

# FSH Society Facioscapulothumeral Muscular Dystrophy [FSHD] 2010 International Research Consortium & Research Planning Meetings

Sponsored by:



**Boston Biomedical Research Institute**  
Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center for FSHD Research



# **FSH Society Facioscapulohumeral Muscular Dystrophy [FSHD] 2010 International Research Consortium & Research Planning Meetings**

Thursday, October 21, 2010

7:30 a.m. – 6:30 p.m.

&

Friday, October 22, 2010

7:30 a.m. – 2:00 p.m.

**Boston Biomedical Research Institute**  
64 Grove Street, Watertown, Massachusetts 02472 USA

**Co-Chairs: Rabi Tawil, M.D.**

*University of Rochester Medical Center, Rochester, New York USA &  
Fields Center for FSHD and Neuromuscular Research*

**Silvère van der Maarel, Ph.D.**

*Leiden University Medical Center, Leiden, the Netherlands &  
Fields Center for FSHD and Neuromuscular Research*

**Organizers: Daniel Paul Perez**

*FSH Society, Inc.*

**Silvère van der Maarel, Ph.D.**

**Rabi Tawil, M.D.**

**Hosted By:**

FSH Society, Inc.

NIH Eunice Kennedy Shriver NICHD Boston Biomedical Research Institute Senator  
Paul D. Wellstone Muscular Dystrophy Cooperative Research Center

**Sponsored By:**

Association Française Contre les Myopathies (AFM)

The Fields Center for FSHD and Neuromuscular Research

FSH Society

FSHD Global Research Foundation

NIH Eunice Kennedy Shriver NICHD Boston Biomedical Research Institute

Senator Paul D. Wellstone MDCRC

Muscular Dystrophy Association United States (MDAUSA)

**October 21-22, 2010**

## **PREFACE**

Dear Colleagues,

Welcome to the FSHD International Research Consortium 2010. Thanks to you, we are seeing exciting developments and increased momentum in every aspect of FSHD basic and clinical research!

Given the potential watershed event of the recent DUX4 finding, we encourage you to share your latest data and findings. Please keep platform presentations to 20 minutes including 5-10 minutes for questions and answers. Platform speakers please consider bringing a poster as well. Together we will review last year's research priorities and reprioritize them in light of recent developments. We are also expanding on the format of the meeting. A series of topical discussions (working sessions) will be lead by a pair of distinguished scientists whose role is to provide a stimulating overview of the topic and facilitate discussion. Working sessions are limited to "chalk talks." Moderators will present and introduce each working session topic and summarize findings in the subsequent overall group discussions. This meeting is a working meeting with experts, developing future plans in the context of what we know now. In the interest of time, we would like to emphasize new data and ideas and to avoid restating data that has already been published. Be sure to connect with funding agencies including the NIH at the workshop. New investigators should consider attending the "NIH Grants Workshop for Beginners," offered by Ljubisa Vitkovic, Ph.D., Program Director, NIH NICHD.

The goal of this meeting is to explore and verify the complex genetic mechanism and various features of FSHD, to allow us to move quickly to the development of potential treatments for FSHD. 2010 has brought quite a significant increase in government, non-profit, and private funding for FSHD. It has also ushered in an international collaboration of volunteer health agencies and FSHD patients working side-by-side with research and clinical communities. It is essential to keep going in this direction, and for the entire community to work together at every level to communicate clearly on programs, developments and needs.

This year's workshop participants include clinicians, scientists, biotechnology companies, pharmaceutical companies, government and non-profit funding agencies, and patients – committed to solving, treating and curing FSHD at this workshop.

This meeting is organized and sponsored by the FSH Society, the U.S. DHHS NIH Eunice Kennedy Shriver NICHD Sen. Paul D. Wellstone BBRI FSHD Muscular Dystrophy Cooperative Research Center, the Association Française Contre les Myopathies (AFM), the Muscular Dystrophy Association (MDAUSA), FSHD Global Research Foundation, and the Fields Center for FSHD and Neuromuscular Research. It is truly a pleasure to come together to accelerate solutions for FSHD!

Thank you for your extraordinary efforts and hard work on behalf of patients and their families.

Sincerely,

Daniel Paul Perez  
FSH Society, Inc., Watertown, Massachusetts, USA

Rabi Tawil, M.D.  
University of Rochester Medical Center, Rochester, New York USA &  
Fields Center for FSHD and Neuromuscular Research

Silvère van der Maarel, Ph.D.  
Leiden University Medical Center, Leiden, the Netherlands &  
Fields Center for FSHD and Neuromuscular Research

*The FSH Society, Inc. (Facioscapulohumeral Muscular Dystrophy) is an independent, non-profit 501(c)(3) and tax-exempt U.S. corporation organized to address issues and needs specifically related to facioscapulohumeral muscular dystrophy (FSHD). Contributions and financial donations are acknowledged for tax purposes. All inquiries should be addressed to: FSH Society, Inc., Daniel Paul Perez, 11 Elmbrook Circle, Bedford, Massachusetts 01730 USA. Phone: (781) 275-7781, fax: (781) 275-7789, e-mail: [daniel.perez@fshsociety.org](mailto:daniel.perez@fshsociety.org), website: <http://www.fshsociety.org>*

## Day 1

Thursday, October 21, 2010

Registration & Continental Breakfast	7:30-8:00	
Welcome	8:00-8:30	
Platform Session 1	8:30-9:30	<b>Genetics</b> (3x20 minutes)
Platform Session 2	9:30-10:30	<b>Genes &amp; Associated Pathophysiology</b> (3x20 minutes)
Break & Poster Session 1	10:30-11:00	
Platform Session 3	11:00-12:20	<b>Models &amp; Therapeutic Targets</b> (4x20 minutes)
Lunch & Poster Session 2	12:20-14:00	[Cafeteria]
Lunch & Grants Workshop	13:00-14:00	<b>NIH Grants Workshop for Beginners</b> [2 <sup>nd</sup> Floor Conf. Room]
Platform Session 4	14:00-15:40	<b>Molecular Biology, Chromatin &amp; Epigenetics</b> (5x20 minutes)
Break & Poster Session 3	15:40-15:50	
Working Sessions 1-4	15:50-16:50	<b>Concurrent Discussion Groups of 20</b>

Working Group 1 [Conference Room 2<sup>nd</sup> Floor]

**What are the genetics telling us?**

Working Group 2 [Conference Room 3<sup>rd</sup> Floor]

**Summarize the genes, cascades and associate with the pathophysiology of FSHD?**

Working Group 3 [Cafeteria]

**What resources are available and what is missing?**

Working Group 4 [Main Lecture Hall]

**What experimental approaches might ultimately be directed toward ameliorative therapies?**

Break & Poster Session 4	16:50-17:10	
Group Reports 1	17:10-18:10	<b>Entire Group Discussion/Recommendations</b>
Wrap Up Day 1	18:10-18:25	<b>Summary</b>
Dinner on your own		

## Day 2

Friday, October 22, 2010

Registration & Continental Breakfast	7:30-8:00	
Platform Session 5	8:00-9:00	<b>Insights from the Clinic</b> (3x20 minutes)
Review Session 1	9:00-10:00	<b>Recap of Last Year's Priorities as Stated by the Group</b>
Break & Poster Session 5	10:00-11:00	
Working Sessions 5-8	11:00-12:00	<b>Concurrent Discussion Groups of 20</b>
Working Group 5 [Main Lecture Hall] <b>What do we know about the pathological progression of disease?</b>		
Working Group 6 [Cafeteria] <b>What are the gaps in our knowledge? Things if known would support or refute our current hypotheses</b>		
Working Group 7 [Conference Room 2 <sup>nd</sup> Floor] <b>Where do we go from here?</b>		
Working Group 8 [Conference Room 3 <sup>rd</sup> Floor] <b>Thinking out of the box</b>		
Lunch & Poster Session 6	12:00-12:30	
Group Reports 2	12:30-13:30	<b>Entire Group Discussion/Recommendations</b>
Wrap Up Day 2	13:30-14:00	
Adjourn		

	<b>First Author</b>	<b>Presenting Author</b>	<b>Session</b>
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**Day 1**

Chairs: van der Maarel & Tupler

8:30-8:50 a.m.	Lemmers	Lemmers	Genetics
8:50-9:10 a.m.	Greco	Tupler	Genetics
9:10-9:30 a.m.	Pollington	Hewitt	Genetics

Chairs: Tapscott & Hartweck

9:30-9:50 a.m.	Geng	Geng	Genes & Associated Pathophysiology
9:50-10:10 a.m.	Tsumagari	Ehrlich	Genes & Associated Pathophysiology
10:10-10:30 a.m.	Leidenroth	Leidenroth	Genes & Associated Pathophysiology

Chairs: Wentworth & Harper

11:00-11:20 a.m.	Bosnakovski	Hartweck	Models & Therapeutic Targets
11:20-11:40 a.m.	Vanderplank	Belayew	Models & Therapeutic Targets
11:40-12:00 p.m.	Wallace	Harper	Models & Therapeutic Targets
12:00-12:20 p.m.	Chamberlain	Chamberlain	Models & Therapeutic Targets

Chairs: Ehrlich & Belayew

14:00-14:20 p.m.	Corona	Rosa	Mol. Biology, Chromatin & Epigenetics
14:20-14:40 p.m.	Geng	Tapscott	Mol. Biology, Chromatin & Epigenetics
14:40-15:00 p.m.	D.G. Miller	D.G. Miller	Mol. Biology, Chromatin & Epigenetics
15:00-15:20 p.m.	Rabaia	Filippova	Mol. Biology, Chromatin & Epigenetics
15:20-15:40 p.m.	Block	Block	Mol. Biology, Chromatin & Epigenetics

**Day 2**

Chairs: Tawil & Padberg

8:00-8:10 a.m.	Lunt	Lunt	Insights from the Clinic
8:10-8:20 a.m.	Upadhyaya	Upadhyaya	Insights from the Clinic
8:20-8:40 a.m.	Heatwole	Heatwole	Insights from the Clinic
8:40-9:00 a.m.	Fabbri	Tupler	Insights from the Clinic

**Posters**

[	Kazakov	Kazakov	Poster
	Rahimov	Rahimov	Poster
	Reed	Reed	Poster
	Reed	Bloch	Poster
	Scionti	Tupler	Poster
	Siebenthal	Siebenthal	Poster
	Tassin	Tassin	Poster
	Tsumagari	Ehrlich	Poster
	Zuch	Zuch	Poster
	]		

## Suggested Topics for FSH Society FSHD Working Groups

### Day One

#### Working Session 1. **What are the genetics telling us?**

[Conference Room 2nd Floor]

Silvère van der Maarel, Ph.D., Co-moderator & Rossella Tupler, M.D., Ph.D. , Co-moderator

1. Overview (25 minutes)
  - a. Clinical findings
  - b. Families
    - i. Penetrance and mosaicism
    - ii. Unlinked
  - c. Allele specificity
2. Why DUX4 ? (25 minutes)
  - a. What are the numbers?
  - b. What inconsistent observations are there?
3. List other encoded features with consistent genetics? (10 minutes)

#### Working Session 2. **What genes might be involved in the pathophysiology of FSHD?**

[Conference Room 3rd Floor]

Charles P. Emerson, Jr., Ph.D. & Stephen J. Tapscott, M.D., Ph.D., Co-moderator

1. DUX4 (20 minutes)
2. FRG1, FRG2 and other 4q35 loci (10 minutes)
4. PitX1, Pax3, Pax7 and other impacted distributed loci (10 minutes)
5. Related cascades and pathways. (10 minutes)
6. Others.... (10 minutes)

#### Working Session 3. **What resources are available and what is missing?**

[Cafeteria]

Scott Harper, Ph.D. Co-moderator & Melanie Ehrlich, Ph.D., Co-moderator

1. Biopsied materials (10 minutes)
2. Cell lines (15 minutes)
  - a. Human
  - b. Mouse
  - c. Others
3. Data (10 minutes)
  - a. Genetic
  - b. Sequence
  - c. Gene expression
  - d. Proteomic
4. Animal models (15 minutes)
  - a. Mice
    - i. Knock outs
    - ii. Knock ins
    - iii. Conditionals
  - b. Others
5. Priorities (10 minutes)
  - a. Antibodies
  - b. Animals
6. Contract Arrangements for production of priority materials (10 minutes)
  - a. Cell facilities
  - b. Animal facilities



Working Session 4. **What experimental approaches could be directed toward ameliorative therapies?**

[Main Lecture Hall]

Lynn Hartweck, Ph.D., Co-moderator & David Glass, M.D., Ph.D., Co-moderator

1. Development of Biomarkers (20 minutes)
2. DUX4 (20 minutes)
3. Ace031 (10 minutes)
4. Others.... (10 minutes)

**Day 2**

Working Session 5. **What do we know about the pathological progression of disease?**

[Main Lecture Hall]

Rabi Tawil, M.D., Co-moderator & Peter Lunt, Ph.D., Co-moderator

1. Summary of pathology findings (20 minutes)
2. Patient definition by clinical criteria vs. molecular criteria (10 minutes)
  - a. Phenocopies
  - b. incomplete penetrance etc.
3. Are the genetic studies confounded by ascertainment bias? (10 minutes)
4. Alternative hypothesis? (20 minutes)

Working Session 6. **What are the gaps in our knowledge ?– things if known would support or refute our current hypotheses.**

[Cafeteria]

Robert J. Bloch, Ph.D., Co-moderator & Jane E. Hewitt, Ph.D., Co-moderator

1. Links between phenomena observed in excised muscle and cellular models (15 minutes)
2. Links between observations in human cell models and animal cell models (15 minutes)
3. Consistency in the central dogma (DNA-> RNA-> Protein) (15 minutes)
4. Muscle biology (15 minutes)

Working Session 7. **Where do we go from here?**

[Conference Room 2nd Floor]

Silvère van der Maarel, Ph.D., Co-moderator & Louis M. Kunkel, Ph.D., Co-moderator

1. Next steps: Canvass all the attendees (15 minutes)
2. What's needed: Canvass all the attendees (15 minutes)
3. If money were no object (15 minutes)
4. Who's doing what? (15 minutes)

Working Session 8. **Thinking out of the box.**

[Conference Room 3rd Floor]

Alexandra Belayew, Ph.D., Co-moderator & Peter Jones, Ph.D., Co-moderator

1. What doesn't add up? (20 minutes)
2. What have we missed? (20 minutes)
3. What isn't being done that could be? (20 minutes)

**Day 1, Thursday, October 21, 2010**

**7:30 a.m.-8:00 a.m.**

**REGISTRATION & CONTINENTAL BREAKFAST**

**8:00 a.m.-8:10 a.m.**

**WELCOME**

William R. Lewis Sr., M.D.  
Chairman of the Board, FSH Society, Monterey, California USA

Daniel Paul Perez  
President & CEO, FSH Society, Watertown, Massachusetts USA

**8:10 a.m.-8:20 a.m.**

**CO-CHAIRS OPENING REMARKS &  
CHARGE TO THE MEETING ATTENDEES**

Rabi Tawil, M.D.  
University of Rochester Medical Center, Rochester, New York USA &  
Fields Center for FSHD and Neuromuscular Research

Silvère van der Maarel, Ph.D.  
Leiden University Medical Center, Leiden, the Netherlands &  
Fields Center for FSHD and Neuromuscular Research

**8:20 a.m.-8:30 a.m.**

**CO-HOST OPENING REMARKS**

Charles P. Emerson, Jr., Ph.D.  
Boston Biomedical Research Institute, Watertown, Massachusetts USA &  
U.S. NIH Eunice Kennedy Shriver NICHD Boston Biomedical Research Institute Senator Paul D. Wellstone Muscular  
Dystrophy Cooperative Research Center

**8:30 a.m.-9:30 a.m.**

**PLATFORM PRESENTATIONS I**

Silvère van der Maarel, Ph.D., Co-moderator  
Leiden University Medical Center, Leiden, the Netherlands &  
Fields Center for FSHD and Neuromuscular Research  
&

Rossella Tupler, M.D., Ph.D. , Co-moderator  
Dipartimento di Scienze Biomediche, Università di Modena e Reggio Emilia, Modena, Italia  
Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester, Massachusetts USA

**GENETICS**

8:30-8:50 a.m.

Richard J.L.F. Lemmers, Ph.D.

Department of Human Genetics, Leiden University Medical Center, the Netherlands

### **A unifying genetic model for facioscapulohumeral muscular dystrophy**

8:50-9:10 a.m.

Rossella Tupler, M.D., Ph.D.

Dipartimento di Scienze Biomediche, Università di Modena e Reggio Emilia, Modena, Italia

Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester, Massachusetts USA

### **Genotype-phenotype correlation in a large cohort of FSHD families reveals the incomplete penetrance of the D4Z4 molecular defect**

9:10-9:30 a.m.

Jane E. Hewitt, Ph.D.

Institute of Genetics, School of Biology, University of Nottingham, Queen's Medical Centre, Nottingham, NG7 2UH United Kingdom

### **Investigating nucleotide variation at D4Z4**

**9:30 a.m.-10:30 a.m.**

#### **PLATFORM PRESENTATIONS II**

Lynn Hartweck, Ph.D., Co-moderator

Lillehei Heart Institute and Department of Pediatrics, University of Minnesota, Minneapolis, Minnesota USA

&

Stephen J. Tapscott, M.D., Ph.D., Co-moderator

Fred Hutchinson Cancer Research Center, Seattle, Washington USA

#### **GENES & ASSOCIATED PATHOPHYSIOLOGY**

9:30-9:50 a.m.

Linda N Geng, Ph.D.

Fred Hutchinson Cancer Research Center, Seattle, Washington USA

### **DUX4 expression in control and FSHD human muscle cells and biopsies**

9:50-10:10 a.m.

Melanie Ehrlich, Ph.D.

Human Genetics Program and the Department of Biochemistry, Tulane Medical School, New Orleans, Louisiana 70112 USA

### **Expression profiling of FSHD and control myogenic precursors reveals disease-linked downregulation of muscle-specific gene expression and identifies new myogenesis genes**

10:10-10:30 a.m.

Andreas Leidenroth, Ph.D.

**Distant cousin, twice removed? Why we should care about *DUX* gene family relationships**

**10:30 a.m.-11:00 a.m.**

**POSTER VIEWING 1 & MORNING BREAK**

**11:00 a.m.-12:20 p.m.**

**PLATFORM PRESENTATIONS III**

Bruce Wentworth, Ph.D., Co-moderator  
Genzyme Corporation, Framingham, Massachusetts USA  
&

Scott Harper, Ph.D. Co-moderator  
Department of Pediatrics, Ohio State University &  
Molecular, Cellular, and Developmental Biology Program, Ohio State University &  
Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio USA

**MODELS & THERAPEUTIC TARPETS**

11:00-11:20 a.m.

Lynn Hartweck, Ph.D.

Lillehei Heart Institute and Department of Pediatrics, University of Minnesota, Minneapolis, Minnesota USA

**New cellular and animal models for FSHD**

11:20-11:40 a.m.

Alexandra Belayew, Ph.D.

Laboratory of Molecular Biology, University of Mons, Belgium

**Suppression of *DUX4* or *DUX4c* protein expression by antisense strategies as a therapeutic approach for FSHD**

11:40 a.m.-12:00 p.m.

Scott Q. Harper, Ph.D.

Department of Pediatrics, Ohio State University &  
Molecular, Cellular, and Developmental Biology Program, Ohio State University &  
Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio USA

**Targeting *FRG1* as proof-of-principle for RNAi therapy of dominant muscular dystrophies**

12:00-12:20 p.m.

Joel R. Chamberlain, Ph.D.

Department of Medicine, University of Washington, Seattle, Washington 98195 USA

**Development of *in vivo* RNAi therapeutics as a treatment for FSHD**

**12:20 p.m.-2:00 p.m.**

**BUFFET LUNCH & POSTER VIEWING 2**

**1:00 p.m.-2:00 p.m.**

**BUFFET LUNCH &**

**NIH GRANTS WORKSHOP FOR BEGINNERS [2<sup>nd</sup> FLOOR CONFERENCE ROOM]**

**2:00 p.m.-3:40 p.m.**

**PLATFORM PRESENTATIONS IV**

Alexandra Belayew, Ph.D., Co-moderator

Laboratory of Molecular Biology, University of Mons, Mons, Belgium

&

Melanie Ehrlich, Ph.D., Co-moderator

Human Genetics Program and the Department of Biochemistry, Tulane Medical School, New Orleans, Louisiana 70112 USA

**MOLECULAR BIOLOGY, CHROMATIN & EPIGENETICS**

2:00-2:20 p.m.

Alberto Luis Rosa, M.D., Ph.D.

Laboratorio de Biología Celular y Molecular, Fundación Allende, Hipólito Irigoyen Córdoba, Argentina

**Multiple protein domains contribute to nuclear entrance of DUX4**

2:20-2:40 p.m.

Stephen J. Tapscott, M.D., Ph.D.

Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington USA

**DUX4 expression in control human tissues**

2:40-3:00 p.m.

Daniel G. Miller, M.D., Ph.D.

Department of Pediatrics, University of Washington USA

Institute for Stem Cell and Regenerative Medicine, Seattle, Washington USA

**Developmental regulation of DUX4 transcription using FSHD-patient derived iPS cells**

3:00-3:20 p.m.

Galina N. Filippova, Ph.D.

Fred Hutchinson Cancer Research Center, Seattle, Washington 98109 USA

University of Washington, Seattle, Washington, 98195 USA

**Role of CTCF in developmentally regulated silencing of D4Z4**

3:20-3:40 p.m.

Gregory J. Block, M.D., Ph.D.

Department of Pediatrics, University of Washington

Institute for Stem Cell and Regenerative Medicine, Seattle, Washington

### **Wnt signaling regulates *DUX4* expression**

3:40-3:50 p.m.

### **POSTER VIEWING 3 & BREAK**

3:50 p.m.-4:50 p.m.

### **WORKING SESSIONS 1-4**

#### **Working Group 1 [Conference Room 2nd Floor]**

Silvère van der Maarel, Ph.D., Co-moderator

Leiden University Medical Center, Leiden, the Netherlands &  
Fields Center for FSHD and Neuromuscular Research

&

Rossella Tupler, M.D., Ph.D., Co-moderator

Dipartimento di Scienze Biomediche, Università di Modena e Reggio Emilia, Modena, Italia

Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester, Massachusetts USA

#### **What are the genetics telling us?**

1. Overview (25 minutes)
  - a. Clinical findings
  - b. Families
    - i. Penetrance and mosaicism
    - ii. Unlinked
  - c. Allele specificity
2. Why DUX4 ? (25 minutes)
  - a. What are the numbers?
  - b. What inconsistent observations are there?
3. List other encoded features with consistent genetics? (10 minutes)

#### **Working Group 2 [Conference Room 3rd Floor]**

Charles P. Emerson, Jr., Ph.D. Co-moderator

Boston Biomedical Research Institute, Watertown, Massachusetts USA &

U.S. NIH Eunice Kennedy Shriver NICHD Boston Biomedical Research Institute Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center

&

Stephen J. Tapscott, M.D., Ph.D., Co-moderator

Fred Hutchinson Cancer Research Center, Seattle, Washington USA

#### **Summarize the genes, cascades and associate with the pathophysiology of FSHD?**

1. DUX4 (20 minutes)
2. FRG1, FRG2 and other 4q35 loci (10 minutes)
3. PitX1, Pax3, Pax7 and other impacted distributed loci (10 minutes)
4. Related cascades and pathways. (10 minutes)
5. Others.... (10 minutes)

### **Working Group 3 [Cafeteria]**

Scott Harper, Ph.D. Co-moderator

Department of Pediatrics, Ohio State University &

Molecular, Cellular, and Developmental Biology Program, Ohio State University &

Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio  
&

Melanie Ehrlich, Ph.D., Co-moderator

Human Genetics Program and the Department of Biochemistry, Tulane Medical School, New Orleans, Louisiana 70112 USA

### **What resources are available and what is Missing?**

1. Biopsied materials (10 minutes)
2. Cell lines (15 minutes)
  - a. Human
  - b. Mouse
  - c. Others
3. Data (10 minutes)
  - a. Genetic
  - b. Sequence
  - c. Gene expression
  - d. Proteomic
4. Animal models (15 minutes)
  - a. Mice
    - i. Knock outs
    - ii. Knock ins
    - iii. Conditionals
  - b. Others
5. Priorities (10 minutes)
  - a. Antibodies
  - b. Animals
6. Contract Arrangements for production of priority materials (10 minutes)
  - a. Cell facilities
  - b. Animal facilities

### **Working Group 4 [Main Lecture Hall]**

Lynn Hartweck, Ph.D., Co-moderator

Lillehei Heart Institute and Department of Pediatrics, University of Minnesota, Minneapolis, Minnesota USA  
&

David Glass, M.D., Ph.D., Co-moderator

Novartis Institutes for Biomedical Research, Inc., Cambridge, Massachusetts

FSH Society, Inc. FSHD International Research Consortium & Research Planning Meeting. October 21-22, 2010 ©FSH Society 2010.

## **What experimental approaches might ultimately be directed toward ameliorative therapies?**

1. Development of Biomarkers (20 minutes)
2. DUX4 (20 minutes)
3. ACE031 (10 minutes)
4. Others.... (10 minutes)

**4:50 p.m.-5:10 p.m.**

### **POSTER VIEWING 4 & BREAK**

**5:10 p.m.-6:10 p.m.**

### **GROUP REPORTS 1 [Main Lecture Hall]**

David Housman, Ph.D., Co-moderator

Massachusetts Institute of Technology, Cambridge, Massachusetts USA

&

Michael Altherr, Ph.D., Co-moderator

Los Alamos National Laboratory, Los Alamos, New Mexico USA

### **Entire Group Discussion/Recommendations**

**6:10 p.m.-6:25 p.m.**

### **SUMMARY and WRAP-UP DAY 1**

David Housman, Ph.D., Co-moderator

Massachusetts Institute of Technology, Cambridge, Massachusetts USA

&

Michael Altherr, Ph.D., Co-moderator

Los Alamos National Laboratory, Los Alamos, New Mexico USA



**DAY 2, Friday, October 22, 2010**

**7:30 a.m.-8:00 a.m.**

**REGISTRATION & CONTINENTAL BREAKFAST**

**8:00 a.m.-9:00 a.m.**

**PLATFORM PRESENTATIONS V**

Rabi Tawil, M.D.

University of Rochester Medical Center, Rochester, New York USA &  
Fields Center for FSHD and Neuromuscular Research  
&

George W.A.M. Padberg, M.D., Ph.D.

UMC St. Radboud, Nijmegen, the Netherlands

**INSIGHTS FROM THE CLINIC**

8:00-8:10 a.m.

Peter Lunt, Ph.D.

Clinical Genetics Department, St. Michael's Hospital, Bristol BS2 8EG, United Kingdom

**Dissecting FSHD pathogenesis**

8:10-8:20 a.m.

Meena Upadhyaya, Ph.D.

Institute of Medical Genetics, Cardiff University, Heath Park, Cardiff CF14 4XN, United Kingdom

**Confirmation that the specific SSLP microsatellite allele 4qA161 segregates with facioscapulohumeral dystrophy in a large cohort of multiplex and simplex FSHD families**

8:20-8:40 a.m.

Chad Heatwole, M.D.

The University of Rochester Medical Center, Rochester, New York

**An update on the FSHD-specific Quality-of-Life outcome measure: identifying what is most important to FSHD patients**

8:40-9:00 a.m.

Rossella Tupler, M.D., Ph.D.

Dipartimento di Scienze Biomediche, Università di Modena e Reggio Emilia, Modena, Italia

Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester, Massachusetts USA

**Unexpected large number of compound heterozygotes revealed by cumulative effects of D4Z4 mutation**

**9:00 a.m.-10:00 a.m.**

## **REVIEW SESSION I**

Rune R. Frants, Ph.D., Co-moderator  
Leiden University Medical Center, Leiden, the Netherlands  
&

Charles P. Emerson, Jr., Ph.D.  
Boston Biomedical Research Institute, Watertown, Massachusetts USA &  
U.S. NIH Eunice Kennedy Shriver NICHD Boston Biomedical Research Institute Senator Paul D. Wellstone Muscular  
Dystrophy Cooperative Research Center

## **DISCUSSION OF LAST YEAR'S PRIORITIES AS IDENTIFIED BY THE GROUP**

1. Clinical trials readiness: In order to proceed efficiently into human trials, there needs to be several elements in place:
  - a. Patient access through registries: National FSHD Registry, FSH Society, TREATNMD, others.
  - b. Appropriate outcome measures: primary and secondary, suitable for early phase trials and efficacy trials (clinically meaningful measures).
  - c. Surrogate markers: Imaging (MRI,CT, DEXA) and/or biomarkers (discussed in session 3)
2. Epigenetics / Genetics. This line of work will be instrumental to pinpoint the real identity of FSHD1 and FSHD2. This information will form the basis for evidence-based intervention.
  - a. Modifying genes for FSHD1 (large inter-individual variation in symptoms)
  - b. Identify the FSHD2 gene (common molecular pathway with FSHD1)
  - c. Further work on the chromatin structure / function relationship
3. Biomarkers. There is obvious need for monitoring intervention
  - a. Systems biology
    - i. transcriptomics, proteomics, metabolomics etc.
  - b. In situ (RNA, protein) to detect cellular heterogeneity
4. Model systems. Urgent need for more specific model systems for mechanistic, intervention work and advancement to clinical trials.
  - a. Cellular
    - i. Biopsies
    - ii. Mosaics
    - iii. iPS
  - b. Animal
    - i. Mouse – inducible / humanized mouse etc.
    - ii. Other species

**10:00 a.m.-11:00 a.m.**

## **POSTER VIEWING 5 & BREAK**

**11:00 a.m.-12:00 p.m.**

## **WORKING SESSIONS 5-8**

### **Working Group 5 [Main Lecture Hall]**

Rabi Tawil, M.D., Co-moderator

University of Rochester Medical Center, Rochester, New York USA &  
Fields Center for FSHD and Neuromuscular Research

&

Peter Lunt, Ph.D., Co-moderator

Clinical Genetics Department, St. Michael's Hospital, Bristol BS2 8EG, United Kingdom

#### **What do we know about the pathological progression of disease?**

1. Summary of pathology findings (20 minutes)
2. Patient definition by clinical criteria vs. molecular criteria (10 minutes)
  - a. Phenocopies
  - b. incomplete penetrance etc.
3. Are the genetic studies confounded by ascertainment bias? (10 minutes)
4. Alternative hypothesis? (20 minutes)

### **Working Group 6 [Cafeteria]**

Robert J. Bloch, Ph.D., Co-moderator

Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland USA &  
U.S. NIH Eunice Kennedy Shriver NICHD Boston Biomedical Research Institute Senator Paul D. Wellstone Muscular  
Dystrophy Cooperative Research Center

&

Jane E. Hewitt, Ph.D., Co-moderator

Institute of Genetics, School of Biology, University of Nottingham, Queen's Medical Centre, Nottingham, NG7 2UH United  
Kingdom

#### **What are the gaps in our knowledge? – Things if known would support or refute our current hypotheses**

1. Links between phenomena observed in excised muscle and cellular models (15 minutes)
2. Links between observations in human cell models and animal cell models (15 minutes)
3. Consistency in the central dogma (DNA-> RNA-> Protein) (15 minutes)
4. Muscle biology (15 minutes)

### **Working Group 7 [Conference Room 2nd Floor]**

Silvère van der Maarel, Ph.D., Co-moderator

Leiden University Medical Center, Leiden, the Netherlands &  
Fields Center for FSHD and Neuromuscular Research

&

Louis M. Kunkel, Ph.D., Co-moderator

Program in Genomics and Division of Genetics, Children's Hospital Boston, Harvard Medical School, Boston,  
Massachusetts;<sup>6</sup>Howard Hughes Medical Institute, Boston, Massachusetts & U.S. NIH Eunice Kennedy Shriver NICHD Boston  
Biomedical Research Institute Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center

## **Where do we go from here?**

1. Next steps: Canvass all the attendees (15 minutes)
2. What's needed: Canvass all the attendees (15 minutes)
3. If money were no object (15 minutes)
4. Who's doing what? (15 minutes)

### **Working Group 8** [Conference Room 3rd Floor]

Alexandra Belayew, Ph.D., Co-moderator

Laboratory of Molecular Biology, University of Mons, Mons, Belgium

&

Peter Jones, Ph.D., Co-moderator

Boston Biomedical Research Institute, Watertown, Massachusetts USA

### **Thinking out of the box**

1. What doesn't add up? (20 minutes)
2. What have we missed? (20 minutes)
3. What isn't being done that could be? (20 minutes)

**12:00 p.m.-12:30 p.m.**

### **BUFFET LUNCH & POSTER VIEWING 6**

**12:30 p.m.-1:30 p.m.**

### **GROUP REPORTS 2**

Robert H. Brown, Jr., M.D., D.Phil., Co-moderator

University of Massachusetts Medical School, Worcester, Massachusetts USA

&

Michael Altherr, Ph.D., Co-moderator

Los Alamos National Laboratory, Los Alamos, New Mexico USA

### **Entire Group Discussion/Recommendations**

**1:30 p.m.-2:00 p.m.**

### **SUMMARY and WRAP-UP DAY 2**

Charles P. Emerson, Jr., Ph.D., Co-moderator

Boston Biomedical Research Institute, Watertown, Massachusetts USA &

U.S. NIH Eunice Kennedy Shriver NICHD Boston Biomedical Research Institute Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center

&

Rune R. Frants, Ph.D., Co-moderator

Leiden University Medical Center, Leiden, the Netherlands

1.

## **A unifying genetic model for facioscapulohumeral muscular dystrophy**

Richard JLF Lemmers<sup>1</sup>, Patrick J. van der Vliet<sup>1</sup>, Rinse Klooster<sup>1</sup>, Sabrina Sacconi<sup>2</sup>, Pilar Camaño<sup>3</sup>, Johannes G. Dauwerse<sup>1</sup>, Lauren Snider<sup>4</sup>, Kirsten R. Straasheijm<sup>1</sup>, Gert Jan van Ommen<sup>1</sup>, George W. Padberg<sup>5</sup>, Daniel G. Miller<sup>6</sup>, Stephen J. Tapscott<sup>4</sup>, Rabi Tawil<sup>7</sup>, Rune R. Frants<sup>1</sup>, Silvère M. van der Maarel<sup>1</sup>

Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands  
Centre de reference pour les maladies Neuromusculaires and CNRS UMR6543, Nice University, Nice, France

Department of Neurosciences, BioDonostia Health Research Institute, Hospital Donostia, San Sebastián, Spain

Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Department of Neurology, Radboud University Medical Center Nijmegen, Nijmegen, The Netherlands

Department of Pediatrics, University of Washington, Seattle, WA, USA

Department of Neurology, University of Rochester Medical Center, NY, USA

Based on several DNA markers, we identified many genetic D4Z4 variants (haplotypes) on chromosome 4q and 10q. We showed that a contraction of the D4Z4 repeat array on some of these haplotypes are causing FSHD, while contractions on many other haplotypes do not. We identified sequence variations in D4Z4 between different haplotypes and hypothesized that these variations are responsible for the difference in the pathogenicity of the haplotypes.

Previously it was shown that each D4Z4 unit contains an evolutionary conserved open reading frame encoding for the double homeobox gene *DUX4*. In contrast to internal units, the distal D4Z4 unit seems to result in a stable *DUX4* transcript as it incorporates sequences distal to the repeat where it detects a polyadenylation (poly(A)) signal in the chromosomal region distal to D4Z4.

We performed detailed genotyping and D4Z4 sequencing of permissive and non-permissive chromosomes and in FSHD patients with an unusual genotype that were selected worldwide after a complex molecular diagnostic outcome. We show that FSHD patients carry specific single nucleotide polymorphisms (SNPs) creating a canonical poly(A) signal which stabilizes *DUX4* transcripts. All permissive chromosomes carry a canonical *DUX4* poly(A) signal, while non-canonical poly(A) signals were detected in non-permissive chromosomes. Transfection studies revealed that *DUX4* transcripts are efficiently polyadenylated and are more stable when expressed from permissive chromosomes. These findings suggest that FSHD arises through a toxic gain of function attributable to the stabilized distal *DUX4* transcript. Our study not only explains the striking haplotype-specificity of the disorder, but also provides a genetic mechanism that unifies all genetic observations in all patients with FSHD.

2.

## **Genotype-phenotype correlation in a large cohort of FSHD families reveals the incomplete penetrance of the D4Z4 molecular defect.**

Francesca Greco<sup>a</sup>, Ilaria Frambolli<sup>a</sup>, Carlo Borsato<sup>b</sup>, Michelangelo Cao<sup>b</sup>, Liliana Vercelli<sup>c</sup>, Maura Servida<sup>d</sup>, Lorenzo Peverelli<sup>d</sup>, Luca Colantoni<sup>e</sup>, Giulia Ricci<sup>f</sup>, Leda Volpi<sup>f</sup>, Rita Di Leo<sup>g</sup>, Claudia Manzoli<sup>h</sup>, Paola Cudia<sup>i</sup>, Ebe Pastorello<sup>l</sup>, Leopoldo Ricciardi<sup>h</sup>, Roberto Frusciante<sup>m</sup>, Isabella Scionti<sup>a</sup>, Monica Govi<sup>a</sup>, Cristina Forghieri<sup>a</sup>, Emanuela Bonifazi<sup>a</sup>, Morandi Lucia<sup>i</sup>, Trevisan Carlo<sup>l</sup> Pietro, Gabriele Siciliano<sup>f</sup>, Giuliana Galluzzi<sup>e</sup>, Enzo Ricci<sup>m</sup>, Di Muzio Antonio<sup>h</sup>, Carmelo Rodolico<sup>g</sup>, Giuliano Tomelleri<sup>n</sup>, Maurizio Moggio<sup>d</sup>, Laura Palmucci<sup>c</sup>, Corrado Angelini<sup>b</sup>, Lucio Santoro<sup>o</sup>, Rossella Tupler<sup>a,p</sup>.

a) University of Modena e Reggio Emilia, Dipartimento Scienze Biomediche, Modena, Italy; b), University of Padua, Department of Neurosciences, Padua, Italy; c) University of Turin, Department of Neurosciences, Turin, Italy; d) IRCCS Fondazione Ospedale Maggiore Policlinico Mangiagalli Regia Elena, Dino Ferrari Center, University of Milan, Department of Neurology, Milan, Italy; e) IRCCS Santa Lucia Foundation, Rome, Italy; f) University of Pisa, Department of Neuroscience, Pisa, Italy; g) University of Messina, Department of Neurosciences, Messina, Italy; h) Neuromuscular Disease Unit, Cenetr of Excellence on Aging, G.D'Annunzio University Foundation, Ospedale SS Annunziata, Neurological Clinic, Chieti, Italy; i) National Neurologica Institute Carlo Besta, Division of Neuromuscular Diseases, Milan, Italy; l) University of Padua, Department of Neurological and Psychiatric Sciences, Padua, Italy; m) Catholic University, Department of Neuroscience, Rome, Italy; n) University of Verona, Department of Neurological Sciences and Vision, Verona, Italy; o) University of Naples Federico II, Department of Neurological Sciences, Napoli, Italy;p) Program in Gene Function and Expression, University of Massachusetts Medical School.

The facioscapulohumeral muscular dystrophy (FSHD) molecular defect consists in reduced number (<10) of a 3.3 kb sequences (D4Z4), tandemly arrayed on chromosome 4q. Since 2008 the Italian Registry for FSHD ([www.fshd.it](http://www.fshd.it)) collected clinical and molecular data from 771 subjects carrying D4Z4 alleles ranging between 1-8 D4Z4 units. FSHD clinical severity was numerically defined by the FSHD score, which results from the functional evaluation of six muscle groups. The FSHD score ranges from 0 (no signs) to 15 (wheelchair bound). The 771 subjects enrolled in the study have been clustered based on the FSHD score, to verify whether D4Z4 units number, age, sex affect FSHD clinical expression.

Analysis of our data, showed:

- 76% of subjects, carrying fewer than 3 D4Z4 units, received FSHD score  $\geq 5$ , indicating an inverse correlation between the D4Z4 allele size and disease severity;
- 30% of patients younger than 45 received FSHD score  $\geq 5$ ; by contrast, 57% of patients older than 45 received FSHD score  $\geq 5$ , suggesting that aging influences FSHD clinical expression;
- 29% of subjects younger than 40 years display no muscle weakness. This high percentage of healthy subjects is maintained in all age classes (22% 41-60 years, 18% 61-90 years);
- 57% of subjects carrying the molecular defect of FSHD, who received FSHD score 0 or 1 are women, indicating that gender might influence the severity of the disease outcome.
- 

Our study also revealed that in the cohort examined the first sign of the disease is facial weakness in 52% of subjects. Besides we detected weakness of scapular girdle, abdominal, distal leg, or upper limbs

muscles in respectively 45,5%, 1,2%, 0,3% and 1% of subjects. Involvement of pelvic girdles appears in all subjects afterwards. The 4q35 haplotype, including 4qA/4qB polymorphism, proximal SSLP and SNP in the p-LAM region, has been characterized in a subgroup of chromosomes 4 carrying FSHD-sized alleles. Interpretation of results obtained from this analysis is discussed.

We are grateful to the FSHD Italian families for their unlimited support. This work has been funded by Telethon-UILDM (Grant GUP08004), Association Française contre les Myopathies.

3.

### **Investigating nucleotide variation at D4Z4**

Jo Pollington, Laura Mitchell, Amanda Hampson, Gemma Dennis, Andreas Leidenroth, John Armour and Jane E Hewitt

Centre for Genetics and Genomics, School of Biology, The University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH, United Kingdom

e-mail address:

JEH: jane.hewitt@nottingham.ac.uk

We have been focusing on the sequence architecture of D4Z4 and the DUX4 coding region. It is clear that D4Z4 deletions are not sufficient for FSHD to occur; these deletions have to arise on a permissive haplotype for the disease to be manifested. Therefore, we have been interested in studying the patterns of nucleotide variation near and within the D4Z4 array. This association of only a subset of 4q35 haplotypes with FSHD suggested that specific variants within or near to D4Z4 might be causal in this disease. Recently, Lemmers *et al.* reported that the causative variant is a non-canonical poly (A) sequence just distal to D4Z4.

In our study we investigated in more detail the extent to which the linkage disequilibrium (LD) previously reported for the p13E-11 region extended into the D4Z4 repeat itself. We also wanted to examine the level of nucleotide variation within individual D4Z4 arrays, particularly within the DUX4 open reading frame. We used PCR to amplify the proximal D4Z4 repeat from a number of haplotypes and cloned and sequenced this region. We also carried out detailed analysis of intra-array nucleotide variation, initially focusing on chromosomes carrying the 4qA161, 10qA166, 4qB168 and 4qB163 haplotypes.

In agreement with the recent publication from Lemmers *et al.*, our data show that the LD extends into the D4Z4 array. However, we did identify some recombinant haplotypes. Sequencing of multiple D4Z4 repeats from within individual arrays identified a number of nucleotide differences, some of which were haplotype-specific. We found no frameshift or nonsense substitutions within DUX4 copies, indicative of selection for the ORF. By RT-PCR, we were able to identify short transcripts from multiple, independent internal repeats. Some of the nucleotide variants that we found introduce coding changes into the DUX4 ORF, although no non-synonymous changes were present within the homeobox regions. We will discuss the relevance of our data to the evolution of D4Z4 and of DUX4.

We would like to thank the Muscular Dystrophy Association, the FSH Society, Inc and the Muscular Dystrophy Campaign for funding our work.



4.

### **DUX4 expression in control and FSHD human muscle cells and biopsies**

Linda N. Geng<sup>1</sup>, Lauren Snider<sup>1</sup>, Ashlee E Tyler<sup>1</sup>, and Stephen J Tapscott<sup>1</sup>

<sup>1</sup> Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109 USA

Double homeobox 4 (DUX4) is one of the leading candidate disease genes for facioscapulohumeral muscular dystrophy (FSHD). It was recently shown the presence of a favorable polyadenylation signal in the 4qA161 haplotype is required for a contracted D4Z4 array to cause FSHD, suggesting that stabilization of the DUX4 mRNA might be a causal molecular mechanism. Although prior studies have reported expression of DUX4 mRNA in FSHD muscle cells, these studies have not assessed the relative expression of alternative splice forms of DUX4 and have not shown a correlation between the abundance of the mRNA and the DUX4 protein. We reported the presence of multiple DUX4 splice forms in muscle cells, including full-length (DUX4-fl) and a short splice form (DUX4-s). The full-length form contains the entire DUX4 open reading frame, while the short form results in truncation of more than two thirds of the coding sequence on the 3-prime end. Our goal was to determine the relationship between the splice forms and disease state. We examined a large set of muscle biopsies and primary cultured muscle cells from FSHD and control individuals and found that the full-length polyadenylated DUX4-fl transcript is only detected in FSHD samples, while the DUX4-s polyadenylated short splice form is found in both FSHD and control samples that contain the 4A161 allele, but not in samples without the 4A161 allele. In order to assess the protein expression of DUX4 in muscle samples, we developed mouse and rabbit monoclonal antibodies against the N- and C-termini of human DUX4, the latter of which is not shared by DUX4c. Lentiviral-mediated expression of the full-length DUX4-fl in control human primary myoblasts resulted in cell death, and immunostaining for DUX4 revealed an initial homogeneous nuclear staining that progressed to nuclear foci as the cells initiated apoptosis. In contrast, similar lentiviral-mediated delivery of the short DUX4-s maintained a relatively homogeneous nuclear staining pattern and did not induce obvious cellular toxicity. Therefore, the C-terminus is likely the region responsible for the cell toxicity associated with full length DUX4. Additional studies to correlate the abundance of DUX4 mRNA and protein in FSHD muscle will be discussed.

## **Expression profiling of FSHD and control myogenic precursors reveals disease-linked downregulation of muscle-specific gene expression and identifies new myogenesis genes**

Koji Tsumagari<sup>1</sup>, Shiao-Chi Chang<sup>1</sup>, Jerry S. Zifodya<sup>1</sup>, Michelle Lacey<sup>2</sup>, Rabi Tawil<sup>3</sup>, Sridar V. Chittur<sup>4</sup>, Zhancheng Zhang<sup>5</sup>, Gregory E. Crawford<sup>5</sup>, and Melanie Ehrlich<sup>1</sup>

<sup>1</sup>Human Genetics Program and the Dept. of Biochemistry, Tulane Medical School, and <sup>2</sup>Department of Mathematics, Tulane University, New Orleans, LA 70112; <sup>3</sup>University of Rochester, School of Medicine and Dentistry, Rochester, NY 14642; <sup>4</sup>Center for Functional Genomics, University at Albany-SUNY, Rensselaer, NY 12144; <sup>5</sup>Institute for Genome Sciences & Policy, Duke University, Durham, NC 27708

Our recent expression profiling has revealed a distinctive gene-expression phenotype in myoblasts and myotubes derived from muscle biopsies of facioscapulohumeral muscular dystrophy (FSHD) patients. Using exon microarrays (Affymetrix), we compared FSHD myoblast and myotube preparations (three each from moderately affected muscle; 2 – 6 repeat units in the pathogenic 4q D4Z4 arrays), normal-control myoblast and myotube preparations (two each), and one disease-control set of myoblasts and myotubes (sporadic inclusion-body myositis). Each batch of cells for analysis had  $\geq 85\%$  myoblasts or  $\geq 72\%$  of the nuclei in myotubes, as determined by immunocytochemistry. The results, which were verified for representative genes on additional FSHD and normal-control cell strains by real time quantitative RT-PCR (qRT-PCR), indicate major defects in molecular pathways in FSHD at the myoblast and myotube stages. Of the  $\sim 17,000$  genes represented on the microarray, the steady-state RNA levels for 296 genes in myoblasts and 798 genes in myotubes displayed at least two-fold FSHD-linked dysregulation ( $p < 0.01$ , adjusted for multiple comparisons, Benjamini-Hochberg correction). The groups of genes exhibiting dysregulation included highly overrepresented pathways that can help explain the pathology of the disease. For example, FSHD myogenic precursors were deficient in upregulation of 82 genes encoding known muscle-associated proteins (fold change  $\geq 2$ , adjusted  $p < 0.01$  for each gene).

Our results are elucidating normal myogenesis as well as the molecular defects in FSHD. For 53 genes not previously linked to muscle in various databases, we found an association with myogenesis from our expression profiling. These genes had  $\geq 4$  fold higher steady-state levels of RNA in control myotubes than in the average of 19 non-muscle cell types and  $\geq 2$  fold higher in control myotubes than in control myoblasts (adjusted  $p < 0.01$ ). Twenty-five of these genes (47%) that we have newly associated with myogenesis were downregulated  $\geq 2$  fold in FSHD myotubes relative to control myotubes (adjusted  $p < 0.01$ ). We conclude that insufficient expression in myogenic precursors of both known and newly identified myogenesis-specific genes, rather than the previously proposed apoptosis, is a major contributor to the FSHD muscle phenotype in moderately affected muscle. (Supported in part by the National Institutes of Health [NS048859 to M.E., HG003169 to G.E.C.], and the Fields Center for FSHD and Neuromuscular Research [R.T]).

6.

### **Distant cousin, twice removed? Why we should care about *DUX* gene family relationships**

Andreas Leidenroth and Jane E Hewitt

Centre for Genetics and Genomics, School of Biology, The University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH, United Kingdom

e-mail addresses:

AL: andreas.leidenroth@gmail.com

JEH: jane.hewitt@nottingham.ac.uk

There is now strong evidence that *DUX4* is causally involved in the molecular pathogenesis of FSHD, thus the need to understand its usual *in vivo* function has become more urgent. Cellular toxicity and low endogenous expression levels make it difficult to study *DUX4* itself, and one possible alternative angle may be the investigation of some of its homologues found not only in humans but many other mammals, including model organisms. Clues about *DUX4* function could emerge from the study of other *DUX* gene family members such as *DUXA*, *DUXB*, *DUXC*, *Duxbl* and rodent *Dux*. However, it is crucial that we understand how these homologues relate to *DUX4*, as their different evolutionary histories are likely to have important consequences on their functional conservation or divergence.

Our analysis of the *DUX* family shows the distribution of different homologues across the mammalian class, including events of secondary loss. Phylogenetic comparison, analysis of gene structures and information from syntenic regions confirm the paralogous relationship of *Duxbl* and *DUXB* and characterize their relationship with *DUXA* and *DUXC*. We further identify *Duxbl* pseudogene orthologues in primates. A survey of non-mammalian genomes identified a single-homeobox gene (*sDUX*) as a likely representative homologue of the mammalian *DUX* ancestor before the homeobox duplication. Based on the gene structure maps, we suggest a possible mechanism for the generation of the *DUX* gene structure.

We will discuss how the relationships between the different *DUX* gene family members are complicated by the loss of particular homologues in certain lineages, and how this can lead to misinterpretation of their true relationships. We will highlight exactly why such information must be considered when interpreting the relevance of functional data from *DUX4* homologues such as *Dux* and *Duxbl* to FSHD.

We would like to thank the Muscular Dystrophy Campaign for funding our work with a Ph.D. studentship for A.L.

7.

### **New cellular and animal models for FSHD**

Darko Bosnakovski<sup>1,2</sup>, Lynn M. Hartweck<sup>1</sup>, Abhijit Dandapat<sup>1</sup>, John Day<sup>3</sup>, Ramiro Nandez<sup>1</sup>, Radbod Darabi<sup>4</sup>, Rita R. Perlingeiro<sup>4</sup>, Lauren Snider<sup>5</sup>, Stephen J. Tapscott<sup>5</sup>, Janet Sowden<sup>6</sup>, Rabi Tawil<sup>6</sup> and Michael Kyba<sup>1</sup>

<sup>1</sup>Lillehei Heart Institute and Department of Pediatrics, University of Minnesota, Minneapolis, MN 55455

<sup>2</sup>Faculty of Technology and Technical Science-Veles, University St. Kliment Ohridski-Bitola, Skopje, R. Macedonia

<sup>3</sup>Paul and Sheila Wellstone Center for Muscular Dystrophy and Department of Neurology, University of Minnesota, Minneapolis, MN 55455

<sup>4</sup>Lillehei Heart Institute and Department of Medicine, University of Minnesota, Minneapolis, MN 55455

<sup>5</sup>Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA 98109

<sup>6</sup>Fields Center for FSHD and Neuromuscular Research and Department of Neurology, University of Rochester, Rochester, NY 14642

There is currently no animal model bearing the actual FSHD mutation (D4Z4 contraction), and the lack of a suitable model system to study the effects of this mutation has severely hampered progress in understanding FSHD. We present data from two approaches to model FSHD: the generation of a mouse bearing a doxycycline-inducible Dux4 gene, and the differentiation of iPS cells derived from myoblasts cultures established from FSHD and control biopsies. We have inserted a construct starting at the MAL initiation of Dux4 and continuing to the end of the EcoRI site, therefore containing the pLAM polyA signal from the FSHD A haplotype, into constitutively open chromatin 5' of the HPRT gene. Even without induction we observed that males are almost never born and carrier females present several pathologies, although not in muscle. Rare males are severely affected, and do not survive past about 1 month of age. In addition to the phenotypes observed in the females, males also present testis abnormalities, including a defect in spermatogenesis. We are currently investigating vascular pathologies, which are especially evident in embryos, and severely enhanced when doxycycline is applied to induce expression of the transgene. Although muscle is not dystrophic, these are the first animals presenting a phenotype due to the presence of human D4Z4 DNA. In a second strategy, we have derived iPS cells from myoblast cultures taken from FSHD patients and controls. We present preliminary data on the ability of these cells to differentiate into muscle in vitro, and on the chromatin state and transcription of DUX4/D4Z4 at different stages of development.

8.

## **Suppression of DUX4 or DUX4c protein expression by antisense strategies as a therapeutic approach for FSHD**

C. Vanderplanck<sup>1</sup>, F. Coppée<sup>1</sup>, E. Anseau<sup>1</sup>, A. Tassin<sup>1</sup>, D. Laoudj-Chenivresse<sup>2</sup>, S.D. Wilton<sup>3</sup> and A. Belayew<sup>1</sup>

<sup>1</sup>Laboratory of Molecular Biology, University of Mons, Belgium ; <sup>2</sup>INSERM ERI 25 Muscle et Pathologies, CHU A. de Villeneuve, University of Montpellier, France ; <sup>3</sup>ANRI - Australian Neuromuscular Research Institute, Nedlands, WA, Australia.

Our group has identified the double homeobox 4 (*DUX4*) gene within each unit of the *D4Z4* repeat array at the FSHD locus (Gabriëls *et al*, 1999). It encodes a transcription factor with a double homeodomain that is expressed in FSHD but not control primary myoblasts (Kowaljew *et al*, 2007; Dixit *et al*, 2007). Using 5' and 3' RACE and RT-PCR, we determined that *DUX4* transcription could initiate in any *D4Z4* unit, but that stable mRNAs comprising the full *DUX4* ORF only derived from the most distal unit and unexpectedly extended within the flanking *pLAM* region that provided an intron and a polyadenylation signal (Dixit *et al*, 2007). The presence of this signal is required to develop FSHD as recently shown by others (Lemmers *et al*, 2010). *DUX4* activation at the FSHD locus initiates a global transcription deregulation cascade leading a.o. to muscle atrophy and differentiation defects (Dixit *et al*, 2007; Bosnakovski *et al*, 2008).

Our group has also identified the homologous *DUX4c* gene located 42-kb upstream of the *D4Z4* locus. It is expressed in muscles from healthy individuals but is also induced in FSHD. *DUX4c* over-expression in human myoblasts induced MYF5 and proliferation suggesting a role in muscle regeneration (Anseau *et al*, 2009).

No specific therapeutic strategies exist to date for FSHD. The rationale of this work is that inhibition of *DUX4* or *DUX4c* expression should prevent the transcription deregulation cascade and restore a control myotube phenotype. In order to evaluate the impact of *DUX4* or *DUX4c* inhibition, we have selected a few downstream biomarkers. These are typical FSHD deregulated genes (mu-crystallin, TP53,...) or characteristic myotubes phenotypes (atrophic/disorganized, see Barro *et al*, 2008). We found that forced *DUX4* expression in control myoblasts leads to formation of atrophied myotubes. In contrast, forced *DUX4c* expression induced  $\beta$ -catenin, abnormal accumulation of nuclei, and formation of disorganized myotubes.

We have used different antisense approaches in human myoblast cultures to either induce messenger RNA destruction by RNA interference (siRNAs) or interfere with splicing by use of specific antisense oligomers (AOs). Decrease in *DUX4* or *DUX4c* protein expression was confirmed by immunodetection on western blot and the biomarker levels changed as expected. These strategies seem promising and could contribute to future development of therapeutic approaches for FSHD.

We acknowledge funding from the AFM. CV had a FRIA (Belgium) graduate fellowship, AT and EA were post-doctoral FNRS (Belgium) associates.

References

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9.

### **Targeting FRG1 as proof-of-principle for RNAi therapy of dominant muscular dystrophies**

Lindsay M. Wallace, Sara E. Garwick, Rosella Tupler, and Scott Q. Harper

Department of Pediatrics, Ohio State University &

Molecular, Cellular, and Developmental Biology Program, Ohio State University &

Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio  
USA

Over the last 15 years, muscular dystrophy gene therapy strategies have been primarily aimed at replacing defective or missing genes underlying recessive disorders, such as Duchenne muscular dystrophy (DMD). However, these gene replacement strategies are not feasible for treating dominant diseases. Instead, patients bearing dominant mutations would likely benefit from reduction or elimination of the abnormal allele. Until very recently, there was no feasible mechanism to reduce or eliminate disease genes, and molecular therapy development for dominant muscular dystrophies was largely unexplored. RNA interference (RNAi) has recently emerged as a powerful tool to suppress any gene of interest in a sequence specific manner. As such, RNAi is a leading candidate strategy to silence dominant disease genes, including those involved in muscular dystrophies such as facioscapulohumeral muscular dystrophy (FSHD).

To demonstrate proof-of-principle for RNAi therapy of dominant myopathies, we developed AAV vectors carrying artificial microRNAs targeting FRG1, and tested their potential to correct myopathic features in FRG1-high mice. Our FRG1-targeted microRNA vectors (called miFRG1) microRNAs significantly reduced FRG1 mRNA and protein in FRG1-high muscle, and improved muscle mass, strength and histopathology. Specifically, miFRG1-transduced muscles were normal in size, showed no fibrosis or fat deposition in muscle, and had no evidence of myofiber degeneration or regeneration. Our results support the feasibility of using RNAi to target other FSHD candidate genes, such as DUX4. Moreover, since mutations in at least 37 different genes cause various dominant myopathies, our work has implications for treating dominant muscular dystrophies in general.

10.

### **Development of *in vivo* RNAi therapeutics as a treatment for FSHD**

Joel R. Chamberlain<sup>1</sup>, Sergia Bortolanza<sup>2,3</sup>, and Davide Gabellini<sup>2,3</sup>

<sup>1</sup>Department of Medicine, University of Washington, Seattle, Washington 98195 USA

<sup>2</sup>Dulbecco Telethon Institute and

<sup>3</sup>Division of Regenerative Medicine, San Raffaele Scientific Institute, Milano, Italy

Facioscapulohumeral muscular dystrophy (FSHD) is a common dominant genetic disease for which no curative therapy exists. The predominant clinical disease feature is upper body muscle weakness resulting from progressive muscle wasting. Recent progress in understanding the underlying cause of FSHD has revealed that disease likely results from both chromatin relaxation, as a consequence of contraction of D4Z4 macrosatellite repeats (FSHD1) or from other factors (FSHD2), and a functional polyadenylation sequence that is permissive for DUX4 expression. DUX4 protein expression in a cultured muscle cell line causes an increased sensitivity to oxidative stress and cell death. Cellular toxicity of DUX4 in conjunction with detection of polyadenylated DUX4 transcripts in FSHD myoblasts, suggests a DUX4 gain-of-function mechanism for FSHD. For dominantly inherited diseases associated with gain-of-function mechanisms, a straightforward therapeutic scheme would be to eliminate the genetic product that governs all pathogenic processes by using RNA interference (RNAi). The *in vivo* model chosen to test our RNAi therapeutic approach is the FRG1 mouse that displays a variety of typical physical and molecular features of FSHD patients and is the first animal model of an FSHD-like disease. Using the FRG1 mouse model we can harness the endogenous cellular RNAi pathway to degrade mRNA from genes expressing a toxic product in a targeted fashion. We have packaged U6+27 short hairpin RNA (shRNA) expression cassettes targeting FRG1 mRNA into adeno-associated virus serotype 6 (AAV6) shuttles for systemic delivery *in vivo*. Recombinant AAV6 is a very attractive delivery system due to its predominant muscle tropism, high efficiency transduction of muscle cells, and long-term expression of transgenes. Intravenous tail vein injection of  $5 \times 10^{12}$  vector genomes of the AAV6 FRG1 shRNAs into dystrophic mice resulted in significant improvements in histological features, including a reduced fiber size, central nucleation, adipose accumulation, and fibrosis. We also observed a significant improvement in muscle function as measured by treadmill running. Accompanying molecular changes included reduction of FRG1 mRNA by 40-60% relative to saline-injected controls. Our data indicate that RNAi-mediated mRNA knockdown is a feasible approach to disease therapy and can be applied after onset of symptoms of an FSHD-like muscular dystrophy in mice to reverse the course of the disease. Application of this method to target relevant components involved in the complex cascade of events leading to disease offers a route to clinical application of RNAi for treatment of FSHD.



## Multiple protein domains contribute to nuclear entrance of DUX4

Edgardo Daniel Corona, Daniela Jacquelin and Alberto Luis Rosa

Laboratorio de Biología Celular y Molecular, Fundación Allende

Hipólito Irigoyen 384 / 8vo Piso – 5000 - Córdoba, Argentina

e-mails:

edanielcorona@yahoo.com.ar; danijacquelin@gmail.com; alrosa@sanatorioallende.com

Twelve years ago<sup>1,2</sup> Belayew's and Rosa's laboratories hypothesized that shortening of the D4Z4 tandem repeat may lead to abnormal expression of a D4Z4-encoded toxic protein (i.e. DUX4) which may cause FSHD<sup>3-4</sup>. Recent elegant cellular and genetic studies from other laboratories supported and extended this toxic gain of function hypothesis<sup>5-6</sup>, dramatically increasing the interest on DUX4<sup>7</sup>. The *DUX4* gene is specifically transcribed in myoblasts from FSHD patients and a DUX4-related protein is endogenously expressed in human muscle derived cells<sup>4</sup>. Stable *DUX4* mRNAs appears to be transcribed from the distal D4Z4 unit of contracted FSHD alleles on "permissive" haplotypes<sup>6</sup>. These *DUX4* mRNAs may have a potential pathogenic role *via* a DUX4-mediated<sup>4</sup> and/or an RNA-mediated<sup>8</sup> toxic effect. DUX4 is a nuclear protein and it was previously suggested that potential nuclear localization signals NLS1 and NLS2 determine its nuclear sorting<sup>9</sup>. Our functional studies of NLS1 (RRRR<sup>23</sup>), NLS2 (RRKR<sup>98</sup>) and NLS3 (RRARHPG<sup>151</sup>), however, suggested that subcellular trafficking of DUX4 is based on alternative and/or additional nuclear sorting pathway(s)<sup>10</sup>. In this report we show that NLS1, NLS2 or NLS3 sequences does not confer nuclear location to a reporter cytoplasmic protein. We also show that nuclear entrance of DUX4 does not follows the classical nuclear import pathway mediated by alpha/beta importins. Interestingly, blockage of this pathway appears to increase the nuclear entrance of some DUX4 mutants. Deletion studies indicate that the C-terminal region of DUX4 contains an alternative domain conferring nuclear localization. A deletion mutant lacking NLS1, NLS2, NLS3 and this C-terminal domain almost exclusively localize to the cytoplasm. This DUX4 mutant represents the first described DUX4 variant mostly residing at the cytoplasm. Potential complementation of the nuclear entrance defect from the various DUX4 mutants was also explored. Results from these studies suggest that DUX4 forms heteromeric molecules able to complement the nuclear sorting defect. We hypothesize that these multiple pathways for DUX4 nuclear entrance are associated to alternative functions of the protein and/or represent the consequence of a strong evolutionary pressure to provide alternative and/or cooperative mechanisms for nuclear entrance.

<sup>1</sup>Gabriëls *et al* *Gene* (1999) 236, 25-32; <sup>2</sup>Kowaljow & Rosa (1998) PhD Thesis Project, University of Cordoba, Argentina; <sup>3</sup>Rosa AL (2003) FSHD IRCM, Los Angeles; <sup>4</sup>Kowaljow *et al* (2007) *NMD* 17, 611-623; <sup>5</sup>Bosnakovski *et al* (2008) *EMBO J* 27, 2766-2779; <sup>6</sup>Lemmers *et al* (2010) *Science* [Epub ahead of print]; <sup>7</sup>Dmitriev *et al* (2009) *NMD* 19, 17-20; <sup>8</sup>Snider *et al* (2009) *HMG* 18, 2414-30; <sup>9</sup>Ostlund *et al* (2005) *Biochemistry* 44, 2378-2384; <sup>10</sup>Corona and Rosa (2009) FSHD IRCM, Boston.

12.

### **DUX4 expression in control human tissues**

Linda N. Geng<sup>1</sup>, Lauren Snider<sup>1</sup>, and Stephen J Tapscott<sup>1</sup>

<sup>1</sup> Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109 USA

Double homeobox 4 (DUX4) is one of the leading candidate disease genes for facioscapulohumeral muscular dystrophy (FSHD). It was recently shown the presence of a favorable polyadenylation signal in the 4qA161 haplotype is required for a contracted D4Z4 array to cause FSHD, suggesting that stabilization of the DUX4 mRNA might be a causal molecular mechanism. There are several compelling reasons to believe that the DUX4 gene does not have a necessary function in normal human biology. For example, only the 4A161 allele contains the permissive polyadenylation sequence necessary to stabilize the DUX4 mRNA and approximately one-fourth of the human population lacks this permissive allele. In addition, DUX4 is a retrogene in an epigenetically repressed macrosatellite repeat and the DUX4 mRNA has not been detected at significant levels in human muscle or other tissues. For these reasons DUX4 has been referred to a “junk” DNA. However, the DUX4 open reading frame has been conserved, either because of an unknown function or simply as a consequence of its arrayed repeat. We have assessed DUX4 mRNA and protein expression in a variety of human tissues in an attempt to identify whether DUX4 might have a normal role in human biology. The results indicate that DUX4 might have a normal role in human biology.

13.

### **Developmental regulation of *DUX4* transcription using FSHD-patient derived iPS cells**

Daniel G. Miller

Department of Pediatrics, University of Washington

Institute for Stem Cell and Regenerative Medicine, Seattle, Washington.

Several observations suggest that disruption of developmentally regulated *DUX4* transcription is an important determinant of FSHD pathology. It was previously shown that FSHD symptoms correlate with D4Z4-associated histone modifications characteristic of chromatin relaxation and transcriptional activation in both FSHD1 and FSHD2-affected individuals. To determine if this epigenetic profile correlates with expression of *DUX4* transcripts during development, we assessed *DUX4* expression and associated histone modifications before and after induced differentiation of IPS cells. The presence of *DUX4* transcripts correlated with histone modifications typical of euchromatin in FSHD IPS cells and differentiated embryoid bodies. In contrast, a transition to histone modifications characteristic of heterochromatin and transcriptional repression occurred as normal IPS cells were induced to differentiate. Since the tissue and regional distribution of FSHD pathology suggests there are additional levels of regulatory control, we constructed *LacZ* reporters and delivered them to Human ES cells using a lentivirus vector so that promoter activity could be evaluated without array-mediated transcriptional influences. We show that transcription from the *DUX4* promoter in this context is repressed in undifferentiated embryonic stem cells but becomes active in a restricted pattern as cells begin to differentiate and we've mapped the location of promoter sequences important for this compartmentalized expression. These experiments demonstrate that *DUX4* transcription requires the presence of factors restricted to specific cell types in addition to a transcriptionally permissive chromatin environment. Thus, tissue and regionally specific FSHD pathology is likely a consequence of both preferential *DUX4* expression in specific muscle groups, and the sensitivity of muscle cells to *DUX4* activity.

Full Author list and affiliations.

Gregory J. Block, Lisa M. Petek, Chrissie Cirovic, Lauren Snyder\*, Natalia Rabaia\*, James M. Moore\*, Galina N. Filippova\*, Stephen J. Tapscott\*, and Daniel G. Miller

Department of Pediatrics, University of Washington

Institute for Stem Cell and Regenerative Medicine, Seattle, Washington.

\*Fred Hutchinson Cancer Research Center, Seattle, WA

14.

### **Role of CTCF in developmentally regulated silencing of D4Z4**

Natalia A. Rabaia, Yvonne D. Krom, James M. Moore, Judit Balog, Jessica C. de Greef, Leslie E. Smith, Rabi Tawil, Stephen J. Tapscott, Daniel G. Miller, Silvere van der Maarel, Galina N. Filippova

Fred Hutchinson Cancer Research Center, Seattle, Washington 98109 USA  
Leiden University Medical Center, Leiden, 2333 ZA, The Netherlands  
University of Washington, Seattle, Washington, 98195 USA

Recent studies have suggested that loss of repressive chromatin marks and DNA methylation at the 4qD4Z4 region in FSHD may result in de-repression of DUX4, the candidate FSHD gene imbedded in the D4Z4 repeat unit. The chromatin insulator protein, CTCF, has been shown to protect genes from epigenetic silencing. CTCF binding is generally inhibited by DNA methylation and CTCF prevents spreading of DNA methylation. We have identified and characterized multiple CTCF binding sites in the D4Z4 repeats and surrounding sequences, and demonstrated both enhanced binding of CTCF to the disease-associated pathogenic D4Z4 alleles and decreased repressive chromatin marks and DNA methylation at these CTCF binding sites in FSHD cells. siRNA-mediated depletion of CTCF in primary myoblasts resulted in reduction of the DUX4 transcripts levels and was accompanied by chromatin changes at the D4Z4 CTCF sites in FSHD cells, suggesting that aberrant CTCF binding at D4Z4 in FSHD may be involved in maintaining the open chromatin structure and active transcription in the region.

Finally, the enhanced CTCF binding to D4Z4 was also observed in pluripotent cells and was lost upon differentiation into embryoid bodies. Using induced pluripotent stem cells from controls and FSHD patients, in collaboration with Dan Miller we showed that D4Z4 undergoes epigenetic silencing in control cells during differentiation into embryoid bodies whereas in FSHD cells it retains its open chromatin structure. We propose that inappropriate CTCF binding to D4Z4 in FSHD might interfere with the setting of the repressive chromatin marks at the D4Z4 repeat array in early development.

15.

### **Wnt signaling regulates *DUX4* expression**

Gregory J. Block

Department of Pediatrics, University of Washington

Institute for Stem Cell and Regenerative Medicine, Seattle, Washington

To understand the regulation of *DUX4* during development, we cloned the *DUX4* promoter region from a single D4Z4 subunit and placed it upstream of the *LacZ* encoding beta-galactosidase (*DUX4*-Pro(*LacZ*)). Beta-galactosidase activity and immunostaining of endogenous *DUX4* protein was localized to a subset of cells on the periphery of human embryonic stem and FSHD-iPS cell colonies. Similar distributions of beta-galactosidase positive cells were seen in mouse embryonic stem cells. *LacZ* expression was tissue restricted in embryoid bodies and teratomas derived from human iPS cells, as well as in transgenic mice expressing *DUX4*-Pro(*LacZ*). The similarity of other wnt-reporter mouse models to the *LacZ* expression pattern we observed, and our cell culture data prompted us to investigate the role of Wnt signaling in *DUX4* regulation. Activation of the Wnt signaling pathway upregulated expression of *DUX4*-Pro(*LacZ*) and endogenous *DUX4* transcripts and protein in FSHD myoblasts. Inhibition of the Wnt signaling pathway abolished *DUX4* expression. Our data suggest that the Wnt signaling pathway is critical for *DUX4* activation, and may explain why *DUX4* transcripts are difficult to detect in culture and *in vivo*. Furthermore, disruption of Wnt signaling may present a novel approach for disease treatment.

#### Full Author list and affiliations

Gregory J. Block, Lisa M. Petek, Linda N. Geng\*, Chrissie Cirovic, Divya Narayanan, Kathryn Davidson, , Randall T. Moon, Rabi N. Tawil\*\*, Gala Filippova\*, Stephen J. Tapscott\*, and Daniel G. Miller

Department of Pediatrics, University of Washington

Institute for Stem Cell and Regenerative Medicine, Seattle, Washington.

\* Fred Hutchinson Cancer Research Center. Seattle, Washington

\*\*University of Rochester Medical Center, Rochester, NY

16.

## Dissecting FSHD pathogenesis

Peter Lunt

Clinical Genetics Department, St. Michael's Hospital, Bristol BS2 8EG, United Kingdom

The recent publication from Lemmers et al. (*Science*. 2010 Aug 19), reporting the stabilisation of any DUX4 RNA transcript from the distal most D4Z4 repeat and immediate distal sequence by the addition of a polyAdenine sequence in patients with a 4qA161-type telomere, identifies a major step in a unifying model for FSHD pathogenesis, and hence a framework on which to speculate different ways in which the same phenotype can be produced.

### FSHD appears to require :

- 1) Relaxation of the chromatin structure at 4q35, or other mechanism associated with hypomethylation to release availability for transcription of the distal D4Z4 repeat.

This can be achieved by :

- i) Deletion to leave  $\leq 10$  residual D4Z4 units  
(degree of effect on chromatin depends on residual copy number)
- ii) Translocation of distal most D4Z4 units and 4q35 telomere to another chromosome (eg. 10qtel)
- iii) Other mechanism associated with hypomethylation of D4Z4 repeats  
(? distal repeat specifically)  
eg. (speculatively) - ? 4q-specific-methylation-gene mutation  
(? specific-methyltransferase encoding gene)  
- ? mutation in distal repeat specifically preventing its methylation

**AND**

- 2) Stabilisation of distal DUX4 transcript, by :

- i) PolyA added to DUX4 transcript from distal most D4Z4 unit
- ii) ? Other speculative mechanism  
eg.- ? stabiliser mutation within distal D4Z4 unit  
- ? defective degradation mechanism  
(if normal degradation is a controlled process)

**AND**

- 3) Toxicity of DUX4 transcript or encoded protein to muscle

This may involve :

- i) Dys-regulation (eg. upregulation) of target muscle genes

- ii) Direct toxicity to muscle cell
- iii) (speculatively) & alternatively, a 'loose-cannon' mutational malfunction of DUX4 target
- iv) DUX4 toxicity will vary according to other variation in muscle cell stability

Study of patients and particularly any families with FSHD2, or of other exceptional patients, as well as a conceptual search for any other condition which could possibly have a related molecular mechanism will be key to further understanding of FSHD.

17.

**Confirmation that the specific SSLP microsatellite allele 4qA161 segregates with facioscapulohumeral dystrophy in a large cohort of multiplex and simplex FSHD families**

Meena Upadhyaya, Hoi-Ping Jim, Gill Spurlock

Institute of Medical Genetics, Cardiff University, Heath Park, Cardiff CF14 4XN, United Kingdom

Several studies have demonstrated that a specific haplotype (4qA161) located at 4q35.2 is tightly associated with FSHD disease expression. The present study analysed haplotype associations in a large cohort of sporadic and familial FSHD families from the UK. The study group of 22 unrelated FSHD families, confirmed by clinical and molecular diagnosis, comprised 96 affected and 107 unaffected individuals. All 96 affected individuals exhibited the 161bp allele that showed the expected inheritance pattern in all informative family and we confirmed a complete association between 4qA161 and disease expression. While the 161 SSLP has no apparent causative role in FSHD, 161 marker analysis should be included in all future diagnostic tests to demonstrate both its complete association with FSHD and also to identify those rare cases in which this allele is not disease associated. In family situations where parental DNA is fully informative (the affected parent is heterozygous for 4qA161 and the normal parent lacks this allele) the SSLP represents a quick and cost-effective test for assessing presymptomatic children. The 4q35-located 161 allele is common in the general population, however this allele may be in linkage disequilibrium with additional SSLPs, SNPs, or other sequence variations, that specifically give rise to the FSHD disease phenotype.



**An update on the FSHD-specific Quality-of-Life outcome measure: identifying what is most important to FSHD patients**

Chad Heatwole, M.D.;<sup>1</sup> Christine Quinn, M.S.;<sup>1</sup> Rabi Tawil, M.D.;<sup>1</sup> Nicholas Johnson, M.D.<sup>1</sup>

<sup>1</sup>The University of Rochester Medical Center, Rochester, New York

**OBJECTIVE:** To identify, quantify, and analyze the themes, issues, and symptoms that have the greatest impact on Facioscapulohumeral Muscular Dystrophy (FSHD) patient health related quality-of-life (HRQOL).

**BACKGROUND:** FSHD is a multisystemic disease capable of impairing the physical, mental, and social health of patients while inflicting significant disability.

**METHODS:** The interviews of twenty genetically confirmed adult FSHD participants were recorded, transcribed, coded, and analyzed using a qualitative framework technique. Each 30-60 minute interview focused on the symptoms and issues that have the greatest impact on an FSHD patient's HRQOL. Once compiled, the data from each interview was familiarized, indexed, mapped, charted, matched, and interpreted via a three investigator consensus qualitative approach.

**RESULTS:** 1375 direct quotes were coded resulting in 250 like themes. These themes were then categorized into 14 subdomains that represent the physical, mental, social, and disease-specific HRQOL of the FSHD population. A disease-specific HRQOL conceptual model was created for FSHD representing the most relevant symptomatic and psychosocial issues in this population. Categories in this model included: 1) mobility and ambulation; 2) fine motor and distal arm weakness; 3) proximal upper extremity and shoulder limitation; 4) truncal weakness; 5) FSHD specific activity impairment; 6) emotional distress; 7) FSHD specific impaired body image; 8) cognitive impairment; 9) social role dissatisfaction; 10) social role limitations; and FSHD specific symptoms of: 11) fatigue; 12) pain; 13) communication difficulties /facial weakness; and, 14) gustatory dysfunction. Difficulty with ambulation, steps, and a reliance on family members were the most frequently mentioned high impact issues in our sample of patients.

**CONCLUSIONS:** FSHD patients cope with a wide variety of physical, mental, social, and disease-specific issues. The composite of these issues has the capability to severely impact FSHD HRQOL. Obtaining the FSHD patient's point of view is a critical first step in the future development of patient-reported outcome measures and functional rating scales for this population. A larger study of 514 FSHD patients is currently underway to validate the most critical issues identified through the above research.

### **Unexpected large number of compound heterozygotes revealed by cumulative effects of D4Z4 mutation**

Greta Fabbri<sup>1</sup>, Chiara Fiorillo<sup>2</sup>, Isabella Scionti<sup>1</sup>, Francesca Greco<sup>1</sup>, Roberto D'Amico<sup>3</sup>, Alberto Termanini<sup>1</sup>, Liliana Vercelli<sup>4</sup>, Emanuela Bonifazi<sup>1</sup>, Luca Colantoni<sup>5</sup>, Giuliano Tomelleri<sup>6</sup>, Giuliana Galluzzi<sup>5</sup>, Laura Palmucci<sup>4</sup>, Carmelo Rodolico<sup>7</sup>, Lucio Santoro<sup>2</sup> and Rossella Tupler<sup>1,8,\*</sup>

<sup>1</sup> Department of Biomedical Sciences, University of Modena and Reggio Emilia, Italy. <sup>2</sup> Department of Neurological Sciences, University "Federico II", Naples, Italy. <sup>3</sup> Department of Oncology, University of Modena and Reggio Emilia, Italy; <sup>4</sup> Center for Neuromuscular Diseases, Department of Neuroscience, University of Turin, Italy. <sup>5</sup> Molecular Genetics Laboratory of UILDM, IRCCS Santa Lucia Foundation, Rome, Italy. <sup>6</sup> Department of Neurological Sciences and Vision, University of Verona, Italy. <sup>7</sup> Department of Neurosciences, Psychiatry and Anesthesiology, University of Messina, Messina, Italy. <sup>8</sup> Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester, MA, USA.

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant myopathy, characterized by progressive atrophy and weakness of selective muscles. Reduction of tandemly arrayed 3.3 kb repeat units (D4Z4) at chromosome 4q35 has been identified as the causative genetic defect of this disease. Association between clinical outcome and residual repeat unit number is expected; however, due to the wide variability in clinical spectrum, even within the same family, such association needs to be ascertained.

The Italian registry comprising 1119 FSHD families was used to identify 23 families in which at least one person carries two D4Z4 alleles of reduced size. Out of the 23 families, eleven families were involved in this study; 111 subjects were clinically examined and molecularly characterized. The FSHD clinical evaluation score was calculated in each subject. Out of 111 subjects examined, fifteen patients carrying two FSHD-sized alleles and 54 carrying one FSHD-sized allele were found. FSHD score was compared between those two groups.

Differences in the distribution of FSHD score were observed between the two groups. People carrying two FSHD-sized alleles tend to have higher score values. However 54% of people with single FSHD-sized allele and 6.7% of those with double FSHD-sized alleles did not show any signs of FSHD.

Our study shows that compound heterozygotes people are significantly more affected than their relatives carrying a single FSHD-sized D4Z4 allele and surprisingly unveils that a high fraction of subjects carrying FSHD-sized D4Z4 alleles have no signs of muscle weakness.

Thus, our analysis suggest that in subjects carrying FSHD-sized allele disease expression can be influenced by other factors besides reduction of tandemly arrayed 3.3 kb repeat units (D4Z4) at chromosome 4q35. To verify the role of subtelomeric polymorphisms in FSHD expression, the 4q35 haplotype, including 4qA/4qB polymorphism, proximal SSLP and SNP in the p-LAM region, has been characterized in each allele segregating in these families.

Interestingly, the frequency of observed compound heterozygous patients is three orders of magnitude higher than expected and this study might update the frequency of the deleted allele in the population. The identification of factors influencing the development of FSHD is going to be a priority

for the future research of this complex disease.

We are grateful to the FSHD Italian families for their unlimited support. This work has been funded by Telethon-UILD (Grant GUP08004), Association Française contre les Myopathies.

### **The Facio-Scapulo-Limb, Type 2 (or the facioscapulo-peroneal) autosomal dominant muscular dystrophy**

Kazakov V., Rudenko D.

Department of Neurology, Pavlov State Medical University, St. Petersburg, Russia

Data show that Erb, Landouzy and Dejerine, and later Davidenkov described an unusual type of MD – *a descending type with a “jump”* which differs greatly from classical FSHD – *a gradually descending type* which was recognised in the first time by Duchenne. Later these various myopathies *with different sequences* of muscle affections *were united into one group*, now called FSHD. Our investigations (1969-2009) allows to suggest that FSHD is a heterogeneous form of muscular dystrophy. The best name for it is *“facioscapulolimb muscular dystrophy (FSLD)”*. On the historical and clinical data FSLD maybe divided into two nosological entities, namely: 1. Facioscapulolimb muscular dystrophy, type 1 (FSLD1), a gradually descending one with initial facio-scapulo-humeral phenotype, autosomal dominant (Duchenne de Boulogne) and 2. Facioscapulolimb muscular dystrophy, type 2 (FSLD2), a descending with a “jump, with initial facio-scapulo-peroneal or (facio)-scapulo-peroneal phenotypes, autosomal dominant (Erb, Landouzy and Dejerine). Data show that FSLD2 is a very special type of MD with a “hard” static and dynamic patterns of muscle weakness, with a slight/severe affection of the isolated facial muscles or their parts and mild course of the disease. The FSP or the (F)SP phenotypes predominate in the clinical picture during a many years. The probe p13E-11 detected EcoRI/BlnI of DNA fragments size (DFS) between 13 – 35 kb (double digestion) cosegregated with the FSLD2 and linked with 4q35. However, the classical FSHD is associated as well with the 4q35-linked EcoRI fragment detected by p13E-11 which equal 40 kb or shorter (Galluzzi et al. 2003). Therefore *taking into consideration the DFS*, FSLD2 are *“absorbed” by FSHD*. Thus, in our opinion *detected DFS cannot be the criterion* for establishing the genetic heterogeneity of FSHD (and FSLD).

Address: Valery Kazakov, M.D., Ph.D., Dr. med. Sc.  
Professor of Clinical Neurology,  
Department of Neurology, Pavlov State Medical University,  
L. Tolstoy Str. 6/8, 197022 filial 1, St. Petersburg, Russia ,  
Tel.: +7 812 510 9381, fax: +7 812 513 26 12,  
e-mail: valerykazakov@mail.ru

**Gene expression profiling in facioscapulohumeral muscular dystrophy**

Fedik Rahimov<sup>1</sup>, Oliver D. King<sup>2</sup>, Jennifer Chen<sup>2</sup>, Kendal Hanger<sup>2</sup>, Leigh C. Warsing<sup>3,4</sup>, Robert J. Bloch<sup>5</sup>, Jeffrey B. Miller<sup>2</sup>, Charles P Emerson, Jr.<sup>2</sup>, Kathryn R. Wagner<sup>3,4</sup>, Louis M. Kunkel<sup>1,6</sup>

<sup>1</sup>*Program in Genomics and Division of Genetics, Children's Hospital Boston, Harvard Medical School, Boston, Massachusetts;* <sup>2</sup>*Boston Biomedical Research Institute, Watertown, Massachusetts;* <sup>3</sup>*The Johns Hopkins University School of Medicine and* <sup>4</sup>*Kennedy Krieger Institute, Baltimore, Maryland;* <sup>5</sup>*University of Maryland School of Medicine, Baltimore, Maryland;* <sup>6</sup>*Howard Hughes Medical Institute, Boston, Massachusetts*

Facioscapulohumeral muscular dystrophy (FSHD) is a progressive myopathy caused by deletions of integral number of repeat elements within the subtelomeric macrosatellite element D4Z4 on chromosome 4q35. Deletion of a critical number of repeats causes overexpression of the polyadenylated *DUX4* transcript from the distal repeat located only on the permissive chromosome. Although the primary cause of the disease is becoming more evident the underlying molecular mechanisms of the FSHD pathogenesis remains poorly understood. In order to identify genes and pathway affected in FSHD, we performed transcriptome expression profiling of biceps and deltoid muscle tissues collected from FSHD patients and unaffected first degree relatives from 14 cohorts using the Affymetrix GeneChip® Gene 1.0 ST array. We identified a number of differentially regulated genes between affected and unaffected muscles. In addition, we performed high-throughput quantitative real-time RT-PCR analysis on 46 muscle growth and differentiation marker genes and several candidate genes residing on chromosome 4q35, including the *DUX4* gene, in muscles and primary cells derived from a subset of these muscle tissues. Our preliminary data show that the expression of polyadenylated and stable *DUX4* mRNA is restricted to affected myotubes derived from 3 FSHD cohorts, but we failed to detect its expression in unaffected cells. We are currently testing its expression in muscle biopsies and additional cell lines. *DUX4*, a double homeobox protein with sequence similarity to *PAX3* and *PAX7* transcription factors, was shown to compete with *PAX3/PAX7* and induce toxicity in muscle cells. *PAX3/PAX7* target genes tended to be upregulated in affected muscles compared with unaffected controls. Genes identified as differentially expressed in this study will be valuable for understanding the molecular etiology of FSHD and assessing the success of clinical trials for this disease.

### **Over-expression of mu-crystallin impedes myotube formation *in vitro***

Patrick W. Reed<sup>1</sup>, Fedik Rahimov<sup>2</sup>, Andrea O'Neill<sup>1</sup>, Jennifer Chen<sup>3</sup>, Oliver King<sup>3</sup>, Kathryn Wagner<sup>4</sup>, Allison Densmore<sup>1</sup>, William Randall<sup>1b</sup>, Jeffrey Boone Miller<sup>3</sup>, Louis M. Kunkel<sup>2</sup>, Charles P. Emerson<sup>3</sup>, and Robert J. Bloch<sup>1</sup>

<sup>1</sup>Departments of Physiology and <sup>1b</sup>Pharmacology, University of Maryland School of Medicine, Baltimore, Maryland 21201; <sup>2</sup>Children's Hospital, Boston, Massachusetts; <sup>3</sup>Boston Biomedical Research Institute, Watertown, Massachusetts 02472; <sup>4</sup>Center for Genetic Muscle Disorders, Kennedy Krieger Institute, Baltimore, Maryland 21205

We have previously reported high expression of mu-crystallin (CRYM) in muscle biopsies from patients with FSHD, but the expression levels in adult muscle biopsies are highly variable. We have therefore characterized the expression of CRYM in myogenic cells cultured from patients with FSHD and unaffected relatives, and see significant changes in its expression as fusion and myogenesis proceed in several of the cohorts. CRYM expression tends to be higher in the FSHD samples of those cohorts than in the cells from unaffected muscle. We next tested if high levels of CRYM can inhibit myogenesis. We infected C2C12 myoblasts with adenovirus (Adv) engineered to express a control protein, mCherry (a fluorescent marker protein), or mCherry and CRYM from a bicistronic viral construct. We grew C2C12 myoblasts in DMEM with 10% FBS to ~70-80% confluency, then infected them with control (Adv CMV-Cherry) or experimental (Adv CMV-Crym/CMV-mCherry) virus immediately before switching them into differentiation media (DMEM with 5% horse serum). After 7 days, we immunolabeled the coverslips with antibodies to desmin. Uninfected C2C12 myoblasts differentiated into thick myotubes by 7 days of differentiation. Cells infected with Adv CMV-mCherry were indistinguishable from controls, showing that adenoviral infection of C2C12 myoblasts does not interfere with myogenesis in culture. In contrast, high levels of CRYM expression, seen in Adv CMV-Crym/CMV-mCherry infected cells, strongly impeded the development of myotubes. Our results suggest that, although the expression of CRYM in FSHD muscle cells in culture is variable, FSHD samples tend to express higher levels than controls, and expression in both varies significantly with developmental stage. Furthermore, over-expression of CRYM in differentiating myoblasts inhibits myotube development *in vitro*, consistent with the possibility that CRYM interferes with muscle fiber regeneration *in vivo*. We suggest that CRYM over-expression may inhibit regeneration of FSHD muscle.

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23. [P]

**Toward quantitative proteomic comparisons of skeletal muscles from FSHD patients and their unaffected, first-degree relatives: an update**

Patrick W. Reed<sup>1</sup>, Kathryn Wagner<sup>4</sup>, Allison Densmore<sup>1</sup>, and Robert J. Bloch<sup>1</sup>

<sup>1</sup>Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201;

<sup>4</sup>Center for Genetic Muscle Disorders, Kennedy Krieger Institute, Baltimore, Maryland 21205

We are studying the changes in the proteome that occur in FSHD and have been using large format, two-dimensional gel electrophoresis in our initial studies. We have introduced several modifications to published methods that improve protein solubility and recovery, minimize proteolysis, reduce the loss of resolution due to contaminants and cross-linking, and enhance analysis of faint spots. Key modifications are: (i) the use of 7M urea + 2 M thiourea, instead of 9M urea, in preparing samples and gels for isoelectric focusing; (ii) the use of a strong reducing agent followed by 4-vinylpyridine, to block sulfhydryl groups and prevent non-specific protein cross-linking; (iii) digital enhancement of spots that are faintly labeled with stain; (iv) large gels that enable all proteins from the first dimension to be resolved in the second dimension without cutting the gel, and proteins with molecular masses less than 30 kDa to be resolved. These changes make 2-D gel electrophoretic analysis of the proteome more comprehensive, reproducible and sensitive, with minimal artifacts. We have begun to use our improved methods to examine fresh, snap frozen, age- and sex-matched biopsies of deltoid and biceps muscles from patients with FSHD and from their first degree affected and unaffected relatives, as well as myoblast cell lines prepared from control and affected samples. In this update, we demonstrate that our quantitative measurements are highly reproducible and thus significant differences likely represent true changes in protein abundance between healthy and FSHD muscle samples.

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24. [P]

### **Unexpected high frequency of FSHD alleles in the general population**

Isabella Scionti<sup>1</sup>, Francesca Greco<sup>1</sup>, Luca Colantuoni<sup>2</sup>, Patricia Arashiro<sup>3</sup>, Giuliana Galluzzi<sup>2</sup>, Mayana Zatz<sup>3</sup>, Rossella Tupler<sup>1,4</sup>

<sup>1</sup> Department of Biomedical Sciences, University of Modena and Reggio Emilia, Italy.

<sup>2</sup> Molecular Genetics Laboratory of UILDM, IRCCS Santa Lucia Foundation, Rome, Italy, Worcester, MA, USA.

<sup>3</sup> Human Genome Research center, Biosciences Institute, University of São Paulo, São Paulo, Brazil.

<sup>4</sup> Program in Gene Function and Expression, University of Massachusetts Medical School,

Facioscapulohumeral muscular dystrophy (FSHD) is the third most common myopathy transmitted as autosomal dominant disease, which has been associated with a reduction of tandemly arrayed D4Z4 repeat units on chromosome 4 (4q35). FSHD molecular diagnosis is based on the detection by probe p13E-11 of EcoRI fragments that contain D4Z4 repeat array. FSHD patients usually carry an EcoRI-p13E-11 allele shorter than 35 Kb. Recent studies have hypothesized that the association of FSHD with 4qA polymorphism distal to D4Z4, 161 SSLP proximal to D4Z4 and a polyadenylation signal within the 4qA allele is necessary to develop FSHD. Recent reports of 23 FSHD compound heterozygous patients carrying two FSHD-sized alleles suggest that FSHD-sized alleles are more frequent than expected. These findings prompted us to investigate the frequency of FSHD alleles in the general population. The 4qter D4Z4 polymorphism was analyzed in 560 Italian and 241 Brazilian unrelated healthy subjects. Remarkably, despite the prevalence of 1 in 20,000 for FSHD, we found that 3% of the subjects analyzed carry EcoRI fragments of size equal or shorter than 35 Kb in both Brazilian and Italian populations.

This observation indicates that the distribution of FSHD-sized alleles in the general population has a frequency of a common polymorphism and is between two and three order of magnitude higher than expected. The 4q35 haplotype, including 4qA/4qB polymorphism, proximal SSLP and SNP in the p-LAM region, has been characterized in this group of chromosomes 4 carrying FSHD-sized alleles. Interpretation of results obtained from this analysis is of particular importance for genetic counseling and prenatal diagnosis of families at-risk for FSHD.

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### **Assaying the nuclear organization of the FSHD locus using 4C-Seq**

Kyle T. Siebenthall and Barbara J. Trask

Division of Human Biology, Fred Hutchinson Cancer Research Center and Department of Genome Sciences, University of Washington School of Medicine, Seattle, Washington

The FSHD locus at 4q35 has previously been shown to be tethered to the nuclear periphery in many cell types, including muscle cells. We are revisiting the hypothesis that pathogenic D4Z4 array contractions alter the location or folding of 4q35 in the nucleus— changes that could have consequences for the mis-regulation of genes in *cis* or *trans*. To address this hypothesis, we have adapted the circular chromosome conformation capture assay for high-throughput sequencing (4C-Seq). This technique identifies regions of the genome (“prey”) that are captured by a locus of interest (“bait”) because of proximity in three-dimensional nuclear space. By using the SSLP as bait in this assay, we can compare the nuclear neighborhoods of the FSHD locus with and without a pathogenic D4Z4 deletion in FSHD and control primary myoblast cells. We will present the initial analysis of our sequence data, including the enrichment of prey fragments in a broad domain proximal to the FSHD locus, as well as in regions of other chromosomes, and the challenges of using the SSLP to distinguish bait alleles.

### Focus on nuclear proteins of FSHD primary myotubes by differential mass spectrometry

A. Tassin<sup>1</sup>, B. Leroy<sup>2</sup>, E. Anseau<sup>1</sup>, E. Pire<sup>1</sup>, J. Faille<sup>1</sup>, T. Leclercq<sup>1</sup>, S. Charron<sup>1</sup>, D. Laoudj-Chenivesse<sup>3</sup>, F. Coppée<sup>1</sup>, R. Wattiez<sup>2</sup> and A. Belayew<sup>1</sup>

Laboratory of Molecular Biology<sup>1</sup> and Dpt of Proteomics and Protein Biochemistry<sup>2</sup>, University of Mons, Belgium ; INSERM, ERI 25 Muscle et Pathologies<sup>3</sup>, CHU A. de Villeneuve, University of Montpellier I, France.

Our group has identified the DUX4 gene at the FSHD locus and shown its expression in affected but not control myoblasts (Gabriels *et al.*, 1999; Kowaljow *et al.*, 2007; Dixit *et al.*, 2007). The DUX4 protein is a transcription factor that targets a number of genes, some of which encode other transcription factors among these, PITX1 (Dixit *et al.*, 2007). Several studies investigated gene/protein expression in FSHD primary myoblasts or muscle biopsies. Since DUX4 is activated upon myoblast differentiation, we chose to compare the proteome of FSHD and control myotubes by gel free differential mass spectrometry analysis (LC-MS/MS) using an isotope coded protein labelling (ICPL). The genes coding differentially expressed proteins are putative DUX4/PITX1 targets. Their promoters were fused to the luciferase reporter to evaluate their response to DUX4/PITX1 in cotransfection experiments.

**1/** With this approach we identified the *GAL1* gene as a PITX1 target: its proximal promoter has a PITX1 binding site. *GAL1* expression is induced in FSHD myotubes, or by forced DUX4 expression in control myoblasts and the protein is secreted.

**2/** We wanted to focus on changes in the expression of transcription factors, splicing factors and other nuclear proteins. Because of the low level of these proteins as compared with the abundant cytoskeletal proteins in FSHD myotubes, we had to improve the method sensitivity. We have first adapted a nuclear extraction compatible with the LC-MS/MS method. Moreover, higher resolution LC separation and a modified version of ICPL was also tested (Leroy *et al.*, 2010). Using this strategy, we have identified 649 proteins including 33% of predicted nuclear proteins involved in transcription regulation (FHL2, STAT1, SOX5), RNA maturation (splicing factors), DNA repair (KU86) or chromatin remodeling (SWI/SNF). Using this method, we also compared the nucleome of disorganized and atrophic myotubes as described by Barro *et al.*, 2008.

**3/** Although we could improve the sensitivity by this method, we have still not been able to detect DUX4 by mass spectrometry. Because of its central role in FSHD, we decided to use the Multiple Reaction Monitoring (MRM) approach to focus on a DUX4-specific signature. To determine DUX4-specific peptides, we analyzed by LC-MS/MS a DUX4-HaloTag protein produced in human muscle cell cultures and purified by affinity chromatography using a Halolink column (Promega). We detected four DUX4 peptides characterized by their m/z value and MS/MS spectra. All these peptides are in the NH2-terminal domain of DUX4, identical to DUX4c. No peptide of the DUX4-specific COOH-terminal domain was detected. Further experiments are in progress to achieve this aim.

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### **Understanding FSHD gene dysregulation and normal myogenesis: genome-wide mapping of open chromatin sites by their DNaseI hypersensitivity**

Koji Tsumagari<sup>1</sup>, Lingyun Song<sup>2</sup>, Darin London<sup>2</sup>, Zhancheng Zhang<sup>2</sup>, Michelle Lacey<sup>3</sup>, Rabi Tawil<sup>4</sup>, Terrence S. Furey<sup>2</sup>, Gregory E. Crawford<sup>2</sup>, and Melanie Ehrlich<sup>1</sup>

<sup>1</sup>Human Genetics Program and the Department of Biochemistry, Tulane Medical School, New Orleans, Louisiana 70112; <sup>2</sup>Institute for Genome Sciences & Policy, Duke University, Durham, North Carolina 27708; <sup>3</sup>Department of Mathematics, Tulane University, New Orleans, Louisiana 70112; <sup>4</sup>University of Rochester, School of Medicine and Dentistry, Rochester, New York 14642

To elucidate the epigenetic regulation of myogenesis in control and facioscapulohumeral muscular dystrophy (FSHD) cells, we profiled open chromatin sites throughout the genome in myoblasts and myotubes using high-resolution mapping of DNaseI hypersensitive sites (DHS). Three normal-control myoblast cell strains, two normal-control myotube preparations, three FSHD myoblast cell strains, and one FSHD myotube preparation consisting of >85% myoblasts or >70% of the nuclei in myotubes were analyzed. The DHS profiles of myoblasts and myotubes were compared to those of seven cell types (HepG2, a hepatocellular carcinoma cell line; K562, a myeloid leukemia cell line; GM12878, a lymphoblastoid cell line; HUVEC, human umbilical vein endothelial cells; H1, an embryonal stem cell line; NHEK, normal human epidermal keratinocytes; and HeLa S3 cells). We also looked for correlations between cell type-specific DHS and approximate steady-state levels of RNA determined by identical expression profiling (Affymetrix exon array) of 19 various non-muscle cell types as well as FSHD and control myoblasts and myotubes.

From the expression profiling, there were 163 genes that displayed at least 4 times higher RNA signal in control myotubes ( $p < 0.01$ ) than in the average of the non-muscle cell cultures and that had been annotated as muscle-associated. We compared DHS sites at these known muscle-specific genes and at 53 genes which had not been annotated as associated with any type of muscle but that showed myogenesis-specific expression in our expression profiling ( $\geq 4$  times more RNA signal in control myotubes than in the average of non-muscle cultures and  $\geq 2$  upregulation from control myoblasts to myotubes). We found that both sets of genes had similar frequencies of associated DHS specific to myoblasts or myotubes. This independent analysis verifies our new identification of myogenesis-specific genes. For example, C6orf142 had ~16-fold higher steady-state levels of its RNA in control myotubes than in the average of the non-muscle cell types and almost six-fold higher in control myotubes than in control myoblasts. In addition, its expression was significantly downregulated in FSHD myotubes. This gene had a myogenesis-specific DHS 9 kb upstream of its annotated start site, confirming its muscle-specificity.

Genes with myoblast/myotube or FSHD/control differences in expression often had myogenesis-specific DHS common to all the analyzed myoblast and myotube samples, as if they are poised for muscle-specific expression irrespective of large differences in actual expression. Our mapping of cell type-specific DHS sites not only helped identify new myogenesis-associated genes, but also revealed numerous probable myogenesis-related enhancers or silencers distant from the genes they control. Most of the very many myogenesis-specific DHS that we found throughout the genome may indicate the locations of previously unknown transcription regulatory sites. For example, the muscle-specific

transcription regulatory MYOD1 gene had myogenesis-specific DHS at known enhancers as well as at many distant upstream sites not previously identified as transcription control elements. Understanding the functionality of such DHS will help elucidate normal and disease-associated myogenesis. (Supported in part by grants from the FSHD Global Research Foundation, the NIH [NS048859 to M.E., HG003169 to G.E.C.], and the Fields Center for FSHD and Neuromuscular Research [R.T]).

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### **Increased alpha actinin-3 in FSHD muscle suggests fiber-type dysregulation and potential involvement in FSHD pathogenesis**

Daniel Zuch<sup>1</sup>, Fedik Rahimov<sup>2</sup>, Oliver King<sup>1</sup>, Kathryn Wagner<sup>3</sup>, Jennifer Chen<sup>1</sup>, Louis M. Kunkel<sup>2,4</sup>, Charles P. Emerson, Jr.<sup>1</sup>

<sup>1</sup>Boston Biomedical Research Institute, Watertown, Massachusetts, <sup>2</sup>Program in Genomics and <sup>4</sup>Howard Hughes Medical Institute, Children's Hospital Boston, Harvard Medical School, Boston, Massachusetts, <sup>3</sup>Kennedy Krieger Institute & The Johns Hopkins University School of Hospital, Baltimore, Maryland

FSHD is a form of muscular dystrophy that is characterized by a weakening of muscles of the face and upper limbs. While the genetic foundation of FSHD has been linked to a contraction in the D4Z4 microsatellite repeat at the subtelomeric region of chromosome 4q, the molecular and cellular pathology of the disease is poorly understood and biomarkers for the disease remain elusive. The Wellstone Center utilizes a collaborative approach to identify novel biomarkers of FSHD pathogenesis by comparing groups of patients who are affected by the disease with unaffected first degree relatives. Candidate biomarker profiles are generated by microarray analysis, and potential biomarkers are followed up with a variety of proteomic studies. Microarray analyses of our unique repository of biopsies and cells have illuminated a potential candidate biomarker, alpha actinin-3. Alpha actinin-3 is an actin-binding protein that is localized to the Z-disc of a skeletal muscle sarcomere, specifically in "fast-twitch" muscle fibers. Most human muscles contain a heterogeneous and variable population of "fast-twitch" and "slow-twitch" muscle fibers, generating inconsistent actinin-3 levels between muscle groups. Despite this inherent variability, microarray data indicated that actinin-3 expression tended to be elevated in muscle affected by FSHD versus control muscle. Histology confirmed that FSHD muscle had a greater percentage of actinin-3 containing fibers on average than control muscle. In addition, histology revealed that actinin-3 was expressed in a small population of slow fibers, and these hybrid fibers seemed to be more prevalent in FSHD muscle. These findings suggest that fiber type dysregulation in FSHD muscle may provide a potential link to understanding the underlying molecular mechanisms of FSHD. *This work is supported by NICHD 1U54HD060848-01.*