Transcriptomic profiling of skeletal muscle from the Ts1Cje mouse model of Down syndrome suggests dysregulation of trisomic genes associated with neuromuscular junction signaling, oxidative stress and chronic inflammation

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Abstract: Ts1Cje is a mouse model of Down syndrome (DS) with partial triplication of chromosome 16, which encompasses a high number of human chromosome 21 (HSA21) orthologous genes. The mouse model exhibits muscle weakness resembling hypotonia in DS individuals. The effect of extra gene dosages on muscle weakness or hypotonia in Ts1Cje and DS individuals remains unknown. To identify molecular dysregulation of the skeletal muscle, we compared the transcriptomic signatures of soleus and extensor digitorum longus (EDL) muscles between the adult Ts1Cje and disomic littermates. A total of 166 and 262 differentially expressed protein-coding genes (DEGs) were identified in the soleus and EDL muscles, respectively. The partial trisomy of MMU16 in Ts1Cje mice has a greater effect on gene expression in EDL. Top-down clustering analysis of all DEGs for represented functional ontologies revealed 5 functional clusters in soleus associated with signal transduction, development of reproductive system, nucleic acid biosynthesis, protein modification and metabolism as well as regulation of gene expression. On the other hand, only 3 functional clusters were observed for EDL namely neuron and cell development, protein modification and metabolic processes as well as ion transport. A total of 11 selected DEGs were validated using qPCR (disomic DEGs: Mansc1; trisomic DEGs: Itsn1, Rcan1, Synj1, Donson, Dyrk1a, Ifnar1, Ifnar2, Runx1, Sod1 and Tmem50b). The validated DEGs were implicated in neuromuscular junction signalling (Its1n, Syn1), oxidative stress (Sod1, Runx1) and chronic inflammation processes (Runx1, Rcan1, Ifnar1, Ifnar2). Other validated DEGs have not been well-documented as involved in the skeletal...
muscle development or function, thus serve as interesting novel candidates for future investigations. To our knowledge, the study was the first attempt to determine the transcriptomic profiles of both soleus and EDL muscles in Ts1Cje mice. It provides new insights on the possible disrupted molecular pathways associated with hypotonia in DS individuals.

Keywords: soleus; extensor digitorum longus; skeletal muscle; microarray; Down syndrome;

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1. Introduction

Down syndrome (DS) is a genetic disorder caused by trisomy of human chromosome (HSA) 21 and is the most common genetic form of intellectual disability. In addition, the disorder is characterised by a collection of more than 80 clinical features affecting almost all organs within the body [1] including characteristic set of facial features, early onset dementia, weak immune system and hypotonia [2]. DS individuals with hypotonia have been shown to have difficulty in postural control [3], instability in trunk control [4], delayed motor function development [5,6], perceptual motor deficits [7] and difficulty in motor planning [8] thus negatively impacting on their occupational and social development as well as quality of life [9-11].

DS individuals demonstrate lower levels of muscle strength as compared to those without DS or intellectual disability [12-14]. In general, although children with DS learn to walk, reach and grasp objects, feed themselves and perform many other fundamental skills, their movements lack in precision, are poorly coordinated and less efficient than the movements of normally developing children [15]. Compared to normal individuals, children and adults with DS are slower and more variable in reaction time tasks [16]. Besides exhibiting clumsy sequences of movements of different parts of the arm and hand, DS individuals also tend to have poorer control in programming actions timing and force reduction [16,17]. Motor development of DS children occurs later than normal children. DS children usually do not walk before the age of two. To compensate for these characteristics, children with DS adopt motor patterns that compensate for what they lack in terms of strength, joint stability and length of their limbs [18]. In the long run, if these patterns are repeated frequently they can lead to abnormalities in the musculoskeletal system and motor disabilities.

The underlying pathology of muscle weakness in DS individuals is not fully understood. Motor development deficits in DS are suggested to be determined by structural and functional alterations of the central nervous system (CNS). Brains of DS individuals have been characterised with hypocellularity of the hippocampus, decreased weight and volume; reduced neuron density especially in the cerebral cortex, hippocampus, cerebellum and brainstem; lower synaptic density and reduced number of neurotransmitters; and delayed myelination [19,20]. These abnormalities in the various brain structures including the midbrain and cerebellum have been linked to muscle tone determination, balance and coordination [21]. Interestingly, reduced cerebellar volume has become a consistent finding in DS individuals [22]. However, despite the differences in the volume of the cerebellum in DS individuals, the reduction is non-progressive and do not further reduce significantly with age [23]. Therefore, age-related decline in fine motor activities in DS is not necessarily the result of reduced cerebellar volume suggesting an impairment of the peripheral innervation or the skeletal muscle of DS individuals also contribute to hypotonia.

The current study reported the transcriptomic analysis on two types of skeletal muscles namely soleus (Type I
slow twitch) and extensor digitorum longus (EDL, Type II fast twitch) harvested from a mouse model known as Ts1Cje. We attempted to understand the effect of extra gene dosage on skeletal muscle function in DS due to the triplication of HSA21 genes. Many DS mouse models have been generated exhibiting similar phenotypes seen in DS individuals [24] including motor dysfunctions, such as delayed motor skill development, poor fine motor movement and impaired motor coordination [25-27]. Orthologs of HSA21 genes are dispersed among 3 mouse (MMU) chromosomes namely the telomeric region of MMU16, internal segments of MMU17 and MMU10 [28]. The Ts1Cje mouse model has a triplicated segment of the MMU16 with approximately 85 genes synteny to genes located on HSA21 [2,29]. The model has been shown to exhibit features similar to DS humans such as hypocellularity of the brain, decrease in cognitive ability as well as behavioural and motor abnormalities [30-34]. A recent study showed that besides being genetically representative to DS in humans, the mouse soleus muscle has a great molecular similarity to human skeletal muscles, independent of the anatomic location or muscle type [35] making it a suitable model for studies on molecular characterisation of muscle weakness in Ts1Cje.

Soleus muscle can be found at the calf at beneath the gastrocnemius, originating from the upper two-thirds posterior surfaces of the tibia and fibula. It is a Type I slow twitch muscle that contract slowly but can contract repeatedly for a long period of time resulting in high resistance to fatigue [36]. Thus, it exhibits high mitochondrial numbers and oxidative enzyme content as well as low glycogen levels and glycolytic enzyme activity. On the other hand, Type II fast twitch muscle of Ts1Cje namely extensor digitorum longus (EDL) which is situated at the lateral part of the leg adjacent to the tibialis anterior muscle. Fast twitch muscles have a faster contraction speed but achieves fatigue earlier as compared to slow twitch muscles [36]. Thus, transcriptomic profiling and comparison of these muscles in Ts1Cje mouse model will lead to the identification of dysregulated genes and molecular networks related to muscle weakness due to the partial trisomy of MMU16. The fundamental knowledge of genotype to phenotype (muscle weakness) correlations in Ts1Cje will enhance the current understanding of mechanisms underlying muscle weakness or hypotonia in DS.

2. Materials and Methods

2.1 Animal Husbandry & Genotyping

Two groups of adult mice, the Ts1Cje and wild type (disomic C57BL/6) at postnatal day 56-70 were used in the study. Ts1Cje and wild type mice were generated by mating the Ts1Cje males with a wild type C57BL/6 female as described previously [29]. All experimental protocols were performed with the approval from the Institutional Animal Care and Use Committee of Universiti Putra Malaysia (UPM/IACUC/AUP-RO03/2014). The disomic littermates served as the control for the study. The mice were identified by tail genotyping using polymerase chain reaction (PCR) to amplify the Grik1 gene in WT mice and both Grik1 and Neo genes in the Ts1Cje mouse as described by [29]. All mice were housed under controlled temperatures with a 12:12 hour light:dark cycle. The mice were given unlimited access to standard animal feed (Altromin 1324, Germany) and sterile water ad libitum.

2.2 Skeletal Muscle Harvesting

The skeletal muscle samples collected in this study were namely soleus and extensor digitorum longus (EDL). The mouse was euthanised by inhalation of 4% mg/kg isoflurane fume chamber followed by cervical dislocation and pinned facing upwards. The muscles were isolated using the tendon to tendon method. The entire length of the hindlimb was exposed by removal of the skin and without damaging the underlying muscles. The EDL was visually located at the anterior compartment of the hindlimb just underneath the tibialis anterior (TA) muscle. The distal tendons of the TA and EDL were cut and both muscles were held by their tendons and pulled gently up towards the proximal end. The EDL muscle was located just underneath the TA muscle by pulling the tendons of the respective muscles in opposite directions. The TA was removed by
cutting at the proximal end to expose the EDL proximal tendon. The proximal tendon of the EDL was cut to obtain the muscle. The mouse was then flipped around and pinned face down on the dissection board. The soleus muscle which was visually located at the posterior compartment of the hind limb just underneath the gastrocnemius (GA) muscle was then isolated in similar manner. Caution was taken to ensure that the exposed tissues do not dry out by flushing with ice cold 1X PBS when required. All muscle tissue samples were snap frozen on dry ice once harvested. The tissue was then kept in -80 ºC until further processing.

2.3 RNA Isolation and Quality Assessment
The muscles were homogenised with an IKA® T10 basic Homogenizer (IKA, USA) and RNA was isolated using the MasterPure™ RNA Purification Kit (Epicentre, USA) with the additional RNase-Free DNase I (Epicentre, USA) step according to the manufacturer’s protocol. Quality and quantification of RNA was measured with the NanoVue™ Plus Spectrophotometer (GE Healthcare, UK) and the integrity confirmed by visualisation on agarose gel electrophoresis. Additional quality and quantification assessment were performed prior microarray analysis with the Agilent 2100 Bioanalyzer system on the Bioanalyzer RNA 6000 Nano chip according to the Eukaryote Total RNA Nano assay protocol (Agilent, USA).

2.4 Microarray sample preparation and analysis
Sample preparation and hybridisation kits from Agilent were used to process each RNA samples and RNA samples were hybridised onto Agilent-SurePrint G3 Mouse GE 8x60K Microarray chip (Agilent, U.S.A) following the manufacturer’s protocol. The samples were prepared with the RNA Spike-In Kit, One-Color Kit (Agilent, USA) according to the manufacturer’s protocol. The samples were then labelled and amplified with the Low Input Quick Amp Labeling Kit, One-Color (Agilent, USA) generating fluorescent complementary RNA (cRNA). The cRNA samples were then purified with the RNeasy Mini Kit (Qiagen, Germany). The purified cRNA was quantified using the NanoVue™ Plus Spectrophotometer (GE Healthcare, UK). The Cyanine 3 dye concentration (pmol/µl), RNA absorbance ration (260 nm/280 nm) and cRNA concentration (ng/µl) were recorded. The yield and specific activity of each reaction was determined and only samples with minimum yield and specific activity of 0.825 µg and 6 pmol Cy3 per µg of cRNA, respectively, were selected for hybridization onto the chip using the Gene Expression Hybridization Kit and Hybridization Gasket Slide Kit (Agilent, USA) according to the manufacturer’s protocol. The array with the hybridization mix with samples was incubated in the Agilent SureHyb chamber hybridized at 60°C for 17 hours while rotating. After hybridization, the slides were washed with the Gene Expression Wash Buffer Kit (Agilent, USA), with the addition of Triton X-102 to the wash buffers. The slides were scanned with the Agilent SureScan Microarray Scanner. The Agilent Feature Extraction (FE) program was used to extract the information from probe features from the scanned microarray data. Information from the images (.tif) are extracted where the FE program assigns a default grid template and protocol automatically for each extraction set. The QC report generated is checked to confirm that the microarray has been successful.

2.5 Analysis of Differential Gene Expression
The microarray expression data extracted with the FE program was analysed with the Agilent GeneSpring Software v.13.1. The data was imported into the software and normalised by percentile shift normalisation at 75th percentile. This is a global normalisation method which adjusts the location of all the spot intensities in the array. Each column is taken independently and the nth percentile of the expression values for this array is computed across all spots, ranging from 0 to 100 and n = 50 as the median. The value is the subtracted from the expression value of each entity. Following this, the entities are filtered by expression. The cut-off percentile used was lower cut-off = 20th percentile and upper cut-off of 100th percentile. The microarray expression data sets were compared between two groups namely wild-type and Ts1Cje with each muscle group data analysed separately. The data was statistically analysed using moderated t-Test with Westfall Young Permutative
multiple testing correction. Genes were considered significantly differentially expressed when the corrected p-values ≤ 0.05 and absolute fold change value of ≥1.5.

2.6 Functional clustering of DEGs
The DEGs were clustered based on functional ontologies using the Database for Annotation, Visualisation and Integrated Discovery (DAVID) software [37]. Stringent classification criteria (a kappa similarity threshold of 0.50, a minimum term overlap of three, three initial and final group memberships with a 0.50 multiple linkage threshold and a modified Fisher-exact p-value on enrichment thresholds of 1.0) was employed in the analysis. The DEGs in each cluster and common genes among the clusters were identified. The list of DEGs for soleus and EDL were compared to determine the genes found on MMU16 as well as those not on MMU16 which were differentially regulated in both muscles.

2.7 RT-qPCR
Complementary DNA (cDNA) was synthesised from 500 ng of high quality total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland) with anchored-oligo(dT)18 primers according to manufacturer’s protocol. The optional step of denaturing the template-primer mixture was performed. Quantitative PCR was performed using the LightCycler® 480 Real-Time PCR System (Roche, Switzerland). RT-qPCR was performed using LightCycler® 480 SYBR Green I Master (Roche, Switzerland) at the following conditions: pre-incubation at 95°C (5 minutes), followed by 45 cycles of 95°C (10 seconds), 60°C (10 seconds) and 72°C (10 seconds) and melting analysis from 65°C to 97°C with continuous signal acquisitions (every +0.2°C). The reaction was then cooled down to 40°C. Relative quantification by standard curve method was performed according to the protocol published previously [38] with the normalisation step performed against any 2 of the 3 of the housekeeping genes; phosphoglycerate kinase 1 (Pgk1), hydroxymethylbilane synthase (Hmbs) and proteasome subunit beta type-2 (Psmb2). See Table 1 for list of primers used in the analysis.

Table 1: List of primers used for RT-qPCR assays

| Gene   | Forward Primer (5’→3’)                     | Reverse Primer (5’→3’)                     | Amplicon Size (nt) |
|--------|--------------------------------------------|--------------------------------------------|--------------------|
| Itsn1  | accagtctgcgctgtatgat                       | aggcggaccaatcttc                              | 18                 |
| Rcan1  | cccgacaacagtctcctcat                        | catgcgtctcacttc                              | 52                 |
| Syn1   | cttcgcagcagacacaa               | ggtctcaggtgttgg                               | 18                 |
| Cdk13  | gcacacaggacgggcacact                       | tggcaactcggcgtgg                            | 66                 |
| Donson | tggcgcctgtttctgtgataaa                 | agcatgtcggcgtgtaa                           | 111                |
| Dyrk1a | tcaggccagcagcacaac                        | ttgctctgggccgatg                             | 65                 |
| Ifnar1 | ttgcagttgtaagcaccacag                      | ggacatagctcttgctg                           | 85                 |
| Ifnar2 | tgacagagattgtgcttc                        | ttcctacgctgctgcaacc                         | 81                 |
| Mansc1 | tggcactcagaggacgaact                       | cgggtgcataagcatccataa                       | 92                 |
| Runx1  | acatcgaggagaatctcactcaca                   | ttgctcagctctcatccag                         | 91                 |
| Sod1   | cagagacgcttttaatatctcaca                   | tgccccagttctccacat                          | 78                 |
| Tmem50b| ccggatgaggtttgtcag                       | catggagttctcatttacgcatgg                    | 126                |
| Pgk1   | tacctgctggctgatg                         | cacaagctggttcatatttct                       | 65                 |
| Psmb2  | aggggccagagttttctta                      | aggtggcagatccagatg                         | 71                 |
| Hmbs   | aaatgccacaccttgcgaat                     | ccaggcaatggcactgaat                        | 98                 |

2.8 Western blotting
The muscle samples were homogenized in ice-cold RIPA buffer (Millipore Corporation, USA) supplemented with a protease inhibitor cocktail (Calbiochem, USA) and phosphatase inhibitors (Calbiochem, USA). A total of 20 μg of protein lysate was separated on 10% SDS-PAGE and transferred to PVDF membranes (BioRad). After blocking with 5% non-fat dried milk, the membrane was...
incubated with mouse monoclonal anti-Gapdh (Abcam, UK; 1:1000 dilution; Catalog ID: ab125247), mouse monoclonal anti-Ifnar1 (Biolegend, USA; 1:500 dilution; Catalog ID: 127322) or goat polyclonal anti-Ifnar2 (Santa Cruz, USA; 1:500 dilution; Catalog ID: sc-20218) primary antibodies at 4°C overnight, followed by goat anti-mouse IgG (Santa Cruz, USA; 1:1000 dilution; Catalog ID: sc-2005) or donkey anti-goat (Santa Cruz, USA; 1:1000 dilution for anti-Ifnar and 1:2500 for anti-Gapdh; Catalog ID: sc-2020) HRP-conjugated secondary antibodies at room temperature for 1 hour. Immunoreactivity of the proteins were detected and visualised using the Advansta WesternBright™ Sirius™ chemiluminescence kit (Avidanstra Corp, USA). Images were taken with a G:BOX F3 Gel Documentation Workstation (Syngene, UK) and pixelation analysis of the bands were performed using ImageJ (http://imagej.nih.gov/ij/) according to the standard protocol. The density of the bands were quantified and normalised against the loading control (housekeeping protein), glyceraldehyde 3-phosphate dehydrogenase (Gapdh).

3. Results

3.1 Differentially Expressed Genes (DEGs)

To investigate the effect of partial trisomy on muscle weakness in Ts1Cje, we performed a microarray analysis on soleus and EDL (n=4 per tissue per group) muscles between Ts1Cje and wildtype disomic littermates using the SurePrint G3 Mouse GE 8x60K Microarray chip (Agilent Technologies, USA). Following filtering and normalisation, a total of 40,932 probes were detected for soleus with 10,316 of them being long non-coding (Inc) RNA whereas 42,399 probes were detected in EDL with 10,959 of them being IncRNA. The expression datasets are publicly accessible from the Gene Expression Omnibus database repository under the series accession number GSE96572. Figure 1a depicts the summary of differentially expressed probes and genes in the skeletal muscles. The differentially expressed probes were selected based on corrected p-values ≤ 0.05 and absolute fold change value of ≥1.5 (Figure 1b). Considering only the coding genes, 166 DEGs and 262 DEGs were identified in the soleus and EDL muscles, respectively. This suggests that the partial trisomy of MMU16 in Ts1Cje mice has a greater effect on gene expression in EDL. Of these DEGs, 35 in soleus and 9 in EDL were trisomic genes found in the Ts1Cje mice (Table 2; Figure 1c; Table S1). When the list of DEGs of both skeletal muscle types were compared, only 6 trisomic DEGs (A930006K02Rik, Donson, Dyrk1a, Runx1, Sod1 and Tmem50b) and 3 disomic DEGs (Cdk13, Dnah11 and Mansc1) were common or consistently dysregulated in both the soleus and EDL (Figure 1c).

The dynein axonemal heavy chain 11 (Dnah11) gene stood out among the DEGs found with a significant upregulation in both soleus and EDL muscles of the Ts1Cje mice with an approximately 460- and 550-fold change in EDL and soleus, respectively (Table S1). The upregulation of Dnah11 is consistent with previously reported microarray expression results of the brain [39,40] and is probably specific to the Ts1Cje mouse model [41]. This is due to the partial monosomy of MMU12 resulting from a partial translocation of MMU16 onto Dnah11 locus leading to a segmental deletion of the gene in the Ts1Cje mouse model [39,42]. Thus, Dnah11 was inferred to be uninvolved in molecular contributions to hypotonia in DS as it is specific to Ts1Cje and not translated to a functional protein resulting from the translocation. While the observation does not have significant to muscle weakness in DS, presence of this overexpression served as a validation of the results from the microarray analysis.

3.2 Functional analysis of DEGs

To determine the disrupted molecular networks at a global level, a top-down functional clustering approach using DAVID [37] was performed. The analysis revealed 5 functional clusters in soleus associated with signal transduction (13 DEGs, 5 ontologies), development of reproductive system (4 DEGs, 6 ontologies), nucleic acid biosynthesis (6 DEGs, 12 ontologies), protein modification and metabolism (13 DEGs, 4 ontologies) as well as regulation of gene expression (20 DEGs, 21 ontologies) (Table 3). From the 45 DEGs clustered, 9 DEGs namely Atp5o, Drd4, Fcer1a, Gm8566, Lyn, Morc3,
Pawr, Sod1 and Tlr3 were found to be clustered into more than one functional annotation cluster. On the other hand, only 3 functional clusters were revealed for EDL namely neuron and cell development (11 DEGs, 14 ontologies), protein modification and metabolic processes (26 DEGs, 5 ontologies) as well as ion transport (10 DEGs, 14 ontologies) as listed in Table 3. From the 42 DEGs clustered, 7 of those DEGs namely Atp6v1e2, Cdh4, Gm8566, Ipomk, Runx1, Sod1 and Tgfbr1 were found to be clustered into more than one functional annotation cluster.

|                  | Soleus | EDL  |
|------------------|--------|------|
| Coding genes     | Probe set | 166  | 262  |
|                  | DEG     | 97‡  | 66†  |
| Long non-coding genes | Probe set | 72   | 150  |
|                  | DEG     | 7†   | 65‡  |
| Total number of DEGs |        | 238  | 412  |

Note: Red up arrows (†) denote upregulation; Green down arrows (‡) denote downregulation; DEG=differentially expressed gene

**Figure 1.** Analysis of differentially expressed genes (DEGs). (a) Summary of differentially expressed genes (DEGs) in both muscles. (b) Volcano plots for differentially expressed probes for soleus and EDL. (c) Venn diagrams depict the distribution of disomic and trisomic DEGs in both muscles. (d) Significant functional clusters associated with trisomic DEGs based on DAVID analysis.
| Probe Name | Full Gene Name | Gene Symbol | Log2 Expression of Ts1Cje-WT |
|------------|----------------|-------------|----------------------------|
| A_52_P447284 | Chloride intracellular channel 6 | Clic6 | 2.0793 |
| A_52_P236705 | Ripply3 homolog (zebrafish) | Ripply3 | 1.4131 |
| A_51_P514085 | Myxovirus (influenza virus) resistance 2 | Mx2 | 1.2231 |
| A_55_P1981739 | Downstream neighbor of SON | Donson | 1.0974 |
| A_55_P2177539 | Superoxide dismutase 1, soluble | Sod1 | 1.0932 |
| A_55_P2101920 | Eva-1 homolog C (C. Elegans) | Eva1c | 1.0822 |
| A_55_P2183438 | Run related transcription factor 1 | Runx1 | 1.0619 |
| A_55_P2206461 | RIKEN cDNA A930006K02 gene | A930006K02Rik | 0.9124 |
| A_51_P313483 | Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1a | Dyrk1a | 0.8927 |
| A_51_P510849 | Transmembrane protein 50B | Tmem50b | 0.8537 |
| A_52_P190405 | Interferon (alpha and beta) receptor 2 | Ifnar2 | 0.8372 |
| A_52_P301223 | Bromodomain and WD repeat domain containing 1 | Brwd1 | 0.7939 |
| A_55_P2042600 | Uncharacterized LOC102641028 | LOC102641028 | 0.7870 |
| A_55_P1955517 | Synaptojanin 1 | Synj1 | 0.7825 |
| A_51_P314521 | Small integral membrane protein 11 | Smim11 | 0.7658 |
| A_51_P481238 | Dopey family member 2 | Dopey2 | 0.7588 |
| A_51_P508191 | Down syndrome critical region gene 3 | Dscr3 | 0.7534 |
| A_55_P2190426 | Single-minded homolog 2 (Drosophila) | Sim2 | 0.7457 |
| A_51_P160907 | Microrchidia 3 | Morc3 | 0.7400 |
| A_55_P2181109 | Interleukin 10 receptor, beta | Il10r | 0.7287 |
| A_66_P100232 | PAX3 and PAX7 binding protein 1 | Paxbp1 | 0.7148 |
| A_52_P58257 | Mitochondrial ribosomal protein S6 | Mrps6 | 0.6874 |
| A_55_P1952788 | Son DNA binding protein | Son | 0.6823 |
| A_55_P1952793 | Son DNA binding protein | Son | 0.6681 |
| A_55_P2032297 | RIKEN cDNA B230307C23 gene | B230307C23Rik | 0.6676 |
| A_55_P2171158 | High mobility group nucleosomal binding domain 1 | Hmgn1 | 0.6601 |
| A_66_P134728 | Proteasome (prosome, macropain) assembly chaperone 1 | Psmg1 | 0.6596 |
| A_55_P2091671 | Proteasome (prosome, macropain) assembly chaperone 1 | Psmg1 | 0.6582 |
| A_55_P2033135 | SH3-binding domain glutamic acid-rich protein | Sh3bgr | 0.6579 |
| A_55_P2055682 | ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit | Atp5o | 0.6551 |
| A_52_P639774 | Phosphoribosylglycinamide formyltransferase | Gart | 0.6464 |
| A_55_P1952915 | SH3-binding domain glutamic acid-rich protein | Sh3bgr | 0.6441 |
| A_55_P1994586 | Intersectin 1 (SH3 domain protein 1A) | Itsn1 | 0.6323 |
| A_55_P1977985 | Phosphatidylinositol glycan anchor biosynthesis, class P | Pigp | 0.6274 |
| A_51_P196395 | RIKEN cDNA 1110004E09 gene | 1110004E09Rik | 0.6076 |
| Probe Name       | Full Gene Name                                      | Gene Symbol | Log_2 Expression of Ts1Cje-WT |
|------------------|-----------------------------------------------------|-------------|--------------------------------|
| A_55_P2438722    | Interferon (alpha and beta) receptor 1              | Ifnar1      | 1.2305                         |
| A_51_P481221     | Beta-site APP-cleaving enzyme 2                     | Bace2       | 1.1339                         |
| A_55_P2177539    | Superoxide dismutase 1, soluble                     | Sod1        | 0.8570                         |
| A_55_P2206461    | RIKEN cDNA A930006K02 gene                          | A930006K02Rik | 0.7994                     |
| A_51_P510849     | Transmembrane protein 50B                           | Tmem50b     | 0.7763                         |
| A_55_P1981739    | Downstream neighbor of SON                          | Donson      | 0.7702                         |
| A_55_P2143195    | Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1a | Dyrk1a   | 0.7598                         |
| A_55_P2183438    | Runt related transcription factor 1                 | Runx1       | 0.7216                         |
| A_66_P106065     | Tetratricopeptide repeat domain 3                   | Ttc3        | 0.6111                         |

These results for soleus and EDL highlighted dysregulation of genes involved in basic molecular function suggesting that muscle weakness in Ts1Cje is not caused by genes involved directly in muscle development and function but by indirect gene dosage effect on signal transmission and basic cell functions in the muscles. Trisomic DEGs implicated in all these clusters regardless of muscle types were Atp5o, Bace2, Brwd1, Cdk13, Dyrk1a, Gart, Ifnar2, Itsn1, Morc3, Runx1, Sim2, Sod1, Synj1 and Ttc3 (Figure 1d). Overall, the analysis indicated that protein modification and metabolism (Clusters 4 and 7) and regulation of gene expression (Cluster 5) functional clusters affected by the greatest number of trisomic DEGs.

Table 3: Functional clustering and gene ontology of differentially expressed genes (DEGs) in soleus and extensor digitorum longus (EDL) muscles

**Soleus**

| Functional Cluster | Genes                                      | Gene ontologies                                                                 |
|--------------------|--------------------------------------------|---------------------------------------------------------------------------------|
| Cluster 1          | Drd4, Fcer1a, Fgd3, Gm8566, Itsn1, Lyn, Pawr, Rgn, Sod1, Sos1, Synj1, Tlr3, Wwox | GO:0009966~Regulation Of Signal Transduction  
GO:0009967~Positive Regulation Of Signal Transduction  
GO:0009150~Purine Ribonucleotide Metabolic Process  
GO:0009144~Purine Nucleoside Triphosphate Metabolic Process  
GO:0009260~Ribonucleotide Biosynthetic Process  
GO:0009134~Purine Nucleotide Biosynthetic Process  
GO:0009135~Atpase Activity, Coupled To Transmembrane Movement Of Ions |
| Cluster 2          | Gm8566, Lepr, Msh4, Sod1                  | GO:0022602~Ovulation Cycle Process  
GO:0008585~Female Gonad Development  
GO:0046545~Development Of Primary Female Sexual Characteristics  
GO:0046660~Female Sex Differentiation  
GO:0008406~ Gonad Development  
GO:0045137~Development Of Primary Sexual Characteristics |
| Cluster 3          | Atp13a4, Atp1b4, Atp5o, Cox17, Fignl1, Gart | GO:0009150~Purine Ribonucleotide Metabolic Process  
GO:0009144~Purine Nucleoside Triphosphate Metabolic Process  
GO:0009260~Ribonucleotide Biosynthetic Process  
GO:0006164~Purine Nucleotide Biosynthetic Process  
GO:0042625~Atpase Activity, Coupled To Transmembrane Movement Of Ions |
GO:0006753~Nucleoside Phosphate Metabolic Process
GO:0034654~Nucleobase, Nucleoside, Nucleotide And Nucleic Acid Biosynthetic Process
GO:0034404~Nucleobase, Nucleoside And Nucleotide Biosynthetic Process
GO:0042626~Atpase Activity, Coupled To Transmembrane Movement Of Substances
GO:0009142~Nucleoside Triphosphate Biosynthetic Process
GO:0015405~P-P-Bond-Hydrolysis-Driven Transmembrane Transporter Activity
GO:0006812~Cation Transport

Cluster 4
Protein Modification &Metabolism
(Enrichment Score: 0.7400)

Atp5o, Bub1b, Camk4, Cdk13, Dyrk1a, Fcer1a, Gm8566, Lyn, Morc3, Pigg, Sod1, Stk35, Uevldv

GO:0016310~Phosphorylation
GO:0004672~Protein Kinase Activity
GO:0006464~Protein Modification Process
GO:0044267~Cellular Protein Metabolic Process

Cluster 5
Regulation Of Gene Expression
(Enrichment Score: 0.5947)

Brwd1, Dna2, Drd4, Elk4, Fcer1a, Foxe3, Gng7, Hdx, Hoxd8, Hoxd9, Icosl, Ifnar2, Lyn, Mcm8, Morc3, Nr5a2, Pawr, Runx1, Sim2, Tlr3

GO:0042108~Positive Regulation Of Cytokine Biosynthetic Process
GO:0010604~Positive Regulation Of Macromolecule Metabolic Process
GO:0031325~Positive Regulation Of Cellular Metabolic Process
GO:0010557~Positive Regulation Of Macromolecule Biosynthetic Process
GO:0031328~Positive Regulation Of Cellular Biosynthetic Process
GO:0042035~Regulation Of Cytokine Biosynthetic Process
GO:0009891~Positive Regulation Of Biosynthetic Process
GO:0002694~Regulation Of Leukocyte Activation
GO:0031326~Regulation Of Cellular Biosynthetic Process
GO:0019219~Regulation Of Nucleobase, Nucleoside, Nucleotide And Nucleic Acid Metabolic Process
GO:0010556~Regulation Of Macromolecule Biosynthetic Process
GO:0006355~Regulation Of Transcription, DNA-Dependent
GO:0051252~Regulation Of RNA Metabolic Process
GO:0045935~Positive Regulation Of Nucleobase, Nucleoside, Nucleotide And Nucleic Acid Metabolic Process
GO:0051173~Positive Regulation Of Nitrogen Compound Metabolic Process
GO:0045449~Regulation Of Transcription
GO:0010468~Regulation Of Gene Expression
GO:0045893~Positive Regulation Of Transcription, DNA-Dependent
GO:0051254~Positive Regulation Of RNA Metabolic Process
GO:0045941~Positive Regulation Of Transcription
GO:0010628~Positive Regulation Of Gene Expression
3.3 Quantitative PCR validation of selected DEGs

RT-qPCR was used to validate the DEGs identified in the microarray analysis. The genes selected for validation were found to be differentially regulated in both skeletal muscles (Cdk13 and Mansc1), trisomic genes or reported in previous studies (Itsn1, Rcan1, Synj1, Donson, Dykr1a, Ifnar1, Ifnar2, Runx1, Sod1 and Tmem50b). Only trisomic genes found to be upregulated in both soleus and EDL were selected for validation. This is to focus on genes found to be
dysregulated regardless of muscle type. In total, all genes except for Cdk13, were validated by RT-qPCR using the same RNA that was used for the microarray analyses, which consisted of soleus and EDL skeletal muscle tissues from Ts1Cje (n=6) and disomic mice (n=7) (Figure 2). The expression profile of a gene was considered validated when both microarray and RT-qPCR data showed a consistent directional change. Our results showed that all trisomic genes were upregulated in the Ts1Cje samples with fold differences ≥ 1.50.

**Figure 2.** RT-qPCR validation of DEGs. Comparison of expression profiles of selected DEG candidates in soleus and EDL muscles between Ts1Cje and wildtype groups. Graph bars represent relative log2 mean ± standard error of mean (normalized against any 2 of the 3 housekeeping genes: Pgk1, Psmb2 or Hmbs). Asterisks denote the statistical significance at p-value<0.05 (*) or <0.01 (***) based on Student’s t-test.
Among the trisomic genes dysregulated, *Ifnar1* and *Ifnar2* were found to be upregulated in the soleus and EDL of Ts1Cje, respectively. Thus, Western blot analysis was performed to evaluate the effect of mRNA levels on protein synthesis of the interferon receptors (Figure 3a). Based on the pixelation analysis of the non-saturated bands (Figure 3), Ifnar1 and Ifnar2 protein expressions were found to be lower in the Ts1Cje soleus by 2.10- and 1.72-fold, respectively (Figure 3b). In the EDL of the Ts1Cje, Ifnar2 protein expression was found to be lower in the Ts1Cje mice when compared to wild type by 1.43-fold. The finding was in contrary to the results obtained from microarray and RT-qPCR analysis where the genes were upregulated in Ts1Cje mice.

![Figure 3](image)

**Figure 3.** Western blot analysis of Ifnar1 and Ifnar2. (a) Ifnar1 (66 kDa) and Ifnar2 (57 kDa) protein expression in both soleus and EDL muscles (n=3 tissue per group) of Ts1Cje and wild-type mice. Gapdh protein expression (37 kDa) was used to normalise any loading errors. Blot 1 and Blot 2 represent independent technical replicates. (b) Densitometry analysis of the bands in (a) depicting the normalised level of Ifnar1 and Ifnar2 in the soleus and EDL muscles. The asterisk denotes the statistical significance at *p*-value <0.05 (*) based on Student’s t-test.

4. Discussion

Hypotonia is one of the clinical phenotypes found in DS individuals and the actual underlying cause of hypotonia in DS remains unknown. Defects within the central or peripheral nervous systems, transmission of signals at the neuromuscular junctions or the muscles itself can be attributed to hypotonia. The underlying cause of these defects however, lies within the effect of additional genetic material in DS. Multiple theories have been formed on the link between the trisomy of HSA21 to the
phenotypes such as the dosage imbalance hypothesis suggesting a 50% increase in expression of genes on chromosome 21q \( ^{2,43} \); the amplified developmental instability hypothesis proposes that the trisomy causes general alteration in development homeostasis leading to the phenotypes \( ^{43-45} \); and the DSCR hypothesis predicting that triplication of the region was sufficient to cause DS phenotypes \( ^{46} \). Thus, this study aims to elucidate the potential cause of hypotonia in DS individuals through global transcriptomic gene expression study to identify genes potentially involved in Ts1Cje muscle weakness.

There are limited studies involving the characterisation of the transcriptomic profile in Ts1Cje skeletal muscle especially according to the muscle type. To our knowledge, this is the first attempt to profile on the transcriptomes of Ts1Cje soleus and EDL muscles. Our findings showed that the gene expression profile between the soleus and EDL muscles were different with more DEGs found in the EDL muscles. A higher number of DEGs were found in EDL suggesting that the fast-twitch muscle may be more affected in Ts1Cje as compared to the slow-twitch muscle, soleus. The current study focused on the DEGs found to be significantly dysregulated in both muscles. This was to further investigate the potential role of the DEGs in muscle weakness while ensuring that the difference in expression was not due to muscle type.

The microarray results showed a significant increase expression of 35 trisomic genes in soleus and 6 trisomic genes in EDL by at least 1.5-fold when compared to their disomic littermates. The data set had similar results to previous studies on soleus muscle in Ts65Dn \( ^{47} \) as well as studies on brains and primary neuro stem cells and progenitor cells of Ts1Cje mice \( ^{40,48-51} \) where there was a significant dosage-dependent over-expression of the genes located on the triplicated region of MMU16. Collectively, the DEGs in the Ts1Cje soleus and EDL were mainly involved in basic molecular function such as signal transduction, protein modification and metabolism, nucleic acid biosynthesis, and regulation of gene expression in agreement with previous studies on Ts65Dn mice \( ^{52} \) and human samples \( ^{53,54} \) suggesting that muscle weakness in Ts1Cje was not caused by genes involved directly in muscle development and function but by indirect gene dosage effect on signal transmission and basic cell functions in the muscles.

Studies have suggested that muscle weakness is a result of defects in the CNS of DS patients. Several neuroanatomical defects affecting the cerebellum and cerebral cortex have been reported in DS individuals \( ^{22,55-57} \). However, motor function involves both the nervous and muscular systems. Defective neuromuscular junction is one of the contributing elements in causing muscle weakness where two trisomic genes \textit{Itsn1} and \textit{Synj1} were found to be overexpressed in Ts1Cje. These genes are critical regulators of endocytosis, synaptic transmission, and cell signalling \( ^{58,59} \). Overexpression of \textit{Itsn1} has been linked to neurodegeneration in mice \( ^{60} \) and \textit{Drosophila} \( ^{61} \) while dysregulation of \textit{Synj1} leads to severe weakness, ataxia, spontaneous epileptic seizures, and poor motor coordination \( ^{62,63} \) as well as abnormal synaptic morphology at the neuromuscular junctions in \textit{Drosophila} homologs of HSA21 \( ^{59} \). \textit{Synj1} has also been linked to Parkinson's disease where mutations of this gene were found to be associated with the disease \( ^{64,65} \). Thus upregulation of these genes may promote neurodegeneration and interference of signal transmission which in turn affects motor function as supported by previous studies \( ^{60-63} \).

\textit{Runx1} and \textit{Sod1} were clustered in more than one functional annotation cluster indicating its role in muscle weakness. However, in the Ts1Cje mice, \textit{Sod1} is part of the proximal break point of the translocated segment of MMU16 to MMU12 causing one of the triplicated copies of \textit{Sod1} gene to be non-functional \( ^{29,48} \). While this is unique to Ts1Cje, \textit{Sod1} has consistently been identified as a marker for DS in different models where it is upregulated. Although Ts1Cje has 100% rather than 150% CuZnSOD activity \( ^{29} \), the consistent upregulation of the mRNA in Ts1Cje model suggests the need to investigate the level of Sod1...
expression or CuZnSOD activity in other DS models presented with muscle weakness phenotype.

A high number of genes involved in the inflammatory response such as cytokines, interleukins and interferon receptors were found to be differentially regulated in Ts1Cje skeletal muscles. Studies have shown that the inflammatory response plays a role in tissue healing and thus is important for skeletal muscle regeneration after injury [66-69]. Runx1, another trisomic gene, was also found to be upregulated in Ts1Cje skeletal muscles. Interestingly, Runx1 also known as AML1 was first identified in acute myeloid leukemia and has been shown to regulate hematopoiesis, angiogenesis, muscle function, and neurogenesis [70-73]. Runx1 was shown to be crucial in muscle regeneration and a decrease in this gene lead to severe muscle atrophy [74] as implicated in Duchenne muscular dystrophy (DMD), myopathy patients [75] and myasthenia gravis [76] suggesting a role in the immune response. A study showed that overexpression of Runx1 triggered inflammation [77] suggesting that upregulation of Runx1 in DS may be linked to hypotonia by decreasing efficiency of muscle regeneration as a respond to excessive anti-inflammatory process.

Rcan1 or Dscr1 was found to be upregulated in Ts1Cje muscles. Expression of RCAN1 was found in the brain, skeletal and cardiac muscle, and the expression was elevated in brains of Alzheimer’s disease patients and Down syndrome foetuses [78-80]. RCAN1-calcineurin-SOD1 pathway was activated during high oxidative stress leading to less cardiovascular risk in normal individuals [81] whereas increased RCAN1 has been found to reduce cancer risk in Down syndrome individuals [82] or reduced tumour growth incidence in Ts65Dn mainly by suppressing the growth of blood vessels that innervate the area [83]. The role of Rcan1 and its upregulation in Ts1Cje muscles remains unknown and it would be intriguing to suggest that upregulated Rcan1 may have reduced the blood supply to muscles leading to perturbed growth or regeneration associated with weaknesses seen in Ts1Cje model. Interestingly, expression of two interferon receptors namely Ifnar1 and Ifnar2 at the mRNA and protein levels in Ts1Cje muscles had contrasting results. The trisomic genes were upregulated in Ts1Cje as expected, but the proteins were found to be downregulated in the muscles. The observation suggests an incomplete penetrance of gene dosage effect at least in the skeletal muscle as compared to the adult brain of Ts1Cje [40]. While the observation could be attributed to post-transcriptional or translational regulation and differences in the tissue being analysed, IFN activity has been shown to regulate proliferation of muscles cells especially by triggering the JAK-STAT signalling pathway [84]. Downregulation of Ifnar1 and Ifnar2 are expected to reduce JAK-STAT signalling pathway which in turn would promote myoblast renewal and regeneration processes [85]. However, a previous study of skeletal muscle stem cells (SMSCs) derived from Ts1Cje and control mice of the same age did not show any differences in the number SMSC as well as their differentiation capability in vitro [86]. Although the finding suggests no apparent differences in myogenesis during normal physiological state of Ts1Cje muscles, it does not preclude the potential involvement of Ifnar1 and Ifnar2 via JAK-STAT signalling pathway during early skeletal muscle development or regeneration state which will become obvious in aging subjects or during muscle injuries.

Other trisomic genes such as Dyrk1a, Donson and Tmem50b were also found upregulated in the brain of Ts1Cje mouse model [40]. Dyrk1a is a well-known DS marker, and mouse models with overexpressed Dyrk1a transgenes exhibited motor abnormalities [26] that were comparable to DS patients [87]. The role of Dyrk1a during muscle development and function has not been well documented but its expression has been shown to be regulated by myocyte specific enhancer 2d (Mef2d), a transcription factor that is important for muscle cell differentiation [88]. On the other hand, no relevant reports were available on the role of Donson and Tmem50b in the skeletal muscle function and their contributions to muscle weakness in Ts1Cje should be investigated further. Similarly, the disomic DEG Mansc1,
was found to be upregulated in both soleus and EDL but no prior study has linked the role of the gene to muscle development or function. Further characterisation of these under-documented novel candidate genes may provide invaluable information on the progression of muscle weakness in Ts1Cje or hypotonia in DS individuals.

5. Conclusions
To the best of our knowledge, there has not been a study to determine the gene profiles of both Type I and Type II skeletal muscles in Ts1Cje mice. This transcriptomic profiling of Ts1Cje skeletal muscles led to the identification of trisomic DEGs that are implicated in signal transmission and basic cell functions, through impairment of neuromuscular junction signalling as well as oxidative stress and chronic inflammation. This provides new insights on the potential disrupted molecular pathways associated with muscle weakness in Ts1Cje and can help elucidate the cause for hypotonia in DS individuals. However, only a subset of genes and proteins were selected for validation. Therefore, additional genes including the non-coding genes should be validated in the future. To further study the role of the dysregulated genes in hypotonia, gain- or loss-of-function studies should be performed to determine the role of selected DEGs on the development, function and regeneration of skeletal muscles. Downstream or upstream targets of the genes in the molecular pathways should also be studied to provide more insight to the underlying mechanism involved.

Supplementary Materials: Table S1: List of all DEGs and Table S2: List of trisomic DEGs are available online at https://www.neurosciern.org/ojs/index.php/nrnotes/article/view/12.

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