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Supplemental information

RBFOX2 regulated EYA3 isoforms partner with SIX4 or ZBTB1 to control transcription during myogenesis

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SUPPLEMENTAL INFORMATION

FIGURE S1

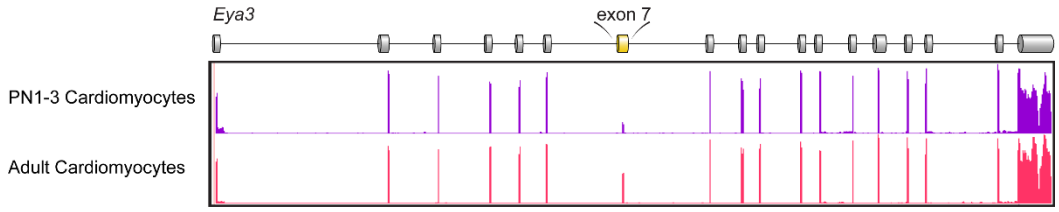

Figure S1. Eya3 splicing patterns are developmentally regulated in cardiomyocytes isolated from neonatal and adult mouse hearts, related to Figure 1. RNA-seq tracks (reference [S1]) from mouse cardiomyocytes freshly isolated from postnatal day 1-3 (PN1-3, purple) and adult heart (pink) tissues at the eyes absent homolog 3 (*Eya3*) locus, visualized in the UCSC genome browser. The alternative exon (exon 7) is shown in yellow.

FIGURE S2

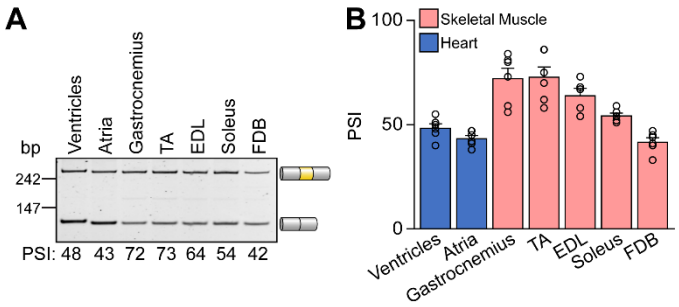

Figure S2. Inclusion levels of Eya3 exon 7 in a panel of adult mouse cardiac and skeletal muscle tissues reveal that Eya3 splicing is striated muscle specific, related to Figure 1. (**A**, **B**) Splicing of Eya3 exon 7 was evaluated by RT-PCR in adult (1-4-month-old) ventricles, atria, and the gastrocnemius, tibialis anterior (TA), extensor digitorum longus (EDL), soleus, and flexor digitorum brevis (FDB) muscles (**A**) and quantified by densitometry (*n*=5-6) (**B**). Cardiac tissues are labeled in blue and skeletal muscle tissues are shown in pink. bp, base pairs. PSI, percent spliced in. Results are shown as mean ± SEM.

FIGURE S3

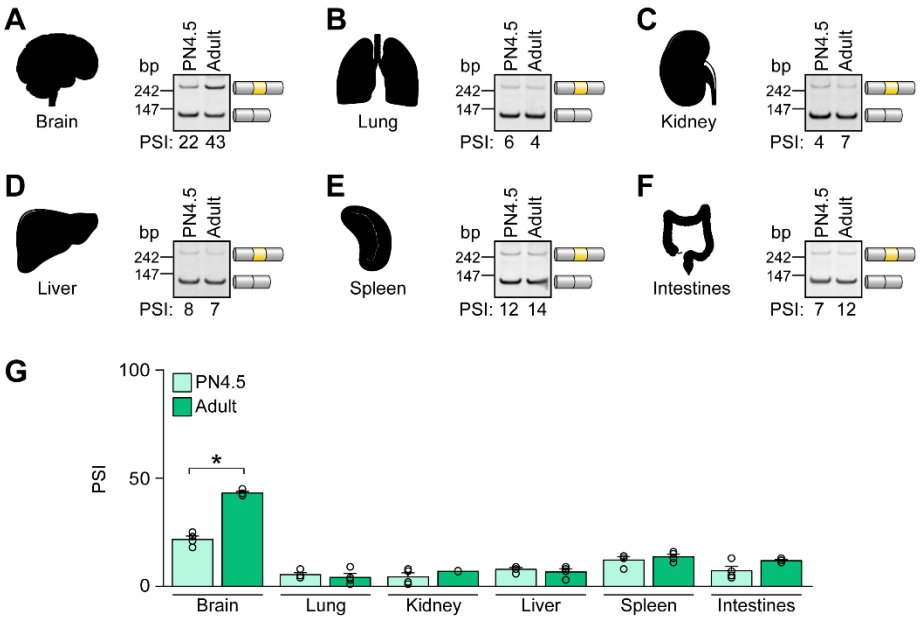

Figure S3. Inclusion levels of Eya3 exon 7 in a panel of neonatal and adult mouse tissues indicate that this exon is developmentally regulated in the brain, but not in other non-striated muscle tissues, related to Figure 1. (**A**-**G**) Splicing of Eya3 exon 7 was evaluated by RT-PCR in neonatal (postnatal day 4.5, PN4.5) and adult (3-4-month-old) mouse brain (**A**), lung (**B**), kidney (**C**), liver (**D**), spleen (**E**), intestines (**F**) and quantified by densitometry (n=4) (**G**). bp, base pairs. PSI, percent spliced in. Results are shown as mean ± SEM. *p < 0.05, Student's T test.

FIGURE S4

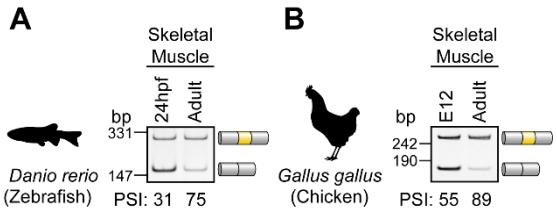

Figure S4. Inclusion levels of Eya3 exon 7 during zebrafish and chicken development reaffirms the evolutionary conservation of exon 7 splicing, related to Figure 1. (**A**, **B**) The inclusion of Eya3 exon 7 was determined by RT-PCR in skeletal muscle tissues of zebrafish (**A**) and chicken (**B**) at indicated developmental timepoints. bp, base pairs. E, embryonic day. hpf, hours post fertilization. PSI, percent spliced in.

FIGURE S5

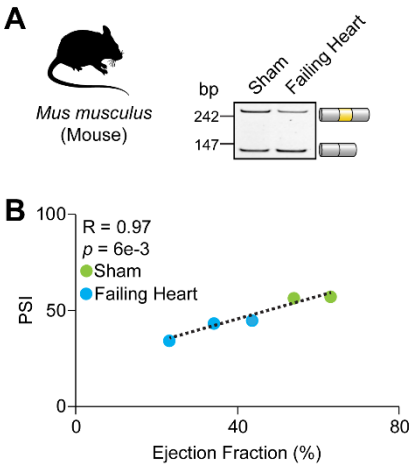

Figure S5. Inclusion levels of Eya3 exon 7 are reduced in response to heart failure induced by transverse aortic constriction (TAC), related to Figure 1. (**A**, **B**) Splicing of Eya3 exon 7 in ventricles was evaluated by RT-PCR after mice were subjected to transverse aortic constriction (failing hearts) or sham surgeries (**A**). Scatterplot displaying the correlation between ejection fraction and Eya3 exon 7 inclusion (**B**). bp, base pairs. PSI, percent spliced in. A significance test for correlation was performed in panel **B**.

FIGURE S6

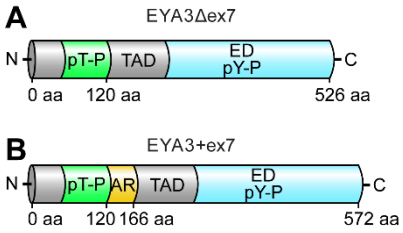

Figure S6. At the protein level, the inclusion of EYA3 exon 7 produces an in-frame insertion within the transactivation domain, related to Figure 2. (**A**, **B**) Schematic of protein domains found within the EYA3 isoform lacking exon 7 (EYA3Δex7) (**A**) and the EYA3 isoform including exon 7 (EYA3+ex7) (**B**). aa, amino acid. AR, alternative region. ED, EYA domain. pT-P, threonine phosphatase domain. pY-P, tyrosine phosphatase domain. TAD, transactivation domain.

FIGURE S7

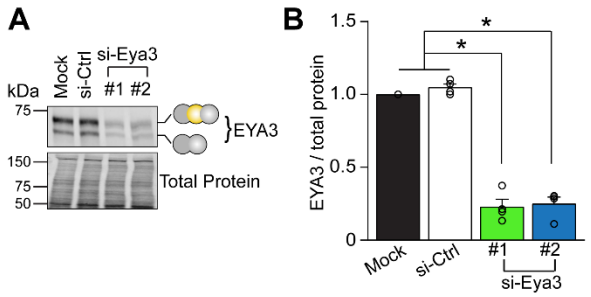

Figure S7. EYA3 knockdown efficiency is maintained following myoblast differentiation, related to Figure 2. C2C12 myoblasts were transfected with control (si-Ctrl) or two Eya3 si-RNAs (si-Eya3 #1, si-Eya3 #2) then differentiated into myotubes for four or five days. EYA3 knockdown efficiency was confirmed in myotubes by western blot analysis (**A**) and quantified by densitometry (**B**). Results are shown as mean ± SEM. *$p < 0.05$ (*versus* si-Ctrl and mock), Student's T test, n=3-4.

FIGURE S8

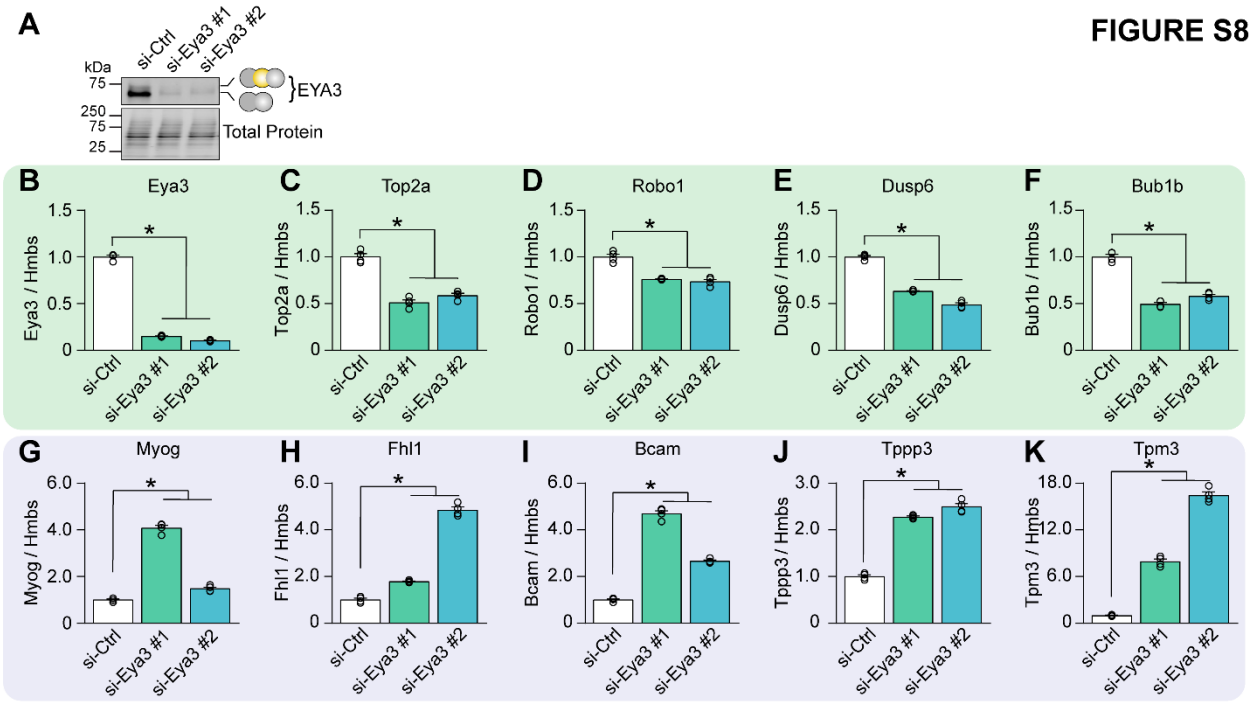

Figure S8. Depletion of Eya3 in undifferentiated C2C12 myoblasts confirms direct targets of EYA3 coregulation, related to Figure 5. C2C12 myoblasts were transfected with control (si-Ctrl) or two Eya3 si-RNAs (si-Eya3 #1, si-Eya3 #2) and analyzed 48 h after si-RNA delivery. (**A**) EYA3 knockdown efficiency was evaluated by western blotting. (**B**-**K**) Real time quantitative PCR (qPCR) analysis following Eya3 depletion (*n*=4): Eya3 (**B**), Top2a, DNA topoisomerase II alpha (**C**), Robo1, roundabout guidance receptor 1 (**D**), Dusp6, dual specificity phosphatase 6 (**E**), Bub1b, BUB1 mitotic checkpoint serine/threonine kinase B (**F**), Myog, myogenin (**G**), Fhl1, four and a half LIM domains 1 (**H**), Bcam, basal cell adhesion molecule (**I**), Tppp3, tubulin polymerization promoting protein family member 3 (**J**), and Tpm3, tropomyosin 3 (**K**). Hmbs, hydroxymethylbilane synthase. Results are shown as mean ± SEM. **p* < 0.05 (*versus* si-Ctrl), Student's T test.

FIGURE S9

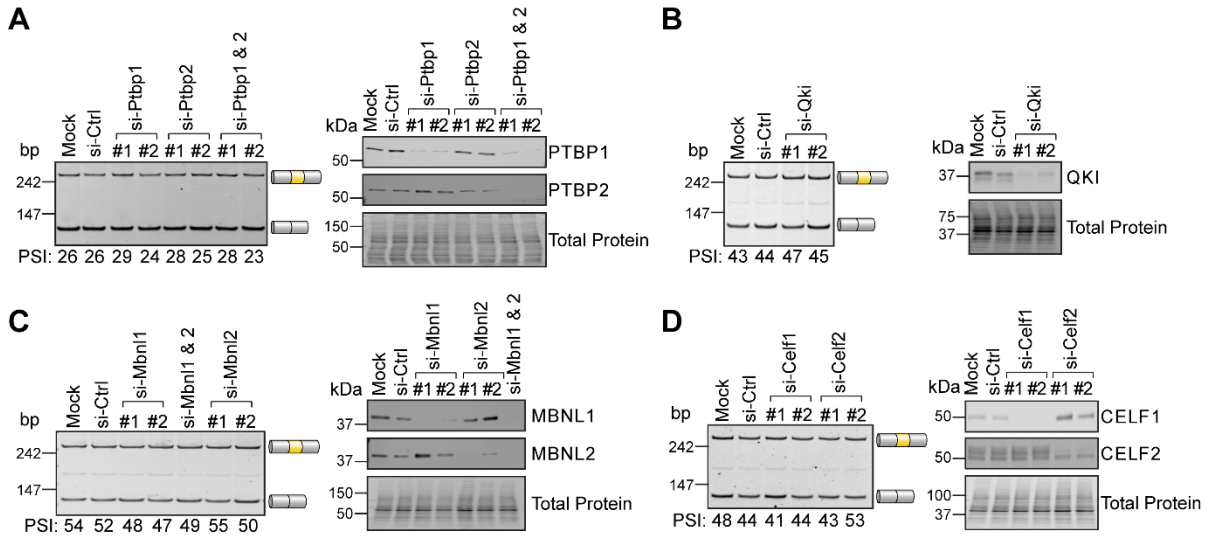

Figure S9. PTBP1, PTBP2, QKI, MBNL1, MBNL2, CELF1, and CELF2 do not regulate splicing of Eya3 exon 7, related to Figure 7. (**A**-**D**) Splicing of Eya3 exon 7 was evaluated by RT-PCR upon knockdown of PTBP1 and/or PTBP2 (**A**, *left*), QKI (**B**, *left*), MBNL1 and/or MBNL2 (**C**, *left*), and CELF1 and/or CELF2 (**D**, *left*). Protein levels of PTBP1 and PTBP2 (**A**, *right*), QKI (**B**, *right*), MBNL1 and MBNL2 (**C**, *right*), and CELF1 and CELF2 (**D**, *right*) were determined via western blotting. bp, base pairs. CELF1, CUGBP Elav-like family member 1. CELF2, CUGBP Elav-like family member 2. MBNL1, muscleblind like splicing regulator 1. MBNL2, muscleblind like splicing regulator 2. PTBP1, polypyrimidine tract binding protein 1. PTBP2, polypyrimidine tract binding protein 2. QKI, KH domain containing RNA binding. PSI, percent spliced in.

Gene symbol	Organism	Genome	Size of the alternative region (nt)	Genomic coordinates of the alternative region	Forward primer	Reverse primer	Expected sizes (nt)
Eya3	***Mouse***	***mm10***	138	chr4:132680756-132680893	TCCTGGGCAGACTCAGTACC	GGTATCAGGCTGGCATTTGT	258, 120
EYA3	***Human***	***hg38***	138	chr1:28027789-28027926	GCAGACTCAACCCTATGCTG	TATCAGGCTGGCATTTGTGC	226, 88
EYA3	***Rabbit***	***oryCun2***	138	chr13:137482356-137482493	GGCAGACTCAGTACCAGACA	TGGTTTGAGATGCTGGCAAC	310, 172
Eya3	***Rat***	***rn6***	138	chr5:150872712-150872849	CACACATCCTCTCGGTTCCT	GTGGATATCAGGCTGGCATT	298, 160
eya3	***Zebrafish***	***danRer11***	159	chr19:24900244-24900402	ATGCCAGTCAAGTGGCTTTC	CTGTGGTCACTGCGGTTG	315, 156
EYA3	***Chicken***	***galGal6***	138	chr23:1601275-1601412	AATGAGCCCTTACCCTGGTC	ACTGGACTGGCATTGGTAGT	267, 129

Table S1, related to Figure 1 and STAR methods. Sequences of the primers (Thermo Fisher Scientific or Sigma) used to analyze Eya3 splicing by RT-PCR assays.

Sample number	Tissue type	Tissue details	Diagnosis	Cause of death	Age	Sex
1	Muscle	skeletal muscle with focal increased interstitial inflammation	Myositis	Unknown	36	Male
2	Heart	left ventricle apex	DM1	Pneumonia, Respiratory failure	47	Male
3	Heart	left ventricle free wall near apex	DM1	Respiratory failure	50	Female
4	Heart	whole without valves	DM1	Anoxia	52	Male
5	Heart	left ventricle apex	DM1	Unknown	Unknown	Unknown

Table S2, related to Figure 1 and STAR methods. Myositis and myotonic dystrophy type 1 (DM1) patient clinical information.

Gene	Probe ID	Organism	Exon boundary	Amplicon length (nt)
Bcam	Mm00522338_m1	mouse	8 – 9	94
Crym	Mm00516679_m1	mouse	7 – 8	70
Fhl1	Mm04204611_g1	mouse	4 – 5	114
Tppp3	Mm01251070_g1	mouse	3 – 4	65
Tpm3	Mm00445589_m1	mouse	1 – 2	65
Igfbp2	Mm00492632_m1	mouse	1 – 2	85
Top2a	Mm01296339_m1	mouse	15 – 13	75
Myo1b	Mm01257265_m1	mouse	27 – 28	65
Myog	Mm00446194_m1	mouse	1 – 2	69
Parm1	Mm00523913_m1	mouse	2 – 3	69
Robo1	Mm00803879_m1	mouse	28 – 29	106
Bub1b	Mm00437811_m1	mouse	5 – 6	79
Ptn	Mm01132688_m1	mouse	3 – 4	61
Dusp6	Mm00518185_m1	mouse	2 – 3	82
Eya3	Mm00438810_m1	mouse	8 – 9	103

Table S3, related to Figures 3 and 5, and STAR methods. qPCR TaqMan® probes (Applied Biosystems).

Gene symbol	Transcripts per million (TPM)
Six2	0
Six4	18
Six5	2

Table S4, related to Figure 3. Transcripts per million (TPM) for SIX family gene members from RNA-seq analysis of si-Ctrl myotube sample 1.

Gene symbol	Catalog No.	Target sequence
Ptbp1	MSS276537	GGUGUGGUCAAAGGCUUCAAGUUCU
Ptbp1	MSS276539	CCUCUGGAGACAGCCAGCCUUCACU
Ptbp2	MSS225938	GGUGGCAAUACAGUCCUGUUGGUUA
Ptbp2	MSS225940	GGGCACUGUGAAAGCAUUUAAGUUU
Qki	MSS208338	GAGCGGUUGAAGAAGUGAAGAAGUU
Qki	MSS276677	GAGCGGCUGCUGGACGAAGAAAUUA
Mbnl1	MSS226392	CCACAGCCAACCAGAUACCCAUAAU
Mbnl1	MSS226393	GCAUUUCUCCCACCAGGCUCAAUAU
Mbnl2	MSS272396	GAGAUUAAUGGGAGGAACAAUUUGA
Mbnl2	MSS200587	GCGUUGCAUGAGGGAGAAAUGCAAA
Celf1	MSS203372	GGACAGAUUGAAGAGUGCCGGAUAU
Celf1	MSS203374	CCAUGAACGGCUUUCAAAUUGGAAU
Celf2	MSS274200	CAGAGUAAAGGUUGUUGUUUCGUAA
Celf2	MSS204012	GCUGGAGCCACUGUCGGAUUGAAUA
Eya3	D-065562-01	GGACCCAACAGUAGUAAUU
Eya3	D-065562-02	UGAGGGAAAUCUACGACAA
Six4	D-057996-01-0002	GCAAGCAAAUGUACUGUGU
Zbtb1	D-057238-02-0002	UAUCAGCGCUGUCAUUUGA
Rbfox2	D-051552-01-0002	GCAAAUGGCUGGAAGUUAA
Rbfox2	D-051552-04-0002	CGAGAAUAGUGCUGAUGCA
luciferase	12935146	GCACUCUGAUUGACAAAUACGAUUU
Non-targeting #1	D-001210-01-20	UAGCGACUAAACACAUCAA

Table S5, related to STAR methods. Sequences of si-RNAs (Invitrogen or Dharmacon) used for depletion experiments.

REFERENCE

[S1] Giudice, J., Xia, Z., Wang, E.T., Scavuzzo, M.A., Ward, A.J., Kalsotra, A., Wang, W., Wehrens, X. H.T.T., Burge, C.B., Li, W., et al. (2014). Alternative splicing regulates vesicular trafficking genes in cardiomyocytes during postnatal heart development. Nature Communications *5*, 3603. 10.1038/ncomms4603.