SUPPLEMENTARY INFORMATION

miR-218 is Essential to Establish Motor Neuron Fate as a Downstream Effector of Isl1-Lhx3

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Supplementary Figures

a ***miR-218-1***

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Human   GTGATAATGTAGCGAGATTTTCTGTTGTGCTTGATCTAACCATGTGGTTGCGAGGTATGAGTAAAACATGGTTCCGTCAAGCACCATGGAACGTCACGCAGCTTTCTACA
Mouse   GTGATAATGGAGCGAGATTTTCTGTTGTGCTTGATCTAACCATGTGCTTGCGAGGTATGAGAAAAACATGGTTCCGTCAAGCACCATGGAACGTCACGCAGCTTTCTACA
Chicken TTGATAATGTAGCGAGATTTTCTGTTGTGCTTGATCTAACCATGTGGTTGTGAGGTATGAGTAAAACATGGTTCTGTCAAGCACCATGGAACGTCACGCAGCTTTCTACA
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gugauaaugga   a   u      u          cu        ---g   gcg
           gcg gau uucugu gugcuugau  aaccaugu    cuu   a
           ||| ||| |||||| |||||||||  ||||||||    |||   g
           cgc cug aaggua cacgaacug  uugguaca    gag   g
acaucuuucga   a   c      c          cc        aaaa   uau
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b ***miR-218-2***

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Human   GACCAGTCGCTGCGGGGCTTTCCTTTGTGCTTGATCTAACCATGTGGTGGAACGATGGAAACGGAACATGGTTCTGTCAAGCACCGCGGAAAGCACCGTGCTCTCCTGCA
Mouse   GACCAGTTGCCGCGGGGCTTTCCTTTGTGCTTGATCTAACCATGTGGTGGAACGATGGAAACGGAACATGGTTCTGTCAAGCACCGCGGAAAGCATCGCTCTCTCCTGCA
Chicken GGC---TGTCCATGGGGTTTTCCTTTGTGCTTGATCTAACCATGTGGTAGAACAATACAAATTGAACATGGTTCTGTCAAGCACCATGGAAGGCTGCATAC-TCCCTGCC
        * *   *  *   *********************************** **** **  *** ***********************  **** **  *   *   *****
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gac   uu -cc   gg      uuu         cu        gguggaacga  g
   cag  g   gcg  gcuuucc  gugcuugau  aaccaugu          ug a
   |||  |   |||  |||||||  |||||||||  ||||||||          ||
   guc  c   cgc  cgaaagg  cacgaacug  uugguaca          gc a
-ac   cu ucu   ua      cgc         uc        --------ag  a
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c

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hsa-miR-218-1   UUGUGCUUGAUCUAACCAUGU- 21
hsa-miR-218-2   UUGUGCUUGAUCUAACCAUGU- 21
mmu-miR-218-1   UUGUGCUUGAUCUAACCAUGU- 21
mmu-miR-218-2   UUGUGCUUGAUCUAACCAUGU- 21
gga-miR-218-1   UUGUGCUUGAUCUAACCAUGU- 21
gga-miR-218-2   UUGUGCUUGAUCUAACCAUGU- 21
xtr-miR-218-1   UUGUGCUUGAUCUAACCAUGU- 21
xtr-miR-218-2   UUGUGCUUGAUCUAACCAUGU- 21
dre-miR-218a-1  UUGUGCUUGAUCUAACCAUGUG 22
dre-miR-218a-2  UUGUGCUUGAUCUAACCAUGUG 22
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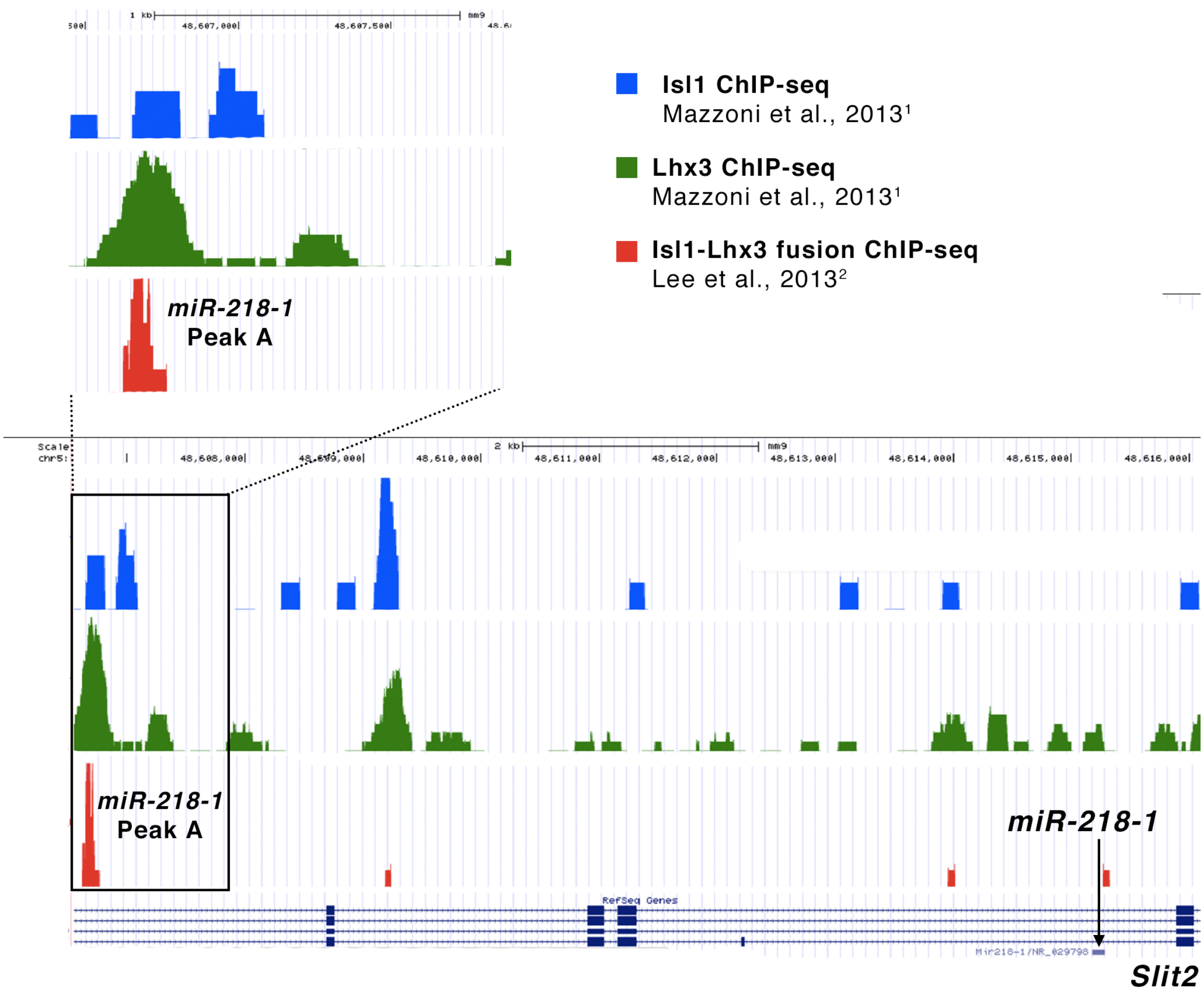
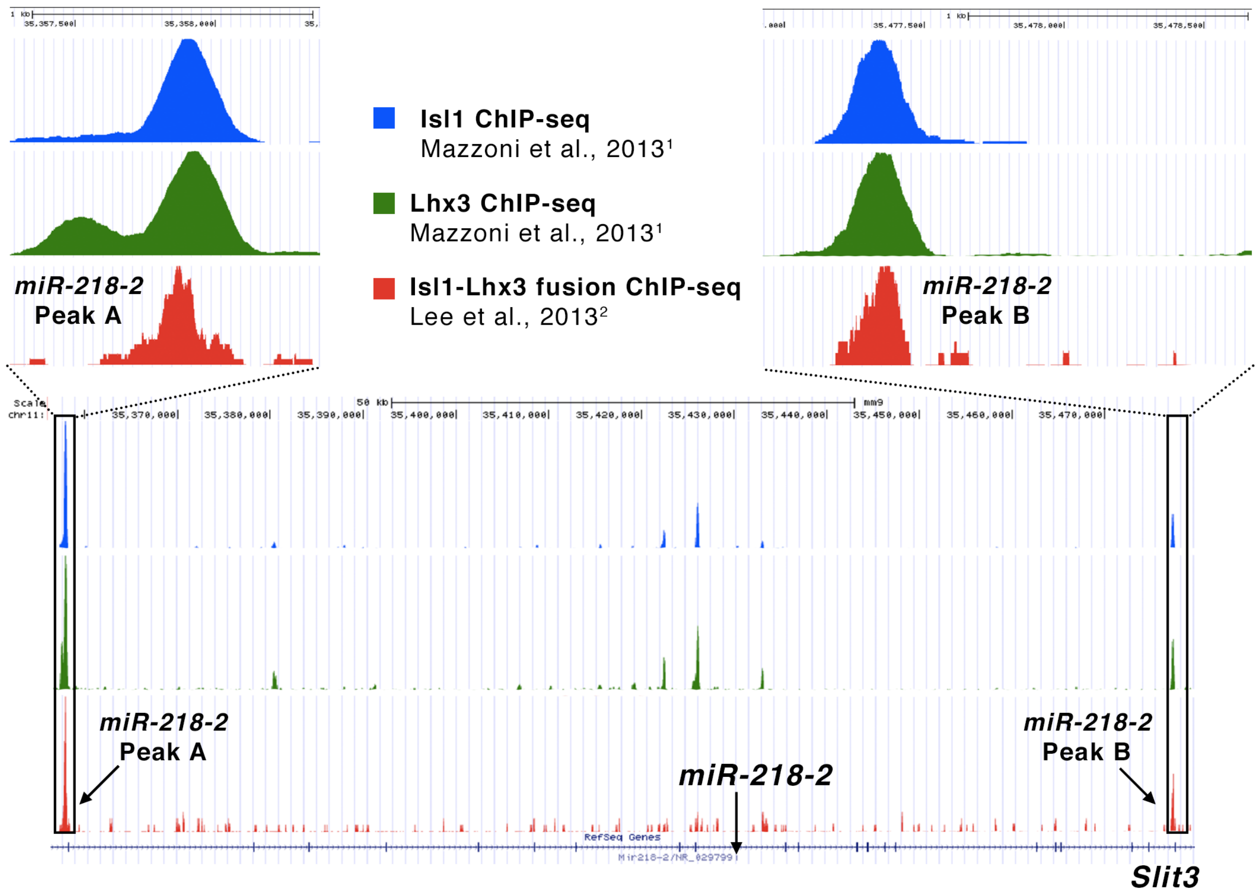
Supplementary Figure 1. miR-218 is an evolutionarily conserved miRNA produced from *miR-218-1* and *miR-218-2* genes.

(a-b) *miR-218-1* and miR-*218-2* genes and hairpin structures in human, mouse and chicken genome.

(c) Sequence alignment shows that the mature miR-218 sequences are highly conserved among multiple species.

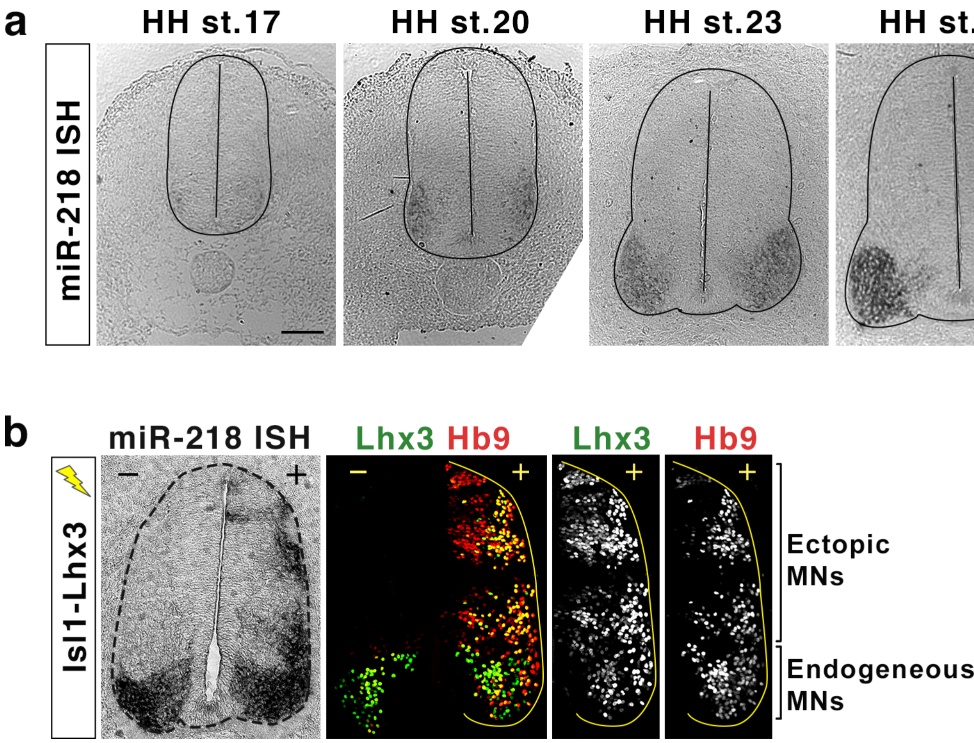


Supplementary Figure 2. ChIP-seq Peak in *miR-218-1/Slit2* locus
Isl1-Lhx3-bound ChIP-seq peak was identified near *miR-218-1* within the intron of *Slit2* from three independent ChIP-seq experiments[1,2].

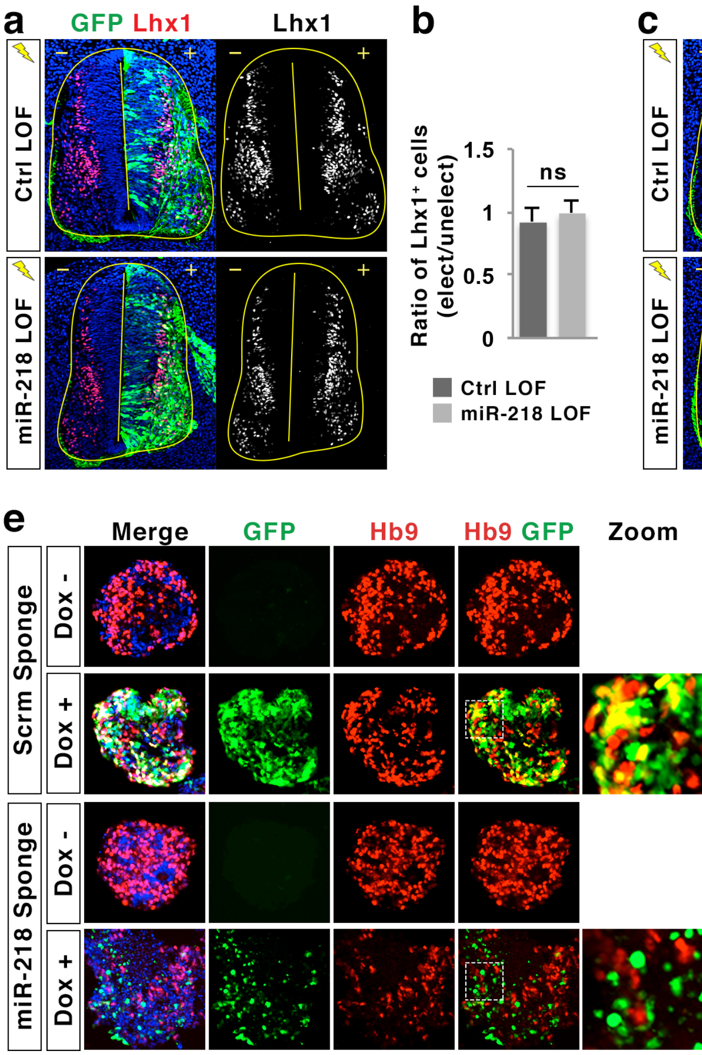


Supplementary Figure 3. ChIP-seq Peaks in *miR-218-2/Slit3* locus

Isl1-Lhx3-bound ChIP-seq peaks were identified near *miR-218-2* within the intron of *Slit3* from three independent ChIP-seq experiments[1,2].



Supplementary Figure 4. miR-218 is upregulated by Isl1-Lhx3 during motor neuron specification.
(a) *In situ* hybridization (ISH) analyses in the developing chick spinal cord show that miR-218 expression begins at the onset of motor neuron differentiation and specifically expressed in motor neurons.
(b) *In situ* hybridization and immunohistochemical analyses on sequential sections of a chick embryo electroporated with Isl1-Lhx3. Misexpression of Isl1-Lhx3 triggers upregulation of miR-218 and the ectopic formation of $Hb9^{+}$ motor neurons (MNs) in dorsal spinal cord. +, electroporated side; -, unelectroporated control side.



Supplementary Figure 5. miR-218 is required for efficient differentiation of motor neurons.

(a,c) Loss of function (LOF) analyses in chicks electroporated with control (Ctrl) LOF conditions (scrambled sponge inhibitor, 2'Ome-inhibitor control, and CMV-GFP reporter) and miR-218 LOF conditions (miR-218 sponge inhibitor, miR-218 2'Ome-inhibitor, and CMV-GFP reporter). Lhx1 antibody labels interneurons and LMC motor neurons, while Olig2 antibody labels motor neuron progenitors in immunohistochemical analyses. +, electroporated side; -, unelectroporated control side.

(b,d) The effect of LOF conditions was quantified by the ratio of $Lhx1^+$ cells or $Olig2^+$ cells on the electroporated (elect) side over the unelectroporated (unelect) side. ns = not significant in two-tailed Student's t-test; n = 12-13 embryos for (b) Lhx1 counts and n = 6-8 embryos for (d) Olig2 counts.

(e) Immunohistochemical analyses in Dox-inducible sponge ESC-derived motor neurons at differentiation day 6. Hb9 antibody labels motor neurons, and GFP labels the cells in which the expression of scramble (Scrm) or miR-218 sponge inhibitor is induced by Dox. miR-218 sponge inhibitor, but not scramble inhibitor, strongly inhibited motor neuron differentiation. Zoom shows the magnified view of the areas marked by dotted lines in Hb9/GFP merged images.

a

b

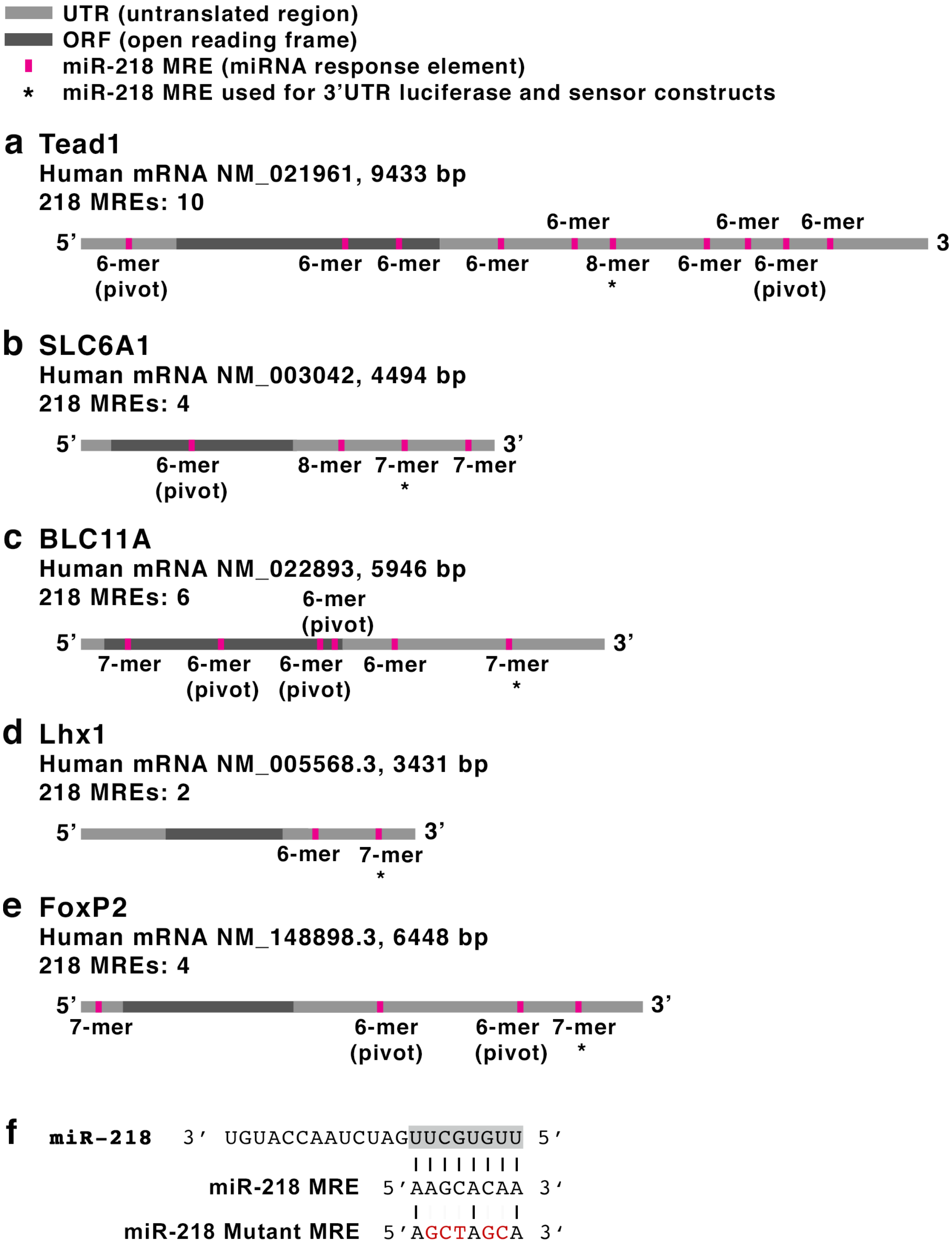
Published miR-218 Target	RISC-trap Fold Change (218 vs. 181)	References
RICTOR	17.83	Uesugi et al., 2011[3]; Venkataraman et al., 2013[4]
ONECUT2	17.5	Simion et al., 2010[5]
GLCE	14.41	Prudnikova et al., 2012[6]
LEF1	8.41	Liu et al., 2012[7]; Tu et al., 2013[8]
CDK6	6.68	Venkataraman et al., 2013[4]
GJA1	5.39	Alajez et al., 2011[9]
LASP1	5.23	Chiyomaru et al., 2012[10]
PIK3C2A	5.12	Mathew et al., 2014[11]
Robo2	5.09	Fish et al., 2011[12]
Robo1	4.27	Fish et al., 2011[12]; Alajez et al., 2011[9]
Cav2	3.52	Yamasaki et al., 2013[13]
Birc5	2.5	Alajez et al., 2011[9]
IKBKB	2.18	Song et al., 2010[14]
BMI1	2.11	Tu et al., 2013[8]; Venkataraman et al., 2013[4]
HMGB1	1.79	Mathew et al., 2014[11]
DTL	1.72	Liu et al., 2013[15]

Supplementary Figure 6. miR-218 RISC-trap Screen identified previously published miR-218 targets as direct target mRNAs for miR-218.
(a) Illustration of miRNA-induced silencing complex (miRISC).
(b) Previously published miR-218 targets that were identified in our miR-218 RISC-trap screen. RISC-trap fold changes for miR-218 vs. miR-181 and references are shown. All targets in this table were significantly enriched in the miR-218 vs. miR-181 RISC-trap analyses with $p < 0.05$.

miR-218 RISC-trap Target mRNA	Spinal Cord Expression	Function	Reference
TEAD1	Progenitor Zone	**Transcription Factor** Neural progenitor proliferation	Cao et al., 2008[16]
SLC6A1 (GAT1)	Interneurons	**GABA Transporter** GABAergic interneuron neurotransmission	Jursky et al., 1999[17] Chen et al., 2004[18]
BCL11A (Ctip1)	Interneurons	**Transcription Factor** Neuronal morphogenesis and sensory circuit formation	Li et al., 2006[19] John et al., 2012[20]
Lhx1	Interneurons	**Transcription Factor** Inhibitory interneuron differentiation	Pillai et al., 2007[21] Huang et al., 2008[22] Brohl et al., 2008[23]
FoxP2	Progenitor Zone V1 Interneurons	**Transcription Factor** Neurogenesis	Morikawa et al., 2009[24] Rousso et al., 2012[25]
Prdm13	Interneurons	**Histone Methyltransferase** Inhibitory interneuron differentiation	Kinameri et al., 2008[26] Chang et al., 2013[27] Hanotel et al., 2014[28]
Sox21	Progenitor Zone V2a Interneurons	**Transcription Factor** Neurogenesis	Uchikawa et al., 1999[29] Sandberg et al., 2005[30]
Pou4f1 (Brn3a)	Interneurons Dorsal Root Ganglia	**Transcription Factor** Sensory and interneuron differentiation	Müller et al., 2002[31] Eng et al., 2007[32] Zou et al., 2012[33]
BMPR1b	Interneurons	**Transcription Factor** Dorsal interneuron differentiation	Wine-Lee et al., 2004[34]

Supplementary Figure 7. Expression and function of the selected miR-218 targets in spinal cord development.

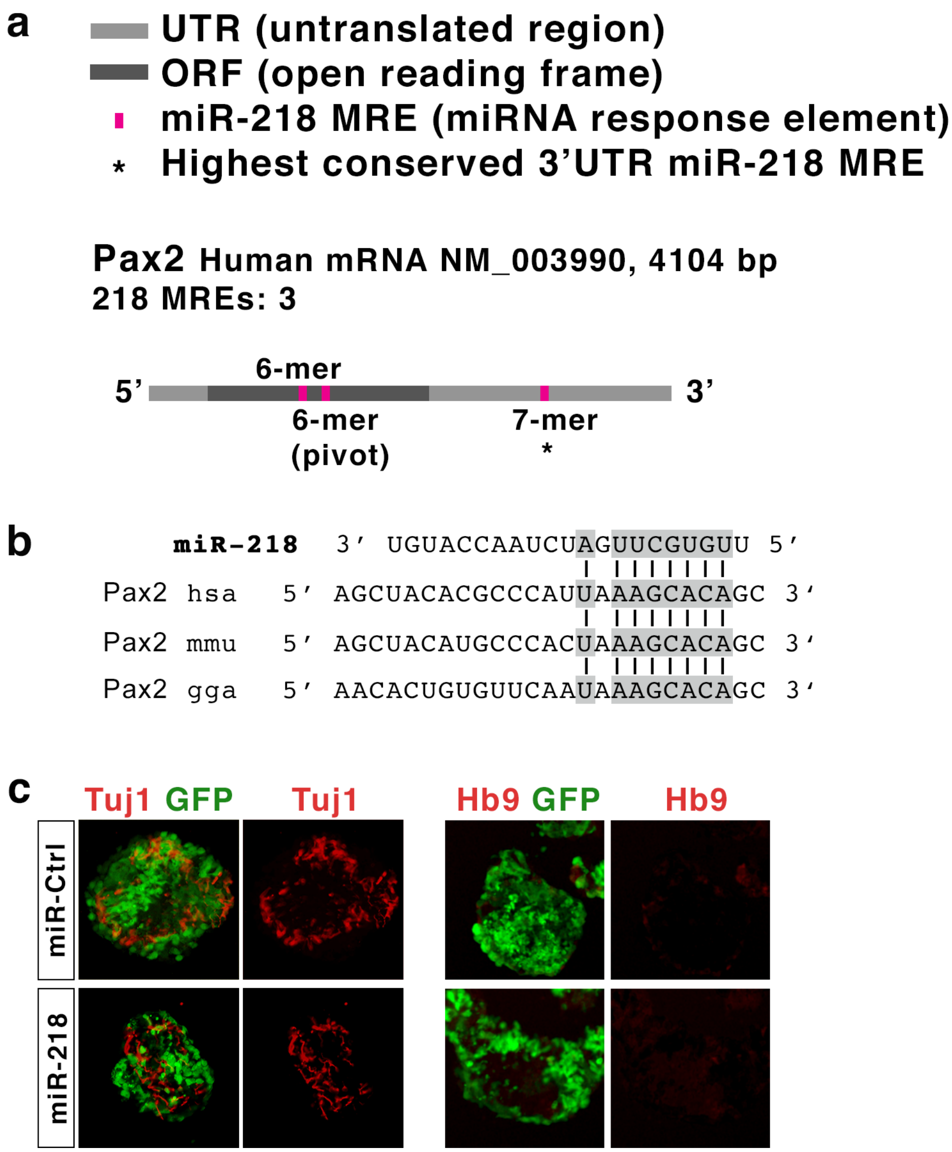
A subset of miR-218 targets identified in the RISC-trap screen play important roles in spinal cord development. Tead1, FoxP2 and Sox21 are known to be important for neurogenesis, while Lhx1, BCL11A, SLC6A1, Foxp2, Pou4f1, Prdm13, Sox21, and BMPR1b play roles for the differentiation and function of spinal interneurons.



Supplementary Figure 8. miR-218 MRE Distribution on miR-218 target mRNAs.

(a-e) Illustrations showing the relative distribution of miR-218 MREs on the selected miR-218 targets. The highest conserved miR-218 MREs in 3'UTR of each gene, marked by asterisk, were selected to generate the 3'UTR sensor constructs. The 6-mer pivot MREs contain 6 perfect matching nucleotides and either a C or G bulge in MRE positions 5-6[35].

(f) Mutation was introduced to the miR-218 MRE as indicated for miR-218 MRE mutant luciferase reporters in Figure 5c.

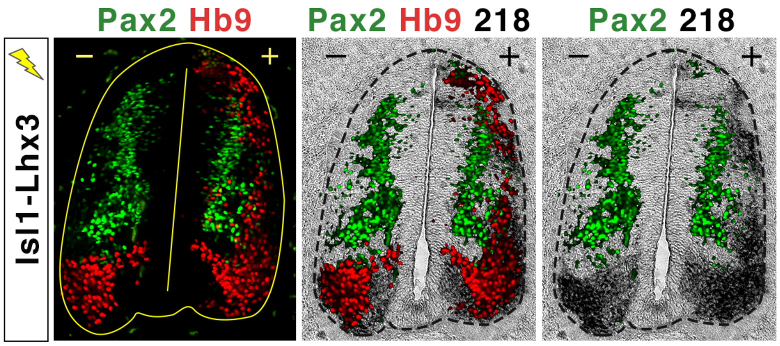


Supplementary Figure 9. Pax2 is a potential miR-218 target and control experiments for miR-218 and miR-Control ESCs

(a) Illustration showing the relative distribution of miR-218 MREs on human Pax2 mRNA. The highest conserved miR-218 MRE in 3’UTR was identified by TargetScan. The 6-mer pivot MREs contain 6 perfect matching nucleotides and either a C or G bulge in MRE positions 5-6[35].

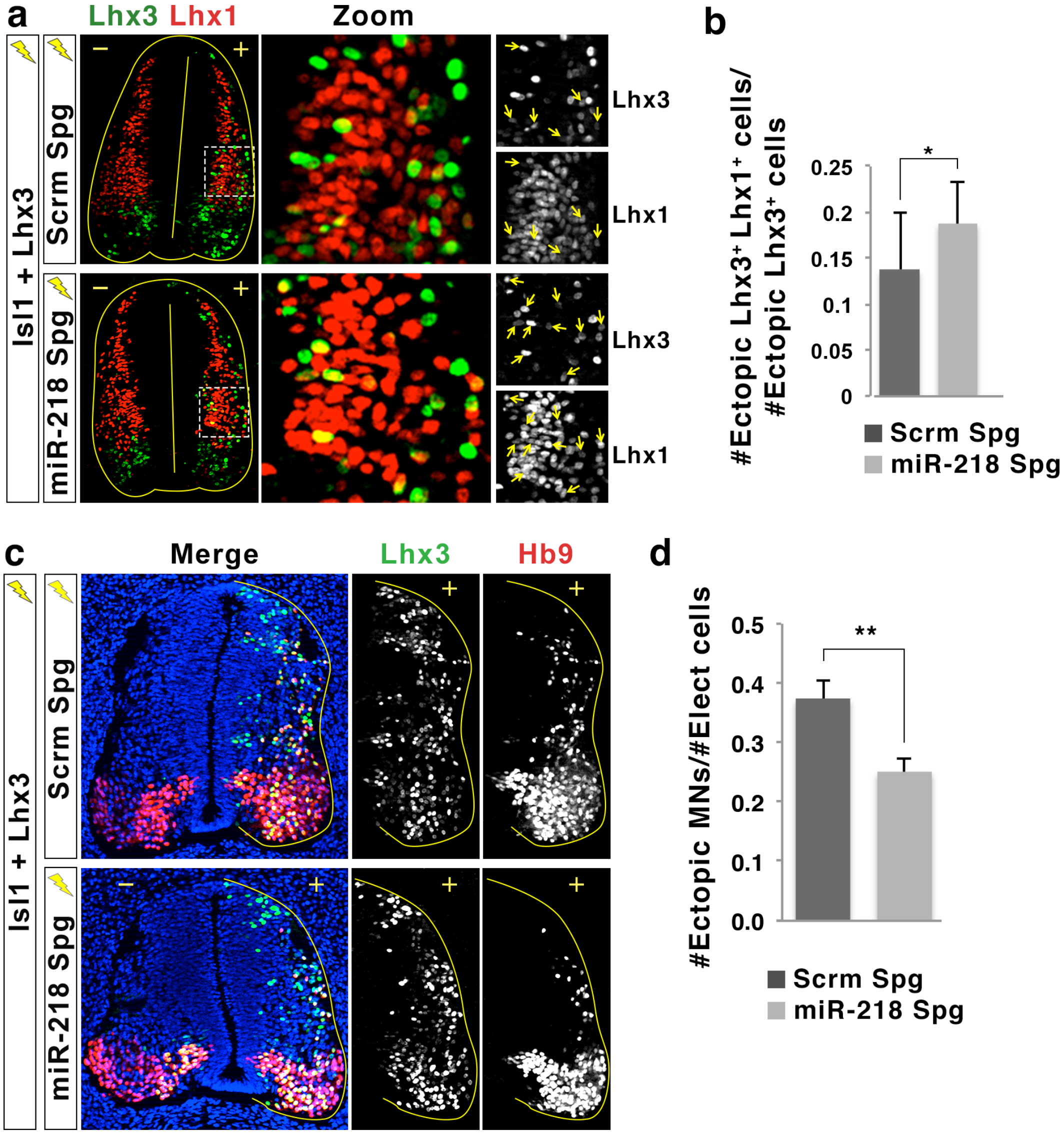
(b) The miR-218 MRE in 3’UTR of the *Pax2* gene is highly conserved among multiple species.

(c) Immunohistochemical analyses of miR-218 and miR-Control (miR-Ctrl) ESCs at differentiation day 6. There was no significant difference in the number of Tuj1$^+$ neurons between miR-218 and miR-Control (miR-Ctrl) ESCs. Hb9$^+$ motor neurons were not formed in either miR-218 or miR-Control ESCs. Weakly Hb9$^+$ cells at the edge of embryoid bodies of miR-Control ESCs are not neurons and appear to represent Hb9 expression in primitive gut tube.



Supplementary Figure 10. Isl1-Lhx3 directs a complete fate transition from interneurons to motor neurons in the dorsal spinal cord.

In situ hybridization for miR-218 and immunohistochemical analyses with Pax2 and Hb9 antibodies on sequential sections of chick embryos electroporated with Isl1-Lhx3. $Hb9^+$ motor neurons are generated at the expense of $Pax2^+$ interneurons. The overlay of Pax2, Hb9 and miR-218 shows that ectopic $Hb9^+$ motor neurons express miR-218, while lacking Pax2 expression. +, electroporated side; -, unelectroporated control side.



Supplementary Figure 11. Interneuron inhibitory action of miR-218, a downstream target of Isl1-Lhx3, is needed for Isl1-Lhx3 to drive efficient motor neuron generation.

(a-d) Immunohistochemical analyses of chick embryos electroporated with Isl1, Lhx3 and either miR-218 sponge inhibitor (miR-218 Spg) or scrambled sponge inhibitor (Scrm Spg). +, electroporated side; -, unelectroporated control side.

(a,b) miR-218 sponge inhibitor significantly increased the proportion of Lhx1-expressing cells among Isl1/Lhx3-electroporated cells, compared to scrambled sponge inhibitor. Zoom panels and arrows show relative prevalence of ectopic Lhx3 and Lhx1 double positive cells. (b) The effect of miR-218 inhibitor on ectopic Lhx3 and Lhx1 double positive cells in (a) was quantified by the ratio of Lhx3 and Lhx1 double positive cells over the number of Lhx3-expressing electroporated cells. Error bars represent the standard error of the mean. *$p < 0.05$, in two-tailed Student's t-test. n = 5-6 embryos.

(c,d) miR-218 sponge inhibitor substantially reduced the efficiency of motor neuron production by the co-expression of Isl1 and Lhx3, compared to scrambled sponge inhibitor. (d) The effect of miR-218 inhibitor on ectopic motor neuron differentiation in (c) was quantified by the ratio of ectopic $Hb9^+$ motor neurons (MNs) over Lhx3-expressing transfected cells (Elect cells). Error bars represent the standard error of the mean. **$p < 0.005$, in two-tailed Student's t-test. n = 5-6 embryos.

ChIP qPCR Primers	Fwd Oligo Sequence (5' -> 3')	Rev Oligo Sequence (5' -> 3')
miR-218-1 peak A	ATATAAAACCCATTAATCCAAGCC	AAGGGTAAATCTAAGCTTCAAGGT
miR-218-2 peak A	AGAGCAGTGACCTCCAATGATTTA	TGCTCTGTCTCTTCTCTCTGACTG
miR-218-2 peak B	GCTATTCTATGGGAAATGGCTTGG	GCTGTACATCCTTCTGGAGAGAGT
RISC-trap qPCR Primers	**Fwd Oligo Sequence (5' -> 3')**	**Rev Oligo Sequence (5' -> 3')**
CycA	GATGCCAGGACCTGTATGCT	GTCTCCTTCGAGCTGTTTGC
Tead1	CACCTGCATCCTCTTGCTCA	GAGAAGCCCACTGGGATGAC
SLC6A1	TGTTCTTCCGTGGAGTGACG	GACGAATCCTGCGAACATGC
BCL11A	GGGAGCACGCCCCATATTAG	GCACAGGCATAGTTGCACAG
Lhx1	TCATCCCCTGGGCTCTACTT	GGTACCGAAACACCGGAAGA
FoxP2	AGTGCAAGACGAGACAGCTC	CGGTCATCCAATGCGTGTTC
GLCE	CGTGCCTTAACAATGTGGCTGTCC	TGCTGTTGCAATGTGGAAGGCAGT
RFT1	TCAGAAGCAGGAGGACGTTG	AGCATGGTCCCTCCGTAGAT
MRE Sensor Oligos	**Fwd Oligo Sequence (5' -> 3')**	**Rev Oligo Sequence (5' -> 3')**
miR-218 MRE	CGCGTACATGGTTAGATCAAGCACAAG	CGCGCTTGTGCTTGATCTAACCATGTA
miR-218* MRE	CGCGCTTGTGCTTGATCTAACCATGTA	CGCGCAACATGGTTAGATCAAGCACAAA
Sensor Primers	**Fwd Oligo Sequence (5' -> 3')**	**Rev Oligo Sequence (5' -> 3')**
Tead1	GCACGCGTGGGAGAGCTGTCTGGTTC	GGCGCGCGGCTCTGGGAAGGCTTCTTT
SLC6A1	GCACGCGTGTGCCCTGTAGCTCCTTAGC	GGCGCGCGGGAAGTGGGACCATGAGAC
BCL11A	GCACGCGTCAAAAGCCCTGGAACGCAAT	GGCGCGCACAGGCAGAGTCAAGTGCT
Lhx1	ACGCGTCAGATTTGCAGGGCTTTCGG	GCGCGCTGCACTGGAGGTCACACAAG
FoxP2	ACGCGTTTTCTGCATCTGCTTTGCGT	GCGCGCACAACTGTGCCACGAATCCA
Ctrl	GCACGCGTTAACTGAGAGGGGACATACAAAGA	GGCGCGCTTGCCAACACCATCATTCCTTCGA
Luciferase Primers	**Fwd Oligo Sequence (5' -> 3')**	**Rev Oligo Sequence (5' -> 3')**
Tead1	GTCTAGAGGGAGAGCTGTCTGGTTC	GACTAGTGGCTCTGGGAAGGCTTCTTT
SLC6A1	GTCTAGAGTGCCCTGTAGCTCCTTAGC	GACTAGTGGGAAGTGGGACCATGAGAC
BCL11A	GTCTAGACAAAAGCCCTGGAACGCAAT	GACTAGTACAGGCAGAGTCAAGTGCT
Lhx1	GTCTAGACAGATTTGCAGGGCTTTCGG	GACTAGTTGCACTGGAGGTCACACAAG
FoxP2	GTCTAGATTTCTGCATCTGCTTTGCGT	GACTAGTACAACTGTGCCACGAATCCA
Luciferase Mutant Primers	**Fwd Oligo Sequence (5' -> 3') (internal primer, overlap extension PCR)**	**Rev Oligo Sequence (5' -> 3') (internal primer, overlap extension PCR)**
Tead1	TTCCAAGCTAGCAAAATACTGG	TTTTGCTAGCTTGGAAAGGA
SLC6A1	ACAATATGCTAGCTAATATTCTGAGG	GAATATTAGCTAGCATATTGTAGAGAAA
BCL11A	TATAGCTAGCACGTGGTACTATTTGC	CGTGCTAGCTATAAATCATATTATTTTC
Lhx1	GTATTGCTAGCTTAATTATTCTATTTGG	TAATTAAGCTAGCAATACTGTAAAGGTG
FoxP2	TGTTGCTAGCTCAGTTTAAAATTT	CTGAGCTAGCAACATCTGTTTATG

Supplementary Table 1. DNA Oligos

Sponge Inhibitor	Oligo Sequence (5' -> 3')
miR-218 Sponge 10x	TAGACAACATGGTTTGGGAAGCACAATAATCAACATGGTTTGGGAAGCACAATAATCAACATGGTTTGGGAAG CACAATAATCAACATGGTTTGGGAAGCACAATAATCAACATGGTTTGGGAAGCACAATAATCAACATGGTTTG GGAAGCACAATAATCAACATGGTTTGGGAAGCACAATAATCAACATGGTTTGGGAAGCACAATAATCAACATG GTTTGGGAAGCACAATAATCAACATGGTTTGGGAAGCACAATAATACTA
Scrm Sponge 10x	TAGAGACTACTATACGAGTAACAGATAATGACTACTATACGAGTAACAGATAATGACTACTATACGAGTAACA GATAATGACTACTATACGAGTAACAGATAATGACTACTATACGAGTAACAGATAATGACTACTATACGAGTA CAGATAATGACTACTATACGAGTAACAGATAATGACTACTATACGAGTAACAGATAATGACTACTATACGAGT AACAGATAATGACTACTATACGAGTAACAGATAATACTA
2'Ome RNA	**Oligo Sequence (5' -> 3')**
Anti-miR-218	mAmCmAmUmGmGmUmUmAmGmAmUmCmAmAmGmCmAmCmAmAmA
Anti-miR-67	mUmCmUmAmCmUmCmUmUmUmCmUmAmGmGmAmGmGmUmUmGmUmGmA
miRNA	**Oligo Sequence (5' -> 3')**
mIR-218 fwd	GATCCACATGGTTAGATCAAGCACAATTCAAGAGATTGTGCTTGATCTAACCATGTTTTTA
mIR-218 rev	AGCTTAAAAACATGGTTAGATCAAGCACAATCTCTTGAATTGTGCTTGATCTAACCATGTG
mIR-Ctrl fwd	GATCCCCGGCTTACGCGTTCTCGTCTTCTCTTGAAAGACGAGAACGCGTAAGCCGGTTTTTA
mIR-Ctrl rev	AGCTTAAAAACCGGCTTACGCGTTCTCGTCTTCTCTTGAAAGACGAGAACGCGTAAGCCGGG

Supplementary Table 2. DNA and RNA Oligos

Supplementary Methods

Quantification of Pixel Intensity

GFP/RFP pixel intensity was determined using an ImageJ analysis script, which was developed by Dr. Greg Scott MD, PhD. Images must be acquired using standard widefield fluorescence microscopy for linear pixel intensity comparisons. To use the ImageJ GFP/RFP pixel intensity script, area selections are made on an 8-bit image stack that contains unadjusted RFP, GFP and DAPI channel images, where RFP = channel 1, GFP = channel 2, and DAPI = channel 3. Using ImageJ software, open an image stack and select the DAPI channel image. Create an area selection using the free-hand selection tool to outline the desired area for pixel intensity measurement. Next, click on the “Plugins” in the toolbar, select “Macros” and then “Run” from the dropdown menus. Select the ImageJ script text shown below (saved as .txt file). Double click on this program text file and it will automatically use the area that was selected in the DAPI panel to define the pixel selection mask in the RFP image. Next, the program will measure the RFP pixel intensity within the selected area, excluding background with a min/max threshold listed in the script (20/255). Next, the program applies the same area selection mask to the GFP channel and measures pixel intensity. The program then calculates the average GFP divided by RFP pixel intensity ratio and provides the output with sample number, area measured, GFP/RFP pixel intensity, as well as minimum and maximum GFP/RFP pixel intensity ratios. The final text in the script closes the remaining open images. The script listed below can be copied and saved as a standard .txt file to be used for image GFP/RFP pixel intensity analysis.

```
//CH1 = RFP, CH2 = GFP, CH3
= DAPI
//Create selection in DAPI
channel before use of this
//8-bit conversion
run("8-bit");
//Grab Titles/Slice Names
parent=getTitle();
setSlice(1);
setMetadata("Label",
"channel1");
imc1=getInfo("slice.label");
setSlice(2);
setMetadata("Label",
"channel2");
imc2=getInfo("slice.label");
setSlice(3);
setMetadata("Label",
"channel3");
imc3=getInfo("slice.label");
//Clear Data Outside DAPI
region selection
run("Create Mask");
mask1=getTitle();
imageCalculator("AND stack",
parent,mask1);
selectImage(mask1);
run("Close");
selectImage(parent);
//run("Clear Outside", "stack");
//Create mask from threshold of
RFP slice
run("Select None");

setSlice(1);
setThreshold(20, 255);
run("Create Selection");
slxn=getInfo("selection.name")
run("Create Mask");
mask2=getTitle();
//Mask image stack with RFP
slice mask
imageCalculator("AND stack",
parent,mask2);
selectImage(parent);
resetThreshold();
run("Select None");
//Delete DAPI channel
setSlice(3);
run("Delete Slice");
//Split channels
run("Stack to Images");
selectImage(imc1);
//divide GFP by RFP
imageCalculator("Divide create
32-bit", imc2,imc1);
quotient=getTitle();
selectImage(quotient);
run("Measure");
//CLOSE REMAINING
IMAgES
selectImage(mask2);
run("Close");
selectImage(imc1);
run("Close");
selectImage(imc2);
run("Close");
selectImage(quotient);
run("Close");

//END OF SCRIPT.  SCRAPS
BELOW

//imc1=getInfo("slice.label");
//print(imc1);
//run("Stack to Images");
//selectimage(imc1);
//joe=getTitle();
//print(joe);
//imid1 = getImageID();
//selectimage();
//run("Create Mask");
//run("Stack to Images");
//setSlice(4);
//run("Delete Slice
```

Supplementary References

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