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Peer Review File

Pre-established ATF4 occupancy and chromatin organization instruct selective transcription activation during integrated stress response

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study reveals that transcriptional responses during the integrated stress response (ISR) are pre-wired by intrinsic chromatin properties. ATF4 pre-binds to many genes under non-stress conditions, priming them for activation during stress. Transcriptional changes rely on the redistribution of CEBPγ to ATF4-bound sites, guided by unique higher-order chromatin structures, rather than changes in chromatin accessibility, H3K27 acetylation, or enhancer-promoter looping.

Main points:

This study offers intriguing insights into how ATF4 occupancy and chromatin organization contribute to selective transcriptional activation during the integrated stress response (ISR). To further contextualize these findings, it would be valuable to compare the identified ATF4 binding sites with previously reported datasets, such as those in PMID: 37906604. Such a comparison could help underscore novel aspects of ATF4's regulatory role during ISR.

The study demonstrates significant transcriptional remodeling under ISR; however, the stress conditions and cell lines used are somewhat limited. Expanding the analysis to additional stress paradigms and a wider variety of cell types would strengthen the generalizability and robustness of the conclusions.

The finding that "11.6% of ATF4-occupied promoters were upregulated" is compelling but may not fully capture the extent of ATF4's impact as a transcription factor. Including all significantly upregulated genes beyond those with a twofold change could provide a broader and more nuanced understanding of ATF4-mediated transcriptional activation.

The study's conclusion that transcriptional changes during ISR are pre-wired by intrinsic chromatin properties is compelling. However, while the focus on ATF4 binding, CEBPγ redistribution, and chromatin structure is impactful, exploring broader interconnections between transcriptome dynamics, epigenetic modifications, and 3D genome organization during ISR could provide a more integrative perspective.

Minor Comments:

Figures 1A and 1B effectively illustrate key concepts related to ATF4 binding and transcriptional activation. However, adding quantitative analysis would enhance the clarity of the observed trends.

Reviewer #2

(Remarks to the Author)
Jiang et.al., studied the role of ATF4 transcription factor during the integrated stress response (ISR) following treatment with Thapsigargin (Thap). The authors have nicely demonstrated that ISR induction triggers widespread transcriptional changes within 6 hours, coinciding with increased binding of ATF4. Interestingly, ATF4 was found to bind hundreds of genes even under control conditions, likely priming them for stronger activation upon stress. The transcriptional changes during ISR do

not seem to correlate with increased H3K27 acetylation, chromatin accessibility, or 3D topological changes. Instead, ATF4-mediated gene activation is linked to the redistribution of CEBPγ from non-ATF4 sites to a subset of ATF4-bound regions. CEBPγ preferentially targets the sites pre-occupied by ATF4 and exhibit a looser 3D organization. Therefore, the authors conclude that the transcriptional responses during ISR are largely pre-wired by intrinsic chromatin properties.

The presented work was done and written very well; however, two critical controls are missing which could bolster the results and the conclusion of this work. 1- Specificity of the ATF4 ChIP-seq peaks are not verified. This can be done either knockdown of ATF4 (by RNAi or degron system) and observing a reduction in ATF4 binding signal in ChIP-seq at true ATF4 binding sites. Not every peak observed in a ChIP-seq experiment is a true binding site, there are many non-specific peaks observed in ChIP-seq experiments. Or, by using a second independent ATF4 antibody and verifying the true ATF4 binding, by requiring peaks to be identified by both antibodies. 2- Necessity of CEBPgamma for the ATF4-dependent ISR induction of genes in ATF4 pre-occupied gene promoters. Again this can be tested by RNAi or degron knock-down CEBPgamma and observing the ISR induced gene expression levels.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my concerns. I have no further questions and recommend accepting this manuscript.

Reviewer #2

(Remarks to the Author)
The authors have sufficiently addressed my concerns.

Response to Reviewers

First and foremost, we would like to thank both reviewers for the positive evaluation of our work and the valuable and insightful comments. We have now revised the manuscript to address all the specific questions raised by the reviewers. We believe that these additional data strengthen the conclusions of our work. Below, we provide detailed, point-by-point responses, with corresponding revisions highlighted in the revised manuscript.

Reviewer #1 (Remarks to the Author):

This study reveals that transcriptional responses during the integrated stress response (ISR) are pre-wired by intrinsic chromatin properties. ATF4 pre-binds to many genes under non-stress conditions, priming them for activation during stress. Transcriptional changes rely on the redistribution of CEBPγ to ATF4-bound sites, guided by unique higher-order chromatin structures, rather than changes in chromatin accessibility, H3K27 acetylation, or enhancer-promoter looping.

We greatly appreciate Reviewer #1's positive evaluation of our work. In the revised manuscript, we have addressed all of the reviewer's questions by conducting additional experiments and data analyses. In particular, we validated our key findings from C2C12 cells using an additional cell line and an alternative ISR-induction approach, thereby enhancing the robustness and generalizability of our conclusions.

Main points:
1. This study offers intriguing insights into how ATF4 occupancy and chromatin organization contribute to selective transcriptional activation during the integrated stress response (ISR). To further contextualize these findings, it would be valuable to compare the identified ATF4 binding sites with previously reported datasets, such as those in PMID: 37906604. Such a comparison could help underscore novel aspects of ATF4's regulatory role during ISR.

To address this concern, we compared our ATF4 CUT&Tag data from C2C12 cells (DMSO-treated) with previously published ATF4 datasets (PMID: 37906604, 33436370, PMC3439153, 26111340, 32299090). Using the UCSC LiftOver tool, we converted the ATF4 peak coordinates from the mouse genome to the human genome and assessed signal enrichment across corresponding regions in the published datasets. Our analysis revealed that ATF4 peaks identified in C2C12 cells also show enrichment of ATF4 binding in all previously published datasets across different cell types, supporting the reliability of our CUT&Tag results. These results and comparisons have been incorporated into the Results section (Lines 181-186) and are presented in Supplementary Fig. 3e.

We would like to note that most previously published datasets profiled ATF4 occupancy

under either unstressed or stressed conditions, but rarely both, which prevents the direct evaluation of whether pre-occupied ATF4 sites prime genes for more robust transcriptional activation. To further validate our conclusion, we performed additional ATF4 profiling in a second cell line, as detailed below in our response to the second comment from Reviewer #1.

2. The study demonstrates significant transcriptional remodeling under ISR; however, the stress conditions and cell lines used are somewhat limited. Expanding the analysis to additional stress paradigms and a wider variety of cell types would strengthen the generalizability and robustness of the conclusions.

We greatly appreciate Reviewer #1 for this important suggestion. In the revised manuscript, we extended our investigation to HeLa cells treated with carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a mitochondrial uncoupler known to activate the ISR (Supplementary Fig. 2a-2d). By integrating RNA-seq, ATAC-seq, and CUT&Tag profiling of ATF4 and CEBPγ, we confirmed that pre-bound ATF4 sites under basal conditions elicit stronger transcriptional activation during ISR in HeLa cells, and the differential activation at ATF4-bound sites correlated with CEBPγ redistribution. Our key findings in HeLa cells are as follows:

RNA-seq revealed temporal patterns of gene expression changes during CCCP-induced ISR that closely mirrored those observed in Thap-treated C2C12 cells (Lines 156-172). After 6 hours of CCCP treatment, 464 genes were upregulated and 184 downregulated, enriched for stress-related GO terms such as “intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress” and “response to endoplasmic reticulum stress” (Supplementary Fig. 2f–2h). At 12 hours, the number of differentially expressed genes increased to 825 upregulated and 628 downregulated (Supplementary Fig. 2i–2k). Importantly, 99 genes were commonly upregulated in both C2C12 and HeLa cells at 6 hours and were enriched in pathways related to protein homeostasis, including “response to endoplasmic reticulum stress,” “intrinsic apoptotic signaling pathway in response to ER stress,” and “response to unfolded protein” (Supplementary Fig. 2l, 2m).

CUT&Tag profiling of ATF4 binding in HeLa cells identified 499 pre-occupied sites under basal (DMSO) conditions and 3,577 gained binding sites upon CCCP treatment (Supplementary Fig.5a). The pre-occupied ATF4 sites exhibited stronger ATF4 binding intensity than the gained ATF4 sites. Genes linked to these pre-occupied ATF4 sites were associated with GO terms including tRNA aminoacylation and unfolded protein response (Supplementary Fig.5d-5g). Importantly, genes associated with pre-occupied ATF4 sites showed significantly greater induction upon ISR activation than those associated with gained sites (Supplementary Fig. 5h), consistent with observations in C2C12 cells, suggesting a conserved role for pre-occupied ATF4 in priming robust transcriptional activation (Lines 233-248).

Moreover, similar to C2C12 cells, activation of these genes in HeLa cells occurred

without substantial changes in H3K27ac or chromatin accessibility, indicating that ATF4 can activate transcription independently of chromatin remodeling (Lines 300-301, Supplementary Fig. 7).

We further examined the role of CEBPγ in HeLa cells by performing the same analysis as for C2C12 cells. We classified ATF4 binding sites in HeLa cells into two categories based on CEBPγ binding during ISR. At class 1 sites, CEBPγ binding increased significantly, whereas at class 2 sites, it remained largely unchanged. Despite similar ATF4 binding across both classes, genes near class 1 sites showed significantly higher upregulation, suggesting that CEBPγ enhances ATF4-mediated transcriptional activation (Lines 426-432, Supplementary Fig.12).

In summary, these results demonstrate strong consistency between C2C12 and HeLa cells, supporting the generalizability of our findings across cell types and ISR-inducing conditions

3. The finding that "11.6% of ATF4-occupied promoters were upregulated" is compelling but may not fully capture the extent of ATF4's impact as a transcription factor. Including all significantly upregulated genes beyond those with a twofold change could provide a broader and more nuanced understanding of ATF4-mediated transcriptional activation.

We thank the reviewer for this valuable suggestion. In the revised manuscript, we expanded our analysis to include all genes that were significantly upregulated and exhibited ATF4 binding ($p < 0.05$), without applying a fold-change cutoff. Gene Ontology analysis of this broader gene set revealed enrichment for terms such as “cellular amino acid metabolic process,” “tRNA aminoacylation for protein translation,” and “cellular response to unfolded protein” (Lines 228-229, Supplementary Fig. 4h–m). These functional categories closely mirror those identified using the more stringent criterion of log2FC > 1. This analysis reinforces the conclusion that ATF4 broadly participates in regulating protein homeostasis and stress responses during the ISR.

4. The study's conclusion that transcriptional changes during ISR are pre-wired by intrinsic chromatin properties is compelling. However, while the focus on ATF4 binding, CEBPγ redistribution, and chromatin structure is impactful, exploring broader interconnections between transcriptome dynamics, epigenetic modifications, and 3D genome organization during ISR could provide a more integrative perspective.

To address this, we quantified the relationship between gene expression changes and epigenetic modifications at the genome-wide scale. Our analysis revealed that genes associated with peaks showing increased H3K27ac or chromatin accessibility exhibited an overall upregulation, whereas those linked to decreased signals were downregulated (Lines 267-273, Supplementary Fig. 6a, 6b). However, because we have shown that increases in H3K27ac or chromatin accessibility at ATF4-bound sites did not correlate

with transcriptional activation at those sites, we infer that the observed genome-wide association between epigenetic marks and transcriptional activity likely reflects secondary transcriptional remodeling downstream of ATF4-mediated transcriptional activation.

We also performed additional analyses to further explore the interplay among 3D genome organization, epigenetic modifications, and gene expression during ISR. We showed that H3K27ac levels and chromatin accessibility remained largely unchanged at both dynamic and stable TAD boundaries (Supplementary Fig. 9a, 9b), and genes within the corresponding TADs exhibited no significant expression changes (Supplementary Fig. 9c). A similar pattern was observed at chromatin loop anchors, where neither epigenetic alterations nor transcriptional changes were detected (Supplementary Fig. 9d–9f). Collectively, these findings suggest that 3D genome reorganization does not directly correlate with changes in chromatin state or gene expression during ISR (Lines 340-345).

Minor Comments:

5. Figures 1A and 1B effectively illustrate key concepts related to ATF4 binding and transcriptional activation. However, adding quantitative analysis would enhance the clarity of the observed trends.

We have added quantitative plots and statistical comparisons (signal intensity profiles and peak enrichments) to Figures 1A and 1B to support our conclusions (Supplementary Fig. 1a, 1b).

Reviewer #2 (Remarks to the Author):

Jiang et.al., studied the role of ATF4 transcription factor during the integrated stress response (ISR) following treatment with Thapsigargin (Thap). The authors have nicely demonstrated that ISR induction triggers widespread transcriptional changes within 6 hours, coinciding with increased binding of ATF4. Interestingly, ATF4 was found to bind hundreds of genes even under control conditions, likely priming them for stronger activation upon stress. The transcriptional changes during ISR do not seem to correlate with increased H3K27 acetylation, chromatin accessibility, or 3D topological changes. Instead, ATF4-mediated gene activation is linked to the redistribution of CEBPγ from non-ATF4 sites to a subset of ATF4-bound regions. CEBPγ preferentially targets the sites pre-occupied by ATF4 and exhibit a looser 3D organization. Therefore, the authors conclude that the transcriptional responses during ISR are largely pre-wired by intrinsic chromatin properties.

The presented work was done and written very well; however, two critical controls are missing which could bolster the results and the conclusion of this work.

We are grateful to Reviewer #2 for the enthusiastic interest in our results and helpful suggestions. We have addressed the specific concerns regarding the specificity of the ATF4 antibody and performed additional functional validation of CEBPγ. These new results further reinforce our conclusions and enhance the overall quality of the manuscript.

1. Specificity of the ATF4 ChIP-seq peaks are not verified. This can be done either knockdown of ATF4 (by RNAi or degron system) and observing a reduction in ATF4 binding signal in ChIP-seq at true ATF4 binding sites. Not every peak observed in a ChIP-seq experiment is a true binding site, there are many non-specific peaks observed in ChIP-seq experiments. Or, by using a second independent ATF4 antibody and verifying the true ATF4 binding, by requiring peaks to be identified by both antibodies.

To further validate the reproducibility and specificity of ATF4 binding sites, we performed CUT&Tag experiments using a second ATF4 antibody (Santa Cruz Biotechnology, #sc-390063, the SCBT antibody) in addition to the antibody used in our primary dataset (Cell Signaling Technology, #11815S, the CST antibody). Both antibodies have been widely used in previous ATF4-related studies.

In DMSO-treated cells, the CST antibody identified 803 peaks, while the SCBT antibody identified 2,756 peaks, with 457 peaks shared between the two datasets (Supplementary Fig. 3a). Multiple lines of evidence indicate that the CST antibody detects ATF4 binding sites more reliably, whereas the SCBT antibody exhibits higher non-specific binding. First, motif enrichment analysis revealed strong enrichment of the canonical ATF4 motif in CST-specific and shared peaks, whereas SCBT-specific peaks showed much weaker ATF4 motif enrichment (Supplementary Fig. 3b-d). Second, signal intensity analysis using independent ATF4 ChIP-seq datasets from other cell types showed that peaks identified using the CST antibody exhibited stronger ATF4 signal enrichment than the peaks identified using the SCBT antibody (Supplementary Fig. 3e, 3f). Furthermore, although the 346 CST-specific peaks were not called as peaks in the SCBT dataset, they displayed clear ATF4 signal enrichment in the SCBT CUT&Tag profile. In contrast, the 2,299 SCBT-specific peaks exhibited minimal ATF4 signal in the CST dataset (Supplementary Fig. 3g–i). Together, these results suggest that CST antibody-derived peaks represent *bona fide* ATF4 binding sites with higher specificity, and we therefore used these peaks for all downstream analyses (Lines 181-186).

2. Necessity of CEBPgamma for the ATF4-dependent ISR induction of genes in ATF4 pre-occupied gene promoters. Again this can be tested by RNAi or degron knock-down CEBPgamma and observing the ISR induced gene expression levels.

We thank the reviewer for bringing up this important point. To further validate the functional role of CEBPγ during ISR, we performed shRNA-mediated knockdown of CEBPγ in C2C12 cells (Supplementary Fig. 13a). Transcriptome analysis revealed that

CEBPγ depletion altered gene expression under both basal (DMSO) and stress (Thap) conditions (Fig. 5j; Supplementary Fig. 13b, 13c). Although genes downregulated upon CEBPγ knockdown were not significantly enriched for stress-related Gene Ontology terms (Supplementary Fig. 13d, 13e), the induction of stress-responsive genes during ISR was markedly attenuated in the absence of CEBPγ (Fig. 5k). Notably, class 1 genes were more strongly affected by CEBPγ knockdown than class 2 genes (Fig. 5l). Together, these findings indicate that the robust transcriptional activation at ATF4-preoccupied sites, as well as the selective activation of both ATF4-preoccupied and ATF4-gained sites, is likely driven by the preferential binding of CEBPγ (Lines 433-444).