Characterization of a Promising DUX4-Regulated Circulating Biomarker for FSHD

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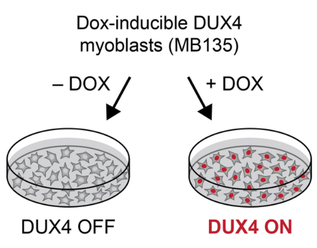
Abstract

Background: FSHD causes progressive muscle weakness, loss of muscle mass, and severe disability due to aberrant DUX4 expression in skeletal muscle. While DUX4-regulated RNAs have been measured in muscle biopsies, the procedure is invasive and only measures DUX4 activity in that specific sample of muscle. Measurement of circulating biomarkers is less invasive and allows assessment of DUX4 activity in the whole-body.

Methods: To discover candidate circulating biomarkers, we performed a mass spec screen for proteins that accumulated in the supernatant of DUX4-inducible immortalized human myoblasts (MB135iDUX4 cells). Hits with >2-fold abundance in the doxycycline-treated cell supernatant and containing putative secretion signals were prioritized for further analysis.

Results: Multiple potential circulating biomarker candidates were identified. The lead biomarker is a DUX4-regulated target found to be elevated in FSHD muscle. Its expression in lysates and supernatants of DUX4-expressing cells upon doxycycline induction was confirmed by immunoblot. Intracellular localization of this protein was also evaluated by immunofluorescence. This DUX4-regulated biomarker was elevated in plasma from individuals with FSHD compared to healthy volunteers.

Figure 1:
Model System of DUX4 Biological Activity
Human MB135 cells with dox-inducible DUX4 (iDUX4)



Identify proteins in supernatant
- Induce DUX4 expression (~4-6hr)
- Change to serum-free media
- Collect supernatant after ~6hrs, prior to cell death or significant toxicity
- Concentrate with Amicon 3kDa filter
- Protein identification by mass spec at FHCC Proteomics Core

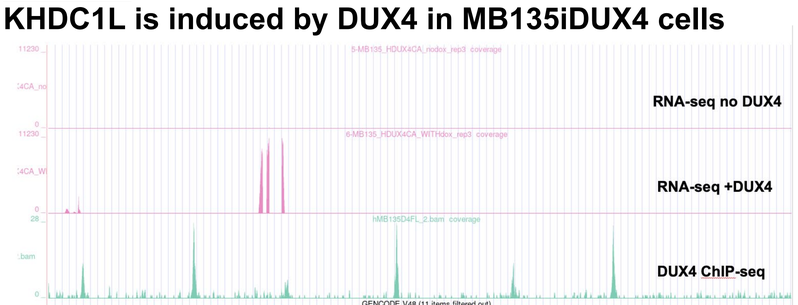
Data filtering criteria included
- DUX4-regulated
- Increased in biological duplicates
- Potentially secreted

Figure 2:
Filtered list of proteins found in DUX4-induced supernatant

Gene Name	Set1: Abundance Ratio (log2)	Set2: Abundance Ratio (log2)	GO:0005615 extracellular space	Conventional Sec. (SignalP)	Unconventional Sec. (SecretomeP)
Ephrin type-A receptor 5 (EPHA5)	**2.58***	2.96			
Putative KHDC1-like protein (KHDC1L)	**2.46***	**5.05***			✓
epidermal growth factor receptor (EGFR)	**2.14***	**4.88***	✓	✓	
Connective tissue growth factor (CTGF)	**2.02***	1.58	✓	✓	
Tumor necrosis factor receptor superfamily member 16 (NGFR)	**1.85***	1.25		✓	
Sodium-dependent phosphate transport protein 2B (SLC34A2)	1.75	**3.08***			
Legumain (LGMN)	**1.59***	3.19	✓	✓	
Cullin-5 (CUL5)	1.54	2.29			
Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA (MAN1A1)	**1.53***	**2.53***	✓		
N-sulphoglucosamine sulphohydrolase (SGSH)	**1.38***	1.7	✓	✓	
Ubiquitin-conjugating enzyme E2 C (UBEC2C)	1.32	4.89			✓
Small nuclear ribonucleoprotein-associated proteins B and B' (SNRPB)	**1.31***	1.51	✓		
HLA class I histocompatibility antigen, B-7 alpha chain (HLA-B)	**1.28***	**5***	✓	✓	
Prosaposin (PSAP)	**1.17***	1.13	✓	✓	
Protocadherin-7 (PCDH7)	**1.14***	1.32		✓	
Protocadherin-9 (PCHD9)	**1.12***	1.94		✓	

Biological duplicates: Set1 and Set2

Figure 3:
KHDC1L is induced by DUX4 in MB135iDUX4 cells



KHDC1L Background
- Member of the KHDC1 family of RNA binding proteins
  - Family with atypical RNA binding KH domain
- KHDC1 family arose in placental mammals
- Mouse KHDC1A localized in the endoplasmic reticulum
- KHDC1L is directly regulated by DUX4
  - RNA is a marker of disease activity in FSHD muscle biopsy

Figure 4:
KHDC1L Expression Profile (Human Protein Atlas)
Not widely expressed in healthy adult somatic tissue

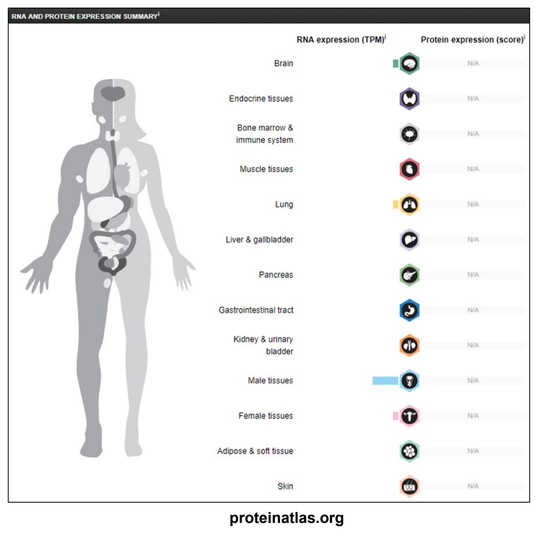
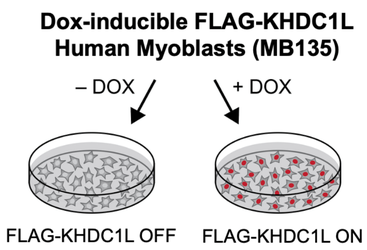


Figure 5:
Is FLAG-KHDC1L secreted into cell supernatants?
Human MB135 cells with dox-inducible FLAG-KHDC1L



- Induce FLAG-KHDC1L with doxycycline
- Collect supernatants after ~18hrs
  - No DUX4 expression
  - No toxicity
- Immunoblot with anti-FLAG antibody

Figure 6:
Induced FLAG-KHDC1L accumulates in MB135 supernatant

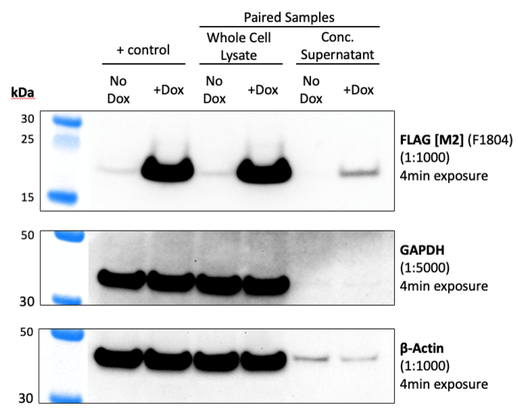


Figure 7:
Generation of mouse monoclonal antibodies to KHDC1L
FHCC Antibody Core

Figure 8:
Antibodies to N- and C-terminus of KHDC1L

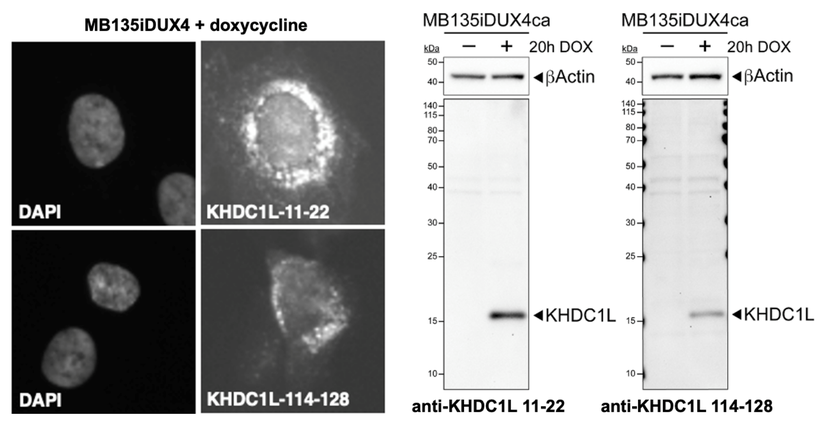


Figure 9:
Confirmation that KHDC1L is in the supernatant of DUX4-expressing muscle cells
MB135iDUX4 myoblasts ~12hrs after induction

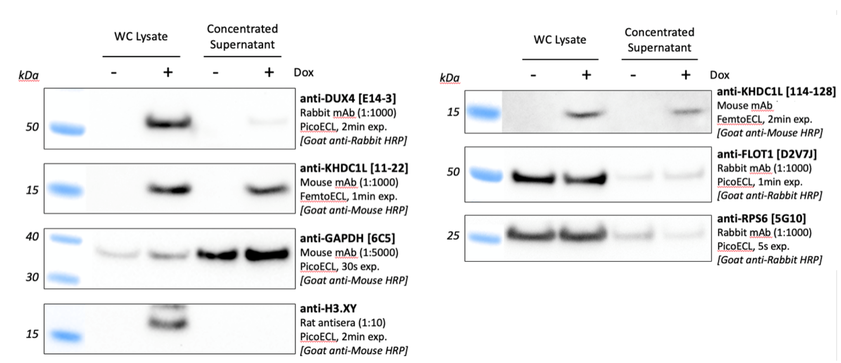


Figure 10:
Preliminary sandwich MSD assay for KHDC1L

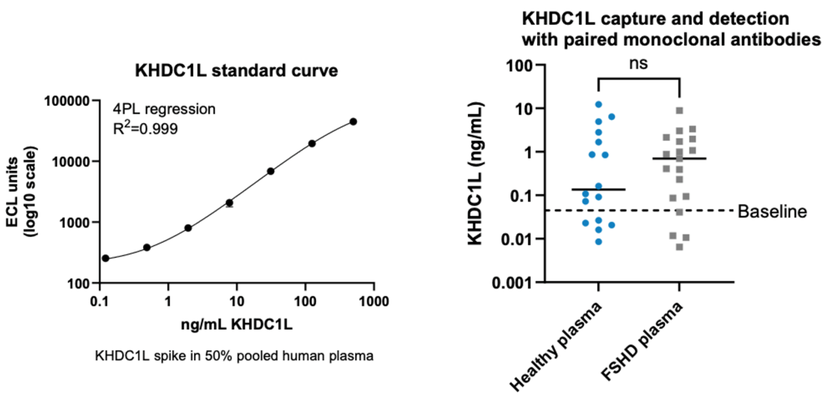


Figure 11:
Significant upregulation of KHDC1L was detected in FSHD vs. healthy plasma in a ligand-binding assay

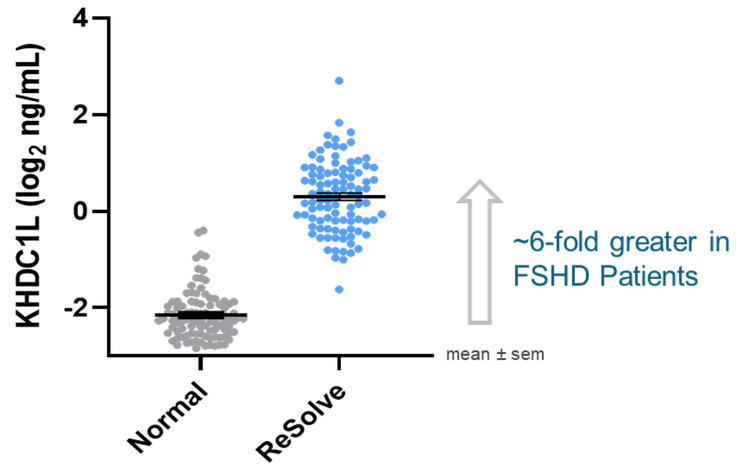
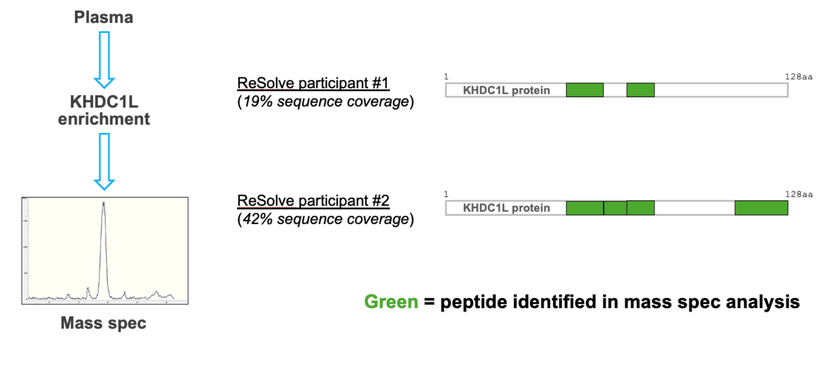


Figure 12:
Orthogonal enrichment/mass spec assay confirms KHDC1L upregulation in FSHD plasma



Conclusions
- KHDC1L is induced by DUX4 in MB135iDUX4 cells and secreted into tissue culture supernatant
- Tool antibodies against KHDC1L confirm induction by DUX4 and suggest cytoplasmic/vesicular localization
- Multiple orthogonal assays demonstrate KHDC1L is upregulated in FSHD plasma compared to healthy plasma
- Plasma KHDC1L could be used to better understand disease biology and support the development of therapies targeting the root cause of FSHD

Acknowledgements
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