



**HAL**  
open science

# Étude fonctionnelle de l'orthologue cristalline $\alpha$ B dans le développement musculaire et la stabilisation du sarcomère chez *Drosophila melanogaster*

Inga Wojtowicz

► **To cite this version:**

Inga Wojtowicz. Étude fonctionnelle de l'orthologue cristalline  $\alpha$ B dans le développement musculaire et la stabilisation du sarcomère chez *Drosophila melanogaster*. Médecine humaine et pathologie. Université d'Auvergne - Clermont-Ferrand I; Wydawnictwo Uniwersytetu Wrocławskiego (Wrocław), 2014. Français. NNT : 2014CLF1MM28 . tel-01849793

**HAL Id: tel-01849793**

**<https://theses.hal.science/tel-01849793>**

Submitted on 26 Jul 2018

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Inga Wójtowicz

**Functional analysis of  $\alpha$ B-crystallin's orthologue in muscle  
development and sarcomere stabilisation in  
*Drosophila melanogaster***

Doctoral dissertation

The thesis has been prepared at the Department of Animal Developmental Biology of  
Experimental Biology Institute, University of Wrocław

and

Genetic Reproduction and Developmental Institute of Faculty of Medicine, INSERM  
U1103, Clermont University,

under the supervision of

dr hab. prof. Małgorzata Daczewska

and

dr hab. Krzysztof Jagła

**Wrocław, 2014**

Inga Wójtowicz

**Analiza funkcjonalna ortologa  $\alpha$ B-kryształiny w rozwoju  
mięśni i stabilizacji sarkomerów  
u *Drosophila melanogaster***

Rozprawa doktorska

Praca została wykonana w Zakładzie Biologii Rozwoju Zwierząt Instytutu Biologii  
Eksperymentalnej, Uniwersytetu Wrocławskiego

oraz

Genetic Reproduction and Developmental Institute of Faculty of Medicine, INSERM

U1103, Clermont University,

pod kierunkiem

dr hab. prof. Małgorzaty Daczewskiej

oraz

dr hab. Krzysztofa Jagła

**Wrocław, 2014**

## Acknowledgements

*Składam serdeczne podziękowania moim promotorom dr hab. prof Małgorzacie Daczeńskiej oraz dr hab. Krzysztofowi Jagle, a także dr hab. Teresie Jagle za umożliwienie mi wykonania tego projektu doktorskiego. Dziękuję rodzinie i przyjaciołom za wielkie pokłady wsparcia i wyrozumiałości oraz za pomoc, bez której ta praca by nie powstała.*

ACKNOWLEDGEMENTS.....	3
LIST OF FIGURES AND TABLES.....	7
ABBREVIATIONS .....	9
GENE SYMBOLS .....	11
SUMMARY .....	13
STRESZCZENIE .....	15
<b><u>1. INTRODUCTION .....</u></b>	<b><u>17</u></b>
<b>1.1. MYOGENESIS MECHANISM IN <i>DROSOPHILA</i>.....</b>	<b>19</b>
1.1.1. MESODERM FORMATION IN <i>DROSOPHILA</i> EMBRYOS .....	20
1.1.2. FROM SOMATIC MESODERM TO MUSCLE FIBRE .....	21
1.1.3. DIVERSIFICATION OF MUSCLE TYPES.....	25
<b>1.2. STRUCTURE OF <i>DROSOPHILA</i> MUSCLE FIBRE .....</b>	<b>26</b>
1.2.1. UNIQUE PROPERTIES AND CHARACTERISTICS OF MUSCLE CELL.....	26
1.2.2. SARCOMERE STRUCTURE AND COMPOSITION .....	27
1.2.3. MYOFIBRIL ASSEMBLY .....	31
<b>1.3. ROLE OF INTERMEDIATE FILAMENTS IN MUSCLE CELL.....</b>	<b>36</b>
1.3.1. CHARACTERISTICS AND CLASSIFICATION OF INTERMEDIATE FILAMENTS.....	36
1.3.2. ROLE OF IFs IN MUSCLE CELLS .....	37
1.3.3. IF-ASSOCIATED DISEASES.....	41
<b>1.4. HEAT SHOCK PROTEINS .....</b>	<b>43</b>
1.4.1. CLASSIFICATION OF HEAT SHOCK PROTEINS.....	43
1.4.2. STRUCTURE AND FUNCTION OF SHSPS.....	45
1.4.3. INVOLVEMENT OF SHSPS IN MUSCLE DEVELOPMENT .....	47
1.4.4. DRM AS A CONSEQUENCE OF <i>AB-CRYSTALLIN</i> MUTATION .....	48
<b><u>2. AIMS OF THE THESIS .....</u></b>	<b><u>50</u></b>
<b><u>3. MATERIAL AND METHODS.....</u></b>	<b><u>52</u></b>
<b>3.1. MATERIAL .....</b>	<b>52</b>
3.1.1. <i>DROSOPHILA</i> STOCKS .....	52
<b>3.2. METHODS .....</b>	<b>53</b>
3.2.1. RNA EXTRACTION AND REVERSE TRANSCRIPTION .....	53
3.2.2. PLASMID CONSTRUCTION AND SITE-DIRECTED MUTAGENESIS .....	53
3.2.3. SEMI-QUANTITATIVE PCR.....	54
3.2.4. IMMUNOFLUORESCENCE STAINING OF <i>DROSOPHILA</i> LARVAL MUSCLES.....	54
3.2.5. MUSCLE MORPHOLOGY OBSERVATIONS AND MUSCLE MEASUREMENTS.....	55

3.2.6.	BEHAVIOURAL ASSAYS .....	55
3.2.7.	VIABILITY TEST .....	56
3.2.8.	WESTERN BLOTTING .....	56
3.2.9.	CO-IMMUNOPRECIPITATION .....	57
3.2.10.	IMMUNOGOLD .....	57
3.2.11.	ANTIBODIES USED IN CONFOCAL LABELLING .....	58
3.2.12.	ANTIBODIES USED IN WESTERN BLOT .....	59
3.2.13.	ANTIBODIES USED IN IMMUNOGOLD.....	59
3.2.14.	MITOTRACKER.....	60
3.2.15.	TEM .....	60
3.2.16.	HEAT SHOCK PROCEDURE.....	60
3.2.17.	STATISTICAL ANALYSIS .....	61
<b>4.</b>	<b>RESULTS .....</b>	<b>62</b>
4.1.	<i>DROSOPHILA DCRYAB</i> GENE IS SPECIFICALLY EXPRESSED IN LARVAL BODY WALL MUSCLES .....	62
4.2.	<i>DCRYAB</i> ATTENUATION LEADS TO FORMATION OF MORPHOLOGICALLY ALTERED MUSCLES.....	63
4.3.	<i>DCRYAB</i> ATTENUATION SPECIFICALLY IN MESODERM LEADS TO IMPAIRED FUNCTION OF LARVAL MUSCLES AND AFFECTS <i>DROSOPHILA</i> LIFE SPAN .....	64
4.4.	<i>dCRYAB</i> CO-LOCALISES WITH PUTATIVE INTERMEDIATE FILAMENTS IN LARVAL MUSCLES .....	65
4.5.	<i>dCRYAB</i> INTERACTS WITH A POTENTIAL INTERMEDIATE FILAMENT PROTEIN .....	67
4.6.	<i>dCRYAB</i> ENSURES STRUCTURAL INTEGRITY OF SOMATIC MUSCLES BY INTERACTING WITH POTENTIAL IF PROTEINS .....	68
4.7.	<i>dCRYAB</i> PARTICIPATES IN THE PUTATIVE INTERMEDIATE FILAMENT MESHWORK FORMATION .....	69
4.8.	MUTATED <i>dCRYAB</i> <sup>R120G</sup> MIMICS EFFECTS OF MUTATIONS IN HUMAN $\alpha$ B-CRYSTALLIN LEADING TO DESMIN-RELATED MYOPATHY.....	70
4.9.	OVEREXPRESSION OF <i>DCRYAB</i> <sup>R120G</sup> LEADS TO FORMATION OF MORPHOLOGICALLY ALTERED MUSCLES.....	71
4.10.	OVEREXPRESSION OF <i>DCRYAB</i> <sup>R120G</sup> LEADS TO IMPAIRED FUNCTION OF LARVAL MUSCLES AND AFFECTS LIFE SPAN.....	72
4.11.	HUMAN $\alpha$ B-CRYSTALLIN INSERTED TO THE <i>DROSOPHILA</i> GENOME MIMICS <i>dCRYAB</i> LOCALISATION AND INTERACTION WITH VIMENTIN-LIKE PROTEIN IN LARVAL MUSCLE CELLS .....	73
<b>5.</b>	<b>DISCUSSION .....</b>	<b>74</b>
5.1.	<i>DCRYAB</i> GENE PLAYS CONSERVED MYOGENIC FUNCTIONS AND <i>dCRYAB</i> PROTEIN CELLULAR LOCALISATION IS REMINISCENT OF ITS VERTEBRATE COUNTERPART .....	77

5.2. dCRYAB CO-LOCALISES AND INTERACTS WITH <i>DROSOPHILA</i> PUTATIVE INTERMEDIATE FILAMENT PROTEINS DETECTED BY ANTI-VIMENTIN ANTIBODY AND <i>DCRYAB</i> ATTENUATION LEADS TO DISRUPTION OF VIMENTIN-LIKE MESHWORK.....	82
5.3. <i>DROSOPHILA</i> IS A PUTATIVE MODEL ORGANISM TO STUDY DRM .....	90
<b>6. CONCLUSIONS AND FUTURE DIRECTIONS .....</b>	<b>96</b>
<b>7. REFERENCES .....</b>	<b>98</b>
<b>8. DOCUMENTATION.....</b>	<b>111</b>

## List of figures and tables

Fig. 1-1.	<i>Drosophila</i> life cycle .....	18
Fig. 1.1.1-1.	Signalling pathways in patterning of <i>Drosophila</i> mesoderm .....	19
Fig. 1.1.2-1.	Pattern of larval muscle abdominal segments A2-A7 .....	23
Fig. 1.1.2-2.	Myogenic cascade in <i>Drosophila</i> embryos .....	24
Fig. 1.2.2-1.	Schematic organisation of sarcomere .....	30
Fig. 1.2.3-1.	Critical role of MHC for formation of muscle striation .....	35
Fig. 1.3.2-1.	Schematic localisation of the intermediate filament scaffold in striated muscle cell .....	40
Fig. 1.4.2-1.	Mechanism of sHsps activation .....	46
Fig. 5.1-1.	<i>Drosophila</i> dCryAB is phylogenetically related to $\alpha$ B-crystallin (CryAB), Hspb1 (Hsp27) and Hspb8 (Hsp22) sHsp families .....	76
Fig. 4.1.1.	dCryAB localisation in the third instar WT larval body wall muscles .....	113
Fig. 4.2.1.	Developmental effects of dcryAB knockdown in third instar larva muscles .....	115
Fig. 4.2.2.	Developmental effects of the dcryAB knockdown on the third instar larval muscle morphology .....	117
Fig. 4.3.1.	Effects of the dcryAB knockdown on the third instar larva motility and life span .....	119
Fig. 4.4.1.	Vimentin-like protein localisation in the third instar larval muscles .....	121
Fig. 4.4.2.	Localisation of the vimentin-like protein inside of the third instar larva muscle nucleus .....	123
Fig. 4.5.1.	Interactions of dCryAB with the vimentin-like protein in <i>Drosophila</i> third instar larval muscle cells .....	125
Fig. 4.6.1.	dCryAB is involved in stabilisation of structural integrity of third instar larval somatic muscles by interacting with putative IF proteins, part1 .....	127
Fig. 4.6.2.	dCryAB is involved in stabilisation of structural integrity of third instar larval somatic muscles by interacting with IF proteins, part 2 .....	129
Fig. 4.7.1.	The vimentin-like meshwork recuperation in third instar larval muscles, after 2 hours of recovery from heat shock .....	131
Fig. 4.8.1.	Comparison of amino acid sequences of $\alpha$ B-crystallin (CryAB)	



	with dCryAB and presentation of the nucleotide sequence of dcryAB containing R120G mutation .....	133
Fig. 4.8.2.	Effects of the R120G mutation in dCryAB on the sarcomere organisation the <i>Drosophila</i> third instar larval somatic muscles .....	135
Fig. 4.8.3.	Effect of the R120G mutation in dCryAB on the accumulation of dCryAB <sup>R120G</sup> aggregates, formation of smaller nuclei and vimentin-like meshwork collapse in <i>Drosophila</i> third instar larval somatic muscles development .....	137
Fig. 4.9.1.	Effects of the <i>dcryAB</i> <sup>R120G</sup> overexpression on the third instar larval muscle morphology .....	139
Fig. 4.10.1.	Effects of the <i>dcryAB</i> <sup>R120G</sup> overexpression on the third instar larva motility and life span .....	141
Fig. 4.11.1.	Localisation in larval muscle cells of human $\alpha$ B-crystallin inserted to the <i>Drosophila</i> genome .....	143
Table 1.3.3-1.	Diseases associated with mutations in IF proteins .....	42
Table 1.4.1-1.	Functions of <i>Drosophila melanogaster</i> Hsps .....	44

## Abbreviations

ALP	actinin-associated protein	LIM	IFM(s)	indirect flight muscle(s)
AMP(s)	adult muscle precursor cell		Ig	immunoglobulin
ATP	adenosine triphosphate		IP	immunoprecipitation
ATPase	adenylpyrophosphatase		kDa	kilodaltons
BHK	baby hamster kidney		M	molar
BLAST	Basic Local Alignment Search Tool		MAPK	mitogen activated protein kinase
BSA	bovine serum albumin		MDa	megadaltons
cDNA	complementary DNA		MF(s)	microfilaments
CI	contractility index		mM	milimolar
CNS	central nervous system		MT(s)	microtubules
DNA	deoxyribonucleic acid		NCBI	The National Center for Biotechnology
DNase	deoxyribonuclease		nM	nanomolar
Dpp	decapentaplegic		p	probability value
DRM	desmin-related myopathy		PBS	phosphate buffered saline
EGF	epidermal growth factor		PCR	polymerase chain reaction
F-actin	filamentous actin		PDZ	post synaptic density protein
FC(s)	founder cell(s)		RNA	ribonucleic acid
FCM(s)	fusion-competent myoblast(s)		RNAi	RNA interference
FGF	fibroblast growth factor		SDS-PAGE	polyacrylamide gel electrophoresis
<i>g</i>	gravitational acceleration		sHsp(s)	small heat shock protein(s)
GFAP	glial fibrillary acidic protein		TALENS	transcription activator-like effector nucleases
GFP	green fluorescent protein		TBS	tris-buffered saline
GTP	guanosine-5'-triphosphate		TEM	transmission electron microscope
Hsp(s)	heat shock protein(s)			
IF(s)	intermediate filament(s)			

TGF	transforming factor	growth	WT	wild type
UAS	upstream sequence	activating	$\mu$ l	microliters

## Gene symbols

<i>act42A</i>	<i>actin42A</i>	<i>hsp22/Hspb8</i>	<i>heat shock protein</i> 22
<i>act5</i>	<i>actin5</i>	<i>hsp23</i>	<i>heat shock protein</i> 23
<i>act57B</i>	<i>actin57B</i>	<i>hsp26</i>	<i>heat shock protein</i> 26
<i>act87E</i>	<i>actin87E</i>	<i>hsp27/Hspb1</i>	<i>heat shock protein</i> 27/ <i>heat shock</i> <i>27kDa protein 1</i>
<i>ap</i>	<i>apterous</i>	<i>hsp40/dHDJ1</i>	<i>heat shock protein</i> 40
<i>capZ</i>	<i>capping protein Z</i>	<i>hsp60</i>	<i>heat shock protein</i> 60
<i>col</i>	<i>collier</i>	<i>hsp60B</i>	<i>heat shock protein</i> 60B
<i>cryAB/αB-crystallin</i>	<i>αB-crystallin</i>	<i>hsp64</i>	<i>heat shock protein</i> 64
<i>dcryAB/ l(2)efl</i>	<i>Drosophila αB-</i> <i>crystallin/ lethal (2)</i> <i>essential of life</i>	<i>hsp67Ba</i>	<i>heat shock protein</i> 67Ba
<i>DES</i>	<i>desmin</i>	<i>hsp67Bb</i>	<i>heat shock protein</i> 67Bb
<i>dFoxo</i>	<i>Drosophila forkhead</i> <i>box O</i>	<i>hsp67Bc</i>	<i>heat shock protein</i> 67Bc
<i>Dm<sub>0</sub></i>	<i>lamin Dm<sub>0</sub></i>	<i>hsp68</i>	<i>heat shock protein</i> 68
<i>duf</i>	<i>dumfounded</i>	<i>hsp70</i>	<i>heat shock protein</i> 70
<i>eve</i>	<i>even-skipped</i>	<i>hsp90</i>	<i>heat shock protein</i> 90
<i>Gal4</i>	<i>yeast transcription</i> <i>activator protein</i> <i>Gal4</i>		
<i>hh</i>	<i>hedgehog</i>		
<i>his3</i>	<i>histone3</i>		
<i>hsc-1</i>	<i>heat shock cognate1</i>		
<i>hsc-2</i>	<i>heat shock cognate2</i>		
<i>hsc-3</i>	<i>heat shock cognate3</i>		
<i>hsc-4</i>	<i>heat shock cognate4</i>		
<i>hsc-5</i>	<i>heat shock cognate5</i>		
<i>hsp20/ Hspb6</i>	<i>heat shock protein</i> 20/ <i>heat shock</i> <i>protein B6</i>		

<i>Hspb2</i>	<i>heat shock 27kDa protein 2</i>	<i>Tn</i>	<i>troponin</i>
<i>Hspb3</i>	<i>heat shock 27kDa protein 3</i>	<i>TnC</i>	<i>troponin C</i>
<i>Hspb7</i>	<i>heat shock 27kDa protein 7</i>	<i>TnI</i>	<i>troponin I</i>
<i>insc</i>	<i>inscuteable</i>	<i>TnT</i>	<i>troponin T</i>
<i>irreC</i>	<i>irregular-chiasm-C</i>	<i>twi</i>	<i>twist</i>
<i>JNK</i>	<i>C-Jun N-terminal protein kinase</i>	<i>wg</i>	<i>wingless</i>
<i>kirre</i>	<i>Kin of Irre C</i>	<i>ZASP</i>	<i>Z-band alternatively spliced PDZ motif protein</i>
<i>Kr</i>	<i>Kruppel</i>		
<i>l'sc</i>	<i>lethal of scute</i>		
<i>lamC</i>	<i>lamin C</i>		
<i>lb</i>	<i>ladybird</i>		
<i>lmd</i>	<i>lame duck</i>		
<i>lmnA</i>	<i>lamin A</i>		
<i>lmnB1</i>	<i>lamin B1</i>		
<i>lmnB2</i>	<i>lamin B2</i>		
<i>Mef2</i>	<i>Myocyte enhancer factor-2</i>		
<i>MHC</i>	<i>myosin heavy chain</i>		
<i>MLC</i>	<i>myosin light chain</i>		
<i>rst</i>	<i>roughest</i>		
<i>SALS</i>	<i>sarcomere length short</i>		
<i>slou</i>	<i>S59/slouch</i>		
<i>slp</i>	<i>sloppy paired</i>		
<i>sna</i>	<i>snail</i>		
<i>sns</i>	<i>sticks and stone</i>		
<i>tin</i>	<i>tinman</i>		
<i>Tm</i>	<i>tropomyosin</i>		
<i>Tmod</i>	<i>tropomodulin</i>		

## Summary

The *Drosophila* CG4533 (*l(2)efl, dcryAB*) gene is an orthologue of vertebrate  $\alpha$ B-crystallin, which encodes a small heat shock protein (sHsp). The most prominent activity of sHsps is binding proteins and protecting them from aggregation, preventing the accumulation of partially denatured or improperly folded proteins in muscle cells.  $\alpha$ B-crystallin is also implicated in the initial phase of skeletal muscle differentiation. It was demonstrated that  $\alpha$ B-crystallin plays a key role in muscle differentiation and its mutated form is involved in human desminopathies. My results revealed that *dcryAB* gene is specifically expressed in larval body wall muscles and the dCryAB expression pattern was reminiscent of the localisation of its human orthologue  $\alpha$ B-crystallin.

Using the generated polyclonal antibodies it was found that dCryAB protein was abundantly expressed in all larval body wall muscles, it was accumulated in a perinuclear area and displayed a striated pattern at the level of M- and Z-lines. To assess the *dcryAB* role in muscle development RNAi-mediated muscle-specific gene attenuation was applied. The *dcryAB* knockdown led to formation of muscles characterised by significant defects in muscle morphology. The majority of the observed larvae exhibited defects in sarcomeric organisation, characterised by an irregular, fuzzy pattern of Z-lines in large muscle segments and smaller number of muscle nuclei. This aberrant sarcomeric pattern was often associated with muscle splitting leading to an altered muscle attachment or to the loss of the affected muscle. Ultrastructural analyses confirmed altered sarcomeres organisation, revealing also mitochondria with barely visible mitochondrial crests and increased amount of glycogen between myofilaments. Moreover, *dcryAB* attenuation specifically in mesoderm led to impaired functionality of larval muscles and affected *Drosophila* life span, implicating *dcryAB* role in muscle development.

Presented studies also revealed that in *Drosophila* larval muscles mouse anti-vimentin antibody detected a protein which co-localised with dCryAB in the Z-line and was present in the nuclear area. I believe that *Drosophila* expresses proteins that correspond to the third class of vertebrate intermediate filaments, which share a similar intracellular distribution with their vertebrate counterparts.

Using co-IP approaches it was confirmed that dCryAB interacts with the vimentin-like protein like  $\alpha$ B-crystallin interacts with desmin. Thus it is expected that the vimentin-like protein may have properties of the third class intermediate filament proteins, enclosing vimentin and desmin. Performed analyses suggest that dCryAB ensures structural integrity of somatic muscles by interacting with potential IF proteins.

In human, R120G substitution in  $\alpha$ B-crystallin leads to the loss of its IF chaperone activity and induces aggregations of desmin in muscles, causing desmin-related myopathy (DRM). The advantage of the  $\alpha$ B-crystallin conservation has been taken to test whether *Drosophila* mutated dCryAB displays similar properties as its vertebrate counterpart. I found that muscle-specific expression of mutated dCryAB<sup>R120G</sup> caused formation of intracellular aggregates containing the vimentin-like protein as well as dCryAB<sup>R120G</sup>. These symptoms led in consequence to muscle weakness, which is characteristic for patients with DRM. My studies revealed that mutated dCryAB<sup>R120G</sup> mimics effects of mutation in human  $\alpha$ B-crystallin, therefore *Drosophila* may represent a suitable model system to study DRM.

## Streszczenie

*Drosophila* gen *CG4533 (l(2)efl, dcryAB)* jest ortologiem genu  $\alpha$ B-krystaliny kręgowców.  $\alpha$ B-krystalina jest małym białkiem szoku cieplnego, które zapobiega akumulacji częściowo zdenaturowanych lub nieprawidłowo złożonych białek, między innymi w komórkach mięśniowych. Ekspresja  $\alpha$ B-krystaliny jest również indukowana podczas początkowej fazy różnicowania się mięśni, wskazując na jej istotną rolę w tym procesie, a także jest zaangażowana w powstawanie desminopatii u ludzi. W rozprawie doktorskiej wykazano, że ekspresja genu *dcryAB* obserwowana jest specyficznym w mięśniach, podobnie do swojego ludzkiego odpowiednika. Wykazano, że białko dCryAB usytuowane jest wokół jąder komórkowych, w linii M oraz na zewnętrznym obszarze linii Z. W celu oceny roli *dcryAB* w rozwoju mięśni, zastosowano mięśniowo specyficzną atenuację genu, przy użyciu interferencyjnego RNA. Wykazano, że obniżona ekspresja genu *dcryAB* prowadzi do nieprawidłowego rozwoju mięśni, przejawiającego się zmniejszonymi rozmiarami mięśni czy rozdzielaniem włókien mięśniowych, mniejszą liczbą jąder oraz sarkomerów. Analiza w TEM włókien mięśniowych ujawniła liczne defekty w strukturze samych sarkomerów jak również obecność mitochondriów o zaburzonej strukturze grzebieni mitochondrialnych. Na obszarze sarkoplazmy włókien mięśniowych zaobserwowano liczne nagromadzenia glikogenu pomiędzy miofibrillami. Ponadto przeprowadzone testy behawioralne ujawniły mniejszą sprawność ruchową oraz krótszy cykl życiowy larw z wyciszonym genem *dcryAB*. Uzyskane wyniki wskazują na istotną rolę produktów genu *dcryAB* w rozwoju mięśni oraz stabilizacji sarkomerów jak również wpływu na długość cyklu życiowego *Drosophila*.

Przy użyciu przeciwciała przeciwko mysiej wimentynie wykazano obecność wimentyno podobnych filamentów pośrednich w mięśniach larwalnych *Drosophila*. Wykazano, że białko wimentyno podobne tworzy sieć na obszarze sarkoplazmy włókien mięśniowych. Rozmieszczenie przestrzenne białka wimentyno podobnego w mięśniach larwalnych swoją lokalizacją przypomina rozmieszczenie włókien desminowych we włóknach mięśniowych kręgowców. Przy zastosowaniu techniki koimmunoprecypitacji wykazano, że białko wimentyno podobne oddziałuje z białkiem *dcryAB*. Przypuszcza się, że białko



wimentyno podobne może posiadać właściwości filamentów III klasy, obserwowanych u kręgowców.

U człowieka, substytucja R120G w  $\alpha$ B-krystalinie powoduje utratę przez nią właściwości opiekuńczych wobec filamentów pośrednich i powstanie w komórkach agregatów zawierających między innymi desminę, co prowadzi do wywołania miopatii zależnej od desminy. W przedstawionej pracy wykorzystano konserwatywność sekwencji  $\alpha$ B-krystaliny do sprawdzenia czy zmutowane białko dCryAB u *Drosophila* wykaże się podobnymi właściwościami co jego odpowiednik u kręgowców. Wykazano, że mięśniowo specyficzna ekspresja zmutowanego białka dCryAB<sup>R120G</sup> powoduje wytworzenie się wewnątrzkomórkowych agregatów, zawierających białko wimentyno podobne, jak również dCryAB<sup>R120G</sup>. U ludzi opisane symptomy prowadzą do osłabienia mięśni, co jest charakterystyczne dla pacjentów z miopatią zależną od desminy. Na podstawie otrzymanych wyników stwierdza się, że *Drosophila* może być użytecznym organizmem modelowym do badań miopatii zależnej od desminy.

# 1. Introduction

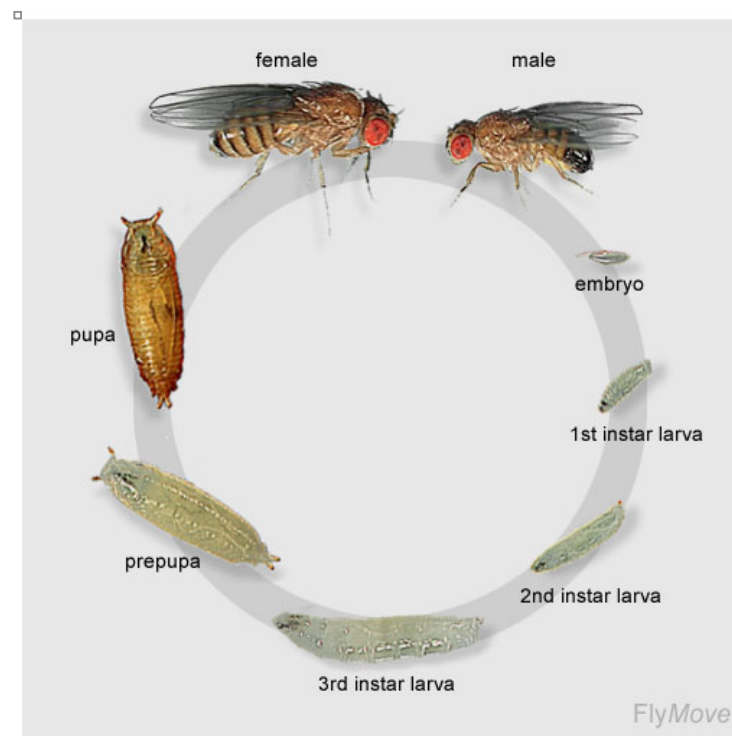
*Drosophila melanogaster* is a model organism commonly used to study development, behaviour, diseases or even evolutionary mechanisms. Thanks to the wide range of available tools to study many of the processes occurring during *Drosophila* life, it has become one of the most important models in the modern biomedical research. Many biological processes in *Drosophila* are analogous to vertebrate ones, both at the cellular and the molecular levels, but the main advantages of the fruit fly over vertebrate models are its short generation time (Fig. 1-1.), a relatively simple development and a less complex genome.

Development of the larval musculature during embryonic stages of the fruit fly is one of the processes analogous to those occurring in vertebrates. As in vertebrates, the mesoderm gives rise to three types of muscle tissues, namely: somatic muscles, also called body wall muscles which enable locomotion, visceral muscles lining the digestive tract and heart muscles building the dorsal vessel and pumping the haemolymph. *Drosophila* myogenesis progresses in two steps: embryonic and adult. During the first step, larval muscles are formed through the fusion between founder cells and fusion-competent myoblasts, whereas during the second step, adult muscle precursor cells serve to develop adult muscles either *de novo* or using larval muscle templates. The developed muscles display unique features due to information provided by identity genes, expressed in the founder cells. Finally, attached and innervated muscles become fully differentiated and functional larval muscles with a completely organised contractile apparatus.

In vertebrates, one part of the striated muscle cytoskeleton consists of an intermediate filament network, which is responsible for stabilisation of the sophisticated sarcomeric organisation, as well as linkage between the contractile apparatus and the cytoplasmic organelles. Small heat shock proteins help to stabilise the muscle cell structure by interacting with the cytoskeleton including intermediate filament components; mutations of any of these proteins can lead to muscle diseases.

Small heat shock proteins also influence the muscle development; for this reason the aim of this study is to investigate the role of  *$\alpha$ B-crystallin's* orthologue

in muscle development and sarcomere stabilisation in *Drosophila melanogaster*. Surprisingly, no intermediate filament proteins have been identified in the *Drosophila* muscles to date. Studying the *Drosophila* counterpart of  $\alpha B$ -crystallin, which is known to interact with the intermediate filament protein, is expected to provide a new insight into this issue.



**Fig. 1-2. *Drosophila* life cycle.** Images represent the developmental stages of *Drosophila*. The development time at 25°C is 8.5 days. Adult females lay up to 400 eggs (embryos), which hatch after 12–15 h. Larvae grow for about 4 days while moulting twice: into 2nd- and 3rd-instar larvae at about 24 and 48 h after hatching. Subsequently, the larvae encapsulate in the puparium and undergo a four-day metamorphosis, after which the adults emerge. After Weigmann et al. (2003).



### 1.1.1. Mesoderm formation in *Drosophila* embryos

Mesoderm formation starts when all the cells of the internal layer of the *Drosophila* embryo express transcription factors Twist (Twi) and Snail (Sna) (Ip et al., 1992; Thisse et al., 1988) induced by a high concentration of Dorsal morphogene. The crucial role of the *twi* and *sna* genes in the mesoderm formation was established by Thisse et al. (1988), who showed that embryos lacking Twist or Snail failed to form mesoderm and died at the end of embryogenesis. Extensive genetic analyses (Baylies and Bate, 1996) and a recent genome-wide study (Sandmann et al., 2007), that Twi activated a large range of genes involved in specification of different mesodermal derivatives. One of its key targets is *tinman* (*tin*), expressed initially in the trunk mesoderm. *Tin* is crucial for mesoderm regionalization and subsequently becomes restricted to its dorsal portion, being required for dorsal fates specification.

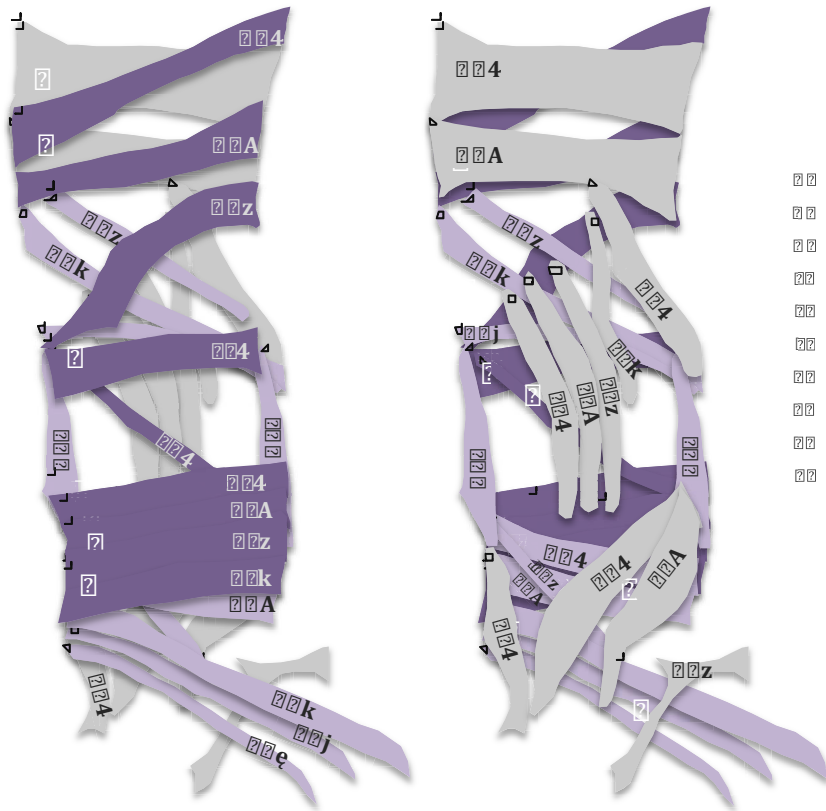
At this point, the mesodermal anlage spreads dorsally and the process requires the function of Heartless, a *Drosophila* FGF receptor homologue (Carmena et al., 1998a). At the same time, unpatterned mesoderm stays under the influence of signals sent from the overlapping ectoderm. The first one, decapentaplegic (Dpp), is a member of the TGF $\beta$  family and defines the dorso-ventral axis of the embryo (Fig.1.1.1-1.), whereas two other ectodermal signals: Wingless (Wg) and Hedgehog (Hh), define the antero-posterior axis (Baylies et al., 1998; Leptin and Grunewald, 1990). Dpp, expressed as a dorso-ventral gradient in ectodermal cells, maintains expression of *tin* in the underlying mesodermal cells and represses expression of ventrally expressed genes such as *pox meso*, a gene required for the proper development of the most ventral and lateral abdominal muscles and for activation of the subset of sidentity genes (Duan et al., 2007). This induction of Dpp signaling divides the mesoderm into dorsal and ventral sectors. The mesoderm partition along the antero-posterior axis also divides each mesoderm segment into two domains. These give rise to the different progenitor cells, due to expression of two segmentation genes: *even-skipped* (*eve*) and *sloppy paired* (*slp*). Consequently, progenitors of the visceral mesoderm and the fat body originate in the *eve* domain, whereas progenitors of the somatic muscles and the heart are produced in the *slp* domain, which is shown in Figure 1.1.1-1. (Azpiazu and Frasch, 1993; Riechmann

et al., 1997). Here, Wg signal amplifies distinctions between cells of the *eve* and *slp* domains, by maintaining a high level of Twist expression in *slp* domain cells, being a crucial element in assigning cells to the somatic myogenesis (Baylies and Bate, 1996). Finally, mesodermal cells of the dorsal part that receive Dpp and Hh from the ectoderm will form the visceral mesoderm in the *eve* domain, while cells from the ventral part will generate a part of the fat body and other mesodermal tissues. Mesodermal cells of the most dorsal part that receive Dpp and Wg signals in the *slp* domain will form the heart, whereas the remaining cells will form the somatic mesoderm (see Figure 1.1.1-1., Baylies 1998).

### **1.1.2. From somatic mesoderm to muscle fibre**

Two waves of myogenesis occur during the *Drosophila* life cycle. During the first, embryonic wave, the body wall muscles are formed to ensure mobility of the larva. In each abdominal hemisegment, 30 different, single and multinucleated muscle fibres are formed (Fig. 1.1.2-1.). Each of them displays unique properties, such as the number of nuclei, position, orientation along antero-posterior and dorso-ventral axes, size, sites of attachment to the exoskeleton and patterns of innervation (Baylies et al., 1998). *Drosophila* somatic muscles are formed by the fusion between two types of cells: founder cells (FC) and fusion-competent myoblasts (FCMs) (Bate, 1990a; Richardson et al., 2008). In spite of the fact that these two types of myoblasts originate from the same competence domain, they have different properties, thanks to progressive specification taking place during successive steps of cell fate determination (Fig. 1.1.2-2.). Initially, the combination of received Wg and Dpp signals makes mesodermal cells, expressing high level of Twi, competent to start myogenic program by induction of expression of the proneural gene, *lethal of scute (l'sc)*, in all the cells of the competent domain (Carmena et al., 1995). During initial steps of diversification (Fig. 1.1.2-2.), the competence domain subdivides into different promuscular clusters. This highly dynamic process involves a combined action of intrinsic fibroblast growth factor (FGF) and epidermal growth factor (EGF), signals which restrict expression of *l'sc* to a spatially defined subset of cells, forming promuscular clusters (Buff et al., 1998; Michelson et al., 1998). Within those clusters, cells start to express muscle

identity genes, which initiate diversification (Carmena et al., 1998), leading to the formation of distinct types of muscles. At the same time, cells from the same promuscular clusters still have potential to become either founder cells or fusion-competent myoblasts. Subsequently, under the action of lateral inhibition process, involving Notch and MAPK pathways, only one cell in each cluster will obtain the identity of muscular progenitor, whereas remaining cells will become FCMs (Fig. 1.1.2-2.). Here, only muscle progenitor cells stay *l'sc* positive, giving rise to all FCs, whereas their surrounding cells express the gene *lame duck (lmd)* which is restricted to the cells fated to become FCMs (Carmena et al., 2002; Duan et al., 2001). Then, each progenitor undergoes symmetric or asymmetric division, giving rise to two FCs or one FC and one adult muscle precursor cell (AMP), which remains undifferentiated throughout embryonic embryogenesis but is required for adult muscle development during the second wave of myogenesis. This asymmetric division takes place thanks to an unequal distribution of Numb and Inscuteable (Insc) proteins, which are localised on the opposite sides of the dividing cell. In consequence, one of the cells inheriting Numb, which is able to repress Notch, becomes a FC of the larval muscle, while the second daughter cell, retaining Notch activity, becomes another FC or an AMP (Carmena et al., 1998b; Leptin and Grunewald, 1990; Ruiz Gómez and Bate, 1997). As a result of the progressive myogenic cascade, three distinct classes of myoblasts are specified, namely FCs, FCMs and AMPs.



- 00 - 0i ly0000f s00
- 00 - 0i ly00i 0naf 00
- 00 - 0i ly00si 0t y00ly00
- 00 - r0s0 0ri0 0naf 00
- 00 - r0s0 0ri0 0t y00ly00
- 00 - r0s0 0ri0 t 0 sf 0 t 0r0
- 00 - c0t si 0000f s00
- 00 - c0t si 00i 0naf 00
- 00 - c0t si 00si 0t y00ly00
- 00 - c0t si 00i t 0 sf 0 t 0r0

0

0 0044bAW00ss0 t 0 0000rc fy000000i c t 0ry00c 0t sy00AWs00r0li 06 0  
 cltl c0j z0j 0W0WzP00j 000dz0j 0W0nI zP0r0 c0t 0zI 00W0r0W0 Dc0W0o0zz0j 0  
 0dz0j 0W0 Dc0W0c00i 00j 0lis 00j 006 ti00j z0j 0W0j 0c00i 00j 0W0I z00j 0000i a0rt Wz0  
 0j z0i ti00t cz0i ti0i t0 0W z0t 0nI z00t 0nr000i t0 0Dre00B0 0e00z0W06' ' ) P0

0  
 0  
 0

0





### 1.1.3. Diversification of muscle types

All larval muscles are composed of a single, multinucleated fibre originated from an individual founder cell. FCs, formed during initial steps of diversification give rise to all embryonic muscles and are thought to carry all the information needed to define unique features of individual muscles. Each FC starts to express identity genes and undergoes a series of myogenic events that direct the formation of functional muscles with unique properties (Fig. 1.2-2.). Identity genes, such as *apterous (ap)*, *collier (col)*, *even-skipped (eve)*, *Kruppel (Kr)*, *ladybird (lb)*, *S59/slouch (slou)* (Tixier et al., 2010), encode developmental regulatory proteins which are expressed in different subsets of FCs. Most of them have been described in detail and their main role in the specification of muscle identities has been well understood. Their expression is determined by a negative regulation with other identity genes (Jagla et al., 2002; Knirr et al., 1999), regulatory interactions with Hox genes (Capovilla et al., 2001) and an influence of extrinsic signals like Wg (Taylor, 2006).

Acquisition of distinct muscle properties includes obtaining specific size, attachment and innervation by individual muscles. All larval muscles are composed of single but multinucleated fibres, which are derived from particular founder cell. The FC fuses with a determined number of FCMs obtained by multiple rounds of fusion, developing multinucleated myotubes (Fig. 1.1.2-2.). A large number of genes controlling different steps of myoblast fusion have been identified (Schejter and Baylies, 2010). This includes genes expressed exclusively in FCs, like *dumfounded/Kin of Irre C (dof/kirre)* (Ruiz Gómez et al., 2000) and *irregular-chiasm-C/roughest (irreC/rst)* (Strünkelnberg et al., 2001), but also genes expressed in FCMs, like *sticks and stone (sns)* (Bour et al., 2000). It has been proposed that Dof and Rst are ligands for Sns, acting here as their receptor, provoking fusion between FCs and FCMs (Baylies et al., 1998; Leptin and Grunewald, 1990). Since muscles differ in their size, they have different numbers of nuclei. This is a result of muscle type-specific regulation of the fusion process, which is dependent on the activity of identity genes (Bataille et al., 2010). Nevertheless, not only identity genes are expressed in a muscle-type specific

manner. Also genes encoding cytoskeletal components or adhesion molecules are expressed differently in individual muscles, revealing their roles in acquisition of distinct muscle properties, other than muscle size. Attachment and innervation types are other unique properties of muscles, regulated by *kon-tiki* (Schnorrer et al., 2007) and *capricious* (Shishido, 1998) genes respectively, and their expression is also restricted to small muscle subsets.

## **1.2. Structure of *Drosophila* muscle fibre**

Muscle cells play various roles in organisms, depending on their structure and localisation. The larval somatic musculature in *Drosophila* is responsible for locomotion, which is crucial for finding food and then a suitable place for pupation. *Drosophila* somatic muscle fibres show many similarities with vertebrate striated muscle cells, and components building contractile apparatus share a lot of homology between these two, very distant animal groups. Their similarities are observed at both, morphological and molecular levels. Organisation of insect striated muscles is remarkably regular and precise which seems to be critical for muscle development and function.

### **1.2.1. Unique properties and characteristics of muscle cell**

Muscle tissue is mainly composed of elongated cells building a muscle. Muscle cells are specialised in changing their length that is contracting and relaxing with changing tension. Diversification process lies in the showing up in the cytoplasm of specific proteins, which take part in contraction. There are three types of muscle tissue: skeletal muscle, smooth muscle and cardiac muscle. In their structure and function the body wall muscles in *Drosophila* resemble the vertebrate skeletal muscle and, moreover, most of vertebrate muscle genes have invertebrate homologues (Sawicki, 2009).

Skeletal muscle tissue consists of long, cylindrical cells, also called fibres because of their elongated shape. Muscle cells contain a contractile apparatus, composed of myofibrils, which are tightly compacted in the whole volume of the cell. Myofibrils are the main cytoplasmic components and are built of proteins

taking part in the contraction. These proteins form thin myofibres, arranged very regularly. Mitochondria, situated between the myofibres, provide energy needed for the generation of a contraction. Nuclei lie on the myofibrils surface, just below the cell membrane in the cytoplasmic space where other cellular organelles are also localised. Skeletal muscle cells also have a well-developed endoplasmic reticulum, which forms many compartments and channels with a characteristic arrangement. Moreover, the cell membrane also creates many big and small channels by its invagination inside the cell, which is important for performing contractions (Sawicki, 2009).

To generate contraction and ensure the proper function of the muscle cell, all its components have to be well organised and form an integrated network. This is sustained, among others, by the intermediate filaments, which are part of the cytoskeleton. Mutations in any sarcomeric or cytoskeletal components of human muscle cell very often cause various neuromuscular diseases, and thus invertebrate studies offer a possibility of improving human diseases therapies (Hooper and Thuma, 2005; Sawicki, 2009).

### **1.2.2. Sarcomere structure and composition**

Sarcomere is the smallest functional contractile unit, building myofibril. Its structure is conserved across the animal kingdom and it is formed from highly organized macromolecular complexes consisting of thin (actin) and thick (myosin) myofilaments, together with their associated proteins (Rui et al., 2010). A single sarcomere (Fig. 1.2.2-1.) is confined by two neighboring Z-lines, also called Z-disks, which are structures made up of various structural proteins such as  $\alpha$ -actinin, Zasp and titin (Hooper and Thuma, 2005; Lin et al., 2013; Zhang et al., 2000). The area on both sides of the Z-line, spanned by thin filaments that do not overlap thick filaments, is called I-band. The Z-line demarcates the middle of the I-band and participates in organisation and stabilisation of the thin filaments, which are cross-linked and anchored to the Z-line by  $\alpha$ -actinin (Sparrow and Schöck, 2009).  $\alpha$ -actinin is directly bound to the Zasp protein, the only *Drosophila* member of the cytoskeletal Alp/Enigma family, which is a group of proteins characterised by an amino-terminal PDZ domain and one or three carboxyl-terminal LIM domains.

Zasp cooperates with  $\alpha$ -actinin in the Z-line in the maintenance and anchoring actin at the Z-line (Jani and Schock, 2007). Another Z-line protein, titin, was predicted by molecular modelling to be a component of a ternary complex of Zasp,  $\alpha$ -actinin and titin, where Zasp and titin bind to different surfaces of  $\alpha$ -actinin (Au et al., 2004; Leptin and Grunewald, 1990). The *Drosophila* titin (D-titin) and its smaller part kettin align and connect the thick and thin filament systems, providing elasticity to the muscle. Their N-termini are found to bind to both  $\alpha$ -actinin and actin in the center and the periphery of the Z-line, whereas their C-termini are associated with the thick filaments. Thanks to that, they can play a role in the organisation of actin-myosin filaments into striated arrays and also in the contribution of the myofibril passive stiffness (Clark et al., 2007; Kulke et al., 2001; Leptin and Grunewald, 1990; Zhang et al., 2000). Moreover, Z-lines are also connected to the plasma membrane. Here, ends of muscle fibres are linked to the tendon matrix on the outside of the muscle via integrin junctions, forming enlarged “modified” Z-lines (Reedy and Beall, 1993).

Thin filaments (Fig. 1.2.2-1.) consist of filamentous (F)-actin associated with tropomyosin (Tm) and troponin (Tn) complex proteins. They are anchored to the Z-line and together are often referred to as the “I-Z-I” complex (Rui et al., 2010; Sparrow and Schöck, 2009). Muscle thin filaments are characterised by a diameter between 6 and 10 nm. Their main component is actin, which forms a double helix made of polymerized actin monomers. Invertebrate actin double helix repeats once every 13-15 monomers, which is every 35-40 nm (Hooper et al., 2008). The fly genome contains six actin gene *loci*. Particular actin proteins differ in only few amino acids, both within species and between distant species such as fly and mouse. *Drosophila* has four muscle-specific actins with different time points of expression. Actin 87E is expressed throughout the fly life, actin 57B is expressed in embryonic and larval muscles and actins 79B and 88F are mostly expressed in pupal and adult muscles. Moreover, other than temporal restriction of expression, the fly actins are also restricted in their spatial expression pattern (Roper et al., 2005). Other components of thin filaments are tropomyosin and troponin complexes, which are required for the proper interactions of thin filaments with myosin. Tropomyosin is a filamentous protein, formed by dimeric tropomyosin subunits, polymerised in the head-to-tail manner (Karlik and Fyrberg, 1986).

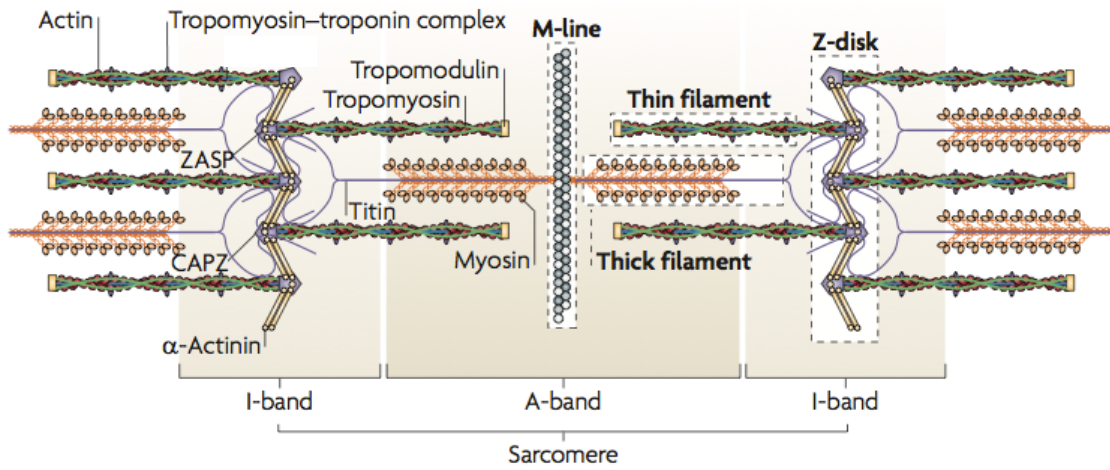
These filaments twist with the actin double helix and sterically block the myosin binding sites during relaxed state, but move away from them in the presence of  $\text{Ca}^{++}$  (Hooper et al., 2008). Every tropomyosin molecule is associated with a troponin complex, which is composed of three proteins, troponin T (TnT), troponin I (TnI) and troponin C (TnC). Each subunit has different function, namely TnT binds to tropomyosin, TnI is involved in inhibition of  $\text{Mg}^{++}$  ATPase activity of actomyosin and TnC binds  $\text{Ca}^{++}$  removing TnI inhibition (Farah and Reinach, 1995). Altogether, actin filaments, tropomyosin and troponin complexes take part in ensuring interactions between thin and thick filaments, which is crucial for generation of muscle contraction.

Muscle thick filaments are composed of myosin and the area of sarcomere that is spanned by such filaments is known as the A-band (Fig. 1.2.2-1.). Three pairs of molecules, the heavy chain (MHC), the essential light chain (MLC) and the regulatory chain build myosin. Heavy chains coil around each other to form a long coiled-coil tail. Hundreds of myosin tails bind to each other to form the scaffold of the thick filament. The opposite end of every heavy chain is bound to one essential light chain and one regulatory chain, forming one of the combined molecules of globular heads. Heads are connected to the tails by a short neck domain and bind to the thin filaments to generate force. The myosin molecules are oriented in the opposite directions with two peripheral regions containing protruding heads and a central region without heads. This organisation is a result of the polarisation of the myosin ends and leads to the formation of cylindrical filaments, which are fixed firmly to the M-line, situated at the centre of A-bands. Another protein, which is comprised in thick filaments, is paramyosin. This protein forms bipolar filaments, which are formed by two-chain  $\alpha$ -helical coiled-coils. Paramyosin creates the core of the thick filament, being surrounded by myosin. It is shown that paramyosin plays a central role in determining the thick filament diameter (Hooper et al., 2008). In *Drosophila* indirect flight muscles (IFM) is also present characteristic protein, called flightin. It is localised in the A-band except for the H-band region (the zone of the thick filaments that is not superimposed by the thin filaments). Flightin binds to the myosin rod in the molar ratio of between 1:1 and 1:2 vv. The presence of flightin is essential for flight, for maintenance of sarcomeric integrity

1. **Struktura i funkcja miofibrila**  
 2. **Skład i organizacja miofibrila**

3. **Proces skurczu mięśnia**  
 4. **Regulacja skurczu**  
 5. **Wzrost i regeneracja**  
 6. **ChOROBY**

- ?
- ?
- ?
- ?



?

7. **ChOROBY**  
 8. **ChOROBY**

### 1.2.3. Myofibril assembly

In regular organisation of filaments, not only compounds they are built of are important. Also maintaining a precise organisation and the size of filaments are critical for the muscle function. It is not completely understood how filaments assemble and attain their stereotyped lengths (Bai et al., 2007). Like the sarcomere structure, the mechanism of precise myofibril assembly seems to be also conserved across animal species.

Four models of myofibril assembly have been proposed to date. The first one suggests that assembly pathways for sarcomeric thin and thick filaments arrays are remarkably independent and in the absence of one filament type, the other can still form crude networks, resembling the sarcomeric arrangement (Beall et al., 1989). Further work (Holtzer et al., 1997) confirmed that I-Z-I complexes and bipolar myosin filaments assembled independently before joining. Free-floating A-bands were detected in the absence of actin, and irregular Z-lines were observed with thin filaments attached in muscles lacking myosin. According to the second model structures called premyofibrils are formed prior to the development of mature myofibrils. Premyofibrils are characterised by interdigitating, banded patterns of I-Z-I complexes and non-muscle myosin. Subsequently, non-muscle myosin filaments are replaced by muscle myosin filaments during the premyofibril to myofibril transition, and discrete aggregates of Z-bodies grow along the premyofibrils, fusing laterally into Z-lines (LoRusso et al., 1997). The third model indicates the role of the giant protein titin during myofibrillogenesis. According to this model, the interdigitation of the I-Z-I complexes with myosin is driven by interaction of the N-terminal domain of titin with the I-Z-I complex and subsequent association of the unfolded C-terminal region with the M-line (Ehler et al., 1999). The fourth model emphasizes the role of integrin adhesion complex as the starting point for myofibrillogenesis, showing that integrins and their extracellular matrix ligands are required for sarcomere assembly and Z-line formation (Sparrow and Schöck, 2009).

More recent studies (Rui et al., 2010) verified those hypotheses and showed that sarcomere assembly was a highly coordinated process, mediated by multiple



latent protein complexes and did not occur in a step-wise fashion. It was confirmed that I-Z-I proteins and myosin filaments were two independent complexes, which interacted with each other, and were responsible for the arrangement of the myofibrils. To clarify how these two complexes assembled, studies on mutant *Mhc<sup>1</sup>* lacking thick filaments were performed (Rui et al., 2010). They showed that homozygous *Mhc<sup>1</sup>* mutant muscles no longer localised in Z-lines (Fig. 1.2.3-1. A, B), indicating that I-Z-I proteins could not align into striation in the absence of MHC. Moreover, immunostaining against zipper, which corresponds to the only non-muscle myosin, showed that zipper is strongly localised to the Z-line but not to the thick filaments and removal of MHC did not result in any obvious zipper periodic pattern (Fig. 1.2.3-1. B). This suggests that in *Drosophila* intermediate non-muscle myosin-containing premyofibrils do not exist during the myofibril assembly. Elimination of MHC also led to a complete disruption of the distribution of other sarcomeric components, like  $\beta$ -integrin (Fig. 1.2.3-1. A), titin and Zasp, whereas there was no effect on the distribution of integrin,  $\alpha$ -actinin, zipper, kettin and Zasp at the attachment sites, suggesting that formation of muscle attachments was independent of MHC. Knocking down *Mhc* by RNAi in later stages, such as the third instar larvae, caused a loss of muscle sarcomere striation correlated with a reduction of MHC expression level, indicating that MHC played a critical role not only in the sarcomere formation but also in its maintenance. The thin filament-associated proteins were also shown to participate in the sarcomere formation. Depletion of *TnT* or *TnI* eliminated the striation pattern, whereas knockdown of *TnC* did not lead to any significant change in MHC/actin/ $\alpha$ -actinin striation. Tropomyosin (Tm) is another critical component participating in the sarcomere assembly, but removal of *TnT*, *TnI* or *Tm* leads to a random distribution of all the remaining proteins, indicating the lack of loading sequence for troponin-tropomyosin complex during the sarcomere assembly. The complex plays an important role in the initial sarcomere assembly by allowing stable interdigitation of thin and thick filaments and is recruited to the sarcomere as a complex (Rui et al., 2010).

I-Z-I complexes are the first identifiable structures during early myofibrillogenesis (Holtzer et al., 1997). To test if MHC assembled into thick filaments and interdigitated with thin filaments to form sarcomeres, each

component of the I-Z-I complex was knocked down (Rui et al., 2010). Depletion of muscle-specific *actins*, *act57B* and *act87E* with incompletely depleted cytoplasmic actins, *act5* and *act42A* caused formation of shorter and thinner striated myofibrils. This suggests that cytoplasmic actins, which are already expressed in myoblasts, may take part in formation of these shortened thin filaments. It allows speculating that the initial sarcomere assembly in muscles utilises cytoplasmic actins as building blocks (Rui et al., 2010). Attaining the stereotyped length of these filaments is not completely understood but it is known that certain capping proteins are required for the sarcomere to reach its proper size. Two ends of sarcomeric actin filaments are lined up in a regular array. The barbed ends (plus), which are cross-linked by  $\alpha$ -actinin at the Z-line, are capped by CapZ protein. The free pointed ends (minus) are capped by other capping proteins, tropomodulin (Tmod) and sarcomere length short (SALS). The length of sarcomeric actin filaments in striated muscles is primarily regulated by modulation of the pointed-end capping activity of Tmod, which limits actin assembly, suggesting that actin filaments can elongate from pointed ends (Fischer and Fowler, 2003; Mardahl-Dumesnil and Fowler, 2001). SALS protein is required for the second phase of actin filament elongation. Most likely, thanks to antagonising Tmod at the pointed ends, SALS takes part in achieving the full length of filaments. SALS is essential for attaining the final thin-filament length but is not critical for the normal organisation of other sarcomeric components (Bai et al., 2007).

Investigating the role of Z-line components showed no significant changes in the striation pattern under the  $\alpha$ -actinin knocked-down, indicating that  $\alpha$ -actinin was not required for the thick filament organisation (Fyrberg et al., 1998). On the other hand, it was shown that  $\alpha$ -actinin mutant muscles contained expanded Z-lines, indicating that  $\alpha$ -actinin was required for the Z-line condensation. Attenuation of *zipper* and *Zasp* expression led to fuzzy but discernable striation, indicating that individual knockdown of these sarcomeric components did not affect the initial formation of sarcomeres. However, in later development stages of *Zasp* mutants, striation began to disappear, suggesting that *Zasp* was required for the sarcomere maintenance. In contrast, *kettin* mutant muscles did not show any striation pattern for any markers (Hakeda et al., 2000; Zhang et al., 2000) and it was proposed that titin/*kettin* acted as a third filament to regulate the sarcomere

plasticity. Subsequently, it was tested if simultaneous removal of *actin* and one of the Z-line components, such as  $\alpha$ -actinin, zipper or Zasp were associated with complete loss of striation. Altogether, these results suggest that myosin thick filaments and I-Z-I complexes assemble independently from each other, but they require each other to be properly ordered (Rui et al., 2010).

It has been also shown that zipper/Zasp/ $\alpha$ -actinin complexes are highly enriched at the muscle attachment sites. They act downstream of integrin, linking the muscle cytoskeleton to the cell membrane. Disruption of integrins is associated with a complete loss of myofibril striation (Volk et al., 1990) but it has been suggested that integrins are not involved in the I-Z-I complexes assembly, as association of actin and  $\alpha$ -actinin occurs even in the absence of integrin. However, the absence of integrins blocks the interdigitation of thin and thick filaments with each other and sarcomeres cannot be formed. It has been proposed that integrins play a very important role in maintaining the tension at the cell periphery for sarcomere structural organisation and serve as anchor points for floating I-Z-I complexes (Rui et al., 2010).



### **1.3. Role of intermediate filaments in muscle cell**

The cytoskeleton in striated muscle cells is composed of a highly complex scaffold of microfilaments (MFs), microtubules (MTs) and intermediate filaments (IFs). Thanks to this cytoskeletal network, striated muscles are able to maintain their sophisticated sarcomeric organisation, as well as integration between contractile apparatus, nucleus and other organelles. MT and MF systems are the two principal elements of eukaryotic cells, being engaged in many basic cellular functions (Herrmann et al., 2007). Despite the fact that mutations in their proteins, like tubulin and actin, are more harmful for a cell than those in proteins of IFs, this study is particularly focused on the intermediate filament part of the cytoskeleton, as they interact with  $\alpha$ B-crystallin (see the following chapter).

#### **1.3.1. Characteristics and classification of intermediate filaments**

Intermediate filaments are the key components, maintaining high organisation inside of the muscle cell, by interacting with various cellular structures (Capetanaki et al., 2007).

Intermediate filaments owe their name to their average diameter of approximately 10 nm, which places them between thin, 7 nm actin filaments and thick of over 20 nm microtubules. IFs are found in many cell types, including cells forming epithelial tissues, such as epidermis, those present in nerve tissue, those comprised in skeletal, smooth and cardiac muscles, as well as cultured cells, such as fibroblasts (Cooke, 1976; Eriksson et al., 2009; Goldman, 1971; Ishikawa et al., 1968; Schmitt, 1968). Thanks to the possibility of IFs isolation and purification, *in vitro* studies provided an opportunity to analyse their unique properties. It was established that IFs were insoluble under physiological conditions and could re-polymerize after disassembly into subunits under conditions that would irreversibly denature many other proteins. Moreover, IFs assembly does not need nucleoside triphosphates such as ATP and GTP and thanks to that they self-assemble under very simple conditions, which is in contrast to actin and tubulin filaments (Zackroff and Goldman, 1980).

All the intermediate filaments share a common tripartite structure which consists of a highly conserved central  $\alpha$ -helical rod domain, a variable N-terminal head domain and a C-terminal tail domain. In contrast to the conserved rod domains, the terminal C- and N- domains are the main source of enormous sequence variability among IF proteins, being responsible for the cell-specific functions of particular proteins of the family (Goldman et al., 1999). Therefore, the IFs classification is based on the similarities in their  $\alpha$ -helical rod domain sequences, but also on the assembly properties (Eriksson et al., 2009). This classification led to distinguish: type I cytoskeletal IF proteins, including the acidic keratin proteins; type II cytoskeletal proteins including the neutral-basic keratin proteins; type III IF proteins, including GFAP, peripherin, vimentin and desmin; type IV IF proteins, including nestin, synemin and the neurofilament triplet proteins; type V IF proteins, including nuclear lamins and type VI IF proteins, including filensin and phakinin (Eriksson et al., 2009). Intermediate filaments of types I-IV are present in the cytoplasm, type V IF proteins are present in the nucleus and type VI proteins are present exclusively in eye lenses (Omary, 2009).

### **1.3.2. Role of IFs in muscle cells**

Thanks to slight differences between various intermediate filaments, different cell types possess different types of cytoskeletal IF proteins, characterising various tissues. Epithelial cells contain mainly keratins; mesenchymal cells: vimentin; neurons: neurofilaments and muscle cells contain desmin (Franke et al., 1978). Some of the cell types express more than one IF protein and their expression is frequently regulated developmentally (Gard et al., 1979; Granger and Lazarides, 1979).

In mature muscles two IF systems exist with different functions and biochemical properties. The first one consists of nuclear lamins and the second one of type III IF proteins. Lamins form a meshwork on the inner surface of the nuclear envelope (Fig. 3.2-1.), creating the nuclear lamina (Stuurman et al., 1998). This filamentous layer provides a structural support to the nuclear membrane and participates in a chromatin organisation inside the nucleus. Three genes encoding lamins: *lmnA*, *lmnB1* and *lmnB2*, are present in the human genome, whereas the

*Drosophila* genome includes only two: *lamin C* and *lamin Dm<sub>0</sub>* (Schulze et al., 2009; Stuurman et al., 1998). Various types of lamins are distinguished in each of the two species: A/C (lamins A and C are alternatively spliced products from *lmnA*) and B in humans and their *Drosophila* equivalents, *lamC* and *Dm<sub>0</sub>*. The classification is based on their structure and expression pattern (Herrmann et al., 2007; Schulze et al., 2009). Moreover, comparison of the gene structures of lamin genes and genes encoding cytoplasmic IFs suggests that all the IF proteins derive from lamin-like progenitors (Steinert and Roop, 1988).

The second group of muscle IFs, aside from keratins (Stone et al., 2007), predominantly consists of cytoplasmic type III IF proteins, enclosing mesodermal vimentin and a muscle-specific IF protein, desmin (Capetanaki et al., 1989; Milner et al., 1996). Other intermediate filaments which are present in muscle cells, like synemin, paranemin, syncoilin and nestin, are not muscle-specific and even if they co-polymerize with type III IFs, their role remains unclear (Granger and Lazarides, 1980; Kachinsky et al., 1994; Newey et al., 2001; Price and Lazarides, 1983; Schweitzer et al., 2001). Generation of specific antibodies and the widespread use of immunofluorescence have enabled to show, that IFs form an extensive and complex network, pervading most of the cytoplasm (Franke et al., 1978; Starger et al., 1978). Even early studies showed that the most remarkable attribute of IFs was their resistance to relatively high concentrations of salt and detergents, suggesting that their role is primarily to maintain mechanical and structural integrity of the cell (Franke et al., 1978). This view got changed with the observations that mutations in genes encoding IF proteins lead to many diseases, not necessarily related to IFs structural functions. Particular attention was then paid to other properties of the IFs, pointing out their involvement in a broader spectrum of functions, such as organisation of cytoplasmic architecture, signalling, and transcription regulation; IFs are highly dynamic structures (Hyder et al., 2008; Ivaska et al., 2007; Prahlad et al., 1998). In muscle cells, desmin filaments are the most abundant intermediate filaments, playing an essential role in maintaining muscle fibre organisation. Desmin filaments (Fig. 1.3.2-1.) are attached to the sarcolemma at costameres where they stabilise the sarcolemma organisation and overlie Z-discs and M-lines, supporting myofibrils and protecting them from mechanical insult (O'Neill et al., 2002). In addition, cytoplasmic filaments are,

directly or indirectly, linked to membranous organelles, like nuclei and mitochondria. For both organelles, this association is important for keeping their proper morphology, positioning or even function (Anesti and Scorrano, 2006; Mermelstein et al., 2006; Shah et al., 2004). Moreover, cytoplasmic IFs are also believed to play a role in coordinating mechanical forces in embryonic development, growth and tissue maturation (Chen et al., 2004).





### 1.3.3. IF-associated diseases

Intermediate filament proteins, together with other cytoskeleton components, form a very effective intracellular network that supports physiological functions promoting cell survival; any anomaly of these filaments either causes or predispose to a human disease (Table 1.3.3-1).

The IF-associated diseases can affect a wide range of tissues and cover the whole spectrum of genetic alterations, including missense and nonsense mutations, deletions and gene duplications (Omary, 2009). In many IF-associated diseases the histopathological and diagnostic hallmarks are characteristic cytoplasmic inclusions. It is still unclear if the formation of such inclusions protects the tissue or perpetuates the disease but their formation is a consequence of a mutation or overexpression of a particular IF (Liem and Messing, 2009; Omary et al., 2009). One such disease, in which the phenotype of inclusions is observed, is a desmin-related myopathy. As this disease is relevant to this study and is caused by mutation either in the desmin gene (*DES*) or in the  $\alpha$ B-crystallin gene (*CryAB*), which is a member the heat shock proteins family (Goldfarb and Dalakas, 2009; Vicart et al., 1998), it will be described in the following chapter.

<b>Diseases associated with mutations in IF proteins</b>	
<b>IF</b>	<b>Examples of diseases</b>
<b>Types I and II</b> Acidic and neutral-basic keratins	Epidermolysis bullosa simplex; epidermolytic hyperkeratosis; Meesman corneal dystrophy; keratodermas
<b>Type III</b> Desmin  GFAP Peripherin Vimentin	Desmin-related myopathy; dilated cardiomyopathy 1A; dilated cardiomyopathy with conduction system deficits; distal myopathy  Alexander disease Amyotrophic lateral sclerosis 1 Dominant cataract
<b>Type IV</b> Neurofilaments	Various Charcot-Marie-Tooth diseases; Parkinson disease; amyotrophic lateral sclerosis 1; neuronal IF inclusion disease
<b>Type V</b> A-type lamins  B-type lamins	Hutchinson-Gilford progeria syndrome; dilated cardiomyopathy 1; restrictive dermopathy; Charcot-Marie-Tooth disorder type 2B1  Acquired partial lipodystrophy; adult-onset leukodystrophy
<b>Type VI</b> Filensin (Bfsp1) Phakinin (Bsfp2)	Autosomal-dominant cataract  Autosomal-dominant cataract

**Table 1.3.3-1. Diseases associated with mutations in IF proteins.** Modified from Eriksson et al. (2009).

## 1.4. Heat shock proteins

Heat shock proteins (Hsps) are a group of proteins first discovered in *Drosophila melanogaster* after heat and chemical induction (Tissières et al., 1974). Subsequently, their response to heat and other stressors (cold, hypoxia, ischemia, endotoxins, heavy metals, organic solvents and reactive oxygen species) was reported in a wide variety of organisms, including bacteria, plants and animals, making it a highly conserved genetic system. Interestingly, expression of Hsps is not exclusive for the response to unfavourable conditions, and some of them are induced developmentally, whereas others are constitutively synthesised in cells, controlling protein transport, folding, unfolding, assembly, disassembly and degradation (Colinet et al., 2010; Lindquist and Craig, 1988; Mymrikov et al., 2010; Sorensen et al., 2003).

### 1.4.1. Classification of heat shock proteins

In *Drosophila*, as well as in humans, five main groups of Hsps have been distinguished. Hsps are classified into families according to their sequence homology and the approximate protein molecular mass. Namely, the Hsp90/100 family includes high-molecular-mass Hsps and in *Drosophila* is represented by only one gene *hsp83* (Cutforth and Rubin, 1994). The complex Hsp/Hsc70 (*hsp68*, *hsp70*, *hsc-1*, *hsc-2*, *hsc-3*, *hsc-4*, *hsc-5*) consists of proteins with molecular mass between 68 and 73 kDa. Members of the family Hsp60 (*hsp60*, *hsp60B*, *hsp64*) are characterised by the molecular mass around 60 kDa, whereas size of 37 kDa is characteristic for members of the family Hsp40 (*dHDI1*) (Kazemi-Esfarjani, 2000; Morrow and Tanguay, 2003; Timakov and Zhang, 2001). The last group of Hsps contains twelve (*hsp22*, *hsp23*, *hsp26*, *hsp27*, *hsp67Ba*, *hsp67Bb*, *hsp67Bc*, *l(2)efl*, *CG14207*, *CG4461*, *CG7409*, *CG13133*) putative small heat shock proteins (sHsps) with molecular mass between 12 and 43 kDa (Carra et al., 2010; Haass et al., 1990; Ingolia and A., 1982; Morrow and Tanguay, 2003; Sun and MacRae, 2005a). Functions of the members belonging to the first four families have been extensively studied, whereas still little is known about the function of the others (Table 1.4.1-1.), thus the objective of this study is functional analysis of *l(2)efl*, a member of the small heat shock protein family.

Family	Genes in <i>Drosophila</i>	Functions
<b>sHsp</b>	<i>hsp22, hsp23, hsp26, hsp27, hsp67Ba, hsp67Bb, hsp67Bc, l(2)efl, CG14207, CG4461, CG7409, CG13133</i>	Thermotolerance Protection against apoptosis
<b>Hsp60</b>	<i>hsp60, hsp60B, hsp64</i>	Involvement in spermatid individualization process Folding of nascent proteins
<b>Hsp70</b>	<i>hsp68, hsp70, hsc-1, hsc-2, hsc-3, hsc-4, hsc-5</i>	Folding of nascent proteins Thermotolerance Involvement in clathrin-mediated endocytosis
<b>Hsp90/100</b>	<i>hsp83</i>	Ensuring proper centrosome function Maintaining of signal transduction pathways and microtubule effectors Capacitor of morphological evolution

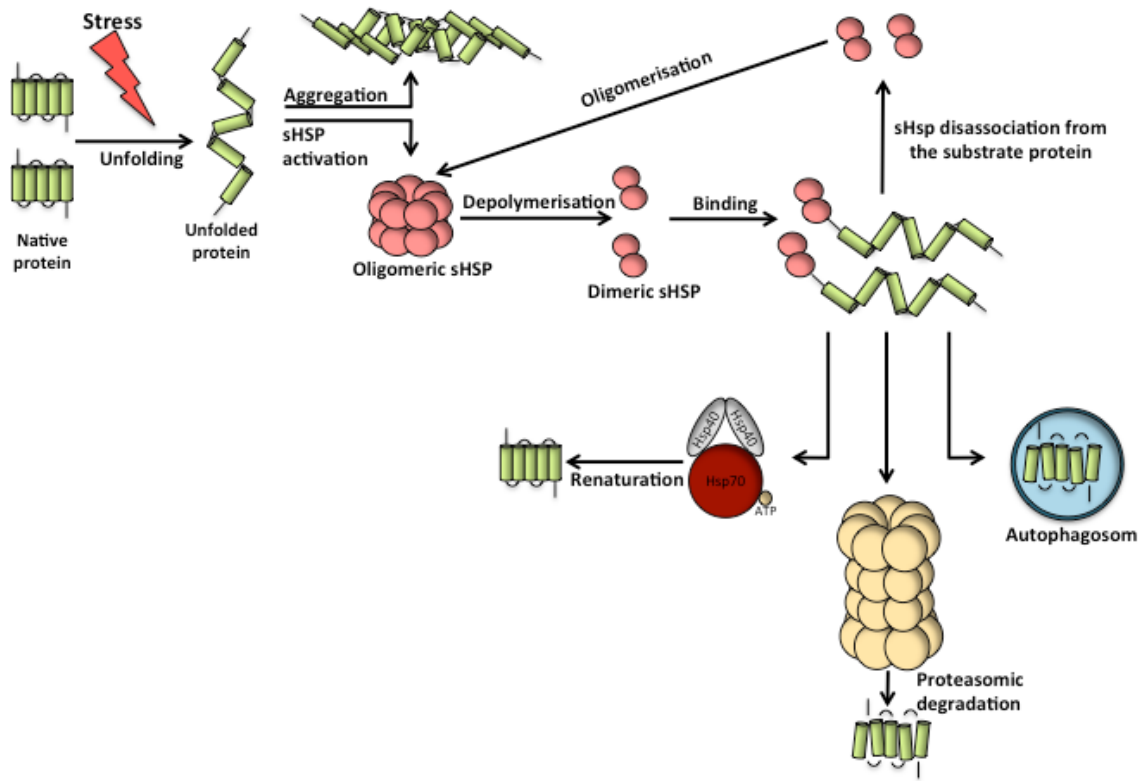
**Table 1.4.1-1. Functions of *Drosophila melanogaster* Hsps.** Modified from Morrow and Tanguay (2003).

### 1.4.2. Structure and function of sHsps

Within the entire group of the sHsps, protein monomers have a similar pattern, with a structure composed of a  $\alpha$ -crystallin rod domain of  $\sim 90$  residues, flanked by a hydrophobic N-terminal region and a C-terminal extension. Intriguingly, sHsps show low overall sequence conservation, except for the  $\alpha$ -crystallin domain, which owes its name to the homology to  $\alpha$ -crystallin from the vertebrate eye lens. In this very conserved region, also called small-heat-shock-protein domain, the observed homology can exceed up to 40%, whereas N- and C-termini vary extensively, both in sequence and in length, with average of 55 and typically  $<20$  residues, respectively (Ingolia and A., 1982; Kim et al., 1998; Lindquist and Craig, 1988). The secondary structure of the  $\alpha$ -crystallin domain is dominated by  $\beta$ -strands with limited  $\alpha$ -helical content.  $\beta$ -strands, organised into a  $\beta$ -sheet sandwich, are responsible for dimers formation, the basic building blocks of most sHsps, which subsequently are able to assemble into large, dynamic complexes with the molecular mass of up to 1 MDa (Sun and MacRae, 2005a; Sun and MacRae, 2005b). The N-terminal extension influences higher-order oligomerisation, subunit dynamics and chaperoning, which is one of the main sHsps activity based on protection of newly made proteins from misfolding. Here, the N-terminal extension is responsible for subunit binding by attaching to one end of the  $\alpha$ -crystallin domain, whereas the C-terminus binds to the other end stabilizing generated oligomers but also promoting solubility and chaperoning (Kim et al., 1998; Sun and MacRae, 2005b). Moreover, different members of the sHsps group are known to form homo- or hetero-complexes, whereas the knowledge about its structural organisation is still insufficient (Arrigo et al., 2007).

sHsps play a crucial role in maintaining protein homeostasis by interacting with partly unfolded, aggregate-prone proteins, by keeping them soluble and thus preventing cell damage. They are able to support folding of nascent proteins, as well as thermally and chemically denatured proteins (Fig. 1.4.2-1.) ranging from at least 4 to 100 kDa. It is thought that the mechanism of substrate binding is based on a change in the hydrophobicity profiles of several sHsps under stress conditions (Haslbeck, 2002). The binding capacity is dependent on its oligomeric state. Under physiological conditions sHsps exist in the cytoplasm in an inactive oligomeric

czt i 21 22 tió H I 2i 22c2j 2l 22bi 2c2j 222t 2Dj t W2222bit z2j c2l 2s 22rcct 2r2z22j zt 2  
 có 2W2i 22ró 2i r222t ó oVd2c2H I r2i 22i 2222V22t 22j 22j tj Q 22r 22bit z2j c222i 26SXS 0  
 6SS22D2c2wD2j zW2211i 212zt j 2bit j 22j z2i ó 22r2z222t ó oVd2c22i 222ni 22z2222t 2l 22  
 2Dz ol 21t ct ó 22i 2bit z22ct ó 2H I 2i 22l 2s 22i 22221i 22222S2I 22i z2i 2i 2t ccr22W2s 2r2  
 zt 2bit r222j tj Q 22r 22bit z2j c2t 22co) H2I I r2i 2j 2l 22bi 2c2j 222t 22222r222V22t 2  
 i 2t W22l 26 2j zt 2z2l 2ni 2j 22r 22cz2z2S22 2r2j 122ni 22z2222z2l 22cD22czi 2z222oit z2j 2z2  
 2221i 2222zt j 2i 22czt i 2zt j 2z2l l 2s 22i 22t 22co 22ró 2i 2rc22V2c2222it ó 2l 222t ó oVd2  
 2j 22i 202cct 2r2z2c2z2t 2z2l 22j 22r 22czt i 2122 tió 2Dj 22i 2ol scit W1r22V22t j 2r2t j c2  
 22 2cV222a 22- HH- P22I 22222c2i r222222i 2o2i tj 22 Dj 2zt j 2t 2c2coc22Di j 122222 LTL2  
 csj z2l 2rc2t 2ot Wo2oz22c22j 22Dj 22i 2czi 2cc22t j 2r2t j c2rc2i 2z2l 2i 212j 2i 2W2j 22  
 Dj co22hr2S2222r2t j 2W222c2oc22i 22j rt W2222j 22r 2i c22co22hr2222W22i 2bit 22cc2c22  
 cl tl 2j 12 cz21202 2j 22 22W2co22hr222 o2z22j c2t 2 2doi 2cc2t j S2 2I r22 22W2co22hr22  
 2doi 2cc2t j 2t 2-D-A-2t 22Di c22Di j 12222r2W2ó 2j z2222W22n 2i 2j z2zt j 2ti 22 z2i 2  
 22r 2zt j 22s 2litl z2l 222t ic22t j 2t 12j 2c22j 22bit zt 0j 2t 12j 2c22j 22r22i 2c2222j 2  
 i 2ot iz2222j 2222 r2222 2j 122t 2ti 12j r2ó c22j 2W22j 122BL-LADR22H 222I L22-02222j 22  
 ó 26 ó 2W222 r2i 2D222j 2222j 1D2s 22 HH8M22 timó tzt 26 ' ' qP22



2 222k2W222 2222t yc 2 22y2oy222s 22s i t 22i 22c2i r2zt j 22222222t 2nr222  
 itó 22 2cV222a 2- HH- P22j 222 só imatr 222222- H6HPS2

### 1.4.3. Involvement of sHsps in muscle development

Early studies showed that in *Drosophila* *shsp* genes were expressed in the absence of stress during embryogenesis and metamorphosis in a tissue-specific manner (Michaud et al., 1997; Michaud and Tanguay, 2003). In other species, like zebrafish, mouse or human, their specific pattern of expression was also established, showing their widespread importance in tissue development, that includes myogenesis (Benjamin et al., 1997; Marvin et al., 2008; Sugiyama, 2000).

In humans seven *sHsps* are expressed in cardiac and skeletal muscles. *Hspb1*,  $\alpha$ B-crystallin, *Hspb6* and *Hspb8* are expressed ubiquitously, whereas *Hspb2*, *Hspb3* and *Hspb7* are tissue-specific. All those sHsps prevent the accumulation of partially denatured or improperly folded proteins in muscle cells, having chaperone-like activity during recovery or/and under normal conditions. Interestingly, only three of them,  $\alpha$ B-crystallin, *Hspb2* and *Hspb3* are induced during the initial phase of skeletal muscle differentiation and their expression is controlled by MyoD, which is a key player in muscle differentiation, committing mesoderm cells to the skeletal lineage (Berkes and Tapscott, 2005; Mymrikov et al., 2010; Sugiyama, 2000). This fact suggests their muscle-specific function in development, which is precisely regulated by myogenic programme. The function of *Hspb1* and  $\alpha$ B-crystallin in myogenesis may be due to their cytoprotective role, which relies on myotube-specific association with actin microfilaments. It was suggested that these two sHsps were possibly involved in the initial organisation of myofibril assembly in myotubes. Moreover, *Hspb1*, *Hspb2* and  $\alpha$ B-crystallin are localised on Z-bands or I-bands of the skeletal and cardiac muscle myofibrils, maintaining and protecting cytoskeletal structures (Mymrikov et al., 2010; Sugiyama, 2000). Additionally,  $\alpha$ B-crystallin stabilises IFs and prevents them from aggregating under various forms of stress but also assists them during developmental reorganisations, suggesting its engagement in muscle formation and further maintenance of its proper structure (Nicholl and Quinlan, 1994)

In the *Drosophila* genome, has been identified a gene called *lethal (2) essential for life (l(2)efl)*, which belongs to the *shsp* family and is homologous to the vertebrate  $\alpha$ B-crystallin (Kurzik-Dumke and Lohmann, 1995). Since the function of L(2)efl protein has not been well described yet, this study is focused on analysing



its role in muscle development, sarcomere stabilisation and eventual involvement in the origin of muscle diseases.

#### **1.4.4. DRM as a consequence of $\alpha$ B-crystallin mutation**

sHsps interact with many essential cell structures and functional disruption or inappropriate association of these molecular chaperones with their substrates can foster disease. One such disease is desmin-related myopathy, a heterogeneous disorder associated with mutations of desmin or  $\alpha$ B-crystallin.

The desmin-related myopathy (DRM) was originally described as a skeletal and cardiac myopathy, morphologically characterised by an abnormal accumulation of desmin within muscle fibres (Goebel, 1995). Early studies showed that mutation in the highly conserved carboxy-terminal end of the desmin rod domain was involved in the onset of skeletal myopathy within two American families (Goldfarb et al., 1998). However, in other studies, mutation in  $\alpha$ B-crystallin was identified as a source of DRM phenotypes in a large French pedigree (Vicart et al., 1998). Myopathic manifestations of diseases caused by either desmin or  $\alpha$ B-crystallin mutations are identical; therefore in both cases the disorder is designated as the desmin myopathy. Consequently, systemic disorders caused by mutations in desmin,  $\alpha$ B-crystallin or perhaps other proteins interacting with desmin and causing myopathy, are named desmin myopathy, whereas the accurate terms desminopathy and  $\alpha$ B-crystallinopathy are used for patients with mutations in desmin and  $\alpha$ B-crystallin, respectively. The inheritance pattern in the familial desmin myopathy is autosomal dominant or autosomal recessive, but many cases have no family history with *de novo* created desmin mutations. The onset of the disease depends on the inheritance and the location of the mutation, but is mostly late, which is related to the accumulation of toxic desmin aggregates inside of the cells during the life time (Goldfarb et al., 2004).

The DRM is characterised by a lower limb muscle weakness slowly spreading and involving trunk, neck flexors, facial, bulbar and respiratory muscles. Symptoms observed in skeletal muscles are often associated with cardiomyopathy, being manifested by conduction blocks and arrhythmias but also with cataract formation. In DRM patients with the missense mutation in *cryAB*, where arginine in

codon 120 in the amino acid sequence is replaced by glycine (R120G), muscle fibres contain intracellular aggregates composed of desmin and  $\alpha$ B-crystallin (Dalakas et al., 2000; Goldfarb and Dalakas, 2009; Goldfarb et al., 2004). The arginine in position 120 is highly conserved and mutation leads to changes in the secondary, tertiary and quaternary structure of the protein.  $\alpha$ B-crystallin<sup>R120G</sup> loses its chaperone activity *in vitro* and directly induces aggregations of type III intermediate filament proteins, like desmin in muscle fibres and vimentin in eye lenses. Deposition of those aggregates is a result of an increased binding capacity of the  $\alpha$ B-crystallin<sup>R120G</sup> and a decreased complex dissociation constant. The altered protein-protein interactions impair significantly the desmin and vimentin network, myofibril alignment, sarcomere architecture, mitochondrial architecture and function, and the ubiquitin/proteasome system. Moreover,  $\alpha$ B-crystallin<sup>R120G</sup> may also affect the cell viability because one of the functions of  $\alpha$ B-crystallin is negative regulation of apoptosis (Bova et al., 1999; Simon et al., 2007; Wettstein et al., 2012).

Thus, the *Drosophila l(2)efl*, being homologous to  $\alpha$ B-crystallin, may have similar functions and can be involved in muscle development and stabilisation of the structural integrity of somatic muscles.

## 2. Aims of the thesis

Up to date, the role of the *Drosophila* gene *lethal (2) essential of life (l(2)efl, dcryAB, CG4533)* has not been well explored. However, it is known that *dcryAB* belongs to the small heat shock proteins family, is orthologous to the human  $\alpha$ B-crystallin gene and may play an important role in the *Drosophila* development. It is well established that some sHsps influence muscle development in humans as well as in *Drosophila* and therefore, the main aim of my thesis was to investigate the role of  $\alpha$ B-crystallin's orthologue in *Drosophila melanogaster* muscle development and sarcomere stabilisation. I chose the third instar larva as the object of my studies, because at this stage of development *Drosophila* muscles are fully developed, functional and relatively easy to observe, making them a perfect material for examinations.

To understand the function of the *dcryAB* gene, first I wanted to describe its expression pattern in larval muscles. Knowing that dCryAB protein was abundantly expressed in all the larval body wall muscles in a very stereotypical pattern, reminiscent of the localisation of its human orthologue  $\alpha$ B-crystallin, I attempted to check if attenuation of its expression would lead to any developmental changes. Morphological alterations and impaired function of larval muscles with decreased dCryAB level in muscle cells raised the questions: 1. could dCryAB, like its human orthologue, stabilise muscle cell structure by interacting with intermediate filaments and, 2. could its point mutation R120G lead to a disease corresponding to the desmin-related myopathy? Since it was suggested that *Drosophila* might possess a counterpart of the protein belonging to the third class of intermediate filaments, I started answering these questions by labelling its larval muscles with anti-vimentin antibody. In this way I visualised a protein which interacted with dCryAB and might be a putative IF in *Drosophila* muscles. Therefore, the next questions were: 1. does dCryAB ensure structural integrity of somatic muscles by interacting with IF proteins, and 2. is dCryAB involved in the pathogenesis of muscle disease corresponding to the human desmin-related myopathy?

To find answers to these questions, I used many different approaches and I hope that my thesis will contribute to our understanding of the role of *dcryAB* in muscle development and sarcomere stabilisation.

### 3. Material and methods

#### 3.1. Material

##### 3.1.1. *Drosophila* stocks

Fly strains were maintained at 25°C in the standard medium in the 12:12h light and dark cycling humidified incubator.

<b>Fly strain</b>	<b>Source/laboratory</b>	<b>Catalogue number</b>
<i>Mef-Gal4</i>	Bloomington Stock Center	27390
<i>UAS-RNAi-dcryAB</i>	Vienna Drosophila RNAi Center	40532
<i>UAS-RNAi-dcryAB</i>	Vienna Drosophila RNAi Center	107305
<i>UAS-dcryAB</i>	H. Jasper (a kind gift)	
<i>UAS-dcryAB-GFP</i>	K. Jagla (created for this study)	
<i>UAS-dcryAB-GFP<sup>R120G</sup></i>	K. Jagla (created for this study)	
<i>UAS-cryAB-GFP</i>	K. Jagla (created for this study)	

## 3.2. Methods

### 3.2.1. RNA extraction and reverse transcription

To obtain *Drosophila* cDNA, total RNA was extracted from fifty 3rd instar larvae of selected lines, using a TRIzol® Reagent (Invitrogen). The remaining DNA was removed by RQ1 DNase (Promega). To obtain human cDNA, total RNA was extracted from human fibroblasts (a kind gift from C. Barriere). 2 µl of each RNA were used for reverse transcription, performed with the Superscript III First strand Synthesis System (Invitrogen) according to manufacturer's instructions.

### 3.2.2. Plasmid construction and site-directed mutagenesis

For plasmid construction two coding sequences were amplified: *dcryAB* using cDNA from WT 3rd instar larvae and *cryAB* using human cDNA via PCR. The following pairs of primers, containing Xba1 and BamH1 (Biolabs) restriction sites (underlined), were used:

- a) for *dcryAB*: forward, 5'-ATATCTAGATCCG TAGTGCCACTGATGTTC-3' with reverse, 5'-TATGATCCCTAGGCGGTGGAGGTCTCC-3'
- b) for *cryAB*: forward, 5'-ATAGGATCCGACATCGCCATCCACCACC-3' with reverse, 5'-ATATCTAGAGAAAGGCATCTATTTCTTGGG-3'.

To generate the point mutation R120G in amplified sequences of *dcryAB* and *cryAB*, the following pairs of primers were used (exchanged nucleotides are underlined and in bold):

for *dcryAB* mutagenesis:

- c) forward, 5'-ATATCTAGATCCG TAGTGCCACTGATGTTC-3' with reverse, 5'-AGCTGGTAGCGTCCGGGAGAACTGGCGGG-3',
- d) forward, 5'-CCC GCCAGTTCTCCGGACGCTACCAGCT-3' with reverse, 5'-TATGGATCCCTAGGCGGTGGAGGTCTCC-3';

for *cryAB* mutagenesis:

- e) forward, 5'-ATAGGATCCGACATCGCCATCCACCACC-3' with reverse, 5'-GTATTTCCGTGGA ACTCCCT-3',

f) forward, 5'-AGGGAGTTCCACCGGGAAATAC-3' with reverse, 5'-ATATCTAGAGAAAGGGCATCTATTTCTTGGG-3'.

PCR products amplified in reactions above were mixed in the following way: 2 µl of the product c) with 2 µl of the product d) and 16 µl of water for *dcryAB*; and 2 µl of the product e) with 2 µl of the product f) and 16 µl of water. Hybridization was performed in water bath by cooling down the mixtures from 99°C to 60°C. After 25 min of incubation at 60°C the mixtures were cooled down to 37°C and subsequently 2 µl of each were amplified via PCR, by using following pairs of primers, containing Xba1 and BamH1 restriction sites (underlined):

g) for *dcryAB*: forward, 5'-ATATCTAGATCCGTAGTGCCACTGATGTTC-3' with reverse, 5'-TATGGATCCCTAGGCGGTGGAGGTCTCC-3',

h) for *cryAB*: forward, 5'-ATAGGATCCGACATCGCCATCCACCACC-3' with reverse, 5'-ATATCTAGAGAAAGGGCATCTATTTCTTGGG-3'.

All DNA amplifications were obtained with a high-fidelity DNA polymerase (Phusion, Biolabs) according to manufacturer's instructions. Obtained sequences were cloned into a BamH1 and Xba1 sites of pUASp-PL-Venus vector (a kind gift from Vincent Mirouse) and injected into a  $w^{118}$  embryos to produce transgenic flies, a step performed by Fly Facility platform (Clermont-Ferrand, France).

### 3.2.3. Semi-quantitative PCR

To compare the level of *dcryAB* expression in selected fly strains, 2 µl of reverse transcription products (described above) were amplified via PCR, by using following pairs of primers: forward, 5'-TCCGTAGTGCCACTGATGTTC-3' with reverse, 5'-CTAGGCGGTGGAGGTCTCC-3'. After 20<sup>th</sup>, 25<sup>th</sup> and 35<sup>th</sup> cycle 5 µl of each PCR product were taken, run on a 1% agarose gel and intensity of the bands was compared. As a control *gapdh1* was used, whose expression remains at a constant level. All the DNA amplifications were obtained with a *Taq* DNA polymerase (Invitrogen) according to manufacturer's instructions.

### 3.2.4. Immunofluorescence staining of *Drosophila* larval muscles

Third instar larvae were dissected as described previously (Budnik et al., 2006) in an ice-cold Ca<sup>2+</sup>-free saline buffer containing 128 mM NaCl, 2 mM KCl, 4

mM MgCl<sub>2</sub>, 1 mM EGTA, 35 mM sucrose and 5 mM HEPES, pH 7.2 (Demontis and Perrimon, 2009). Buffer without EGTA was used for measurements of contracted muscles. Body wall muscles were fixed with 4% formaldehyde in PBS for 15 minutes and then rinsed three times for 5 minutes in PBS with 0.5% Tween20 (PBT). The material was blocked for 30 minutes in 20% horse serum in PBT at room temperature. Primary antibodies (see Material and Methods 3.2.11.) were applied over night at 4°C. After three washes adequate secondary antibodies (see Material and Methods 3.2.11.) were used for 2 hours at room temperature with or without phalloidin-TRITC (1:1000; Sigma P1951) when appropriate. Specimens labelled and mounted in Fluoromount-G antifade reagent (Southern Biotech) were analysed using a FV300 (Olympus), SP5 or SP8 (Leica) confocal microscopes. Three-dimensional models of labelled muscle fibres were constructed by using Imaris software.

### **3.2.5. Muscle morphology observations and muscle measurements**

Muscles VL3, VL4 and SBM of abdominal segments 3 or 4 were analysed in 15-22 dissected and fixed 3rd instar larvae, as described above. Observations of muscle morphology, as well as measurements of muscle length, diameter, number and size of sarcomeres were carried out on muscles visualised with phalloidin. For counting, muscle nuclei were visualised using anti-Mef2 antibody (see Material and Methods 3.2.11.). The fibre contractility index (CI) was calculated from the following formula:  $CI = (\text{size of relaxed fibres} - \text{size of contracted fibres}) / \text{size of relaxed fibres}$ . FV300 (Olympus) confocal microscope was used for imaging and Fluoview programme (Olympus) for muscle measurements.

### **3.2.6. Behavioural assays**

Behavioural assays were carried out on fifteen 3rd instar larvae per genotype and repeated 3 times for each individual. For all locomotor assays larvae were placed for at least 120 s on a Petri dish filled with solidified 3% agarose for acclimation before being tested. In the righting assay, larvae were placed on a Petri dish with 3% agarose in the dorsal position and the time needed to reverse to the ventral position was measured. For the journey test a 2 mm wide, 5 mm deep and



60 mm long track was made on a Petri dish containing 3% agarose. Fresh yeasts were placed behind the end line as stimuli. Larvae were placed on the test track and the time taken to crawl 50 mm distance was recorded but not longer than 420 s. Larvae which turned backward during the trial were returned to the starting line and the timing was resumed. In motility test larvae were crawling on a Petri dish with 3% agarose and the peristaltic movements were counted during 30 s.

### **3.2.7. Viability test**

In the viability test, 200 embryos were placed on a Petri dish containing 3% agarose supplemented with grape juice and with yeast paste. Subsequently, 1st instar larvae were moved to vials with a standard medium. Individuals achieving each stage of development (3rd larval instar, pupa, imago) were counted.

### **3.2.8. Western Blotting**

For immunoblotting, 50 dissected and frozen larvae were homogenized at 4°C in an ice-cold TSA buffer (10mM Tris pH 8,0, 140 mM NaCl and protease inhibitors from Roche). To obtain lysis, TritonX-100 and NaCl were added, up to the final concentration of 1% and 300 mM, respectively. After 30 min of incubation on ice, the lysate was centrifuged at 17000 *g* for 5 min at 4°C. Equal volumes of supernatant were boiled for 10 min in a loading buffer, containing 10% SDS and 10% β-mercaptoethanol, incubated on ice for 2 min and centrifuged at 2000 *g* for 5 min. Samples were resolved on 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Whatmann). The membrane was blocked for 1 h at room temperature with 3% non-fat milk. Western blotting was performed with rat anti-dCryAB antibody (see Material and Methods 3.2.12.). Following overnight incubation in a primary antibody solution, the membrane was washed 3x20 min in TBST (TBS + 0.5% Tween20). Subsequently, the membrane was incubated in anti-rat HRP-conjugated secondary antibody (see Material and Methods 3.2.12.) for 1 h at room temperature. After 3x20 min washes in TBST, signal was detected by ECL Plus (Amersham) and visualised using Amersham system on the X-ray Film (Kodak). Western blot with rabbit anti-β3-tubulin (see Material and Methods

3.2.12.) was performed as a control. The strength of signal was quantified using the ImageJ 1.47 u.

### **3.2.9. Co-immunoprecipitation**

For co-immunoprecipitation assay, 100 dissected and frozen larvae were homogenized at 4°C in 500 µl harsher extraction buffer (HEB: 20 mM Tris-HCl [pH 7.6], 140 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% [v/v] NP-40, 0.5% [w/v] sodium deoxycholate), described previously (Perng et al., 2006) and supplemented with a protease inhibitor cocktail from Roche. 50 µl of protein G sepharose beads (Amersham Pharmacia Biotech AB) were washed 3 times with 100 µl of PBS1X and centrifuged at 2000 *g* for 5 min after each washing. After 30 min of incubation on ice, lysate was centrifuged at 17000 *g* for 20 min at 4°C, diluted up to a total volume of 500 µl with Ab binding & Washing buffer (Invitrogen) and pre-incubated for 1 h at 4°C with washed protein G sepharose to eliminate proteins that bind non-specifically to the protein G sepharose beads. The protein G sepharose beads were spun down by centrifuging at 17000 *g* for 5 min at 4°C and the supernatant was incubated for 3-4 h at 4°C with 5 µl rat anti-dCryAB antibody. Ab-protein complex was incubated with pre-cleaned beads over night at 4°C on the cradle as well as negative control containing 5% rat serum and 50 µl of washed protein G sepharose in Ab binding & Washing buffer (Invitrogen). The samples were centrifuged at 2000 *g* for 5 min at 4°C. The supernatant from IP was collected and used in the Western Blotting step as an IP efficiency control. Combined complex and negative control were washed 3 times for 5 min at 4°C with a WB washing buffer (Invitrogen). The pellet from both samples as well as the lysate before IP and the IP efficiency control were ran in Western Blot procedure described above with mouse anti-vimentin antibody (see Material and Methods 3.2.12.).

### **3.2.10. Immunogold**

Dissected larvae (as described above) were fixed for 1.5 h at room temperature in 0.5% glutaraldehyde in PBS mixed with 4% paraformaldehyde as 1:1. Post fixation was performed after overnight incubation in 2%

paraformaldehyde at 4°C. Following 3x5 min washes in PBS with 0.5% TritonX (PBT), the material was dehydrated in a graded alcohol series (35%, 50%, 75%, 85%). Subsequently, larvae were incubated in a mixture of 85% ethanol and LRwhite (London Resin Company Ltd), mixed in ratios 2:1, 1:1 and 1:2 for 1 h in each. The material was impregnated in LRwhite 2 times for 12 h at room temperature and once overnight at 4°C and subsequently embedded in LRwhite in gelatin capsules. Polymerization was performed for 24 h at 59°C and subsequently for a few days at room temperature. The capsules were removed in warm water and the material was cut using a microtome (ReihertUltracut E). Ultrathin sections were collected on a nickel mesh and blocked in 1% BSA in PBS for 30 min. Primary antibodies anti-vimentin, anti-β-actin, anti-myosin and anti-dCryAB (see Material and Methods 3.2.13.) were diluted with a buffer containing 1% BSA, 0.1% Tween20, 0.1% TritonX-100, 0.02% sodium azide in PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na<sub>2</sub>HPO<sub>4</sub> • 2H<sub>2</sub>O, 2 mmol/L KH<sub>2</sub>PO<sub>4</sub>). The sections were incubated on a cradle (Stuart mini gyro-rocker SSM3) for 2 h at room temperature. After three 10 min washes in PBT, adequate secondary antibodies conjugated with gold particles were added. The secondary antibodies were diluted in the same buffer as the primary antibodies. After three washes in PBS for 10 min and 2 washes in distilled water for 2 min, the sections were contrasted in lead citrate and observed in electron microscope Zeiss EM 900.

### 3.2.11. Antibodies used in confocal labelling

<b>Antigen/ Antibody</b>	<b>Conjugated moiety</b>	<b>Host species</b>	<b>Dilution</b>	<b>Vendor/ Source</b>
GFP	-	Rabbit	1:500	Biogenesis
dCryAB	-	Rat	1:500	K. Jagla
Mef2	-	Rabbit	1:1000	A. Nguyen
Kettin	-	Rat	1:25	Abcam
Vimentin	-	Mouse	1:40	Sigma
LamC28.26	-	Mouse	1:1000	DSHB
Rabbit IgG	Alexa 488	Donkey	1:300	Jackson Immuno- Research

Rabbit IgG	Cy5	Donkey	1:300	Jackson	Immuno- Research
Rat IgG	Alexa 488	Donkey	1:300	Jackson	Immuno- Research
Rat IgG	Cy5	Donkey	1:300	Jackson	Immuno- Research
Mouse IgG	Cy3	Donkey	1:300	Jackson	Immuno- Research

### 3.2.12. Antibodies used in western blot

<b>Antigen/ Antibody</b>	<b>Conjugated moiety</b>	<b>Host species</b>	<b>Dilution</b>	<b>Vendor/ Source</b>
dCryAB	-	Rat	1:2000	K. Jagla
Vimentin	-	Mouse	1:400	Sigma
$\beta$ 3-tubulin	-	Rabbit	1:0000	R. Renkawitz Pohl
Rat IgG	Horseradish peroxidase	-	1:10000	Pierce
Mouse IgG	Horseradish peroxidase	-	1:10000	Pierce
Rabbit IgG	Horseradish peroxidase	-	1:10000	Pierce

### 3.2.13. Antibodies used in immunogold

<b>Antigen/ Antibody</b>	<b>Conjugated moiety</b>	<b>Host species</b>	<b>Dilution</b>	<b>Vendor/ Source</b>
dCryAB	-	Rat	1:500	K. Jagla
Vimentin	-	Mouse	1:50	Sigma
Myosin	-	Rat	1:1000	Invitrogen
$\beta$ -actin	-	Mouse	1:10	Invitrogen
Rat IgG	18 nm gold particles	Goat	1:20	Jackson Immuno- Research

Rat IgG	10 nm gold particles	Goat	1:100	Sigma
Mouse IgG	10 nm gold particles	Goat	1:100	Jackson Immuno-Research

#### **3.2.14. Mitotracker**

Dissected larvae (as described above) were incubated for 15 min at 37°C in 1500 nM Mitotracker®Red CMXRos (Invitrogen) diluted in an ice-cold Ca<sup>2+</sup>-free saline buffer. Subsequently, larvae were fixed in 4% formaldehyde for 15 min, washed in PBS three times for 10 min, mounted in Fluorescent Mounting Medium (Dakocytomation) and observed in confocal microscope FV1000 (Olympus).

#### **3.2.15. TEM**

Dissected larvae (as described above) were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer. The material was repeatedly rinsed in the same buffer and post-fixed for 2 h in 1% OsO<sub>4</sub> (Sigma–Aldrich) in 0.1 M phosphate buffer. Following rinsing in phosphate buffer, the material was dehydrated first in a graded alcohol series and then in acetone, and embedded in epoxy resin Epon 812 (Sigma–Aldrich). The Epon blocks were cut on a Reichert Ultracut E ultramicrotome (Leica, Wetzlar, Germany). Ultrathin sections were contrasted with uranyl acetate and lead citrate according to the standard protocol (Reynolds, 1963) and examined in a transmission electron microscope (Zeiss EM 900; Carl Zeiss AG, Oberkochen, Germany) at an accelerating voltage of 80 kV.

#### **3.2.16. Heat shock procedure**

Third instar larvae were incubated in the water bath for 10 min at 37°C. To recover after the heat shock, larvae were incubated for 2 h at 25°C in the normal feeding conditions (Colinet et al., 2010). Subsequently, they were dissected and stained with antibodies (see section 3.2.4.) or collected to the protein extraction and western blot procedures (see section 3.2.8.).

### 3.2.17. Statistical analysis

Statistical analysis was carried out using the GraphPad Prism5 software. T-test non-parametric or one-way ANOVA were used for comparisons of the lines phenotypes. The results are shown in the graphs as a standard error of mean;  $p < 0.05$  is regarded.

### 3.2.18. Software

<b>Name</b>	<b>Purpose</b>
ImageJ 1.47u	Export and processing of microscopy data files
AdobePhotoshop CS5	Processing images
Imaris	Three-dimensional modelling
GraphPad Prism5	Statistics
Fluoview	Muscle measurements

## 4. Results

### 4.1. *Drosophila dcryAB* gene is specifically expressed in larval body wall muscles

According to the FlyBase data base, the *Drosophila* gene *l(2)efl* (CG4533) is orthologous to the human  $\alpha$ B-crystallin (*cryAB*) gene, and the amino acid alignment of their products shows 44% of identity (NCBI/BLAST). Following these assumptions, this study is based on the hypothesis that the *l(2)efl* product has a function similar to that of the human  $\alpha$ B-crystallin. For this reason I propose to name it *Drosophila*  $\alpha$ B-crystallin (dCryAB) and accordingly, to call the gene *dcryAB*.

Several Hsp genes play specific developmental functions (Haass et al., 1990). To determine a potential role of *dcryAB* in the *Drosophila* development, its expression pattern in the third instar larva was first analysed. Using the generated polyclonal antibodies it was found that dCryAB protein was abundantly expressed in all the larval body wall muscles (Fig. 4.1.1, A). Within an individual larval muscle dCryAB protein was accumulated in a perinuclear area and displayed a striated pattern at the level of M and Z-lines (Fig. 4.1.1, B-E). Three-dimensional iso-surface modelling of labelled muscles showed that the Z-line-associated dCryAB was mainly detected at the external level of Z-lines but also in the M-line (Fig. 4.1.1, F-H). Images of ultrathin sections of larval muscles, labelled with antibodies combined with gold particles, confirmed the dCryAB localisation in Z-lines (Fig. 4.1.1, I). As a control for the specificity of immunogold reaction, staining against myosin was used (Fig. 4.1.1, J), showing that even in the presence of abundant protein, the number of accessible epitopes in the ultrathin sections was relatively small but the signal was properly localised.

This first analysis showed that the dCryAB expression pattern was reminiscent of the localisation of its human orthologue  $\alpha$ B-crystallin (Dubin et al., 1990; Golenhofen et al., 1999), suggesting that *dcryAB* might play conserved myogenic functions.

#### 4.2. *dcryAB* attenuation leads to formation of morphologically altered muscles

To assess the role of *dcryAB* in formation of larval muscles, RNAi-mediated muscle-specific gene attenuation was applied, and the muscle morphology was analysed using phalloidin staining. Third instar larvae of two RNAi-*dcryAB* *Drosophila* lines, driven by a MefGal4 driver, were used in the analysis. Three classes of somatic muscle defects were observed: defects in sarcomere organisation, defects in muscle morphology and defects in sarcomere organisation and muscle morphology occurring together (Fig. 4.2.1, A-F). The majority of observed larvae (58% of line 40532 and 40% of line 107305) exhibited defects in sarcomeric organisation, characterised by an irregular, fuzzy pattern of Z-lines in large muscle segments (Fig. 4.2.1, D, E). This aberrant sarcomeric pattern was often associated with muscle splitting (37% of line 40532 and 40% of line 107305) leading to an altered muscle attachment or to the loss of the affected muscle (Fig. 4.2.1, D-F). Finally, in some isolated cases (about 5% in line 40532 and 20% in line 107305) muscle splitting was detected independently of sarcomeric alterations (Fig. 4.2.1, B-C, F). To confirm that RNAi-mediated muscle-specific *dcryAB* gene attenuation was efficient, *Drosophila* muscles were labelled with specific anti-dCryAB antibodies and phalloidin (Fig. 4.2.1, G, H). The localisation pattern of dCryAB in RNAi-*dcryAB* lines was significantly affected compared to the one described in the WT. The level of dCryAB decreased considerably, with preserved expression level of F-actin. Western blot analysis of the muscle lysate also showed a reduced content of dCryAB protein in RNAi-*dcryAB* line, compared to the WT, without observed reduction of  $\beta$ 3-tubulin expression.

Considering that each of the larval muscles displays specific properties including size, shape, number of sarcomeres and number of nuclei (Bate, 1990b), changes in any of those attributes in *dcryAB* knockdown context can give an insight into its myogenic functions. Thus, several muscle parameters were measured in larvae with muscle-targeted *dcryAB* RNAi. The measurements focused on three distinct muscles: VL3, VL4 and SBM. In all the analysed cases, muscle-specific attenuation of *dcryAB*, compared to the wild type, led to the formation of significantly shorter (VL3: 17 and 29%; VL4: 19 and 27%; SBM: 11 and 15%; in



line 40532 and 107305, respectively, Fig. 4.2.2, A) and thinner muscles (VL3: 22 and 47%; VL4: 19 and 37%; SBM: 7 and 34%; in line 40532 and 107305, respectively, Fig. 4.2.2, B). The number of sarcomeres in the muscle fibres was also reduced (VL3: 10 and 7%; VL4: 11 and 5%; SBM: 12 and 17%; in line 40532 and 107305, respectively, Fig. 4.2.2, C) indicating that the sarcomere assembly could be affected. However, except the fuzzy pattern areas (see Fig. 4.2.1, D), even when the size of sarcomeres was affected compared to the control muscles (VL3: 6 and 24% shorter; VL4: 8 and 23% shorter; SBM: 1 and 5% longer; in line 40532 and 107305, respectively), the observed differences were statistically insignificant (Fig. 4.2.2, D). In some of the RNAi-*dcryAB* line muscles, a smaller number of nuclei was observed (VL3: 19 and 0%; VL4: 20 and 20%; SBM: 20 and 0%; in line 40532 and 107305, respectively, Fig. 4.2.2, E), indicating that embryonic function of dCryAB might be directly or indirectly involved in promoting myoblast fusion events.

#### **4.3. *dcryAB* attenuation specifically in mesoderm leads to impaired function of larval muscles and affects *Drosophila* life span**

To test whether described morphological alterations affected the motility of larvae, three behavioural assays were carried out. In the righting assay the time needed for each larva to revert from the dorsal-“wrong” position to the ventral-“right” position was measured. In this test, *dcryAB*-attenuated individuals of two RNAi-mediated lines needed over twice and five times longer, respectively, to revert to the right position (Fig. 4.3.1, A). In the journey test, the speed of larvae crawling has been measured and it has been registered that *dcryAB*-RNAi larvae move slower and needed about 30% and 500% more time to go through the appointed, 5 cm distance (Fig. 4.3.1, B). In the motility test the number of peristaltic movements of crawling larvae was counted, and during 30 seconds the number of peristaltic movements executed by *dcryAB*-RNAi larvae was by over 20 and 50% smaller comparing to the control (Fig. 4.3.1, C). To confirm the contraction disturbance of muscles in tested larvae, the contractility index was calculated by comparing the length of relaxed (by EDTA) and contracted muscle fibres. Muscles of RNAi-*dcryAB* individuals had a significantly lower contractility index (VL3: 2.5 and 2.1; VL4: 3 and 2.5; SBM: 5.9 and 5.2 times lower, in line 40532

and 107305, respectively), suggesting that muscles did not contract properly (Fig. 4.3.1, D). Altogether, results of behavioural assays and contractility test indicate that *dcryAB* knockdown affects muscle contraction and induces motility defects.

It was also assessed whether *dcryAB* had an influence on survival and life span. The viability tests revealed that *dcryAB*-RNAi flies were characterised by a markedly higher mortality rate, compared to the control, at every stage of development, namely: embryo (by 30 and 31% higher), larva (by 22 and 24% higher) and pupa (by 26 and 27% higher), in line 40532 and 107305, respectively (Fig. 4.3.1, E). The life span of the surviving adult *dcryAB*-RNAi flies was also significantly shorter (up to three weeks). Thus, the muscle *dcryAB* expression is involved in control of life span and acts downstream of JNK/dFoxo pathway (Wang et al., 2005).

#### **4.4. dCryAB co-localises with putative intermediate filaments in larval muscles**

As previously described (see section 4.1.), dCryAB localises in Z-lines, M-lines and around nuclei. Moreover, three-dimensional images show that the Z line-associated dCryAB localises mainly at the surface of sarcomeres, just beyond the muscle membrane. This sarcomeric dCryAB localisation is reminiscent of that of its vertebrate orthologue,  $\alpha$ B-crystallin and suggests that dCryAB might co-localise with intermediate filaments that are known to link sarcomeres with sarcolemma. As the existence of intermediate filaments in *Drosophila* with homology to vimentin had already been suggested by Walter and Biessmann (1984) and dCryAB was detected at extensions of Z-lines, which is an expected location of intermediate filaments (Konieczny et al., 2008), it was decided to test whether an intermediate filament protein could be detected in *Drosophila* muscles, using antibodies directed against mouse vimentin.

Interestingly, in *Drosophila* larval muscles anti-vimentin antibody detects a protein which co-localises with dCryAB in the Z-line and is present in the nuclear area (Fig. 4.4.1, A-D). The visualised dots, slightly above the sarcomeric pattern of the detected protein, seem to attach to the sarcolemma (Fig. 4.4.1, C). In this study, the *Drosophila* protein, which is spaced at the predicted location of intermediate

filaments and has been detected by anti-vimentin antibody, will be called vimentin-like protein. As the vimentin-like position is equivalent to the position of desmin filaments in vertebrate muscles (Goldfarb and Dalakas, 2009), it is expected that the vimentin-like protein may have properties of the third class intermediate filament proteins, including vimentin. To better visualise subcellular localisation of the vimentin-like protein, three-dimensional reconstructions and iso-surface modelling were performed from confocal scans through larval muscles. They showed that the detected protein, just as dCryAB, mainly localised on the external level of the Z-line in the sarcomeres (Fig. 4.4.1, E). Immunogold staining against the vimentin-like protein confirmed its localisation in the Z-line (Fig. 4.4.1, F) but also showed that antibodies conjunct with gold particles appeared to filamentous structures (Fig. 4.5.1,C).

The vimentin-like protein as well as desmin filaments are also present in the perinuclear area. Immunocytochemistry reaction, visualised in confocal microscope, shows that the vimentin-like protein is present in the nuclear envelope area but also inside the nucleus (Fig. 4.4.2, A). To check if the vimentin-like protein was present inside the nucleus, co-staining with antibodies against histone3 was used. Confocal images (Fig. 4.4.2, B-C) show co-localisation of the vimentin-like protein and histone3, suggesting that the vimentin-like protein is indeed present inside the nucleus. Immunogold reaction against the vimentin-like protein confirmed its distribution on the external side of the nuclear envelope and inside the nucleus, being probably associated with chromatin (Fig. 4.4.2, D).

Following Walter and Biessmann (1984), it is suggested in this study that *Drosophila* expresses proteins which correspond to the third class of vertebrate intermediate filaments and that these proteins share a similar intracellular distribution with their vertebrate counterparts.

#### 4.5. dCryAB interacts with a potential intermediate filament protein

Analysis of dCryAB amino acid alignment shows that dCryAB, like  $\alpha$ B-crystallin and other small heat shock proteins, contains in its sequence a conserved -crystallin rod domain. Inside this crystallin domain, dCryAB has the <sup>77</sup>EKFEVILDVQQFS<sub>89</sub> motif which corresponds to the intermediate filament interaction domain (Fig. 4.5.1, A) of other organisms (Houck et al., 2011b). This finding suggests that dCryAB has an ability to interact with the intermediate filament protein, which potentially is the vimentin-like protein in *Drosophila* muscles. To verify the occurrence of possible interactions between dCryAB and the vimentin-like protein, muscle-specific gain of function of *dcryAB* fused with GFP protein was performed using UAS/GAL4 system. Indeed, in third instar larval muscles, overexpression of *dcryAB* led to formation of large cytoplasmic accumulations of dCryAB-GFP. Aggregates of dCryAB-GFP contained also the vimentin-like protein, suggesting that they interacted with each other (Fig. 4.5.1, B-D). It was shown that cytoplasmic aggregates occurred in 75% of observed UAS-*dcryAB*-GFP individuals and, at the mean time, in all visualised larvae when dCryAB-GFP aggregates were present, they contained also vimentin-like protein (Fig. 4.5.1,E). To confirm detected protein-protein interactions, co-immunoprecipitation reaction was performed using dCryAB antibodies and isolated proteins with the size around 40, 70 and 170 kDa were visualised on nitrocellulose membrane using anti-vimentin antibody. The antibody detected two bands of about 70 and 72 kDa in the protein extract from third instar larval muscles. One of these bands, corresponding to 72 kDa, was immunoprecipitated by anti-dCryAB antibody. However, the major IP-detected 170 kDa band was not seen in Western blot with anti-vimentin. This suggests that IP reaction leads to a high enrichment in 170 kDa vimentin-like protein interacting with dCryAB and/or to a formation of a highly stable protein complex of such a size. Moreover, a low intensity 40 kDa band could be detected in the IP material. The lack of this band in western blot can be explained by the enrichment resulting from the IP reaction. Described interactions are related to the fact that dCryAB is probably necessary for correct formation of vimentin-like meshwork, which was described in the sections below.

#### **4.6. dCryAB ensures structural integrity of somatic muscles by interacting with potential IF proteins**

In vertebrates, intermediate filaments form a very well organised meshwork inside muscle cells, taking part in stabilisation of their intracellular components and in positioning of organelles (Shah et al., 2004). Desmin filaments are stabilised by crystallin (Goldfarb and Dalakas, 2009), therefore I hypothesised that dCryAB maintained the stability of vimentin-like filaments in *Drosophila* muscle cells. To determine whether dCryAB was involved in stabilisation of structural integrity of constituent elements of somatic muscles, by interacting with IF proteins, the structure of WT larval body wall muscle cells was compared with that of *dcryAB* knocked-down ones. Confocal projections of muscle fibres, stained for the vimentin-like protein, showed that in WT muscle cells the vimentin-like protein formed a regular meshwork anchored to the sarcolemma, surrounding sarcomeres at the level of Z-bands, extending from one Z-disc to the other and finally reaching the perinuclear area (Fig. 4.6.1, A). Under RNAi *dcryAB* conditions, the vimentin-like protein loses its proper arrangement, observed as a decreased attendance of filaments around myofibrils with a fuzzy pattern occurring also around nuclei (Fig. 4.6.1, B). This fuzzy pattern can be caused by disorganisation of the filamentous formation of the vimentin-like protein, as a result of the loss of function of dCryAB. Electron microscopy observations of immunogold reaction results confirmed that in WT muscles the vimentin-like protein was organised in tight filaments (Fig. 4.6.1, C), whereas vimentin-like filaments in *dcryAB* knocked-down muscles seemed to be misarranged, forming a loose meshwork (Fig. 4.6.1, D). Considering that in vertebrates desmin intermediate filaments are linked to myonuclei and mitochondria, being responsible for their positioning and function (Capetanaki, 2002; Maloyan et al., 2005; Shah et al., 2004), it was checked if the vimentin-like protein had the same properties in *Drosophila* larval muscles. As expected, the disorganisation of vimentin-like filaments caused by the absence of dCryAB in muscle cells, leads to disturbance in myonuclear positioning. In WT VL3 and VL4 muscles nuclei are disposed relatively regularly along muscle fibres (Fig. 4.6.1, E). Under RNAi *dcryAB* conditions nuclei were clearly displaced to one side of the muscle fibre (Fig. 4.6.1, F), suggesting a lack of force keeping them in the

proper position. Besides, electron microscopy showed that in muscles with *dcryAB* knocked-down, interfibrillar mitochondria associated with Z-discs were swollen, comparing to the WT mitochondria (Fig. 4.6.2, A-B). They also exhibited damaged cristae (Fig. 4.6.2, A-B), suggesting that they were no longer functional. To investigate this hypothesis, I used MitoTrackerRed to follow the mitochondria pattern. The detected signal of active mitochondria in the muscles of RNAi *dcryAB* line appeared to be significantly reduced, indicating an impaired respiratory function of mitochondria positioned around nuclei, as well as those in the Z-line area (Fig. 4.6.2, C-D). Additionally, between myofibrils, increased deposits of glycogen were observed, supporting the respiratory disturbance in the tested muscles of RNAi *dcryAB* line (Fig. 4.6.2, E-F). Moreover, observed pathological changes included also misaligned Z-discs, visualised in electron microscopy images (Fig. 4.6.2, E-F). All those morphological and biochemical abnormalities suggest that dCryAB plays an important role in maintaining a properly organised vimentin-like network, and in ensuring a normal position and function of investigated muscle components.

#### **4.7. dCryAB participates in the putative intermediate filament meshwork formation**

To verify whether dCryAB was involved in the assembly of the vimentin-like meshwork, RNAi *dcryAB* larvae were exposed to heat shock conditions to provoke increased expression of *dcryAB*. *Drosophila* larvae expressing RNAi against *dcryAB* have a smaller amount of intracellular dCryAB and are characterised by a fuzzy pattern of the vimentin-like protein (see sections 4.2. and 4.5.). After 2 h of recovery from 10 min heat shock at 37°C, the dCryAB protein level in *dcryAB*-RNAi flies, as well as in WT flies, increased significantly, compared to the normal conditions (Fig. 4.7.1, D). Western blot procedure also showed that anti-dCryAB antibodies recognised an additional protein, which might correspond to another sHsp protein induced by the heat shock and not expressed in the larva in normal conditions (Fig. 4.7.1, D). Muscle immunostaining also showed an increased level of dCryAB in RNAi-*dcryAB* larvae, compared to the normal conditions (Fig. 4.7.1, A and 4.2.1, H). It was observed that in muscle cells dCryAB was restored in

sarcomeric localisation and accumulated in the cytoplasm with the vimentin-like protein. In the mean time, a recovery of the vimentin-like sharp pattern in Z-lines and in the perinuclear area was registered (Fig. 4.7.1, B-C), highlighting that dCryAB is required for the proper organisation of intermediate filament meshwork in muscle cells.

#### **4.8. Mutated dCryAB<sup>R120G</sup> mimics effects of mutations in human $\alpha$ B-crystallin leading to desmin-related myopathy.**

Desmin-related myopathy is caused by the missense mutation R120G in  $\alpha$ B-crystallin. It results in intracellular aggregations of type III intermediate filaments, such as desmin and vimentin, accompanied by  $\alpha$ B-crystallin. Formation of inclusion bodies in this disorder is due to misfolding of the mutated  $\alpha$ B-crystallin<sup>R120G</sup> protein (Simon et al. 2007; Zobel et al. 2003). Comparison of amino acid alignment of human  $\alpha$ B-crystallin and dCryAB shows conservation of arginine in codon 120 (Fig. 4.8.1, A). To check if the *dcryAB* R120G mutation could cause the same pathological phenotypes, a fly line expressing specifically *dcryAB*<sup>R120G</sup> in its muscles was developed. The required mutation was achieved by site-directed mutagenesis where adenine from codon 124, adequate to the codon 120 in human, was replaced by guanine, resulting in exchanging arginine for glycine in the amino acid sequence of the protein (Fig. 4.8.1, B). DNA sequencing confirming the presence of desirable mutation in *dcryAB* gene was performed by GATC Biotech. It was visualised in confocal images that dCryAB<sup>R120G</sup>-GFP occurred in the sarcomeres but its distribution was not as regular as that of the normal form of the protein. The mutated protein was present in some sarcomeres but lacking in sarcomeres of some muscle parts (Fig. 4.8.2, A). In the sarcomeres devoid of dCryAB<sup>R120G</sup>-GFP, actin filaments were not assembled in Z-lines and seemed to not be organised in any particular order (Fig. 4.8.2, B, C). Moreover, dCryAB<sup>R120G</sup>-GFP was localised ectopically in sarcomeres, meaning that dCryAB<sup>R120G</sup>-GFP protein did not occur in Z-lines of some sarcomeres, being present only in the middle of the M-lines, whereas in other sarcomeres dCryAB<sup>R120G</sup>-GFP appeared in Z-lines and in the edges of I-bands (Fig. 4.8.2, D, E). A Z-line shift in sarcomeres was also observed (Fig. 4.8.2, E). Ultrathin sections of larval muscle observed in TEM (Fig. 4.8.2, F)

showed misaligned and shifted Z-line indicating disturbance in its organisation. Elongated mitochondria with reduced cristae and glycogen deposits were also observed between myofibrils. In muscle fibres dCryAB<sup>R120G</sup>-GFP may also form aggregates, which do not incorporate actin filaments (Fig. 4.8.3, A, B). Moreover, smaller nuclei were observed in these muscles, apart from normal-sized ones (Fig. 4.8.3, B). Compared to the control (Fig. 4.8.3, C), in a third instar larva, expressing *dcryAB<sup>R120G</sup>*, a loss of proper localisation of the vimentin-like protein was observed. The vimentin-like protein showed a fuzzy sarcomeric pattern and irregular disposal inside muscle cells, accumulating among others around nuclei (Fig. 4.8.3, D, E). Moreover, dCryAB<sup>R120G</sup> and the vimentin-like protein commonly form intrasarcoplasmic aggregates which accumulate together around nuclei and can even localise inside of them, which is better visualised on three-dimensional modelling images (Fig. 4.8.3, F). Muscles expressing the mutated form of dCryAB showed alterations in the shape and arrangement of nuclei. In these larval muscles, nuclei were not arranged regularly but were located randomly or/and were compacted to each other. They seemed to be smaller than nuclei of control muscles with irregular shape and varying size. 85% of tested individuals possessed delocalised dCryAB and the vimentin-like protein, always occurring together (Fig. 4.8.3, G). At the same time, 45% of larvae had deformed and displaced nuclei, which was always combined with disarrangement of the vimentin-like protein (Fig. 4.8.3, G). These results suggest that dCryAB<sup>R120G</sup> does not localise like the wild type form of the protein and its sarcomeric localisation is required for normal localisation of actin filaments. Moreover, dCryAB<sup>R120G</sup> and the vimentin-like protein strongly interact with each other, provoking loss of function of both proteins and muscle defects, which is similar to observations in muscles of DRM patients.

#### **4.9. Overexpression of *dcryAB<sup>R120G</sup>* leads to formation of morphologically altered muscles**

Considering that the WT form of dCryAB participated in muscle formation and its attenuation led to formation of impaired muscle fibres, it was investigated if mutation R120G in dCryAB protein could also cause similar changes in larval



muscles. Thus, several muscle parameters were measured in larvae with muscle-specific overexpression of *dcryAB<sup>R120G</sup>*. Since in the experiments *dCryAB<sup>R120G</sup>* was coupled with the GFP protein, in addition to the WT condition, the *Drosophila* line overexpressing the WT form of *dCryAB* also fused with GFP, was used as the second control for tests investigating its effect on muscle development. Measurements of three distinct muscles, VL3, VL4 and SBM, were taken. Larval muscles containing the mutated form of the *dCryAB* protein were significantly shorter (VL3: 25%; VL4: 24%; SBM: 4%; Fig. 4.9.1, A) and had an altered diameter (thinner: VL3: 36%; VL4: 29%; and thicker: SBM: 27%; Fig. 4.9.1, B), compared to muscles overexpressing *dCryAB*-GFP. The number of sarcomeres inside the muscle fibres was reduced (VL3: 22%; VL4: 21%; SBM: 0%; Fig. 4.9.1, C), with statistically insignificantly reduced length (VL3: 9%; VL4: 9%; SBM: 0%; Fig. 4.9.1, D). The differences in the measured morphological parameters of the muscle confirmed that the native *dCryAB* protein was necessary for the proper muscle development and for the sarcomere assembly. Moreover, muscles of the *dcryAB<sup>R120G</sup>-GFP* line displayed a smaller number of nuclei (VL3: 19%; VL4: 20%; SBM: 20 and 10%; Fig. 4.9.1, E), indicating a reduced number of fusion events, in which *dCryAB* may be directly or indirectly involved.

#### **4.10. Overexpression of *dcryAB<sup>R120G</sup>* leads to impaired function of larval muscles and affects life span**

Three behavioural assays were carried out in order to ascertain if the described morphological alterations affected locomotive abilities of the larvae: righting assay, journey test and motility test (for description of the tests see section 4.3). In the righting assay larvae with muscle-specific overexpression of *dcryAB<sup>R120G</sup>-GFP* needed over four times more time to revert their body, comparing to muscles overexpressing the WT form of *dCryAB* fused with GFP (Fig. 4.10.1, A). In the journey test, *UAS-dcryAB<sup>R120G</sup>* larvae were crawling significantly slower and took almost four times more time to go through the appointed, 5 cm distance (Fig. 4.10.1, B). In the motility test *UAS-dcryAB<sup>R120G</sup>* larvae executed over 50% less peristaltic movements, comparing to the control (Fig. 4.10.1, C). The contraction disturbance of muscles in the tested fly line was confirmed by the values of the

contractility index. The muscles of the UAS-*dcryAB*<sup>R120G</sup> individuals had a significantly lower contraction ability (VL3: 3.1; VL4: 2.3; SBM: 1.1 times lower, respectively), which explains their lower performance in behavioral assays, compared to the control (Fig. 4.10.1, D). Altogether, results of behavioral assays and contractility test indicate that the muscle-specific overexpression of *dcryAB*<sup>R120G</sup> affects muscle contraction and induces motility defects.

The presence of *dcryAB*<sup>R120G</sup> affected also the *Drosophila* survival and life span. The performed viability tests revealed that UAS-*dcryAB*<sup>R120G</sup> flies showed a markedly higher mortality rate compared to the control at every stage of development, namely, embryo (by 29% higher), larva (by 31% higher) and pupa (by 29% higher) (Fig. 4.10.1, E). The life span of the surviving adult *dcryAB*-RNAi flies was also significantly shorter (up to two weeks), compared to the control flies. This indicates that muscle aggregates of the mutated form of dCryAB, together with the vimentin-like protein, may induce cell toxicity, which in turn affects life span.

#### **4.11. Human $\alpha$ B-crystallin inserted to the *Drosophila* genome mimics dCryAB localisation and interaction with vimentin-like protein in larval muscle cells**

To test whether human  $\alpha$ B-crystallin (CryAB) could have the same function in *Drosophila* muscle cells as dCryAB, a fly line overexpressing human  $\alpha$ B-crystallin, coupled with GFP, specifically in muscles was generated. In the third instar larval muscle cells both proteins, human and *Drosophila* CryAB, occurred together in the Z-line, in the perinuclear area.  $\alpha$ B-crystallin was present in the M-line and inside the nucleus but also in all the muscle cytoplasm (Fig. 4.11.1, A-A', C-C'). Figures 4.11.1, B-B' and C-C' show that  $\alpha$ B-crystallin attached to the vimentin-like meshwork in Z-lines and was also present together with the vimentin-like protein inside the nucleus. The observed distribution of these three proteins suggests that dCryAB and  $\alpha$ B-crystallin share similar subcellular localisation and thus, potentially similar functions in muscle cells, stabilising the intermediate filament network. However, further research is needed to confirm this hypothesis.

## 5. Discussion

Already in 1990 Haass et al. (1990) found that sHsps were present in unstressed and yet transcriptionally inactive early embryos, indicating the importance of sHsps from the very beginning of embryonic life. However, it was also shown that upon cellularisation the intensity of the sHsp27, sHsp26 and sHsp23 immuno-signal was strongly reduced and restricted to the embryo's cortex. In the next stages of embryonic development the sHsps expression increased, being again detectable in the cytoplasm of the embryonic cells, continuing to increase in the elongating germ band. Nevertheless, in fully developed embryos sHsps were strictly confined to the CNS (Haass et al., 1990). These findings directed the attention to understand the role of sHsps in human development, as well as in development of other organisms.

The main role of Hsps is to act as a chaperone by a temporary association to other polypeptides. Through this transient binding, Hsps can modulate cellular functions, for instance limiting activation of the apoptotic cascade, cell proliferation, antigen presentation or cytoskeleton organisation by influencing the ultimate function of their partners. Hsps become developmentally important already during gametogenesis, protecting male and female gametes. Dix et al. (1996) showed that male mice with disrupted Hsp70 expression lacked postmeiotic spermatids, mature sperm and were infertile, suggesting its protective role during the prophase of the first meiotic division. Another example of the Hsp importance during spermatogenesis is the role of Hsp27 in cytoskeleton dynamics of Sertoli cells which surround germ cells (Welsh et al., 1996). Here, Hsp27 co-localises with F-actin bundles helping to maintain the germ cells migration to the tubule lumen. The reported presence of Hsp27 in ovaries during normal development suggests its role also in oogenesis (Joanisse et al., 1998; Marin and Tanguay, 1996). A wide variety of studies demonstrated the Hsps expression during organogenesis. For example, Hsp26 is expressed during *Drosophila* development in the epithelium, proventriculus, larval brain and ventral ganglion (Marin and Tanguay, 1996), whereas Hsp90, Hsp70, Hsp25 and  $\alpha$ B-crystallin are required for formation of mouse cartilage and bone tissues (Loones and Morange, 1998). Even up to seven sHsps (Hsp27,  $\alpha$ B-crystallin, Hsp20, Hspb2, Hspb3, cvHsp

and Hspb8) are expressed at relatively high levels during development and differentiation in mammalian heart and skeletal muscles (Davidson et al., 2002), implying their role in muscle formation. It is not known whether all these genes diverged from a common ancestor with developmental functions, however phylogenetic analysis performed in our laboratory indicates that sHsps, Hsp27, Hspb8 and  $\alpha$ B-crystallin, involved in muscle development, have indeed a common ancestor (Fig. 5.1-1.). In human cardiac and skeletal muscles  $\alpha$ B-crystallin, Hspb2 and Hspb3 are induced during the initial phase of skeletal muscle differentiation, controlled by MyoD, suggesting their muscle-specific function in development (Sugiyama, 2000). It was established that Hsps function as developmental buffers in different manners, most likely by preventing apoptosis or/and stabilising cytoskeleton. Nevertheless, their expression patterns vary among particular sHsps and among species.



### **5.1. *dcryAB* gene plays conserved myogenic functions and dCryAB protein cellular localisation is reminiscent of its vertebrate counterpart**

The *Drosophila* sHsp genes are clustered within the 67B region on the left arm of the third chromosome (Corces et al., 1980) except *dcryAB (l(2)efl)* located on the right arm of the second chromosome at locus 59F4 (Kurzik-Dumke and Lohmann, 1995). Kurzik-Dumke and Lohmann (1995) found that *dcryAB (l(2)efl)* was expressed during *Drosophila* embryogenesis, and also during larval stages, reaching its maximum expression during the third larval instar, whereas its role remained unknown. This prompted me to try to define its function in *Drosophila*. It was confirmed in our laboratory that dCryAB was expressed specifically in unstressed muscles of *Drosophila* embryos (data not shown) as well as in larvae. Immunocytochemical analysis showed that the dCryAB protein was abundantly expressed in all the larval body wall muscles, accumulating in the perinuclear area and displaying a striated pattern at the level of M-lines and extremities of Z-lines, which was also confirmed by immunogold and iso-surface modelling analyses. The mammalian counterpart of dCryAB,  $\alpha$ B-crystallin is expressed, among others, in unstressed pig, mouse and human lenses, heart and skeletal muscles (Brady et al., 2001; Goldfarb and Dalakas, 2009; Golenhofen et al., 1999). In eye lenses,  $\alpha$ B-crystallin helps to maintain their transparency, whereas in muscles it acts as chaperone for desmin, vimentin and actin, helping to maintain cytoskeletal integrity. During pig (Tallot et al., 2003), rat and human (Klemenz et al., 1993) development, constant levels of  $\alpha$ B-crystallin were observed in heart muscles. In pig (Tallot et al., 2003) and rat (Inaguma et al., 1993) skeletal muscles,  $\alpha$ B-crystallin and Hsp27 levels were found to increase from foetal to adult stage, suggesting their essential role in muscle physiology. Moreover, Ito et al. (2001) showed that during differentiation of mouse C2C12 cells the level of  $\alpha$ B-crystallin was elevated 10-fold and  $\alpha$ B-crystallin played a crucial role in preventing apoptotic cell death induced during the differentiation process. It seems that the role of two closely related proteins,  $\alpha$ B-crystallin and Hsp27, is the most similar to that of dCryAB, whereas it is not completely certain if their role is displayed due to involvement in the modulation of cytoskeleton or inhibition of apoptosis. Because  $\alpha$ B-crystallin and dCryAB are to a high extent homologous and both are localised in

Z-lines and around nuclei (Djabali et al., 1997), one could expect that they play similar roles in muscle cells.

As at the embryonic stages the body plan is formed and during the pupal stage the key metamorphosis steps occur, the higher *hsps* expression in embryo, larva and early pupae coincides with major developmental events, including muscle formation. To determine if dCryAB stabilised the muscle cell homeostasis buffering developmental variations, I applied RNAi-mediated muscle-specific *dcryAB* gene attenuation and observed muscle morphology of the third instar larvae. Obtained results show that *dcryAB* is expressed specifically in muscles and the dCryAB protein is localised around nuclei, in Z-lines and M-lines, suggesting its role in stabilisation of the structural integrity of muscle fibres. Thus, it was not surprising to observe that muscles with a attenuation of dCryAB during development displayed various morphological defects. One of them was a disturbance in the actin cytoskeleton organisation, observed in confocal microscope as a fuzzy pattern of phalloidin-stained sarcomeres, muscle splitting which in some cases led to altered muscle attachment or even to the loss of the affected muscle. DCryAB, like other sHsps, including  $\alpha$ B-crystallin, has an actin-binding domain in its  $\alpha$ -crystallin fragment. Association of  $\alpha$ B-crystallin with actin filaments and its role in preventing formation of actin aggregates was already established by Bennardini et al. (1992). Mounier and Arrigo (2002) proposed that direct actin-sHsp interaction occurred to inhibit actin polymerization and to participate in the regulation of actin filament dynamics (Bennardini et al., 1992; Mounier and Arrigo, 2002). They suggested that protection of the actin cytoskeleton would be a result of an F-actin-sHsp interaction in which microfilaments would be coated by small oligomers of phosphorylated sHsps. According to this study, some sHsps would behave with the actin cytoskeleton as actin-binding proteins. Here, sHsps would be capable of either capping a microfilament or stabilizing and protecting the microfilament when organized in small, phosphorylated oligomers. Therefore, deficiency of sHsps committed in protection of actin filaments can cause their destabilisation and affect cellular processes in which they are involved, which is also true for dCryAB knockdown, leading to muscle morphology defects caused by irregular distribution of F-actin.

Assuming that each larval muscle was characterised by specific properties including size, number of sarcomeres and number of nuclei (Bate, 1990b), several muscle parameters were measured in larvae with muscle-targeted *dcryAB* knockdown. Confocal observations of muscle morphology of the third instar larva with a deficiency of dCryAB during development allowed me to notice a significant reduction of mentioned muscle parameters. The smaller muscle size is not surprising, knowing that many members of the Hsps family, like Hsp90, Hsp70, Hsp27 or  $\alpha$ B-crystallin are known to play a vital role in the normal cell growth and development (Hartl, 1996). Andley et al. (1996) observed that  $\alpha$ A-crystallin knockout epithelial cells of mouse lens were characterised by decreased growth. This effect could be due to a direct interaction of  $\alpha$ A-crystallin with cell cycle regulators or to functional absence of  $\alpha$ B-crystallin, which normally occurs in a complex with  $\alpha$ A-crystallin. Recent results of Middleton et al. (2013) showed that in zebrafish morphant embryos expressing reduced levels of Hsp27 the size of craniofacial muscles was smaller. They suggested that Hsp27 was required for optimal growth of these muscles by influencing expression or/and assembly of cytoskeletal proteins forming myofibres (Middleton and Shelden, 2013). Considering that Hsp27 and dCryAB have a common ancestor both results seem to be consistent, emphasising the role of dCryAB in the maintenance of cytoskeletal network. Moreover, muscles in third instar larvae of *Drosophila*, like vertebrate striate muscles, grow by addition of sarcomere units (Rui et al., 2010). Thus, I counted sarcomeres in three different muscle fibres (VL3, VL4, SBM), to check if the smaller muscle size was due to a lower number of sarcomeres. Indeed, in all cases analysed the number of sarcomeres was slightly reduced, whereas, except the fuzzy pattern areas, their size was not affected. These results allow concluding that dCryAB plays a role in larval muscle growth, which occurs by the addition of sarcomere units. Observed lower muscle size can also be compatible with a lower number of sarcomeres. A possible hypothesis explaining this aberration is impaired myoblast fusion, which is formation of myotubes of multinucleated skeletal muscle that is essential for formation of contractile skeletal muscle tissue. In the absence of fusion, FCs differentiate in tiny and mononucleate muscles at appropriate locations and express their normal complement of genes (Rushton et al., 1995). The process of cell-cell fusion requires several distinct cellular



behaviours. Initially, FCs or myotubes and FCMs have to recognise and adhere to each other. This results in a series of cellular events that are necessary to bring the cell membranes of the two cells in close proximity to one another. Subsequently, pores are formed between the plasma membranes, and their expansion leads to fusion of the two myoblasts (Schejter and Baylies, 2010). Actin filaments participate actively in this process by forming cortical actin walls in skeletal muscle myoblast cultures (Duan and Gallagher, 2009). These results demonstrated that described actin walls underwent a dramatic reorganisation prior to “vesicle paring” and fusion pore formation. According to these findings, actin filament disorganisation can lead to disturbance of myoblast fusion. Since the smaller number of nuclei in muscles of the RNAi-*dcryAB* third instar larva could be a consequence of disturbed myoblast fusion, and since dCryAB may be involved in stabilisation and reorganisation of actin filaments, the smaller number of nuclei in the observed muscles and the related smaller muscle size and lower number of sarcomeres may be consequences of impaired microfilament organisation resulting from dCryAB deficiency. To verify this hypothesis it would be advisable to check if dCryAB interacts with actin filaments in cortical actin walls during early muscle differentiation and to construct flies with inactive actin binding domain inside the dCryAB rod domain and investigate if interactions between dCryAB and actin would be affected (work in progress).

Since *Drosophila* third instar larvae have fully developed locomotor behavior (Mudher et al., 2004), it was possible to perform three different behavioural assays on individuals lacking *dcryAB* expression, to assess if described muscle defects affected their motility. Obtained results revealed severe motility problems and a lower contractility index, indicating a reduced ability to perform muscle contraction. The easiest way to record motility defects is observation of unicellular organism and it has been shown by Montagna et al. (2012) that *hsp20(-)* *Plasmodium* sporozoites display aberrant cell motility. Observed changes in their speed are due to impaired microfilament regulation caused by the lack of Hsp20 (Montagna et al., 2012). Thus, in *Drosophila* improper muscle contraction causing impaired motility may also be provoked by dCryAB deficiency-dependent actin filament disorganisation, observed as sarcomere fuzzy pattern. On the other hand, overexpression of *dcryAB* resulted in an increased muscle mass and increased

number of nuclei. Thus, it would be interesting to see if crossing flies overexpressing *dcryAB* with RNAi-*dcryAB* flies could restore the proper phenotype in terms of correct muscle size and number of nuclei, as well as the appropriate localisation of the entire set of sarcomeric proteins, allowing normal motility. Unfortunately, construction of desirable fly strains failed, and the experiment has to be repeated.

Despite of the reported importance of the sHsps in the development, it seems that their deficiency is not always lethal. Depletion of one sHsp is usually not noxious, due to a certain level of redundancy between the functions of each small heat shock protein (Morrow and Tanguay, 2012). According to many authors (Colinet et al., 2010; Hao et al., 2007; Michaud and Tanguay, 2003; Morrow et al., 2004), some of the sHsps do not play any significant role in *Drosophila* development under laboratory conditions. Their role is rather preventive, as flies depleted of Hsp22, Hsp23 or Hsp27 were viable but had a reduced resistance to certain types of stress and a reduced longevity. It was thus interesting to check if the proposed name of the gene: *lethal (2) essential for life* (Kurzik-Dumke and Lohmann, 1995), correctly suggested that its normal expression was indispensable in *Drosophila* development. Because a complete knockout of *l(2)efl* gene is not yet available and the work to create it using TALENS approach is still in progress in our laboratory, to verify this hypothesis I created conditions of attenuated *l(2)efl* expression in muscles during development by muscle-specific RNAi expression against the gene product. In these circumstances I was able to obtain fertile adult flies. However, their life span was affected and I observed a higher mortality rate at every stage of development. This is consistent with results of Brady et al. (2001) who used knockout approach to show that  $\alpha$ B-crystallin and Hspb2 were not essential for viability or reproduction of their mouse model but their disturbance could lead to apparent degeneration of some skeletal muscles. Therefore I assume that *l(2)efl* expression is also not particularly “*essential for life*” but this has to be confirmed by observations of the fly null mutant. Nevertheless, the *l(2)efl* has an knockdown influence on the fly longevity and muscle morphology. Because I assume that the L(2)efl protein has a similar function to the human  $\alpha$ B-crystallin (see below, section 5.2.), I propose to call the protein drosophila- $\alpha$ B-crystallin (dCryAB) and the gene: *dcryAB*.

In summary, I conclude that observed sarcomere and full muscle morphology defects, like smaller muscle size, lower number of sarcomeres and nuclei, as well as larval motility defects may have the same origin, namely impaired microfilament organisation triggered by deficiency of dCryAB which is required for the proper muscle development, probably as a cytoskeleton stabiliser.

## **5.2. dCryAB co-localises and interacts with *Drosophila* putative intermediate filament proteins detected by anti-vimentin antibody and *dcryAB* attenuation leads to disruption of vimentin-like meshwork**

The cytoskeleton in striated muscle cells is composed of a highly complex scaffold of microfilaments, microtubules and intermediate filaments. Thanks to this cytoskeletal network, striated muscles are able to maintain their sophisticated sarcomeric organisation, as well as integration between contractile apparatus, nucleus and other organelles (Herrmann et al., 2007). Intermediate filaments are the key components maintaining this precise organisation by interacting with many cellular structures (Capetanaki et al., 2007). In humans at least 65 genes, which are expressed in a tissue-specific manner, encode intermediate filament proteins (Herrmann et al., 2007). In vertebrate mature muscle cells the IF cytoskeleton is predominantly composed of desmin, muscle-specific type III IF proteins, whereas in myoblasts the main type III protein is vimentin (Capetanaki et al., 2007). A continuous cytoskeletal network maintains its integrity by interacting with small heat shock proteins, which supports the idea that sHsps may play a crucial role in maintaining the stability of cell structures.

One of the main physiological functions of sHsps is their ability to regulate the cytoskeleton dynamics (Wettstein et al., 2012). All sHsps share a similar structural pattern: a rod  $\alpha$ -crystallin domain flanked by a variable hydrophobic N-terminal region and a C-terminal extension, where their amino acid sequence, in the corresponding regions, is similar to  $\alpha$ -crystallin in over 40% of their length. Houck et al. (2011) described multiple sites in  $\alpha$ B-crystallin, which modulate its interactions with desmin filaments. They demonstrated that substitution of those three sequences in  $\alpha$ B-crystallin, situated in  $\beta$ 3-strand,  $\beta$ 8-strand and C-terminus (<sub>73</sub>DRFSVNLDVKHFS<sub>85, 131</sub>LTITSSLS<sub>138</sub> and <sub>155</sub>PERTIPITREE<sub>165</sub>, respectively), could

alter the interactions of  $\alpha$ B-crystallin with desmin. It suggests that described sequences contribute to the interaction of  $\alpha$ B-crystallin with desmin filaments (Houck et al., 2011a). Similarly, dCryAB possesses  $_{77}\text{EKFEVILDVQQFS}_{89}$  motif, which corresponds to the intermediate filament interaction domain in  $\beta$ 3-strand of human  $\alpha$ B-crystallin,  $\alpha$ A-crystallin but also to *C. elegans* Hsp12.2 (Ghosh et al., 2006). Assuming that dCryAB contains an intermediate filament interaction domain, it could be well expected that the *Drosophila* cell should contain a protein partner to interact with this motif.

Except nuclear lamins, no gene encoding cytoplasmic filaments has been reported in *Drosophila* so far (Schulze et al. 2009). However, in 1981 Falkner et al. and subsequently Walter and Biessmann (1984) described two proteins (46 and 40 kDa) that appeared to form a very fine meshwork inside tissue culture cells and salivary gland cells and shared an epitope with the vertebrate intermediate filament protein vimentin (56 kDa) (Walter and Biessmann, 1984). These proteins contained antigenic determinants also found in vimentin and desmin of baby hamster kidney cells and authors suggested that the 46 kDa-sized protein was an invertebrate homologue of vimentin (Falkner et al., 1981; Walter and Biessmann, 1984). Knowing that  $\alpha$ B-crystallin shares a high homology to dCryAB and interacts with IFs, stabilising them, I succeeded in finding putative IFs in *Drosophila* muscle cells, which could be a target for dCryAB. Using antibody directed to the mouse vimentin, I stained and visualised in confocal microscope a very well organised meshwork inside muscle cells, resembling the organisation of vertebrate desmin filaments. Since it was visualised with anti-vimentin antibodies, I called it vimentin-like protein. On confocal longitudinal sections of third instar larval muscle fibres, the vimentin-like protein was situated around nuclei, co-localised with actin and dCryAB in Z-lines and was associated with the sarcolemma, which was observed as a dotted pattern above the sarcomeric part of the fibre. Iso-surface modelling showed that in sarcomeres, vimentin-like protein is situated particularly on the external part of the Z-line, and immunogold assays confirmed its localisation in Z-lines and around nuclei. This suggests that the vimentin-like protein may form filaments spread between the sarcolemma and nuclei, wrapping myofibrils at the height of the Z-lines, which is consistent with the distribution of desmin filaments in vertebrate muscle cells (Goldfarb and Dalakas, 2009).

Moreover, confocal projections confirmed by electron microscopy showed that the vimentin-like protein entered nuclei. It is believed that cytoplasmic IFs play a role in coordinating mechanical forces in embryonic development, growth and maturation of tissues. Vimentin is expressed in the precursor cells and in myoblasts becoming undetectable in adult skeletal muscles. Moreover, desmin and vimentin filaments enter nuclei, possibly affecting gene activity (Capetanaki et al., 2007; Goldfarb and Dalakas, 2009; Herrmann et al., 2007). Therefore, it is possible that the vimentin-like protein in *Drosophila* can also have mechanical influence on gene activity apart from its structural role, but this function has to be verified. Nevertheless, the identical position of dCryAB and the vimentin-like protein on the extremities of Z-lines and around nuclei, as well as the presence of intermediate filament domain in the dCryAB sequence, may indicate that dCryAB interacts with the vimentin-like protein, stabilising the formed network, like  $\alpha$ B-crystallin does with desmin and vimentin filaments (Goldfarb and Dalakas, 2009).

To verify if dCryAB interacts with the vimentin-like protein I observed third instar larval muscles overexpressing dCryAB fused with GFP protein. I visualised desirable proteins by appropriate antibodies in confocal microscope. I registered a normal distribution of dCryAB-GFP in sarcomeres but a surplus of dCryAB-GFP was clearly accumulated with the vimentin-like protein in the cytoplasm, especially around nuclei. This suggested that both proteins formed tight complexes, which was confirmed by the coIP assays. In Western blot immunoprecipitation I detected proteins with the size around 40kDa, 72kDa and 170kDa. I assume that the one with small molecular mass is the vimentin-like protein, which I detect in muscle cells. Moreover, this protein may correspond to the protein described by Falkner et al. (1981), which formed filamentous meshwork inside the *Drosophila* cells and shared the epitope with vertebrate intermediate filament proteins. Considering that the IF proteins form oligomers (Cooper, 2000) and interact with sHsps (Goldfarb and Dalakas, 2009), I suggest that detected higher mass proteins are simply two different and highly stable protein complexes formed by the vimentin-like protein subunits, possibly containing also dCryAB. Already in 1979, Kibbelaar et al. suggested possible interactions between calf eye lens actin, vimentin, and  $\alpha$ B-crystallin (Kibbelaar et al., 1979). Bennardini et al. (1992) investigated the role of  $\alpha$ B-crystallin in rat

cardiomyocytes and found that it was bound to actin and desmin filaments in Z-lines. Moreover, these interactions became even stronger during an ischemic episode, thus authors concluded that  $\alpha$ B-crystallin could stabilise the proper conformation of various filaments protecting them from rupture by mechanical damage and from degradation by oxidative or proteolytic processes, and thus preventing aggregation. Djabali et al. (1997), studying interactions between  $\alpha$ B-crystallin and IFs, showed by co-sedimentation assay that mouse and bovine  $\alpha$ B-crystallin interacts with peripherin and vimentin in the temperature dependent manner, where interactions between the tested proteins were increasing with the temperature. They also showed that in cultured fibroblasts,  $\alpha$ B-crystallin was expressed constitutively and distributed throughout the cell, with no specific subcellular localisation and did not overlap the vimentin network extending from the nuclear surface to the plasma membrane in normal growth conditions. Nevertheless, when cells were submitted to different stress conditions,  $\alpha$ B-crystallin appeared to be distributed along tracks coinciding with vimentin filaments (Djabali et al., 1997). Inconsistent with these results but consistent with mine was the report of Perng et al. (1999) who showed that Hsp27 and  $\alpha$ B-crystallin in cultured cells interacted with the IF network of both stressed and unstressed cells. They showed that Hsp27 associated with the full range of filament arrangements found in cells, from bundles to the very fine filament distributions, and this association was not restricted to any particular intermediate filament type, because it bound to type I and II keratins, as well as type III IFs, including vimentin and GFAP. They also found that these two sHsps formed mixed soluble complexes, suggesting that they shared at least some physiological targets and possible functions (Perng et al., 1999). All these results imply that sHsps, by interacting with IFs, help to maintain their homeostasis.

To ascertain if dCryAB was involved in maintaining structural integrity of somatic muscles by interacting with the vimentin-like protein, I checked if there were any morphological changes in muscle fibres, similar to those observed in vertebrates, when interactions between Hsps and IFs were impaired. To obtain desirable conditions I applied muscle-specific attenuation of *dcryAB* expression and observed muscle morphology of third instar larvae. Immunocytochemical reaction visualised in confocal microscope, and immunogold reaction visualised in

electron microscope revealed an altered, fuzzy and amorphous arrangement of the vimentin-like network in sarcomeres and around nuclei. Perng et al. (1999) showed that sHsps associated not only with filaments but also with the soluble fraction of intermediate filament proteins. This might help to maintain the intermediate filament protein in an assembly competent state. Moreover, IFs formed a gel in the absence of sHsps, whereas inclusion of  $\alpha$ B-crystallin or Hsp27 prevented the gel formation but not the filament assembly. Other studies showed that implication of desmin to the human adrenal carcinoma cell line, which normally does not express any cytoplasmic IFs, led to formation of cytoplasmic aggregates with some filaments and short rod-like structures in these cells, whereas transfection of these cells with desmin and wild type  $\alpha$ B-crystallin significantly decreased formation of cellular aggregates (Perng et al., 2004). These results suggest that *de novo* formation of the IF network involves sHsps which play a role in its further management. Thus, I assume that in wild type *Drosophila* muscle cells the vimentin-like meshwork is stabilised by dCryAB and the protein is involved in formation of the vimentin-like meshwork, whereas these functions are impaired by the dCryAB deficiency in cells.

Other observed morphological changes in muscle cells are related to disorganisation of cellular organelles and mechanical integration of the cell. It is well known that cytoplasmic IFs associate with the nuclear envelope and participate in shaping and positioning of nuclei. In 1994 Sarria et al. showed that human vimentin-null cultured cells or cells expressing mutant vimentin led in some cases to nuclear invaginations (Sarria et al., 1994). Shah et al. (2004) showed that in desmin-null mice nuclear shapes, as well as links to sarcomeres, were altered, indicating the role of desmin in organelles organisation (Shah et al. 2004). Also epidermis of the keratin-10-null mice was characterised by unordered spacing of enlarged nuclei (Reichelt and Magin, 2002), indicating the role of different kinds of IFs in the organelles orchestration. Gilbert et al. (2012) showed that cytoskeleton organisation affected by depletion of Hsp27 in HeLa cells also led to grave nucleus abnormalities, namely loosing the spherical shape (Gibert et al., 2012). Similarly, in my experiments, disrupting the vimentin-like network by attenuation of *dcryAB* expression led to altered positioning of nuclei, observed in confocal microscope as their irregular arrangement, being displaced to one side of

the cell. This provides evidence for the effect of the vimentin-like network on the shape and position of the nuclei. Desmin IFs also take part in stabilisation of mitochondrial morphology, their distribution in areas of high-energy demand and in their respiratory function via influencing mitochondrial membrane behaviour in muscles (Capetanaki et al., 2007; Rappaport et al., 1998). My results show that disruption of the vimentin-like network led to similar effects. Namely, mitochondrial morphology was damaged which was observed in electron microscope as swollen mitochondria with no cristae. This suggested that damaged mitochondria could be functionally impaired, which was then confirmed by Mitotracker reaction showing the presence of large numbers of respiratory inactive mitochondria in the intermyofibrillar area. Milner et al. (2000) have demonstrated that lack of desmin in cardiac and skeletal muscles led to mitochondrial swelling and degeneration of the mitochondrial matrix, as well as significant respiration impairment. The proper mitochondria distribution, number, morphology, and function was lost indicating that desmin IFs play a significant role in maintaining mitochondrial homeostasis (Milner et al., 2000). Kostareva et al. (2008) confirmed these findings by similar observations of mouse skeletal and cardiac muscles expressing desmin carrying L345P mutation, which led to desmin malformations characteristic for myopathies. Using electron microscopy they showed striking changes in mitochondria, including reduced density of cristae and significant vacuolization of the mitochondrial matrix, resulting in formation of circular membranous structures and giant mitochondria. Moreover, the motility of the tested mice was disturbed (Kostareva et al., 2008), which is consistent with results of behavioral assays of *Drosophila* larvae with attenuated *dcryAB* expression. Hnia et al. (2011) established that not only ablation of desmin filaments or its mutation leads to the collapse of organelles organisation but also absence of desmin-binding protein myotubularin can cause similar effects. Knockout or knockdown of myotubularin expression led to disruption of the myotubularin-desmin complex promoting desmin aggregation and in consequence, to abnormal mitochondrial positioning, morphology and dynamics (Hnia et al., 2011). The elevated glycogen deposits in muscle cells I observed in electron microscope might be related to the dysfunction of mitochondria. The glycogen storage was observed in different pathological cases of patients with Becker



muscular dystrophy (Rose et al., 1993) or ocular myopathy with altered mitochondria (Scelsi et al., 1984). Thus, I assume that disruption of the vimentin-like meshwork can lead to glycogen storage, caused by inability to use it by dysfunctional mitochondria. Moreover, I confirmed in electron microscope disarrangement of sarcomeres described above and observed as a fuzzy pattern of actin filaments and irregular Z-lines. This misalignment was most probably due to the lack of dCryAB causing disturbance of cytoskeleton organisation, either actin filaments (see above) or IF network. It is well known that IFs, especially desmin filaments, are responsible for supporting of myofibrils at the level of Z-line (Goldfarb and Dalakas, 2009). Desmin mutations or absence, which lead to its dysfunction are not lethal but disturb the organisation of Z-discs and costameres, affecting the integrity of the cellular IF network (Goldfarb et al., 1998; O'Neill et al., 2002). In more advanced stages, fragmented desmin filaments aggregate to form insoluble deposits, which can lead to disruption of myofibrils, breaking connections between them and impairing their binding to cellular organelles, causing myopathy (Goldfarb and Dalakas, 2009). These findings suggest that in *Drosophila* muscle cells the vimentin-like protein plays a role of IF proteins, including the role of desmin filaments in organisation of cellular organelles and stabilisation of myofibrils. Therefore I assume that the described conserved motif interacts with this invertebrate homologue of vimentin, which plays the role of intermediate filament protein in *Drosophila* muscle cells and that dCryAB, by this domain, integrates the putative intermediate filament meshwork, stabilising it.

As mentioned above, under *dcryAB* RNAi conditions I observed a lower number of nuclei in muscle cells, suggesting a smaller number of fusion events during muscle formation. The decreased number of fusion events could be caused by disturbance of either microfilament or intermediate filament network. As desmin deficiency blocks myoblast fusion (Li et al.) the impaired formation of the vimentin-like network also may have an impact on disturbed fusion process. Cytoplasmic IFs are also believed to play a role in coordinating mechanical forces in embryonic development, growth and maturation of tissues. Vimentin is expressed in precursor cells and in myoblasts and becomes undetectable in adult skeletal muscles (Capetanaki et al., 2007; Goldfarb and Dalakas, 2009; Herrmann et al., 2007). It is thus very likely that dCryAB may have an influence on muscle

growth and maturation not only through interactions with actin filaments but also through orchestration of putative IF network composed of the vimentin-like protein.

Assuming that sHsps not only stabilise the formed IF network but also play a role in its *de novo* formation (Perng et al., 1999), I checked if the primary misfolded vimentin-like network caused by dCryAB deficiency in muscle cells during development could be restored by restoration of dCryAB expression. As sHsps are very often expressed under stress conditions (Feder and Hofmann, 1999) and their expression is temperature-dependent (Djabali et al., 1997), to induce dCryAB expression I placed *dcryAB-RNAi* third instar larvae under heat shock conditions. Subsequently, after two hours of recovery, I observed an elevated level of dCryAB protein, which was visualised in muscle fibres on confocal sections. The protein was observed in sarcomeres and around nuclei and its increased expression was confirmed by the western blot assay. Nevertheless, the most important observation was the restoring of the vimentin-like meshwork sharp pattern in Z-lines and around nuclei, probably due to formation of normal interactions between the vimentin-like protein and dCryAB, confirming that dCryAB was required for its proper formation. High overexpression of dCryAB caused by heat shock led to the deposition of the protein surplus in the cytoplasmic area around the nuclei. The deposited dCryAB occurred together with the vimentin-like protein, resembling the accumulations of these two proteins in *UAS-dcryAB* larvae, suggesting that dCryAB could bind to the vimentin-like protein even after the muscle development had been completed. This is consistent with previous results of Djabali et al. (1997), showing that *in vivo* interaction between vimentin and  $\alpha$ B-crystallin was clearly detected when cells were submitted to different types of stress. Moreover, when cells were subjected to heat shock treatment, authors have observed a complete rearrangement of the vimentin network and  $\alpha$ B-crystallin positioning at the same localisation as vimentin. The recovery of the stressed cells led to redistribution of the vimentin filaments, which slowly re-extended through the cytoplasmic space (Djabali et al., 1997). Perng et al. (2004) showed that transfection of desmin protein into mouse lens cells resulted in a pronounced tendency to form aggregates, whereas cotransfection of desmin together with  $\alpha$ B-crystallin significantly reduced this tendency. Thus, I conclude that overexpression

of dCryAB protein provoked by heat shock is helpful in the vimentin-like network restoration and stabilisation.

Altogether, obtained results allow me to conclude that the vimentin-like protein is a putative *Drosophila* intermediate filament protein, taking over functions of vertebrate type III intermediate filaments. Moreover, dCryAB is required for the proper formation of the vimentin-like meshwork, and impaired interactions between them do not cause the vimentin-like protein to aggregate but cause changes in muscle morphology, sarcomeric pattern, morphology and functioning of some muscle organelles. Therefore, the primary task now is to identify the vimentin-like protein using mass spectrometry approach (work in progress). It would also be advisable to investigate the effect of loss of function of the intermediate filament domain of dCryAB on the vimentin-like meshwork. To answer this question I constructed flies containing *dcryAB* gene with inactivated intermediate filament binding domain and now the effect of this mutation needs to be verified.

### **5.3. *Drosophila* is a putative model organism to study DRM**

*Drosophila melanogaster* is a very well studied model organism for understanding molecular mechanisms of human diseases. Many discoveries in genetics were first developed in the fly and it was shown that a big variety of basic biological, physiological and neurological properties are conserved between *D. melanogaster* and mammals. The vast majority of genes involved in all aspects of *Drosophila* development were found to be essential also for normal mammalian development (Pandey and Nichols, 2011). Moreover, *D. melanogaster* was the first major complex organism having its genome sequenced (Adams, 2000), and nearly 75% of human disease-causing genes have functional homologues in *Drosophila* (Reiter et al., 2001). Even if the compared total identity of the fly and mammal protein sequence is usually approximately 40% between homologues at the nucleotide level of the conserved functional domains, it can rise up to 80 to 90% or even higher. Thus, the fruit fly can be considered as a multi-purpose model organism, with specific advantages to each developmental stage, where the embryo is often used in fundamental developmental studies, the larva frequently

serves to study developmental and physiological processes as well as some aspects of behaviour. The adult fly is used to understand functioning of the large range of fully developed organs, having their equivalents in mammals (Pandey and Nichols, 2011). Thanks to the development of a very wide range of tools to perform different genetic manipulations, *Drosophila* became a very important model organism, used to understand mechanisms of various mammalian diseases (Afsari et al., 2014; Bellen et al., 2010; Niwa and Niwa, 2011; Picchio et al., 2013). Therefore, in the light of my results it seems possible to consider *Drosophila* as a putative model of desmin-related myopathy.

In the course of some human diseases the IF network does not assemble correctly, and anomalous protein aggregates are formed inside the cells. Normally, in these pathological cases  $\alpha$ B-crystallin can reorganise such abnormal inclusion bodies into a normal filamentous network (Koyama and Goldman, 1999). However, some mutations in  $\alpha$ B-crystallin can lead to the loss of this chaperone activity, and up to date fifteen mutations of  $\alpha$ B-crystallin causing markedly different features were reported (Berry et al., 2001; Chen et al., 2009; Del Bigio et al., 2011; Devi et al., 2008; Forrest et al., 2011; Inagaki et al., 2006; Liu et al., 2006a; Liu et al., 2006b; Pilotto et al., 2006; Reilich et al., 2010; Sacconi et al., 2012; Safieh et al., 2009; Selcen and Engel, 2003; Vicart et al., 1998). The WT  $\alpha$ B-crystallin forms dimers (Bagn eris et al., 2009), and some of its mutations such as R120G (Vicart et al., 1998) or D109H are claimed to affect dimerisation of  $\alpha$ B-crystallin (Sacconi et al., 2012). These mutations lead to the loss of  $\alpha$ B-crystallin chaperone activity and ultimately to pathological muscle defects. Interestingly, protein sequence alignment revealed that both these residues (R120 and D109) were conserved in dCryAB, thus it was interesting to check if these particular sites had similar functions in both proteins. Therefore, by site directed mutagenesis I generated a *Drosophila* strain carrying a mutation in one of the *dcryAB* residues corresponding to the  *$\alpha$ B-crystallin<sup>R120G</sup>* mutation. In muscle cells of third instar larvae of *Drosophila* I observed in confocal microscope an irregular localisation of dCryAB<sup>R120G</sup>-GFP protein in sarcomeres. Namely, dCryAB<sup>R120G</sup>-GFP did not occur in sarcomeres of some muscle parts, which was associated with accumulations of actin without perceptible sarcomeric organisation. Considering that  $\alpha$ B-crystallin stabilises actin filaments (see above), it was not surprising that in muscle parts

lacking dCryAB the actin filament pattern was severely affected. Nevertheless, observation of the relatively normal sarcomeric organisation of actin filaments in muscle parts containing dCryAB<sup>R120G</sup>-GFP indicates that even its mutated form is sufficient to stabilise actin filaments in Z-lines. However, a closer confocal view of sarcomeres containing dCryAB<sup>R120G</sup>-GFP protein revealed its altered sarcomeric localisation compared to the wild type protein. In some groups of sarcomeres dCryAB<sup>R120G</sup>-GFP did not occur in Z-lines but appeared in the middle of the M-lines, whereas in other sarcomeres it was present in Z-lines but also accumulated on the edges of the I-band. In TEM I also observed a misalignment of Z-lines, typical for humans with DRM (Goldfarb and Dalakas, 2009) but also observed in mice (Wang et al., 2001). Wang et al. (2001) showed in ultrastructural analyses that in  $\alpha$ B-crystallin<sup>R120G</sup> transgenic mouse hearts the alignment of adjacent myofibrils at the Z-band was markedly perturbed. Golenhofen et al. (1999) studied  $\alpha$ B-crystallin binding to pig cardiac myofibrils under normal and stress conditions. They have shown that  $\alpha$ B-crystallin occurred in the soluble protein fraction of the cytoplasm of normal state cells, whereas during reversible damaging ischemia  $\alpha$ B-crystallin translocated completely to the Z-line area of myofibrils. Moreover, after irreversibly damaging ischemia  $\alpha$ B-crystallin was shifted from the Z-line to the I-band area. Authors also observed that the  $\alpha$ B-crystallin shift to the I-band correlated with displacement of the T12 epitope of titin from the vicinity of Z-lines into I-bands. They suggested that the primary binding sites for  $\alpha$ B-crystallin might also be located in juxtaposition to Z-lines and move into the I-bands during extreme sarcomeric stress. All these changes could be due to breakage of actin filaments at the Z-line level during overstretching and subsequent translocation of the filaments together with titin and  $\alpha$ B-crystallin towards I-bands by residual myosin function (Golenhofen et al., 1999). Similarly, in the *Drosophila* larval muscles alterations of the actin filament organisation caused by overexpression of the dCryAB<sup>R120G</sup>-GFP may explain shifting of  $\alpha$ B-crystallin into the I-bands, and all these irregularities indicate that mutation R120G of dCryAB as well as  $\alpha$ B-crystallin leads to reducing of their chaperone-like function in relation to actin filaments.

Ultrathin sections examined in TEM allowed observing changed mitochondrial morphology and glycogen deposits in muscle fibres, and confocal

projections visualised changed mitochondrial size, features similar to those observed in larval muscles expressing RNAi against dCryAB. Therefore, observed phenotypes could be explained by the loss of  $\alpha$ B-crystallin activity following the fact that the  $\alpha$ B-crystallin mutation R120G alters the dynamics of  $\alpha$ B-crystallin, causing a classical “loss of function” of the protein (Bagn ris et al., 2009). Moreover, described alterations, as well as the appearance of protein aggregates are known as typical signs observed on muscle biopsies from DRM patients (Goldfarb and Dalakas, 2009). In muscle cell of the third instar larvae of *Drosophila* in confocal microscope as well as on three-dimensional projections, I observed aggregates of dCryAB<sup>R120G</sup>-GFP mostly containing also the vimentin-like protein. Similar cytoplasmic or perinuclear aggregates were observed by Vicart et al. (1998) in two desmin-producing cell lines: C2.7 myoblasts and BHK 21 kidney smooth muscle cells transfected with  *$\alpha$ B-crystallin<sup>R120G</sup>* cDNA. Both cell lines contained  $\alpha$ B-crystallin- and desmin-labelled aggregates, which were not found in cells transfected with the wild-type  *$\alpha$ B-crystallin* cDNA. Wang et al. (2001) also observed desmin and  $\alpha$ B-crystallin-reactive aggregates in cardiomyocytes of transgenic mice carrying R120G mutation in  *$\alpha$ B-crystallin* gene. Moreover, in my studies, the vimentin-like network observed in confocal microscope in muscle cells containing dCryAB<sup>R120G</sup>-GFP protein lost its regularity, like it was observed in RNAi-*dcryAB* muscles. This is consistent with findings showing that expression of  *$\alpha$ B-crystallin<sup>R120G</sup>* led to a collapse of IF network in cells (Goldfarb et al., 1998). The muscle measurements (muscle size, number and length of sarcomeres, number of nuclei) showed that larvae expressing dCryAB<sup>R120G</sup>-GFP achieved muscle parameters comparable to those of larvae expressing RNAi against *dcryAB*, which again could be explained by the loss of dCryAB function. All these observations allow concluding that the expression of *dcryAB<sup>R120G</sup>-GFP* leads to formation of protein aggregates similar to those observed in mammalian cells in DRM. Moreover, it is very likely that the vimentin-like protein plays a role of desmin filaments in *Drosophila* muscle cells and the observed disturbances are the consequence of impaired vimentin-like network, caused by the lack or mutation of dCryAB.

I also noticed that in the behavioural assays the *Drosophila* larvae expressing *dcryAB<sup>R120G</sup>* had severe motility problems, confirmed by lower

contractility index, which could be the cause of muscle weakness, being a typical symptom of the DRM (Vicart et al., 1998). The DRM is a disease caused by mutations in *desmin* or  $\alpha$ B-crystallin genes, characterised by lower limb muscle weakness, slowly spreading and involving trunk, neck flexors, facial, bulbar and respiratory muscles. Symptoms observed in skeletal muscles are often associated with a cardiomyopathy, manifested by conduction blocks and arrhythmias but also with a cataract formation (Dalakas et al., 2000; Goldfarb and Dalakas, 2009; Goldfarb et al., 2004). Moreover, the adult flies had a shorter life span, which also is a consequence of DRM. The observed shorter life span is consistent with a recent study of Elliott et al. (2013) who found that alteration of specific interactions between  $\alpha$ B-crystallin and desmin filaments had an impact on filament aggregation and cell viability (Elliott et al., 2013). The second explanation for the shorter life span of larvae expressing dCryAB<sup>R120G</sup>-GFP may be its loss of ability to regulate apoptosis.  $\alpha$ B-crystallin is a negative regulator of apoptosis and when its mutated form is expressed, the cell viability becomes affected (Bova et al., 1999; Simon et al., 2007; Wettstein et al., 2012). Thus, it is possible that dCryAB also functions as an apoptosis regulator and may have a similar effect to its mammalian orthologue, but this conjecture requires further investigation. A third explanation for the shorter life span may be a cellular toxicity of observed aggregates, like in other diseases which present proteins accumulations (Der Perng et al., 2006).

Apart from the DRM, sHsps have a relationship to many other disorders, and such diseases may be associated with changes to sHsps occurring either as a result of mutation, posttranslational changes or changes in the amounts of sHsps. The latter are not accompanied by a structural change in the protein *per se* and are observed in cancers and neurological diseases such as Alzheimer's, Alexander's, Creutzfeldt-Jakob, amyotrophic lateral sclerosis, Parkinson's and multiple sclerosis. In different cancer types the sHsps role varies and while some studies show that elevated expression of Hsp27 indicates good prognosis, other results show that increased sHsps indicates aggressive tumor behaviour and rather poor prognosis. Moreover, sHsps modulate metastasis and tumor progression or even drug resistance in patients undergoing cancer chemotherapy. Most of mentioned neurological diseases are associated with a tissue-specific formation of protein aggregates termed amyloids, and these protein deposits may be harmful, beneficial

or of no consequence but very often are the markers for diagnosis (Sun and MacRae, 2005a). Diseases caused by posttranslational changes are provoked by truncation, deamidation, oxidation, glycation, phosphorylation or isomerization and lead to cataract. Mutations of sHsps also lead to cataract ( $\alpha$ A-crystallin<sup>R116C</sup>,  $\alpha$ A-crystallin<sup>R49C</sup>,  $\alpha$ B-crystallin<sup>R120G</sup>), desmin-related myopathy ( $\alpha$ B-crystallin<sup>R120G</sup>,  $\alpha$ B-crystallin<sup>DC13</sup>,  $\alpha$ B-crystallin<sup>DC25</sup>), desmin-related cardiomyopathy ( $\alpha$ B-crystallin<sup>R120G</sup>), distal hereditary motor neuropathies (Hsp25<sup>S135F</sup>, Hsp25<sup>R127W</sup>, Hsp25<sup>T151I</sup>, Hsp25<sup>P182L</sup>, Hsp22<sup>K141N</sup>, Hsp22<sup>K141E</sup>) and Charcot–Marie–Tooth disease (Hsp25<sup>S135F</sup>, Hsp25<sup>R136W</sup>, Hsp22<sup>K141N</sup>). The wide range of sHsp-related diseases shows the importance of further research on the mechanism of sHsp action. Thus, I introduced the human  $\alpha$ B-crystallin gene into the fly genome and observed in confocal microscope that its expression pattern in muscle cells was similar to the one in vertebrate cells. It was localised in the cytoplasm but was also incorporated in the vimentin-like network showing functional conservation of dCryAB and its partners. The next step would be to compare this expression to R120G mutated form of the  $\alpha$ B-crystallin implicated in *Drosophila* larval muscles with its WT and with dCryAB<sup>R120G</sup> (work in progress).

This work is the first study showing developmental importance of *Drosophila* sHsp, dCryAB. It demonstrates similarities between dCryAB and  $\alpha$ B-crystallin role in muscle formation but also that dCryAB interacts with the putative cytoplasmic intermediate filaments, which have not been described in *Drosophila* to date. It also shows that the muscle-specific expression of dCryAB carrying pathological R120G mutation results in DRM-like muscle defects. In future, *Drosophila* could be used as a new model to study DRM, helping to understand molecular mechanisms involved in its origin but also in drug screening. Moreover, establishing that *Drosophila* possesses putative cytoplasmic IFs gives wide perspectives to study many other IFs-related diseases.



## 6. Conclusions and future directions

*Drosophila* has been established as a model organism commonly used to study a wide variety of biological processes, including some diseases related to human. This study is an attempt at assessing the role of *dcryAB* gene, which is a human orthologue of  $\alpha B$ -crystallin, in muscle development and sarcomere stabilisation.

My studies revealed that the expression pattern of dCryAB protein was reminiscent of the  $\alpha B$ -crystallin localisation, suggesting that *dcryAB* might play conserved myogenic roles. Indeed, dCryAB is involved in muscle formation and may be directly or indirectly involved in promoting myoblast fusion events. Its attenuation specifically in mesoderm leads to impaired function of larval muscles and affects the *Drosophila* life span. Moreover, *Drosophila* expresses proteins, which correspond to the third class of vertebrate IFs and have a similar intracellular distribution as their vertebrate counterparts. I showed that dCryAB co-localised with the putative IFs in larval muscles, interacting with them, which is related to the fact that dCryAB plays an important role in maintaining a proper organisation of the vimentin-like network, and in ensuring a normal position and function of the investigated muscle components. It is noteworthy that dCryAB also participates in the formation of the described putative intermediate filament meshwork.

Mutation of dCryAB, where the equivalent arginine of position 120 in  $\alpha B$ -crystallin is replaced by glycine, mimics effects of mutations in human  $\alpha B$ -crystallin leading to the desmin-related myopathy. dCryAB<sup>R120G</sup> is delocalised, compared to the wild type form of the protein, and strongly binds to the vimentin-like protein to form aggregates in muscle fibres. Overexpression of *dcryAB*<sup>R120G</sup> causes loss of function of both proteins, leading to muscle defects and affected life span, which is similar to symptoms observed in DRM patients. Implicating human  $\alpha B$ -crystallin in the *Drosophila* genome leads to the expression of  $\alpha B$ -crystallin in muscle cells. Here,  $\alpha B$ -crystallin incorporates in dCryAB and the vimentin-like proteins, forming complexes with dCryAB as well as with the vimentin-like protein, suggesting that dCryAB shares with its human counterpart not only a

sequence homology but also its function. Thus, *Drosophila* may be a suitable model organism to study desmin-related myopathy.

Now it would be advisable to: 1) create a null mutant for *dcryAB*, to confirm its role in muscle cells; 2) confirm roles of dCryAB actin and IF binding domains by mutating them, provoking loss of their function in order to analyse specifically the requirements of those domains in muscle structure and function; 3) identify other proteins which dCryAB interacts with and which might have human orthologues; 4) identify the gene encoding the vimentin-like protein; and 5) assess the role of  $\alpha$ B-crystallin<sup>R120G</sup> in *Drosophila* muscle cells at the molecular level in order to get an insight into desmin-related myopathy. Such a complete set of analyses, started in the present study, should improve our knowledge of the mechanism of the disease.

## 7. References

- Adams, M.D. 2000. The Genome Sequence of *Drosophila melanogaster*. *Science*. 287:2185-2195.
- Afsari, F., K.V. Christensen, G.P. Smith, M. Hentzer, O.M. Nippe, C.J. Elliott, and A.R. Wade. 2014. Abnormal visual gain control in a Parkinson's Disease model. *Hum Mol Genet*:10.1093/hmg/ddu1159.
- Anesti, V., and L. Scorrano. 2006. The relationship between mitochondrial shape and function and the cytoskeleton. *Biochimica et biophysica acta*. 1757:692-699.
- Arrigo, A.P., S. Simon, B. Gibert, C. Kretz-Remy, M. Nivon, A. Czekalla, D. Guillet, M. Moulin, C. Diaz-Latoud, and P. Vicart. 2007. Hsp27 (HspB1) and alphaB-crystallin (HspB5) as therapeutic targets. *FEBS letters*. 581:3665-3674.
- Au, Y., R.A. Atkinson, R. Guerrini, G. Kelly, C. Joseph, S.R. Martin, F.W. Muskett, A. Pallavicini, G. Faulkner, and A. Pastore. 2004. Solution structure of ZASP PDZ domain; implications for sarcomere ultrastructure and enigma family redundancy. *Structure*. 12:611-622.
- Azpiazu, N., and M. Frasch. 1993. tinman and bagpipe: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes & Development*. 7:1325-1340.
- Bagn eris, C., O.A. Bateman, C.E. Naylor, N. Cronin, W.C. Boelens, N.H. Keep, and C. Slingsby. 2009. Crystal structures of alpha-crystallin domain dimers of alphaB-crystallin and Hsp20. *J Mol Biol*. 392:1242-1252.
- Bai, J., J.H. Hartwig, and N. Perrimon. 2007. SALS, a WH2-domain-containing protein, promotes sarcomeric actin filament elongation from pointed ends during *Drosophila* muscle growth. *Developmental cell*. 13:828-842.
- Bataille, L., I. Delon, J.P. Da Ponte, N.H. Brown, and K. Jagla. 2010. Downstream of identity genes: muscle-type-specific regulation of the fusion process. *Developmental cell*. 19:317-328.
- Bate, M. 1990a. The embryonic development of larval muscles in *Drosophila*. *Development*. 110:791-804.
- Bate, M. 1990b. The embryonic development of larval muscles in *Drosophila*. *Development*. 110:791-804.
- Baylies, M.K., and M. Bate. 1996. twist: a myogenic switch in *Drosophila*. *Science*. 272:1481-1484.
- Baylies, M.K., M. Bate, and M. Ruiz Gomez. 1998. Myogenesis: a view from *Drosophila*. *Cell*. 93:921-927.
- Beall, C.J., M.A. Sepanski, and E.A. Fyrberg. 1989. Genetic dissection of *Drosophila* myofibril formation: effects of actin and myosin heavy chain null alleles. *Genes & Development*. 3:131-140.
- Bellen, H.J., C. Tong, and H. Tsuda. 2010. 100 years of *Drosophila* research and its impact on vertebrate neuroscience: a history lesson for the future. *Nat Rev Neurosci*. 11:514-522.
- Benjamin, I.J., J. Shelton, D.J. Garry, and J.A. Richardson. 1997. Temporospatial expression of the small HSP/alpha B-crystallin in cardiac and skeletal muscle during mouse development. *Dev Dyn*. 208:75-84.
- Bennardini, F., A. Wrzosek, and M. Chiesi. 1992. Alpha B-crystallin in cardiac tissue. Association with actin and desmin filaments. *Circ Res*. 71:288-294.

- Berkes, C.A., and S.J. Tapscott. 2005. MyoD and the transcriptional control of myogenesis. *Semin Cell Dev Biol.* 16:585-595.
- Berry, V., P. Francis, M.A. Reddy, D. Collyer, E. Vithana, I. MacKay, G. Dawson, A.H. Carey, A. Moore, S.S. Bhattacharya, and R.A. Quinlan. 2001. Alpha-B crystallin gene (CRYAB) mutation causes dominant congenital posterior polar cataract in humans. *Am J Hum Genet.* 69:1141-1145.
- Bour, B.A., M. Chakravarti, J.M. West, and S.M. Abmayr. 2000. Drosophila SNS, a member of the immunoglobulin superfamily that is essential for myoblast fusion. *Genes Dev.* 14:1498-1511.
- Bova, M.P., O. Yaron, Q. Huang, L. Ding, D.A. Haley, P.L. Stewart, and J. Horwitz. 1999. Mutation R120G in alphaB-crystallin, which is linked to a desmin-related myopathy, results in an irregular structure and defective chaperone-like function. *Proceedings of the National Academy of Sciences of the United States of America.* 96:6137-6142.
- Brady, J.P., D.L. Garland, D.E. Green, E.R. Tamm, F.J. Giblin, and E.F. Wawrousek. 2001. AlphaB-crystallin in lens development and muscle integrity: a gene knockout approach. *Invest Ophthalmol Vis Sci.* 42:2924-2934.
- Budnik, V., M. Gorczyca, and A. Prokop. 2006. Selected Methods for the Anatomical Study of Drosophila Embryonic and Larval Neuromuscular Junctions. 75:323-365.
- Buff, E., A. Carmena, S. Gisselbrecht, F. Jiménez, and A.M. Michelson. 1998. Signalling by the Drosophila epidermal growth factor receptor is required for the specification and diversification of embryonic muscle progenitors. *Development.* 125:2075-2086.
- Capetanaki, Y. 2002. Desmin cytoskeleton: a potential regulator of muscle mitochondrial behavior and function. *Trends Cardiovasc Med.* 12:339-348.
- Capetanaki, Y., R.J. Bloch, A. Kouloumenta, M. Mavroidis, and S. Psarras. 2007. Muscle intermediate filaments and their links to membranes and membranous organelles. *Exp Cell Res.* 313:2063-2076.
- Capetanaki, Y., S. Starnes, and S. Smith. 1989. Expression of the chicken vimentin gene in transgenic mice: efficient assembly of the avian protein into the cytoskeleton. *PNAS.* 86:4882-4886.
- Capovilla, M., Z. Kambris, and J. Botas. 2001. Direct regulation of the muscle-identity gene *apterous* by a Hox protein in the somatic mesoderm. *Development.* 128:1221-1230.
- Carmena, A., M. Bate, and F. Jimenez. 1995. Lethal of scute, a proneural gene, participates in the specification of muscle progenitors during Drosophila embryogenesis. *Genes & Development.* 9:2373-2383.
- Carmena, A., E. Buff, M.S. Halfon, S. Gisselbrecht, F. Jimenez, M.K. Baylies, and A.M. Michelson. 2002. Reciprocal regulatory interactions between the Notch and Ras signaling pathways in the Drosophila embryonic mesoderm. *Developmental biology.* 244:226-242.
- Carmena, A., S. Gisselbrecht, J. Harrison, F. Jiménez, and A.M. Michelson. 1998a. Combinatorial signaling codes for the progressive determination of cell fates in the Drosophila embryonic mesoderm. *Genes Dev.* 12:3910-3922.
- Carmena, A., B. Murugasu-Oei, D. Menon, F. Jiménez, and W. Chia. 1998b. Inscuteable and numb mediate asymmetric muscle progenitor cell divisions during Drosophila myogenesis. *Genes Dev.* 12:304-315.

- Carra, S., A. Boncoraglio, B. Kanon, J.F. Brunsting, M. Minoia, A. Rana, M.J. Vos, K. Seidel, O.C. Sibon, and H.H. Kampinga. 2010. Identification of the *Drosophila* ortholog of HSPB8: implication of HSPB8 loss of function in protein folding diseases. *The Journal of biological chemistry*. 285:37811-37822.
- Chen, C.S., J. Tan, and J. Tien. 2004. Mechanotransduction at cell-matrix and cell-cell contacts. *Annual review of biomedical engineering*. 6:275-302.
- Chen, Q., J. Ma, M. Yan, M.E. Mthobi, Y. Liu, and F. Zheng. 2009. A novel mutation in CRYAB associated with autosomal dominant congenital nuclear cataract in a Chinese family. *Mol Vis*. 15:1359-1365.
- Clark, K.A., J.M. Bland, and M.C. Beckerle. 2007. The *Drosophila* muscle LIM protein, Mlp84B, cooperates with D-titin to maintain muscle structural integrity. *J Cell Sci*. 120:2066-2077.
- Colinet, H., S.F. Lee, and A. Hoffmann. 2010. Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult *Drosophila melanogaster*. *The FEBS journal*. 277:174-185.
- Cooke, P. 1976. A filamentous cytoskeleton in vertebrate smooth muscle fibers. *The Journal of cell biology*. 68:539-556.
- Cooper, G.M. 2000. *The Cell: A Molecular Approach*. 2nd edition. *Sinauer Associates*:<http://www.ncbi.nlm.nih.gov/books/NBK9839>.
- Corces, V., R. Holmgren, R. Freund, R. Morimoto, and M. Meselson. 1980. Four heat shock proteins of *Drosophila melanogaster* coded within a 12-kilobase region in chromosome subdivision 67B. *PNAS*. 77:5390-5393.
- Cutforth, T., and G.M. Rubin. 1994. Mutations in Hsp83 and cdc37 impair signaling by the sevenless receptor tyrosine kinase in *Drosophila*. *Cell*. 77:1027-1036.
- Dalakas, M.C., K.Y. Park, C. Semino-Mora, H.S. Lee, K. Sivakumar, and L.G. Goldfarb. 2000. Desmin myopathy, a skeletal myopathy with cardiomyopathy caused by mutations in the desmin gene. *N Engl J Med*. 342:770-780.
- Davidson, S.M., M.T. Loones, O. Duverger, and M. Morange. 2002. The developmental expression of small HSP. *Prog Mol Subcell Biol*. 28:103-128.
- Del Bigio, M.R., A.E. Chudley, H.B. Sarnat, C. Campbell, S. Goobie, B.N. Chodirker, and D. Selcen. 2011. Infantile muscular dystrophy in Canadian aboriginals is an alphaB-crystallinopathy. *Ann Neurol*. 69:866-871.
- Demontis, F., and N. Perrimon. 2009. Integration of Insulin receptor/Foxo signaling and dMyc activity during muscle growth regulates body size in *Drosophila*. *Development*. 136:983-993.
- Der Perng, M., M. Su, S.F. Wen, R. Li, T. Gibbon, A.R. Prescott, M. Brenner, and R.A. Quinlan. 2006. The Alexander disease-causing glial fibrillary acidic protein mutant, R416W, accumulates into Rosenthal fibers by a pathway that involves filament aggregation and the association of alpha B-crystallin and HSP27. *Am J Hum Genet*. 79:197-213.
- Devi, R.R., W. Yao, P. Vijayalakshmi, Y.V. Sergeev, P. Sundaresan, and J.F. Hejtmancik. 2008. Crystallin gene mutations in Indian families with inherited pediatric cataract. *Mol Vis*. 14:1157-1170.
- Djabali, K., B. de Néchaud, F. Landon, and M.M. Portier. 1997. AlphaB-crystallin interacts with intermediate filaments in response to stress. *J Cell Sci*. 110:2759-2769.
- Duan, H., J.B. Skeath, and H.T. Nguyen. 2001. *Drosophila* Lamé duck, a novel member of the Gli superfamily, acts as a key regulator of myogenesis by

- controlling fusion-competent myoblast development. *Development*. 128:4489-4500.
- Duan, R., and P.J. Gallagher. 2009. Dependence of myoblast fusion on a cortical actin wall and nonmuscle myosin IIA. *Developmental biology*. 325:374-385.
- Dubin, R.A., A.H. Ally, S. Chung, and J. Piatigorsky. 1990. Human alpha B-crystallin gene and preferential promoter function in lens. *Genomics*. 7:594-601.
- Ehler, E., B.M. Rothen, S.P. Hämmerle, M. Komiyama, and J.C. Perriard. 1999. Myofibrillogenesis in the developing chicken heart: assembly of Z-disk, M-line and the thick filaments. *J Cell Sci*. 112:1529-1539.
- Elliott, J.L., M. Der Perng, A.R. Prescott, K.A. Jansen, G.H. Koenderink, and R.A. Quinlan. 2013. The specificity of the interaction between alphaB-crystallin and desmin filaments and its impact on filament aggregation and cell viability. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*. 368:20120375.
- Eriksson, J.E., T. Dechat, B. Grin, B. Helfand, M. Mendez, H.M. Pallari, and R.D. Goldman. 2009. Introducing intermediate filaments: from discovery to disease. *The Journal of clinical investigation*. 119:1763-1771.
- Farah, C.S., and F.C. Reinach. 1995. The troponin complex and regulation of muscle contraction. *FASEB J*. 9:755-767.
- Feder, M.E., and G.E. Hofmann. 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol*. 61:243-282.
- Fischer, R.S., and V.M. Fowler. 2003. Tropomodulins: life at the slow end. *Trends in Cell Biology*. 13:593-601.
- Forrest, K.M., S. Al-Sarraj, C. Sewry, S. Buk, S.V. Tan, M. Pitt, A. Durward, M. McDougall, M. Irving, M.G. Hanna, E. Matthews, A. Sarkozy, J. Hudson, R. Barresi, K. Bushby, H. Jungbluth, and E. Wraige. 2011. Infantile onset myofibrillar myopathy due to recessive CRYAB mutations. *Neuromuscular disorders : NMD*. 21:37-40.
- Franke, W.W., E. Schmid, M. Osborn, and K. Weber. 1978. Different intermediate-sized filaments distinguished by immunofluorescence microscopy. *PNAS*. 75:5034-5038.
- Fyrberg, C., A. Ketchum, E. Ball, and E. Fyrberg. 1998. Characterization of lethal *Drosophila melanogaster* alpha-actinin mutants. *Biochem Genet*. 36:299-310.
- Gard, D.L., P.B. Bell, and E. Lazarides. 1979. Coexistence of desmin and the fibroblastic intermediate filament subunit in muscle and nonmuscle cells: identification and comparative peptide analysis. *PNAS*. 76:3894-3898.
- Ghosh, J.G., M.R. Estrada, S.A. Houck, and J.I. Clark. 2006. The function of the  $\beta 3$  interactive domain in the small heat shock protein and molecular chaperone, human  $\alpha B$  crystallin. *Cell Stress Chaperones*. 11:187-197.
- Gibert, B., B. Eckel, L. Fasquelle, M. Moulin, F. Bouhallier, V. Gonin, G. Mellier, S. Simon, C. Kretz-Remy, A.P. Arrigo, and C. Diaz-Latoud. 2012. Knock down of heat shock protein 27 (HspB1) induces degradation of several putative client proteins. *PloS one*. 7:e29719.
- Goebel, H.H. 1995. Desmin-related neuromuscular disorders. *Muscle Nerve*. 18:1306-1320.

- Goldfarb, L.G., and M.C. Dalakas. 2009. Tragedy in a heartbeat: malfunctioning desmin causes skeletal and cardiac muscle disease. *The Journal of clinical investigation*. 119:1806-1813.
- Goldfarb, L.G., K.Y. Park, L. Cervenáková, S. Gorokhova, H.S. Lee, O. Vasconcelos, J.W. Nagle, C. Semino-Mora, K. Sivakumar, and M.C. Dalakas. 1998. Missense mutations in desmin associated with familial cardiac and skeletal myopathy. *Nat Genet*. 19:402-403.
- Goldfarb, L.G., P. Vicart, H.H. Goebel, and M.C. Dalakas. 2004. Desmin myopathy. *Brain*. 127:723-734.
- Goldman, R.D. 1971. The role of three cytoplasmic fibers in BHK-21 cell motility. I. Microtubules and the effects of colchicine. *The Journal of cell biology*. 51:752-762.
- Goldman, R.D., Y.H. Chou, V. Prahlad, and M. Yoon. 1999. Intermediate filaments: dynamic processes regulating their assembly, motility, and interactions with other cytoskeletal systems. *FASEB J*. 13:S261-265.
- Golenhofen, N., P. Htun, W. Ness, R. Koob, W. Schaper, and D. Drenckhahn. 1999. Binding of the stress protein alpha B-crystallin to cardiac myofibrils correlates with the degree of myocardial damage during ischemia/reperfusion in vivo. *J Mol Cell Cardiol*. 31:569-580.
- Granger, B.L., and E. Lazarides. 1979. Desmin and vimentin coexist at the periphery of the myofibril Z disc. *Cell*. 18:1053-1063.
- Granger, B.L., and E. Lazarides. 1980. Synemin: a new high molecular weight protein associated with desmin and vimentin filaments in muscle. *Cell*. 22:727-738.
- Gretchen, A., and J.O. Vigoreaux. 2003. Flightin is a myosin rod binding protein. *Cell Biochem Biophys*. 38:41-54.
- Haass, C., U. Klein, and P.M. Kloetzel. 1990. Developmental expression of Drosophila melanogaster small heat-shock proteins. *J Cell Sci*. 96:413-418.
- Hakeda, S., S. Endo, and K. Saigo. 2000. Requirements of kettin, a giant muscle protein highly conserved in overall structure in evolution, for normal muscle function, viability, and flight activity of Drosophila. *The Journal of cell biology*. 148:101-114.
- Hao, X., S. Zhang, B. Timakov, and P. Zhang. 2007. The Hsp27 gene is not required for Drosophila development but its activity is associated with starvation resistance. *Cell stress & chaperones*. 12:364-372.
- Hartl, F.U. 1996. Molecular chaperones in cellular protein folding. *Nature*. 381:571-579.
- Haslbeck, M. 2002. sHsps and their role in the chaperone network. *Cellular and molecular life sciences : CMLS*. 59:1649-1657.
- Herrmann, H., H. Bar, L. Kreplak, S.V. Strelkov, and U. Aebi. 2007. Intermediate filaments: from cell architecture to nanomechanics. *Nat Rev Mol Cell Biol*. 8:562-573.
- Hnia, K., H. Tronchere, K.K. Tomczak, L. Amoasii, P. Schultz, A.H. Beggs, B. Payrastre, J.L. Mandel, and J. Laporte. 2011. Myotubularin controls desmin intermediate filament architecture and mitochondrial dynamics in human and mouse skeletal muscle. *The Journal of clinical investigation*. 121:70-85.
- Holtzer, H., T. Hijikata, Z.X. Lin, Z.Q. Zhang, S. Holtzer, F. Protasi, C. Franzini-Armstrong, and H.L. Sweeney. 1997. Independent assembly of 1.6 microns long bipolar MHC filaments and I-Z-I bodies. *Cell Struct Funct*. 22:83-93.

- Hooper, S.L., K.H. Hobbs, and J.B. Thuma. 2008. Invertebrate muscles: thin and thick filament structure; molecular basis of contraction and its regulation, catch and asynchronous muscle. *Progress in neurobiology*. 86:72-127.
- Hooper, S.L., and J.B. Thuma. 2005. Invertebrate muscles: muscle specific genes and proteins. *Physiol Rev*. 85:1001-1060.
- Houck, S.A., A. Landsbury, J. Clark, and R.A. Quinlan. 2011a. Multiple sites in  $\alpha$ B-crystallin modulate its interactions with desmin filaments assembled in vitro. *PloS one*. 6:e25859.
- Houck, S.A., A. Landsbury, J.I. Clark, and R.A. Quinlan. 2011b. Multiple sites in  $\alpha$ B-crystallin modulate its interactions with desmin filaments assembled in vitro. *PloS one*. 6:e25859.
- Hyder, C.L., H.M. Pallari, V. Kochin, and J.E. Eriksson. 2008. Providing cellular signposts--post-translational modifications of intermediate filaments. *FEBS letters*. 582:2140-2148.
- Inagaki, N., T. Hayashi, T. Arimura, Y. Koga, M. Takahashi, H. Shibata, K. Teraoka, T. Chikamori, A. Yamashina, and A. Kimura. 2006. Alpha B-crystallin mutation in dilated cardiomyopathy. *Biochemical and biophysical research communications*. 342:379-386.
- Inaguma, Y., S. Goto, H. Shinohara, K. Hasegawa, K. Ohshima, and K. Kato. 1993. Physiological and pathological changes in levels of the two small stress proteins, HSP27 and alpha B crystallin, in rat hindlimb muscles. *J Biochem*. 114:378-384.
- Ingolia, T.D., and C.E. A. 1982. Four small Drosophila heat shock proteins are related to each other and to mammalian alpha-crystallin. *Proc Natl Acad Sci*. 79:2360-2364.
- Ip, Y.T., R.E. Park, D. Kosman, K. Yazdanbakhsh, and M. Levine. 1992. dorsal-twist interactions establish snail expression in the presumptive mesoderm of the Drosophila embryo. *Genes & Development*. 6:1518-1530.
- Ishikawa, H., R. Bischoff, and H. Holtzer. 1968. Mitosis and intermediate-sized filaments in developing skeletal muscle. *The Journal of cell biology*. 38:538-555.
- Ivaska, J., H.M. Pallari, J. Nevo, and J.E. Eriksson. 2007. Novel functions of vimentin in cell adhesion, migration, and signaling. *Exp Cell Res*. 313:2050-2062.
- Jagla, T., Y. Bidet, J.P. Da Ponte, B. Dastugue, and K. Jagla. 2002. Cross-repressive interactions of identity genes are essential for proper specification of cardiac and muscular fates in Drosophila. *Development*. 129:1037-1047.
- Jani, K., and F. Schock. 2007. Zasp is required for the assembly of functional integrin adhesion sites. *The Journal of cell biology*. 179:1583-1597.
- Joanisse, D.R., S. Michaud, Y. Inaguma, and R.M. Tanguay. 1998. The small heat shock proteins of Drosophila: Developmental expression and functions. *J. Biosci*. 23:369-376.
- Kachinsky, A.M., J.A. Dominov, and J.B. Miller. 1994. Myogenesis and the intermediate filament protein, nestin. *Developmental biology*. 165:216-228.
- Karlik, C.C., and E.A. Fyrberg. 1986. Two Drosophila melanogaster tropomyosin genes: structural and functional aspects. *Mol Cell Biol*. 6:1965-1973.
- Kazemi-Esfarjani, P. 2000. Genetic Suppression of Polyglutamine Toxicity in Drosophila. *Science*. 287:1837-1840.
- Kibbelaar, M.A., A.M. Selten-Versteegen, I. Dunia, E.L. Benedetti, and H. Bloemendal. 1979. Actin in mammalian lens. *Eur J Biochem*. 95:543-549.



- Kim, K.K., R. Kim, and S.-H. Kim. 1998. Crystal structure of a small heat-shock protein. *Nature*. 394:595-599.
- Klemenz, R., A.C. Andres, E. Fröhli, R. Schäfer, and A. Aoyama. 1993. Expression of the murine small heat shock proteins hsp 25 and alpha B crystallin in the absence of stress. *The Journal of cell biology*. 120:639-645.
- Knirr, S., N. Azpiazu, and M. Frasch. 1999. The role of the NK-homeobox gene slouch (S59) in somatic muscle patterning. *Development*. 126:4525-4535.
- Konieczny, P., P. Fuchs, S. Reipert, W.S. Kunz, A. Zeold, I. Fischer, D. Paulin, R. Schroder, and G. Wiche. 2008. Myofiber integrity depends on desmin network targeting to Z-disks and costameres via distinct plectin isoforms. *The Journal of cell biology*. 181:667-681.
- Kostareva, A., G. Sjöberg, J. Bruton, S.J. Zhang, J. Balogh, A. Gudkova, B. Hedberg, L. Edstrom, H. Westerblad, and T. Sejersen. 2008. Mice expressing L345P mutant desmin exhibit morphological and functional changes of skeletal and cardiac mitochondria. *Journal of muscle research and cell motility*. 29:25-36.
- Koyama, Y., and J.E. Goldman. 1999. Formation of GFAP cytoplasmic inclusions in astrocytes and their disaggregation by alphaB-crystallin. *Am J Pathol*. 154:1563-1572.
- Kulke, M., C. Neagoe, B. Kolmerer, A. Minajeva, H. Hinssen, B. Bullard, and W.A. Linke. 2001. Kettin, a major source of myofibrillar stiffness in Drosophila indirect flight muscle. *The Journal of cell biology*. 154:1045-1057.
- Kurzik-Dumke, U., and E. Lohmann. 1995. Sequence of the new Drosophila melanogaster small heat-shock-related gene, lethal(2) essential for life [(2)efl], at locus 59F4,5. *Gene*. 154:171-175.
- Leptin, M., and B. Grunewald. 1990. Cell shape changes during gastrulation in Drosophila. *Development*. 110:73-84.
- Liem, R.K., and A. Messing. 2009. Dysfunctions of neuronal and glial intermediate filaments in disease. *The Journal of clinical investigation*. 119:1814-1824.
- Lin, C., X. Guo, S. Lange, J. Liu, K. Ouyang, X. Yin, L. Jiang, Y. Cai, Y. Mu, F. Sheikh, S. Ye, J. Chen, Y. Ke, and H. Cheng. 2013. Cypher/ZASP is a novel A-kinase anchoring protein. *The Journal of biological chemistry*. 288:29403-29413.
- Lindquist, S., and E.A. Craig. 1988. The heat-shock proteins. *Annu Rev Genet*. 22:631-677.
- Liu, M., T. Ke, Z. Wang, Q. Yang, W. Chang, F. Jiang, Z. Tang, H. Li, X. Ren, X. Wang, T. Wang, Q. Li, J. Yang, J. Liu, and Q.K. Wang. 2006a. Identification of a CRYAB mutation associated with autosomal dominant posterior polar cataract in a Chinese family. *Invest Ophthalmol Vis Sci*. 47:3461-3466.
- Liu, Y., X. Zhang, L. Luo, M. Wu, R. Zeng, G. Cheng, B. Hu, B. Liu, J.J. Liang, and F. Shang. 2006b. A novel alphaB-crystallin mutation associated with autosomal dominant congenital lamellar cataract. *Invest Ophthalmol Vis Sci*. 47:1069-1075.
- Loones, M.T., and M. Morange. 1998. Hsp and chaperone distribution during endochondral bone development in mouse embryo. *Cell stress & chaperones*. 3:237-244.
- LoRusso, S.M., D. Rhee, J.M. Sanger, and J.W. Sanger. 1997. Premyofibrils in spreading adult cardiomyocytes in tissue culture: evidence for reexpression of the embryonic program for myofibrillogenesis in adult cells. *Cell Motil Cytoskeleton*. 37:183-198.

- Maloyan, A., A. Sanbe, H. Osinska, M. Westfall, D. Robinson, K. Imahashi, E. Murphy, and J. Robbins. 2005. Mitochondrial dysfunction and apoptosis underlie the pathogenic process in alpha-B-crystallin desmin-related cardiomyopathy. *Circulation*. 112:3451-3461.
- Mardahl-Dumesnil, M., and V.M. Fowler. 2001. Thin filaments elongate from their pointed ends during myofibril assembly in *Drosophila* indirect flight muscle. *The Journal of cell biology*. 155:1043-1053.
- Marin, R., and R.M. Tanguay. 1996. Stage-specific localization of the small heat shock protein Hsp27 during oogenesis in *Drosophila melanogaster*. *Chromosoma*. 105:142-149.
- Marvin, M., D. O'Rourke, T. Kurihara, C.E. Juliano, K.L. Harrison, and L.D. Hutson. 2008. Developmental expression patterns of the zebrafish small heat shock proteins. *Developmental Dynamics*. 237:454-463.
- Mermelstein, C.S., L.R. Andrade, D.M. Portilho, and M.L. Costa. 2006. Desmin filaments are stably associated with the outer nuclear surface in chick myoblasts. *Cell and tissue research*. 323:351-357.
- Michaud, S., R. Marin, and R.M. Tanguay. 1997. Regulation of heat shock gene induction and expression during *Drosophila* development. *Cellular and molecular life sciences : CMLS*. 53:104-113.
- Michaud, S., and R.M. Tanguay. 2003. Expression of the Hsp23 chaperone during *Drosophila* embryogenesis: association to distinct neural and glial lineages. *BMC Developmental Biology*. 3.
- Michelson, A.M., S. Gisselbrecht, Y. Zhou, K.H. Baek, and E. Buff. 1998. Dual functions of the heartless fibroblast growth factor receptor in development of the *Drosophila* embryonic mesoderm. *Dev Genet*. 22:212-229.
- Middleton, R.C., and E.A. Shelden. 2013. Small heat shock protein HSPB1 regulates growth of embryonic zebrafish craniofacial muscles. *Exp Cell Res*. 319:860-874.
- Milner, D.J., M. Mavroidis, N. Weisleder, and Y. Capetanaki. 2000. Desmin cytoskeleton linked to muscle mitochondrial distribution and respiratory function. *J Cell Sci*. 150:1283-1298.
- Milner, D.J., G. Weitzer, D. Tran, A. Bradley, and Y. Capetanaki. 1996. Disruption of muscle architecture and myocardial degeneration in mice lacking desmin. *The Journal of cell biology*. 134:1255-1270.
- Montagna, G.N., K. Matuschewski, and C.A. Buscaglia. 2012. Small heat shock proteins in cellular adhesion and migration: evidence from *Plasmodium* genetics. *Cell Adh Migr*. 6:78-84.
- Morimoto, R.I. 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes & Development*. 12:3788-3796.
- Morrow, G., S. Battistini, P. Zhang, and R.M. Tanguay. 2004. Decreased lifespan in the absence of expression of the mitochondrial small heat shock protein Hsp22 in *Drosophila*. *The Journal of biological chemistry*. 279:43382-43385.
- Morrow, G., and R.M. Tanguay. 2003. Heat shock proteins and aging in *Drosophila melanogaster*. *Seminars in Cell & Developmental Biology*. 14:291-299.
- Morrow, G., and R.M. Tanguay. 2012. Small heat shock protein expression and functions during development. *The international journal of biochemistry & cell biology*. 44:1613-1621.

- Mounier, N., and A.P. Arrigo. 2002. Actin cytoskeleton and small heat shock proteins: how do they interact? *Cell stress & chaperones*. 7:167-176.
- Mudher, A., D. Shepherd, T.A. Newman, P. Mildren, J.P. Jukes, A. Squire, A. Mears, J.A. Drummond, S. Berg, D. MacKay, A.A. Asuni, R. Bhat, and S. Lovestone. 2004. GSK-3beta inhibition reverses axonal transport defects and behavioural phenotypes in *Drosophila*. *Molecular psychiatry*. 9:522-530.
- Mymrikov, E.V., A.S. Seit-Nebi, and G.N. B. 2010. Large potentials of small heat shock proteins. *Physiol Rev*. 91:1123-1159.
- Newey, S.E., E.V. Howman, C.P. Ponting, M.A. Benson, R. Nawrotzki, N.Y. Loh, K.E. Davies, and D.J. Blake. 2001. Syncoilin, a novel member of the intermediate filament superfamily that interacts with alpha-dystrobrevin in skeletal muscle. *The Journal of biological chemistry*. 276:6645-6655.
- Nicholl, I.D., and R.A. Quinlan. 1994. Chaperone activity of alpha-crystallins modulates intermediate filament assembly. *The EMBO journal*. 13: 945-953.
- Niwa, R., and Y.S. Niwa. 2011. The Fruit Fly *Drosophila melanogaster* as a Model System to Study Cholesterol Metabolism and Homeostasis. *Cholesterol*. 2011:176802.
- O'Neill, A., M.W. Williams, W.G. Resneck, D.J. Milner, Y. Capetanaki, and R.J. Bloch. 2002. Sarcolemmal organization in skeletal muscle lacking desmin: evidence for cytokeratins associated with the membrane skeleton at costameres. *Molecular biology of the cell*. 13:2347-2359.
- Omary, M.B. 2009. "IF-pathies": a broad spectrum of intermediate filament-associated diseases. *The Journal of clinical investigation*. 119:1756-1762.
- Omary, M.B., N.O. Ku, P. Strnad, and S. Hanada. 2009. Toward unraveling the complexity of simple epithelial keratins in human disease. *The Journal of clinical investigation*. 119:1794-1805.
- Pandey, U.B., and C.D. Nichols. 2011. Human disease models in *Drosophila melanogaster* and the role of the fly in therapeutic drug discovery. *Pharmacological reviews*. 63:411-436.
- Perng, M.D., L. Cairns, P. van den IJssel, A. Prescott, A.M. Hutcheson, and R.A. Quinlan. 1999. Intermediate filament interactions can be altered by HSP27 and alphaB-crystallin. *J Cell Sci*. 112:2099-2112.
- Perng, M.D., M. Su, S. Wen Fang, R. Li, T. Gibbon, A.R. Prescott, M. Brenner, and R.A. Quinlan. 2006. The Alexander Disease-Causing Glial Fibrillary Acidic Protein Mutant, R416W, Accumulates into Rosenthal Fibers by a Pathway That Involves Filament Aggregation and the Association of  $\alpha$ B-Crystallin and HSP27. *Am J Hum Genet*. 79:197-213.
- Perng, M.D., S.F. Wen, I.P. van den, A.R. Prescott, and R.A. Quinlan. 2004. Desmin aggregate formation by R120G alphaB-crystallin is caused by altered filament interactions and is dependent upon network status in cells. *Molecular biology of the cell*. 15:2335-2346.
- Picchio, L., E. Plantie, Y. Renaud, P. Poovthumkadavil, and K. Jagla. 2013. Novel *Drosophila* model of myotonic dystrophy type 1: phenotypic characterization and genome-wide view of altered gene expression. *Hum Mol Genet*. 22:2795-2810.
- Pilotto, A., N. Marziliano, M. Pasotti, M. Grasso, A.M. Costante, and E. Arbustini. 2006. alphaB-crystallin mutation in dilated cardiomyopathies: low prevalence in a consecutive series of 200 unrelated probands. *Biochemical and biophysical research communications*. 346:1115-1117.

- Prahlad, V., M. Yoon, R.D. Moir, R.D. Vale, and R.D. Goldman. 1998. Rapid movements of vimentin on microtubule tracks: kinesin-dependent assembly of intermediate filament networks. *The Journal of cell biology*. 143:159-170.
- Price, M.G., and E. Lazarides. 1983. Expression of intermediate filament-associated proteins paranemin and synemin in chicken development. *The Journal of cell biology*. 97:1860-1874.
- Rappaport, L., P. Oliviero, and J.L. Samuel. 1998. Cytoskeleton and mitochondrial morphology and function. *Mol Cell Biochem*. 184:101-105.
- Reedy, M.C., and C. Beall. 1993. Ultrastructure of developing flight muscle in *Drosophila*. II. Formation of the myotendon junction. *Developmental biology*. 160:466-479.
- Reichelt, J., and T.M. Magin. 2002. Hyperproliferation, induction of c-Myc and 14-3-3sigma, but no cell fragility in keratin-10-null mice. *J Cell Sci*. 115:2639-2650.
- Reilich, P., B. Schoser, N. Schramm, S. Krause, J. Schessl, W. Kress, J. Muller-Hocker, M.C. Walter, and H. Lochmuller. 2010. The p.G154S mutation of the alpha-B crystallin gene (CRYAB) causes late-onset distal myopathy. *Neuromuscular disorders : NMD*. 20:255-259.
- Reiter, L.T., L. Potocki, S. Chien, M. Gribskov, and E. Bier. 2001. A systematic analysis of human disease-associated gene sequences in *Drosophila melanogaster*. *Genome research*. 11:1114-1125.
- Reynolds, E. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *The Journal of cell biology*. 17:208-212.
- Richardson, B., K. Beckett, and M. Baylies. 2008. Visualizing new dimensions in *Drosophila* myoblast fusion. *BioEssays : news and reviews in molecular, cellular and developmental biology*. 30:423-431.
- Riechmann, V., U. Irion, R. Wilson, R. Grosskortenhaus, and M. Leptin. 1997. Control of cell fates and segmentation in the *Drosophila* mesoderm. *Development*. 124:2915-2922.
- Roper, K., Y. Mao, and N.H. Brown. 2005. Contribution of sequence variation in *Drosophila* actins to their incorporation into actin-based structures in vivo. *J Cell Sci*. 118:3937-3948.
- Rose, M.R., R.S. Howard, S.A. Genet, C.J. McMahon, A. Whitfield, and J.A. Morgan-Hughes. 1993. A case of myopathy associated with a dystrophin gene deletion and abnormal glycogen storage. *Muscle Nerve*. 16:57-62.
- Rui, Y., J. Bai, and N. Perrimon. 2010. Sarcomere formation occurs by the assembly of multiple latent protein complexes. *PLoS genetics*. 6:e1001208.
- Ruiz Gómez, M., and M. Bate. 1997. Segregation of myogenic lineages in *Drosophila* requires numb. *Development*. 124:4857-4866.
- Ruiz Gómez, M., N. Coutts, A. Price, M.V. Taylor, and M. Bate. 2000. *Drosophila* dumbfounded: a myoblast attractant essential for fusion. *Cell*. 102:189-198.
- Rushton, E., R. Drysdale, S.M. Abmayr, A.M. Michelson, and M. Bate. 1995. Mutations in a novel gene, myoblast city, provide evidence in support of the founder cell hypothesis for *Drosophila* muscle development. *Development*. 121:1979-1988.
- Sacconi, S., L. Feasson, J.C. Antoine, C. Pecheux, R. Bernard, A.M. Cobo, A. Casarin, L. Salviati, C. Desnuelle, and A. Urtizbera. 2012. A novel CRYAB mutation

- resulting in multisystemic disease. *Neuromuscular disorders : NMD*. 22:66-72.
- Safieh, L.A., A.O. Khan, and F.S. Alkuraya. 2009. Identification of a novel CRYAB mutation associated with autosomal recessive juvenile cataract in a Saudi family. *Mol Vis*. 15:980-984.
- Sandmann, T., C. Girardot, M. Brehme, W. Tongprasit, V. Stolc, and E.E. Furlong. 2007. A core transcriptional network for early mesoderm development in *Drosophila melanogaster*. *Genes Dev*. 21:436-449.
- Sarria, A.J., J.G. Lieber, S.K. Nordeen, and R.M. Evans. 1994. The presence or absence of a vimentin-type intermediate filament network affects the shape of the nucleus in human SW-13 cells. *J Cell Sci*. 107:1593-1607
- Sawicki, W. 2009. Histologia. *Wydawnictwo Lekarskie PZWL. Warszawa*. V:241-251.
- Scelsi, R., C. Marchetti, L. Faggi, G. Sandrini, and B. Rocchelli. 1984. An ocular myopathy with glycogen storage and abnormal mitochondria in muscle fibres. Histochemical and ultrastructural findings. *Eur Neuro*. 20:440-444.
- Schejter, E.D., and M.K. Baylies. 2010. Born to run: creating the muscle fiber. *Current opinion in cell biology*. 22:566-574.
- Schmitt, F.O. 1968. Fibrous proteins--neuronal organelles. *PNAS*. 60:1092-1101.
- Schnorrer, F., I. Kalchhauser, and B.J. Dickson. 2007. The transmembrane protein Kon-tiki couples to Dgrip to mediate myotube targeting in *Drosophila*. *Developmental cell*. 12:751-766.
- Schulze, S.R., B. Curio-Penny, S. Speese, G. Dialynas, D.E. Cryderman, C.W. McDonough, D. Nalbant, M. Petersen, V. Budnik, P.K. Geyer, and L.L. Wallrath. 2009. A comparative study of *Drosophila* and human A-type lamins. *PLoS one*. 4:e7564.
- Schweitzer, S.C., M.W. Klymkowsky, R.M. Bellin, R.M. Robson, Y. Capetanaki, and R.M. Evans. 2001. Paranemin and the organization of desmin filament networks. *J Cell Sci*. 114:1079-1089.
- Selcen, D., and A.G. Engel. 2003. Myofibrillar myopathy caused by novel dominant negative alpha B-crystallin mutations. *Ann Neurol*. 54:804-810.
- Shah, S., J. Davis, N. Weisleder, I. Kostavassili, A.D. McCulloch, E. Ralston, Y. Capetanaki, and R.L. Lieber. 2004. Structural and Functional Roles of Desmin in Mouse Skeletal Muscle during Passive Deformation. *Biophys J*. 86:2993-3008.
- Simon, S., J.M. Fontaine, J.L. Martin, X. Sun, A.D. Hoppe, M.J. Welsh, R. Benndorf, and P. Vicart. 2007. Myopathy-associated alphaB-crystallin mutants: abnormal phosphorylation, intracellular location, and interactions with other small heat shock proteins. *The Journal of biological chemistry*. 282:34276-34287.
- Sorensen, J.G., T.N. Kristensen, and V. Loeschcke. 2003. The evolutionary and ecological role of heat shock proteins. *Ecology Letters*. 6:1025-1037.
- Sparrow, J.C., and F. Schöck. 2009. The initial steps of myofibril assembly: integrins pave the way. *Nat Rev Mol Cell Biol*. 10:293-298.
- Starger, J.M., W.E. Brown, A.E. Goldman, and R.D. Goldman. 1978. Biochemical and immunological analysis of rapidly purified 10-nm filaments from baby hamster kidney (BHK-21) cells. *The Journal of cell biology*. 78:93-109.
- Steinert, P.M., and D.R. Roop. 1988. Molecular and cellular biology of intermediate filaments. *Annu. Rev. Biochem*. 57:593-625.

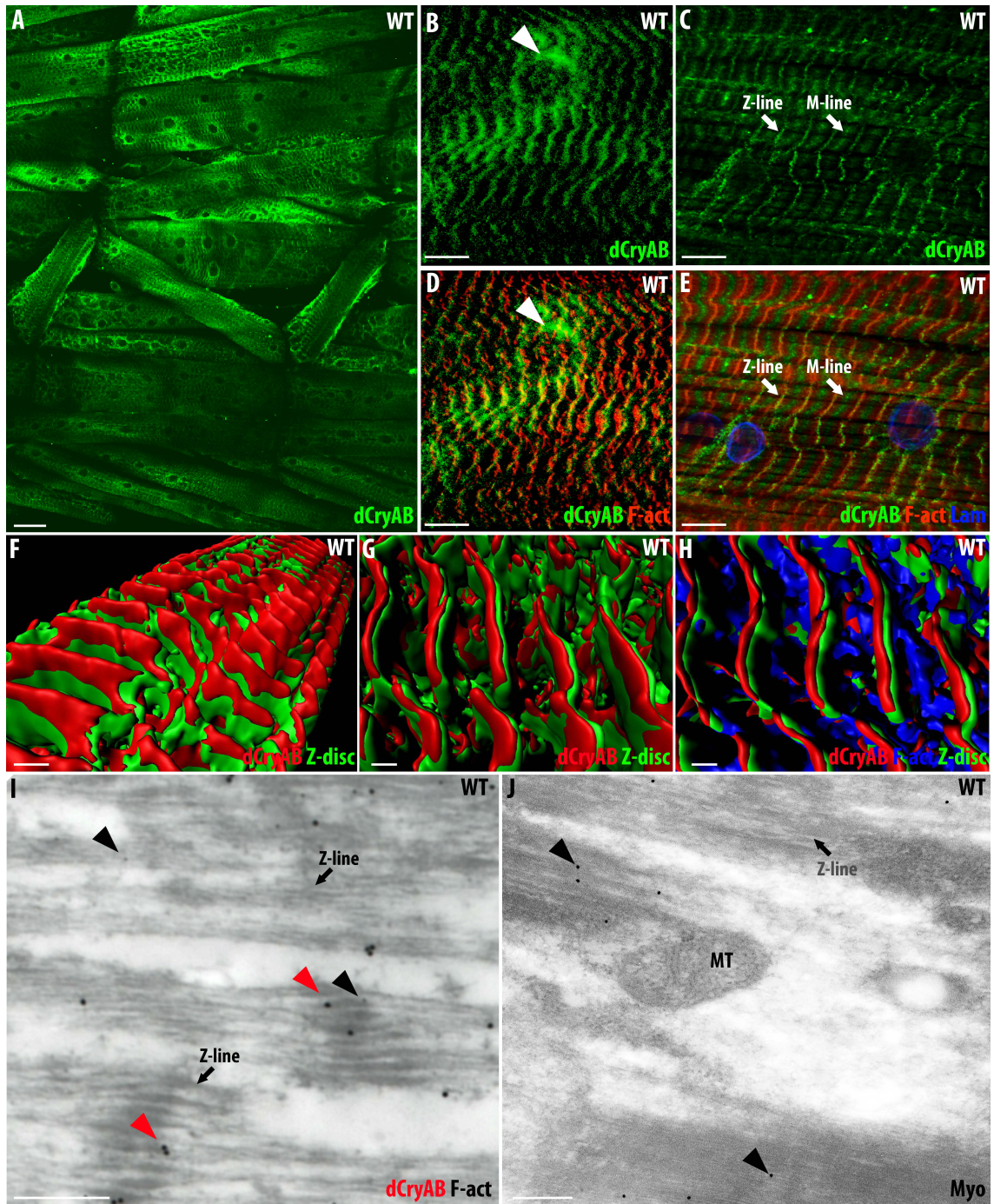
- Stone, M.R., A. O'Neill, R.M. Lovering, J. Strong, W.G. Resneck, P.W. Reed, D.M. Toivola, J.A. Ursitti, M.B. Omary, and R.J. Bloch. 2007. Absence of keratin 19 in mice causes skeletal myopathy with mitochondrial and sarcolemmal reorganization. *J Cell Sci.* 120:3999-4008.
- Strünkelberg, M., B. Bonengel, L.M. Moda, A. Hertenstein, H.G. de Couet, R.G. Ramos, and K.F. Fischbach. 2001. *rst* and its paralogue *kirre* act redundantly during embryonic muscle development in *Drosophila*. *Development.* 128:4229-4239.
- Stuurman, N., S. Heins, and U. Aebi. 1998. Nuclear lamins: their structure, assembly, and interactions. *J Struct Biol.* 122:42-66.
- Sugiyama, Y. 2000. Muscle Develops a Specific Form of Small Heat Shock Protein Complex Composed of MKBP/HSPB2 and HSPB3 during Myogenic Differentiation. *Journal of Biological Chemistry.* 275:1095-1104.
- Sun, Y., and T.H. MacRae. 2005a. The small heat shock proteins and their role in human disease. *The FEBS journal.* 272:2613-2627.
- Sun, Y., and T.H. MacRae. 2005b. Small heat shock proteins: molecular structure and chaperone function. *Cellular and molecular life sciences : CMLS.* 62:2460-2476.
- Tallot, P., J.F. Grongnet, and J.C. David. 2003. Dual perinatal and developmental expression of the small heat shock proteins [FC12]αB-crystallin and Hsp27 in different tissues of the developing piglet. *Biol Neonate.* 83:281-288.
- Taylor, M. 2006. Comparison of Muscle Development in *Drosophila* and Vertebrates. *Muscle development in Drosophila.* Helen Sink.:169-190.
- Thisse, B., C. Stoetzel, C. Gorostiza-Thisse, and F. Perrin-Schmitt. 1988. Sequence of the *twist* gene and nuclear localization of its protein in endomesodermal cells of early *Drosophila* embryos. *EMBO J.* 7:2175-2183.
- Timakov, B., and P. Zhang. 2001. The *hsp60B* gene of *Drosophila melanogaster* is essential for the spermatid individualization process. *Cell Stress Chaperones.* 6:71-77.
- Tissières, A., H.K. Mitchell, and U.M. Tracy. 1974. Protein synthesis in salivary glands of *Drosophila melanogaster*: Relation to chromosome puffs. *Journal of Molecular Biology.* 84:389-392.
- Tixier, V., L. Bataillé, and K. Jagla. 2010. Diversification of muscle types: recent insights from *Drosophila*. *Exp Cell Res.* 316:3019-3027.
- Vicart, P., A. Caron, P. Guicheney, Z. Li, M.C. Prévost, A. Faure, D. Chateau, F. Chapon, F. Tomé, J.M. Dupret, D. Paulin, and M. Fardeau. 1998. A missense mutation in the αB-crystallin chaperone gene causes a desmin-related myopathy. *Nat Genet.* 20:92-95.
- Volk, T., L.I. Fessler, and J.H. Fessler. 1990. A role for integrin in the formation of sarcomeric cytoarchitecture. *Cell.* 63:525-536.
- Walter, M.F., and H. Biessmann. 1984. A monoclonal antibody that detects vimentin-related proteins in invertebrates. *Molecular and Cellular Biochemistry.* 60:99-108.
- Wang, M.C., D. Bohmann, and H. Jasper. 2005. JNK extends life span and limits growth by antagonizing cellular and organism-wide responses to insulin signaling. *Cell.* 121:115-125.
- Wang, X., H. Osinska, R. Klevitsky, A.M. Gerdes, M. Nieman, J. Lorenz, T. Hewett, and J. Robbins. 2001. Expression of R120G- B-Crystallin Causes Aberrant

- Desmin and B-Crystallin Aggregation and Cardiomyopathy in Mice. *Circulation Research*. 89:84-91.
- Welsh, M.J., W. Wu, M. Parvinen, and R.R. Gilmont. 1996. Variation in expression of hsp27 messenger ribonucleic acid during the cycle of the seminiferous epithelium and co-localization of hsp27 and microfilaments in Sertoli cells of the rat. *Biol Reprod*. 55:141-151.
- Wettstein, G., P.S. Bellaye, O. Micheau, and P. Bonniaud. 2012. Small heat shock proteins and the cytoskeleton: an essential interplay for cell integrity? *The international journal of biochemistry & cell biology*. 44:1680-1686.
- Zackroff, R.V., and R.D. Goldman. 1980. In vitro reassembly of squid brain intermediate filaments (neurofilaments): purification by assembly-disassembly. *Science*. 208:1152-1155.
- Zhang, Y., D. Featherstone, W. Davis, E. Rushton, and K. Broadie. 2000. Drosophila D-titin is required for myoblast fusion and skeletal muscle striation. *J Cell Sci*. 113:3103-3115.

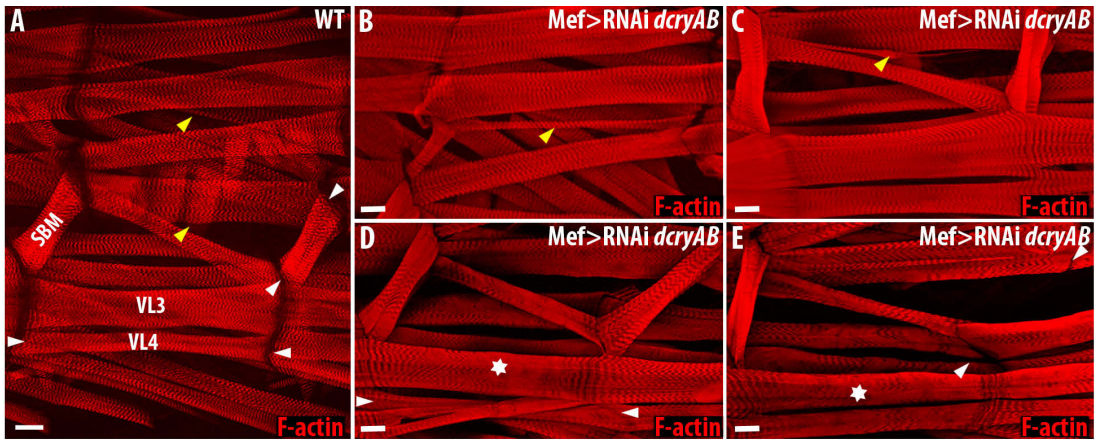
## **8. Documentation**



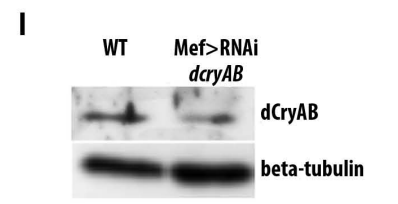
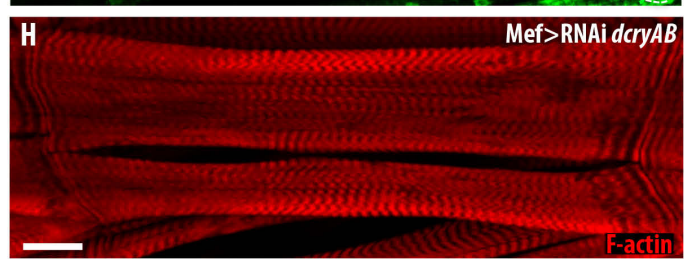
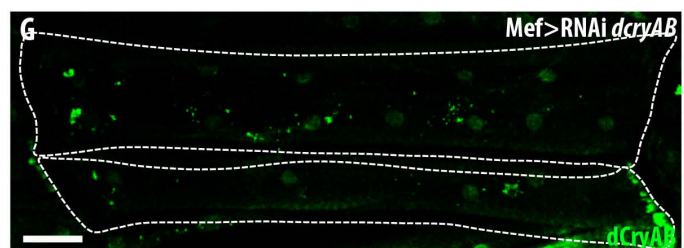
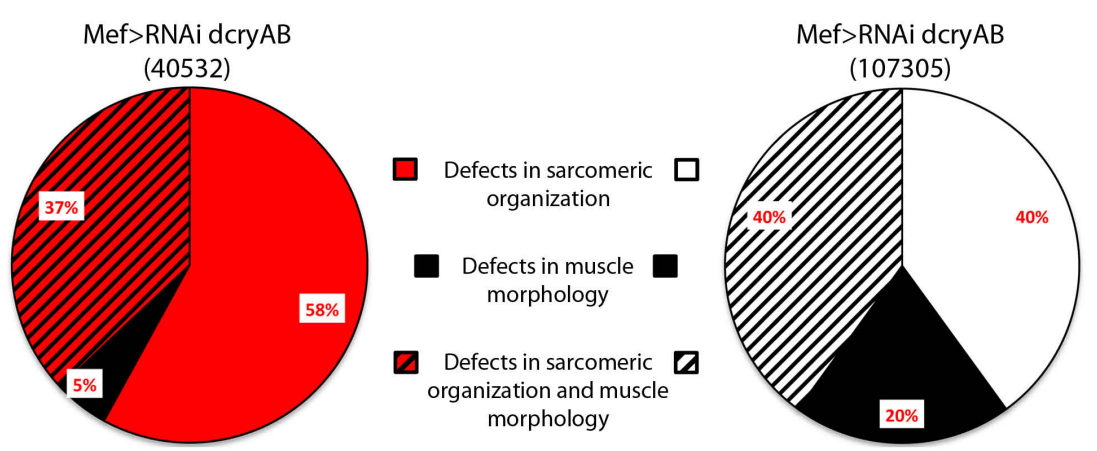
**Fig. 4.1.1. dCryAB localisation in the third instar WT larval body wall muscles.** Immunocytochemical reaction, visualised in confocal microscope show that dCryAB protein is expressed in all the muscles of abdominal hemisegment of the WT *D. melanogaster* 3<sup>rd</sup> instar larva **(A)**. dCryAB is localised in Z-lines (arrows), M-lines (arrows) and perinuclear areas (arrowheads), inside of the muscle fibre **(B-E)**. Three-dimensional modelling of larval muscle fibre, previously stained with adequate antibodies and visualised in the confocal microscope, show dCryAB situated at the surface of sarcomeres **(F-H)**. Ultrathin sections, stained in the immunogold reaction and observed in TEM show dCryAB at the Z-line area (red arrowheads) in the neighbourhood of F-actin filaments, pointed by black arrowheads **(I)** and myosin filaments **(J)**. MT-mitochondrion. Scale bars: 50  $\mu\text{m}$  (A), 20  $\mu\text{m}$  (B-E), 5  $\mu\text{m}$  (F-H), 0.4  $\mu\text{m}$  (I-J).



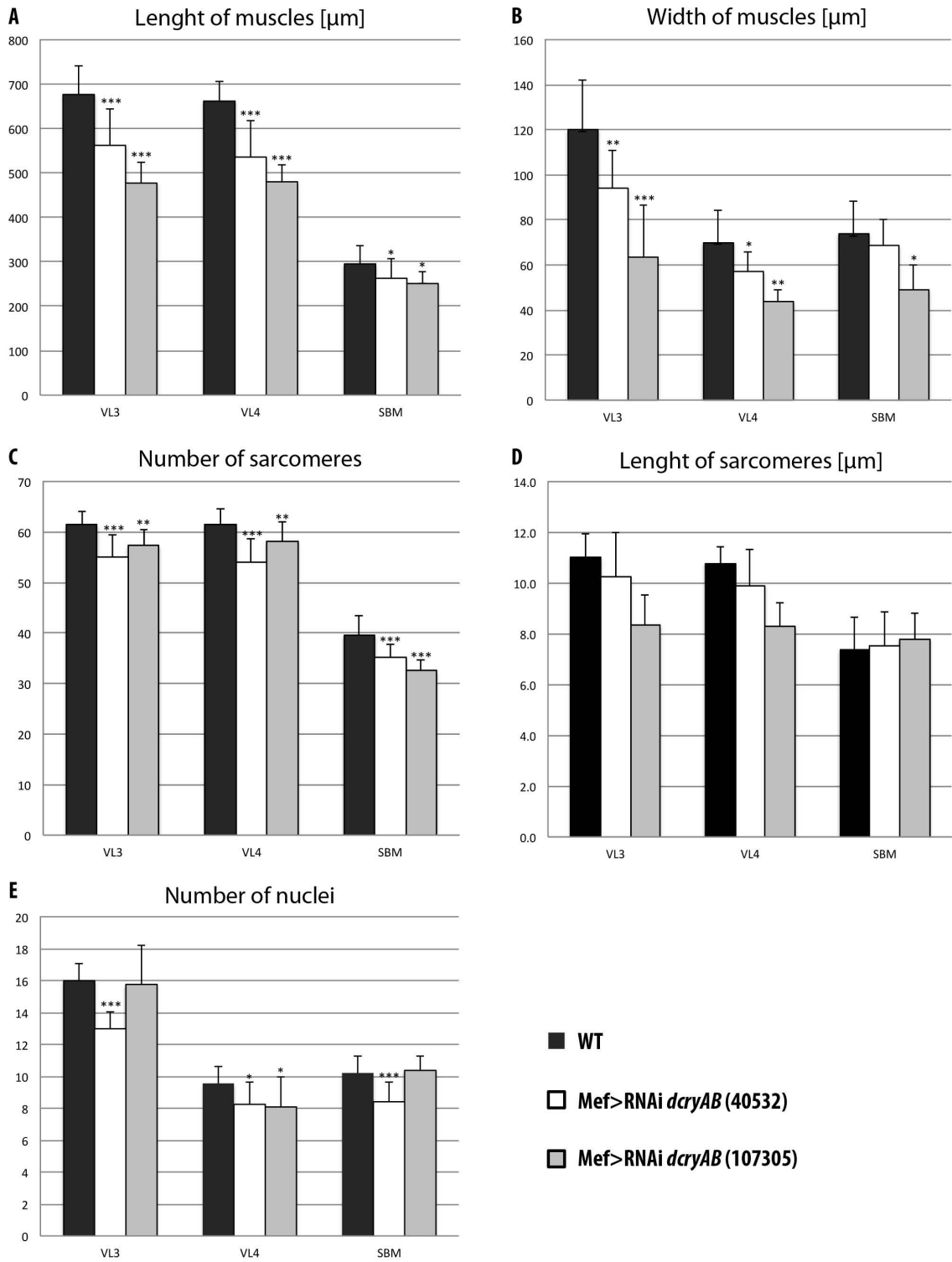
**Fig. 4.2.1. Developmental effects of *dcryAB* knockdown in third instar larva muscles.** Normal shape of muscle fibres and correct sarcomere organisation in a wild type stereotypical abdominal hemisegment of *D. melanogaster* 3<sup>rd</sup> instar larva **(A)**. RNAi *dcryAB* knockdown leads to muscle splitting **(B-C, arrowheads)** and changing attachment sites **(D, arrowheads)** or lack of some muscles **(E, arrowheads)** Arrowheads on the image presenting WT muscles correspond to the same muscles, pointed by arrowheads on images showing *Mef>RNAi dCryAB* muscles. Some of the muscles show misarranged, pinched sarcomere pattern, pointed by asterisks **(D, E)**. Distribution of observed muscle and sarcomere defects in muscles expressing RNAi against *dcryAB* **(F)**. Decreased dCryAB expression level in muscle fibres VL3 and VL4, which contours are marked by a dashed line **(G)**. The same muscles preserve expression level of F-actin **(H)**. Western Blot confirms lower level of dCryAB protein in *Mef>RNAi dcryAB* line **(I)**. Muscles on images A-E and H were stained with phalloidin. All the muscle projections were obtained in the confocal microscope. Scale bars: 50  $\mu$ m (A-E, G-H).



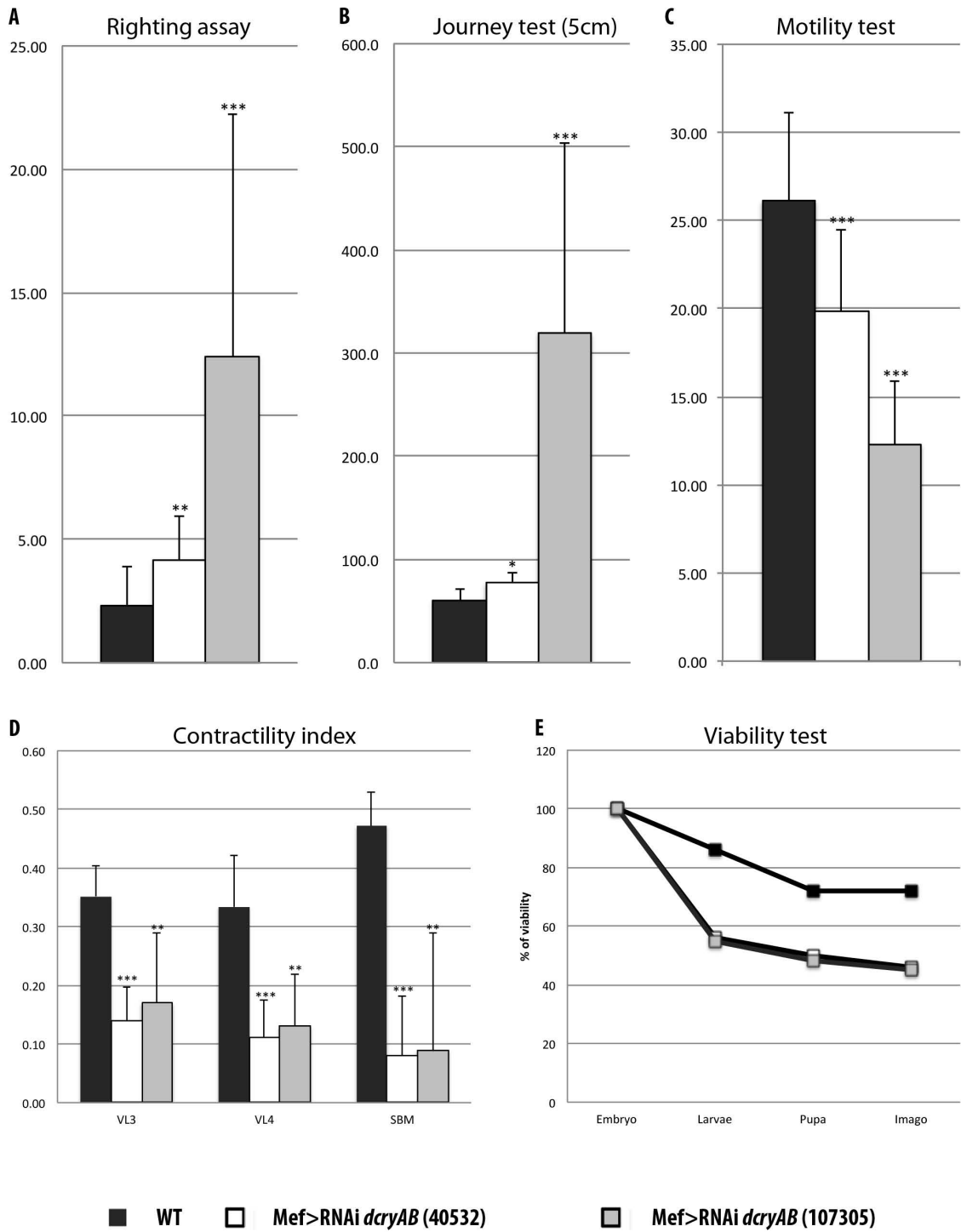
**F** Muscle defects in the third instar larva



**Fig. 4.2.2. Developmental effects of the *dcryAB* knockdown on the third instar larval muscle morphology.** Diagrams A-E present results of adequate muscle measurements. Muscle measurements show that muscles with attenuated *dcryAB* expression have smaller length **(A)**, width **(B)**, number of sarcomeres **(C)**, insignificantly reduced sarcomere size **(D)** and contain lower number of nuclei **(E)**, comparing to the WT. Asterisks indicate statistical level of significance of observed differences: \* $p < 0.1$ , \*\* $p < 0.05$ , \*\*\* $p < 0.001$ .

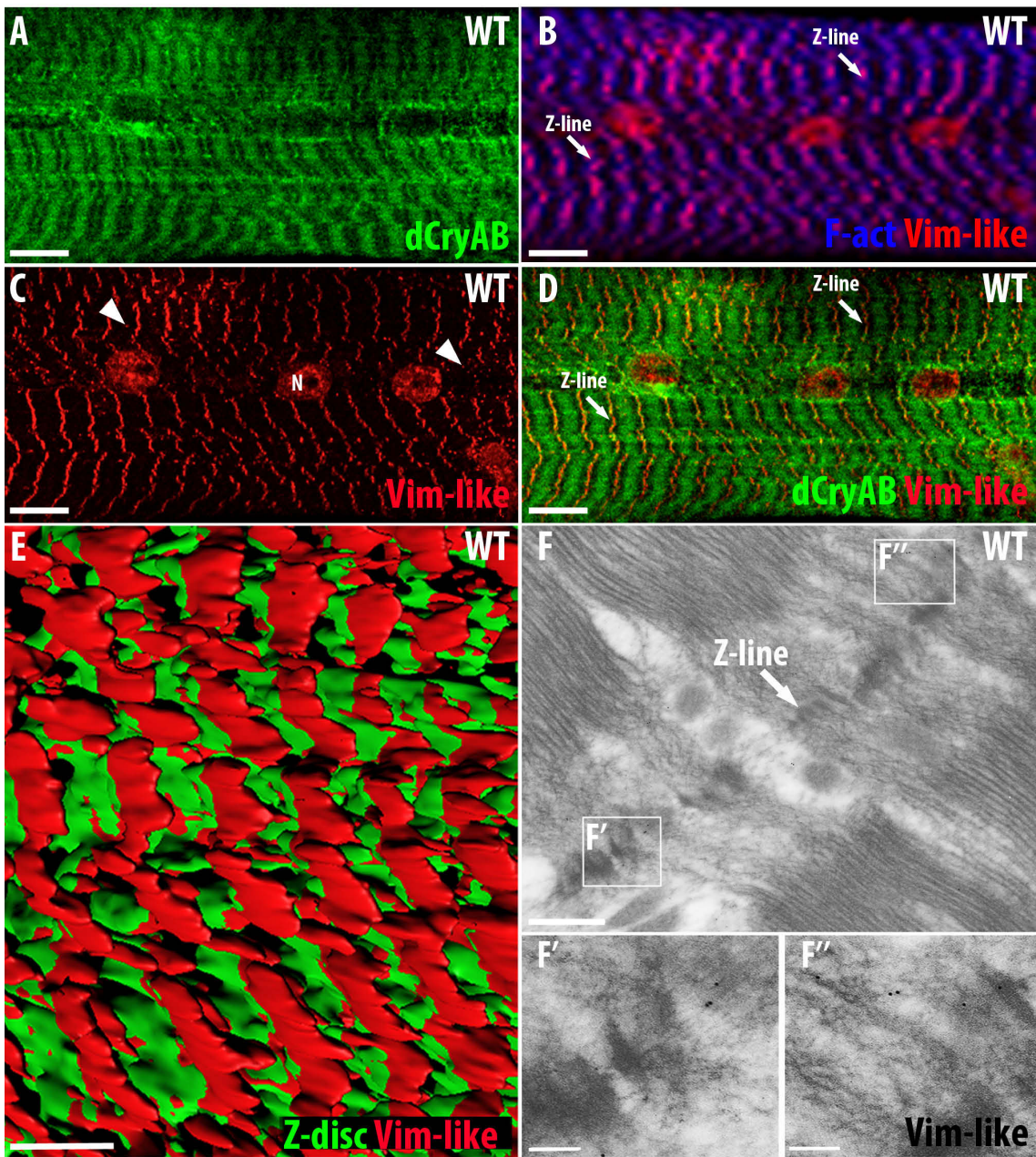


**Fig. 4.3.1. Effects of the *dcryAB* knockdown on the third instar larva motility and life span.** Behavioural assays indicate that movement ability of Mef>RNAi *dCryAB* larvae are significantly affected. They need significantly more time to revert their body in the righting assay **(A)**, to crawl 5 cm the distance in the journey test **(B)** and execute less peristaltic movements during the time of 30 seconds **(C)**. Muscles of the RNAi *dCryAB* larvae are characterised by lower contractility index **(D)** and Mef>RNAi *dCryAB* flies show higher mortality rate **(E)**. Asterisks indicate statistical level of significance of observed differences: \* $p < 0.1$ , \*\* $p < 0.05$ , \*\*\* $p < 0.001$ .

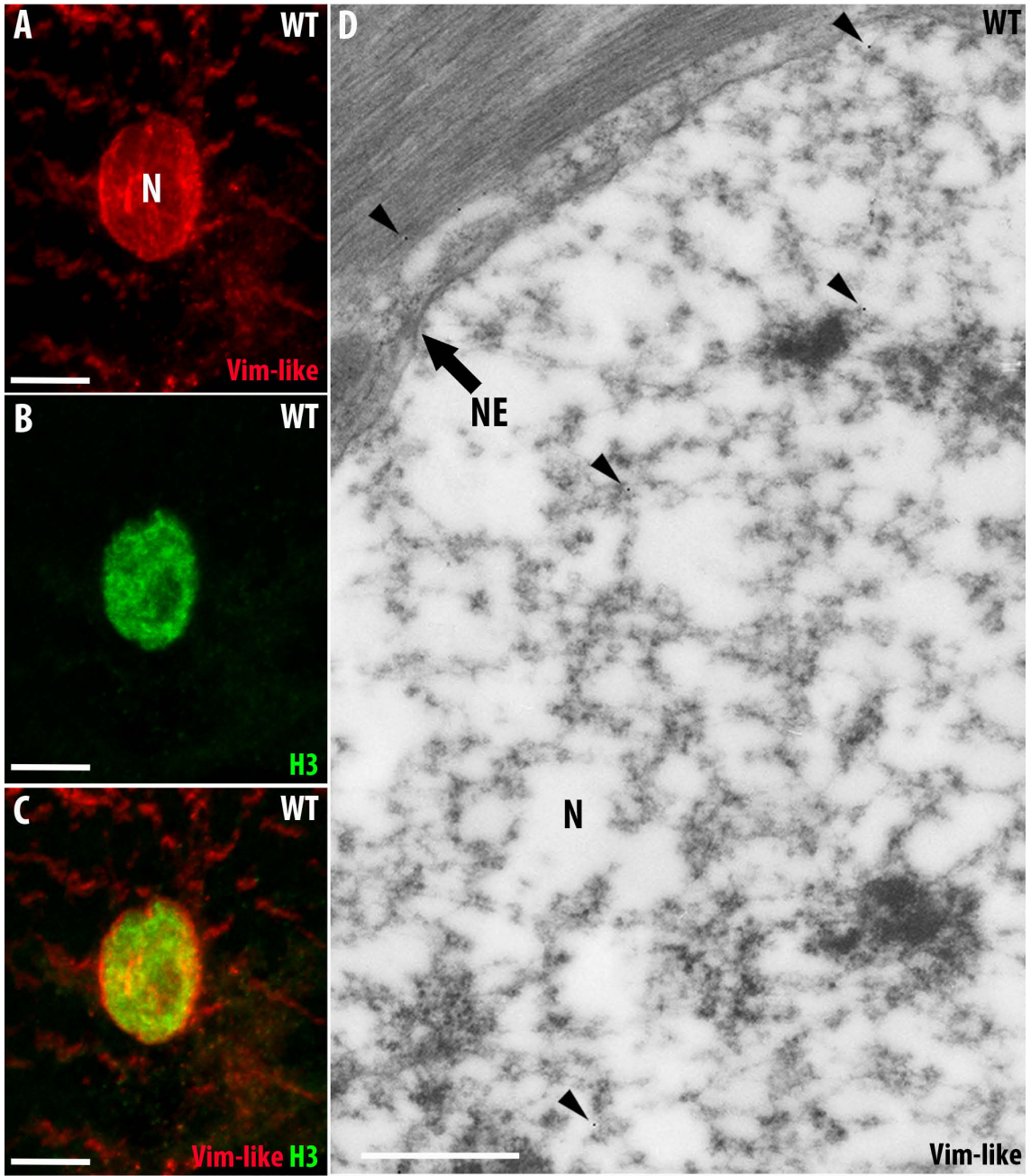




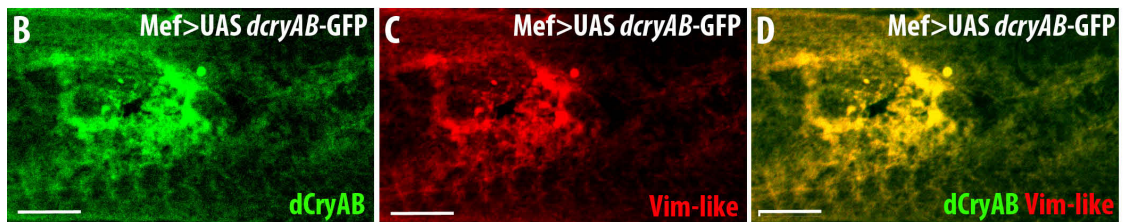
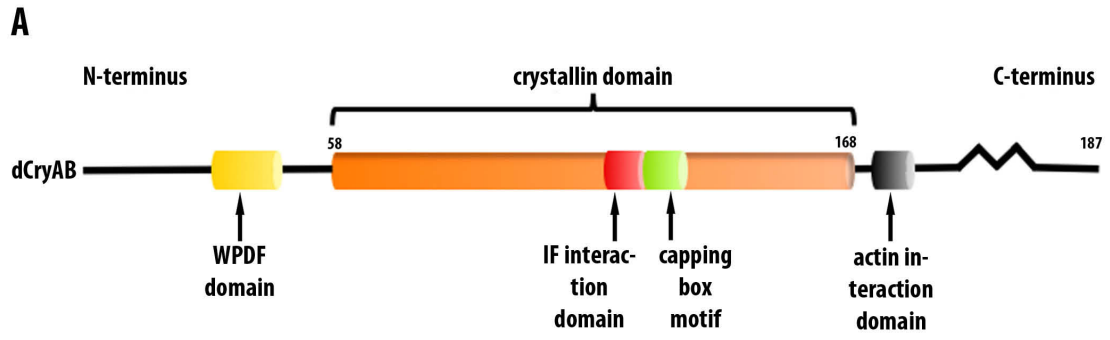
**Fig. 4.4.1. Vimentin-like protein localisation in the third instar larval muscles.** Confocal scans show third instar larval muscles labeled with adequate antibodies: anti-dCryAB, anti-vimentin and phalloidin, visualising F-actin **(A-D)**. The vimentin-like protein is localised in Z-lines **(B)**, in the nuclear area and is associated to the sarcolemma (dotty pattern, pointed by arrowheads) **(C)**. The vimentin-like protein co-localises with dCryAB in Z-lines and around the nucleus **(D)**. Three-dimensional modelling of larval muscle fibres, previously stained with adequate antibodies and visualised in the confocal microscope, show the vimentin-like protein situated at the surface of sarcomeres **(E)**. Ultrathin sections, stained in the immunogold reaction and observed in TEM confirm a presence of the vimentin-like protein in the Z-line area **(F)**. N-nucleus. Scale bars: 20  $\mu\text{m}$  (A-D), 10  $\mu\text{m}$  (E), 0,4  $\mu\text{m}$  (F), 0.1  $\mu\text{m}$  (F', F'').



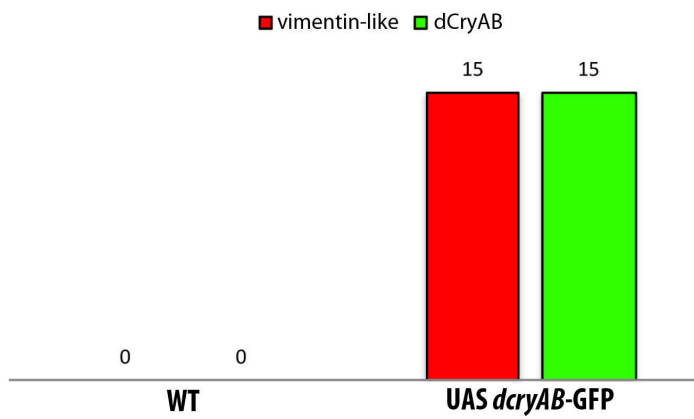
**Fig. 4.4.2. Localisation of the vimentin-like protein inside of the third instar larva muscle nucleus.** Confocal scans show third instar larval muscles labeled with adequate antibodies: anti-vimentin and anti-histone 3 (H3) **(A-C)**. The vimentin-like protein is present in the perinuclear area and inside of the nucleus **(A)**. The vimentin-like protein co-localises with histone 3 inside of the nucleus **(C)**. Ultrathin sections, stained in the immunogold reaction and observed in TEM show the vimentin-like protein around and inside of the nucleus, in the neighbourhood of the chromatin. Gold particles, corresponding to the vimentin-like protein are pointed by black arrowheads **(D)**. N-nucleus, NE-nuclear envelope. Scale bars: 10  $\mu\text{m}$  (A-C), 0.1 $\mu\text{m}$  (D).



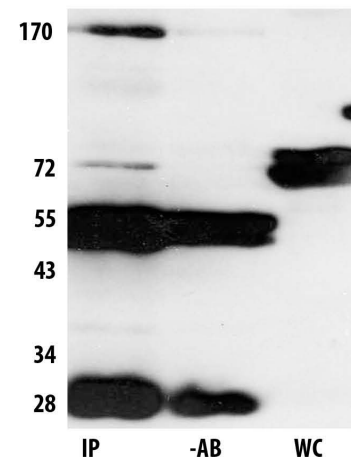
**Fig. 4.5.1. Interactions of dCryAB with the vimentin-like protein in *Drosophila* third instar larval muscle cells.** Schematic alignment of dCryAB protein indicates the  $\alpha$ -crystallin rod domain containing an intermediate filament interaction domain, N- and C-termini **(A)**. Confocal projections show accumulations of dCryAB occurring together with the vimentin-like protein **(B-D)**. Diagram shows number of observed third instar larvae possessing in their muscles delocalised dCryAB and vimentin-like proteins **(E)**. Western blot shows proteins with the size around 40, 72 and 170kDa labeled by anti-vimentin antibodies after co-immunoprecipitation reaction between dCryAB and vimentin-like **(F)**. IP-immunoprecipitation, -AB-negative control, WC-whole cell lysate. N-nucleus. Scale bars: 20  $\mu$ m (B-D).



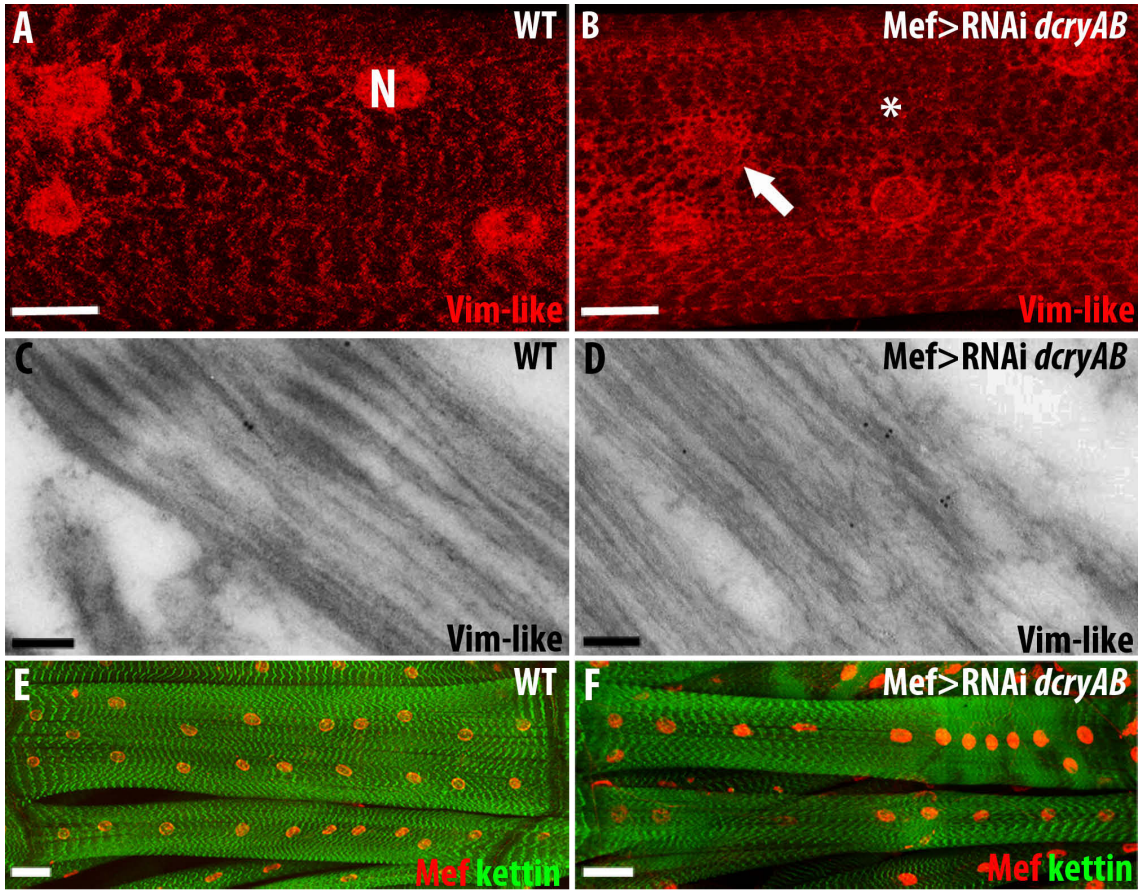
**E** Number of third instar larvae with vimentin-like and dCryAB mislocalization (n=20)



**F**

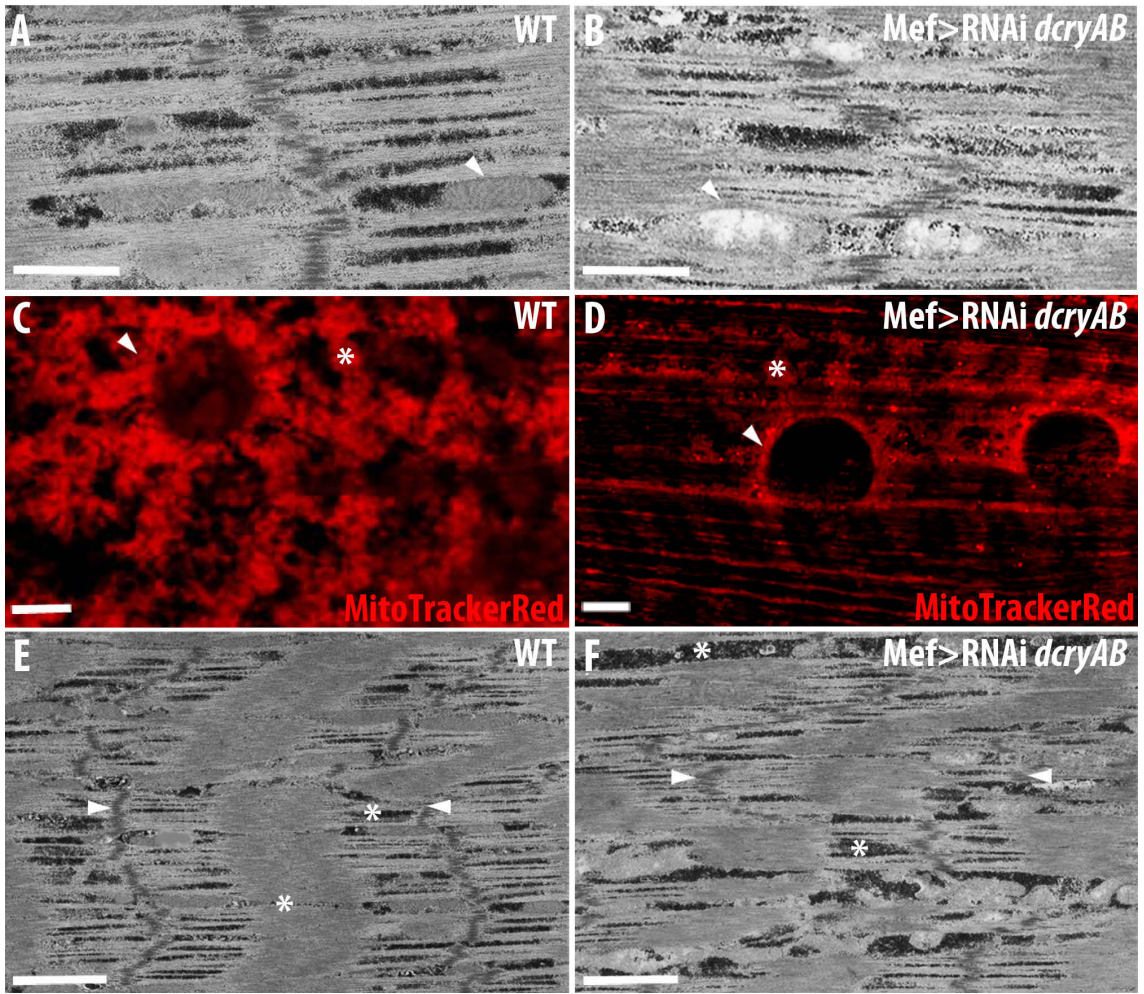


**Fig. 4.6.1. dCryAB is involved in stabilisation of structural integrity of third instar larval somatic muscles by interacting with putative IF proteins, part1.** Confocal scans show anti-vimentin-like staining using immunocytochemistry method **(A, B)** and TEM visualisation of an immunogold procedure **(C, D)**. Images show that in normal muscles the vimentin-like protein creates regular meshwork on extensions of Z-lines and around nuclei **(A, C)**, whereas muscles with attenuated *dcryAB* expression have altered arrangement of the vimentin-like protein around the nucleus (arrow) and in sarcomeres (asterisk) **(B, D)**. In muscles with *dcryAB* knockdown, visualised in the confocal microscope, nuclei position is altered **(F)** comparing with the control **(E)**. N-nucleus. Scale bars, 20  $\mu\text{m}$  (A, B), 0.2  $\mu\text{m}$  (C, D), 50  $\mu\text{m}$  (E, F).

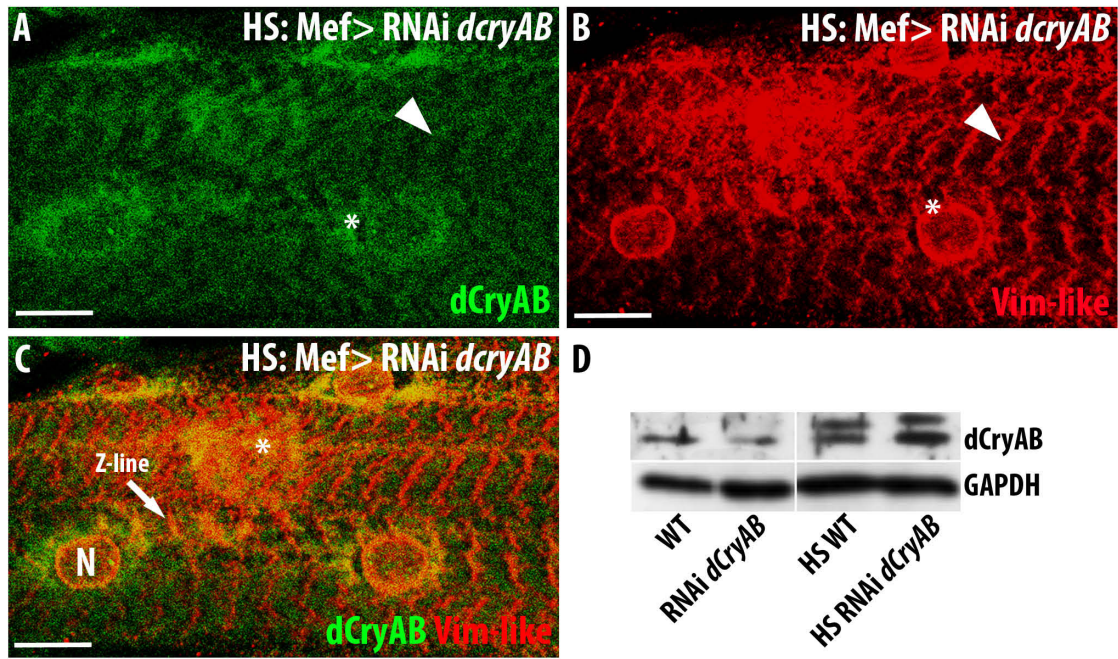




**Fig. 4.6.2. dCryAB is involved in stabilisation of structural integrity of third instar larval somatic muscles by interacting with IF proteins, part 2.** TEM images show in RNAi *dcryAB* line swollen mitochondria (arrowheads) with barely visible mitochondrial crests, comparing to the WT **(A, B)**. MitoTrackerRed reaction, visualised in confocal microscope, detects a lower number of active mitochondria around the nucleus (arrowheads) and in the Z-line area (asterisks), comparing to the wild type **(C, D)**. TEM images show increased deposits of the glycogen (asterisks) and shifted Z-lines (arrowheads) in muscles of RNAi *dcryAB* line, comparing to the control **(E, F)**. Scale bars : 1  $\mu\text{m}$  (A, B), 5  $\mu\text{m}$  (C, D), 2.5  $\mu\text{m}$  (E, F).



**Fig. 4.7.1. The vimentin-like meshwork recuperation in third instar larval muscles, after 2 hours of recovery from heat shock.** Immunocytochemical reaction, visualised in confocal microscope, show that dCryAB is present in larval muscles in sarcomeres (arrowhead) and in the perinuclear area (asterisk) **(A)**. The vimentin-like protein restores its normal sharp pattern in Z-line (arrowhead) and in the perinuclear area (asterisk) **(B)**. dCryAB and the vimentin-like protein accumulate together in muscle cells (asterisk) **(C)**. Western blot shows dCryAB level in muscle cells in normal conditions and after 2 hours of recovery from heat shock **(D)**. Scale bars: 20  $\mu\text{m}$  (A-C).



**Fig. 4.8.1. Comparison of amino acid sequences of  $\alpha$ B-crystallin (CryAB) with dCryAB and comparison of the nucleotide sequence of *dcryAB* with and without mutation R120G.** Comparison of amino acid sequences of human  $\alpha$ B-crystallin with drosophila dCryAB, in red pointed conserved arginine in codon 120th **(A)**. DNA sequence of *dcryAB* and *dcryAB<sup>R120G</sup>*, mutation R120G marked in red **(B)**.

**A**

<b>CryAB</b>	9	WIRRPFFPFHSPSRLFDQFFGEHLLSDLFPTSTLSLSPFYLRPPSFLRAPSWFDTGL---	65
		W    FP + SRL DQ FG+ L    DL + + P LR +LR W    L	
<b>dCryAB</b>	11	WWDELDFPMRT-SRLLDQHFGQGLKRDDLMSVWNSRPTVLRG-YLRP--WHTNSLQKQ	66
<b>CryAB</b>	66	---SEMRLKDRFSVNLVDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHRY	122
		S + ++ ++F V LDV+ FSP E+ VKV    + V GKHEE+QDEHG++SR+F R+Y	
<b>dCryAB</b>	67	ESGSTLNIDSEKFEVILDVQQFSPSEITVKVADKVFVIVEGKHEEKQDEHGYVSRQFSRY	126
<b>CryAB</b>	123	RIPADVDPPLTITSSSLSSDGVLTVNGPRKQVSGP--ERTIPITR	163
		++P+DV+P T+TSSLSSDG+LT+ P K + P ER + IT+	
<b>dCryAB</b>	127	QLPSDVNPDTVTSSLSSDGLLTIKAPMKALPPPQTERLVQITQ	169

**B**

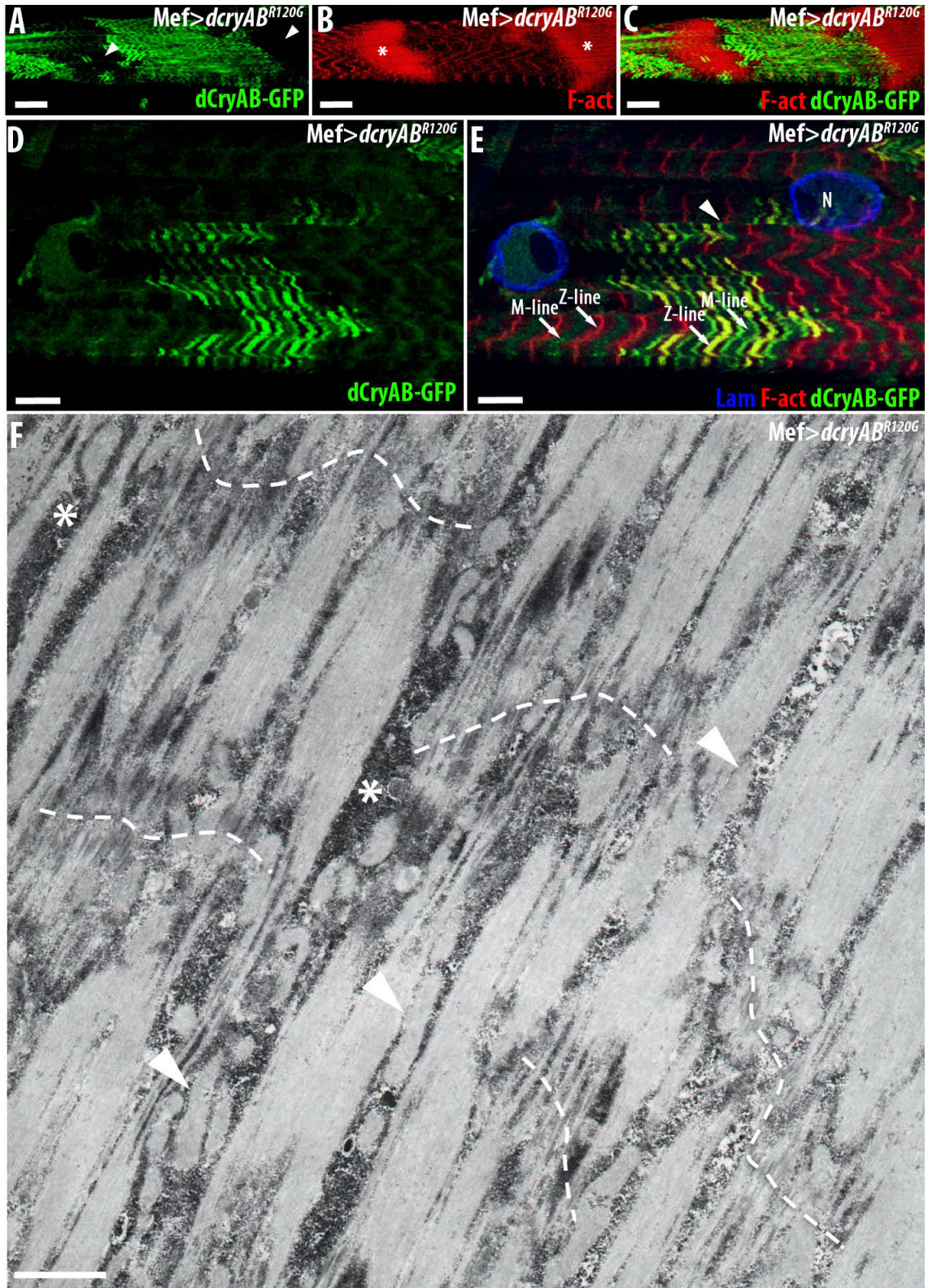
WT: **AGG**     $\longrightarrow$     Mutant: **GGG**

R120     $\longrightarrow$     G120

R=arginin

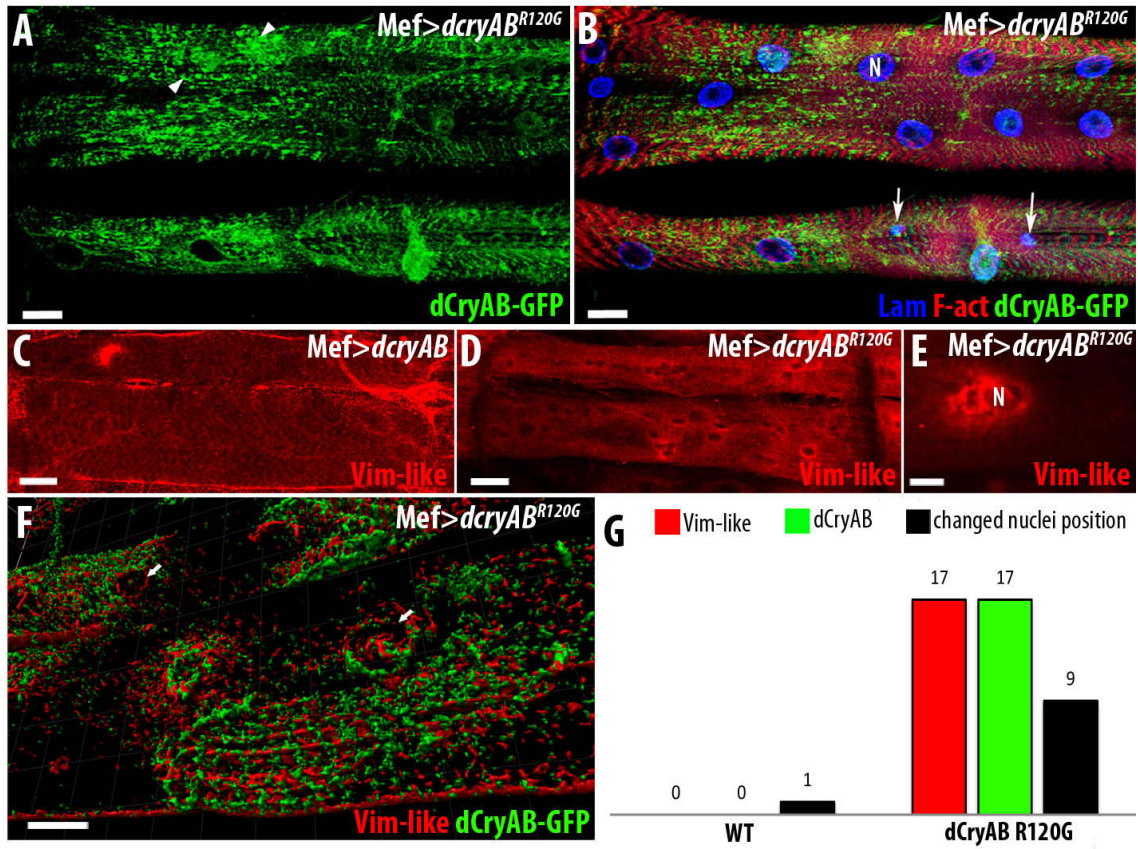
G=glycine

**Fig. 4.8.2. Effects of the R120G mutation in dCryAB on the sarcomere organisation in *Drosophila* third instar larval somatic muscles.** Confocal images show the lack of dCryAB<sup>R120G</sup>-GFP in sarcomeres of some muscle parts, indicated by arrowheads (**A**). Muscle parts with the lack of dCryAB<sup>R120G</sup>-GFP contain accumulations of actin (asterisks) without perceptible sarcomeric organisation (**B, C**). dCryAB<sup>R120G</sup>-GFP localises ectopically in muscle fibres: in some sarcomeres dCryAB<sup>R120G</sup>-GFP does not occur in Z-lines but appears in the middle of M-lines, whereas in other sarcomeres it is present in Z-lines but also accumulates on edges of the I-band; Z-line shift (arrowhead) is also observed (**D, E**). Ultrathin section of larval muscle observed in TEM shows misaligned and a shifted Z-line (dashed line marks Z-lines), elongated mitochondria with a reduced cristae (arrowheads) and a glycogen deposits (asterisks). N-nucleus. Scale bars, 20  $\mu\text{m}$  (A-E), 2  $\mu\text{m}$  (F).

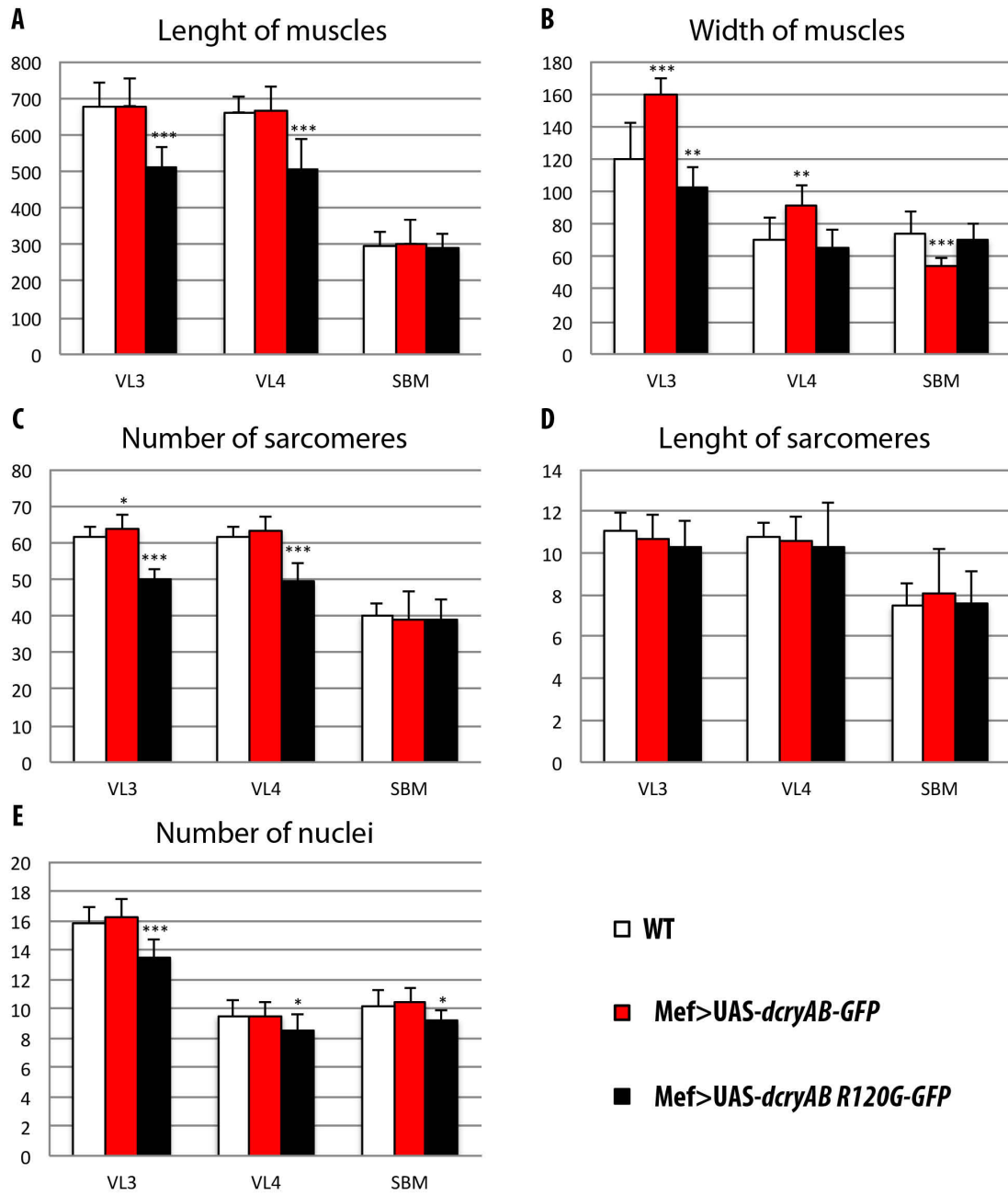




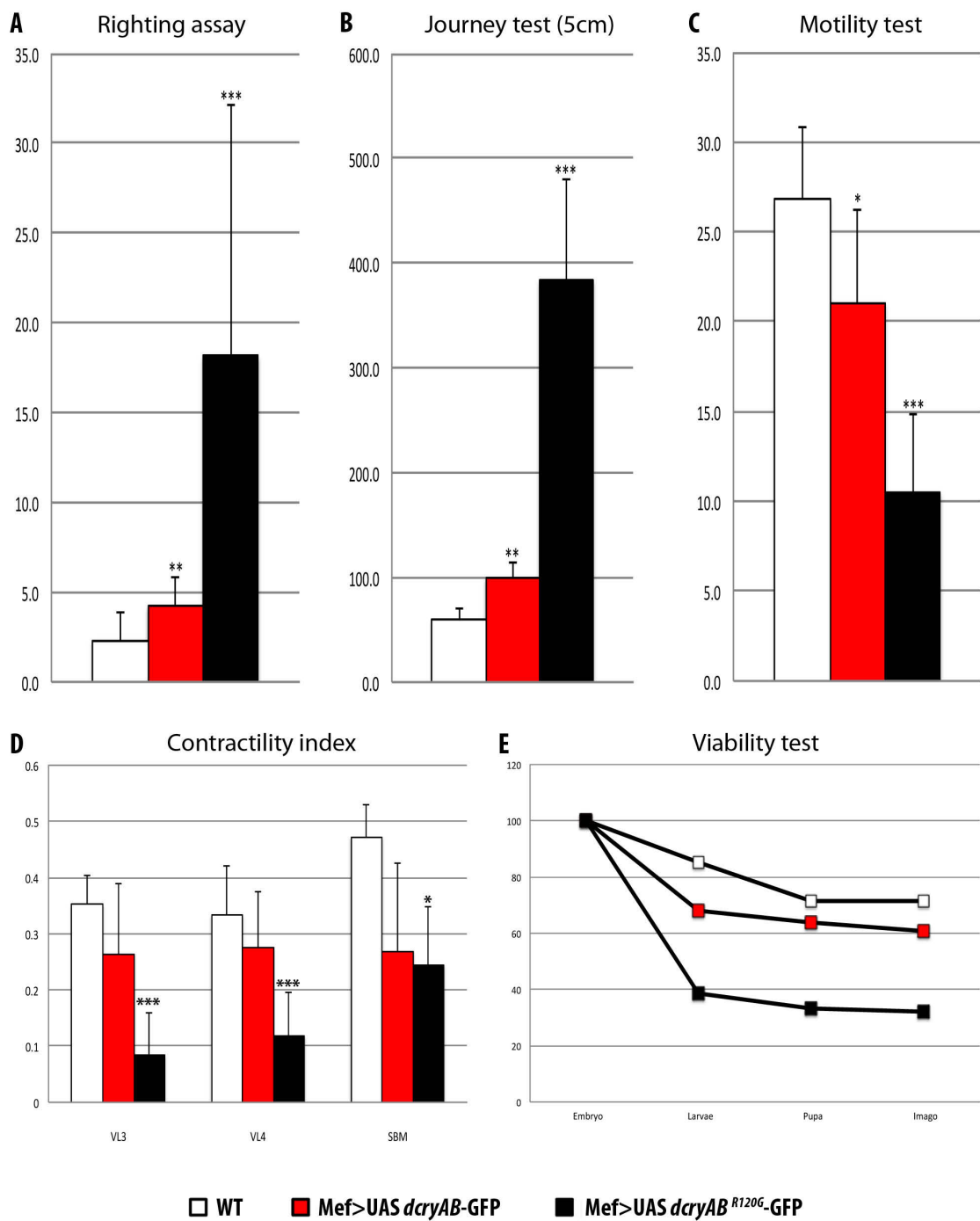
**Fig. 4.8.3. Effect of the R120G mutation in dCryAB on the accumulation of dCryAB<sup>R120G</sup> aggregates, formation of smaller nuclei and a vimentin-like meshwork collapse in *Drosophila* third instar larval somatic muscles development.** Confocal images show accumulations of dCryAB<sup>R120G</sup>-GFP (arrowheads) in sarcomeres **(A)** and presence of small nuclei (arrowheads) in muscle fibres **(B)**. In larval muscles, expressing *dcryAB* with provided R120G mutation the vimentin-like protein losses regular sarcomeric localisation and accumulates around nucleus **(D, E)**, comparing to the control **(C)**. Three-dimensional modelling of created muscles labeled by adequate antibodies, show complexes compound of dCryAB<sup>R120G</sup>-GFP and the vimentin-like protein positioned around and inside of nuclei (arrows), and in the sarcomeric part of the muscle fibre **(F)**. The diagram shows a number of individuals with misarranged nuclei position and visible changes of dCryAB and the vimentin-like protein localisation, observed in *Mef>dcryAB<sup>R120G</sup>* larvae, comparing to the WT **(G)**. N-nucleus. Scale bars, 20  $\mu\text{m}$  (A, B, E, F), 50  $\mu\text{m}$  (C, D).



**Fig. 4.9.1. Effects of the *dcryAB<sup>R120G</sup>* overexpression on the third instar larval muscle morphology.** Diagrams A-E present results of adequate muscle measurements. Muscle measurements show that muscles with overexpressed *dcryAB<sup>R120</sup>* have a smaller length **(A)**, width **(B)**, number of sarcomeres **(C)**, insignificantly changed sarcomere size **(D)** and contain a lower number of nuclei **(E)**, comparing to the WT. Asterisks indicate statistical level of significance of observed differences: \* $p < 0.1$ , \*\* $p < 0.5$ , \*\*\* $p < 0.001$ .



**Fig. 4.10.1. Effects of the *dcryAB<sup>R120G</sup>* overexpression on the third instar larva motility and life span.** Behavioural assays indicate that the movement ability of *Mef>UASdcryAB<sup>R120R</sup>-GFP* larvae are significantly affected. Diagrams show that they need significantly more time to revert their body in the righting assay **(A)**, to crawl 5 cm the distance in the journey test **(B)** and execute less peristaltic movements during the time of 30 seconds **(C)**. Muscles overexpressing *dcryAB<sup>R120G</sup>-GFP* larvae are characterised by a lower contractility index **(D)**. *dcryAB<sup>R120R</sup>-GFP* flies show a higher mortality rate **(E)**. Asterisks indicate a statistical level of significance of observed differences: \* $p < 0.1$ , \*\* $p < 0.5$ , \*\*\* $p < 0.001$ .



**Fig. 4.11.1. Localisation in larval muscle cells of human  $\alpha$ B-crystallin inserted to the *Drosophila* genome.** Confocal images show larval muscles overexpressing human  $\alpha$ B-crystallin and labeled with adequate antibodies.  $\alpha$ B-crystallin occurs together with dCryAB in the Z-line and in the perinuclear area, is present in the M-line and inside of the nucleus but also localises unspecifically in all muscle cytoplasm area (**A, A', C, C'**).  $\alpha$ B-crystallin attaches to the vimentin-like meshwork in the Z-line but also is present together with the vimentin-like protein inside of the nucleus (**B, B', C, C'**). N-nucleus. Scale bars: 20  $\mu$ m (A,B,C), 10  $\mu$ m (A', B', C').

