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Cell-stress in sIBM muscle: α B-crystallin correlates to APP and accumulation of β -amyloid

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ABSTRACT

Background: In the pathology of sIBM, the relevance of cell-stress molecules such as the heat-shock-protein α B-crystallin, particularly in healthy appearing muscle fibres, has remained elusive.

Methods: 10 muscle biopsies from sIBM patients were serially stained for H&E, trichrome, and multi-immunohistochemistry for neural-cell-adhesion-molecule (NCAM), α B-crystallin, amyloid-precursor-protein (APP), desmin, major-histocompatibility-complex (MHC)-I, β -amyloid, ubiquitin. Corresponding areas of all biopsies were quantitatively analyzed for all markers. Primary myotube cultures were exposed to pro-inflammatory cytokines IL-1 β +IFN- γ .

Results: In human myotubes exposed to IL-1 β +IFN- γ , overexpression of APP was accompanied by upregulation of α B-crystallin. In sIBM muscle biopsies, over 20% of all fibres displayed accumulation of β -amyloid or vacuoles/ inclusions. A clearly larger fraction of the fibres were positive for α B-crystallin or APP. In contrast to the accumulation of β -amyloid in atrophic fibres, a major part of fibres positive for APP or α B-crystallin showed no morphological abnormalities. The expression of APP and α B-crystallin significantly correlated with each other and most double-positive fibres displayed accumulations of β -amyloid, vacuoles or an atrophic morphology. In almost all of these fibres, other markers of degeneration/regeneration such as NCAM and desmin were evident as additional indicators of a cell-stress response. Some fibres double-positive for APP and α B-crystallin displayed an infiltration by inflammatory cells.

Conclusion: Our results suggest that α B-crystallin is associated with overexpression of APP in sIBM muscle and that upregulation of α B-crystallin precedes accumulation of β -amyloid. The data help to better understand early pathologic changes and underscore that a network of cell-stress, inflammation and degeneration is relevant to sIBM.

INTRODUCTION

Sporadic inclusion body myositis (sIBM) is an acquired myopathy of unknown cause that occurs in older patients.^{1,2} No treatment is available to effectively ameliorate the disease progression related to continuous degeneration of the skeletal muscle.³

Along with a chronic inflammatory response of the muscle fibres and their invasion by cytotoxic CD8+ T-cells, aberrant proteins accumulate within the muscle fibres.⁴ The most common abnormal protein present in muscle fibres that carry vacuoles or filamentous inclusions is β -amyloid, which is cleaved from amyloid precursor protein (APP). Upregulated APP has been demonstrated in sIBM muscle⁵ and its experimental overexpression has been shown to be associated with an IBM-like vacuolar transformation of muscle cells *in vitro*⁶ as well as *in vivo*.^{7,8} Therefore, overexpression of APP is considered an early event in sIBM pathogenesis.⁹ As another molecule with relevance to early sIBM pathology, α B-crystallin has recently been demonstrated in healthy-appearing muscle fibres.¹⁰ α B-crystallin is a heat-shock protein known to chaperone proteins including actin- and desmin-filaments and its lack of function is associated with protein aggregation in skeletal muscle¹¹ as well as β -amyloid in Alzheimer disease.^{12,13} Furthermore, α B-crystallin has been shown to be a crucial anti-inflammatory mediator in experimental autoimmune demyelination of the CNS.¹⁴ In spite of recent evidence of a specific interplay between accumulation of β -amyloid and inflammation in skeletal muscle of sIBM patients,⁵ α B-crystallin may serve as a key molecule in this network of inflammatory and degenerative pathomechanisms.

METHODS

Patients and muscle biopsies

Diagnostic biopsies were used from skeletal muscle of 10 patients with sIBM, who each fulfilled typical clinical, electrophysiological and histopathologic criteria of the disease.¹ The

patients were admitted to the National Institutes of Health (NIH) Clinical Center and studied under Institutional Review Board (IRB)-approved clinical protocols.

Staining of tissue

For immunohistochemistry, 5 μ m frozen sections of all muscle biopsies were fixed either in 4% paraformaldehyde for 10 min. (for β -amyloid) or in acetone at -20°C for 10 min. (for all other primary antibodies). Unspecific binding was reduced by 30 min. incubation with 5% bovine serum albumin (BSA) and 3% goat serum (all from Jackson ImmunoResearch, West Grove, PA) in PBS. All primary and secondary reagents were diluted in 1% BSA. Following primary antibodies were used at the respective concentration with an incubation time of one hour at room temperature unless stated otherwise: β -amyloid (mouse clone 6E10, Signet, Dedham, MA) at 10 μ g/ml for 24 hours at 4°C; MHC class I (rat clone YTH 862.2 from Serotec, Oxford, UK) at 5 μ g/ml; APP (goat polyclonal from R&D, Minneapolis, MN) at 10 μ g/ml; desmin (rabbit polyclonal from Abcam, Cambridge, MA) at 30 μ g/ml; NCAM (mouse clone Eric-1 from Labvision/Neomarkers, Fremont, CA) at 2 μ g/ml; α B-crystallin (rabbit polyclonal from Serotec) at 1:1000 dilution; ubiquitin (rabbit polyclonal from DAKO, Carpinteria, CA) at 15 μ g/ml. Serial sections were double- or triple-labelled by fluorescent staining for 1) NCAM and α B-crystallin; 2) APP, desmin and MHC-I; 3) β -amyloid and ubiquitin. Secondary reagents were Alexa 594, Alexa 488, or Alexa 350-conjugated secondary antibodies from goat. Nuclear counterstaining was performed by DAPI (1:50.000), followed by mounting in Fluoromount G (Electron Microscopy Sciences, Hatfield, PA). Adjacent serial sections were stained by 4) hematoxylin/ eosin and 5) trichrome histochemistry. Immunofluorescent and bright-field microscopy and digital photography was performed on a Zeiss Axiophot microscope (Zeiss, Göttingen, Germany), using appropriate filters for green (488nm), red (594nm) and blue (350nm) fluorescence, a cooled CCD digital camera (Retiga 1300, Qimaging, Burnaby, BC, Canada) and the ImageProPlus software

(MediaCybernetics, Bethesda, MD). Microphotographs of representative and corresponding areas of all serial sections were taken from each patient's sample, yielding between 80 and 299 muscle fibres per patient. The expression of the respective markers was manually identified in a total of 1,418 fibres of all serial sections. All statistics were calculated using GraphPad Prism V4.0 (San Diego, CA) and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as significant values.

Cell culture

As approved by the local ethics committee of the University of Göttingen, a small piece of skeletal muscle was taken during routine surgery from patients after written consent. Muscle cell progenitors (satellite cells) were grown using a recently established protocol.⁵ After thorough mincing with a scissor and washing in PBS, the muscle was trypsinized in three subsequent fractions of 15 min. each at 37°C. All fragments were collected and seeded in a 25cm² flask in DMEM with pyruvate, high glucose and glutamine (Gibco/Invitrogen, Carlsbad, CA), supplemented with 10% FCS (Cambrex Bio Science, Walkersville, MD), penicillin, streptomycin (all from Gibco/Invitrogen) and 0.5% chick embryo extract (Accurate chemicals, Westbury, NY). After 2-3 subcultures over 2-3 weeks, myoblasts were labelled with anti CD56 (NCAM) MicroBead-antibody (Miltenyibiotec, Bergisch Gladbach, Germany) for MACS sorting following the supplier's protocol. After expansion during 2-3 subcultures over 2-3 weeks, myoblasts were seeded in 8-chamber slides (LabTek II from Nunc, Rochester, NY) and 24-well plates (Nunc). At 80% confluence, fusion was induced by switching to DMEM supplemented with 2% horse serum (Gibco/Invitrogen), penicillin and streptomycin. After 3 to 5 days, multinucleated myotubes had formed that typically reached 95% purity as revealed by immunohistochemical staining for the muscle marker desmin. Chamber slides and culture wells were exposed to the cytokines IFN- γ (300 U/ml) or IL-1 β (20ng/ml), (all from Chemicon International via Millipore, Schwalbach, Germany), alone or

in combinations, for 24 hours to 72 hours in serum free medium X-vivo 15 (Gibco/Invitrogen). Controls were kept in X-vivo 15 only. After washing in prewarmed PBS, the chamber slides were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) at room temperature or in acetone at -20°C and stored at -80°C until staining (see below).

RESULTS

In vitro cell-stress in muscle cells

Human primary muscle cell cultures were used to address overexpression of APP and a cell-stress response including α B-crystallin at a functional level. Using a recently established *in vitro* model,⁵ immunocytochemistry with subsequent computerized grey-scale analysis revealed a significant overexpression of APP upon exposure of muscle cells to IFN- γ and IL- 1β for 40h and 72h (***P < 0.001 or **P < 0.01 vs. controls) (fig 1), which was well in accord with our previous observations.⁵ Along with the overexpression of APP, α B-crystallin was upregulated in response to the same pro-inflammatory cytokines after 40h and 72h (**P < 0.01 vs, controls) (fig 1). This suggests that an inflammatory environment in muscle cells causes overexpression of APP along with an upregulation of cell-stress molecules including α B-crystallin.

Markers of inflammation, cell-stress and β -amyloid

In serial sections of sIBM muscle, tissue immuno-labelling displayed overexpression of degenerative markers such as β -amyloid, APP and ubiquitin, the regenerative/degenerative markers NCAM and desmin and the cell-stress marker α B-crystallin (fig 2A). Quantitative assessment of serial sections by photo-microscopy of all patients revealed accumulation of β -amyloid in $22.6 \pm 9.3\%$ of the fibres (all data indicated as mean+ standard deviation), whereas APP was present in $37.4 \pm 12.8\%$ and the tagging molecule ubiquitin in $21.8 \pm 9.6\%$ (fig 2B). Desmin ($32.0 \pm 8.3\%$), NCAM ($25.5 \pm 9.0\%$) and α B-crystallin ($28.3 \pm 9.4\%$) were noted to a

similar degree in the fibres. By H&E and trichrome staining, $37.8 \pm 11.8\%$ atrophic fibres were observed and $21.9 \pm 10.0\%$ of all fibres contained intracellular vacuoles or inclusions, whereas $7.6 \pm 4.1\%$ were invaded by inflammatory cells. Signs of hypertrophy ($14.1 \pm 10.3\%$) or necrosis ($0.4 \pm 0.6\%$) were present in a smaller fraction of the fibres.

Association between α B-crystallin and degenerative markers

α B-crystallin has previously been demonstrated as one of the major cell-stress molecules present in skeletal muscle.¹⁰ Exploring the significance of α B-crystallin for sIBM pathology, co-localization analysis revealed that degenerative markers such as APP as well as degenerative/regenerative such as NCAM and inflammatory such as MHC-I co-localized to α B-crystallin (fig 3A). Furthermore, quantitative analysis revealed a significant correlation between the rate of muscle fibres positive for α B-crystallin and APP as well as between α B-crystallin and other degenerative markers such as β -amyloid, ubiquitin, NCAM and desmin (fig 3B). Accordingly, α B-crystallin significantly correlated with muscle fibres that contained vacuoles/inclusions, displayed atrophy or inflammatory infiltrates.

α B-crystallin in normal appearing muscle fibres

To further characterize changes indicative of early changes in sIBM pathology, a serial section analysis of normal appearing muscle fibres was performed. This revealed that a noticeable part of normal appearing fibres was positive for α B-crystallin ($15.8\% \pm 8.5$), which is in line with previous observations¹⁰ (fig 4A). APP was noted to a similar degree ($19.4\% \pm 16.2$). Overexpression of all other pathologic markers including NCAM ($4.8 \pm 4.4\%$), desmin ($10.2 \pm 7.8\%$), and ubiquitin ($9.6 \pm 7.1\%$) remained at a lower level. **Further subset analysis revealed that only a small fraction of $2.8 \pm 4.6\%$ of the α B-crystallin-positive, normal appearing fibres -previously called “X-fibres”- were surrounded by inflammatory cells,**

whereas $17.6 \pm 19.8\%$ were positive for APP and $8.6 \pm 12.2\%$ were positive for APP and β -amyloid (fig. 4A).

An early sIBM pathology may centre around co-overexpression of α B-crystallin and APP: the majority of the fibres positive for α B-crystallin showed an overexpression of APP ($56.0 \pm 11.7\%$, not shown). Serial section analysis of fibres double positive for α B-crystallin and APP revealed an accumulation of β -amyloid in $60.8 \pm 14.5\%$ of the fibres (fig 4B).

Similar fractions were positive for all other markers of degeneration/ regeneration.

Inflammatory infiltrates were noted in $18.3 \pm 13.5\%$ of these fibres (fig. 4B).

β -amyloid associated pathology

To further elucidate the relevance of overexpression of APP and accumulation of β -amyloid in sIBM pathology, APP-positive as well as β -amyloid-positive fibres were analyzed in serial stainings. $45.3 \pm 14.2\%$ of APP-positive fibres displayed accumulations of β -amyloid (not shown). On the other hand, the predominant part of fibres with accumulations of β -amyloid were also positive for APP ($74.5 \pm 13.3\%$; fig 5). The majority of β -amyloid positive muscle fibres were also positive for most markers of cell-stress/inflammation and degeneration/ regeneration. Similar values of co-overexpression of markers of degeneration and cell-stress were observed in fibres that were APP-positive as well as those that displayed vacuoles or inclusions (not shown). To further address associations between accumulation of APP/ β -amyloid and other markers of degeneration as well as cell-stress, a correlation analysis of the serial section assessment was performed. This revealed a significant correlation between the labelling for APP/ β -amyloid and ubiquitin ($***P < 0.001$), desmin ($***P < 0.0001$) and NCAM ($**P < 0.01$) (data not shown). Moreover, APP/ β -amyloid correlated with morphological signs of atrophy ($***P < 0.001$) and vacuoles/inclusions ($***P < 0.001$). In line with recent observations,⁵ we found a correlation between β -amyloid and inflammation ($**P < 0.01$).

DISCUSSION

In a cell-culture model that shares features of sIBM, accumulation of β -amyloid upon pro-inflammatory cell-stress was preceded by upregulation of APP and α B-crystallin. To explore the relationship between these and all other markers relevant to sIBM pathology, a quantitative assessment of multi-labelling and serial-staining immunohistochemistry was performed in sIBM muscle. We demonstrate that there is a positive correlation between overexpression of α B-crystallin and β -amyloid-associated markers. Many normal appearing muscle fibres displayed a co-localization of α B-crystallin and APP. Furthermore, in these fibres, subsequent accumulation of β -amyloid and overexpression of regeneration/degeneration-associated markers such as NCAM and desmin was noted. A significant co-localization between APP/ β -amyloid and markers of cell-stress and regeneration was observed. Collectively, this suggests that α B-crystallin, together with APP, may be an early pathologic marker that indicates intracellular cell-stress, which subsequently is associated with accumulation of β -amyloid in sIBM muscle.

In sIBM muscle, α B-crystallin has previously been demonstrated in normal appearing muscle fibres, which had been termed “X fibres”.¹⁰ Our data confirm this finding of a noticeable overexpression of α B-crystallin in normal appearing muscle fibres. Banwell et al. had reported a mean of 9.8% of α B-crystallin positive fibres. In our 10 patients, about a two-fold higher rate of these fibres was noted. This may be explained by the fact that, whereas none of our patients had received treatment at the time of the biopsy, 5 of the 11 patients in the previous report received prednisolone, alone or in combination with other immunosuppressive or immunomodulatory treatments. Moreover, a higher rate of inflammation in our samples may have contributed to a higher rate of α B-crystallin signal. This is of interest in view of the induction of α B-crystallin by pro-inflammatory cytokines in our muscle cell culture model. Lastly, in view of a dim signal of α B-crystallin in almost all fibres in our analysis as well as in the previous report by Banwell,¹⁰ a different arbitrary cut-

off value for “overexpression” or a stronger labelling by a different antibody may also have contributed to the discrepancy. In the present study, the cut-off value used for each marker was similar to the one established in a previous report by our group.⁵

In view of a similar magnitude of overexpression of α B-crystallin and detection of abnormalities such as vacuoles, β -amyloid, neurofilament/hyperphosphorylated tau and ubiquitin, it has been hypothesized that α B-crystallin is associated with aggregation of aberrant proteins in sIBM.¹⁰ However, the relevance of α B-crystallin in normal-appearing muscle fibres regarding overexpression of APP and subsequent accumulation of β -amyloid has so far remained elusive. This aim could not be addressed using a mere protein analysis by Western blot. Therefore, we employed an approach with extensive multi- and serial immunohistochemical staining. It has recently been demonstrated that *in vitro* overexpression of APP induces overexpression of α B-crystallin.¹⁵ Furthermore, a physical association of APP/ β -amyloid had been demonstrated by immunoprecipitation, which led to the assumption that α B-crystallin could be upregulated in response to intracellular generation of oligomers of β -amyloid in still normal appearing muscle fibres. Our data are in line with this assumption and, by serial and multi-labelling, extend these findings on the immunohistochemical level by demonstrating a correlation and co-localization of α B-crystallin and APP/ β -amyloid. However, the reason that APP and α B-crystallin are co-overexpressed in healthy appearing muscle fibres may not solely be explained by an overexpression of α B-crystallin as a secondary response to an upregulation of APP. It is possible that an inflammatory or general underlying stress-response contributes to the simultaneous upregulation of α B-crystallin and generation of APP as well as accumulation of β -amyloid. This is corroborated by our *in vitro* data where we show that in muscle cells, pro-inflammatory cell-stress induces upregulation of α B-crystallin as well as of APP. Subsequently, accumulation of β -amyloid was noted, which is in accord with recent observations by us⁵ as well as by others.¹⁶ An association between protein aggregation and α B-crystallin has previously been demonstrated *in vitro*¹³ and in

skeletal muscle *ex vivo*.¹⁷ Furthermore, the expression of α B-crystallin correlated to other markers of degeneration/regeneration such as NCAM and desmin, which is in line with previous observations.⁵

In muscle fibres that displayed accumulation of β -amyloid, there was a high degree of co-labelling with α B-crystallin, APP and markers of degeneration/ regeneration as well as vacuoles/ inclusions. This is in line with previous results¹⁰ and suggests that overexpression of α B-crystallin together with APP precedes accumulation of β -amyloid. Inflammation may be a primary event that can trigger or aggravate accumulation of β -amyloid. This is corroborated by the co-localization of MHC-I and α B-crystallin, which is in line with observations by others.¹⁷ Further, this is sustained by our previous observations of a co-localization between β -amyloid and IL-1 β in sIBM.⁵ Not all of the fibres with β -amyloid depositions displayed an overexpression of α B-crystallin and APP. This can be explained by a reduced capacity to upregulate a cell-stress response in end-stage degenerative fibres, which supports the interpretation that α B-crystallin and APP are rather early markers of the disease.

The fact that some of the fibres without morphological abnormalities were also positive for NCAM and desmin is denotative for an early degeneration/regeneration progress, which may serve as another indication of a cell-stress reaction. A similar association between desmin and α B-crystallin has recently been demonstrated,¹⁸ but not in a preceding report.¹⁰ This discrepancy may be explained by a higher degree of cell-stress in our samples, which may be due to lack of treatment and a higher grade of inflammation as discussed above. Although it has previously been suggested that myofibres in sIBM may have an inherent ability to over-respond to pro-inflammatory conditions,⁵ it is possible that the present findings are not specific to sIBM. However, since degenerative markers such as vacuoles and accumulation of β -amyloid are not present in polymyositis and dermatomyositis, the current multi-parameter analysis with serial stainings is only possible in sIBM, but not in disease controls.

Taken together, our data shed light on the recently described “X fibres” in sIBM muscle and solidify evidence that α B-crystallin is part of an early cell-stress response in skeletal muscle fibres. A significant association between APP/ β -amyloid and α B-crystallin was demonstrated in sIBM muscle fibres including those that appeared normal by morphological criteria. The co-localization of an inflammatory response and α B-crystallin together with an induction of *in vitro* overexpression of α B-crystallin upon pro-inflammatory cell-stress underscores the relevance of inflammation in sIBM muscle, which may trigger or aggravate accumulation of β -amyloid and vacuolar degeneration. The results further our understanding of sIBM pathology and may help to identify novel treatment strategies.

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Competing interests: None.

Ethics approval: The local ethics committee approved the study.

FIGURE LEGENDS

Fig. 1: Human primary myotube cultures exposed to 10 ng/ ml IL-1 β and 300 U/ ml IFN- γ for 40 to 72 hours. By fluorescent immunocytochemical staining, overexpression of APP and α B-crystallin was noted after 40 hours as well as after 72 hours, which was statistically significant as revealed by computerized grey-scale analysis (**P < 0.01; ***P < 0.001). One of three experiments with similar results is shown.

Fig. 2: Serial stainings in sIBM tissue. **A)** Representative serial sections of a muscle biopsy from a patient with sIBM. Serial sections were stained histochemically for H&E and trichrome, as well as immunohistochemically for β -amyloid, NCAM, desmin, ubiquitin, α B-crystallin, and APP. **B)** Quantitative assessment of all fibres in all serial sections (80 to 299 per patient). Data indicate the mean +SD of all 10 patients (total of 1,418 fibres).

Fig. 3: **A)** Co-localization of α B-crystallin, APP, MHC-I and NCAM in sIBM muscle fibres. Two of the serial sections in one representative patient are demonstrated with staining for 1) α B-crystallin (red) and MHC-I (green) in the upper row and 2) APP (red) and NCAM (green) in the lower row. Panels on the right depict co-localization (yellow) in the merged images. Nuclear counterstaining by DAPI (blue). **B)** Correlation analysis of the quantitative assessment of histochemical and immunohistochemical stainings of all biopsies (data as in **Fig. 2B**). A significant correlation is observed between the rate of muscle fibres that overexpress α B-crystallin and all major β -amyloid- and degeneration-associated markers. Spearman correlation analysis using *P < 0.05, **P < 0.01, ***P < 0.001 as significant values.

Fig. 4: Multi-parameter analysis of all fibres in all serial sections. All data given as mean +SD of all 10 patients. **A)** Percentage of presence of each marker in muscle fibres that display no

morphological abnormalities. The dashed frame highlights a subset analysis of fibres that were positive for α B-crystallin and showed no morphological abnormalities (*previously termed “X-fibres”). **B)** Percentage of presence of each marker in muscle fibres that are double-positive for APP and α B-crystallin.

Fig. 5: Multi-parameter analysis of all fibres in all serial sections similar to **Fig. 4**. All data given as mean +SD of all 10 patients. Percentage of presence of each marker in muscle fibres that display β -amyloid positive accumulations.

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Fig. 1

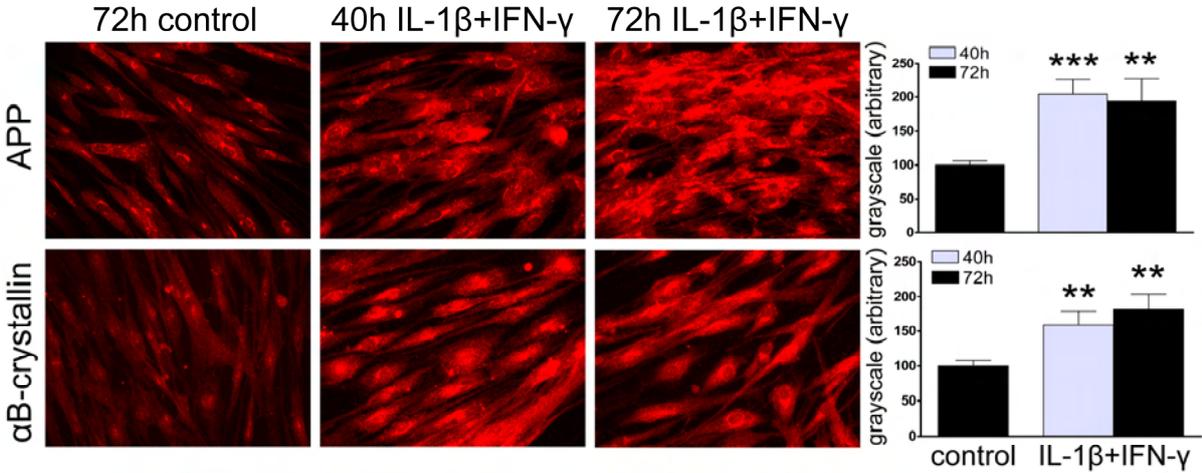
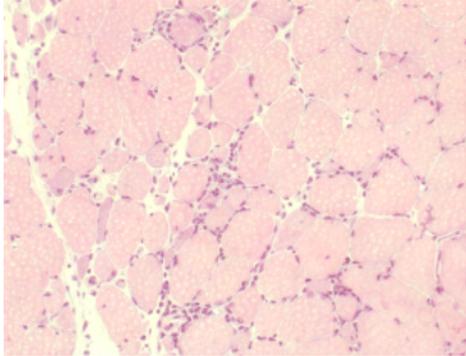


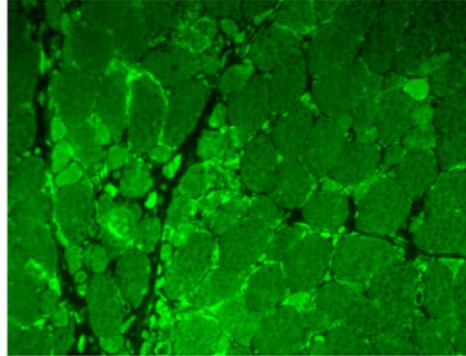
Fig. 2

A

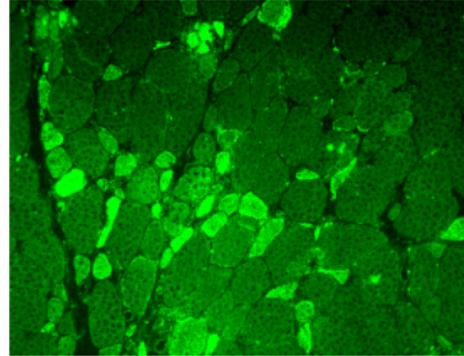
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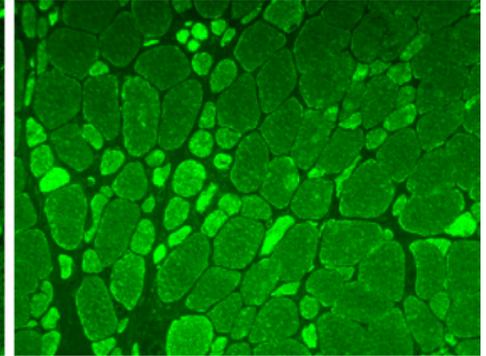
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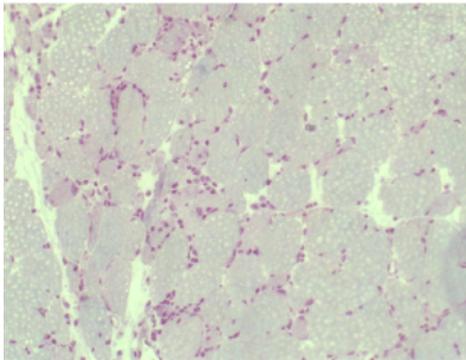
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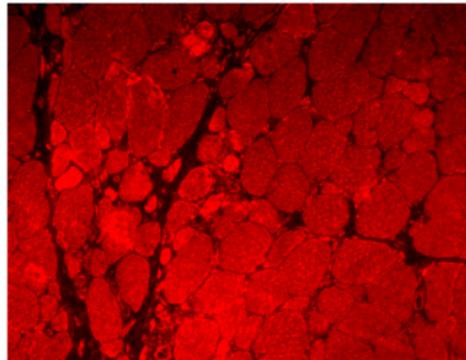
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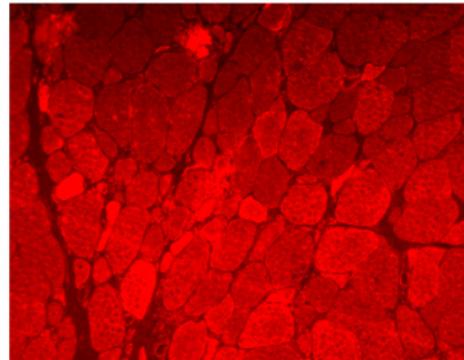
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α B-crystallin



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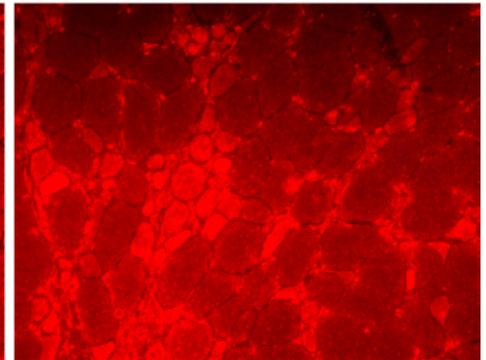


Fig. 2
B

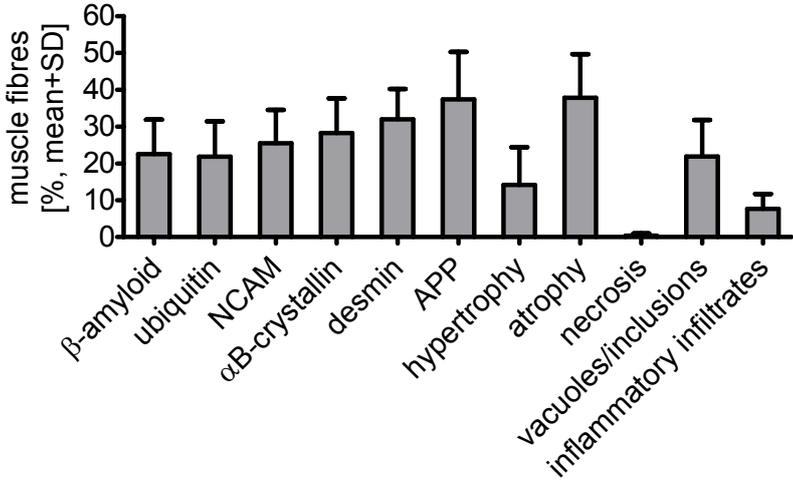
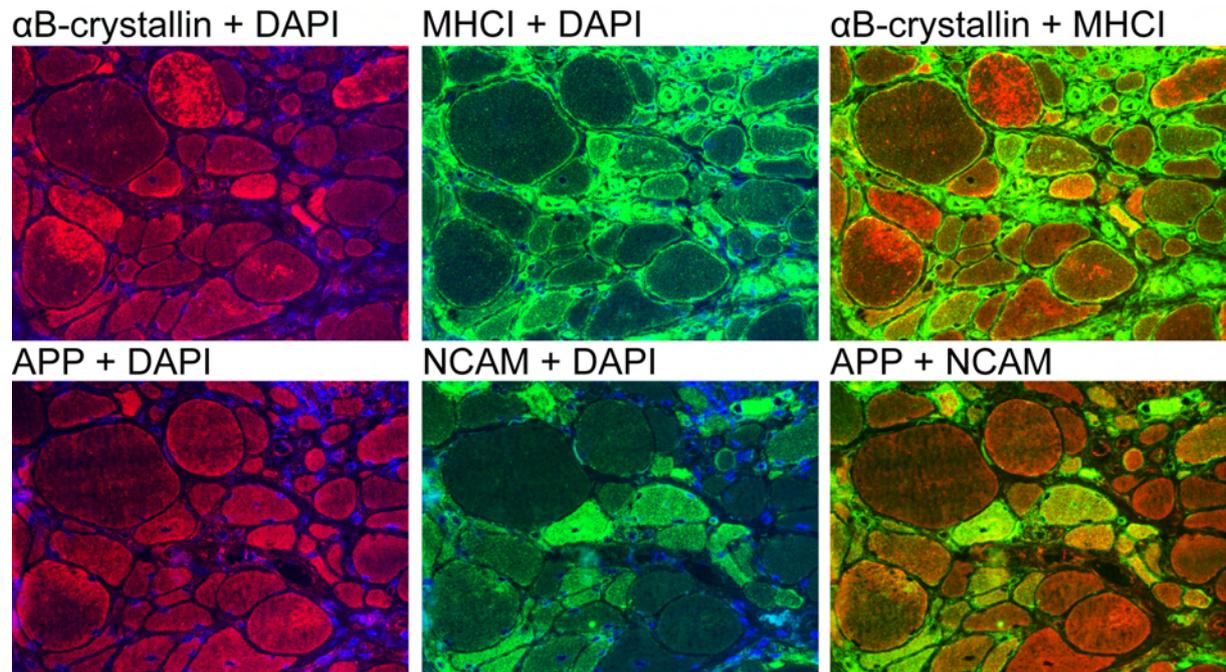


Fig. 3
A



B

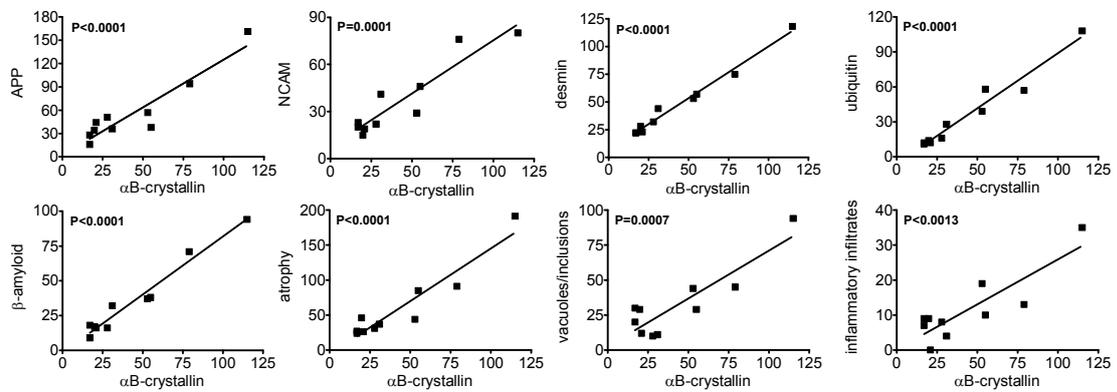
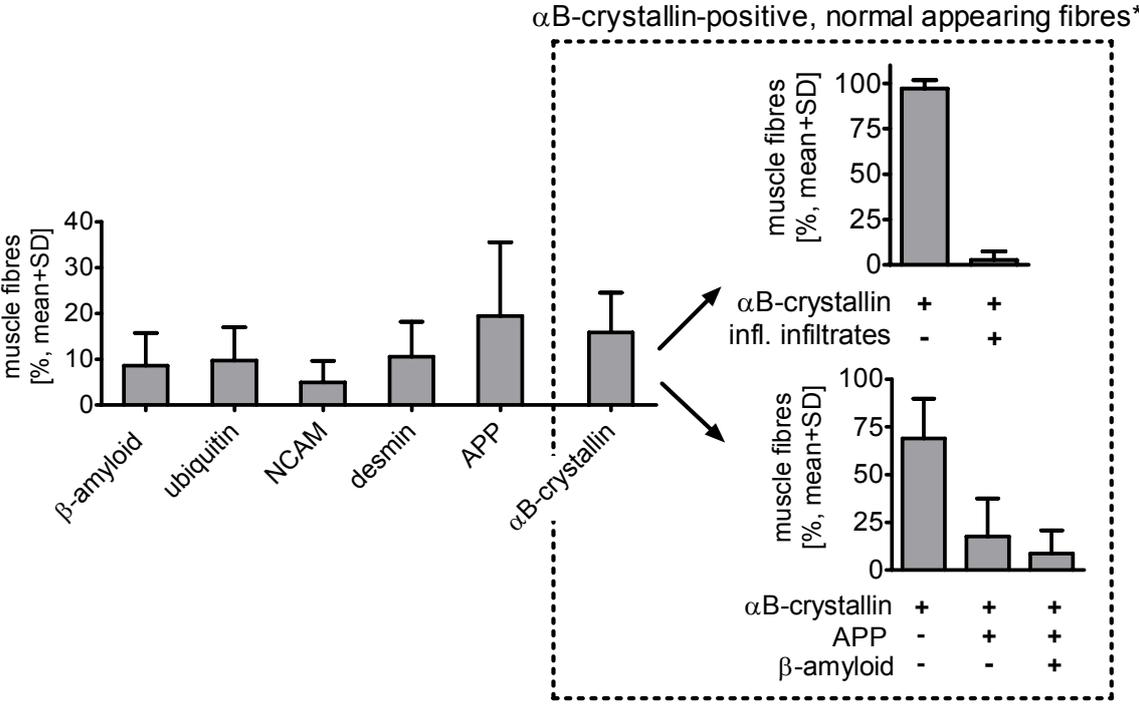


Fig. 4

A



B

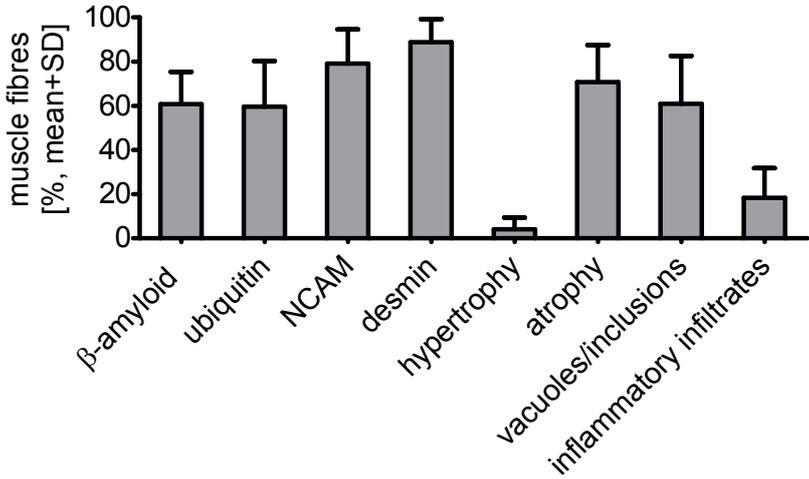


Fig. 5

