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A Double Jeopardy: Loss of FMRP Results in DSB and Down-regulated DNA Repair

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Abstract

Our understanding of the molecular functions of the nucleocytoplasmic FMRP protein, which, if absent or dysfunctional, causes the fragile X syndrome (FXS), largely revolves around its involvement in protein translation regulation in the cytoplasm. Recent studies have begun honing in on the nuclear and genomic functions of FMRP. We have shown that during DNA replication stress, cells derived from FXS patients sustain increased level of R-loop formation and DNA double strand breaks. Here, we describe a transcriptomic analysis of these cells in order to identify those genes most impacted by the loss of FMRP with and without replication stress. We show that FMRP loss causes transcriptomic changes previously reported in untreated conditions. Importantly, we also show that replication stress, in addition to causing excess of DSB, results in down-regulation of transcription in virtually all DNA repair pathways. This finding suggests that despite normal DNA damage response, FXS patient-derived cells experience R-loop-induced DNA breakage as well as impaired DNA repair functions, effectively a double jeopardy. We suggest that it is imperative to deepen the understanding of the nuclear functions, particularly a genome protective function, of FMRP, which will lead to discoveries of novel therapeutic interventions for the FXS.

Keywords: *DNA double strand breaks (DSBs); DNA repair; FMR1; FMRP; fragile X Syndrome (FXS); Genome instability; R-loop*

Introduction

Fragile X syndrome (FXS) is a neurodevelopmental disorder affecting 1 in 7000 males and 1 in 11,000 females (National Fragile X foundation). FXS is the most common cause for inherited intellectual disability and developmental delay [1]. At the molecular level, FXS patient-derived cells when cultured in folate deficient medium present a secondary constriction in the long arm of the X chromosome [2,3]. This abnormality defined the first rare fragile site associated with a genetic disorder, FRAXA (Figure 1A) [4]. It also constitutes the most frequent monogenic cause for autism spectrum disorder [5,6]. FXS patients display a multitude of behavioral problems such as anxiety, aggression, and attention deficit hyperactivity disorder [7]. FXS patients have limited treatment options with no cure and life-long dependency on psychopharmacological drugs to manage the behavioral problems [8].

FXS is primarily caused by CGG repeat expansion in the 5'UTR of the FMR1 gene, resulting in epigenetic silencing and lack of FMRP expression [9-12]. It is also less frequently caused by mutations in the coding region of FMR1 and thus dysfunctional FMRP [13,14]. FMRP is an RNA-binding protein and has multifaceted functions. It regulates key neuronal pathways by sequestering specific mRNA substrates and controlling signaling cascades across several cellular membrane receptors such as the metabotropic-glutamate receptor (mGluR), AMPA, NMDA, dopamine and cannabinoid receptors [15]. FMRP loss affects dendrite morphogenesis, neuronal circuit integration and axon guidance [16]. FMRP also interacts with pre-synaptic ion channels in hippocampal and cortical excitatory neurons and modulate neurotransmitter release and synaptic transmission [17-21]. Among FMRP's multi-faceted functions, the best understood is the mGluR-mediated long-term depression (LTD) pathway in which FMRP functions as a translation repressor [22]. Loss of FMRP causes an exaggerated mGluR-LTD and reduced synaptic strength [22-25]. However, despite the rescue of AMPA receptor trafficking defects in cultured neurons and behavior phenotypes in animal models, mGluR antagonists did not show expected efficacy in clinical trials [24,26-28]. Importantly, only a few of the mRNA targets of FMRP show high levels of protein expression in its absence and increased protein levels does not correlate with pathogenicity [29].

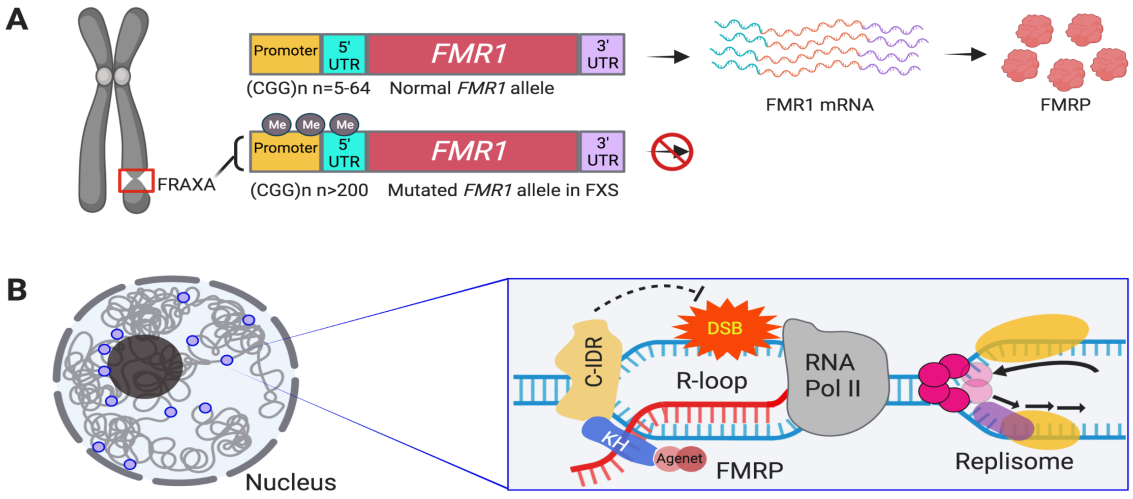


Figure 1: Overview of our current understanding of FXS. (A) A constriction in the long arm of the X chromosome marked by a red box represents the FRAXA site which is recurrently observed in FX cells under folate deficiency. The same site bears the mutated *FMR1* gene. The 5'-UTR of the *FMR1* gene has greater than 200 repeats for a full mutation. (B) A proposed genome protective role of FMRP (depicted by its protein domains including the N-terminal Agenet domain, KH domains and the C-terminal intrinsically disordered region) as a novel R-loop regulator. FMRP inhibits R-loop mediated replication-transcription collision. FMRP interacts with the chromatin, binds R-loop directly and may engage R-loop resolvases to initiate resolution, thereby preventing DSBs. Images were created with BioRender.com.

Therefore, it stands to reason that FMRP may have translation regulation-independent functions which underlie FXS disease etiology.

Since the discovery of FMRP as an mRNA binding protein, there has been an explosion of studies aiming to determine cell type- and sequence-specific binding of the mRNA targets of FMRP. The initial studies applied FXS mouse models with isolated brain regions (forebrains, hippocampus, cortex, cerebellum), followed by Purkinje cells and CA1 neurons, using RNA pull-down assays coupled with microarray or high-throughput sequencing [23,30-34]. Among these studies, it was reported that the FMRP mRNA targets were enriched in G-quadruplex sequences and/or long coding sequences and 3'UTRs. However, these studies in the mouse model do not correlate well with those using human counterparts in the majority of brain development [35]. Therefore, studies investigating FMRP mRNA targets in HEK293 cells and in adult post-mortem brain were conducted [36, 37], which led to the report of approximately 6000 human mRNA targets of FMRP [36]. A more recent study used human induced pluripotent stem cells differentiated into dorsal and ventral forebrain neural progenitor cells, arguably the most relevant cell types affected in FXS [38]. It showed that the FMRP tends to bind coding sequences instead of 3'UTRs, contrary to the mouse model, and preferably in long genes. Altogether, these studies did not reach an agreement on the mRNA sequence motifs that FMRP recognizes, suggesting that the recognition is structure- rather than sequence-specific, and is determined by the cell type. Importantly, genes whose mRNAs are FMRP binding targets participate in pathways that involve synaptic development, cell signaling, RNA transport, actin cytoskeleton, transcription, and epigenetic function [16,39]. Additionally, these genes are implicated in autism, thereby associating their binding by FMRP to potential disease mechanisms [23,37]. But what steps during mRNA regulatory or metabolic pathways other than translation regulation in which does FMRP function?

Studies in various model systems have now shown that FMRP functions in pre-mRNA splicing [40], mRNA stability [29,41], mRNA editing [42,43], and miRNA regulation [44,45]. In addition, studies have described nuclear and genomic functions of FMRP in DNA damage response, etc., which are not well understood [46-50]. We recently reported that lymphoblastoid cells derived from an FXS patient (FX cells) sustained genome-wide DNA double-strand breaks (DSBs) when undergoing DNA replication stress by aphidicolin (APH, a DNA polymerase inhibitor) [51]. Moreover, DSBs occurred near sequences that are prone to forming DNA:RNA hybrids called R-loops during gene transcription [51]. We also demonstrated that these FX cells have an intact DNA damage response [51]. These findings suggested a new co-transcriptional function of FMRP, which mitigates R-loop-induced DSBs during replication stress, thereby maintaining

genome stability (Figure 1B). To further investigate this function, we asked if and how FMRP loss impacts the transcriptome upon replication stress in the FX lymphoblastoids in which we have analyzed DSB formation. Transcriptomic studies have primarily been conducted using brain tissue or cells from animal models of FXS. Due to cell heterogeneity, these studies have reported only subtle changes in mRNA levels [29,41,52], though single cell transcriptomics revealed dysregulation of cellular and molecular networks in the mouse model of FXS [41]. In humans, access to brain tissue is limited to adult post-mortem brain which does not model the neurodevelopmental role of FMRP. We note that peripheral blood cells have been used for molecular and phenotypic analyses of the FXS, as well as other autism spectrum disorders [1,53-55]. Our studies thus far have demonstrated that they are also a useful system for studying the genomic functions of FMRP.

Method

RNA-seq: FX cells (GM03200) and normal control NM (GM06990) cells were either treated with DMSO, 0.3 μM APH or left untreated for 24 h before harvest. $3x10^6$ cells were harvested for RNA-seq. RNA was extracted using the Qiagen RNeasy Plus Mini Kit. The RNA was run on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano Chip to assess RNA quality and quantity. 1 μg of total RNA was used as input to the Illumina TruSeq Stranded Total RNA Library Prep Kit Ribo Zero Gold H/M/R. Library size was assessed using the DNA 1000 chip on the Bioanalyzer, and the libraries were quantified using a Qubit fluorometer. Pair-end sequencing was run on an Illumina NextSeq 500 instrument. A total of four replicates were processed for treatment/conditions out of which three were biological replicates.

RNA-seq data analysis: Raw reads were obtained from Illumina Base space and pair-end reads were merged. Merged sequence reads were then aligned to the UCSC human genome assembly, GRCh37/hg19 using STAR-fusion aligner. The BAM files generated by STARfusion were then subjected to featureCounts [56] for the generation of read counts per gene. RNA-seq expression count obtained from featureCounts was Log2 transformed, mean normalized, and value trimmed prior to differential gene expression analysis. Mean normalization was performed by calculating the mean expression of every given sample. The mean of the sample means for each unique cell type and condition was then calculated. A correction coefficient was calculated by dividing a sample's gene expression mean by their cell type and condition's mean. Each sample was then multiplied by this correction coefficient. A cut-off value of 2(2^2 = 4 for raw counts) was used to determine genes which are not expressed as compared to genes that are expressed. Fold change was calculated by subtracting Log2 mean expression values and then setting 2 to the power of this value. Significance was determined by one-way ANOVA. The Benjamini and Hochberg method was used to calculate false discovery rate (FDR). Significant differentially expressed genes (DEGs) are determined by a p-value <= 0.05. Up-regulated and down-regulation of genes is determined as having a fold-change of >1 and <1 respectively.

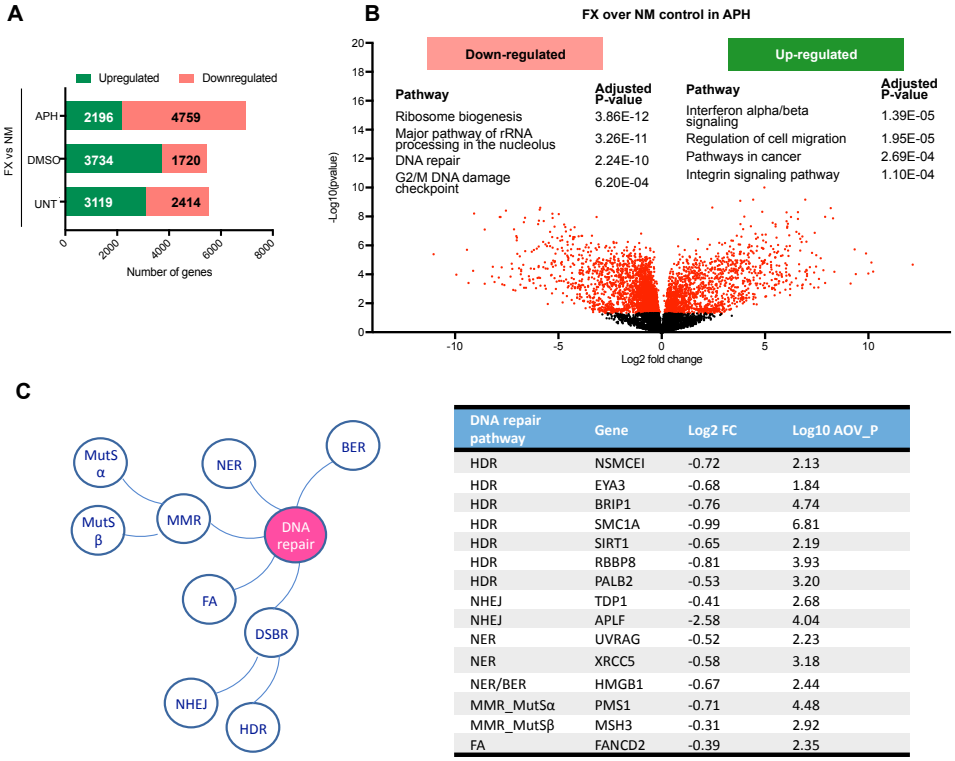
Gene ontology analysis: Pathway analysis was performed using Enrichr [57,58]. Tables were generated using all significant DEGs, as well as significant up and down regulated DEGs. Databases used for this analysis include GO Molecular Function 2018, GO Cellular Component 2018, GO Biological Process 2018, WikiPathways 2019 Human, KEGG 2019 Human, Reactome 2016, InterPro Domains 2019, and Panther 2016. Pathways analysis was also performed on FDR significant (FDR <= 0.05) genes for each pair. Heatmaps were produced using Morpheus (https://software.broadinstitute.org/morpheus).

Results

We conducted a transcriptome analysis using total RNA isolated from FX and normal control (NM) lymphoblastoids, with and without replication stress by APH. We aimed to comprehensively define the transcriptomic changes due to FMRP loss and to address the increased DNA DSB phenotype in our previous studies. We performed differential gene expression analysis, comparing transcript counts in FX over NM cells. We categorized genes based on their transcriptional status ("on" *vs.* "off") or expression level ("up-regulated" *vs.* "down-regulated") in FX cells with respect to NM cells. Specifically, "on" corresponds to gene expression only in FX cells and not in NM cells, and vice versa for "off" genes. Similarly, "up- or down-regulated" correspond to genes expressed in both cell lines and with increased or decreased expression in FX cells compared to NM cells, respectively. First, there were more "on" than "off" genes in all conditions, suggesting a significant increase of transcriptional induction due to the loss of FMRP. Second, there were more up-regulated than down-regulated genes in both untreated and DMSO-treated conditions; however, the APH treatment caused a sharp increase of down-regulated genes by approximately 3-fold (Figure 2A). These results together suggest that, despite increasing transcriptional induction (without APH) in FX cells, replication stress by APH reduced the levels of gene expression. This is consistent with the notion that DNA damage itself has a negative impact on gene transcription [59] and that FX cells sustain higher level of DNA damage.

We next asked what biological pathways were enriched in the DEGs. In all conditions, genes up-regulated in cancer, such as "interferon alpha/beta signaling", were up-regulated in FX cells (Figure 2B and not shown). For example, IFITM3 (Interferon-inducible Transmembrane Protein 3) has been recently associated with bone metastasis of prostate cancer cells [60].

Currently it is unclear if and how the up-regulated pathways impact FXS pathology, as cohort studies have reported conflicting conclusions as to whether FXS patients have increased risk for cancer [61,62]. However, we note that antiepileptic drug use, which is a common medical intervention among FXS patients, has been linked to increased risk for cancer [63]. Thus, it is challenging to delineate the cause for the observed up-regulation of cancer genes in FX cells. Additional up-regulated pathways include 'immune response', 'Cytokine signaling' and 'Actin cytoskeleton regulation', as reported by previous transcriptome studies [64,65]. On the other hand, genes involved in translation, including "eukaryotic translation elongation", "3'-UTR-mediated translational regulation", "major pathway of rRNA processing in the nucleolus" and "ribosome biogenesis", were down-regulated in FX cells, presumably as a response to increased translational burden in the absence of FMRP. Notably, APH caused down-regulation of 101 DNA repair genes and 29 G2/M checkpoint genes in FX cells (Figures 2B&C). This observation recapitulated a previous studies reporting down-regulated expression of DNA damage/repair pathway transcripts in FXS patient lymphoblastoids even without replication stress [55,65]. These results suggest that FX cells are inflicted with a double jeopardy during replication stress—that is—increased R-loop/DSB formation and down-regulated DNA repair.



DNA repair pathway	Gene	Log2 FC	Log10 AOV_P
HDR	NSMCEI	-0.72	2.13
HDR	EYA3	-0.68	1.84
HDR	BRIP1	-0.76	4.74
HDR	SMC1A	-0.99	6.81
HDR	SIRT1	-0.65	2.19
HDR	RBBP8	-0.81	3.93
HDR	PALB2	-0.53	3.20
NHEJ	TDP1	-0.41	2.68
NHEJ	APLF	-2.58	4.04
NER	UVRAG	-0.52	2.23
NER	XRCC5	-0.58	3.18
NER/BER	HMGB1	-0.67	2.44
MMR_MutSα	PMS1	-0.71	4.48
MMR_MutSβ	MSH3	-0.31	2.92
FA	FANCD2	-0.39	2.35

Figure 2: Emerging molecular players in FXS pathology identified by RNA-seq analysis. NM and FX cells were treated with DMSO, 0.3 μM APH or nothing for 24 h before harvest. $3x10^6$ cells were used for RNA-seq using the Illumina TruSeq Stranded Total RNA Library Prep Kit Ribo Zero Gold H/M/R, with pair-end sequencing on Illumina NextSeq 500. Four replicates were processed. Detailed RNA-seq data analysis and raw data are accessible from the GEO accession number GSE124403. (A&B) Summary of gene expression from RNA-seq analysis. (A) Number of genes up- or down-regulated in FX cells when compared to NM cells with or without APH. (B) Volcano plot of -Log10 (p-value for significance in differential expression) versus Log2 (fold change of transcript levels of FX_APH to NM_APH) for all genes. Relative to NM_APH, significantly different genes in FX_APH with -Log10 p-value greater than 1.3 are shown in red. Top biological pathways that are enriched for those genes significantly down- or up-regulated in FX_APH cells relative to NM_APH are shown. (C) Representative down-regulated DNA repair genes in FX_APH cells. Log2 (fold change of expression of FX to control). AOV_P, differential expression ANOVA test P value. HDR, homologous DNA recombination; NHEJ, non-homologous end joining; NER, nucleotide excision repair; BER, base excision repair, MMR, mismatch repair; FA, Fanconi anemia pathway.

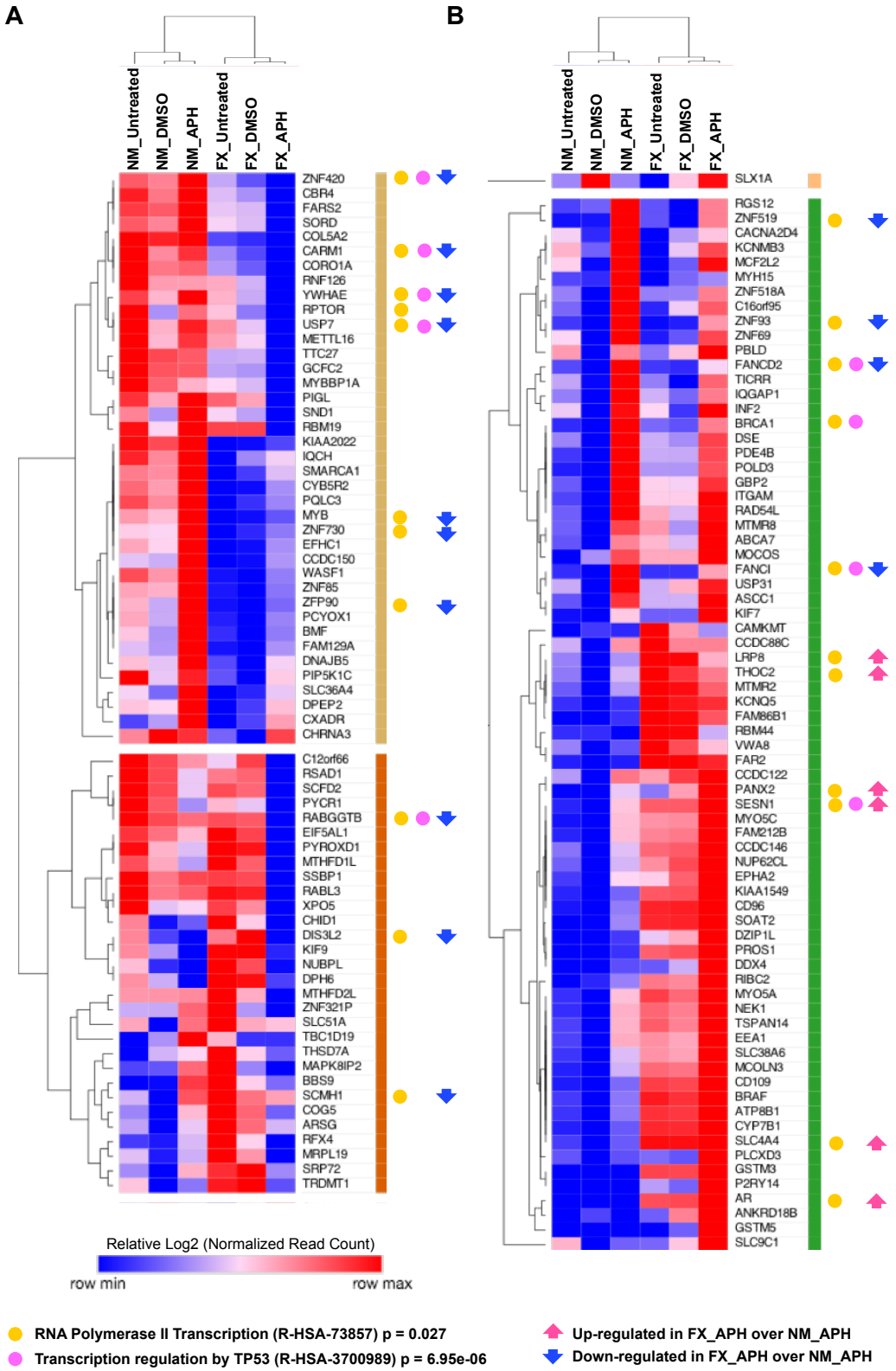


Figure 3: TP53-regulated transcription pathway genes are down-regulated in FX cells undergoing APH-induced DSBs. Heat map analysis and hierarchical clustering of gene expression from 142 protein coding genes suffering DSBs in FX cells specifically under APH treatment [51]. Log2 transformed normalized read counts were used to perform the analysis using Morpheus (https://software.broadinstitute.org/morpheus). Reactome pathways enriched for DEGs in APH-treated FX cells (FX_APH) relative to the NM_APH cells are indicated with solid circles. Up- and down-regulated genes are indicated by up and down arrows, respectively.

Discussion

Our previous study led us to conclude that the FX genome suffers from R-loop-associated DSBs induced by replication stress [51]. Among the DSB hotspots are many genes involved in neuronal development and synaptic regulation, suggesting that these genes are protected by FMRP in addition to being translationally regulated by it [51]. Thus, it appears that FMRP controls all aspects of RNA metabolism including co-transcriptional regulation. In this study we further demonstrated that APH-treated FX cells show down-regulated expression of genes in virtually all DNA repair pathways. This is a result that recapitulated previous findings of the FX cells without replication stress, though only a selected few DNA repair pathways were previously reported [55,65]. In addition, it has been shown that mouse embryonic fibroblasts from an FXS mouse model showed defective single-stranded DNA repair during meiotic DSB formation [46].

In contrast to the previous gene expression studies which used microarray-based gene expression data, we found that there were more up-regulated genes in FX cells compared to the control cells, suggesting that FX cells have heightened transcriptional response as a result of FMRP deficiency. The up-regulated genes are enriched in oncogenic pathways such as 'Ras protein signaling transduction' including MAPKAPK3, RAB genes, TIAM1, INFα/β and KRAS. Moreover, MDM2 and XIAP, which prevents p53 accumulation and inhibits apoptosis, respectively, are also up-regulated in our current study. Consistent with our finding, recent RNA-seq studies using neuronal cells differentiated from human embryonic stem cells or induced pluripotent stem cell models of FXS also reported up-regulated expression of PI3K-AKT and ERK/MAPK pathways, both of which are downstream to RAS signaling and controlled by the RAS proteins with implications of cancer-like transformations [64,66-69]. Interestingly, we also observed an increased expression of Amyloid β-precursor protein (APP) in FX cells compared to control without replication stress (Log2 fold change values 0.44 and 0.50 for untreated and DMSO-treated, respectively). Upon APH treatment the differential expression dropped to Log 2 fold change of 0.14. APP is an integral membrane protein that is ubiquitously expressed but enriched in the brain [70]. APP undergoes proteolytic cleavage by three types of proteases that results in the shedding of the extracellular domain. The type of proteolytic processing can result in neuroprotective or neurotoxic consequences as observed in Alzheimer's disease with the accumulation of Aβ-peptide [71]. FMRP has been shown to bind APP mRNA directly, and through the miRNA pathway suppress its translation [71,72]. Consequently, APP and its cleavage products were found to be up-regulated in Fmr1 KO mice. Moreover, APP haploinsufficiency resulted in the rescue of repetitive behavior, hyperactivity, mGluR-LTD and spine morphology in a mice model of FXS [72]. Similarly, APP, sAPPα and Aβ peptides are shown to be up-regulated in post-mortem brain and in the blood plasma of FXS children [71,72]. Our findings suggest that the APP mRNA is regulated by FMRP both transcriptionally and translationally, in the absence of replication stress.

Treatment of the FX-patient derived cells with APH resulted in a shift in the mRNA expression pattern such that more genes were down-regulated because of DNA damage. Notably, we observed down-regulation of genes in virtually all DNA repair pathways.

Conclusion

In conclusion, our results suggest that the FX genome undergoes a double jeopardy of sustaining R-loop-induced DSBs and reduced DNA repair as a result of replication stress. APH treatment led to more genes showing down-regulated expression compared to vehicle control cells, possibly due to DNA damage of these genes. Indeed, 60% of the DEGs that also sustained DSBs in APH-treated cells showed decreased expression in APH. We envision that such genome instability may profoundly impact cellular functions of neuronal cells when FMRP is absent. It has not escaped our attention that post-mitotic neurons are unlikely subjected to DNA replication stress. However, we note that R-loop formation can be induced by chemicals/reagents that perturb gene transcription, thus still necessitating FMRP to resolve R-loops and maintain genome integrity. Future work would be dedicated to understanding of the mechanisms of FMRP protection of the mRNA substrates, particularly DNA repair genes, during transcription. It will also be dedicated to the determination of neuronal activities upon the loss and gain of FMRP's genomic substrates that have been identified in the lymphoblastoid cells. In turn, these effort would likely lead to better targets for therapeutic interventions of FXS.

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Authors' Contributions

Conceptualization: AC & WF. Data curation: AC. Formal analysis: AC, AG & WF. Funding acquisition: VAK & WF.

Methodology: AC & WF. Project administration: WF. Visualization: AC & AG. Writing – original draft: AC. Writing – review & editing: AC & WF.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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