Neuromuscular Biology and Disease
Histopathology/Pathophysiology overview

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Experimental & Clinical Neuromuscular Laboratories

MUSCLE TISSUE PROCESSING & STAINS

- Tissue blocks of skeletal muscle, **frozen** in isopentane cooled in liquid nitrogen. 12 µm thick sections are cut using a cryostat.
- The following *routine* stains are done :
- Basic histopathological stains: H & E and Gomori trichrome
- Special Stains:, oil red O, PAS, Congo red.
- Enzyme Histochemistry: NADH, SDH, COX, and ATPase, at pH 9.4, 4.6, 4.2. (Myophosphorylase, MAD, acid phosphatase if needed)
- Immune staining: carried out if needed
  - CD3, CD4, CD8, CD20 and CD68 cell markers, MAC
  - dystrophin (dys 1, 2, 3), sarcoglycans (α, β, γ, δ), dystroglycans (α, β), dysferlin, caveolin 3, laminin alpha 2 (merosin), utrophin, spectrin , collagen VI
  - specific antibodies for protein aggregates

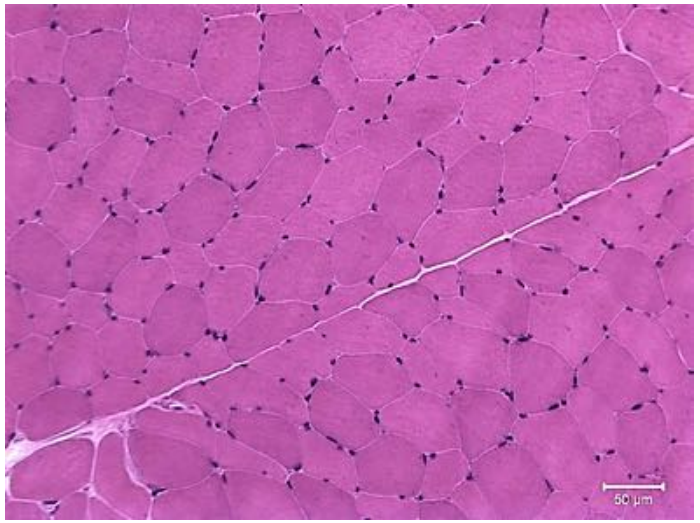
- EM piece placed in glutaraldehyde for further processing
- A separate piece of muscle frozen for biochemical/genetic studies

H&E and Gomori Trichrome

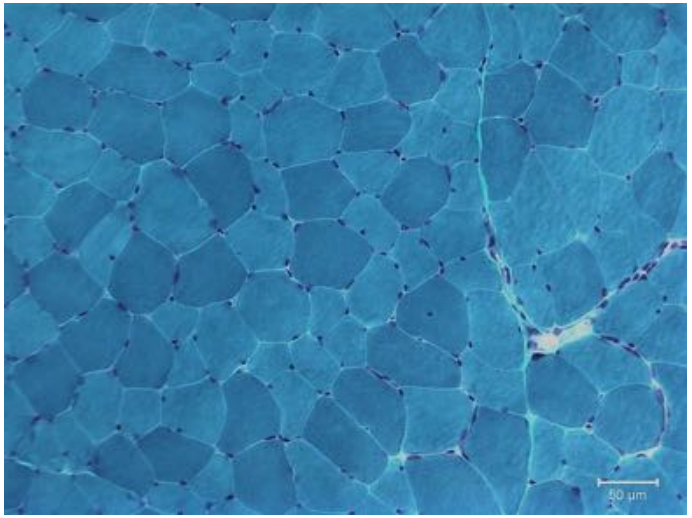
Give wide range of information for **general pathological reactions** :

- ✓ Necrosis
- ✓ Regeneration
- ✓ Fiber size – atrophy/hypertrophy
- ✓ Inflammation
- ✓ Fibrosis
- ✓ Structural changes
- ✓ Organelle changes

Hematoxylin & Eosin (Gill's)



Modified Gomori Trichrome



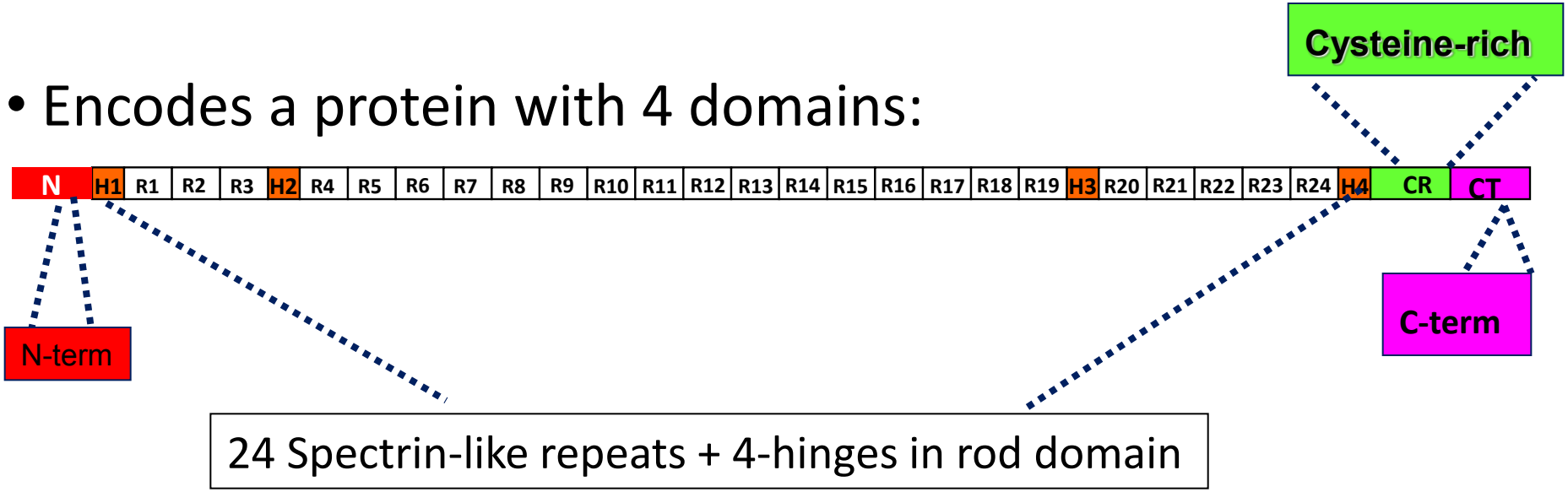
Pathogenesis of DMD

- 1987 the DMD gene was cloned
  - Opened up new avenues for potential treatment

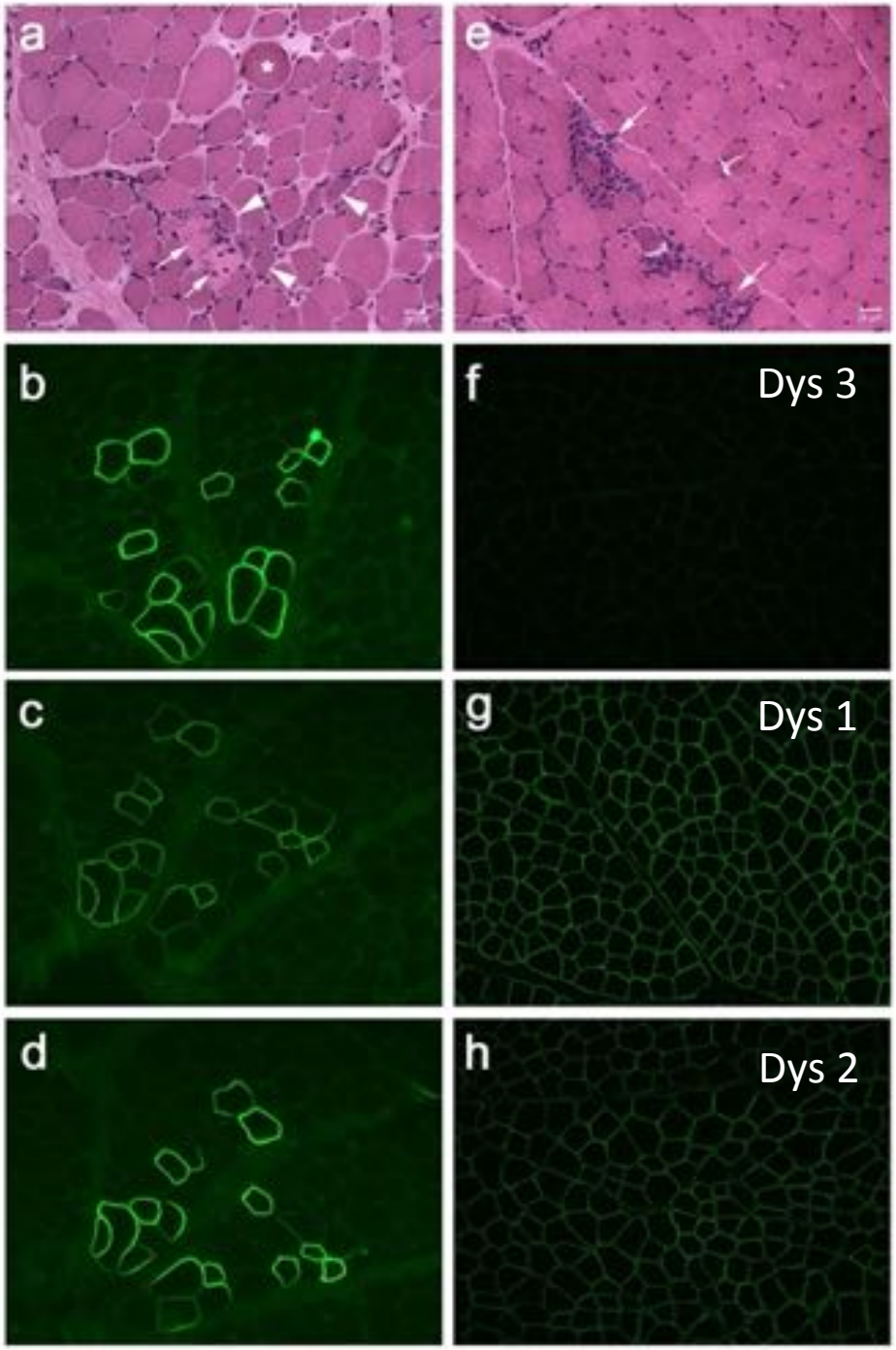
The largest gene in human genome – 2.6 m bp

-a critical obstacle for molecular manipulation

- Encodes a protein with 4 domains:

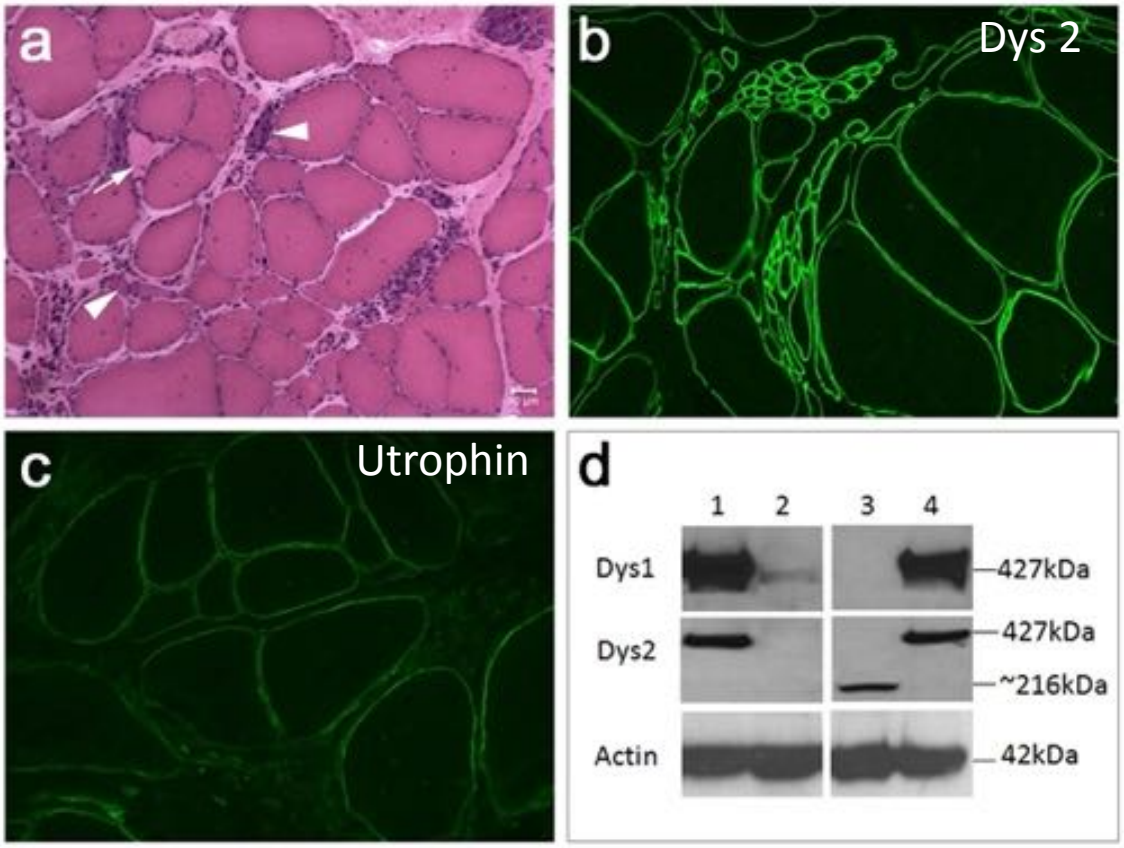


Immune stains:
Dystrophinopathies



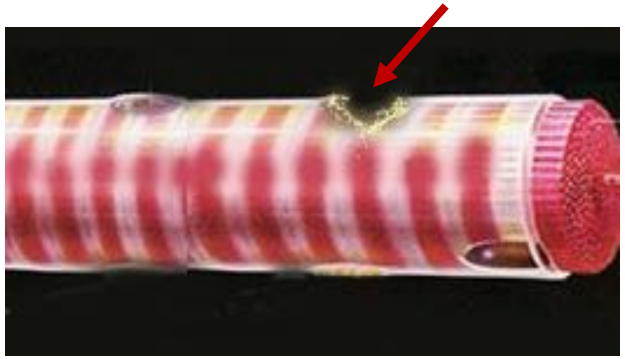
DMD
Exon 55-63 duplication

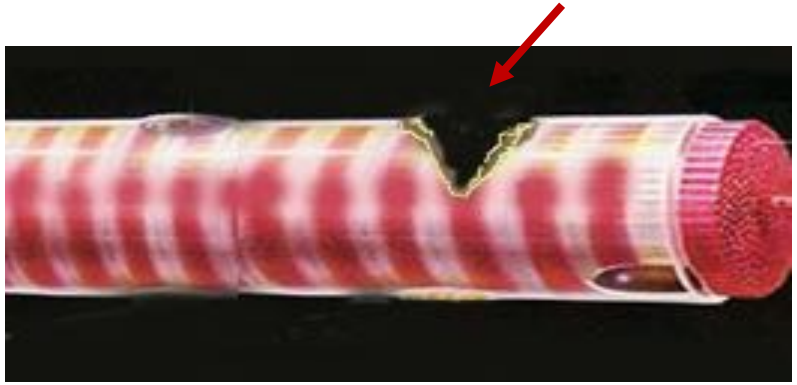
BMD
Exon 19-29 duplication

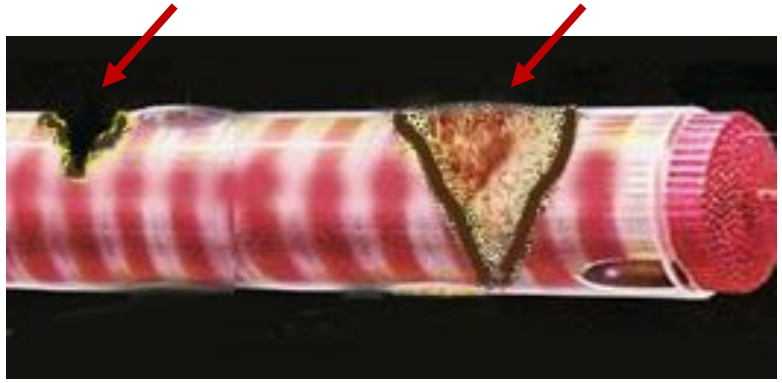


Twelve y/o boy with BMD who has exons 10-44 and promoters Dp260 and Dp240 in-frame deletion

Consequences of Absent Dystrophin







Reduced or absent dystrophin

↓

Mechanically weakened plasma membrane, prone to focal tears during contractile activity

↓

Massive influx of extracellular calcium, activation of proteolytic enzymes

→

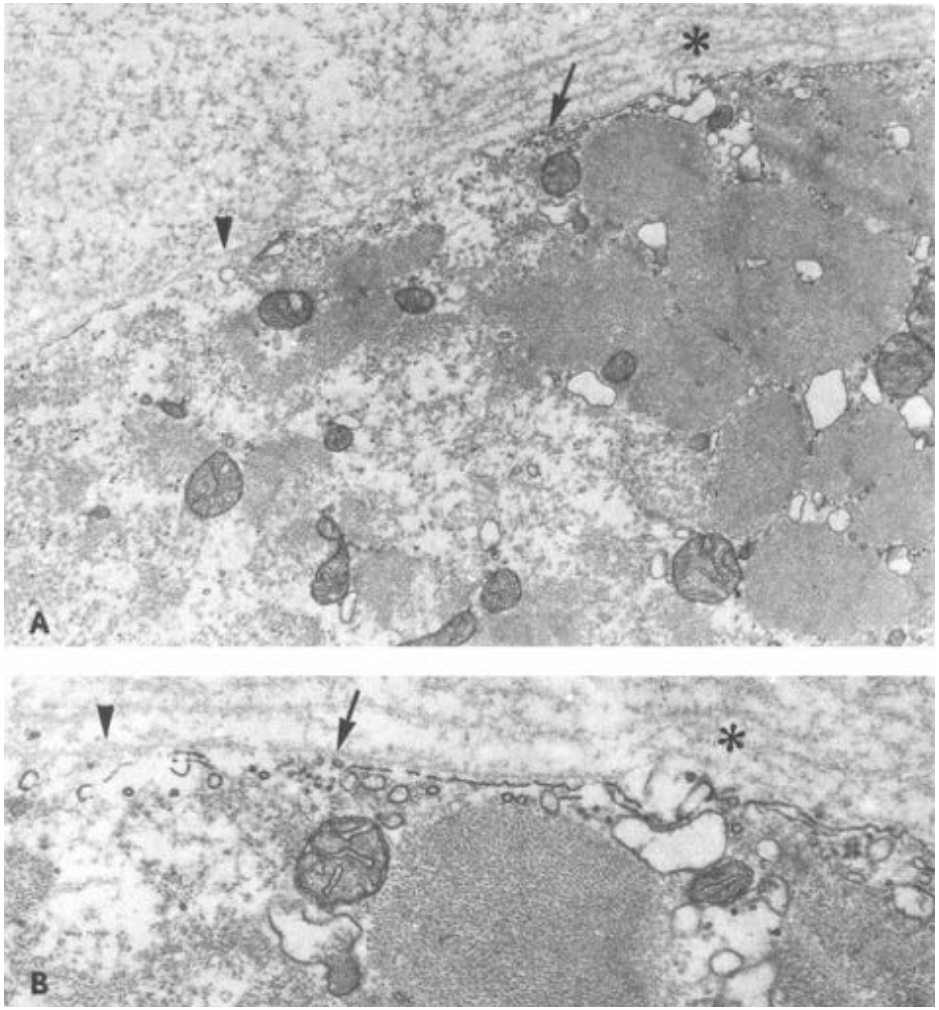
Segmental necrosis in muscle cell

Landmark Article:

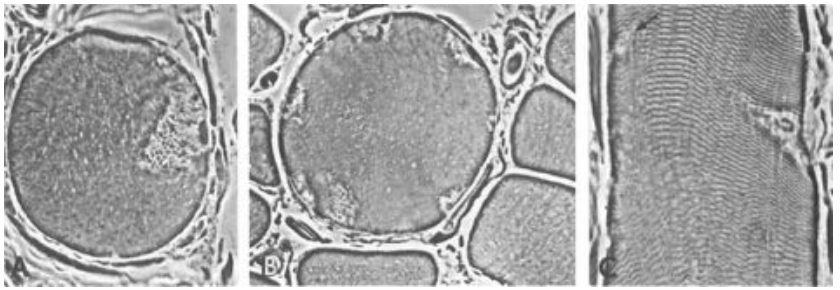
Duchenne dystrophy: Electron microscopic findings pointing to a basic or early abnormality in the plasma membrane of the muscle fiber

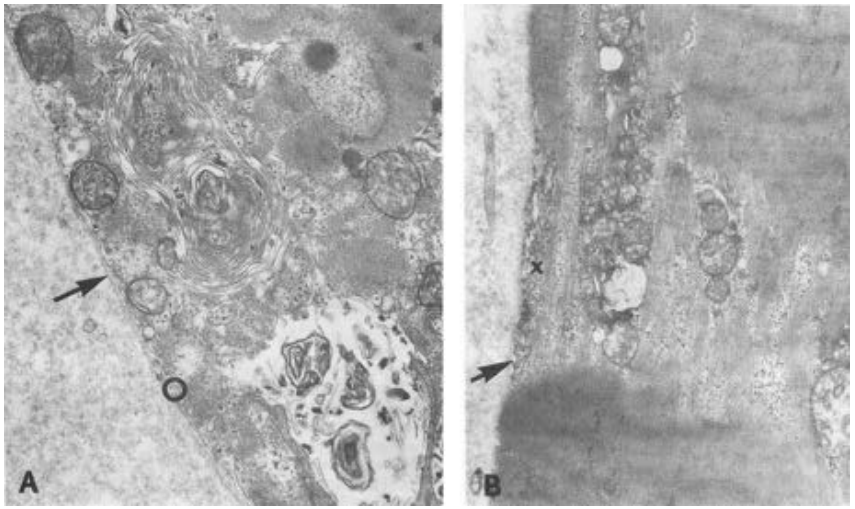
Bahram Mokri, MD; and Andrew G. Engel, MD

Neurology 1975;25:1111-1120

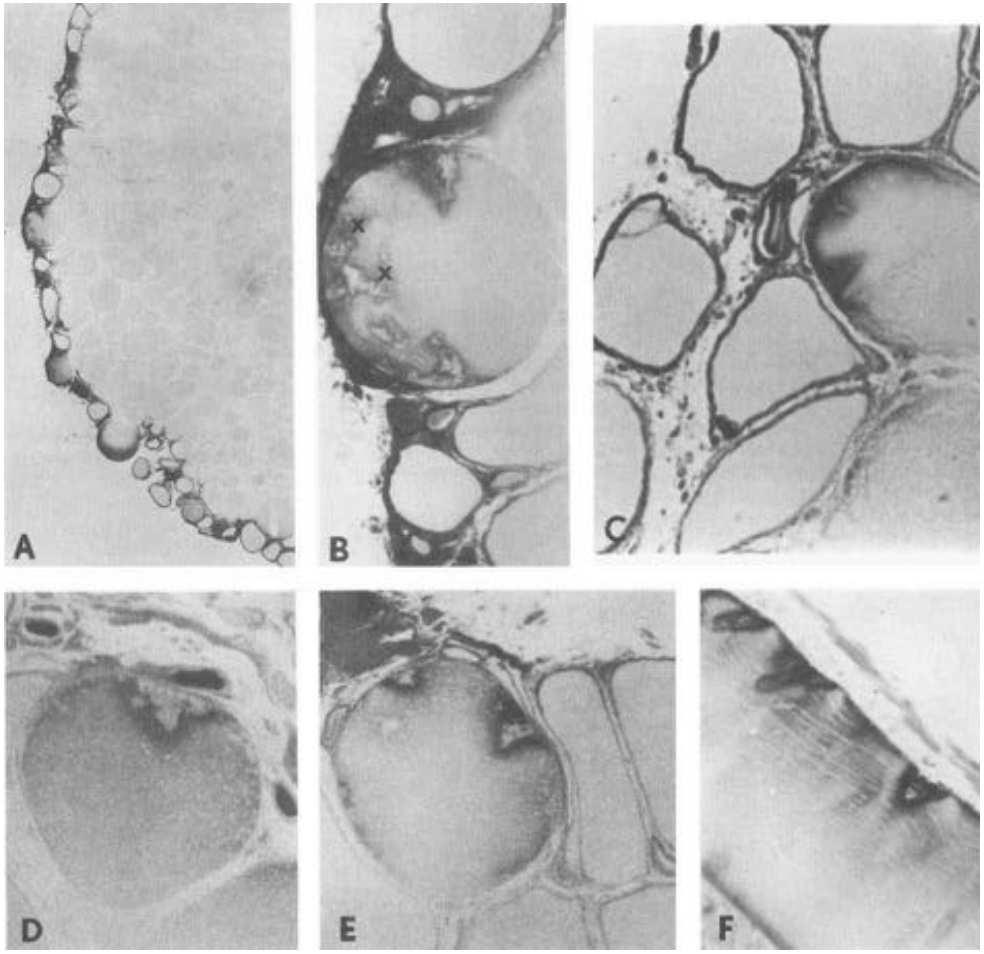


High-resolution phase microscopy





Ingress of the peroxidase-containing extracellular fluid into the delta lesions



"These findings suggested that the membrane lesions were an ineffective barrier to the ingress of extracellular fluid, and we confirmed this by demonstrating the focal penetration of peroxidase-labeled extracellular fluid into the "delta lesions."

" We concluded our report by stating that ". . . if the cause of the structural defects resided in the membrane itself (as it very well might), it could be caused by an abnormal lipid component **or** by a defective structural protein in the membrane. Further studies directed at the molecular architecture of the muscle fiber plasma membrane will clarify these questions."

Intracellular calcium accumulation in Duchenne dystrophy and other myopathies: A study of 67,000 muscle fibers in 114 biopsies

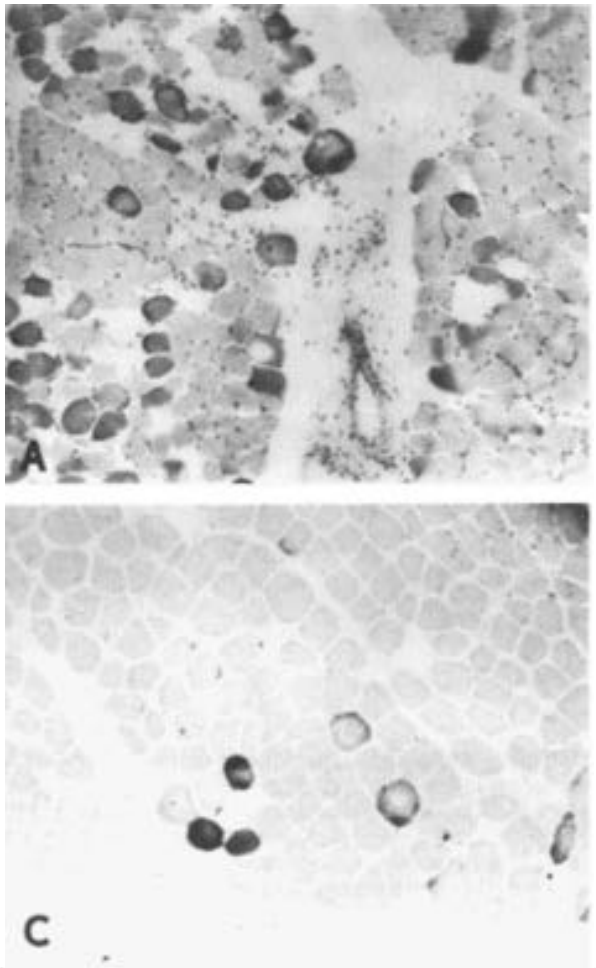
JOHN B. BODENSTEINER, M.D., and ANDREW G. ENGEL, M.D.

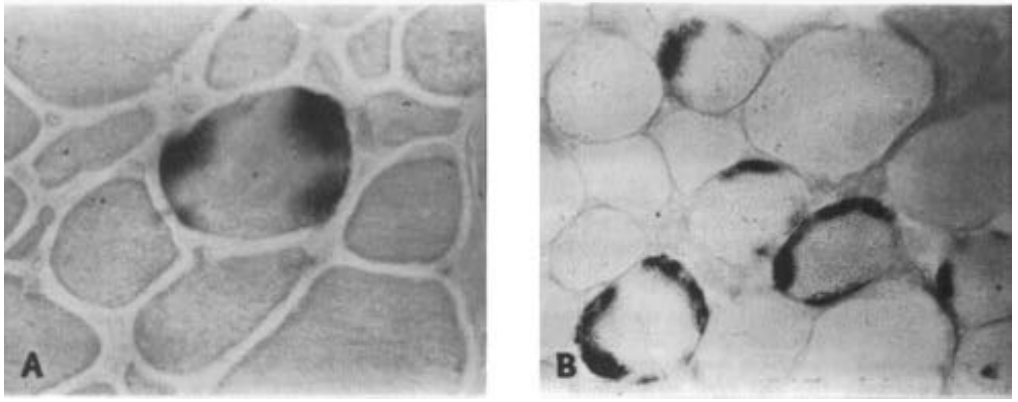
NEUROLOGY 28:439-446, **May 1978**

EM studies have shown plasmalemmal defects in a proportion of non-necrotic muscle fibers in DMD, suggesting that intracellular Ca++ overloading may be an important mechanism of muscle fiber degeneration. To investigate this the localization of calcium with the von Kossa method, with alizarin red, and with GBHA were done in serial, fresh-frozen sections from biopsy specimens

- In DMD, non-necrotic calcium-positive fibers occurred with a mean frequency of 4.83 %. For all other groups, the corresponding value was 0.57 % [0.21 % in normal to 1.76 % in scleroderma, **p** < 0.001].
- 43% of the fibers were calcium-positive in DMD, whereas calcium-positive large-dark fibers were extremely rare in the other cases.

Diagnosis	No. of Cases	Fibers analyzed
Duchenne dystrophy	24	122,348
Duchenne dystrophy carrier	2	9,861
Becker dystrophy	2	8,114
Myotonic dystrophy	18	50,456
Limb-girdle dystrophy	4	20,458
Ocular-limb muscle dystrophy	2	1,537
Facioscapulohumeral dystrophy	1	10,859
Polymyositis	32	160,239
Dermatomyositis	8	78,217
Unclassified collagen vascular disease	5	28,907
Scleroderma	2	7,424
Phosphorylase deficiency	3	11,682
Normal controls	11	57,451
Total	114	567,553

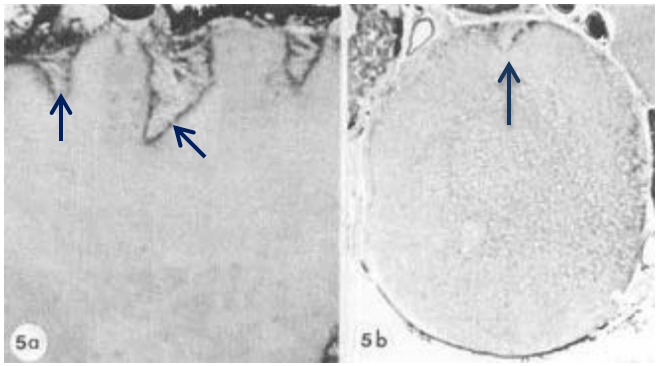




Ann N Y Acad Sci. 1979;317:409-30.

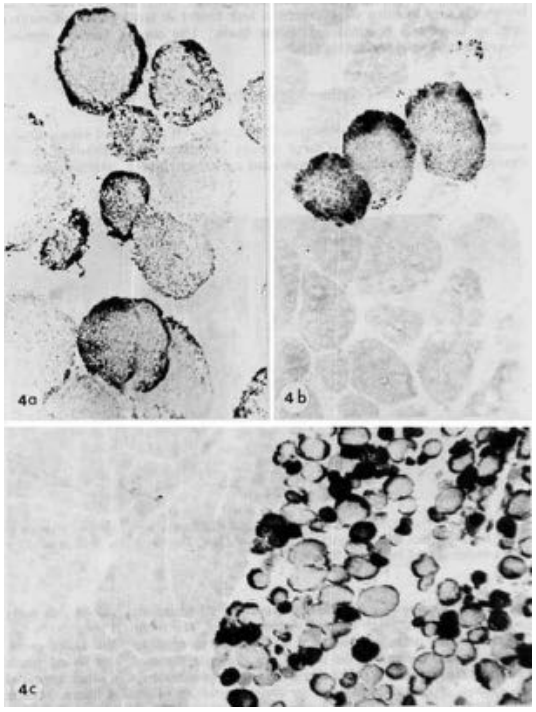
Relevance of genetic animal models of muscular dystrophy to human muscular dystrophies.

Mendell JR, Higgins R, Sahenk Z, Cosmos E.

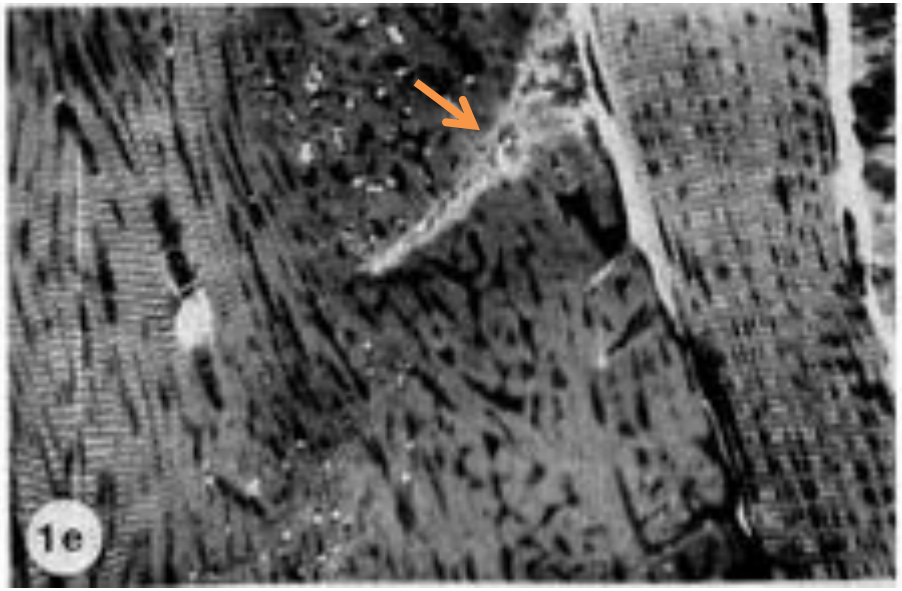


Syrian Hamster model
(δ-sarcoglycan-deficient)

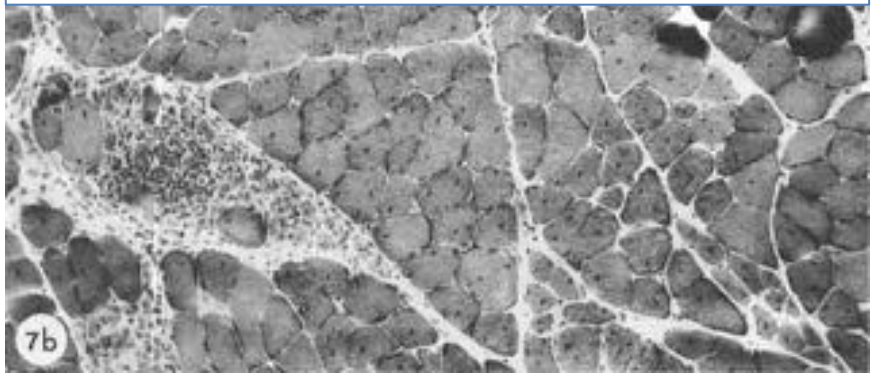
HRP-leak into delta lesions in 1 µm Thick sections

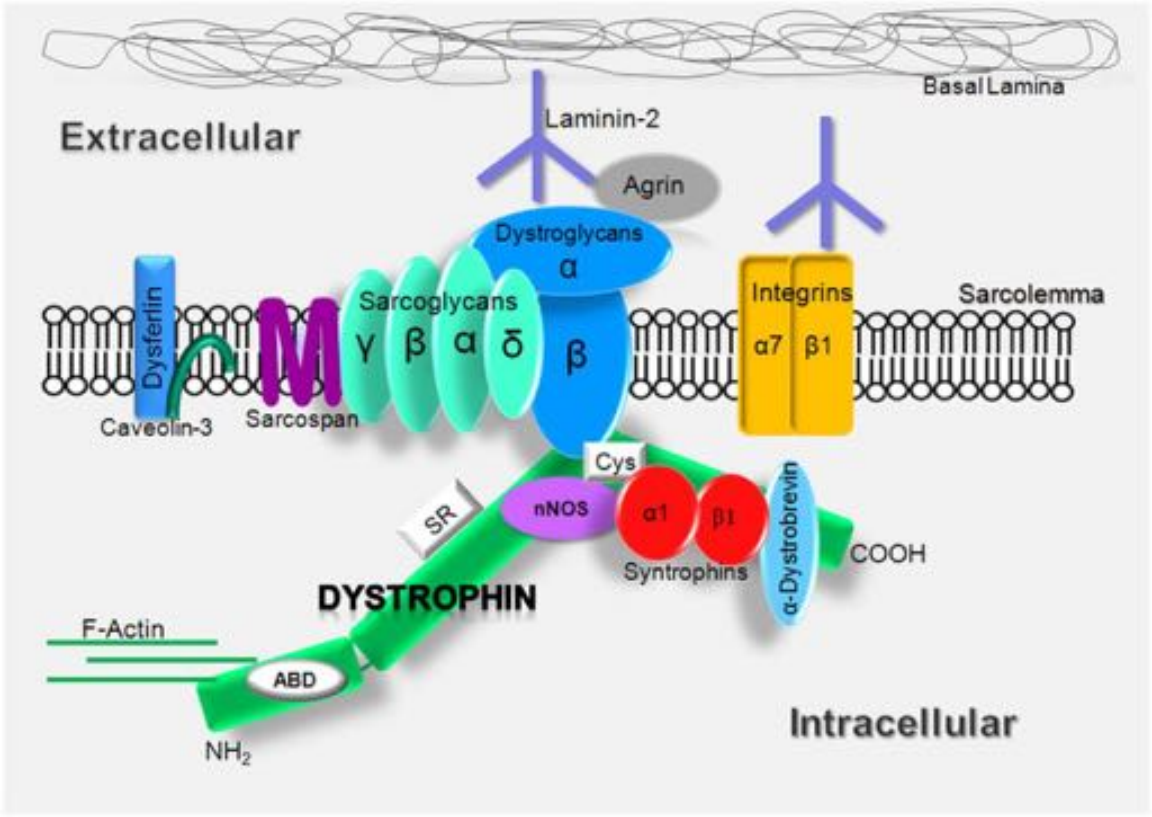


Calcium Stains

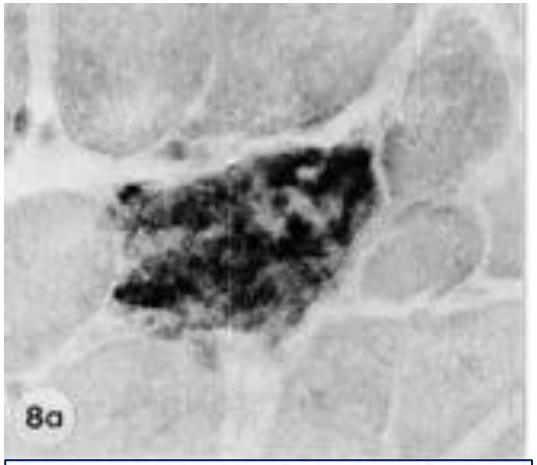


Spectrum of changes in the soleus muscle; trichrome



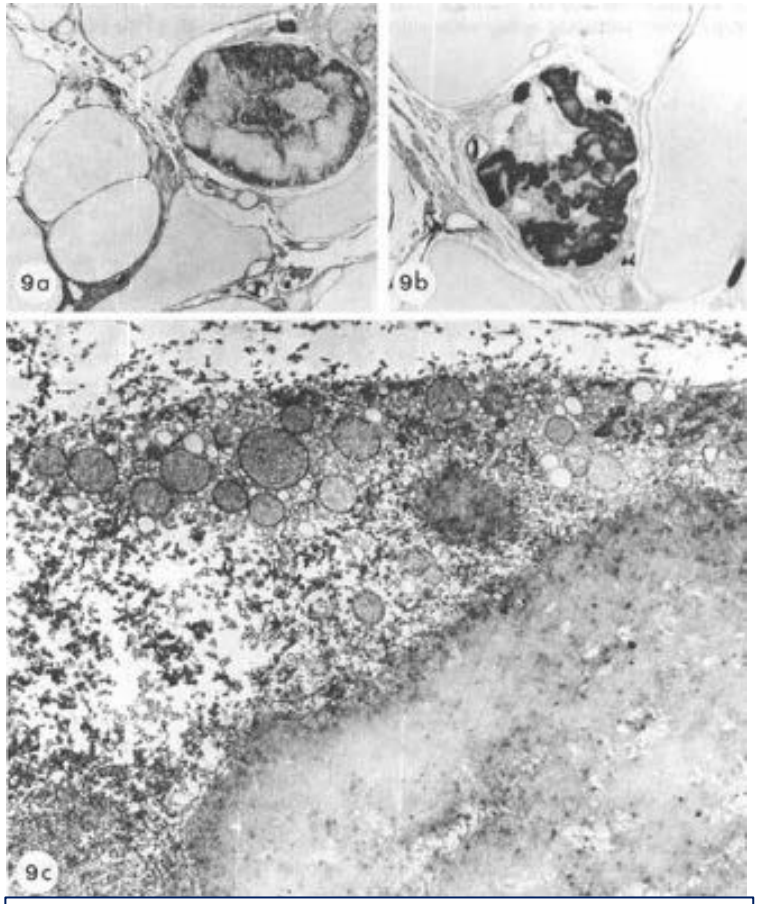

Basal Lamina
Extracellular
Laminin-2
Agrin
Dystroglycans
α
Integrins
α7
β1
Sarcolemma
Dysferlin
Sarcoglycans
γ
β
α
δ
β
Caveolin-3
Sarcospan
Cys
SR
nNOS
α1
β1
Syntrophins
α-Dystrobrevin
COOH
DYSTROPHIN
F-Actin
ABD
NH2
Intracellular

Dystrophica Muscularis mouse model (*dy/dy and dy^{2J}/dy^{2J}*)
(genetic defects in laminin α2-chain)

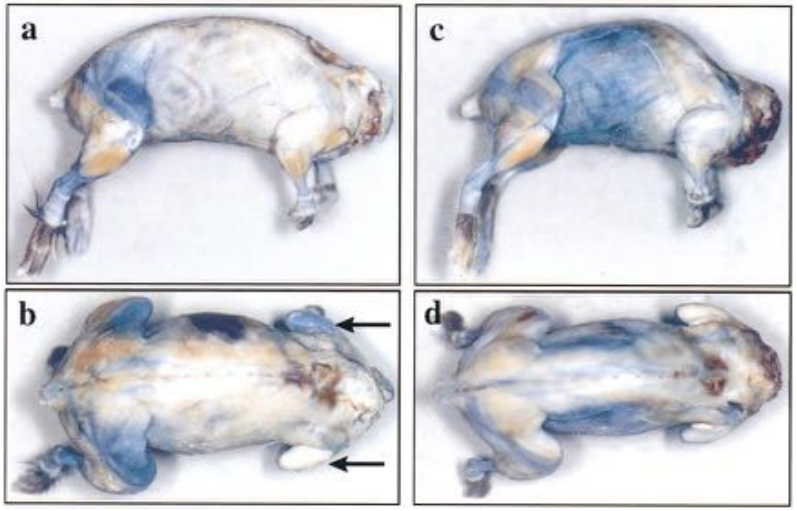


Diffuse alizarin red staining for calcium

*"….the percentage and the pattern of leakage of both calcium and **HRP** suggested that the dystrophic process in the mouse may more closely simulate a kind of neuromuscular disease(s) other than Duchenne human dystrophy."*

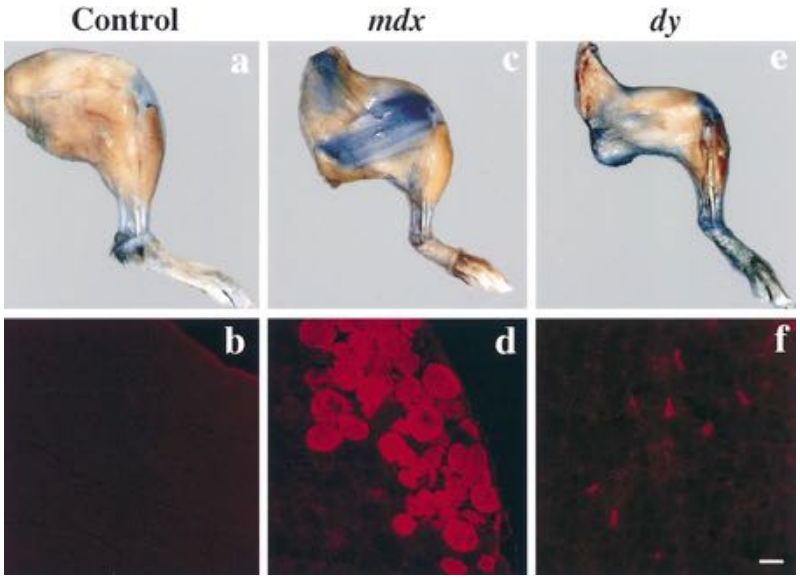


Extensive HRP leakage across the entire cross-sectional diameter of the fibers

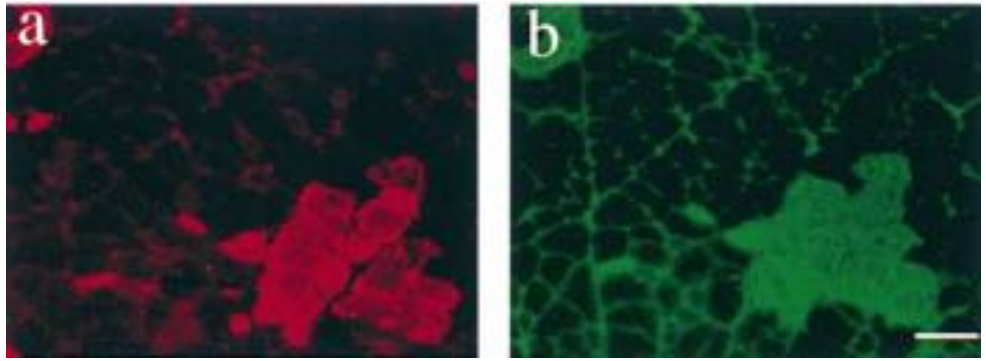


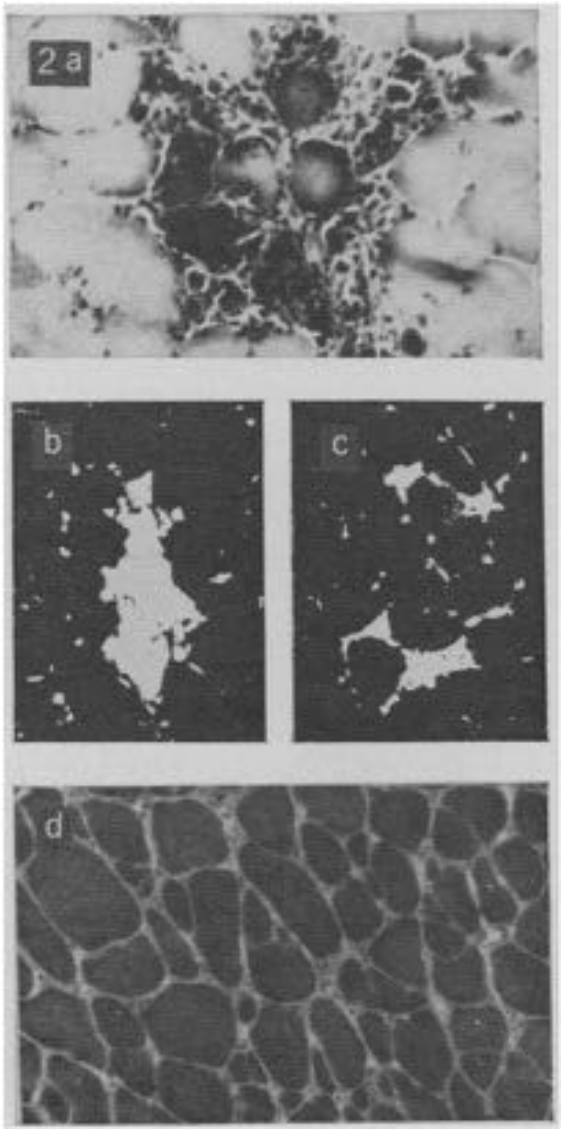
J Cell Biol. 1997 Oct 20;139(2):375-85.
Animal models for muscular dystrophy show different patterns of sarcolemmal disruption.
Straub V, Rafael JA, Chamberlain JS, Campbell KP

- Loss of Sarcolemmal Integrity in the *Mdx* Mice
- Negligible membrane defect in dy/dy mice



Uptake of EBD into hind leg muscles 6 h after injection



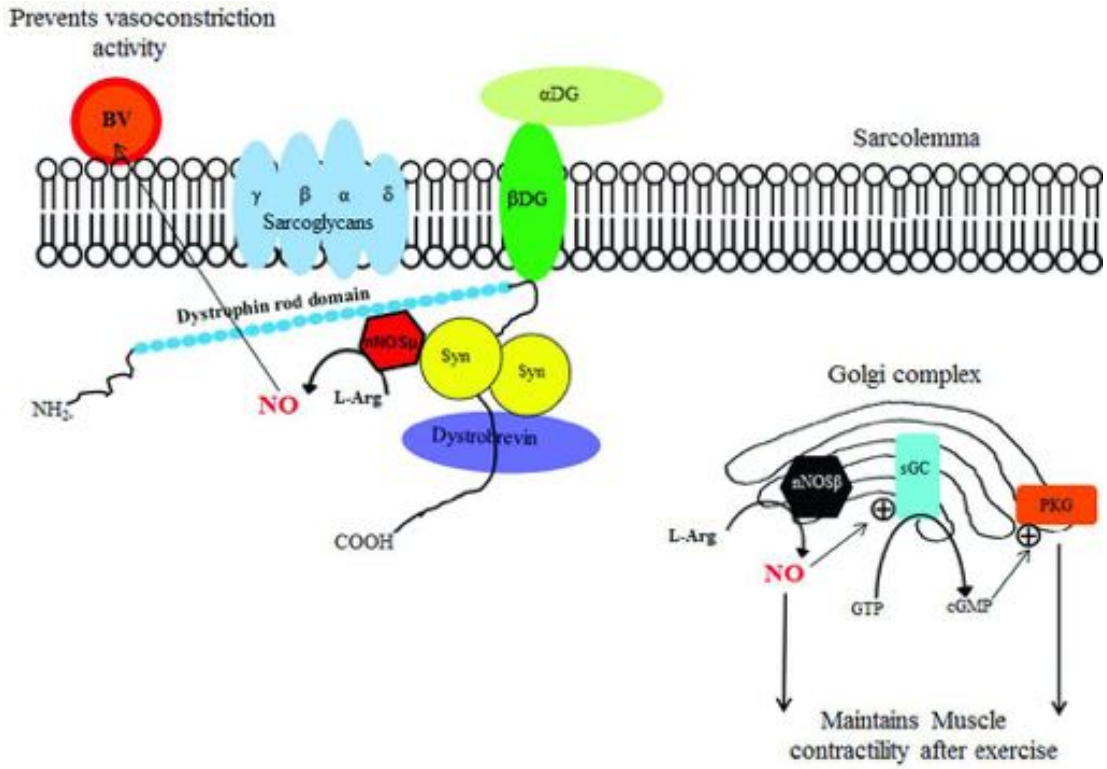


Duchenne Muscular Dystrophy: Functional Ischemia Reproduces Its Characteristic Lesions

Author(s): J. R. Mendell, W. King Engel and E. C. Derrer

Source: **Science**, New Series, Vol. 172, No. 3988 (Jun. 11, 1971), pp. 1143-1145

Abstract. *The highly characteristic early and midstage histological lesions of Duchenne dystrophy were reproduced experimentally in the rat by the combination of a vascular abnormality, aortic ligation, which does not affect the structure of the intramuscular blood vessels, and the humoral vasoactive substance 5-hydroxytryptamine. Neither ligation nor injection of 5-hydroxytryptamine alone causes changes in the muscle fibers. This result establishes the possibility of a similar combined mechanism for a nonstructural ischemia pathogenesis* in *Duchenne dystrophy. The proposed pathogenesis is contrary to the generally held idea that the cause is an intrinsic abnormality of muscle fiber metabolism.*



The role of different isoforms of nNOS, namely nNOSμ and nNOSβ in skeletal muscle, is illustrated. Two binding sites of nNOSμ can be seen: at the dystrophin rod domain and at α-syntrophin (a member of dystrophin-glycoprotein complex). NO derived from nNOSμ enhances blood flow to muscle during activity by preventing vasoconstriction of nearby blood vessels during muscle contraction; the increased blood flow is a source of oxygen supply to the muscle during exercise. In contrast, nNOSβ signaling at the Golgi complex regulates force generation during and after exercise generating cGMP dependent protein kinase G (PKG). (Adapted with permission of the American Society for Clinical Investigation from Percival et al. 2010 [117]). Abbreviations: nNOS- nitric oxide synthase; BV-blood vessel; aDG-alpha-dystroglycan; b-DG- beta-dystroglycan; NO- nitric oxide; Syn–syntrophin; GTP- guanosine triphosphate; sGC- soluble guanylyl cyclase; cGMP- cyclic guanosine monophosphate.

Malik, Rodino-kalpac & Mendell. Expert Opin Emerg Drugs, 2012

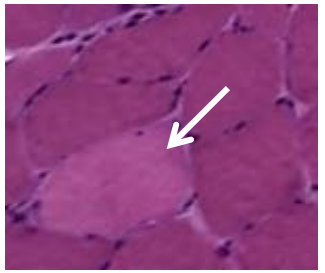
Necrosis

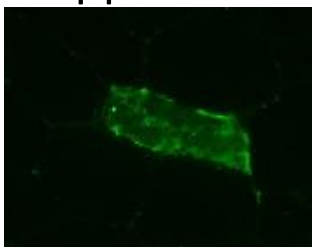
Factors triggering necrosis in muscle cells:

- Lengthening contractions
  - dystrophic muscle particularly vulnerable
- Ischemia
  - dermatomyositis
- Energy deprivation
  - Glycolytic defects
- Toxic agents
  - Cardiotoxin, neutoxin, statins

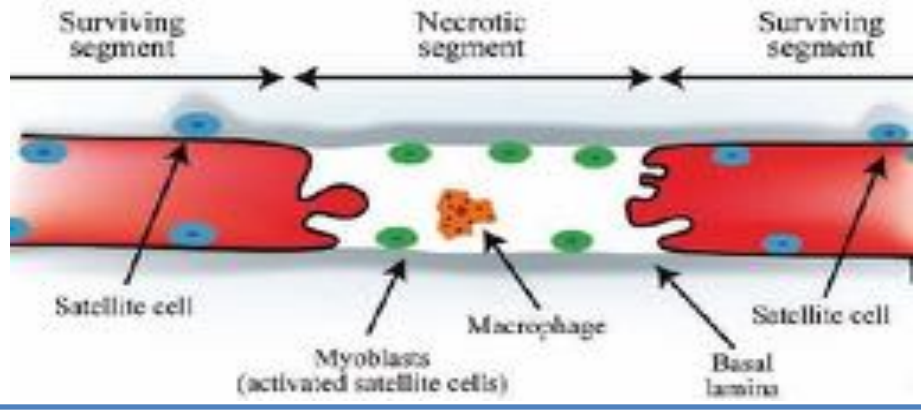
In the course of necrosis:

- Plasma membrane becomes permeable
  -- Ca^{++} entry, activation of phospholipases, proteases (calpains)
- Some DAG complex- lost early; by 24 hrs dys lost
- Activation of compliment cascade, diffuse cytoplasmic appearance of lytic C5-9 (MAC) within muscle

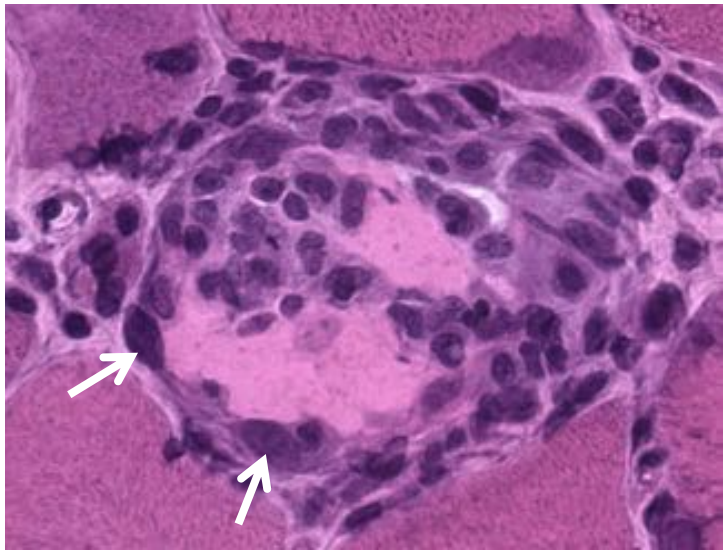


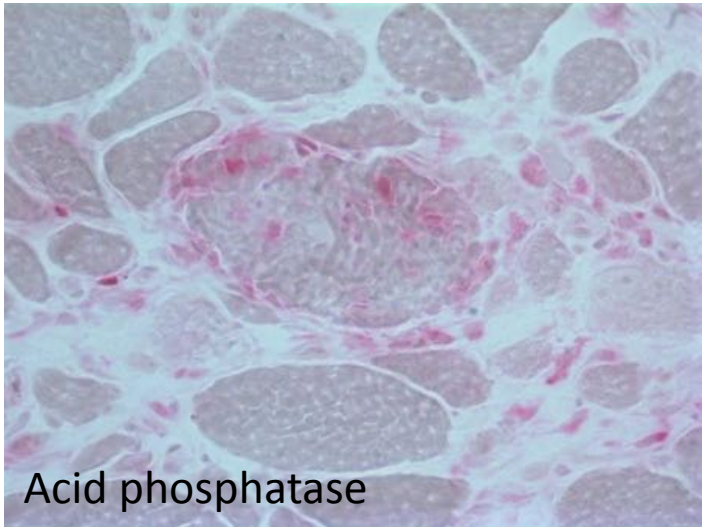


Segmental Necrosis



Phagocytosis



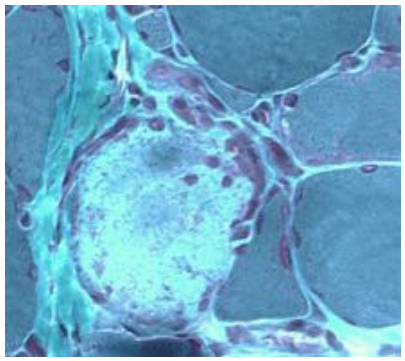


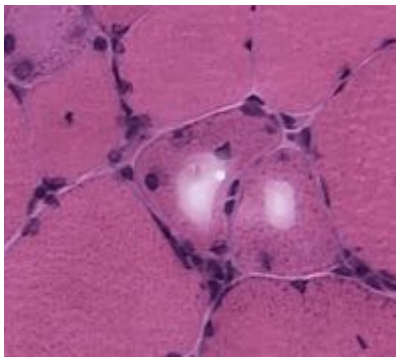
- Starts ~ 6 to 8 hrs after the fiber passed the "point of no return"

  -- sarcolemmal and myonuclear dissolution

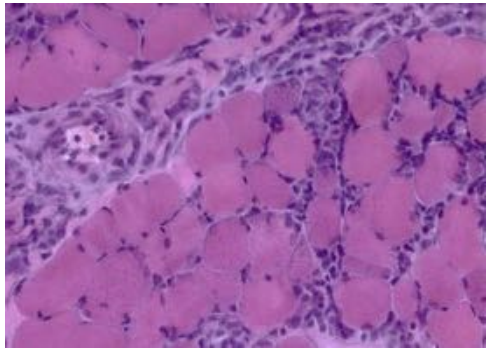
  (earliest change), followed by gradual dissolution of contractile elements

  what is not destroyed: Basal Lamina & Satellite Cells

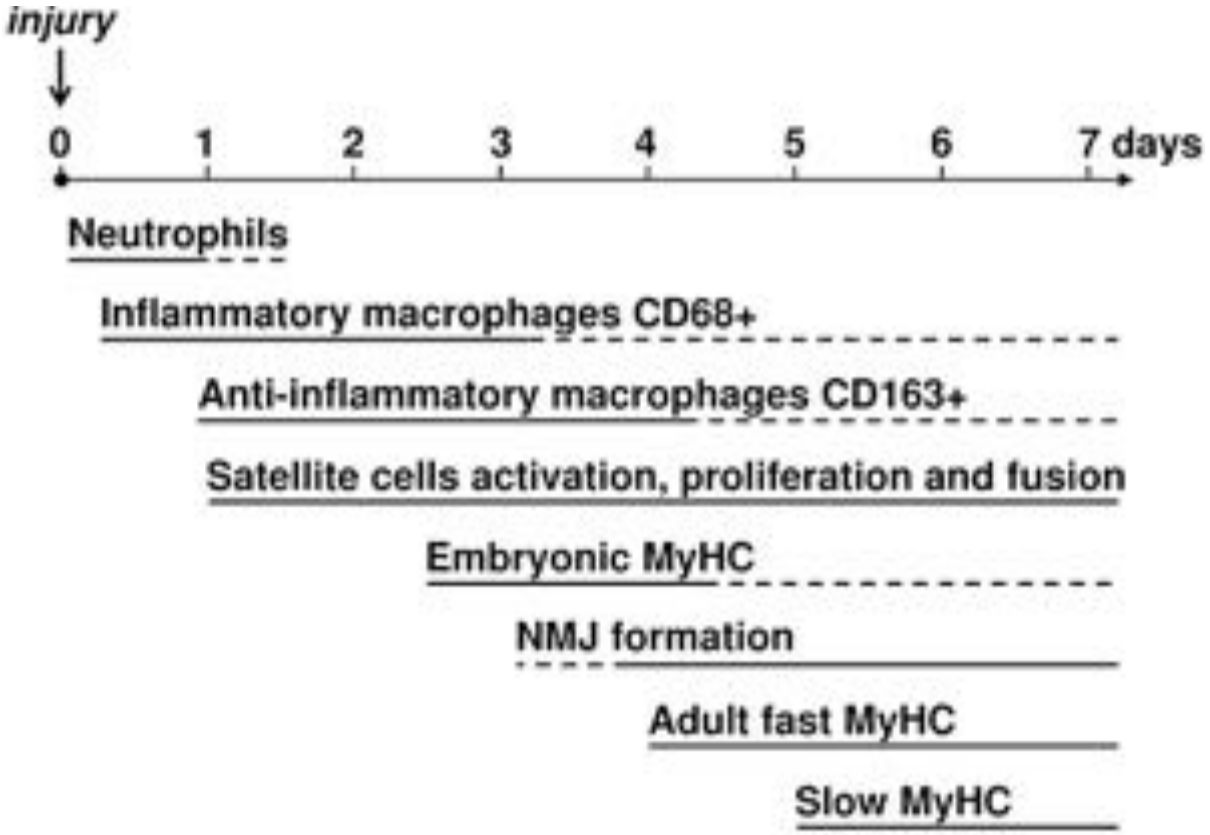




- In surviving stumps- T tubule dilatation
- Abundant macrophages within endomysium

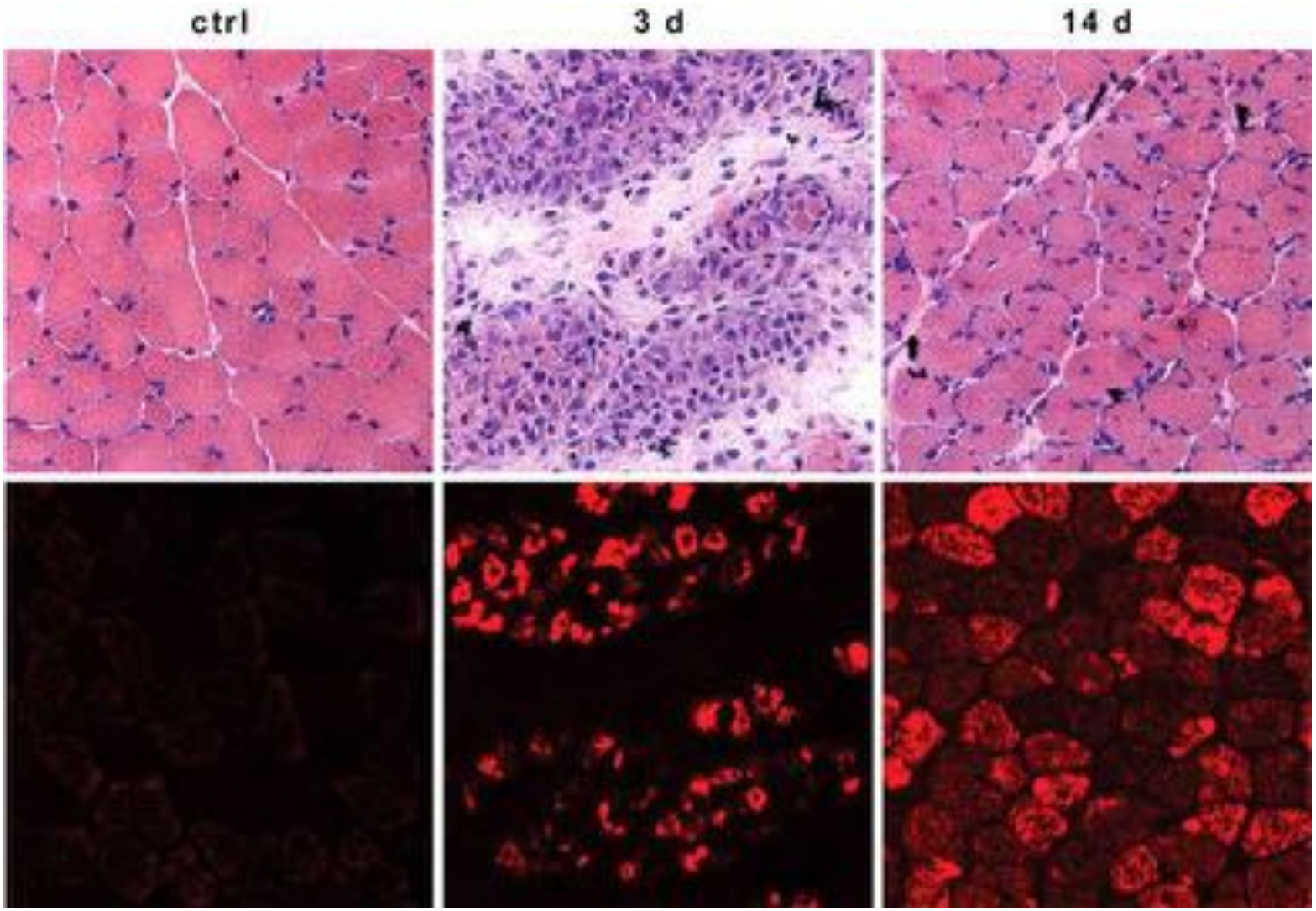


Temporal sequence of inflammatory and regenerative events following muscle injury:



Ciciliot S., Curr. Pharmaceutical Design, 2010

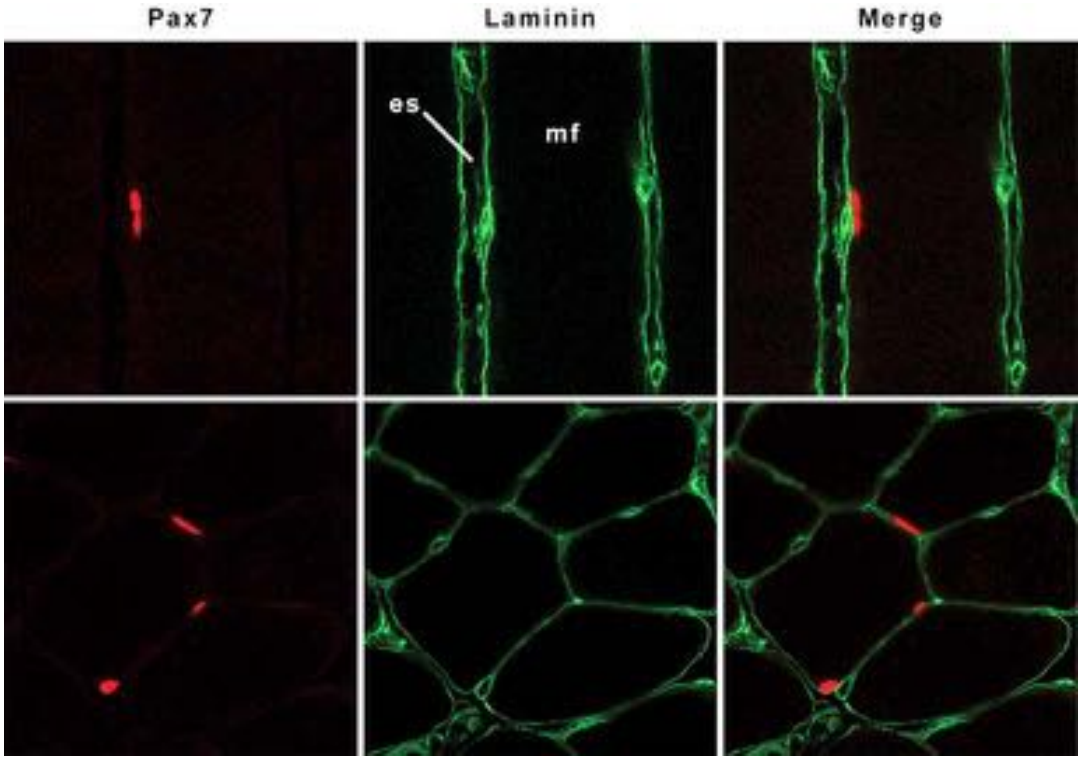
Myofiber growth and embryonic MyHC expression in regenerating skeletal muscle



Ciciliot S., Curr. Pharmaceutical Design, 2010

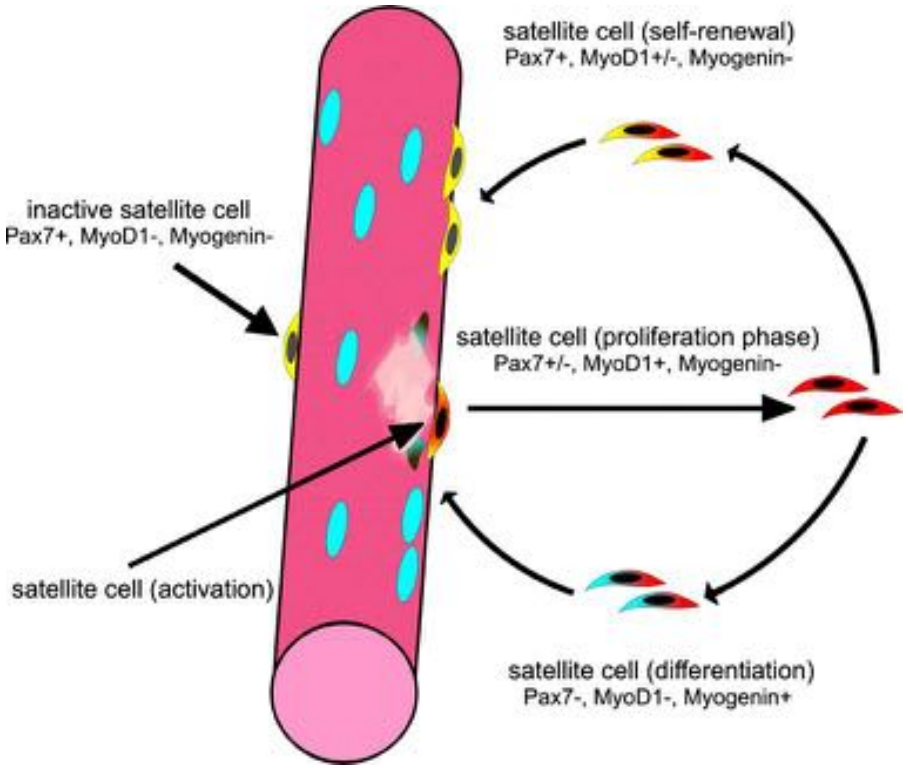
Satellite Cells

- Muscle specific stem cells located beneath the basal lamina of the myofiber
- Pax7, N-CAM, m-cadherin, CD34 useful marker for quiescent SCs
- Prevalence = r S/M
- Major role in
  - Natural growth
  - Muscle maintenance, work hypertrophy
  - Regeneration
- Proliferative/differentiating processes lead transformation into myoblast/myotubes in necrotic segments
- Limit of their mitotic cycles?

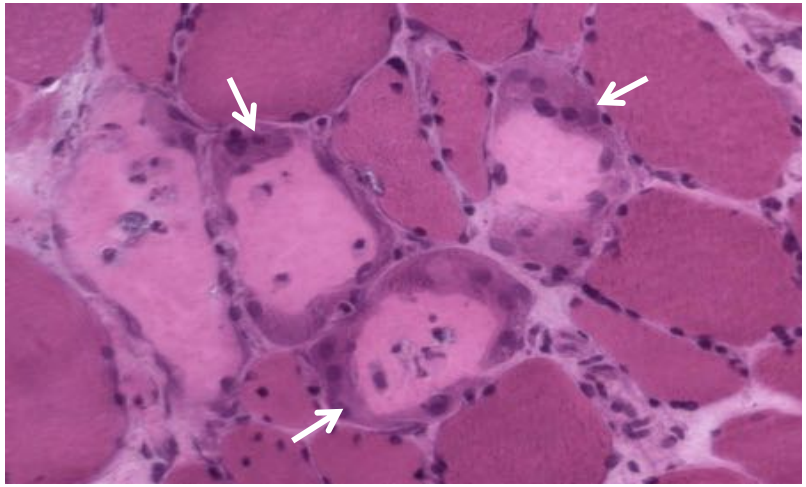


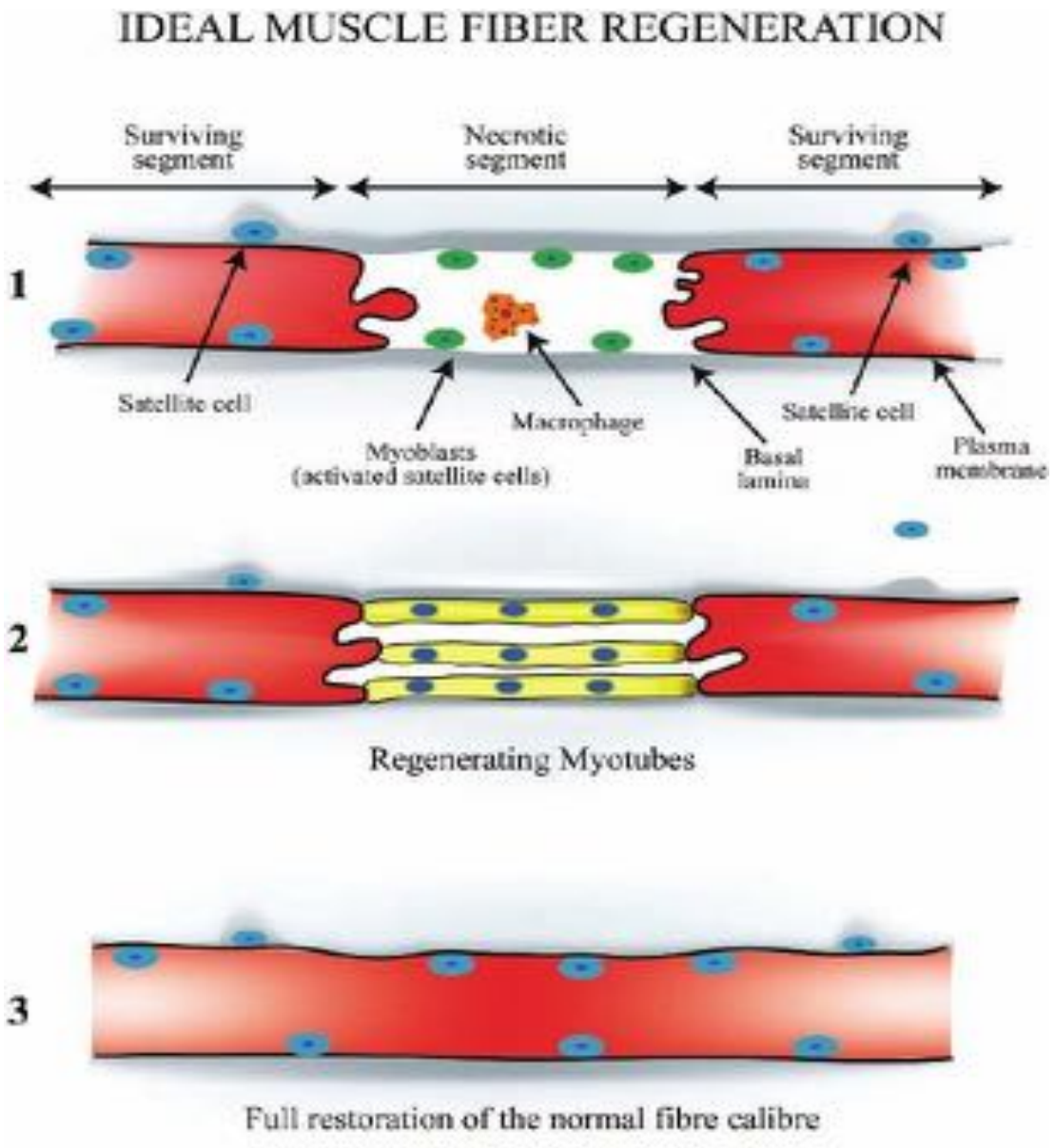
Ciciliot S., Curr. Pharmaceutical Design, 2010

Model for satellite cell self-renewal and differentiation



Activated satellite cells in necrotic fibers

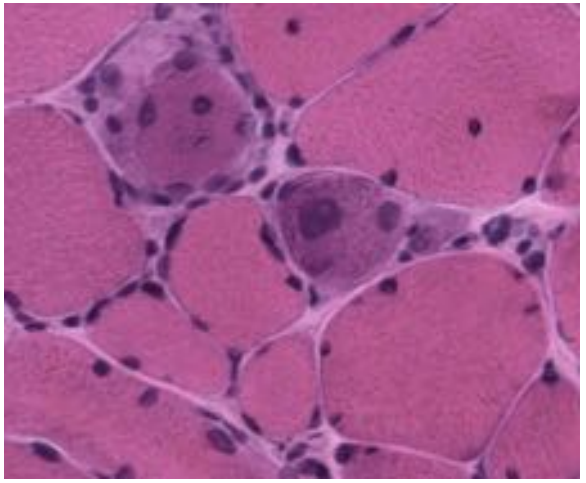




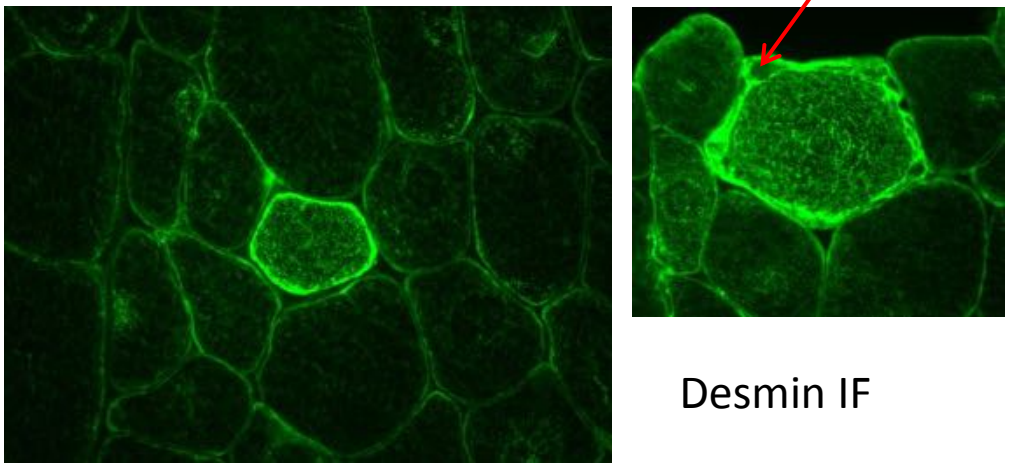
Karpati, G; 2008

Histological Features of Regenerating Muscle

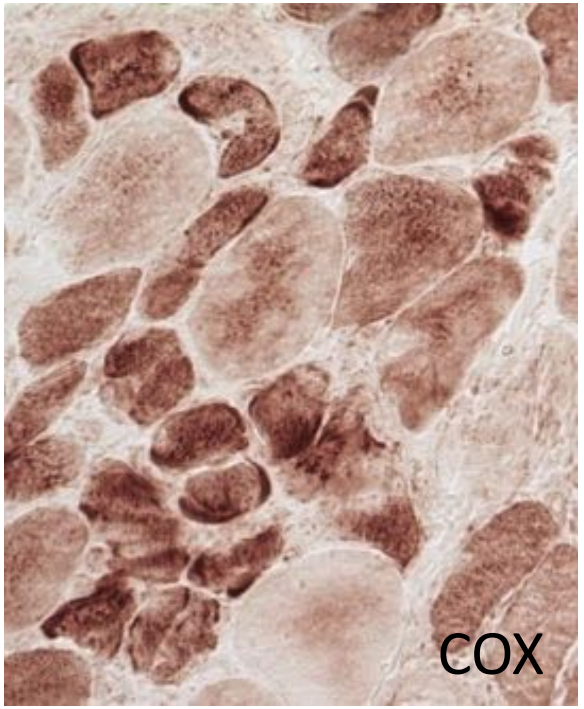
- Basophilic cytoplasm, reflecting high content of ribosomes
- Nuclei tend to be pale and large
- Relative excess of glycogen and mitochondria (early)
- Emb & Neo forms of myCH
- Diffuse cytoplasmic desmin stain

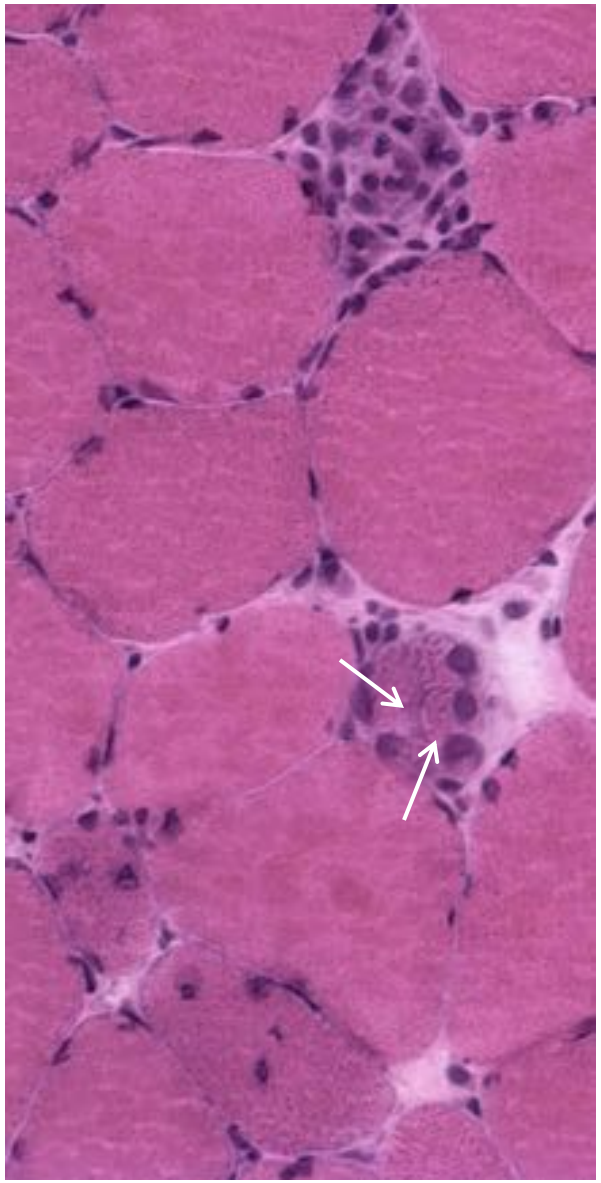


Satellite cell

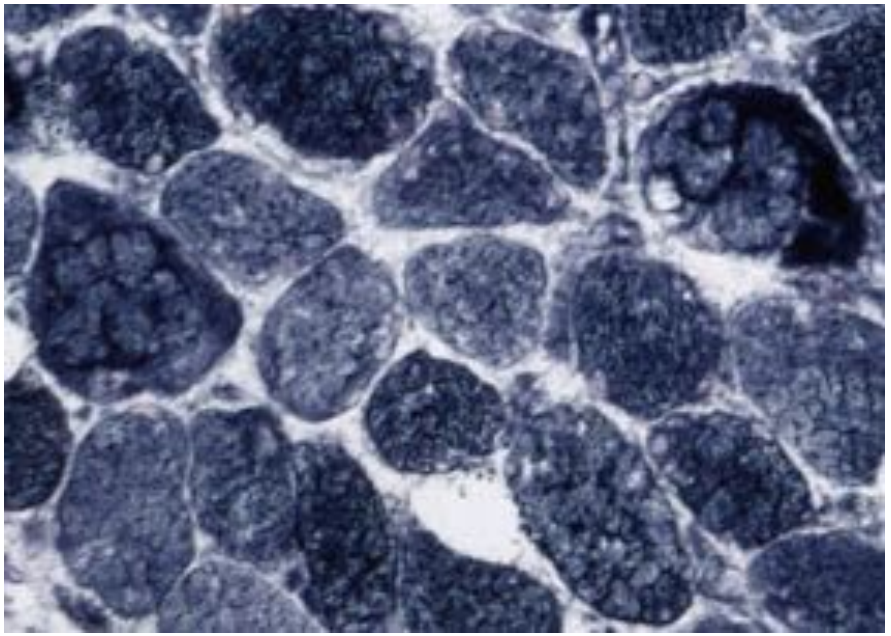


Desmin IF





Muscle Fiber Regeneration



ABERRATIONS OF MUSCLE FIBER REGENERATION

1

Regenerated segment is of smaller caliber than the rest of the fiber

2

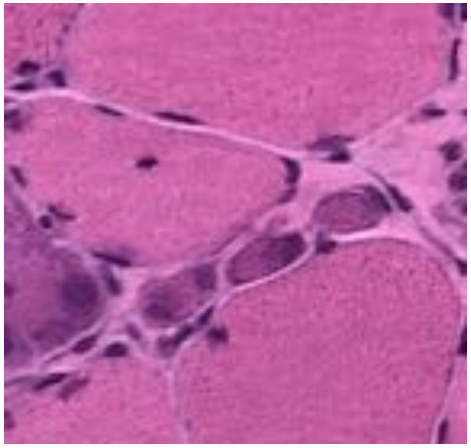
Forked fibers due to incomplete lateral fusion of myotubes

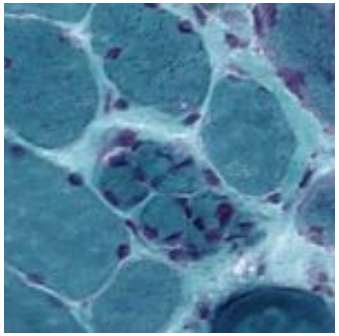
3

Muliple independent fibers due to lack of fusion of myotubes with the surviving stump

4

Empty basement membrane sleeve due to lack of regeneration





Histopathological spectrum of dystrophic process

Early stage
Prominent necrosis/regeneration, inflammation
Minimal fibrosis

→

Late stage
Prominent fibrosis
Minimal necrosis/regeneration, inflammation

- Endomysial fibrosis is an end-stage consequence of muscle fiber loss

-often explained by a putative failure of muscle fiber regeneration that requires a stepwise process: activation, of SCs with proliferation & differentiation followed by lateral fusion of myotubes with each other and with surviving stumps

- Muscle-specific miRs play important posttranscriptional regulatory roles in this process

-miR-1, miR-206 facilitate SC differentiation

-down-regulation or inhibition of miR-1, miR-206 enhances SC proliferation and increases Pax7 protein levels *in vivo*

J Clin Invest. 2012 Jun 1;122(6):2054-65. doi: 10.1172/JCI62656. Epub 2012 May 1.

microRNA-206 promotes skeletal muscle regeneration and delays progression of Duchenne muscular dystrophy in mice.

Liu N, Williams AH, Maxeiner JM, Bezprozvannaya S, Shelton JM, Richardson JA, Bassel-Duby R, Olson EN.

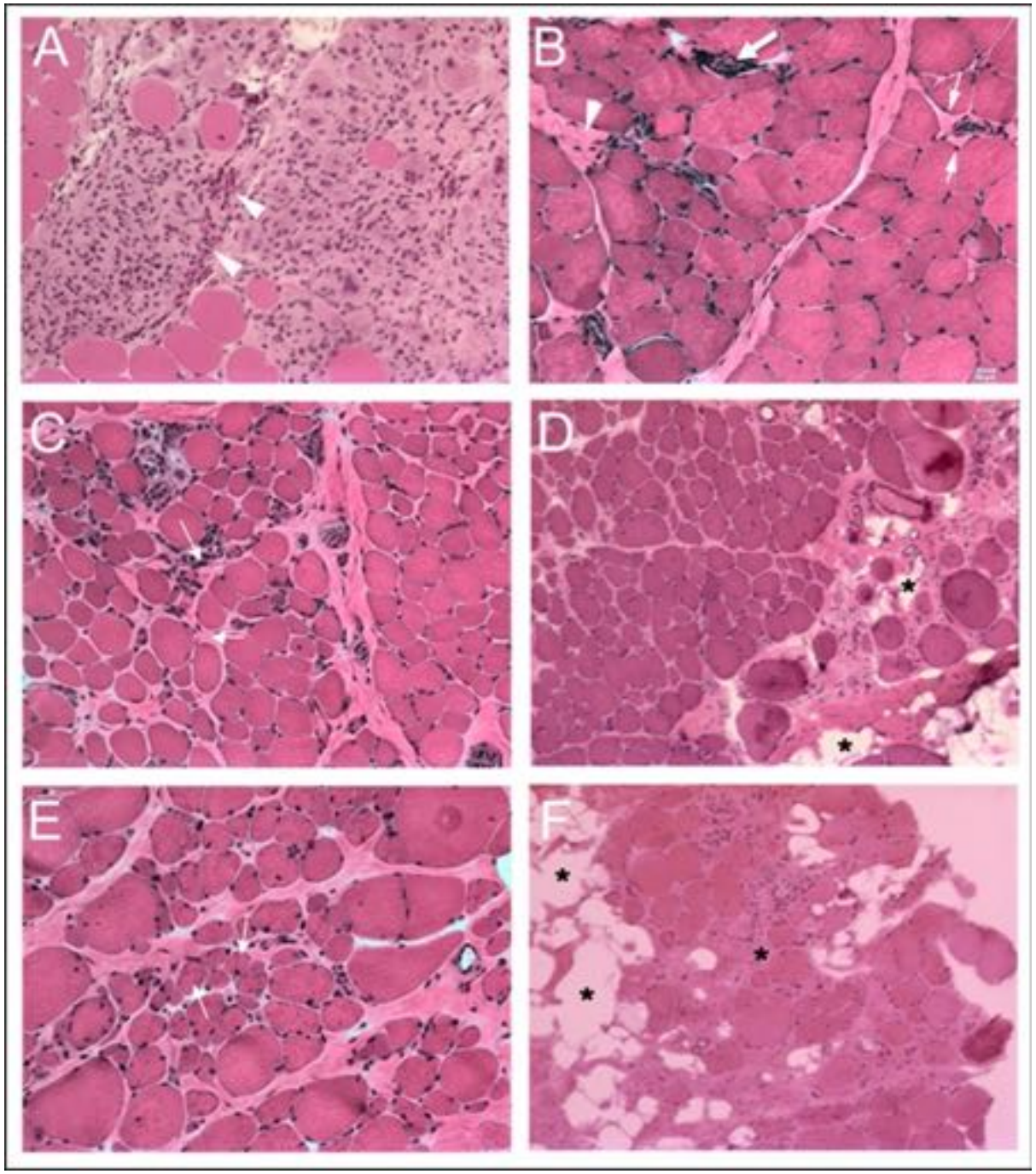
- Genetic deletion of miR-206 in mice substantially delayed regeneration induced by cardiotoxin injury.
- Loss of miR-206 accelerated and exacerbated the dystrophic phenotype in a mouse model of Duchenne muscular dystrophy.
- miR-206 acts to promote satellite cell differentiation and fusion into muscle fibers through suppressing a collection of negative regulators of myogenesis.
- These findings reveal an essential role for miR-206 in satellite cell differentiation during skeletal muscle regeneration and indicate that miR-206 slows progression of Duchenne muscular dystrophy.

Dystrophic process and Satellite cells

LGMD2A:

- caused by mutations in the *CAPN3*, encoding Ca^{2+} - activated cysteine protease
- role in sarcomere assembly, turnover and maintenance
- in Calpainopathy there is a good correlation between age, duration of symptoms and degree of fibrosis
- microRNA dysregulation leads to inability of Pax7-positive SCs to transit from proliferation to differentiation resulting in impaired regeneration and fibrosis in LGMD 2A

Rosales et al., Muscle &Nerve, 2013



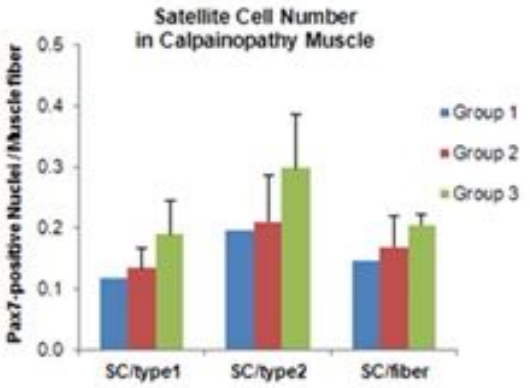
Satellite Cells in Dystrophic Process (calpainopathy)

A

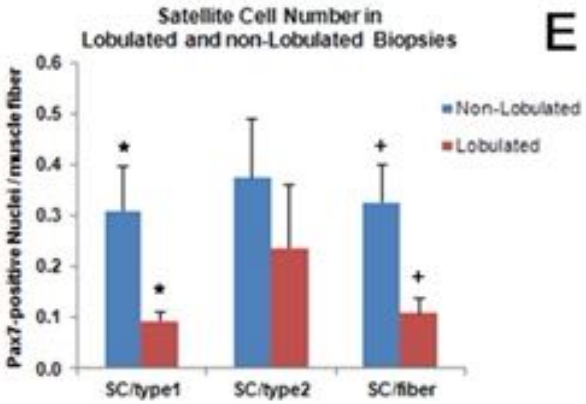
Fiber type specific distribution of satellite cells in calpainopathy biopsies

Biopsies	n	Age	DD	FG	SC /type1	SC/type2	SC/fiber
Group 1	1	11	1	1	0.117	0.196	0.147
Group 2	3	19.7 ± 2.7	7.7 ± 3.3	1 ± 0.0	0.134 ± 0.032	0.210 ± 0.076	0.168 ± 0.051
Group 3	9	37.8 ± 4.8	19.7 ± 3.6	3.1 ± 0.2	0.189 ± 0.054	0.298 ± 0.087	0.205 ± 0.052
Control	3	45.7 ± 5.0			0.081 ± 0.001	0.056 ± 0.010	0.065 ± 0.006

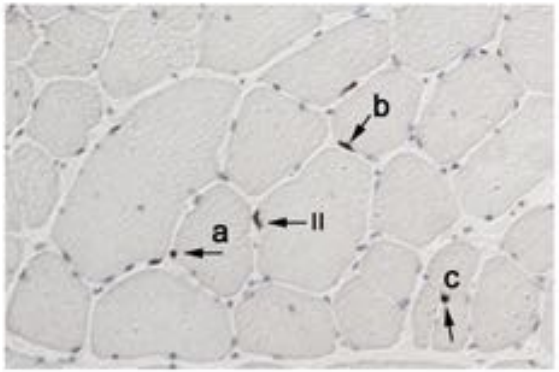
B



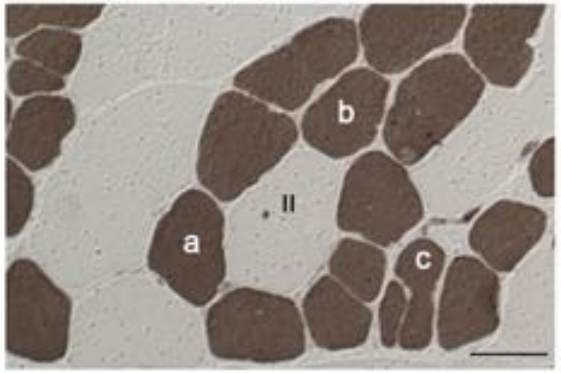
C

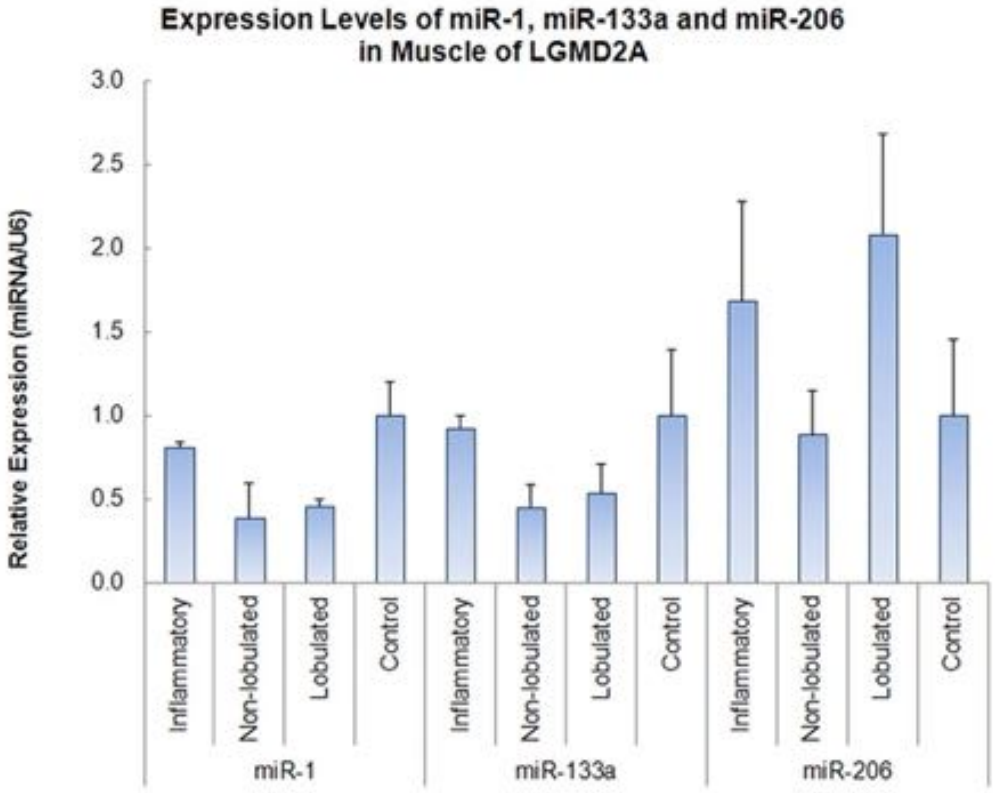


D



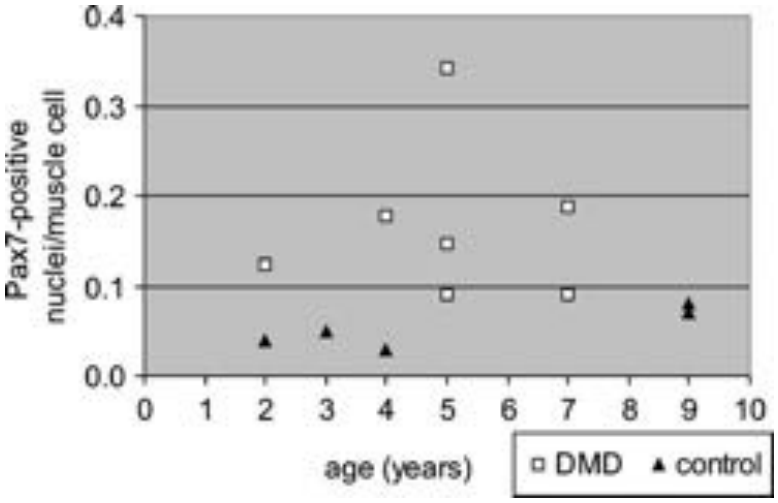
E



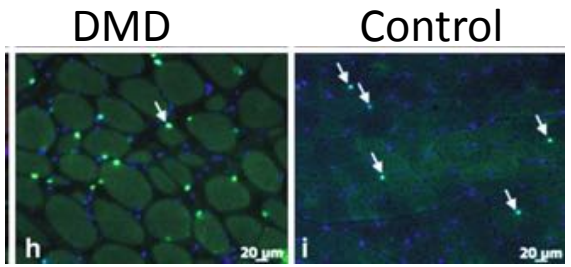

Expression Levels of miR-1, miR-133a and miR-206 in Muscle of LGMD2A
Relative Expression (miRNA/U6)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Inflammatory
Non-lobulated
Lobulated
Control
miR-1
miR-133a
miR-206

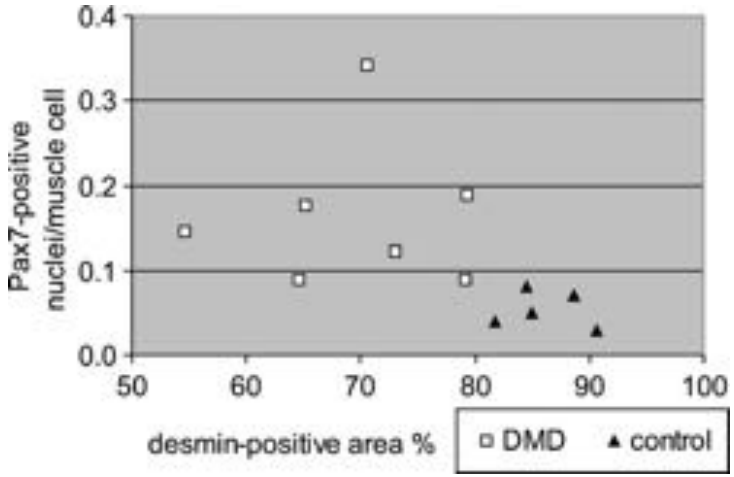
Elevated satellite cell number in Duchenne muscular dystrophy

Michael Kottlors & Janbernd Kirschner Cell Tissue Research, 2010



Pax7-positive nuclei number per muscle cell of DMD and control muscle at the age from 2 to 9 years showing the higher number of Pax7-positive nuclei in DMD muscle in comparison with control muscle





Pax7-positive nuclei number per muscle cell of DMD and control muscle plotted against desmin-positive area showing the increased Pax7-positive nuclei numbers in DMD, despite decreasing desmin-positive area (increasing fibrosis)

- Pax7-positive SC number is elevated in DMD in comparison to controls, even in advanced stage of dystrophy
- The expression of myogenin is not correlated with fibrosis or age suggesting variable factors influencing the differentiation of satellite cells

DMD Pathophysiology Overview

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Associate Professor

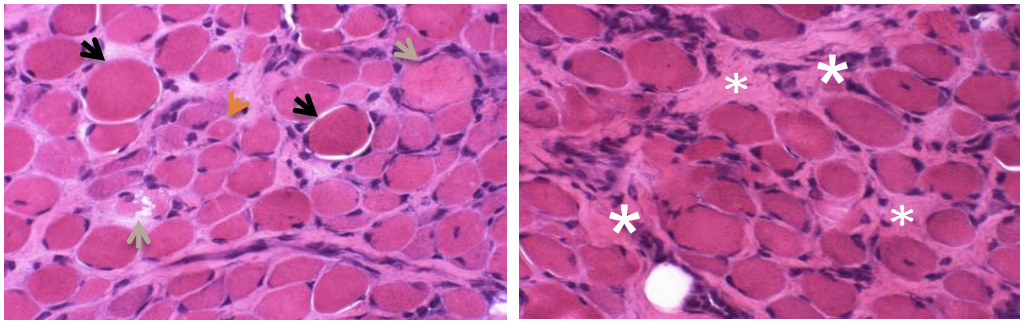
Dept. Molecular and Cellular Biochemistry

(Physiology & Cell Biology, Cardiology)

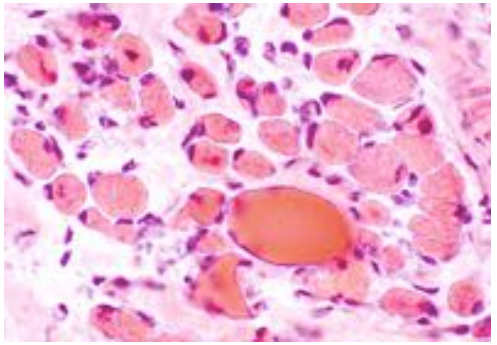
Wexner Medical Center

Skeletal muscle pathology

- Muscle weakness
- Myofiber degeneration; regeneration; focal inflammation
- Regenerated muscle fibers display a large variation in diameter and have centrally located nuclei
- Gradual replacement of fibers with adipose and connective tissue (fibrosis)



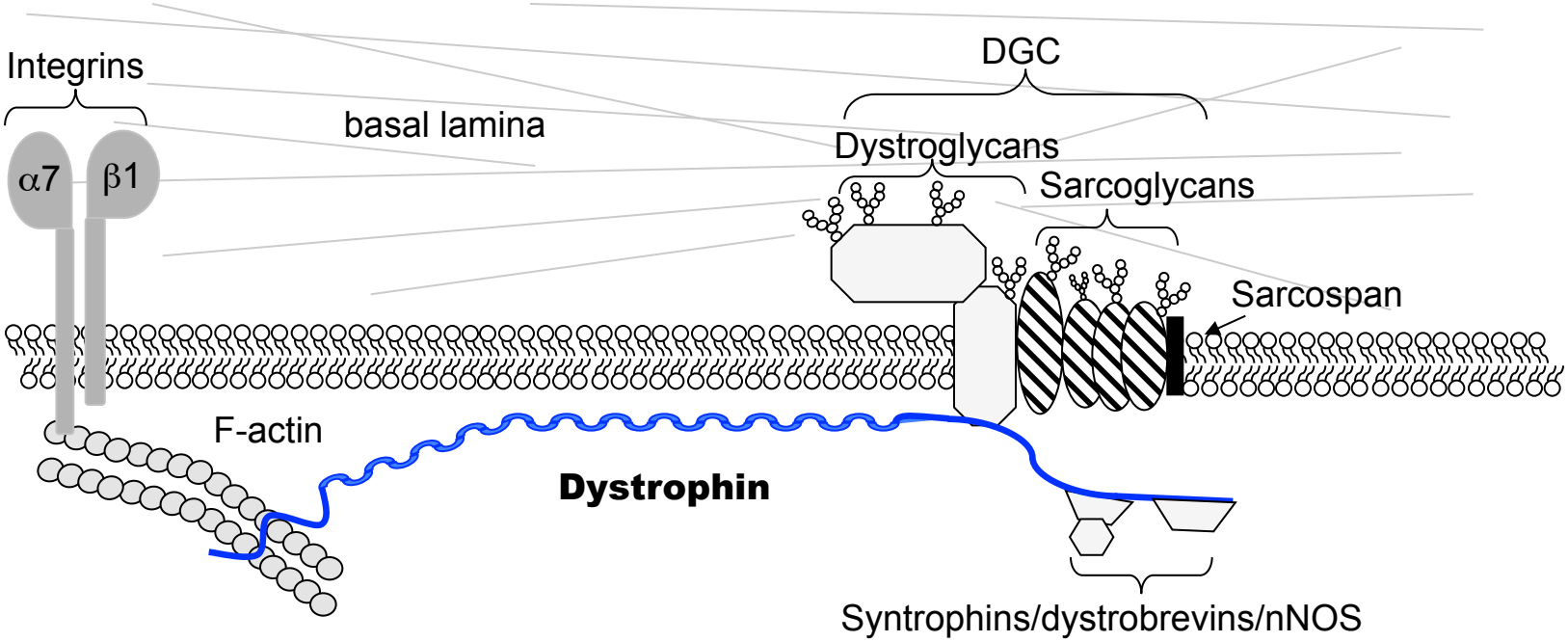
18 month-old DMD biopsy



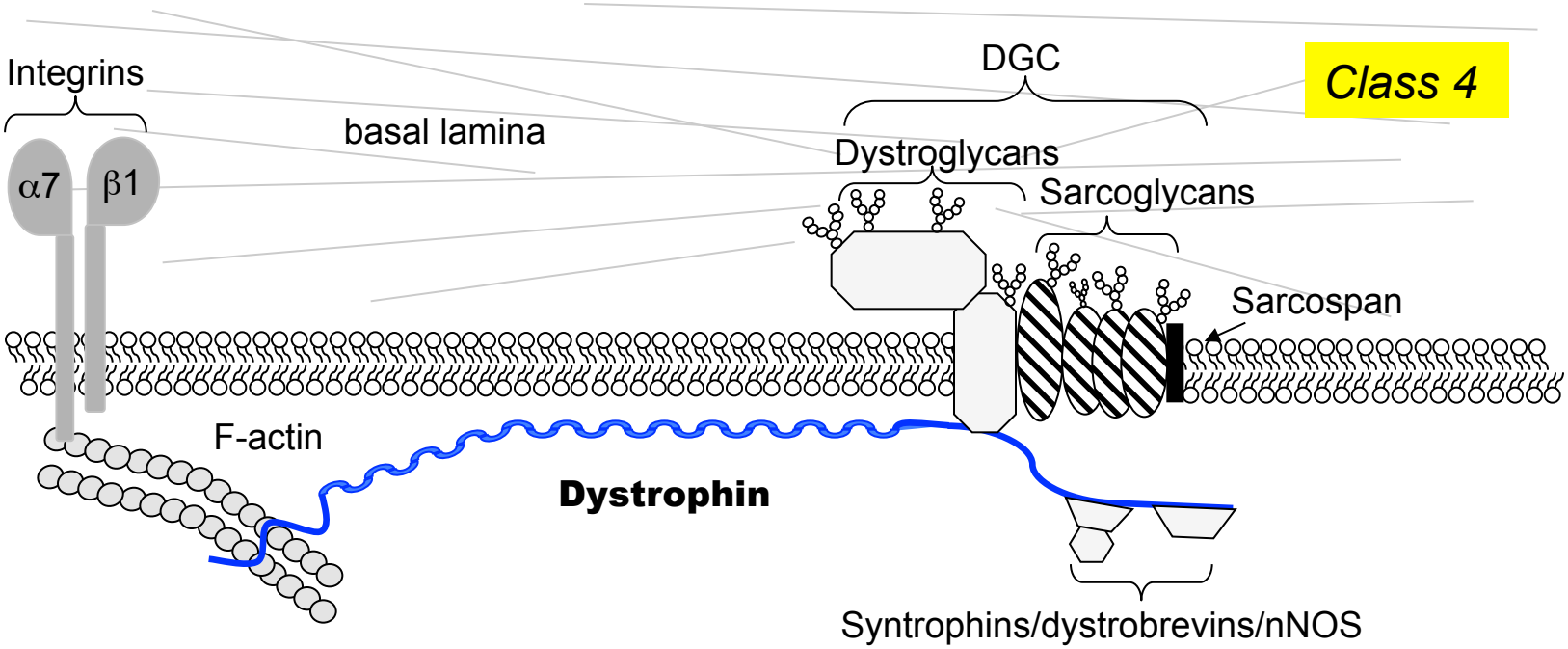
late stage DMD

(L. Zhou; neuromuscular.wustl.edu)

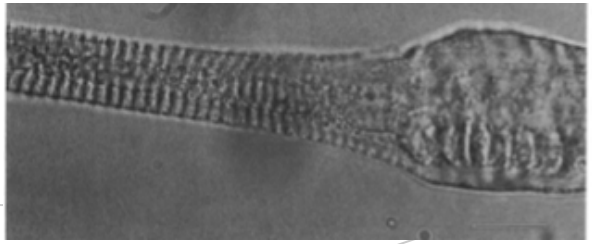
Dystrophin and the muscle membrane



Dystrophin and the muscle membrane



DMD and the muscle membrane



Integrins

basal lamina

α7 β1

F-actin

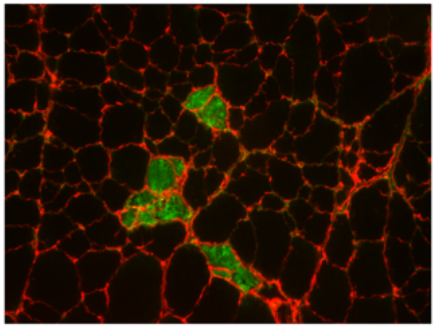
Membrane damage

Loss of actin-ECM connection

Free radicals

Ca^{2+}

nNOS



Activation of proteases
Mitochondrial dysfunction
Downstream signaling changes

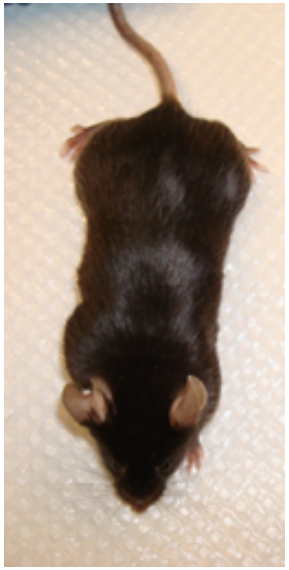
Class 11

(Williams, J.Physiol, 1993)

Mouse models

mdx
Dystrophin-deficient

- normal mouse lifespan (2 yrs)
- mild skeletal muscle fibrosis
- mild cardiomyopathy

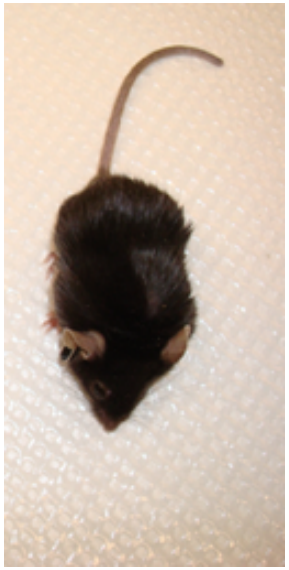


Het
Dystrophin-deficient; missing 1 copy of utrophin

- normal mouse lifespan (2 yrs)
- severe skeletal muscle fibrosis
- Cardiomyopathy progression more similar to DMD patients

dko
Dystrophin/utrophin-deficient

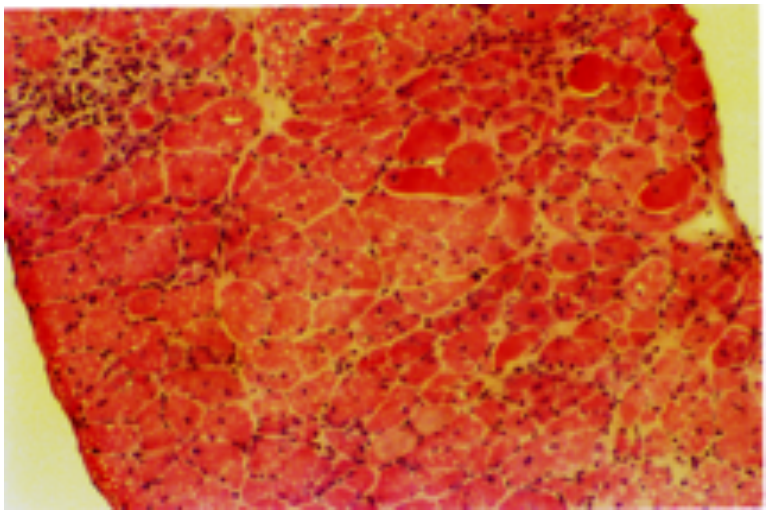
- Dies 10-12 weeks-of-age
- mild skeletal muscle fibrosis
- severe cardiomyopathy



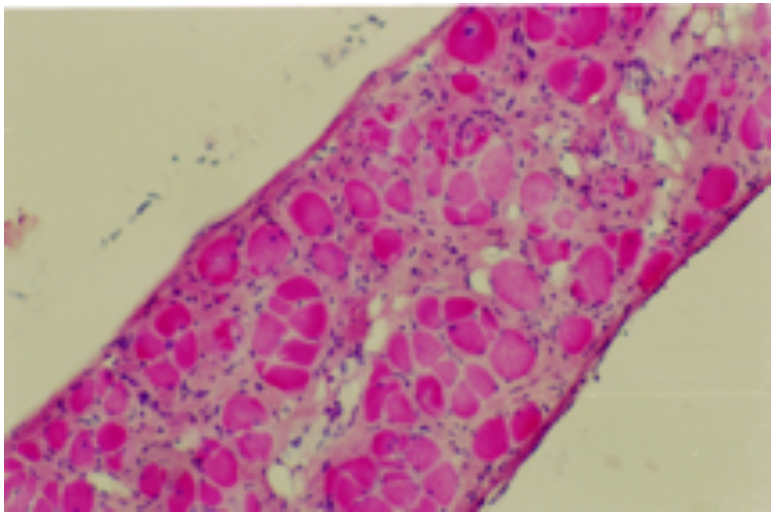
Class 7

Dystrophic histopathology

10 week-old *mdx* diaphragm



24 month-old *mdx* diaphragm



Membrane damage — *Class 8*

↓

Inflammation — *Class 10*

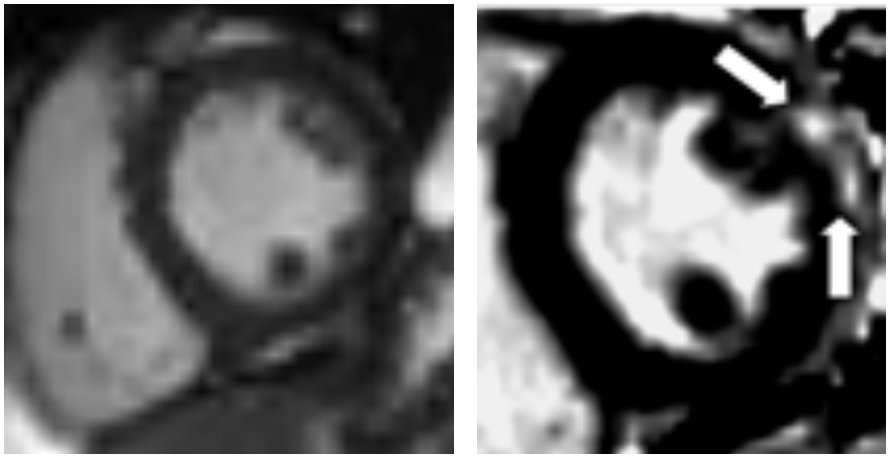
↓

Regeneration — *Class 12*

↓

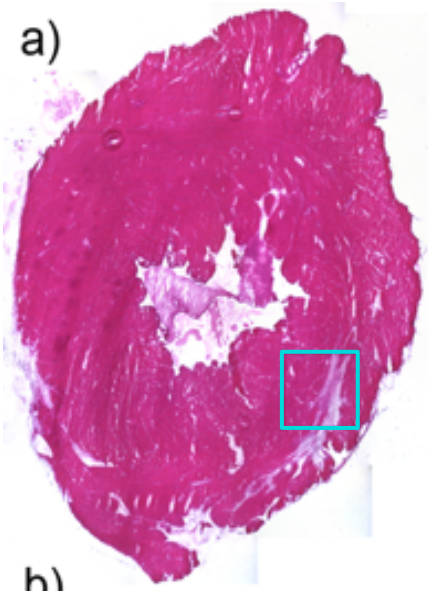
Fibrosis

In vivo Cardiac MRI



Normal Function **Preclinical Scar**

10 wk dko



14 yr old DMD patient

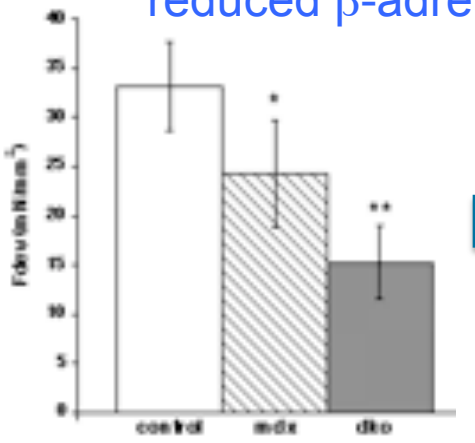
Class 5

DMD and utrophin/dystrophin-deficient mouse hearts show the same pattern of scarring with NORMAL ejection fraction

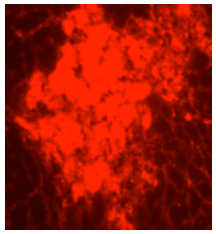
(Delfin, NMD, 2012)

Dystrophic hearts show common indicators of heart failure

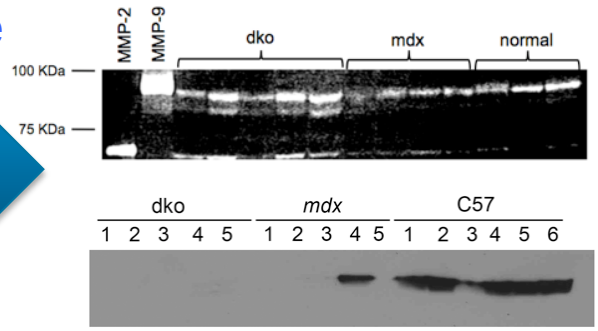
•Step 1: Cardiac contractile dysfunction / reduced β-adrenergic response



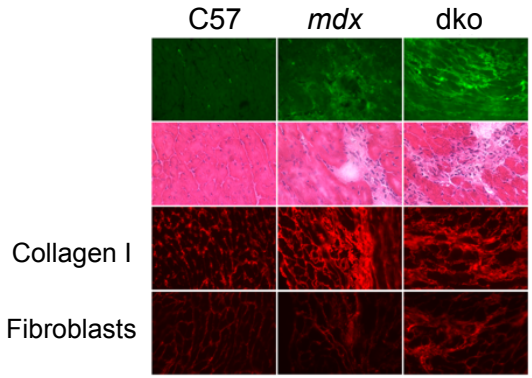
•Step 2: Cardiomyocyte damage



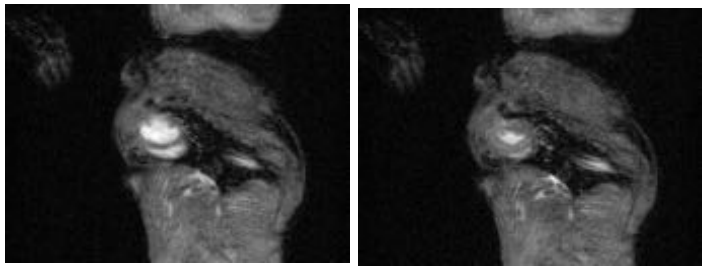
•Step 3: Unregulated MMP remodeling



•Step 4: Collagen scarring



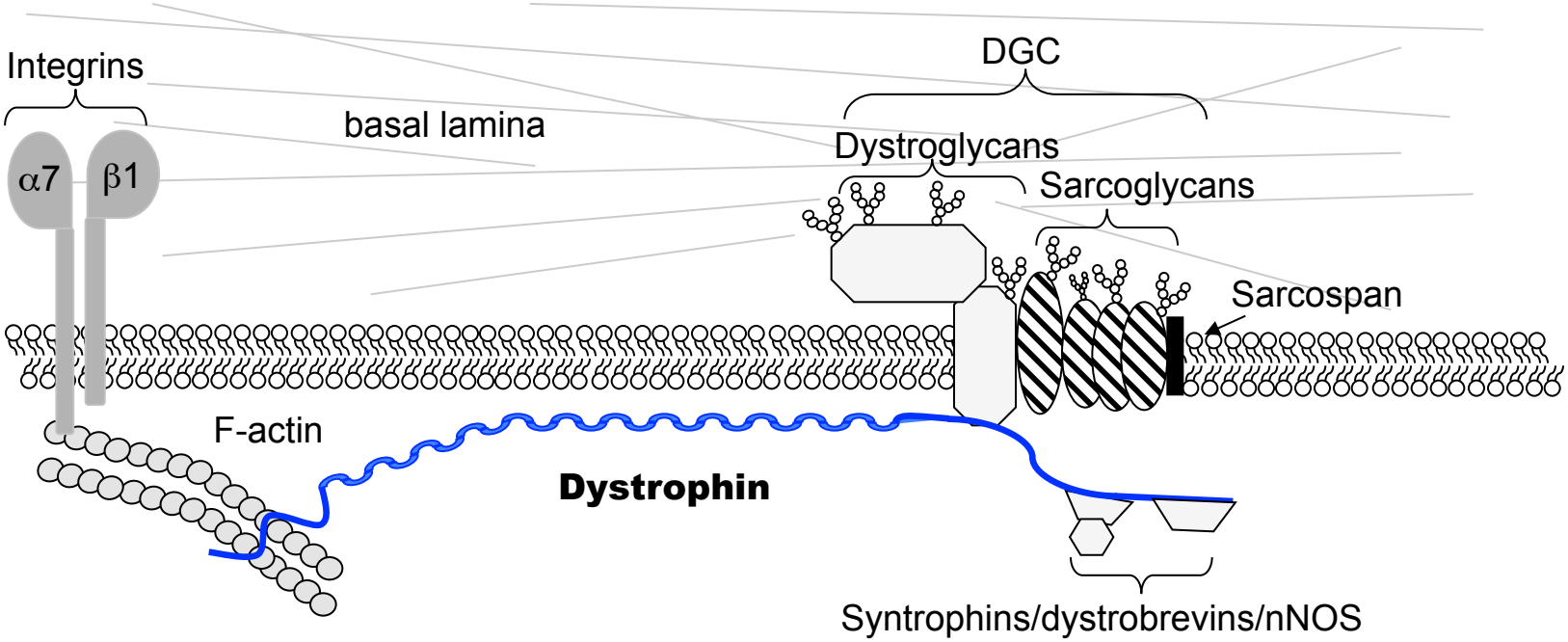
•ALL PRESENT WHILE NORMAL EJECTION FRACTION IS PRESERVED



Wexner Medical Center

Understanding the pathogenesis provides therapeutic opportunities…

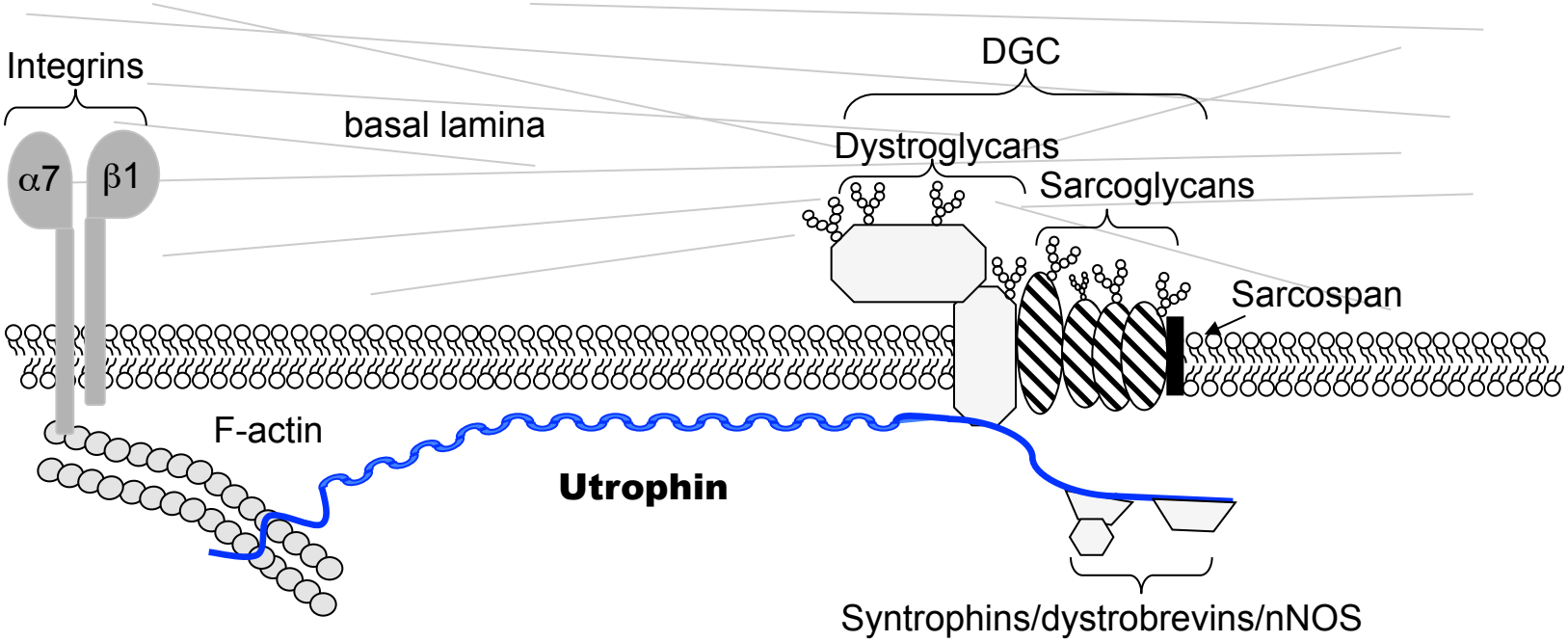
Dystrophin Replacement



- Dystrophin gene therapy
- Exon skipping
- Stop codon readthrough

Class 13

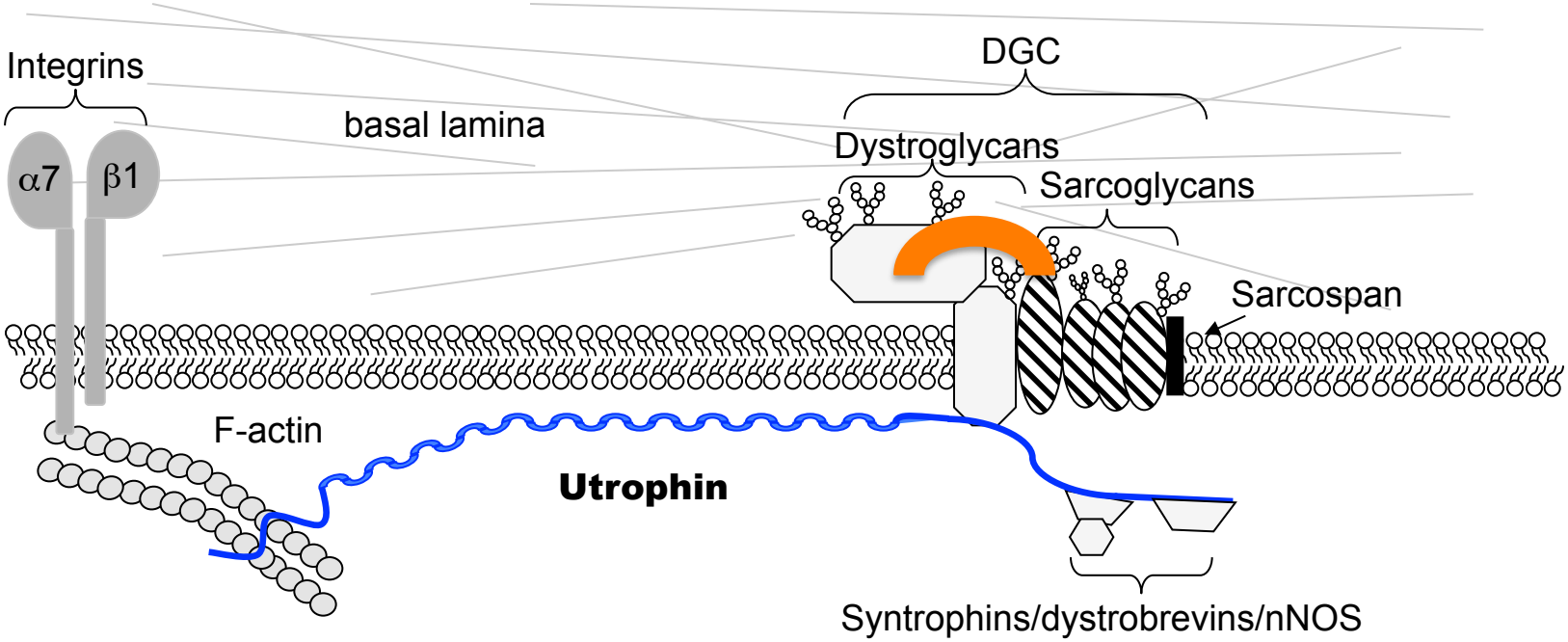
Upregulation of compensatory linkages



- Upregulation of utrophin

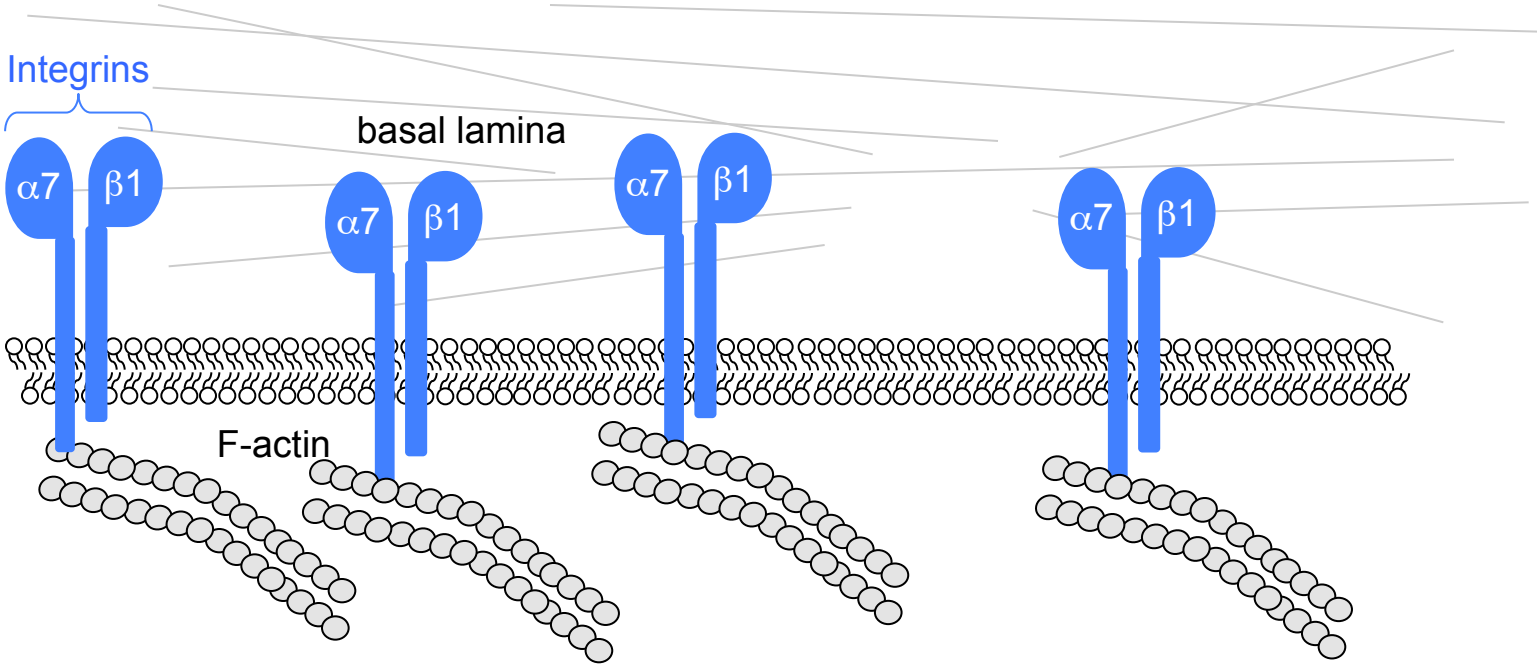
Class 7

Upregulation of compensatory linkages



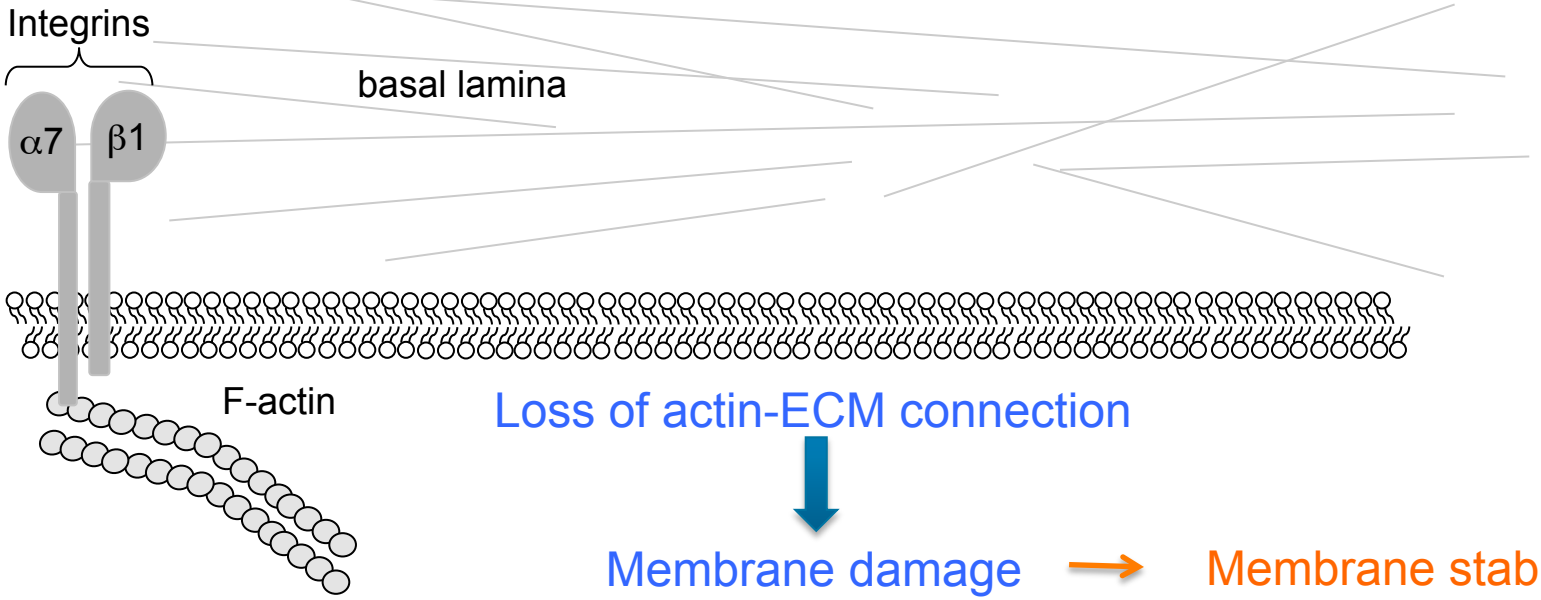
- Upregulation of biglycan

Upregulation of compensatory linkages



- Upregulation of integrins / laminins

Pathophysiology and therapeutic approaches



Loss of actin-ECM connection

Membrane damage → Membrane stabilization

Class 9

Inflammation → Anti-inflammatory

Class 10

Glucocorticoids – clinically delays disease progression with side-effects

- NFκB inhibition - effects on inflammatory cells and muscle

Class 11

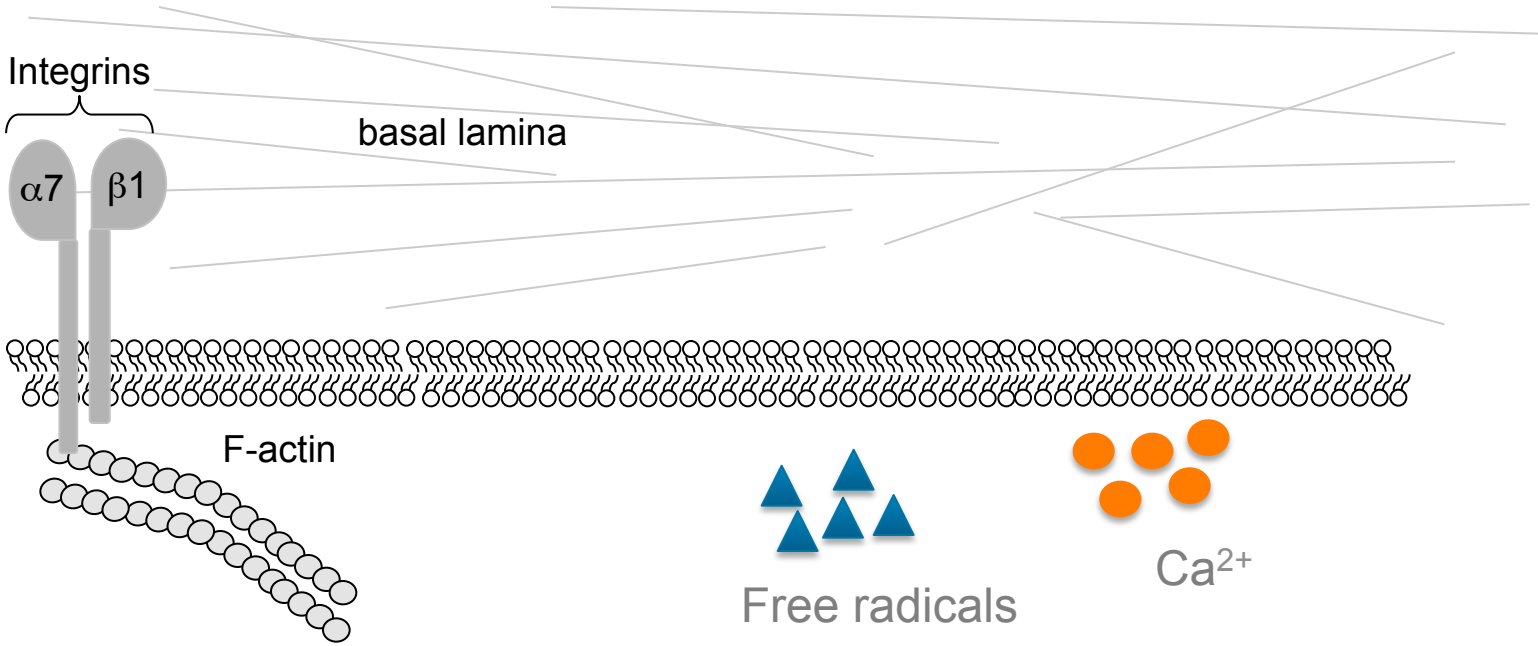
Regeneration

- Muscle growth
- Stimulate regeneration
- Improve muscle differentiation

Fibrosis → Antifibrotics

Class 12

Pathophysiology and therapeutic approaches



- Activation of proteases
- Mitochondrial dysfunction
- Signaling pathway changes

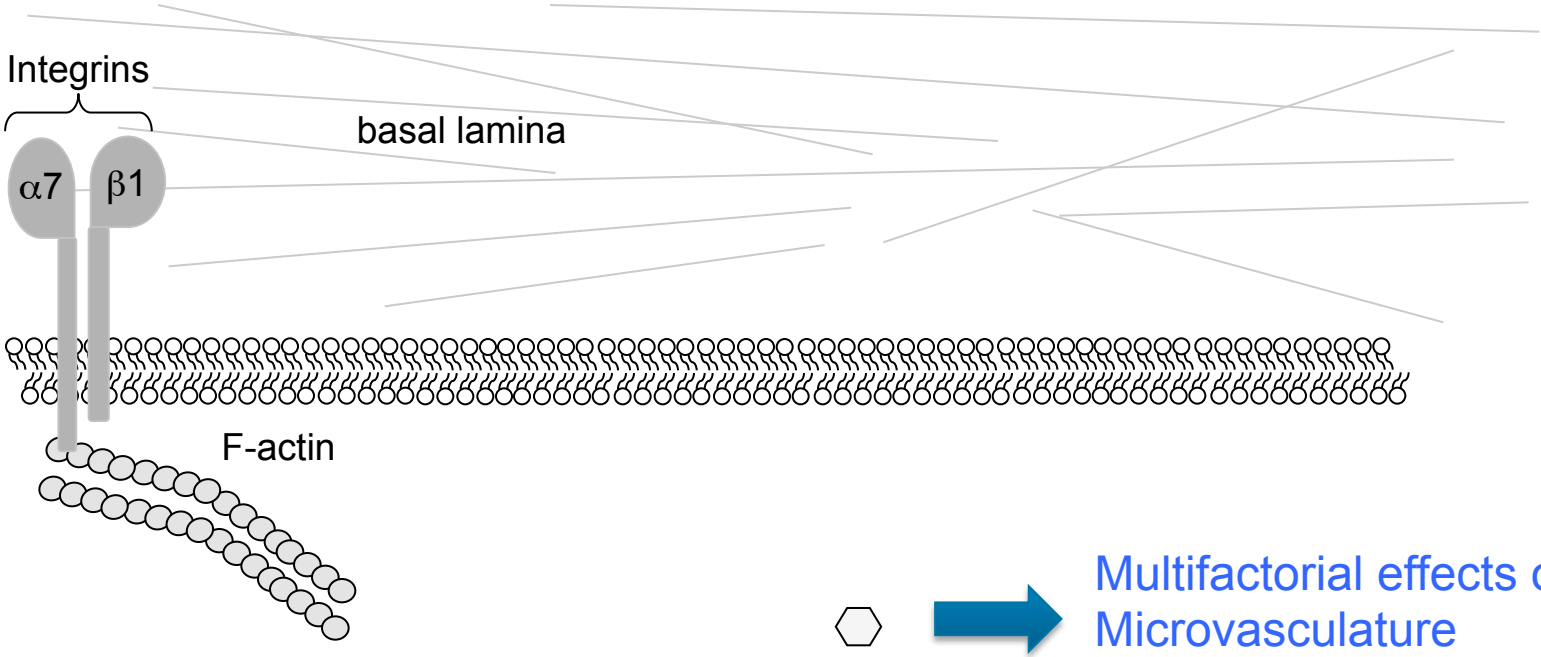
Class 11

Calcium handling

Protease inhibitors

Oxidative stress

DMD and the muscle membrane



nNOS → Multifactorial effects on:
Microvasculature
Signaling / muscle strength?

↓

PDE5 inhibitors

Class 11

Translation to DMD patients

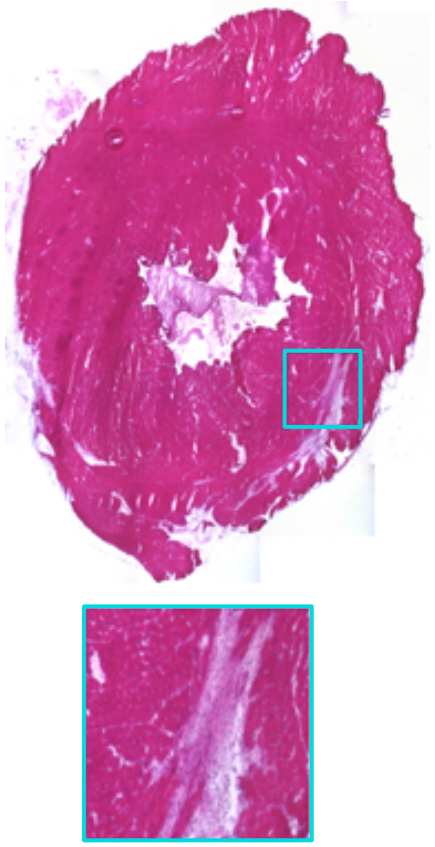
- Vast majority of pathophysiology is from animal models
  - Histopathology verified in patients
- Improvements in which parameters in mice translate to clinical improvements in longevity or quality of life?
- What percentage improvement in mice is therapeutic for patients?
  - Statistically significant improvements are often small
- Need for useful patient biomarkers

Questions?

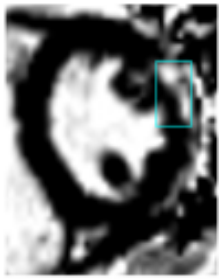
Wexner Medical Center

DMD and utrophin/dystrophin-deficient mouse hearts show the same pattern of scarring with NORMAL ejection fraction

10 wk dko



14 yr old DMD patient



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