

# ***THE XI MYOGENESIS SYMPOSIUM***

***Paris***

***2 & 3 February 2009***

**Myology Institute, Batiment Babinski,  
Groupe Hospitalier Pitié-Salpêtrière  
47-83, Boulevard de l'Hôpital , 75013 Paris**

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## **Programme**

20 minute presentations (in French or English, Mac or PC) +5 minutes of questions

### **Monday 2nd February**

#### **Development, signalisation and stem cells**

13:45-14:10 Sylvia Brunelli: Role of necdin in the differentiation and regeneration of the skeletal muscle

14:10-14:35 Didier Montarras: The transcriptome of skeletal muscle satellite cells

14:35-15:00 Fabien Le Grand: The Planar Cell Polarity Pathway Regulates the Symmetric Expansion of Satellite Stem Cells

15:00-15:25 Anne-Sophie Armand: Apoptosis-Inducing Factor Regulates Skeletal Muscle Progenitor Cell Number and Self-Renewal

15:25-15:50 Alice Pannérec: PW1 interstitial cells (PICs): a new source of myogenic progenitors

15:50-16:15 Romain Gheradi: Muscle resident macrophages of epi/perimysium crucially control the inflammatory cell reaction to myoinjury

#### **40 minute break**

16:55-17:20 Ramkumar Sambasivan: Distinct regulatory cascades govern extraocular and branchiomeric muscle progenitor cell fates.

17:20-17:45 Keren Bismuth: Myogenic factors and CDKI: regulation of cell cycle exit during in vivo muscle development

17:45-18:10 Claire Niro: Six1 and Six4 gene expression is required to activate the fast-type muscle gene program in the mouse myotome.

18:10-18:35 Laure Stochlic: Rôle de Wnt4 dans la mise en place de la Jonction Neuromusculaire

18:35-19:00 Alfredo Csibi: MAFbx/Atrogin-1 controls eIF3-f activity in skeletal muscle atrophy by targeting multiple C-terminal lysines

19:00-19:35 Delphine Duteil: Characterisation of glucocorticoid signaling in skeletal muscle.

19:35-20:00 Marie-Catherine Le Bihan: Characterization of the Human Myoblast "secretome" during in vitro differentiation

#### **20:00- Buffet**

## **Tuesday 3rd February**

### **Signalisation and muscle diseases**

9h-9h25: Sophie Charrasse: Involvement of M-cadherin, Rac1 and ARF6 signaling pathways during myoblast fusion

9h25-9h50: Bruno Cadot: Nuclear positioning during myofiber formation

9h50-10h15: Johan Boehm: Invivo imaging of muscle fibers

10h15-10h40: Lama Al-Qusairi: Defective excitation-contraction coupling in muscle fibres from a mouse model of X-linked myotubular myopathy

10H40-11H05: Laetitia Guevel: Identification des perturbations protéiques globales par spectrométrie de masse dans le modèle canin de la dystrophie musculaire de Duchenne

### **30 minute break**

11h35-12h: Tayebah Soheili: Specific Inhibition of Protein Quality Control System as a Therapeutic Approach for Treatment of Sarcoglycanopathies

12h-12h25: Celine Vanderplanck: DUX4 / DUX4c expression and inhibition in FSHD.

12h25-12h50: Matthieu Pasco: Recherche de gènes modificateurs de la Dystrophie Musculaire OculoPharyngée

12h50-13h15: Adeline Vulin: Dystrophin rescue by using exon-skipping and new technique of systemic delivery in dog

## Abstracts

### **Role of Necdin in the differentiation and regeneration of the skeletal muscle**

**Silvia Brunelli.**

*Division of Regenerative Medicine Dibit-HSR. Milano, Italy*

Regeneration of muscle fibers, lost during pathological muscle degeneration or after injuries, is sustained by the production of new myofibers. Necdin a member of the MAGE family of proteins member, is expressed in satellite cell-derived myogenic precursors during perinatal growth. We have shown that transgenic mice overexpressing necdin in skeletal muscle have accelerated muscle regeneration, a process dependent on proliferation and differentiation of the satellite cells. Satellite cells from these mice showed an increased ability to differentiate and decreased apoptosis. On the other hand the necdin-null mice show a considerable defect in muscle healing following muscle injury. We have also demonstrated that necdin is selectively expressed in the atrophic muscles of cachectic mice (tumor induced cachexia) and formally proved that its expression is causally linked to a protective response of the tissue against tumor-induced wasting, inhibition of myogenic differentiation and fiber regeneration. Necdin plays this role mainly via interference with TNF- $\alpha$  signaling at various levels, including regulation of expression of TNFR1, of p53 and of the activity of caspase 3 and 9. These results suggested that necdin can play an important role both in the in myoblast differentiation and survival in response to external stimuli. These data prompted us to investigate whether Necdin could be exploited also to enhance myogenic differentiation and inhibit cell death of other types of stem cells. Mesoangioblasts (MABs) are vessel-associated stem cells that have been shown to be ideal candidates for stem cell therapy of muscular dystrophies. However, despite of the identification of MABs as potential source of skeletal muscle, detailed studies on their molecular pathways are needed to improve their ability to reconstitute skeletal muscle and act as pool of resident stem cells. Preliminary data showed that muscle of  $\alpha$ Sarcoglyan KO dystrophic mice injected with MABs overexpressing Necdin showed a greater restoration of the  $\alpha$ -SG expression.

# The transcriptome of skeletal muscle satellite cells

**Didier Montarras, Giorgia Pallafacchina, Ana Cumano\*, Margaret Buckingham**

*CNRS URA 2578, Unité de Génétique Moléculaire du Développement ; Institut Pasteur, Paris, (\*) Unité de Développement des Lymphocytes ; Institut Pasteur, Paris*

Muscle satellite cells are responsible for post-natal skeletal muscle growth and regeneration. These quiescent cells, located under the basal lamina of muscle fibres, become activated upon injury, proliferate and differentiate into new muscle fibres. During regeneration, the satellite cell pool is also reconstituted. Thus, satellite cells display two hallmarks of stem cells: lineage-specific differentiation and self-renewal. Elucidating the mechanisms which govern quiescence and activation of satellite cells requires access to these cell states *in vivo*. In the last few years, strategies have been developed, which permit prospective isolation of satellite cells from adult muscles. They rely upon the direct isolation of cells by flow cytometry on the basis of the expression of surface markers or satellite cell targeted reporter gene expression. The *Pax3<sup>GFP/+</sup>* knock in line permitted us to achieve this goal, since *Pax3*, as well as *Pax7* is expressed in satellite cells of numerous muscles. These genes encode paired/homeo-domain transcription factors that are important upstream regulators of myogenesis.

Determining the transcriptomes of quiescent and activated satellite cells *in vivo* is an important step since it should provide novel insights into the genes expressed in these cells under physiological conditions. Previous gene expression profiling analyses were either based on whole muscle tissue in the course of development or regeneration or had used activated satellite cells in culture ; the former provides a picture resulting from the response of several cell types, while culture leads to artefactual changes in gene expression.

We have taken this analysis an important step further by comparing the transcriptomes of quiescent to activated states *in vivo*. The approach we developed depends on the *Pax3<sup>GFP/+</sup>* mouse strain that permits purification of *Pax3* expressing satellite cells by flow cytometry. Such cells from adult skeletal muscle, were compared to those from 1 week postnatal muscle where muscle growth involves extensive satellite cell activation, and from adult *mdx* mice in which the dystrophic muscle is undergoing repeated cycles of degeneration and regeneration, with satellite cell activation. This analysis provides novel insights into the genes expressed in quiescent versus activated muscle satellite cells *in vivo*.

# **The Planar Cell Polarity Pathway Regulates the Symmetric Expansion of Satellite Stem Cells**

**Fabien Le Grand, Anthony Scimè, Andrew Jones, Vanessa Seale and Michael Rudnicki**

*Sprott Center For Stem Cell Research, Ottawa Health Research Institute  
Ottawa, Canada*

Satellite cells in skeletal muscle are a heterogeneous population of stem cells and committed progenitors. We found that quiescent Pax7<sup>+</sup>/Myf5<sup>-</sup> stem cells expressed the Wnt-receptor Fzd7, and that its candidate ligand Wnt7a was upregulated during muscle regeneration. Notably, while Wnt7a did not affect the growth or differentiation of myoblasts, Wnt7a markedly stimulated the symmetric expansion of satellite stem cells on cultured myofibers. Silencing of Fzd7 abrogated Wnt7a activity and binding. Wnt7a signaling induced the redistribution of the polarity effector Vangl2 and silencing of Vangl2 blocked redistribution and inhibited Wnt7a stimulation of stem cell expansion. Lastly, Wnt7a overexpression significantly enhanced muscle regeneration and increased both the number of satellite cells and the proportion of satellite stem cells. Therefore, Wnt7a signaling through the planar cell polarity pathway controls the homeostatic level of satellite stem cells and hence regulates the regenerative potential of muscle.

# Apoptosis-Inducing Factor Regulates Skeletal Muscle Progenitor Cell Number and Self-Renewal

**Anne-Sophie Armand<sup>1,2§</sup>, Iman Laziz<sup>2 §</sup>, Anne T. Bertrand<sup>1</sup>, Olivier Biondi<sup>2</sup>, Christophe Chanoine<sup>2\*</sup>, Leon J. De Windt<sup>1,3\*</sup>**

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<sup>2</sup> *UMR 7060 CNRS, Equipe Biologie du Développement et de la Différenciation Neuromusculaire, Centre Universitaire des Saints-Pères, Université Paris Descartes, Paris, France;*

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Apoptosis Inducing Factor (AIF) is a highly conserved, ubiquitous flavoprotein localized in the mitochondrial intermembrane space. In vivo, AIF provides protection against neuronal and cardiomyocyte apoptosis induced by oxidative stress. Conversely in vitro, AIF has been demonstrated to have a proapoptotic role upon induction of the mitochondrial death pathway, once AIF translocates to the nucleus where it facilitates chromatin condensation and large scale DNA fragmentation. Given that the aif hypomorphic harlequin (Hq) mutant mouse model displays severe sarcopenia, we examined skeletal muscle from the aif hypomorphic mice in more detail. Adult AIF-deficient skeletal myofibers display a severe form of atrophy, associated with a fast to slow fiber type switch, both in "slow" muscles such as soleus, as well as in "fast" muscle such as extensor digitorum longus. This fiber type switch was conserved in regenerated soleus and EDL muscles of Hq mice subjected to cardiotoxin injection. In addition, muscle regeneration in soleus and EDL muscles of Hq mice was severely delayed. Freshly cultured myofibers from Hq mice displayed a decreased satellite cell pool, which could be rescued by pretreating aif hypomorphic mice with the manganese-salen free radical scavenger EUK-8. Satellite cell activation was delayed in Hq primary culture compared to controls. However, AIF deficiency did not affect myoblast cell proliferation and differentiation. Thus, AIF protects skeletal muscles against oxidative stress-induced damage primarily by protecting satellite cells against oxidative stress and maintaining skeletal muscle stem cell number and activation.

## **PW1 interstitial cells (PICs): a new source of myogenic progenitors**

**Pannérec A, Mitchell K.J, Marazzi G, Sassoon D., Myology Group  
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Non-satellite cell muscle progenitors can participate in muscle regeneration, however their precise anatomical location remains unclear. During post-natal growth, PW1/Peg3 is expressed in satellite cells and a sub-population of muscle interstitial cells, termed PICs (PW1 interstitial cells), which do not express any known lineage marker. PICs can be isolated from muscle by FACS using stem cell-surface markers. Thus, we obtain a population highly enriched in PICs (PW1<sup>+</sup>/Pax7<sup>-</sup>, >80%), which is distinct from the satellite cell population (PW1<sup>+</sup>/Pax7<sup>+</sup>, >70%). In culture, PICs show two main phenotypes: smooth muscle and skeletal muscle. In the presence of satellite cells, PICs convert readily to the myogenic lineage (PW1<sup>+</sup>/Pax7<sup>+</sup>/MyoD<sup>+</sup>), and fuse together or with existing fibers. Interestingly, in the Pax7 mutant, PICs cannot be recruited to the myogenic lineage whereas pure satellite cells show pronounced myogenic potential. These data suggest that PICs are muscle progenitor cells that require Pax7 to switch to a myogenic phenotype. To better understand the relationship between PICs and satellite cells, we performed Affymetrix-based analyses using RNA extracted from freshly sorted cells. As expected, Pax7 and MyoD were detected at high levels in satellite cells. In contrast, PICs express genes involved in embryonic development as well as stem cell-related genes (Meox, Runx). In addition, several growth factors and their receptors were differentially expressed, suggesting a paracrine relationship between PICs and satellite cells, and this was confirmed *in vitro*. Lastly, to discern the lineage relationship between PICs and satellite cells, we used the Pax3-cre mouse crossed to the floxed Rosa lac Z mouse. We find that PICs do not originate from the Pax3 lineage, therefore PICs are originate independently from satellite cells.

# **Muscle resident macrophages of epi/perimysium crucially control the inflammatory cell reaction to myoinjury**

**Madly Brigitte<sup>1</sup>, Clementine Shilte<sup>2</sup>, Anne Plonquet<sup>3</sup>, Yasmine Baba-Amer<sup>1</sup>, Adeline Henri<sup>4</sup>, Shhragim Tajbakhsh<sup>5</sup>, Matthew Albert<sup>2</sup>, Romain K.Gherardi<sup>1,\*</sup>, Fabrice Chrétien<sup>1,5\*</sup>.**

Skeletal muscle represents a specific but incompletely characterized immunologic microenvironment. In particular, very little is known about muscle resident macrophages (MPs) and dendritic cells (DCs), and how these cells are recruited into injured muscle. We report that the connective tissue surrounding mouse muscle and muscle fascicles (epi/perimysium) hosts a population of resident  $CD11b^+CD11c^-F4/80^+Ly-6C^-CX3CR1^-$  MPs, and is used as a privileged migration pathway by incoming/outgoing leukocytes after notexin myoinjury. Using BM transplantation experiments to discriminate resident from exudate MPs, cytokine screening, and selective resident MP depletion in GFP+BM chimeric *Tg:CD11bDTR* mice, we observed that resident MPs concentrate in the epimysium after myoinjury, selectively release KC and MCP-1, and crucially contribute to massive recruitment of neutrophils and monocytes (MOs) from blood. The paradigmatic myoinjury-induced inflammation is characterized by early appearance of  $Ly-6C/Gr1^{hi}CX3CR1^{lo}CD11c^-$  cells in the epimysium corresponding to recruited MOs, and progressive substitution by  $Ly-6C/Gr1^{lo}CX3CR1^{hi}$  cells, a subset previously shown to enhance myogenic cell differentiation and now found to be mostly composed of  $CD11c^{int}$  cells. These  $CD11c^{int}$  cells mainly derive from circulating  $CCR2^+$ MOs, functionally behave as immature APCs, and migrate to draining LNs where they acquire mature DC phenotype ( $CD11c^+Ia^+CD80^+$ ). In conclusion, resident epi/perimysial MPs play a crucial role in the inflammatory cell reaction to myoinjury which is both integral to myorepair and links innate and adaptive immunity.

## **Distinct regulatory cascades govern extraocular and branchiomic muscle progenitor cell fates.**

**Ramkumar Sambasivan<sup>1</sup>, Barbara Gayraud-Morel<sup>1</sup>, Gérard Dumas<sup>1</sup>, Clémire Cimper<sup>2</sup>, Sylvain Paisant<sup>1</sup>, Robert Kelly<sup>3</sup> and Shahragim Tajbakhsh<sup>1\*</sup>**

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Genetic regulatory networks governing skeletal myogenesis in the body are well understood, yet their hierarchical relationships in the head remain unresolved. We show that extraocular muscles have an obligate requirement for *Myf5* or *Mrf4* for initiating myogenic fate. In contrast, *Mrf4* as well as *Pax7* are dispensible for initiating pharyngeal muscle progenitor fate. Notably, essentially all branchiomic muscles are absent in *Tbx1:Myf5* double mutants indicating that *Tbx1* and *Myf5* act synergistically in this location. As in the body, *Myod* acts epistatically to the initiating cascades in the head. Thus, complementary pathways, governed by *Pax3* in body, and *Tbx1*, in pharyngeal muscles, but absent in extraocular muscles, activate the core myogenic network (MRFs) for initiating myogenesis. Interestingly, adult muscle stem cells maintain their ontogenic regulatory signatures. In spite of this "developmental memory", heterotopic transplantations of extraocular satellite cells to limb muscle failed to generate eye muscle specific markers, although robust engraftment was observed. These results indicate that satellite cells alone do not dictate the unique muscle phenotypes. This study identifies unique genetic relationships in head myogenesis that may provide insights into myopathies which often affect only subsets of muscles.

# Myogenic factors and CDKI: regulation of cell cycle exit during in vivo muscle development

**Bismuth K<sup>1</sup>, Vincent S<sup>2</sup>, Sambasivan R<sup>3</sup>, Yao Z<sup>4</sup>, Tapscott S<sup>4</sup>,  
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Muscle cell formation is a coordinated process of tissue-specific gene expression, proliferation and differentiation. In order to safeguard the developing muscle cells against a prolonged proliferation or a premature differentiation, cell cycle exit has to be tightly regulated. In the myogenic lineage, irreversible cell cycle exit is controlled by the Cyclin Dependent Kinase Inhibitors (CDKIs) p21 and p57. Mice lacking these two genes harbor severe muscle defect characterized by a failure to exit cell cycle and an increased apoptosis.

To address the role of MRFs in the regulation of growth arrest, we have investigated the expression of CDKI throughout development in a series of MRFs mutant mice. We found that none of the MRFs per se are necessary to trigger cell cycle exit, rather we hypothesize that inducing growth arrest might be another shared common feature of all MRFs. Moreover, at E10,5 and E11,5 in *Myf5<sup>nLacZ/+</sup>* we found less myoblasts that have exited the cell cycle in the head than in the trunk, indicating that the timing of growth arrest and/or the program used to exit cell cycle are different in these two regions. Furthermore, analysis of *MyoD* null mice reveal that forelimb myoblasts that only expressed MYF5 were able to exit cell cycle, suggesting first that cell cycle exit occurs at the cell determination stage and not at the differentiation stage, as previously thought, and second the existence of an uncoupling between cell cycle exit and cell differentiation. We are currently investigating the molecular aspect of growth arrest and the identification of p21 and p57 muscle specific regulatory element used during muscle development.

# **Six1 and Six4 gene expression is required to activate the fast-type muscle gene program in the mouse myotome.**

**Claire Niro<sup>1</sup>, Josiane Demignon<sup>1</sup>, Stéphane Vincent<sup>2</sup>, Julien Giordani<sup>1</sup>, Maryline Favier<sup>1</sup>, Yubing Lu<sup>3</sup>, Isabelle Guillet-Deniau<sup>1</sup>, Alexandre Blais<sup>3</sup> and Pascal Maire<sup>1</sup>**

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Genesis of muscle fiber-type diversity during mammalian development remains unexplained. We provide data showing that Six homeoproteins are required to activate the fast-type muscle program in the mouse primary myotome. The first mouse myocytes, that activate the expression of muscle structural genes, express both fast-type and slow-type muscle genes independently of their medio-lateral position. The presence of Six1 and Six4 proteins allow these myocytes to activate their fast-type program: in *Six1*<sup>-/-</sup>/*Six4*<sup>-/-</sup> (*SixdKO*) embryos fast-type muscle gene expression is no longer detected by in situ hybridization from E9.5. We further demonstrate that *Pax3*, *Eya1* and *Eya2* genes, that belong to the myogenic Six/Eya/Pax genetic network, are not required for the observed early fast-type fiber induction. We also demonstrate that *MRF4* and *myogenin*, two muscle regulatory factors controlled by Six in the embryo, are dispensable for fast-type muscle genes activation in synergy with Six homeoproteins. Thus, these data identify Six homeoproteins as key molecules, necessary in the earliest differentiated myogenic cells present in the embryo, to directly activate a network of fast-type muscle fiber specific genes in synergy with Myf5.

# Rôle de Wnt4 dans la mise en place de la Jonction Neuromusculaire

**Laure Strohlic et Claire Legay**

*INSERM UMR 686 Biologie des Jonctions Neuromusculaires  
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Le développement de la synapse neuromusculaire implique une communication via divers processus de signalisation réciproques entre les motoneurones et leurs cibles musculaires. L'un des acteurs clés de la formation des connections synaptiques est la voie de signalisation Wnt. Les molécules Wnts sont des glycoprotéines sécrétées jouant un rôle essentiel au cours du développement et dans un grand nombre de processus physiologiques. De nombreuses études ont montré l'implication de la voie de signalisation Wnt dans la formation de la jonction neuromusculaire (JNM) chez les invertébrés, mais à ce jour, il existe très peu de données dans la littérature concernant l'identité et le rôle des facteurs Wnts endogènes dans les étapes précoces de la formation de la JNM des vertébrés. Parmi les 19 molécules Wnts répertoriées chez la souris, 10 sont exprimées par la cellule musculaire. Des études de puces à ADN et de RT-PCR quantitative ont montré que le facteur Wnt4 présente un profil d'expression qui varie au cours de la différenciation musculaire, fortement exprimé à un stade très précoce (juste après la fusion des myoblastes en myotubes) au moment où les premiers contacts synaptiques se forment *in vivo* et réprimé aux stades plus tardifs de la différenciation musculaire. Par ailleurs, l'analyse des JNM réalisée sur des embryons de souris knock-out pour Wnt4 à un stade E18.5 du développement embryonnaire a révélé d'importants défauts d'innervation : en effet, les terminaisons axonales reconnaissent peu les agrégats de récepteurs nicotinique de l'acétylcholine (RACHs) et poussent de manière excessive le long du muscle; la largeur de la bande d'agrégats de RACH et la surface des agrégats de RACH sont fortement augmentés comparé aux embryons sauvages. L'accumulation à la JNM de protéines synaptiques clés telles que l'acétylcholinésterase, la rapysne et MuSK n'est pas perturbée. L'ensemble de ces résultats indique que Wnt4 est impliqué dans la mise en place de la JNM et montrent pour la première fois *in vivo* le rôle d'un facteur Wnt dans le processus d'innervation des synapses périphériques.

# **MAFbx/Atrogin-1 controls eIF3-f activity in skeletal muscle atrophy by targeting multiple C-terminal lysines**

**Alfredo CSIBI, Karen CORNILLE, Marie Pierre LEIBOVITCH, Lionel A. TINTIGNAC and Serge A. LEIBOVITCH.**

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Skeletal muscle (SM) mass depends upon a dynamic balance between anabolic and catabolic processes. SM hypertrophy is characterized by an increase of the diameter of muscle fibers and increased protein synthesis, mainly by activation of the IGF1/Akt/mTOR pathway. Muscle loss occurs as the result of a number of disparate conditions including cancer, diabetes, AIDS, sepsis, renal failure, aging, cachexia, and other systemic diseases. These diverse conditions result in reduced protein synthesis and increased protein breakdown. The process of atrophy is characterized by the activation of the ubiquitin-proteasome proteolysis pathway. The E3-ligase MAFbx is upregulated in multiple models of atrophy and appears to be essential for accelerated muscle protein loss. Recently, we showed that MAFbx interacts with the initiation factor eIF3-f for polyubiquitylation and further proteasome-mediated degradation during SM atrophy. eIF3-f is a regulatory subunit of the eIF3 complex that interacts directly with mTOR and S6K1 to coordinate the assembly of the preinitiation complex. Furthermore, overexpression of eIF3-f in SM induces a marked hypertrophy associated with an increase of sarcomeric proteins. Thus, the specific targeting of eIF3-f by MAFbx may account for the decreased protein synthesis observed in multiple types of SM atrophy. In the present work, we have mapped the region of eIF3-f responsible for its proteolysis. We showed that six lysines located in the C-terminal domain are required for fully MAFbx-mediated polyubiquitylation and degradation by the proteasome. In addition, site-directed mutagenesis of these six lysines (mutant K<sub>5-10</sub>R) displayed hypertrophic activity *in cellulo* and *in vivo* and was able to protect against starvation-induced SM atrophy. Taken together, our data demonstrate that the C-terminal modifications, believed to be critical for proper eIF3-f regulation, are essential and contribute to a fine-tuning mechanism that plays an important role for eIF3-f function in SM.

# Characterisation of glucocorticoid signaling in skeletal muscle.

**Delphine Duteil, Céline Chambon, Pierre Chambon and Daniel Metzger**

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Skeletal muscle is a dynamic tissue that regulates its size in response to various external factors, including mechanical load, neural activity, hormones and growth factors, stress and nutritional status. Muscle protein serves as a reserve of amino acids that can be mobilized during fasting for hepatic gluconeogenesis. An important physiological adaptation to fasting is an increase of the overall rate of muscle protein breakdown, leading to rapid loss of muscle mass. A similar muscle atrophy is a common debilitating feature of aging and of many systemic diseases, including cancer, sepsis, hyperthyroidism, uremia and diabetes, and occurs in specific muscles upon disuse or nerve injury. Interestingly, many pathological conditions characterized by muscle atrophy are associated with increased circulating glucocorticoid levels, and muscle atrophy is one of the major side effect of glucocorticoids used as anti-inflammatory drugs.

Most of the effects of glucocorticoids are mediated by the glucocorticoid receptor (GR), a ligand-dependent transcription factor, member of the nuclear receptor superfamily.

To understand the molecular and cellular basis of glucocorticoid-induced skeletal muscle atrophy, we generated mice in which the glucocorticoid receptor is selectively ablated in skeletal muscle myocytes of adult mice ( $GR^{(i)skm^{-/-}}$  mice). The analysis of such mice revealed that their muscle mass and strength is increased. Moreover,  $GR^{(i)skm^{-/-}}$  mice are resistant to fasting-induced muscle atrophy, thus indicating that glucocorticoids induce muscle atrophy by activating their nuclear receptor in myocytes.

Detailed analysis of our mutant mice should allow to identify the pathways controlled by glucocorticoids in skeletal muscle myocytes, and may facilitate the development of potent anti-inflammatory glucocorticoids without debilitating effects on skeletal muscle.

# Characterization of the Human Myoblast “secretome” during *in vitro* differentiation

***Marie-Catherine Le Bihan<sup>1</sup>, Adelina Rogowska-Wrzesinska<sup>2</sup>, Søren Skov Jensen<sup>2</sup>, Jeanne Lainé<sup>3</sup>, Anne Bigot<sup>1</sup>, Prashanth Kumar Kandalla<sup>1</sup>, Ole Nørregaard Jensen<sup>2</sup>, Vincent Mouly<sup>1</sup>, Denis Furling<sup>1</sup>, Gary R. Coulton<sup>4</sup> & Gillian Butler-Browne<sup>1</sup>***

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Extracellular factors play critical roles in regulating myogenesis during pre- and post-natal development. Growth and cytokines have been shown to regulate every aspect of adult satellite cells myogenesis including activation, proliferation and differentiation.

The main objective of the current study was to characterize the secretome of differentiating myoblasts derived from human muscle biopsies. For that purpose, we adopted a combined proteomic strategy using 2D gel electrophoresis coupled to mass spectrometry (2DE-MS) and a gel-free tandem mass spectrometry based approach (HPLC-ESI-MS/MS) for the separation and identification of protein elements of the conditioned medium (CM) following the induction of myoblast fusion in serum free media. This discovery based proteomic study was performed in parallel with a multi-analyte Luminex-based immunoassay for the detection of low abundance cytokines and growth factors.

By combining 3 complementary and powerful proteomic strategies, 965 non redundant proteins were identified. To our knowledge, the proteins reported here constitute one of the largest catalogue of any cell secretome to date. The identified proteins were further submitted to stringent computational analysis for predicting secretory pathways. Classical and leaderless secreted molecules are defining the “soluble” secretome as released “free” in the extracellular space and represented a total of 257 proteins, of which 85 were extracellular matrix components.

A number of these “soluble secreted” proteins has been involved previously in biological processes related to skeletal muscle homeostasis & myogenesis such as Galectin-1 & IGF-II key regulators of myoblast fusion and differentiation. Among the “non secretory” proteins, computational analysis revealed that 434 of them had been found in extracellular vesicles. Further characterization of these muscle derived secretory vesicles is currently under going.

# **Involvement of M-cadherin, Rac1 and ARF6 signaling pathways during myoblast fusion**

**Sophie Charrasse**

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Myoblast fusion results from an ordered sequence of events, including cell migration, alignment, recognition, adhesion and membrane merging but the regulatory circuits are poorly known. In mammalian cells, molecules from the cadherin family are involved in cell-cell recognition and adhesion and they act as adhesion-activated signaling receptors which regulate Rho family GTPases. Rho GTPases promote the reorganization of the actin cytoskeleton, an essential process during myoblast cell fusion. Recently, we have showed that M-cadherin-dependent adhesion activates the Rho GTPase Rac1 *via* the Rho-GEF Trio at the time of myoblast fusion. Cadherins form a large complex at the plasma membrane, and by co-immunoprecipitation experiments using C2C12 myoblasts, we found that ARF6 GTPase is associated with M-cadherin and Rac1 at the fusion time. During the differentiation process of C2C12 myoblast, the ARF6 GTPase is activated just prior Rac1, an event required for myoblast fusion. *ARF6* knockdown decreases myotube formation and modifies myoblast morphology. Analysis of myotube morphology and number and measurement of the fusion index after transfection of Rac1 and/or ARF6 in their different activation states (WT, DN) confirms that both GTPases are involved in myotube formation. Moreover ARF6 and Rac1 activities were both detected during skeletal muscle regeneration. In conclusion we show that ARF6 participates in myoblast fusion into myotubes in combination with Rac1.

# **Nuclear positioning during myofiber formation**

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Nuclear positioning within cells is known to occur during the formation of a multinucleated myofiber, which is formed by the fusion of mononucleated myoblasts . After fusion, nuclei regroup at the center before moving to the periphery of the myotube during myofiber maturation. Furthermore, some nuclei become anchored near the neuromuscular synapse. It has been proposed that distribution of nuclei in the mature myofiber forms distinct functional domains and this is probably important for muscle function since misdistribution of nuclei is observed in some muscular dystrophies. The question of how position of nucleus is established in muscle cells and what are the molecular mechanisms responsible for the position of the nucleus in muscle cells has not been addressed. The goal of our research is to determine, through time lapse microscopy and biochemical studies, which mechanisms are involved in the nuclear movements during myotube formation. We are investigating the role of two cytoskeleton networks, microtubules and actin, through the use of specific inhibitors or mutants expressing vectors. We found that microtubules are required for nuclear movement after fusion. We are currently investigating the role small GTPases ( Cdc42, Rho and Rac) and their effectors.

## **Invivo imaging of muscle fibers**

**Johann BÖHM, Marc KOCH, Yves LUTZ, Jean-Luc VONESCH, Didier HENTSCH, Jean-Louis MANDEL and Jocelyn LAPORTE**

In skeletal muscle, positioning of the nuclei is an active process, but the underlying mechanisms are not well understood. Centronuclear myopathies (CNM) comprise 3 classes of congenital myopathies with abnormal centralization of the nuclei in muscle fibres: the severe X-linked form with neonatal onset (XLCNM), the autosomal recessive form with childhood onset (ARCNM), and the autosomal dominant form with adult onset (ADCNM). The mutated genes are involved in membrane trafficking and signalling. The IGBMC imaging center has developed a confocal microscope offering the possibility to follow fluorescent markers in the living mouse. With this innovative technology we aim to analyze the general cellular organization of muscle fibers both under normal conditions and in mice models for centronuclear myopathies during the appearance of the clinical signs. Here we present preliminary data on the application and the detection of fluorophores in murine skeletal muscle. By focussing series of z-stacks we are able to virtually travel across single muscle fibers. Based on the 3Dreconstructions of the z-stacks we can determine the position and the size of subcellular structures. This project provides a direct application of the confocal macroscopy technology and might therefore suggest further scientific and medical approaches.

# Defective excitation-contraction coupling in muscle fibres from a mouse model of X-linked myotubular myopathy

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We have investigated the mechanisms leading to X-linked myotubular myopathy (XLMTM), a severe congenital disorder due to loss of function mutations in the *MTM1* gene, encoding myotubularin, a phosphoinositide phosphatase thought to have a role in plasma membrane homeostasis and endocytosis. Using a mouse model of the disease, we report that *Mtm1*-deficient muscle fibres have a decreased number of triads and abnormal longitudinally oriented T-tubules. In addition, sarcoplasmic reticulum (SR) Ca<sup>2+</sup> release elicited by voltage-clamp depolarisations is strongly depressed in myotubularin-deficient muscle fibres, with the voltage-sensing steps of E-C coupling, myoplasmic Ca<sup>2+</sup> removal and SR Ca<sup>2+</sup> content essentially unaffected. At the molecular level, *Mtm1*-deficient myofibres exhibit a 3-fold reduction in type 1 ryanodine receptor (RyR1) protein level. These data reveal a critical role of myotubularin in the proper organisation and function of the E-C coupling machinery, and strongly suggest that defective RyR1-mediated SR Ca<sup>2+</sup> release is responsible for the failure of muscle function in myotubular myopathy.

# Identification des perturbations protéiques globales par spectrométrie de masse dans le modèle canin de la dystrophie musculaire de Duchenne

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Dans la dystrophie musculaire de Duchenne, l'absence d'une protéine, la dystrophine, entraîne une délocalisation du DGC (complexe glyco-protéiques qui lui est normalement associé), l'instabilité du sarcolemme, l'entrée de calcium et l'activation de calpaïnes. Ce processus a pour conséquence directe une nécrose et une atrophie progressive de l'ensemble du système musculaire. Cependant ces éléments ne suffisent pas à expliquer le phénotype dégénératif observé car de nombreux dérèglements cellulaires touchant des voies de signalisation semblent impliqués. Les analyses protéomiques du muscle dystrophique versus normal présentent un grand intérêt afin d'identifier et de caractériser de nouveaux bio-marqueurs protéiques associés à dystrophie musculaire de Duchenne dans un modèle animal, très proche de l'homme, le chien GRMD. Récemment, une première étude utilisant des puces à protéines nous a permis de comparer le profil de phosphorylation de 19 kinases, révélant ainsi que Akt1, GSK3 $\alpha$  et p70S6K présentent toutes une phosphorylation réduite dans le muscle du chien GRMD (Féron et al., A.J.Pathol 2009). D'autres voies sont également perturbées, et une large étude protéomique, non biaisée, est nécessaire afin d'identifier les voies métaboliques globales dérégulées dans la dystrophie de Duchenne. Cependant, pour une étude globale du protéome, la difficulté des expérimentations reste liée à la complexité cellulaire et moléculaire du tissu musculaire, et il apparaît aujourd'hui indispensable de fractionner et simplifier les extraits protéiques du muscle squelettique. A partir de fractions subcellulaire cytosoliques puis d'une isolation du phosphoprotéome, nous avons utilisé une approche protéomique quantitative, l'ICAT, couplée à la spectrométrie de masse (ICAT/MS), pour identifier et quantifier les protéines dérégulées dans les muscles canins affectés par la myopathie de Duchenne. Des différences quantitatives ont été identifiées, 54 protéines sont significativement sur-exprimées, 30 sont sous-exprimées dans le muscle GRMD. Les protéines dérégulées ont ensuite été classées selon la classification "Gene Ontology" afin d'identifier les différences, d'un point de vue fonctionnel et selon la localisation subcellulaire des protéines. Les protéines mitochondriales et membranaires sont globalement sous exprimées dans le muscle GRMD, soulignant la fragilité de ces compartiments. Parmi les protéines sous exprimées, il apparaît clairement une majorité de protéines appartenant au métabolisme glucidique, énergétique ou présentant une activité oxydo-réductase. De plus, les protéines impliquées dans l'apoptose, la voie de signalisation calcique ou les protéines du développement musculaire montrent une surexpression dans le muscle dystrophique. L'ensemble de ces résultats permettra d'identifier de nouveaux marqueurs biologiques et de nouvelles cibles potentiellement utilisables en thérapie pharmacologique.

# **Specific Inhibition of Protein Quality Control System as a Therapeutic Approach for Treatment of Sarcoglycanopathies.**

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Sarcoglycanopathies are recessive muscular disorders caused by defects in a group of transmembrane proteins, known as sarcoglycans, and part of the dystrophin-associated complex. Mutations in the  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  sarcoglycan genes lead to a similar phenotype and are referred as limb-girdle muscular dystrophies type 2D, 2E, 2C and 2F (LGMD2D, 2E, 2C and 2F) respectively. Dysfunction of one of the sarcoglycan destabilizes the whole sarcoglycan complex, leading to a partial or complete disappearance of the other sarcoglycans at the membrane. To date, no treatment exists for these diseases. The most frequently reported mutation in the  $\alpha$ -sarcoglycan gene is the substitution of an arginine in position 77 by a cysteine ( $\alpha$ -R77C). We demonstrated that this mutation encodes a misfolded protein that fails to be delivered to its proper sarcolemmal localization due to blockade in the endoplasmic reticulum (ER). The quality control system of the ER is an important monitoring mechanism in the protein maturation process, which ensures export of properly folded proteins from the ER. Incorrectly or incompletely folded proteins are retained in the ER for refolding or translocated in the cytosol for degradation by the ER associated proteasome (ERAD). We hypothesized that, by blocking the ER quality control system, we should be able to rescue the misfolded  $\alpha$ -sarcoglycan protein at the cell membrane. Consistently, results of our experiments using an heterologous cellular model of sarcoglycan complex formation showed that the  $\alpha$ -mannosidase I inhibitors prevent  $\alpha$ -R77C degradation by proteasome/ERAD and restore correct localization of the protein. Furthermore, this membrane targeting allows the assembly of the sarcoglycan complex. Consequently, we were able to rescue other sarcoglycan mutations using the same approach. Overall, these results suggests a therapeutic approach for LGMD2 patients carrying mutations that impair sarcoglycan trafficking.

## DUX4 / DUX4c expression and inhibition in FSHD.

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Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant disorder linked to contractions of the *D4Z4* repeat array in the 4q35 subtelomeric region. In non-affected individuals the array comprises 11-100 tandem copies of a 3.3 kb element named *D4Z4*. Patients only have 1-10 copies, and the disease is more severe with smaller copy numbers. FSHD is a disorder of gene regulation induced by alteration of the chromatin structure associated to the repeat array. A strong transcriptional enhancer mapped in the 5' part of the *D4Z4* unit is normally blocked by a nuclear matrix attachment site (MAR) that splits the repeat array and neighbour genes in 2 chromatin loops. In most FSHD muscle cells the MAR is weakened yielding a single chromatin loop where the *D4Z4* enhancer might up-regulate any gene (1).

Our group has identified the *DUX4* gene within each *D4Z4* element and the homologous *DUX4c* gene 42 kb of the *D4Z4* repeat array. We only detected the expression of the *DUX4* protein in FSHD muscle biopsies, in derived primary myoblasts and in immortalized FSHD primary myoblasts. *DUX4* is a transcription factor that targets a large set of genes. Its expression in C2C12 cells recapitulates key features of the FSHD molecular phenotype, including activation of the *PITX1* gene (specifically induced in FSHD muscles and involved in muscle atrophy; 2), repression of *MyoD* and its target genes, repression of glutathione redox pathway components, and sensitivity to oxidative stress (3).

The *DUX4c* protein is expressed in control myoblasts and muscles and induced in FSHD. Forced *DUX4c* expression in human TE671 cells induced a strong proliferation that is maintained in differentiation medium (2% serum). Since *DUX4c* specifically induced the MYF5 transcription factor, these findings suggested a role in muscle regeneration. We propose that as well an excess (in patients with FSHD) as a reduced amount (in families where the *D4Z4* deletion removes the *DUX4c* gene) of *DUX4c* expression could affect muscle regeneration and contribute to the FSHD pathology.

We want to explore the role of *DUX4* and *DUX4c* in human primary myoblasts and develop tools to inhibit their expression as an approach towards a therapeutic strategy. We generated recombinant lentiviruses expressing *DUX4c* or *DUX4*. We transduced control human immortalized myoblasts with these lentiviral vectors and induced their differentiation to compare their morphology with FSHD myotubes. *DUX4/DUX4c* expression has confirmed by immunofluorescence and western blot. In addition we could define siRNAs that interfere with the expression of *DUX4* or *DUX4c* as evaluated by western blot and immunofluorescence. We constructed and encapsidated shRNA-*DUX4/DUX4c* lentiviral vectors to express the most efficient siRNAs. We will test this tool in FSHD myoblasts/myotubes to assess whether a control phenotype can be recovered and maintained at long term.

- (1) Petrov *et al* (2008). A nuclear matrix attachment site in the 4q35 locus has an enhancer-blocking activity in vivo: Implications for the facioscapulohumeral muscular dystrophy. *Genome Res.* 18: 39-45.
- (2) Dixit *et al* (2007.) *DUX4*, a candidate gene of FSHD, encodes a transcriptional activator of paired-like homeodomain transcription factor 1. *Proc. Natl. Acad. Sci. U.S.A.* 104, 18157-18162.
- (3) Bosnakovski *et al* (2008). An isogenetic myoblast expression screen identifies *DUX4*-mediated FSHD-associated molecular pathologies. *EMBO J.* 27:2776-79.

# Recherche de gènes modificateurs de la Dystrophie Musculaire OculoPharyngée.

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Nous utilisons un modèle *Caenorhabditis elegans* de la Dystrophie Musculaire OculoPharyngée (OPMD). La surexpression dans les cellules musculaires du nématode exprimant *myo-3*, de la protéine fluorescente GFP et d'un transgène exprimant au choix un allèle de PABPN1 (A0, A10 ou A13), permet de détecter une perte des signaux GFP en présence de l'allèle muté PABPN1-A13. Nous proposons d'étudier l'environnement génétique de ce modèle de pathologie humaine en utilisant une banque d'interférents à ARN. Les vers sont incubés dans des plaques de 96 puits en présence d'un clone bactérien ARNi. Des photographies de chaque puits sont analysées pour le nombre moyen de signaux GFP par ver et par clone. Nous essayons ainsi de déterminer le potentiel modificateur de chaque gène et d'isoler des composantes génétiques majeures impliquées dans les mécanismes cytopathologiques induits par la forme mutée de PABPN1.

# **Dystrophin rescue by using exon-skipping and new technique of systemic delivery in dystrophic dog.**

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Duchenne Muscular Dystrophy (DMD) is an X-linked recessive disorder due to mutations in the gene that encodes dystrophin. Most of these mutations consist in large genomic deletions, although their extent is not directly correlated with the severity of the phenotype. Out-of-frame deletions lead to abortion of translation, dystrophin deficiency and severe DMD phenotypes, while internal deletions that produce in frame mRNAs leading to shorter proteins are responsible for a milder myopathy known as Becker Muscular Dystrophy (BMD). About 80% of the out-of-frame mutations could be theoretically rescued after restoring the translational frame by using exon skipping strategies. Here we used gene transfer in a large animal model of DMD, the Golden Retriever Muscular Dystrophy (GRMD) dog, to achieve the precise skipping of multiple exons spaced over 125,000 bp of the dystrophin pre-mRNA and the re-expression of a functional protein. Exon skipping was obtained with U7snRNAs (U7smOPT) carrying antisense sequences designed to mask determinants of exon 6 and 8 definition. These U7smOPT were introduced into skeletal muscle fibres by using Adeno Associated Viral (AAV2/1) vectors. This led to sustained correction of the dystrophic phenotype in extended muscle areas but the practical application of a clinically relevant gene therapy for DMD requires the treatment of the whole skeletal and cardiac musculature. Only a systemic injection would allow to approach this objective, as demonstrated by initial trials in murine models. Such a method is not without risk and requires long and expensive developments. First of all, it is essential to be able to produce the required quantity of vector to treat many patients. Then, the systemic mode of administration needs to be developed and its safety should be guaranteed. We are currently testing the feasibility of a systemic delivery of an AAV\_U7 vector in the GRMD dog model, in order to anticipate, on a rational basis, the adaptation of such protocols in man. The first results are promising and thus represent a critical milestone for the development of clinical trials in human patients.