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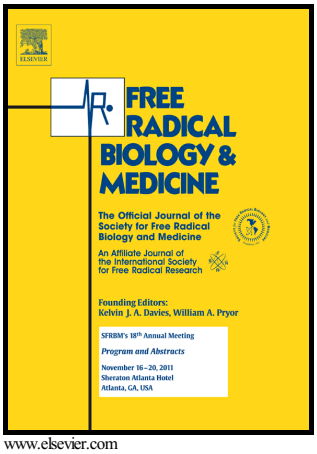

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Titin isoforms are increasingly protected against oxidative modifications in developing rat cardiomyocytes

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Abbreviations: BSA, bovine serum albumin; CI, carbonylation index; DTDP, dithiodipyridine; DTT, dithiothreitol; ECL, enhanced chemiluminescence; $F_{passive}$, cardiomyocyte passive tension; HSP, heat shock protein; ISO, isolating solution; LV, left ventricular; MHC, myosin heavy chain; N2B, stiff titin isoform; N2BA, compliant titin isoform; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SH, sulfhydryl; sHSPs, small heat shock protein, SL, sarcomere length

Abstract

During the perinatal adaptation process N2BA titin isoforms are switched for N2B titin isoforms leading to an increase in cardiomyocyte passive tension ($F_{passive}$). Here we attempted to reveal how titin isoform composition and oxidative insults (i.e. sulfhydryl (SH)-group oxidation or carbonylation) influence $F_{passive}$ of left ventricular cardiomyocytes during rat heart development. Moreover, we also examined a hypothetical protective role for titin associated small heat shock proteins (sHSPs), Hsp27 and αB-crystallin in the above processes. Single, permeabilized left ventricular (LV) cardiomyocytes of the rat (at various ages following birth) were exposed either to 2,2'-dithiodipyridine (DTDP) to provoke SH-oxidation or Fenton reaction reagents (iron(II), hydrogen peroxide (H_2O_2), ascorbic acid) to induce protein carbonylation of cardiomyocytes *in vitro*. Thereafter, cardiomyocyte force measurements for $F_{passive}$ determinations and Western immunoblot assays were carried out for the semiquantitative determination of oxidized SH-groups or carbonyl-groups of titin isoforms and to monitor sHSPs' expressions. DTDP or Fenton reagents increased $F_{passive}$ in 0- and 7-day-old rats to relatively higher extents than in 21-day-old and adult animals. The degrees of SH-group oxidation or carbonylation declined with cardiomyocyte age to smiliar extents for both titin isoforms. Moreover, the above characteristics were mirrored by increasing levels of HSP27 and αB-crystallin expressions during cardiomyocyte development. Our data implicate a gradual build-up of a protective mechanism against titin oxidation through the upregulation of HSP27 and αB-crystallin expressions during postnatal cardiomyocyte development.

Keywords: passive tension, titin isoform, oxidation, carbonylation, modifications, isolated cardiomyocytes, heat shock protein

Introduction

Transition from fetal to adult myocardium is coupled to specific changes in the expression pattern of sarcomeric protein isoforms, e.g. troponin-I and troponin-T [1-3], α-actin [4], myosin heavy chain [5] and titin [6, 7], in association with developmental changes in cardiac function [8, 9]. In this context, the biophysical characteristics of titin are considered to be decisive for the viscoelastic properties of the sacromere [10, 11]. Titin is a big protein, spanning a sarcomere from Z-disc to M-line, it may contain as many as 34.350 amino acids correponding to a molecular weight up to 3800 kDa [12]. Titin is encoded by a single gene of 363 exons with a molecular weight of ~3000-3800 kDa [13]. In mammalian hearts two titin isoforms are coexpressed resulting from multiple alternative splicing steps: the smaller and stiffer N2B (~3000 kDa) and larger, more compliant N2BA isoform (~3200-3800 kDa) [14]. In newborns, a highly compliant large fetal N2BA-type titin isoform is also expressed, and together with the N2BA isoform, they are gradually repleaced by the stiffer N2B isoform [6]. The N2BA titin isoform contains 513 cysteins whereas the N2B isoform contains 355 cysteins [15].

Oxidative modification of proteins by reactive oxygen species (ROS) range from protein fragmentation to different types of posttranslational modifications [16-18]. ROS induced contractile dysfunctions [19-22] have been implicated in different cardiovascular diseases including chronic heart failure [23, 24], myocardial ischemia and reperfusion [25], diabetic cardiomyopathy [26], and cardiac hyperthrophy [27, 28]. Titin oxidation modulates diastolic stiffness [29] through specific chemical changes of titin amino acid residues in a process depending on the chemical characteristics of the oxidative agent together with the three dimensional conformation of the interacting titin domains. For example, oxidation of six cysteinyl SH-groups resulted in three disulphide bridges in the cardiac specific N2B-unique sequence (N2-Bus) in titin, thereby increasing the rigidity of titin and cardiomyocyte passive

tension ($F_{passive}$) [30]. Exposures of human permeabilized cardiomyocytes to the SH-oxidant 2,2'-dithiodipyridine (DTDP) also increased cardiomyocyte $F_{passive}$ [20]. Conversely, S-glutathionylation of cryptic cysteines in Ig domains of the I-band region of titin resulted in a substantial drop in $F_{passive}$ in overstreched sarcomeres (*i.e.* at a sarcomere length of 2.6-2.7 μm) [22]. Protein carbonylation - the commonly considered biomarker of oxidative stress [31] - may also bear with functional consequence on sarcomeric funtion [32-34]. Protein carbonylation of lysine, arginine, proline or threonine residues develops in response to a hydroxyl radical mediated reaction involving reactive aldehydes (or ketones), through the participation of hydrogen peroxide (H_2O_2) and iron(II) (*i.e.* Fenton reaction) [35-37]. Interestingly, similarly to the *in vitro* effects of DTDP, incubations in the presence of Fenton reagents also increased $F_{passive}$ of human cardiomyocytes [38].

Recently it has been revealed that titin function is not only regulated by direct (i.e. posttranslational modifications of titin itself) but also by indirect mechanisms in the heart. In particular, two small heat shock proteins (sHSPs), the HSP27 and αB-crystallin contributes to the regulation of $F_{passive}$. sHSPs translocate to I-band region of titin springs and contribute to the maintaince of normal $F_{passive}$ under a variety of pathophysiological conditions [39]. Over the last decade studies have also addressed the influnce of age and oxidative stress on the function of HSPs [40, 41].

Here we focused on titin oxidation and changes in $F_{passive}$ of developing cardiomyocytes and related the mechanical alterations to titin isoform expressions/oxidations and to sHSPs expression. SH-specific oxidation or carbonylation were provoked by the SH-specific oxidative agent, DTDP or Fenton reagents *in vitro*. Dithiothreitol (DTT) was used to test reversibility. $F_{passive}$ was determined before and after exposures to oxidative and reducing agents in permeabilized cardiomyocyte-sized preparations of 0-, 7-, 21-day-old and adult (8-week-old) healthy rats at a wide range of sarcomere lengths. Finally, biochemical and

immunohistochemical assays were performed to correlate the age-dependent characteristics of titin isoform and sHSPs expressions with those of oxidative $F_{passive}$ changes.

Our results revealed the existince of an age-dependent protective mechanism to maintain physiological $F_{passive}$ under oxidative stress which was apperently mediated by changes in the expression of sHSPs.

Materials and Methods

Experimental specimens and ethical statement

All procedures were approved by the Ethical Committee of the University of Debrecen (Ethical Statement No. 24/2013/DE MÁB). Left ventricular (LV) myocardium was collected from 0-, 7-, 21-day-old and adult (8 week-old) healthy control Wistar rats. The animals were housed in standard cages at 20±2°C controlled room temperatures with natural day and night exchange. Standard laboratory chow and tap water were available *ad libitum*. The hearts were quickly excised and the left ventricles were dissected in cold isolating solution (ISO) (1 mM $MgCl_2$, 100 mM KCl, 2 mM EGTA, 4 mM ATP, 10 mM imidazole; pH 7.0, 0.5 mM phenylmethylsulfonyl fluoride, 40 μM leupeptin and 10 μM E-64, all from Sigma-Aldrich, St. Louis, MO, USA), snap frozen in liquid nitrogen, and stored at −80°C.

Passive force measurements in permeabilized cardiomyocytes and in vitro application of oxidative and reducing agents

Deep-frozen LV tissue samples were mechanically disrupted in ISO and thereafter permeabilization was performed with 0.5% Triton X-100 detergent for 5 min, as described by Papp et al. [42]. Briefly, single permeabilized cardiomyocytes were mounted with silicone adhesive (DAP 100% all-purpose silicone sealant; Baltimore, MD, USA) to two stainless steel insect needles, which were connected to a sensitive force transducer (SensoNor, Horten,

Norway) and to an electromagnetic high-speed lenght controller (Aurora Scientific Inc., Aurora, Canada) in ISO at 15°C. $F_{passive}$ measurements were performed at sarcomere lengths (SLs) of 1.9-2.5 µm in relaxing solution (10 mM N,N-Bis (2-hydroxyethyl)-2-aminoethanesulfonic acid, 37.11 mM KCl, 6.41 mM $MgCl_2$, 7 mM EGTA, 6.94 mM ATP, 15 mM creatine phosphate; pH 7.2 all from Sigma-Aldrich, St. Louis, MO, USA) in the presence of protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride, 40 µM leupeptin and 10 µM E-64. The Ca^{2+}-independent $F_{passive}$ was approximated by shortening of the preparations to 80% of the original lengths for 8 sec. $F_{passive}$ was normalized for the cardiomyocyte cross-sectional area, calculated from the width and height of the cardiomyocyte.

To modulate the SH-group content or carbonylation status of titin isoforms isolated cardiomyocytes were incubated at room temperature in 200 µl relaxing solution supplemented with the SH-oxidant DTDP (10 mM, Sigma-Aldrich, St. Louis, MO, USA) for 2 min or Fenton reagents (50 µM $FeSO_4$, 1.5 mM H_2O_2, 6 mM ascorbic acid, Sigma, St. Louis, MO, USA) for 7 min at a SL of 2.3 µm. Reversibility of the oxidative effects was tested by the sequential application of the thiol-groups reductant DTT (10 mM, Sigma-Aldrich, St. Louis, MO, USA) for 30 min.

Sodium dodecyl sulfate-polyacrilamid gel electrophoresis (SDS-PAGE)

Agarose-strengthened 2% SDS-polyacrilamide gels were used to separate titin isoforms. Separation of myosin heavy chain (MHC) small heat shock proteins (HSP27 and αB-crystallin) or myocardial porteins with relatively low molecular weights were carried out in 4%, 8% or 12% polyacrilamide gels. Proteins were visualized by Coomassie blue staining or by Western immunoblotting.

Ellman's reaction

Myofilament SH-group contents were determined by the Ellman's reaction. Permeabilized LV cardiomyocytes were treated with ISO (time control) or with ISO supplemented with DTDP and DTT as described for the functional and biochemical experiments. Protein homogenates were washed and incubated with the Ellman's reagent for 15 min at RT (5,5′-dithiobis(2-nitrobenzoic acid Sigma–Aldrich, St. Louis, MO, USA), which reacts with protein SH groups and produces yellow 2-nitro-5-thiobenzoic acid (NTB). The absorbance of NTB was measured by a NOVOStar microplate reader (BMG Labtech GmbH, Offenburg, Germany) at 412 nm. N-acetyl-l-cysteine (NAC; Sigma–Aldrich, St. Louis, MO, USA) was used to calibrate the NTB absorbance in relation to the amount of SH groups. The calibration curves were fitted to a single exponential, and SH-groups contents of LV heart samples were calculated. Measurements were performed in triplicate.

Determination of SH-group oxidation in titin isoforms

Cardiomyocytes were treated in relaxing solution containing DTDP (10 mM, for 2 min) or DTT (10 mM, for 30 min). Reduced SH-groups of titin isoforms of cardiomyocytes were labeled by EZ-Link Iodoacetyl-LC-Biotin or biotin-iodoacetamide (BIAM) (Thermo Scientific, Rockford, IL, USA, for 60 min in darkened conditions at room temperature) in a reaction buffer (containing 5 mM EDTA, 50 mM Tris–HCl, pH 8.3 and 0.1 mg/ml biotin) according to the manufacturer's instructions (biotin was dissolved in dimethyl formamide (Sigma-Aldrich, St. Louis, MO, USA) and diluted in reaction buffer to 0.1 mg/ml). After biotinylation, proteins of permeabilized cardiomyocytes were solubilized in sample buffer (containing 8 M urea, 2 M thiourea, 3% (w/v) sodium dodecyl sulfate (SDS), 75 mM DTT, 50 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 0.5% bromophenol blue, 40 μM leupeptin and 10 μM E-64) [43]. Following 45 min continuous agitation protein concentration was measured from the supernatant with a dot-blot based method, where different dilutions from bovine

serum albumine (BSA) served as standard, and protein concentration was adjusted to 2 mg/ml. Following SDS- PAGE proteins were transfered onto an Immobilon-FL PVDF membrane (Millipore, Billerica, MA, USA). Membranes were blocked by 2% BSA diluted in PBS containing 0.1% Tween 20 (PBST) for 30 min. Biotin-labeled SH-groups were probed with phycoerytrin-conjugated (PE) streptavidin (Jackson ImmunoResearch, West Grove, PA, USA, cat.no: 016-110-084) during 45 minuntes long incubations. Total protein amounts were quantitated with the super sensitive blot staining (UD-GenoMed, Debrecen, Hungary). Fluorescent signals were detected with a gel documentation system (MF-ChemiBIS 3.2, DNR Bio-Imaging Systems, Jerusalem, Israel), and subsequently normalized for those assessed with the super sensitive blot stain.

Determination of titin isoform carbonylation by oxyblot assay

The carbonyl group content of titin isoforms was determined using an oxyblot protein detection kit (Millipore, Billerica, MA, USA). Permeabilized cardiomyocytes were treated in 200 µl relaxing solution containing Fenton reagents (50 µM $FeSO_4$, 1.5 mM H_2O_2, 6 mM ascorbic acid, Sigma, St. Louis, MO, USA). Cardiomyocytes were solubilized in sample buffer (containing 8 M urea, 3% SDS, 50 mM Tris–HCl (pH 6.8), 40 µM leupeptin and 10 µM E-64), and the carbonyl groups were derivatized to 2,4-dinitrophenylhydrazone (DNPH) through a reaction with 2,4-dinitrophenylhydrazine (DNPH). After the derivatization process (15 min), samples were centrifuged (1000g for 1 min) and the pellet was dissolved in a sample buffer (8 M urea, 2 M thiourea, 3% (w/v) SDS, 75 mM DTT, 50 mM Tris-base (pH 14), 10% (v/v) glycerol, 0.5% bromophenol blue, 40 µM leupeptin and 10 µM E-64) [43]. Protein concentrations of the supernatants were determined by the dot-blot technique using a BSA standard and the concentration of samples was adjusted to 2 mg/ml. SDS-PAGE was used to seperate the derivatized titin isoforms. After gel electroforesis proteins were

transferred onto Immobilon-FL PVDF membrane. Proteins in PVDF membranes were visualized with a membrane stain. Then membranes were blocked with 2% (w/v) BSA in PBST for 30 min and probed with primary (rabbit anti-DNPH antibody 1:150, 1 h, cat.no: S7150) and biotin conjugated secondary antibodies (goat anti-rabbit IgG 1:1000 45 min) diluted in 1% (w/v) BSA/PBST. Biotin-labeled carbonyl-groups were probed with phycoerytrin-conjugated (PE) streptavidin during 45 minutes long incubations. Bands were normalized for those assessed with a super sensitive stain. Data are given relative to signal intensities of untreated controls (carbonylation index, CI).

Western immunoblot analysis of HSP27 and αB-crystallin

Isolated, permeabilized LV cardiomyocytes were used during Western immunoblot analyses. Cardiomyocyte proteins were solubilized in SDS sample buffer (Sigma, St. Louis, MO, USA) supplemented with 10 μM E-64, and 40 μM leupeptin. Protein concentration was measured from the supernatant with the dot-blot based method, as mentioned above, and was adjusted to 2 mg/ml. Proteins were loaded onto 8% SDS-polyacrylamide gels. Proteins were transfered onto nitrocellulose membranes. Membranes were blocked with 5% BSA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in PBST and probed with primary (anti-HSP27 (1:300) or anti-αB-crystallin (1:1000) Abcam, Cambridge, UK, cat.no: ab2790 and ab76467) and secondary antibodies (peroxidase- conjugated goat anti-mouse (1:100000) or goat anti-rabbit (1:300), Sigma-Aldrich, St. Louis, MO, USA). Bands were visualized by the chemiluminescence (ECL) method. Total protein amounts were quantified with the super sensitive membrane stain.

Immunohistochemistry

Cardiomyocytes were isolated from frozen LV heart tissues the same way as for the functional measurements. Cardiomyocytes were permeabilized in 0.5% Triton X-100 and samples were fixed with cold acetone for 10 min and were washed twice with PBS (containing 150.7 mM NaCl, 3.22 mM KCl, 0.735 mM KH_2PO_4, 8.6 mM Na_2HPO_4; pH 7.4 all from Sigma-Aldrich, St. Louis, MO, USA) on microscope slides. Then the samples were incubated in methanol for 20 min, followed by a washing step with PBS for 5 min and blocked in normal goat sera (1.5% in PBS, Sigma-Aldrich, St. Louis, MO, USA) for 20 min. Next, tissue samples were incubated with primary antibodies (anti-HSP27 (1:50, Abcam, Cambridge, UK, cat.no: ab2790), anti-αB-crystallin (1:300, Abcam, Cambridge, UK, cat.no: ab76467) and anti-titin (1:50, T11, Sigma-Aldrich, St. Louis, MO, USA, and T63 by *Wolfgang A. Linke* from Bochum, Germany) diluted in blocking buffer (1.5% goat serum in PBS) overnight at 4°C, and thereafter secondary antibodies were applied (goat-anti-rabbit Cy2 and Cy3 and biotinylated goat-anti-mouse (1:200, Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at room temperature. Biotinylated antiobodies were detected by Cy2- and Cy3- conjugated streptavidine (1:500, Jackson, Bar Harbor, ME, USA). Images were captured by a Scion Corporation (Frederick, MA, USA) digital camera attached to a Nikon Eclipse 80i fluorescent microscope (Nikon, Tokyo, Japan). Recorded myocyte images were processed by ImageJ software.

Data analysis, statistics

$F_{passive}$ values are expressed in kN/m^2. Signal intensities of protein bands were quantified by using the ImageJ, image processing program (National Institutes of Health, Bethesda, Maryland, USA) and Magic Plot (Magicplot Systems, Saint Petersburg, Russia) softwares. Statistical significance was tested by analysis of variance (ANOVA followed by Bonferroni's post hoc test). GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) software

was used to evaluate the results. Values are given as mean±SEM. The limit for statistical significance was $P < 0.05$.

Results

1. SH oxidation of titin isoforms increases $F_{passive}$ in an age-dependent manner

Permeablized LV cardiomyocytes of 0-, 7-, 21-day-old, or adult rats were characterized by gradually increasing passive stiffness ($F_{passive}$) along with decreasing expression levels of the N2BA titin isoform and increasing expression levels of the N2B titin isoform (Supplemantary Material, Figure S1) as expected. The concentration dependent effects of DTDP (between 1 mM and 50 mM) on free SH-group contents of N2BA and N2B titin isoforms and $F_{passive}$ were screened in permeabilized cardiomyocytes of 7-day-old animals, where we found marked responses in the above parameters following incubations in the presence of 10 mM DTDP (Supplementary Material, Figure 2S).

Following exposures to the SH-oxidant DTDP F_{passive} increased significantly in all age-groups, but to relatively higher extents in the newborn (0-day-old) and 7-day-old age groups than in the 21-day-old and adult age groups (F_{passive} changed from 0.09±0.02 kN/m^2 to 0.18±0.03 kN/m^2 in the newborn group; from 0.19±0.02 kN/m^2 to 0.31±0.04 kN/m^2 in the 7 day-old age group; from 0.45±0.07 kN/m^2 to 0.60±0.07 kN/m^2 in the 21 day-old group and from 2.35±0.11 kN/m^2 to 2.76±0.16 kN/m^2 in cardiomyocytes of adult rats, SL: 2.3 µm, P<0.05 *vs.* control in all age groups, n=6-8 cardiomyocytes from at least three different hearts, Figure 1). DTT was applied after DTDP treatments to test the reversibility of SH oxidation. DTDP mediated increases in $F_{passive}$ were reversed upon DTT treatment in all age groups ($F_{passive}$ following DTT were: 0.10±0.01 kN/m^2; 0.21±0.02 kN/m^2; 0.51±0.08 kN/m^2 and 2.28±0.12 kN/m^2 in 0, 7, 21 day-old and adult rat cardiomyocytes, respectively, SL: 2.3 µm; n=6-8 cardiomyocytes from at least three different hearts; Figure 1). To test a hypothetical SL

dependent influence on DTDP-evoked $F_{passive}$ changes, 0 day-old and adult cardiomyocytes (7 cardiomyocytes in both groups) were also exposed to DTDP at a reduced SL (*i.e.* at 1.9 μm). However, increases in $F_{passive}$ following DTDP incubations at 1.9 μm or 2.3 μm SLs were indistinguishable from each other when tested at a SL of 2.3 μm ($P>0.05$) in both age groups (data not shown). Hence, these experitments argued against a hypothetical SL dependent effect of DTDP (within a range of SLs between 1.9 μm and 2.3 μm).

A biotinylation-based assay was included to evaluate the SH oxidative changes of N2BA and N2B titin isoforms following DTDP exposures. DTDP decreased the free SH-group content of N2BA and N2B titin isoforms by similar extents (normalized SH-group content: 100% before DTDP exposures; control) in permeabilized cardiomyocytes of the 0-, 7-, 21-day-old and adult age groups (*i.e.* by: 80±1%, 71±1%, 64±1% and NA in the N2BA titin isoform; and by: 74±1%, 62±2%, 53±2% and 32±2% in the N2B titin isoform; $P<0.05$ *vs.* control, for the above age groups, respectively, n=8-23 independent determinations, Figure 2). The free SH-group content of N2BA titin isform in the adult age group could not be assessed (NA) due to the low expression level of this titin isoform. Free SH-group sensitive signal intensities returned to control levels following DTT applications, moreover, DTT alone had no effect on cardiomyocyte SH-group oxidation (Figure 2). Figure S3A illustrates the SH-contents of the two titin isoforms in control and DTDP-treated cardiomyocytes in blots including all four age groups. In this study, functional and biochemical studies were performed in defrosted permeabilized cardiomyocytes from animals of different age groups. Using the SH-sensitive Ellman's reaction and biotinylation assays revealed no statistically significant differences between the free SH-contents of frozen/thawed and freshly isolated samples (Supplementary Material, Figure S4). Results of additional assays by the SH-sensitive biotin-iodoacetamide (BIAM) [44] gave qualitatively similar results (Supplementary

Material, Figure S5) to those obtained by EZ-Link-Iodoacetyl-LC-Biotin (Figures 2, S3A, S4A, S6A).

2. Carbonylation of titin isoforms increases $F_{passive}$ in an age-dependent manner

The effects of protein carbonylation on $F_{passive}$ were investigated in permeabilized LV cardiomyocytes in different age groups following incubations in the presence of Fenton reagents. Similarly as above, attempts were made to reverse the Fenton effects by the application of DTT. Figure 3 illustrates that Fenton reagents increased $F_{passive}$ significantly in 0-, 7-, 21-day-old and adult groups, but gradually to less and less relative extents with cardiomyocyte age (*i.e.* to: from 0.11±0.01 kN/m^2 to 0.22±0.01 kN/m^2 in 0-day-old, from 0.26±0.02 kN/m^2 to 0.44±0.05 kN/m^2 in 7-day-old, from 0.49±0.03 kN/m^2 to 0.69±0.06 kN/m^2 in 21-day-old, from 2.17±0.05 kN/m^2 to 2.59±0.10 kN/m^2 in adult rats, respectively, $P<0.05$ *vs.* control, n=6-7 cardiomyocytes from at least three different hearts). Moreover, the above mechnical effects were fully reversed by the application of DTT in the 0-,7- and 21-day-old groups, but not in adult group ($F_{passive}$: 0.11±0.01 kN/m^2; 0.22±0.02 kN/m^2; 0.35±0.03 kN/m^2 and 2.94±0.15 kN/m^2, in the above age groups, respectively, $P<0.05$ *vs.* control, n=6-7 cardiomyocytes from at least three different hearts).

In paralell biochemical investigations oxyblot assays were performed to reveal the extents of carbonyl group formations of N2BA and N2B titin isoforms in myocardial tissue homogenates following incubations in the presence of Fenton reagents. Figure 4 illustrates that the levels of carbonyl groups (carbonylation index of untreated control: 1 AU) increased significantly following exposures to Fenton reagents, although in an apparent negative correlation with age (*i.e.* to 2.57±0.06 AU, 2.37±0.02 AU, 1.35±0.03 AU, in 0-, 7-, 21-day-old cardiomyocytes, respectively, in N2BA titin isoforms; and to: 2.90±0.10 AU, 2.57±0.04 AU, 1.79±0.03 AU, 1.51±0.02 AU, in 0-, 7-, 21-day-old and adult cardiomyocytes,

respectively, in N2B titin isoforms, P<0.05, n=9-18 independent determinations from at least three different hearts, Figure 4). Protein carbonylation of the N2BA titin isform in the adult age group could not be assessed due to the low expression level of this titin isoform. Titin carbonylations were generarly reversed by DTT, except in adult cardiomyocytes. Figure S3B illustrates the carbonylations of the two titin isoforms of control and Fenton-treated cardiomyocytes in blots including all four age groups. Figure S6B illustrates the age dependence of protein carbonylation for additional myofilament proteins.

3. Gradual increases in the expression levels of HSP27 and αB-crystallin proteins in developing cardiomyocytes

To characterize the levels of HSP27 and αB-crystallin expressions in permeabilized cardiomyocytes tissue homogenates from 0-, 7-, 21-day-old and adult rat hearts were analyzed by Western immunblotblotting. These investigations revealed, that the relative expression levels of HSP27 (1.04±0.08 AU, 2.14±0.30 AU, 3.04±0.49 AU, 4.83±0.54 AU in 0-, 7-, 21-day-old and adult rats, respectively, P<0.05 *vs.* day 0 (d0, reference level:1), n=9-17 determinations from at least three different hearts; Figure 5A), and αB-crystallin (1.00±0.11 AU, 1.61±0.19 AU, 3.23±0.86 AU, 4.42±0.96 AU in 0-, 7-, 21-day-old and adult rats, respectively, P<0.05 *vs.* d0, n=8-15 determinations from at least three different hearts; Figure 6B) gradually increased with cardiomyocyte age. Figure 5C illustrates the increasing levels of sHSPs compared to the amounts of titin isoform expressions (total titin) during cardiac developement. (Expression levels of both sHSPs were normalized to actin expression, while relative titin levels are given relative to MHC expression. The ratio of MHC to actin expression appeared to be constant in all age groups (data not shown).

In accordance with the biochemical data, immunohistochemical staining of LV cardiomyocytes revealed progressive HSP27 and αB-crystallin expressions with remarkbale colocalizations with titin proteins during cardiomyocyte maturation (Figure 6).

4. Correlations between oxidative changes of cardiomyocyte $F_{passive}$, oxidative changes of titin isoforms and expression levels of HSP27 and αB-crystallin during cardiomyocyte development

Strong negative correlations were noted when protein oxidation evoked changes in $F_{passive}$, oxidized SH contents or carbonylation indices of titin isoforms were contrasted to the expression levels of HSP27 or αB-crystallin at different cardiomyocyte ages (Figure 7).

Discussion

Major novelties of the present study are that titin oxidation and carbonylation have similar effects on N2BA and N2B titin isoforms, and myofibrillum associated heat shock proteins (HSP27 and αB-crystallin) may confer protection against the above forms of oxidative modifications. Interestingly, this putative HSP-mediated protection builds up gradually with postnatal cardiomyocyte age, without the influence of experimental stress of any forms (*i.e.* in control, untreated animals).

In this study, F_{passive} was probed by exposures to oxidative agents in isolated, permeabilized cardiomyocytes at physiologically relevant sarcomere lengths and at various postnatal developmental stages. This approach also allowed the investigation of a hypothetical link between titin isoform oxidation and sHSPs (*i.e.* HSP27 and αB-crystallin) expression. Here we report that cardiomyocyte development is accompained by a gradual decline in the sensitivity of both N2BA and N2B titin isoforms to oxidative challenges, and consequently F_{passive} became more and more resistant against oxidative insults in developing

cardiomyocytes. This finding implicates the build up of a protective mechanism against titin oxidation (not discriminating between the two titin isoforms) during postnatal cardiomyocyte maturation.

Our present data on the relationship between titin isoform switching and $F_{passive}$ production are consistent with those of earlier investigations [45] [6]. $F_{passive}$ increased with cardiomyocyte age at a wide range of sarcomere lengths due to an increase in the N2B/N2BA titin isoform expression ratio together with increases in total sarcomeric titin expression. To compensate for the effects of changes in total titin expression $\Delta F_{passive}$ has been normalized for baseline $F_{passive}$ when the mechanisms of oxidative challenges were analyzed. Besides titin isoform composition [46] cardiomyocyte $F_{passive}$ can be also increased by dephosphorylation ([47]) and oxidation (including SH-group oxidation [30], and carbonylation [38]) of titin isoforms. Nevertheless, till now little information became available on the oxidation of titin isoforms and on their functional consequences in the developing heart.

In our present work, SH-group oxidation of titin isoforms was provoked by an SH-group specific oxidative agent, DTDP *in vitro*. DTDP can react and oxidize thiol groups of proteins, thereby generating intra- or intermolecular disulfide bridges or mixed disulfides between the cysteinil residues and one-half of the DTDP molecules [48]. We observed that the DTDP evoked increments in $F_{passive}$ (when normalized for baseline $F_{passive}$) declined with age in permeabilized cardiomyocytes of the rat in a reversible fashion. Moreover, the extent of DTDP evoked SH-group oxidation also declined with age in both N2BA and N2B titin isoforms (Figure 8). To avoid aspecific reactions with histidyl side chains, pH was set to 8.3 during SH-group labelling by EZ-Link-Iodoacetyl-LC-Biotin. It is to be stressed, that at pH 8.3 more thiols are labelled by EZ-Link-Iodoacetyl-LC-Biotin than those being sensitive to oxidation at a lower pH, and consequently this approach represents a limitation from a quantitative viewpoint. Nevertheless, results of additional assays where reactive cysteine

thiols were specifically labelled by BIAM at pH 6.5 were consistent with those obtained by EZ-Link-Iodoacetyl-LC-Biotin labelling, thereby confirming the main finding of this work on the age dependent decline in oxidant sensitive thiol contents of the two titin isoforms.

In addition to SH-group oxidation, ROS induced myofilament protein carbonylation can also contribute to contractile dysfunction in the heart [49]. Hydroxyl radicals are highly reactive and thus react with divergent cell components [50]. In this study, Fenton reagents were used to produce hydroxyl radicals and to induce titin carbonylation *in vitro*. Fenton reagents increased F_{passive} in all age groups. Moreover, similarly to the effects of DTDP, the increases in F_{passive} (when normalized for baseline F_{passive}) declined with age. Furthermore, the extent of titin carbonylation also diminished with age (Figure 8). Interestingly, DTT exposures following Fenton treatments could restore F_{passive} in 0-, 7- and 21-day-old cardiomyocytes, but not in adult cardiomyocytes. Moreover, carbonyl content mirrored these mechanical effects. These findings are consistent with those of *Wong et al.* who reported that carbonylated protein content can be decreased by thiol-group reductants resulting in protein decarbonylation [51, 52].

Taken together, for oxidative challenges of identical strengths, the extents of titin SH-oxidations or carbonylations declined with age for both titin isoforms. This effect apparently did not depend on the N2B/N2BA titin isoform expression ratio and paralleled the changes of normalized $\Delta F_{passive}$, suggestive for a cause-effect relationship. Moreover, these findings are also suggestive for a gradual decline in the oxidative sensitivities of cardiomyocyte F_{passive} with cardiomyocyte maturation. Interestingly, for a given $\Delta F_{passive}$ the involvements of titin isoforms by SH-oxidation or carbonylation were remarkably similar (Figure 8). Collectively, these findings implicated the gradual build up of a protective mechanism being irrespective to the chemical nature of oxidative insults (*i.e.* SH-oxidation *vs.* protein carbonylation) or to titin isoform expression.

Previous experimental studies furnished with evidence on the protective roles for sHSPs for sarcomeric proteins such as actin, desmin, myosin, tropinin-I, troponin-T and titin [53-56]. sHSPs have been implicated in promoting physiological protein folding and preventing misfolding, thereby protecting the function of native macromolecules, and limiting the effects of oxidative stress in general [57]. In the above context misfolded proteins have been associated with higher sensitivities for carbonylation than those of their native forms [31]. Two myocardial sHSPs, HSP27 and αB-crystallin are known to be induced by heat stress, ageing, end-stage heart failure or ishemic injury [58, 59]. Moreover, these proteins are also known to be involved in preventing the effects of oxidative stress [60] and in the regulation of cellular redox states [61]. Although many sHSPs are associated with sarcomeric proteins, it has been postulated that HSP27 is not bound to actin, actin linked proteins, myosin or desmin in zebrafish cardiomyocytes [62]. However, HSP27 and αB-crystallin can both colocalize with I-band titin in cardiac and skeletal muscles, translocate and bind to the spring elements of titin isoforms under stress conditions [39] [56]. A potential contribution of sHSPs to normal passive elasticity and a role in the maintenance of normal function of cardiac and skeletal muscles have been also implicated [39, 56, 63, 64]. Interestingly, Kötter et al. demonstrated that the interaction between titin and sHSPs is enhanced by the expression of the N2B titin isoform. Consistently, we found strong co-localiozation between sHSPs and the sarcomeric cross striation pattern at more advanced ages when N2B titin isoform expression was high [39]. Moreover, it has been argued, that in the presence of sHSPs, Ig domains of titin are prevented from unfolding [56] and that the unfolded Ig regions of titin (disordered titin spring elemnts: N2-Bus and PEVK) are probably protected against aggregation [39]. In neonatal cardiomyocytes the more compliant N2BA is the dominant isoform, and sHSPs might not bind efficiently to compliant titin [39]. Accordingly, we could not find prominent co-localization between sHSPs and titin in neonatal cardiomyocytes, thereby hypothetically

leaving more functional groups of titin molecules accessible for oxidative insults. Our present findings extend the above observations, and suggest that the increase in sHSPs expression can gradually outweight the increase in titin expression during cardiomyocyte development, thereby increasing the probability for interactions between sHSPs and titin isoforms. Moreover, our data did not support major distinctions between the oxidative characteristics of N2BA and N2B titin isoforms. Based on remarkably pronounced correlations between the expression levels of HSP27 and αB-crystallin and oxidative changes in mechanical characteristics of cardiomyocytes (*i.e.* in $F_{passive}$) and biochemical (*i.e.* SH oxidation or carbonylation) properties of titin isoforms, we postulate a link between the oxidative sensitivities of titin isoforms and the expression levels of HSP27 and αB-crystallin.

Sarcomeric protein oxidation is the function of chemical and spatial characteristics of the employed oxidative agent and of the interacting proteins. In this context SL appears as a crucial determinant for titin oxidation by modulating exposures of oxidation sensitive amino acid residues through protein folding/unfolding [22]. In this study, mechanical responses on SH-group oxidation where not different when cardiomyocytes of newborn and adult rats were exposed to DTDP at a SL of 1.9 μm or 2.3 μm, suggesting that in this range of SLs the above effect had negligable influence. Nevertheless, we cannot exclude that oxidative manipulations by other oxidative agents and at higher sarcomere lengths than employed here may expose additional, so called cryptic cysteinil residues of titin isoforms thereby leading to distinct functional effects. The hypothetical involvement of sHSPs in preventing the oxidation of titin isoforms is supported only by correlative associations here, and hence we have to stress that additional molecular mechanisms can be also involved in the above processes.

In summary, this work implicated relationships between titin isoform oxidation, cardiomycyte F_{passive} and HSP27/αB-crystallin expression in the developing rat heart. Two different types of oxidative modifications: SH-group oxidation and carbonylation of titin

isoforms influenced $F_{passive}$ of cardiomyocytes in a similar fashion suggesting common molecular mechanisms for their coordinated effects. Our data suggest that during cardiac development the oxidative sensitivity of titin-based $F_{passive}$ declines due to the build-up of a protective mechanism preventing the oxidation of titin. We propose that this protection is mediated (at least in part) by sHSPs, such as HSP27 and αB-crystallin.

In this investigation we focused on titin, which is probably the most important determinant of cardiomyocyte $F_{passive}$. The employed oxidative interventions were arbitrarily set to levels severe enough to provoke significant changes in cardiomyocyte $F_{passive}$, and to allow quantification of titin alterations from technically challenging gel procedures. One may argue that under these conditions several cardiomyocyte proteins are affected, and therefore the recorded mechanical alterations might be complemented by protein changes other than titin. As a matter of fact, age-dependent characteristics appeared to be similar to those of the two titin isoforms for some (e.g. at the molecular weights of MHC, α-actinin, actin and MLC), but not all cardiomyocyte proteins (e.g. at the molecular weights of desmin, tropomyosin and cTnI). In addition, age dependent oxidant sensitivity of reactive thiols may also be the function of thiol:protein abundance ratio, a paramater not systematically screened here. It is clear from the above limitations, that further efforts are needed to address the age dependence of cardiomyocyte protein oxidation and their contribution to cardiomoycyte function under more physiological conditions. An additional major limitation of our study is that we have not employed a model system where plasmid- or virus-mediated HSP27 or αB-crystallin are overexpressed in a controlled way, and the impact of this on titin oxidation is determined. An interventional study such as this one may help address this major limitation.

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Figure legends

Figure 1. Effects of DTDP on cardiomyocyte $F_{passive}$ generation in different age groups. Bar graphs illustrate the magnitudes of changes in $F_{passive}$ after DTDP treatments (10 mM) in permeabilized cardiomyocytes of 0- **(A)**, 7- **(B)**, 21-day-old **(C)** and adult **(D)** animals followed by the application of the thiol-group reductant DTT (10 mM) (n=6-8 cardiomyocytes in each group) at a sarcomere length of 2.3 μm. Data are expressed as mean±SEM, *P<0.05 *vs.* control.

Figure 2. DTDP-induced SH-group oxidation of titin isoforms during postnatal heart development. After DTDP treatments, free SH-group content of N2BA and N2B titin isoforms was determined by a biotinylation assay and total protein content of proteins were quantitated with blot staining in all groups. The ratios of the optical densities of the bands (free SH-group content/total protein) were normalized to untreated control for each membrane (n=10-16 hearts). T2 is a degradation product of titin, that mainly contains the A-band segment of titin [6]. Symbols illustrate means±SEM, *P<0.05 in all graphs *vs.* csontrol **(A-D)**. SH-group oxidatin of N2BA titin isoform could not be detected in adult rat cardiomyocytes due to its low expression level **(D)**. Discontinuities within the membranes are signified by continous lines.

Figure 3. The effects of protein carbonylation on $F_{passive}$ in cardiomyocytes in different age groups. $F_{passive}$ in LV cardiomyocytes were determined in relaxing solution before (control), after exposures to Fenton reagents (Fenton) in the four age-groups (n=6-7 cardiomyocytes in each group), and after incubations in the presence of DTT (10 mM) **(A-D)**. Data are expressed as mean±SEM, *P<0.05 *vs.* untreated control.

Figure 4. Fenton reaction-induced carbonylation of titin isoforms during postnatal heart development. Myocardial samples of different age groups were exposed to Fenton reagents in relaxing solution and protein carbonylation was subsequently assayed by the oxyblot method (n=9-16 hearts). Total protein amounts were determined with blot staining. Carbonylation is given in relative terms as carbonylation index (CI = 1, based on signal intensities in relaxing solution, control) **(A-D)**. Data are expressed as mean±SEM, *P< 0.05. Carbonylation of the N2BA titin isoform could not be detected in adult rat cardiomyocytes due to its low expression level **(D)**.

Figure 5. The expression levels of HSP27 and αB-crystallin increase during cardiac development. Representative examples of Western immunoblot assays demonstrate the expression levels of HSP27 **(A)** and αB-crystallin **(B)** in permeabilized cardiomyocytes during rat heart development. HSP27 and αB-crystallin were labelled with specific antibodies and actin was visualized by blot staining following its identification based on its molecular mass. Both sHSPs amounts were contrasted to the expression levels of actin. The expression levels of total titin isoforms (same data as in Figure S1D), HSP27 and αB-crystallin were also contrasted to each other as a function of cardiomyocyte age **(C)**. Symbols illustrate means±SEM, and asterisks (*) depict significant differences *vs.* 0-day-old rat hearts (Control) (n=6-8 hearts).

Figure 6. Binding of HSP27 and αB-crystallin to titin molecules. Cardiomyocytes of different age groups were labeled with ant-titin T11 (green) and anti-HSP27 (red) **(A)** or titin T63 (green) and anti-αB-crystallin (red) antibodies **(B)**. Cy2- or Cy3-conjugated secondary antibodies were used for development. Merged images are given in the right columns. Similar observations were obtained in four independent immunohistochemical assays.

Figure 7. Expression levels of sHSPs correlated with oxidative changes in $F_{passive}$ and oxidative modifications of titin isoforms. Strong correlations were found between the expression levels of HSP27 or αB-crystallin and $\Delta F_{passive}$ of DTDP- or $\Delta F_{passive}$ of Fenton-treated cardiomyocytes **(A-D)**. The oxidized SH contents and carbonylation indices of N2B and N2BA titin isoforms correlated reasonably with the expression levels of HSP27 or αB-crystallin in all groups. **(E-H).** Correlations were assessed by approximating the values by linear regression. Data points represent means±SEM.

Figure 8. Relative extents of DTDP-evoked or Fenton-evoked changes in cardiomyocyte $F_{passive}$ values together with oxidized SH-groups or carbonyl contents of titin isoforms in developing rat hearts. Cardiomyocyte $F_{passive}$ values (expressed relative to the control values following DTDP or Fenton treatments in the four age groups, see Figure 1 and Figure 3 for absolute values) illustrate gradually decreasing oxidative sensitivities for developing cardiomyocytes following birth ($\Delta F_{passive}$ of DTDP: 99±26 % in 0-, *vs.* 65±23 % in 7-, *vs*. 39±21 % in 21-day old and 18±7 % in adult groups. $\Delta F_{passive}$ of Fenton: 108±12 %; 69±10 %; 40±5 % *vs.* 21±5% 0-, 7-, 21-day-old and adult groups, SL: 2.3μm $P<0.05$) **(A-B)**. Oxidized SH-group contents of N2BA and N2B isoforms were calculated from free SH-group contents of titin isoforms (Figure 2) following DTDP treatments (N2BA: 80±1%, 71±1%, 64±1%, for

the first three age groups, respectively; N2B: 74±1%, 62±2%, 53±2%, 32±2%,; for the four age groups, respectively, n=10-16 hearts, P<0.05) **(C)**, and panel **D** illustrates the carbonylation indices (CI=1, intesities of all bands were compared with those of the controls on the same membranes) following Fenton treatments (N2BA: 2.57±0.06 AU, 2.34±0.04 AU, 1.35±0.03 AU, for the first three age groups, respectively; N2B: 2.9±0.1 AU, 2.58±0.04 AU, 1.74±0.04 AU, 1.53±0.05 AU for the four age groups, respectively; P<0.05, n=9-16 hearts) (Figure 4) with ageing. Data are means±SEM, and asterisks illustrate significant differences *vs.* data at 0-day-old cardiomyocytes (control) in all graphs. Biochemical data of N2B titin isoforms could be analyzed in adult rat cardiomyocytes only.

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Highlights

- Oxidative sensitivity of $F_{passive}$ gradually declines with cardiomyocytes age
- The susceptibilities of N2BA and N2B titin isoforms for oxidative modifications are similar at a given cardiomyocyte age
- The expression levels of myofibrillum associated sHSPs increase gradually in the developing heart
- sHSPs may confer protection against oxidative titin modifications

Figure 1

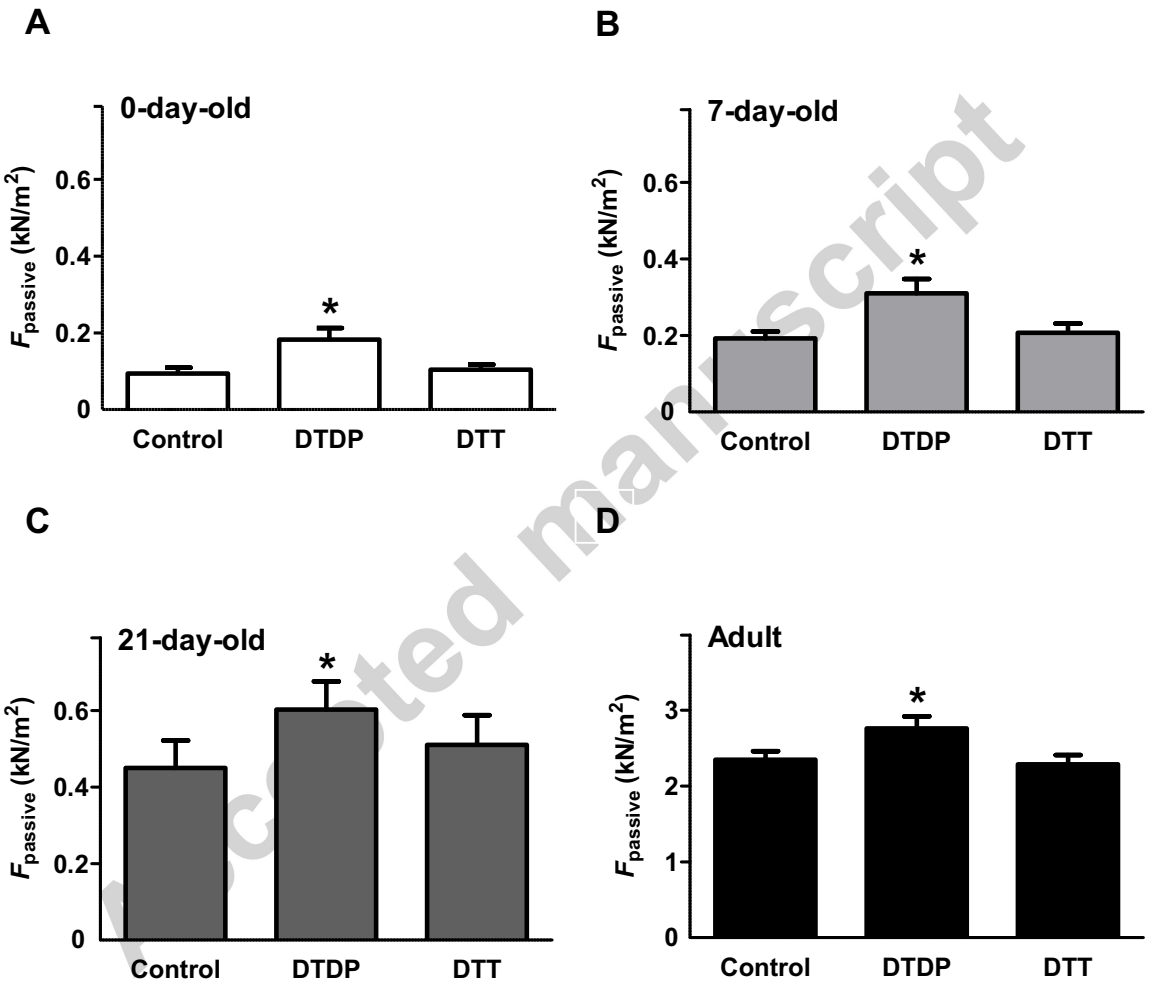

Figure 2

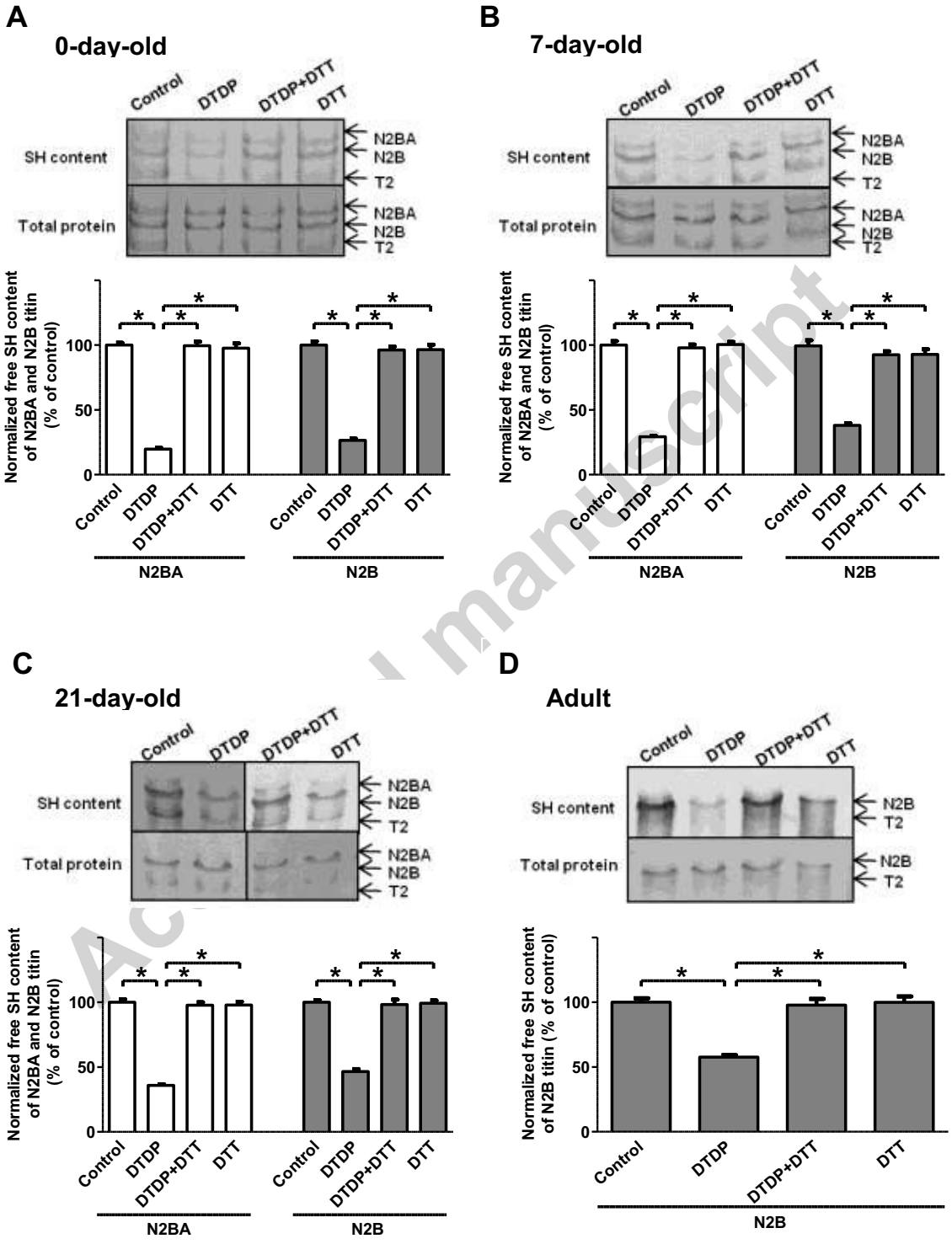
A
0-day-old
Control
DTDP
DTDP+DTT
DTT
SH content
Total protein
N2BA
N2B
T2
Normalized free SH content of N2BA and N2B titin (% of control)
N2BA
N2B
B
7-day-old
C
21-day-old
D
Adult

Figure 3

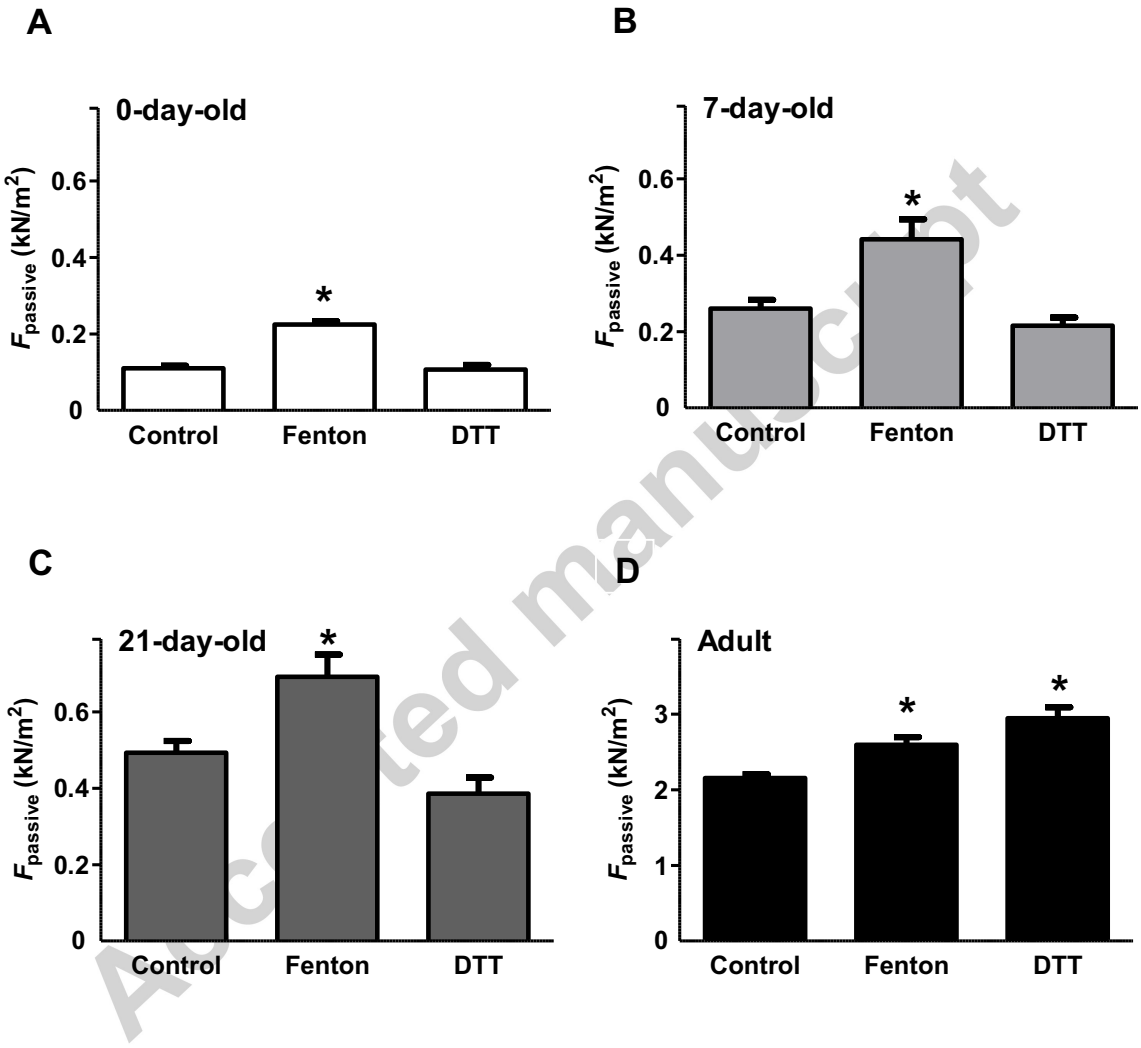

Figure 4

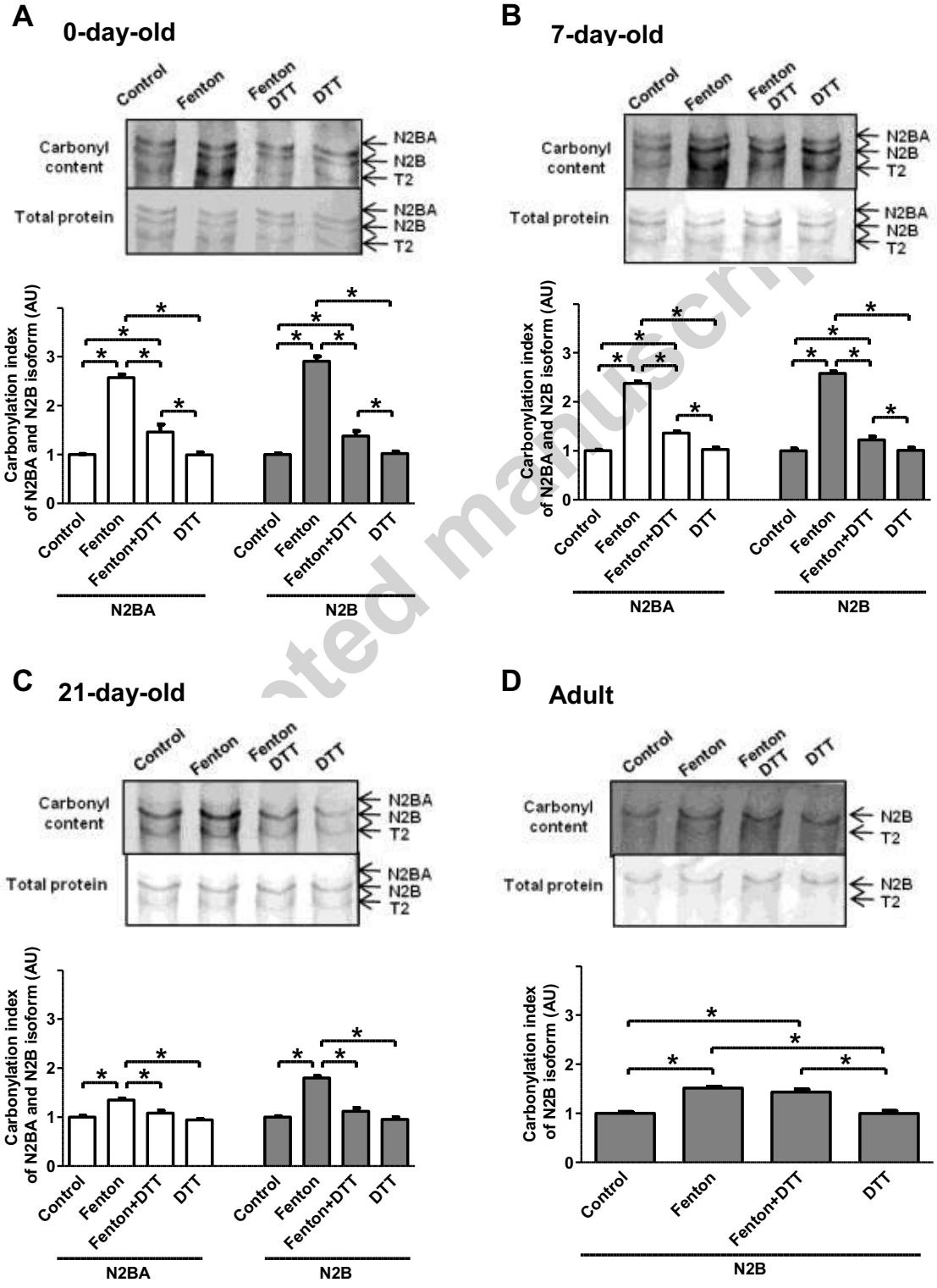

Figure 5

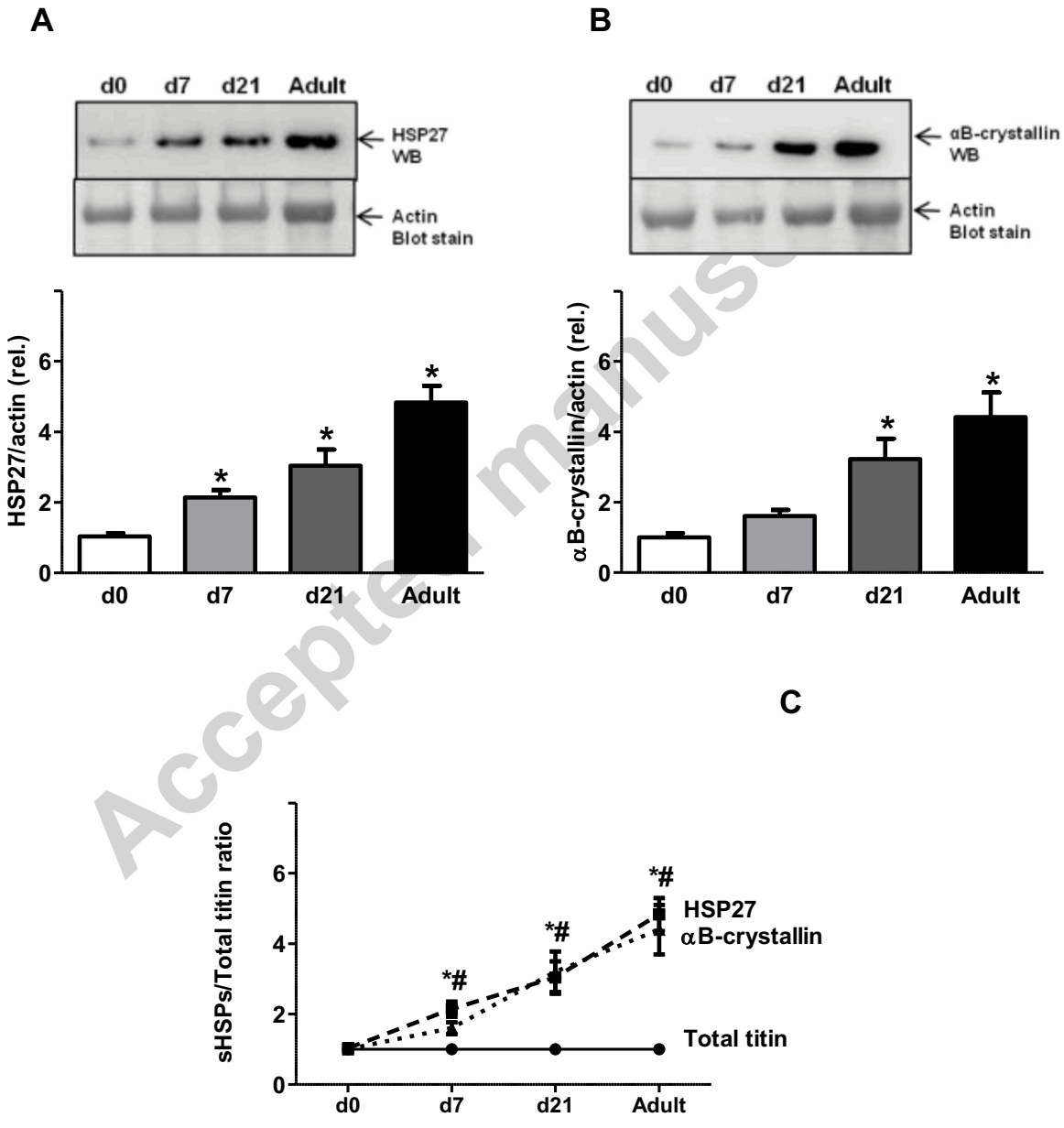

Figure 6

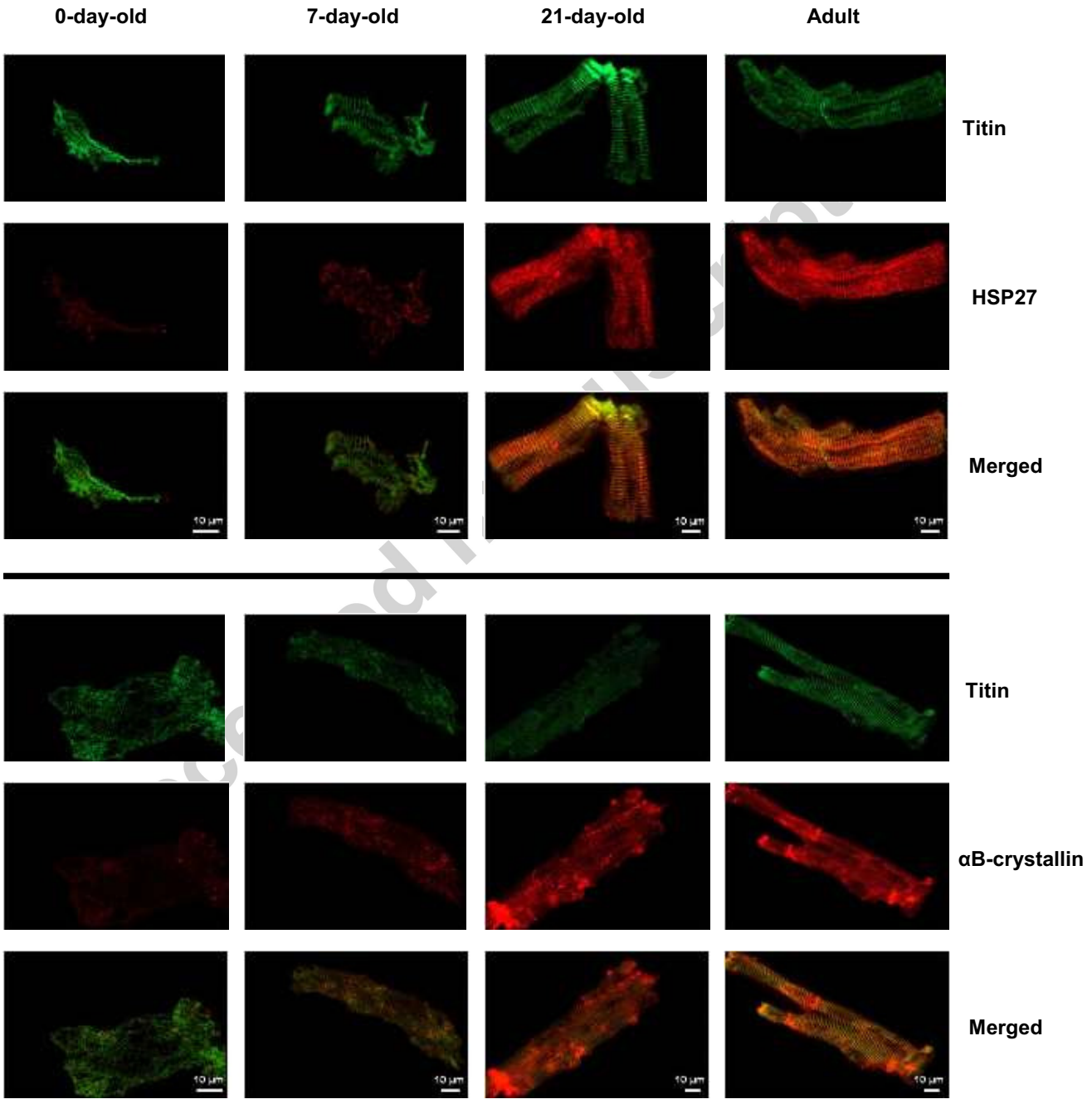
0-day-old
7-day-old
21-day-old
Adult
Titin
HSP27
Merged
10 μm
Titin
αB-crystallin
Merged
10 μm

Figure 7

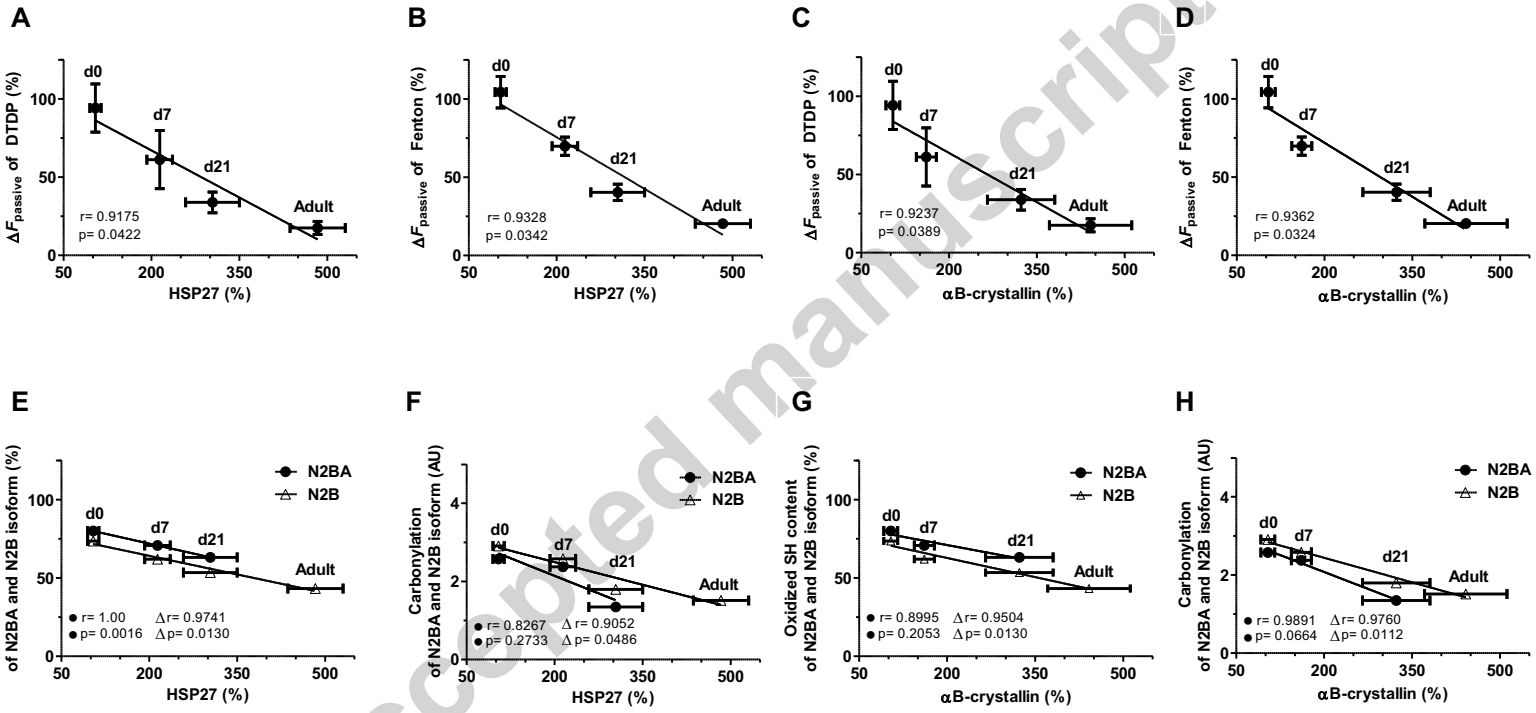

Figure 8