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Supplemental Information

Circular RNA SNX29 Sponges miR-744 to Regulate Proliferation and Differentiation of Myoblasts by Activating the Wnt5a/Ca^{2+} Signaling Pathway

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Figure S1

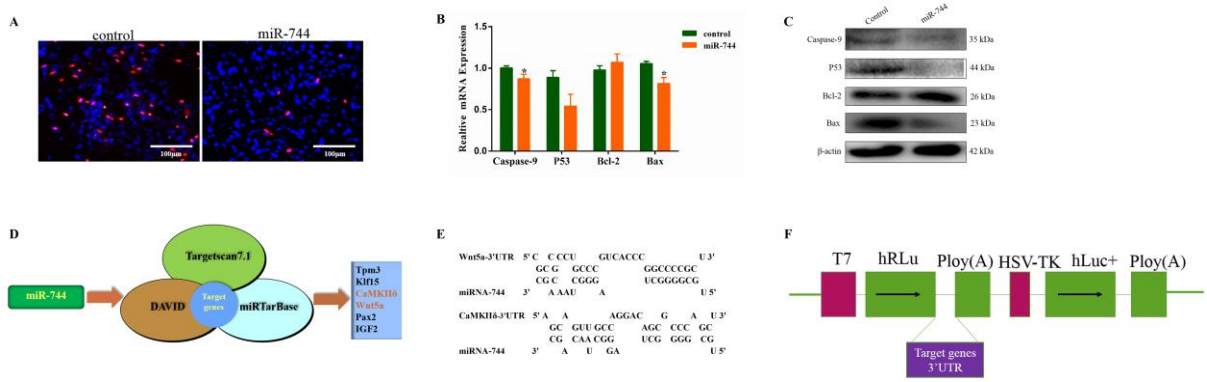

Figure S2

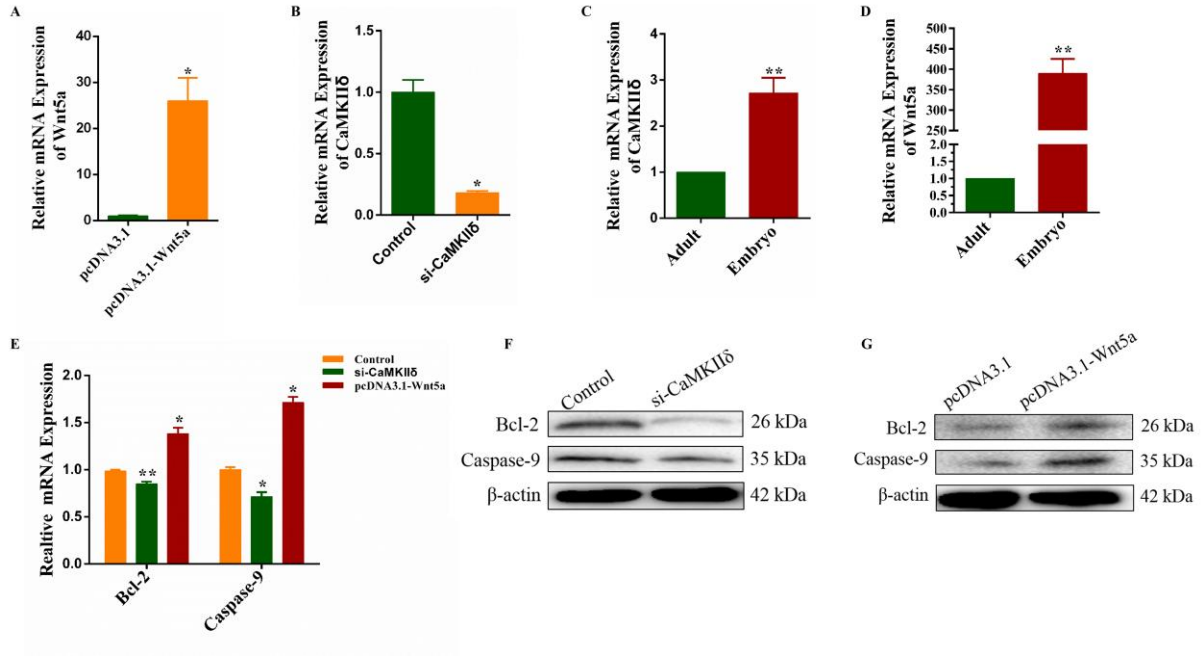

Figure S3

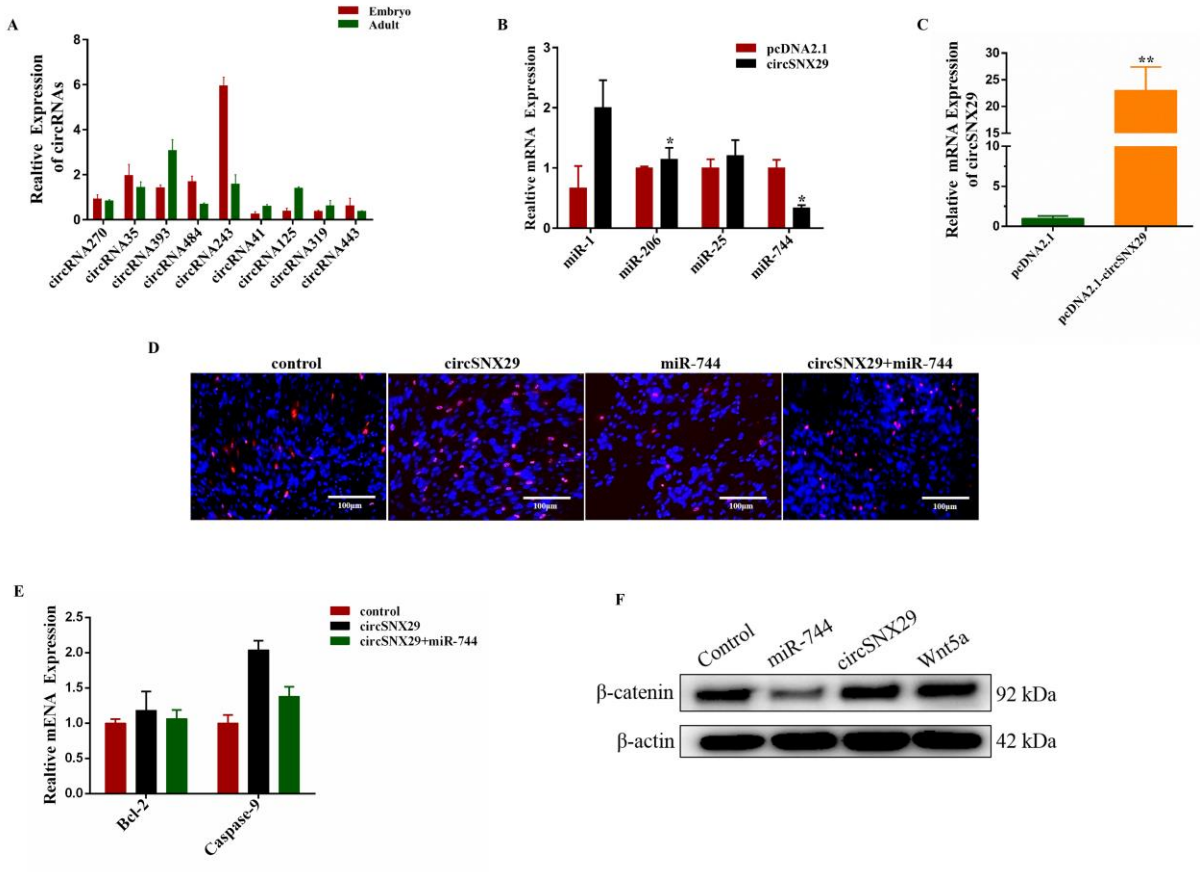
A
Embryo
Adult
Realtive Expression of circRNAs
circRNA270
circRNA35
circRNA393
circRNA484
circRNA243
circRNA41
circRNA125
circRNA319
circRNA443
B
pcDNA2.1
circSNX29
Realtive mRNA Expression
miR-1
miR-206
miR-25
miR-744
C
Relative mRNA Expression of circSNX29
pcDNA2.1
pcDNA2.1-circSNX29
D
control
circSNX29
miR-744
circSNX29+miR-744
100μm
E
control
circSNX29
circSNX29+miR-744
Realtive mENA Expression
Bcl-2
Caspase-9
F
Control
miR-744
circSNX29
Wnt5a
β-catenin
92 kDa
β-actin
42 kDa

Figure S4

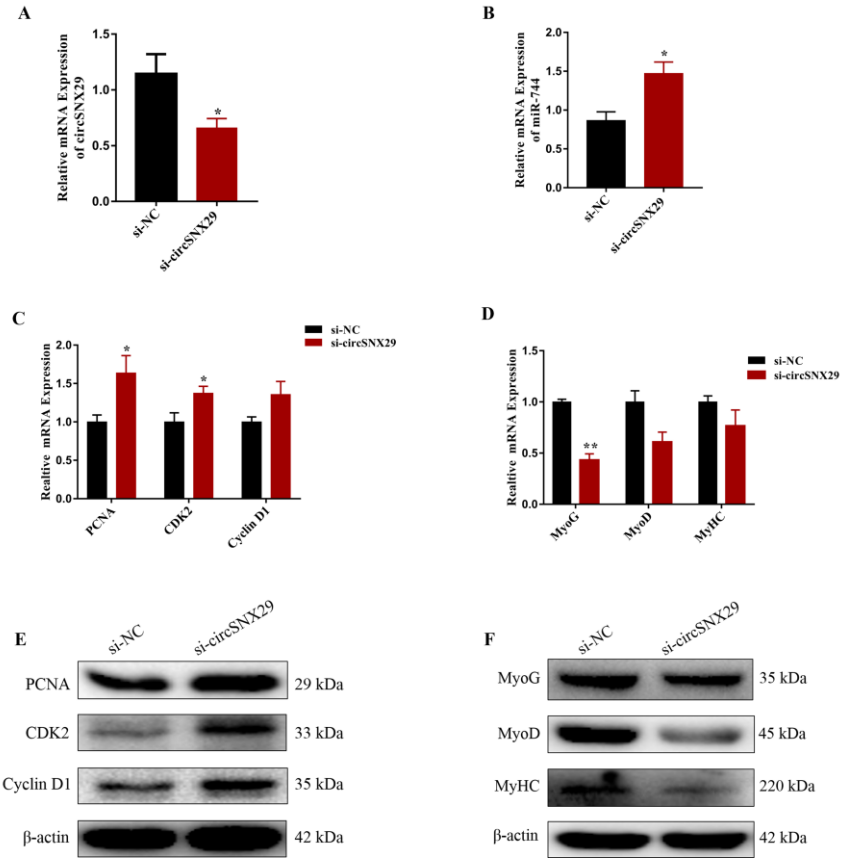
A
Relative mRNA Expression of circSNX29
1.5
1.0
0.5
0.0
*
si-NC
si-circSNX29
B
Relative mRNA Expression of miR-744
2.0
1.5
1.0
0.5
0.0
*
si-NC
si-circSNX29
C
si-NC
si-circSNX29
Realtive mRNA Expression
2.0
1.5
1.0
0.5
0.0
*
*
PCNA
CDK2
Cyclin D1
D
si-NC
si-circSNX29
Realtive mRNA Expression
1.5
1.0
0.5
0.0
**
MyoG
MyoD
MyHC
E
si-NC
si-circSNX29
PCNA
29 kDa
CDK2
33 kDa
Cyclin D1
35 kDa
β-actin
42 kDa
F
si-NC
si-circSNX29
MyoG
35 kDa
MyoD
45 kDa
MyHC
220 kDa
β-actin
42 kDa

Figure Legends

Supplementary Figure 1. Overexpression of miR-744 Inhibits Apoptosis of Bovine Primary Myoblasts. (A-C) Myoblasts were transfected with pcDNA3.1 or pcDNA3.1-miR-744. (A) Cell apoptosis was determined by Hoechst 33342/PI dual staining assays; the scale bar represents 100 μm. (B, C) mRNA and protein levels of apoptosis marker genes (Caspase-9, P53, Bcl-2, and Bax) was detected by quantitative real-time PCR (qPCR) and Western blotting assay. (D) The schematic illustration of the proposed model depicting of 6 putative target genes of miR-744 as mentioned above from 3 microRNA prediction databases, TargetScan.org, DAIVD.org, and miRtarbase.org. (E) The binding sites analysis in CaMKIIδ and Wnt5a 3'UTR. (F) The sketch map of psi-Check2 vector in which CaMKIIδ and Wnt5a 3'UTR and mutation 3'UTR were inserted into the 3' end of Renilla luciferase gene (hRluc). Values are mean ± SEM for three biological replicates, $*P < 0.05$.

Supplementary Figure 2. Effects of Wnt5a Overexpression and CaMKIIδ Silencing on the Cell Apoptosis. (A, B) Myoblasts were treated by pcDNA3.1-Wnt5a or si-CaMKIIδ, and the mRNA expression of Wnt5a and CaMKIIδ were measured by real-time qPCR. (C, D) The expression of Wnt5a and CaMKIIδ in skeletal muscle of Qinchuan cattle at the embryonic stage compared with the adult stage is shown. (E-G) The expression of apoptosis marker genes Bcl-2 and caspase-9 was measured by qPCR and Western blotting. Values are mean ± SEM for three biological replicates, $*P < 0.05$; $**P < 0.01$.

Supplementary Figure 3. Overexpression of circSNX29 Promotes Cell Apoptosis by Targeting miR-744. (A) Validation of differential expression levels of selected circRNAs at embryonic and adult stages. (B) Effect of circSNX29 on the abundance of miR-1, miR-206, miR-25 and miR-744. (C) Visualization of the efficiency of circSNX29 overexpression vector pcDNA2.1-circSNX29 by qPCR. (D) Cell apoptosis was determined by Hoechst 33342/PI dual staining assays; the scale bar represents 100 μm.（E) the mRNA expression of apoptosis marker genes (Bcl-2 and Caspase-9) was detected using qPCR. (F) Nuclear β-catenin levels of cells transfected with circSNX29 and Wnt5a were detected Western blotting. Values are mean ± SEM for three biological replicates, $*P < 0.05$; $**P < 0.01$.

Supplementary Figure 4. Knockdown of circSNX29 Suppresses Cell Differentiation while Promoting Proliferation. (A) The efficiency of circSNX29 knockdown were evaluated by qPCR. (B) The effects of circSNX29 silencing on the expression of miR-744 in myoblasts. (C and D) Real-time qPCR analysis of the

proliferation/differentiation-related genes. (E and F) Western blot detection of PCNA, CDK2, CyclinD1, MyOG, MyOD, and MyHC protein levels with β-actin treatment. Values are mean ± SEM for three biological replicates, *$P < 0.05$; **$P < 0.01$.

Table S1 Stem-loop primers for reverse-transcribed miRNAs

miRNA ID	stem-loop primer
bta-miR-1	gtcgtatccagtgcagggtccgaggtattcgcactggatacgacATACATA
bta-miR-206	gtcgtatccagtgcagggtccgaggtattcgcactggatacgacCCACACA
bta-miR-101	gtcgtatccagtgcagggtccgaggtattcgcactggatacgacTTCAGTT
bta-miR-423-5p	gtcgtatccagtgcagggtccgaggtattcgcactggatacgacAAAGTCT
bta-miR-7f	gtcgtatccagtgcagggtccgaggtattcgcactggatacgacAACTATA
bta-miR-25	gtcgtatccagtgcagggtccgaggtattcgcactggatacgacTCAGACC
bta-miR-128	gtcgtatccagtgcagggtccgaggtattcgcactggatacgacAAAGAGA
bta-miR-744	gtcgtatccagtgcagggtccgaggtattcgcactggatacgacTGCTGTT
bta-miR-140	gtcgtatccagtgcagggtccgaggtattcgcactggatacgacTCCGTGG
bta-miR-126	gtcgtatccagtgcagggtccgaggtattcgcactggatacgacCGCATTA
bta-miR-134	gtcgtatccagtgcagggtccgaggtattcgcactggatacgacCCACTCT
bta-miR-150	gtcgtatccagtgcagggtccgaggtattcgcactggatacgacACACTGG

Table S2 Primers for qPCR

miRNA ID	Forward primer 5'→3'	Reverse primer 5'→3'
bta-miR-1	gcgcgTGGAATGTAAAGAAG	GCAGGGTCCGAGGTATTC
bta-miR-206	gcgcTGGAATGTAAGGAAG	GCAGGGTCCGAGGTATTC
bta-miR-101	gcgcgcgcTACAGTACTGTGATA	GCAGGGTCCGAGGTATTC
bta-miR-423-5p	atttTGAGGGGCAGAGAGC	GCAGGGTCCGAGGTATTC
bta-miR-7f	gcgcgcacaTGAGGTAGTAGATTG	GCAGGGTCCGAGGTATTC
bta-miR-25	gcgcCATTGCACTTGTCTC	GCAGGGTCCGAGGTATTC
bta-miR-128	gcgcTCACAGTGAACCGGT	GCAGGGTCCGAGGTATTC
bta-miR-744	atataTGCGGGGCTAGGGCT	GCAGGGTCCGAGGTATTC
bta-miR-140	gcgcgcTACCACAGGGTAGAA	GCAGGGTCCGAGGTATTC
bta-miR-126	gcgcaaCGTACCGTGAGTAAT	GCAGGGTCCGAGGTATTC
bta-miR-134	agcgcgcTGTGACTGGTTGACC	GCAGGGTCCGAGGTATTC
bta-miR-150	gcgcgcTCTCCCAACCCTTGT	GCAGGGTCCGAGGTATTC
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
Wnt5a	CAACTGGCAGGACTTTCTCAA	CATCTCCGATGCCGGAACT
CaMKIIδ	ACACCCGTGGATCTGTCAAC	ACACTCGAACTGGACTTCCT
Calcineunrin	CGGAGGAGACCAAGCAGAAG	TGTAGTCCTCGTCCTCGTCC
NFATC1	TTTAACCAGAGCGACGAGGG	GTCCACTGTCCCGCCATAG
β-actin	CATCCTGACCCTCAAGTA	CTCGTTGTAGAAGGTGTG
GAPDH	ACCACTTTGGCATCGTGGAG	GGGCCATCCACAGTCTTCTG

MyHC	GGAGGCTGATGAACAAGCCAA	GAAGTCTCGGGTCTTTGCTCT
MyoG	AGGGGATCATCTGCTCCCAG	ATCCCGGCAGACAATCTCAG
MyoD	ACGGCATGATGGACTACAGC	AGGCAGTCGAGGCTCGACA
BCL-2	ATGACCGAGTACCTGAAC	CATACAGCTCCACAAAGG
P53	CACCAGCCAAAGAAGAAAC	CTCTCGGAACATCTCATAG
Caspase9	TGGTGGTCATCCTGTCTC	CATCCATCTGTGCCATAAAC
BAX	GAGATGAATTGGACAGTAACA	TTGAAGTTGCCGTCAGAA
CDK2	AGGGAACGTACGGAGTTGTG	GACATCCAGCAGCTTGACAAT
PCNA	TCCAGAACAAGAGTATAGC	TACAACAGCATCTCCAAT
CyclinD1	ATGAAGGAGACCATCCCCCT	CGCCAGGTTCCACTTGAGTT
CircSNX29	ACAGTGTGGAAGCCAGTCCT	TCCAGCGGCTAACTGTCATGG
SNX29	TGTTACGAAGGTCCAGGCAC	TGGGACAACTTCGGCTGTTT

Table S3 Primers for vector construction

Name	Primer sequence 5'→3'
$pcDNA_{2.1}$-circSNX29-F	CGGGATCCCAGAGTCCATGACAGTTA
$pcDNA_{2.1}$-circSNX29-R	GTACCGAGGCAGAAGGTAACAGGC
$pcDNA_{3.1}$-Wnt5a(CDS)-F	GGGGTACCCCATGAAGAAGTCGATTGGAATATTAA
$pcDNA_{3.1}$-Wnt5a(CDS)-R	CGGGATCCCG CTACTTGCAGACGAACTGGTCCA
Psi-CHECK-circSNX29-W-F	GCTCGAGGCCGCTGGACGCAGGCACCTG
Psi-CHECK-circSNX29-W-R	GGCGGCCGCTAACTGTCATGGACTCTGGTACC
Psi-CHECK-circSNX29-Mut-F	GCTCGAGGCCGCTGGACGCAGGCACCTG
Psi-CHECK-circSNX29-Mut-R	GGCGGCCGCTAACTGTCATGGACTCTGGTACC
Psi-CHECK-CaMKIIδ-3'UTR-W-F	GCTCGAGCGTCTTACTGAGTGGAGCAGTTG
Psi-CHECK-CaMKIIδ-3'UTR-W-R	GGCGGCCGCAGCCCCACAGGAATGGAATAAA
Psi-CHECK-CaMKIIδ-3'UTR-Mut-F	GCTCGAGCCCTTAATTATCCTGCGTGAT
Psi-CHECK-CaMKIIδ-3'UTR-Mut-R	GGCGGCCGAGCCCCACAGGAATGGAATAAA
Psi-CHECK-Wnt5a-3'UTR-W-F	GCTCGAGGCTCCCAGGACCCACTTATTTATA
Psi-CHECK-Wnt5a-3'UTR-W-R	GGCGGCCGCGGGATATGAGATTCTTGTTCTCAA
Psi-CHECK-Wnt5a-3'UTR-Mut-F	GCTCGAGGCTCCCAGGACCCACTTATTTATA
Psi-CHECK-Wnt5a-3'UTR-Mut-R	GGCGGCCGCTTATAATATTATTAACGCGAGAGTTCTC

Notes: The nucleotides with underline is the restriction enzyme cutting site.

Text S1: CircSNX29 sequence

CircSNX29>**GCCGCTGG**ACGCAGGCACCTGCCTCTCCCAGATGCATGGCTGGGCC

CCGCTGCAAGTGCTGCACGGCCACGCCGAGGTGCTCTTCCCCGTCAGCGGCGTGG

GCTCCTACGGGCCTGCAGATGCCCCCCTCGGGAGCCTGGAGAACGGGACGGGAC

CTGAGGACCACATCCTCCCGGAGCCGGGACCCCGGTACAGTGTGGAAGCCAGTC

CTCCAGGCCAGGAGAGTCCTCTGAGCAGCCTGTTACCTTCTGCCTCGGTACCAGA

GTCCAT**GACAGTTA**

ABBREVIATIONS: circRNAs, Circular RNAs; *SNX29*, sorting nexin 29; CaMKIIδ, calmodulin-dependent protein kinase; ceRNAs, competing endogenous RNAs; KEGG, kyoto encyclopedia of genes and genomes; MyoD, myogenic differentiation antigen; Myf5, myogenic factor 5; MyoG, myogenin; EIciRNAs, exon-intron circRNAs; HDAC4/5, histone deacetylase 4/5; MEF2, myocyte enhancer factor 2; HEK293T, human embryonic kidney 293; CCK-8, cell counting kit-8; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; EdU, 5-ethynyl-2′-deoxyuridine; MyHC, myosin heavy Chains; Bcl-2, B-cell lymphoma 2; Bax, bcl2-associated x; P53, tumor suppressor gene; CDK2, cyclin-dependent kinase 2; PCNA, proliferating cell nuclear antigen; cell cycle protein d1; siRNA, small interfering RNA; qRT-PCR, quantitative RT-PCR.