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**Convergent and divergent molecular pathways in *FMR1*-, *TSC2*- and *FMR1/TSC2*
knockout neurons**

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Abstract

Background

Fragile X syndrome (FXS) and tuberous sclerosis complex (TSC) are common monogenic causes of autism spectrum disorder (ASD). FXS arises from *FMR1* silencing, while TSC results from mutations in *TSC1* or *TSC2*, both converging on dysregulated ERK and mTORC1 signaling. Animal knockout models suggest opposing effects on synaptic plasticity, with reciprocal compensation in double knockouts (dKO). However, clinical case with dual mutations shows severe neurodevelopmental deficits; here, we explored a human cellular model to dissect the shared and divergent mechanisms.

Methods

We generated isogenic human pluripotent stem cell (hPSC)-derived models of *FMR1*KO, *TSC2*KO, and *FMR1/TSC2* dKO neurons. Neuronal transcriptomes were profiled by RNA-seq, integrating ERK, mTOR, FMRP targets, and ASD risk genes. Validation via qPCR of key genes, protein synthesis, proliferation assays and microelectrode array was performed.

Results

The *FMR1/TSC2* dKO neural progenitor cells (NPCs) demonstrated high DNA damage response but normalized proliferation. Convergent transcriptomic pathways across *FMR1*KO, *TSC2*KO and dKO neurons included upregulated extracellular matrix and stress responses, and downregulated synaptic and neurotransmission-related pathways. *TS2*KO and dKO neurons showed greater similarity transcriptionally and functionally. Translational pathways and global protein synthesis were oppositely regulated in *TSC2*KO and dKO versus *FMR1*KO neurons. The dKO neurons showed hyperexcitable network activity, mTOR hyperactivation, with distinct dysregulated FMRP targets and ASD risk gene expression.

Conclusion

Unlike mouse models, *FMR1/TSC2* dKO hPSC-derived neurons did not show rescue of synaptic gene expression. Rather, dKO neurons predominantly resembled *TSC2*KO neurons with translational, synaptic and neurotransmission abnormalities. These findings highlight complex interplay between FMRP and TSC, providing a foundation for future studies of ASD-relevant mechanisms.

Introduction

Fragile X syndrome (FXS) and tuberous sclerosis complex (TSC) are major monogenic syndromes that cause autism spectrum disorder (ASD). FXS, affecting 1 in 4000 males and 1 in 4000-8000 females, is caused by expansion of CGG repeats in the 5' UTR of the *FMR1* gene(1). This leads to epigenetic silencing of the locus and loss of its protein product, FMRP, an RNA-binding protein that regulates translation of numerous synaptic mRNAs(2). TSC, occurs in 1 in 6000 births, is caused by mutations in *TSC1/TSC2*, resulting in hyperactivation of the mTORC1 signaling pathway, a central regulator of cell growth and protein synthesis(3, 4).

FXS and TSC both disrupt ERK and mTOR signaling but are proposed to have opposite effects on translation and synaptic plasticity. *Fmr1/Tsc* double knockout mouse model demonstrated bidirectional rescue, normalising hippocampal mGluR-long term depression (LTD), synaptic protein synthesis and normalized behaviour(5-7). In contrast, fibroblasts from a female with co-occurring *FMR1* mutation and heterozygous *TSC1* mutation exhibited severe intellectual disability, with persistently elevated mTOR signaling and global protein synthesis, highlighting a gap between mouse models and clinical observations(8).

To address this, we performed a direct comparison of transcriptomic and cellular phenotypes in human pluripotent stem cell (hPSC)-derived neurons lacking *FMR1*, *TSC2*, or both (isogenic male dKO model with complete loss of *FMR1* and homozygous *TSC2* knockout to assess maximal functional effects). We also integrated common differentially expressed genes (DEGs) with ERK, mTOR, FMRP targets and ASD risk genes to identify shared and divergent disease-relevant signatures.

Methods

Cell line generation

Isogenic *TSC2KO* hPSC lines were generated with SpCas9/sgRNAs (Streptococcus pyogenes Cas9/single guide RNA) targeting exon 3 (Synthego) from H1/WA01 controls

(details on sgRNA sequences in Supplemental Methods). *TSC2/FMR1* dKO were generated using same multi-gRNAs for *TSC2*KO from *H1-FMR1*KO previously generated in Utami et al., 2020. *hPSCs* were cultured and differentiated into neural progenitor cells (NPCs) and neurons as described in Supplemental Methods.

Protein assays

Immunofluorescence and immunoblot were performed as previously described(9) (protocols in Supplementary Table 1 and Supplemental Methods).

Neural progenitor assays

For proliferation assays, ~70% confluent NPCs were pulse-labeled with 50 μ M BrdU (bromodeoxyuridine) for 6h and fixed in 4% paraformaldehyde. Details for CCK8 (cell counting kit-8) assay to assess proliferation are described in the Supplemental Methods.

RNA-sequencing profiling and bioinformatic analysis

RNA-sequencing data from three independent biological replicates per genotype (~20M paired-end 150 bp reads/sample, HiSeq 4000) were mapped to hg38 with Salmon, analyzed for differential expression using DESeq2 (*FMR1*KO, *TSC2*KO and dKO versus controls; $\text{abs}(\text{Log}_2\text{FC}) > 1.5$, $\text{adj.p} < 0.05$), and subjected to gene ontology (GO) pathway enrichment by over representation analysis (ORA) with *clusterProfiler* package (false discovery rate (FDR) < 0.05). RNA-sequencing data is deposited in Gene Expression Omnibus (GEO database) under GSE306594. Differentially expressed genes were compared across various datasets including ERK/mTOR-related genes(10, 11), FMRP targets(12), and SFARI gene database(13) (Supplemental Methods). Key gene validation was conducted using quantitative real-time PCR (qRT-PCR), detailed in the Supplemental Methods.

Functional assays

Global protein synthesis (SUnSET) assay(14) and microelectrode array were performed as previously described(9, 14) (Supplemental Methods).

Results

mTOR hyperactivation is specific to the loss of TSC2 protein

*FMR1*KO, *TSC2*KO, dKO cell lines were generated from H1 hPSCs, and differentiated into NPCs and neurons (Figure 1A, 1B). Immunoblotting confirmed loss of FMRP (fragile X messenger ribonucleoprotein), TSC2, or both proteins in the respective cell lines, with reduced trend of FMRP expression in *TSC2*KO hPSCs (Figure 1C, Figure S1). mTOR pathway activation, assessed by p70S6K1/S6K1 levels, was elevated only in *TSC2*KO and dKO NPCs, confirming that mTOR hyperactivation is specific to *TSC2* loss (Figure S2A, 2B).

Early neural phenotypes reveal increase in DNA damage response and NPC proliferation

We evaluated cellular stress by measuring DNA damage response in hPSC-derived NPCs with γ H2Ax. Levels were elevated in *TSC2*KO and *FMR1*KO, peaking in dKO NPCs versus controls (Figure 1D, 1E). NPC proliferation, assessed by BrdU and Ki67 (labels cells in S-phase and Ki67 localization) (Figure 1F,1G), as well as CCK8 (assessing net population expansion) (Figure S3), were elevated in *FMR1*KO and *TSC2*KO NPCs relative to controls. The proliferation rate of dKO NPCs was normalized to control levels, suggesting possible compensatory mechanism that mitigate excessive proliferation in dKO NPCs.

Transcriptional profiling reveals convergent and divergent gene signatures across genotypes

Transcriptome principal component analysis showed that *TSC2*KO and dKO neurons clustered together, indicating more similarity to each other than to *FMR1*KO or control neurons (Figure S4, S5A-5B). DEGs (adj. $p < 0.05$, abs(LFC) > 1.5) were identified in *FMR1*KO vs control (1,156 DEGs), *TSC2*KO vs control (2,459 DEGs) and dKO vs controls (2,996 DEGs) (Figure S5C-E, Supplementary Table 1). As a quality control, we checked and found ~50% reduction

of *FMR1* gene expression in *FMR1KO* and dKO, and ~80% reduction of *TSC2* gene expression in dKO and *TSC2KO* (Figure S6A-B). Approximately 70-80% of DEGs overlapped between *TSC2KO* vs control and dKO vs control neurons, indicating that *TSC2* loss predominantly drives transcriptional changes in dKO (Figure 2A).

In addition, 571 DEGs were shared across *FMR1KO*, *TSC2KO*, and dKO, vs control: 314 genes commonly upregulated (Figure S7A, S7C), 216 genes commonly downregulated (Figure S7B, S7C), and 41 genes differentially regulated in opposite direction (Figure S7D). We validated several of the common DEGs across the three genotypes involved in synaptic and neurotransmission functions, including *NRXN3*, *SYN3*, *NEUROG2*, *POU3F3*, *GRIN3B*, and *WNT3A* by qPCR, and found similar directional changes of these transcripts with our transcriptomic dataset (Figure S8).

Transcriptional profiling reveals convergent and divergent enriched pathways across genotypes

ORA GO analysis identified shared pathways across all three genotypes vs control: upregulated extracellular matrix (ECM) organization (*FMR1KO* vs control adj. $p=6.33\times 10^{-19}$; *TSC2KO* vs control adj. $p=3.32\times 10^{-35}$; dKO adj. $p=3.13\times 10^{-24}$) and endoplasmic reticulum (ER) stress pathways (*FMR1KO* vs control adj. $p=2.69\times 10^{-11}$, *TSC2KO* vs control adj. $p=4.61\times 10^{-13}$, dKO vs control adj. $p=2.69\times 10^{-11}$), and downregulated synaptic function and neurotransmission pathways (*FMR1KO* vs control adj. $p=5.97\times 10^{-22}$; *TSC2KO* vs control adj. $p=5.66\times 10^{-34}$; dKO vs control adj. $p=2.95\times 10^{-34}$) (Figure 2B, Supplementary Table 2). In contrast, translation-related pathways were in the opposite direction; upregulated in *FMR1KO* and downregulated in *TSC2KO* and dKO neurons. We compared these transcriptomic pathway findings with those from an additional *FMR1KO* and *TSC2KO* neurons from previously generated RNAseq data from different clones and observed consistent trends and pathway enrichment (Figure S9).

Functional assessment of global protein synthesis and network connectivity

Given the divergent translational pathways in transcriptomic profiling in *TSC2KO*, *dKO* versus *FMR1KO* neurons, we examined global protein synthesis. Protein synthesis was significantly elevated in *TSC2KO* and *dKO* neurons, with an increased trend in *FMR1KO* without statistical significance (Figure S10). Due to synaptic enrichment found in *dKO* neuron transcriptomics, we evaluated their network connectivity using a microelectrode array. The *dKO* neurons demonstrated significantly increased firing activity (number of spikes and weighted mean firing rate) compared to control, demonstrating a hyperexcitable network activity (Figure S11).

Divergent ERK and mTOR pathway activation in *FMR1KO*, *TSC2KO*, and *dKO* neurons

ERK and mTOR control synaptic protein synthesis in FXS and TSC, contributing to abnormal neurodevelopmental signalling. We profiled the expression of key ERK- and mTOR-related genes (Figure S12A). *FMR1KO* vs control neurons showed upregulation of ERK-associated genes (*MAPK1*, *MAPK3*, *RAF*) (Figure S12B), whereas *TSC2KO* and *dKO* vs control neurons exhibited upregulation of mTOR-related genes (*EIF4EBP1*, *RPS6KA1*, *RPTOR*). *FMR1KO* appears to favour ERK hyperactivation, whereas *TSC2KO*, alone or in context of *dKO*, predominantly drives mTOR hyperactivation.

Dysregulated FMRP targets and ASD risk gene expression across *FMR1KO*, *TSC2KO*, and *dKO* neurons

FMRP regulates translation of numerous brain mRNAs, defined as its targets. We intersected 571 common DEGs across all three genotypes vs control with 842 established FMRP target genes(12, 15), identifying 28 dysregulated FMRP targets (Figure 2C, Supplementary Table 3). Common upregulated FMRP targets are involved in neurodevelopmental patterning and synaptic plasticity, while commonly downregulated FMRP targets regulate circuit excitability, neurite growth and synaptic architecture. Notably, *TRPM3* (ion channel), *SPARCL1* (synapse-organising matrix protein), *KCNQ3* (potassium channel subunit), *GRIN2A* (NMDA receptor

subunit), and *ZFHX2* (a transcription factor expressed in differentiating neurons) showed opposite regulation in *FMR1KO* versus *TSC2KO/dKO*.

Within the 571 common DEGs across the three genotypes, 53 overlapped with ASD risk genes from SFARI database(13) (Figure S13), 40 were downregulated and enriched in synaptic and neurotransmitter pathways (GABA receptor and calcium channel genes). Homeobox transcription factors *LHX2* and *MSX2* (regional brain patterning), *TRPM3*, *ANXA1* (microglial anti-inflammatory mediator), and *SPARCL1* showed divergent trends between *FMR1KO* and *TSC2KO/dKO*.

Discussion

We identified a convergent ASD-risk molecular signature centred on synaptic function and neurotransmission across *FMR1KO* and *TSC2KO* neurons. However, the *FMR1/TSC2* dKO line in our human hPSC model did not reproduce the rescue effect observed in mouse models. The dKO NPCs exhibited the highest DNA damage response, likely driven by mTORC1 hyperactivation and ER stress; suggesting additive effects of *TSC2* and *FMRP* loss. The dKO neurons exhibited a transcriptomic profile influenced by *TSC2KO*, with persistent translational, synaptic, and stress-related abnormalities. The dKO neurons showed increased global protein synthesis, hyperexcitable network activity, mTOR hyperactivation, dysregulated *FMRP* targets and ASD gene expression (proposed mechanistic pathway Figure S14).

Transcriptomic profiling across all three genotypes revealed convergent upregulation of ECM and ER stress pathways, and downregulation of synaptic function and neurotransmission, consistent with previous FXS/TSC neuronal studies(9, 16, 17). We found divergent translation regulation between FXS and TSC, also observed in other studies(18, 19). Global protein translation was significantly elevated in *TSC2KO* and dKO, but not in *FMR1KO*, whereas translation-related pathways were downregulated in *TSC2KO* and dKO on RNA sequencing and upregulated in *FMR1KO* neurons. This discordance may reflect chronic mTORC1 activation in *TSC2KO/dKO* driving elevated global protein-level translation while

triggering feedback suppression of translation-gene transcription(20, 21). Loss of FMRP likely removes a transcript-specific translational repressor with lesser effects on mTORC1, so the transcriptional upregulation in *FMR1*KO does not translate into increased global protein synthesis(15). The dKO neurons also showed hyperexcitable network activity, closely resembling *TSC2*KO neurons in previous studies(22).

ERK and mTOR are key regulators of synaptic protein synthesis in both FXS and TSC(23). We found that *FMR1* loss preferentially drives ERK hyperactivation, while *TSC2* deficiency and dKO predominantly drives mTOR hyperactivation. Consistent with prior studies, FXS shows upregulation of both pathways due to FMRP loss, whereas TSC exhibits variable ERK activity but robust mTOR activation from loss of *TSC1/2* inhibition(23). Next, we investigated the impact of *FMR1* and *TSC2* loss on FMRP target genes. Prior research has demonstrated that loss of TSC leads to a decrease in FMRP target transcripts due to increased degradation(18). In our study, we found that dysregulated FMRP and ASD targets converge on synaptic and excitatory-inhibitory regulatory (GABAergic, and calcium channel signaling) pathways but vary across genotypes. Several ASD risk genes important for brain development and cell growth, showed distinct pattern between *FMR1*KO and *TSC2*KO/dKO, suggesting that genotype-specific signatures underlie phenotypic differences between FXS and TSC.

Our results align more closely with clinical observations from the patient exhibiting dual mutations(8) than with the murine rescue model(7); however, it is important to consider the potential influence of species-, gender-, gene- (*TSC1* vs *TSC2*), and dosage-dependent effects. Importantly, dKO transcriptome reflected *TSC2* dominance with no normalization of translation as previously suggested(7), indicating that co-targeting both pathways along the protein translation axis may offer limited therapeutic benefit. Our findings indicate that therapies for syndromic ASD should not focus solely on translation regulation, as this may overlook important molecular complexities. As the interaction between mTOR and ERK pathways is critical in ASD, treatment should target their interplay and convergent downstream pathways to achieve greater efficacy.

The study's limitations include its use of a single isogenic line, with emphasis on transcriptional changes. Functional assessments, such as evaluations of synaptic or ciliary activity, are lacking. Additional validation in other cell lines including proliferation assays (cell-cycle kinetics, mitotic index), oxidative stress assays, electrophysiological, synaptic analyses, proteomics, and ribosome profiling should be considered. Because this study mainly examines early neural progenitors and immature neurons, it may not capture features of later circuit maturation.

Conclusion

We identified a convergent ASD-risk molecular signature centred on synaptic function and neurotransmission across *FMR1*KO and *TSC2*KO neurons. However, in our human hPSC model, *FMR1/TSC2* dKO neurons did not demonstrate “rescue” synaptic gene expression previously observed in mice(7). The dKO neurons, were predominantly shaped by the effects of *TSC2*KO with persistent translational, synaptic and stress-related deficits. These findings highlight the complex interplay between FMRP and mTOR in syndromic ASD.

Declarations:

Ethics approval and consent to participate

NA

Consent for publication

NA

Data availability

The RNA-sequencing has been deposited in GEO under the identifier GSE306594.

Conflict of interest

317 The authors declare no conflict of interest.

318

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323 **Author contributions**

324 Conceptualization was done by V.X.H. and K.H.U. Experiments were performed by N.A.B.M.
325 and K.H.U. Analysis was done by J.T., A.R., Y.R, N.T. and V.X.H, K.H.U. Resources were
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Figure legends

Figure 1. Generation and Characterization of Isogenic *FMR1KO*, *TSC2KO*, and *dKO* hPSC-Derived Neuronal Models

- a) Schematic overview of the experimental workflow. CRISPR/Cas9 genome editing was applied to control hPSCs to generate isogenic *FMR1KO*, *TSC2KO*, and double knockout (*dKO*) lines, and they were subsequently differentiated into neurons.
- b) Representative immunofluorescence images of differentiation of hPSCs into NPCs and neurons. NPCs were labeled with Nestin and Pax6, while neurons were marked by MAP2 (microtubule-associated protein 2) and TUJ1 (class III β -Tubulin).
- c) Immunoblot analysis confirming the absence of TSC2 protein in *TSC2KO* and *dKO* lines, and loss of FMRP in *FMR1KO* and *dKO* lines. Calnexin was used as a loading control.
- d) DNA damage response assessed by immunoblot detection of phosphorylated γ H2AX. Calnexin served as a loading control.
- e) Quantification of phosphorylated γ H2AX expression, indicating elevated DNA damage response in mutant lines (*Cohen's D* Control vs *TSC2KO* = 2.55; Control vs *FMR1KO* = 2.87; Control vs *dKO* = 9.62; *P* values: Control vs *TSC2KO* = 0.1761; Control vs *FMR1KO* = 0.0156; Control vs *dKO* = 0.0007; *** *P* < 0.001, * *P* < 0.05).
- f) Immunocytochemistry of hPSC-derived NPCs labeled with BrdU and stained for proliferation markers Ki67 and BrdU.
- g) Quantification of Ki67 and BrdU-positive cells, showing increased proliferation in *FMR1KO* and *TSC2KO* NPCs, with comparable levels in *dKO*. (*** *P* < 0.001, ** *P* < 0.01, * *P* < 0.05)

Figure 2. Transcriptomic Profiling Reveals Convergent and Divergent Signatures Across *FMR1KO*, *TSC2KO*, and *dKO* Neurons

- a) Venn diagram illustrating the number of DEGs that are shared or unique across *FMR1KO*, *TSC2KO*, and *dKO* datasets.

b) Functional enrichment analysis of DEGs visualized as a dot plot, highlighting both shared and genotype-specific dysregulated pathways. Across the 3 genotypes, there was upregulated extracellular matrix and endoplasmic reticulum stress pathways and downregulated synaptic pathways, but divergent translational, cilia organization and axoneme assembly pathways.

c) Heatmap showing expression patterns of intersected DEGs and FMRP targets(10) across all three genotypes.

377 **Supplementary Materials**

378 Figure S1: FMRP expression in TSC2KO hPSCs

379 Figure S2: mTOR hyperactivation in TSC2KO and dKO NPCs

380 Figure S3: Longitudinal CCK-8 (cell counting kit-8) measurements across the three genotypes.

381 Figure S4: RNA-seq quality control metrics across genotypes

382 Figure S5: Differentially expressed genes identified through transcriptomic analysis

383 Figure S6: TSC2 and FMR1 expression in RNA-seq dataset

384 Figure S7: Shared and distinct differentially expressed genes (DEGs) between the three
385 genotypes

386 Figure S8: qPCR validation of common differentially expressed genes (DEGs)

387 Figure S9: Overlap of gene ontology enrichment analysis across FMR1KO, TSC2KO and dKO
388 neurons and the *FMR1*KO and *TSC2*KO neuron clones

389 Figure S10: SUnSET assay assessing global translation rate via puromycin incorporation

390 Figure S11: Increased network activity in dKO neurons revealed by microelectrode array
391 recordings

392 Figure S12: Intersection of common differentially expressed genes (DEGs) with ERK and
393 mTOR pathway genes

394 Figure S13: Intersection of common differentially expressed genes (DEGs) with SFARI genes

395 Figure S14: Proposed mechanistic pathway of FMRP-TSC2 loss in human neurons

396 Supplementary Table 1: List of differentially expressed genes

397 Supplementary Table 2: List of over representation analysis gene ontology (ORA GO) terms

398 Supplementary Table 3: Overlap of dysregulated transcriptome with FMRP and SFARI genes

399 Supplemental Methods

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