Dyrk1b is a key Regulatory Kinase Integrating Fgf, Shh and mTORC1 signaling in Skeletal Muscle Development and Homeostasis

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Dyrk1b: Dual-specificity Tyrosine-regulated kinase 1B

SO: sarcopenic obesity

MetS: metabolic syndrome

myoD1: myoblast determination protein 1

Fgf: Fibroblast growth factor

Shh: Sonic Hedgehog

mTORC1: mechanistic target of rapamycin C1

4e-bp1: Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1

ABSTRACT

The advent of human genetics has provided unprecedented opportunities for discovery of novel disease pathways. Mutations in *DYRK1B* have been associated with metabolic syndrome and sarcopenic obesity in humans, underscoring the critical role of the encoded protein in skeletal muscle development and homeostasis. By the novel creation of Dyrk1b knockout zebrafish models we demonstrate that Dyrk1b kinase activity is critical for specification of the paraxial *myoD*. Mechanistically, Dyrk1b mediates and amplifies Fgf signaling in the paraxial domain by the transcriptional suppression of its negative feedback inhibitor *sprouty1*. In the adaxial *myoD* domain, Dyrk1b amplifies Shh signaling and partially rescues defects caused by its disruption. The investigations of C2C12 terminal differentiation revealed that Dyrk1b also plays a critical role in myofiber fusion. Combined biochemical and proteomic analysis of C2C12 myoblasts undergoing differentiation showed that Dyrk1b kinase activation is induced by shh inhibition, and triggers differentiation by inhibiting mTOR, subsequent upregulation of 4e-bp1 and induction of autophagy. In conclusion, we demonstrate that Dyrk1b plays a critical role in sustaining myocyte specification and differentiation by integrating Fgf, Shh and mTORC1 signaling pathways.

Keywords: *dyrk1b*, *myoD*, myogenesis, sarcopenic obesity, Shh, Fgf, human genetics, fate determination, inherited metabolic syndrome.

INTRODUCTION

The syndrome of Sarcopenic Obesity (SO), characterized by a combination of truncal obesity and reduced skeletal muscle mass is an emerging risk factor for the metabolic syndrome (MetS), (1) and is a greater risk for cardiovascular diseases, and type2 diabetes than obesity alone. Accordingly, the burden of type 2 diabetes is most rapidly increasing in populations with low to normal body mass index but high prevalence of sarcopenic obesity (2, 3). The rapid trend of aging population and the growing sedentary life styles are other impetus for the recent surge in sarcopenia (4), which has prompted universal efforts to reduce its burden. Unfortunately, strategies to improve skeletal muscle mass have been largely unsuccessful due to the limited understanding of myogenesis as an integrated process in humans.

SO has strong genetic components that are linked to the risk for type2 diabetes and atherosclerosis (5-9). The advent of human genome project has provided an excellent opportunity for the discovery of novel disease genes and their cognate pathways. Specifically, major progress has been made in genetic studies of single gene disorders. This in turn has led to the discovery of key driver nodes of complex biological networks involved in development and disease. Most recently, independent, rare and novel genetic variants with large effect sizes in *DYRK1B* gene have been shown to underlie atherosclerosis, MetS and sarcopenic obesity by using whole exome sequencing (10). This discovery provided a unique opportunity to investigate developmental and disease pathways of the human skeletal muscle.

DYRK1B protein is a dual-specificity kinase, with the tyrosine phosphorylation primarily limited to auto-phosphorylation (Y271/273) and an arginine-directed serine/threonine kinase activity towards its substrates (10, 11). It is a pancellular kinase (9, 12), that is highly expressed in the skeletal muscle and has been reported to promote *in vitro* differentiation of myofibers in C2C12 myoblast cells (13-15). The mechanisms by which Dyrk1b promote myofiber differentiation are relatively unexplored. Surprisingly, no *in vivo* genetic model of DYRK1B and its characterization has been reported to this date. Consequently, its roles during skeletal muscle specification, prior to myoblast differentiation have never been explored. We embarked on investigating its role during early myogenesis in zebrafish due to the ease of genetic engineering, external fertilization and the transparency of embryonic development. Although, the skeletal muscle in zebrafish is

predominantly made of fast twitch fibers (16, 17), the fundamental pathways involved in regulation of myogenic specification are highly conserved across metazoans (18).

The myogenesis across metazoans is regulated by the transcription factors (TF) called Muscle regulatory factors (MRFs) which includes MyoD, Myf5, Myogenin and MRF proteins (19, 20). The basic helix-loop-helix TF MyoD is the central protein sufficient to orchestrate differentiation of numerous cell types to skeletal muscle fate (21, 22). The MRFs cooperate with the coregulatory proteins of Mef2 class of transcription factors to stimulate myofiber differentiation (23). During zebrafish embryonic myogenesis, *myf5*, *myoD* and *myogenin* are co-expressed in cells adjacent to the axial mesoderm (aka. adaxial *myoD*) which differentiate into the slow muscle fibers (Wolff et al., 2003, Reifers et al., 1998). In the paraxial myotomal somitic domain, *myoD* (aka. paraxial *myoD*) and *myogenin* are upregulated as somitogenesis proceeds (17) and these cells differentiate into fast-twitch fibers. Based on earlier *in vivo* studies, the adaxial and paraxial *myoD* expression is regulated by distinct signaling pathways: Shh and Fgf, respectively (24). In zebrafish, the knockout of Shh canonical receptor *smoothed* results in loss of adaxial *myoD* with no detectable effect on paraxial *myoD* (25). Conversely, loss of Fgf8 leads to complete loss of the paraxial *myoD* with no change reported in the adaxial domain (Groves et al., 2005). Thus, Shh and Fgf act in a mutually exclusive but coordinated fashion to drive expression of *myoD* in these distinct domains within the somites. However, the downstream effectors of Shh and Fgf pathways during muscle specification are largely unknown.

During the subsequent stage of differentiation of myoblasts into myofibers, Fgf (26) and Shh (27) signaling pathways play much more different, complex and dynamic roles. While data from some investigators supports the role of Fgf signaling in myotube fusion (28, 29) other studies have either contradicted these findings (30, 31) or have suggested stage specific function of FGF during myotube differentiation (32, 33) (34). Similarly, opposite results for the role of Shh on myoblast fusion has been reported (27, 35). These discrepancies could be due to the variability of the dosage, timing and duration of its pharmacological manipulations, necessitating more in-depth investigations. In addition, pathways downstream from DYRK1B in regulation of myogenesis were relatively unknown.

We comprehensively investigated the role of Dyrk1b kinase in myogenic specification, and differentiation and examined its role in integrating different myogenic pathways into a coordinated developmental process. We generated a CRISPR/Cas9-mediated Dyrk1b zebrafish knockout

model. We found that this kinase is indispensable for survival as *Dyrk1b*^{-/-} embryos did not survive beyond 72hpf. We subsequently examined the role of *Dyrk1b* in muscle specification and differentiation in the context of pharmacological and genetic perturbation of Shh and Fgf pathways and delineated its role in 1) regulation of paraxial and adaxial domains of *myoD* expression in zebrafish, and in 2) myofiber differentiation in mouse C2C12 cells. We find that in zebrafish myogenic progenitors and C2C12 myoblasts, *Dyrk1b* interact with Fgf and Shh signaling in strikingly contrasting ways. In zebrafish, *Dyrk1b* acts downstream of the Fgf signaling to specify paraxial *myoD* domain and increases Shh signaling to specify adaxial *myoD* cells. In C2C12 cells, *Dyrk1b* kinase activation is enhanced by Shh inhibition, which results in myofiber differentiation. Using proteomics analysis in C2C12 cells, we identified genes regulated by DYRK1B during myofiber differentiation and uncovered a network of co-regulated pathways that are activated upon *Dyrk1b* inhibition of mTORC1 and are associated with autophagy. Strikingly, the induction of excessive autophagy by DYRK1B^{R102C} mutation was coupled with impaired myogenesis in C2C12 cells. Altogether, we demonstrate the role of *Dyrk1b* as a key node integrating Fgf, Shh, and mTORC1 signaling during myogenesis.

RESULTS:

***Dyrk1b* is expressed in the somitic mesoderm, that harbors the precursors of skeletal muscles**

We chose zebrafish as a vertebrate model to study the dynamic process of skeletal muscle development due to the ease in creating genomic alterations, the external fertilization, the transparency of the embryos and the high levels of sequence identity (80%) and similarity (94%) in the kinase domains of zebrafish and human *Dyrk1b*. We first examined the expression of *dyrk1b* in the zebrafish by whole mount *in-situ* hybridization (WISH), using two digoxigenin-labeled anti-sense probes and a control sense RNA probe. We performed WISH at various stages of embryogenesis starting from single cell stage to 24 hpf. Examination of mRNA expression of *dyrk1b* revealed that transcripts are deposited maternally, and the gene is expressed from 1-cell stage through 4 and 8-cell stages (Fig. 1A-D, n=30 embryos at each stage). After the onset of zygotic transcription, the expression is broadly seen throughout the embryo at 9hpf (Fig. 1E), both ventrally and dorsally. At mid-somitogenesis (14 hpf), the transcripts are expressed in the entire dorsal region, but were excluded from the posterior axial domain of the embryo (red asterisk, Fig. 1F, n=30 embryos at each stage). At 24-hpf stage, the *dyrk1b* transcripts are localized in the

anterior neuroectoderm including the forebrain, midbrain and hindbrain (Fig. 1G, n=30 embryos at each stage). The cross sections in the anterior embryonic domains revealed expression in the forebrain and eyes (Fig. 1H) at 24 hpf. In the posterior domain, *dyrk1b* is expressed in mesodermally derived somites (red asterisks, in Fig. 1I), but excluded from the notochord (red asterisk in Fig. 1G and red arrow in Fig. 1I) and posterior neural tube (Fig. 1I NT, sections were examined at 100um intervals for n=5 embryos for each stage). The sense-control probe showed no staining (Fig. 1K) when compared to the antisense probe (Fig. 1J). The second anti-sense probe generated against *dyrk1b*, showed an identical expression pattern by WISH (SFig.1).

Fig.1

Figure 1. Knockdown of *dyrk1b* in the zebrafish causes loss of paraxial *myoD*, the central regulator of embryonic myogenesis. A-I WISH for *dyrk1b* transcripts using an anti-sense probe at different stages of zebrafish embryonic development as indicated. Arrows on A and B indicate transcripts at one and two cell stages, respectively. Insets on C and D show animal pole view. A-E lateral views, F-G dorsal views, H and I are cross-sections at regions indicated by red lines on G. Red asterisks on F and G indicate the unstained axial domain. Red asterisks on I denote somites, red arrow denotes notochord, NT=neural tube. J,K are group shots of zebrafish embryos stained with anti-sense and the control sense probe. L. Schematic diagram of the *dyrk1b* locus in the zebrafish genome. Boxes indicate the exons (not drawn to scale). The dotted lines from second exon indicate the translation start site (in red). Guide RNA is highlighted in yellow and PAM region in green. wildtype and *dyrk1b*^{+/-} sequence are shown, 7bp insertion induced by CRISPR/Cas9 is highlighted in blue. M Schematic showing Dyrk1b CRISPR/Cas9 injection and transmission of induced mutation. N T7 assay with one band for Wildtype and two bands for *dyrk1b*^{+/-}, indicating the heteroduplex formed by CRISPR/Cas9 activity. O. Sanger sequencing images of amplified segments of wildtype and *dyrk1b*^{+/-}, demonstrating the site of 7bp insertion (red arrow) and the ensuing frame shift. P. *dyrk1b* mRNA expression levels (mean $\pm$ s.e.m) in the wildtype and Dyrk1b^{+/-} zebrafish embryo at 24hpf (** $p \leq 0.001$, Students ttest, 2 tailed, n=30 pooled embryos). Q Micrographs of 3-month-old adult heterozygous Dyrk1b^{+/-} fish showing lack of caudal fin (red arrow) and jaw malformation (red arrowhead). R, S Lateral views of wildtype and Dyrk1b^{-/-} embryos at 24hpf showing shortened body axis and defective somites in Dyrk1b^{-/-} fish. T, U Ventral views of 24 hpf wildtype and Dyrk1b^{-/-} embryos, red asterisk in R indicates cyclopic eyes. V, W Immuno- fluorescent staining (IF) for Myosin Heavy Chain1 on cross-section of 24hpf Dyrk1b^{-/-} and control embryos. X, Y WISH for *myoD* at 14hpf in *dyrk1b* CRISPR/Cas9-injected fish, red asterisks in Y indicate loss of paraxial *myoD* (dorsal views). Z-A.7 WISH for different markers of genes in the myogenic pathway, as indicated. Z-A.3 tail bud view at 14hpf, A.4-A.5 dorsal views, 14 hpf, A.6-A.7 lateral views at ~80% epiboly, *indicates $p \leq 0.05$, ** $p \leq 0.01$, Students ttest, 2 tailed. Scale bars indicate 200 μ m.

SFig.1

Figure S1. WISH of two anti-sense probes for *Dyrk1b* exhibiting identical expression pattern A-C Group shots of 14hpf embryos revealing *Dyrk1b* transcripts by WISH. D-F individual embryos from the respective in-situ (lateral view).

***Dyrk1b* is required for normal myogenesis, brain and eye development in zebrafish**

To determine the function of *dyrk1b* during zebrafish development, we employed CRISPR/Cas9 mediated gene editing to create stable knockouts (36). The major caveats of *dyrk1b* knockdown, previously attempted using anti-sense morpholino (37) includes widely reported off-target effects of the morpholinos and the lack of comparison of the observed phenotypes with a stable knockout (38). In this study, we generated stable *dyrk1b* knockout zebrafish, using two different guide RNAs (gRNA): one targeting the translation start site in exon 2 (Fig. 1L, SFig. 2A, for gRNA design and off-target analysis see the Materials and Methods) and another gRNA targeting the kinase domain of the *dyrk1b* gene (SFig. 2B). Both gRNAs successfully and independently targeted the *dyrk1b* gene, generated indels and resulted in identical phenotypic traits. We employed three different methods to confirm the efficient disruption of the *dyrk1b* gene in the random clutch of wild type zebrafish embryos. First, 6 FAM (Fluorescein amidite) labeled primers were used, which revealed insertions and deletions (indels) created by the gRNA activity. Second, the T7 endonuclease activity assay was performed to further confirm the presence of indels and final confirmation was obtained by sanger sequencing. These different steps will be discussed again

below for generation of stable F2 zebrafish knockouts. The F0 embryos showed the following consistent and striking phenotypes at 24hpf: cyclopic eyes, shortened and curved body axis. Nearly 30% of embryos were lethal. We raised the remaining ca. seventy percent surviving embryos to adulthood and screened for the founder fish (F0). The F0 fish, were inherently mosaic carrying several different *dyrk1b* mutations in different cells (indicated by a multicolored fish in schematic Fig. 1M) and were crossed to wild-type to generate F1 fish (Fig. 1M). After confirming the successful transmission of the mutation by T7 assay (Fig. 1N), the F1 fish were sequenced to identify the nature of the mutation. We identified three independent mutations in the *dyrk1b* gene in F1 fish (SFig. 2A). For the purpose of the study, we focused on the carrier fish that had an insertion of 7 bp near the translation start site of *dyrk1b* gene (Fig 1O). The total length of Dyrk1b is 751 amino acids, and this insertion caused a premature stop codon at the codon 59. The *dyrk1b* mRNA levels in the heterozygotes were about 50% of the wild type siblings as expected (Fig. 1P). The F1 heterozygote fish carrying this 7 bp insertion were intercrossed to obtain the F2 generation (schematic Fig. 1M). The adult heterozygote F1 fish showed protuberance of lower jaw, complete loss of the caudal fin and exhibited swimming defects (Fig.1Q).The F2 homozygous embryos showed similar traits as observed in F0 generation at 24hpf: shortened and curved body axis (Fig.1 R, S), visible loss of somites, cyclopic eyes (Fig. 1T, U) and were embryonic lethal and did not survive beyond 72 hpf.

Fig.S2

Figure S2. Reference DNA sequence flanking translation start site and kinase domain and the sequences of 3 mutations generated using CRISPR/Cas9. **A.** A schematic diagram of the *dyrk1b* locus in the zebrafish genome. Black boxes indicate exons (not drawn to scale). Dotted lines from the second exon indicate the translation start site (in red). Guide RNA is highlighted in yellow and PAM region is in green. The reference sequence is shown on the top and the 3 mutations are shown underneath **B.** CRISPR/Cas9 targeting the kinase domain creates a 4bp insertion and one-base-pair change (in red). **C.** T7 assay with one band for Wildtype (WT) and two bands for *dyrk1b*^{+/-}, indicating the heteroduplex formed by CRISPR/Cas9 activity targeting exons carrying translation start site and kinase domain respectively.

Dyrk1b regulates *myoD* expression in paraxial mesoderm

The expression of *dyrk1b* in the somites (Fig. 1I), and the shortened body axis (Fig. 1R, S) of *Dyrk1b* homozygous knockout F2 embryos at 24hpf, prompted the examination of skeletal muscle fibers, that are specified from the myotomal segments of somites (39). Immunofluorescent (IF) staining for Myosin heavy chain1 (MyHC1) in the *Dyrk1b*^{-/-} F2 embryos revealed a drastic

reduction of myofibers at 24hpf (Fig.1 V, W, n=10 embryos of each genotype were sectioned, and cross sections in the trunk region were examined at intervals of 100um per embryo with identical results). To characterize if the loss of myofibers results from the molecular events occurring at earlier stages, we performed WISH for several unique but partially overlapping myogenic markers starting from mid-gastrulation through somitogenesis. This investigation reproducibly revealed complete loss of myogenic differentiation (*myoD*) expression in the paraxial mesoderm in F2 *Dyrk1b* ^{-/-} embryos (Fig.1X, Y, n=50 in each group for each experiment, n=3 biological replicates). Based on three independent breeding experiments, an average of 26.3 embryos from a clutch of total 99.3 F2 embryos were homozygous (*Dyrk1b*^{-/-}) and showed complete loss of paraxial *myoD* at 14 hpf (bilateral *myoD* loss in 1Y and Table S1). Next, we examined expression of mesogenin1 (*msg1*), myogenic factor 5 (*myf5*), myogenin1 (*mygn1*) and T-box transcription factor 6 (*tbx6*). *mesogenin* (*msg1*), expressed in the posterior paraxial presomitic mesodermal progenitors (40), *tbx6*, expressed bilaterally in the somitic precursors anterior to *msg1* (41) and *myogenin*, expressed primarily in adaxial muscle precursors showed either no change or incremental expansion in the expression domains, in the *Dyrk1b*^{-/-}-vs. wildtype embryos at 14hpf (Fig.1Z-A.3, SFig.3A, n=50 embryos for each genotype, from two independent experiments). These expression patterns suggest that somitic progenitors are specified normally in the absence of *Dyrk1b* (39). Next, we examined the notochord marker, *shh* (42) and *wnt8a*, a marker of early mesodermal patterning (43) which showed no major changes in the *Dyrk1b*^{-/-} embryos at the end of gastrulation (Fig 1A.4-A.7, n=50 embryos for each genotype, from two independent experiments). Interestingly, myogenic factor 5 (*myf5*) expressed in the myoblasts and the posteriorly localized somitic progenitors was upregulated in *Dyrk1b*^{-/-} vs. wildtype embryos (SFig.3A, n=50 embryos for each genotype, from two independent experiments). As we will discuss later, the expansion in the *myf5* expression domain was also observed upon loss of Fgf signaling (see below). Altogether, these data reveal that *Dyrk1b* is dispensable for the specification of the presomitic progenitors but plays an essential function at the onset of *myoD* expression, the latter being the central regulator of embryonic and adult myogenesis.

The kinase activity of *Dyrk1b* is essential for paraxial *myoD* specification.

We next examined whether kinase activity of *Dyrk1b* is required for specification of *myoD* positive cells in zebrafish. To this end, we misexpressed human DYRK1B in zebrafish. We noted about 3-

fold increase in *dyrk1b* transcripts upon its overexpression (Fig.2A). The injection of wildtype *DYRK1B* mRNA into wild-type zebrafish at one-cell stage extended the expression of *myoD* in each somite in the anterior-posterior axis. (Fig. 2B, C). To test whether kinase-activity of Dyrk1b was necessary, we mutated two residues in Dyrk1b: tyrosine 273 residue (Y273F) that undergoes cis-auto phosphorylation and the Lysine 140 in the ATP binding region of the catalytic domain (K140R) (10, 11). The *DYRK1B*^{Y273F, K140R} mRNA overexpression failed to expand paraxial *myoD* domain, indicating that kinase activity of Dyrk1b is important for myogenic specification (Fig. 2D, red arrow) and may have a dominant negative effect as suggested before (44).

SFig.3

Figure S3. **A** Expression by RT-qPCR of selected myogenic differentiation factors in *Dyrk1b* knockout zebrafish embryos at 14hpf. **B** RT-qPCR showing *Dyrk1b* levels in the C2C12 cells transfected with the indicated constructs. **C** RT-qPCR of the indicated genes in the Fgf signaling pathway in *Dyrk1b* knockout zebrafish embryos. **D** The expression of Shh effectors *Gli2* and *Gli3* in *Dyrk1b* knockout embryos compared to control zebrafish embryos at 24hpf. **E** The expression of pDyrk1b, using pHIPK2 antibody (45) in primary cells treated with different concentrations of dmsol or AZ191.

Fig.2

Figure 2. Dyrk1b mediates Fgf and Shh signaling in the paraxial and adaxial expression domains respectively. **A** *dyrk1b*, normalized to *b-actin*, is elevated in zebrafish overexpressing human wildtype *DYRK1B*. **B-D** WISH for *myoD* (dorsal views) in wild type embryos injected with plasmids containing no cDNA (empty), human wildtype *DYRK1B*, and kinase-defective (*Y273F, K140R*), and exposed to dmsol, at the same concentration at SU5402, at 14hpf. Inset in B and C are magnifications of *myoD* expression for better visualization. **E, F** WISH for *myoD*, showing loss of paraxial *myoD* after treatment FGF inhibitor SU5402 (E) and its rescue by *DYRK1B* overexpression in SU5402 treated embryos at 14hpf. **G** mRNA expression of Fgf syn-expression genes, *myoD* and *dyrk1b* in dmsol or SU5402 treated embryos at 14hpf. SU5402 was added at the end of gastrulation. **H** mRNA expression of *sprouty1* in *dyrk1b* knockout embryos at 14hpf. **I, J** WISH and quantification (by Image J) for *dyrk1b* in SU5402 treated embryos. **K** the

model depicting that Dyrk1b mediates Fgf signaling and also amplifies it by suppressing *sproutyl*, the feed-back inhibitor of the Fgf pathway. **L-M** WISH(dorsal views) for *myoD* in 14hpf embryos, showing loss of adaxial *myoD* in cyclopamine treated embryos **N** Rescue of adaxial *myoD* by human *DYRK1B* overexpression in cyclopamine treated embryos. **O, P** Western blot images and their quantification (mean $\pm$ s.e.m) showing the expression of Shh effectors Gli1, Gli2 and Gli3 in control wild type embryos, Dyrk1b knockout and overexpressing zebrafish embryos at 14hpf. **B-F**, **L-N** dorsal views, **I** lateral views *indicates $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, Students ttest, 2 tailed.

Dyrk1b mediates Fgf signaling of the paraxial *myoD* domain in zebrafish

Paraxial *myoD* was previously reported to be transcriptionally regulated by fibroblast growth factor (Fgf) signaling (24). Fgf8 mutant embryos (*acerebellar*) as well as embryos treated with FgfR-specific tyrosine kinase inhibitor SU5402 show complete loss of *myoD* expression in the paraxial somites (24). Since Dyrk1b^{-/-} embryos phenocopy the Fgf mutants' loss of *myoD* in the paraxial domain (Fig. 1X, Y) we decided to explore the epistatic interaction between Fgf and Dyrk1b in zebrafish. Note, that *myf5* expression domains are expanded in Fgf8 mutants and SU5402 treated embryos (24) similar to the Dyrk1b^{-/-} embryos (SFig.3A). We inhibited Fgf signaling by administrating SU5402 to the embryos post gastrulation to avoid potential interference from effects of Fgf disruption during gastrulation on subsequent myogenesis (24). The embryos treated with SU5402 showed loss of *myoD* in paraxial domain and recapitulated *acerebellar* mutants (Fig. 2E) when compared to dmsol control (Fig. 2B). To determine whether Dyrk1b mediates Fgf signaling during paraxial *myoD* specification, *DYRK1B*^{WT} was overexpressed at one-cell stage and treated with SU5402 post-gastrulation. Strikingly, the entire domain of paraxial *myoD* was rescued in this condition (Fig. 2F) (86.3% $\pm$ 3.8% embryos were rescued; P-value= 0.001, average 35 $\pm$ 10 embryos per experiment, three independent experiments). This finding demonstrates the critical role of Dyrk1b in mediating Fgf signaling during specification of paraxial *myoD*. Next, we examined whether Fgf signaling regulates *dyrk1b* transcription as a mechanism for specification of paraxial *myoD*. The expression levels of Fgf8 synexpression group genes *pea3*, *sprtyl*, and *sef* (46, 47) and the expression of *myoD* were reduced, ensuring a sufficient blockage of the Fgf signaling pathway with SU5402 (Fig. 2G). Strikingly, the inhibition of Fgf led to 50% reduction in the *dyrk1b* expression levels, as assessed by both RT-qPCR (Fig. 2G) and WISH (Fig. 2 I, J). These data indicate that *dyrk1b* mediates Fgf signaling in the paraxial *myoD* domain. Next, we investigated whether Dyrk1b mediates Fgf

signaling in the paraxial domain by regulating expression of Fgf effector genes. The positive regulators of Fgf signaling *pea3*, *sef* and *erm* remained unchanged (SFig.3C) while the negative regulator of Fgf signaling, *spry1* was significantly upregulated in *Dyrk1b*^{-/-} embryos (Fig.2H). This finding is consistent with a previous study that demonstrated the expansion of paraxial *myoD* domain by disruption of *spry4* in zebrafish (48), indicating a stringent negative feedback mechanism on Fgf signaling to spatially limit the paraxial *myoD* domain (48). Altogether, these data suggest the following model: Fgf signaling in the paraxial domain transcriptionally regulates *dyrk1b*, which further amplifies Fgf signaling by downregulating its feedback inhibitor, *spry1*, leading to specification of paraxial *myoD* domain (Fig.2K).

Dyrk1b is not necessary but sufficient for adaxial *myoD* expression

The phenotypic similarity of cyclopic eyes between *Dyrk1b*^{-/-} and the Sonic hedgehog (Shh)^{-/-} embryos, and the essential role of Shh in specification of adaxial *myoD* domain (25, 49) led us to investigate if *Dyrk1b* interacts with Shh signaling to induce adaxial *myoD* expression. Additionally, DYRK1B has been shown to modulate switch between autocrine to paracrine Hedgehog signaling in pancreatic cancer cells (50). The adaxial cells are precursors of slow muscle fibers specified adjacent to the notochord and eventually migrate to the most lateral part of the somites (51). Since the adaxial *myoD* domain appeared intact in the *Dyrk1b*^{-/-} embryos (Fig.1 X, Y), we investigated whether the sensitized background of Shh inhibition would reveal any novel interactions of Shh with *Dyrk1b*. We performed a series of experiments, starting with Shh inhibition using the well-established smoothened inhibitor cyclopamine (52). We treated the embryos at 10hpf to circumvent any effects of Shh inhibition during germ-layer formation in gastrulation. Cyclopamine treatment of embryos resulted in loss of adaxial *myoD* with only a small reduction of paraxial *myoD* (Fig. 2L, M) and recapitulated the *myoD* expression in smoothened mutants (25). To determine if *Dyrk1b* could rescue the adaxial *myoD* expression, we injected human *dyrk1b* mRNA at one-cell stage and treated with cyclopamine at 10hpf followed by examination of *myoD* at 10 somite stage. Strikingly, human *dyrk1b* mRNA considerably rescued the adaxial *myoD* (Fig.2N) (68.9% ± 1.37% were rescued, P-value =0.0003, average 40 ± 5 embryos, n=3 independent experiments). To explore the mechanisms by which *Dyrk1b* interacts with Shh signaling pathway, we first examined the expression of Shh ligand itself which revealed

no significant difference in Shh expression in the notochord between *Dyrk1b*^{-/-} and controls by WISH (Fig. 1A.4-A.5), suggesting that *Dyrk1b* does not affect the transcription of the Shh ligand. Likewise, the mRNA expression of *Dyrk1b* was unaffected upon cyclopamine treatment, suggesting that Shh signaling does not transcriptionally regulate *Dyrk1b* (data not shown). Next, we examined the effect of *dyrk1b* genetic manipulation on Shh effectors, Gli1, Gli2 and Gli3. In the *Dyrk1b* knockdown embryos at 14hpf, the protein levels of Gli1, and full length (active forms) Gli2 and Gli3 were significantly reduced as assayed by western blot (Number of independent experiments =2, technical replicates=2, pooled 50 embryos, Fig.2O, P). Similar patterns in Shh downstream effectors were obtained for *Dyrk1b* knockout embryos at 24hpf (SFig.3D). Conversely, the overexpression of *Dyrk1b* increased the protein levels of Gli1 at 14hpf (Number of independent experiments =2, technical replicates=2, pooled more than 50 embryos, Fig. 2O, P). These findings indicate that *Dyrk1b* rescued adaxial *myoD* in Shh-inhibited embryos by increasing the protein levels of Gli1. The suppression of Shh effectors in *Dyrk1b* knockout embryos, did not cause any substantial changes in the adaxial *myoD* domain leading to the conclusion that *Dyrk1b* is not necessary, but sufficient for adaxial *myoD* expression.

The investigation of myocyte differentiation in C2C12 cells

In parallel with studies in zebrafish, we examined the interaction of *Dyrk1b* with Fgf and Shh signaling pathways in the mouse myoblast C2C12 cell line. C2C12 cells were previously isolated from 2-month old normal C3H mice, 70 hours post crush injury of the thigh muscle. The injury caused a rapid proliferation of myogenic mono-nucleated progenitors in the primary cultures from the muscle explants. These cells under low-serum conditions differentiated and fused into multi-nucleated myofibers within 3-4 days (53). These proliferating myogenic progenitors have been also described in the literature as “satellite cells” that can be stimulated to differentiate in low-serum conditions (54). Note that in zebrafish we examined the function of *Dyrk1b* in specification of myoblasts from pre-myogenic precursors while in C2C12 cells we are examining differentiation of committed myoblasts to myofibers. This may explain some of the key differences in the interaction of *Dyrk1b* with Fgf and Shh signaling pathways, as described below.

Fig.3

Figure 3: Interaction of Dyrk1b with Fgf and Shh signaling during specification of myofibers from myoblasts in C2C12 cells. A-D IF staining for myosin heavy chain (MyHC1) in C2C12 cells after 7 days of differentiation. The cells were transfected with plasmids containing no external cDNA but GFP, $DYRK1B^{WT}$, $DYRK1B^{R102C}$ and $DYRK1B^{Y273F, K140R}$ (kinase-defective) before differentiation was ensued. E Quantification for % area of MyHC1 (mean $\pm$ s.e.m) and nuclei per myofiber (mean $\pm$ s.e.m) on 7th day of differentiation in the indicated conditions. F-H IF staining of MyHC1 expression on 7th day of C2C12 differentiation in cells treated with dms0 (H), SU5402

(I) and SU5402 + *DYRK1B*^{WT}. **I.** Quantification of the nuclei (mean $\pm$ s.e.m) per myofiber in the indicated conditions. **J** Percentage area (mean $\pm$ s.e.m) of MyHC1 expression on 7th day of differentiation. The cells were transfected before beginning the differentiation and treated with low dose of SU5402 for all seven days of differentiation. **K-N** IF staining of MyHC1 at 7th day of differentiation in C2C12 cells in dmsu control (K), cyclopamine transfected with empty vector (no cDNA) (L); cyclopamine and wild type *DYRK1B* (M) or cyclopamine and kinase defective *DYRK1B* (N). **O.** Western blot and quantification of phosphorylated DYRK1B and 4e-bp1 in cyclopamine treated C2C12 cells. **P-Q,** IF staining of MyHC1 in C2C12 cells treated with inhibitor AZ191 alone or co-treated with inhibitor AZ191 and cyclopamine. **R-S** % area (mean $\pm$ s.e.m) of MyHC1(R) and quantification of % nuclei (mean $\pm$ s.e.m) of myofibers for the panels K-N **T.** quantification of % nuclei (mean $\pm$ s.e.m) per myofibers for the panels P, Q. * indicates $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Students ttest, 2 tailed. Scale bar = 200 μ m.

The kinase activity of Dyrk1b is essential for differentiation of C2C12 myoblasts to myofibers

We transfected C2C12 cells with vectors containing GFP, *DYRK1B*^{WT}, *DYRK1B*^{R102C} the most common human disease-causing mutation (10), and the kinase defective *DYRK1B*^{Y273F, K140R} at the beginning of differentiation and examined Myosin heavy chain 1 (MyHC1) expression at day 7 of differentiation. Dyrk1b levels were elevated in *DYRK1B*^{WT}, *DYRK1B*^{R102C}, *DYRK1B*^{Y273F, K140R} relative to GFP controls (SFig3B). A 2.69-fold higher percentage area of MyHC1 and 2.00 fold elevated nuclei per myofiber were observed in *DYRK1B*^{WT} compared to controls (Fig. 3A-E, n=3 independent experiments). In the *DYRK1B*^{Y273F, K140R} transfected C2C12 cells, the percentage area of MyHC1 and number of nuclei per myofiber were similar to the control cells (Fig.3 A, D, E). These data indicate that DYRK1B promotes differentiation of myoblasts to myofibers in a kinase-dependent fashion. *DYRK1B*^{R102C} transfected C2C12 cells, however, exhibited near complete abrogation of muscle differentiation as visualized by percent MyHC1 and nuclei per myofiber (Fig. 3C, E, n=3 independent experiments) and suggested loss of function as a potential mechanism for sarcopenia in *DYRK1B*^{R102C} mutation carriers. The more severe phenotype of *DYRK1B*^{R102C}, which demonstrated only a 50% reduction in the kinase activity (44), is likely due to the kinase-independent effects of the mutation that has been previously shown in cultured HepG2 cells (10). Overall, these findings suggest a contribution of a kinase-independent function of Dyrk1b in regulation of myofiber differentiation. This was not further explored due to lack of available methods to target the kinase independent function for translational intervention.

Exclusion of Dyrk1b as an effector of Fgf signaling in myofiber differentiation

We then investigated the interaction between Fgf and Dyrk1b during muscle differentiation in C2C12 myoblasts. We inhibited FGF signaling from day1 to day 7 of differentiation, after which cells were fixed for IF staining (number of independent experiments=3). To prevent toxicity, we used 1 μ M of SU5402, a substantially lower dose compared to those used in previous studies (55). This dose of SU5402 was sufficient to suppress myoblast differentiation as assessed by percentage of MyHC1 positive cells and myotubule fusion evident by nuclei per myofiber (Fig. 3F, G, I, J). The overexpression of *DYRK1B*^{WT} was unable to rescue loss of MyHC1 and myoblasts fusion (Fig. 3 H, I, J, n=3 independent experiments) in SU5402 treated cells. This finding suggests that either 1. other potent downstream effectors, in addition to Dyrk1b, mediate the effects of Fgf signaling during C2C12 myogenesis 2. Fgf and Dyrk1b independently regulate myoblast differentiation; with Fgf being a stronger stimulator of myogenesis.

Dyrk1b is activated by Shh inhibition and promotes myotube fusion

The relationship between Dyrk1b and Shh in the context of myoblast fusion and differentiation is not known. Both inhibitory and stimulating effects of Shh activation on myoblast fusion have been suggested (27, 35), which is likely due to different experimental conditions. We treated C2C12 myoblasts with cyclopamine at a dose of 1 μ M for all 7 days of differentiation. This cyclopamine treatment of C2C12 myoblasts resulted in increased MyHC1 levels and excessive myoblast fusion with the number of nuclei per fiber increasing by about 10-fold on day7 of differentiation compared to vehicle alone (DMSO= 4.42 $\pm$ 0.6 nuclei, Cyclopamine= 46.44 $\pm$ 8.2 nuclei per fiber, respectively, p= 9.0X10⁻⁴, number of independent experiments=3, Fig. 3 K,L, R, S). To determine if myoblast fusion induced by cyclopamine is dependent on kinase activity of Dyrk1b, we overexpressed control vector, *DYRK1B*^{WT}, and *DYRK1B*^{Y273F, K140R} in C2C12 cells along with cyclopamine. The excess myoblast fusion induced by cyclopamine was blocked in C2C12 cells expressing *DYRK1B*^{Y273F, K140R} but not with *DYRK1B*^{WT} (Fig. 3 M, N, R, S). To further confirm the role of Dyrk1b kinase activity in myoblast fusion, we co-treated C2C12 cells with cyclopamine and AZ191, a specific kinase inhibitor of Dyrk1b (56) for all 7 days of differentiation. We tested several concentrations of AZ191 and found 800nM to be the lowest concentration that reduces pDyrk1b levels (SFig.3E). Co-treatment of cyclopamine with AZ191 reduced the number of nuclei per myofiber by 75% compared to cyclopamine alone (Fig. 3 P, Q, T). Altogether, these findings

suggest that Shh inhibition increased myotube fusion in a manner dependent on the kinase function of Dyrk1b. Next, we hypothesized that increased auto-phosphorylation of Dyrk1b via Shh-inhibition is likely responsible for its enhanced catalytic activity since the formation of catalytic active Dyrk1b is dependent on its auto-phosphorylation (57). We examined phospho-Dyrk1b (p-Y273) levels in C2C12 cells after treatment with cyclopamine. The antibody against p-HIPK2 cross-reacts with the phospho-Y273 residue of the Dyrk1b protein and was previously used to assess Dyrk1b activation (45). WB analysis in cyclopamine treated C2C12 cells revealed higher Dyrk1b auto-phosphorylation upon Shh inhibition compared to untreated cells (Fig. 3O). Collectively, our findings indicate that myofiber differentiation of C2C12 cells requires auto-phosphorylation of Dyrk1b, which can be achieved by inhibiting Shh signaling.

The proteomics analysis identifies the augmented muscle differentiation pathways in Dyrk1b overexpressing C2C12 cells

The downstream effectors of Dyrk1b during skeletal muscle differentiation are largely unknown. To this end, we carried out proteomic analysis of C2C12 cells in DYRK1B^{WT} and the corresponding controls. The expression of 362 proteins was significantly altered by DYRK1B overexpression in C2C12 cells compared to the controls (Table1, Fig. 4 A, B, n=3 biological replicates, unique peptides >2, ttest ≤ 0.05 , FDR ≤ 0.05 , Benjamini-Hochberg correction, fold change ≤ 0.5 and ≥ 1.5). The Principal Component Analysis showed segregation of the 6 samples into 2 distinct groups: one group for the 3 controls and the other for 3 Dyrk1b overexpression samples indicating that these two sets of samples are distinct in their expression patterns. Ingenuity Pathway Analysis was used to decipher the changes in the pathways upon Dyrk1b overexpression in C2C12 cells. The top canonical pathways with p-value of < 0.05 and z-score of ≤ -2 or ≥ 2 , included activation of Glycolysis, AMPK Signaling, Rho-mediated Signaling, Actin Cytoskeletal Signaling and Integrin Signaling in DYRK1B^{WT} (Table2, Fig.4C). Prominent proteins most relevant to muscle differentiation in these pathways included Pard3 (elevated 1897 fold), SRC non-receptor kinase (elevated 8.89), Focal Adhesion Protein1 (elevated 7.87 fold), Arhgef17 (elevated 5.9 fold), Protein Phosphatase 1B (reduced 1.85), Cofilin1 (reduced 1.14), and elevation of several isoforms of myosin: Myosin Light chain 1/3 (slow twitch, elevated 2.32) and Troponin

T (slow twitch, elevated 4.42), Troponin T (fast twitch fiber, elevated 1.77) (Table1). These changes in the myosin and troponin isoforms are consistent with microarray analysis previously reported in C2C12 cells when Dyrk1b was knocked down (13). IPA additionally found Egf, Vegf and chemokine signaling, which have been implicated in increasing growth and metabolic capacity of the myocytes (39, 58), to be activated in Dyrk1b^{WT} cells. Further, skeletal muscle contractility, cell movements and glycolysis were predicted to be increased and myofiber degeneration was predicted to be decreased (Fig.4D, Table3) in Dyrk1b^{WT} cells. The proteomic analysis also predicted the potential upstream regulators of the proteins that were significantly altered (Fig.4E). Strikingly, MyoD (z-score 2.71) and Mef2c (z-score 2.09), the two important transcription factors for muscle differentiation, were identified to be activated in Dyrk1b^{WT} C2C12 cells (Table4, Fig.4E). Remarkably, p38 MAPK (z-score 2.77) (59), EgfR (z-score 2.48) (60) and Insulin (2.01), previously implicated in muscle differentiation, were also predicted to be activated (Table4). Altogether, a complex set of several regulators of muscle differentiation were altered upon elevated Dyrk1b expression in C2C12 cells.

Fig.4

Figure 4: Proteomic analysis of C2C12 cells overexpressing Dyrk1b. **A** The significantly altered proteins in empty-vector transfected (N=3 biological replicates) and DYRK1B^{WT} transfected C2C12 cells (N=3 biological replicates) were hierarchically clustered using Qlucore-omics software. The significantly altered proteins were sorted by following thresholds: $p \leq 0.05$, false discovery rate (FDR) ≤ 0.05 , Benjamini-Hochberg corrected, fold change ≤ 0.5 or ≥ 1.5 fold. The cells were serum-starved prior to proteomics analysis. The coloring legend is besides the heat-map. **B** Volcano plot showing the spread of highly significantly altered proteins displayed in panel A (fold change $< \text{or} > 10$, Anova $P < 10^{-4}$). **C, D** All the significantly altered proteins (from panel A) were input into Ingenuity Pathway Analysis (IPA) and the top significantly altered pathways (C) and functions (D), with the z-scores (≥ 2 or ≤ -2) are shown. **E** The most relevant selected upstream regulators, as predicted by IPA, of the significantly altered proteins are displayed. The orange color denotes activation of the molecule.

Dyrk1b inhibits mTORC1, upregulates 4e-bp1 and activates autophagy

One of the most striking findings of the proteomics analysis was more than 300-fold increase in protein levels of eukaryotic translation initiation factor 4E (EIF4E), binding protein 1 (4e-bp1) in DYRK1B^{WT} vs. GFP transfected C2C12 cells (Fig.4A, B, Table1). Under normal conditions, 4e-bp1 inhibits the cap-dependent translation of mRNAs unless phosphorylated by mTORC1 complex (61). Paradoxically, elevated 4e-bp1 activity in the mouse skeletal muscle leads to increased MyHC1 expression and protects against high-fat induced Insulin resistance (62). We confirmed by WB that 4e-bp1 levels were elevated in C2C12 cells expressing Dyrk1b^{WT} and Dyrk1b^{R102C} relative to GFP-expressing controls on day 7 of differentiation (Fig. 5A, C, n=3 biological replicates). No difference was observed in *4e-bp1* mRNA levels between C2C12 cells overexpressing DYRK1B-WT vs. GFP-vector (data not shown), suggesting that Dyrk1b post-transcriptionally regulates 4e-bp1. We then examined whether 4ebp1 mediates the effects of Dyrk1b in promoting muscle differentiation in C2C12 cells. Indeed, the knockdown of 4e-bp1 using siRNA (Fig.5D) at the onset of differentiation prevented the increased myotubule formation in Dyrk1b^{WT} C2C12 cells (Fig. 5E-H, n=3 independent experiments). We further confirmed our findings *in-vivo*, where a complete loss of total 4e-bp1 protein (Fig. 5B, C) levels was observed in 24hpf Dyrk1b Crispr/Cas9 knockout zebrafish embryos (n=50 pooled embryos for WB). The overall protein synthesis was not previously reported to be affected when 4e-bp1 was overexpressed in the mouse skeletal muscle (62, 63). Altogether, these series of experiments indicate Dyrk1b increases myofiber specification by increasing 4e-bp1 protein expression.

Fig.5

Figure 5: Dyrk1b inhibits mTORC1 signaling leading to increased 4ebp1, autophagy, PGC1a and nuclear Foxo1. **A-C** Western blot and quantification of (mean $\pm$ s.e.m) 4e-bp1 expression in C2C12 cells overexpressing empty vector, DYRK1B^{WT} or DYRK1B^{R102C}. **B** WB showing 4e-bp1 expression in zebrafish wild type embryos or Dyrk1b knockout embryos. **C** Quantification of the blots shown in A-B. **D** RT-qPCR showing reduction of 4e-bp1 expression using 4ebp1 siRNA compared to cells transfected with control siRNA. **E-G** IF against MyHC1 expression in C2C12 cells transfected with DYRK1B^{WT} alone, DYRK1B^{WT}+ control siRNA, DYRK1B^{WT}+ 4e-bp1 siRNA. The cells were transfected before the onset of differentiation and MyHC1 expression was examined on day7 of differentiation. **H** The percentage of MyHC1 area calculated for the conditions shown in panels E-G. **I-M** LC3 expression by WB (I, M) and IF (J-L) in the indicated backgrounds. **N-Q** RT-qPCR analysis of the proteins in the autophagic pathway in the C2C12 transfected with the indicated constructs. **R-S** WB displaying expression of indicated proteins collected from C2C12 cells that were transfected with the indicated constructs. The cells were transfected before the onset of differentiation and were collected on day 7 of differentiation in their basal state (R) or after serum-starvation (S). The top panels in S shows nuclear Foxo1 and the bottom panels shows cytoplasmic Foxo1. **T** Seahorse assay to examine mitochondrial respiration and glycolysis in C2C12 transfected with indicated constructs. **U** The model depicting Dyrk1b induced myofiber differentiation in C2C12 cells: during skeletal muscle differentiation in C2C12 cells, Shh inhibition promotes Dyrk1b phosphorylation and stimulates myoblast differentiation. Dyrk1b inhibits mTORC1 signaling that activates an interconnected network of increased translational inhibition by 4e-bp1, elevated autophagy, Foxo1 nuclear translocation and increased PGC1a.

Previous reports have suggested that 4e-bp1 activates a cross-regulatory interconnected network of autophagic pathways, PGC1a and Foxo1, during adult skeletal muscle homeostasis (Figure 5U) (62, 64-66). The autophagic flux was reported to be necessary and highly dynamic during C2C12 differentiation, peaking up at the beginning of C2C12 differentiation, and gradually tapering off towards the end of differentiation (Sin et al., 2016). This ensures timely degradation of ineffective mitochondria and production of mitochondria better suited for oxidative phosphorylation in myofibers. These events are in-turn co-incident with elevated mitochondrial biogenesis mediated by PGC1a (62, 64-66). Consistent with these reports, we found that elevated 4e-bp1 expression observed in DYRK1B^{WT} C2C12 cells is associated with increased autophagic flux as visualized by increased LC3II/LC3I ratio by WB (Fig.5 I, M, the experiment was done on n=4 biological replicates with identical results) and increased punctate LC3 immunostaining by the end of differentiation in C2C12 cells (Fig.5 J-L, n=2 biological replicates for each condition). The elevation was more pronounced in Dyrk1b^{R102C} relative to Dyrk1b^{WT} cells. Accordingly, the RNA expression of genes in the autophagic pathway such as Atg1, Atg4, LC3 and Lamp2b were increased in Dyrk1b^{WT} and even to a greater degree in Dyrk1b^{R102C} cells vs. GFP-transfected cells

(Fig.5 N-Q). The increased autophagic flux was associated with elevated PGC1a expression in Dyrk1b^{WT} and Dyrk1b^{R102C} cells versus GFP-transfected cells (Fig.5R, n=2 biological replicates with identical results). This increased PGC1a expression in Dyrk1b^{R102C} myotubes correlated with significantly increased basal glycolysis and mitochondrial oxidation (Fig.5T, n=11 technical replicates for each transfection condition, n=2 independent experiments). Foxo1 has been previously associated with increasing autophagy and PGC1a expression (67, 68). We found that cytosolic Foxo1 levels were reduced (Fig.5R, S, n=2 independent experiments), while nuclear levels were elevated in Dyrk1b^{WT} and Dyrk1b^{R102C} cells versus GFP-transfected cells (Fig.5S). We next considered the possibility that Dyrk1b may suppress mTORC1 activity, which would encompass the molecular changes manifested as elevated 4ebp1, elevated autophagy, and elevated total Foxo1 levels (61). Accordingly, both pS6K and pS6 levels were reduced in Dyrk1b^{WT} and to a greater degree in Dyrk1b^{R102C} C2C12 versus GFP-transfected cells (Fig.5R, n=2 independent experiments). Consistent with these data, the proteomic analysis independently predicted activation of AMPK signaling, which counteracts mTOR pathways and induces autophagy (Fig.4C). Taken together, our findings reveal that Dyrk1b mediates suppression of mTORC1 signaling and subsequently activates an interconnected network of 4ebp1, autophagy, PGC1a, and Foxo1 (Fig.5U).

DISCUSSION

By using a comprehensive *in vivo* and *in vitro* approaches, we demonstrate here the role of Dyrk1b as a key regulatory node that integrates multiple pathways involved in skeletal muscle development.

In vertebrates, skeletal muscle precursors in the segmental plate consists of two distinct adaxial and paraxial populations with unique fates. The adaxial cells are localized adjacent to the notochord and contribute to the slow muscle fibers while paraxial cells differentiate into fast-twitch fibers (Wolff et al., 2003, Reifers et al., 1998). Myogenesis in both cell populations is regulated by the master transcription factor (TF) MyoD. The *myoD* expression in adaxial cells is dependent on Shh signaling activity (69-71), while paraxial *myoD* positive cells are temporally and spatially regulated by Fgf8. The mechanisms that coordinate the activities of Fgf and Shh pathways was previously not known. Importantly, the downstream effectors of Fgf8 in myogenesis had not been explored. Using combined genetics and pharmacological approaches, we show here that Dyrk1b plays a critical role during early skeletal muscle specification by mediating FGF signaling in the

paraxial domain and coordinating with Shh in the adaxial domain to induce *myoD* expression and initiate muscle specification. Accordingly, loss of Dyrk1b by gene editing in zebrafish led to loss of *myoD* in paraxial mesoderm while the overexpression of human Dyrk1b cDNA rescued loss of paraxial and adaxial *myoD* caused by Fgf and Shh inhibition, respectively. Similar interface of Shh and Fgf signaling is evident in the zebrafish retina where Fgf from dorsal forebrain specifies dorsal optic vesicle and Shh from ventral forebrain specifies ventral optic vesicle(72). Future work aims to determine how Dyrk1b may interact with these pathways during forebrain patterning, considering high expression of Dyrk1b in these domains and the cyclopic eye phenotype.

Further, our investigations of muscle differentiation in C2C12 mouse myoblasts revealed that Dyrk1b stimulates myogenic differentiation in a kinase-dependent manner. In this phase, Shh inhibition leads to excessive myogenic differentiation by promoting the Dyrk1b auto-phosphorylation. This finding shows again that Dyrk1b integrates Shh signaling, although in different ways, to advance the process of muscle differentiation into its completion. These functions of Dyrk1b make it an optimal candidate for development of therapeutics against sarcopenia.

Prior to our study very little was known about the downstream effectors of Dyrk1b. The proteomics analysis, supported by *in vivo* studies identified 4e-bp1 as one of the key targets of Dyrk1b. Strikingly, 4e-bp1 was found to be almost entirely absent in Dyrk1b-CRISPR knockout zebrafish embryos, indicating a cross-species conservation of 4e-bp1 regulation by Dyrk1b. Numerous reports indicate that 4e-bp1 activates a network of interconnected pathways to maintain skeletal muscle homeostasis by upregulation of PGC1a, Foxo1 and induction of autophagy in mice (62) and drosophila (65). Accordingly, C2C12 cells overexpressing Dyrk1b exhibited higher PGC1a protein levels, increased autophagy and elevated nuclear Foxo1 levels compared to the controls. Interestingly, the molecular changes orchestrated by elevated 4e-bp1 levels suggested reduced mTORC1 activity. Accordingly, mTORC1 activity was dramatically lower upon Dyrk1b overexpression in C2C12 cells. These results were also consistent with the increased activation of AMPK as an upstream regulator by proteomic analysis. Overall, these findings indicate the important role of DYRK1B in regulation of genes that play critical roles in skeletal muscle homeostasis.

One major strength of our study is its link to a human disease gene and hence the relevance of the discovery of Dyrk1b-dependent molecular networks to human skeletal muscle development and disease. The autophagy-PGC1a-4ebp1 network was considerably more activated in Dyrk1b^{R102C} vs, Dyrk1b^{WT} expressing cells, indicating a gain-of function effect as reported previously (10). The lack of muscle differentiation in Dyrk1b^{R102C} C2C12 cells indicates that a dynamic and fine-tuned regulation of the autophagy-PGC1a-4ebp1 network is required for proper myofiber differentiation. During the course of C2C12 differentiation, autophagic pathways are regulated in a dynamic fashion: LC3II/LC3I is elevated on day1 and reduces through the course of differentiation, concomitant with mitophagy and gradual generation of younger mitochondria with increased capacity for oxidative respiration of glucose (Sin et al., 2016). Functional characterization of Dyrk1b and its recognition as a key regulatory node for multiple myogenic pathways provides mechanistic insights into human skeletal muscle development and disease. Future genetic studies will determine whether mutations in gene encoding different components of Shh, Fgf and mTOR-4ebp1 pathways underlie disorders of musculoskeletal system.

In conclusion, our comprehensive characterization of Dyrk1b *in vivo* and *in vitro* identifies its role as a novel key regulatory node that integrates multiple pathways involved in myogenic differentiation and regeneration and a potential target for therapeutic interventions for the aging population with limited ability for muscle regeneration.

MATERIALS AND METHODS

Zebrafish lines and maintenance

Zebrafish strain WT^{CF} were raised and maintained according to protocols approved by the Yale University Institutional Animal Care and Use Committee (IACUC protocol number 2015-20070). Embryonic staging and husbandry were performed as described (73, 74).

Whole-mount *in-situ* mRNA hybridization

Sense and antisense Dig-labeled probes were designed against zebrafish Dyrk1b transcripts. Antisense probe generated through PCR that amplified an exonic region of approximately 420 bp in

the three Dyrk1b transcripts using the primers GCAGAGCCCACAAGCTCCGTCTCAGCAGC and TAATACGACTCACTATAGGGGGCCAGGTGGAGGTTGGAGGAGCCGTAC.

Second anti-sense probe spanning ~ 550 bp was generated using TAATACGACTCACTATAGGGTCCCTGCGGCGGGTTGTTATTG and GGGACAGCAGCATGGGTCAGCT Sense probe was generated using GGCCCAGGTGGAGGTTGGAGGAGCCGTAC and TAATACGACTCACTATAGGGGCAGAGCCCACAAGCTCCGTCTCAGCAGC. (underlined region is the T7 promoter for In vitro transcription) These primers amplified exonic region of Dyrk1b and the product was cleaned using PCR purification kit (Qiagen) and cloned into pGEM®-T Easy Vector Systems (Promega). The sequence was confirmed through sanger sequencing. In vitro transcription was performed on the pcr product with Ampliscribe T7- flash kit with Dig RNA labeling mix from Roche(75). In situ hybridization with both the anti-sense probes yielded the same results.

CRISPR/Cas9 gene editing, identification of On-target mutagenesis and Off-target analysis

gRNA sequence was generated through CHOPCHOP against the start codon and kinase domain. The gRNA sequence GCTGCTGGTCGGCCATGGAGTGG was designed to against the start codon and GAGAGCGGTAAAACCGGCTCTTGG against the kinase domain respectively. PAM region is underlined and is excluded from the gRNA sequence. Generation of gRNA, Cas9 mRNA and zebrafish injections was done as described(36). To confirm the gRNA activity, genomic DNA was isolated using a DNeasy Blood and Tissue kit (Qiagen) from a clutch of 15-20 embryos at 24 hpf injected with gRNA and Cas9. 50 ng/μl or fin-clip of adult fish. This served as the template for pcr amplification of approximately 200-300bp region surrounding the intended target. Mutations were detected through a T7 endonuclease I assay (NEB)(76). To confirm the disruption of endogenous locus, the pcr product was cloned into pGEM®-T Easy Vector Systems (Promega) and sequence was analyzed after sanger sequencing. Once the mutation was detected, the rest of the embryos from the same clutch were raised to adulthood. PCR Fragment Analysis was also performed to analyze the indels generated through gRNA.

Primer sequences for T7 endonuclease assay and pcr fragment analysis

Forward and reverse primer for amplifying region surrounding the start codon are TCCCTATGAAGGTGGAAGCA and CGGATGAGACAGTGAAACAAAGA respectively. Forward and reverse primer for amplifying region surrounding the kinase domain are TGTGTGTTTGTGCATGTCTTTC and CATCGTGTAACCGTACCTCATT respectively. The FAM were added to the forward primers for pcr fragment analysis.

Off-target analysis

The gRNA sequence GCTGCTGGTCGGCCATGGAG was used as input in Chopchop (77-79) and CAS offfinder (CRISPR RGEN tools) (79) and no similar sequences were identified in the zebrafish genome that had 1, 2 or 3 bp mismatches.

GRNA	Potential Off targets with mismatch of		
	1bp	2bp	3bp
GCTGCTGGTCGGCCATGGAG	0	0	0
GAGAGCGGTAAAACCGGCTC	0	0	0

Plasmids for *DYRK1B* overexpression, site directed mutagenesis for *DYRK1B*

The generation of plasmids for Dyrk1b and Dyrk1b^{R102C} has been described (10). To generate Dyrk1b^{Y273F, K140R}, primers were designed with the desired point mutations flanked on either side by approx. 20 base pairs. The primers were PAGE purified and the template was PCR amplified by Turbo Taq DNA polymerase along with the mutated primers. The reactions with the forward and reverse primers were done separately, followed by Dpn1 digestion of parent plasmid, and transformation of plasmids into the Ultracompetent cells (Agilent). The plasmid was sequenced to verify the point mutation. Following primers were used for creating point mutations:

K140R: Forward: GACCCAGGAGCTTGTGGCCATCAGAATCATCAAGAACAAAAAGGC
Reverse: GCCTTTTTGTTCTTGATGATTCTGATGGCCACAAGCTCCTGGGTC

Y273F: Forward: TGGCCAGAGGATCTACCAGTTCATCCAGAGCCGCTTCTACCG
Reverse: CGGTAGAAGCGGCTCTGGATGAACTGGTAGATCCTCTGGCC

mRNA synthesis and one-cell stage injections into zebrafish embryos of human Dyrk1b and Dyrk1b^{R102C} or Dyrk1b^{Y273F, K140R}

mRNA was synthesized after restricting digestion of the plasmid with XbaI and purified using phenol-chloroform extraction and ethanol precipitation. This purified product was in vitro transcribed with T7. The mRNA was purified using RNeasy Mini kit (Qiagen, Catalog number 74104). Zebrafish embryos at the one-cell stage injected with 2 nanoliters of the solution containing 150 ng/μl of the mRNA and phenol red for visualization.

Quantitative PCR of zebrafish embryos

Single cell stage embryos were injected with CRISPR/Cas9 against Dyrk1b locus or mRNA for Dyrk1b overexpression (o/e), Dyrk1b^{R102C} or Dyrk1b^{Y273F, K140R}. Embryos were raised to the required stage and RNA was isolated using 300μl of RLT-beta-mer (1 ml RLT buffer and 10 μl beta-mercaptoethanol). The embryos were dissociated by passing them through a syringe and needle (0.5mm X 22mm, catalog number 305124, BD). The embryos were then passed through a QIA shredder (Catalog number 79654) and purified using RNeasy Mini kit. cDNA synthesis was performed using iScript cDNA synthesis kit (Bio-Rad, 1708891). qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad 172-5121). All reactions were done in triplicates and quantified in relation to beta-actin.

Primers for qPCR in zebrafish:

Genes

<i>myoD</i>	Forward: ACTATCCCGTTCTGGAACATTAC
	Reverse: GTCATAGCTGTTCCGTCTTCTC
<i>dyrk1b</i>	Forward: AGCAGGTGCTGTCAGATA
	Reverse: GATGAGATCGACTGAGAGTTT
<i>pea3</i>	Forward: CCCGCCTGTGAAGATTAAGAA
	Reverse: CTCATAGGCACTGGCGTAAA
<i>sprouty</i>	Forward: CGTGCATGTGTTTGGTGAAG
	Reverse: ACTGCGGATGCGATAATGAA
<i>sef</i>	Forward: CACCTTCGGCTTCTCCATTTA
	Reverse: CATACGTTCTTGGAGTGACATC
<i>b actin</i>	Forward: CGAGCTGTCTTCCCATCA

Reverse: TCACCAACGTAGCTGTCTTTCTG

Differentiation and transfection of C2C12 cells:

The C2C12 cells were maintained in 10%FBS+ DMEM+1X Pen-Strep. The differentiation was induced by shifting the media to 2%horse serum+ DMEM+ 1X Pen-Strep. The cell culture media was changed every day until the end of differentiation at day7. The plasmids containing human wildtype *DYRK1B*, *DYRK1B*^{R102C}, *DYRK1B*^{Y273F, K140R} or GFP were transfected before ensuing the differentiation, using lipofectamine 3000 (Invitrogen) using manufacturers' guidelines with a slight modification: The DNA-lipofectamine mix was added to C2C12 cells in suspension post trypsin treatment. For immunostaining, the transfected cells were cultured on glass coverslips and subjected to the standard protocols. For western blot, the cells were harvested in cell lysis buffer+protease inhibitors (Cell Signaling). The cell fractionation into nuclear and cytoplasmic fractions was done using NE-PER extraction method (Thermo Fisher Scientific).

Inhibition of Shh and Fgf pathways

Inhibition of Shh was done through Cyclopamine (LC laboratories) as described (80).

Inhibition of FGF8 was done through incubation of embryos in the drug SU5402, dilution of the drug was described(81). The cyclopamine and SU5402 treatments were added at a final concentration of 1µM from a stock of 10mM for each. Equivalent amount of DMSO was added to control cultures. The treatment was done for 7 days in the differentiation media and the media was changed every day.

Western Blot for zebrafish embryos

The zebrafish lysates at 14hpf and 24hpf were prepared in 0.063M Tris, pH 6.8, 10% glycerol, 0.5ml Beta-mercaptoethanol (in a total of 10mls), 3.5% SDS (zebrafish book). For each sample, 40-50 embryos were pooled together. The lysates were heated at 95°C for 10 minutes prior to running the SDS-PAGE gel. Western blot was carried by standard procedures. All quantifications were done normalized to beta-actin.

Antibodies for Western Blot and Immunostaining:

The F59 and A4.1025 (MyHC1) antibodies were obtained from Developmental Studies Hybridoma Bank. Gli1 (Abcam, ab49314), Gli2a (Genetex, GTX128280), Gli3 (Genetex,

GTX26050), MyoD (Santa Cruz, sc-302, Zfin), pHIPK2 (PA513045, Life technologies), beta actin (Abcam, ab8227), LC3 (Cell signaling, 2775)Pgc1a (Novus Biological NBP1-04676) antibodies were all selected and purchased based on their prior verifications. The western blots were quantified by ImageJ.

Proteomics: Tissue processing for proteomics

The tissues were provided to the Yale proteomics core and suspended in 500uL RIPA buffer contains protease (Pierce; 100X:cat#87786) and phosphatase (Pierce; 100X:cat#78420) inhibitor cocktail. Three bursts (10% amp) followed by two bursts (15% amp) of sonication for 15 seconds each were carried out to burst the cells open. Suspension was centrifuged at 14K rpm for 10 minutes, and 150uL of the supernatant was removed. Chloroform:methanol:water protein precipitation was performed, and dried protein pellet was resuspended in 100uL 8M urea containing 400mM ABC, reduced with DTT alkylated with iodoacetamide, and dual enzymatic digestion with LysC and trypsin (carried out at 37 C for overnight and 6 hrs., respectively). Digestion was quenched (with 0.1% formic acid) during the de-salting step with C₁₈ MacroSpin columns (The Nest Group). The effluents from the de-salting step were dried and re-dissolved in 5 µl 70% FA and 35 µl 0.1% TFA. An aliquot was removed from each sample to determine peptide concentrations via Nanodrop, and diluted to 0.05 µg/µl with 0.1% TFA. 1:10 dilution of 10X Pierce Retention Time Calibration Mixture (Pierce; Cat#88321) was added to each sample prior to injecting on the UPLC Q-Exactive Plus mass spectrometer to check for retention time variability the normalization during LFQ data analysis.

Label-Free Quantitation (LFQ) LC MS/MS data collection was performed on a Thermo Scientific Q-Exactive Plus Mass spectrometer connected to a Waters nanoACQUITY UPLC system equipped with a Waters Symmetry® C18 180 µm × 20 mm trap column and a 1.7-µm, 75 µm × 250 mm nanoACQUITY UPLC column (35°C). 5 µl of each digests (in triplicates) at 0.05 µg/µl concentration was injected in block randomized order. To ensure a high level of identification and quantitation integrity, a resolution of 60,000 was utilized for MS and 15 MS/MS spectra was acquired per MS scan using HCD. All MS (Profile) and MS/MS (centroid) peaks were detected in the Orbitrap. Trapping was carried out for 3 min at 5 µl/min in 99% Buffer A (0.1% FA in water) and 1% Buffer B [(0.075% FA in acetonitrile (ACN))] prior to eluting with linear gradients

that will reach 30% B at 140 min, 40% B at 155 min, and 85% B at 160 min. Two blanks (1st 100% ACN, 2nd Buffer A) will follow each injection to ensure against sample carry over. The LC-MS/MS data was processed with Progenesis QI software (Nonlinear Dynamics, version 2.4) with protein identification carried out using in-house Mascot search engine (2.4). The Progenesis QI software performs chromatographic/spectral alignment (one run is chosen as a reference for alignment of all other data files to), mass spectral peak picking and filtering (ion signal must satisfy the 3 times standard deviation of the noise), and quantitation of peptides and proteins. A normalization factor for each run was calculated to account for differences in sample load between injections as well as differences in ionization. The normalization factor was determined by calculating a quantitative abundance ratio between the reference run and the run being normalized, with the assumption being that most proteins/peptides are not changing in the experiment so the quantitative value should equal 1. The experimental design was setup to group multiple injections (technical and biological replicates) from each run into each comparison sets. The algorithm then calculates the tabulated raw and normalized abundances, Anova *p* values for each feature in the data set. The MS/MS spectra was exported as .mgf (Mascot generic files) for database searching. Mascot Distiller was used to generate peak lists, and the Mascot search algorithm was used for searching against the Swiss Protein database with taxonomy restricted to *Mus musculus*; and carbamidomethyl (Cys), oxidation (Met), Phospho (Ser, Thr, Tyr), and Deamidation (Asn, Gln), Acetyl (Lys) were entered as variable modifications. Two missed tryptic cleavages were allowed, precursor mass tolerance was set to 10ppm, and fragment mass tolerance was set to 0.02 Da. The significance threshold was set based on a False Discovery Rate (FDR) of 1%. The Mascot search results was exported as .xml files and then imported into the processed dataset in Progenesis QI software where peptides ID'ed were synched with the corresponding quantified features and their corresponding abundances. Protein abundances (requiring at least 2 unique peptides) were then calculated from the sum of all unique normalized peptide ions for a specific protein on each run.

A label free quantitation approach utilizing Progenesis QI (Nonlinear Dynamics) similar to that found in (82) was utilized to obtain quantitative information on peptides and proteins which can be referenced in the method.

Three independent samples from each genotype: empty vector and DYRK1B-wt transfected C2C12 cells were analyzed for proteomics analysis. The cells were serum starved prior to submission for proteomics analysis. The fold changes corresponding to each protein were calculated and subjected to the following filters: unique peptides >2 , p-value ≤ 0.05 , 2-tailed unpaired ttest, FDR ≤ 0.05 , Benjamini Hochberg (B-H) correction. The significantly changed proteins were used as inputs for Ingenuity Pathway Analysis (IPA, Version 03-13, Qiagen). The IPA predicted the altered pathways, molecular and cellular functions, and potential upstream regulators which are reported. For the latter, the data was filtered using p-value ≤ 0.05 and $-2 < z\text{-score} < 2$. The hierarchical clustering of the variables and the heat-maps for differentially expressed proteins were created by Qlucore Omics Explorer 3.4.

Statistical information: All statistical analyses were carried out using 2 tailed Student's ttest for pairwise and Anova one-way analysis for multiple comparisons using GraphPad Prism 6 Project software (GraphPad). $P \leq 0.05$ was used as statistical threshold.

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AUTHOR CONTRIBUTIONS

N.B. (**Neha Bhat Ph. D**) and A.N., (**Anand Narayanan Ph. D**) were primarily responsible for performing the experiments and the analysis and helped with the study, design and writing of the manuscript

A.M. (**Arya Mani M.D.**) designed the study and oversaw its implementation, supervised all aspects of the project from performing the experiments to the analysis of all data and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests

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Table1: The list of significantly proteins altered in C2C12 cells upon Dyrk1b overexpression. The cells were serum starved on day 7 of differentiation prior to subjecting them to proteomics by LC-MS/MS.

	empty-1	empty-2	empty-3	Dyrk1b oe-1	Dyrk1b oe-2	Dyrk1b oe-3	fold change	P-value	B-H corrected
PARD3	679.268	0.000	0.000	479859.94 9	444529 .864	364856.2 45	1897.992	0.006	0.019
SRGP2	1793.996	5228.258	0.000	968698.06 0	965377 .359	851072.2 94	396.617	0.002	0.012
4EBP1	0.000	44.442	6061.202	866305.06 5	678827 .896	512678.9 69	337.034	0.022	0.034
TM115	137.816	141.055	10758.33 3	1300886.9 38	816816 .544	806444.5 91	264.936	0.027	0.037
FBXW8	439.615	4453.868	1037.798	513374.05 8	435118 .284	293258.3 15	209.356	0.024	0.035
UBP10	69499.301	8234.846	43915.71 9	1590438.1 91	203195 0.109	1534370. 979	42.390	0.008	0.021
RM53	352.841	4072.329	1771.562	85716.778	55232. 935	55276.68 3	31.666	0.024	0.035
OAS1A	4580.182	150.117	1065.096	67358.694	56193. 442	45466.18 8	29.164	0.011	0.024
CCD43	13727.464	189132.53 7	103766.7 66	3343574.1 26	259788 2.498	1868422. 170	25.470	0.026	0.037
RABL6	9692.356	9243.969	13208.40 3	273248.48 4	234403 .272	256816.1 48	23.782	0.002	0.013
MPC2	8293.358	2238.299	3524.422	69176.705	101597 .030	140949.1 36	22.177	0.040	0.044
SZRD1	66553.305	112598.03 8	63217.99 0	2235824.2 78	182614 9.567	1143884. 324	21.479	0.035	0.042
AAKB2	11503.808	6591.234	42434.30 7	309998.34 5	296164 .391	521290.4 76	18.627	0.037	0.043
BTF3	384827.788	242426.96 3	263865.2 87	5507413.7 69	739796 2.061	3604114. 698	18.527	0.041	0.045
CX6A1	537894.509	530282.55 2	531813.6 18	11674390. 368	994188 3.303	7698285. 446	18.322	0.015	0.028
MAP1S	56743.571	107531.51 6	108146.1 46	2102590.0 39	141695 4.015	1203523. 802	17.337	0.031	0.040
GLO2	52313.445	11606.076	29713.98 4	699851.60 6	521091 .063	394519.9 30	17.253	0.027	0.037
NOL3	15675.270	1419.734	2041.776	103344.05 4	122061 .257	93595.23 6	16.669	0.002	0.012
RBM7	10612.589	28963.339	10035.02 0	312699.16 9	224580 .994	256634.7 15	16.003	0.008	0.021
CIAO1	13264.694	10506.011	6458.051	189522.22 8	139206 .944	131768.3 26	15.234	0.015	0.028
SYNPO	105298.054	171082.01 6	210844.8 16	2988051.2 30	201544 9.540	2269025. 895	14.926	0.015	0.028
HTSF1	149502.329	34803.291	50817.71 9	1245634.1 12	970474 .854	960379.2 68	13.510	0.004	0.017

STK3	33915.354	46305.559	96688.347	756642.823	827053.607	706344.311	12.945	0.000	0.007
NUP88	46971.434	9371.786	18785.385	302353.033	324721.664	304558.857	12.401	0.000	0.005
TOP2B;T OP2A	424178.160	722988.594	694984.049	9002297.995	946142.9231	4363991.935	12.392	0.050	0.050
MECP2	367233.497	173887.542	250422.422	2657858.379	342977.9.613	3107965.503	11.617	0.004	0.017
FBP1L	151490.348	460624.417	99890.094	3270143.416	339482.0.101	1527201.286	11.506	0.049	0.050
IFIH1	123990.956	60901.098	43052.157	1112649.104	881782.334	617624.718	11.459	0.028	0.037
CRIP2	10158549.015	14138805.082	13368147.619	180789372.776	153641388.860	86918587.075	11.187	0.044	0.046
APOO	447259.665	678180.836	519295.482	8335345.532	5993143.480	3922258.672	11.096	0.049	0.049
OGFR	103690.806	117790.974	89549.422	1181689.969	976676.122	807877.145	9.537	0.014	0.028
TAOK3	85857.500	91462.651	88414.390	957201.498	874323.495	646373.442	9.325	0.015	0.028
SRC	336633.322	304162.949	311831.862	2602676.009	3040000.579	2832144.937	8.896	0.002	0.013
CRIP1	37176422.610	20896835.306	22245157.309	208508798.706	231456162.263	225969029.451	8.291	0.000	0.004
MAN1	15127.085	4123.116	2425.068	68288.493	60759.181	48045.603	8.170	0.003	0.015
TYY1	228396.219	210436.541	289089.865	2478796.957	1985886.413	1295394.396	7.913	0.039	0.044
FAK1	112078.324	34582.888	21258.792	441261.065	467153.920	413685.110	7.873	0.001	0.010
IMPCT	170733.374	317894.405	365849.082	2475476.048	2214028.121	1965872.800	7.789	0.002	0.013
PIN1	177410.799	48570.704	89052.103	720388.604	770476.511	839616.057	7.398	0.000	0.006
F134C	10125.595	32917.475	12682.765	153549.569	104512.212	144933.819	7.232	0.007	0.020
TTC1	301034.655	744661.644	326342.888	3052718.976	3709371.250	3074290.225	7.169	0.001	0.009
CO6A1	37029.346	11811.636	18272.468	145718.883	165568.568	164180.604	7.085	0.000	0.006
BIG1	38244.193	13692.939	15218.385	136002.532	157747.528	180068.954	7.056	0.002	0.013
SETD2	43803.058	129973.845	105994.149	642948.512	674081.324	649240.764	7.028	0.001	0.009
RGPA1	95671.284	12776.126	87253.864	491112.411	415178.148	444905.637	6.904	0.000	0.008
RM38	62026.367	67432.852	23890.492	376507.369	329913.895	328052.354	6.746	0.000	0.006
CCD50	116327.840	448511.862	145713.880	1297995.539	1562846.483	1579762.050	6.249	0.001	0.010
RANG	3434373.390	3885090.305	7706432.710	35021596.473	30223736.776	25165096.131	6.017	0.005	0.017
ARHGH	37981.201	27925.885	35442.288	214258.545	201329.754	190986.677	5.985	0.000	0.007
PABP2	600710.850	496001.740	283734.556	3010110.289	3144612.090	2072956.400	5.960	0.016	0.029
NFIC;NFI A;NFI FTO	48261.849	63853.427	57528.245	316722.677	302442.399	358297.795	5.762	0.002	0.013
	13680.806	942.627	3221.398	28455.550	32024.434	41910.434	5.738	0.007	0.020
CPSF7	145634.106	110484.934	170242.635	1065131.458	801660.654	566625.631	5.707	0.042	0.045
CHMP5	109070.191	26605.182	93752.292	479592.107	485616.330	283689.238	5.444	0.024	0.035
RBGPR	52521.289	17806.341	13593.308	189402.255	121032.553	146358.930	5.443	0.010	0.023
ADA10	704181.097	674327.473	576338.547	3901158.489	3566425.626	3055272.386	5.383	0.006	0.019

ACTY	233297.292	45047.906	36634.408	756265.368	597718.453	337786.714	5.371	0.044	0.046
TISD	209711.762	330378.260	112840.801	1009988.050	126669.2438	1210498.119	5.341	0.001	0.010
PSMD8	337690.724	27452.240	90596.629	943279.008	805657.477	597341.965	5.148	0.010	0.024
DHI1	42143.883	24594.542	33073.492	147174.440	173927.796	191212.579	5.133	0.004	0.016
SFSWA	408221.218	358614.840	304918.508	2085178.210	173859.0873	1597972.814	5.059	0.008	0.021
NCBP1	290301.700	115397.814	205471.565	1126921.145	977697.610	916051.550	4.942	0.001	0.009
UBE4B	65315.789	39758.854	46338.859	269670.258	227934.397	242742.816	4.890	0.000	0.008
NRAP	506613.665	594519.348	731534.134	3678786.614	290434.0490	2356906.843	4.878	0.023	0.034
GIT1	88487.381	104287.339	59960.704	400906.798	374746.146	420862.796	4.734	0.000	0.005
SYNC	3233919.547	3957874.381	3612109.794	17529864.818	161544.28.110	1551409.5.893	4.554	0.001	0.009
TNNT1	235129.549	154700.413	198477.200	990892.746	910145.961	699775.138	4.421	0.012	0.025
LASP1	4346579.602	4703636.272	5397472.138	19265450.436	229805.71.292	2064481.6.587	4.353	0.003	0.014
PHLB2	513357.994	306678.211	380663.945	2286367.984	171992.2.241	1217270.522	4.350	0.045	0.047
K1522	118709.489	193654.205	109968.878	729361.182	566918.346	534819.599	4.336	0.008	0.020
MCM5	18150.942	7475.227	3385.784	55084.595	31248.343	39419.632	4.335	0.024	0.035
FKB15	595366.504	749484.705	821676.404	3813357.596	283262.5.747	2698567.810	4.313	0.018	0.031
UBP8	103907.382	116805.563	94337.452	443955.623	452213.017	453360.384	4.284	0.000	0.004
COPB2	7976430.089	5211499.551	6811101.839	24786331.232	307407.32.740	2948635.8.953	4.251	0.002	0.013
AKAP2	1661709.827	1662392.086	1405698.016	6884477.227	676345.7.707	6110163.055	4.177	0.001	0.009
TMF1	231286.439	215063.303	138264.365	908030.996	797787.924	722554.607	4.154	0.002	0.013
PAK2;PAK3	1087028.188	1047971.334	1441129.824	4618299.800	462542.2.381	5289721.212	4.064	0.001	0.009
NCKP1	18599.601	5349.497	5970.629	42126.474	37895.809	41108.117	4.049	0.014	0.027
WFS1	392725.795	249859.061	209072.804	1195211.320	118054.7.660	1021239.305	3.989	0.000	0.008
JUN	95005.250	13918.855	25786.443	136525.681	153407.479	237880.963	3.918	0.033	0.041
TPSN	3962857.125	5610873.126	6997434.449	22878831.933	228803.43.898	1881424.9.078	3.897	0.001	0.011
CASQ2	3299064.899	1766056.540	2395635.810	10055370.988	106658.64.369	8154959.083	3.870	0.003	0.014
CO8A1	121808.300	18807.628	19506.623	244977.435	190709.668	183514.036	3.867	0.027	0.037
MAVS	141836.208	125818.653	118425.165	592486.350	503621.232	381490.226	3.827	0.026	0.036
MYCBP	303841.454	365772.876	205976.101	1310733.362	115914.5.321	872144.118	3.817	0.015	0.028
TMX3	1847847.489	2320752.118	2372258.700	8128685.320	886363.6.912	7924100.260	3.809	0.000	0.006
STAT1	550097.001	302080.394	456055.166	1673125.218	168740.9.378	1593234.336	3.787	0.001	0.010
CT2NL	76229.206	54262.595	55245.910	231572.990	223646.791	245728.064	3.774	0.000	0.005
NID2	2204639.783	2380140.648	2236777.654	9619710.766	860404.6.962	7397748.473	3.756	0.010	0.023
WWTR1	552463.394	451382.981	445402.515	1646211.934	191047.0.214	1757084.711	3.667	0.001	0.010
IPO9	38456.534	8807.167	15764.083	97604.296	69949.941	62149.879	3.644	0.018	0.030

FCL	54565.683	8117.371	12042.34	105257.60	87832.	78391.86	3.633	0.029	0.038
			8	6	997	9			
PRDBP	110990.518	68438.114	36666.80	298690.61	265586	220156.8	3.630	0.004	0.016
			0	1	.386	59			
DUS3L;S	144155.810	132211.18	112267.0	467163.57	492487	423083.7	3.558	0.001	0.010
MG1		9	26	5	.031	20			
TNS2	62448.077	209171.87	133731.3	566152.40	418925	423283.1	3.474	0.007	0.020
		3	73	6	.108	75			
PPA6	701155.266	704709.01	765815.8	2613783.9	267369	2227777.	3.461	0.005	0.018
		3	28	93	3.193	258			
C560	102885.005	30676.059	39140.26	211535.09	207613	176180.8	3.447	0.012	0.026
			0	7	.370	68			
AL3B1	84336.594	27516.205	48589.54	189204.48	177716	184243.9	3.435	0.013	0.027
			2	5	.868	35			
RD23A	12503.061	42585.207	25286.11	79611.983	95422.	97969.53	3.397	0.006	0.018
			7		633	1			
IF4B	6396613.41	6084848.1	5497364.	18901264.	213879	2055417	3.384	0.001	0.010
	9	30	920	980	97.214	6.127			
PXDN	120197.144	49431.515	42140.00	221471.42	261506	228646.9	3.360	0.010	0.023
			5	3	.366	61			
EIF3M	168352.743	54063.410	77264.75	444596.63	322328	228407.9	3.321	0.045	0.047
			8	7	.879	02			
IFIT3	68563.906	102615.29	109395.0	275607.77	304815	343984.8	3.295	0.002	0.012
		7	16	2	.327	65			
KLC4	73916.593	84594.241	89503.04	272479.06	276730	267760.6	3.294	0.000	0.004
			3	3	.307	24			
GYS1	153297.320	270964.15	97882.05	447461.45	618002	644491.3	3.275	0.009	0.022
		3	4	3	.578	31			
AAK1	140786.512	51752.610	71407.88	319988.30	308794	233416.7	3.267	0.006	0.019
			3	6	.260	87			
PGAM1	19098881.2	12117231.	1504022	53345875.	508636	4651059	3.258	0.000	0.006
	20	374	8.780	702	79.123	2.250			
HDAC2	3942761.17	6276665.4	5299043.	16812504.	172415	1646381	3.255	0.001	0.012
	5	18	222	105	33.212	5.368			
STRN	282469.204	217653.30	140045.2	722776.38	669542	604893.1	3.120	0.001	0.011
		7	22	1	.846	25			
EXTL3	43536.770	119751.39	87987.13	283621.67	256710	233445.8	3.079	0.005	0.017
		8	0	1	.037	74			
TM1L2	63518.381	117040.96	55301.98	273507.05	240632	210196.9	3.071	0.004	0.016
		5	4	9	.471	23			
ATE1	52751.621	46190.325	29153.44	167046.68	125397	98218.09	3.050	0.037	0.043
			8	8	.245	3			
UN45B	103352.973	32612.881	61914.08	200859.80	184178	200965.2	2.961	0.019	0.031
			1	6	.717	81			
GDIR2	1586031.95	1593095.0	1705656.	5562715.5	504710	3684008.	2.926	0.030	0.038
	4	73	296	34	7.960	367			
SR140	4972.662	13595.106	11283.50	31539.738	25342.	29945.94	2.909	0.005	0.018
			6		837	0			
NCOR1	189115.130	70507.862	157725.6	336285.28	436038	435763.0	2.895	0.006	0.018
			55	4	.642	96			
HMCS1	487000.992	384333.57	315568.6	1372534.9	123316	828895.3	2.894	0.035	0.042
		8	70	46	8.410	40			
GRIN3	83092.644	49103.033	71848.31	200968.52	183683	204765.5	2.889	0.001	0.010
			9	5	.468	19			
DLGP4	971901.406	1057325.1	942940.0	3520686.8	286840	2193184.	2.888	0.039	0.044
		51	43	84	1.878	291			
PFKAL	632866.659	508933.03	499951.5	1755960.5	157559	1401319.	2.883	0.004	0.017
		1	58	63	8.370	993			
BNI3L	373241.398	513827.77	356291.4	1130995.2	126566	1160987.	2.861	0.000	0.007
		1	45	45	4.651	559			
MLEC	4962012.67	4469091.9	4557307.	13526529.	139326	1233544	2.845	0.001	0.012
	7	10	811	707	34.332	1.072			
ITPR3	383574.821	296673.13	342292.6	1207809.2	886525	776128.6	2.807	0.037	0.043
		2	42	26	.336	18			
ATAD1	2238505.22	2332416.5	1328951.	5915466.9	534025	5195938.	2.788	0.001	0.012
	5	24	718	89	3.139	734			
POSTN	390339.549	424514.32	535488.1	1332271.3	119075	1237152.	2.785	0.000	0.006
		3	73	08	7.035	380			
PGAM2	51250.326	25648.435	59017.58	138440.17	110181	127437.1	2.767	0.004	0.017
			4	4	.910	44			

BAP31	5106823.05 7	4380420.2 22	6028119. 162	14962621. 093	136992 41.654	1419402 9.886	2.762	0.000	0.006
ZO1	6796742.61 7	9903693.4 56	6984112. 567	23009275. 758	220525 59.916	2013969 5.007	2.753	0.001	0.009
CCAR2	1050031.64 0	758296.84 8	1074097. 707	2793224.6 53	269192 9.806	2352369. 877	2.719	0.001	0.009
HYOU1	5348272.85 4	5276819.0 40	4755788. 140	15167150. 210	143692 39.814	1218996 3.552	2.713	0.008	0.021
UBA6	237647.525	571440.01 1	287858.3 63	992804.51 4	103529 3.642	942617.5 95	2.708	0.021	0.033
SFPQ	12638226.0 96	18297404. 156	1630961 0.910	48550970. 697	431877 66.549	3574479 3.867	2.698	0.009	0.022
F177A	175210.511	91716.709	134814.8 70	379698.61 4	383871 .154	292731.0 59	2.629	0.005	0.018
YTHD1; YTHD2	198651.291	155880.41 4	149960.1 54	385537.41 4	453528 .337	485864.9 05	2.626	0.004	0.016
LMAN1	3680336.97 4	1493063.5 89	2419692. 171	8563303.2 81	658959 9.683	4697003. 336	2.614	0.046	0.048
TIF1B	10931255.1 79	10649751. 707	1018629 2.719	32234592. 961	283585 76.196	2183132 7.481	2.595	0.030	0.038
PPP6	73320.231	49797.790	88524.11 6	126523.40 0	203638 .712	217157.7 26	2.586	0.043	0.046
M4K4;MI NK1	704629.176	548413.14 6	712450.3 80	1382250.3 46	183505 7.762	1829825. 264	2.568	0.013	0.027
LAMC1	547921.391	297965.81 2	486135.0 41	1273430.9 52	112439 3.459	1013781. 308	2.561	0.003	0.014
MY18A	1296222.86 8	1888220.0 18	2127385. 476	5131401.4 77	452252 0.356	3914271. 467	2.554	0.004	0.017
TOM1	801861.015	606119.78 7	575628.6 95	1698326.7 83	163810 9.391	1727419. 501	2.553	0.002	0.013
BROX	6080169.64 4	3267694.5 37	4513341. 271	11250750. 071	121447 89.356	1198081 8.278	2.552	0.007	0.020
PUR8	2362703.96 5	1577309.4 22	1747741. 152	5157789.3 56	483317 8.163	4497172. 480	2.547	0.001	0.009
ACTN2	429776.864	449350.21 2	441931.2 72	1220804.3 59	114844 4.215	970755.2 54	2.528	0.012	0.025
XIRP1	6128864.79 9	5865227.1 33	6465301. 475	18542363. 839	156157 08.632	1227997 9.326	2.516	0.035	0.042
AMOT	194796.885	289772.91 7	108061.0 20	442024.82 7	475791 .389	569466.8 60	2.510	0.012	0.026
PLCC	110561.698	172279.66 5	53615.82 6	260965.02 1	300970 .313	281264.1 88	2.506	0.029	0.037
RT22	429775.767	195618.26 4	199041.3 93	782257.05 8	711744 .421	566788.2 79	2.500	0.016	0.029
AN32B	977315.332	911469.97 0	724000.0 10	2728047.9 80	185496 0.871	1806849. 483	2.446	0.045	0.047
TEBP	3201746.20 3	1521555.5 78	2003455. 662	6405645.5 66	546508 0.246	4508306. 358	2.435	0.012	0.026
PB1	265894.191	319935.84 6	223486.0 20	621293.28 7	697752 .288	647085.3 46	2.429	0.001	0.009
GDIA	449988.644	339449.65 8	262256.0 49	899600.73 5	800983 .937	845982.7 42	2.421	0.004	0.016
GLRX1	352107.245	142189.68 0	246836.4 51	582423.89 9	558183 .819	653456.3 92	2.421	0.015	0.028
HDAC1	2022538.89 8	3662695.7 11	2886740. 652	7140909.2 23	669859 7.643	6894507. 367	2.419	0.009	0.023
GAMT	144670.707	144360.72 0	99913.89 1	365453.85 9	279065 .984	296092.2 81	2.418	0.008	0.021
CAMP1	156811.909	194548.47 1	97209.31 6	351107.66 8	418775 .398	309507.5 57	2.406	0.008	0.021
F195B	1427537.95 8	1173077.6 44	966630.7 65	2894311.7 37	349491 6.526	2126141. 406	2.387	0.042	0.045
KLH40	490373.596	498375.03 3	303570.0 20	1133597.7 69	102025 2.136	929616.6 18	2.386	0.002	0.013
LUC7L	26213.349	21816.054	21767.50 6	41470.665	60059. 808	64444.33 1	2.378	0.040	0.044
PFKAM	430415.804	323222.64 0	321684.2 45	851013.20 6	921409 .065	773012.5 45	2.367	0.001	0.010
TRI72	1258137.30 4	1528684.5 84	1716484. 665	4118329.7 18	351046 4.138	2992638. 919	2.359	0.014	0.028

HYEP	6372198.19 9	7763060.6 64	7312868. 839	20295649. 254	165717 88.487	1365255 7.536	2.355	0.032	0.041
MYG1	171927.216	86883.564	108668.9 17	247156.96 7	289691 .479	327434.1 69	2.352	0.009	0.022
MYL1;PL CL1	6376975.77 2	6615813.4 35	8019408. 256	17105661. 069	169210 58.470	1492332 7.243	2.330	0.001	0.009
PCBP2;P CBP3	4540899.70 1	4307797.1 98	5116022. 020	11383034. 747	111023 82.648	9591824. 099	2.297	0.003	0.015
EF1D	14347677.5 89	21384815. 072	1557367 9.650	36256027. 223	391680 25.713	4153257 3.757	2.280	0.002	0.013
SMCA5	1255064.91 1	1378515.1 23	1357633. 153	3370233.7 23	298606 5.620	2675721. 722	2.263	0.012	0.025
EHIL1	1550649.85 4	1102993.4 10	1618299. 464	3268572.7 96	327501 7.598	3123688. 879	2.263	0.005	0.017
RLA2	11450228.1 93	4677671.4 164	6237082. 807	18187538. 164	180313 42.046	1399383 5.106	2.245	0.025	0.036
MIF	6600552.02 5	2529145.1 77	3772914. 835	9715783.3 70	988817 8.215	9344128. 017	2.244	0.045	0.047
CTBP1	685989.554 2	318018.45 57	424467.9 99	1147113.9 99	114127 9.446	914441.2 77	2.242	0.015	0.028
FABP5	1102246.87 8	1211431.3 91	1064042. 070	2881157.3 84	249973 1.045	2190521. 927	2.242	0.016	0.029
VAPB	2288124.56 7	1022521.3 33	1586685. 206	3892921.0 38	348544 4.937	3556062. 729	2.233	0.022	0.034
NXP20	352978.775 9	132565.24 9	217070.6 03	520872.72 0	554816 .641	481338.1 55	2.216	0.037	0.042
SRBS1;I NSR	4718919.74 5	3303858.2 67	3912922. 640	8600306.6 12	869266 0.020	8746917. 716	2.182	0.007	0.020
CYTB	4347184.79 2	2787682.4 24	2577758. 123	6955792.4 85	719030 7.078	6956698. 568	2.173	0.019	0.032
MTX2	211136.099 4	308978.42 34	205138.0 2	440856.94 2	560575 .868	573449.7 20	2.171	0.007	0.020
SY2L	3813641.75 7	2904832.0 41	3654410. 837	9001360.3 58	720310 0.389	6244373. 174	2.164	0.028	0.037
DENR	123835.824 0	122408.19 0	100470.3 78	261899.79 7	259171 .136	229192.0 02	2.164	0.001	0.009
LRC47	305327.028 7	408928.21 63	420346.6 5	949021.73 5	810844 .350	687261.8 95	2.157	0.015	0.028
MYH8	134588.572 8	139269.95 8	123907.1 06	284065.67 1	293214 .757	276220.6 46	2.146	0.000	0.004
G6PE	90004.808 8	41037.315 8	60569.10 2	148030.83 2	127790 .901	132592.1 26	2.131	0.023	0.035
NDUB7	2465843.34 3	2205918.8 96	2527556. 182	6199339.3 74	465659 8.654	4335675. 599	2.110	0.040	0.044
ZC11A	307627.234 5	503811.23 5	291214.8 36	835496.31 7	734126 .803	753426.1 06	2.107	0.015	0.028
LGUL	226856.132 89	92167.989 89	135500.9 6	329197.34 6	335863 .143	291752.0 09	2.105	0.040	0.044
RFOX2;R FOX1;RF OX3	275090.531 9	151502.96 9	182097.7 96	437961.23 3	457857 .873	382984.3 90	2.101	0.011	0.025
PTGR1	304761.925 2	169101.59 64	165960.1 5	543173.37 5	460618 .798	339364.6 08	2.099	0.038	0.043
PA1B3	80789.243 7	69233.358 7	60313.60 7	150948.29 8	145484 .889	144646.1 73	2.097	0.003	0.014
FXR1	2570134.45 5	4094320.8 02	4814617. 920	7698123.8 73	812290 3.521	8182357. 733	2.091	0.020	0.032
DCPS	497903.615 7	357159.22 40	441398.2 40	829457.96 5	101868 9.454	857923.3 61	2.087	0.004	0.017
SYSC	3258754.25 5	2593883.7 07	2984964. 655	6745418.9 68	639560 1.072	5244986. 105	2.080	0.010	0.024
LUZP1	122222.452 0	171773.45 0	109633.9 23	331847.59 6	259864 .233	247196.1 75	2.078	0.014	0.027
XRN2	3675197.24 4	3519295.2 18	4133414. 523	7803864.0 34	801718 7.844	7654276. 808	2.072	0.000	0.006
EIF3A	11428637.6 80	12311141. 078	9527309. 528	22117241. 681	241486 07.722	2262491 6.831	2.071	0.000	0.008
RL6	18666484.6 87	20614112. 730	2178938 9.269	48356391. 022	446174 63.677	3330164 3.745	2.068	0.036	0.042

FUBP1	2863882.70 8	4479619.8 95	3371139. 736	7322863.4 92	809857 6.352	6610376. 349	2.056	0.004	0.017
AGM1	22863.393	13869.345	10410.76 3	32756.955	33983. 721	30110.42 0	2.054	0.037	0.043
PRKRA	969560.866	1209236.1 61	740031.6 55	1774760.2 37	209042 2.442	2120016. 224	2.051	0.005	0.017
DCTN1	658000.262	351468.66 2	600476.6 90	1293252.6 02	108293 4.909	922870.5 57	2.049	0.017	0.030
GDIR1	15139182.7 51	14840625. 847	1389290 3.084	31064916. 769	301184 11.806	2794020 8.146	2.031	0.001	0.011
TOP1	2258417.15 7	3363161.9 47	2663458. 551	5421489.9 95	559948 5.999	5737514. 493	2.023	0.009	0.022
CSTF3	3299868.30 9	5228924.1 83	2811312. 273	6974740.2 06	796559 4.156	7935932. 676	2.017	0.021	0.033
DPM1	142763.865	238841.27 5	115739.8 03	323146.43 9	384397 .271	288690.1 37	2.003	0.027	0.037
TMX4	903009.799	1464388.3 81	1009189. 822	2273816.3 67	226562 0.267	2204957. 169	1.997	0.021	0.033
HCDH	555245.481	282722.20 5	296445.4 16	880941.42 3	728186 .031	652967.3 73	1.994	0.031	0.039
DGCR8	78637.846	108861.68 3	100484.6 67	206217.95 1	202097 .455	163075.1 96	1.984	0.007	0.020
SPEE	415089.029	352554.77 5	273382.6 06	619809.39 6	739847 .193	701256.9 59	1.980	0.004	0.015
CDC5L	413716.085	229889.32 1	187947.0 21	640281.79 7	507539 .020	498041.4 21	1.979	0.038	0.043
HMG5	562515.586	827475.12 6	706377.2 21	1301821.1 04	159366 4.037	1247986. 821	1.977	0.009	0.022
MLRS	8860682.32 4	9893523.1 05	9688159. 785	18560314. 083	194726 61.774	1800571 0.844	1.970	0.000	0.005
SNX3	219937.207	187342.51 6	171017.7 82	313464.51 5	357865 .099	461721.7 81	1.959	0.041	0.045
SRCA	2447946.80 5	1619798.7 63	2117339. 677	4082588.4 39	403919 5.162	3935907. 936	1.949	0.013	0.026
OX2G	384912.733	278531.58 0	261251.8 66	542003.23 5	626611 .989	632186.1 21	1.947	0.005	0.017
NIPS2	415849.457	450491.50 8	403058.6 96	872533.10 0	754230 .453	840636.5 54	1.944	0.003	0.015
PLIN2	748190.535	760146.85 8	733581.1 69	1413049.9 99	139373 7.905	1465467. 150	1.906	0.000	0.007
PYGM;P YGB;PY GL	523556.739	466636.33 0	468421.8 37	885822.96 1	937804 .633	950297.3 29	1.902	0.000	0.005
DNMT1	94296.976	125074.02 9	83098.90 6	161485.13 0	201927 .502	211626.7 62	1.901	0.011	0.025
NUP98	1529316.66 4	912522.65 4	1303361. 834	2514742.7 84	235403 3.925	2177560. 597	1.881	0.012	0.025
GPTC8;Z 804A	421565.687	518544.83 2	395954.1 14	778764.38 8	859031 .503	871542.6 29	1.878	0.001	0.012
PTK7	260084.824	207910.16 3	278574.7 90	552331.33 6	444282 .754	400003.3 34	1.871	0.025	0.036
EHD4	716913.012	484177.54 5	541884.2 92	1071647.8 86	120149 8.488	982073.2 81	1.868	0.006	0.019
PRUN2	2316641.64 2	1901451.7 39	2250497. 869	4263126.7 09	376998 1.991	3978482. 425	1.857	0.001	0.009
ARLY	160534.940	115384.70 5	153181.6 58	250334.59 6	266865 .302	272635.7 58	1.841	0.005	0.018
CYBP	698532.487	430798.89 5	531773.2 55	1028491.2 91	984461 .403	1032848. 945	1.834	0.024	0.035
IBP5	1281254.32 7	1258815.9 57	1330210. 085	2335811.7 90	252199 4.621	2216513. 541	1.828	0.005	0.017
LDB3	614283.056	665065.55 5	600828.3 08	1224599.9 50	126513 4.194	943131.8 30	1.826	0.032	0.040
MA7D1	647372.647	420117.47 9	474043.6 15	898437.56 3	911333 .770	996348.1 32	1.820	0.014	0.027
ENPP1	522104.881	703640.26 6	534398.6 62	1209128.2 70	104642 0.409	899011.2 19	1.792	0.017	0.030
TFP11	1425056.37 2	1300887.1 00	1962448. 097	2337062.7 32	260797 0.956	3416187. 233	1.783	0.042	0.045

MATR3	9791642.74 6	8736625.7 78	8431487. 125	17609926. 587	157986 22.910	1450975 1.897	1.777	0.007	0.020
EIF3G	4676272.12 6	5870516.6 32	5134159. 453	9610283.1 00	104822 03.609	7775811. 949	1.777	0.023	0.034
TNNT3	19637772.3 46	18621092. 426	1828621 2.592	34664214. 918	340666 56.546	3168860 5.816	1.776	0.001	0.010
RL4	5740578.08 2	4170647.8 91	4521568. 569	9536304.8 57	830113 0.394	7573453. 416	1.761	0.009	0.022
MIC25	152332.696 2	141123.85 2	151975.2 11	268631.42 9	272743 .286	241548.9 04	1.758	0.003	0.015
DYSF	172946.013 3	190571.00 3	181194.2 21	304072.78 8	347446 .591	301771.4 92	1.750	0.007	0.019
DJB11	1359294.40 4	1378035.5 91	891095.2 03	2408123.3 08	201772 1.279	1920019. 726	1.749	0.014	0.028
NDRG1	2798704.81 1	3512715.7 63	2766395. 627	5173690.2 04	538068 6.966	5309678. 624	1.748	0.008	0.021
REEP5	588824.632 9	467667.69 9	608685.5 95	914680.69 7	953485 .227	1039249. 872	1.746	0.002	0.013
KANK2	1190993.98 1	1654178.1 70	1594660. 354	2793419.0 14	254040 8.816	2385768. 006	1.739	0.005	0.017
DCTN4	240464.765 4	136600.28 4	155323.5 10	340572.90 3	311143 .682	265050.5 13	1.722	0.036	0.042
PIPNA	405270.606 4	572411.86 4	630019.5 46	995292.99 8	983737 .387	787147.9 55	1.721	0.016	0.028
INF2	881352.501 90	1021456.6 90	1151534. 801	2004504.2 80	165848 1.630	1583916. 478	1.718	0.014	0.027
UBXN4	657379.300 9	523065.00 9	551494.3 90	1091413.8 03	101041 7.631	869446.1 20	1.716	0.009	0.022
MOT4	256274.514 2	175074.90 2	260399.4 54	393701.19 9	435360 .181	354815.3 24	1.711	0.011	0.025
ABCE1	311893.816 5	281923.78 5	305809.6 81	500231.20 0	498062 .270	526299.8 10	1.695	0.000	0.005
PLCG1	468084.566 2	320572.45 2	469151.8 80	661448.34 7	753107 .614	707358.3 00	1.687	0.014	0.027
GALK1	469922.768 3	507799.14 3	507823.5 50	801837.64 2	854374 .464	849235.1 01	1.687	0.000	0.006
ATX2	604282.915 8	437334.68 8	574553.8 60	898169.31 3	952562 .585	868612.5 78	1.683	0.009	0.022
FLII	990086.983 2	661851.61 2	694234.6 44	1385319.0 65	127684 0.130	1275918. 845	1.679	0.026	0.037
RBBP4	6155712.38 1	7626422.5 79	7160175. 906	12407671. 348	118422 73.388	1084509 3.326	1.676	0.002	0.012
GMPPB	284474.721 1	328253.22 1	250157.2 73	434084.80 0	517152 .078	492996.2 00	1.674	0.005	0.017
S12A7	282175.413 8	175007.94 8	203208.0 69	334325.61 7	435913 .981	333184.0 96	1.671	0.034	0.042
PURA2	168981.857 3	109594.79 3	96861.91 2	234058.21 6	212333 .530	180672.4 44	1.670	0.042	0.045
TOR3A	689945.905 7	828569.70 7	590049.0 63	1177320.5 51	116055 4.377	1178538. 956	1.668	0.020	0.032
CNN2	1860420.82 1	1380430.0 33	1554688. 543	2807369.1 13	254225 8.886	2599718. 925	1.658	0.006	0.019
CAD13	3274025.59 4	4748858.5 79	3763804. 254	6879925.9 97	636398 8.869	6204021. 268	1.650	0.015	0.028
KAD1	1993305.03 6	1380382.1 09	1743311. 531	2865573.7 56	281308 3.734	2749345. 912	1.647	0.022	0.034
LG3BP	2538320.86 5	1807392.1 34	1990102. 272	3993101.5 92	363394 6.344	2807598. 185	1.647	0.039	0.044
PPIA	63395649.8 54	39596642. 085	5265335 0.118	87991133. 413	865623 27.760	8165359 6.352	1.646	0.032	0.040
EHD1;EH D3	564938.387 9	516281.15 9	553571.7 43	871134.01 0	770371 .471	1031087. 240	1.635	0.041	0.044
PTN12	953381.542 3	796839.07 3	960101.8 65	1446124.0 78	147441 4.643	1506334. 081	1.633	0.005	0.017
LIPB1;LI PB2	1176197.99 1	1016808.4 20	1431900. 884	2235034.7 97	176323 5.581	1908721. 745	1.630	0.015	0.028
MYH1	3412313.08 6	3826143.0 83	3737559. 725	5510835.4 54	643951 3.486	5881494. 777	1.625	0.006	0.018
EIF3L	1858117.16 4	1589740.8 27	1726365. 882	2879144.5 45	292526 9.369	2590999. 638	1.623	0.002	0.012

AT1A2;A	332186.978	299285.03	309552.5	527197.87	521667	469562.3	1.614	0.003	0.014
TIA3		5	21	8	.880	20			
UBA5	90451.204	93409.855	84147.64	127482.58	151791	153158.5	1.614	0.015	0.028
			1	4	.890	78			
CAPG	5074828.36	3998987.8	5164381.	7567898.7	760877	7783459.	1.613	0.014	0.028
	2	01	619	86	0.121	347			
MYEF2;	2157581.19	1973380.5	1595909.	3030412.4	305770	3116725.	1.607	0.018	0.030
NSD1	5	32	132	04	9.182	845			
CUL4B	481315.190	462412.40	454521.8	801213.84	726408	718573.0	1.606	0.005	0.018
		5	41	8	.858	16			
CO1A1	3142505.04	4443533.7	4140698.	6470037.8	658369	5746033.	1.603	0.011	0.024
	9	12	343	30	9.960	114			
IRS1	2086837.65	1908892.2	2145422.	2742816.4	368881	3407279.	1.602	0.041	0.045
	7	05	113	49	5.008	959			
MIA3	592162.119	439809.25	483585.4	850030.61	844098	733831.9	1.602	0.007	0.020
		4	04	9	.776	57			
CSN8	402278.434	266390.61	453790.3	632382.03	593033	568273.2	1.598	0.046	0.047
		7	32	0	.250	59			
IF5A1;IF	10889837.2	15835629.	1333368	23435374.	213273	1915174	1.596	0.014	0.028
5A2	39	627	8.978	997	94.768	9.365			
ODPX	311977.468	281290.38	262228.5	472882.81	470326	414787.1	1.587	0.003	0.014
		3	33	2	.286	93			
CN166	1176464.19	781019.67	824924.0	1443195.9	152959	1440604.	1.586	0.043	0.045
	3	9	02	50	8.921	773			
TPM3	6368168.34	5725120.3	5446617.	10342346.	984713	7565047.	1.582	0.047	0.048
	8	96	003	938	2.706	296			
PLST;PL	7413567.15	7470563.2	8045661.	12249797.	119791	1199213	1.580	0.000	0.008
SI	5	54	536	995	66.088	9.235			
TNNC2	506575.815	645331.42	598046.8	916146.30	927253	920260.3	1.579	0.014	0.027
		1	03	0	.181	05			
NRDC	338685.510	442904.80	482793.0	653402.39	663137	671948.2	1.573	0.029	0.037
		7	24	4	.932	68			
MBOA5	77809.786	82868.189	69803.16	130671.14	119656	111283.7	1.569	0.005	0.017
			6	8	.023	99			
FAM21	2868084.54	4361563.2	3370096.	6526410.0	523177	4855923.	1.567	0.041	0.045
	4	69	030	82	6.087	456			
HSP72	101540.606	77614.829	67539.43	113846.59	123925	148686.5	1.567	0.032	0.040
			1	1	.027	80			
PRAF1	478507.305	330911.74	351097.4	540611.39	640009	636670.5	1.566	0.022	0.034
		0	58	7	.125	82			
PROF1	11774500.3	8123663.8	1099763	17174541.	159989	1519090	1.565	0.019	0.031
	86	53	9.940	785	54.103	9.109			
DDX58	469368.073	742327.25	681788.3	1048978.5	939450	967392.7	1.561	0.036	0.042
		6	97	81	.822	29			
NID1	575406.893	528008.17	601838.0	858308.77	896847	903667.2	1.559	0.001	0.009
		7	16	6	.469	24			
BPNT1	119413.142	96660.535	113845.7	166791.46	181630	165187.9	1.557	0.003	0.014
			56	0	.419	87			
PUR4	392712.163	301389.07	294789.5	474812.24	546142	512931.1	1.551	0.012	0.026
		0	16	5	.550	51			
GT251	2236308.24	1876649.2	1854068.	3581780.6	301564	2633332.	1.547	0.042	0.045
	8	04	241	20	3.216	067			
HEM6	300145.932	325653.92	357618.0	467883.11	533379	518662.8	1.546	0.003	0.014
		3	43	4	.794	73			
LDHA;L	25298202.4	31023494.	3452636	50018240.	475577	4226108	1.539	0.010	0.024
DHB;LD	17	550	4.699	275	74.064	7.681			
HC									
44081.00	4986918.33	6168257.9	5546258.	8800598.2	822344	8560824.	1.532	0.005	0.017
	7	23	026	98	5.764	433			
AT2A1	5228206.82	5961535.6	5804592.	8806148.0	903091	8100382.	1.526	0.001	0.012
	5	11	609	85	0.426	104			
RS28	4918218.65	6488913.9	7241952.	9390370.2	101711	8884512.	1.525	0.023	0.035
	4	60	771	34	98.557	124			
SPEG	114395.293	104362.00	123648.4	168449.39	201676	150509.9	1.521	0.045	0.047
		9	33	6	.691	75			
ACTS	16614776.5	13980927.	1501752	23028366.	249214	2121571	1.516	0.005	0.018
	40	967	9.329	530	10.005	8.455			
ABRAL	689750.347	590797.59	644435.6	991998.32	926118	988167.0	1.510	0.001	0.010
		1	56	7	.226	35			

XPO2	663205.166	627703.554	528498.375	888550.345	921924.866	931596.174	1.507	0.011	0.024
SRC8	10794881.539	13225510.396	1316974.2643	19760287.973	182316.04.982	1799289.2.797	1.505	0.004	0.017
KCRM	3585150.830	5153916.644	4812569.121	7668228.246	674498.1.427	5976426.521	1.505	0.029	0.038
ARL3	143215.680	141753.435	119815.233	192943.654	198710.116	216576.985	1.503	0.003	0.014
P66B	259583.541	199867.062	190360.797	285796.722	328243.097	361919.991	1.502	0.024	0.035
PGK1;PGK2	33648066.755	34607340.844	3866111.8957	56832164.710	545721.49.617	4907341.8.723	1.501	0.005	0.017
CASC3	2030574.039	1479141.874	1916120.948	883346.171	907549.937	900374.574	0.496	0.032	0.040
S10AA	28439396.360	34574314.790	3671090.6.843	15787815.103	164666.98.400	1711822.0.964	0.495	0.019	0.032
PHB	17769223.144	23208265.707	2352779.9.577	10279239.131	107468.35.437	1079726.7.213	0.493	0.027	0.037
MXRA7	3864038.366	3024435.125	4305596.441	1458116.073	213970.3.711	1901182.671	0.491	0.020	0.032
CCD47	766109.860	1002937.677	973033.298	386798.956	479931.818	474704.468	0.489	0.014	0.028
SERC1	332700.852	386029.364	345583.242	134379.510	194007.520	191874.707	0.489	0.002	0.013
YBOX1;YBOX2	271494652.876	240472736.452	192053772.383	77448143.784	113126.934.32	153295790.526	0.488	0.020	0.032
LAMP2	17106947.205	23494893.216	2331099.1.233	9271273.840	104719.44.969	1130729.2.672	0.486	0.027	0.037
VAMP8	2022347.383	1902167.862	2003868.010	806670.019	993663.512	1043379.824	0.480	0.001	0.010
CHAP1	951849.131	1423602.724	1199355.068	493179.109	578989.557	639230.601	0.479	0.035	0.042
RUXF	9542022.973	10075672.021	9496765.718	4028025.957	489111.7.866	5003850.086	0.478	0.000	0.008
VASP	23751001.051	25687776.161	2014989.6.048	9343355.069	111234.74.139	1277754.5.898	0.478	0.006	0.018
CTGE5	363935.956	332364.427	352793.329	130624.511	187172.449	178426.967	0.473	0.003	0.014
PLP2	7630567.121	6759129.753	8900610.655	3054508.831	438010.3.706	3536983.957	0.471	0.008	0.021
CYB5	2038172.205	2146878.117	2333482.333	923860.714	972564.654	1163612.125	0.469	0.001	0.009
HNRPL	87088909.395	72445891.456	6687916.8.671	35362882.725	345170.40.919	3635658.1.174	0.469	0.021	0.033
AT2B1;A2B2	21112887.217	24427434.430	2377855.3.563	10899952.171	112936.30.737	1028128.2.901	0.468	0.004	0.017
GRDN	228917.110	235854.879	268589.354	131997.125	101782.438	108590.301	0.467	0.001	0.012
UBC9	1222392.385	1552855.431	1717149.216	625883.892	652587.734	777190.881	0.458	0.022	0.034
ZDH20	177528.389	182492.149	215338.013	92880.413	79620.071	90059.999	0.456	0.007	0.020
OSTP	2024601.158	1667934.529	1845210.279	703728.794	891507.407	927720.047	0.456	0.002	0.013
UBP7	220229.024	297234.072	363311.444	157912.646	121243.787	111125.111	0.443	0.046	0.048
CWC15	638877.946	469956.611	564830.938	198082.273	292530.801	246391.959	0.440	0.010	0.024
CDK18	1169531.117	886352.754	809283.000	398811.822	445694.884	399943.927	0.434	0.036	0.042
EIF3C	19726919.237	22113263.289	2397406.1.436	9208266.476	902649.0.218	1023830.2.666	0.433	0.006	0.018
SPT6H	506723.520	511358.305	502727.397	188608.622	224667.225	239767.071	0.429	0.002	0.013
ZN207	178859.412	269771.594	259049.050	101254.911	91592.417	109747.331	0.428	0.039	0.044
MFGM	1313999.699	1590597.192	1754028.495	700572.070	642108.687	644715.330	0.427	0.018	0.031

RS27L	5184424.51 7	6814373.1 30	6114585. 277	2443601.0 53	256315 8.064	2712133. 375	0.426	0.016	0.029
THRB	721147.551	566925.83 5	620670.0 63	313974.69 6	274423 .795	218725.2 38	0.423	0.004	0.017
STML2	896264.995	805017.31 8	942012.5 50	408040.53 9	329750 .001	372278.2 07	0.420	0.001	0.011
CQ085	583663.529	870831.93 3	669491.7 97	276838.05 8	323778 .290	280768.9 41	0.415	0.036	0.042
BORG5	726624.542	820659.92 8	942623.4 13	281412.29 4	328425 .713	374771.1 18	0.395	0.007	0.020
CK5P3	69601.246	90547.614	82756.07 3	31935.872	27050. 658	34387.92 1	0.384	0.009	0.022
VCAM1	2076721.82 2	1305437.2 21	1812016. 314	589858.91 2	759400 .396	611812.8 98	0.378	0.035	0.042
RFC2	53382.681	83691.967	60315.53 0	25053.130	19786. 398	29349.77 8	0.376	0.037	0.043
FLOT2	1363414.52 9	1566243.9 37	1242215. 675	529753.07 7	578334 .704	427845.3 56	0.368	0.004	0.017
RCOR1	142676.158	82069.467	122158.4 11	35584.031	40991. 332	48508.07 1	0.361	0.048	0.049
K22E	7284199.66 4	10030411. 005	1028258 5.678	3190329.7 52	331842 3.310	3195630. 464	0.352	0.025	0.036
RBM14	18374943.1 80	15678863. 604	2068486 0.701	5804899.9 95	627898 5.481	6811596. 608	0.345	0.012	0.025
A2M;A2 MP	2110401.14 9	3221149.7 29	3243464. 902	973401.21 5	977210 .017	991845.6 62	0.343	0.037	0.043
EM55	2230183.66 8	2903893.3 37	2342354. 673	702353.35 6	843719 .528	1014848. 201	0.343	0.008	0.020
SRSF9	3789998.79 8	2268607.3 39	3150270. 293	982230.33 5	926940 .799	1203321. 430	0.338	0.040	0.044
PIEZ1	365704.915 5	319363.05 5	421603.9 95	121529.22 7	134566 .353	110569.4 48	0.331	0.011	0.024
RNT2	2562758.78 6	2579773.0 32	2896043. 254	759054.49 7	927721 .505	946524.6 33	0.328	0.001	0.009
NIPBL	2725214.91 1	2440489.7 43	2772378. 152	686062.17 4	866885 .877	1029672. 178	0.325	0.000	0.006
REQU	726019.831	684490.02 8	923362.5 53	196565.03 3	225616 .470	294423.6 90	0.307	0.010	0.023
RRAGC	3960014.11 1	3674429.4 65	4648874. 111	1028689.2 21	117960 2.418	1277515. 880	0.284	0.007	0.020
BPPL	706441.743	778090.61 6	884195.3 41	225416.22 9	194837 .367	213977.4 63	0.268	0.007	0.019
PLMN	412509.902	574708.13 4	588945.2 76	121334.47 7	141177 .388	132673.9 33	0.251	0.019	0.032
ARGL1	8494441.77 3	5853769.0 39	7109923. 403	1625014.2 58	169419 9.636	1993716. 052	0.248	0.018	0.030
BUD31	96159.430	164056.40 7	139760.0 43	24626.194 43	24840. 636	38332.93 4	0.220	0.029	0.038
NVL	1198671.03 0	903746.04 4	1097189. 954	212971.52 9	215099 .342	261108.9 02	0.215	0.009	0.022
GFPT1;G FPT2	6431928.41 3	4305730.4 38	5899021. 275	1128352.7 36	110989 1.125	1321058. 705	0.214	0.020	0.032
GFRA1	86917.277	137106.83 3	152020.4 68	21652.149	13633. 774	39626.49 1	0.199	0.024	0.035
H2AW	6023249.64 5	5861375.7 27	6873147. 503	1083578.4 05	121756 9.682	1411293. 454	0.198	0.002	0.013
GALM	544101.790	348214.91 1	350681.6 09	115879.63 9	64521. 876	62170.49 6	0.195	0.029	0.038

Table2: The top canonical pathways predicted by Ingenuity Pathway Analysis. The significantly altered proteins (FDR ≤ 0.05 , fold change ≤ 0.05 , ≥ 1.5) were used as inputs. The Pathways were then filtered again by their p-values and z-scores of >2 , <-2 .

Ingenuity Canonical Pathways	$-\log(p\text{-value})$	P-value	Ratio	z-score	Molecules
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Activation of IRF by Cytosolic Pattern Recognition Receptors	3.58	0.000	0.095	2.449	DDX58,IFIH1,JUN,MAVS,PIN1,STAT1
Glycolysis I	3.34	0.000	0.154	2	PFKL,PFKM,PGAM1,PGAM2
Agrin Interactions at Neuromuscular Junction	3.05	0.001	0.076	2.236	ACTA1,CTTN,JUN,LAMC1,PTK2,SRC
EGF Signaling	2.97	0.001	0.091	2.236	ITPR3,JUN,PLCG1,SRC,STAT1
Actin Cytoskeleton Signaling	2.95	0.001	0.046	2.333	ACTA1,ACTN2,F2,GIT1,MYH1,MYH8,MYLPF,NCKAP1,PFN1,PTK2
Integrin Signaling	2.46	0.003	0.042	2.828	ACTA1,ACTN2,CTTN,GIT1,PFN1,PLCG1,PTK2,SRC,VASP
AMPK Signaling	2.45	0.004	0.042	2	AK1,CKM,EIF4EBP1,GYS1,IRS1,PBRM1,PFKL,PFKM,PRKAB2
Signaling by Rho Family GTPases	2.09	0.008	0.037	2.121	ACTA1,ARHGEF17,CDC42EP1,CDH13,JUN,MYLPF,PARD3,PTK2,SEPTIN7
VEGF Signaling	1.88	0.013	0.051	2.236	ACTA1,ACTN2,PLCG1,PTK2,SRC
ILK Signaling	1.73	0.019	0.037	2.646	ACTA1,ACTN2,IRS1,JUN,MYH1,MYH8,PTK2
Renin-Angiotensin Signaling	1.58	0.026	0.042	2	ITPR3,JUN,PLCG1,PTK2,STAT1
Chemokine Signaling	1.57	0.027	0.050	2	JUN,PLCG1,PTK2,SRC
Prolactin Signaling	1.55	0.028	0.049	2	IRS1,JUN,PLCG1,STAT1
PDGF Signaling	1.47	0.034	0.047	2	JUN,PLCG1,SRC,STAT1
Regulation of Actin-based Motility by Rho	1.35	0.045	0.043	2	ACTA1,ARHGDI1,MYLPF,PFN1

Table3: The cellular and molecular functions altered by Dyrk1b overexpression in C2C12 cells. The different categories were filtered by their p-values ≤ 0.05 and z-scores of >2 , <-2 .

Diseases or Functions Annotation	p-value	Predicted Activation State	Activation z-score
Size of body	0.00000	Increased	4.511
Infection by HIV-1	0.00101	Increased	4.087
Infection of cells	0.00002	Increased	4.068
Infection by RNA virus	0.00057	Increased	3.94
Quantity of cells	0.00002	Increased	3.442
Infection of epithelial cell lines	0.00009	Increased	3.299
Infection of kidney cell lines	0.00014	Increased	3.299
Infection of embryonic cell lines	0.00014	Increased	3.299
Viral Infection	0.00000	Increased	3.176
Cell survival	0.00000	Increased	2.674
Contractility of skeletal muscle	0.00000	Increased	2.414
Cell movement of epithelial cell lines	0.00041	Increased	2.37
Cell viability	0.00001	Increased	2.293
Cell movement of stem cells	0.00131	Increased	2.226
Antiviral response of cells	0.00063	Increased	2.219
Biosynthesis of purine ribonucleotide	0.00109	Increased	2.219
Hydrolysis of ATP	0.00075	Increased	2.213
Contractility of muscle	0.00000	Increased	2.21
Glycolysis of cells	0.00092	Increased	2.2
Migration of epithelial cell lines	0.00009	Increased	2.151
Cell cycle progression	0.00001	Increased	2.143
Quantity of blood cells	0.00078	Increased	2.139

Interaction of protein	0.00035	Increased	2.095
Invasion of breast cancer cell lines	0.00013	Increased	2.084
Metabolism of nucleic acid component or derivative	0.00097	Increased	2.032
Metabolism of nucleotide	0.00161	Increased	2.032
Degeneration of myofiber	0.00010	Decreased	-2
Cell death of cancer cells	0.00126	Decreased	-2.04
Necrosis	0.00000	Decreased	-2.086
Necrosis of muscle	0.00046	Decreased	-2.099
Cell death of osteosarcoma cells	0.00065	Decreased	-2.121
Degeneration of muscle cells	0.00012	Decreased	-2.219
Movement Disorders	0.00022	Decreased	-2.368
Cell death of tumor cells	0.00012	Decreased	-2.401
Motor dysfunction or movement disorder	0.00008	Decreased	-2.73
Growth Failure	0.00032	Decreased	-4.333
Organismal death	0.00000	Decreased	-6.008

Table4: The predicted upstream regulators of proteins altered by Dyrk1b overexpression in C2C12 cells. The potential candidates were sorted by p-values ≤ 0.05 and z-scores of >2 , <-2 .

Upstream Regulator	Expr Fold Change	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap	Target Molecules in Dataset
SMARCA4		transcription regulator	Activated	2.968	0.00116	ABRACL,ACTA1,ARHGDIB,ATP2A1,BNIP3L,CKM,COL1A1,CUL4B,DYSF,GYS1,IGFBP5,JUN,MFGE8,MYLPP,PLIN2,SPP1,TAOK3,TNNC2,TNNT3,YY1
CD38		enzyme	Activated	2.959	0.000642	BNIP3L,COL1A1,CRIP1,EIF4EBP1,GALK1,NDRG1,PFKL,SLC16A3,VCAM1
ATF4		transcription regulator	Activated	2.945	0.0000304	CPOX,EIF4EBP1,GYS1,HYOU1,IGFBP5,JUN,NARS1,NDRG1,NID2,SARS1,WFS1
XBP1		transcription regulator	Activated	2.894	0.000647	ADAM10,COPB2,DNAJB11,GMPBP,HYOU1,LMAN1,PGM3,RABAC1,TOPI,VCAM1,WFS1
IFNA2		cytokine	Activated	2.786	0.00098	CYB5A,DDX58,ENPP1,HMGCS1,IFIH1,IFIT3,LGALS3BP,OAS1,OGFR,STAT1,VCAM1
P38 MAPK		group	Activated	2.77	0.00142	BNIP3L,GDI1,IGFBP5,JUN,LDB3,MIF,MYLPP,POSTN,PTPN12,SPP1,STAT1,TNNC2,TNNT3,VCAM1
MYOD1		transcription regulator	Activated	2.718	5.63E-09	ACTA1,ATP2A1,CKM,DYSF,GYS1,HADH,IGFBP5,LDB3,MYLPP,PFKM,POSTN,PTK2,SRCT,STAT1,TJP1,TNNC2,TNNT1,TNNT3,TOPI
MYC		transcription regulator	Activated	2.645	8.61E-13	ABCE1,ACTA1,COL1A1,COL8A1,COX6A1,CRIP1,Crip2,CSE1L,CSTB,DNMT1,EIF3G,EIF4EBP1,F2,FABP5,GAMT,GDI1,H6PD,HDAC2,IFIH1,IFIT3,IMPACT,IRS1,JUN,LAMP2,MCM5,MIF,MYLPP,NARS1,NCKAP1,NDRG1,OAS1,PFAS,PFKL,PFKM,PGAM1,PHB,PPIA,RANBP1,RBBP4,RPL4,RPL6,RPLP2,R

						PS27L,RPS28,S100A10,SLC16A3,SPP1,SRM,STAT1,TNNT3,UBE2I,VCAM1,YY1
SP1		transcription regulator	Activated	2.623	0.0225	BNIP3L,COL1A1,COL8A1,HDAC1,HDAC2,JUN,MECP2,NDRG1,PDHX,PRKRA,RANBP1,SP1,SRM,STAT1,YY1
STAT4		transcription regulator	Activated	2.621	0.0489	ACP6,ADSS2,DDX58,IFIH1,LOC7L,NDRG1,STAT1
HIF1A		transcription regulator	Activated	2.601	0.0262	ADAM10,BNIP3L,CYB5A,IGFBP5,JUN,MIF,NDRG1,PFKL,PP1A,SLC16A3,VASP,VCAM1
IFN Beta		group	Activated	2.591	0.00567	DDX58,IFIH1,IFIT3,OAS1,PLCG1,STAT1,TAPBP
IRF3		transcription regulator	Activated	2.588	0.0293	DDX58,IFIH1,IFIT3,NRAP,OAS1,STAT1,USP8
IRF7		transcription regulator	Activated	2.587	0.0134	DDX58,IFIH1,IFIT3,NRAP,OAS1,STAT1,USP8
SRF		transcription regulator	Activated	2.549	0.0143	ACTA1,CKM,CNN2,JUN,LDB3,LPCAT3,MYH1,MYLPF,PPIA,PTGR1,TNNT1
EGFR		kinase	Activated	2.484	0.00871	ACTA1,COL1A1,COL6A1,COL8A1,IGFBP5,JUN,PLCG1,POSTN,PPIA,RANBP1,TNNT3,TPST1,XRN2
IFNL1		cytokine	Activated	2.434	0.00168	DDX58,IFIH1,IFIT3,LGALS3BP,OAS1,STAT1
IL5		cytokine	Activated	2.345	0.028	BNIP3L,CRIP1,EIF4EBP1,GALK1,NDRG1,SFPQ,SLC16A3,SUPT6H,TMF1
IL3		cytokine	Activated	2.236	0.000573	ARHGDI1,BNIP3L,CTTN,EIF3G,HSPA2,JUN,MCM5,NARS1,PFKL,RANBP1,RPL4,RPL6,VASP,VCAM1
IRF5		transcription regulator	Activated	2.203	0.0000458	COL1A1,DDX58,IFIH1,IFIT3,OAS1,PRKRA,STAT1,USP8
Ifnar		group	Activated	2.157	0.0000735	DDX58,IFIH1,IFIT3,OAS1,SPP1,STAT1,TAPBP,VCAM1
MEF2C		transcription regulator	Activated	2.092	0.00000441	ACTA1,ACTN2,ATP2A1,CASQ2,CKM,COL1A1,JUN,MYH1,MYLPF,POSTN,TNNT1
RB1		transcription regulator	Activated	2.091	0.000103	ACTN2,ADAM10,CKM,COX6A1,DCTN4,DNMT1,KANK2,LAMC1,MCM5,MRPL53,MYH8,PGAM2,RANBP1,SDHC,SMARCA5,STK3,TNNC2,TNNT1,TRIM72
Insulin		group	Activated	2.011	0.0281	ACTA1,ADAM10,COL1A1,COL6A1,HSD11B1,IFIT3,IRS1,JUN,LGALS3BP,PPIA,PTK2,STAT1,TJP1
JAK		group	Activated	2	0.00358	DDX58,IFIH1,IFIT3,STAT1
ELAVL1		other	Activated	2	0.0111	HSPA2,IFIH1,LGALS3BP,OAS1,STAT1,TJP1,VAPB,VCAM1
Gm15807/Hmgn5	1.977	other	Activated	2	0.00118	ACTA1,ATP2A1,TNNC2,TNNT3
FADD		other	Activated	2	0.0421	DDX58,IFIH1,JUN,STAT1
ACKR2		G-protein coupled receptor	Inhibited	-2	0.00358	DDX58,IFIT3,OAS1,STAT1
PTCH1		transmembrane receptor	Inhibited	-2	0.000809	ACTA1,MYH1,TNNT1,TNNT3
mir-29		microRNA	Inhibited	-2.19	0.00959	COL1A1,DNMT1,LAMC1,NID1,PRKRA
mir-122		microRNA	Inhibited	-2.219	0.012	ADAM10,CACYBP,GIT1,GYS1,LAMC1

SOCS1		other	Inhibited	-2.222	0.00121	DDX58,IFIH1,IFIT3,IRS1,JUN,OAS1,STAT1,VCAM1
SMTNL1		other	Inhibited	-2.236	0.0000587	ACTA1,GLO1,PFKM,TNNC2,TNNT3
miR-338-3p (miRNAs w/seed CCAGCAU)		mature microRNA	Inhibited	-2.236	0.00013	COL1A1,COL8A1,LAMC1,NID1,NID2
miR-335-3p (miRNAs w/seed UUUUCAU)		mature microRNA	Inhibited	-2.236	0.0000492	COL1A1,COL8A1,LAMC1,NID1,NID2
Esrra		transcription regulator	Inhibited	-2.236	0.0208	GALM,GYS1,PFKL,PGAM2,SPPI
SP110		transcription regulator	Inhibited	-2.333	0.000243	FLI1,HDAC2,IFIH1,IFIT3,OAS1,PLCG1,PTPN12,STAT1,TRIM28
miR-16-5p (and other miRNAs w/seed AGCAGCA)		mature microRNA	Inhibited	-2.345	0.00351	ADSS2,ARHGDIA,F2,JUN,LAMC1,LUZP1,PHLDB2,SLC16A3,TPM3
miR-29b-3p (and other miRNAs w/seed AGCACCA)		mature microRNA	Inhibited	-2.374	0.00189	COL1A1,COL8A1,LAMC1,NID1,NID2,YY1
TRIM24		transcription regulator	Inhibited	-2.393	0.0012	DDX58,IFIH1,IFIT3,LGALS3BP,OAS1,SPPI,STAT1
let-7a-5p (and other miRNAs w/seed GAGGUAG)		mature microRNA	Inhibited	-2.602	0.000407	CAPG,COL1A1,COL8A1,F2,GYS1,HYOU1,LAMC1,NID1,NID2,PXDN
PNPT1		enzyme	Inhibited	-2.646	0.00000799	DDX58,IFIH1,IFIT3,LGALS3BP,OAS1,STAT1,TOR3A
RICTOR		other	Inhibited	-2.985	0.0195	COX6A1,FABP5,NDUFB7,PSMD8,RPL4,RPL6,RPLP2,SDHC,VCAM1
EGLN		group	Inhibited	-2.985	0.000671	BPNT1,EIF4EBP1,GYS1,MIF,NOL3,PFKL,PGAM1,SLC16A3,WWTR1
KDM5A		transcription regulator	Inhibited	-3	0.000132	ACTN2,COX6A1,MRPL53,MYH8,PGAM2,SDHC,TNNC2,TNNT1,TRIM72
MAP4K4		kinase	Inhibited	-3	0.0000622	AGPAT3,ALDH3B1,GYS1,H6PD,HADH,NDUFB7,PDHX,PFKM,PGAM1

Table. S1. Homozygous and heterozygous embryos from a clutch of Dyrk1b^{+/-} intercross

Number of embryos in a clutch of ~ 100 F2 embryos			
Homozygous	Heterozygous	Wild type	Total number of embryos
23	48	24	95
26	40	32	98
30	35	40	105