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DNA replication stress differentially regulates G1/S genes via Rad53-dependent inactivation of Nrm1

Anna Travesa, Dwight Kuo, Robertus A.M. de Bruin, Tatyana I.Kalashnikova, Marisela Guaderrama, Kevin Thai, Aaron Aslanian, Marcus B. Smolka, John R. Yates III, Trey Ideker, Curt Wittenberg

Corresponding author: Curt Wittenberg, The Scripps Research Institute

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Transaction Report:

(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 12 September 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. I am sorry about the delayed evaluation, which was largely due to the referees requiring additional time to assess two co-submitted manuscripts during this vacation period. We have now received all three sets of comments, which I am forwarding you in this email. These reports indicate that the identification of checkpoint-regulated G1/S gene transcription in yeast (and of the underlying mechanisms) is potentially important and interesting. Nevertheless, all three referees raise a number of substantive issues, culminating in the key concerns that the proposed competition-based SBF/MBF transcription factor switch is not decisively supported by the present set of data (see referee 2 point 3, referee 3 points 2 and 3), and that the physiological importance of Rad53 regulation of Nrm1 has not been definitively demonstrated (cf referee 2's main point, referee 3 point 1). Furthermore, referee 1 raises two additional major criticisms, that the G1/S group of transcripts analyzed was too broadly defined to allow conclusive insight, and that the essential expression profiling datasets would need to available in order to assess the validity of the results and derived conclusions (a point also seconded by referee 3).

In light of these substantial concerns and potential caveats, I am afraid I currently see little choice but to conclude that we will not be able to invite a revision of the study. While the study remains certainly a potential candidate for an EMBO J paper, the amount of time and experimental work required to resolve the key issues is considerable and would exceed the three months period we usually aim for with our major revisions; and it is furthermore not clear whether the essential experiments requested by the reviewers would eventually consolidate or confound the present conclusions. We would however remain open to looking at a new submission on this topic, should future work allow you to decisively address the essential issues and to validate the main conclusions. As mentioned above, the key points to be decisively answered for such a new version

to be considered further and returned to the referees will be those raised by referees 1 and 3, as well as referee 2's major point related to referee 3's point 1. I should also stress that since such an extended manuscript would be treated as a new submission, we will have to take into account the novelty of your findings at the time of resubmission.

Should you decide to resubmit an improved manuscript to The EMBO Journal, please make sure to indicate the original submission number and to include a point-by-point response letter. In the meantime, I hope you will in any case find our referees' detailed comments and suggestions helpful.

Yours sincerely,
Editor
The EMBO Journal

Referee reports:

Referee #1 (Remarks to the Author):

These authors report the induction of a large group of G1/S transcripts in response to DNA damage and replication stress. The induction of about half of these genes is Rad53-dependent and has been traced to the Rad53-dependent phosphorylation and subsequent removal of the repressor Nrm1. This indicates conservation of a pathway already described in fission yeast. It is an important and solid piece of work. The targets of this regulation were identified with RNA microarrays, which were carried out on G1-synchronized cells released into DNA damage or hydroxyurea to halt S phase. This strategy enabled them to identify several different transcriptional responses and many induced transcripts that have not been identified previously. They repeated the analysis with a checkpoint-deficient rad53 strain to identify those that are coordinately induced by the checkpoint pathway. Additional microarrays were carried out to identify those transcripts that were Nrm1 regulated.

My gravest concern about this manuscript is that they are not disclosing the data upon which they are basing their conclusions. As a result, their conclusions must be taken at face value. Not only are the microarray data sets not available with this manuscript (to be presented elsewhere), but neither is the subset of data used to assess whether or not they are Rad53- induced or Nrm1-repressed. Those data have been reduced to an X on a supplementary table. Heat maps are shown, but the genes are not identified and many of the effects are very modest. Without the data, one cannot tell a strongly regulated transcript from a borderline false positive. So in spite of the "global" approach, all we can truly evaluate are the five transcripts that they characterized further.

In addition, they identified the G1/S transcripts based on a crude subtraction method. They include genes that peak at 30 or 45 minutes and are repressed at 60 minutes after release from alpha factor. By this approach they have really defined a broad set of genes, which includes about half the G1/S genes identified by previous studies and many others that would be considered S phase transcripts. By lumping these genes together, many of which are known to be regulated by completely different mechanisms, they see a diversity of dependency relationships. With this limited analysis, they were able to identify a pattern they expected to find (Rad53-induced and Nrm1-repressed), but not much else.

Reporting conclusions without having to disclose the data upon which those conclusions are based is unacceptable. If the regulatory circuit they have identified is important and data has been generated for all genes, then it seems appropriate for this manuscript to report the findings for all genes, without excessive filtering so that the reader can evaluate them.

Referee #2 (Remarks to the Author):

Review of Travesa et al. (EMBOJ)
In this paper, Travesa et al. use a budding yeast model to explore mechanisms regulating the transcriptional response to genotoxic stress (HU, MMS and CTP). The authors focus on genes that are regulated by the G1/S-specific transcription factors, SBF and MBF, since many of the genes controlled by SBF and MBF are involved in DNA replication and the DNA damage response. In an initial test, they discovered that expression of an MBF-regulated gene (RNR1) was induced and maintained upon HU treatment and this response was dependent on the Rad53 checkpoint kinase. In contrast, expression of an SBF target gene (CLN2) was comparable in the presence or absence of HU. To more broadly explore this transcriptional response, the authors used microarray experiments to show that DNA damage and replication stress induced expression of a broad array of G1/S-regulated genes, many of which contained binding sites for SBF and/or MBF in their promoters. Induction of this group of G1/S genes was dependent on Rad53 and regulated by Nrm1, a repressor of MBF. Importantly, the authors discovered that expression of MBF target genes was induced and maintained upon genotoxic stress during S-phase by Rad53-dependent phosphorylation of Nrm1 -- elimination of Rad53 resulted in loss of Nrm1 binding to MBF target promoters and thus induction of transcription. Further, the authors show that the promoter of the SBF target gene SWE1, which encodes the Wee1 homolog of budding yeast, undergoes a SBF/MBF transcription factor exchange resulting in repression of SWE1 expression by Nrm1/MBF, which is relieved upon treatment with HU.

This is an interesting paper that describes a number of discoveries that ought to appeal to the broad EMBO J readership. A main message is that Rad53-dependent phosphorylation of Nrm1 results in its eviction from promoters resulting in induction of gene expression upon HU treatment. However, at this stage, there is no direct evidence for involvement of Nrm1 phosphorylation in regulating its promoter binding. I appreciate that experiments to directly link Rad53 to Nrm1 (or any kinase to a substrate for that matter) are not trivial - however, ideally phosphosites on Nrm1 should be identified (or inferred by motif scanning?), mutated and the resulting Nrm1 derivative tested for eviction from promoters after HU treatment.

Other comments:
1. Some figures are not high quality - e.g. the coomassie gels in fig 5C - and there are no error bars in any experiment.

2. Figures 5A and B are not aligned with the figure legends.

3. It would be informative to know more about the nature of gene repression of upon MBF binding post S-phase. For example, is there any involvement of known repressors/activators of cell cycle-regulated genes like SWI/SNF, RSC and Rtt106? If yes, is there any change in promoter binding upon HU-treatment?

Referee #3 (Remarks to the Author):

The article by Travesa et al describes a transcriptomic analysis of the cellular response to genotoxic stress in budding yeast. In addition to expected Rad53-upregulated genes, they identify a set of genes belonging to the G1/S regulon and mainly controlled by MBF. Although their upregulation by genotoxic stress requires Rad53, the downstream effector Dun1 is not involved. The authors show that the loss of the Nrm1 co-repressor does not affect upregulation by genotoxic stress but mimics its consequences in the absence of stress. Moreover, the authors show that Nrm1 is phosphorylated during genotoxic stress, and that Rad53 is able to phosphorylate Nrm1 in vitro, which lead them to

propose a model where Rad53 would derepress responsive genes by phosphorylation-dependent inactivation of Nrm1 as an MBF repressor. The authors show that upregulation of SWE1, a gene strongly regulated by SBF during an unperturbed G1/S transition, involves MBF. Since it shows hybrid sequences to those bound by SBF and MBF, the authors propose a TF switch at the promoter of SWE1. The observations shown by the authors are interesting and sustain properly their main conclusions. Although the key concept of the article has been already demonstrated in fission yeast, the results shown in this paper point to a direct molecular mechanism that would link MBF-dependent transcription to Rad53 and genotoxic stress. In my view, the functional relevance of the proposed mechanism should be demonstrated if this article intends to be of interest to a wide scientific audience.

Specific points that I find particularly important:

1. The functional relevance of Nrm1 phosphorylation by Rad53 should be demonstrated with non-phophorylatable Nrm1 mutants.

2. The SBF to MBF switch is another example of TF substitution at promoters, but I think it is an interesting point per se that could contribute to explain TF diversity within the G1/S regulon. If the model proposed by the authors is correct, Swi4 might need to be inactivated for TF switching. I would suggest exploring this possibility by analyzing the transcriptional response to HU in the absence of Clb proteins (not just activity of Cdc28-Clb complexes by Sic1P). To reinforce the notion of TF switching, I would also suggest the authors to test synthetic SCC, MCC and hybrid SCC-MCC enhancers in a heterologous promoter context.

3. That MBF is required for transcriptional upregulation by genotoxic stress should be demonstrated in Mbp1-deficient cells.

4. I would include the complete set of transcriptomic data.

Resubmission 15 December 2011

Detailed Response to Reviewer's Comments

We would like to start by thanking the Reviewers for their general endorsement of the work. All three Reviewers recognized the importance of the findings, the soundness of the experimental data and its likely appeal to the audience of EMBO Journal. That said, each of the Reviewers had concerns that we have endeavored to address either through additional experiments, additional access to available data or further explanation. We are hopeful that the Reviewers find those remedies satisfactory and can now endorse the report in its entirety.

Because several issues appear in more than one Review and/or were raised as significant concerns by the Editor, we will address them at the outset.

1. Reviewers 1 and 3, along with the Editor, raised the issue of the access to the full database. Although we recognized that this was a requirement for publication, we did not anticipate the desire of reviewers to access the raw data set and, therefore, did not make it available at this stage in the process. We appreciate their request and have made the full dataset used to derive the heat maps in Figures 2 and 4 available to Reviewers via the NCBI Gene Expression Omnibus through GEO Series accession number GSE33695 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=fbkpfcgeqcgmana&acc=GSE33695). This access is now referred to in the text and will be accessible by all readers.

2. Reviewers 2 and 3, along with the Editor, were concerned that we did not established that the phosphorylation of Nrm1 by Rad53 has a functional consequence in terms of regulation of MBF binding, transcriptional regulation or physiological consequence (varies among comments). We have now identified multiple S/T residues in Nrm1 that are phosphorylated by Rad53 either in vivo or in vitro. Those residues have been mutated to alanine to prevent phosphorylation and the *NRM1-4A* mutant expressed as the only source of Nrm1. The mutant protein is expressed and functions normally to regulate transcription of MBF targets in the absence of DNA replication stress. However, cells expressing the mutant gene show diminished induction of MBF targets in response to hydroxyurea (HU) (presented in the new Figure 6). The decrease in induction is associated with a failure of HU to promote dissociation of Nrm1 from MBF target promoters. Consistent with the partial effect of the Nrm1 mutant on transcriptional induction by the checkpoint during S phase, the phosphorylation of the mutant protein is reduced, but not eliminated in vivo and in vitro. Furthermore, we suspect that this partial effect explains our failure to detect HU sensitivity in the *NRM1-4A* mutant. Nevertheless, the transcriptional phenotype and defect in checkpoint regulation of Nrm1 binding to MBF target promoters clearly establishes the role of direct phosphorylation of Nrm1 by Rad53 in the transcriptional response to DNA replication stress.

3. Reviewers 2 and 3, along with the Editor, requested further analysis of the phenomenon of “transcription factor switching”. Although we consider this an interesting and exciting observation that came out of our genome-wide expression analysis, it is the subject of the study of the companion paper that was cosubmitted with this one and that has been revised and resubmitted along with it. The two studies have some overlap but emphasize distinct aspects of this regulation. Because we have now expanded our study to address the fundamental mechanism of the checkpoint response in greater detail to address the reviewers comments, we have eliminated the prior Figure 6 and replaced it with that analysis. Similar data to that presented in the prior version of our paper is presented and studied in more detail in the manuscript by Bastos de Olivera et al. In the end, we believe that this change represents an improvement in our paper both in terms of focus and depth.

Other issues raised by the Reviewers are addressed in the specific comments below:

Response to Reviewer 1

The primary concern of this Reviewer, that the full gene expression data set was not made available to reviewers, is addressed by Comment 1 above.

The second concern, echoed by the Editor, was that the basis for selection of G1/S genes was too broad and prevented important observations from being derived from that data.

The basis for the Reviewer’s concern about our selection method is not clear to us. In fact, we implemented a relatively broad definition of “interesting” genes because our intent was to capture a set of genes likely to encompass all SBF and MBF targets along with other genes that might show significant regulation by the checkpoint during G1/S phase. Because SBF and MBF gene expression have a relatively broad distribution of peak expression (see Eser and Skotheim, 2011 for a detailed study) it was necessary to select genes showing peak expression over a relatively broad timeframe. In doing so, we captured a gene set significantly correlated with criteria associated with the gene set of interest (see Table S1) and are more strongly correlated to those criteria than two other well-regarded G1/S gene sets from the literature. Because we then applied other criteria to select MBF-regulated gene and Rad53-dependent genes from among the parent gene set, spurious members of this data set were eliminated. All of this is not to say that other interesting, though likely unrelated, discoveries can be made from this gene set (something we are interested in pursuing) but, rather, that this was an appropriate approach for studying the topic addressed in this manuscript, the induction of G1/S gene expression by genotoxic stress.

Response to Reviewer 2

This Reviewer's primary concern, that "there is no direct evidence for involvement of Nrm1 phosphorylation in regulating its promoter binding", has been addressed as outlined in Comment 2 above.

We have addressed the Reviewer's "Other Comments" as follows:

1. The Reviewer was concerned with two aspects of data presentation:
   The quality of the coomassie blue-stained gel in Figure 5 is poor both because of the abundance of IgG in some lanes of the gel and because the image of the dried gel on paper was difficult to capture. Nevertheless, we think our intention to show the mobility and relative abundance of Rad53 and Nrm1 from those images has been reasonably achieved. We have now included a similar analysis in the new Figure 6 with a "better" image that should address any concerns regarding the panel in the previous Figure.
   All of the RNA and ChIP experiments were performed using triplicate samples and were only used if the error among triplicates was less than 0.1 cycle. Each experiment is representative of at least three independent analyses. Variability in the timing of cell cycle progression out from the mating pheromone arrest and in timepoints sampled in individual experiments precludes presentation of statistical analysis of biological replicates. Nevertheless, our data presentation complies with published data of this type in this and other journals.

2. Figures 5A and B are now aligned with their figure legends.

3. The Reviewer asked for additional information regarding the mechanism of repression of gene expression post-S phase. Although we are certainly interested in both the mechanism by which Nrm1 imposes repression on MBF-regulated genes and of Nrm1-independent repression of those genes later in the cell cycle, we consider this analysis outside of the scope of this paper which focuses on checkpoint regulation of expression via Nrm1 rather than general mechanisms of MBF gene regulation during the cell cycle.

Response to Reviewer 3

The concerns of this Reviewer outlined in the "Specific points" have been addressed as follows:

1. The concern that "The functional relevance of Nrm1 phosphorylation by Rad53 should be demonstrated with non-phophorylatable Nrm1 mutants"

has been addressed as described in Comment 2 above.

2. The interest of the Reviewer in further exploration of transcription factor switching has been addressed as described in Comment 3 above.

3. We have provided new data addressing the issue raised by the Reviewer in Figure S1. We show MBF is required for transcriptional upregulation by genotoxic stress by analyzing expression of MBF targets in an *mbp1*Δ mutant with and without HU. This result may be confusing because transcription of MBF targets in those cells is high at all times. This is a consequence of the fact that MBF acts largely as a transcriptional repressor outside of G1, in part due to the activity of Nrm1 (de Bruin et al, 2006). Nevertheless, the result is as expected.

4. The request to include a complete microarray data set has been addressed as described in Comment 1 above.

Acceptance letter 20 January 2012

Thank you again for submitting a new version of your earlier manuscript (EMBOJ-2011-79007) for our consideration. I have now had a chance to carefully look through it and to assess your responses to the comments raised by the original reviewers; in addition, it has in the meantime also been assessed once more by one of the original referees (see comments below). I am happy to inform you that there are no further objections towards publication in The EMBO Journal, and we will thus be able to proceed with acceptance and production of the study at this point.

You shall receive a formal letter of acceptance shortly.

Yours sincerely,
Editor
The EMBO Journal

Referee #1

(Remarks to the Author)
The authors have added new experimental data that properly answer most questions raised by this reviewer, so I recommend publication of the revised manuscript as it is.

