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BRPF3-HBO1 regulates replication origin activation and histone H3K14 acetylation

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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.)

Editor: Hartmut Vodermaier

1st Editorial Decision 24 March 2015

Thank you for submitting your manuscript on BRPF3-HBO1 roles in replication origin firing for consideration by The EMBO Journal. It has now been assessed by three expert referees, whose reports you will find copied below. Although all three reviewers in principle consider the new post-licensing HBO1 role involving H3 targeting by a distinct cofactor potentially interesting, they currently remain unconvinced that all of the main conclusions are fully supported by sufficiently compelling evidence. As you will see, especially referees 1 and 3 raise a number of specific points that in their view require additional experimentation. The overriding key concern, stated explicitly by referees 1 and 2, is the the need to demonstrate a direct and local effect of the identified BRPF3-HBO1-H3K14Ac-Cdc45 axis at selected origins. In the absence of such demonstration, I am afraid it would seem premature for us to commit to eventual future acceptance and publication in The EMBO Journal at this stage.

Nevertheless, should further experimental efforts allow you to decisively rule out indirect/general effects and to establish causal involvement of the proposed mechanism and factors at specific loci, we would remain open to considering a revised manuscript further for publication. Since it is our policy to to allow only a single round of major revision, it will however be essential to carefully respond to both these conceptual and the various more specific points raised during this round; I

would in this case be open to discussing an extension of our standard three-months revision time if necessary. As always at The EMBO Journal, competing manuscripts published elsewhere during such an official revision period would have no negative impact on our final assessment of your revised study. When revising the paper, I would furthermore urge you to carefully proof-read and edit the manuscript before resubmission, including a more explicit early introduction of some BRPF-related concepts and precedent findings that had already been reported by some of your current co-authors. Finally, I should point out that we now require a completed 'author checklist' to be submitted with all revised manuscripts - see below for more detail.

Thank you again for the opportunity to consider this work for The EMBO Journal, and please do not hesitate to contact me should you have any comments or questions regarding the referee reports or this decision. I look forward to your eventual revision.

REFEREE REPORTS

Referee #1:

In this manuscript, Feng et al performed siRNA screen for genes affected DNA replication using RPA-GFP as readout. They identified several genes and focused on characterization of BRPF3, a protein containing PHD and bromodomain. They showed that depletion of BRPF3 affected DNA replication (BrdU incorporation, DNA coming/fiber assays) and origin firing. In addition, BRPF3 binds HBO1 and BRPF3-HBO1 acetylates histone H3 and H4 in vitro and the specificity depends on in part the substrates (core histone vs nucleosomes). Depletion of BRPF3 also affects the levels of H3K14ac as well as Cdc45 chromatin binding. BRPF3 co-localizes with Orc1, HBO1 and H3K14ac. Overall, the authors presented interesting results. However, most of these results are correlative in nature and little or no direct evidence supports the model that BRPF3-HBO1 affects H3K14ac, which in turn affects Cdc45 chromatin binding. To provide direct evidence supporting this model, the authors need to show results at several origins the effect of BRPF3 depletion on HBO1 binding, H3K14ac levels, Cdc45 chromatin binding and BrdU incorporation.

Other concerns

1) PCNA positive cells are not altered in cells depleted with candidate genes (Fig. S1) However, BrdU is reduced in cells depleted with some of the genes. In normal cell cycle progression, PCNA and BrdU should co-localize. What is the explanation?

2) what is knock-out efficiency for other siRNA of BRPF3 (d and e) and siRNAs for BRPF1 and 2? It appears that some of siRNA for BRPF1 and 2 affects RPA-GFP (Fig S3A).

3) Fig. 2E. BRPF3-containing HAT complex in vitro specificity for lysine residue should be tested using either Western blot or mass spectrometry analysis.

4) Fig. 2F. what is the effect of HBO1 depletion on H3K14ac?

5) Fig. 2G. Why expression of BRPF3 cannot rescue the H3K14ac in cells BRPF3 depleted cells using siRNA (b)?

6) Fig. 2D. The effect of BRPF3 depletion on Cdc45 chromatin binding is very mild and is within the experimental error of Western blot. Therefore, additional assays such as Cdc45 ChIP should be used to directly test whether Cdc45 is reduced at dormant origins. In addition, the effect of HBO1 depletion on Cdc45 binding under the same conditions should be presented.

7) Figure 2A. If BRPF3 is required for dormant origin activation, one would expect that depletion of BRPF3 suppressed the activation of dormant origins induced by UCN-01.

8) Figure 3B. It appears that the majority of HBO1 sites co-localizes with BRPF3 sites and yet HBO1 also affects origin firing through H4 acetylation, which is independent of BRPF3. Is this reasonable?

9) Figure 4. Is it known whether origins close to TSS are dormant? Is it possible to perform BrdU ChIP to determine whether BRPF3 is localized at dormant origins?

10) Fig 5B would argue that BRPF3 is a negative regulator of DNA replication instead of a positive one. It is hard for me to understand the meaning of the result

Referee #2:

In this manuscript, Feng and colleagues describe the identification of the chromatin scaffold protein BRBF3 in an siRNA screen for mutants that impact DNA replication. Loss of BRBF3 results in reduced replication (EdU incorporation by 30%) and decreased inter-origin distance (DNA fiber analysis). Loss of BRBF3 was also associated with moderately decreased levels of H3K13Ac (~20%). Interestingly, BRBF3 was found to be in a complex with the HAT HBO1 and, as a complex, they exhibited specificity for nucleosomal histone H3. The authors also used genomic approaches to analyze the genome-wide distribution of BRBF3 and found that it was enriched at TSSs and overlapped with H3K18Ac and HBO1 localization. A minor subset also overlapped with ORC1. Finally, the defect in origin activation in the absence of BRBF3 appears to be due to a defect in Cdc45 loading. Aside from the role of BRBF3 in DNA replication there are a couple of interesting and novel observations that will be important for the field. First, BRBF3 acts a specificity factor for HBO1 targeting it to histone H3 and not the canonical HBO1 target of histone H4. Second, loss of the BRBF3/HBO1 complex impairs an origin activation step, Cdc45 loading, and not pre-RC assembly (a step previously shown to be dependent on HBO1), which suggests that HBO1 regulates origin function at multiple distinct steps. The experiments were well executed and convincing despite the moderate effects. However, a word of caution is warranted -- It is not trivial to assign a direct role and local role for a specific histone modification in regulating the DNA replication program. For example, loss of the HDAC Rpd3 was originally thought to promote origin activation via the accumulation of hyperacetylated H3 in S. cerevisiae. However, it was recently demonstrated by the Pasero group that this was not due to the local impact of histone acetylation at origins, but rather was mediated in trans by the effect of Rpd3 on the the multicopy rDNA locus which sequestered key replication factors (Yoshida et al., Mol Cell, 2014). The reviewers could significantly strengthen their conclusions by demonstrating the local loss of H3K18Ac in the absence of BRPF3 at a few select origins and a corresponding loss of Cdc45 or origin specific replication intermediates.

Minor:

There are several HATs that potentially acetylate H3K18. Do loss of any of these HATs phenocopy loss of BRPF3?

Origin 'firing' is a colloquial expression. It would be better to use 'activation'.

Statements like 'BRPF3 "is required for" DNA replication and H3K14Ac.' should be replaced with "regulates" or "modulates". The effect is moderate at best.

The genomic data needs to be deposited in a public data repository .

Referee #3:

In this manuscript, the authors performed a large SiRNA screen to identify proteins that are required for HU-induced ssDNA exposure. They found twenty potential factors and focused their study on BRPF3. From their data, they conclude that BRPF3 forms a complex with HB01, which in turn acetylates H3K14 and regulates the efficient activation of replication origins.
I found the results of rather unequal quality. Several aspects of BRPF3 role are analyzed, but for a number of them the quality and quantity of the data are not convincing enough. It is also not clear whether the defects observed in BRPF3-deficient cells are linked to checkpoint defects or directly to

the normal process of replication origin activation. Overall, this study remains preliminary for several reasons.
1) Figure 2 A and B analyze EdU intensity as a marker of DNA replication after BRPF3 silencing. Here, a FACS analysis, also involving BrdU incorporation, is crucially missing. Florescence intensity is clearly not sufficient to conclude that BPF3 is required for DNA replication. In addition, the defects shown are rather weak.
2) In Figure 2C, like in several other Figures of this manuscript, the analysis is performed in cells transiently transfected to express tagged recombinant proteins, and not on the endogenous proteins. Therefore, the level of the proteins of interest could be abnormally increased, leading to abnormal effects.
3) In Figure 3C, the authors measure RPA loading based on the fluorescence intensity. Here, it would have been more convincing to perform a western blot analysis of chromatin.
4) Figure 3D. It is not clear what 1x and 2x are meaning. If it is the volume loaded on the gels, then the BRBF3 and histone H4 signals cannot be interpreted, as there is a huge increase of the signal for 2x as opposed to 1x.
5) Figure 4. The correlation with replication origins is very preliminary. It would have been better to compare these results with the data by Besnard et al (Nature Struct Biol. 2012), or better by Picard et al, (PLOS Genet. 2014) all obtained from human cells.
6) Figure 5 should be much better explained. I guess the FACS was performed 9 hrs after the release. It would be more convincing to perform intermediate FACS analyses, for example 4 hrs after the release, and also much later to determine whether the cell cycles are completed. For these analyses, BrdU-FACS is required.

1st Revision - authors' response 22 September 2015

Please find our response to the reviewers' comments in italics below.

Referee #1:
In this manuscript, Feng et al performed siRNA screen for genes affected DNA replication using RPA-GFP as readout. They identified several genes and focused on characterization of BRPF3, a protein containing PHD and bromodomain. They showed that depletion of BRPF3 affected DNA replication (BrdU incorporation, DNA coming/fiber assays) and origin firing. In addition, BRPF3 binds HBO1 and BRPF3-HBO1 acetylates histone H3 and H4 in vitro and the specificity depends on in part the substrates (core histone vs nucleosomes). Depletion of BRPF3 also affects the levels of H3K14ac as well as Cdc45 chromatin binding. BRPF3 co-localizes with Orc1, HBO1 and H3K14ac. Overall, the authors presented interesting results. However, most of these results are correlative in nature and little or no direct evidence supports the model that BRPF3-HBO1 affects H3K14ac, which in turn affects Cdc45 chromatin binding. To provide direct evidence supporting this model, the authors need to show results at several origins the effect of BRPF3 depletion on HBO1 binding, H3K14ac levels, Cdc45 chromatin binding and BrdU incorporation.

We have performed ChIP analysis in BRPF3 depleted cells and find that H3K14ac is reduced at several replication origins (selected based on H3K14ac and BRPF3 occupancy at origins identified by SNS and ORC1) (new Fig. 4E). We do not propose that BRPF3 is responsible for HBO1 recruitment at these sites, but rather that BRPF3 directs HBO1 towards H3K14 when bound as part of a HBO1-ING4/5-EAF6-BRPF3 complex. Consistent with this, HBO1 ChIP at a selected origin was not changed upon BRPF3 depletion (see Fig. R1).

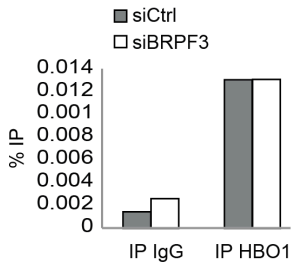

Figure R1. ChIP-qPCR of HBO1 in siRNA treated RKO cells. The figures show immunoprecipitated material relative to input measured at the Lamin B2 origin.

Further, in support of our model we find that HBO1 depletion also reduced H3K14ac at several

origins. Together with our data showing reduced EdU incorporation and impaired origin activation (single molecule analysis) in response to BRPF3 depletion, this argues that BRPF3-HBO1 acetylation of H3K14ac impacts on origin activity. While BrdU and CDC45 ChIP are routinely used to measure origin activity in yeast, this is not a method applicable to assay origin efficiency in mammalian cells where origin activation occurs in broad initiation zones and asynchronously (Gilbert, 2010). We have attempted to analyse CDC45 loading by ChIP, but these experiments were not successful for technical reasons. To our knowledge ChIP of CDC45 has not been used convincingly to study activation of replication origins in mammalian cells. CDC45 travels with the fork and should be present throughout the genome in an asynchronous population. In a synchronized population (e.g. released into HU), CDC45 could in theory be present at early firing origins. A previous paper found a ChIP signal from CDC45 at an early replicating site; however, CDC45 was only detected in cells treated with HU and checkpoint inhibitors and, as MCM3 was lost under these conditions, it was not clear how CDC45 would be bound at this locus (Karnani & Dutta, 2011). In this light, we hope the reviewer will agree that cell fractionation and western blotting is the most solid approach to assess CDC45 loading in mammalian cells, as done in recent key publication on mammalian replication initiation (Aparicio et al, 2009; Ballabeni et al, 2009; Boos et al, 2013; Gerhardt et al, 2015; Rondinelli et al, 2015). We have now carried out DNA fiber analysis in BRPF3 depleted cells treated with UCN-01 and find that dormant origin activation is significantly reduced (new Fig. 3C), correlating with the impaired CDC45 loading onto chromatin assayed by chromatin fractionation and western blotting (Fig. 3E and new Fig 3G).

Other concerns

1) PCNA positive cells are not altered in cells depleted with candidate genes (Fig. S1) However, BrdU is reduced in cells depleted with some of the genes. In normal cell cycle progression, PCNA and BrdU should co-localize. What is the explanation?
BRPF3 is not required for cells to enter S phase and thus the number of PCNA positive cells remain largely unaltered in BRPF3 depleted cells (Supplementary Fig S1A). However, in S phase BRPF3 depleted cells activate fewer origins compared to control cells. This explains why the rate of EdU incorporation is reduced without affecting the number of PCNA positive cells. We did not observe any general correlation between the level of PCNA and BrdU incorporation, which probably reflects that PCNA loading and unloading is a regulated process that does not necessarily correlate with the number of active origins (Gomes & Burgers, 2001; Lee et al, 2013; Mejlvang et al, 2014). In example, ASF1 depleted cells arrest in S phase with strongly reduced BrdU incorporation and elevated levels of PCNA on chromatin (Groth et al, 2007).

2) what is knock-out efficiency for other siRNA of BRPF3 (d and e) and siRNAs for BRPF1 and 2? It appears that some of siRNA for BRPF1 and 2 affects RPA-GFP (Fig S3A).
We had verified all siRNAs, and we apologize that not all siRNA knockdown data were included in the manuscript. A new western blot has been included, showing that all our siRNAs against BRPF3 are working efficiently (new supplementary Fig. S3B). In addition, the siRNAs targeting BRPF1 and 2 also worked efficiently as demonstrated (Supplementary Fig. S4A).

Compared to our positive control (siRNA against Asf1), 1 out of 3 siRNAs against BRPF2 reduced accumulation of GFP-RPA1 (Supplementary Fig S3A). Our screen scores the combined effect of several siRNAs and this explains why BRPF2 did not score. Importantly, none of the siRNAs against BRPF1 and BRPF2 impaired DNA replication (Fig. 2A). We noted that BRPF2 depletion moderately reduced H3K14ac (consistent with a recent report (Mishima et al, 2011)), suggesting that BRPF2-HBO1 might make a minor contribution to H3K14ac at origins. However, accidentally we had used a siRNA that targeted both BRPF2 and BRPF3 and this co-depletion did not enhance the replication defect compared to BRPF3 depletion alone (Fig R2). Together this argues that the replication initiation defect is specific to BRPF3 depleted cells.

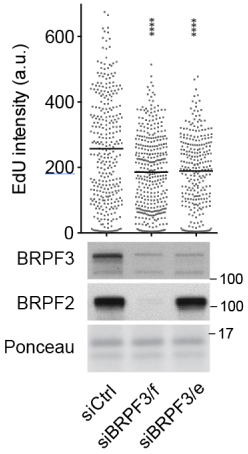

*Figure R2. Effect of BRPF2/3 co-depletion on DNA replication. U2OS cells were treated with indicated siRNAs for 48 h prior to (top) analysis of EdU incorporation and (bottom) western blot. Intensities of EdU were analysed in EdU-positive cells. n > 250. Mann-Whitney: **** $P < 10^{-4}$. a.u. arbitrary unit.*

3) Fig. 2E. BRPF3-containing HAT complex in vitro specificity for lysine residue should be tested using either Western blot or mass spectrometry analysis.
We have now analysed the specificity of the BRPF3 and BRPF1 complexes on H3 peptides (new Fig. 2F). This analysis shows that the BRPF3-HBO1 complex mainly targets H3 peptides spanning amino acids 1-21, but that it has no activity on a peptide carrying K14ac. This provides additional evidence that the BRPF3-HBO1 complex targets H3K14. In contrast BRPF1-MOZ prefers peptides spanning H3K23. This result is consistent with our in vivo analysis, showing reduced H3K14ac (not H3K23ac or H4ac) in BRPF3 depleted cells and reduced H3K23ac (not H3K14ac or H4ac) in BRPF1 depleted cells (Fig. 2G and supplementary Fig. S4A). This is now further confirmed by ChIP-qPCR showing decreased H3K14ac signals at replication origins in BRPF3 and HBO1 depleted cells (New Fig. 4E)

4) Fig. 2F. what is the effect of HBO1 depletion on H3K14ac?
H3K14ac is reduced upon HBO1 depletion as measured by Western blot and ChIP-qPCR at origins (New Fig. 4E and supplementary Fig. S4A), consistent with a previous report showing loss of H3K14ac in HBO1 knockout mice (Kueh et al, 2011).

5) Fig. 2G. Why expression of BRPF3 cannot rescue the H3K14ac in cells BRPF3 depleted cells using siRNA (b)?
Exogenous BRPF3 was engineered to be resistant only to siBRPF3/a, not siBRPF3/b. Therefore, siBRPF3/b reduced BRPF3 protein expression and downregulated H3K14ac. We now clarify this in the legend.

6) Fig. 2D *(we believe that the reviewer refers to 3D)*. The effect of BRPF3 depletion on Cdc45 chromatin binding is very mild and is within the experimental error of Western blot. Therefore, additional assays such as Cdc45 ChIP should be used to directly test whether Cdc45 is reduced at dormant origins. In addition, the effect of HBO1 depletion on Cdc45 binding under the same conditions should be presented.
We agree with the reviewer that the effect on CDC45 is within the experimental error of a Western blot experiment, which is why we have quantified CDC45 loading relative to histone H4 levels in three independent experiments. This shows that there is a statistically significant reduction in CDC45 loading (Fig. 3F). As described above it has not been feasible to measure CDC45 loading by ChIP, and we would strongly argue that cell fractionation and western blotting is the most solid approach to assess CDC45 loading in mammalian cells as has been done previously (Aparicio et al, 2009; Ballabeni et al, 2009; Boos et al, 2013; Gerhardt et al, 2015; Rondinelli et al, 2015).

We also investigated the loading of CDC45 upon knocking down HBO1 as suggested by the reviewer. Upon UCN-01 treatment, CDC45 loading was reduced in HBO1 depleted cells (New Fig. 3G). In addition, HBO1 depletion also reduced MCM2 loading onto chromatin, consistent with previous reports (Iizuka et al, 2006; Miotto & Struhl, 2008; Miotto & Struhl, 2010).

7) Figure 2A *(we believe that the reviewer refers to 3A)*. If BRPF3 is required for dormant origin

activation, one would expect that depletion of BRPF3 suppressed the activation of dormant origins induced by UCN-01.
We have now tested this prediction and found by DNA fibre analysis that BRPF3 depletion indeed reduces the inter CldU track distances in cells treated with UCN-01. This new result further supports that BRPF3 is required for dormant origin firing and it has been incorporated into our manuscript as Fig. 3C and supplementary Fig. S4D.

8) Figure 3B (*we believe that the reviewer refers to* 4B). It appears that the majority of HBO1 sites co-localizes with BRPF3 sites and yet HBO1 also affects origin firing through H4 acetylation, which is independent of BRPF3. Is this reasonable?
Yes, we apologize for not clarifying this point. BRPF3 and JADE1 are alternative scaffold proteins that hold together HBO1 and ING4/5 (this work; (Doyon et al, 2006; Lalonde et al, 2013)). We propose that BRPF3 and JADE1 control substrate specificity of HBO1-ING4/5 against H3K14 and H4, respectively (this work; (Foy et al, 2008; Lalonde et al, 2013)). Recruitment of these complexes to chromatin relies on a complex combinatorial effect of the many chromatin-binding domains (including those in the ING4/5 proteins) (Saksouk et al, 2009). Genome-wide analyses of BRPF3, HBO1 and JADE1 argue that the two complexes (HBO1-BRPF3-ING4/5 and HBO1-JADE1-ING4/5) are recruited to the same regions surrounding TSS (this work; (Avvakumov et al, 2012; Lalonde et al, 2013; Saksouk et al, 2009)), where we propose they can facilitate two consecutive steps (origin licensing and activation) in replication initiation.

9) Figure 4. Is it known whether origins close to TSS are dormant? Is it possible to perform BrdU ChIP to determine whether BRPF3 is localized at dormant origins?
Characteristics of dormant origins remain unclear, however they are supposedly scattered throughout the genome and found in vicinity of active origins also surrounding TSS. Since BRPF3 localization is overlapping significantly with ORC1 binding sites (of which only a fraction will act as active origins) (Fig. 4B), we expect that BRPF3 is also present at dormant origins.
A previous study has identified clusters of potentially dormant origins by releasing cells into HU in the presence of BrdU and performing HU-BrdU ChIP-Chip (Karnani & Dutta, 2011). From the 178 HU-BrIP sites identified, 34 overlap with BRPF3 peaks (19 %). However, most of the HU-BrIP sites cluster in regions of less than 10 kb, which means that not more than 29 clusters of fired origins were identified in the whole genome. This illustrates the complication of mapping dormant origins in human cells. However, of the 29 clusters, 17 overlap with BRPF3 and H3K14ac peaks (58 %), supporting that BRPF3-HBO1 could regulate dormant origin activation.

10) Fig 5B would argue that BRPF3 is a negative regulator of DNA replication instead of a positive one. It is hard for me to understand the meaning of the result.

This result was also surprising to us and we included it because it illustrates an important point; namely that a moderate replication initiation defect in asynchronous cells can protect cells from replication stress induced DNA damage.
Our data show that BRPF3 depleted cells have fewer (and faster) active replication forks as compared to control cells (Fig. 2A and 2B). In response to HU treatment, these cells show reduced ssDNA formation (Fig. 1C and supplementary Fig S3A) and less DNA damage signalling (Fig. 5A). The most parsimonious explanation is that fewer active forks stall and collapse. This view is also in line with recent reports showing that new origin firing and exhaustion of RPA pools can contribute to fork collapse (Dungrawala et al, 2015; Toledo et al, 2013). Once the replication inhibitor is removed, cells must recover from the damage and surprisingly we found that BRPF3 depleted cells now showed an advantage over control cells (Fig. 5B, 5C and new supplementary Fig. S6). In our eyes, the simplest explanation is that BRPF3 cells suffered less DNA damage in response to HU (Fig. 5A).
Reduced licensing upon partial depletion of MCM2-7 increases sensitivity to replication stress and replication stress induced DNA damage signalling (Ge et al, 2007; Ibarra et al, 2008). However, that situation differs from BRPF3 depletion as cells with partial MCM2-7 depletion are only impaired in dormant origin firing and do not have fewer active forks prior to HU treatment. Our data thus argues that reducing licencing and deficient origin firing are not necessarily comparable. Although mechanistically clearly different, this has some resemblance to the observation that defects in origin licensing in Meier-Gorlin syndrome mutations (ORC1, CDT1, CDC6) do not show genome

instability or sensitivity to replication stress (Alver et al, 2014). Thus in our eyes, the recovery data presented in Fig.5 and new supplementary Fig. S6 brings an important addition to understand how the balance between origin usage, fork speed and dormant origin activation affect susceptibility to replication stresses, which has potential implication for cancer treatment.

Referee #2:

In this manuscript, Feng and colleagues describe the identification of the chromatin scaffold protein BRBF3 *(BRPF3)* in an siRNA screen for mutants that impact DNA replication. Loss of BRBF3 results in reduced replication (EdU incorporation by 30%) and decreased inter-origin distance (DNA fiber analysis). Loss of BRBF3 *(BRPF3)* was also associated with moderately decreased levels of H3K13Ac *(H3K14ac)* (~20%). Interestingly, BRBF3 was found to be in a complex with the HAT HBO1 and, as a complex, they exhibited specificity for nucleosomal histone H3. The authors also used genomic approaches to analyze the genome-wide distribution of BRBF3 and found that it was enriched at TSSs and overlapped with H3K18Ac *(K14ac)* and HBO1 localization. A minor subset also overlapped with ORC1. Finally, the defect in origin activation in the absence of BRBF3 appears to be due to a defect in Cdc45 loading. Aside from the role of BRBF3 in DNA replication there are a couple of interesting and novel observations that will be important for the field. First, BRBF3 acts a specificity factor for HBO1 targeting it to histone H3 and not the canonical HBO1 target of histone H4. Second, loss of the BRBF3/HBO1 complex impairs an origin activation step, Cdc45 loading, and not pre-RC assembly (a step previously shown to be dependent on HBO1), which suggests that HBO1 regulates origin function at multiple distinct steps. The experiments were well executed and convincing despite the moderate effects. However, a word of caution is warranted -- It is not trivial to assign a direct role and local role for a specific histone modification in regulating the DNA replication program. For example, loss of the HDAC Rpd3 was originally thought to promote origin activation via the accumulation of hyperacetylated H3 in S. cerevisiae. However, it was recently demonstrated by the Pasero group that this was not due to the local impact of histone acetylation at origins, but rather was mediated in trans by the effect of Rpd3 on the multicopy rDNA locus which sequestered key replication factors (Yoshida et al., Mol Cell, 2014). The reviewers could significantly strengthen their conclusions by demonstrating the local loss of H3K18Ac *(K14ac)* in the absence of BRPF3 at a few select origins and a corresponding loss of Cdc45 or origin specific replication intermediates.

We are pleased that the reviewer highlights that our work include several interesting findings that will be important for the field and further states that our work is well executed and convincing. We agree that caution should be taken in assigning a histone modification to regulate the replication program. We take care in our discussion to say that we cannot exclude non-histone targets and we now also refer to the Rpd3 story from the Pasero lab. On that note, it is important to state that we also went a long way to address whether BRPF3 might regulate replication through indirect effects on transcription, but found no evidence to support that (Fig. 4F and supplementary Fig. S5D).

We have performed ChIP analysis in BRPF3 depleted cells and find that H3K14ac is reduced at several replication origins (selected based on H3K14ac and BRPF3 occupancy at origins identified by SNS and ORC1) (new Fig. 4E). We propose that BRPF3 directs HBO1 towards H3K14 when bound as part of an HBO1-ING4/5-EAF6-BRPF3 complex. In support of this model, we find that HBO1 depletion also reduced H3K14ac at these origins. Together with our data showing reduced EdU incorporation and impaired origin activation (single molecule analysis) in response to BRPF3 depletion, this argues that BRPF3-HBO1 acetylation of H3K14 impacts on origin activity. As also described in our response to reviewer 1: While BrdU and CDC45 ChIP are routinely used to measure origin activity in yeast, this is not a method applicable to assay origin efficiency in mammalian cells where origin activation occurs in broad initiation zones and asynchronously (Gilbert, 2010). We have attempted to analyse CDC45 loading by ChIP, but these experiments were not successful for technical reasons. To our knowledge ChIP of CDC45 has not been used convincingly to study activation of replication origins in mammalian cells. CDC45 travels with the fork and should be present throughout the genome in an asynchronous population. In a synchronized population (e.g. released into HU), CDC45 could in theory be present at early firing origins. A previous paper found a ChIP signal from CDC45 at an early replicating site; however, CDC45 was only detected in cells treated with HU and checkpoint inhibitors and, as MCM3 was

lost under these conditions, it was not clear how CDC45 would be bound at this locus (Karnani & Dutta, 2011). In this light, we hope the reviewer will agree that cell fractionation and western blotting is the most solid approach to assess CDC45 loading in mammalian cells, as done in recent key publication on mammalian replication initiation (Aparicio et al, 2009; Ballabeni et al, 2009; Boos et al, 2013; Gerhardt et al, 2015; Rondinelli et al, 2015). We have now carried out DNA fiber analysis in BRPF3 depleted cells treated with UCN-01 and find that dormant origin activation is significantly reduced (new Fig. 3C), correlating with the impaired CDC45 loading onto chromatin assayed by chromatin fractionation and western blotting (Fig. 3E and new Fig 3G).

Minor:

There are several HATs that potentially acetylate H3K18 *(K14ac)*. Do loss of any of these HATs phenocopy loss of BRPF3?
HBO1 knockout in mice led to a dramatic reduction of H3K14ac (Kueh et al, 2011), suggesting that HBO1 is a main HAT for H3K14ac. However, as HBO1 can also mediate histone H4ac (Supplementary Fig. S4A), involved in licencing, the phenotype is different from knockdown of BRPF3 alone (see text and new Figure 3G for details). It is plausible that other HATs like GCN5 and p300/CBP acetylate H3K14 at some positions in the genome, but the in vivo specificities of different H3 HATs remain poorly defined despite extensive investigations.
We are not aware that they regulate replication initiation and if so whether this happens through indirect or direct effects (these are key regulators of transcription that target multiple H3 lysines). However, artificial recruitment of GCN5 to a late replication origin can force premature activation.

KAT5 (TIP60) and KAT8 (MOF), which catalyse H4K16ac, were identified in our siRNA screen (Fig. 1B and supplementary table 2) and found to be required for ssDNA accumulation and normal rate of DNA replication (Fig 1C, 1D, supplementary Fig S2E and S2F). This suggests that also H4K16ac could be important for replication, but the mechanism remains to be explored.

We hope this answer illustrates the large nature of the question and why it is beyond the scope of our work to screen H3 HATs for a role in H3K14ac and DNA replication initiation.

Origin 'firing' is a colloquial expression. It would be better to use 'activation'.
We appreciate reviewer's suggestion and we now generally use "activation" in our revised manuscript.

Statements like 'BRPF3 "is required for" DNA replication and H3K14Ac.' should be replaced with "regulates" or "modulates". The effect is moderate at best.
We have changed these statements; we did not want to give the impression that BRPF3 is absolutely required for replication, but rather that BRPF3 is required for normal rates of DNA replication and normal levels of H3K14ac.

The genomic data needs to be deposited in a public data repository.
All genomic data in the manuscript are deposited in public databases, and will be released upon publication of the manuscript.

Referee #3:

In this manuscript, the authors performed a large siRNA screen to identify proteins that are required for HU-induced ssDNA exposure. They found twenty potential factors and focused their study on BRPF3. From their data, they conclude that BRPF3 forms a complex with HB01, which in turn acetylates H3K14 and regulates the efficient activation of replication origins.
I found the results of rather unequal quality. Several aspects of BRPF3 role are analysed, but for a number of them the quality and quantity of the data are not convincing enough. It is also not clear whether the defects observed in BRPF3-deficient cells are linked to checkpoint defects or directly to the normal process of replication origin activation. Overall, this study remains preliminary for several reasons.

We present evidence that checkpoint signalling is not activated in BRPF3 depleted cells (Fig. 5A,

supplementary Fig. S1B, and new Fig. S3B), thus this cannot explain the reduced fork density and elevated fork speed in BRPF3-depleted cells. However, BRPF3 depletion largely phenocopies cdc7 mutants in yeast, which also show reduced origin firing, fewer but faster forks and reduced Rad53 signalling (Zhong et al, 2013).

We would also like to make the reviewer aware that many of the controls requested below were already included in the manuscript (as detailed below), thus we feel the strong statement regarding the quality of our work is not justified.

1) Figure 2 A and B analyze EdU intensity as a marker of DNA replication after BRPF3 silencing. Here, a FACS analysis, also involving BrdU incorporation, is crucially missing. Florescence intensity is clearly not sufficient to conclude that BPPF3 is required for DNA replication. In addition, the defects shown are rather weak.

Both FACS analysis and microscopy rely on fluorescence and modern high-content microscopy is quantitative and fully comparable to FACS. EdU is also equivalent to BrdU and now the standard choice for replication analysis in human cells, because it can be detected without a need for denaturing DNA.

We would like to underscore that EdU intensity is reduced in BRPF3 depleted cells, considering either the total cell population or S phase cells specifically (selected based on PCNA staining) (Fig. 2A and supplementary Fig. S3C; a new panel in S3C highlights this). Thus, differences in S phase population are not influencing the result, if this is what the reviewer is concerned about. To further illustrate this point, we now also show diagrams of EdU intensity plotted against DAPI staining (obtained by high-content microscopy, but comparable to FACS). This further confirms the rate of DNA replication is reduced in the absence of BRPF3 (new supplementary Fig. S3D).

2) In Figure 2C, like in several other Figures of this manuscript, the analysis is performed in cells transiently transfected to express tagged recombinant proteins, and not on the endogenous proteins. Therefore, the level of the proteins of interest could be abnormally increased, leading to abnormal effects.
Our description of the experimental settings used for investigating BRPF3-HBO1 interaction must unfortunately have been insufficient. To clarify:
- Data in Fig. 2C was obtained using a single copy ZFN integrated transgene at the AAVS1 locus, which is fundamentally different from overexpression. Importantly, the distinct sets of interactions obtained for BRPF1 and 3 (e.g. MOZ with BRPF1 and HBO1 with BRPF3) reflect the specificity in this system.
- Data in Fig. 2B, 2H, supplementary Fig. S3E and S3F is based on a stable expression of Flag-BRPF3 in a cell line generated by lenti-viral transduction.
- Fig. 2D and supplementary Fig. S3G are indeed transient transfection. However, compared to according controls, the specificity of BRPF3 interaction was clearly demonstrated.

Collectively, using different approaches, including mass spectrometry and IP (Fig. 2C, 2D, supplementary Fig. S3F and S3G), we have shown that BRPF3 specifically interacts with HBO1 and ING4/5 and EAF6.

3) In Figure 3C, the authors measure RPA loading based on the fluorescence intensity. Here, it would have been more convincing to perform a western blot analysis of chromatin.
This experiment is included in Fig. 3E (previously 3D), showing that depletion of BRPF3 with two independent siRNAs reduced RPA loading on chromatin.

4) Figure 3D. It is not clear what 1x and 2x are meaning. If it is the volume loaded on the gels, then the BRBF3 and histone H4 signals cannot be interpreted, as there is a huge increase of the signal for 2x as opposed to 1x.
We apologize for not defining this in the legend. 1x and 2x indicate the amount of cell extracts loaded on the gels. We routinely use this as a semi-quantitative approach to evaluate western-blotting results because the signals are often not linear (Alabert et al, 2014; Groth et al, 2007; Huang et al, 2015; Jasencakova et al, 2010; Mejlvang et al, 2014). It is common to see that antibodies differ widely in their linear range but this does not question the specificity of the antibody.

5) Figure 4. The correlation with replication origins is very preliminary. It would have been better to compare these results with the data by Besnard et al (Nature Struct Biol. 2012), or better by Picard et al, (PLOS Genet. 2014) all obtained from human cells.
We appreciate the suggestion of the reviewer and have now compared our results with those data carefully. Both datasets show a high overlap in identified origins (99.7% of Picard origins also identified by Besnard). However, considering that Picard 2014 solves resolution issues found previously in SNS analysis, we have decided to focus our analysis on this data set. Using the HeLa replication origins described in Picard et al. PLoS Genet. 2014, we found a better correlation between origins and BRPF3 binding sites (68% overlap versus the 29% observed with ORC1) (new supplementary Fig S6B and S6C). Furthermore, using the Picard et al., data as a reference for replication origins we also see a large overlap with HBO1 and enrichment of H3K14ac (new supplementary Fig S6B and S6C). Thus this further supports a role of BRPF3-HBO1 and H3K14ac at replication origins.

6) Figure 5 should be much better explained. I guess the FACS was performed 9 hrs after the release. It would be more convincing to perform intermediate FACS analyses, for example 4 hrs after the release, and also much later to determine whether the cell cycles are completed. For these analyses, BrdU-FACS is required.

As the reviewer suggested, we now include more time points for this analysis (new supplementary Fig. S6A and S6B) and analysed both cell cycle profiles and EdU incorporation by high content microscopy. Consistent with our previous experiments (Fig. 5C), BRPF3 depleted cells recovered from HU faster than control cells. In particular, EdU incorporation was more efficient 4 hours after release in BRPF3 depleted cells (new Fig. 5B) as predicted by the reviewer and at later time points (12-18 hours) these cells showed more efficient progression into the next cell cycle as compared to controls (new supplementary Fig. S6A and S6B).

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2nd Editorial Decision 19 October 2015

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Before we will able to send you a formal letter of acceptance, there are a number of editorial issues that need to be taken care of:

* please introduce the minor presentational change requested by referee 1

* the supplementary files will need altering in accordance with our new format for these types of information: see http://emboj.embopress.org/authorguide#expandedview
- Tables 1-5 should be turned into EV (expanded view) files and cited as such in the text. Each one needs a short legend contained with an additional sheet of the respective Excel file
- Tables 6-9 should be added to the 'Appendix' (previously: Supplementary Information) PDF file containing the supplementary figures. These figures (and the tables 6-9) too need relabelling as 'Appendix Figure S#'/'Appendix Table S#' both within that file and within the main text wherever they are referenced

* please suggest 2-5 one-sentence 'bullet points', containing brief factual statements that summarize key aspects of the paper - they will form the basis of a 'Synopsis' accompanying the online version of the article'. Please see the latest research articles on our website (emboj.embopress.org) for examples.

* In addition, I would encourage you to also provide an image for the Synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size is fixed to 550 pixels in width and 150-400 pixels in height. In this case,

the easiest would be to utilize the schematic from Figure 6 of the paper, just slightly rearranging it so as to smugly fit into the 550x400 pixel 'landscape' format of our synopses.

I am therefore returning the manuscript to you once more for a final round of modification, allowing you to introduce these changes. Please don't hesitate to contact our office in case there should be any confusion around the supplementary information policies. Once we will have received your final files, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

REFEREE REPORTS:

Referee #1:

This revised manuscript has addressed most of my concerns and the revised manuscript has improved substantially. I support it publication. I have only one minor change. Figure 2F, label the residue number of H3K27me peptide to help readers.

Referee #2:

I am satisfied with the revised manuscript and the response from the author.

2nd Revision - authors' response 30 October 2015

I am happy that our reviewers supported publication of our manuscript and I hereby send you a revised version amended to all editorial concerns.

- Peptide residues have now been indicated in Figure 2F
- Supplementary tables 1-5 are included as Table EV1-5 and are refer-enced accordingly in the text.
- Supplementary tables 6-9 have been added to the Appendix and are referenced accordingly in the text.
- Highlights summarizing the key findings are included
- Image for the synopsis is included

We have moved part of the Materials and Methods section to the Ap-pendix, shortening the manuscript to fit EMBO J guidelines

I thus hope we meet all editorial points and the manuscript can now be accepted for publication in EMBO Journal.