Elucidating DUX4-mediated molecular mechanisms underlying FSHD pathophysiology using multiomics approaches

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Chapter 1

General introduction

Facioscapulohumeral muscular dystrophy

General introduction to FSHD

Facioscapulohumeral muscular dystrophy (FSHD) represents a prominent inherited myopathy, ranking as the third most common form of muscular dystrophy after Duchenne/Becker muscular dystrophy and myotonic dystrophy(*1*). The prevalence of FSHD likely varies across different geographical regions. An Italian study estimated a prevalence rate of 4.4 per 100,000 individuals in a northeastern Italian population sample(*2*). Deenen et al. reported a prevalence rate of 12 per 100,000 individuals in the Netherlands(*3*). More recently, Wang et al. conducted an epidemiological study on FSHD1 in China, estimating a prevalence rate of 0.075 per 100,000 individuals(*4*). FSHD is characterized by progressive skeletal muscle weakness and wasting, manifesting through a distinct pattern of muscle involvement that distinguishes it from other muscular dystrophies(*1*). Currently, while no curative therapy exists for FSHD, various treatment and intervention strategies have been developed to ease symptoms, restore muscle function, and improve patients' quality of life(*5*). Moreover, trials with medications targeting the root cause of the disease, DUX4, are currently in progress(*6*).

The pathological manifestation of FSHD follows a characteristic pattern of muscle involvement, initially affecting the facial musculature and scapulohumeral region, with subsequent progression to the upper arms, abdominal, axial, and lower limb muscles(*7-9*). The FSHD-specific pathology is further characterized by the presence of mostly subclinical extramuscular manifestations, including retinal vasculopathy (Coats' disease) and sensorineural hearing loss, perhaps suggesting a multisystemic nature of FSHD pathogenesis(*10-13*).

Clinical heterogeneity is a hallmark of FSHD, showing diverse patterns of disease onset, progression, and severity between and within affected families. While symptom onset typically occurs during the second or third decade of life, the temporal spectrum spans from early childhood to late adulthood(*14, 15*). The early-onset form, presenting before age 10, often exhibits notably increased severity compared to classical presentations(*14*). Disease severity demonstrates remarkable variability: ~20% of mutation carriers remain asymptomatic, while another ~20% experience severe progression necessitating wheelchair assistance(*16*). The remarkable diversity in clinical manifestations suggests complex regulatory mechanisms extending beyond conventional monogenic inheritance patterns.

The phenotypic variability observed in FSHD reflects a complex interplay of molecular and cellular mechanisms that regulate disease progression. Genetic, epigenetic, developmental, and environmental factors collectively contribute to the variable disease severity and incomplete penetrance. Although FSHD does not typically reduce life expectancy, it significantly affects patient quality of life through chronic pain, fatigue, and progressive muscle weakness(*17-19*). These diverse disease mechanisms and their clinical manifestations highlight the need for continued research to develop targeted therapeutic strategies.

Genetic basis of FSHD

FSHD comprises two genetically distinct subtypes, FSHD type 1 (FSHD1) and FSHD type 2 (FSHD2), which, though sharing similar clinical and (epi)genetic features, arise through distinct molecular mechanisms. Both forms are associated with the D4Z4 macrosatellite repeat array at

the chromosome 4q35 subtelomeric region, where epigenetic dysregulation leads to the aberrant expression of the double homeobox 4 (*DUX4*) gene in skeletal muscle(*20, 21*) (Figure 1).

FSHD1, accounting for approximately 95% of cases, is characterized by a contraction of the D4Z4 repeat array on chromosome 4q35(*3, 22-24*). Normally, the repeat array contains 8-100 D4Z4 repeat units in the European population, each unit spanning 3.3 kb and containing the *DUX4* gene(*22, 25*). In FSHD1 patients of European ancestry, the array is contracted to 1-10 repeat units on one 4q chromosome(*22, 23*). However, the contraction alone is not sufficient for disease manifestation. The presence of a specific disease-permissive 4qA haplotype is required(*24, 26, 27*). It contains a somatic polyadenylation signal that stabilizes the *DUX4* transcript, enabling its expression in skeletal muscle(*20, 28*) (Figure 1).

Population studies have revealed variations in D4Z4 repeat array sizes across different ethnic groups. A recent study on FSHD in the Indian population showed that the mean repeat array size in this cohort was 4.9 units(*29*). Research in Japanese and Korean populations indicated mean repeat array sizes of 3.4 units and 3.8 units, respectively(*30, 31*). Chinese studies revealed that the mean repeat sizes in Chinese pediatric and adult patients are 3.6 units and 4.7 units, respectively, which are smaller than the mean of 5.2 units in Dutch pediatric patients and 5.8 units in Dutch adult patients(*4*). Such ethnic variation implies that populations with different genetic backgrounds have varying susceptibilities to D4Z4 contraction.

FSHD2, representing approximately 5% of cases, is characterized by pathogenic variants in genes encoding epigenetic regulators responsible for maintaining D4Z4 chromatin repression in somatic tissue(*32*) (Figure 1). Most FSHD2 cases result from pathogenic variants in *SMCHD1*, a gene encoding a chromatin remodeling factor critical for DNA methylation and epigenetic regulation(*32*). Additional genetic contributors of FSHD2 include pathogenic variants in *DNMT3B*, a DNA methyltransferase gene, with heterozygous pathogenic variants contributing to FSHD2, while biallelic pathogenic variants cause ICF1 syndrome (Immunodeficiency, Centromeric instability, and Facial anomalies syndrome type 1)(*33*). More recently, homozygous pathogenic variants in *LRIF1*, encoding the ligand-dependent nuclear receptor-interacting factor 1, a protein partner of SMCHD1, have been identified as another genetic determinant of FSHD2, further highlighting the complex epigenetic regulatory network underlying FSHD pathogenesis(*34*).

Like FSHD1, FSHD2 requires a permissive 4qA haplotype for disease manifestation(*20, 27*). However, the two forms of the disease differ in D4Z4 repeat size. Although the original classification defined FSHD1 as having 1-10 D4Z4 units and FSHD2 as having more than 11 units, recent studies suggest that these cutoffs are not absolute. The D4Z4 repeat size in FSHD2 patients overlaps with both the FSHD1 population and healthy controls, indicating a more complex disease threshold(*35, 36*). Typically, FSHD2 patients have an intermediate D4Z4 repeat size ranging from 8 to 20 units, with a mean of 12 units(*33, 37, 38*). However, recent findings have also identified FSHD2 patients with D4Z4 repeats exceeding 20 units who exhibit moderate-to-severe disease, suggesting that additional genetic modifiers may influence disease severity(*38*).

Notably, the genetic complexity of FSHD extends beyond simple repeat contractions or pathogenic variants in D4Z4 chromatin modifiers and may include D4Z4 translocations and

duplications(*39-42*). The relationship between genetic changes and disease severity is not strictly linear, as evidenced by cases of asymptomatic individuals carrying contracted D4Z4 arrays and the presence of identical twins with discordant phenotypes(*43-47*). The observed phenotypic heterogeneity implies that additional factors, including epigenetic modifications, developmental timing, and environmental influences, play crucial roles in determining disease manifestation and severity.

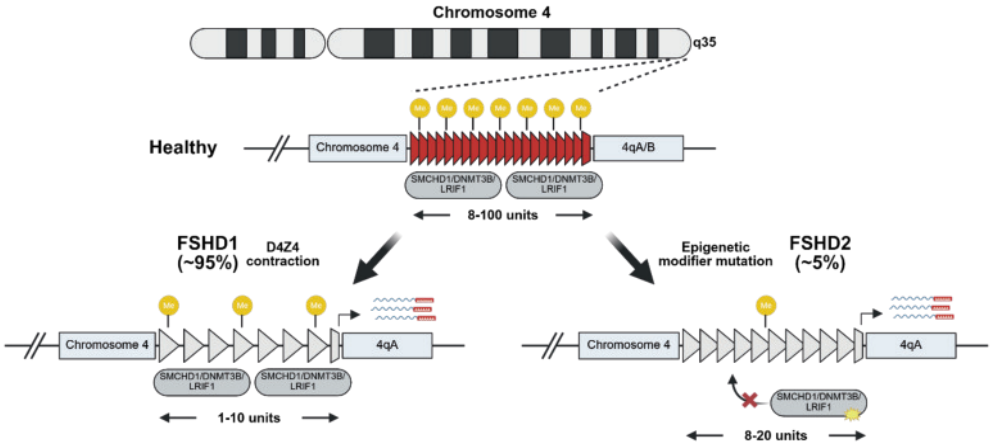

Figure 1. Schematic overview of molecular and genetic basis of FSHD1 and FSHD2.

The retrogene *DUX4* is located in the D4Z4 macrosatellite repeat array on chromosome 4q35. A triangle represents an individual repeat unit. In healthy individuals, the D4Z4 repeat array contains 8-100 units showing hypermethylation (Me) and maintaining transcriptional silencing of the *DUX4* gene. FSHD1 (~95% of cases) results from D4Z4 contractions to 1-10 units, leading to chromatin relaxation and partial loss of methylation. FSHD2 (~5% of cases) occurs due to mutations in epigenetic modifiers SMCHD1/DNMT3B/LRIF1, resulting in hypomethylation of a normal-sized D4Z4 array (8-20 units). The aberrant expression of DUX4 in the presence of a permissive 4qA allele leads to the FSHD phenotype. The red triangular patterns represent the D4Z4 repeat units, yellow circles indicate methylation sites, and wavy lines with red bars represent DUX4 transcription.

DUX4

Physiological role of DUX4

DUX4 is a germline and early embryonic transcription factor encoded within the D4Z4 macrosatellite repeat array on chromosome 4q35(*48-53*). It contains two DNA-binding homeodomains and a C-terminal transcriptional activation domain, enabling it to regulate a broad network of downstream genes and repetitive elements(*54-56*) (Figure 2). Under physiological conditions, DUX4 expression is tightly regulated, exhibiting activity during early embryonic development and in the germline while being epigenetically suppressed in most somatic tissues(*57-59*).

The evolutionary origins of *DUX4* trace back to an ancient duplication event that gave rise to *DUXC*, an intron-containing gene in the common ancestor of placental mammals(*60*). Over time, retrotransposition and genomic rearrangements led to the emergence of *DUX4*, now localized to the subtelomeric regions of chromosomes 4q and 10q in humans(*61, 62*). Unlike *DUXC*, which has been retained in species such as dogs, cows, and dolphins, *DUX4* evolved as an intronless gene unique to primates. The presence of additional human DUX family members, such as DUXA and DUXB, which are transcriptional targets of DUX4 and retain introns, suggests a complex regulatory network controlling early embryogenesis(*63*). At the molecular level, DUX4 binds DNA through two distinct homeodomains, each recognizing specific sequences: homeodomain 1 preferentially binds the TAAT motif, whereas homeodomain 2 recognizes the TGAT sequence in a head-to-head configuration(*55, 64, 65*). In humans, alternative splicing of DUX4 generates two functionally distinct isoforms. The full-length DUX4 (DUX4-fl) protein, consisting of 424 amino acids, contains both homeodomains and the C-terminal transactivation domain(*28*). In contrast, the truncated DUX4 isoform (DUX4-s), composed of 159 amino acids, retains the DNA-binding domains but lacks the transactivation region, limiting its regulatory potential(*50*).

DUX4 functions as a master driver of zygotic genome activation (ZGA), orchestrating the maternal-to-zygotic transition in early embryogenesis. It is expressed at the oocyte stage, and its expression reaches the peak level in the four-cell stage, with transcriptional targets becoming active between the two- and eight-cell stages(*51, 53, 58*) (Figure 2). The timing of DUX4 activation coincides with the major wave of ZGA, which shifts embryonic control from maternal transcripts to zygotic transcription, occurring at the two-cell stage in mice and between the four- and eight-cell stages in humans(*53*). By activating cleavage-stage genes such as *ZSCAN4*, *KDM4E*, and *PRAMEF* family members, DUX4 establishes an epigenetic environment necessary for early embryonic development(*51*). The DUX4-induced factors contribute to chromatin remodeling as well. ZSCAN4 regulates heterochromatin stability and KDM4E, a histone H3 lysine 9 (H3K9) demethylase, modulates epigenetic states essential for preimplantation progression(*66-68*).

As a pioneer transcription factor, DUX4 demonstrates unique chromatin-binding properties, facilitating transcriptional activation in both accessible and inaccessible genomic regions(*49*). In addition to binding DNase-accessible chromatin enriched in H3K27 acetylation (H3K27ac), DUX4 can access DNase-inaccessible H3K27ac-depleted MaLR repeat-enriched chromatin(*49, 69*). DUX4 achieves the chromatin penetration through the recruitment of histone acetyltransferases, including p300 and CBP, which remodel local chromatin architecture by inducing H3K27ac at target loci(*56*).

Beyond regulating zygotic gene expression, DUX4 also plays a critical role in controlling the activity of repetitive elements. During early embryogenesis, it preferentially activates retrotransposons such as HERVL, contributing to the establishment of early transcriptional networks(*51*). The control of repetitive elements appears evolutionarily conserved, as similar roles have been observed for mouse Dux and canine DUXC, suggesting that a core ancestral mechanism has been preserved across mammalian species. Despite its conserved function, species-specific variations in DUX4 and its homologs may represent adaptive modifications

that fine-tune transcriptional programs in primate embryogenesis, integrating conserved developmental processes with lineage-specific innovations(*70*).

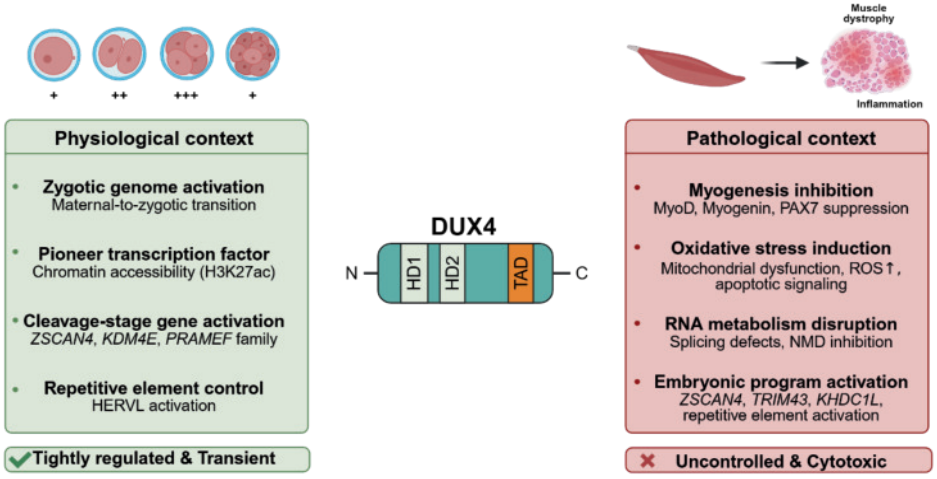

Figure 2. Dual roles of DUX4 in physiological and pathological contexts.

DUX4 contains two homeodomains (HD1 and HD2) at the N-terminus and a transactivation domain (TAD) at the C-terminus. In physiological contexts (left panel, green), *DUX4* expression is tightly regulated and transient during early embryonic development, peaking at the 4-cell stage (+++) and declining in the 8-cell stage (+). DUX4 serves essential functions during early embryogenesis. In pathological contexts (right panel, red), aberrant *DUX4* expression in skeletal muscle leads to uncontrolled and cytotoxic effects.

Pathological role of DUX4 in FSHD

The aberrant expression of DUX4 in skeletal muscle is considered a primary driver of FSHD pathology, initiating a cascade of molecular events that disrupt cellular homeostasis and ultimately lead to cell death and muscle wasting. As a transcription factor, DUX4 profoundly alters gene expression programs genome-wide, leading to apoptosis, response to oxidative stress, proteotoxicity, dysregulation of RNA metabolism, and the inappropriate activation of embryonic gene networks(*56, 71-80*) (Figure 2). Understanding the DUX4-induced pathological mechanisms is essential for developing targeted therapeutic strategies.

Disruption of myogenesis and muscle regeneration

DUX4 disrupts myogenesis by triggering a cascade of pathological processes, including aberrant immune response activation, enhanced apoptotic signaling, and elevated oxidative stress, ultimately compromising muscle development. It has been reported that DUX4 and the DUX4-induced cascade result in the suppression of the myogenic regulatory factor network, including MyoD, myogenin, and MRF4, thereby dysregulating critical differentiation pathways(*78, 79, 81, 82*). In satellite cells, DUX4 represses genes essential for differentiation and fusion, while competitively inhibiting PAX7 function through shared DNA binding motifs,

disrupting early myogenic programming(*81, 83*). DUX4 expression demonstrates temporal regulation, increasing during myogenic differentiation coincident with SMCHD1 downregulation, a key epigenetic suppressor of DUX4 in somatic cells(*84*). The pathological impact is further amplified by DUX4's downstream targets, such as Double homeobox 4 centromeric (*DUX4C*), also named *DUX4L9* (DUX4-like 9). When overexpressed, DUX4C causes severe myotube disorganization characterized by abnormal nuclear clustering, aberrant accumulation of β-Catenin, and mislocalization of cytoskeletal and contractile proteins, including α-Tubulin and Troponin T(*85*). The observed myotube abnormalities suggest that DUX4C significantly contributes to compromised muscle regeneration in FSHD, representing a secondary pathogenic mechanism triggered by primary DUX4 expression. At the structural level, DUX4 compromises cytoskeletal integrity through perturbation of desmin organization, resulting in impaired myoblast fusion and sarcomeric disorganization(*86*). The inhibition of DUX4 or DUX4C expression attenuates these pathological phenotypes, providing mechanistic validation of their central regulatory role in FSHD myogenic dysfunction(*87*).

Induction of oxidative stress and apoptotic pathways

Skeletal muscle, one of the body's most metabolically active tissues, depends on a precise balance between energy production and reactive oxygen species (ROS) homeostasis to maintain cellular function(*88*). In FSHD, compelling evidence indicates that mitochondrial dysfunction represents a major contributor to disease pathology. Studies have demonstrated that altered mitochondrial morphology, disrupted membrane potential, and impaired oxidative phosphorylation, collectively, lead to excessive ROS accumulation and oxidative stress(*89-91*). The consequent metabolic imbalance creates a toxic cellular environment that renders FSHD myoblasts particularly vulnerable to oxidative damage and apoptotic cell death(*76, 92, 93*).

Transcriptomic analyses have consistently identified dysregulation of oxidative phosphorylation pathways, oxidative stress responses, and hypoxia signaling as characteristic features of FSHD muscle(*89, 94*). Mechanistically, aberrant DUX4 expression has been causally linked to increased ROS production and increased sensitivity to oxidative stress through two primary mechanisms: disruption of the glutathione redox system and activation of hypoxia-inducible factor 1-alpha (HIF1α) signaling(*75, 76, 89, 95, 96*). Complementing these findings, functional studies have demonstrated significant mitochondrial bioenergetic perturbations in FSHD muscle, including reduced cytochrome C oxidase activity and diminished adenosine triphosphate production(*89*). The observed bioenergetic defects further exacerbate oxidative stress and metabolic dysfunction. At the molecular level, these changes compromise cellular homeostasis while simultaneously activating apoptotic signaling cascades through enhanced RAGE-NF-κB activity and mitochondrial dysfunction-associated cell death pathways(*97*).

The central role of oxidative stress in FSHD pathophysiology has prompted exploration of antioxidant-based therapeutic strategies. Multiple *in vitro* studies have demonstrated that antioxidant compounds can attenuate DUX4-induced toxicity by reducing oxidative DNA damage and inhibiting apoptotic cascades(*94, 98*). Translating these findings to clinical applications, trials evaluating antioxidant supplementation regimens (including vitamin C, vitamin E, zinc gluconate, and selenomethionine) in FSHD patients have reported moderate

improvements in secondary functional outcomes, though effects on primary clinical endpoints have been more limited(*99, 100*). Recent developments suggest that mitochondria-targeted antioxidants may offer superior therapeutic efficacy by neutralizing ROS at its primary cellular source, thereby more effectively restoring redox balance and enhancing myogenic differentiation capacity in FSHD muscle cells(*90*). Future research should focus on optimizing antioxidant delivery systems and investigating combinatorial approaches that simultaneously target multiple aspects of oxidative stress-driven muscle degeneration in FSHD.

Dysregulation of RNA processing and post-transcriptional regulation

Extensive studies indicate that DUX4-induced influence on the transcriptome extends far beyond transcriptional activation(*72, 74, 80, 82, 101, 102*). DUX4 orchestrates a complex dysregulation of gene expression through its multifaceted effects on RNA processing and post-transcriptional mechanisms, creating a cascade of molecular aberrations that contribute to FSHD pathology. The dysregulation of biological processes associated with RNA metabolism, including RNA splicing and RNA processing, represents a hallmark of ectopic DUX4 expression in muscle cells. Extensive gene expression analyses have revealed that DUX4 induces widespread alterations in alternative splicing across genes critical for muscle structure and function, including *MYL6*, *TPM2*, and *PALLD*, partially explaining how DUX4 impairs muscle development(*82*). Notably, genes encoding core components of the splicing machinery themselves undergo alternative splicing following DUX4 expression, suggesting that DUX4 establishes a self-amplifying regulatory circuit that progressively distorts the cellular splicing landscape(*82, 101*). By altering the splicing of splicing regulators, DUX4 creates a cascade effect that compromises RNA processing throughout the transcriptome, potentially accounting for the broad dysregulation observed in FSHD muscle.

Beyond splicing alterations, DUX4 profoundly disrupts RNA quality control mechanisms, particularly the nonsense-mediated decay (NMD) pathway. NMD serves as the most evolutionarily conserved mRNA surveillance system, selectively targeting transcripts containing premature termination codons or unusually long 3' UTRs for degradation(*103*). Feng et al. demonstrated that DUX4 expression leads to the proteolytic degradation of UPF1, a central component of the NMD machinery, without affecting its mRNA levels(*80*). Concurrently, transcripts encoding other NMD factors, including *UPF3B* and *SMG7*, show compensatory upregulation in response to *UPF1* depletion. The consequence of inhibited NMD is significant. Approximately, 13% of transcripts normally targeted for degradation persist at elevated levels in DUX4-expressing myogenic cells. More concerning, many of these aberrant transcripts are actively translated into truncated proteins, including truncated RNA-binding proteins that may further dysregulate RNA processing. A particular example is an NMD-targeted isoform of *SRSF3*, which produces a truncated protein that contributes to cytotoxicity in FSHD muscle cells(*104*). The production of truncated proteins may have profound implications for cellular homeostasis, potentially functioning as dominant-negative inhibitors of full-length counterparts, forming protein aggregates, or generating neoantigens that trigger inflammatory responses.

Intriguingly, DUX4 itself is subject to NMD regulation(*80*). The *DUX4* mRNA contains a constitutively spliced intron within its 3'UTR, making it a canonical NMD substrate. By

promoting UPF1 degradation, DUX4 establishes a negative feedback loop that stabilizes its own mRNA, potentially exacerbating its pathogenic effects in FSHD. Such self-reinforcing circuit may represent a physiological mechanism that prevents unwanted accumulation of DUX4 in healthy cells, which becomes dysregulated in FSHD muscle.

DUX4's post-transcriptional effects extend to altered mRNA stability for specific transcripts. For instance, DUX4 expression increases the stability of *MYC* mRNA by approximately twofold in DUX4-expressing FSHD muscle cells(*74*). Given that MYC overexpression has been linked to NMD inhibition, enhanced MYC stability might represent another mechanism by which DUX4 disrupts RNA surveillance pathways. Furthermore, DUX4 expression has been associated with perturbations in protein homeostasis, including altered distribution of ubiquitinated proteins and abnormal aggregation of RNA-binding proteins such as TDP-43(*105*).

The complex interplay between DUX4 and post-transcriptional regulation represents a critical aspect of FSHD pathophysiology that warrants detailed investigation. In chapter 4 of this thesis, we explore the splicing alterations induced by DUX4 and their consequences in detail. Understanding these regulatory networks may reveal novel therapeutic targets and biomarkers for FSHD, potentially enabling more precise interventions that target the fundamental molecular dysregulation underlying this complex muscular dystrophy.

Activation of embryonic programs

DUX4 plays a critical role in early embryonic development as one of the master regulators for ZGA. During early embryogenesis, DUX4 activates the expression of hundreds of genes essential for the maternal-to-zygotic transition, including numerous cleavage-stage-specific transcripts and repetitive elements(*51, 53*). Notably, the transcriptional program triggered by DUX4 in FSHD muscle cells is similar to embryonic development(*106*). DUX4 expression in FSHD myocytes induces a subset of germline-specific genes that are normally expressed during early embryonic development(*49, 69*), including well-characterized markers such as *ZSCAN4*, *TRIM43*, *MBD3L2*, and *KHDC1L*. The core DUX4 target genes show substantial overlap with genes activated during ZGA(*52*). The ectopic expression of them in adult muscle tissue serves as a molecular hallmark of FSHD and contributes to the unique pathophysiology of the disease.

Beyond protein-coding genes, DUX4 also activates various repetitive elements in both embryonic and pathological contexts. In FSHD muscle cells, DUX4 binds to and activates LTR elements, particularly from the HERVL family, using them as alternative promoters to drive expression of novel spliced isoforms(*49, 69*). During early embryogenesis, DUX4 activates transposable elements, where such activation is essential for early development. In FSHD muscle cells, their reactivation represents another aspect of the embryonic-like state induced by DUX4.

Comprehensive gene expression analyses further support the activation of embryonic programs in FSHD. Our study using single-nucleus RNA-sequencing (snRNA-seq) (chapter 2) has demonstrated significant enrichment of early developmental programs in DUX4-expressing nuclei of primary myotube cultures derived from FSHD patients(*77*). Additionally, a previous study has shown that DUX4 overexpression in stem cells is able to revert cellular states toward

an 8-cell embryo-like (8C-like) phenotype(*107*). The observed developmental reprogramming collectively suggests that DUX4 maintains conserved functional capabilities across different cellular contexts, activating core aspects of its embryonic program even in adult somatic cells.

In chapters 2 and 5 of this thesis, we present detailed comparisons of DUX4-induced processes between myogenic and embryonic contexts, highlighting both commonalities and differences at the gene and isoform levels, respectively. Exploring how DUX4 activity is conserved or diverges across contexts may uncover therapeutic targets for FSHD.

DUX4 expression in FSHD

The expression pattern of DUX4 in FSHD presents unique challenges for both research and therapeutic development due to its stochastic and highly variable nature (Figure 3). Understanding these patterns across different contexts, both *in vitro* and *in vivo*, has been critical for elucidating FSHD pathogenesis and improving detection strategies.

In vitro studies using FSHD patient-derived myogenic cells have demonstrated that DUX4 expression is sporadic, with detectable protein found in only a small fraction of myonuclei (~ 1 in 2,000), varying significantly between patient cell lines and culture conditions(*108, 109*). The burst-like activation of DUX4 fluctuates dramatically even within the same culture, necessitating highly sensitive detection methods such as single-molecule RNA-fluorescence in situ hybridization (RNA FISH) and sensitive reverse-transcription quantitative PCR (RT-PCR). Analyses of FSHD muscle biopsies suggest that DUX4 expression *in vivo* follows a similarly rare and variable pattern, suggesting that its sporadic nature is an intrinsic feature of FSHD pathology(*110*). Despite its apparently low abundance, even transient bursts of DUX4 expression appear sufficient to trigger downstream pathological cascades that drive muscle degeneration(*71, 111*).

Due to the technical challenges associated with detecting DUX4 directly, the analysis of its target genes, such as *ZSCAN4*, *MBD3L2*, and *TRIM43*, has emerged as a valuable alternative approach(*49, 112*). They exhibit more stable and robust expression patterns, serving as reliable molecular markers of DUX4 activity and providing insights into its spatial and temporal dynamics in FSHD muscle.

A particularly significant aspect of DUX4 expression is its upregulation during myogenic differentiation of FSHD muscle cells. As proliferating myoblasts transition into differentiated myotubes, DUX4 expression increases, while SMCHD1 protein levels at the D4Z4 repeats decrease. The simultaneous upregulation of DUX4 and reduction of SMCHD1 during differentiation highlights a key mechanism underlying the aberrant activation of DUX4 in FSHD muscle. Supporting this, experiments in control myoblasts with the FSHD-permissive 4qA allele demonstrated that depletion of SMCHD1 results in robust activation of DUX4 expression. Notably, knockdown of other chromatin repressors, such as SUV39H1 and cohesin proteins, did not lead to DUX4 activation, further emphasizing the unique role of SMCHD1 in regulating DUX4 expression in FSHD(*84*).

DUX4-inducible cellular model systems for FSHD

To overcome the limitations caused by the sporadic nature of DUX4 in FSHD, researchers have developed inducible cellular systems that enable controlled DUX4 activation, facilitating reproducible analysis of its transcriptional regulatory networks and downstream pathological cascades.

Lentiviral-based systems have been used to generate DUX4-inducible (DUX4i) models using healthy donor myoblasts, allowing selective expression of both DUX4-s and DUX4-fl isoforms(*49, 82*). The biological validity of these models is reinforced by the consistent induction of established DUX4 target genes, including *ZSCAN4*, *MBD3L2*, and *TRIM43*, and the disruption of skeletal muscle homeostasis through multiple mechanisms, including activation of germline-specific transcriptional programs, expression of retrotransposable elements, and perturbation of myogenic differentiation pathways. All of which closely resemble the transcriptional profiles observed in FSHD muscle biopsies.

Recent technological advances using tetracycline-responsive promoter systems (TetO/rtTA) have enabled precise temporal control over DUX4 expression. Jagannathan et al. established doxycycline-inducible myoblast models that facilitate synchronized DUX4 activation across cell populations(*101*) (Figure 3). Comparative transcriptomic analyses of these models and myogenic cells from FSHD patients have revealed highly consistent gene expression signatures and transcriptomic programs. These engineered systems significantly enhance the signal-to-noise ratio in molecular analyses, enabling clear discrimination between primary transcriptional responses and secondary effects of DUX4 expression.

The development of these controllable expression systems provides essential experimental platforms that complement studies of endogenous DUX4 in patient-derived cells. These models serve as valuable tools for investigating the molecular mechanisms underlying FSHD pathogenesis and evaluating potential therapeutic interventions through standardized screening approaches.

FSHD modeling using 3D culture

Drug discovery and therapeutic development for muscular dystrophies, including FSHD, have traditionally relied on two-dimensional (2D) muscle cell culture systems and animal models. Despite providing valuable insights, these approaches typically cannot recapitulate the complex three-dimensional (3D) structure and mechanical properties of human skeletal muscle(*113*). Skeletal muscle is a complex tissue composed of multinucleated muscle cells and various resident cell types, including satellite cells and fibro-adipogenic progenitors (FAPs)(*114*). Monolayer primary myoblast cultures cannot recreate this intricate muscle tissue architecture(*115, 116*). Consequently, they lack the true 3D environment present in native tissue, including cell-cell and cell-matrix interactions involving the 3D extracellular matrix(*117*). Additionally, 2D cultures lack the structural organization and mechanical signals crucial for proper muscle function(*116, 118*). Under these conditions, cells cannot mature sufficiently to form functional sarcomere structures or generate contractile force, while muscle contractility is essential for recapitulating certain disease characteristics. For instance, standard 2D cell cultures cannot model sarcolemmal damage(*119*). Meanwhile, animal models frequently do not

fully recapitulate human disease due to species-specific differences in muscle biology, immune response, and pathogenic mechanisms(*120*). Additionally, these models present ethical challenges while being both expensive and limited in throughput capacity(*121*).

Tissue engineering provides a viable solution for modeling skeletal muscle diseases(*122-124*). Using specialized fabrication methods, human-based 3D muscle models replicate the complex tissue structure by growing cells on engineered scaffolds. Structural support from scaffolds leads to more accurate modeling of muscle pathophysiological conditions(*125-127*). Consequently, such 3D muscle cultures develop functional properties, responding to electrical signals and generating contractions(*128, 129*). To date, this technology has been widely applied in research on muscular disorders. For example, Tejedera-Villafranca et al. developed a patient-derived functional 3D skeletal muscle model for Duchenne muscular dystrophy (DMD) that exhibits contractile functionality and disease functional phenotypes(*119*), providing a more advanced platform for studying muscular dystrophies.

Beyond recapitulating muscle tissue characteristics, 3D tissue cultures hold significant promise for preclinical research, drug testing, small molecule screening, and other applications(*130, 131*). While technologies such as organoids, microtissue cultures, and multicellular co-cultures have been widely established in oncology as excellent platforms for precision and personalized medicine, they are now gaining traction in muscle disease research(*132*). Ebrahimi et al. created 3D human skeletal muscle microtissues (hMMTs) from immortalized myoblast clones derived from both healthy and DMD patients. Their 3D system revealed dystrophin-positive myotubes that remained undetectable in conventional 2D culture conditions. The researchers also showed that β1-integrin activating antibody treatment enhanced contractile protein maturation and structural stability in DMD hMMTs, demonstrating how these 3D platforms could serve as effective tools for drug testing and therapeutic research(*133*).

Our group previously established a protocol using optimized hydrogel formulations and culture conditions to create highly functional 3D skeletal muscle tissues (Figure 3). Human induced pluripotent stem cells (hiPSCs) offer an advantage over primary myoblasts by providing an endless supply of cells for these differentiation studies(*134*). With this advantage, van der Wal et al.(*134*) established a long-term stable 3D culture platform for preclinical research and other applications. The 3D-based models provide a more reliable platform than 2D cultures for evaluating small molecules and candidate therapies, in a setting that better reflects the complexities of the *in vivo* environment. The ability to generate patient-specific 3D muscle constructs enables personalized drug testing and deeper investigation into FSHD disease mechanisms. Moreover, these models support long-term culture and repeated drug a administration, allowing for extended evaluation of therapeutic effects on muscle function, gene expression, and disease-related pathways.

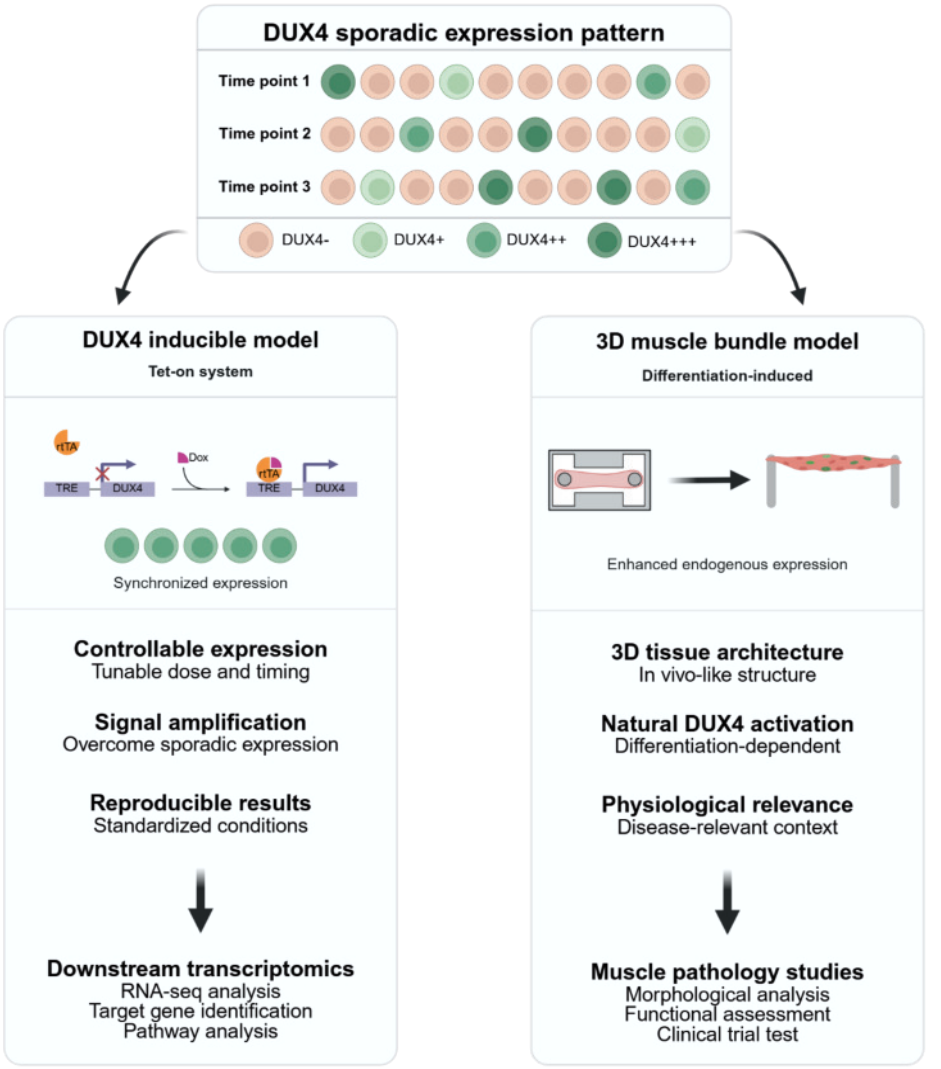

Figure 3. Experimental approaches to study DUX4 sporadic expression and its downstream effects.

The top panel demonstrates the stochastic and sporadic expression pattern of DUX4, where individual cells within a population show seemingly random and heterogeneous expression levels (DUX4-, DUX4+, DUX4++, DUX4+++) at any given time point. The DUX4 inducible model (left panel) uses a Tet-on system where DUX4 expression is placed under the control of a tetracycline-responsive element (TRE), allowing doxycycline (Dox)-induced synchronized expression across cell populations. The 3D muscle bundle model (right panel) employs differentiation-induced systems that recapitulate physiological muscle architecture, providing enhanced endogenous DUX4 expression in a 3D tissue context that mimics *in vivo*-like structure.

Application of high-throughput sequencing technology in FSHD research

High-throughput sequencing (HTS) has developed rapidly over the past decades, gaining favor in biomedical research due to its ability to simultaneously and accurately sequence DNA on a large scale, generating massive amounts of sequencing data quickly and at relatively low cost. New technologies continue to be created, developed, improved, and commercialized. Currently, HTS primarily includes next-generation sequencing (NGS) based on short-read sequencing and third-generation sequencing based on long-read technologies(*135*). The application of HTS has greatly facilitated clinical diagnosis of FSHD and enhanced our comprehensive understanding of this complex genetic disorder at the genetic, epigenetic, and transcriptomic levels(*136, 137*).

HTS and Genetic Aspects of FSHD

The primary screening for FSHD aims to confirm the D4Z4 repeat size and haplotype, with most laboratories using Southern blotting(*138*). To improve availability of FSHD testing, molecular combing (MC) and single-molecule optical mapping (SMOM) have been introduced for FSHD diagnosis(*139-141*). MC technology employs fluorescent labeling to visualize and map genetic sequences like D4Z4 by directly examining individual DNA molecules at approximately 1 kb resolution. SMOM, on the other hand, maps individual DNA molecules by using fluorescently labeled nucleotides at restriction enzyme sites, allowing high-resolution fluorescence imaging.

Recent advances in long-read sequencing technologies, including Oxford Nanopore Technologies (ONT), allow analysis of single DNA molecules spanning thousands of bases and offer significant benefits for resolving repetitive genomic sequences(*142*). Long-read genomic sequencing methods can detect pathogenic FSHD1 alleles by sequencing the complete D4Z4 repeat array in a single read, thereby enabling detection of the permissive 4qA haplotype(*143, 144*). Combining short-read and long-read genome sequencing on a suspected FSHD family, Li et al. identified a pathogenic FSHD allele containing the 4qA permissive haplotype and 5 repeat units at the D4Z4 locus, demonstrating the superior accuracy and completeness achieved through third-generation sequencing applications(*145*).

Long-read sequencing is now also being explored for prenatal diagnosis. Wang et al. reported a case where a mother with a family history of FSHD underwent amniocentesis at seventeen weeks(*146*). ONT analysis of fetal DNA from amniotic fluid identified the contracted D4Z4 array and 4qA allele inheritance pattern. These findings were validated through haplotype analysis. Since the fetus was diagnosed with FSHD1, the study proved that ONT sequencing can serve as a direct and reliable method for prenatal FSHD1 diagnosis.

Whole-genome sequencing (WGS) and whole-exome sequencing (WES) have also been widely used in FSHD diagnosis and research(*147-149*). Short-read sequencing-based WGS and WES cannot directly benefit FSHD2 research because the D4Z4 repeat size and haplotype cannot be determined. However, numerous studies using these sequencing technologies have identified a large number of pathogenic variants of disease modifiers, providing excellent insights for further understanding the genetic factors of FSHD. Recently, a study including 126 patients with clinical symptoms of FSHD characterized them through D4Z4 sizing, methylation analysis, and WES(*147*). Analysis of WES data revealed 20 variants of interest: 14 were found in

established genetic modifiers of D4Z4 (*SMCHD1*, *DNMT3B*, and *LRIF1*), while 6 occurred in candidate genes (*CTCF*, *DNMT1*, *DNMT3A*, *EZH2*, and *SUV39H1*). The majority of these variants appeared alongside permissive short D4Z4 alleles (4-7 repeats) or borderline/long alleles (8-20 repeats), suggesting that various genes might influence disease variability when combined with an FSHD-permissive genetic background.

Long-read sequencing-based WGS overcomes the technical limitations in repeat coverage. Huang et al. used an ONT-based WGS to identify 59 haplotypes (35 4qA and 24 4qB) and D4Z4 repeat size in 29 samples, showing 100% concordance with the Optical Genome Mapping (OGM) results(*150*). Additionally, WES can easily correct misdiagnoses, as demonstrated by Leidenroth et al., who corrected a misdiagnosis from FSHD2 to limb girdle muscular dystrophy 2A through whole-exome analysis(*151*). These studies clearly demonstrate the unique advantages of HTS technologies in the clinical diagnosis of FSHD and in deciphering the complexity of pathogenic genes.

HTS and Epigenetic Aspects of FSHD

Several clinical features of FSHD, such as variable penetrance, gender differences in severity, asymmetric muscle atrophy, and discordance in identical twins, suggest that FSHD development involves epigenetic factors(*152*). The D4Z4 array is a large satellite DNA element rich in CpG (73%)(*153*). Decreased methylation in this region contributes to FSHD pathogenesis and provides a dependable diagnostic marker for the disease(*154*).

Bisulfite sequencing is widely applied for high-resolution methyl-CpG analysis of the D4Z4 repeat in FSHD. The distinct distribution patterns of methylation levels can differentiate between healthy individuals and patients with FSHD1 or FSHD2(*155-157*). Bisulfite sequencing assays measure methylation levels at the terminal D4Z4 repeat unit within permissive haplotype alleles. It also characterizes the methylation profile across the complete D4Z4 repeat array on chromosome 4q35. Based on methylation profiles, individuals diagnosed with FSHD1 have only distal hypomethylation, whereas those exhibiting both overall and distal hypomethylation are diagnosed with FSHD2. Beyond its role as a diagnostic marker, methylation is also considered an indicator of disease severity(*157, 158*). FSHD2 patients show a qualitative correlation between disease severity and D4Z4 methylation levels(*156*).

The ONT-based approach has been used to infer methylation levels in FSHD as well. Since the first attempt to apply ONT sequencing for FSHD suggested that the sequencing coverage of D4Z4 arrays is not sufficient, an advanced approach by combining ONT sequencing with Cas9-targeted enrichment of 4q and 10q D4Z4 arrays was further employed for comprehensive genetic analysis(*25, 143, 159, 160*). Use of the Cas9-targeting strategy can determine the 4q and 10q D4Z4 repeat sizes at base-pair resolution. The researchers also found uneven methylation patterns that vary with array length in both 4q and 10q D4Z4 sequences, with peak methylation occurring around 10 D4Z4 repeat units, matching the established pathogenic contraction threshold. High-resolution analysis of individual D4Z4 repeat methylation revealed hypomethylated regions near CTCF/insulator regions and hypermethylated regions preceding the DUX4 transcription start site(*159*). Thus, nanopore sequencing opens the door for comprehensive sequencing of D4Z4 arrays and enables the determination of CpG methylation within the arrays, which is a crucial aspect for further advancing FSHD genetic analyses.

HTS and Transcriptomic Aspects of FSHD

Application of short-read RNA sequencing in FSHD

Gene expression studies in FSHD have evolved from low-throughput quantification methods, such as RT-qPCR, to a probe-based microarray approach and, eventually, to comprehensive NGS technologies, which provide unprecedented insights in both *in vitro* and *in vivo* research. Gene expression analyses based on short-read RNA-seq (SR RNA-seq) have been conducted extensively in FSHD, as discussed in the previous sections. These transcriptome-based studies provide critical insights into the molecular mechanisms underlying FSHD pathogenesis and the cytotoxicity mediated by DUX4.

When applied to patient muscle biopsies, bulk SR RNA-seq initially faced challenges in detecting DUX4 target genes due to the dilution effect of heterogeneous cellular populations and focal pathology (Figure 4). Despite these limitations, transcriptome analyses of FSHD muscle tissue identified differentially expressed genes involved in immunity and extracellular matrix organization(*161*). Moreover, SR RNA-seq studies have successfully identified transcriptional signatures associated with disease activity as well. Research has shown that DUX4 signatures are significantly upregulated in STIR-positive muscles (indicating active pathology), while PAX7 signatures appear to be valuable markers for disease progression(*161, 162*). Another study from our group using SR RNA-seq on 39 FSHD and 24 control muscle biopsies revealed that DUX4 and PAX7 signatures serve as partially overlapping biomarkers in FSHD, each exhibiting unique expression profiles in affected muscle biopsies. When correlated with imaging-based parameters, like Turbo Inversion Recovery Magnitude (TIRM) hyperintensity and fat fraction, these signatures appear to reflect distinct aspects of disease activity and progression. This not only suggests that non-invasive imaging-based biomarkers could enhance detection of FSHD-associated transcriptional changes in clinical trials, but also demonstrates the value of SR RNA-seq in identifying and validating molecular biomarkers for FSHD(*110*). Wong et al. identified numerous differentially expressed genes in mildly FSHD-affected muscles compared to controls, with a subset capable of distinguishing affected muscles with high accuracy (AUC-ROC: 0.9), highlighting progression-specific gene expression features(*163*). The well-defined molecular signature provides a valuable tool for early disease detection and monitoring, offering potential endpoints for clinical trials and new targets for therapeutic intervention.

Application of long-read RNA sequencing in FSHD

While SR RNA-seq has significantly advanced transcriptomic studies, it presents fundamental limitations in capturing full-length transcripts. Assembling short sequencing reads (100-150 bp) creates significant challenges for accurately identifying isoforms, particularly when a single locus produces multiple overlapping transcript variants(*164*). SR RNA-seq cannot clearly determine the connectivity between distant exons, and it struggles with accurately resolving genes with complex structures or repetitive elements(*165*).

The implementation of long-read RNA sequencing (LR RNA-seq) technologies, including Pacific Biosciences (PacBio) and ONT platforms, addresses these limitations through sequencing the full-length transcripts. PacBio Iso-Seq generates high-fidelity (HiFi) reads

exceeding 10 kb, while ONT platforms enable real-time sequencing of native RNA molecules with lengths surpassing 20 kb(*166, 167*). These advanced technological capabilities facilitate improved transcript annotation accuracy and enhanced detection of alternative splicing events.

The application of LR RNA-seq in FSHD research has enabled high-resolution characterization of DUX4-induced transcriptional perturbations. Mitsuhashi et al. demonstrated the utility of ONT-based direct RNA sequencing in DUX4-inducible rhabdomyosarcoma cell lines, facilitating the detection of differential expression patterns in known DUX4-regulated genes and the identification of differentially expressed repeat loci proximal to DUX4 binding sites(*168*). The long-read based approach revealed hundreds of previously uncharacterized gene-ERV fusion transcripts in addition to the ones described in an earlier study(*69*), highlighting the complexity of DUX4-mediated transcriptional dysregulation and providing deeper insights into FSHD molecular pathogenesis. In chapter 5, we established an isoform-resolved transcriptome specific to DUX4-expressing myoblasts using PacBio Iso-seq, which allows for uncovering the transcriptional changes mediated by DUX4 at the isoform level (Figure 4).

Furthermore, the capability of ONT direct RNA sequencing to sequence native RNA molecules enables simultaneous analysis of transcript structure and RNA modifications at single-nucleotide resolution(*169, 170*). It works by recording electric intensity changes as RNA passes through nanopores, with modifications causing distinctive shifts that allow computational identification of modified bases. RNA modifications like N6-methyladenosine (m6A) are vital for post-transcriptional regulation, yet no studies have examined their role in FSHD pathogenesis(*171*). The application of ONT direct RNA sequencing technology presents an opportunity to investigate potential epitranscriptomic regulatory mechanisms in FSHD, potentially revealing novel layers of molecular complexity in disease progression. Integration of these epitranscriptomic insights with existing molecular and cellular analyses could provide a more comprehensive understanding of FSHD pathophysiology, potentially identifying novel therapeutic targets within the RNA modification landscape.

Summary

The application of HTS in FSHD has significantly enhanced our understanding of the disease mechanisms and identified potential therapeutic targets. By uncovering the specific molecular mechanisms involved in derepression of DUX4 and the molecular pathways dysregulated by DUX4 expression, these studies have guided the development of targeted therapies aimed at silencing DUX4 or mitigating its downstream effects. Finding reliable biomarkers using these methods could enhance clinical tracking of disease progression and therapeutic response in upcoming treatment studies.

As sequencing technologies continue to evolve, emerging methodologies such as single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics are providing even finer resolution of muscle biology. These approaches enable the dissection of transcriptomic heterogeneity within skeletal muscle, offering new insights into how specific cell populations contribute to disease progression. Despite their many challenges, such as high cost and computational complexity, the integration of multi-omics data with RNA-seq is expected to further advance

our understanding of muscular disorders, ultimately accelerating the development of targeted therapies.

Single-fiber RNA-seq in muscle biology

Bulk RNA-seq has significantly expanded our understanding of skeletal muscle biology and disease. However, its application to complex muscle tissues is limited by the heterogeneous composition of skeletal muscle, which consists of multinucleated myofibers alongside amongst others satellite cells, fibroblasts, endothelial cells, and immune cells(*172*). Because bulk RNA-seq captures an aggregate transcriptional profile from these diverse cell populations, it often dilutes muscle fiber-type-specific signals and obscures disease-relevant molecular changes. This limitation is particularly problematic in disorders with focal pathology or sporadic gene expression, such as FSHD, where key molecular alterations may be masked by the dominant signal from unaffected fibers.

Single-fiber RNA sequencing (sfRNA-seq) may overcome some of these challenges by enabling transcriptomic profiling at the level of individual myofibers (Figure 4). This approach allows for classifying fibers based on molecular signatures, investigating fiber-type-specific transcriptional programs, and detecting subtle yet functionally significant gene expression alterations that would otherwise be lost in bulk analyses. A study using sfRNA-seq in whole muscle from young and old mice has identified fiber-type-specific transcriptional networks, elucidated differential responses to aging, and revealed adaptive molecular changes(*173*). Similarly, recent application of sfRNA-seq in exercise physiology has demonstrated distinct transcriptional responses between fast and slow fibers following high-intensity interval training, revealing temporal gene expression patterns that extend beyond traditional fiber-type classifications and contribute to our understanding of exercise-induced adaptations at the single fiber level(*174*).

We anticipate that applying sfRNA-seq to FSHD research could yield deeper insights into the molecular consequences of DUX4 activation at the resolution of individual muscle fibers. By isolating and profiling the transcriptomes of single fibers, researchers can accurately identify DUX4-positive fibers and characterize their specific gene expression program and fiber type. While current sfRNA-seq protocols are limited in tracking spatial relationships between fibers, careful sample annotation may help preserve some contextual information. Similarly, although true temporal resolution is not yet achievable with this method, analyzing fibers at defined stages of disease or differentiation could provide indirect insights into disease progression. Importantly, it is to be expected that sfRNA-seq offers a powerful tool for evaluating therapeutic strategies targeting DUX4, enabling precise molecular readouts of treatment efficacy at the single-fiber level.

SfRNA-seq is poised to become an indispensable tool in muscle disease research. Its ability to resolve fiber-specific transcriptomic changes, particularly in disorders characterized by sporadic gene expression such as FSHD, represents a major advancement over traditional bulk RNA-seq approaches. Future studies integrating sfRNA-seq with spatial transcriptomics and single-nucleus RNA-seq will further refine our understanding of muscle biology and FSHD pathology, enabling the mapping of fiber-type-specific regulatory networks and distinguishing

primary from secondary disease effects. These advancements will not only deepen our knowledge of muscular dystrophies but also accelerate therapeutic development.

Single-cell and single-nucleus RNA sequencing in muscle biology

ScRNA-seq and snRNA-seq are essential techniques in muscle biology, enabling researchers to examine gene expression at single-cell or single-nucleus resolution. These technologies overcome the limitations of bulk RNA-seq, which averages gene expression across diverse cell populations, masking cell-type-specific signals and rare cellular states. Given the cellular complexity of skeletal muscle including amongst others multinucleated myofibers, satellite cells, FAPs, endothelial cells, and immune cells, single-cell and single-nucleus approaches are crucial for uncovering distinct transcriptional programs in muscle development, regeneration, and disease(*172, 175, 176*).

For mononuclear muscle cells, scRNA-seq thus provides a rapid, high-throughput and high-resolution approach that can decipher muscle development muscle regeneration processes and capture rare cell populations or transitional cell states during specific process windows. ScRNA-seq has identified transcriptional signatures of myogenic progenitors, FAPs, and immune cells during muscle regeneration, revealing key regulatory networks and signaling pathways that drive tissue repair(*177*). It has also elucidated the dynamic transitions of satellite cells, highlighting their molecular heterogeneity and identifying distinct subpopulations involved in muscle regeneration. Additionally, scRNA-seq has uncovered rare and transient cell states contributing to myogenesis, such as previously unrecognized regenerative intermediate cell populations(*175, 178*). For mononuclear muscle cells, scRNA-seq thus provides a rapid, high-throughput, and high-resolution approach that can decipher muscle development, muscle regeneration processes, and capture rare cell populations or transitional cell states during specific process windows.

SnRNA-seq is an alternative approach, particularly valuable for studying skeletal muscle, specifically addressing challenges raised by the multinucleated nature of fully differentiated myofibers and mature muscle tissue. It has revealed remarkable heterogeneity within myonuclei, demonstrating that nuclei within the same myofiber can express distinct transcriptional programs. Beyond characterizing myogenic nuclei, snRNA-seq has revealed specialized myonuclear domains at critical functional interfaces. Studies have identified distinct transcriptional signatures in nuclei positioned at neuromuscular junctions (NMJ) and myotendinous junctions (MTJ), which express unique gene sets like *Ache*, *Etv5*, and *Col22a1* that support specialized functions(*176, 179*). While the application of snRNA-seq in muscle biology presents unique challenges, such as the inability to determine which nuclei originate from the same myofiber, it has emerged as an invaluable tool for high-resolution transcriptomic analysis of mature skeletal muscle, advancing our understanding of myonuclear heterogeneity.

The application of these technologies to FSHD research has been particularly valuable (Figure 4). In FSHD, scRNA-seq and snRNA-seq have enhanced the characterization of DUX4-mediated pathogenesis. The sporadic and low-frequency expression of DUX4 in affected muscle tissue makes it challenging to use bulk RNA-seq. Single-cell and single-nucleus sequencing allow for precise identification and molecular characterization of rare DUX4-expressing myoblasts and nuclei, offering new insights into disease mechanisms. Two studies

have employed these approaches to facilitate the identification of DUX4-expressing cells or nuclei responsible for pathogenic transcriptional changes, enabling detailed comparisons between affected and unaffected muscle cells in FSHD(*102, 180*). In chapter 2, we applied snRNA-seq to fully differentiated myotube cultures derived from a healthy control individual and three FSHD patients, revealing two DUX4-affected nuclear populations exhibiting distinct transcriptomic features in the FSHD myotube cultures. Our snRNA-seq analysis provides novel insights into cellular heterogeneity and understanding DUX4-mediated cytotoxicity in FSHD. Furthermore, growing evidence suggests that integration of scRNA-seq and snRNA-seq data can reveal complex cellular hierarchies and regulatory networks and lead to the identification of potential therapeutic targets, which could be beneficial for FSHD research. Moreover, these technologies are invaluable for assessing therapeutic efficacy, enabling researchers to evaluate treatment responses at a cellular level and determine the impact of interventions on specific muscle cell populations.

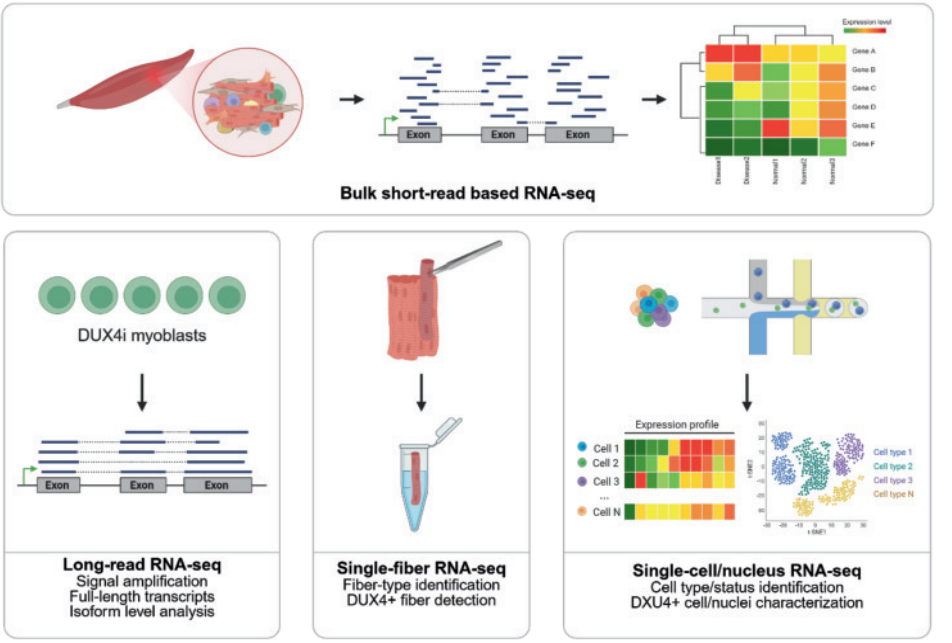

Figure 4. RNA sequencing technologies used in this thesis.

This figure illustrates the comprehensive RNA sequencing strategies used in this thesis to address the limitations of conventional bulk RNA-seq in detecting DUX4-mediated effects. Bulk short-read based RNA-seq (top panel) demonstrates the limitation of analyzing heterogeneous muscle tissue, where RNA extraction from mixed cell populations results in averaged expression signals that mask the true DUX4-induced effects due to the sporadic nature of DUX4 expression. To overcome these limitations, three specialized RNA-seq approaches are employed. LR RNA-seq (bottom left) uses DUX4i myoblasts to achieve synchronized DUX4 expression and signal amplification, enabling detection of full-length transcripts and comprehensive isoform-level analysis of DUX4-induced effects. Single-fiber RNA-seq (bottom middle) employs manual isolation of individual muscle fibers to enable fiber-type identification and specific detection of DUX4-affected fibers, circumventing the signal dilution problem by analyzing discrete fiber units. Single-nucleus RNA-seq (bottom right) performs nuclear isolation and single-nucleus capture to generate expression profiles for individual nuclei, enabling precise identification of DUX4-affected nuclei and their transcriptomic characterization. These targeted approaches provide enhanced resolution and sensitivity to detect authentic DUX4-mediated transcriptomic changes that would otherwise be obscured in bulk tissue analysis.

Multi-Omics approaches in FSHD

The advent of high-throughput technologies has revolutionized our ability to study biological systems at unprecedented molecular resolution. Multi-omics represents an integrative approach that combines different "omics" technologies to provide a comprehensive view of biological processes. Unlike single-omics approaches focusing on one molecular level like transcriptomics

or proteomics, multi-omics integrates data from multiple molecular layers to capture the complex interplay between different biological components and establish a comprehensive picture(*181*). Thus, the multi-omics approach is particularly valuable for studying complex diseases like FSHD, where pathogenic mechanisms span multiple regulatory levels.

In this thesis, we focus on three key omics layers that follow the central dogma of molecular biology, creating a framework that enables us to trace the flow of genetic information from chromatin regulation to protein synthesis. The dynamic interplay between these layers provides insights that would be impossible to obtain from any single approach alone.

FSHD represents a unique genetic disorder where aberrant DUX4 expression affects multiple molecular processes, making it an ideal candidate for multi-omics investigation. DUX4 functions as a pioneer transcription factor that orchestrates widespread changes across several regulatory layers. It is known that DUX4 influences chromatin status(*58, 71*). Transcriptionally, DUX4 activates hundreds of normally silenced genes, including other transcription factors that create regulatory cascades and feedback loops(*49, 69, 101, 182*). DUX4 simultaneously affects RNA processing, extending its impact to genes not directly targeted. These changes ultimately manifest at the translational and protein levels, altering biological functions in affected muscle cells(*82, 104*). The multi-level regulatory impact makes FSHD particularly suited for multi-omics analysis as no single molecular approach could capture how these diverse perturbations collectively contribute to muscle pathology. The comprehensive nature of DUX4's effects requires an equally comprehensive analytical strategy.

Epigenomics studies the regulatory mechanisms that control gene expression without changing the underlying DNA sequence, such as DNA methylation, chromatin accessibility, and histone modifications, which together decide which genes can be transcribed. In FSHD research, epigenomic approaches have been instrumental in understanding the molecular basis of the disease. ChIP-seq (Chromatin Immunoprecipitation followed by sequencing) has been applied to study DUX4 binding profiles. It is able to identify DNA binding sites by first cross-linking proteins to their bound DNA, then fragmenting the chromatin, and finally using antibodies to selectively enrich for DNA fragments bound to the protein of interest. Extensive DUX4 ChIP-seq studies have identified numerous target genes and revealed that DUX4 can bind to repetitive elements, providing crucial insights into its transcriptional regulatory network(*49, 51, 53*). Histone modification ChIP-seq studies, which use antibodies specific to modified histones, have demonstrated alterations in repressive histone H3 lysine 9 trimethylation (H3K9me3) and activating H3 lysine 27 acetylation (H3K27ac) marks in FSHD muscle cells, helping to explain the dysregulated transcriptional environment characteristic for the disease(*56, 183, 184*). Reduced H3K9me3 levels at the D4Z4 array have been specifically linked to FSHD development. Zeng et al. demonstrated that SUV39H1-dependent H3K9 trimethylation normally present at D4Z4 in healthy cells is absent in FSHD patients. This chromosome 4q/10q-specific epigenetic change disrupts SMCHD1 recruitment to D4Z4, resulting in chromatin activation and elevated DUX4 expression(*183*).

ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing) represents another technology in epigenomic research, identifying open chromatin regions that may function as regulatory elements(*185*). In the FSHD field, there is currently limited evidence from ATAC-

seq studies demonstrating DUX4's regulatory impact on chromatin accessibility. Future research using ATAC-seq will be essential for understanding DUX4's influence on chromatin states, particularly in the context of skeletal muscle cells, where chromatin remodeling plays a crucial role during cell fusion and multinucleated myotube formation. More robust applications of ATAC-seq have been demonstrated in early embryonic development, where footprinting analysis of ATAC-seq data from human preimplantation embryos has revealed stage-specific chromatin accessibility patterns associated with the presence of DUX4. This study shows clear DUX4 footprints during the 2-cell to 4-cell stages that progressively diminish at the 8-cell stage and disappear in later stages(*186*), perfectly aligning with DUX4's expression pattern during ZGA. Furthermore, ATAC-seq studies of Dux overexpression in mouse embryonic stem cells demonstrated that Dux/DUX4 not only increases chromatin accessibility at its target sites but also decreases accessibility at binding sites for pluripotency factors like *Nanog*, *Oct4*, and *Sox2*. These findings highlight DUX4's fundamental role in regulating chromatin architecture and reveal how it orchestrates genome-wide accessibility changes during early development, potentially informing our understanding of aberrant DUX4 activity in FSHD.

The evaluation of methylation levels is also a key factor for FSHD. Reduced representation bisulfite sequencing (RRBS) is a technique that converts unmethylated cytosines to uracils while leaving methylated cytosines unchanged before sequencing(*187*). It has been employed to characterize global methylation patterns in FSHD2 patients. Mason et al. observed reduced methylation levels associated with a loss of SMCHD1 binding and increased expression for certain loci, advancing our understanding of epigenetic dysregulation genome-wide(*188*). These epigenomic analyses serve as the foundation for understanding the upstream regulatory changes that drive aberrant gene expression in FSHD.

Building on these epigenetic insights, transcriptomics analysis reveals the consequent alterations in gene expression. As extensively discussed in previous sections, transcriptomic profiling has been invaluable in identifying DUX4 target genes, characterizing expression patterns in different cellular contexts, and revealing the heterogeneity of DUX4 expression in FSHD muscle tissue. The greatest value of transcriptomics within a multi-omics framework lies in its ability to connect epigenetic changes to downstream functional consequences, serving as the critical bridge between regulatory mechanisms and phenotypic outcomes. Integrating transcriptomic data with epigenomic information allows us to establish direct links between chromatin states and gene expression patterns, thereby constructing a more coherent picture of the molecular cascade initiated by DUX4 activation in FSHD.

The translatome represents the next layer in this cascade, capturing the subset of mRNAs that are actively being translated into proteins and thereby bridging the gap between transcriptomics and proteomics. This layer reveals post-transcriptional regulation and translational control, which have emerged as crucial factors in FSHD pathophysiology. Recent ribosome profiling sequencing (Ribo-seq), a technique that sequences only the mRNA fragments protected by ribosomes to provide a genome-wide snapshot of active translation, has been applied in DUX4-inducible myoblast cell lines, uncovering that DUX4 dramatically reshapes the translational landscape. Ribo-seq analysis revealed that abnormal RNAs accumulating during DUX4 expression are translated into truncated proteins, particularly shortened RNA-binding proteins

(RBPs) and splicing factors, which then disrupt RNA processing and translation patterns while contributing to cell death(*104*). Furthermore, combined RNA-seq and polysome profiling analyses, where mRNAs are separated based on the number of bound ribosomes to assess translation efficiency, have demonstrated that DUX4 mediates cellular reprogramming through differential translational regulation, simultaneously suppressing global protein synthesis while maintaining preferential translation of DUX4-induced transcripts associated with early developmental pathways(*111*). This reprioritization of the cellular translational machinery represents a previously underappreciated mechanism through which DUX4 exerts its pathogenic effects.

The integration of epigenomics, transcriptomics, and translatomics provides a comprehensive model of FSHD pathogenesis that reveals DUX4's cascading effects across molecular networks. The integrated approach offers several key advantages. Multi-omics integration identifies critical points where DUX4's effects amplify into pathological outcomes, distinguishing primary from secondary effects and highlighting potential therapeutic targets. The approach may offer explanations for FSHD's variable penetrance and asymmetric presentation by revealing how molecular alterations at different levels contribute to pathology in diverse contexts. The integration may enable the creation of comprehensive biomarker panels that may provide reliable indicators of DUX4 activity, facilitating patient stratification and therapeutic monitoring. By mapping the flow of information from chromatin changes to translational alterations, we can anticipate that multi-omics would reveal how initial DUX4 expression progresses to muscle cell damage through interconnected molecular pathways. Furthermore, the approach may highlight potential intervention points at multiple regulatory levels, from epigenetic silencing of DUX4 to blocking downstream translational dysregulation. In conclusion, integrated multi-omics provides a powerful framework for understanding FSHD pathology and holds significant potential for advancing precision medicine approaches for this complex muscular dystrophy.

Thesis outline

The transcription factor DUX4 activates a broad network of target genes and repetitive elements in skeletal muscle, triggering a cascade of downstream events that ultimately lead to cell death and muscle wasting. Understanding the transcriptional and post-transcriptional changes induced by DUX4 is therefore crucial for unraveling its pathological impact. In this thesis, by applying multiple high-throughput sequencing technologies in different DUX4-expressing contexts, we provide a comprehensive view of how DUX4 influences gene regulation and cell fate. Our findings offer critical insights into why DUX4, despite its normally repressed state and sporadic expression in FSHD, can drive such severe muscle degeneration. Notably, DUX4 exerts profound effects across multiple molecular layers, from transcriptional regulation to epigenetic modifications and translational control, acting much like a multifunctional Swiss Army knife. These discoveries deepen our understanding of DUX4-mediated cytotoxicity and its role in FSHD pathology.

In chapter 2, we employed snRNA-seq to comprehensively profile transcriptional heterogeneity in primary myotube cultures derived from FSHD patients and healthy donors. This high-

resolution approach revealed two distinct populations of DUX4-affected nuclei with distinct transcriptional profiles. These populations exhibited heterogeneity in oxidative stress responses and myogenic inhibition, two key FSHD-associated pathways. Additionally, DUX4-affected nuclei shared transcriptomic features with early embryonic cells, extending beyond the cleavage stage into later developmental stages. The comprehensive analyses of snRNA-seq highlight the complexity of DUX4-induced transcriptional states in FSHD muscle at the single-nucleus level and provides important insight into the cellular diversity underlying disease pathology.

In chapter 3, we employed sfRNA-seq to investigate DUX4-positive fibers within muscle biopsies from FSHD patients. SfRNA-seq approach enabled the isolation and transcriptomic profiling of individual muscle fibers, allowing for the distinction between affected and unaffected fibers within the same tissue sample. While it does not provide full spatial context, the method retains partial anatomical information, offering a unique window into the localized impact of DUX4 activation. Our analysis successfully detected sporadic DUX4-affected fibers within FSHD muscle tissue and demonstrated the feasibility of capturing their transcriptomic profiles, providing a foundation for future studies to explore fiber-level pathological mechanisms.

In chapter 4, to address the limitations of traditional 2D cell culture systems, a three-dimensional tissue-engineered skeletal muscle (3D-TESM) model was developed from iPSC-derived myogenic progenitors of FSHD patients to better recapitulate disease pathology *in vitro*. Compared to controls, 3D-TESMs from FSHD-affected cells exhibited enhanced DUX4 target gene expression, smaller myofiber diameters, and reduced contractile force. 3D TESMs, therefore, provide a good and more physiologically relevant model of FSHD than 2D cultures alone. Drug testing in 3D-TESMs revealed potential limitations of compounds identified in 2D screens, highlighting the model's utility for preclinical research and future drug development. This work demonstrates the importance of tissue architecture in modulating cellular behavior and DUX4-mediated effects, providing a more physiologically relevant platform for investigating FSHD pathogenesis.

In chapter 5, the integration of PacBio Iso-Seq and SR RNA-seq on DUX4-inducible myoblast cell lines allows for the construction of a comprehensive isoform-resolved transcriptome, featuring numerous unannotated isoforms and novel intergenic loci activated by DUX4. Through comparative analysis with 4-cell stage embryonic cells, we established, for the first time, the context-specific nature of DUX4-mediated isoform regulation. The comparison analysis reveals distinct transcriptional programs activated by DUX4 in different cellular contexts, providing crucial insights into its dual roles in early embryonic development and FSHD pathology.

Lastly, in chapter 6, I provide a comprehensive summary of the main findings from all studies and discuss their implications and future perspectives. Additionally, the limitations of these studies are discussed in this chapter.

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