**Abstract**

- FSHD is caused by the loss of repression at the D4Z4 locus leading to aberrant DUX4 expression in skeletal muscle, activation of its early embryo transcriptional program and muscle fiber death.
- While some progress toward understanding the signals driving DUX4 expression has been made, the factors and pathways involved in the transcriptional activation of this gene remain largely unknown.
- Using optimized myotube culture conditions, we identified p38 MAPK as a key regulator of DUX4 expression.
- RNA-seq studies revealed that only a small number of genes were differentially expressed after treatment with losmapimod, ~90% of these are targets of DUX4.
- Fulcrum Therapeutics has selected losmapimod, a specific p38α/β inhibitor, for clinical development.

**2. Fulcrum’s approach to target identification and validation**

(A) Schematic of the loss in gene repression caused by contraction of D4Z4 repeats that leads to DUX4 expression in the muscle of FSHD1 patients.

(B) Schematic describing the downstream consequences of DUX4 expression in skeletal muscle.

**3. Identification of a drug target that inhibits DUX4 expression**

(A) Cell-based assay schematic.

(B) Expression of DUX4 and DUX4 target gene MBD3L2 during FSHD myotube differentiation in vitro. (C) DUX4 target gene selection for HTS. (D) 96-well assay format allows for identification of targets modulating the expression of DUX4. (E) Hits identified showed high correlation in between biological replicates. (F) Losmapimod reduces expression of DUX4 in a concentration dependent manner in FSHD myotubes. (G) Losmapimod reduces p38α/β activity in FSHD myotubes. (H) P38a knockout reduces activity of DUX4 in FSHD myotubes.

**4. Losmapimod reduces DUX4 activation and its downstream consequences**

(A) Losmapimod treatment does not affect in vitro differentiation of myotubes. (B) RNA-seq analysis of myotubes indicates that losmapimod selectively inhibits DUX4 and its downstream program expression in a concentration dependent manner with minimal impact across the transcriptome of FSHD myotubes. <100 differentially expressed genes (abs(FC)>4, FDR<0.001)

(C) Losmapimod reduces cleaved caspase-3 signal detected in FSHD myotubes indicating reduction of cell death.

**5. DUX4 activity and apoptosis in primary myotubes**

(A) MBD3L2 expression across a variety of primary FSHD patient- and non-FSHD (WT) derived myotubes. (B) Detection of cleaved caspase-3 in primary FSHD1 and FSHD2 patient-derived myotubes.

**6. Studying p38 inhibition across FSHD genotypes**

(A) Table describing the genotypes of cells used in this study. (B) MBD3L2 expression was reduced across all FSHD1 and FSHD2 patient-derived myotubes after losmapimod treatment. (C) Inhibition of p38αβ MAPK pathway using a structurally distinct inhibitor (FTX-2965) results in reduction of DUX4 expression and inhibition of cell death in vitro across FSHD1 and FSHD2 genotypes.

**6. Conclusions**

- Using an in vitro model of FSHD, we identify novel regulators of aberrant DUX4 expression.
- Losmapimod is a selective p38αβ inhibitor that reduces DUX4 expression in FSHD myotubes.
- Further in vitro characterization demonstrates that the DUX4-driven gene expression and cell death are inhibited in FSHD myotubes exposed to losmapimod.
- Losmapimod reduced expression of DUX4 in all FSHD1 and FSHD2 cell lines tested.
- Reduction of DUX4 expression resulted in inhibition of cell death across all genotypes tested.