A multiprotein complex necessary for both transcription and DNA replication at the beta globin locus

Subhradip Karmakar, Milind C. Mahajan, Vincent Schulz, Gokul Boyapaty and Sherman M. Weissman

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 17 March 2010

Thank you for submitting your manuscript for consideration at The EMBO Journal. It has now been seen by two referees, whose comments are shown below. The referees find the study to be potentially interesting but they require some further experimental analysis before it can be further considered at the EMBO Journal.

The referees find that the biochemistry identifying DAART to be relatively convincing, referee #1 would like to see a sucrose gradient to determine if a single complex or several sub complexes exist. However, both referees raise concerns with the data showing that the complex plays a role in replication origin activation and beta-globin transcription. In addition to several technical concerns these issues need to be addressed, should be able to address these issues we would be willing to consider a revised version of the manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.
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Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The submitted paper addresses the interesting and important question of how the human beta globin locus control region (LCR) interacts with downstream target genes. Understanding the molecular interactions that regulate gene expression at a distance is a fundamental question in cell biology. The beta globin locus is a particularly appropriate model to study this question and previous studies have yielded some insights into the possible role of LCR in regulating transcription, chromatin remodeling and DNA replication in that locus. The current submission reports that the LCR binds a protein complex (DAART) including the enhancer binding proteins ILF2 and ILF3. The DAART complex also includes proteins involved in chromatin remodeling and DNA repair and proteins associated with replication forks, for example, the MCM helicase complex. In erythroid cells, ILF2 recruits the complex to the LCR and possibly to downstream promoter regions. Depletion of ILF2 affects erythroid differentiation, gene expression, chromatin modifications and DNA replication patterns for the entire beta globin locus.

The submitted paper provides sufficient evidence to support the existence of DAART and its interaction with the LCR. It also provides interesting information about effects of ILF2 depletion. However, further corroboration is needed to support the conclusion that the effects of ILF2 depletion are mediated by DAART interaction with LCR, and there are some concerns regarding the replication data. This work can potentially contribute to understanding the regulation of gene expression at a distance after the concerns listed below are addressed.

Critique:

1. Evidence is presented that IFL2 depletion alters gene expression and chromatin modifications at the human beta globin locus. Evidence is also presented that IFL2 is a member of the DAART complex. However, it is not clear whether the downstream effects of IFL2 depletion are mediated by the DARRT complex. The reported effects of IFL2 on replication rates and on chromatin might result from the documented differentiation program activated by IFL2.

2. More data are needed to support the suggestion that ILF2 plays a role in replication origin activation. The presented data suggest that replication did not initiate at all within the entire locus under conditions that depleted IFL2, despite the fact that there was no pronounced effect on the frequency of cells in S-phase. IFL2 depletion lowered replication rates, as expected given that IFL2 interacts primarily with proteins associated with replication forks (MCM is the replicative helicase). The effect on origin activity is unexpected (see point #3 below) and there is a concern that the methodology used to measure replication initiation might reflect chromatin structure rather than origin activity. The method involves a prolonged incubation at an intermediate temperature and under such conditions nascent DNA strands might not separate from condensed chromatin as efficiently as from "open" chromatin.

3. How do the observations reported in the submitted paper reconcile with published data from the Groudine lab that HS4 deletion is not essential for establishing replication and open chromatin patterns in human beta globin loci?

4. The colocalization of EdU foci and ILF2 should report a statistical analysis to evaluate how frequently the two signals colocalize (i.e. how many cells exhibit colocalization and the percentage of foci that exhibit colocalization within cells).
5. A complex between ILF2 and DNA repair proteins were reported previously (Biol Chem. 1998 273:2136-45) and this fact should be cited.

Referee #2 (Remarks to the Author):

In this manuscript, the authors identified a multi-protein complex that binds to the \(\beta\) globin locus control region (particularly, the HS4 site). They show that this complex contains factors involved in transcription, replication, repair and chromatin assembly.

The authors have carried out many independent experiments to validate their data and their efforts are praiseworthy. However, I found that several results are technically not convincing enough. I will mention below some suggestions to improve this work.

1. It might not be clear to a non-specialist reader why the authors selected the complex formed with the HS4 sequence in the experiment shown in Figure 1B. I appreciate that it is because HeLa and K562 cells patterns differ precisely at this position. This should be better explained. Moreover, this Figure is not clearly described. What is the meaning of lanes 1 to 9? If this represents the relative position of the oligos along the \(\beta\) globin cluster, this should be indicated in both Figure 1A and in its legend. Figure 1B is also incorrectly numbered. If the band shown in Lane 8 (3' end) is the one selected, it does not fit with the 5' end position of H54 in Figure 1A.

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7. I have a major problem with the nascent strand assay for the localization of replication origins. In both the Figure legend and the Methods section it is claimed that 10 ng NS DNA are used for each qPCR reaction. It can be calculated that no more than 10 ng nascent strand DNA will be present in 108 cells (see also Cadoret et al, PNAS 2008, 2008 105, 15837-42). In this manuscript, the authors used only 2 x 107 cells and claimed to use 10 ng NS DNA per qPRC reaction. This might suggest that most of the DNA they isolated as NS could be contaminating broken DNA pieces. This leads to question whether they did isolate NS DNA or whether they are mapping sites sensitive to breakage.

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10. Several experiments of this manuscript are not enough explained and could be misunderstood by readers less familiar with this field.

In conclusion, I appreciate that the authors have done a considerable effort of investigation. However, my general feeling is that more experiments are necessary and a new version of the manuscript should include only solid data from well described experiments in order to make this paper convincing.

We sincerely thank you and the referees for the helpful suggestions in regard to our manuscript. We attach specific responses to each of the points raised by the referees together with additional supplementary data figures.

In response to the points raised in your letter we have performed a sucrose gradient analysis (Supplementary Figure 9) that shows that seven components of the DARRT complex for which we have antibodies predominantly sediment as a single complex.

With regard to questions as to whether the DARRT complex plays a role in globin gene transcription and initiation of DNA replication, in our original manuscript we have shown that knockdown of ILF2, a DNA binding component of DARRT complex, inhibits the association of the DARRT complex with its cognate HS4 DNA sequence as shown by electrophoretic mobility shift assay (EMSA) (Figure 4 in main manuscript) and abolishes recruitment of MCM5 and p300 as shown by the ChIP experiments (Figure 4 in the main manuscript). These data suggest that upon knockdown of ILF2 in K562 cells, DARRT is not recruited to the HS4 region of the beta globin LCR. Our data in the main manuscript also shows that knockdown of ILF2 and other components of DARRT such as MCM5 and ORC2 specifically inhibit the accumulation of gamma and epsilon globin gene mRNA and not ILF3 that served as a control, and that knockdown of ILF2 in murine erythroleukemia cells also markedly reduces globin gene transcript levels (Figure 5 in the main manuscript). In the revised manuscript, we have carried out additional experiments to show that ILF2 knockdown in K562 cells does not affect the transcription of any of twelve transcription factors that are either erythroid specific or necessary for globin mRNA production or erythroid differentiation (Supplementary Figure 7). These additional experiments support the experiments in the main manuscript that suggest that DARRT complex is involved in the transcription of the beta like globin genes in K562 cells. Also, we have now used several additional reagents to slow K562 cell growth by targeting various aspects of the cell cycle (Supplementary Figure 11). In no case do these treatments result in the selective depression of globin synthesis seen after knockdown of the ILF2, MCM5, or ORC2 components of DARRT.

Knockdown of ILF2 also inhibits the origin of DNA replication specifically at the beta globin locus and not at the lamin B2 locus (Figure 6 in the main manuscript). In the revised manuscript, we have carried out additional experiments to show that the methodology used in this study specifically enriched the DNA-RNA hybrids at transcriptionally inactive “closed” chromatin in three other non-erythroid cell lines as well as at the transcriptionally active “open” chromatin in the beta globin locus of erythroleukemic K562 cells (Supplementary figures 8 and 10). The major beta globin origin is the active origin in each cell type, consistent with other reports in the literature and independent of the types of histone modification present. Therefore the silencing of the globin origin of replication when we knock down ILF2 (the DNA localizing component of DARRT) is not secondary to inactive histone modifications. Thus, the effects of ILF2 knockdown on the beta globin origin of replication are specific rather than secondary to the decreased transcriptional activity or change in chromatin structure of the locus.

In response to the excellent suggestions given by the referees, we have carried out all the required additional experiments and hope that the revised manuscript will be accepted for publication in EMBO Journal. In the following pages please find our response to referees’ comments.

Referee #1 (Remarks to the Author):

The submitted paper addresses the interesting and important question of how the human beta globin locus control region (LCR) interacts with downstream target genes. Understanding the molecular
interactions that regulate gene expression at a distance is a fundamental question in cell biology. The beta globin locus is a particularly appropriate model to study this question and previous studies have yielded some insights into the possible role of LCR in regulating transcription, chromatin remodeling and DNA replication in that locus. The current submission reports that the LCR binds a protein complex (DAART) including the enhancer binding proteins ILF2 and ILF3. The DAART complex also includes proteins involved in chromatin remodeling and DNA repair and proteins associated with replication forks, for example, the MCM helicase complex. In erythroid cells, ILF2 recruits the complex to the LCR and possibly to downstream promoter regions. Depletion of ILF2 affects erythroid differentiation, gene expression, chromatin modifications and DNA replication patterns for the entire beta globin locus.

The submitted paper provides sufficient evidence to support the existence of DAART and its interaction with the LCR. It also provides interesting information about effects of ILF2 depletion. However, further corroboration is needed to support the conclusion that the effects of ILF2 depletion are mediated by DAART interaction with LCR, and there are some concerns regarding the replication data. This work can potentially contribute to understanding the regulation of gene expression at a distance after the concerns listed below are addressed.

Critique:

Comment 1. Evidence is presented that IFL2 depletion alters gene expression and chromatin modifications at the human beta globin locus. Evidence is also presented that IFL2 is a member of the DAART complex. However, it is not clear whether the downstream effects of IFL2 depletion are mediated by the DARRT complex. The reported effects of IFL2 on replication rates and on chromatin might result from the documented differentiation program activated by IFL2.

Response: We thank the reviewer for raising this concern. In murine erythroleukemic (MEL) cells we have observed inhibition of DMSO induced differentiation upon ILF2 knockdown (Figure 5 in the main manuscript and Supplementary Figure 3). Hence, we feel that referee’s comment “differentiation program activated by ILF2” is actually “differentiation program inactivated by ILF2 knockdown”.

Co-immunoprecipitation and immunodepletion experiments (Figure- 4 in the main manuscript) along with sucrose gradient fractionation (Supplementary figure 9), carried out as suggested by Referee-2, show that ILF2 in K562 cells is complexed with the components of DARRT. The ChIP experiments and EMSA experiments with specific antibodies show that several components of DARRT in addition to ILF2 bind to the same site in HS4 region of the beta globin cluster, and knockdown of ILF2 abolishes the HS4 associated DARRT EMSA bands and significantly inhibits the recruitment of other DARRT components to HS4, as shown by ChIp-PCR (Figure 2 - 4 in the main manuscript). We have carried out additional experiments in the revised manuscript (Supplementary figure 7) that show that, in K562 cells, the effects of ILF2 knockdown on transcription of key erythroid and non-erythroid transcription factors is minimal and not sufficient to account for the selective effects on globin transcription. These additional experiments together with above mentioned experiments in the original manuscript strengthen our suggestion that in human K562 cells, knockdown of ILF2 effects the transcription of beta globin genes by inhibiting the binding and function of DARRT.

Further, knockdown of ILF2 in K562 cells does not effect the transcription of key erythroid genes such as GATA-1, NF-E2, EKLF, p18MAF, LMO2 etc indicating that ILF2 depleted K562 cells retain their erythroid character (Supplementary figure 7). In these cells, effect of ILF2 depletion on the origin of DNA replication may not be due to the change in the erythroid differentiation status. Our experiments in the revised manuscript demonstrate that the effect on the globin replication origin does not appear to be a result of a change in differentiation program as several other types of cells differentiated along different non-erythroid lineages, still continue to use the beta globin origin, unlike the ILF2 knockdown K562 cells (Supplementary Figure 8 in revised manuscript and Figure 6 in the main manuscript).

However, in Mouse Erythroleukemic (MEL) cells, knockdown of ILF2 partly prevents DMSO mediated erythroid differentiation. In this situation, transcription of several erythroid specific genes including globin genes is down regulated. We have not investigated DNA replication or DARRT recruitment in the murine system.
Although we cannot absolutely exclude that ILF2 knockdown acts through effects on some undiscovered factor elsewhere in the genome that is necessary for use of the globin origin of replication and transcription of the globin genes, it appears simpler and more plausible to propose that the effects are a result of actions of the DARRT complex containing ILF2 directly at the globin LCR, where we know it binds.

Comment 2. More data are needed to support the suggestion that ILF2 plays a role in replication origin activation. The presented data suggest that replication did not initiate at all within the entire locus under conditions that depleted IFL2, despite the fact that there was no pronounced effect on the frequency of cells in S-phase. IFL2 depletion lowered replication rates, as expected given that IFL2 interacts primarily with proteins associated with replication forks (MCM is the replicative helicase). The effect on origin activity is unexpected (see point #3 below) and there is a concern that the methodology used to measure replication initiation might reflect chromatin structure rather than origin activity. The method involves a prolonged incubation at an intermediate temperature and under such conditions nascent DNA strands might not separate from condensed chromatin as efficiently as from "open" chromatin.

Response: We understand and appreciate the reviewers concern. As described earlier, our data with the knockdown of ILF2 in K562 cells shows inhibition of the binding of DARRT to its cognate HS4 DNA sequence as seen with the EMSA assay. This knockdown also significantly reduces the recruitment of MCM5 throughout the beta globin sequence including HS4 (Figure 4 in the main manuscript). In this situation, we see inhibition of the use of DNA replication origins at the beta globin locus. These data suggest that ILF2 participates in the initiation of DNA replication by recruiting the MCM complex on DNA. ILF2 mediated recruitment of MCM complex does not appear to occur at all the replication origins in the genome as we do not see an effect of ILF2 depletion on the origin of DNA replication at Lamin B2 origin. Inhibition of a subset of ILF2 associated replication origins such as the beta globin replication origin in ILF2 knocked down K562 cells may reduce the rate of cell growth instead of arresting the cell cycle at a particular stage. We also note that the results we see with respect to slowing replication rate without substantially changing the distribution of cells in the various phases of the cell cycle are quite similar to the effects of ILF2 knockdown noted in HeLa cells by Matthews and colleagues (Guan D, et al, Mol Cell Biol. 2008, 28:4629-4641). In addition, in another study knock down of MCM5 in 293T cells did not result in the cell cycle arrest (Snyder M, Huang XY, Zhang JJ. J Biol Chem. 2009, 284(20): 13466-72).

In the revised manuscript, we have performed several additional controls for the origin of replication studies. We have used three non-erythroid human cell lines namely JEG-3, MCF-7 and LNCaP that do not express beta globin genes and have the histone H3K9Me2 mark at the beta globin locus. This mark is well known to be associated with the transcriptionally silent condensed chromatin. At the same locus in these cell lines, there is an absence of the H3K4Me2 mark that is a mark of transcriptionally active "Open" chromatin (Supplementary figure 8). In these cells we assayed for the origin of DNA replication at the beta globin using the same methodology that we have used in case of K562 cells for the isolation of nascent replicating strands. As described in the Methods section of the main manuscript, this procedure involves isolation of single stranded regions from the genomic DNA on BND-cellulose column followed by the isolation of less than 1Kb of the l-exonuclease resistant ssDNA fragments to determine their enrichment at the beta globin locus. Each of three non-erythroid cell types that show marks of condensed chromatin over the beta globin cluster (Supplementary Fig. 8 ) still used the beta globin locus origin(s) of replication as do K562 cells (Figure 6 in the main manuscript). This argues strongly that our methodology used in this study efficiently detects the l-exonuclease resistant nascent DNA strands at the DNA replication origins at the transcriptionally active "open" chromatin as well as transcriptionally silent condensed chromatin. Further, treatment of the putative nascent DNA strands from K562 cells with RNAse prior to l-exonuclease digestion abolished the signals over the origin(s) of DNA replication, confirming that these signals arose from RNA-DNA hybrid molecules. Again, we note that the major origin of replication we see is the same as that noted by other groups including recent studies showing this origin by both the l-exonuclease and the BrdU labeling assays for mapping DNA origins in HeLa cells (Karnani N, Taylor CM, Malhotra A, Dutta A.Mol Biol Cell. 2010 Feb;21(3):393-40).
Comment 3. How do the observations reported in the submitted paper reconcile with published data from the Groudine lab that HS4 deletion is not essential for establishing replication and open chromatin patterns in human beta globin loci?

Response: We thank the reviewer for raising this point. Although LCR deletion studies by Groudine lab did not show significant effect on the chromatin structure and origin of DNA replication at the beta globin locus, the naturally occurring Hispanic deletion of LCR and a large portion of DNA 5’ to LCR keeps the rest of the beta globin locus chromatin in a closed configuration. This Hispanic deletion also silenced the origin of DNA replication at the beta globin locus (Aladjem MI, et al Science. 1995, 270(5237):815-9). To the best of our knowledge, the Groudine lab did not study the effects of deletions of only HS4 on DNA replication, but rather the deletion of the entire LCR. Moreover, HS4 deletions abolished the late firing of beta globin replication origin in non-erythroid cells (Simon I, Tenzen T, Mostoslavsky R, Fibach E, Lande L, Milot E, Gribnau J, Grosveld F, Fraser P, Cedar H; 2001, EMBO J 20: 6150-6157). These differences in effects of experimental and naturally occurring cis DNA element deletions may be explained by functional redundancies in the system. Indeed, our ChIP data in primary erythroid cells described in the present study shows recruitment of ILF2 and its associated DARRT components not only at HS4 but also to other regions of the beta globin locus that includes the widely known replication origin (Beta Rep-1) at the beta globin gene (Figure 3 in main manuscript).

Comment 4. The colocalization of EdU foci and ILF2 should report a statistical analysis to evaluate how frequently the two signals colocalize (i.e. how many cells exhibit colocalization and the percentage of foci that exhibit colocalization within cells).

Response: We thank the referee for this excellent suggestion. However, following the suggestion of the referee-2, we have now omitted the immunofluorescent microscopy experiments from this manuscript.

Comment 5. A complex between ILF2 and DNA repair proteins were reported previously (Biol Chem. 1998 273:2136-45) and this fact should be cited.

Response: We thank the referee for this reference and have now included the reference in the first paragraph of the Discussion.

Referee #2 (Remarks to the Author):

In this manuscript, the authors identified a multi-protein complex that binds to the ß globin locus control region (particularly, the HS4 site). They show that this complex contains factors involved in transcription, replication, repair and chromatin assembly. The authors have carried out many independent experiments to validate their data and their efforts are praiseworthy. However, I found that several results are technically not convincing enough. I will mention below some suggestions to improve this work.

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Response: We agree with the reviewers comment. We have now expanded the explanations for rational behind selecting HS4 oligo-9 for the isolation of DARRT complex in “Introduction” and “Result” section. We have also described all sections of Figure-1 for clarity so that we can effectively describe our experiments to the non-specialist reader. We have expanded the description of the figure to clarify that lanes 1 to 10 in Fig. 1B refer to positions of 35 mer oligonucleotides within the HS4 site. We have also added arrows in the figure to make this point.
Comment 2. Figure 1C should also be better described. The reader has to read the Methods section to understand it. The rationale of the choice to analyze the 0.4 M salt eluate and not other fractions is also not explained.

Response: We agree with the reviewers comment and worked according to his/her suggestions to improve the clarity. We have expanded the explanation in the text, and have mentioned that the erythroid specific gel shift that we see with crude nuclear extract is entirely accounted for by protein complexes eluted at 0.42M salt from the heparin agarose column. Specifically no gel shift was seen with fractions eluted from the column at either lower (0.2M) or higher (0.6 M) salt concentrations, in experiments with stepwise elution of proteins from Heparin Sepharose columns. In addition, we have also explained that we have tested for the HS4 oligo-9 DNA binding activity in all the stepwise salt elutions of DEAE-Sephacel and oligoaffinity columns and collected the protein fractions that showed positive DNA binding activity with HS4 oligo-9.

Comment 3. As for the complex, a sucrose gradient should be performed and a western blot carried out using antibodies against the proteins expected to be present in the complex. The positions and the shapes of the peaks should tell whether a single or several sub-complexes are present.

Response: This experiment has been performed and confirms that the various components of DARRT are associated together as a multiprotein complex (Supplementary Figure 9).

Comment 4. The authors mention that tagged ILF2 associates with the same partners that form the complex. This is an important piece of information and it should be shown.

Response: We agree with the reviewers comment and now have included a supplementary table-1 with this data.

Comment 5. In Figure 2B, the supershifts are not obvious. Where is the ILF2 lane? Overall, this experiment is technically poor.

Response: We understand the concern of the reviewer. In the original figure ILF2 was labeled as NF45, an alternative name for the factor. We have changed the labeling to be consistent throughout the manuscript. The supershift of the EMSA bands with antibody against MCM5 is subtle, but reproducible. The antibodies against p300 and RAD50 disrupted the EMSA bands rather than shifting them, suggesting that the EMSA bands are formed by the interaction of DARRT with its cognate HS4 oligonucleotide sequence. This experiment together with the ChIP experiments and the inhibition of EMSA bands by ILF2 knock down (Figure 3 & 4 in the main manuscript) shows the in vitro and in vivo binding of the DARRT to the HS4 sequence.

Comment 6. Preliminary data about the use of anti-ORC2 antibodies should be either shown or not mentioned.

Response: As per the reviewer’s suggestion, we have omitted the mention of the use of anti-ORC2 antibodies for ChIP-sequencing experiments.

Comment 7. I have a major problem with the nascent strand assay for the localization of replication origins. In both the Figure legend and the Methods section it is claimed that 10 ng NS DNA are used for each qPCR reaction. It can be calculated that no more than 10 ng nascent strand DNA will be present in 108 cells (see also Cadoret et al, PNAS 2008, 2008 105, 15837-42). In this manuscript, the authors used only 2 x 107 cells and claimed to use 10 ng NS DNA per qPCR reaction. This might suggest that most of the DNA they isolated as NS could be contaminating broken DNA pieces. This leads to question whether they did isolate NS DNA or whether they are mapping sites sensitive to breakage.

Response: We note that K562 cells in mid log phase are rapidly replicating with a relatively large fraction of the cells in S phase (Supplementary Figure 11). Our initial estimates of DNA concentration were performed by a nanodrop method and the levels of DNA in our samples was at the lowest end of the range of detectability. We calibrated the nanodrop versus the more accurate Qubit estimate of DNA and find that the nanodrop method overestimated by 3 to 5 fold the amount...
of DNA in dilute samples (see Table 1 below). We have therefore corrected the estimate in the text, and thank the referee for drawing our attention to this.

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<th>DNA type</th>
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Table 1. Comparison of DNA content estimates by nanodrop and Qubit methods, showing that nanodrop overestimated the amount of DNA in samples with concentrations of DNA similar to those used in the present study.

We further wished to rule out the possibility suggested by the reviewer that in our assay conditions, replication origin peaks appeared because of broken DNA fragments. To address this issue, and to show that we have indeed specifically isolated nascent RNA-DNA hybrids that enrich at the beta globin origin of replication, we carried out more control experiments in the revised manuscript. First, in one set of experiment, we carried out DNase free RNase digestion of the material prior to the l-exonuclease treatment. When this was done, we did not get any enrichment of the nascent replicating strands at the beta globin sites, although l-exonuclease digestion without prior RNase treatment resulted in strong enrichment of nascent strands (Supplementary figure 10). There was a diffuse background of DNA in both the RNase treated and untreated l-exonuclease digests and this background may have contributed to the total amount of DNA recovered. However, the background did not obscure the specific peaks. In addition, we have isolated four sizes of single stranded DNA after l-exonuclease digestion. Among these, we see significant enrichment of nascent strands with fragments that are less than 1.8 kb in size and was most prominent with DNA fragments in the range of 300-600 bases in length. Peaks of nascent DNA strands from l-exonuclease resistant fragments was not seen when fragments larger than 1.8 kb were analyzed (Supplementary Figure 10). Recently, the nascent replicating strand enrichment at the b Rep-1 region of the beta globin locus has been shown in HeLa cells using both the l-exonuclease digestion and BrdU labeling methods for mapping origins (Karnani N, Taylor CM, Malhotra A, Dutta A. Mol Biol Cell. 2010, 21:393-340) These results are all consistent with these fragments truly deriving from nascent strands at the origin of replication (Supplementary figure-11).

Comment 8. Figure 7 is of extremely poor value. How can co-localization of two objects be claimed when one object is staining the whole nucleus? ILF2 staining spread all over the nuclei and therefore could colocalize with anything. This data should be deleted.

Response: We thank reviewer for this suggestion. Following the advice, we have eliminated this Figure and its description in “Results” and “Discussion” sections.

Comment 9. Knock down of MCM5, ORC or ICF2 resulted in inhibition of β globin transcription (Figure 5A). However, this also caused inhibition of cell division. The explanation given by the authors to exclude a secondary effect due to reduced cell growth on transcription is not convincing. They claimed that serum starvation did not alter transcription of β globin. However, knock down of ORC and MCM5 are expected to arrest cell division at a specific stage that might be very different from that of cells inhibited in their growth. This leads to question whether MCM5 (or ORC) really plays a specific role in transcription of the β globin cluster.

Response: We thank the reviewer for raising this concern. We have now used three additional treatments of K562 cells with nocadazole, roscovitine and hydroxyurea acting at different phases of
the cell cycle to impair cell growth. In no case was there an effect on globin transcription similar to that seen with ILF2, MCM5 and ORC2 knockdown (Figure 5 in the main text and Supplementary Figure 11). Hydroxyurea treatment would be expected to block the cell cycle at the step of initiation of DNA synthesis and, as has been observed in other systems in the literature, actually caused an increase in gamma globin transcription. This is consistent with the widely known fact that hydroxyurea increase the transcription of the gamma globin gene. Hydroxyurea has actually been used as a therapeutic agent in patients with sickle-cell disease. These data suggest that inhibition of gamma and epsilon globin transcription by knockdown of MCM5 and ORC2 may be due to their participation in transcription and not as a secondary effect of the cell cycle arrest. As mentioned above, earlier work of others (see above, response to Comment 2 of Referee 1) showed that knock down of MCM5 (OR ILF2) did not arrest the cells at any single stage of the cell cycle. The data further support the duel role for the DARRT complex in the regulation of DNA replication origin usage and gene transcription.

Comment 10. Several experiments of this manuscript are not enough explained and could be misunderstood by readers less familiar with this field.

Response: We completely agree with the reviewer’s suggestion and have now expanded the explanation of a number of the experiments presented in the text. In the final paragraph in “Introduction” we have explained the background and rational for the present investigation keeping in mind the readers less familiar with the field. We have modified the descriptions of our experiments in the “Results” section for clarity of our experimental designs for the general reader. We have also inserted the description of the Supplementary figures 7 to 11 that contains additional new data to explain our results more clearly and effectively.

Comment 11. In conclusion, I appreciate that the authors have done a considerable effort of investigation. However, my general feeling is that more experiments are necessary and a new version of the manuscript should include only solid data from well described experiments in order to make this paper convincing.

Response: We have added a considerable number of additional experiments showing integrity of DARRT on sucrose gradient fractionation, re-evaluating the yield of DNA in the origin detection experiments, comparing the effects of various means of decreasing cell proliferation on globin transcription, showing that the effect of ILF2 knockdown on globin transcription is not a result of silencing of any of the genes for common erythroid transcription factors in K562, showing that the assay we used clearly demonstrates the major globin origin(s) of replication in several non-erythroid cells in which the globin chromatin is in a closed configuration, and providing evidence for the specificity of the assay for origins of replication by means including the dependence of this assay on intact RNA (attached to the nascent DNA strands). We have expanded the description of the experiments and their purpose, and revised figure legends for clarity and omitted the figure and discussion of the immuno-microscopy results in accordance with the referee’s suggestion. We sincerely thank the reviewers for their excellent suggestions, guidance and critical comments to improve our manuscript. We have carried out all the experiments suggested by the reviewer and have described our experiments, methodology and figures for more clarity of purpose for the non-specialist reader.

2nd Editorial Decision 16 July 2010

Your revised manuscript has been reviewed by one of the original referees who finds that you have answered the majority of the concerns raised but also finds that a couple of issues remain that must be addressed prior to publication. Pending satisfactory minor revision, we would be willing to publish your manuscript in the EMBO Journal.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For
more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The revision addressed most of the issues raised in the previous review. The controls performed to establish that the DAART complex is indeed a single complex and to validate the nascent strand abundance assay are helpful. There are two remaining minor issues:

1. The data in the current form of the article might still indicate an indirect effect of IFL-2 on DNA replication and transcription. This conclusion was also discussed by the authors in their point-by-point response. Because this issue is of interest and could form the basis for further investigation, it will be good to highlight in the Discussion section the possibility of an indirect action as an alternative to the direct involvement of DAART in the replication program of the beta globin locus.

2. The current form of Figure 1B as it appears on my computer screen and in print shows a supershift with HS4-10 not HS4-9. It is important to correct the alignment since the supershift is the main rationale for the choice of the HS4-9 oligo for further investigation. Other panels in Figure 1 also have minor alignment issues.

2nd Revision - Authors' Response 27 July 2010

Thank you for favorably considering our manuscript for publishing in EMBO Journal pending a couple of minor revisions as suggested by the referee. We have completed these, and in the following pages please find point-by-point response for the modifications suggested by the referee. We hope that this revised version will be accepted for publication in EMBO J.

Referee #1 (Remarks to the Author):

The revision addressed most of the issues raised in the previous review. The controls performed to establish that the DAART complex is indeed a single complex and to validate the nascent strand abundance assay are helpful. There are two remaining minor issues:

Comment 1. The data in the current form of the article might still indicate an indirect effect of IFL-2 on DNA replication and transcription. This conclusion was also discussed by the authors in their point-by-point response. Because this issue is of interest and could form the basis for further investigation, it will be good to highlight in the Discussion section the possibility of an indirect action as an alternative to the direct involvement of DAART in the replication program of the beta globin locus.

Response: We thank the reviewer for the excellent suggestion to discuss the possibility of an alternate indirect role of ILF2 in globin transcription and replication. Accordingly we have added the following lines at the end of fourth paragraph of the “Discussion” section in the manuscript. As the referee has pointed out, these lines reflect our discussion in the “Letter of Response: Response to Referee #1’s Comment # 1).”
“Although we cannot absolutely exclude the alternative possibility that ILF2 knockdown acts indirectly through effects on some undiscovered factor elsewhere in the genome that is necessary for use of the globin origin of replication and transcription of the globin genes, it appears simpler to propose that the effects are a result of actions of the DARRT complex containing ILF2 directly at the globin locus where we know it binds.”

Comment 2. The current form of Figure 1B as it appears on my computer screen and in print shows a supershift with HS4-10 not HS4-9. It is important to correct the alignment since the supershift is the main rationale for the choice of the HS4-9 oligo for further investigation. Other panels in Figure 1 also have minor alignment issues.

Response: We again thank the referee in pointing out the need to align the HS4 oligo numbers in Figure 1B and suggest fine tuning the alignments in Figure 1. Accordingly we have made the following changes in Figure 1.

Figure 1B: We have replaced HS4 oligonucleotide numbers 1 to 10 written on top of EMSA lanes with HS4-1 to HS4-10 that clearly show that K562 specific EMSA bands are seen with HS4-9 oligo. All the HS4-1 to HS4-10 oligo names are aligned exactly on top of their respective EMSA lanes. These modifications explicitly identify the EMSA lanes representing corresponding HS4 oligonucleotide number, wherein the K562 specific EMSA bands are clearly seen to be associated with HS4-9 oligo.

Additional alignments carried out in Figure 1:

Figure 1A: The line is resized and properly aligned over HS1 through HS5 of the LCR. All the black bars representing various globin genes are aligned properly to look even.

Figure 1C: All the arrow marks for standard molecular weight marks are fine tuned for even alignment.

Figure 1D: The “names” and “open triangles” on top of the gels are realigned for minute adjustments over their respective lanes.

We hope that these modifications are to your and referee’s satisfaction. We once again thank you and the referee for additional critical comments to enhance the quality of the figures and manuscript. We thank you for considering publishing this manuscript in EMBO J with these minor modifications.