Functional assessment of spontaneous and evoked activity in iPSC-derived Fragile X neurons

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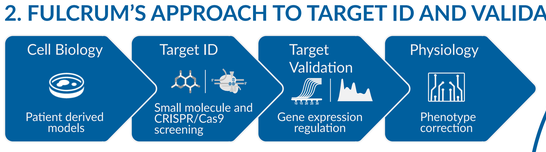
ABSTRACT

• Fragile X Syndrome (FXS) is a neurodevelopmental disorder caused by >200 repeat CGG expansion in the 5'-UTR of the Fragile X mental retardation gene *FMR1*, leading to hypermethylation and silencing of the *FMR1* transcript, thereby reducingFMRP protein .
• To date, there has been no quantitative asssessment of the relationship between varying levels of *FMR1* / FMRP expression and effects on spontaneous neuronal activity.
• We have developed patient-specific iPSC-derived neurons that retain the pathological repeat expansion, have decreased FMRP levels, and demonstrate increased spontaneous activity in in vitro neuronal networks as measured by multielectrode arrays (MEAs).
• Using CRISPR/Cas9, we have also generated a FMR1 knockout line, as well as isogenic Fragile X cell lines with truncated numbers of CGG repeats that demonstrate increased spontaneous activity in the absence of FMRP and attenuated activity when FMRP is restored to near normal levels.
• We further show through FMR1 mRNA transient transfections and titration of isogenic control neurons into a Fragile X neuronal network, that low levels of FMRP (<10%) partially attenuate FMRP-induced hyperexcitability, with higher levels of FMRP (>10%) leading to full normalization of increased levels of spontaneous activity seen in Fragile X neuronal networks.

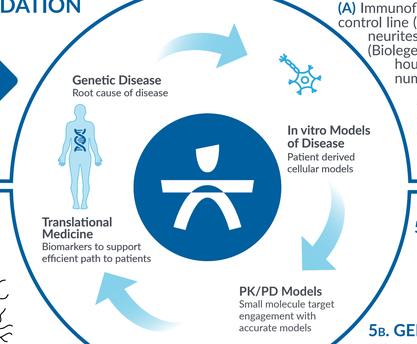
1. FRAGILE X SYNDROME (FXS)

(A) Schematic of the heterochromatin formation and silencing of FMR1 in neurons of FXS patients. **(B)** Schematic describing the downstream consequences of activation of FMR1/FMRP

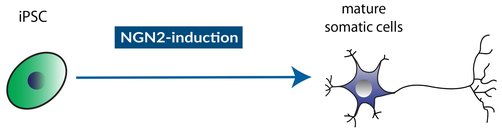
2. FULCRUM'S APPROACH TO TARGET ID AND VALIDATION



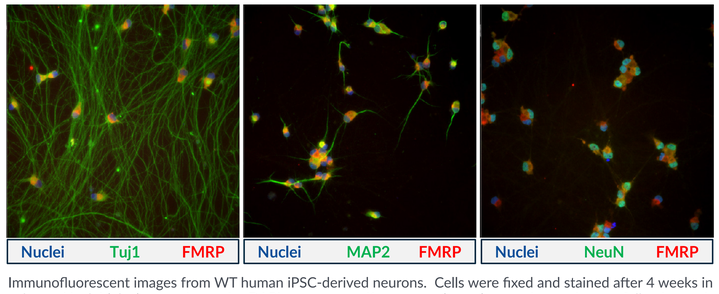
Cell-based screening drives Fulrum's target identification engine



3. HUMAN IPSC-DERIVED NEURONS

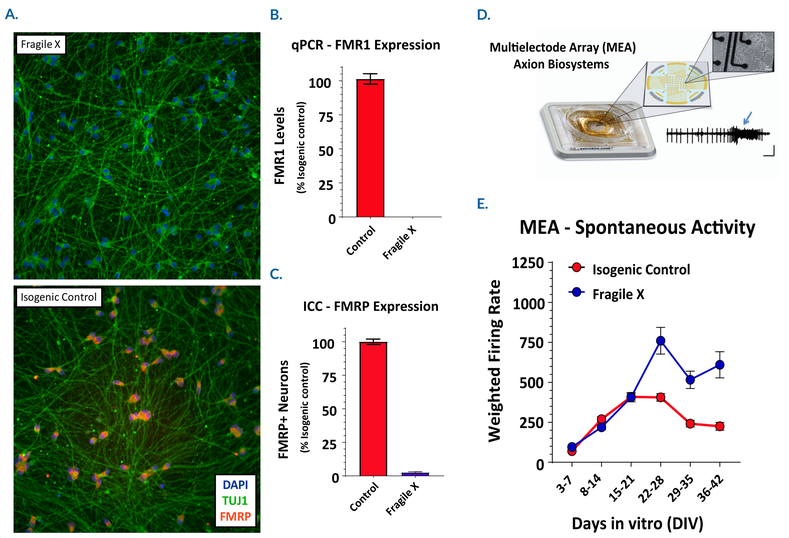


Patient-specific iPS cells are differentiated into excitatory glutamatergic neurons using a modified protocol adapted from Zhang et al. (2013), "Rapid Single-Step Induction of Functional Neurons from Human Pluripotent Stem Cells", *Neuron*.



Immunofluorescent images from WT human iPSC-derived neurons. Cells were fixed and stained after 4 weeks in culture. Nuclei (blue) are stained with DAPI, neuron-specific markers (green) are stained using the following antibodies: **(A)** Tuj1, Aves, TUJ89947984; **(B)** MAP2, Aves, MAP717984; and **(C)** NeuN, Abcam, ab177487. FMRP (red) is stained using the 5C2 antibody from Biolegend. As can be seen from the images, FMRP can only be detected in the soma and not in the neurites.

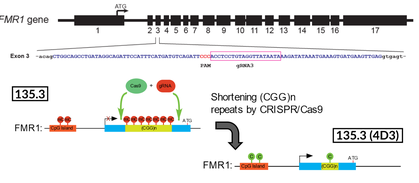
4. FXS NEURONS HAVE REDUCED *FMR1*/FMRP AND INCREASED ACTIVITY

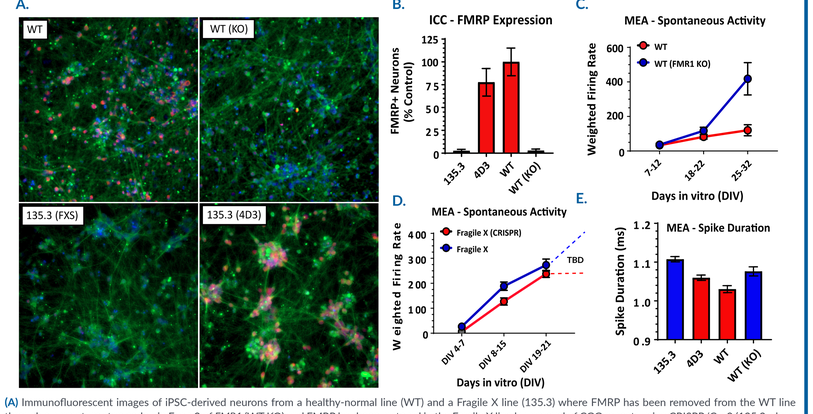


(A) Immunofluorescent images of iPSC-derived neurons from a Fragile X cell line (SW_FXS) and an isogenic control line (SW_C1_2). Neurons were fixed 4 weeks post-induction. Nuclei (blue) are stained using DAPI, neurites (green) are stained with a Tuj1 antibody (Aves), and FMRP (red) is stained using a FMRP antibody (Biolegend, 5C2). Quantification of *FMR1* mRNA levels are shown in panel **B.** Ct values are normalized housekeeping gene POP4, then normalized to the mean of the control values. Quantification of the number of FMRP+ neurons are shown in panel **C** and normalized to the mean of the control values. Example image of a multielectrode array with an image of neurons plated directly onto the electrode array along with an example recording of spontaneous activity. **(E)** Quantification of the weighted firing rate (average spike rate within a well multiplied by the number of active electrodes in the well) for both Fragile X and isogenic control neurons over a period of 6 weeks. As can be seen from the graph, Fragile X neurons begin exhibiting hyperactivity starting 3 weeks post differentiation. Data represented as mean +/- SEM.

5A. GENERATION OF FMRP KO NEURONS

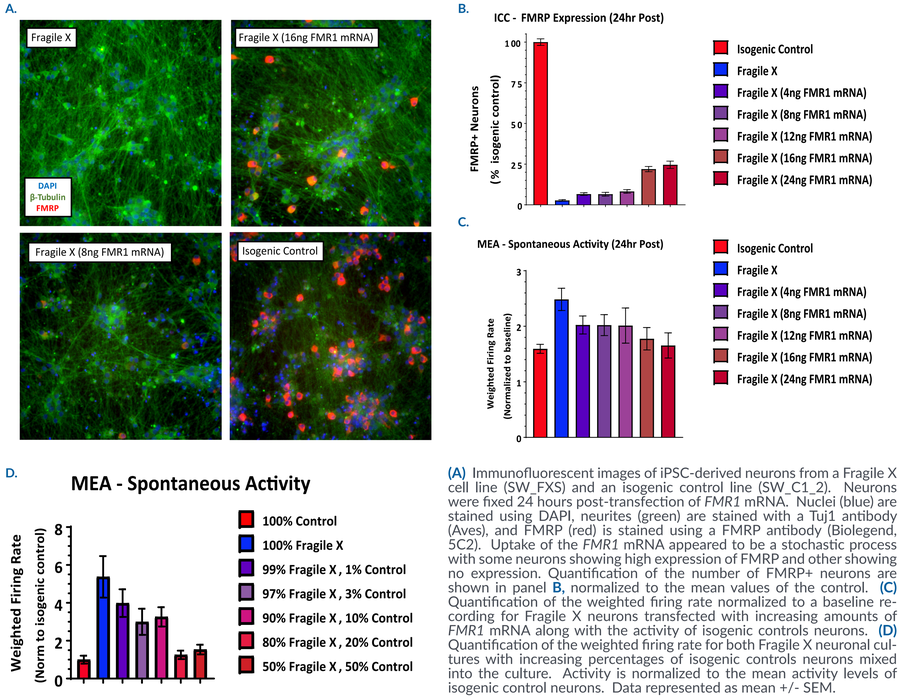
5B. GENERATION OF CRISPR-CORRECTED FXS NEURONS





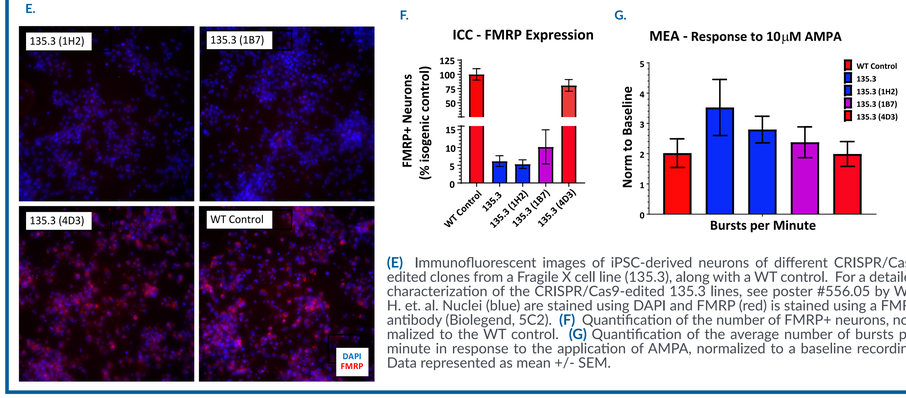
(A) Immunofluorescent images of iPSC-derived neurons from a healthy-normal line (WT) and a Fragile X line (135.3) where FMRP has been removed from the WT line through a premature stop codon in Exon 3 of *FMR1* (WT KO) and FMRP has been restored in the Fragile X line by removal of CGG repeats using CRISPR/Cas9 (135.3, clone 4D3). Quantification of the number of FMRP+ neurons are shown in panel **B. (C)** Quantification of spontaneous activity for WT and KO neurons over 4 weeks in culture showing increased activity in neurons lacking FMRP. **(D)** Quantification of spontaneous activity for both Fragile X and CRISPR-corrected neurons over 3 weeks in culture showing decreased activity in neurons re-expressing FMRP. **(E)** Average spike duration of spontaneous spikes for all four lines. Data represented as mean +/- SEM.

6. HOW MUCH FMRP IS NEEDED TO NORMALIZE ACTIVITY?



(A) Immunofluorescent images of iPSC-derived neurons from a Fragile X cell line (SW_FXS) and an isogenic control line (SW_C1_2). Neurons were fixed 24 hours post-transfection of *FMR1* mRNA. Nuclei (blue) are stained using DAPI, neurites (green) are stained with a Tuj1 antibody (Aves), and FMRP (red) is stained using a FMRP antibody (Biolegend, 5C2). Uptake of the *FMR1* mRNA appeared to be a stochastic process with some neurons showing high expression of FMRP and other showing no expression. Quantification of the number of FMRP+ neurons are shown in panel **B,** normalized to the mean values of the control. **(C)** Quantification of the weighted firing rate normalized to a baseline recording for Fragile X neurons transfected with increasing amounts of *FMR1* mRNA along with the activity of isogenic controls neurons. **(D)** Quantification of the weighted firing rate for both Fragile X neuronal cultures with increasing percentages of isogenic controls neurons mixed into the culture. Activity is normalized to the mean activity levels of isogenic control neurons. Data represented as mean +/- SEM.

DIFFERENT CRISPR/CAS9-EDITED CLONES RESULT IN DIFFERENT FMRP LEVELS



(E) Immunofluorescent images of iPSC-derived neurons of different CRISPR/Cas9 edited clones from a Fragile X cell line (135.3), along with a WT control. For a detailed characterization of the CRISPR/Cas9-edited 135.3 lines, see poster #556.05 by Wu, H. et. al. Nuclei (blue) are stained using DAPI and FMRP (red) is stained using a FMRP antibody (Biolegend, 5C2). **(F)** Quantification of the number of FMRP+ neurons, normalized to the WT control. **(G)** Quantification of the average number of bursts per minute in response to the application of AMPA, normalized to a baseline recording. Data represented as mean +/- SEM.

CONCLUSIONS

• We have developed patient-specific iPSC-derived neurons that retain the pathological repeat expansion, have decreased FMRP levels, and demonstrate increased spontaneous activity in *in vitro* neural networks as measured by multielectrode arrays.
• Using CRISPR/Cas9, we have also generated an *FMR1* knockout line, as well as a Fragile X cell line with a truncated number of CGG repeats that demonstrates increased spontaneous activity in the absence of FMRP and attenuated activity when FMRP is restored to near normal levels.
• We further show through FMR1 mRNA transient transfections and titration of isogenic control neurons into a Fragile X neuronal network, that low levels of FMRP (<10%) partially attenuate FMRP-induced hyperexcitability, with higher levels of FMRP (>10%) leading to full normalization of increased levels of spontaneous activity seen in Fragile X neuronal networks.