FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY (FSHD)

AN (EPI-)GENETIC DISEASE

THE ROLE OF THE lncRNA DBE-T IN FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY (FSHD)

FSHD: http://www.omim.org/entry/158900

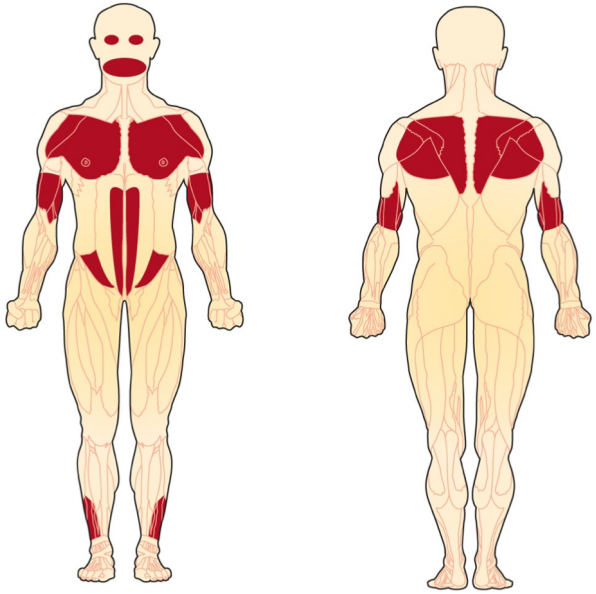
Facioscapulohumeral muscular dystrophy (FSHD) is a genetic muscle disorder that starts in the second decade. Frequency 1:200.000

Characterized by progressive muscle weakness
Initially: facial , scapular and humeral muscles
Later: abdominal muscles and muscles of the lower limb and feet, with time formation of asymmetric body posture

The long name comes from facies, the Latin word and medical term for face; scapula, the Latin word and anatomical term for shoulder blade; and humerus, the Latin word for upper arm and the anatomical term for the bone that goes from the shoulder to the elbow.

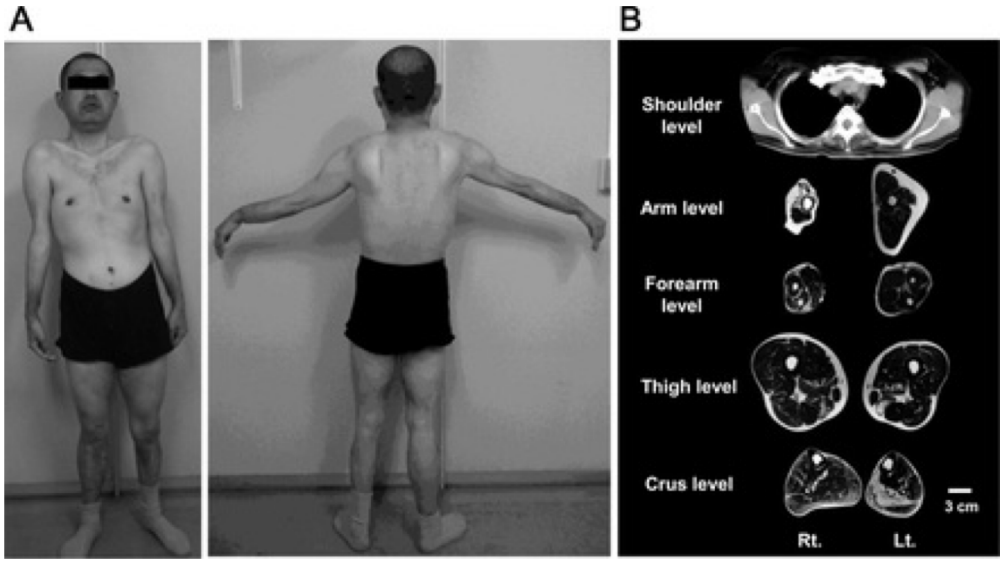
The term muscular dystrophy means progressive muscle degeneration, with increasing weakness and atrophy (loss of bulk) of muscles. In FSHD, weakness first and most seriously affects the face, shoulders and upper arms, but the disease usually also causes weakness in other muscles.

Muscles affected by wasting in FSHD patients



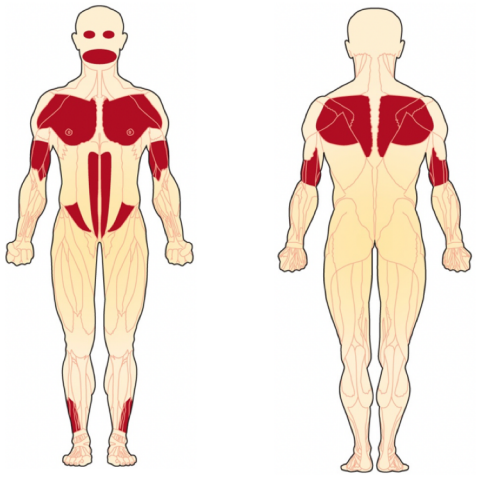
Genetic alteration of repeats number:
Aberrant expression of genes in vicinity to subtelomeric D4Z4 repeats, including DUX4, ANT1, FRG1, FRG2 in FSHD patients are thought to mediate the syndrome (have a “toxic” effect**)**
Mechanism of altered gene expression?

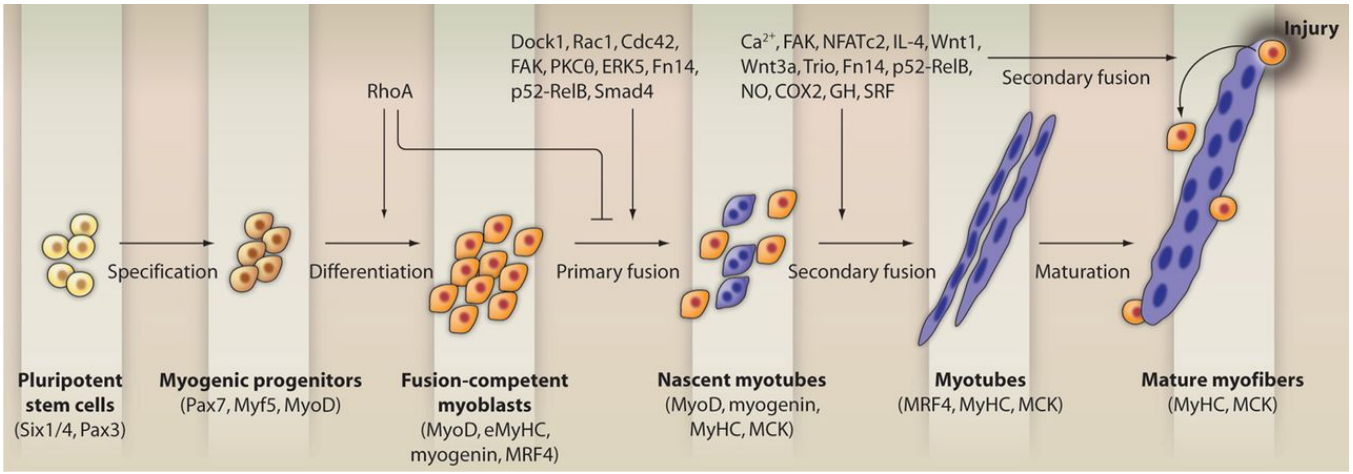
Genetic disease: variable amongst family members

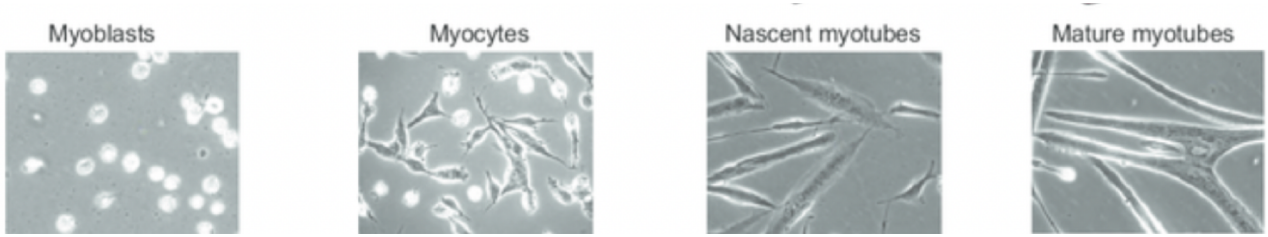


Myotube differentiation from myoblasts: a powefull in vitro model of muscle differentiation

FSHD: Analyse muscle cell differentation of patient myoblasts to myotubes







Muscle fibers generally form through the fusion of precursor **myoblasts** into multinucleated fibers called **myotubes**.

IN VIVO: In the early development of an embryo, myoblasts can either proliferate, or differentiate into a myotube

IN VITRO: If placed in cell culture, most **myoblasts** will proliferate if enough fibroblast growth factor (FGF) is present in the medium. When the growth factor runs out, the myoblasts cease division and undergo terminal differentiation into myotubes containing multiple nuclei per cell.

Myoblast differentiation proceeds in stages chacaerized by defined gene expression programs

FSDH impairs muscle cell function

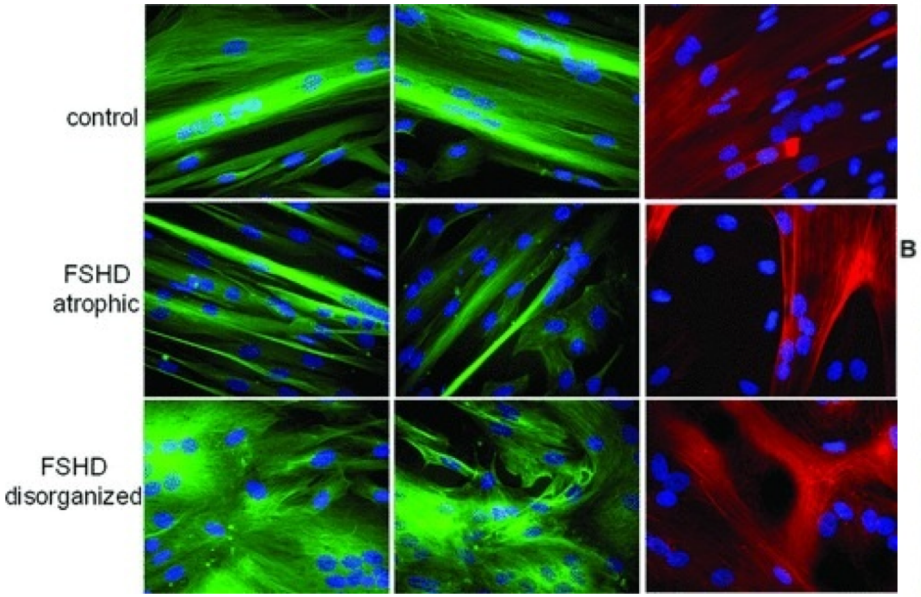
In patients affected with FSHD, it is quite common to observe the co-existence of affected and apparently healthy muscles.

Myoblasts, which were obtained from muscle typically affected in FSHD, manifested an increased susceptibility to oxidative stress during proliferation.

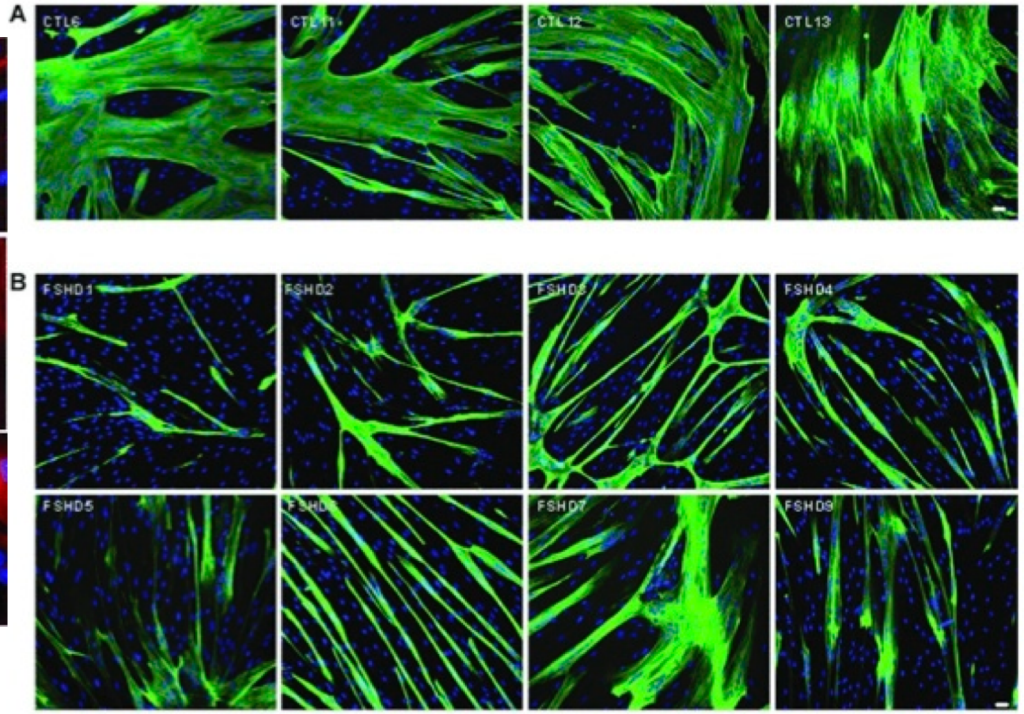
Myotubes obtained from patient myblasts show abnormal morpholgy and muscle marker expression → differentiation defect

Myoblasts = muscle cell progenitors that proliferate and fuse to form myotubes

Anti actin/tubulin immunostainingof myotubes obtained by differentiating myblasts cells isolated from healthy or FSHD patients



anti-troponinT immunostaining of myotubes obtained by differentiating myblasts cells isolated from healthy or FSHD patients



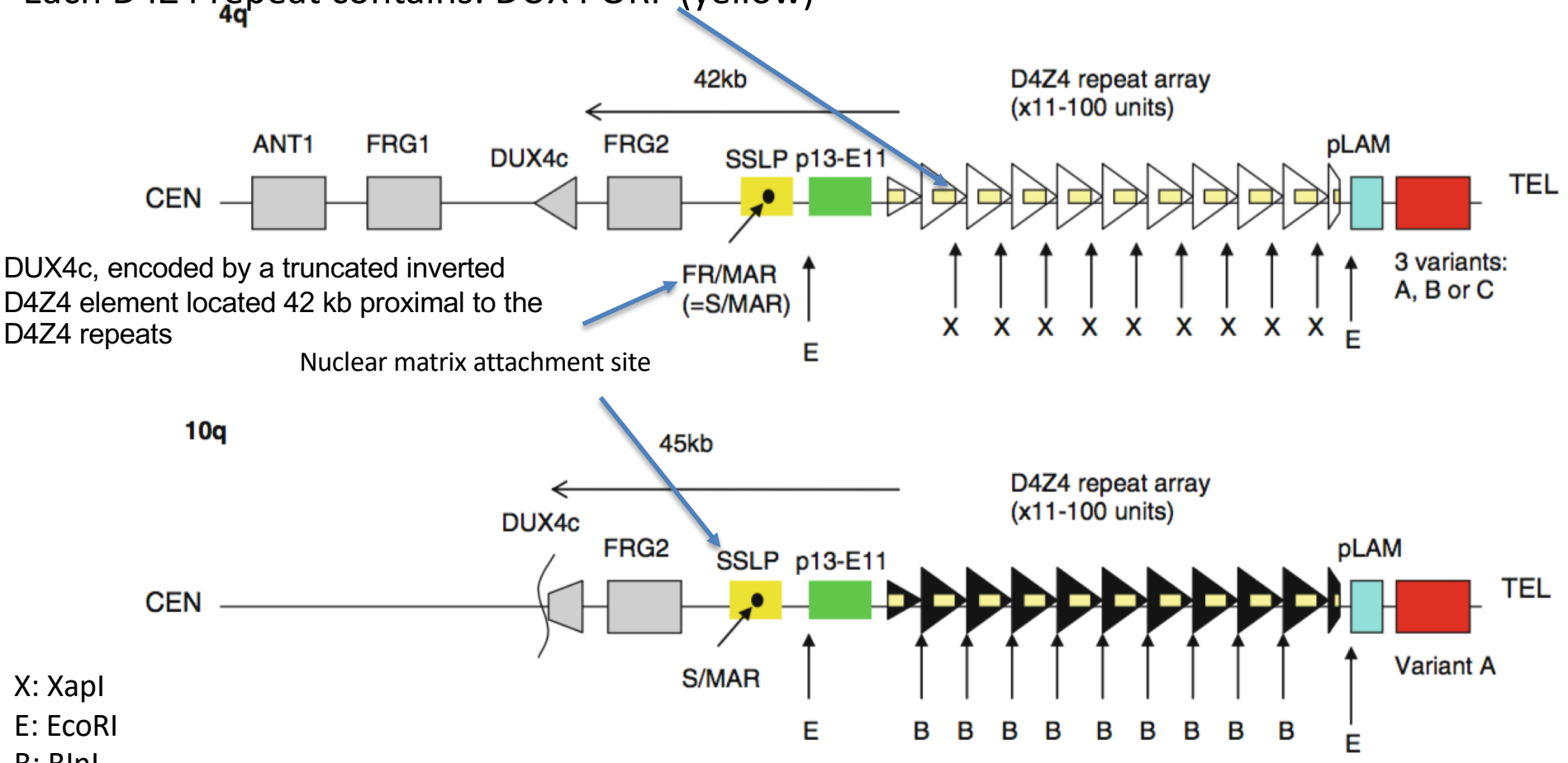
Common feature: aberrant expression of genes in vicinity to subtelomeric D4Z4 repeats, including DUX4, ANT1, FRG1, FRG2 in FSHD patients → "toxic" effect

The genetics if FSDH

FSDH is linked with aberrant D4Z4 repeat numbers at subtelomeric repeats of Chr4q

D4Z4: 3,3kb, repeats oriented head-to-tail, **11-100 repeat in healthy individuals** - polymorphic
Located on **Chr4q** → disease relevant
Located on **Chr10q** → not disease relevant (99% identical to Chr4q D4Z4 repeats);
no proximal DUX4, ANT1, FRG1, FRG2genes

Each D4Z4 repeat contains: DUX4 ORF (yellow)



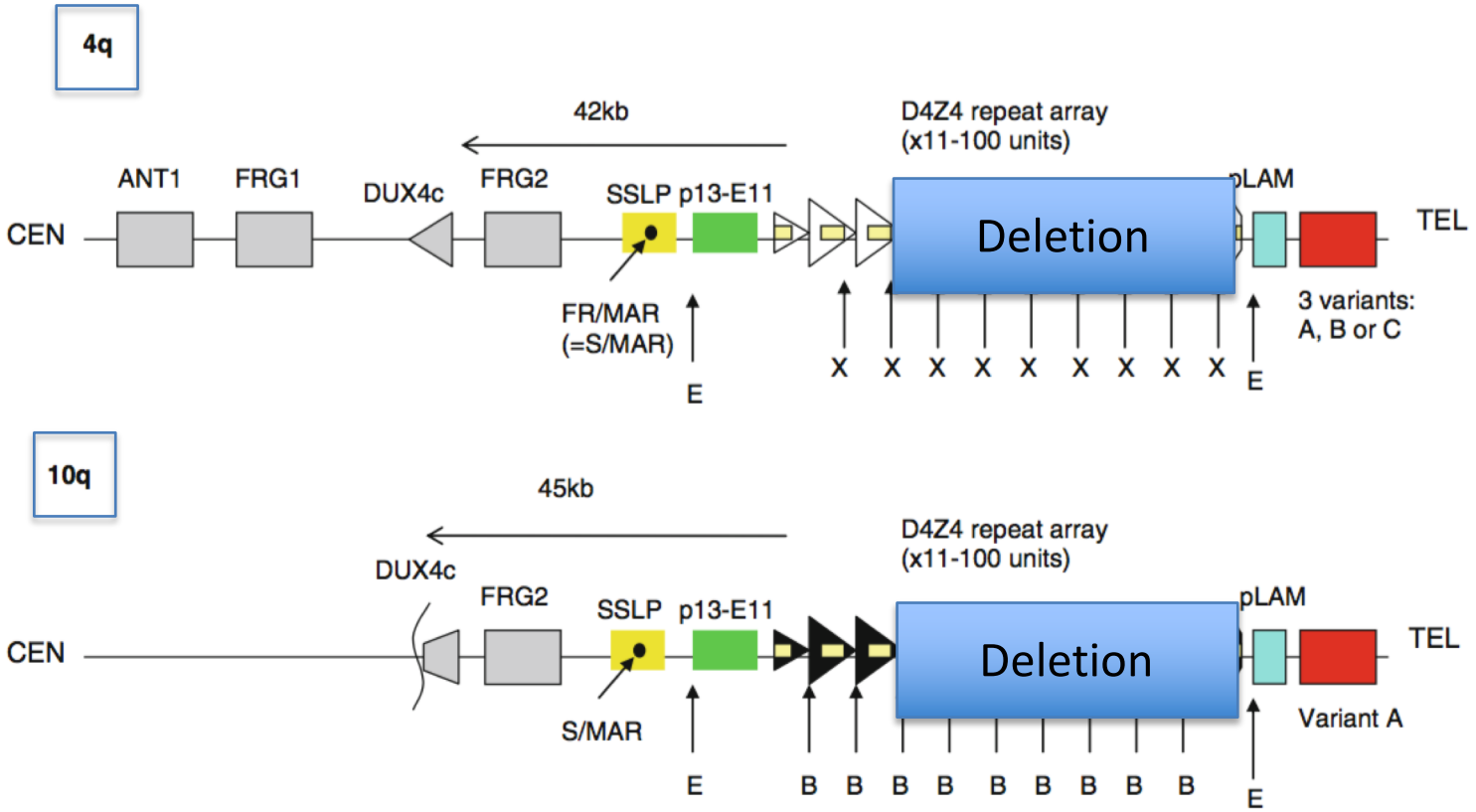
DUX4c, encoded by a truncated inverted D4Z4 element located 42 kb proximal to the D4Z4 repeats

Nuclear matrix attachment site

X: XapI
E: EcoRI
B: BlnI

The genetics of FSDH

Facioscapulohumeral muscular dystrophy-1 (FSHD1) is associated with contraction of the D4Z4 macrosatellite repeat in the subtelomeric region of **chromosome 4q35**.

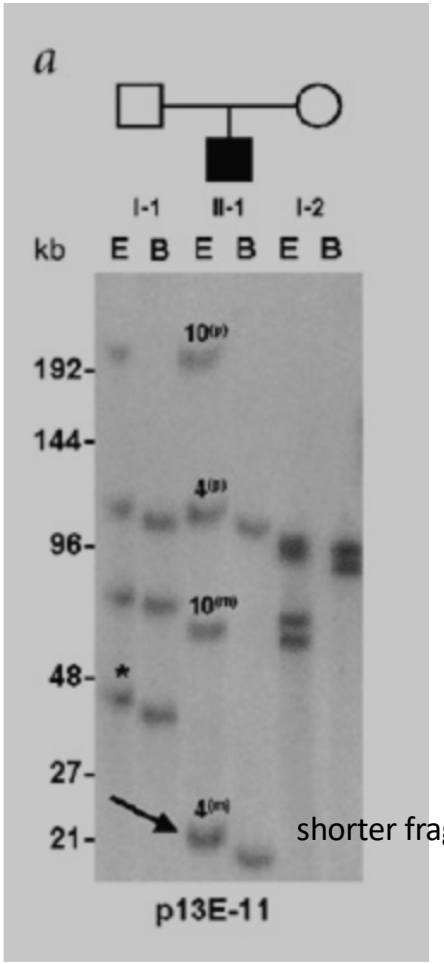


1-10 repeats:
→ FSDH

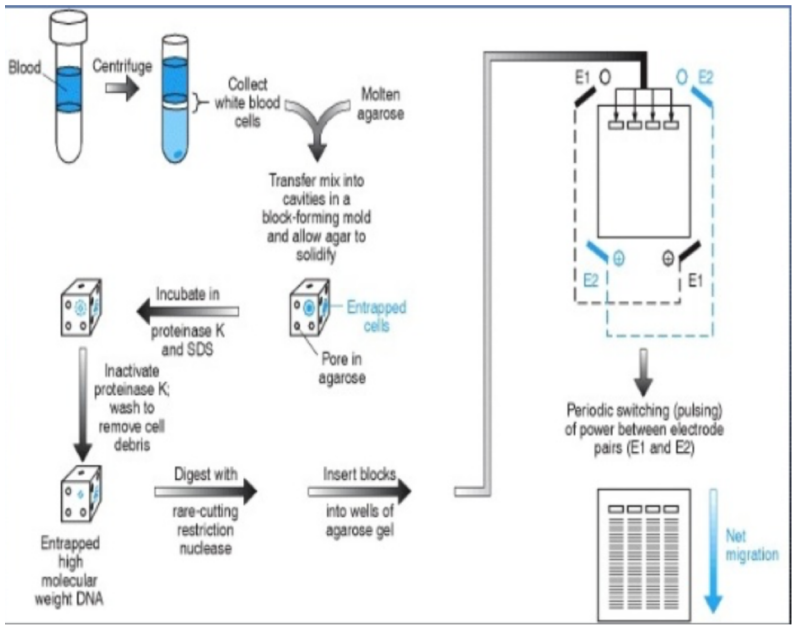
1-10 repeats:
→ NO DISEASE

The genetics of FSDH

Repeat retractions can be detected by pulsed field gel electrophoresis and southern blotting

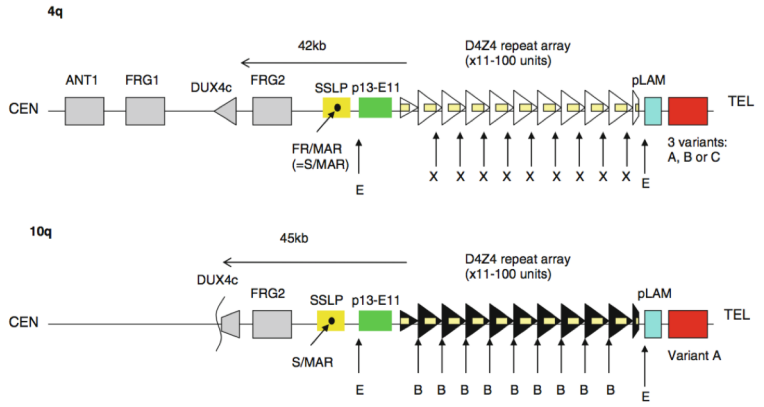


shorter fragment



Southern blot

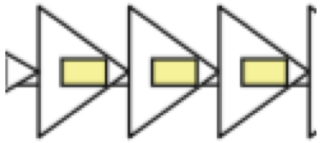
Probe: p13E-11

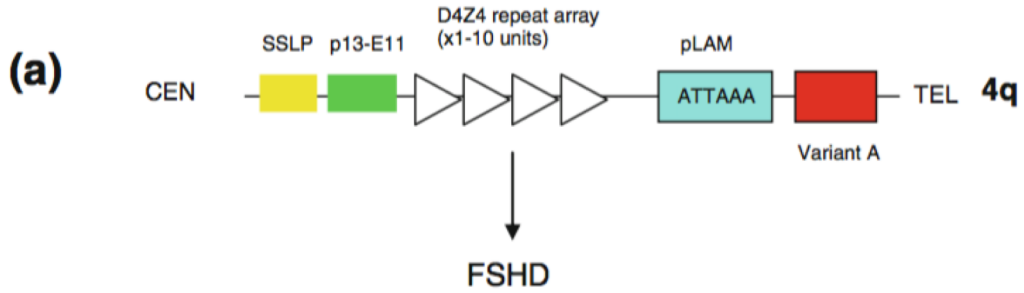


Pulsed-field gel electrophoresis (PFGE) analysis of kindred 25 affected with FSHD using probe **p13E-11**. a, DNA was digested with EcoRI and HindIII (E) and with EcoRI and BlnI (B), separated fragments by PFGE and hybridized them with p13E-11 (left panel). **A de novo fragment of 21 kb is visible for individual II-1** (arrow).

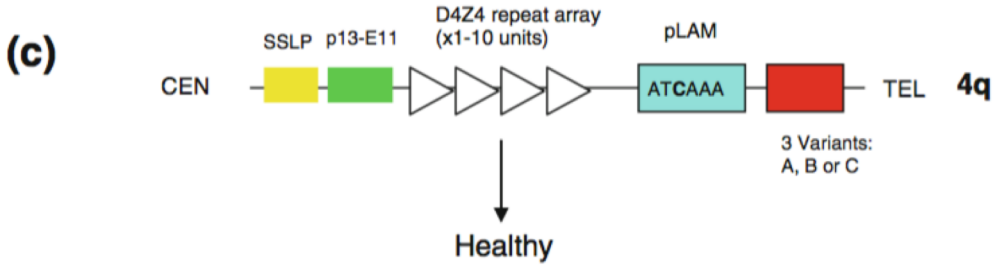
The genetics of FSDH

DUX4 ORF DUX4 ORF DUX4 ORF

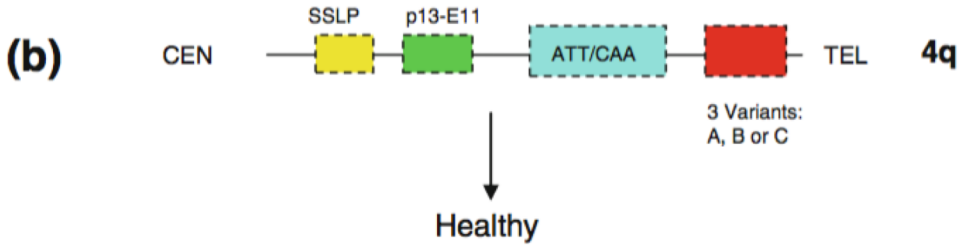




Deletion of repeats → reduction to 1-10 repeats,
SNP in pLAM box generates a Poly A site
combined with VARIANT A
→FSDH



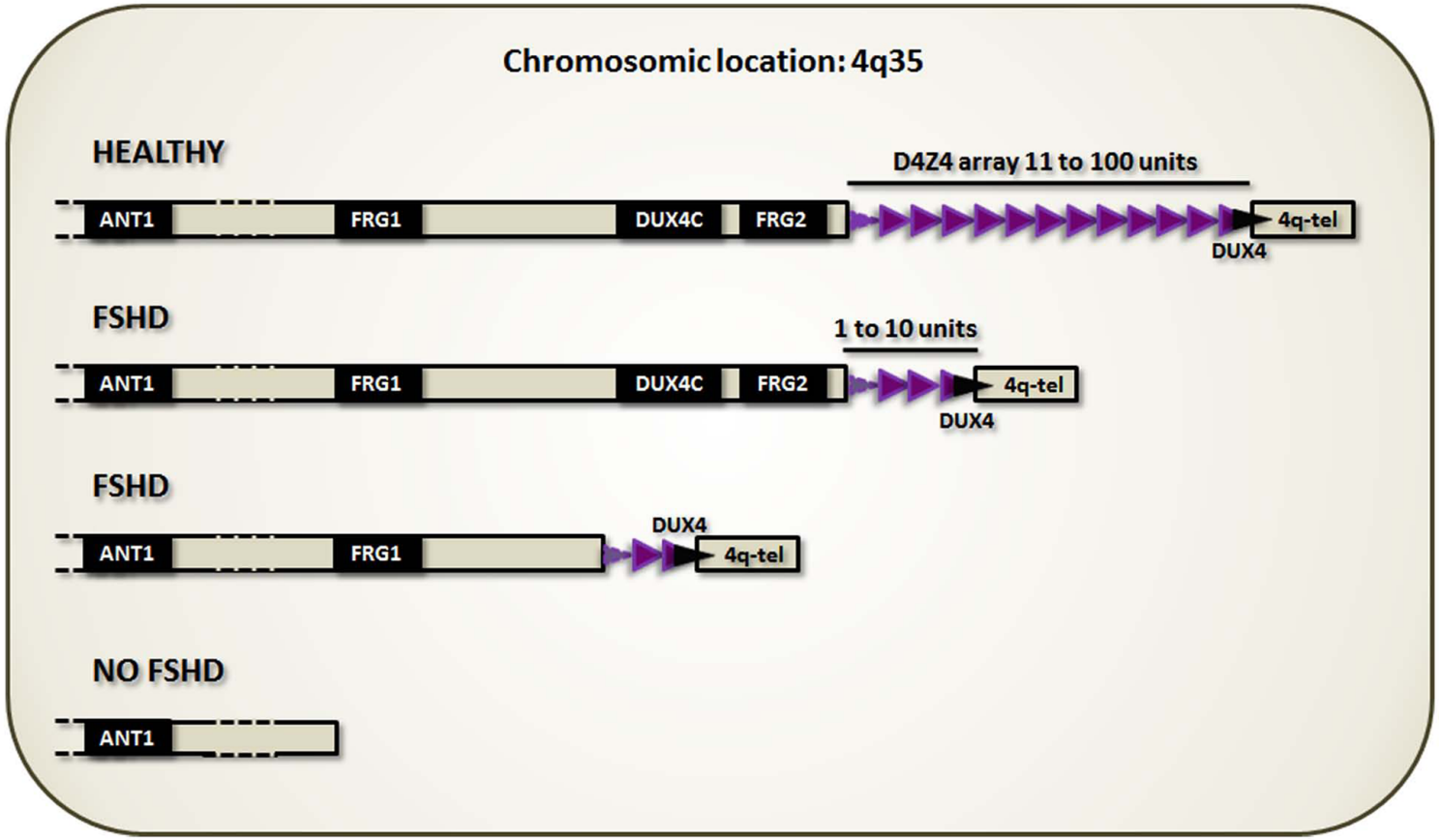
Deletion of repeats → reduction to 1-10 repeats
NO Poly-A sequence in pLAM boc
combined with VARIANT A, B, C
→no FSDH



Deletion of all repeats,
Poly-A sequence in pLAM boc
combined with VARIANT A, B, C
→no FSDH

Variant A: ATTAAA stabilizes most distal DUX4 mRNA by directling the formation of a polyA tail

The epi-genetics of FSDH



Upregulation of ANT1, 2, FRG1

Upregulation of ANT1, 2, FRG1

No DUX4 in D4Z4 repats

Observation: repeat restriction leads to upregulation of ANT1, FRG1,and FRG2 genes:

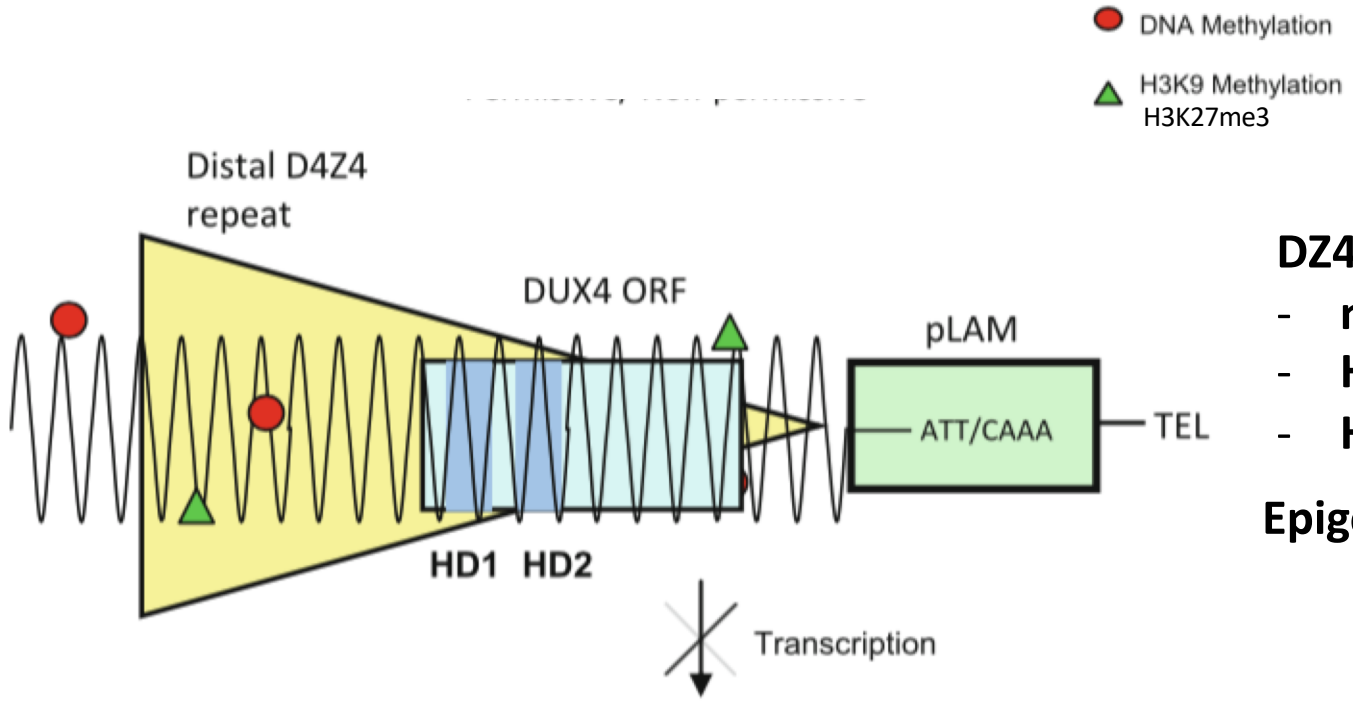
→ Events take place in heterochromatic regions in sub-telomeres
→ Epigenetic effect that allows the expression of a DUX4 mRNA and releases silencing of ANT1, ANT2, FRG2?

MISS-EXPRESSION OF DUX4 and ANT1, ANT2 and FRG2 causes "cell wasting" of muscles

The epi-genetics of FSDH

NORMAL SITUATION 11-100 DZ4Z repeat units

Figure shows most distal (close to telomere) DZ4Z unit



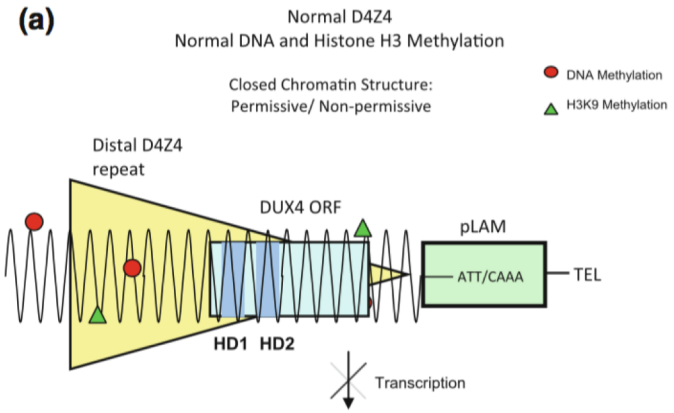
DZ4Z repeats contain

- **methylated CpG islands**
- **H3K9me3, HP1**
- **H3K27me3/Polycomb**

Epigenetic silencing of DUX4

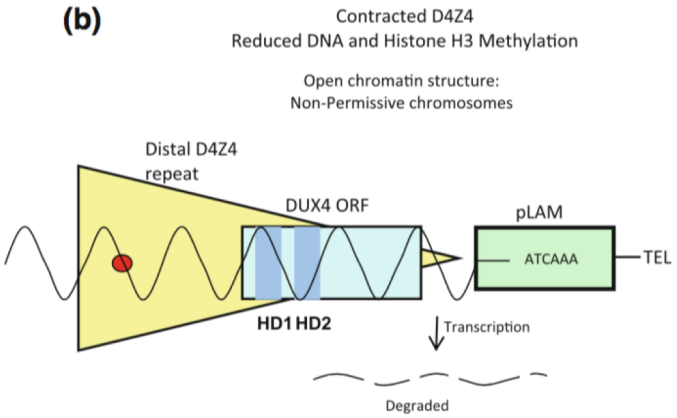
DUX4: early embryonic transcription factor, particularly in zygotic genome activation. Abnormal expression in adult somatic cells, specifically muscle cells, is the cause of FSHD. In FSHD, the DUX4 gene is inappropriately activated, leading to a cascade of toxic effects in muscle cells, including the activation of inappropriate genes, induction of apoptosis, and disruption of muscle differentiation

The epi-genetics of FSDH



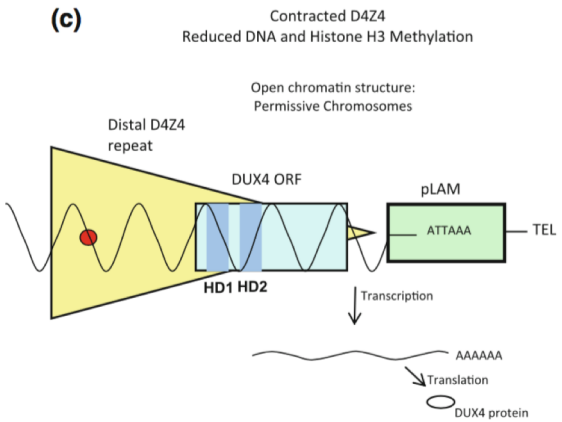
Normal situation/No disease:

H3K27me3
H3K9me3-HP1



No disease:

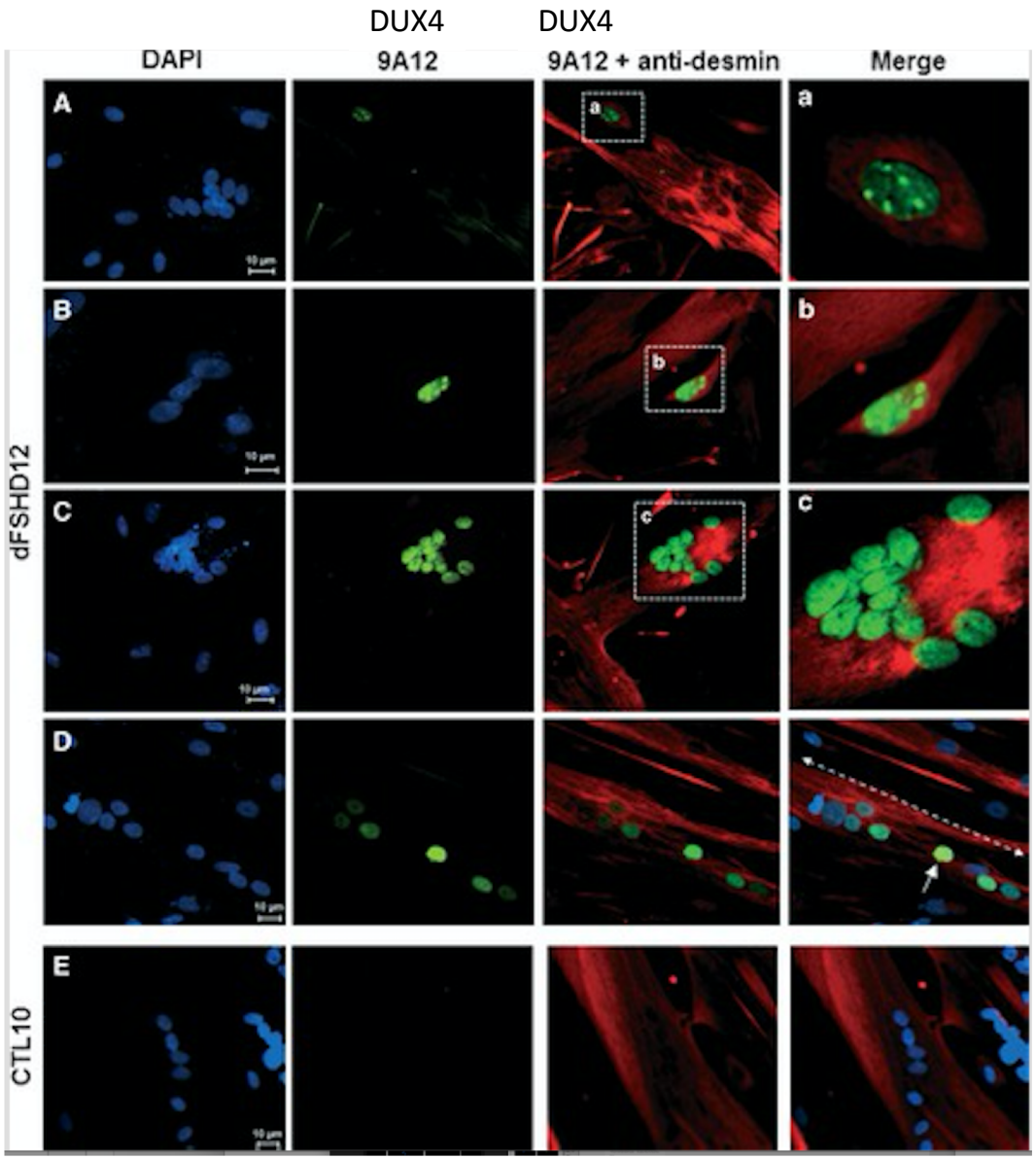
Reduced D4Z4 repeats
- No polymorphism in pLAM box
- **No poly-A site** for most distal DUX4 ORF
- RNA degradation

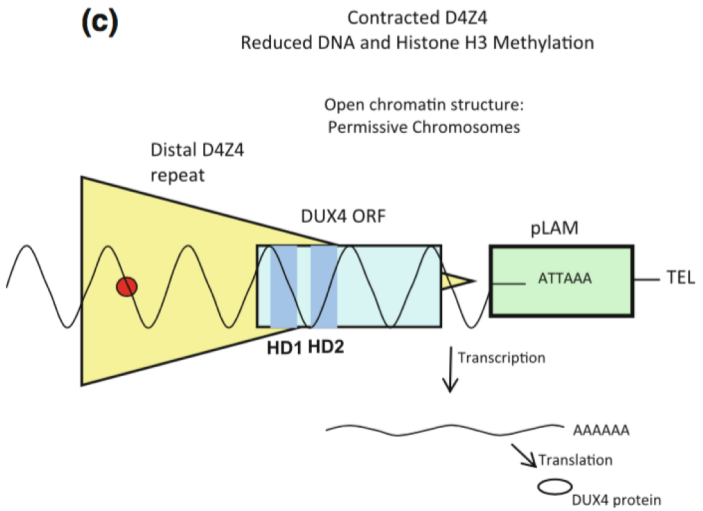


Disease:

Reduced D4Z4 repeats
- polymorphism in pLAM box
- poly-A site for most distal DUX4 ORF avalable
- DUX4 mRNA stable
- abberrant DUX4 protein expression

The epi-genetics of FSDH



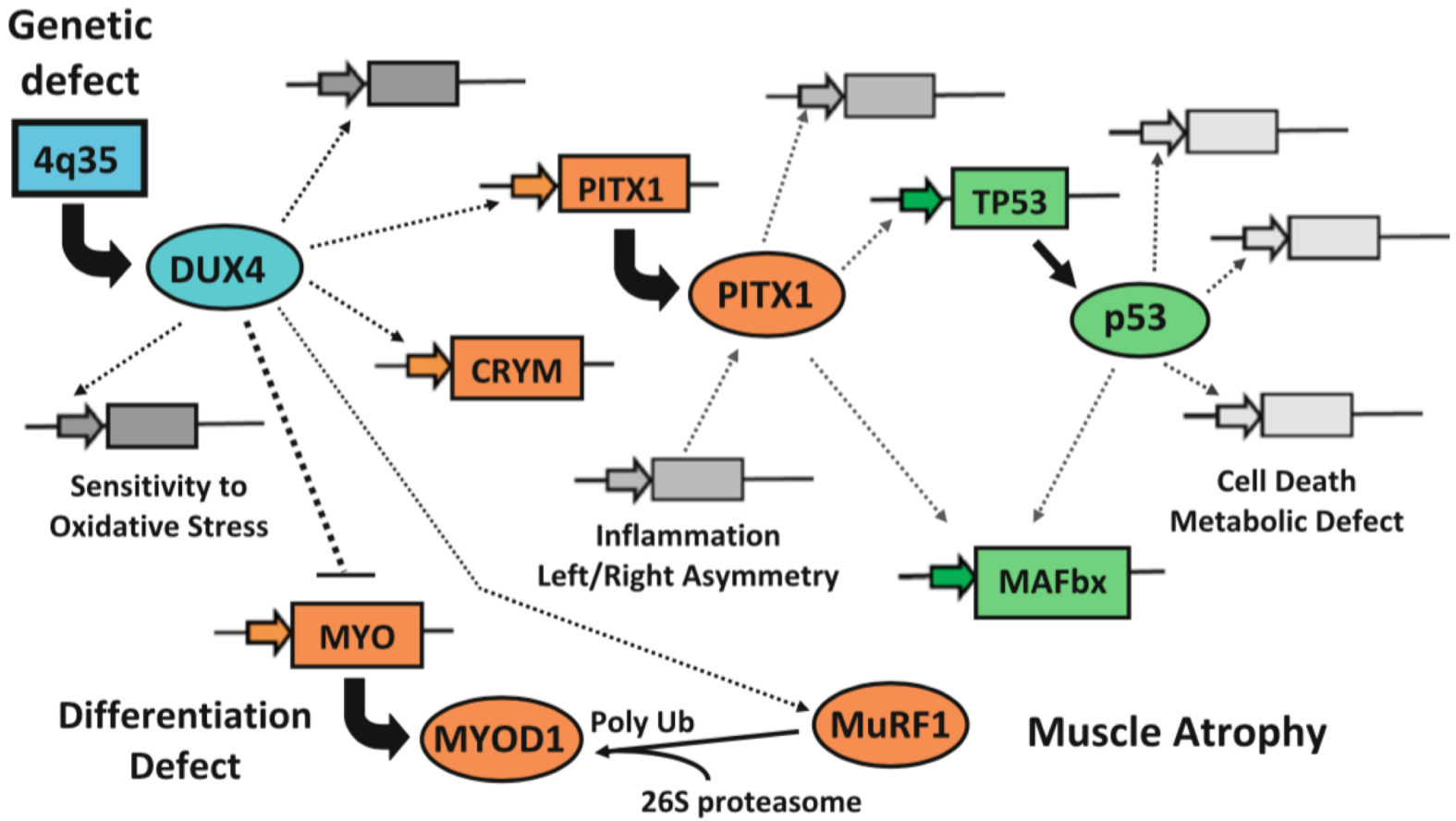


DUX4 expression:

- → **DUX4 is a TF that contains homeobox domains (HD1, HD2)**
- → **Transcription factor**
- → Interferes with muscle differentiation
- → Impairs muscle for muscle regeneration
- → wasting

DUX4 is expressed in facioscapulohumeral muscular dystrophy (FSHD) myoblasts and in consecutive nuclei in FSHD myotubes. Co-immunofluorescence with MAb 9A12 = DUX4 (green) and a rabbit serum directed against desmin = muscle marker (red) on FSHD (dFSHD12) and control (CTL10) primary myotubes, 5 days after the induction of differentiation. a, b and c correspond to enlarged fields from the left boxes. Arrows indicate the most stained nuclei and the dotted arrows the intensity gradient of the DUX4 staining (D: merge panel). DAPI (blue) was used to visualize nuclei.

Molecular Pathology: The transcriptional cascade driven by DUX4 in FSDH

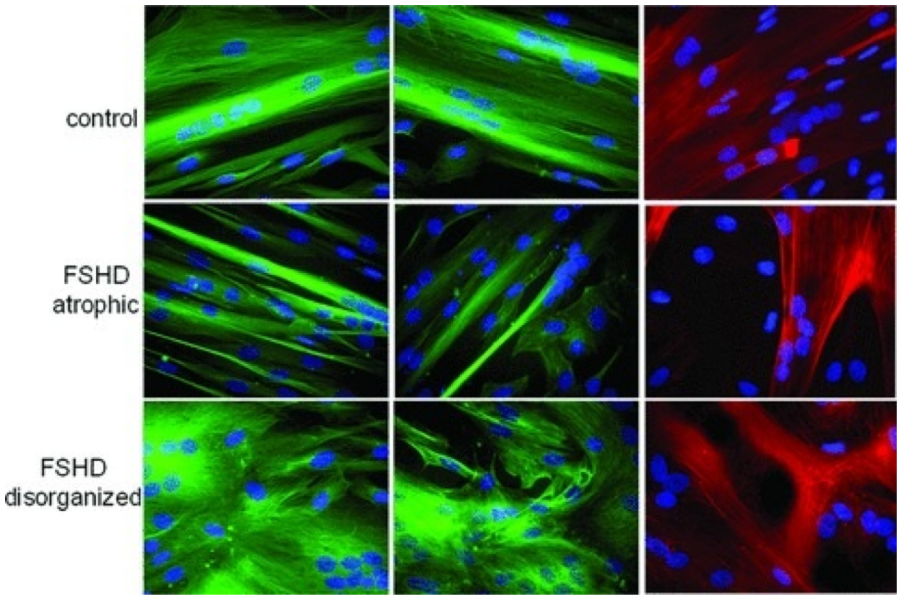


A transcription dysregulation cascade in FSHD. The DUX4 gene mapped in the D4Z4 repeated element at 4q35 is activated either by the pathogenic deletion that contracts the repeat array, or by another uncharacterized mutation that leads to chromatin opening of normal sized repeat arrays. The chromatin changes allow transcription of the DUX4 gene. On permissive alleles that carry the poly-A signal in the pLAM region this results in a stable mRNA that can be translated. The expressed DUX4 protein is a transcription factor that may directly or indirectly interact with a set of target genes. Among those, DUX4 expression results in the inhibition of the MyoD gene which encodes the transcription master switch of muscle differenti-ation thus **causing inhibition of the MyoD target genes in FSHD**. DUX4 over-expression also **inhibits the expression of genes involved in response to oxidative stress,** and probably inducing the lcrystallin (CRYM) gene whose promoter carries a DUX4 binding site. A direct DUX4 target gene is PITX1 at 5q31 which encodes a transcription factor that is the **master switch for hindlimb** development in embryogenesis. PITX1 is specifically induced in FSHD muscles as compared to 11 neuromuscular disorders; it induces E3 ubiquitin ligase which is linked to atrophy in adult skeletal muscles and is involved in inflammation. Among the PITX1 target genes is TP53 which has major roles in the control of DNA repair, cell cycling and apoptosis as well as in multiple levels of cell metabolism and muscle atrophy.

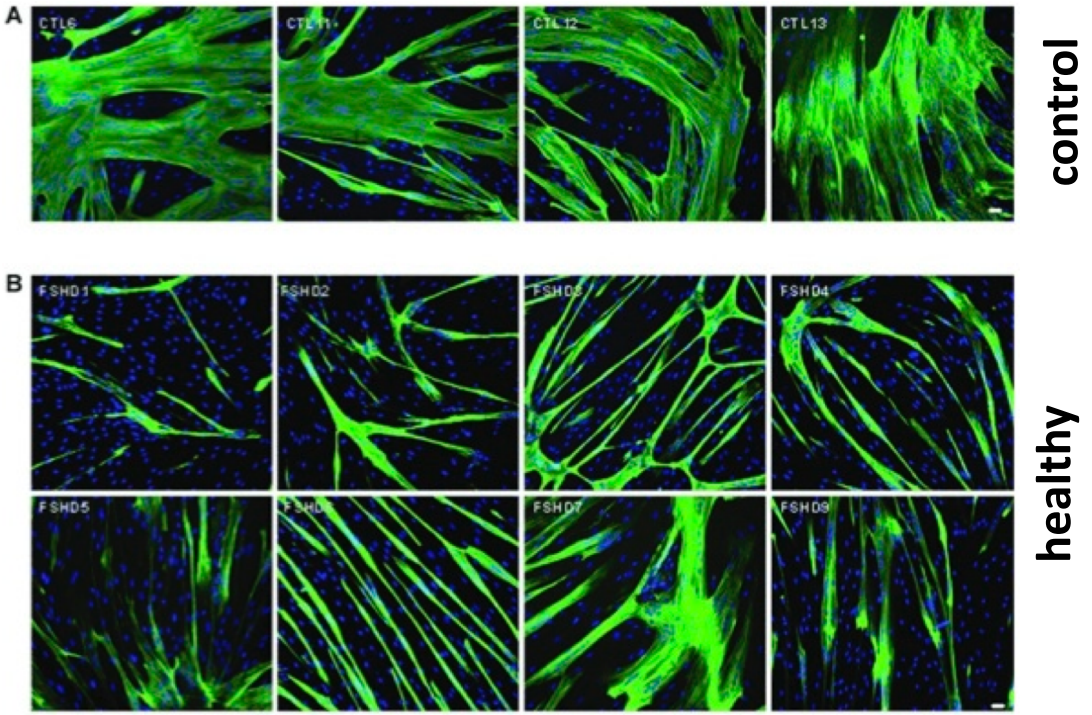
FSDH impairs muscle cell function

Moreover, in patients affected with FSHD, it is quite common to observe the co-existence of affected and apparently healthy muscles. Myoblasts, which were obtained from muscle typically affected in FSHD, manifested an increased susceptibility to oxidative stress during proliferation. Myotubes obtained from patient/healthy myblasts show abnormal morpholgy and muscle marker expression

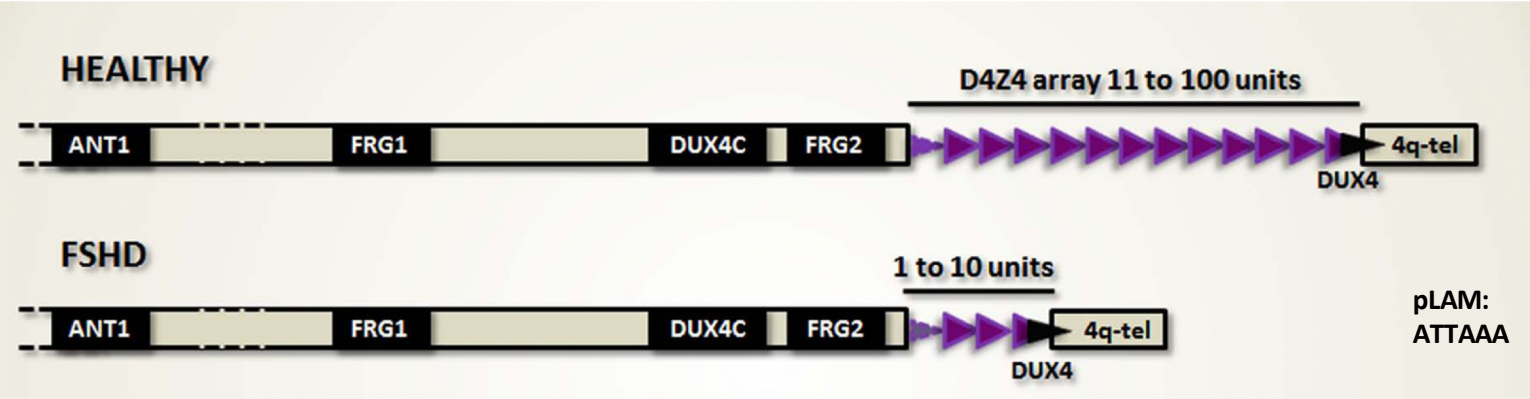
Anti actin/tubulin immunostainingof myotubes obtained by differentiating myblasts cells isolated from healthy or FSHD patients



anti-troponinT immunostainingof myotubes obtained by differentiatin myblasts cells isolated from healthy or FSHD patients



Scientific question:



Loss of heterochromatin is not sufficient to mediate efficient activation of gene transcription:

- Is there a pathway that drives the formation of an active chromatin structure at subtelomeres in FSDH patients?

- How does this pathway activate more distal genes involved in the disease (ANT1, FRG1, FRG2)

A Long ncRNA Links Copy Number Variation to a Polycomb/Trithorax Epigenetic Switch in FSHD Muscular Dystrophy

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DOI 10.1016/j.cell.2012.03.035

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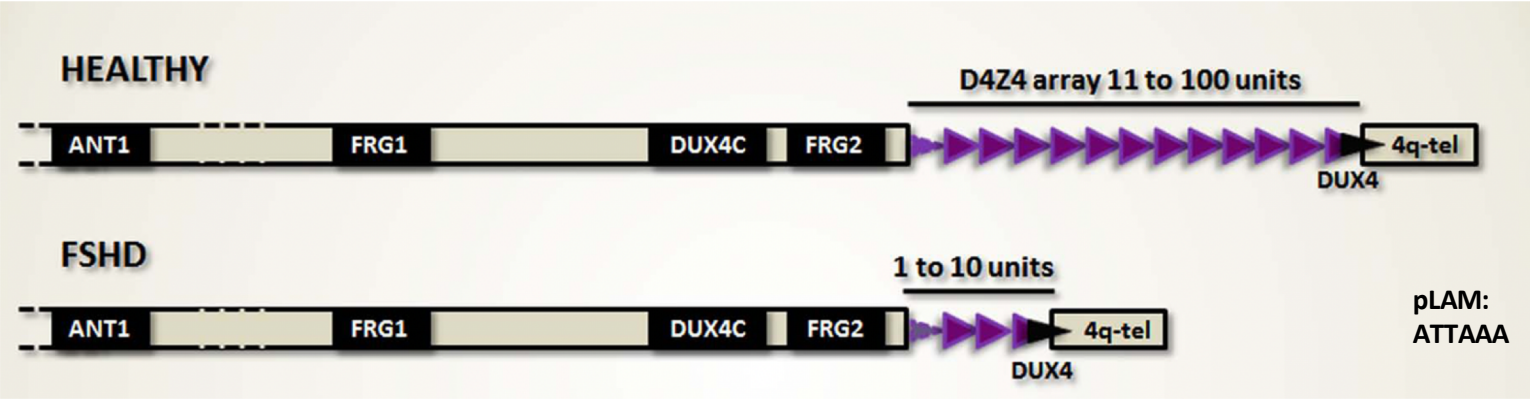

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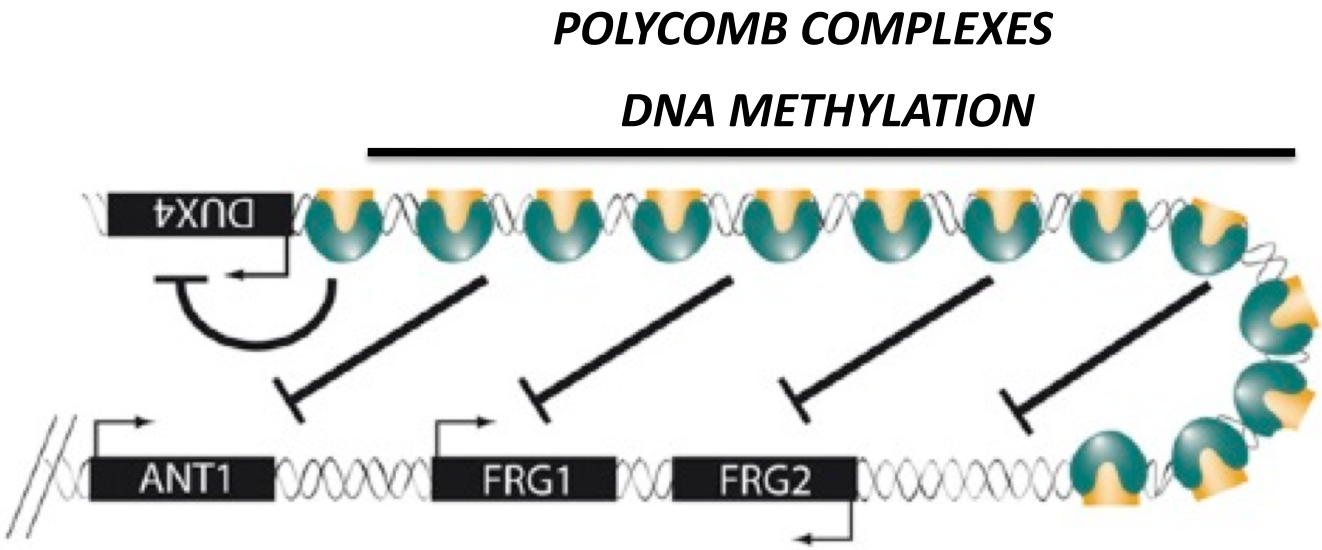
HETEROCHROMATIN AT D4Z4 REPEATS SILENCES LOCAL GENE EXPRESSION

HEALTHY

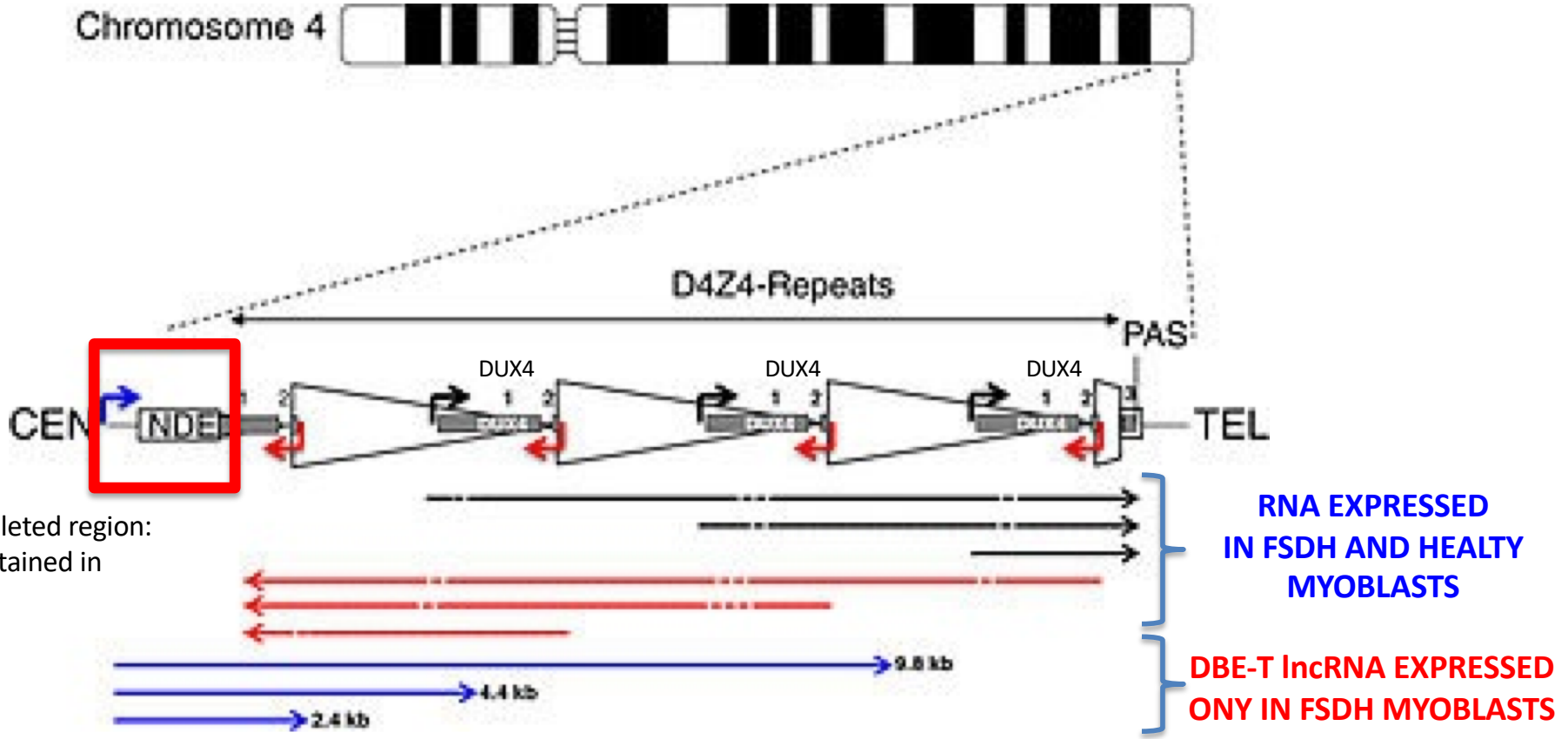
11 to 100 D4Z4 repeats

4q35 gene repression



Formation of a loop structure that supports silencing of DUX4, ANT1, FRG1, FRG2

Observation: D4Z4 repeats in Chr. 4q subtelomeres give rise to various transcripts



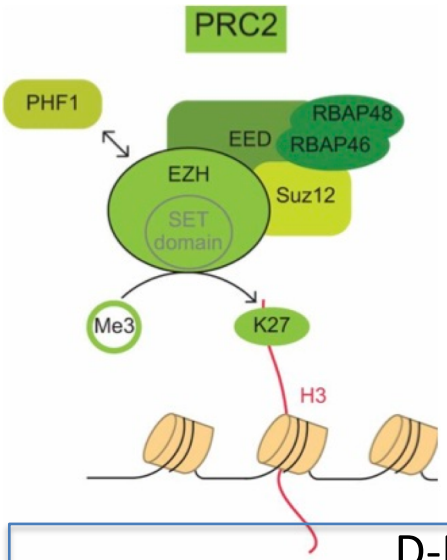
NDE: non deleted region: always maintained in patients

A schematic of D4Z4 locus on chromosome 4: The D4Z4 locus is in the sub-telomeric region of 4q. The figure shows a three repeat D4Z4 array. CEN indicates the centromeric end and TEL indicates the telomeric end. The DUX4 gene is shown as a gray rectangle with exon 1 and exon 2 in each repeat and exon 3 in the pLAM region telomeric to the last partial repeat (numbered 1, 2, and 3). PAS indicates the polyadenylation site on the permissive 4qA allele that is not present on the non-permissive 4qB allele or on chromosome 10. The arrowed lines represent: Blue, DBE-T transcripts (2.4, 4.4, and 9.8 kb) found in FSHD cells and reported to de-repress DUX4 expression; Black and red, transcripts in the sense and antisense direction were detected in both FSHD and control cells and might originate from the mapped sense promoters (black) and anti-sense promoters (red) with dashed lines indicating areas that might be degraded or produce si-like small RNAs. NDE, non-deleted element identified as the transcription start site for the DBE-T transcripts. Always present in patient DNA.

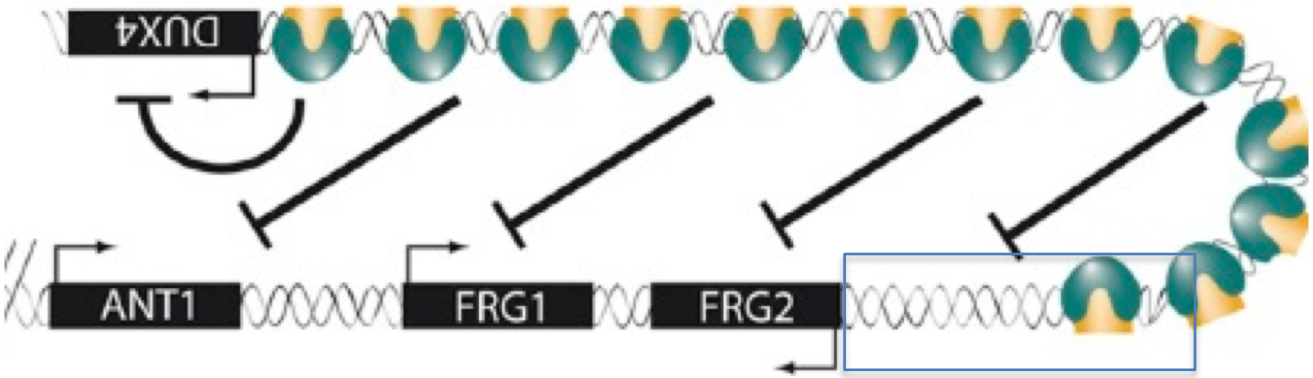
Observation: PRC2 is present at D4Z4 repeats

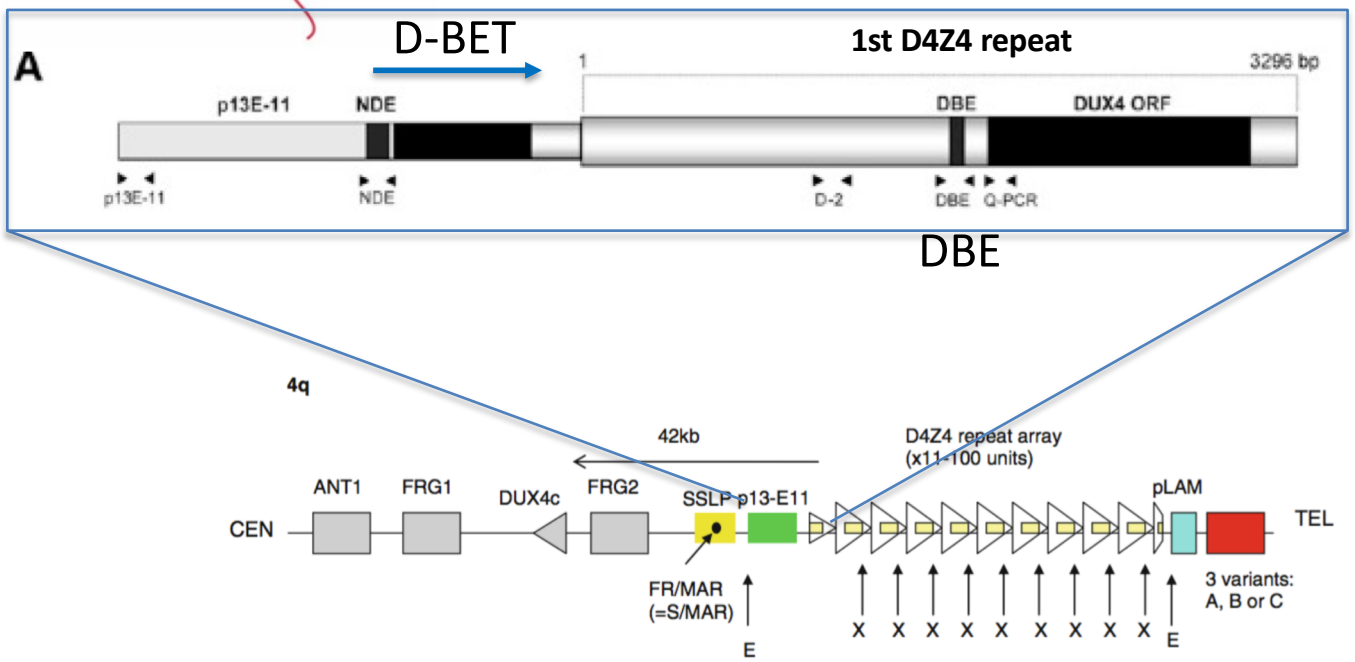
IS SILENCING AT D4Z4 REPEATS IMPAIRED IN FSDH PATIENTS??

PRC2 → H3K27me3



POLYCOMB COMPLEX 2



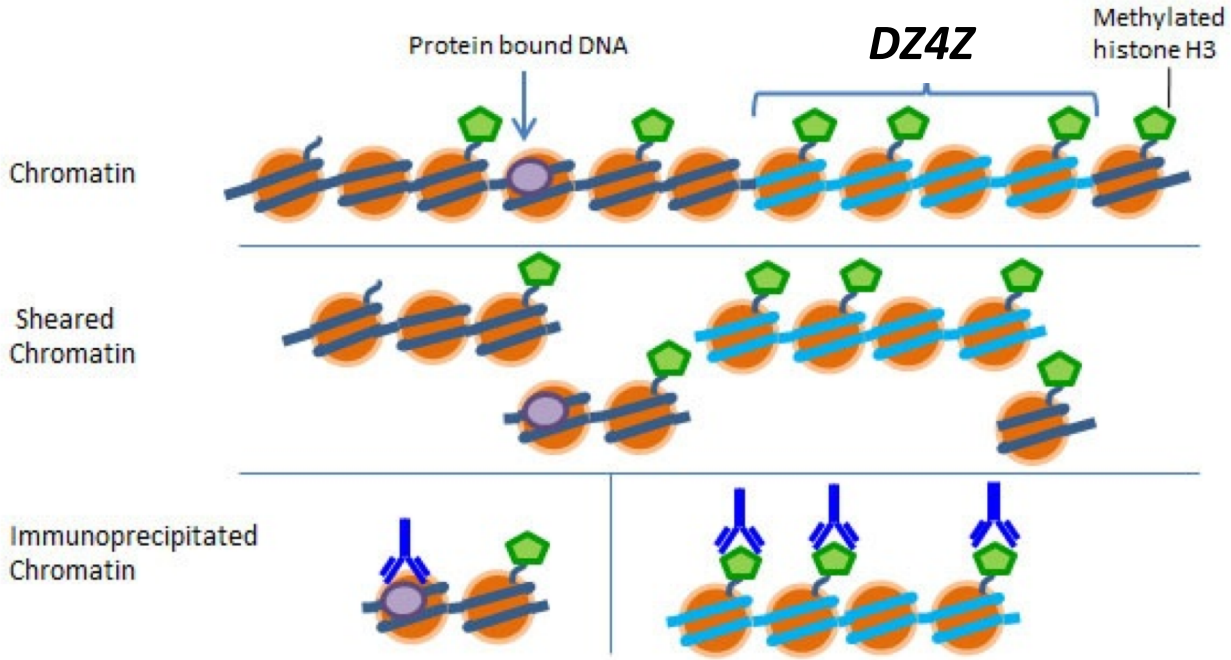


DBE (D4Z4 binding element), a region necessary and sufficient to confer copy-number-dependent repressive activity due to its ability to recruit YY1, EZH2, and HMGB2 (human homologs of the *Drosophila* PcG proteins Pho and E(z) and the PcG recruiter Dsp1, respectively)

IS SILENCING IMPAIRED IN FSDH PATIENTS??

CHEMICALLY CROSSLINKED CHROMATIN ISOLATED FROM

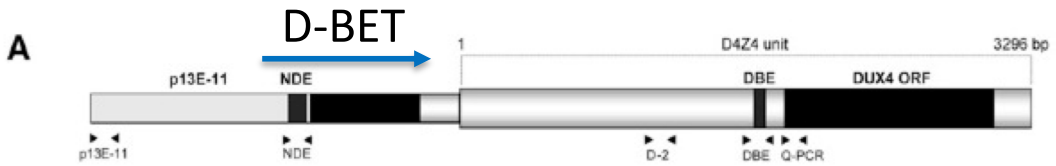
a. **Patient primary muscle cells**
b. **Normal primary muscle cells**



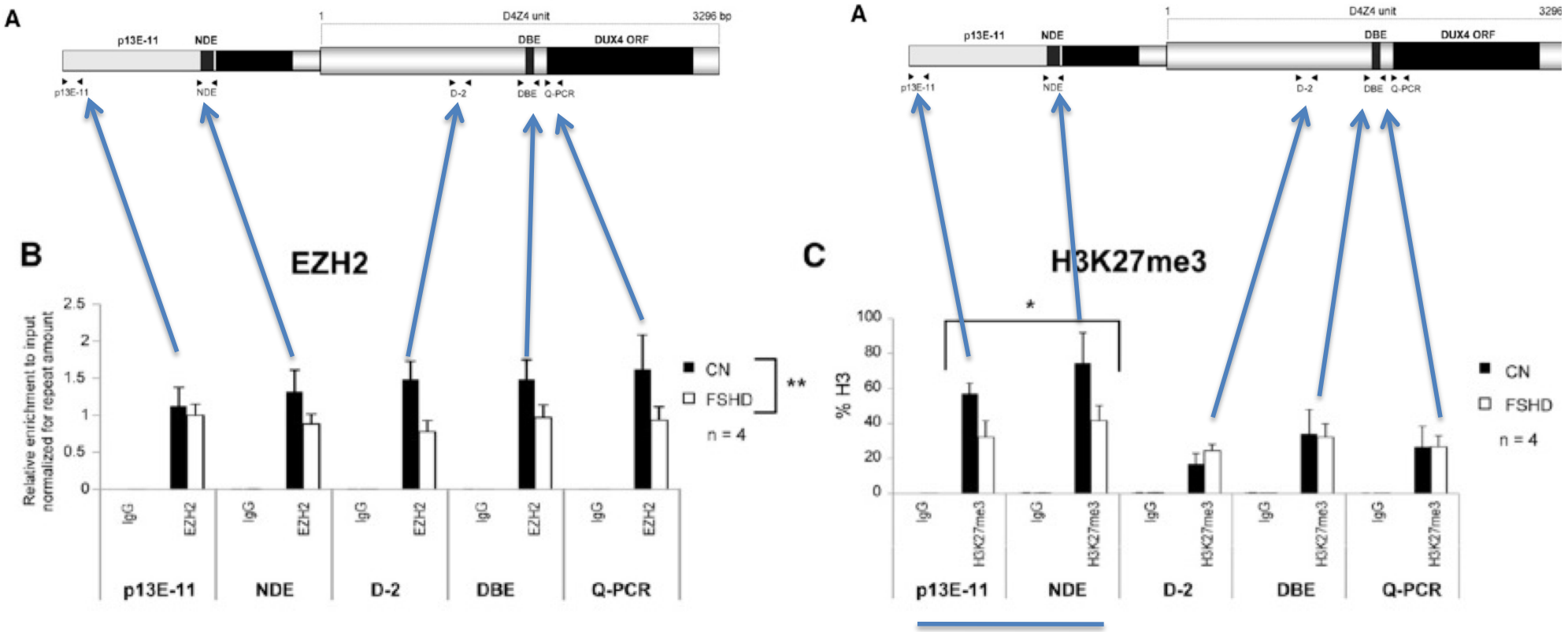
crosslinked chromatin

Precipitate-Ab-Chromatin complex with beads that bind heavy chain of antibody

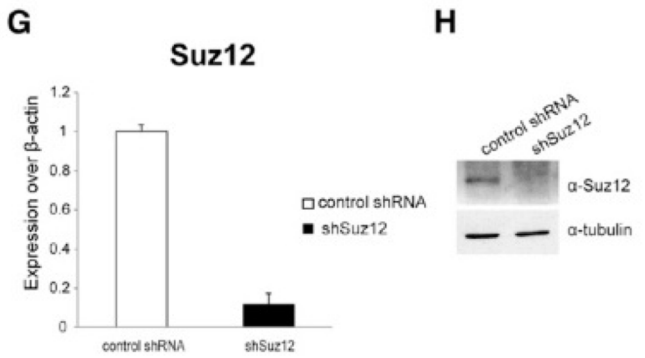
Make PCR with primers that amplify specific regions in D4Z4 repeats

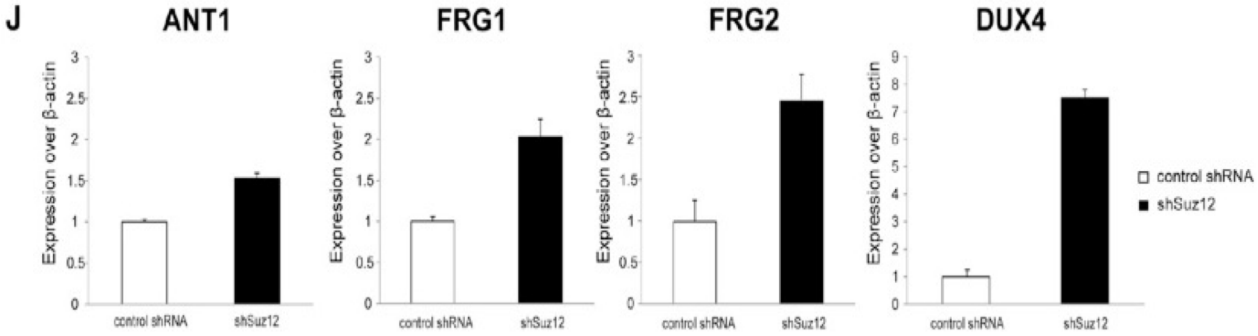


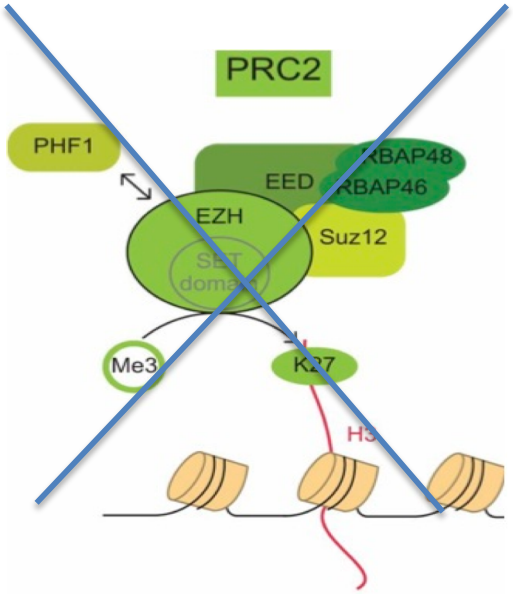
FSDH IS LINKED WITH LOSS OF PRC2 FUNCTION AT DZ4Z REPEATS

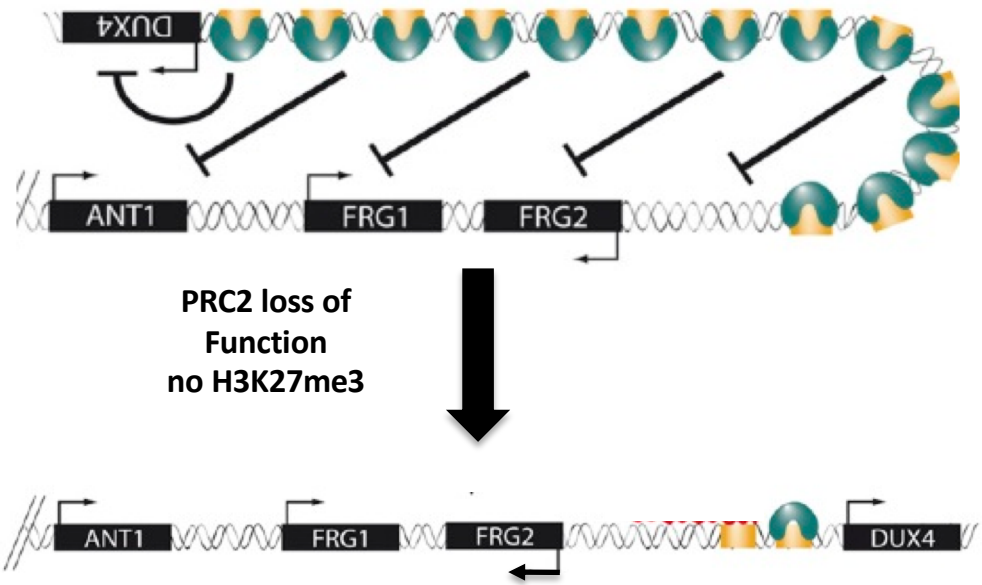


LOSS OF PRC2 FUCNTION INCREASES ANT1, FRG1, FRG2 and DUX4 EXPRESSION

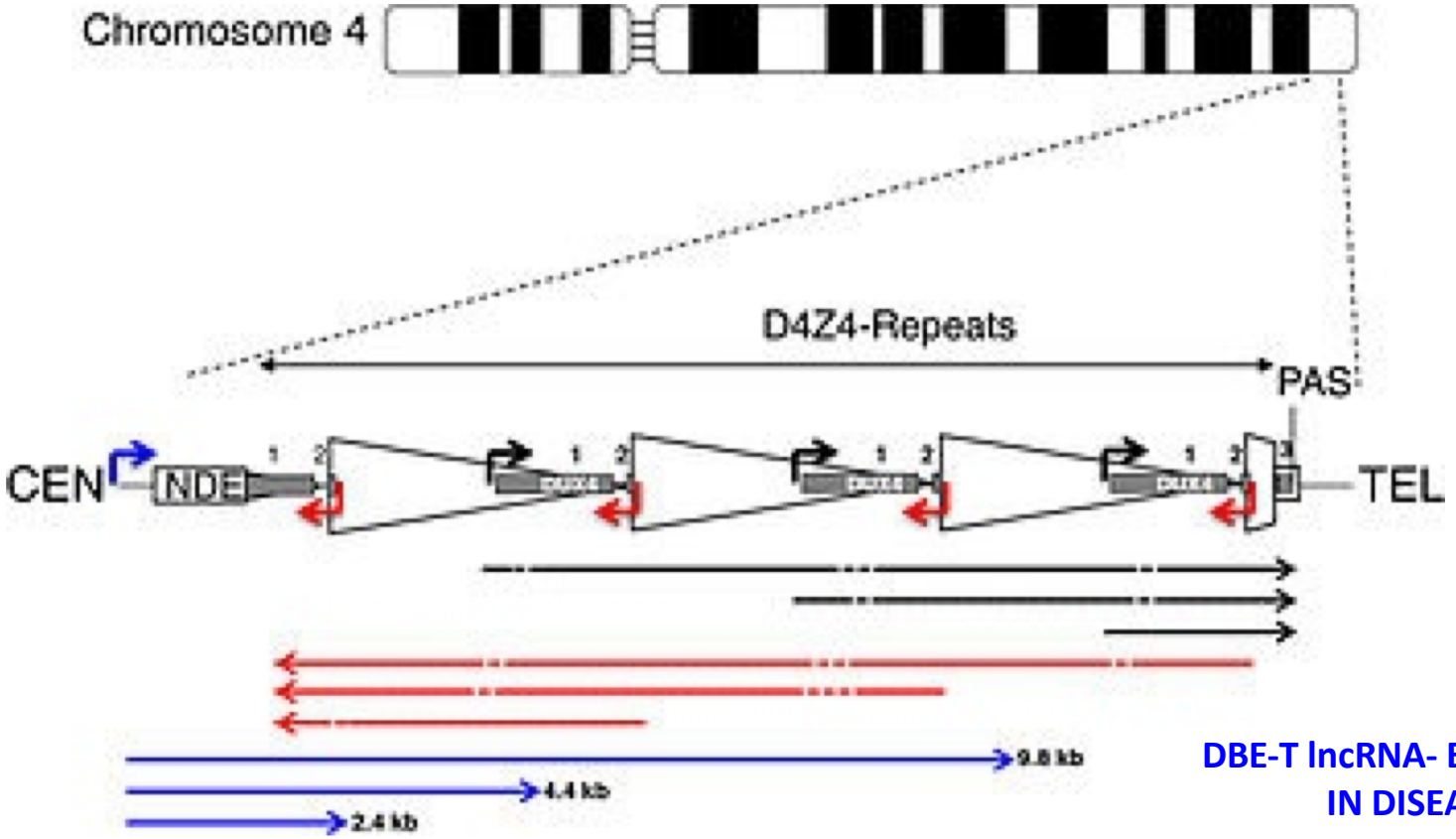








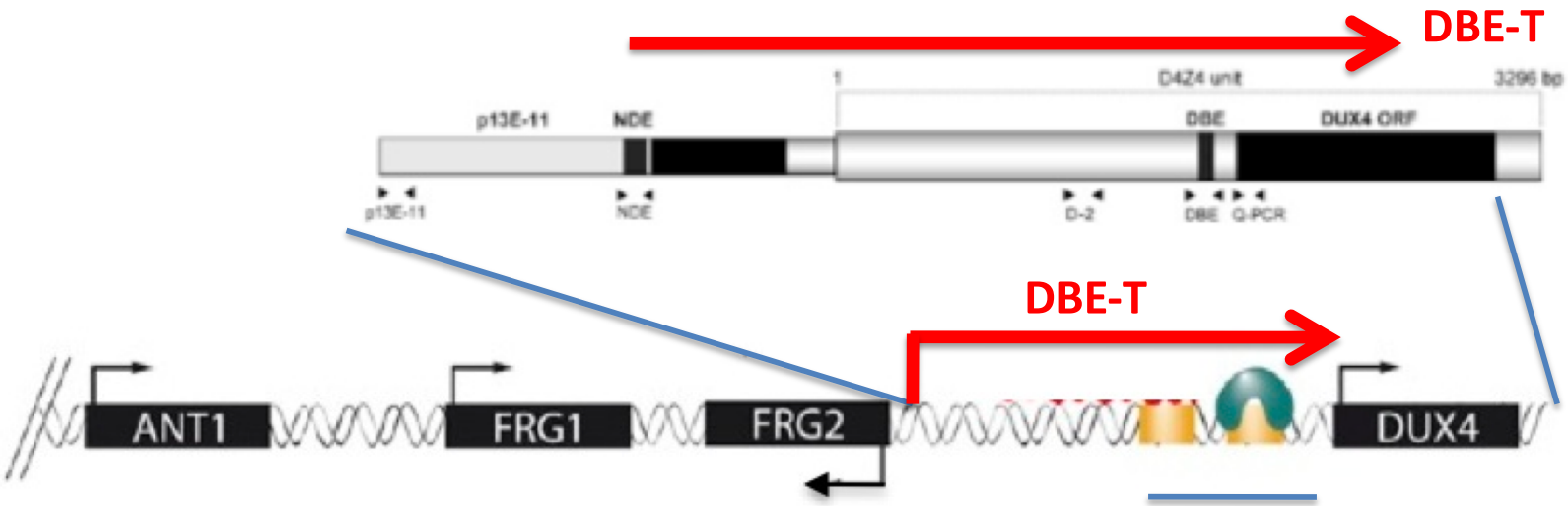
D4Z4 repeats in Chr. 4q subtelomeres give rise to various transcripts



DBE-T lncRNA- EXPRESSED IN DISEASE

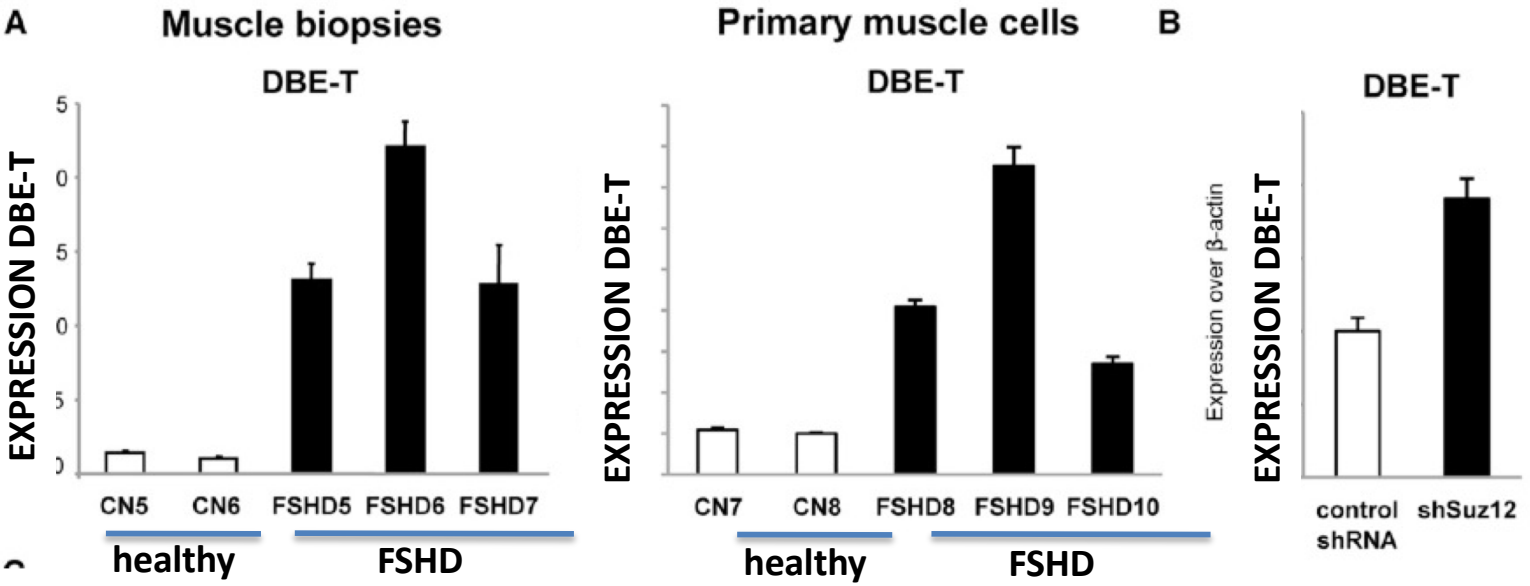
A schematic of D4Z4 locus on chromosome 4: The D4Z4 locus is in the sub-telomeric region of 4q. The figure shows a three repeat D4Z4 array. CEN indicates the centromeric end and TEL indicates the telomeric end. The DUX4 gene is shown as a gray rectangle with exon 1 and exon 2 in each repeat and exon 3 in the pLAM region telomeric to the last partial repeat (numbered 1, 2, and 3). PAS indicates the polyadenylation site on the permissive 4qA allele that is not present on the non-permissive 4qB allele or on chromosome 10. The arrowed lines represent: Blue, DBE-T transcripts (2.4, 4.4, and 9.8 kb) found in FSHD cells and reported to de-repress DUX4 expression; Black and red, transcripts in the sense and antisense direction were detected in both FSHD and control cells and might originate from the mapped sense promoters (black) and anti-sense promoters (red) with dashed lines indicating areas that might be degraded or produce si-like small RNAs. NDE, non-deleted element identified as the transcription start site for the DBE-T transcripts.

LOSS OF PRC2 FUCNTION CAUSES AN UPREGUALTION OF A NOVEL lncRNA – DBE-T



1st D4Z4 repeat

Check DBE-T EXPRESSION IN 2 CONDITIONS: FSDH Patients; loss of PRC2 function

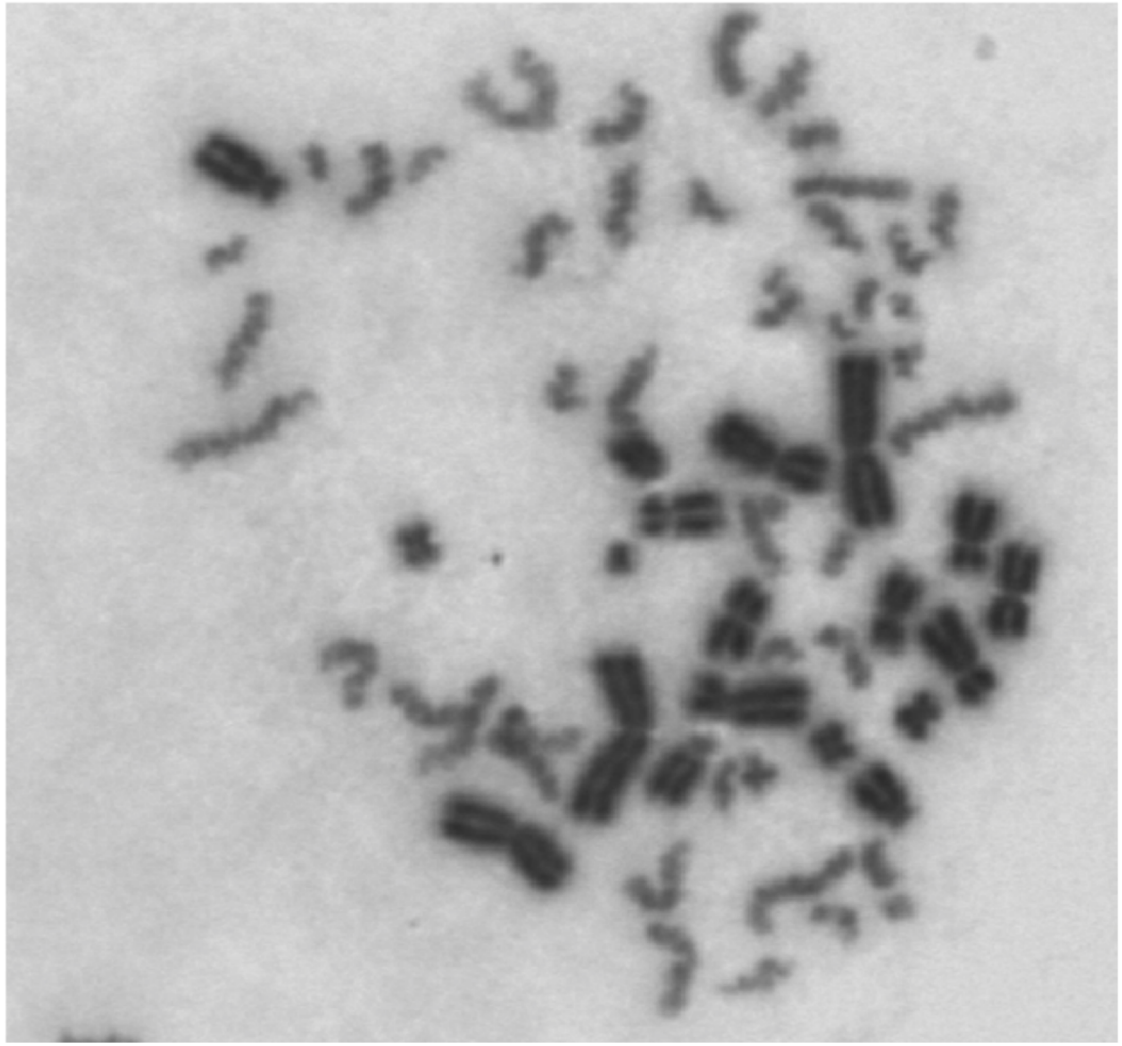


HOW TO SHOW D-BET lncRNA at chromosomes

Fuse ovary hamster cells with FSDH cells

Why? Use hamster cell to maintain chromosome 4 or FSDH patient cell

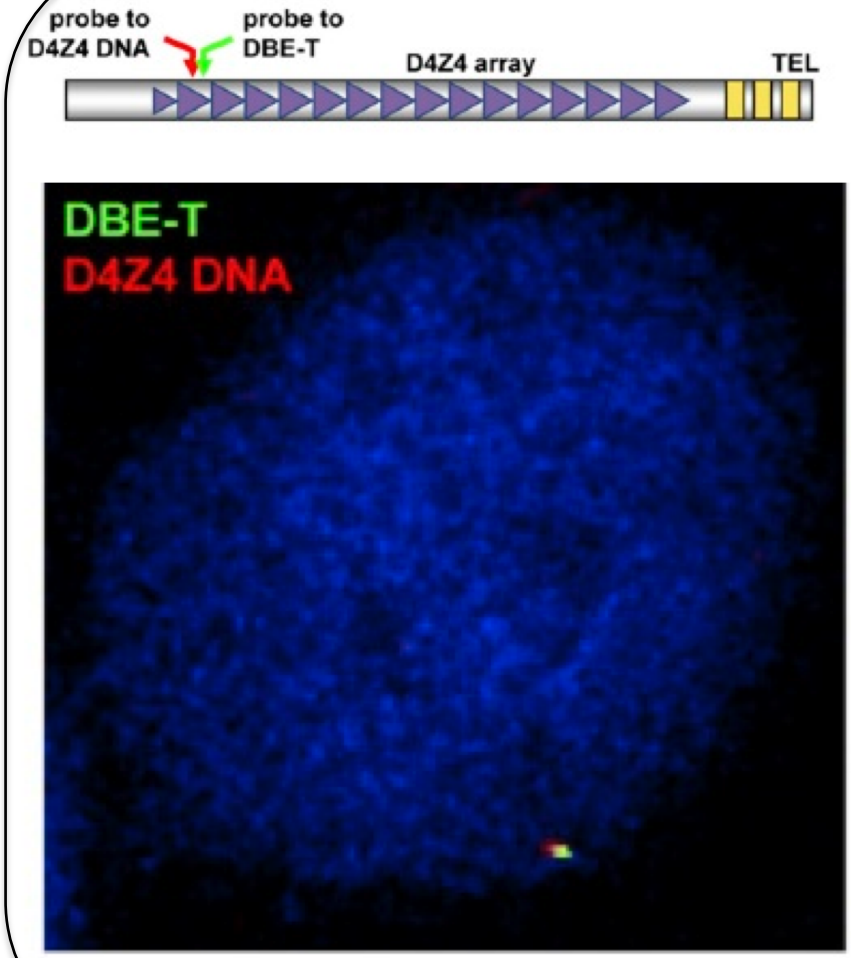
Fusion of a mitotic Chinese hamster ovary cell (large, dark stained chromosomes with visible double chromatids) with an interphase human lymphocyte (smaller, less brightly stained chromosomes).



→ Overcome poor proliferation of FSDH cells
→ allows to study FSHD Chr4 in a replicating cell model

DBE-T lncRNA COLOCALIZES TO D4Z4 REPEATS

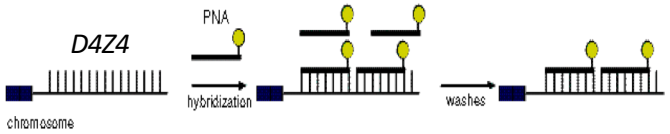
Fuse diseased human cell with hamser cell lines and select cell fusion product that carries human D4Z4 repeats



COMBINED DBE-T RNA-FISH AND D4Z4 DNA FISH

1. FIX CELLS
2. HYBRIDIZE A DBE-T PROBE (fluorescently labelled green)
3. CAPUTURE IMAGE WITH MICROSCOP
4. Start next staining (DNA-FISH
5. WASH
6. DENATURE DNA (HEAT)
7. HYBRIDIZE D4Z4 PROBE (fluorescently labelled – red)
8. CAPUTURE IMAGE
9. Merge IMAGES

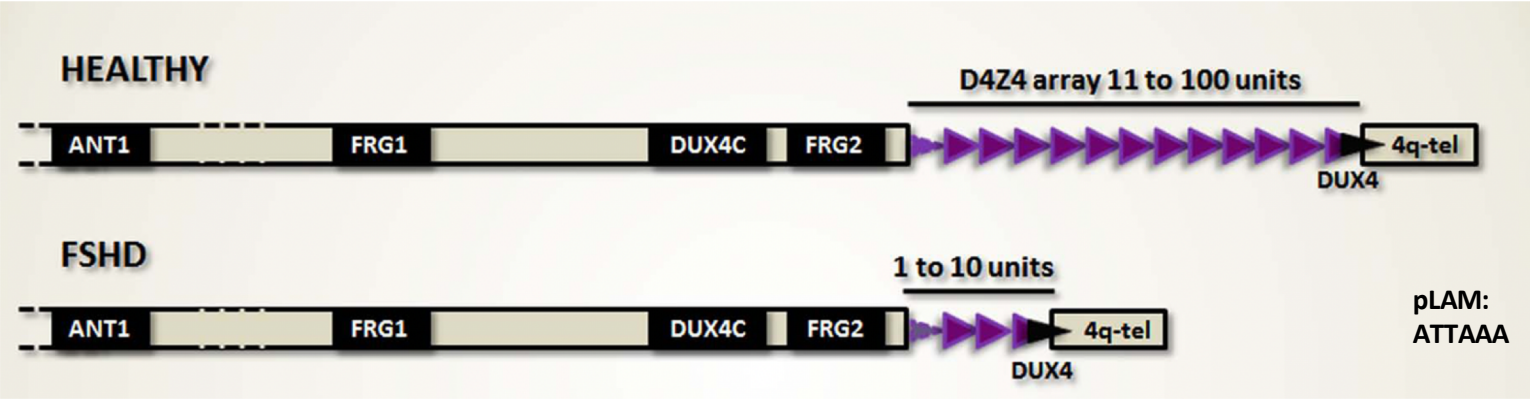
Fluorescence in situ hybridization



D4Z4 repeats

DBE-T remains associated with chromatin in cis; does not diffuse away from site of production

Scientific question:



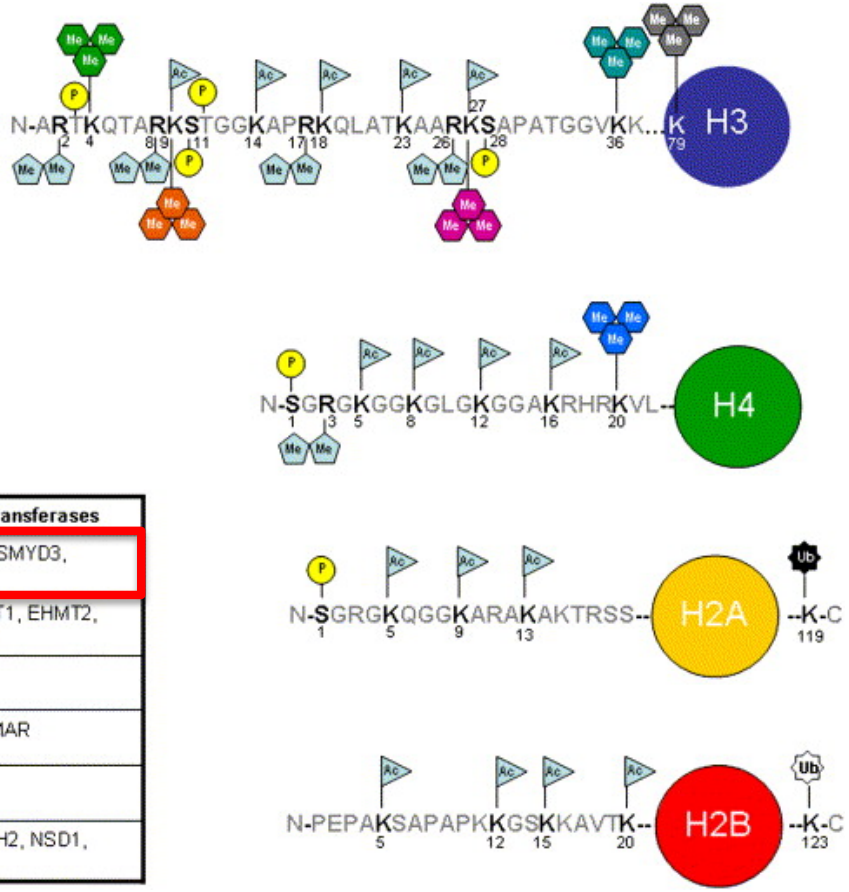
Loss of heterochromatin is not sufficient to mediate efficient activation of gene transcription:

- Is there a pathway that drives the formation of an active chromatin structure at subtelomeres in FSDH patients?

- **How does this pathway activate more distal genes involved in the disease (ANT1, FRG1, FRG2)**

HMTases that tri-methylate H3K4 activate RNA Pol II promoters:

A



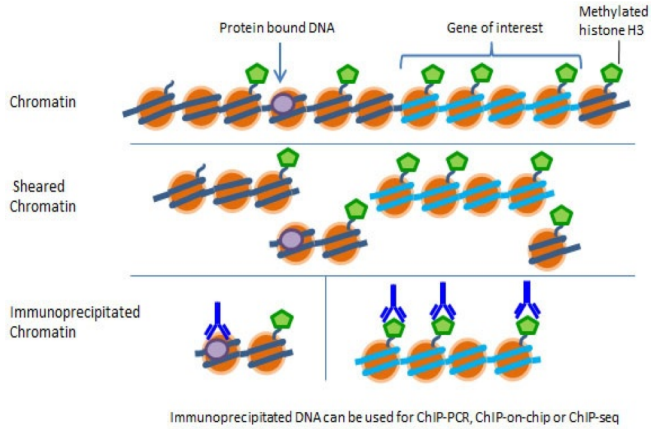
B

Substrate	Histone lysine methyltransferases
H3K4	SET9, SET1, MLL, ASH1L, SMYD3, PRDM9, SETMAR
H3K9	SUV39H1, SUV39H2, EHMT1, EHMT2, SETDB1, PRDM2, ASH1L
H3K27	EZH2, EHMT2
H3K36	NSD1, SETD2/HYPB, SETMAR
H3K79	DOT1L
H4K20	SET8, SUV420H1, SUV420H2, NSD1, ASH1L

Most famous:
MLL
ASH1L

DOES LOSS OF A HMTase LOCALIZE TO D4Z4 REPEATS AND PREVENT UPREGULATION OF DBE-T and SUBTELOMERIC GENES?

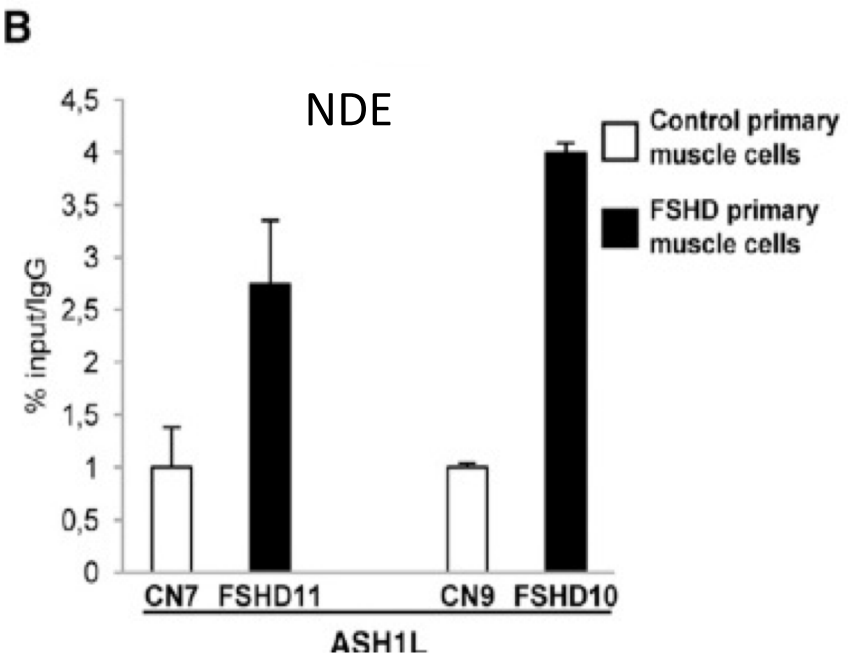
THE HMTase Ash1L localizes to DZ4Z REPEATS



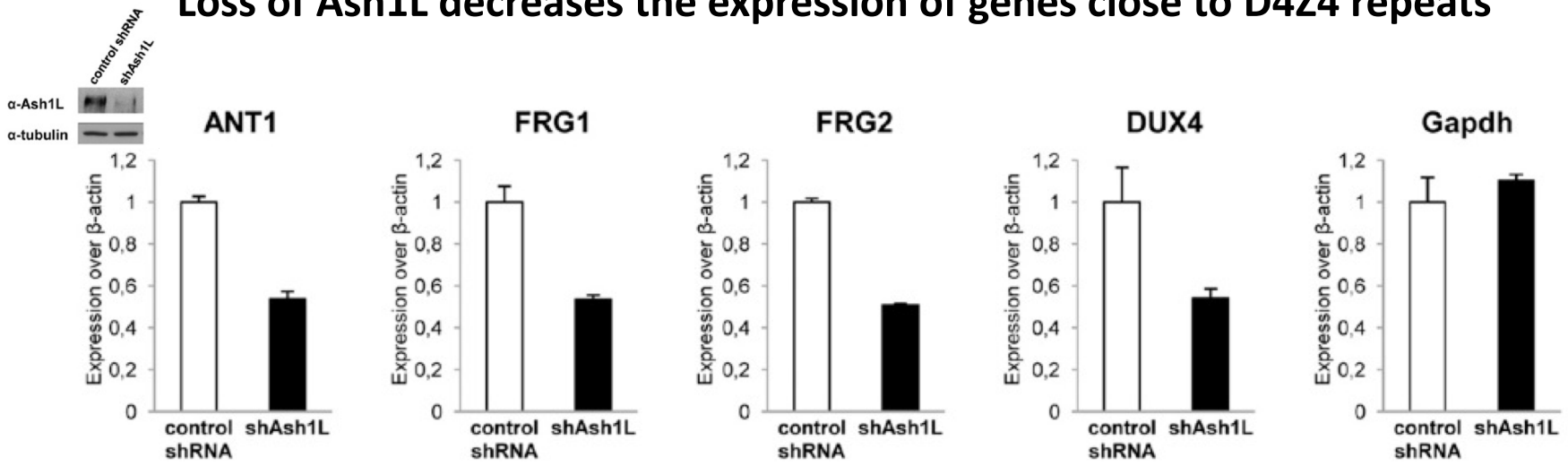
Ashl1: Histone methyltransferase

→H3K4me3 **ACTIVATES**

→H3K36m2 **TRANSCRIPTION**



Loss of Ash1L decreases the expression of genes close to D4Z4 repeats

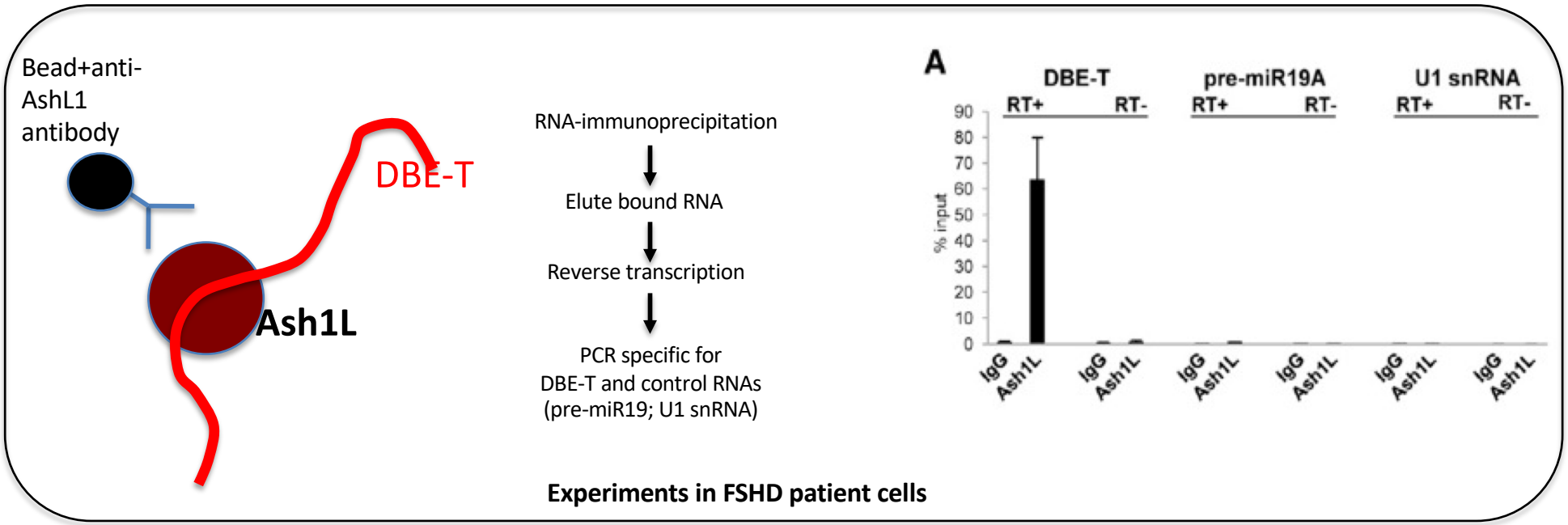


Ashl1 shRNA experiments in FSDH patient cells

DBE-T INTERACTS WITH Ash1L

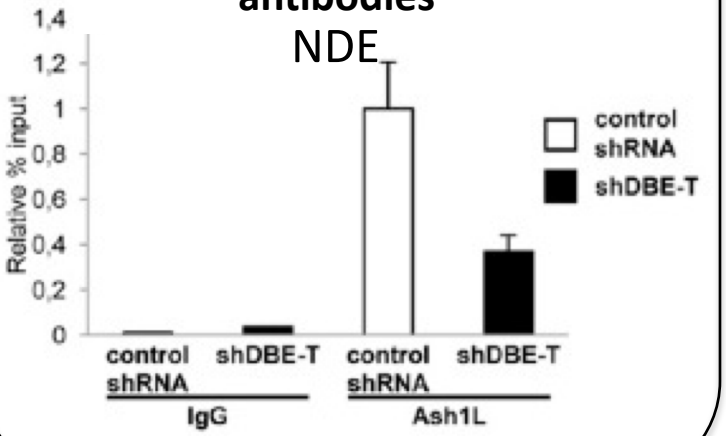
RNA immunoprecipitation:

use antibodies to immunuprecipitate protein-RNA complex
- «soft» crosslinked lysates
- Immunoprcpaitation
- Reverse crosslink
- RNA purification and cDNA production
- Target specific PCR (or high-throughput sequancing)



DBE-T BRINGS Ash1L TO D4Z4 REPEATS

ChIP using anti-Ash1L antibodies

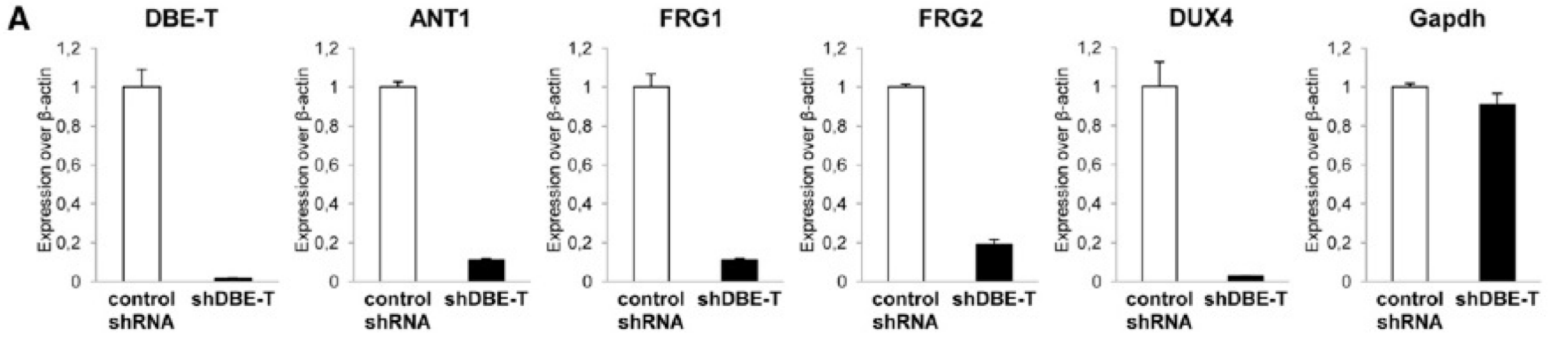


si DBE-T (reduction of DBE-T)

Loss of DBE-T results in increased expression of D4Z4 neighboring genes

Gene expression RT-PCR

control and si DBE-T (reduction of DBE-T)



Ashl1 shRNA experiments in FSHD patient cells

A lincRNA IS USED TO TRANSMIT D4Z4 REPEAT NUMBER INTO A DISEAS RELEVANT MECHANISM