to early CFU-E). These normal avian progenitors, referred to as T2ECs, can be induced to synchronously enter into terminal differentiation in erythrocytes by withdrawal of TGF-α, TGF-β and dexamethasone and addition of anaemic chicken serum plus insulin. The synchronous progression of differentiation was confirmed by a decrease in the expression of the immature-specific MEP26 antigen and an increase in the expression of the mature-specific antigen JS4 (Figure S1A). As shown in Figure S1B, activation of globin genes started during the first cell division after induction of differentiation.

To map replication origins within the chicken FR/β-globin region, we quantified the abundance of short nascent strands at different positions along this region before and after induction of differentiation in two independent batches of cells (Fig. 1A and 1C). We used as a negative control a primer pair located upstream of the lysozyme locus, a region confirmed to lack initiation activity (Prioleau et al., 2003). So far we have not found regions that give a lower enrichment than this locus (unpublished data). The fold of enrichment of short nascent strands was therefore calculated with respect to the lysozyme negative control, which was

taken as background. Only enrichments reproducibly higher than 4-fold have been considered significant.

Replication initiation profile of undifferentiated T2ECs

In self-renewing T2ECs (Figure 1, dark circles), the enrichment of nascent strands ranged from 1- to 3-fold over the FR gene, the condensed chromatin region and 3' of the β-globin gene, suggesting a lack of significant initiation over all these regions. Four discrete peaks, however, of significant initiation (black arrows above black circles, Figure 1) were reproducibly observed at the 5'HS4 insulator (76-fold enrichment in Fig 1A and 47-fold enrichment in Fig 1C); upstream (50- and 44-fold) and downstream (100- and 100-fold) of the ρ-globin gene; and over the β^A-globin gene and promoter (128- and 80-fold). In addition, most of the remaining primer pairs located between HS4 and the ε gene showed enrichments of 4- to 12-fold, raising the possibility that the entire β- globin locus is a single large initiation zone. The similarity of the profiles shown in Fig 1A and 1C illustrates the high reproducibility of the results. Furthermore, the initiation profile of undifferentiated T2ECs was virtually identical to that of the transformed 6C2 cell line (Prioleau et al., 2003), showing that it is not affected by cellular transformation.

Activation of tissue-specific origins during differentiation of T2ECs

After only 48 hrs of differentiation, which corresponds to two cell cycles, an increase in the number of potential initiation sites was observed (gray triangles, Figure 1). Whereas the four major sites of initiation observed before differentiation were maintained (black arrows), three previously "dormant" origins were activated (gray arrows) upstream (12- and 8-fold) and downstream (12- and 8-fold) of the folate receptor gene, and 5' (18- and 17-fold) of HS4. In addition, a three-fold increase in nascent strand enrichment was observed at the previously

active site located 3' of HS4 (final enrichments, 28- and 21-fold). Nascent strand enrichment in the 16 kb condensed chromatin region and 3' of the β-globin gene remained in the 1- to 5-fold range, suggesting a lack of significant initiation, as in undifferentiated T2ECs. All these constitutive and tissue-specific origins have been confirmed by analyzing the 1.0-1.5 kb nascent strand fraction from the same batch of differentiating T2ECs as in Fig. 1A (Figure S2). We also reproducibly observed an increase in nascent strand enrichment upstream (primer pair 27) and downstream (primer pair 31) of the β^A-globin gene (Figure 1). The increased enrichments observed around 5'HS4 and the β^A-globin gene might be interpreted as a broadening of origin usage within the β-globin initiation zone rather than as an activation of a small number of discrete sites. Given the already high density of probes we used, testing these alternative interpretations appears beyond resolution of currently available techniques. A high resolution mapping study of the 55 kb CHO DHFR "initiation zone" has previously suggested that it consists of a minimum of 20 (and possibly much more) inefficient initiation sites (Dijkwel et al., 2002).

The strong initiation signal over the β^A-globin gene was confirmed by probing 0.8-1.2 kb and 1.0-1.5 kb nascent strands from two independent batches of differentiated T2ECs with primer pair 29 (Figure 2A and S2). As this amplicon is located in the middle of the 1.5 Kb β^A-globin gene, this result demonstrates that initiation occurred within the coding region. Therefore, induction of β^A expression did not repress initiation within the gene, in contrast with the situation observed for the CHO DHFR gene (Mesner and Hamlin, 2005). Our data are consistent with previous observations that transcription does not restrict initiation inside the coding region of the murine β-globin locus (Aladjem et al., 2002).

Increase in nascent strand abundance over the ρ gene during differentiation of T2ECs

We previously observed that, in 6C2 cells, the abundance of nascent strands up to 5 kb in size is very low over the body of the ρ-gene, despite the close proximity of two flanking origins, and we interpreted this to suggest that replication forks oriented in either direction pause at several positions inside the ρ-globin gene (Prioleau et al 2003). We observed a similar, anomalously low enrichment of short nascent strands with primer pair 23 in undifferentiated T2ECs, suggesting that replication forks also pause inside the ρ-globin gene in these cells (black double arrow below the graphs, Figure 1). The sharpness of the peaks flanking the putative RFP is nevertheless surprising. If forks moving to the right from marker 22 and to the left from marker 24 are arrested inside the ρ-gene, then the only way nascent strands initiated around primers 22 and 24 could grow to 2.0-3.5-kb is by growing past primers 21 and 25, respectively. However, primers 21 and 25 did not amplify their target sequences to an equal extent to primers 22 and 24 (Fig. 1), although they did so when 4.0-5.0 kb nascent strands were analyzed (Prioleau et al., 2005). This discrepancy suggests that nascent strands from this region may migrate faster than their estimated size in sucrose gradients, or that nascent strands may turn over very rapidly from less than 2.0 kb to more than 3.5 kb on both sides of marker 23. In differentiating T2ECs, however, a strong (37- and 35-fold) enrichment of nascent strands was detected with primer pair 23, suggesting that replication forks then progressed unimpeded inside the ρ-globin gene (Figure 1, grey triangles). Identical results were obtained with primer pair 23' (table S1) located 3' of primer pair 23 and overlapping it by 5 bp (Figure 1C and 2B). Analysis of a 0.8-1.2 kb and 1.0-1.5 kb nascent strands showed that this change did not result from activation of a new origin inside the ρ-globin gene, because initiation was only detected 5' and 3' of the ρ-globin gene (Figure 2B and S2). Although we cannot formally exclude that some unknown factors may affect the stability of replication intermediates, we suggest that a replication fork pausing region inside the ρ gene is downregulated during erythroid cell differentiation. Developmental

regulation of multiple RFPs has been previously observed in Xenopus rDNA at the gastrula and neurula stages (Maric et al., 1999).

Changes in histone acetylation associated with differentiation of T2ECs

Histone tails are extensively modified along the β-globin locus during red blood cell differentiation. It was reported that only 5'HS4 is hyperacetylated in 6C2 cells, whereas in mature erythrocytes hyperacetylated histone H3 and H4 are distributed more or less uniformly over the whole locus (Litt et al., 2001b). How these modifications are established during the last six cell cycles before G1 arrest is not known. We therefore mapped diacetylated K9 and K14 histone H3 in self-renewing and differentiating T2ECs by chromatin immunoprecipitation using two independently derived batches of cells (Fig. 3A and B). In undifferentiated T2ECs, four regions of increased diacetylation of histone H3 were observed around (i) the folate receptor promoter, (ii) the 5'HS4 insulator, (iii) the HS321 region, and (iv) the β^A-gene and downstream sequences in two independent experiments (Figure 3, dark circles). The histone acetylation pattern around the folate receptor promoter and the 5'HS4 insulator did not change upon differentiation (Figure 3, grey triangles). We have therefore arbitrarily set the relative recovery of diacetylated histone H3 at 100% for primer pair 5. In contrast, the relative recovery of diacetylated histone H3 over the HS321 region and the β^A-gene increased 2- to 8- and 8- to 13-fold, respectively, upon differentiation of T2ECs (the slight variation in the basal level of acetylation in undifferentiated cells probably reflects the variable background of spontaneously differentiating cells). In order to evaluate the coordination of replication origin activation with histone H3 diacetylation, we placed on Fig 3A black and gray vertical arrows corresponding to constitutive and differentiation-activated origins, respectively. As shown by the star, the enhanced activity of the origin located between HS4 and HS3 was concomitant with an increase in histone H3 diacetylation.

Activation of the three previously dormant origins, however, was not accompanied by any change in histone H3 diacetylation. Two of them (3' of the folate receptor gene, 5' of HS4 insulator) were associated with very low levels of histone H3 diacetylation in both undifferentiated and differentiating T2ECs, whereas the third one (within the FR gene upstream sequences) was significantly acetylated in both. Finally, we noted that the replication fork pausing region located over the ρ gene and downregulated during differentiation, was also associated with a very low level of histone H3 diacetylation in both undifferentiated and differentiating T2ECs.

Our results provide, for the first time, direct evidence for both a tissue-specific broadening of replication origin usage and the repression of a replication fork pausing region in a vertebrate *ex-vivo* differentiation system. Our results also suggest that origin activation may or may not, depending on the origin considered, be concomitant with an increase in histone H3 diacetylation.

The differentiation of chicken erythrocytic progenitors into erythrocytes activates at least four origins inside and upstream of the β-globin locus. Interestingly, the four origins that are active prior to differentiation remain active upon differentiation even when located inside a transcribed coding region. This differentiation-induced broadening of origin usage thus contrasts with the developmental restriction of initiation in Xenopus and Drosophila, from random sites in non-trancribing, early embryos, to intergenic zones, when nearby genes become expressed after the midblastula transition (Hyrien et al., 1995; Sasaki et al., 1999).

We also show that during differentiation, four out of eight regions of initiation are associated with undetectable levels of diacetylated histone H3. These results are consistent with our previous observation that in the 6C2 cell line three out of four sites of initiation are associated with low levels of histone acetylation (Prioleau et al., 2003). Although these results do not formally exclude a role for histone acetylation in the origin transitions observed here,

taken together they suggest that histone acetylation is not crucial for origin specification in either undifferentiated or differentiating cells. Other histone modifications have been mapped at the chicken ß-globin locus (di-Ac K9&K14/H3, di-Me K4/H3, di-Me K9/H3, Ac K8/H4, Ac K9/H4, Ac K12/H4 and multi-acetylated H3 and H4 in red blood cell precursors and in erythrocytes by (Litt et al., 2001a; Litt et al., 2001b); acetylation of H2B and H2AZ by (Bruce et al., 2005; Myers et al., 2003); di-Me R3/H4 by (Huang et al., 2005). Whereas interesting correlations and anticorrelations between these various modifications have been reported, we have been unable to detect any convincing correlation between these histone modifications and origin usage.

Basic Methods

Nascent strands preparation and quantification

Nascent strands were purified and quantified by real-time quantitative PCR as described previously (Prioleau et al., 2003). Primer pair 24 was used as a standard in each PCR experiment.

Chromatin Immunoprecipitation

Formaldehyde cross-linking and immunoprecipitation were performed as described in supplementary material and methods with anti-diacetylated K9&K14 H3 antibodies (Upstate Biotechnology). Two independent immunoprecipitations were performed on two independent batches of differentiation. Real-time quantitative PCR was performed by using primer pair 5 as a standard in each PCR experiment.

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Figure legends

Figure 1: Activation of tissue-specific origins during erythroid differentiation

(A and C) Fold of enrichments of 2.0-3.5 kb (A) and 2.0-3.0 kb (C) nascent strands extracted from two independently prepared batches of either self-renewing T2ECs (black circles) or 48 hour differentiating T2ECs (gray triangles) and quantified by real-time PCR. Each quantification was repeated at least twice. Error bars indicate standard deviation for samples quantified four or six times. The scale corresponds to fold of enrichments over the background. The abscissa scale is map position (nucleotide number). Black and gray arrows in the graph point towards constitutive and differentiation-activated origins, respectively. The

horizontal double arrow over the βA-globin gene indicates a 1.5 kb zone of initiation. The position of a bi-directional replication fork pausing site inside the ρ-globin gene in self-renewing T2ECs is shown below each graph (double black arrowheads). The positions of primer pairs 10, 20 and 30 are shown further below. Additional primer pairs (18', 23', 36, 37, 38 and 39) were used for the differentiation shown in (C). The position of primer pairs 18', 23', 27 and 31 is shown inside the graph. (B) Map of the FR gene, condensed chromatin region, β–globin domain and olfactory receptor gene (COR3'). DNase I HSs (HSA, HS1 to-3, HS βA/ε, 5'HS4 and 3'HS) are indicated. Expression of globin genes in self-renewing and differentiated T2ECs is indicated below the map. +/- and ++++ indicate weak and very strong expression, respectively.

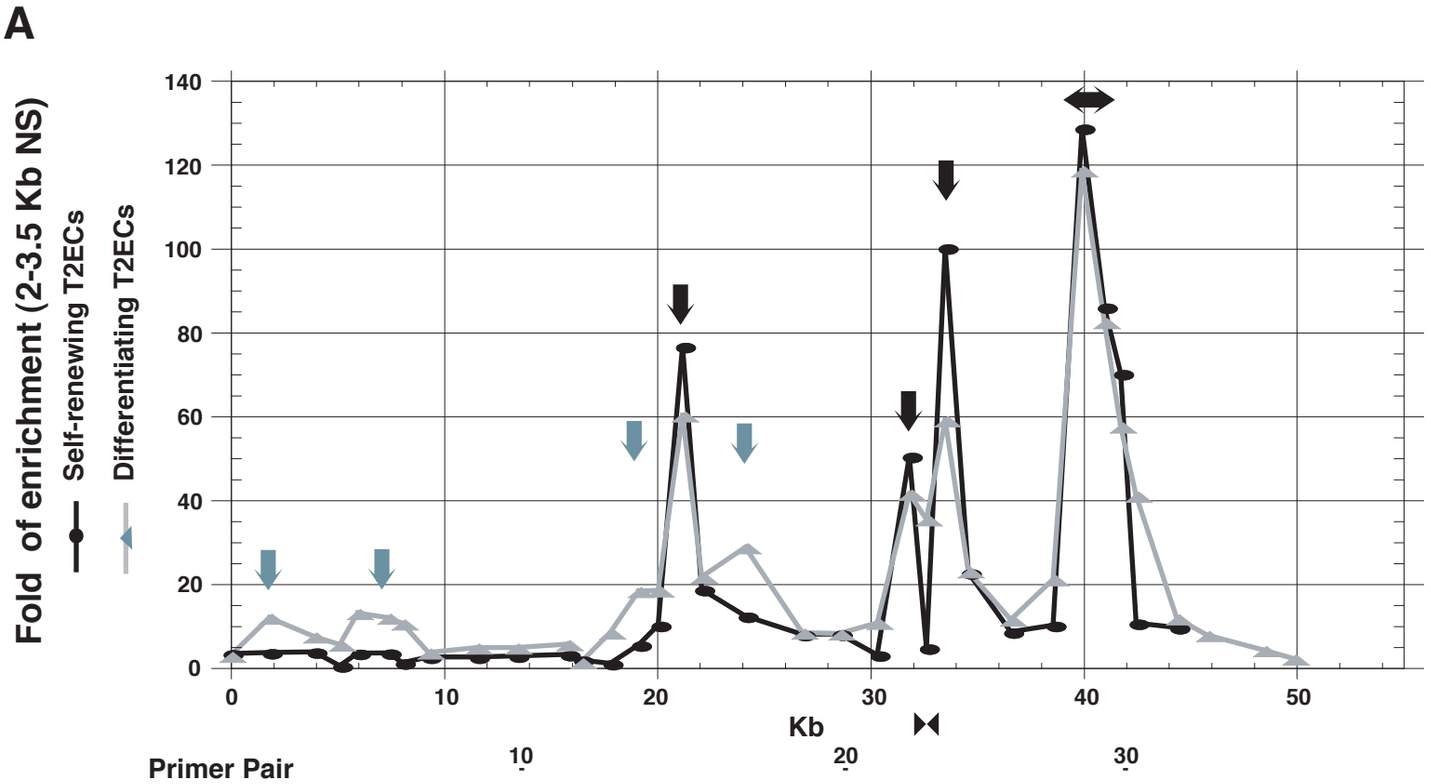
Figure 2: Nascent-strand maturation analysis around the ρ- and βA-globin genes.

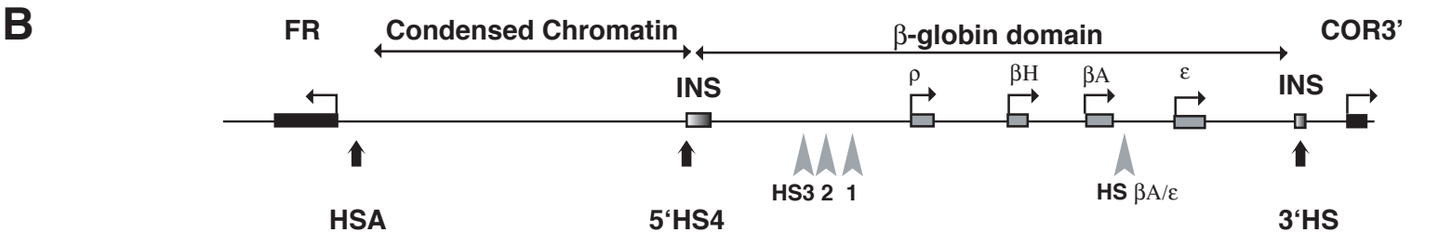
Fold of enrichments of 0.8-1.2 kb (black triangles) and 2.0-3.0 kb (gray triangles) nascent strands extracted from the same batch of differentiating T2ECs as in Fig 1B and quantified with primer pairs inside and around the β^A gene (A) or the ρ gene (B). Each quantification was performed twice. Below the graph are shown the coding regions of ρ and β^A globin genes. Gray rectangles, exons 1 to 3. Black rectangle, $\beta^{A/\varepsilon}$ enhancer. Black bars, amplicons used for real-time PCR quantifications. Gray bars inside graphs delineate 1kb regions devoid of origin activity because centered on a primer pair that gives enrichment close to background level. Vertical and horizontal black arrows indicate origins and initiation zones, respectively. Panel A and B demonstrate continued initiation within the ßA gene and downregulation of the ρ gene replication fork barrier, respectively, in differentiating cells.

Figure 3: Changes in histone H3 diacetylation during differentiation and comparison with replication changes.

Relative recovery of cross-linked protein-DNA complexes recovered after immunoprecipitation with anti-K9K14 diacetylated histone H3 antibody from self-renewing T2ECs (black circles) or 48 hours differentiating T2ECs (gray triangles) as determined by real-time PCR with 27 primer pairs (1 to 7 and 13 to 33). Results with two independent batches of cells are shown (A and B). Each data point is the mean of two different immunoprecipitations from the same sample of cross-linked chromatin. The ordinate is a relative recovery (%Ac/K9&K14 H3) normalized with respect to primer pair 5 (arbitrarily set as 100%). The abscissa is map position (nucleotide number). Black and gray arrows in the graph point towards constitutive and differentiation-activated origins of replication, respectively. The star above one gray arrow indicates a differentiation-activated origin localized within a region where acetylation increases during differentiation. The position of the bi-directional replication fork pausing site inside the ρ-globin gene of undifferentiated cells is indicated (double black arrowheads).

Figure 1





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Expression in Differentiating T2ECs	+	++	++++	+

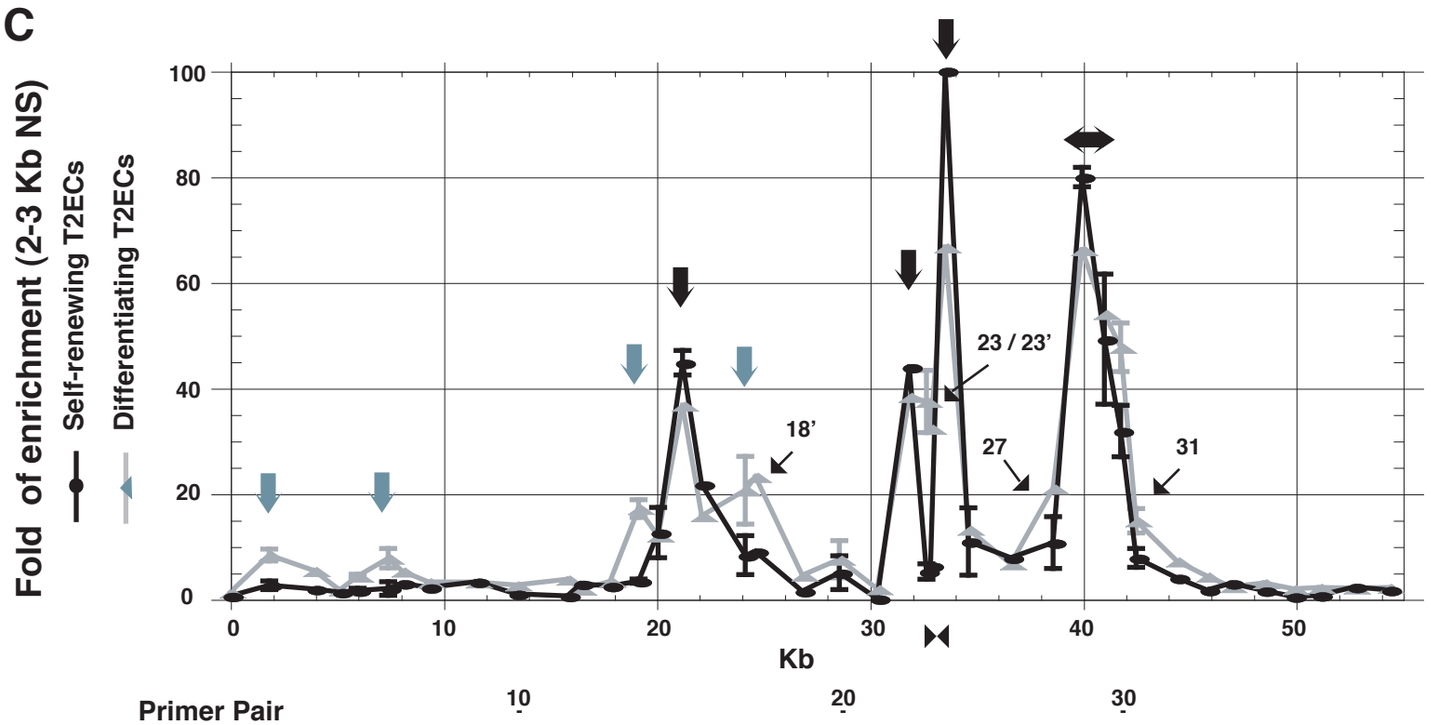
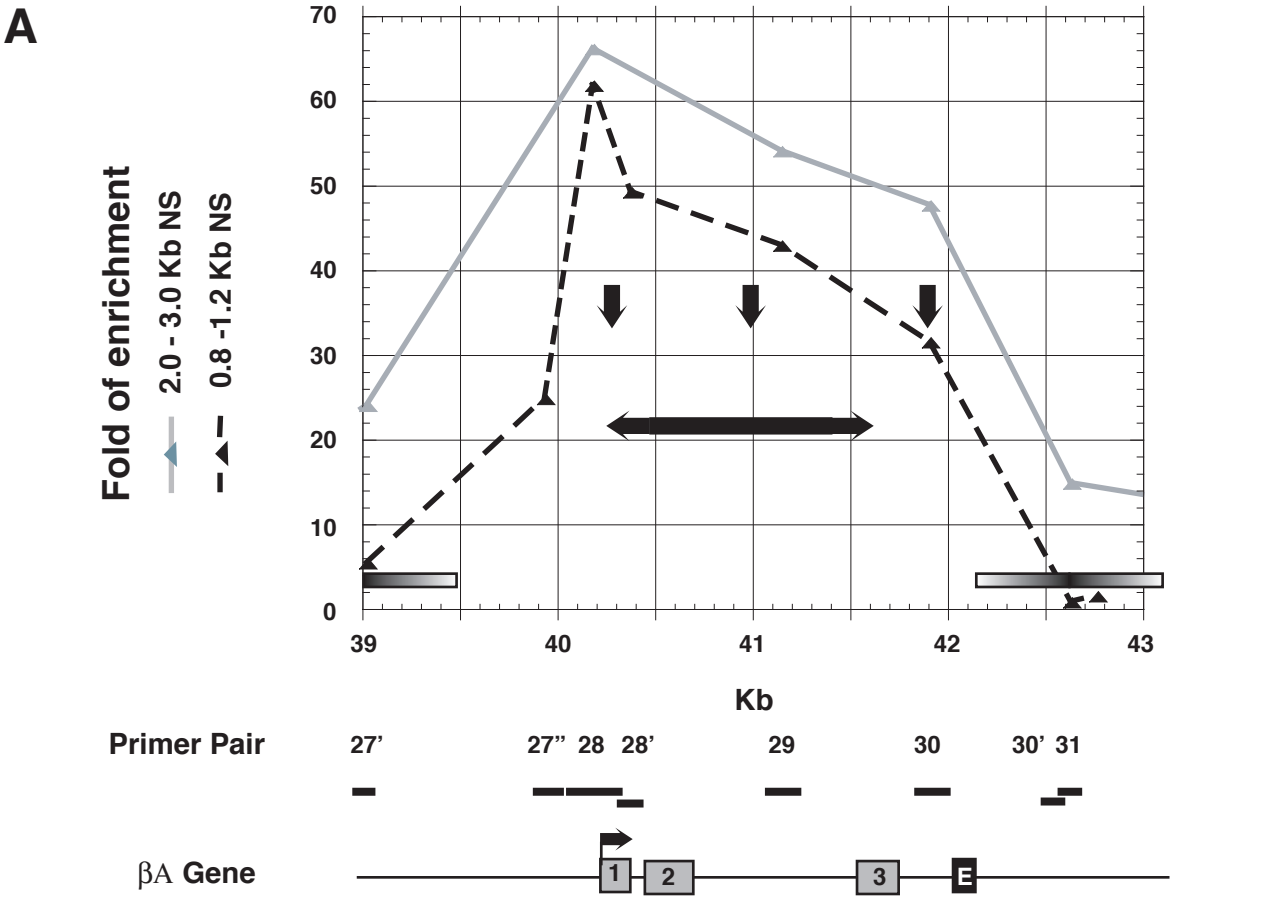


Figure 2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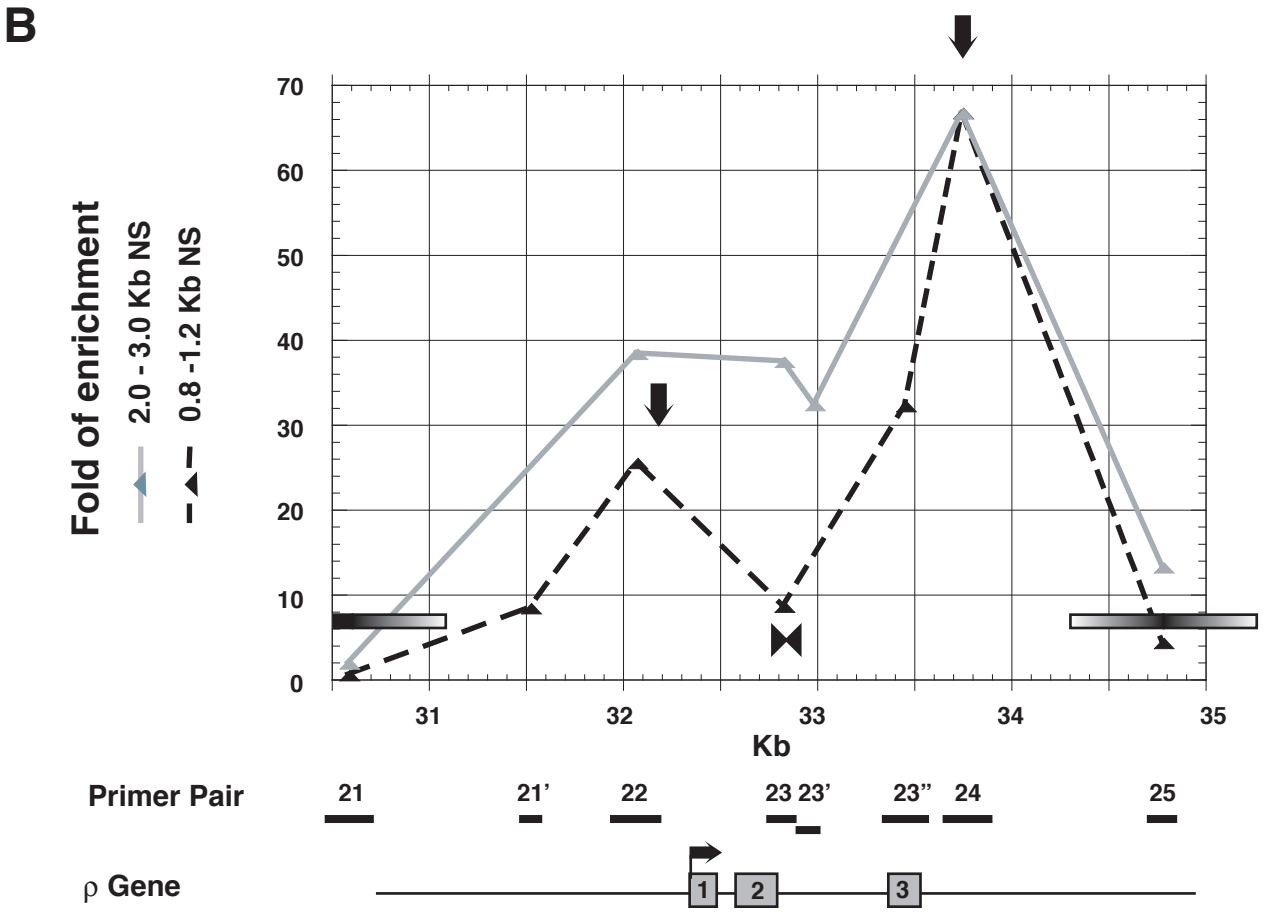
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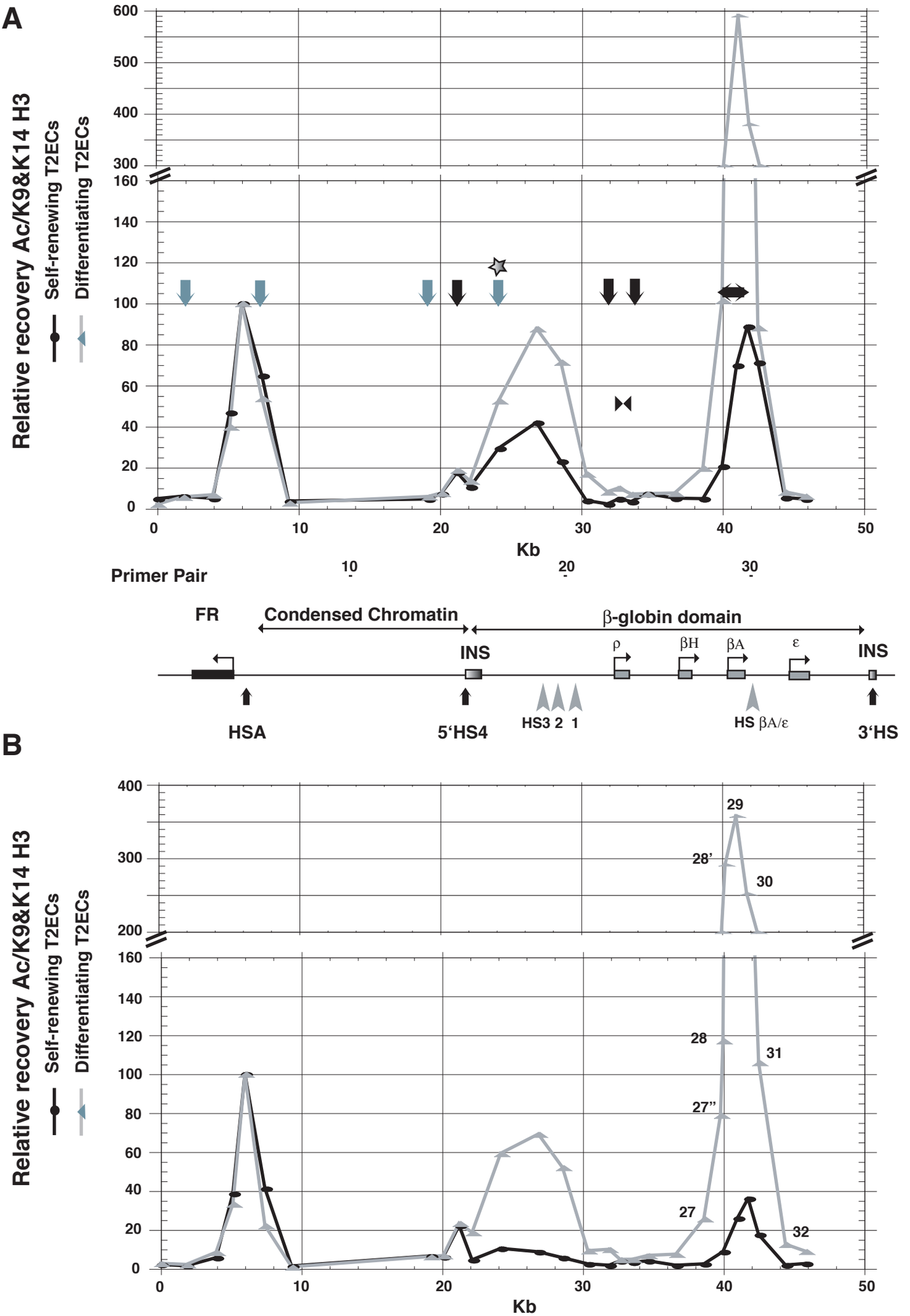
Figure 3


A
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ρ
βH
βA
ε
INS
HSA
5'HS4
HS3 2 1
HS βA/ε
3'HS
B
Relative recovery Ac/K9&K14 H3
Self-renewing T2ECs
Differentiating T2ECs
400
300
200
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
Kb
29
28'
30
28
31
27"
27
32

Supplementary Results

Differentiation parameters of T2ECs

We followed the progression of differentiation by monitoring the surface expression of molecules characteristic of either immature or mature erythrocytes. As expected, a strong reduction in the expression of the immature-specific MEP26 antigen and an increase in the frequency of cells expressing the mature-specific JS4 antigen were detected after 48 hrs of differentiation (Figure S1, panel A). Furthermore, the percentage of haemoglobin expressing cells, as detected by benzidine staining, reached 30 to 40% after two days of differentiation (data not shown). Accumulation of haemoglobin more specifically reflected the transcriptional induction of βH and βA inside the β-globin cluster, as βH and βA transcripts increased 4 to 6 fold whereas embryonic gene transcripts increased only 2 fold (Figure S1, panel B). Cell cycle analysis by flow cytometry before and after 48 hrs of differentiation showed that both populations contained the same proportion of G1, S and G2 cells (Figure S1, panel C).

Supplementary Methods

Cell culture

Self-renewing T2ECs and differentiating T2ECs were generated from SPAFAS white leghorn chicken (PA12 line from INRA, Tours, France) as previously described (Damiola et al., 2004; Dazy et al., 2003; Gandrillon et al., 1999). Because large amount of cells were necessary to perform replication origin localization, we made slight modifications. Mainly exponentially self-renewing T2ECs were suspended at 3-6x10^5 cells/ml in the differentiation medium, which includes anaemic chicken serum (ACS). Different batches of Anaemic Chicken Serum (ACS) were prepared and tested for the minimal concentration required to induce robust cell differentiation, as monitored by blue acidic benzidine staining of haemoglobin as described (Quadrillion and Samara, 1998). To control for potential variability associated with ACS we decided to differentiate two independent preparations of T2ECs using two batches of ACS effective at very different concentrations (batch 1, 5% ACS; batch 2, 15% ACS). We observed that the replication initiation profile of both undifferentiated and differentiating cells was very reproducible (compare Fig 1A and 1C).

Flow cytometric analysis

We performed cell surface antigen detection using either MEP26 (early progenitors) or JS4 (late progenitors) antibodies as previously described (Dazy et al., 2003; Gandrillon et al., 1999). Cell cycle analysis was performed on cells washed in PBS, fixed in 75% cold ethanol, treated with RNAse at 1mg/ml and stained with propidium iodide at a final concentration of 50 μg/ml. Fluorescence data acquisition was done using a FACSCalibur flow cytometer and analysis was performed using the CellQuest program (both from Becton-Dickinson). MEP26 antibody was a kind gift of Dr Kelly McNagny.

Chromatin Immunoprecipitation

$25x10^6$ cells were fixed 10 min at room temperature with 0.1 volume of fixation buffer (PBS 11% formaldehyde 0.1M NaCl, 0.05M Hepes pH 7.9). Fixation was stopped by adding glycine at a final concentration of 0.125M. Cells were then washed twice in cold PBS. The pellet was suspended 10 min at 4°C in 2mL of lysis buffer (50mM Tris-HCl pH8.0, 10mM EDTA pH8.0, 1%SDS, 1/25 complete mini EDTA-free protease inhibitor cocktail (Boehringer)). The lysed cell suspension was frozen quickly in liquid nitrogen and kept at -80°C before chromatin sonication. Sonication conditions were established to shear DNA to an average size of 300 bp. Immunoprecipitation was performed overnight at 4°C in 1ml of 20mM Tris-HCl pH 8.0, 2mM EDTA, 150 mM NaCl and 0,25% Triton-X100 on an amount of chromatin corresponding to 10 μg of DNA using 5 μl of anti-diacetylated K9&K14 H3 antibodies (Upstate Biotech). Immunocomplexes were recovered and processed as described previously (Chen et al., 1999). Two independent immunoprecipitations were performed on two independent batches of undifferentiated or differentiating cells. Real-time quantitative PCR was performed by using a LightCycler and the QuantiTect SYBR GreenPCR Mix in accordance with the recommendations of the manufacturer (QIAGEN). Primer pair 5 was used as a standard in each PCR experiment.

Nascent strands preparation and quantification

Nascent strands were purified as described previously (Prioleau et al., 2003). Quantitative real-time PCR was performed by using a LightCycler and the QuantiTect SYBR GreenPCR Mix in accordance with the recommendations of the manufacturer (QIAGEN). Primer pair 24 was used as a standard in each PCR experiment. Primers were synthesized for each of the target sites to be amplified (table 1).

RNA isolation and cDNA synthesis

RNA extraction and reverse transcription assay were performed as previously described (Damiola et al., 2004; Dazy et al., 2003; Gandrillon et al., 1999). Sequences of the primers used for real-time PCR were the following: Beta-globin Adult GAACACCTTCTCCCAACTGTCC3' and CTTTGGTGCTGGTGCTTAGTGG; Beta-globin Hatching TTTGCCCAGCTGAGCAAACTGC and CTGCCAGGCAGCCTGGCTGGCG; Beta-globin rho GAGAACTTCAGGCTCCTGGGGAAC and ACTTTCACACTGTGTCCTGCTCTGG; Beta-globin epsilon GAACACCTACGCCAAGCTGTCG and TTGGGGGCTATGGCCACGGCTG.

The ratio of expression (R) of each beta-globin gene was calculated as described (Damiola et al., 2004) using as a standard the invariant BBC1 gene (Dazy et al., 2003). The biological condition of reference was self-renewing T2ECs. The tested biological conditions were 24h or 48h differentiating T2ECs. Similar results were obtained with another invariant gene EF1 (data not shown) (Damiola et al., 2004).

Supplementary References

Chen, H., Lin, R.J., Xie, W., Wilpitz, D. and Evans, R.M. (1999) Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell*, 98, 675-686.

Damiola, F., Keime, C., Gonin-Giraud, S., Dazy, S. and Gandrillon, O. (2004) Global transcription analysis of immature avian erythrocytic progenitors: from self-renewal to differentiation. *Oncogene*, 23, 7628-7643.

Dazy, S., Damiola, F., Parisey, N., Beug, H. and Gandrillon, O. (2003) The MEK-1/ERKs signalling pathway is differentially involved in the self-renewal of early and late avian erythroid progenitor cells. *Oncogene*, 22, 9205-9216.

Gandrillon, O. and Samarut, J. (1998) Role of the different RAR isoforms in controlling the erythrocytic differentiation sequence. Interference with the v-erbA and p135gag-myb-ets nuclear oncogenes. *Oncogene*, 16, 563-574.

Supplementary Figure Legend

Figure S1: Differentiation parameters of T2ECs.

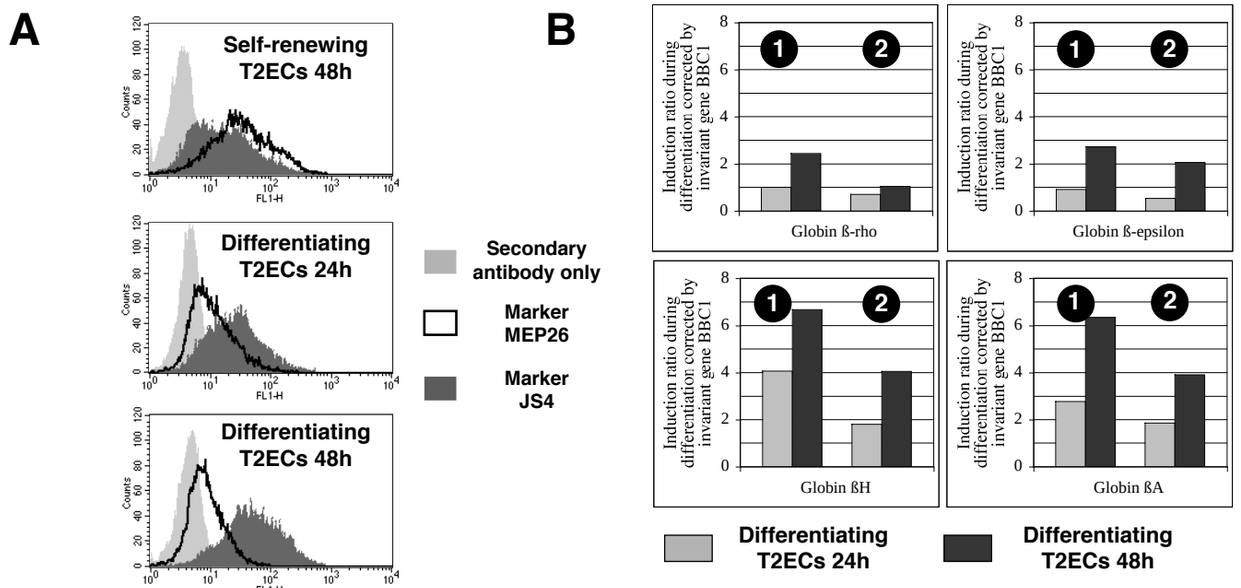
(A) Expression of differentiation antigens. T2ECs grown for 48 hours in self-renewing medium and for 24 and 48 hours in differentiating medium were analyzed by cell sorting for the expression of the immature specific MEP26 (black line) and the mature-specific JS4

antigens (Dark gray shaded profiles). Fluorescence profiles of cells incubated with FITC labelled goat anti-mouse secondary antibody only are indicated (Pale grey shaded profiles) as a control of staining. (B) Expression of embryonic (β-rho, β-epsilon, upper panels) and adult (βH, βA, bottom panels) globin genes in differentiating T2ECs. Quantifications of the β-globin transcripts were performed by real-time PCR on the same batches of cells (1 and 2 white numbers) as used in Fig. 1-3. The ratio of β-globin transcripts present in differentiating T2ECs (24h, pale grey; 48h dark grey) to transcripts present in self-renewing T2ECs are shown. Each PCR quantitation was performed in duplicate. (C) Cell cycle analysis. Histograms of propidium iodide (PI) staining intensity of self-renewing and differentiating T2ECs in batches 1 and 2 (white numbers).

Figure S2: 1-1.5 Kb Nascent enrichments in differentiated T2ECs confirm origins found with longer nascent strands:

1.0-1.5 Kb nascent strands prepared from differentiated cells (batch 1) were quantified by real-time PCR. The scale corresponds to fold of enrichments over the background. The abscissa scale is map position (nucleotide number). Black and gray arrows in the graph point towards constitutive and differentiation-activated origins, respectively. The positions of primer pairs 10, 20 and 30 are shown below and primer pairs 21', 23" and 29 are indicated inside the graph.

Figure S1


A
Self-renewing T2ECs 48h
Differentiating T2ECs 24h
Differentiating T2ECs 48h
Counts
FL1-H
Secondary antibody only
Marker MEP26
Marker JS4
B
Induction ratio during differentiation corrected by invariant gene BBC1
Globin ß-rho
Globin ß-epsilon
Globin ßH
Globin ßA
Differentiating T2ECs 24h
Differentiating T2ECs 48h

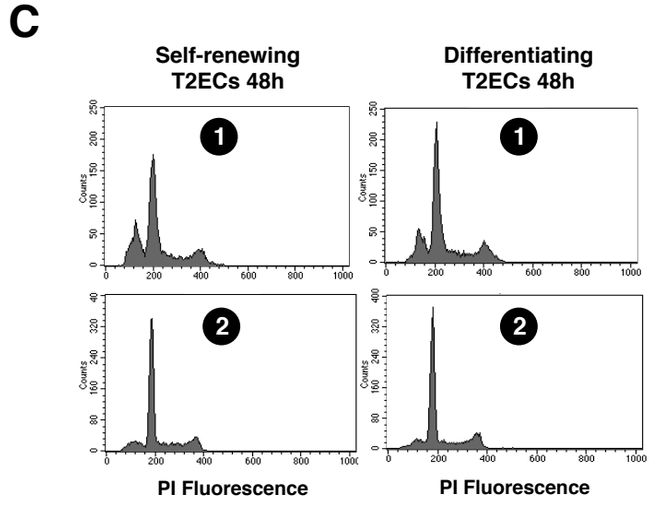
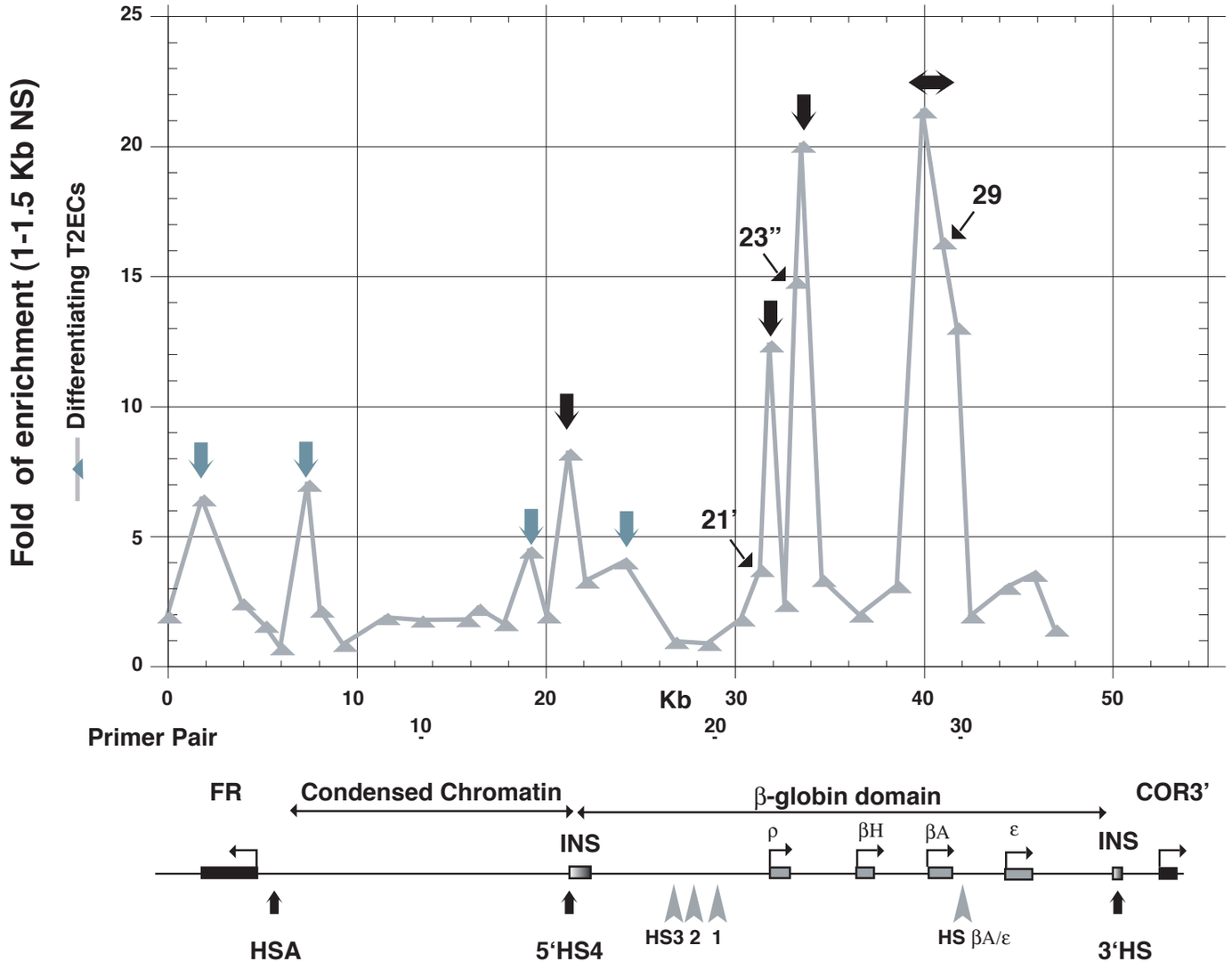

C
Self-renewing T2ECs 48h
Differentiating T2ECs 48h
Counts
PI Fluorescence
PI Fluorescence

Figure S2


Fold of enrichment (1-1.5 Kb NS)
Differentiating T2ECs
25
20
15
10
5
0
0
10
20
Kb
30
40
50
10
20
30
Primer Pair
23"
29
21'
FR
Condensed Chromatin
β-globin domain
COR3'
INS
ρ
βH
βA
ε
INS
HSA
5'HS4
HS3 2 1
HS βA/ε
3'HS

Supplementary Table 1 : List of primer sequences

Name Sequence 5' to 3'	Map position	Name Sequence 5' to 3'	Map position
Primers 1		**Primers 11**	
TGAGCTGCTGACTCTGCCCA	80	TGGCACCGATGGGATCACAT	15895
CATGTCCTCATGGCCAACAC	382	CACTGATACTGCAGCCTCTT	16189
Primers 2		**Primers 12**	
CAATGCAGCTCTGGCAGTCT	1904	GGTGTTCAATAATCAGACTGTC	16535
GTTACTCGTGTCCCTGCATG	2075	TCACATTTCAGCTGGCGCTC	16824
Primers 3		**Primers 13**	
CAGCAAGATGGTGTTGAAGG	4016	TCTCACTGTGCAGATGATGG	17905
CAGTGGTGTGGGTAATTGGA	4322	CATGGTGTCATCACAGCATC	18159
Primers 4		**Primers 14**	
GCCTCCTGACACAGCAGAGC	5217	GGATCCTGAAGCTGTTTGGC	19230
GCAGGTTTGAGGCAAGTTAG	5512	CTCTGCTGGGTTTCAGCAC	19363
Primers 5		**Primers 15**	
CTGAATGGTCTTTGTGGTCCC	6064	ACGGGATGGTGAAGGCACAG	20165
CTTGCTGTGGGAGACCTGCT	6348	TTAGCTCCCATTCCCCCACA	20472
Primers 6		**Primers 16**	
TGGGAGATAATTGTTAGCCAC	7499	GCCTCCAGGAAACCACTC	21423
TATGCTGGGAACAGCTATAC	7651	GACCTTCCAGGAAAGCCTG	21538
Primers 7		**Primers 17**	
GCCATACCCATTCACTCCAT	8166	GGACGTGGACATGCAGGTG	22200
AATGGGACACGCAGTCCCAG	8391	CCAGCCTTCATGATTTGACG	22508
Primers 8		**Primers 18**	
GCATCCTTCATCCAGCTG	9380	TGGGCAGTTTTCTAAGGGAA	24240
CAGTGAGCAGACTGTGAAGC	9533	GTGCTGGCAGGGCATTCCAA	24554
Primers 9		**Primers 18'**	
CTGTGGTCTGGTTTGTTTGG	11649	TGAGAGCTGAGCTGCTCCTG	24720
CAGCCTCTCCACTCATGCAC	11956	CAGACTGCAGCTGAAGGCA	24911
Primers 10		**Primers 19**	
CTGAGCTGTGACAGTGCC	13489	CAGCTCTGAGCACAGCACTG	26910
AATGGTCTTTGCCTGGGTCA	13652	CAGGGAATTCCTTCTGGGG	27216

Name	Map position
Sequence 5’ to 3’	
Primers 20	
CTGTCTGAATATCCTGGCTC	28642
GTGATTCAATGTCAGGCACT	29 021
Primers 21	
GTGAGAGGGGCACTCCAGG	30441
GCAGTGCTCCGATAATGCC	30739
Primers 21’	
TGCACAGGGGCACCATTTTG	31444
CCTTGCATAAGGACAGCAGG	31610
Primers 22	
GAAGGGTGAGGGAAGTGCC	31941
TCAGTGTGCACAAGGTGTGG	32211
Primers 23	
GAACACCTACGCCAAGCTGT	32738
TGCTGCACCTTGTTCCACAC	32925
Primers 23’	
CAGCAGATGAAGGAGGGAAG	32920
GTCAGTTCCATGTCTGCCTG	33060
Primers 23’’	
TTACCCCATTGCTCCCCTTC	33317
ACAGCCCTGAGCCCTCTTTC	33600
Primers 24	
GACGGTCAGGTTTGCCAAAG	33620
TCCTGAGGATACGTTTTTCAG	33887
Primers 25	
CTGGGAGCAAAGACACTGAC	34701
TGGTCACTCTGATTGCAGCA	34869
Primers 26	
GATACGCACTGAGCTCTCGT	36700
CACCCATGATCTCGTAGCCA	37000
Primers 27	
GAACAAGTCATTGCACAACGG	38690
GGCAGTGAAACCAAGTGCTC	38994

Name	Map position
Sequence 5’ to 3’	
Primers 27’	
GACAGAGCACTTGGTTTCAC	38970
GATGCTGCGAGCCACATCTG	39208
Primers 27’’	
GAGTGCTGTGGTTTGGAACT	39870
CAGCTCTGCAGCTCTATAC	40010
Primers 28	
TGGTGTGGCCACGGATCTG	40040
GTGATGAGCTGCTTCTCCTC	40333
Primers 28’	
GAGGAGAAGCAGCTCATCAC	40310
GTGTACACCTTCAACTGCAC	40453
Primers 29	
CATAGAGCAAGGGACGGTG	41070
TACTGTGGGAAGAGTAGCTC	41231
Primers 30	
AGCGCTTTGTGCTCAGTGG	41835
ATGACTCCTCTGTGTCAGTGA	41989
Primers 30’	
TCCCCTGACTCACTGCTGG	41835
ACACGGAGTGTCCTCCTATG	42700
Primers 31	
CATAGGAGGACACTCCGTGT	42681
TACCCTTTTCCTTCCGGCTG	42857
Primers 32	
GCAGCTCCGCTCCAAGCTCT	44490
GGCTGGAGAGGTTCCCAAAG	44810
Primers 33	
TCCAAGCAGCACTAACCCTG	45900
ACGTTGACCAGCTTCTGCCA	46039
Primers 34	
GCTGAATGCTGTGCCTCTGG	47020
CCATCAACCTGCTAGAGAAG	47318

Primers 35
CCCAGATTTGCTTCATCAGGAG 48560
GAGATGTGATAACAGACTGCCA 48758

Primers 36
CACTTCTGCTCTACAAGGCC 49950
ACTGACATGGAAACACACGG 50103

Primers 37
GCAGTGCAGACCCATCCCT 51170
TCTTTCTGTGCGTCATCAGC 51455

Primers 38
CCAAGCAAGTCCAGACAGAG 52817
GCAAGCCTACATTCCTCCC 52996

Primers 39
TCATGAACTCCCAGTACCAG 54424
TGCACACTGATCAGTAGGTC 54567

Primers LYS :
CGGGTATCATTAGTGCCGAG
CTGCCAGTATATCCTGGCAAA