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AAV9-mediated *SMN* gene therapy rescues cardiac desmin but not lamin A/C and elastin dysregulation in *Smn*^{2B/-} spinal muscular atrophy mice

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Abstract

Structural, functional and molecular cardiac defects have been reported in spinal muscular atrophy (SMA) patients and mouse models. Previous quantitative proteomics analyses demonstrated widespread molecular defects in the severe Taiwanese SMA mouse model. Whether such changes are conserved across different mouse models, including less severe forms of the disease, has yet to be established. Here, using the same high-resolution proteomics approach in the less-severemilder *Smn*^{2B/-} SMA mouse model, 277 proteins were found to be differentially produced-abundant at a symptomatic timepoint (post-natal day (P) 18), 50 of which were similarly dysregulated in severe Taiwanese SMA mice. Bioinformatics analysis linked many of the differentially produced-abundant proteins to cardiovascular development and function, with intermediate filaments highlighted as an enriched cellular compartment in both datasets. Lamin A/C was increased in cardiac tissue whilst another intermediate filament protein, desmin, was reduced. The extracellular matrix (ECM) protein, elastin, was also robustly decreased in the heart of *Smn*^{2B/-} mice. AAV9-*SMNI*-mediated gene therapy rectified low levels of survival motor neuron (SMN) protein and restored desmin levels in heart tissues of *Smn*^{2B/-} mice. In contrast AAV9-*SMNI* therapy failed to correct lamin A/C or elastin levels. Intermediate filament proteins and the ECM have key roles in cardiac function and their dysregulation may explain cardiac impairment in SMA, especially since mutations in genes encoding these proteins cause other diseases with cardiac aberration. Cardiac pathology may need to be considered in the long-term care of SMA patients, as it is unclear whether currently available treatments can fully rescue peripheral pathology in SMA.

Introduction

Spinal muscular atrophy (SMA) arises from insufficient levels of survival motor neuron (SMN) protein due to a homozygous mutation/deletion in the *SMN1* gene (1). Although humans possess the *SMN2* gene (2), this generates only a limited amount of functional SMN (~10%) with the majority (~90%) being an unstable, truncated form of the protein (3). SMA patients present with a range of clinical phenotypes with varying severity, which generally, but not always, have an inverse relationship with *SMN2* copy number (4). Recent advances in SMA treatments aim to increase the levels of full-length SMN by either upregulating the *SMN2* gene or by delivering the *SMN1* gene directly to the cells via a viral vector (5). Neither treatment option is fully effective and evidence is emerging of new patient phenotypes with long-term treatment outcomes remaining unknown (5).

Although SMA is typically considered a motor neuron disease, the ubiquitous nature of SMN production has prompted research into the effects of reduced levels of SMN in peripheral tissues and organs, in both patients and animal models of SMA. Evidence demonstrating that SMA is a multisystem disease has been accruing (6,7) and impairment of cardiac function in mouse models of SMA and in SMA patients described (8–15). Examples of cardiac dysfunction include observations of bradycardia, dilated cardiomyopathy, and decreased contractility in *SMN Δ 7* mice (12), and [electrocardiogram](#) ECG abnormalities and thickened myocardium in patients with milder forms of SMA (9,16); whilst a retrospective study of patients with SMA Type I identified ~24% of patients with severe symptomatic bradycardia (10). Congenital heart defects, such as septal defects, in severe Type I patients (11) and in the severe Taiwanese SMA mouse model (14) have also been noted. A study in which the incidence of health insurance claims in Type I to III patients pre-SMA diagnosis were compared to control patients found valve disorders, cardiomyopathies, septal defects and premature beats to be increased (17).

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3 Furthermore, a systematic review of more than 70 studies in which 264 SMA patients were
4 identified with cardiac pathology found a tendency for structural abnormalities to occur in the
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6 more severe form of SMA (Type I) whilst in patients with less severe SMA, cardiac rhythm
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8 disturbances were more common (18).
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13 SMN protein is found in both the cytoplasm and nucleus of cells, and typically associated with
14 structures called Gemini of the coiled bodies (Gems) in the nucleus. There is also evidence that
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16 SMN localises to structural components such as the sarcomere in striated muscle fibres (19)
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18 and the Z-disc in mice cardiac myofibrils (20), but its function in cardiac muscle remains
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20 unknown. Most studies have focussed on the more severe form of SMA, including our previous
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22 quantitative proteomics study in which we demonstrated widespread molecular defects in heart
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24 tissue from the severe Taiwanese SMA mouse model compared to healthy controls (15). In
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26 particular, a robust increase in the intermediate filament protein, lamin A/C, was observed,
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28 which we hypothesised may contribute to the impairment of cardiac function by causing
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30 nuclear stiffness in the cardiomyocytes.
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40 A recent study that focussed on the FoxO family of transcription factors in heart tissues from
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42 the less-severe ~~milder~~ *Smn*^{2B/-} SMA mouse model, found no significant pathology related to
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44 these factors or their downstream targets of proteosomal and autophagosomal degradation (21),
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46 but the full extent of molecular consequences and their implications remain unknown for this
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48 less-severe SMA mouse model. A recent transcriptome study of cardiomyocytes isolated from
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50 a severe SMA mouse model and cardiomyocytes generated from induced pluripotent stem cells
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52 (iPSCs) derived from an SMA Type II patient, however, found that SMN deficiency disrupts
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54 muscle cell and fibre development, muscle function and Ca²⁺ handling (22). Cell studies are
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56 extremely useful, but cell isolation and subsequent culture changes the physiological stresses
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3 that they are exposed to in comparison to those experienced *in vivo*. Thus, we undertook a
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5 quantitative proteomic comparison of heart tissues from the [less-severemilder](#) *Smn*^{2B/-} SMA
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7 mouse model and age-matched wild-type (WT) mice and compared these findings with those
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9 identified in our previous study of heart tissues from the severe Taiwanese SMA mouse model
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11 (15).
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17 We report evidence of widespread protein dysregulation in *Smn*^{2B/-} mouse hearts compared to
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19 age-matched WT mice, some of which was common to the Taiwanese SMA mouse model. In
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21 agreement with our previous findings from the Taiwanese model (15), the intermediate
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23 filament protein, lamin A/C, was increased in expression in hearts from the [less-severemilder](#)
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25 *Smn*^{2B/-} SMA mouse model, whereas desmin, another intermediate filament protein, was found
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27 to be decreased in heart tissues in both mouse models. The extracellular matrix (ECM) protein,
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29 elastin, was also significantly decreased in the heart tissues of the *Smn*^{2B/-} mouse. Whilst
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31 AAV9-mediated *SMN1* delivery restored desmin levels in the *Smn*^{2B/-} SMA mouse model to
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33 those seen in WT mice, this treatment did not rescue the increased levels of lamin A/C or the
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35 decreased levels of elastin, even though levels of SMN were significantly enhanced when a
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37 codon-optimised transgene was administered. Together these findings suggest that post-natal
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39 AAV9-mediated *SMN1* delivery may not rectify all the dysregulated proteins [found in the](#)
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41 [Smn^{2B/-} mouse model of associated with](#) SMA, and since these proteins have the potential to
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43 detrimentally impact heart function, additional cardiac monitoring may prove useful in the
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45 long-term care of SMA patients.
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54 Results

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57 **Quantitative proteomics analysis of heart tissue from a mouse model of [less-](#)**
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59 **[severeintermediate](#) SMA [severity](#) reveals widespread molecular defects**
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3 To determine the molecular consequences of SMN depletion in heart tissue from the
4 [intermediateless-severe](#) *Smn*^{2B/-} SMA mouse model, a relative quantitation proteomic
5 comparison against age-matched WT mouse hearts was undertaken using iTRAQ™ mass
6 spectrometry analysis at the symptomatic time-point of P18. This approach identified 3105
7 proteins in total (Supplementary Table 1) after removing proteins identified from 1 or more
8 peptides and those which matched to the decoy search (i.e., prefixed with “REVERSED...”).
9
10 Of these, 2479 were identified with a 5% local false-discovery rate. From these, 277 proteins
11 met the specified criteria (an unused ProtScore (conf) > 0.05, detected by ≥2 unique peptides,
12 and a statistically significant (p≤0.05) fold change of ≥1.25 or ≤0.8) for differential expression
13 in the *Smn*^{2B/-} mouse heart compared to age-matched WT hearts, of which 156 proteins were
14 increased and 121 were decreased in expression in SMA mouse hearts (Supplementary Table
15 2).

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33 To guide the direction of further studies, we were interested to understand whether any of the
34 differentially [expressed-abundant](#) proteins identified in heart tissue from the [milderless-severe](#)
35 *Smn*^{2B/-} SMA mouse model were conserved with those identified previously from the severe
36 Taiwanese SMA mouse model (15). The iTRAQ data pertaining to the Taiwanese model were
37 derived in the same experiment as the *Smn*^{2B/-} data reported here, but only the Taiwanese section
38 of the data was published previously (15). Compared to their respective age-matched WT mice
39 (i.e., P18 for the *Smn*^{2B/-} & P8 for the Taiwanese), and using the same criteria (p≤0.05, [fold](#)
40 [change of >1.25 or ≤0.8, FC ≥ 0.25](#), >1 unique peptide), 50 proteins were found to be commonly
41 dysregulated in both mouse models of SMA (Supplementary Table 3). Of these, 30 were up-
42 regulated and 20 down-regulated with statistical significance in both models (Figure 1A) with
43 a similar fold-change in most dysregulated proteins (Figure 1B).

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3 The list of differentially ~~expressed~~ abundant proteins from both mouse models was also
4 compared using the comparison analysis function of the curated bioinformatics platform,
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6 Ingenuity Pathway Analysis (IPA®). This two-way comparison in which the enriched terms
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8 were ranked by absolute *p*-value, demonstrated a similar enrichment of terms for “diseases &
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10 disorders, physiological system development & function” after removal of cancer related terms.
11
12 In particular, “abnormal morphology” and “dilated cardiomyopathy” were highly enriched in
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14 both mouse models (*Smn*^{2B/-} mouse model, *p*-value range 3.72 x 10⁻¹⁹ to 2.65 x 10⁻⁷; Taiwanese
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16 mouse model, *p*-value range 9.52 x 10⁻²⁴ to 4.69 x 10⁻⁸) (Figure 1C). Similarly, for “molecular
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18 & cellular functions”, terms relating to “translation, synthesis and metabolism of proteins” and
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20 “cell & organismal death” were significantly enriched among the differentially ~~expressed~~
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22 abundant proteins common to both mouse models (*Smn*^{2B/-} mouse model, *p*-value range 5.01 x
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24 10⁻⁴⁰ to 2.65 x 10⁻⁷; Taiwanese mouse model, *p*-value range 9.52 x 10⁻²⁴ to 4.69 x 10⁻⁸) (Figure
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26 1D). Various canonical pathways already linked to SMA such as “EIF2 signaling” (239) and
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28 “mitochondrial dysfunction” (2409) were also enriched in the *Smn*^{2B/-} and Taiwanese mouse
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30 models (Figure 1E). In addition, the “dilated cardiomyopathy signaling pathway” was
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32 significantly enriched in both models (*Smn*^{2B/-} mouse model, *p*=6.52 x 10⁻¹¹, *z*-score = 1.043;
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34 Taiwanese mouse model, *p*=2.72 x 10⁻¹⁸, *z*-score = -0.667).
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45 Using the Database for Annotation, Visualization and Integrated Discovery (DAVID)
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47 bioinformatics tool, the cellular components with which the significantly dysregulated proteins
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49 in the *Smn*^{2B/-} mouse model were associated was ascertained (Figure 1F and Supplementary
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51 Table 4A). Of particular interest were those cellular components pertinent to cardiomyocyte
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53 function including the Z-disk (12 proteins; *p*=4.7x 10⁻⁶), the costamere (6 proteins; *p*=5.4 x 10⁻⁶),
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55 focal adhesion (12 proteins; *p*=7.9 x 10⁻⁵) and adherens junction (10 proteins; *p*=3.2x10⁻⁴),
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57 the sarcolemma (9 proteins; *p*=5.4 x 10⁻⁴), the intercalated disc (6 proteins; *p*=1.4 x 10⁻³), and
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3 intermediate filaments (6 proteins; $p=2.5 \times 10^{-2}$). When the 50 proteins significantly
4 dysregulated in both the *Smn*^{2B/-} and Taiwanese SMA mouse models were considered in the
5 DAVID analysis, “intermediate filament” (3 proteins; $p=4.8 \times 10^{-2}$) remained an enriched
6 cellular component term (Supplementary Table 4B).
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15 **The intermediate filament proteins, lamin A/C and desmin, are differentially ~~expressed~~**
16 **abundant in heart tissue from two mouse models of SMA**
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19 The finding that intermediate filament proteins are differentially ~~expressed~~ abundant in both
20 SMA mouse models is of particular interest with regards to heart conditions. Mutations
21 affecting both lamin and desmin (type V and type III intermediate filament proteins,
22 respectively), commonly give rise to conditions associated with cardiomyopathy and heart
23 failure (4125). Of additional relevance is that mutations in *LMNA* -the lamin A/C gene- have
24 been attributed to adult-onset conditions with an SMA-like phenotype (426,2743). Lamin A
25 was identified by iTRAQ as being increased in both the Taiwanese (15) and *Smn*^{2B/-} SMA
26 mouse models (Supplementary Table 1) (albeit it just missed the criteria for differential
27 expression in the *Smn*^{2B/-} with $p=0.056$). Desmin, on the other hand, was decreased in
28 expression in both the Taiwanese (15) and *Smn*^{2B/-} SMA mouse models, but only met the
29 criteria for differential expression when peptides quantified with 99% confidence were used to
30 calculate the ratio, resulting in $p=0.049$).
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49 To gain further insights into the involvement of these intermediate filament proteins in SMA,
50 we verified the differential expression of lamin A/C and desmin in heart tissue from the *Smn*^{2B/-}
51 and Taiwanese SMA mice. Quantitative western blotting confirmed a 1.94-fold and 2.06-fold
52 increase in lamin A and C expression, respectively, in heart tissue extracts from *Smn*^{2B/-} mice
53 compared to age-matched WT mice ($p=0.014$ and $p=0.030$, respectively) (Supplementary File
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3 Figure 1A). Immunohistochemistry analysis also confirmed increased lamin A
4 immunoreactivity in heart tissues from the *Smn*^{2B/-} mouse model compared to those from WT
5 mice (1.74-fold increase, $p=0.007$; Supplementary File Figure 1B). This aligned with our
6 previous findings (15) where lamin A/C levels were increased in heart tissue from the severe
7 Taiwanese SMA mouse model. Immunohistochemistry analysis of *Smn*^{2B/-} mouse heart
8 sections revealed few lamin A/C positive cells in the ventricle lumen (Supplementary File
9 Figure 1C), in line with our previous observation from the Taiwanese SMA mouse model (15).
10 Although we cannot rule out a minor contribution, this result confirms that the increased lamin
11 A/C levels cannot be solely attributed to circulating blood cells. For reference, SMN levels in
12 heart tissue extracts from the *Smn*^{2B/-} mice were reduced to 0.08-fold ($p=0.007$) of that found
13 in WT mice (Supplementary File Figure 1A).
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31 Decreased desmin expression was also confirmed in both mouse models of SMA by western
32 blotting, with levels being reduced in the *Smn*^{2B/-} mice compared to age-matched (P18) WT
33 mice to 0.73-fold ($p=0.033$) and in the Taiwanese mice compared to age-matched (P8) WT
34 mice to 0.76-fold ($p=0.007$) (Supplementary File Figure 2A). These findings were corroborated
35 by immunohistochemistry analysis (Supplementary File Figure 2B) with desmin
36 immunoreactivity in *Smn*^{2B/-} mice showing a decrease to 0.41-fold ($p=0.033$) compared to WT
37 (P18) mice and desmin staining in Taiwanese mice demonstrating a decrease to 0.26-fold
38 ($p=0.019$) compared to (P8) WT mice (Supplementary File Figure 2C).
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51 **AAV9-mediated *SMN1* delivery to increase SMN expression in the hearts of *Smn*^{2B/-} SMA** 52 **mice** 53

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56 To establish the impact of currently approved gene therapy treatments, which are designed to
57 increase SMN levels in SMA patients, on a peripheral tissue such as the heart, we examined
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3 the extent to which AAV9-mediated *SMNI* treatment restored expression levels of lamin A/C
4 and desmin towards control mice. For these experiments, we chose to focus only on the less-
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8 severe *Smn*^{2B/-} SMA model as these mice have a longer pre-symptomatic period compared to
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10 the Taiwanese mouse model allowing for a better understanding of developmental defects
11 secondary to motor neuron loss. In addition, to validate our findings further, two distinct
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15 vectors that have previously been used in *Smn*^{2B/-} mice were utilised (3128,329), in particular
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17 the one vector results in enhanced expression of the *SMNI* gene (3128). SMN levels were
18 confirmed by quantitative western blotting to be significantly increased in extracts of heart
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21 tissues from *Smn*^{2B/-} mice following AAV9-mediated *SMNI* replacement (Figure 2). As
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23
24 expected (3128), delivery of the optimised cDNA transgene AAV9_*Co-hSMNI* resulted in
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26 enhanced levels of SMN (169-fold; $p=0.0002$) compared to untreated *Smn*^{2B/-} mice with
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28 increased levels of SMN beyond that expected in WT mice (~13.50-fold). Although the
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30 scAAV9-*SMNI*-mediated delivery also resulted in an SMN increase (2.4-fold; $p=0.0003$)
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32 compared to untreated *Smn*^{2B/-} mice, SMN levels remained below that found in WT mice
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34 (~0.32-fold) (Figure 2A). There was no statistically significant difference in SMN expression
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36 levels between untreated *Smn*^{2B/-} mice and vehicle controls (i.e., AAV9_*eGFP* (2.1-fold;
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38 $p=0.14$); scAAV9-*GFP* (1.8-fold; $p=0.08$) (Figure 2A). Immunohistochemistry analysis of
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40 heart tissues from *Smn*^{2B/-} mice revealed increased SMN immunoreactivity following SMN
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42 replacement, particularly in those treated with AAV9_*Co-hSMNI* (Figure 2B).
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49 **AAV9-mediated *SMNI* delivery restores desmin expression but does not correct** 50 **increased lamin A/C expression in the hearts of *Smn*^{2B/-} SMA mice**

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52 Quantitative western blotting and immunohistochemistry analysis revealed that AAV9-
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54 mediated *SMNI* treatment did not correct the increased production of lamin A/C in *Smn*^{2B/-}
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56 mouse hearts. On western blots, lamin A expression levels following delivery of AAV9_*Co-*
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3 *hSMN1* were 0.80-fold ($p=0.36$) compared to untreated *Smn*^{2B/-} mice and 1.56-fold ($p=0.04$)
4 compared to WT mice. Following scAAV9-*SMN1*-mediated delivery lamin A levels were 0.96-
5 fold ($p=0.44$) compared to untreated *Smn*^{2B/-} mice and 1.56-fold ($p=0.003$) compared to WT
6 mice (Figure 3A). Similarly, lamin C expression levels following delivery of AAV9_*Co-*
7 *hSMN1* were 0.77-fold ($p=0.26$) compared to untreated *Smn*^{2B/-} mice and 1.39-fold ($p=0.13$)
8 compared to WT mice and following scAAV9-*SMN1*-mediated delivery were 0.96-fold
9 ($p=0.50$) compared to untreated *Smn*^{2B/-} mice and 1.54-fold ($p=0.004$) compared to WT mice
10 (Figure 3A). There was no statistically significant difference in lamin A and C expression levels
11 between untreated *Smn*^{2B/-} mice and vehicle controls (i.e., AAV9_*eGFP* (1.06-fold ($p=0.46$)
12 for lamin A and 1.21-fold ($p=0.06$) for lamin C); scAAV9-*GFP* (0.96-fold ($p=0.59$) for lamin
13 A and 0.84-fold ($p=0.06$) for lamin C) (Figure 3B).

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31 In heart sections from WT mice, lamin A/C immunoreactivity was largely confined to the
32 nuclear membrane, as expected, with a small proportion being localised to the sarcomere as
33 confirmed by double-label immunohistochemistry with alpha-actinin 2, a sarcomere marker
34 (Figure 3C & 3D). In heart sections from the *Smn*^{2B/-} mice, lamin A/C was significantly
35 increased in expression at the nuclear membrane alongside a strikingly increased distribution
36 of lamin A/C at the sarcomeres (Figure 3C). When quantified across each section, lamin A/C
37 immunoreactivity in heart tissues from *Smn*^{2B/-} mice showed a significant increase in lamin A/C
38 expression compared to age-matched (P18) WT mice (3.29 ± 1.13 -fold; $p=0.004$; Figure 3C),
39 and after treatment with AAV9_*Co-hSMN1* or scAAV9-*SMN1* lamin A/C levels in *Smn*^{2B/-}
40 mice remained increased compared to the untreated *Smn*^{2B/-} mice (3.66 ± 0.70 -fold ($p=0.54$)
41 and 4.25 ± 0.45 -fold ($p=0.13$), respectively (Figure 3C).

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3 Western blot and immunohistochemistry analysis revealed that AAV9-mediated *SMNI*
4 treatment did, however, restore the decreased expression of desmin in *Smn*^{2B/-} mouse hearts
5 towards WT levels. From western blots, desmin expression levels following delivery of
6 AAV9_*Co-hSMNI* were 1.74-fold ($p=0.0095$) compared to untreated *Smn*^{2B/-} mice and 1.06-
7 fold ($p=0.44$) compared to WT mice and following scAAV9-*SMNI*-mediated delivery were
8 1.84-fold ($p=0.04$) compared to untreated *Smn*^{2B/-} mice and 1.31-fold ($p=0.13$) compared to
9 WT mice (Figure 4A). There was no statistically significant difference in desmin expression
10 levels between untreated *Smn*^{2B/-} mice and vehicle controls (i.e., AAV9_*eGFP*) (0.99-fold
11 ($p=0.88$)) and scAAV9-*GFP* (1.16-fold ($p=0.19$)) (Figure 4B). Desmin immunoreactivity in
12 heart sections from WT mice was typically present at the Z-disc (Figure 4C). Desmin
13 immunoreactivity in heart sections from the *Smn*^{2B/-} mice was clearly reduced ([Figure 4C, high](#)
14 [magnification images](#)), although some striations could still be seen, the staining appearing to
15 be variable in intensity and disorganised. Quantification of desmin immunoreactivity across
16 each heart section from the *Smn*^{2B/-} mice found desmin expression to be significantly decreased
17 compared to age-matched (P18) WT mice (0.61 ± 0.12 -fold ($p<0.001$)) (Figure 4C), but
18 treatment with AAV9_*Co-hSMNI* or scAAV9-*SMNI* confirmed that desmin levels in *Smn*^{2B/-}
19 mice were increased compared to the untreated *Smn*^{2B/-} mice (1.75 ± 0.19 -fold ($p<0.0001$) and
20 1.28 ± 0.22 -fold ($p=0.035$)), respectively (Figure 4C).

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 **Decreased elastin expression in *Smn*^{2B/-} mouse heart is also refractory to AAV9-mediated** 48 ***SMNI* delivery**

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51 Lamin A/C is a significant regulator of cell stability and dynamics ([3044](#)), and its expression
52 correlates with the stiffness of tissue ([4531](#)). Increased rigidity of cardiomyocytes enhances
53 passive tension *in vitro* and *in vivo*, resulting in functional heart defects ([4632,4733](#)). To gain
54 insights into the wider impact of increased lamin A/C levels on the contractile apparatus of
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3 cardiomyocytes and overall stiffness of heart tissues in the *Smn*^{2B/-} SMA mouse model, we
4 examined the levels of elastin, a protein essential for the elastic properties of many tissues.
5 Elastin was identified by iTRAQ analysis to be significantly reduced to 0.01-fold ($p=0.003$) in
6 heart tissue from the *Smn*^{2B/-} SMA mouse model compared to WT. This finding was confirmed
7 qualitatively by immunohistochemical analysis of van Gieson staining (i.e., a modified Miller's
8 stain which differentially stains elastin from collagen and muscle), and quantitatively by
9 higher-power immunofluorescence analysis (0.30 ± 0.14 -fold ($p<0.00001$) in *Smn*^{2B/-} vs WT)
10 (Figure 5). Elastin levels in *Smn*^{2B/-} mice following treatment with either AAV9_*Co-hSMN1* or
11 scAAV9-*SMN1* remained comparable to those observed in untreated *Smn*^{2B/-} mice (0.37 ± 0.16 -
12 fold ($p=0.41$), and 0.30 ± 0.13 -fold ($p=0.24$)) of WT levels respectively) (Figure 5).
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29 Discussion

30 Over the last two decades, evidence has built to suggest that in severe forms of SMA, structural
31 and functional abnormalities in heart tissues exist (14,18,22), whilst in patients with less severe
32 types, a potentially milder impairment of cardiac function is suggested (9,16). The aim of this
33 study was to conduct a proteomic comparison of heart tissues from the [less-severemilder](#)
34 *Smn*^{2B/-} SMA mouse model and age-matched wild-type (WT) mice and compare these findings
35 with those identified in our previous study of heart tissues from the severe Taiwanese SMA
36 mouse model (15) to determine whether a conserved response to reduced SMN levels is
37 evident. We also investigated whether dysregulated proteins with a known role in heart
38 function are impacted and whether their levels could be rescued by increasing SMN levels in
39 the [less-severemilder](#) *Smn*^{2B/-} mouse model.
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56 In each SMA mouse model, hundreds of proteins were found to be dysregulated compared to
57 age-matched WT mice. Bioinformatics analysis revealed a highly significant enrichment of
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3 terms relating to “abnormal morphology” and “dilated cardiomyopathy” in reference to
4
5 “diseases & disorders, physiological system development & function” in both dysregulated
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7 datasets. Previously, cardiac defects such as thinning of the interventricular septum and
8
9 cardiomyocyte disorganisation have been noted in the severe Taiwanese SMA mouse model
10
11 (14) as have atrial or ventricular septal defects (11) and thickened myocardium (9) in patients
12
13 with SMA type I. Out of 42 SMA type II and III patients, one patient had mitral valve prolapse,
14
15 7 had sinus tachycardia, and more than half had an elevated minimum 24-hour heart rate (16).
16
17 When “molecular and cellular functions” were considered, terms relating to the “translation,
18
19 synthesis and metabolism of proteins”, and “cell and organismal death” were highly significant.
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21 Most of these terms are typically associated with SMA, in particular, SMN is known to be key
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23 for protein homeostasis (348). Both *in vitro* and *in vivo* studies provide support for impaired
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25 translation in SMA (4935,5036) and a comprehensive study involving rat primary neuron
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27 culture, fibroblasts from SMA Type I patients and neurons from a severe mouse model of SMA
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29 (*Smn*^{-/-}; *SMN2*^{tg/0}) demonstrated reduced *de novo* protein synthesis linked to mTOR signalling
30
31 (5137). In an earlier study, microfilament metabolism was found to be impacted by reduced
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33 levels of SMN (5238), and with evidence building for a role of mitochondrial dysfunction in
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35 SMA (240,539–5541), all these factors may contribute to altered protein metabolism. Most of
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37 the significantly enriched “canonical pathways” common to both mouse models have also been
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39 implicated in SMA, such as EIF2 signaling (239), mitochondrial dysfunction (240) and mTOR
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41 signaling (5137), but of particular interest was the significant enrichment of “dilated
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43 cardiomyopathy” and the “dilated cardiomyopathy signaling pathway” in both mouse models
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45 of SMA.
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56 While differentially expressed-abundant proteins from the *Smn*^{2B/-} dataset were associated with
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58 several cellular components pertinent to cardiomyocyte function, the only cellular component
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3 to be significantly enriched among proteins common to both mouse models was intermediate
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5 filaments. Genetic mutations of the intermediate filaments lamin A/C and desmin are known
6
7 contributors of cardiomyopathies and heart failure ([4125,5642](#)). Both proteins form networks
8
9 within the cardiomyocyte and help maintain intra- and intercellular structure, with lamin A/C
10
11 at the nuclear envelope ([5743](#)) and desmin within the cytoplasm. In addition, lamin and desmin
12
13 have important functions in nucleo-cytoskeletal coupling and mechano-transduction, gene
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15 regulation, metabolism, mitochondrial homeostasis and cardiomyocyte differentiation and
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17 survival ([5743-459](#)), all of which are necessary for a fully functioning heart.
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24 As previously confirmed in the Taiwanese mouse model of severe SMA (15), levels of lamin
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26 A/C were significantly increased in heart tissues from the *Smn*^{2B/-} mouse model of [less-](#)
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28 [severemilder](#) SMA. Increased levels of lamin A/C were localised to the nuclear envelope and
29
30 to the sarcomere of *Smn*^{2B/-} mouse hearts, and further support the concept that appropriate lamin
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32 A/C production is SMN-dependent (15,[4327,460](#)). Previously, increased lamin A/C levels have
33
34 been described in muscle ([6147](#)) and Schwann cells from SMA mice ([6248](#)) and in motor
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36 neurons obtained from Type I SMA patients ([6349](#)). In terms of impact on heart conditions and
37
38 development of dilated cardiomyopathy, impairment of the lamin A/C gene is a well-known
39
40 factor ([6450](#)) and results in conduction problems, arrhythmias, atrioventricular block and
41
42 sudden cardiac death ([651-6955](#)) with at least 260 *LMNA* mutations having been linked to
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44 cardiac diseases ([7056](#)). In several studies, *LMNA* mutations have also resulted in an SMA-like
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46 phenotype with cardiac involvement ([426,4327](#)).
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54 An increase in lamin A/C may result in a “stiffer” nucleus which will alter the biomechanics
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56 of the cardiomyocyte, and thus the heart tissue, and may be in response to increased stiffness
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58 of the heart tissue ([4531](#)). Conditions where increased accumulation of lamin A at the nuclear
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3 envelope is known to occur are Hutchinson-Gilford progeria syndrome and restrictive
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5 dermopathy. Both conditions arise from improper post-translational processing of prelamin A,
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7 the former from an *LMNA* mutation which results in a mutant farnesylated prelamin A
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9 (progerin) whilst the latter is due to reduced levels of ZMPSTE24, the enzyme necessary for
10
11 processing prelamin A (574). Although not verified by immuno techniques in the current study,
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13 ZMPSTE24 was significantly increased in the mass spectrometry data from both SMA mouse
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15 models which may imply dysregulation of lamin A processing. In the current study, lamin A
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17 was also prevalent at the sarcomere, especially in *Smn^{2B/-}* mice where it co-localised with alpha-
18
19 actinin 2. This may indicate an increase in unprocessed lamin A in SMA, as previously
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21 prelamin A was identified at the sarcomere along with alpha-actinin 2 (6955). Since it has been
22
23 suggested that prelamin A may be toxic and could promote dilated cardiomyopathy (6955),
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25 further investigations into understanding whether lamin A processing is defective in SMA may
26
27 prove useful. Additionally, the phosphorylation status of lamin A in SMA is worthy of
28
29 investigation as this may have implications for the regulation of lamin A levels since hypo-
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31 phosphorylation renders lamin A more stable and less likely to be degraded (7258) and has
32
33 occurred in mesenchymal stem cells when subjected to increased tissue stiffness (7359).
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42 Lamin A/C is the main provider of nuclear membrane mechanical strength and is key to
43
44 transmitting mechanical force from the ECM to the nucleus (7460,7561). Deficiency or
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46 mutations in lamin A/C can cause mechano-transduction impairment (762) or impact whole-
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48 cell biomechanical properties (7763). In a lamin A-deficient mouse model, the desmin network
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50 was disrupted and detached from the nuclear surface (7864), suggesting defective force
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52 transmission due to loss of lamin interactions with desmin and subsequent loss of cytoskeletal
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54 tension. In another study of lamin A-deficient mice, desmin accumulation was increased in
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56 both muscle and cardiac tissues (7965). It may prove useful to consider whether the reverse is
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3 true in SMA cardiomyocytes whereby decreased levels of desmin result in increased lamin A
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5 or *vice versa*.
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10 Desmin is located throughout the cardiomyocyte and assists in transmitting force across the
11 ECM, sarcomere and cytoskeleton (8066), and has been implicated in SMA (8167). In
12 combination with microtubules and microfilaments, desmin helps to maintain the cytoskeleton
13 structure and organisation of sub-cellular organelles (682) and is thought to provide
14 cardiomyocyte nuclei with tension (8369). Structurally, desmin links individual myofibrils at
15 the Z-discs (8470,8571), connects Z-discs to the intercalated disc (8672), forms part of the
16 desmosomes, and is linked to the costamere enabling adhesion to the ECM (873). Overall,
17 desmin is key to the functioning of sarcomeres in efficient syncytia. Mutations often result in
18 loss of sarcomere integrity and myofibrillar structure (8874) and depletion causes muscle
19 architecture disruption (8975), which is not impacted during embryogenesis and myofibrillar
20 assembly but affects muscle regeneration resulting in weaker mice in a desmin knock-out
21 model (9076). In other studies, lack of desmin impacted contractile function (9177) and
22 increased fibrosis (9278) with muscle fibres lacking desmin being more susceptible to physical
23 damage (793) and desmin loss causing increased passive stiffness in muscle (9480).
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45 Mechano-sensing within cardiomyocytes is a “two-way” system, with the nucleus being an
46 important mechanosensory organelle in the cell and intimately connected to the cytoskeleton,
47 and the latter in turn to the ECM. For optimal heart performance, both electrical and mechanical
48 stimuli are required with cardiomyocytes being subjected to cyclic contraction as the heart
49 beats (9581). Cardiomyocytes also experience passive mechanical stimuli such as that exerted
50 by the cardiac ECM. The cardiac ECM is a combination of elastin bundles, collagen and
51 interconnected basement membranes (9682), with intra- and extracellular components
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3 contributing to overall myocardial stiffness (9581). Although collagen is the main extracellular
4 protein in myocardial tissue, elastin, which is found in the epicardium, endomysium and
5 structures such as arteries, atria and ventricles of the heart (9783), provides elasticity upon
6 mechanical demand (984). Elastin is a structural glycoprotein and an essential component of
7 myocardial stiffness (9985), so any alterations in its levels will impact heart function.
8 Typically, elastin is very stable with a considerably low turnover rate, with degradation or
9 damage indicating pathological remodelling of the tissue and cardiovascular disease (10086).
10 Reduced levels of elastin, as found in this study, will increase the heart tissue's passive
11 stiffness, potentially reducing cardiac function, since cardiomyocytes cultured on a stiff,
12 fibrotic-like matrix fail to beat properly (10187). Typically, following myocardial
13 infarction/ischaemia, elastin levels will decrease (10288), promoting ECM remodelling which
14 further increases the stiffness of the cardiac tissue and impairment of cardiac function (9985).
15 In Williams-Beuren syndrome, impairment of the elastin *ELN* gene results in 4 out of 5 patients
16 having cardiovascular abnormalities (10389), with mutations also resulting in congenital heart
17 disease (1904). Elastin fibres are crucial for the proper functioning of Purkinje's fibres (9105)
18 and blood vessels (10692), and impairment can cause arrhythmias (10793) or altered blood
19 flow respectively, again impacting heart function.
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44 Two AAV9-mediated *SMN1* treatments were used in this study, both of which have previously
45 been used in *Smn*^{2B/-} mice (3128,329), but the novel codon-optimised cDNA transgene
46 demonstrated significantly enhanced expression of the human *SMN1* gene in *Smn*^{2B/-} mice
47 hearts. Both treatments restored desmin levels to that of WT mice, but lamin A/C levels
48 remained elevated and elastin levels decreased even though the codon-optimised cDNA
49 transgene generated SMN levels more than 10-fold greater than those found in WT mice. The
50 lack of effect of increased SMN levels on lamin A in this study may be due to several reasons:
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3 (4i) lamin A is independent of SMN levels; (2ii) the impact of low SMN levels on lamin A is
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5 greatest during embryonic development so post-natal treatment is too late; (3iii) cardiac
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7 remodelling is influencing lamin A levels; and/or (4iv) dysregulation of proteins responsible
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9 for post-translation processing or regulating the stability of lamin A are not independent of
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11 SMN levels. In contrast, desmin levels were increased by both AAV9-mediated *SMN1*
12
13 treatments, indicating a direct relationship between these two proteins. Previously,
14
15 abnormalities in the Z-bands of a patient with SMA were identified (10894). Since low levels
16
17 of desmin appeared to be associated with sarcomere damage in a rat model of diabetes mellitus
18
19 (1095) and alpha-actinin staining showed disrupted sarcomere/costamere/z-disc striations in
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21 desmin-null mice (11096), it is coherent that the improved levels of desmin in the AAV9-
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23 treated *Smn*^{2B/-} mice result in improved sarcomere structure, although higher resolution
24
25 microscopy is required to confirm this. Interestingly, SMN has been found to be localised to
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27 the I- and M-bands of sarcomeres in normal human skeletal fibres (11197) and the Z-disc in
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29 mice (20) which suggests that sarcomeric SMN may directly or indirectly interact with the
30
31 cytoskeleton and thus be involved in sarcomere structure (11197), or its maintenance (20). In
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33 addition, during heart development, desmin and lamin A are ~~expressed abundant~~ in the cardiac
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35 conduction systems; desmin preferentially in the myocardium of the central conduction system
36
37 and lamin A more localised to the peripheral conduction system, then progressively to the
38
39 central conduction system (11298). It may be possible that AAV9-mediated treatment does not
40
41 rectify lamin A/C levels due to inherent problems with the central conduction system that are
42
43 established prior to treatment being administered. Additionally, abnormalities in conduction
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45 usually occur before dilated cardiomyopathy development (11399) with elevated heart rate
46
47 being associated with cardiomyopathy in late adolescence and increased heart failure risk
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49 (10014). In desmin mutant mice, distribution of lamin A/C was nucleoplasmic whilst in WT
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3 mice, lamin A/C was mostly at the nuclear periphery (8672). Since AAV9-mediated treatment
4 restored desmin levels, lamin A/C's nuclear distribution may improve over time.
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10 Other researchers have found the outcome of AAV9 treatment in SMA to be variable. A study
11 focussed on fibrosis, remodelling, vascular integrity and oxidative stress in two mouse models
12 of differing severity only found a partial rescue of cardiovascular defects in their mouse model
13 of severe SMA and although structural defects were rectified, heart function incorporating heart
14 rate, stroke volume and cardiac output remained significantly lower than WT mice, as did
15 lifespan (13). In the SMN Δ 7 mouse model, scAAV9 treatment resolved the bradycardia issues,
16 but "cardiac output" remained similar to untreated mice, probably as a result of "stroke
17 volume" remaining decreased following scAAV9 treatment (12). In addition, contractility
18 remained lower than WTs following treatment. Another study using the SMN Δ 7 SMA mouse
19 model used the histone deacetylase inhibitor, trichostatin A, which is known to improve the
20 phenotype of SMA mice. Although heart function improved, these mice did not survive as well
21 as the WT mice (1015). Typically, levels of lamin A/C in mouse cardiomyocytes, desmin in
22 rat heart tissues and the elastin component of connective tissues decline with age (102-104),
23 although in male F344/BN hybrid rats, desmin levels were found to increase in aging skeletal
24 muscle (105). In future studies, it will therefore be important to characterise the temporal
25 regulation of the proteins investigated throughout the disease time-course in the *Smn*^{2B/-} mouse
26 model, with and without AAV-9 mediated *SMN1* replacement, and to establish whether
27 restoring levels of these proteins would have an impact on cardiac function of the mouse model.
28
29 SMN may be essential for heart development prior to birth and thus scAAV9 treatment may
30 not correct key development milestones involving SMN. Additionally, other factors such as
31 lung and metabolic function or scoliosis may contribute to continued impairment of heart
32 function in SMA. Prior to the introduction of treatments, such as, nusinersen and risdiplam
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3 which modify *SMN2* gene regulation to increase functional SMN expression, the impact on
4 concomitant health issues in SMA patients following respiratory assistance, and thus extended
5 life expectancy, has been previously highlighted (10) and included development of heart
6 arrhythmia (bradycardia).
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14 In conclusion, accumulating evidence suggests that cardiac assessment in the long-term care
15 of SMA patients is warranted. We only investigated three of the dysregulated proteins
16 identified from mass spectrometry analysis in the *Smn*^{2B/-} mouse model, but there are many
17 more that warrant further investigation, especially those pertinent to heart function. AAV9-
18 *SMNI* gene therapy only corrected one of the proteins investigated, and although severe
19 symptoms may be rare in SMA, subtle differences in cardiac performance in combination with
20 current treatments increasing longevity in these patients, may impact cardiac health in future
21 years. Thus, additional cardiac therapies may prove useful in optimising treatment regimens
22 for SMA patients.
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38 **Materials & Methods & Materials**

39 **SMA mouse models**

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42 The *Smn*^{2B/-} and Taiwanese (original strain from Jackson Laboratories, No. 005058) mouse
43 models were housed at Keele and Edinburgh Universities, respectively. As previously
44 described (23106), the less-severe *Smn*^{2B/-} mouse model is maintained on a pure C57BL/6
45 genetic background with creation of the 2B mutation via a 3-nucleotide substitution within the
46 exon splicing enhancer of exon 7 (24107,25108). The severe Taiwanese mouse model is
47 heterozygous for the *SMN2* transgene on a *Smn* null background (*Smn*^{-/-}; *SMN2*^{tg/+}) (26109).
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3 experimental procedures on Taiwanese mice were authorized and approved by the University
4 of Edinburgh Animal Welfare Ethical Review Body (AWERB) and UK Home Office (Project
5 Licence 60/4569) in accordance with the Animals (Scientific Procedures) Act 1986. For the
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8 *Smn*^{2B/-} mice, experimental procedures were authorized and approved by the Keele University
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11 ~~Animal Welfare Ethical Review Body (AWERB)~~ and UK Home Office (Project Licence
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14 P99AB3B95) in accordance with the Animals (Scientific Procedures) Act 1986.
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19 **Protein extraction and preparation for quantitative proteomic analysis**

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21 Total protein extracts were prepared as previously described (27110) from the hearts of P18
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23 *Smn*^{2B/-} and age-matched WT animals (both n = 3) and isobaric tags for relative and absolute
24
25 quantitation (iTRAQ) quantitative mass spectrometry analysis were added. Briefly, proteins
26
27 were digested with trypsin, then peptides tagged with iTRAQTM reagents as described in the
28
29 iTRAQ kit protocol and sample groups assigned as follows: 116-P18 WT and 117-P18 *Smn*^{2B/-}.
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33 **High pH reverse-phase liquid chromatography fractionation and liquid** 34 35 **chromatography–tandem mass spectrometry analysis**

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37 Fractionation and mass spectrometry analysis were carried out as previously described (15). In
38
39 brief, the iTRAQ-labelled peptides were combined, concentrated, resuspended in buffer A (10
40
41 mm ammonium formate [NH₄HCO₂], 2% acetonitrile [MeCN], pH 10.0) then fractionated
42
43 using high pH reverse-phase liquid chromatography (C18 column). Following rinsing of the
44
45 column with 96% buffer A until the optical density returned to baseline, a gradient was run
46
47 from 4% to 28% of buffer B (10 mm NH₄HCO₂, 90% MeCN, pH 10.0) for 30 min followed
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49 by another from 28–50% buffer B for 6 min. Buffer B at 80% was used to rinse the column for
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51 5 min and the column then re-equilibrated with 4% buffer B for 11 min. Fractions (0.5 ml)
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53 were collected every 30 s and their UV chromatograms analysed. Fractions with similar peptide
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3 concentrations were combined resulting in 12 fractions which were vacuum dried then
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5 resuspended in 30 μ l of 0.1% formic acid.
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10 For the mass spectrometry analysis, one-third of each fraction was analysed by mass
11 spectrometry. Following separation of the peptides via liquid chromatography, peptides were
12 loaded with buffer A (2% MeCN, 0.05% TFA in ultrapure water) and bound to an Acclaim
13 PepMap100 AQ6 trap (100 μ m \times 2 cm) (Thermo Fisher Scientific) which was then washed for
14 10 min with buffer A. The analytical solvent system consisted of buffer A and buffer B (98%
15 MeCN, 0.1% FA in ultrapure water) at 300 nl/min flow rate. To elute the peptides the following
16 gradient was utilized: linear 2–20% of buffer B over 90 min, linear 20–40% of buffer B for 30
17 min, linear 40–98% of buffer B for 10 min, isocratic 98% of buffer B for 5 min, linear 98–2%
18 of buffer B for 2.5 min and isocratic 2% buffer B for 12.5 min. Eluent was sprayed with a
19 NANOSpray II source (electrospray ionization) into the TripleTOF 5600+ tandem mass
20 spectrometer (AB Sciex) under the control of Analyst® TF software (AB Sciex). The mass
21 spectrometer was operated as previously described (15) and the raw mass spectrometry data
22 files analyzed by ProteinPilot software, version 5.0.1.0 (Applied Biosystems) in addition to the
23 Paragon™ database search and Pro Group™ algorithm using the UniProtKB/Swiss-Prot
24 FASTA database. Paragon search analysis parameters for sample were: type “iTRAQ4plex
25 (Peptide Labeled),” cysteine alkylation “MMTS,” digestion “trypsin,” instrument “TripleTOF”
26 and species “Mouse” with processing parameters described as “quantitative,” “bias correction,”
27 “background correction,” “thorough ID” and “biological modifications.” A protein threshold
28 of >5 was used in the Pro Group algorithm to calculate the relative protein expression with the
29 generation of an error factor and *p*-value, and Proteomics System Performance Evaluation
30 Pipeline used to determine false discovery rate. The mass spectrometry proteomic data can be
31 accessed at the following DOI: 10.6084/m9.figshare.23256374
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Bioinformatics analysis

Data that met the criteria for differential expression in the *Smn*^{2B/-} mice were analysed using Database for Annotation, Visualization and Integrated Discovery (DAVID) Functional Annotation Bioinformatics 2021 (28111,29112) and Ingenuity Pathway Analysis software (IPA; QIAGEN Inc., <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>) (1139). Proteins were deemed differentially **expressed-abundant** in the *Smn*^{2B/-} mice in comparison to age-matched WT mice after the following filtering criteria were applied and proteins removed if; (i) they were identified from just one peptide, (ii) had expression changes of less than 25% (SMA vs WT) or (iii) had *p*-values of >0.05 assigned to their expression changes. Only terms with at least three annotated proteins and a *p*-value of ≤0.05 were noted for the DAVID analysis.

SMN replacement in *Smn*^{2B/-} mice via viral vector treatment

For SMN replacement, AAV9 vectors were used from two sources, RY (single stranded AAV9_ *eGFP* and AAV9_ *Co-hSMNI*) (3128) and BS (self-complementary scAAV9-*GFP* and scAAV9-*SMNI*) (329) and administered through the facial vein at P0 (5E10 vg/pup-7E10 vg/pup). The vector sourced from RY uses a codon-optimised cDNA transgene which has been developed to enhance levels of the functional SMN protein produced both *in vitro* and *in vivo* (3128) whilst the vector from BS used standard cloning procedures as previously described (33114). Following CO₂ anaesthesia and exsanguination, heart tissues were harvested from the following groups at the symptomatic time-point of P18: untreated WT mice (n=4), untreated *Smn*^{2B/-} mice (n=6), *Smn*^{2B/-} mice treated with the vector plus GFP (RY, n=5; BS, n=4) and *Smn*^{2B/-} mice treated with the vector plus *SMNI* (RY, n=5; BS, n=5).

Protein extraction and western blotting

All extraction steps were carried out on ice and as previously described (34115). Heart tissues were diced then homogenized with pellet pestles in 2× modified RIPA buffer (2% NP-40, 0.5% Deoxycholic acid, 2 mM EDTA, 300 mM NaCl and 100 mM Tris-HCl (pH 7.4)) plus protease inhibitor cocktail at 1:100 (Sigma-Aldrich; P8340), left on ice for 5 min then sonicated briefly at 5 microns for 10 s. This process was repeated an additional two times. The samples were centrifugated at 13,000 RPM (MSE, Heathfield, UK; MSB010.CX2.5 Micro Centaur) for 5 min at 4°C and protein concentration determined via the BCA protein assay (Pierce™, 23227). The concentration of the protein extracts was adjusted to approximately 2mg/ml, and they were heated in 2× SDS sample buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.125 M Tris-HCl (pH 6.8) and bromophenol blue) for 3 min at 95 °C prior to loading onto 4-12% Bis-Tris polyacrylamide gels (Life Technologies, Warrington, UK) for SDS-PAGE (Biorad, Hercules, CA, USA). A horizontal slice of the gel was excised and stained with Coomassie blue as an internal loading control for total protein, if necessary, protein concentrations were further adjusted to ensure even loading of the samples. The proteins in the remaining gel were transferred overnight via western blot onto a nitrocellulose membrane. Following blocking with 4% powdered milk, the membranes were incubated with primary monoclonal antibodies against: mouse anti-SMN (MANSMA12 2E6; 1:100 (35116)); rabbit anti-lamin A/C (Abcam; ab169532; 1:2000); rabbit anti-desmin (Abcam; ab227651; 1:1000) in dilution buffer (1% FBS, 1% horse serum (HS), 0.1% bovine serum albumin (BSA) in PBS with 0.05% Triton X-100) for 2 h at RT or O/N at 4°C. Membranes were then incubated with HRP-labelled rabbit anti-mouse Ig (DAKO, P0260) or goat anti-rabbit Ig (DAKO, P0488) at 0.25 ng/ml in dilution buffer for 1 h followed by either West Pico or West Femto (both ThermoFisher) and bands imaged with the ChemiDoc™ Touch Imaging Gel Image Documentation sSystem (Bio-Rad).

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3 Using ImageJ Fiji software (v1.51) ([36117](#)) densitometry measurements of both antibody
4 reactive bands and Coomassie-stained gel bands were undertaken.
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10 **Immunofluorescence microscopy**

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12 Following excision, heart tissues [incorporating the left and right ventricles](#) were flash frozen
13 in liquid nitrogen and stored at -80°C then sectioned (7 µm) on a rotary cryostat, collected onto
14 polylysine-coated slides and stored at -20°C. Prior to staining, slides were brought to RT. All
15 subsequent steps were carried out at RT and PBS used for each wash step (3 x 5 min) whilst
16 blocking buffer (1% FBS, 1% HS, 0.1% BSA in PBS) was used for antibody dilution. Sections
17 were washed, then blocked for 30 min prior to being incubated either for 2 h at RT or O/N at
18 4°C with primary antibodies against mouse anti-SMN (MANSMA12 2E6; 1:4 ([35116](#))), rabbit
19 polyclonal anti-lamin A/C (NOVUS; NBP2-19324; 1:100), rabbit monoclonal anti-desmin
20 (Abcam; ab227651; 1:100), rabbit polyclonal anti-elastin (Fisher Scientific; PA5-99418;
21 1:100) or mouse anti-alpha Actinin 2 (Gene Tex; GTX632361; 1:200). Following washing, 5
22 µg/ml of secondary antibody (Molecular Probes; goat anti-rabbit IgG ALEXA Flour 488;
23 A11034) was applied for 1 h. Sections were washed prior to being stained with 4',6-diamidino-
24 2-phenylindole (DAPI; D9542; 0.4 µg/mL) for 10 min, then washed, mounted and imaged with
25 a Leica TCS SP5 spectral confocal microscope (Leica Microsystems, Milton Keynes, UK).
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47 Images generated were analysed via ImageJ. For lamin A, images obtained with the 63x
48 objective with an additional four-fold magnification were utilised, whereas images taken with
49 the 63x objective were used for desmin and elastin analysis. For the lamin A sections, a
50 minimum of 100 cells were analysed whereas for desmin and elastin a minimum of 200 cells
51 were analysed per condition. For each analysis the scale function was removed, so only the
52 pixels either staining for DAPI or the protein (lamin A, desmin and elastin) were counted.
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3 Using the threshold function ([37118](#)), the highlighted pixels were analysed, and protein
4 staining was corrected for cells present by dividing by the measure obtained for the positively
5 stained DAPI areas. The fold difference between the SMA mouse models and their relevant
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WTs were then determined.

Histochemical staining of elastin

Heart tissue sections were also stained for elastin with the Miller's Elastic Van Gieson ([EVG](#))
Stain Kit (Atom Scientific Ltd; RRSK11-100) using the protocol provided. All wash steps were
carried out with tap water unless otherwise stated. Briefly, sections were washed then treated
with acidified potassium permanganate (0.5%) for 5 min. Following further washing the
sections were bleached with oxalic acid (1%) for 1 min, washed, then rinsed in 95% alcohol
and submerged in Miller's elastin stain for 3 h at RT. Sections were then rinsed with 95%
alcohol until the slides were clear, washed, and counterstained with filtered Van Gieson stain
for 2 min, then blotted, dehydrated, cleared and mounted.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism version 9.0.0 ([121](#)) for Windows,
GraphPad Software, San Diego, California USA, www.graphpad.com. Data distribution was
assessed with the Shapiro–Wilk test and the unpaired two-tailed t-test and Mann-Whitney U
test applied to parametric and non-parametric data respectively. Western blot densitometry
measurements were assessed using an unpaired, one-tailed or two-tailed t-test with Welch's
correction (2-sample equal variance), depending on the null-hypothesis that was tested in each
case. Venn diagrams were generated via a web-based tool ([38119](#)). Data are presented as mean
 \pm standard deviation with a *p*-value of ≤ 0.05 being considered as significant for all analyses.

Supplementary Material

Supplementary File (Word document containing Figures 1 and 2)

Supplementary Tables (Excel document containing supplementary Tables 1-4)

Data Availability Statement: The mass spectrometry proteomic data can be accessed for reviewing purposes at <https://figshare.com/s/72b949c0934737a15b51>

Following publication, the mass spectrometry proteomic data can be accessed at the following DOI: 10.6084/m9.figshare.23256374

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Conflicts of Interest Statement

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3 THG has served on advisory boards for SMA Europe, LifeArc, Roche and Novartis.
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Figures & Legends to Figures

Figure 1. Summary and bioinformatics analysis of the proteomic profiles of heart tissues differentially expressed-abundant in the *Smn*^{2B/-} and Taiwanese mice models of SMA compared to age-matched WT mice. (A) Venn diagram showing an overlap of 50 proteins identified as significantly up- or down-regulated in both *Smn*^{2B/-} and Taiwanese mouse models of SMA (an unused ProtScore (conf) > 0.05, detected by ≥2 unique peptides, and a statistically significant (p≤0.05) fold change of ≥1.25 or ≤0.8). (B) Heat map illustrating the size of fold-change in proteins found to be dysregulated in both mice models of SMA. Two-way comparisons generated in IPA® in which the following were ranked by -log(p-value) for each SMA mouse model: (C) Disease & disorders, physiological system development & function; (D) Molecular & cellular functions and (E) Canonical pathways. (F) Schematic illustrating the cellular components impacted by the significantly dysregulated proteins of the *Smn*^{2B/-} mouse model of SMA as determined following DAVID analysis. Image created with BioRender.com.

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3 **Figure 2. Levels and distribution of SMN in heart tissues from *Smn*^{2B/-} mice following**
4 **AAV9-mediated *SMNI* delivery at P0. (A)** Representative western blots of SMN levels in
5 heart tissues from untreated *Smn*^{2B/-} mice, *Smn*^{2B/-} mice following AAV9-mediated treatment
6 with and without *SMNI* from two sources plus a corresponding age-matched WT mouse (P18).
7 The bar graph represents average SMN levels expressed relative to the corresponding WT
8 mouse. **(B)** Representative [immunofluorescent images \(IMFs\)](#) for SMN staining within heart
9 tissues from untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following treatment with AAV9-mediated
10 *SMNI* from two sources. ****p*<0.001. Scale bars represent 75 μm.

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24 **Figure 3. Levels and distribution of lamin A/C in heart tissues from *Smn*^{2B/-} mice**
25 **following AAV9-mediated *SMNI* delivery at P0. (A)** Representative western blots of lamin
26 A/C levels in heart tissues from WT mice (P18), untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice
27 following AAV9-mediated treatment with *SMNI* from two sources. The bar graph represents
28 average lamin A/C levels expressed relative to WT mice. **(B)** Representative western blots of
29 lamin A/C levels in heart tissues from untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following
30 AAV9-mediated treatment without *SMNI* (vehicle control) from two different vector sources.
31 The bar graph represents average lamin A/C levels expressed relative to untreated *Smn*^{2B/-} mice.
32 **(C)** Representative IMFs for lamin A/C staining within heart tissues from WT mice (P18),
33 untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following AAV9-mediated treatment with *SMNI* from
34 two vector sources. Corresponding bar graph reflects the area of cells stained for lamin A/C
35 corrected for number of cells present (DAPI stain) as determined by ImageJ analysis and
36 expressed relative to corresponding WT mice. Dashed line represents the average lamin A/C
37 levels in WT mice and error bars represent the standard deviation from the mean. **(D)** Images
38 demonstrating dual staining of sarcomeres with alpha-actinin 2 (red) and lamin A/C (green) in
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3 WT mice. * $p < 0.05$; ** $p < 0.01$. Scale bars represent 25 μm except the lower panel of (D) where
4 they represent 5 μm . Δ represents a combined sample not included in the analysis.
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10 **Figure 4. Levels and distribution of desmin in heart tissues from *Smn*^{2B/-} mice following**
11 **AAV9-mediated *SMNI* delivery at P0. (A)** Representative western blots of desmin levels in
12 heart tissues from WT mice (P18), untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following AAV9-
13 mediated treatment with *SMNI* from two sources. The bar graph represents average desmin
14 levels expressed relative to WT mice. **(B)** Representative western blots of desmin levels in
15 heart tissues from untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following AAV9-mediated
16 treatment without *SMNI* (vehicle control) from two different vector sources. The bar graph
17 represents average desmin levels expressed relative to untreated *Smn*^{2B/-} mice. **(C)**
18 Representative IMFs for desmin staining within heart tissues from WT mice (P18), untreated
19 *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following AAV9-mediated treatment with *SMNI* from two
20 vector sources. Zoomed images to highlight the reduction in desmin positive striations in
21 untreated *Smn*^{2B/-} mice in comparison to WT mice and AAV9-mediated *SMNI* treated *Smn*^{2B/-}
22 mice. Corresponding bar graph reflects the area of cells stained for desmin corrected for
23 number of cells present (DAPI stain) as determined by ImageJ analysis and expressed relative
24 to corresponding WT mice. Dashed line represents the average desmin levels in WT mice and
25 error bars represent the standard deviation from the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
26 Scale bars represent 75 μm (panels a-d), 25 μm (panels e-g) and 10 μm (panel h).
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51 **Figure 5. Levels and distribution of elastin in heart tissues from *Smn*^{2B/-} mice following**
52 **AAV9-mediated *SMNI* delivery at P0. (Panels a-d)** Representative heart sections stained
53 with Miller's Elastin van Gieson from WT mice (P18), untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice
54 following AAV9-mediated treatment with *SMNI* from two different vector sources. Elastic
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3 fibres (blue/black); mature collagen (red); other tissues such as muscle and red blood cells
4 (yellow). **(Panels e-l)** Representative IMFs for elastin staining within heart tissues from WT
5 mice (P18), untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following AAV9-mediated treatment with
6 *SMN1* from two vector sources. Corresponding bar graph reflects the area of cells stained for
7 elastin corrected for number of cells present (DAPI stain) as determined by ImageJ analysis
8 and expressed relative to corresponding WT mice. Dashed line represents the average elastin
9 levels in WT mice and error bars represent the standard deviation from the mean. ****p*<0.001.
10 Black scale bars represent 10 μm (panels a-d). White scale bars represent 75 μm (panels e-h)
11 and 10 μm (panels i-l).

Abbreviations

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AWERB Animal Welfare Ethical Review Body

DAVID Database for Annotation, Visualization and Integrated Discovery

ECM Extracellular matrix

Gems Gemini of the coiled bodies

IMF Immunofluorescent image

IPA Ingenuity Pathway Analysis

iPSCs Induced pluripotent stem cells

iTRAQ Isobaric tags for relative and absolute quantitation

P Post-natal day

SMA Spinal muscular atrophy

SMN Survival motor neuron

WT Wild type

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3 **AAV9-mediated *SMN* gene therapy rescues cardiac desmin but**
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6 **not lamin A/C and elastin dysregulation in *Smn*^{2B/-} spinal**
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9 **muscular atrophy mice**
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Abstract

Structural, functional and molecular cardiac defects have been reported in spinal muscular atrophy (SMA) patients and mouse models. Previous quantitative proteomics analyses demonstrated widespread molecular defects in the severe Taiwanese SMA mouse model. Whether such changes are conserved across different mouse models, including less severe forms of the disease, has yet to be established. Here, using the same high-resolution proteomics approach in the less-severe *Smn*^{2B/-} SMA mouse model, 277 proteins were found to be differentially abundant at a symptomatic timepoint (post-natal day (P) 18), 50 of which were similarly dysregulated in severe Taiwanese SMA mice. Bioinformatics analysis linked many of the differentially abundant proteins to cardiovascular development and function, with intermediate filaments highlighted as an enriched cellular compartment in both datasets. Lamin A/C was increased in cardiac tissue whilst another intermediate filament protein, desmin, was reduced. The extracellular matrix (ECM) protein, elastin, was also robustly decreased in the heart of *Smn*^{2B/-} mice. AAV9-*SMN1*-mediated gene therapy rectified low levels of survival motor neuron (SMN) protein and restored desmin levels in heart tissues of *Smn*^{2B/-} mice. In contrast AAV9-*SMN1* therapy failed to correct lamin A/C or elastin levels. Intermediate filament proteins and the ECM have key roles in cardiac function and their dysregulation may explain cardiac impairment in SMA, especially since mutations in genes encoding these proteins cause other diseases with cardiac aberration. Cardiac pathology may need to be considered in the long-term care of SMA patients, as it is unclear whether currently available treatments can fully rescue peripheral pathology in SMA.

Introduction

Spinal muscular atrophy (SMA) arises from insufficient levels of survival motor neuron (SMN) protein due to a homozygous mutation/deletion in the *SMN1* gene (1). Although humans possess the *SMN2* gene (2), this generates only a limited amount of functional SMN (~10%) with the majority (~90%) being an unstable, truncated form of the protein (3). SMA patients present with a range of clinical phenotypes with varying severity, which generally, but not always, have an inverse relationship with *SMN2* copy number (4). Recent advances in SMA treatments aim to increase the levels of full-length SMN by either upregulating the *SMN2* gene or by delivering the *SMN1* gene directly to the cells via a viral vector (5). Neither treatment option is fully effective and evidence is emerging of new patient phenotypes with long-term treatment outcomes remaining unknown (5).

Although SMA is typically considered a motor neuron disease, the ubiquitous nature of SMN production has prompted research into the effects of reduced levels of SMN in peripheral tissues and organs, in both patients and animal models of SMA. Evidence demonstrating that SMA is a multisystem disease has been accruing (6,7) and impairment of cardiac function in mouse models of SMA and in SMA patients described (8–15). Examples of cardiac dysfunction include observations of bradycardia, dilated cardiomyopathy, and decreased contractility in *SMN Δ 7* mice (12), and electrocardiogram abnormalities and thickened myocardium in patients with milder forms of SMA (9,16); whilst a retrospective study of patients with SMA Type I identified ~24% of patients with severe symptomatic bradycardia (10). Congenital heart defects, such as septal defects, in severe Type I patients (11) and in the severe Taiwanese SMA mouse model (14) have also been noted. A study in which the incidence of health insurance claims in Type I to III patients pre-SMA diagnosis were compared to control patients found valve disorders, cardiomyopathies, septal defects and premature beats to be increased (17).

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3 Furthermore, a systematic review of more than 70 studies in which 264 SMA patients were
4 identified with cardiac pathology found a tendency for structural abnormalities to occur in the
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6 more severe form of SMA (Type I) whilst in patients with less severe SMA, cardiac rhythm
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8 disturbances were more common (18).
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13 SMN protein is found in both the cytoplasm and nucleus of cells, and typically associated with
14 structures called Gemini of the coiled bodies (Gems) in the nucleus. There is also evidence that
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16 SMN localises to structural components such as the sarcomere in striated muscle fibres (19)
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18 and the Z-disc in mice cardiac myofibrils (20), but its function in cardiac muscle remains
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20 unknown. Most studies have focussed on the more severe form of SMA, including our previous
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22 quantitative proteomics study in which we demonstrated widespread molecular defects in heart
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24 tissue from the severe Taiwanese SMA mouse model compared to healthy controls (15). In
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26 particular, a robust increase in the intermediate filament protein, lamin A/C, was observed,
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28 which we hypothesised may contribute to the impairment of cardiac function by causing
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30 nuclear stiffness in the cardiomyocytes.
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40 A recent study that focussed on the FoxO family of transcription factors in heart tissues from
41 the less-severe *Smn*^{2B/-} SMA mouse model, found no significant pathology related to these
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43 factors or their downstream targets of proteosomal and autophagosomal degradation (21), but
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45 the full extent of molecular consequences and their implications remain unknown for this less-
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47 severe SMA mouse model. A recent transcriptome study of cardiomyocytes isolated from a
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49 severe SMA mouse model and cardiomyocytes generated from induced pluripotent stem cells
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51 (iPSCs) derived from an SMA Type II patient, however, found that SMN deficiency disrupts
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53 muscle cell and fibre development, muscle function and Ca²⁺ handling (22). Cell studies are
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55 extremely useful, but cell isolation and subsequent culture changes the physiological stresses
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3 that they are exposed to in comparison to those experienced *in vivo*. Thus, we undertook a
4 quantitative proteomic comparison of heart tissues from the less-severe *Smn*^{2B/-} SMA mouse
5 model and age-matched wild-type (WT) mice and compared these findings with those
6 identified in our previous study of heart tissues from the severe Taiwanese SMA mouse model
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17 We report evidence of widespread protein dysregulation in *Smn*^{2B/-} mouse hearts compared to
18 age-matched WT mice, some of which was common to the Taiwanese SMA mouse model. In
19 agreement with our previous findings from the Taiwanese model (15), the intermediate
20 filament protein, lamin A/C, was increased in expression in hearts from the less-severe *Smn*^{2B/-}
21 SMA mouse model, whereas desmin, another intermediate filament protein, was found to be
22 decreased in heart tissues in both mouse models. The extracellular matrix (ECM) protein,
23 elastin, was also significantly decreased in the heart tissues of the *Smn*^{2B/-} mouse. Whilst
24 AAV9-mediated *SMN1* delivery restored desmin levels in the *Smn*^{2B/-} SMA mouse model to
25 those seen in WT mice, this treatment did not rescue the increased levels of lamin A/C or the
26 decreased levels of elastin, even though levels of SMN were significantly enhanced when a
27 codon-optimised transgene was administered. Together these findings suggest that post-natal
28 AAV9-mediated *SMN1* delivery may not rectify all the dysregulated proteins found in the
29 *Smn*^{2B/-} mouse model of SMA, and since these proteins have the potential to detrimentally
30 impact heart function, additional cardiac monitoring may prove useful in the long-term care of
31 SMA patients.
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54 **Results**

55 **Quantitative proteomics analysis of heart tissue from a mouse model of less-severe SMA** 56 **reveals widespread molecular defects** 57 58 59 60

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3 To determine the molecular consequences of SMN depletion in heart tissue from the less-severe
4 *Smn*^{2B/-} SMA mouse model, a relative quantitation proteomic comparison against age-matched
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6 WT mouse hearts was undertaken using iTRAQTM mass spectrometry analysis at the
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8 symptomatic time-point of P18. This approach identified 3105 proteins in total (Supplementary
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10 Table 1) after removing proteins identified from 1 or more peptides and those which matched
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12 to the decoy search (i.e., prefixed with “REVERSED...”). Of these, 2479 were identified with
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14 a 5% local false-discovery rate. From these, 277 proteins met the specified criteria (an unused
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16 ProtScore (conf) > 0.05, detected by ≥ 2 unique peptides, and a statistically significant ($p \leq 0.05$)
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18 fold change of ≥ 1.25 or ≤ 0.8) for differential expression in the *Smn*^{2B/-} mouse heart compared
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20 to age-matched WT hearts, of which 156 proteins were increased and 121 were decreased in
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22 expression in SMA mouse hearts (Supplementary Table 2).
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31 To guide the direction of further studies, we were interested to understand whether any of the
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33 differentially abundant proteins identified in heart tissue from the less-severe *Smn*^{2B/-} SMA
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35 mouse model were conserved with those identified previously from the severe Taiwanese SMA
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37 mouse model (15). The iTRAQ data pertaining to the Taiwanese model were derived in the
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39 same experiment as the *Smn*^{2B/-} data reported here, but only the Taiwanese section of the data
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41 was published previously (15). Compared to their respective age-matched WT mice (i.e., P18
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43 for the *Smn*^{2B/-} & P8 for the Taiwanese), and using the same criteria ($p \leq 0.05$, fold change of
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45 ≥ 1.25 or ≤ 0.8 , >1 unique peptide), 50 proteins were found to be commonly dysregulated in
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47 both mouse models of SMA (Supplementary Table 3). Of these, 30 were up-regulated and 20
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49 down-regulated with statistical significance in both models (Figure 1A) with a similar fold-
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51 change in most dysregulated proteins (Figure 1B).
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3 The list of differentially abundant proteins from both mouse models was also compared using
4 the comparison analysis function of the curated bioinformatics platform, Ingenuity Pathway
5 Analysis (IPA®). This two-way comparison in which the enriched terms were ranked by
6 absolute p -value, demonstrated a similar enrichment of terms for “diseases & disorders,
7 physiological system development & function” after removal of cancer related terms. In
8 particular, “abnormal morphology” and “dilated cardiomyopathy” were highly enriched in both
9 mouse models ($Smn^{2B/-}$ mouse model, p -value range 3.72×10^{-19} to 2.65×10^{-7} ; Taiwanese
10 mouse model, p -value range 9.52×10^{-24} to 4.69×10^{-8}) (Figure 1C). Similarly, for “molecular
11 & cellular functions”, terms relating to “translation, synthesis and metabolism of proteins” and
12 “cell & organismal death” were significantly enriched among the differentially abundant
13 proteins common to both mouse models ($Smn^{2B/-}$ mouse model, p -value range 5.01×10^{-40} to
14 2.65×10^{-7} ; Taiwanese mouse model, p -value range 9.52×10^{-24} to 4.69×10^{-8}) (Figure 1D).
15 Various canonical pathways already linked to SMA such as “EIF2 signaling” (23) and
16 “mitochondrial dysfunction” (24) were also enriched in the $Smn^{2B/-}$ and Taiwanese mouse
17 models (Figure 1E). In addition, the “dilated cardiomyopathy signaling pathway” was
18 significantly enriched in both models ($Smn^{2B/-}$ mouse model, $p=6.52 \times 10^{-11}$, z -score = 1.043;
19 Taiwanese mouse model, $p=2.72 \times 10^{-18}$, z -score = -0.667).
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45 Using the Database for Annotation, Visualization and Integrated Discovery (DAVID)
46 bioinformatics tool, the cellular components with which the significantly dysregulated proteins
47 in the $Smn^{2B/-}$ mouse model were associated was ascertained (Figure 1F and Supplementary
48 Table 4A). Of particular interest were those cellular components pertinent to cardiomyocyte
49 function including the Z-disk (12 proteins; $p=4.7 \times 10^{-6}$), the costamere (6 proteins; $p=5.4 \times 10^{-6}$),
50 focal adhesion (12 proteins; $p=7.9 \times 10^{-5}$) and adherens junction (10 proteins; $p=3.2 \times 10^{-4}$),
51 the sarcolemma (9 proteins; $p=5.4 \times 10^{-4}$), the intercalated disc (6 proteins; $p=1.4 \times 10^{-3}$), and
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3 intermediate filaments (6 proteins; $p=2.5 \times 10^{-2}$). When the 50 proteins significantly
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5 dysregulated in both the *Smn*^{2B/-} and Taiwanese SMA mouse models were considered in the
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7 DAVID analysis, “intermediate filament” (3 proteins; $p=4.8 \times 10^{-2}$) remained an enriched
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9 cellular component term (Supplementary Table 4B).
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14 **The intermediate filament proteins, lamin A/C and desmin, are differentially abundant** 15 **in heart tissue from two mouse models of SMA** 16 17

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19 The finding that intermediate filament proteins are differentially abundant in both SMA mouse
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21 models is of particular interest with regards to heart conditions. Mutations affecting both lamin
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23 and desmin (type V and type III intermediate filament proteins, respectively), commonly give
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25 rise to conditions associated with cardiomyopathy and heart failure (25). Of additional
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27 relevance is that mutations in *LMNA* -the lamin A/C gene- have been attributed to adult-onset
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29 conditions with an SMA-like phenotype (26,27). Lamin A was identified by iTRAQ as being
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31 increased in both the Taiwanese (15) and *Smn*^{2B/-} SMA mouse models (Supplementary Table
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33 1) (albeit it just missed the criteria for differential expression in the *Smn*^{2B/-} with $p=0.056$).
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35 Desmin, on the other hand, was decreased in expression in both the Taiwanese (15) and *Smn*^{2B/-}
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37 SMA mouse models, but only met the criteria for differential expression when peptides
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39 quantified with 99% confidence were used to calculate the ratio, resulting in $p=0.049$.
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47 To gain further insights into the involvement of these intermediate filament proteins in SMA,
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49 we verified the differential expression of lamin A/C and desmin in heart tissue from the *Smn*^{2B/-}
50
51 and Taiwanese SMA mice. Quantitative western blotting confirmed a 1.94-fold and 2.06-fold
52
53 increase in lamin A and C expression, respectively, in heart tissue extracts from *Smn*^{2B/-} mice
54
55 compared to age-matched WT mice ($p=0.014$ and $p=0.030$, respectively) (Supplementary File
56
57 Figure 1A). Immunohistochemistry analysis also confirmed increased lamin A
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3 immunoreactivity in heart tissues from the *Smn*^{2B/-} mouse model compared to those from WT
4 mice (1.74-fold increase, $p=0.007$; Supplementary File Figure 1B). This aligned with our
5
6 previous findings (15) where lamin A/C levels were increased in heart tissue from the severe
7
8 Taiwanese SMA mouse model. Immunohistochemistry analysis of *Smn*^{2B/-} mouse heart
9
10 sections revealed few lamin A/C positive cells in the ventricle lumen (Supplementary File
11
12 Figure 1C), in line with our previous observation from the Taiwanese SMA mouse model (15).
13
14 Although we cannot rule out a minor contribution, this result confirms that the increased lamin
15
16 A/C levels cannot be solely attributed to circulating blood cells. For reference, SMN levels in
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18 heart tissue extracts from the *Smn*^{2B/-} mice were reduced to 0.08-fold ($p=0.007$) of that found
19
20 in WT mice (Supplementary File Figure 1A).
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30 Decreased desmin expression was also confirmed in both mouse models of SMA by western
31 blotting, with levels being reduced in the *Smn*^{2B/-} mice compared to age-matched (P18) WT
32 mice to 0.73-fold ($p=0.033$) and in the Taiwanese mice compared to age-matched (P8) WT
33 mice to 0.76-fold ($p=0.007$) (Supplementary File Figure 2A). These findings were corroborated
34
35 by immunohistochemistry analysis (Supplementary File Figure 2B) with desmin
36
37 immunoreactivity in *Smn*^{2B/-} mice showing a decrease to 0.41-fold ($p=0.033$) compared to WT
38
39 (P18) mice and desmin staining in Taiwanese mice demonstrating a decrease to 0.26-fold
40
41 ($p=0.019$) compared to (P8) WT mice (Supplementary File Figure 2C).
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50 **AAV9-mediated *SMNI* delivery to increase SMN expression in the hearts of *Smn*^{2B/-} SMA** 51 **mice**

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53 To establish the impact of currently approved gene therapy treatments, which are designed to
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55 increase SMN levels in SMA patients, on a peripheral tissue such as the heart, we examined
56
57 the extent to which AAV9-mediated *SMNI* treatment restored expression levels of lamin A/C
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3 and desmin towards control mice. For these experiments, we chose to focus only on the less-
4
5 severe *Smn*^{2B/-} SMA model as these mice have a longer pre-symptomatic period compared to
6
7 the Taiwanese mouse model allowing for a better understanding of developmental defects
8
9 secondary to motor neuron loss. In addition, to validate our findings further, two distinct
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11 vectors that have previously been used in *Smn*^{2B/-} mice were utilised (28,29), in particular the
12
13 one vector results in enhanced expression of the *SMN1* gene (28). SMN levels were confirmed
14
15 by quantitative western blotting to be significantly increased in extracts of heart tissues from
16
17 *Smn*^{2B/-} mice following AAV9-mediated *SMN1* replacement (Figure 2). As expected (28),
18
19 delivery of the optimised cDNA transgene AAV9_*Co-hSMN1* resulted in enhanced levels of
20
21 SMN (169-fold; $p=0.0002$) compared to untreated *Smn*^{2B/-} mice with increased levels of SMN
22
23 beyond that expected in WT mice (~13.50-fold). Although the scAAV9-*SMN1*-mediated
24
25 delivery also resulted in an SMN increase (2.4-fold; $p=0.0003$) compared to untreated *Smn*^{2B/-}
26
27 mice, SMN levels remained below that found in WT mice (~0.32-fold) (Figure 2A). There was
28
29 no statistically significant difference in SMN expression levels between untreated *Smn*^{2B/-} mice
30
31 and vehicle controls (i.e., AAV9_*eGFP* (2.1-fold; $p=0.14$); scAAV9-*GFP* (1.8-fold; $p=0.08$)
32
33 (Figure 2A). Immunohistochemistry analysis of heart tissues from *Smn*^{2B/-} mice revealed
34
35 increased SMN immunoreactivity following SMN replacement, particularly in those treated
36
37 with AAV9_*Co-hSMN1* (Figure 2B).
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47 **AAV9-mediated *SMN1* delivery restores desmin expression but does not correct** 48 49 **increased lamin A/C expression in the hearts of *Smn*^{2B/-} SMA mice**

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51 Quantitative western blotting and immunohistochemistry analysis revealed that AAV9-
52
53 mediated *SMN1* treatment did not correct the increased production of lamin A/C in *Smn*^{2B/-}
54
55 mouse hearts. On western blots, lamin A expression levels following delivery of AAV9_*Co-*
56
57 *hSMN1* were 0.80-fold ($p=0.36$) compared to untreated *Smn*^{2B/-} mice and 1.56-fold ($p=0.04$)
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3 compared to WT mice. Following scAAV9-*SMN1*-mediated delivery lamin A levels were 0.96-
4 fold ($p=0.44$) compared to untreated *Smn*^{2B/-} mice and 1.56-fold ($p=0.003$) compared to WT
5 mice (Figure 3A). Similarly, lamin C expression levels following delivery of AAV9_*Co-*
6 *hSMN1* were 0.77-fold ($p=0.26$) compared to untreated *Smn*^{2B/-} mice and 1.39-fold ($p=0.13$)
7 compared to WT mice and following scAAV9-*SMN1*-mediated delivery were 0.96-fold
8 ($p=0.50$) compared to untreated *Smn*^{2B/-} mice and 1.54-fold ($p=0.004$) compared to WT mice
9 (Figure 3A). There was no statistically significant difference in lamin A and C expression levels
10 between untreated *Smn*^{2B/-} mice and vehicle controls (i.e., AAV9_*eGFP* (1.06-fold ($p=0.46$))
11 for lamin A and 1.21-fold ($p=0.06$) for lamin C); scAAV9-*GFP* (0.96-fold ($p=0.59$) for lamin
12 A and 0.84-fold ($p=0.06$) for lamin C) (Figure 3B).

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29 In heart sections from WT mice, lamin A/C immunoreactivity was largely confined to the
30 nuclear membrane, as expected, with a small proportion being localised to the sarcomere as
31 confirmed by double-label immunohistochemistry with alpha-actinin 2, a sarcomere marker
32 (Figure 3C & 3D). In heart sections from the *Smn*^{2B/-} mice, lamin A/C was significantly
33 increased in expression at the nuclear membrane alongside a strikingly increased distribution
34 of lamin A/C at the sarcomeres (Figure 3C). When quantified across each section, lamin A/C
35 immunoreactivity in heart tissues from *Smn*^{2B/-} mice showed a significant increase in lamin A/C
36 expression compared to age-matched (P18) WT mice (3.29 ± 1.13 -fold; $p=0.004$; Figure 3C),
37 and after treatment with AAV9_*Co-hSMN1* or scAAV9-*SMN1* lamin A/C levels in *Smn*^{2B/-}
38 mice remained increased compared to the untreated *Smn*^{2B/-} mice (3.66 ± 0.70 -fold ($p=0.54$)
39 and 4.25 ± 0.45 -fold ($p=0.13$), respectively (Figure 3C).

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Western blot and immunohistochemistry analysis revealed that AAV9-mediated *SMN1*
treatment did, however, restore the decreased expression of desmin in *Smn*^{2B/-} mouse hearts

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3 towards WT levels. From western blots, desmin expression levels following delivery of
4 AAV9_*Co-hSMNI* were 1.74-fold ($p=0.0095$) compared to untreated *Smn*^{2B/-} mice and 1.06-
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6 fold ($p=0.44$) compared to WT mice and following scAAV9-*SMNI*-mediated delivery were
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8 1.84-fold ($p=0.04$) compared to untreated *Smn*^{2B/-} mice and 1.31-fold ($p=0.13$) compared to
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10 WT mice (Figure 4A). There was no statistically significant difference in desmin expression
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12 levels between untreated *Smn*^{2B/-} mice and vehicle controls (i.e., AAV9_*eGFP*) (0.99-fold
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14 ($p=0.88$)) and scAAV9-*GFP* (1.16-fold ($p=0.19$)) (Figure 4B). Desmin immunoreactivity in
15
16 heart sections from WT mice was typically present at the Z-disc (Figure 4C). Desmin
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18 immunoreactivity in heart sections from the *Smn*^{2B/-} mice was clearly reduced (Figure 4C, high
19
20 magnification images), although some striations could still be seen, the staining appearing to
21
22 be variable in intensity and disorganised. Quantification of desmin immunoreactivity across
23
24 each heart section from the *Smn*^{2B/-} mice found desmin expression to be significantly decreased
25
26 compared to age-matched (P18) WT mice (0.61 ± 0.12 -fold ($p<0.001$)) (Figure 4C), but
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28 treatment with AAV9_*Co-hSMNI* or scAAV9-*SMNI* confirmed that desmin levels in *Smn*^{2B/-}
29
30 mice were increased compared to the untreated *Smn*^{2B/-} mice (1.75 ± 0.19 -fold ($p<0.0001$) and
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32 1.28 ± 0.22 -fold ($p=0.035$)), respectively (Figure 4C).
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42 **Decreased elastin expression in *Smn*^{2B/-} mouse heart is also refractory to AAV9-mediated** 43 ***SMNI* delivery**

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46 Lamin A/C is a significant regulator of cell stability and dynamics (30), and its expression
47
48 correlates with the stiffness of tissue (31). Increased rigidity of cardiomyocytes enhances
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50 passive tension *in vitro* and *in vivo*, resulting in functional heart defects (32,33). To gain
51
52 insights into the wider impact of increased lamin A/C levels on the contractile apparatus of
53
54 cardiomyocytes and overall stiffness of heart tissues in the *Smn*^{2B/-} SMA mouse model, we
55
56 examined the levels of elastin, a protein essential for the elastic properties of many tissues.
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3 Elastin was identified by iTRAQ analysis to be significantly reduced to 0.01-fold ($p=0.003$) in
4 heart tissue from the *Smn*^{2B/-} SMA mouse model compared to WT. This finding was confirmed
5
6 qualitatively by immunohistochemical analysis of van Gieson staining (i.e., a modified Miller's
7
8 stain which differentially stains elastin from collagen and muscle), and quantitatively by
9
10 higher-power immunofluorescence analysis (0.30 ± 0.14 -fold ($p<0.00001$) in *Smn*^{2B/-} vs WT)
11
12 (Figure 5). Elastin levels in *Smn*^{2B/-} mice following treatment with either AAV9_*Co-hSMNI* or
13
14 scAAV9-*SMNI* remained comparable to those observed in untreated *Smn*^{2B/-} mice (0.37 ± 0.16 -
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16 fold ($p=0.41$), and 0.30 ± 0.13 -fold ($p=0.24$)) of WT levels respectively) (Figure 5).
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24 Discussion

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26 Over the last two decades, evidence has built to suggest that in severe forms of SMA, structural
27
28 and functional abnormalities in heart tissues exist (14,18,22), whilst in patients with less severe
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30 types, a potentially milder impairment of cardiac function is suggested (9,16). The aim of this
31
32 study was to conduct a proteomic comparison of heart tissues from the less-severe *Smn*^{2B/-} SMA
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34 mouse model and age-matched wild-type (WT) mice and compare these findings with those
35
36 identified in our previous study of heart tissues from the severe Taiwanese SMA mouse model
37
38 (15) to determine whether a conserved response to reduced SMN levels is evident. We also
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40 investigated whether dysregulated proteins with a known role in heart function are impacted
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42 and whether their levels could be rescued by increasing SMN levels in the less-severe *Smn*^{2B/-}
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44 mouse model.
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51 In each SMA mouse model, hundreds of proteins were found to be dysregulated compared to
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53 age-matched WT mice. Bioinformatics analysis revealed a highly significant enrichment of
54
55 terms relating to “abnormal morphology” and “dilated cardiomyopathy” in reference to
56
57 “diseases & disorders, physiological system development & function” in both dysregulated
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3 datasets. Previously, cardiac defects such as thinning of the interventricular septum and
4
5 cardiomyocyte disorganisation have been noted in the severe Taiwanese SMA mouse model
6
7 (14) as have atrial or ventricular septal defects (11) and thickened myocardium (9) in patients
8
9 with SMA type I. Out of 42 SMA type II and III patients, one patient had mitral valve prolapse,
10
11 7 had sinus tachycardia, and more than half had an elevated minimum 24-hour heart rate (16).
12
13 When “molecular and cellular functions” were considered, terms relating to the “translation,
14
15 synthesis and metabolism of proteins”, and “cell and organismal death” were highly significant.
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17 Most of these terms are typically associated with SMA, in particular, SMN is known to be key
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19 for protein homeostasis (34). Both *in vitro* and *in vivo* studies provide support for impaired
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21 translation in SMA (35,36) and a comprehensive study involving rat primary neuron culture,
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23 fibroblasts from SMA Type I patients and neurons from a severe mouse model of SMA (*Smn*^{-/-};
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25 *SMN2*^{tg0}) demonstrated reduced *de novo* protein synthesis linked to mTOR signalling (37).
26
27 In an earlier study, microfilament metabolism was found to be impacted by reduced levels of
28
29 SMN (38), and with evidence building for a role of mitochondrial dysfunction in SMA (24,39–
30
31 41), all these factors may contribute to altered protein metabolism. Most of the significantly
32
33 enriched “canonical pathways” common to both mouse models have also been implicated in
34
35 SMA, such as EIF2 signaling (23), mitochondrial dysfunction (24) and mTOR signaling (37),
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37 but of particular interest was the significant enrichment of “dilated cardiomyopathy” and the
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39 “dilated cardiomyopathy signaling pathway” in both mouse models of SMA.
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49 While differentially abundant proteins from the *Smn*^{2B/-} dataset were associated with several
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51 cellular components pertinent to cardiomyocyte function, the only cellular component to be
52
53 significantly enriched among proteins common to both mouse models was intermediate
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55 filaments. Genetic mutations of the intermediate filaments lamin A/C and desmin are known
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57 contributors of cardiomyopathies and heart failure (25,42). Both proteins form networks within
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3 the cardiomyocyte and help maintain intra- and intercellular structure, with lamin A/C at the
4 nuclear envelope (43) and desmin within the cytoplasm. In addition, lamin and desmin have
5 important functions in nucleo-cytoskeletal coupling and mechano-transduction, gene
6 regulation, metabolism, mitochondrial homeostasis and cardiomyocyte differentiation and
7 survival (43–45), all of which are necessary for a fully functioning heart.
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17 As previously confirmed in the Taiwanese mouse model of severe SMA (15), levels of lamin
18 A/C were significantly increased in heart tissues from the *Smn*^{2B/-} mouse model of less-severe
19 SMA. Increased levels of lamin A/C were localised to the nuclear envelope and to the
20 sarcomere of *Smn*^{2B/-} mouse hearts, and further support the concept that appropriate lamin A/C
21 production is SMN-dependent (15,27,46). Previously, increased lamin A/C levels have been
22 described in muscle (47) and Schwann cells from SMA mice (48) and in motor neurons
23 obtained from Type I SMA patients (49). In terms of impact on heart conditions and
24 development of dilated cardiomyopathy, impairment of the lamin A/C gene is a well-known
25 factor (50) and results in conduction problems, arrhythmias, atrioventricular block and sudden
26 cardiac death (51–55) with at least 260 *LMNA* mutations having been linked to cardiac diseases
27 (56). In several studies, *LMNA* mutations have also resulted in an SMA-like phenotype with
28 cardiac involvement (26,27).
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47 An increase in lamin A/C may result in a “stiffer” nucleus which will alter the biomechanics
48 of the cardiomyocyte, and thus the heart tissue, and may be in response to increased stiffness
49 of the heart tissue (31). Conditions where increased accumulation of lamin A at the nuclear
50 envelope is known to occur are Hutchinson-Gilford progeria syndrome and restrictive
51 dermatopathy. Both conditions arise from improper post-translational processing of prelamin A,
52 the former from an *LMNA* mutation which results in a mutant farnesylated prelamin A
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3 (progerin) whilst the latter is due to reduced levels of ZMPSTE24, the enzyme necessary for
4 processing prelamin A (57). Although not verified by immuno techniques in the current study,
5
6 ZMPSTE24 was significantly increased in the mass spectrometry data from both SMA mouse
7
8 models which may imply dysregulation of lamin A processing. In the current study, lamin A
9
10 was also prevalent at the sarcomere, especially in *Smn^{2B/-}* mice where it co-localised with alpha-
11
12 actinin 2. This may indicate an increase in unprocessed lamin A in SMA, as previously
13
14 prelamin A was identified at the sarcomere along with alpha-actinin 2 (55). Since it has been
15
16 suggested that prelamin A may be toxic and could promote dilated cardiomyopathy (55),
17
18 further investigations into understanding whether lamin A processing is defective in SMA may
19
20 prove useful. Additionally, the phosphorylation status of lamin A in SMA is worthy of
21
22 investigation as this may have implications for the regulation of lamin A levels since hypo-
23
24 phosphorylation renders lamin A more stable and less likely to be degraded (58) and has
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26 occurred in mesenchymal stem cells when subjected to increased tissue stiffness (59).
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35 Lamin A/C is the main provider of nuclear membrane mechanical strength and is key to
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37 transmitting mechanical force from the ECM to the nucleus (60,61). Deficiency or mutations
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39 in lamin A/C can cause mechano-transduction impairment (62) or impact whole-cell
40
41 biomechanical properties (63). In a lamin A-deficient mouse model, the desmin network was
42
43 disrupted and detached from the nuclear surface (64), suggesting defective force transmission
44
45 due to loss of lamin interactions with desmin and subsequent loss of cytoskeletal tension. In
46
47 another study of lamin A-deficient mice, desmin accumulation was increased in both muscle
48
49 and cardiac tissues (65). It may prove useful to consider whether the reverse is true in SMA
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51 cardiomyocytes whereby decreased levels of desmin result in increased lamin A or *vice versa*.
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3 Desmin is located throughout the cardiomyocyte and assists in transmitting force across the
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5 ECM, sarcomere and cytoskeleton (66), and has been implicated in SMA (67). In combination
6
7 with microtubules and microfilaments, desmin helps to maintain the cytoskeleton structure and
8
9 organisation of sub-cellular organelles (68) and is thought to provide cardiomyocyte nuclei
10
11 with tension (69). Structurally, desmin links individual myofibrils at the Z-discs (70,71),
12
13 connects Z-discs to the intercalated disc (72), forms part of the desmosomes, and is linked to
14
15 the costamere enabling adhesion to the ECM (73). Overall, desmin is key to the functioning of
16
17 sarcomeres in efficient syncytia. Mutations often result in loss of sarcomere integrity and
18
19 myofibrillar structure (74) and depletion causes muscle architecture disruption (75), which is
20
21 not impacted during embryogenesis and myofibrillar assembly but affects muscle regeneration
22
23 resulting in weaker mice in a desmin knock-out model (76). In other studies, lack of desmin
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25 impacted contractile function (77) and increased fibrosis (78) with muscle fibres lacking
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27 desmin being more susceptible to physical damage (79) and desmin loss causing increased
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29 passive stiffness in muscle (80).
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38 Mechano-sensing within cardiomyocytes is a “two-way” system, with the nucleus being an
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40 important mechanosensory organelle in the cell and intimately connected to the cytoskeleton,
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42 and the latter in turn to the ECM. For optimal heart performance, both electrical and mechanical
43
44 stimuli are required with cardiomyocytes being subjected to cyclic contraction as the heart
45
46 beats (81). Cardiomyocytes also experience passive mechanical stimuli such as that exerted by
47
48 the cardiac ECM. The cardiac ECM is a combination of elastin bundles, collagen and
49
50 interconnected basement membranes (82), with intra- and extracellular components
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52 contributing to overall myocardial stiffness (81). Although collagen is the main extracellular
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54 protein in myocardial tissue, elastin, which is found in the epicardium, endomysium and
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56 structures such as arteries, atria and ventricles of the heart (83), provides elasticity upon
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3 mechanical demand (84). Elastin is a structural glycoprotein and an essential component of
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5 myocardial stiffness (85), so any alterations in its levels will impact heart function. Typically,
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7 elastin is very stable with a considerably low turnover rate, with degradation or damage
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9 indicating pathological remodelling of the tissue and cardiovascular disease (86). Reduced
10
11 levels of elastin, as found in this study, will increase the heart tissue's passive stiffness,
12
13 potentially reducing cardiac function, since cardiomyocytes cultured on a stiff, fibrotic-like
14
15 matrix fail to beat properly (87). Typically, following myocardial infarction/ischaemia, elastin
16
17 levels will decrease (88), promoting ECM remodelling which further increases the stiffness of
18
19 the cardiac tissue and impairment of cardiac function (85). In Williams-Beuren syndrome,
20
21 impairment of the elastin *ELN* gene results in 4 out of 5 patients having cardiovascular
22
23 abnormalities (89), with mutations also resulting in congenital heart disease (90). Elastin fibres
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25 are crucial for the proper functioning of Purkinje's fibres (91) and blood vessels (92), and
26
27 impairment can cause arrhythmias (93) or altered blood flow respectively, again impacting
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29 heart function.
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38 Two AAV9-mediated *SMN1* treatments were used in this study, both of which have previously
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40 been used in *Smn*^{2B/-} mice (28,29), but the novel codon-optimised cDNA transgene
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42 demonstrated significantly enhanced expression of the human *SMN1* gene in *Smn*^{2B/-} mice
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44 hearts. Both treatments restored desmin levels to that of WT mice, but lamin A/C levels
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46 remained elevated and elastin levels decreased even though the codon-optimised cDNA
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48 transgene generated SMN levels more than 10-fold greater than those found in WT mice. The
49
50 lack of effect of increased SMN levels on lamin A in this study may be due to several reasons:
51
52 (i) lamin A is independent of SMN levels; (ii) the impact of low SMN levels on lamin A is
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54 greatest during embryonic development so post-natal treatment is too late; (iii) cardiac
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56 remodelling is influencing lamin A levels; and/or (iv) dysregulation of proteins responsible for
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3 post-translation processing or regulating the stability of lamin A are not independent of SMN
4 levels. In contrast, desmin levels were increased by both AAV9-mediated *SMNI* treatments,
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6 indicating a direct relationship between these two proteins. Previously, abnormalities in the Z-
7
8 bands of a patient with SMA were identified (94). Since low levels of desmin appeared to be
9
10 associated with sarcomere damage in a rat model of diabetes mellitus (95) and alpha-actinin
11
12 staining showed disrupted sarcomere/costamere/z-disc striations in desmin-null mice (96), it is
13
14 coherent that the improved levels of desmin in the AAV9-treated *Smn*^{2B/-} mice result in
15
16 improved sarcomere structure, although higher resolution microscopy is required to confirm
17
18 this. Interestingly, SMN has been found to be localised to the I- and M-bands of sarcomeres in
19
20 normal human skeletal fibres (97) and the Z-disc in mice (20) which suggests that sarcomeric
21
22 SMN may directly or indirectly interact with the cytoskeleton and thus be involved in
23
24 sarcomere structure (97), or its maintenance (20). In addition, during heart development,
25
26 desmin and lamin A are abundant in the cardiac conduction systems; desmin preferentially in
27
28 the myocardium of the central conduction system and lamin A more localised to the peripheral
29
30 conduction system, then progressively to the central conduction system (98). It may be possible
31
32 that AAV9-mediated treatment does not rectify lamin A/C levels due to inherent problems with
33
34 the central conduction system that are established prior to treatment being administered.
35
36 Additionally, abnormalities in conduction usually occur before dilated cardiomyopathy
37
38 development (99) with elevated heart rate being associated with cardiomyopathy in late
39
40 adolescence and increased heart failure risk (100). In desmin mutant mice, distribution of lamin
41
42 A/C was nucleoplasmic whilst in WT mice, lamin A/C was mostly at the nuclear periphery
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44 (72). Since AAV9-mediated treatment restored desmin levels, lamin A/C's nuclear distribution
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46 may improve over time.
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3 Other researchers have found the outcome of AAV9 treatment in SMA to be variable. A study
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5 focussed on fibrosis, remodelling, vascular integrity and oxidative stress in two mouse models
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7 of differing severity only found a partial rescue of cardiovascular defects in their mouse model
8
9 of severe SMA and although structural defects were rectified, heart function incorporating heart
10
11 rate, stroke volume and cardiac output remained significantly lower than WT mice, as did
12
13 lifespan (13). In the SMN Δ 7 mouse model, scAAV9 treatment resolved the bradycardia issues,
14
15 but “cardiac output” remained similar to untreated mice, probably as a result of “stroke
16
17 volume” remaining decreased following scAAV9 treatment (12). In addition, contractility
18
19 remained lower than WTs following treatment. Another study using the SMN Δ 7 SMA mouse
20
21 model used the histone deacetylase inhibitor, trichostatin A, which is known to improve the
22
23 phenotype of SMA mice. Although heart function improved, these mice did not survive as well
24
25 as the WT mice (101). Typically, levels of lamin A/C in mouse cardiomyocytes, desmin in rat
26
27 heart tissues and the elastin component of connective tissues decline with age (102-104),
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29 although in male F344/BN hybrid rats, desmin levels were found to increase in aging skeletal
30
31 muscle (105). In future studies, it will therefore be important to characterise the temporal
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33 regulation of the proteins investigated throughout the disease time-course in the *Smn*^{2B/-} mouse
34
35 model, with and without AAV-9 mediated *SMN1* replacement, and to establish whether
36
37 restoring levels of these proteins would have an impact on cardiac function of the mouse model.
38
39 SMN may be essential for heart development prior to birth and thus scAAV9 treatment may
40
41 not correct key development milestones involving SMN. Additionally, other factors such as
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43 lung and metabolic function or scoliosis may contribute to continued impairment of heart
44
45 function in SMA. Prior to the introduction of treatments, such as, nusinersen and risdiplam
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47 which modify *SMN2* gene regulation to increase functional SMN expression, the impact on
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49 concomitant health issues in SMA patients following respiratory assistance, and thus extended
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3 life expectancy, has been previously highlighted (10) and included development of heart
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5 arrhythmia (bradycardia).
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10 In conclusion, accumulating evidence suggests that cardiac assessment in the long-term care
11
12 of SMA patients is warranted. We only investigated three of the dysregulated proteins
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14 identified from mass spectrometry analysis in the *Smn*^{2B/-} mouse model, but there are many
15
16 more that warrant further investigation, especially those pertinent to heart function. AAV9-
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18 *SMN1* gene therapy only corrected one of the proteins investigated, and although severe
19
20 symptoms may be rare in SMA, subtle differences in cardiac performance in combination with
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22 current treatments increasing longevity in these patients, may impact cardiac health in future
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24 years. Thus, additional cardiac therapies may prove useful in optimising treatment regimens
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26 for SMA patients.
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33 **Materials & Methods**

34 **SMA mouse models**

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36 The *Smn*^{2B/-} and Taiwanese (original strain from Jackson Laboratories, No. 005058) mouse
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38 models were housed at Keele and Edinburgh Universities, respectively. As previously
39
40 described (106), the less-severe *Smn*^{2B/-} mouse model is maintained on a pure C57BL/6 genetic
41
42 background with creation of the 2B mutation via a 3-nucleotide substitution within the exon
43
44 splicing enhancer of exon 7 (107,108). The severe Taiwanese mouse model is heterozygous
45
46 for the *SMN2* transgene on a *Smn* null background (*Smn*^{-/-}; *SMN2*^{tg/+}) (109). Tissues were
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48 harvested at a symptomatic time point of post-natal day (P) 18 and P8 for the *Smn*^{2B/-} and
49
50 Taiwanese models, respectively, in addition to age-matched WT mice. All experimental
51
52 procedures on Taiwanese mice were authorized and approved by the University of Edinburgh
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54 Animal Welfare Ethical Review Body (AWERB) and UK Home Office (Project Licence
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3 60/4569) in accordance with the Animals (Scientific Procedures) Act 1986. For the *Smn*^{2B/-}
4 mice, experimental procedures were authorized and approved by the Keele University AWERB
5 and UK Home Office (Project Licence P99AB3B95) in accordance with the Animals
6 (Scientific Procedures) Act 1986.
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15 **Protein extraction and preparation for quantitative proteomic analysis**

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17 Total protein extracts were prepared as previously described (110) from the hearts of P18
18 *Smn*^{2B/-} and age-matched WT animals (both n = 3) and isobaric tags for relative and absolute
19 quantitation (iTRAQ) quantitative mass spectrometry analysis were added. Briefly, proteins
20 were digested with trypsin, then peptides tagged with iTRAQTM reagents as described in the
21 iTRAQ kit protocol and sample groups assigned as follows: 116-P18 WT and 117-P18 *Smn*^{2B/-}.
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28 **High pH reverse-phase liquid chromatography fractionation and liquid** 29 **chromatography–tandem mass spectrometry analysis** 30 31

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33 Fractionation and mass spectrometry analysis were carried out as previously described (15). In
34 brief, the iTRAQ-labelled peptides were combined, concentrated, resuspended in buffer A (10
35 mm ammonium formate [NH₄HCO₂], 2% acetonitrile [MeCN], pH 10.0) then fractionated
36 using high pH reverse-phase liquid chromatography (C18 column). Following rinsing of the
37 column with 96% buffer A until the optical density returned to baseline, a gradient was run
38 from 4% to 28% of buffer B (10 mm NH₄HCO₂, 90% MeCN, pH 10.0) for 30 min followed
39 by another from 28–50% buffer B for 6 min. Buffer B at 80% was used to rinse the column for
40 5 min and the column then re-equilibrated with 4% buffer B for 11 min. Fractions (0.5 ml)
41 were collected every 30 s and their UV chromatograms analysed. Fractions with similar peptide
42 concentrations were combined resulting in 12 fractions which were vacuum dried then
43 resuspended in 30 µl of 0.1% formic acid.
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3 For the mass spectrometry analysis, one-third of each fraction was analysed by mass
4 spectrometry. Following separation of the peptides via liquid chromatography, peptides were
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6 loaded with buffer A (2% MeCN, 0.05% TFA in ultrapure water) and bound to an Acclaim
7
8 PepMap100 AQ6 trap (100 μm \times 2 cm) (Thermo Fisher Scientific) which was then washed for
9
10 10 min with buffer A. The analytical solvent system consisted of buffer A and buffer B (98%
11
12 MeCN, 0.1% FA in ultrapure water) at 300 nl/min flow rate. To elute the peptides the following
13
14 gradient was utilized: linear 2–20% of buffer B over 90 min, linear 20–40% of buffer B for 30
15
16 min, linear 40–98% of buffer B for 10 min, isocratic 98% of buffer B for 5 min, linear 98–2%
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18 of buffer B for 2.5 min and isocratic 2% buffer B for 12.5 min. Eluent was sprayed with a
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20 NANOSpray II source (electrospray ionization) into the TripleTOF 5600+ tandem mass
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22 spectrometer (AB Sciex) under the control of Analyst® TF software (AB Sciex). The mass
23
24 spectrometer was operated as previously described (15) and the raw mass spectrometry data
25
26 files analyzed by ProteinPilot software, version 5.0.1.0 (Applied Biosystems) in addition to the
27
28 Paragon™ database search and Pro Group™ algorithm using the UniProtKB/Swiss-Prot
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30 FASTA database. Paragon search analysis parameters for sample were: type “iTRAQ4plex
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32 (Peptide Labeled),” cysteine alkylation “MMTS,” digestion “trypsin,” instrument “TripleTOF”
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34 and species “Mouse” with processing parameters described as “quantitative,” “bias correction,”
35
36 “background correction,” “thorough ID” and “biological modifications.” A protein threshold
37
38 of >5 was used in the Pro Group algorithm to calculate the relative protein expression with the
39
40 generation of an error factor and *p*-value, and Proteomics System Performance Evaluation
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42 Pipeline used to determine false discovery rate. The mass spectrometry proteomic data can be
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44 accessed at the following DOI: 10.6084/m9.figshare.23256374
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56 **Bioinformatics analysis**

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3 Data that met the criteria for differential expression in the *Smn*^{2B/-} mice were analysed using
4 Database for Annotation, Visualization and Integrated Discovery (DAVID) Functional
5 Annotation Bioinformatics 2021 (111,112) and Ingenuity Pathway Analysis software (IPA;
6 QIAGEN Inc., <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>)
7 (113). Proteins were deemed differentially abundant in the *Smn*^{2B/-} mice in comparison to age-
8 matched WT mice after the following filtering criteria were applied and proteins removed if;
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10 (i) they were identified from just one peptide, (ii) had expression changes of less than 25%
11 (SMA vs WT) or (iii) had *p*-values of >0.05 assigned to their expression changes. Only terms
12 with at least three annotated proteins and a *p*-value of ≤0.05 were noted for the DAVID
13 analysis.
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29 **SMN replacement in *Smn*^{2B/-} mice via viral vector treatment**

30 For SMN replacement, AAV9 vectors were used from two sources, RY (single stranded
31 AAV9_*eGFP* and AAV9_*Co-hSMN1*) (28) and BS (self-complementary scAAV9-*GFP* and
32 scAAV9-*SMN1*) (29) and administered through the facial vein at P0 (5E10 vg/pup-7E10
33 vg/pup). The vector sourced from RY uses a codon-optimised cDNA transgene which has been
34 developed to enhance levels of the functional SMN protein produced both *in vitro* and *in vivo*
35 (28) whilst the vector from BS used standard cloning procedures as previously described (114).
36 Following CO₂ anaesthesia and exsanguination, heart tissues were harvested from the
37 following groups at the symptomatic time-point of P18: untreated WT mice (n=4), untreated
38 *Smn*^{2B/-} mice (n=6), *Smn*^{2B/-} mice treated with the vector plus GFP (RY, n=5; BS, n=4) and
39 *Smn*^{2B/-} mice treated with the vector plus *SMN1* (RY, n=5; BS, n=5).
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56 **Protein extraction and western blotting**

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3 All extraction steps were carried out on ice and as previously described (115). Heart tissues
4 were diced then homogenized with pellet pestles in 2× modified RIPA buffer (2% NP-40, 0.5%
5 Deoxycholic acid, 2 mM EDTA, 300 mM NaCl and 100 mM Tris-HCl (pH 7.4)) plus protease
6 inhibitor cocktail at 1:100 (Sigma-Aldrich; P8340), left on ice for 5 min then sonicated briefly
7 at 5 microns for 10 s. This process was repeated an additional two times. The samples were
8 centrifugated at 13,000 RPM (MSE, Heathfield, UK; MSB010.CX2.5 Micro Centaur) for 5
9 min at 4°C and protein concentration determined via the BCA protein assay (Pierce™, 23227).
10 The concentration of the protein extracts was adjusted to approximately 2mg/ml, and they were
11 heated in 2× SDS sample buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.125 M
12 Tris-HCl (pH 6.8) and bromophenol blue) for 3 min at 95 °C prior to loading onto 4-12% Bis-
13 Tris polyacrylamide gels (Life Technologies, Warrington, UK) for SDS-PAGE (Biorad,
14 Hercules, CA, USA). A horizontal slice of the gel was excised and stained with Coomassie
15 blue as an internal loading control for total protein, if necessary, protein concentrations were
16 further adjusted to ensure even loading of the samples. The proteins in the remaining gel were
17 transferred overnight via western blot onto a nitrocellulose membrane. Following blocking
18 with 4% powdered milk, the membranes were incubated with primary monoclonal antibodies
19 against: mouse anti-SMN (MANSMA12 2E6; 1:100 (116)); rabbit anti-lamin A/C (Abcam;
20 ab169532; 1:2000); rabbit anti-desmin (Abcam; ab227651; 1:1000) in dilution buffer (1% FBS,
21 1% horse serum (HS), 0.1% bovine serum albumin (BSA) in PBS with 0.05% Triton X-100)
22 for 2 h at RT or O/N at 4°C. Membranes were then incubated with HRP-labelled rabbit anti-
23 mouse Ig (DAKO, P0260) or goat anti-rabbit Ig (DAKO, P0488) at 0.25 ng/ml in dilution
24 buffer for 1 h followed by either West Pico or West Femto (both ThermoFisher) and bands
25 imaged with the ChemiDoc™ Touch Imaging System (Bio-Rad). Using ImageJ Fiji software
26 (v1.51) (117) densitometry measurements of both antibody reactive bands and Coomassie-
27 stained gel bands were undertaken.
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Immunofluorescence microscopy

Following excision, heart tissues incorporating the left and right ventricles were flash frozen in liquid nitrogen and stored at -80°C then sectioned ($7\ \mu\text{m}$) on a rotary cryostat, collected onto polylysine-coated slides and stored at -20°C . Prior to staining, slides were brought to RT. All subsequent steps were carried out at RT and PBS used for each wash step (3 x 5 min) whilst blocking buffer (1% FBS, 1% HS, 0.1% BSA in PBS) was used for antibody dilution. Sections were washed, then blocked for 30 min prior to being incubated either for 2 h at RT or O/N at 4°C with primary antibodies against mouse anti-SMN (MANSMA12 2E6; 1:4 (116)), rabbit polyclonal anti-lamin A/C (NOVUS; NBP2-19324; 1:100), rabbit monoclonal anti-desmin (Abcam; ab227651; 1:100), rabbit polyclonal anti-elastin (Fisher Scientific; PA5-99418; 1:100) or mouse anti-alpha Actinin 2 (Gene Tex; GTX632361; 1:200). Following washing, $5\ \mu\text{g}/\text{ml}$ of secondary antibody (Molecular Probes; goat anti-rabbit IgG ALEXA Flour 488; A11034) was applied for 1 h. Sections were washed prior to being stained with 4',6-diamidino-2-phenylindole (DAPI; D9542; $0.4\ \mu\text{g}/\text{mL}$) for 10 min, then washed, mounted and imaged with a Leica TCS SP5 spectral confocal microscope (Leica Microsystems, Milton Keynes, UK).

Images generated were analysed via ImageJ. For lamin A, images obtained with the 63x objective with an additional four-fold magnification were utilised, whereas images taken with the 63x objective were used for desmin and elastin analysis. For the lamin A sections, a minimum of 100 cells were analysed whereas for desmin and elastin a minimum of 200 cells were analysed per condition. For each analysis the scale function was removed, so only the pixels either staining for DAPI or the protein (lamin A, desmin and elastin) were counted. Using the threshold function (118), the highlighted pixels were analysed, and protein staining was corrected for cells present by dividing by the measure obtained for the positively stained

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3 DAPI areas. The fold difference between the SMA mouse models and their relevant WTs were
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5 then determined.
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10 **Histochemical staining of elastin**

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12 Heart tissue sections were also stained for elastin with the Miller's Elastic Van Gieson Stain
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14 Kit (Atom Scientific Ltd; RRSK11-100) using the protocol provided. All wash steps were
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16 carried out with tap water unless otherwise stated. Briefly, sections were washed then treated
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18 with acidified potassium permanganate (0.5%) for 5 min. Following further washing the
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20 sections were bleached with oxalic acid (1%) for 1 min, washed, then rinsed in 95% alcohol
21
22 and submerged in Miller's elastin stain for 3 h at RT. Sections were then rinsed with 95%
23
24 alcohol until the slides were clear, washed, and counterstained with filtered Van Gieson stain
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26 for 2 min, then blotted, dehydrated, cleared and mounted.
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33 **Statistical analysis**

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35 Statistical analyses were carried out using GraphPad Prism version 9.0.0 for Windows,
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37 GraphPad Software, San Diego, California USA, www.graphpad.com. Data distribution was
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39 assessed with the Shapiro–Wilk test and the unpaired two-tailed t-test and Mann-Whitney U
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41 test applied to parametric and non-parametric data respectively. Western blot densitometry
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43 measurements were assessed using an unpaired, one-tailed or two-tailed t-test with Welch's
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45 correction (2-sample equal variance), depending on the null-hypothesis that was tested in each
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47 case. Venn diagrams were generated via a web-based tool (119). Data are presented as mean ±
48
49 standard deviation with a *p*-value of ≤ 0.05 being considered as significant for all analyses.
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56 **Supplementary Material**

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58 Supplementary File (Word document containing Figures 1 and 2)
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3 Supplementary Tables (Excel document containing supplementary Tables 1-4)
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8 **Data Availability Statement:** The mass spectrometry proteomic data can be accessed for
9 reviewing purposes at <https://figshare.com/s/72b949c0934737a15b51>
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12 Following publication, the mass spectrometry proteomic data can be accessed at the following
13 DOI: 10.6084/m9.figshare.23256374
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20
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51 **Conflicts of Interest Statement**

52 THG has served on advisory boards for SMA Europe, LifeArc, Roche and Novartis.
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Legends to Figures

Figure 1. Summary and bioinformatics analysis of the proteomic profiles of heart tissues differentially abundant in the *Smn*^{2B/-} and Taiwanese mice models of SMA compared to age-matched WT mice. (A) Venn diagram showing an overlap of 50 proteins identified as significantly up- or down-regulated in both *Smn*^{2B/-} and Taiwanese mouse models of SMA (an unused ProtScore (conf) > 0.05, detected by ≥2 unique peptides, and a statistically significant (p≤0.05) fold change of ≥1.25 or ≤0.8). **(B)** Heat map illustrating the size of fold-change in proteins found to be dysregulated in both mice models of SMA. Two-way comparisons generated in IPA® in which the following were ranked by -log(p-value) for each SMA mouse model: **(C)** Disease & disorders, physiological system development & function; **(D)** Molecular & cellular functions and **(E)** Canonical pathways. **(F)** Schematic illustrating the cellular components impacted by the significantly dysregulated proteins of the *Smn*^{2B/-} mouse model of SMA as determined following DAVID analysis. Image created with BioRender.com.

Figure 2. Levels and distribution of SMN in heart tissues from *Smn*^{2B/-} mice following AAV9-mediated *SMN1* delivery at P0. (A) Representative western blots of SMN levels in heart tissues from untreated *Smn*^{2B/-} mice, *Smn*^{2B/-} mice following AAV9-mediated treatment with and without *SMN1* from two sources plus a corresponding age-matched WT mouse (P18). The bar graph represents average SMN levels expressed relative to the corresponding WT mouse. **(B)** Representative immunofluorescent images (IMFs) for SMN staining within heart tissues from untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following treatment with AAV9-mediated *SMN1* from two sources. ***p<0.001. Scale bars represent 75 μm.

Figure 3. Levels and distribution of lamin A/C in heart tissues from *Smn*^{2B/-} mice following AAV9-mediated *SMN1* delivery at P0. (A) Representative western blots of lamin

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3 A/C levels in heart tissues from WT mice (P18), untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice
4 following AAV9-mediated treatment with *SMNI* from two sources. The bar graph represents
5 average lamin A/C levels expressed relative to WT mice. **(B)** Representative western blots of
6 lamin A/C levels in heart tissues from untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following
7 AAV9-mediated treatment without *SMNI* (vehicle control) from two different vector sources.
8 The bar graph represents average lamin A/C levels expressed relative to untreated *Smn*^{2B/-} mice.
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10 **(C)** Representative IMFs for lamin A/C staining within heart tissues from WT mice (P18),
11 untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following AAV9-mediated treatment with *SMNI* from
12 two vector sources. Corresponding bar graph reflects the area of cells stained for lamin A/C
13 corrected for number of cells present (DAPI stain) as determined by ImageJ analysis and
14 expressed relative to corresponding WT mice. Dashed line represents the average lamin A/C
15 levels in WT mice and error bars represent the standard deviation from the mean. **(D)** Images
16 demonstrating dual staining of sarcomeres with alpha-actinin 2 (red) and lamin A/C (green) in
17 WT mice. * $p < 0.05$; ** $p < 0.01$. Scale bars represent 25 μm except the lower panel of **(D)** where
18 they represent 5 μm . Δ represents a combined sample not included in the analysis.

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40 **Figure 4. Levels and distribution of desmin in heart tissues from *Smn*^{2B/-} mice following**
41 **AAV9-mediated *SMNI* delivery at P0. (A)** Representative western blots of desmin levels in
42 heart tissues from WT mice (P18), untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following AAV9-
43 mediated treatment with *SMNI* from two sources. The bar graph represents average desmin
44 levels expressed relative to WT mice. **(B)** Representative western blots of desmin levels in
45 heart tissues from untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following AAV9-mediated
46 treatment without *SMNI* (vehicle control) from two different vector sources. The bar graph
47 represents average desmin levels expressed relative to untreated *Smn*^{2B/-} mice. **(C)**
48 Representative IMFs for desmin staining within heart tissues from WT mice (P18), untreated
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3 *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following AAV9-mediated treatment with *SMNI* from two
4 vector sources. Zoomed images to highlight the reduction in desmin positive striations in
5 untreated *Smn*^{2B/-} mice in comparison to WT mice and AAV9-mediated *SMNI* treated *Smn*^{2B/-}
6 mice. Corresponding bar graph reflects the area of cells stained for desmin corrected for
7 number of cells present (DAPI stain) as determined by ImageJ analysis and expressed relative
8 to corresponding WT mice. Dashed line represents the average desmin levels in WT mice and
9 error bars represent the standard deviation from the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
10 Scale bars represent 75 μm (panels a-d), 25 μm (panels e-g) and 10 μm (panel h).
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24 **Figure 5. Levels and distribution of elastin in heart tissues from *Smn*^{2B/-} mice following**
25 **AAV9-mediated *SMNI* delivery at P0. (Panels a-d)** Representative heart sections stained
26 with Miller's Elastin van Gieson from WT mice (P18), untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice
27 following AAV9-mediated treatment with *SMNI* from two different vector sources. Elastic
28 fibres (blue/black); mature collagen (red); other tissues such as muscle and red blood cells
29 (yellow). **(Panels e-l)** Representative IMFs for elastin staining within heart tissues from WT
30 mice (P18), untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following AAV9-mediated treatment with
31 *SMNI* from two vector sources. Corresponding bar graph reflects the area of cells stained for
32 elastin corrected for number of cells present (DAPI stain) as determined by ImageJ analysis
33 and expressed relative to corresponding WT mice. Dashed line represents the average elastin
34 levels in WT mice and error bars represent the standard deviation from the mean. *** $p < 0.001$.
35 Black scale bars represent 10 μm (panels a-d). White scale bars represent 75 μm (panels e-h)
36 and 10 μm (panels i-l).
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54 Abbreviations

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58 AWERB Animal Welfare Ethical Review Body
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3	DAVID	Database for Annotation, Visualization and Integrated Discovery
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5	ECM	Extracellular matrix
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7	Gems	Gemini of the coiled bodies
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10	IMF	Immunofluorescent image
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12	IPA	Ingenuity Pathway Analysis
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14	iPSCs	Induced pluripotent stem cells
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17	iTRAQ	Isobaric tags for relative and absolute quantitation
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19	P	Post-natal day
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21	SMA	Spinal muscular atrophy
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24	SMN	Survival motor neuron
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26	WT	Wild type
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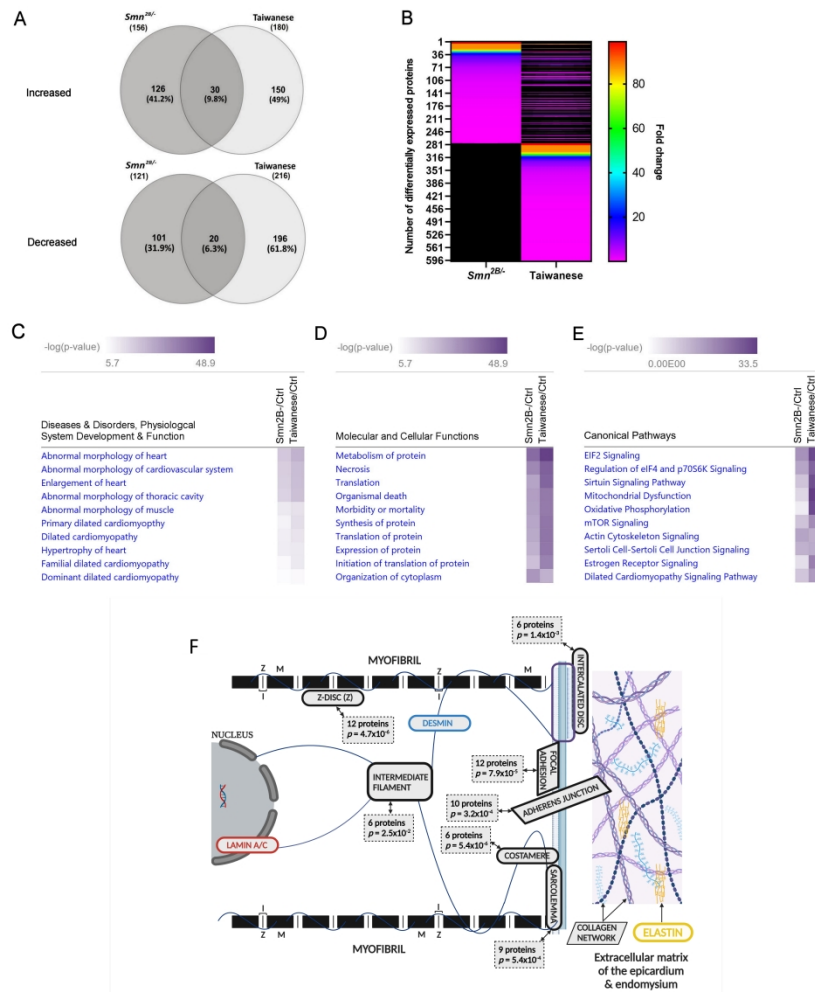


Figure 1. Summary and bioinformatics analysis of the proteomic profiles of heart tissues differentially abundant in the *Smn2B^{-/-}* and Taiwanese mice models of SMA compared to age-matched WT mice. (A) Venn diagram showing an overlap of 50 proteins identified as significantly up- or down-regulated in both *Smn2B^{-/-}* and Taiwanese mouse models of SMA (an unused ProtScore (conf) > 0.05, detected by ≥ 2 unique peptides, and a statistically significant ($p < 0.05$) fold change of >1.25 or <0.8). (B) Heat map illustrating the size of fold-change in proteins found to be dysregulated in both mice models of SMA. Two-way comparisons generated in IPA® in which the following were ranked by $-\log(p\text{-value})$ for each SMA mouse model: (C) Disease & disorders, physiological system development & function; (D) Molecular & cellular functions and (E) Canonical pathways. (F) Schematic illustrating the cellular components impacted by the significantly dysregulated proteins of the *Smn2B^{-/-}* mouse model of SMA as determined following DAVID analysis. Image created with BioRender.com.

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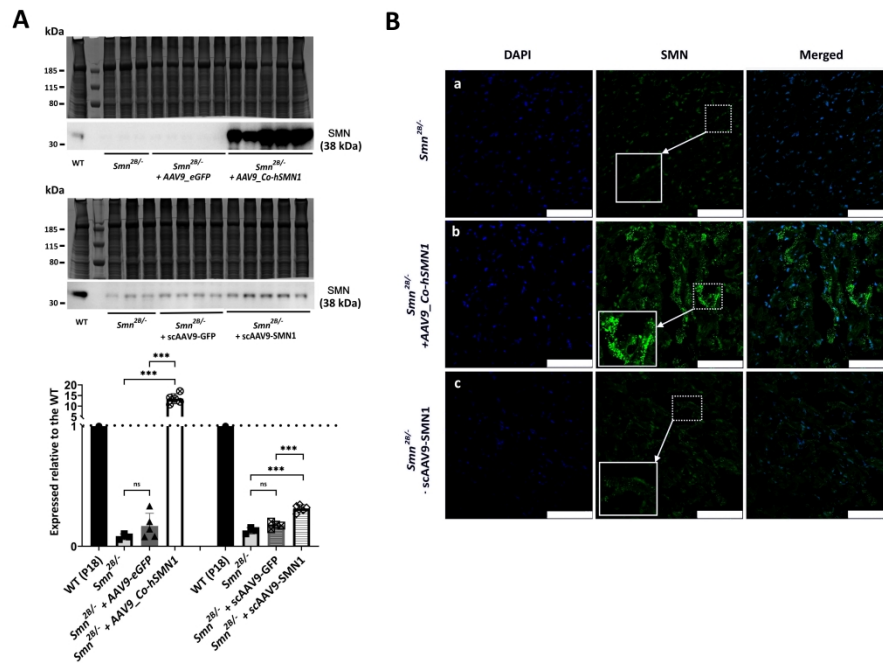


Figure 2. Levels and distribution of SMN in heart tissues from *Smn2B/-* mice following AAV9-mediated SMN1 delivery at P0. (A) Representative western blots of SMN levels in heart tissues from untreated *Smn2B/-* mice, *Smn2B/-* mice following AAV9-mediated treatment with and without SMN1 from two sources plus a corresponding age-matched WT mouse (P18). The bar graph represents average SMN levels expressed relative to the corresponding WT mouse. (B) Representative immunofluorescent images (IMFs) for SMN staining within heart tissues from untreated *Smn2B/-* mice and *Smn2B/-* mice following treatment with AAV9-mediated SMN1 from two sources. *** $p < 0.001$. Scale bars represent 75 μ m.

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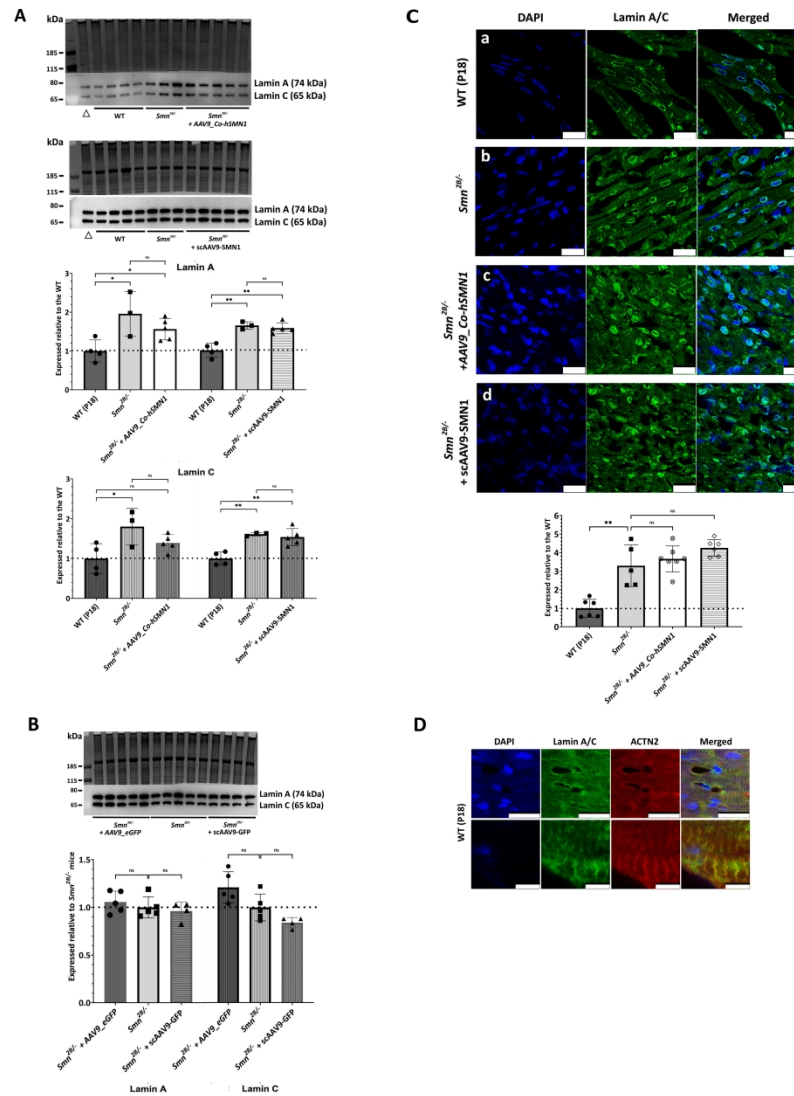


Figure 3. Levels and distribution of lamin A/C in heart tissues from *Smn2B/-* mice following AAV9-mediated SMN1 delivery at P0. (A) Representative western blots of lamin A/C levels in heart tissues from WT mice (P18), untreated *Smn2B/-* mice and *Smn2B/-* mice following AAV9-mediated treatment with SMN1 from two sources. The bar graph represents average lamin A/C levels expressed relative to WT mice. (B) Representative western blots of lamin A/C levels in heart tissues from untreated *Smn2B/-* mice and *Smn2B/-* mice following AAV9-mediated treatment without SMN1 (vehicle control) from two different vector sources. The bar graph represents average lamin A/C levels expressed relative to untreated *Smn2B/-* mice. (C) Representative IMFs for lamin A/C staining within heart tissues from WT mice (P18), untreated *Smn2B/-* mice and *Smn2B/-* mice following AAV9-mediated treatment with SMN1 from two vector sources. Corresponding bar graph reflects the area of cells stained for lamin A/C corrected for number of cells present (DAPI stain) as determined by ImageJ analysis and expressed relative to corresponding WT mice. Dashed line represents the average lamin A/C levels in WT mice and error bars represent the standard deviation from the mean. (D) Images demonstrating dual staining of sarcomeres with alpha-actinin 2 (red) and lamin A/C (green) in WT mice. * $p < 0.05$; ** $p < 0.01$. Scale bars represent 25 μm except the lower panel of (D)

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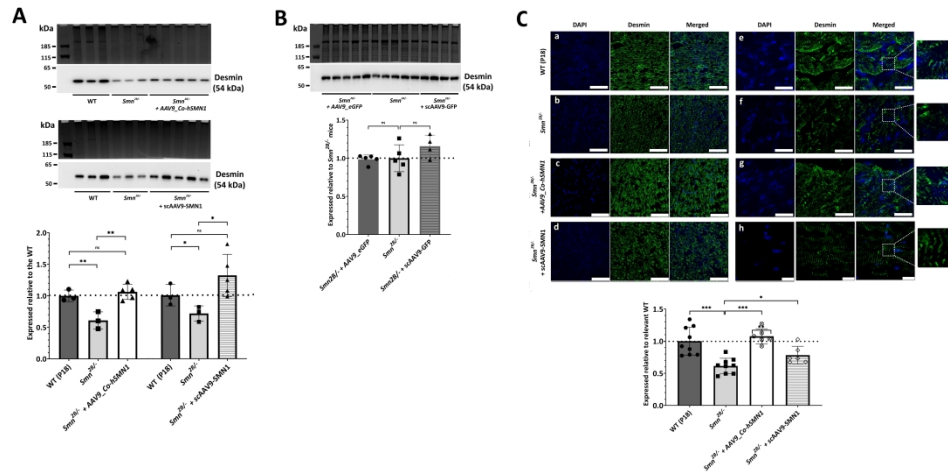


Figure 4. Levels and distribution of desmin in heart tissues from *Smn2B*^{-/-} mice following AAV9-mediated SMN1 delivery at P0. (A) Representative western blots of desmin levels in heart tissues from WT mice (P18), untreated *Smn2B*^{-/-} mice and *Smn2B*^{-/-} mice following AAV9-mediated treatment with SMN1 from two sources. The bar graph represents average desmin levels expressed relative to WT mice. (B) Representative western blots of desmin levels in heart tissues from untreated *Smn2B*^{-/-} mice and *Smn2B*^{-/-} mice following AAV9-mediated treatment without SMN1 (vehicle control) from two different vector sources. The bar graph represents average desmin levels expressed relative to untreated *Smn2B*^{-/-} mice. (C) Representative IMFs for desmin staining within heart tissues from WT mice (P18), untreated *Smn2B*^{-/-} mice and *Smn2B*^{-/-} mice following AAV9-mediated treatment with SMN1 from two vector sources. Zoomed images to highlight the reduction in desmin positive striations in untreated *Smn2B*^{-/-} mice in comparison to WT mice and AAV9-mediated SMN1 treated *Smn2B*^{-/-} mice. Corresponding bar graph reflects the area of cells stained for desmin corrected for number of cells present (DAPI stain) as determined by ImageJ analysis and expressed relative to corresponding WT mice. Dashed line represents the average desmin levels in WT mice and error bars represent the standard deviation from the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Scale bars represent 75 μm (panels a-d), 25 μm (panels e-g) and 10 μm (panel h).

660x330mm (300 x 300 DPI)

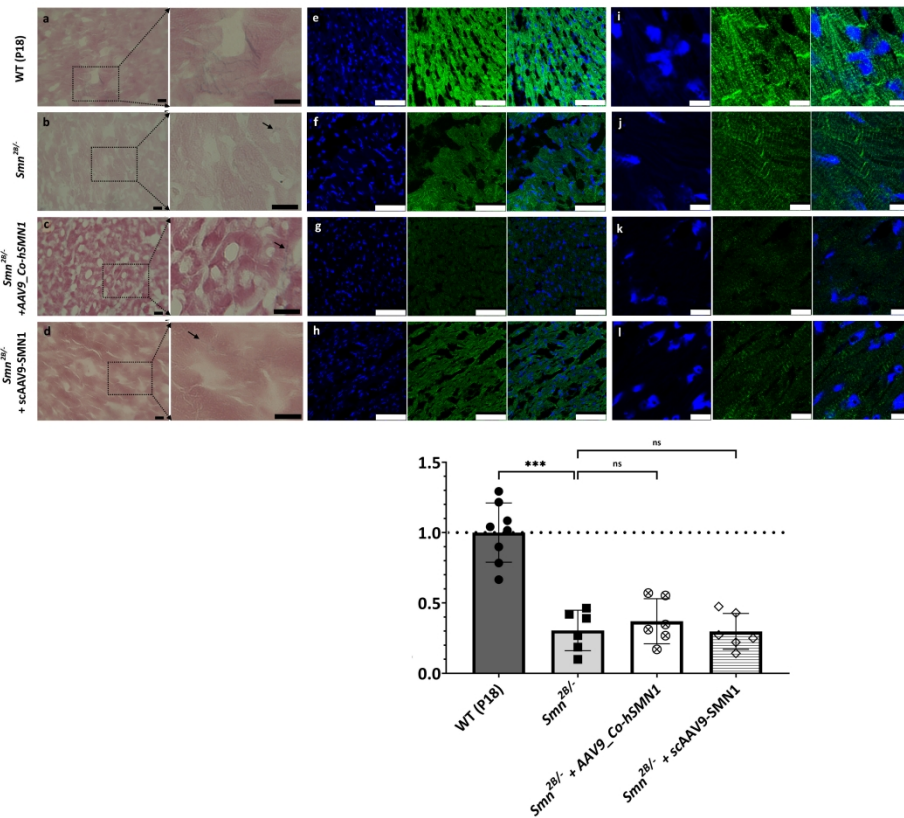


Figure 5. Levels and distribution of elastin in heart tissues from *Smn*^{2B/-} mice following AAV9-mediated SMN1 delivery at P0. (Panels a-d) Representative heart sections stained with Miller's Elastin van Gieson from WT mice (P18), untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following AAV9-mediated treatment with SMN1 from two different vector sources. Elastic fibres (blue/black); mature collagen (red); other tissues such as muscle and red blood cells (yellow). (Panels e-l) Representative IMFs for elastin staining within heart tissues from WT mice (P18), untreated *Smn*^{2B/-} mice and *Smn*^{2B/-} mice following AAV9-mediated treatment with SMN1 from two vector sources. Corresponding bar graph reflects the area of cells stained for elastin corrected for number of cells present (DAPI stain) as determined by ImageJ analysis and expressed relative to corresponding WT mice. Dashed line represents the average elastin levels in WT mice and error bars represent the standard deviation from the mean. ****p* < 0.001. Black scale bars represent 10 μ m (panels a-d). White scale bars represent 75 μ m (panels e-h) and 10 μ m (panels i-l).

1645x1516mm (96 x 96 DPI)