HHS Public Access

Author manuscript

Mutat Res. Author manuscript; available in PMC 2016 June 01.

Published in final edited form as:

Mutat Res. 2015 June ; 776: 40–47. doi:10.1016/j.mrfmmm.2015.01.006.

An epigenomic role of Fe65 in the cellular response to DNA damage

Seungjin Ryu[a], **Francesca Teles**[d], **Giuseppina Minopoli**[e], **Tommaso Russo**[e], **Michael G. Rosenfeld**[d], and **Yousin Suh**[a,b,c,*]

[a]Department of Genetics, Albert Einstein College of Medicine, Bronx, NY 10461, USA

[b]Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461, USA

[c]Institute of Aging Research, Guangdong Medical College, Dongguan, China

[d]Howard Hughes Medical Institute and Graduate Program in Biomedical Sciences, University of California at San Diego, School of Medicine, La Jolla, CA 92093

[5]Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, Napoli, Italy

Abstract

Previous findings describe Fe65 as a key protein in the cellular response to genotoxic stress. However, the precise molecular mechanism by which Fe65 contributes to DNA damage signaling remains unclear. In this study, we hypothesized that the transcriptional activity of Fe65 may contribute to DNA damage pathways by regulating gene expression patterns activated in response to genotoxic stress. To address this hypothesis, we mapped the global binding profile of Fe65 by chromatin immunoprecipitation (ChIP)-sequencing in the SK-N-SH cells exposed to genotoxic stress. Unexpectedly, the genome-wide location analysis showed a substantial enrichment of Fe65 in the promoter regions of coding genes linked to DNA damage signaling pathways. To further investigate the role of Fe65 in the transcriptional regulation of putative coding target genes identified by ChIP-seq, we performed microarray assays using wild-type (WT) or Fe65 deficient mouse embryonic fibroblasts (MEFs) exposed to oxidative stress with multiple recovery times. Gene ontology analysis of the Fe65-depedent transcriptome suggested that Fe65 modulate the expression of genes critical for DNA damage response. Motif enrichment analysis of regulatory regions occupied by Fe65 revealed a strong correlation with key transcription factors involved in DNA damage signaling pathways, including E2F1, p53, and Jun. Comparison of ChIP-sequencing results with microarray results ultimately identified 248 Fe65-depedent target genes, the majority of which were known regulators of cell cycle, cell death, and DNA replication and repair pathways. We validated the target genes identified by *in silico* analysis by qPCR experiments.

[*]Corresponding author: Yousin Suh, PhD, Albert Einstein College of Medicine, Departments of Genetics and Medicine, 1301 Morris Park Avenue, Bronx, NY 10461, *Tel.: 718-678-1111; fax: 718-678-1113;*yousin.suh@einstein.yu.edu.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

Collectively, our results provide strong evidence that Fe65 plays a role in DNA damage response and cell viability by epigenomic regulation of specific transcriptional programs activated upon genotoxic stress.

Keywords

Fe65; transcription; DNA damage response; Microarray; Chromatin immunoprecipitation-sequencing

1. Introduction

The Fe65s are a family of adaptor proteins mediating assembly of multimolecular complexes through protein interaction domains: a WW domain and two PTB domains [1]. Initially, Fe65 was isolated as a protein interacting with cytosolic domain of amyloid precursor protein (APP), which is implicated in the pathophysiology of Alzheimer's disease (AD) [2]. Further studies revealed that Fe65 is involved in a complex network of protein-protein interactions, including proteins involved in cytoskeleton dynamics (c-Abl [3] and Mena [4]); proteins functional in transcriptional regulation (Tip60 [5], SET[6], LSF [7]), but also components of DNA damage signaling pathways (H2AX [8], c-Abl, Tip60, and BML [9]).

The finding that Fe65 interacts with the intracellular domain of APP (AICD) released by the gamma-secretase proteolytic activity, has invigorated the scientific interest for its potential contribution to the pathology of AD, but the biological relevance of this protein complex in disease pathways is still unclear. One intriguing hypothesis is the role of Fe65/AICD complex in the regulation of gene expression patterns. This hypothesis is fascinating for its correlation with other documented pathways of transcriptional regulation by gamma-secretase-dependent regulated intramembrane proteolysis (RIP), such as the Notch pathway [10]. Interestingly, it has been reported that AICD is stabilized by Fe65 and is translocated into the nucleus as a complex AICD-Fe65 [11, 12]. This complex together with Tip60 and SET can activate the transcription of a reporter gene and it can be recruited on the chromatin at the *kai1* promoter [13]. Further studies are needed to clarify the mechanism by which Fe65 modulates transcription.

Intriguingly, a compelling body of evidence suggests that Fe65 plays a role in DNA damage signaling pathways. It has been reported that nuclear translocation of Fe65 is facilitated by multiple genotoxic stimuli, including DNA double-strand breaks induced by etoposide or oxidative stress triggered by hydrogen peroxide treatment, and that the ablation of Fe65 lead to DNA damage accumulation upon stress [14]. Moreover, it has been further demonstrated that Fe65 modulates the double-strand break repair pathway by regulating the acetylation of histone H4 that induces recruitment of Tip60 to DNA damage sites [15]. These reports suggested that Fe65 has a crucial role in DNA damage response.

Even though previous studies have uncovered partially the mechanisms by which Fe65 takes part to DNA damage response, the contribution of its transcriptional activity remains elusive. Here, we investigated the transcriptional function of Fe65 and its correlation with

DNA damage signaling pathways. We used powerful contemporary technologies based on chromatin immunoprecipitation (ChIP)-sequencing and microarray gene expression assays to investigate the function of Fe65 at a genome-wide level. Our results revealed an unprecedented widespread recruitment of Fe65 to regulatory promoter regions of coding genes linked to DNA damage signaling. *In silico* analysis of the Fe65 cistromes uncovered a link with key regulators of cellular pathways activated upon genotoxicity. Furthermore, transcriptional profiles of cells deficient for Fe65 confirmed that this adaptor protein is required for proper response to genotoxic stimuli. Finally, we validated specific target genes confirming our hypothesis that Fe65 contributes to DNA damage response through regulation of gene expression.

2. Materials and Methods

2.1. Cell culture

SK-N-SH cell-line utilized for ChIP-sequencing are differentiated by incubation of retinoic acid and grown in DMEM supplemented with 5% fetal bovine serum, 1% of penicillin, and streptomycin in a 5% CO_2 atmosphere at 37°C. Fe65 knockout (KO) MEF and Wild-type (WT) MEF were generous gift from Dr. Tommaso Russo. Those cells are generated and maintained as reported previously [14].

2.2. ChIP-sequencing and analysis

For preparation of samples for ChIP-sequencing, differentiated SK-N-SH cells with and without treatment of etoposide (10 nM) for 1 hr were subjected to standard ChIP protocol using a specific antibody recognizing Fe65 (kindly provided by Russo, T), and the ChIP-sequencing has been performed once in each condition. The ChIP-ed DNA was purified and utilized for libraries preparation with Sample Preparation kit (Illumina) and massively parallel sequencing on a Illumina Genome Analyzer II. For data analysis, sequencing reads were aligned to UCSC Homo Sapiens reference genome (hg18) using Bowtie. HOMER and MACS were employed for peak finding, Motif enrichment was computed by HOMER, and Gene Ontology and pathway analysis were performed by DAVID/EASE [16].

2.3. Microarray and analysis

To prepare RNAs for microarray, we utilized RNA isolation kit (Qiagen) for Fe65 KO and WT MEF upon hydrogen peroxide (100 uM) treatment for 1.5 hr followed by recovery time of 0 hr, 2 hr and 12 hr, which is removal of hydrogen peroxide, along with untreated control cells. 100 ng of RNAs from each condition were used for microarray with GeneChip Mouse Gene 1.0 ST Array (Affymetrix). Hybridization has been performed in Einstein genomics core. Data analysis has been performed after normalization by RMA method utilizing R program in Einstein computational core. Genes with more than 1.5 fold change compared to non-treated condition in each Fe65 KO and WT MEF at any time points were selected and used for heat map of gene expression change. Then, we calculated a ratio between Fe65 KO and WT MEFs to select genes with different fold change upon stress. It is a fold change of Fe65 KO MEF divided by fold change of WT MEF at each time point (0 hr, 2 hr, and 12 hr). Genes with more than 50% increased or decreased fold change at each time point in Fe65 KO MEF were selected and used for GO/pathway analysis. GoMiner program [17] was

utilized to identify GOs enriched in genes of each increased fold change and decreased fold change compared to WT MEF. DAVID/EASE program was utilized for KEGG pathway analysis. The p-values were indicated as heat map by utilizing values of $\log_{10}$ (p-value) for genes with decreased fold change, and $-\log_{10}$ (p-value) for genes with increased fold change in Fe65 KO MEF. The selected genes also used for upstream regulator prediction by IPA program (Ingenuity® Systems). p-values about each regulator were calculated based on number of target genes, only significant regulators ($p < 1.0$ E-10) at any time point were selected. Z-score was calculated to indicate the predicted activity of regulators. It reflects state of target genes affected by regulators that are based on experimental report in IPA program. All heat maps were drawn by GENE-E program.

2.4. Quantitative real time PCR (qPCR)

The identical RNAs used in microarray were utilized for qPCR to validate the expression change in microarray. The RNA was reverse-transcribed using SuperScript III reverse transcriptase (Life Technologies). SYBR Green Real-Time PCR Master Mix (Life Technologies) has been utilized for qPCR with gene specific forward and reverse primers (Supplementary Table S3). The assay was carried out with StepOnePlus real-time PCR machine (Life Technologies). Three independent experiments were performed and average was shown with standard deviation.

3. Results

3.1. Identification of Fe65 binding sites upon genotoxic stress at a genome-wide level

A large body of evidence suggesting a role of Fe65 in DNA damage response incited us to investigate the potential involvement of the transcriptional activity of Fe65 in these signaling pathways. To explore the global contribution of Fe65 to epigenomic events triggered by genotoxic stress, we performed ChIP-sequencing with a specific antibody for Fe65 using differentiated neuronal SK-N-SH cell-line exposed 10 nM etoposide for 1 hr, which is a compound extensively used to induce double-strand breaks. Analysis of ChIP-seq peaks revealed that etoposide treatment lead to a substantial reprogramming of Fe65 genome-wide binding program. We observed 2612 peaks in absence of genotoxic stress, and 4655 peaks upon exposure to etoposide, suggesting that genotoxic stress largely promotes the Fe65 recruitment to target genomic loci (Fig. 1A). We performed ChIP-seq with IgG control using SK-N-SH cell-line and couldn't detect overlapping patterns suggesting that this result is not a non-specific effect (data not shown). Further analysis revealed that Fe65 binding sites were preferentially enriched in regulatory promoter regions (68%) compared to terminator (18%) and intergenic regions (14%) (Fig. 1B). We analyzed the possible biological roles of all the Fe65 targeted promoters performing a functional annotation analysis by mapping our list of genes to associated Gene Ontology terms and using DAVID/EASE [16]. Interestingly, the most enriched GO terms ($p<0.001$) were those involved in a typical DNA damage response, such as programmed cell death, ubiquitin cycles, regulation of cell cycle and DNA repair (Fig. 1C). This analysis strongly correlates with the previously described role of Fe65 in the cellular response to DNA damage and defines this action as a newly discovered transcriptional regulatory function.

3.2. Transcriptional profiling of Fe65-dependent genes implicated in the response to genotoxic stimuli

To ultimately identify Fe65 target genes involved in the transcriptional response to genotoxic stress, we carried out transcriptional profiling by microarray assays. First, we identified many up and down-regulated genes with more than 1.5 fold change in Fe65 KO MEF compared to wild-type MEF in a condition without stress (Supplementary Table S1). Then, we compared transcriptomes of WT or Fe65 KO MEFs during a time-course of recovery upon oxidative stress treatment induced by 100 uM hydrogen peroxide for 1.5 hour. Specifically, we analyzed RNA at 0, 2, and 12 hours after treatment (Fig. 2A). To evaluate stress-mediated gene expression changes, we calculated fold change of expression level between the non-treated vs. treated Fe65 KO and WT MEFs at each time point (0 hr, 2 hr and 12 hr) (Fig. 2A). A heatmap showing patterns of gene expression changes (>1.5 fold) between WT and deficient Fe65 transcriptomes revealed substantial divergence in the transcriptional output of these two datasets (Fig. 2B). In particular, Fe65 knockout led to decreased expression of genes linked to DNA repair, cellular response to stress and cell cycle. Conversely, Fe65 ablation led to increased expression of genes linked to immune response and apoptosis (Red boxes in Fig. 2B). To identify stress-regulated genes, the expression of which was dependent on the presence of Fe65, we calculated a ratio between fold change of Fe65 KO MEF and WT MEF at each time point (0 hr, 2 hr, and 12 hr). Using this strategy, we detected several genes with more than 50% increased or decreased fold change at each time point. The GO analysis of these genes, confirmed our previous observation linking pathways DNA repair and DNA damage response to the genes with decreased fold-change, while immune response and programmed cell death were significantly enriched in increased fold-change of Fe65 KO MEFs (Fig. 2C). Correspondingly, we classified our list of genes according to the KEEG pathway database and we found significant correlations (p-value < 0.01) with several pathways of cellular response to stress as shown in Figure 2D. Pathways of cell cycle, p53 signaling pathway, and homologous recombination were significantly enriched in decreased fold change, whereas Toll-like receptor pathway and complement and coagulation cascades were significantly enriched in increased fold change of Fe65 KO MEF upon stress.

3.3. *De novo* motif enrichment analysis predicted novel co-regulators of Fe65 in the transcriptional regulation of cellular response to genotoxic stress

Fe65 doesn't have a DNA binding motif as a transcriptional co-factor and require transcription factors to bind genomic regions. To explore the putative transcription factors involved in the Fe65-dependent transcriptional programs, we performed *de novo* element motif enrichment analysis of Fe65 binding sites identified by ChIP-sequencing. Our *in silico* analysis of motifs enriched in binding sites occupied by Fe65 independently of genotoxic stimuli, showed that the E2F1 binding motif was significantly over-represented (p-value : 2.857 e-24) (Fig. 3A). Conversely, the analysis of Fe65 binding sites gained upon stress showed significant enrichment of motifs recognized by the transcription factor p53 (p-value : 2.063 e-27). Surprisingly, the Fe65 binding sites lost during DNA damage response revealed a significant enrichment in the JUN recognition motif (p-value : 2.798 e-17) (Fig. 3A). Furthermore, we used a similar strategy, executed by the IPA software, to analyze

genes that showed more than 50% increased or decreased fold change at each time point in Fe65 KO versus WT MEFs. Among significant regulators in any stress conditions (p-value < 1.0 e-10), E2F1, TP53, and JUN were top ranked based on p-values (data not shown) and showed the clearly changed activation state upon stress predicted by z-score (Fig. 3B). The activity of p53 was predicted to be augmented, while the activity of JUN was predicted to be compromised, upon stress in Fe65 KO MEFs compared to WT MEFs, which is in accord with results from Fig 3A. Based on our preliminary analysis, we suggest that E2F1, p53, and Jun are components of the transcriptional machinery regulated by Fe65 in mechanisms of DNA damage response, given their interaction with Fe65 and dysregulated expression in Fe65 knockout celllines.

3.4. Identification of candidate Fe65 target genes linked to DNA damage response

To ultimately identify the potential Fe65 target genes, we compared our genomic and transcriptomic datasets generated under DNA damage conditions. We selected 3446 coding genes based on the recruitment of Fe65 by ChIP-seq on their proximal promoter regions independent of treatment. Then, we selected 2497 genes that showed more than 50% increased or decreased fold change upon stress at any time point (0hr, 2hr, and 12hr) in microarrays obtained from Fe65 KO MEFs. When we overlapped these two gene sets, we identified 248 common genes (Fig. 4A, Supplementary Table S2) that showed Fe65 binding and Fe65-dependent transcriptional regulation under stress conditions. Further *in silico* analysis of this selected dataset confirmed our previous prediction. The top significantly (p-value < 1.27 E-02) enriched functions were cell cycle, cell death, and DNA replication, and recombination and repair (Table 1). Based on these results (Table 1), we generated an interaction map showing how many Fe65 target genes were linked to multiple functions and inter-connected (Fig. 4B).

3.5. Validation of Fe65 target genes

Based on the gene expression pattern identified as Fe65-depdent transcriptome, we used the IPA program to predict the activation state of Fe65 KO MEF compared to WT MEF. *De novo* predicted functions with z-score above 2 (increased) or below −2 (decreased) in Fe65 KO MEF included decrease of DNA repair (p-value : 8.25 E-05, z-score : −2.157), decrease of viability (p-value : 2.64 E-03, z-score : −2.144) and increase of sensitivity of cells (p-value : 2.96 E-04, z-score : 2.017) (Table 2). Finally we validated the Fe65 target genes identified by the latest analysis (red character in Table 2) using qPCR. When we measured changes in expression levels upon induction of stress in Fe65 KO MEF compared to of WT MEF, we confirmed our initial finding based on microarray assays (Fig. 5A). Together our results provide strong evidence that Fe65 contributes to DNA damage response by regulating gene transcription of key genes involved in DNA repair and sensitivity of cell upon stress.

4. Discussion

In this study, we provide strong evidence that Fe65 plays an important role in DNA damage response mediated by transcriptional regulation. Even though transcriptional role of Fe65 has been investigated by previous studies, the genome-wide global profile of Fe65 has not

been investigated. Using contemporary technology, we profiled the global binding pattern of Fe65 in cells exposed to genotoxic stress. Unexpectedly, we observed a large recruitment of Fe65 to regulatory promoter regions of many genes implicated in DNA damage signaling. Moreover, we noted a dramatic remodeling of the Fe65 binding program upon stress, indicating that Fe65 recruitment to its genomic loci is dynamically changed in a signaling-dependent manner (Fig. 1). A large fraction of Fe65 binding sites (1937) were conserved under different conditions, in according with previous studies reporting that Fe65 interacts with chromosome in both non-stress and stress condition [15]. One possible scenario is that transcriptional activity of Fe65 at these conserved binding sites may be regulated by post-translational modifications of Fe65. Interestingly, it has been reported that c-Abl mediated the phosphorylation of Fe65 at Tyr547 and that this event is able to increases the transactivational activity [3]. It will be of future interest to explore how c-Abl kinase activity modulates the Fe65 target genes. To take into account the well-established role of Fe65 in DNA repair mechanisms, we carried out experiments during a time course of recovering times points [15] (Fig. 2).

Here, for the first time we investigated how Fe65 contributes to gene expression changes observed under stress conditions. Until now the identity of specific Fe65 gene targets has been a controversial debate due to the variety of cellular and reporter systems used in distinct laboratories.[13, 18–20].

In this study, we utilized not only complete knockout condition using Fe65 deficient MEFs, but also explored the signal-dependent transcriptional regulation mediated by Fe65. Interestingly, we identified E2F1, p53, and Jun as potential co-regulators of Fe65-mediated transcriptional function upon stress (Fig. 3). In a previous report it has been shown that, p53 co-localize with Fe65 and is dysregulated by the over-expression of Fe65 [21]. Different study reported that the AICD, which is well-known binding partner of Fe65, interacts with p53 and regulates the transcriptional activity of p53 [22]. Therefore, our finding that p53 is potentially involved in the transcriptional machinery recruited by Fe65 is concordant with the previous literature. Interestingly, a novel link between E2F1 and Fe65 has emerged by our analysis. Based on the motif enrichment analysis, E2F1 binding motif was associated with Fe65 binding events independently of stress conditions. Further experiments are required to explore these relationships. Interestingly, the acetyltransferase Tip60, which is a known binding partner of Fe65, was also reported to functionally interact with E2F1, which is necessary for Tip60-mediated H4 acetylation [23]. Both p53 and E2F1 are key player in many cellular signaling pathways, including apoptosis, cell cycle and DNA damage response [24].

To ultimately identify the Fe65 target genes linked to genotoxicity, we performed extensive comparative analysis between gene data sets obtained from ChIP-seq and microarray assays. In ChIP-sequencing assays, we used differentiated human neuronal cell-line that mimic the expression level of Fe65 in the brain and we used etoposide as a genotoxic stressor. For the microarray assays, we took advantage of Fe65 KO and WT mouse embryonic fibroblasts for complete knockout of Fe65 and used hydrogen peroxide treatment as a genotoxic stimulus. We identified common regulated genes that are conserved between species and cell types. Both etoposide and hydrogen peroxide have been previously used as genotoxic stimulus that

are able to increase the nuclear translocation of Fe65 in a similar fashion [14], suggesting that this strategy could be successful in the identification of Fe65 target genes in DNA damage response. The *in silico* functional analysis, which was performed with 248 target genes (Table 1), showed that the significantly enriched functions included cell cycle, DNA repair, and cell death. Interestingly, those functions were confirmed previous results obtained from the ChIP-seq (Fig. 1C) and microarray studies (Fig. 2B, C). Our findings provide evidence that the Fe65 plays an important role in transcriptional regulation of genes linked to DNA damage response.

Moreover, the lack of Fe65 correlated to increased vulnerability to stress and decreased viability (Table 2), which might be due to defects in DNA repair pathways previously observed in Fe65 knockout cells [14], suggesting that Fe65 transcriptional activity might contribute to these phenotypes. Given the well-established implication of Fe65/APP complex in the pathogenesis of Alzheimer's disease, our results correlating Fe65 to cell survival generates a great interest in further investigating this pathway in the context of brain functions. Accordingly, APP interaction with Fe65 is required for both Fe65-dependent transcriptional activity [25], and for DNA repair [15].

5. Conclusions

In this study, we provide strong evidence that Fe65 transcriptional activity participates to signaling cascades triggered by genotxic stress. Fe65 target genes identified in this study are associated with cell cycle, cell death, and DNA repair pathways. The next-generation sequencing approach of this study uncovered an unexpected broad binding program regulated by Fe65. Further investigation need to explore the contribution of different target genes to specific functions linked to Fe65-depedent regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank YGW for critical reading of the manuscript. This work was funded by NIH grant AG024391, AG027734, and AG17242. YS is the recipient of a Glenn Award for Research in Biological Mechanisms of Aging.

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Highlights

- Genotoxic stress promotes the recruitment of Fe65 on promoter of DNA damage responsive genes.
- Fe65 target genes are functionally related to pathways of DNA damage response.
- Motif analysis of ChIP-seq binding sites identified p53, E2F1, and Jun as co-regulators of Fe65.
- 248 genes differentially expressed upon stress were identified as Fe65 transcriptional targets
- Alteration in Fe65 expression level might lead to compromised DNA repair and cell survival.

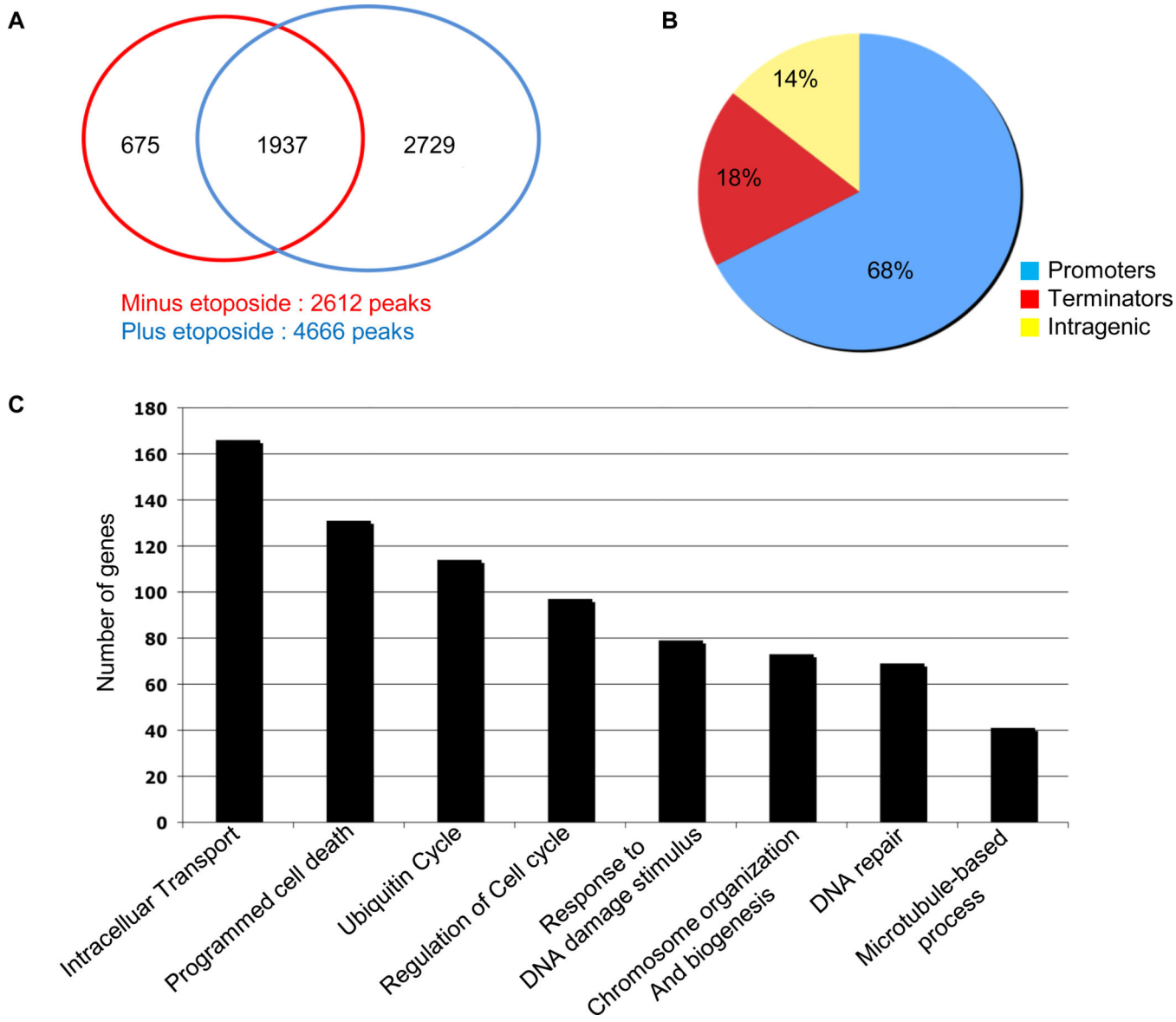

Fig. 1. Characterization of Fe65 binding genomic region upon genotoxic stress by ChIP-sequencing

(A) Distribution of Fe65 binding sites with and without etoposide (10 nM) treatment for 1 hr in differentiated SK-N-SH cell-line was indicated as numbers of binding sites from ChIP sequencing. (B) Proportion of Fe65 binding sites mapping to genomic region which includes promoter, terminator, and intragenic regions. (C) GO analysis of Fe65 binding genes. Genes within 500bp of binding sites were annotated for GO analysis. Significant (p-value < 0.001) GOs were selected and indicated.

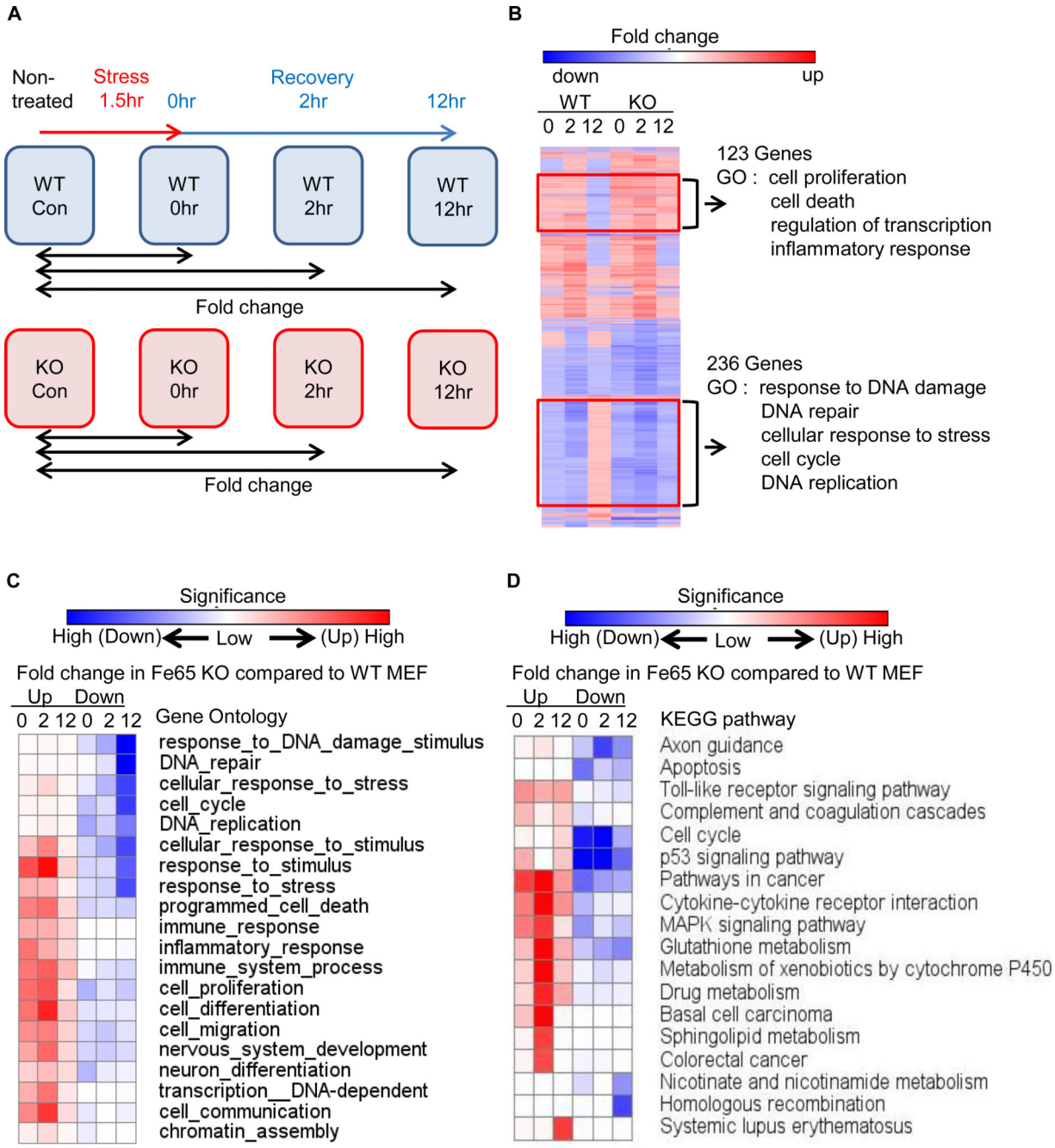

Fig. 2. Characterization of genes with Fe65 mediated expression change upon oxidative stress by microarray

(A) Schematic overview of microarray experiment. Each Fe65 KO MEF and WT MEF was treated with 100 uM hydrogen peroxide for 1.5 hr and recovered by removal of hydrogen peroxide for 0 hr, 2 hr, and 12 hr. Fold changes compared to non-treated condition were calculated at each time point for further analysis. (B) Different pattern of gene expression fold change upon stress between Fe65 KO and WT MEFs. Genes more than 1.5 fold change in Fe65 KO and WT MEF at any time point were selected and used for heat map. Parts of heat map with contrasting difference (red boxes) between Fe65 KO and WT MEFs were

indicated with GOs analyzed by DAVID/EASE. (C, D) GOs and KEGG pathways over-represented in Fe65 KO MEF compared to WT MEF upon stress were indicated. For analysis, the ratio has been calculated to divide fold change of Fe65 KO MEF by fold change of WT MEF for analysis. Genes with more than 50% increased (up) or decreased (down) fold change at each time point in Fe65 KO MEF were selected for analysis. GOs were analyzed by GoMiner and KEGG pathways were analyzed by DAVID/EASE. Only significant GOs and pathways ($p < 0.01$) were indicated. GOs, pathways based on genes with decreased (down) fold change in Fe65 KO MEF were indicated as blue color and $\log_{10}$ (p-value) has been used to indicate the significance. GOs, pathways based on genes increased (up) fold change in Fe65 KO MEF were indicated as red color and $-\log_{10}$ (p-value) has been used to indicate the significance. The stronger color means the higher significance.

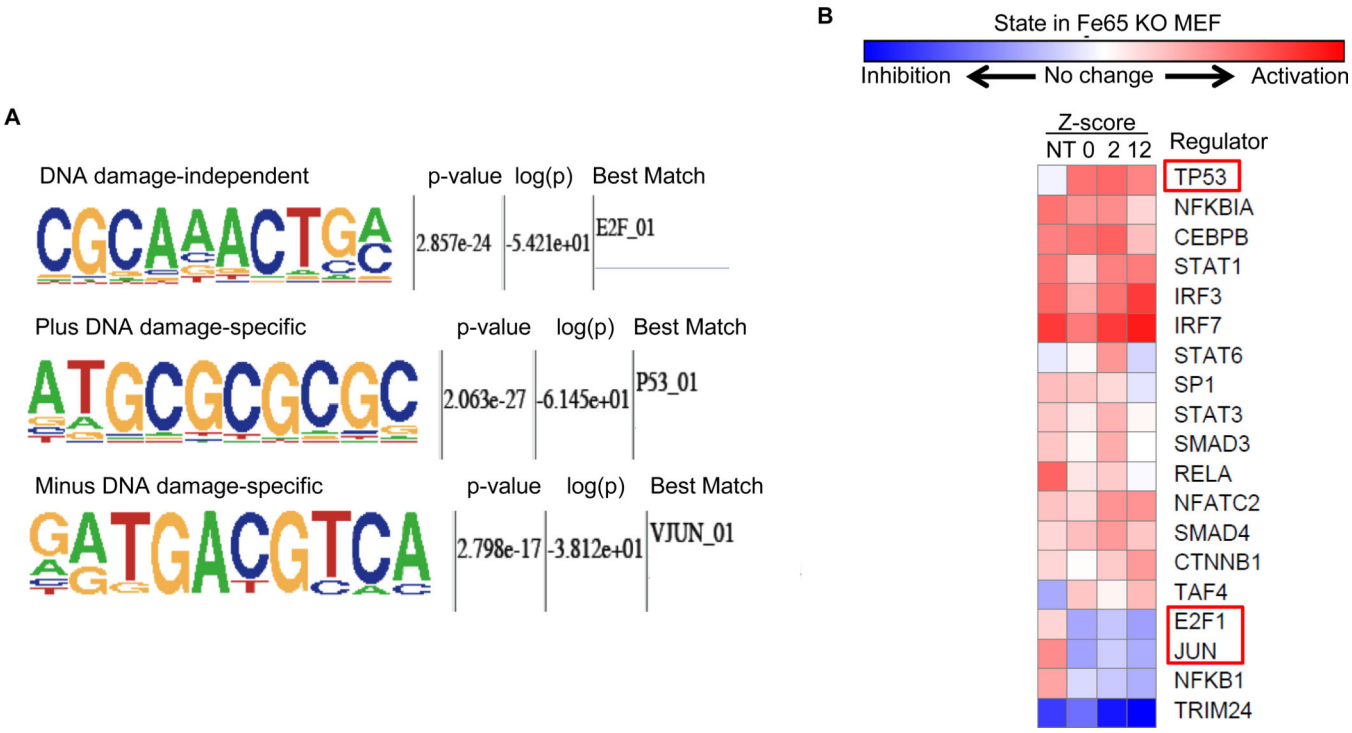

Fig. 3. Predicted co-regulators of Fe65 mediated transcriptional regulation upon stress

(A) Top results from motif enrichment analysis with ChIP-sequencing result. The result indicates significantly enriched conserved motifs of chromatin interacting regulatory element with p-values, log (p-values) among Fe65 interacting sequence analyzed by HOMER software. (B) Predicted upstream regulator activity using microarray result by Ingenuity IPA program. Z-score, which is a score of predicted activity, of transcriptional regulators from microarray result was calculated for Fe65 KO MEF with genes of more than 50% increased or decreased fold change compared to WT MEF at each time point including non-treated condition. We selected significant co-regulators (p-value < 1.0 E-10), and z-scores were used for heat map. Z-score > 0 indicates activated state of regulators (red) and z-score < 0 indicates inhibited state of regulators (blue). Red boxes indicate the regulators indicated in Fig. 3A.

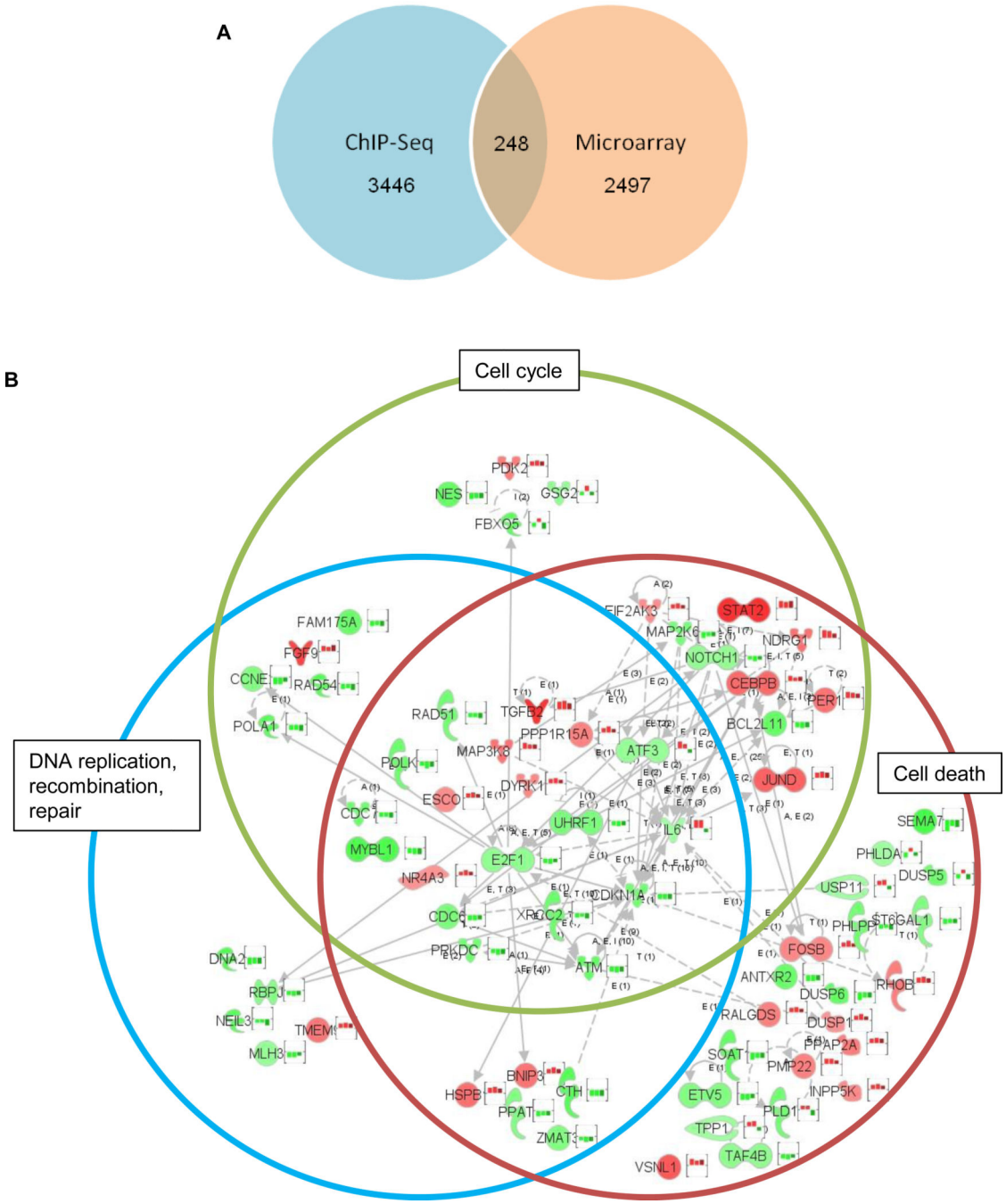

Fig. 4. Identification of Fe65 target genes by comparing results from ChIP-sequencing with microarray

(A) 3446 genes interacting with Fe65 with or without stress were selected from ChIP-sequencing. 2497 genes of more than 50% increased or decreased fold change in Fe65 KO MEF compared to WT MEF at any time points were selected from microarray. Overlapping 248 genes between ChIP sequencing and microarray indicate candidates for Fe65 target genes. (B) Overall interaction map of Fe65 target genes with significantly enriched function from Table. 1. Genes with each function are located within each labeled circle area. Genes with multiple functions were located in overlapped regions. Color of each gene indicates

increased (red) or decreased (green) fold change in Fe65 KO MEF compared to WT MEF. This figure indicates the time point of 12 hr after recovery. Small bar-graph beside genes indicates expression change at each time point in an order of 0 hr, 2 hr, and 12 hr. Connected lines indicate the transcriptional regulation such as activation (A), inhibition (I), expression (E), and transactivation (T) between genes.

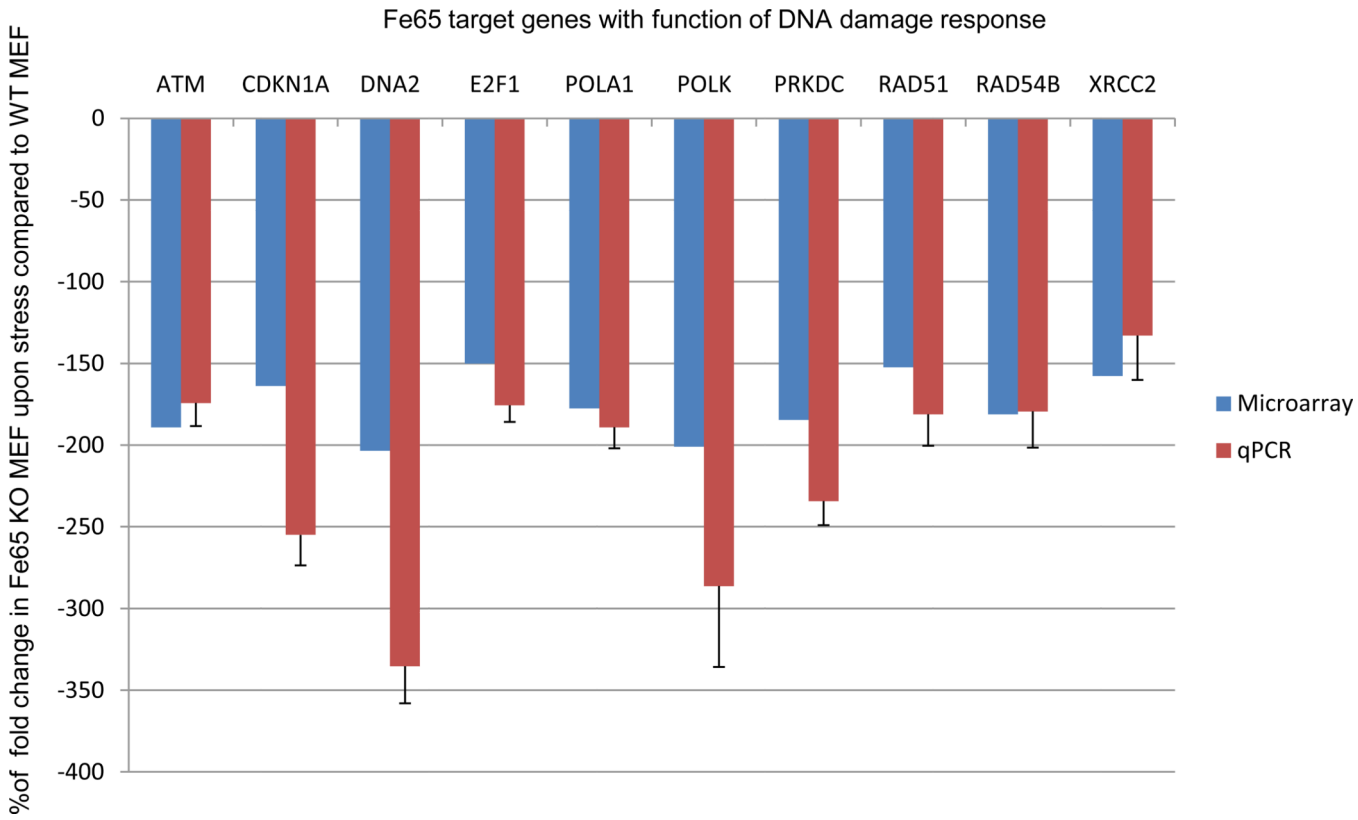

Fig. 5. Validation of expression change in Fe65 target in DNA damage response by an independent qPCR

Fe65 target genes contributing to predicted functional status in Fe65 KO MEF upon stress were selected for validation of expression change from microarray. qPCR has been performed for selected genes with the identical RNAs used for microarray. The minimum ratio (percentage) of fold change in Fe65 KO MEF compared to fold change in WT MEFs across all time points in microarray (blue) and qPCR (red) were indicated in a graph.

Table 1

Significantly over-represented function of Fe65 target genes by IPA functional analysis.

Function Category	p-value	Number of Molecules
Cell Cycle	1.23E-07 - 1.27E-02	37
DNA Replication, Recombination, and Repair	1.10E-05 - 1.27E-02	34
Cell Death	2.19E-05 - 1.27E-02	84

With 248 Fe65 target genes (Fig. 4A), we analyzed the significantly over-represented function by IPA program. Top 3 functions by p-value have been indicated.

Table 2

Predicted functional state in Fe65 KO MEF upon stress by expression change of Fe65 target gene.

Functions	p-value	Predicted Activation State	Activation z-score	Molecules
Repair of DNA	8.25E-05	Decreased	−2.157	ATM, CDKN1A, DNA2, E2F1, FAM175A, NEIL3, POLA1, POLK, PRKDC, RAD51, RAD54B, XRCC2
Viability	2.64E-03	Decreased	−2.144	ATM,E2F1, NOTCH1, PRKDC, XRCC2
Sensitivity of cells	2.96E-04	Increased	2.017	ATM, CDKN1A, MAP2K6, POLK, PRKDC, RAD51, RAD54B, XRCC2

Z-score > 2 or < −2 each indicates decreased or increased activation state. Red character indicates the genes to be confirmed by qPCR about difference of expression identified from microarray (Fig. 5).