Therapeutic targeting of circ-CUX1/EWSR1/MAZ axis inhibits glycolysis and neuroblastoma progression

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Abstract

Aerobic glycolysis is a hallmark of metabolic reprogramming in tumor progression. However, the mechanisms regulating glycolytic gene expression remain elusive in neuroblastoma (NB), the most common extracranial malignancy in childhood. Herein, we identify that CUT-like homeobox 1 (CUX1) and CUX1-generated circular RNA (circ-CUX1) contribute to aerobic glycolysis and NB progression. Mechanistically, p110 CUX1, a transcription factor generated by proteolytic processing of p200 CUX1, promotes the expression of enolase 1, glucose-6-phosphate isomerase, and phosphoglycerate kinase 1, while circ-CUX1 binds to EWS RNA-binding protein 1 (EWSR1) to facilitate its interaction with MYC-associated zinc finger protein (MAZ), resulting in transactivation of MAZ and transcriptional alteration of CUX1 and other genes associated with tumor progression. Administration of an inhibitory peptide blocking circ-CUX1-EWSR1 interaction or lentivirus mediating circ-CUX1 knockdown suppresses aerobic glycolysis, growth, and aggressiveness of NB cells. In clinical NB cases, CUX1 is an independent prognostic factor for unfavorable outcome, and patients with high circ-CUX1 expression have lower survival probability. These results indicate circ-CUX1/EWSR1/MAZ axis as a therapeutic target for aerobic glycolysis and NB progression.

Keywords circular RNA; CUT-like homeobox 1; EWS RNA-binding protein 1; MYC-associated zinc finger protein; tumor progression

Introduction

Neuroblastoma (NB), a malignant tumor arising from primitive neural crest, accounts for 15% of cancer-related mortality in childhood (Brodeur, 2003). For high-risk NB, the clinical outcome remains poor in despite of multimodal therapeutic approaches (Brodeur, 2003). To support their tumorigenecity and aggressiveness, tumor cells uptake and convert a large amount of glucose into lactic acid even in the presence of adequate oxygen, which is known as aerobic glycolysis or Warburg effect (Hanahan & Weinberg, 2011). Activation of oncoproteins (c-Myc) or inactivation of tumor suppressors (p53) contributes to aberrant expression of transporters and metabolic enzymes of aerobic glycolysis (Shim et al, 1997; Bensad et al, 2006; Yang et al, 2014). However, identification of transcriptional regulators for aerobic glycolysis in NB still remains to be determined.

Circular RNAs (circRNAs) are a novel class of endogenous noncoding RNAs that are generated from exons or introns, and may function as microRNA (miRNA) sponges, regulators of transcription and splicing, or partners of RNA-binding protein (RBP) (Lasda & Parker, 2014; Li et al, 2015b). For example, circRNA antisense to cerebellar-degeneration-related protein 1 (Cdr1as) harbors 70 binding sites for miR-7 to regulate its transport in neurons (Piwrecka et al, 2017). A special class of exon–intron circRNAs, such as circEIF3J and circPAP2, is predominantly localized in the nucleus and enhance transcription of their parental genes (Li et al, 2015b). In addition, intronic circRNAs, such as ci-ankrds52, are able to regulate transcription efficiency of parental genes by binding to RNA polymerase II (Zhang et al, 2013). However, the roles of circRNAs in aerobic glycolysis during tumor progression remain largely elusive.

In this study, we identify CUT-like homeobox 1 (CUX1) as a transcription factor facilitating aerobic glycolysis and tumor progression in NB. We also reveal the oncogenic functions of a CUX1-generated intron-containing circular RNA (circ-CUX1) in tumorigenesis and aggressiveness. Elevated circ-CUX1 promotes the aerobic glycolysis, growth, and aggressiveness of NB cells by binding to EWS RNA-binding protein 1 (EWSR1) and facilitating its interaction with MYC-associated zinc finger protein (MAZ), resulting in MAZ transactivation and transcriptional alteration of CUX1 and other genes associated with tumor progression, suggesting...
circ-CUX1/EWSR1/MAZ axis as a therapeutic target for aerobic glycolysis and NB progression.

Results

CUX1 facilitates aerobic glycolysis and tumor progression

Comprehensive analysis of a microarray dataset (GSE16476) (Mole-naar et al, 2012) of 88 NB cases identified 8 differentially expressed glycolytic genes (fold change > 2.0, P < 0.05) that were consistently associated with death, advanced international neuroblastoma staging system (INSS) stages, and clinical progression (Fig 1A). Similarly, we also found 52 transcription factors consistently associated with these clinical features (Fig 1A), which were subjective to further over-lapping analysis with potential transcription factors regulating all of 8 glycolytic genes revealed by Genomatix program (http://www. genomatix.de/solutions/genomatix-software-suite.html). The results indicated CUX1 as the top transcription factor ranking by number of potential targets (Fig 1A). Higher transcript levels of CUX1 isoform p200 were noted in NB cell lines SH-SYSY, IMR32, and SK-N-AS, while p75 (Goulet et al, 2002) was expressed at very low levels (Appendix Fig S1A). Consistently, elevated levels of p200 CUX1 and its proteolytically processed isoform p110 were noted in these NB cells, cervical cancer HeLa cells, colon cancer LoVo cells, and prostate cancer PC-3 cells, than those of non-transformed normal MCF10A cells (Appendix Fig S1A). However, both transcript and protein levels of CD5/cut alternatively spliced cDNA (CAS) (Gillingham et al, 2002) were not differently expressed between normal and tumor cells (Appendix Fig S1A). In an independent cohort of 54 primary NB tissues, the transcript levels of p200 CUX1, but not of CASP, were higher than those in normal fetal adrenal medulla (P < 0.05, Appendix Fig S1A), especially in cases with poor stroma (P = 0.0002) or advanced INSS stages (P = 0.007), without association with MYCN amplification (P = 0.56, Appendix Fig S1B).

Notably, ectopic expression or knockdown of p200 CUX1 (referred as CUX1) increased and decreased the levels of p110 CUX1, enolase 1 (ENO1), glucose-6-phosphate isomerase (GPI), or phosphoglycerate kinase 1 (PGK1), but not of aldolase, fructose-bisphosphate A (ALDOA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hexokinase 2 (HK2), lactate dehydrogenase A (LDHA), or pyruvate kinase M (PKM), in IMR32 and SH-SYSY cells (Fig 1B and C, Appendix Fig S1C and D). Treatment with E64D, an inhibitor of cathepsin L (Goulet et al, 2004), abolished the up-regulation of p110 CUX1, ENO1, GPI, and PGK1 induced by CUX1 over-expression (Appendix Fig S1E). Ectopic expression of p110 CUX1 increased the levels of ENO1, GPI, or PGK1 in IMR32 cells (Appendix Fig S1E). Meanwhile, knockdown of CASP, a Golgi-localized CUX1 isoform (Gillingham et al, 2002), did not affect the transcript and protein levels of these glycolytic genes in SH-SYSY cells (Appendix Fig S1F and G). The CUX1 enrichment and promoter activity of ENO1, GPI, and PGK1 were increased and decreased by p110 CUX1 over-expression, CUX1 knockdown, or E64D treatment in IMR32 and SH-SYSY cells, respectively (Appendix Fig S1H, Fig 1D and E). Over-expression of p110 CUX1 increased the extracellular acidification rate (ECAR) and reduced the oxygen consumption rate (OCR) in IMR32 cells, while CUX1 knockdown or E64D treatment significantly attenuated the glycolytic process (Fig 1F and G). Accordingly, p110 CUX1 over-expression, CUX1 knockdown, or E64D treatment increased and decreased the glucose uptake, lactate production, ATP levels, anchorage-independent growth, and invasion of IMR32 cells, respectively (Appendix Fig S2A–D). Treatment with 2-deoxy-glucose (2-DG), an established glycolysis inhibitor (Zhang et al, 2014), abolished the increase in these features of IMR32 cells induced by p110 CUX1 over-expression (Appendix Fig S2A–D). In public datasets, there was positive expression correlation between CUX1 and ENO1, GPI, or PGK1 in NB, colon cancer, or prostate cancer tissues (Appendix Fig S2E), and their levels were associated with poor survival of tumor patients (Appendix Fig S3). Multivariate Cox regression analysis revealed CUX1 as an independent prognostic factor [hazard ratio = 2.105, 95% confidence interval = 1.087–3.243, P = 0.038] for poor survival of NB patients. These findings indicated that CUX1 was a transcription factor facilitating aerobic glycolysis and tumor progression.

Circ-CUX1 is up-regulated in NB tissues and cell lines

The copy number of CUX1 gene, locating at chr7: 101460882-101901513, was neither significantly altered in NB (Appendix Fig S4A) nor associated with death, MYCN amplification, INSS stages, or survival of NB cases derived from Oncogenomics database (Appendix Fig S4A and B). There were no genetic variants of CUX1 gene in 563 NB cases of public datasets (Appendix Fig S4C). Among 37 potential circRNAs generated from CUX1 gene in circBase (Glazar et al, 2014), 15 had more than 2 read scores, while further RT–PCR validation revealed 7 detectable circRNAs in IMR32 cells (Fig EV1A)

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**Figure 1. CUX1 facilitates aerobic glycolysis and tumor progression in NB.**

A. Venn diagram indicating the identification of differentially expressed glycolytic genes and transcription factors (fold change > 2.0, Student's t-test, P < 0.05) in 88 NB cases (GSE16476), and over-lapping analysis with potential transcription factors regulating glycolytic genes revealed by Genomatix program.

B, C Real-time qRT–PCR (B, normalized to ß-actin, n = 5) and Western blot (C) assays revealing the expression of CUX1 and glycolytic genes in IMR32 cells stably transfected with empty vector (mock), p200 CUX1, scramble shRNA (sh-Scb), sh-CUX1 #1, or sh-CUX1 #2. Student's t-test, one-way ANOVA, *P < 0.05 versus mock or sh-Scb.

D, E ChIP and qPCR using Flag and CUX1 antibodies (D) and dual-luciferase (E) assays indicating the p110 CUX1 enrichment and promoter activity of ENO1, GPI, and PGK1 in IMR32 and SH-SYSY cells stably transfected with mock, Flag-tagged p110 CUX1, sh-Scb, sh-CUX1 #1, or sh-CUX1 #2, and those treated with E64D (10 μM) for 24 h (n = 5). Student's t-test, one-way ANOVA, *P < 0.05 versus mock, sh-Scb, or DMSO.

F, G Seahorse tracing curves (F), ECAR and OCR (G) of IMR32 cells stably transfected with mock, p110 CUX1, sh-Scb, sh-CUX1 #1, or sh-CUX1 #2, and those treated with E64D (10 μM) for 24 h (n = 5). Student's t-test, one-way ANOVA, *P < 0.05 versus mock, sh-Scb, or DMSO.

Data information: Data are presented as mean ± SEM. Exact P values are specified in Appendix Table S4. Source data are available online for this figure.
Figure 1.

A. Glycolysis

Stage 2 vs. 4
Stage 4S vs. 4

Death
Progression

Identified TFs regulating glycolytic genes (GSE16476)

| TFs     | Glycolytic genes |
|---------|------------------|
| CUX1    | ALDOA, ENO1, GAPDH, GPI, HK2, LDHA, PGK1, PKM |
| ENO1    | GAPDH, GPI, HK2, LDHA, PGK1, PKM |
| GAPDH   | HK2, LDHA, PGK1, PKM |
| GPI     | HK2, LDHA, PGK1, PKM |
| HK2     | LDHA, PGK1, PKM |
| LDHA    | PGK1, PKM |
| PGK1    | PKM |
| PKM     |              |
| E2F3    | ENO1, LDHA, PGK1 |
| MAZ     | ENO1, GAPDH, GPI, PKM |
| SRF     | ENO1, GAPDH, GPI, HK2, LDHA, PGK1, PKM |
| SP4     | ENO1, GAPDH, GPI, HK2, LDHA, PGK1, PKM |
| VAX2    | ENO1, HK2, LDHA, PGK1 |

B. IMR32

Relative transcript levels (by qRT-PCR)

C. Transcription factor

p200 CUX1
p110 CUX1
ENO1
GPI
PGK1
β-actin

D. Relative CUX1 binding to promoter (by qPCR)

E. Relative luciferase activity (Firefly/Renilla ratio)

F. Glycolytic function

ECAR (mP/min)

Time (min)

G. OCR (pM/min)

Mock
p110 CUX1
sh-Scb
sh-CUX1 #1
sh-CUX1 #2
DMSO
E64D

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and B). Three of them were differentially expressed between normal fetal adrenal medulla and NB tissues (Fig EV1C). Knockdown of \( hsa_{\text{circ}}_{0005253} \) (termed as \( \text{circ-CUX1} \)), but not of \( hsa_{\text{circ}}_{0132813} \) or \( hsa_{\text{circ}}_{0005253} \), attenuated the CUX1 transactivation in IMR32 and SH-SY5Y cells (Fig EV1D). The 393-nt \( \text{circ-CUX1} \), consisting of exon 2 and partial intron 2 of \( \text{CUX1} \) (Fig 2A), was detected by RT–PCR with divergent primers and Sanger sequencing (Fig 2B), and its expression levels were significantly elevated in many tumor cell lines (Fig 2C and D). Endogenous \( \text{circ-CUX1} \) was resistant to RNase R digestion (Fig 2D) and localized within nucleus of IMR32 cells, which was further confirmed by ectopic expression of \( \text{circ-CUX1} \) (Fig 2D–F). Notably, \( \text{circ-CUX1} \) levels were higher in tissues of NB, colon cancer, and prostate cancer, than normal fetal adrenal medulla or adjacent normal tissues (Fig 2G). In addition, \( \text{circ-CUX1} \) levels were positively correlated with those of \( \text{CUX1} \) in tissues of NB (\( R = 0.590, P < 0.0001 \)), colon cancer (\( R = 0.868, P < 0.0001 \)), or prostate cancer (\( R = 0.619, P = 0.0023 \); Fig 2G). In 54 primary NB tumors, higher \( \text{circ-CUX1} \) levels were observed in cases with poor pT status (\( P = 0.0083 \)) or advanced INSS stages (\( P = 0.0017 \)), without association with MYCN amplification (\( P = 0.1532 \); Fig EV1E). Patients with high \( \text{circ-CUX1} \) expression had lower survival probability (Fig EV1F). These results indicated that \( \text{circ-CUX1} \) was up-regulated in NB tissues and cell lines.

**Circ-CUX1 enhances CUX1 expression at transcriptional level**

To investigate the effects of \( \text{circ-CUX1} \) on expression of parental gene \( \text{CUX1} \), \( \text{circ-CUX1} \) or two independent short hairpin RNAs (shRNAs) targeting junction site of \( \text{circ-CUX1} \) (sh-circ-CUX1) were stably transfected into tumor cell lines. Transfection of \( \text{circ-CUX1} \) vector, but not of that with mutant back-splicing elements (\( \text{circ-CUX1-Mut} \)), resulted in obvious \( \text{circ-CUX1} \) production in IMR32 cells, which was resistant to RNase R digestion (Appendix Fig SSA). Transfection of sh-circ-CUX1 #1 and sh-circ-CUX1 #2 increased the enrichment of Argonaute 2 (AGO2) on \( \text{circ-CUX1} \), but not on \( \text{CUX1} \) mRNA, in IMR32, SH-SY5Y, LoVo, and PC-3 cells (Appendix Fig SSB). In a luciferase reporter-based assay monitoring shRNA specificity (Bramsen et al, 2010), transfection of sh-circ-CUX1 #1 or sh-circ-CUX1 #2 decreased the activity of \( \text{circ-CUX1} \) reporter, without impact on that of \( \text{CUX1} \) reporter (Appendix Fig SSC). Notably, stable transfection of \( \text{circ-CUX1} \), but not of \( \text{circ-CUX1-Mut} \), into IMR32, SH-SY5Y, LoVo, and PC-3 cells resulted in its over-expression (Fig EV2A), increased \( \text{CUX1} \) promoter activity (Fig EV2B), and elevated levels of \( \text{CUX1} \) isoforms p200 and p110, but not of \( \text{CASP} \) (Figs EV2C and 2H). The stability of \( \text{CUX1} \) mRNA was not altered in IMR32 cells stably transfected with \( \text{circ-CUX1} \) (Fig EV2D). Meanwhile, stable knockdown of \( \text{circ-CUX1} \) led to decrease in \( \text{CUX1} \) promoter activity and expression of p200 and p110 in tumor cells, without effects on \( \text{CUX1} \) mRNA stability or \( \text{CASP} \) levels (Figs EV2A–D and 2H). These results illustrated that \( \text{circ-CUX1} \) enhanced \( \text{CUX1} \) expression at transcriptional level in tumor cells.

**Circ-CUX1 exerts an oncogenic role in tumor progression**

We further observed the potential effects of \( \text{circ-CUX1} \) on biological features of tumor cells. The ECAR was increased and decreased in IMR32, SH-SY5Y, LoVo, and PC-3 cells stably transfected with \( \text{circ-CUX1} \) or sh-circ-CUX1, along with reduced and enhanced OCR, while transfection of \( \text{circ-CUX1-Mut} \) did not affect these features (Fig EV2E). Notably, ectopic expression of \( \text{circ-CUX1} \) increased the glucose uptake, lactate production, and ATP levels of IMR32 cells, which was attenuated by 2-DG treatment (Appendix Fig S6A). Stable over-expression or knockdown of \( \text{circ-CUX1} \) increased and decreased the anchorage-independent growth and invasion of IMR32 and SH-SY5Y cells, respectively (Fig 3A and B). Consistently, stable transfection of \( \text{circ-CUX1} \) or sh-circ-CUX1 #1 into IMR32 cells resulted in a significant increase or decrease in growth, tumor weight, Ki-67 proliferation index, CD31-positive microvessels, glucose uptake, lactate production, and ATP levels of subcutaneous xenograft tumors in nude mice (Fig 3C–E). Athymic nude mice treated with tail vein injection of IMR32 cells with stable over-expression or knockdown of \( \text{circ-CUX1} \) displayed more or less lung metastatic colonies, with lower or greater survival probability, respectively (Fig 3F). These results indicated that \( \text{circ-CUX1} \) exerted an oncogenic role in tumorigenesis and aggressiveness.

**Circ-CUX1 directly interacts with EWSR1 protein in NB cells**

To explore the protein partner of \( \text{circ-CUX1} \), RNA pull-down was performed using biotin-labeled probes generated by ligation of linear transcript in vitro (Petkovic & Muller, 2015) or synthesized as oligonucleotides targeting junction site (Fig 4A). Mass spectrometry revealed 47 proteins consistently pulled down by exogenous \( \text{circ-CUX1} \) and antisense probe targeting endogenous \( \text{circ-CUX1} \), but not by linear transcript or sense probe, and 18 of them were RBPs defined by RBPDB (http://rbpdb.ccbr.utoronto.ca). Further comprehensive analysis of protein interacting with transcription factors of \( \text{CUX1} \) promoter revealed by Genomatix and BioGRID database.
Figure 2.
(https://thebiogrid.org) indicated three potential circ-CUX1-interacting partners (Fig 4A), including EWSR1, ELAV-like RNA-binding protein 1 (ELAVL1), and synaptotagmin-binding cytoplasmic RNA-interacting protein (SYNCRIP). Further validating RNA pull-down assay using circ-CUX1 probes revealed the specific enrichment of endogenous or exogenous circ-CUX1, but not p200 CUX1 or CASP transcript, and the physical interaction of circ-CUX1 with EWSR1, but not with ELAVL1 or SYNCRIP, in non-transfected IMR32 cells (Fig 4B). Endogenous binding of EWSR1 protein to circ-CUX1, but not to p200 CUX1 or CASP transcript, was also observed in SH-SY5Y cells, which was facilitated by transfection of circ-CUX1 (Fig 4C). In NB cell lines SH-SY5Y, SK-N-AS, BE(2)-C, and IMR32, PCR assay indicated no fusion of NB cell lines SH-SY5Y, SK-N-AS, BE(2)-C, and IMR32, PCR assay indicated no fusion of circ-CUX1 with Flt1 gene (Appendix Fig S6B). Three-dimensional (3D) confocal images of dual RNA fluorescence in situ hybridization (RNA-FISH) and immunofluorescence assay confirmed endogenous co-localization of circ-CUX1 and EWSR1 in IMR32 cells, which was facilitated by transfection of circ-CUX1 (Fig 4D and Movie EV1). Consistently, RNA electrophoretic mobility shift assay (EMSA) showed that circ-CUX1 interacted with EWSR1 protein within nuclear extracts of SH-SY5Y cells (Fig 4E). The RNA recognition motif (RRM) domain [361–448 amino acids (aa)], but not amino- or carboxyl-terminus, of glutathione S-transferase (GST)-tagged EWSR1 protein was necessary for its interaction with circ-CUX1, but not with p200 CUX1 or CASP transcript (Fig 4F). Mutation of 394–397 or 406–410 aa of RRM domain, potential interacting regions analyzed by catRAPID (Agostini et al., 2013), abolished the interaction of EWSR1 with circ-CUX1 (Fig 4F). These results suggested that circ-CUX1 directly interacted with EWSR1 protein in NB cells.

Circ-CUX1 facilitates EWSR1-mediated MAZ transactivation

To further investigate target genes of circ-CUX1, RNA sequencing (RNA-seq) was performed and revealed 781 up-regulated and 434 down-regulated genes (fold change > 1.5, \( P < 0.05 \)) in IMR32 cells upon circ-CUX1 over-expression (Fig 5A). Transcription factors regulating these genes were analyzed by ChIP-X program (Lachmann et al., 2010), which revealed top five potential ones, including E12, lymphoid enhancer-binding factor 1 (LEF1), MAZ, nuclear factor of activated T cells (NFAT), and specificity protein 1 (SP1) (Fig 5B). Further over-lapping analysis with EWSR1-interacting proteins derived from BioGRID database revealed that MAZ protein was the only transcription factor involved in this process (Fig 5B).

Endogenous interaction between EWSR1 and MAZ was observed in IMR32 cells (Appendix Fig S6C). The RRM domain (361–448 aa), but not transactivation domain (TAD), Arg-Gly-Gly (RGG) 1, RGG2, or RGG3 domain, of Myc-tagged EWSR1 was necessary for its interaction with MAZ protein (Appendix Fig S6D). Meanwhile, zinc finger (ZNF) domain (198–477 aa), but not N-terminus or C-terminus, of Flag-tagged MAZ was necessary for its interaction with EWSR1 (Appendix Fig S6E). Ectopic expression or knockdown of circ-CUX1 increased and decreased the interaction between EWSR1 and MAZ in IMR32 and SH-SY5Y cells, respectively (Fig 5C and D, and Appendix Fig S6F).

Notably, higher MAZ levels were observed in NB tissues than those in normal fetal adrenal medulla (\( P < 0.0001 \)), especially in those with poor stroma (\( P = 0.0205 \)) or advanced INSS stages (\( P = 0.0097 \)), without association with MYCN amplification (\( P = 0.6445 \), Appendix Fig S7A). Among 60 MAZ target genes derived from RNA-seq results and ChIP-X analysis, the expression of circ-CUX1, S100 calcium-binding protein A9 (S100A9), mucin 4 (MUC4), Kruppel-like factor 10 (KLF10), or thioredoxin-interacting protein (TXNIP) was significantly correlated with that of MAZ in 54 NB cases (Appendix Fig S7B). In addition, higher expression of EWSR1, MAZ, S100A9, or MUC4 and lower expression of KLF10 or TXNIP were associated with poor survival of NB patients (GSE16476, Appendix Fig S7C). In RNA pull-down and chromatin isolation by RNA purification (ChIRP) (Chiu & Chang, 2016) assays using biotin-labeled circ-CUX1 junction probe, circ-CUX1 was associated with EWSR1 and MAZ protein, and promoters of target genes (CUX1, S100A9, MUC4, KLF10, or TXNIP), but not with transcripts of downstream genes in SH-SY5Y cells (Appendix Fig S8A). Ectopic expression or knockdown of circ-CUX1 enhanced and reduced the binding of MAZ to these target gene promoters in IMR32 and SH-SY5Y cells, while silencing or over-expression of EWSR1 abolished these effects (Fig 5E, and Appendix Fig S8B). The activity of wild-type CUX1 promoter, but not of that with mutant MAZ-binding site, was increased and decreased by ectopic expression or knockdown of circ-CUX1 (Fig 5F and Appendix Fig S8C). In addition, the levels of CUX1, S100A9, MUC4, KLF10, or TXNIP were significantly altered in IMR32 and SH-SY5Y cells stably transfected with circ-CUX1 or sh-circ-CUX1 #1 (Fig 5G and H, Appendix Fig S8D and E). Knockdown or ectopic expression of EWSR1 or MAZ rescued tumor cells from these changes (Fig 5F–H, Appendix Figs S8C–E and S9A). These data indicated that circ-CUX1 facilitated EWSR1-mediated MAZ transactivation and transcriptional alteration of target genes in NB cells.

Figure 3. Circ-CUX1 exerts an oncogenic role in tumorigenesis and aggressiveness.

A, B Soft agar (A) and Matrigel invasion (B) assays showing the anchorage-independent growth and invasion capability of IMR32 and SH-SY5Y cells stably transfected with empty vector (mock), circ-CUX1, scramble shRNA (sh-Scb), or sh-circ-CUX1 (\( n = 5 \)). Scale bar: 100 \( \mu m \). Student’s t-test, one-way ANOVA, *\( P < 0.05 \) versus mock or sh-Scb.

C Representative fluorescence images, in vivo growth curve, and weight at the end points of subcutaneous xenograft tumors formed by IMR32 cells stably transfected as indicated in nude mice (\( n = 5 \) for each group). Student’s t-test, one-way ANOVA, *\( P < 0.05 \) versus mock or sh-Scb.

D, E Immunohistochemical staining showing the expression of Ki-67 and CD31 (D) and glucose uptake, lactate production, and ATP levels (E) within subcutaneous xenograft tumors formed by IMR32 cells stably transfected as indicated (\( n = 5 \) for each group). Scale bar: 100 \( \mu m \). Student’s t-test, **\( P < 0.01 \) versus mock or sh-Scb.

F Representative images, HE staining (arrowheads), quantification of lung metastatic colonization, and Kaplan–Meier curves of nude mice treated with tail vein injection of IMR32 cells stably transfected as indicated (\( n = 5 \) for each group). Scale bar: 100 \( \mu m \). Student’s t-test, **\( P < 0.01 \) versus mock or sh-Scb. Log-rank test for survival comparison.

Data information: Data are presented as mean \( \pm \) SEM. Exact \( P \) values are specified in Appendix Table S4.
Figure 3.
Therapeutic peptide blocking the circ-CUX1-EWSR1 interaction

Based on the importance of RRM domain (especially 394–397 or 406–410 aa) of EWSR1 in interacting with circ-CUX1, we further designed a cell-penetrating peptide, named as EWSR1 inhibitory peptide of 22 amino acids (EIP-22), that might potentially block circ-CUX1-EWSR1 interaction (Fig 6A). Treatment of SH-SY5Y cells with EIP-22 resulted in its obvious aggregation within the nucleus (Fig 6B). Biotin-labeled peptide pull-down assay revealed the binding of EIP-22 to endogenous circ-CUX1 in SH-SY5Y cells (Appendix Fig S9B). In addition, EIP-22 treatment reduced the interaction between circ-CUX1 and EWSR1, but not that of pri-miR-222 and EWSR1 (Ouyang et al, 2017) or circACC1 and AMP-activated protein kinase beta 1 (AMPKβ1) (Li et al, 2019; Fig 6C and D). Administration of EIP-22 inhibited the viability, anchorage-independent growth, and invasion of SH-SY5Y cells (Appendix Fig S9C, Fig 6E and F), with alteration of circ-CUX1 downstream gene expression (Appendix Fig S9D). In contrast, EIP-22 treatment resulted in no significant alteration in the viability of MCF 10A, non-transformed normal cells with very low circ-CUX1 expression (Fig 2C and Appendix Fig S9C). Notably, EIP-22 treatment synergized the suppressing effects of glycolysis inhibitors, 2-DG and 3-bromopyruvate (3-BP) (Cardaci et al, 2012; Zhang et al, 2014), on the viability, growth, and invasion of IMR32 and SH-SY5Y cells (Appendix Fig S9E–G). Intravenous administration of EIP-22 significantly reduced the growth, tumor weight, Ki-67 proliferation index, and CD31-positive microvessels, altered circ-CUX1 target gene expression, and decreased the glucose uptake, lactate production, and ATP levels in subcutaneous xenograft tumors formed by injection of SH-SY5Y cells (Fig 6G and Appendix Fig S10A–C). Moreover, administration of EIP-22 via tail vein reduced the lung metastatic colonies and prolonged the survival time of athymic nude mice received tail vein injection of SH-SY5Y cells (Fig 6H). These data suggested that EIP-22 suppressed NB progression by blocking circ-CUX1-EWSR1 interaction.

Therapeutic lentivirus-mediated circ-CUX1 knockdown in vivo

We further explored the therapeutic efficiencies of circ-CUX1 knockdown on athymic nude mice bearing xenograft tumors formed by subcutaneous or tail vein injection of IMR32 cells. Lentivirus-mediated knockdown of circ-CUX1 significantly reduced the growth, tumor weight, Ki-67 proliferation index, and CD31-positive microvessels of subcutaneous xenograft tumors (Appendix Fig S11A and B), with altered expression of circ-CUX1 target genes (Appendix Fig S11C). The glucose uptake, lactate production, and ATP levels were significantly decreased in xenograft tumors of nude mice received tail vein injection of lentivirus carrying sh-circ-CUX1 (Appendix Fig S11D). In addition, administration of lentivirus carrying sh-circ-CUX1 #1 decreased the lung metastatic colonies and prolonged the survival time of nude mice (Appendix Fig S11E). These results indicated that lentivirus-mediated circ-CUX1 knockdown inhibited aerobic glycolysis and NB progression in vivo.

Discussion

Recent studies show that although LDHA and LDHB promote tumorigenicity, they are dispensable for aerobic glycolysis in NB (Dorneburg et al, 2018), suggesting the involvement of other glycolytic genes in this process. So far, interrogative screening of transcriptional regulators of aerobic glycolysis in NB remains unknown. In this study, we identified CUX1 as a transcription factor facilitating the expression of glycolytic genes ENO1, GPI, and PGK1 in NB. We demonstrate that circ-CUX1 interacts with EWSR1 protein to promote MAZ transactivation, which subsequently regulates the transcription of CUX1 and other genes associated with tumor progression in cis and in trans (Fig 6I), such as S100A9 (Lim et al, 2016), MUC4 (Rowson-Hodel et al, 2017), KLF10 (Weng et al, 2017), and TXNIP (Shen et al, 2015). The discovery of circ-CUX1/EWSR1/MAZ axis represents a promising step for therapeutic intervention against tumors.

CUX1 is a transcription factor involved in embryonic development (Michl et al, 2005; Harada et al, 2008) and regulates cellular proliferation, migration, and epithelial-to-mesenchymal transition, suggesting its emerging roles in tumorigenesis and aggressiveness (Michl et al, 2005). Elevated CUX1 expression has been documented in many tumors and is associated with poor survival of patients (Liu et al, 2013). Full-length p200 CUX1 binds rapidly but only transiently to DNA (Liu et al, 2013), while its proteolytic product p110 isoform activates gene transcription (Harada et al, 2008; Kedinge et al, 2009). In this study, our results indicated that CUX1 was an independent prognostic marker for progression and poor outcome of NB. In addition, p110 CUX1 promoted the expression of target genes...
Figure 4.
**A**

![Graph showing ChIP-X results with TFs and MAZ](image)

**B**

![Graph showing relative CUX1 promoter activity](image)

**C**

![Western blot results for EWSR1 and MAZ](image)

**D**

![Diagram showing EWSR1 and MAZ interactions](image)

**E**

![Relative MAZ binding to promoter](image)

**F**

![Relative CUX1 promoter activity](image)

**G**

![Relative transcript levels](image)

**H**

![Western blot for EWSR1 and MAZ](image)

Figure 5.
EN01, GPI, and PGK1 in NB cells. As a glycolytic enzyme, EN01 acts as a metabolic tumor promoter by contributing to Warburg effect (Chen et al., 2018). GPI is a housekeeping cytosolic enzyme responsible for catalytic interconversion between glucose-6-phosphatase and fructose-6-phosphate, and plays a key role in glycolytic pathway (Zdralievic et al., 2017). During the glycolytic process, PGK1 contributes to ATP generation and participates in tumor progression (Li et al., 2016). Our gain- and loss-of-function studies indicated that CUX1 promotes the aerobic glycolysis, growth, and invasiveness of NB cells, suggesting its oncogenic roles in NB progression.

Human CUX1 gene locates at chromosome 7q22, a region associated with copy number gain that contributes to multidrug resistance in NB (Mazzocco et al., 2015). However, we found no alteration of copy number or genetic variants of CUX1 in NB cohorts, indicating other mechanisms facilitating its over-expression. CircRNAs play important roles in regulating gene expression at post-transcriptional or transcriptional levels (Hansen et al., 2013; Li et al., 2015b). For example, circS7-7 and circSry serve as sponges of miR-7 and miR-138 in the cytoplasm (Hansen et al., 2013). Exonic circRNAs also exert regulatory functions in the cytoplast by forming a ribonucleoprotein complex with miRNA and AGO protein (Lasda & Parker, 2014). Meanwhile, exon–intron circRNAs are predominantly localized in the nucleus and regulate their parent gene expression in a cis-acting manner through specific RNA–protein interaction (Li et al., 2015b). In this study, circ-CUX1 was identified as an intron-containing circRNA up-regulated in tumor tissues and cells. Circ-CUX1 enhanced the expression of CUX1 at transcriptional level, and tumor-promoting functions of circ-CUX1 were mediated, at least in part, through interacting with EWSR1 protein in NB cells.

As one member of EWS family of RNA-binding proteins, EWSR1 participates in gene transcription, splicing, and miRNA processing (Luo et al., 2015). Chromosomal translocation of EWSR1 has been discovered in Ewing sarcoma (Sohn et al., 2010). However, our results revealed no EWS-Fli1 gene fusion in NB cells. Due to lack of DNA-binding domain, EWSR1 usually acts as a potent transcriptional cofactor in tumor progression via interacting with transcriptional regulatory proteins, such as CREB-binding protein and p300 (Chakravarti et al., 1996). In this study, we found that RRM domain of EWSR1 was necessary for its interaction with ZNF domain of MAZ. As a ubiquitously expressed transcription factor, MAZ binds to GC-rich cis-elements through its C2H2-type ZNF motif (Parks & Shenk, 1996) and activates transcription of KRAS and vascular endothelial growth factor (VEGF) in pancreatic cancer, cervical cancer, and glioblastoma cells (Smits et al., 2012; Cogoi et al., 2013). Our evidence indicated that through interplay with its cofactor EWSR1, MAZ regulated the transcription of CUX1, S100A9, MUC4, KLF10, or TNNIP in NB cells. Notably, circ-CUX1 bound to RRM region of EWSR1, resulting in EWSR1-mediated MAZ transactivation, suggesting the oncogenic roles of circ-CUX1/EWSR1/MAZ axis in aerobic glycolysis and tumor progression.

In summary, we demonstrate that elevated CUX1 and its generated circ-CUX1 are associated with poor outcome of NB patients.
Figure 6.
and exert oncogenic roles in aerobic glycolysis and tumor progression. Mechanistically, circ-CUX1 binds to EWSR1 protein to facilitate MAZ transactivation, resulting in transcriptional alteration of CUX1 and other genes associated with NB progression. An inhibitory peptide (EIP-22) blocking circ-CUX1-EWSR1 interaction or lentivirus-mediated circ-CUX1 knockdown suppresses the aerobic glycolysis, tumorigenesis, and aggressiveness of NB cells. Combinational administration of EIP-22 and glycolysis inhibitors (2-DG or 3-BP) targeting HK2, GPI, or GAPDH (Cardaci et al., 2012; Zhang et al., 2014) exerts synergistic effects in suppressing growth and aggressiveness of NB cells. Due to limited size of cohort, the prognostic value of circ-CUX1/EWSR1/MAZ axis may be a potential therapeutic target for NB.

Materials and Methods

Cell culture

Human MCF 10A (CRL-10317), HeLa (CCL-2), SH-SY5Y (CRL-2266), IMR32 (CRL-127), SK-N-AS (CRL-1237), BE(2)-C (CRL-2268), SK-N-MC (HTB-10), LoVo (CCL-229), PC-3 (CRL-1435), HEK293, HEK293 (CRL-1573), and HEK293T (CRL-3216) cells were obtained from American Type Culture Collection (Rockville, MD), authenticated by short tandem repeat profiling, and used within 6 months after resuscitation of frozen aliquots. Mycoplasma contamination was regularly examined using Lookout Mycoplasma PCR Detection Kit (Sigma, St. Louis, MO). Tumor cells, HEK293, and HEK293T cells were cultured in RPMI1640 supplied with 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD), while MCF 10A cells were cultured in DMEM/F12 medium containing 5% horse serum (Invitrogen, Carlsbad, CA) and 20 ng ml⁻¹ epidermal growth factor (PeproTech, Rocky Hill, NJ) at 37°C, and treated with E64D, actinomycin D (ActD), 2-DG, or 3-BP (Sigma).

RT–PCR and real-time quantitative RT–PCR

Nuclear, cytoplasmic, and total RNA was extracted using RNA Subcellular Isolation Kit (Active Motif, Carlsbad, CA) or RNeasy Mini Kit (Qiagen Inc., Redwood City, CA), with or without RNase R (3 U µg⁻¹, Epicenter, Madison, WI) digestion at 37°C for 15 min. Reverse transcription and real-time PCR were performed using Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN), SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and primers (Appendix Table S1). The transcript levels were analyzed by 2⁻ΔΔCt method. De novo RNA synthesis was blocked by ActD (5 µg ml⁻¹) treatment, while mRNA stability was examined by transcript levels at indicated time points.

Northern blot

The non-junction and junction probes specific for circ-CUX1 were synthesized and labeled by digoxigenin (DIG, Appendix Table S2). For Northern blot, 20 µg of total RNA was separated on 3-(N-morpholino)propanesulfonic acid-buffered 2% (w/v) agarose gel containing 1.2% (v/v) formaldehyde under denaturing condition at 80 V for 4 h, and transferred to Hybond-N+ membrane (Pall Corp., Port Washington, NY). Hybridization was performed at 65°C for 16–18 h in DIG Easy Hyb solution (Roche) and detected by anti-DIG antibody (1:500 dilution) and chemiluminescence substrate CSPD (Roche).

Western blot

Tissue or cellular protein was extracted with 1× cell lysis buffer (Promega, Madison, WI). Western blot was performed as previously described (Zhang et al., 2012; Zhao et al., 2016; Li et al., 2018b), with antibodies (1:500 dilution) specific for CUX1 (sc-514008, Santa Cruz Biotechnology, Santa Cruz, CA), ENU1 (ab155102), GPI (ab66340), PGK1 (ab38007), EWSR1 (ab93837), ELAVL1 (ab136542), SYNGN1 (ab184946), MAZ (ab85725), MUC4 (ab60720), S100A9 (ab92507), KLF10 (ab73537), TXNIP (ab188865), AMPKβ1 (ab32112), β-actin (ab8227), Flag (ab18230), Myc (ab9106, Abcam Inc., Cambridge, MA), GST (sc-33614), or histone H3 (sc-10809, Santa Cruz Biotechnology).

Gene over-expression and knockdown

Human circ-CUX1 linear sequence (393 bp) was obtained from NB tissues by PCR (Appendix Table S2) and inserted into pLCDH-ciR (Geenseed Biotech Co., Guangzhou, China). Human p200 CUX1 construct was provided by Dr. George Stratigopoulos, while p110 CUX1 was released by digestion or amplified using primers (Appendix Table S2), and subcloned into pcDNA3.1 (Invitrogen) or pCMV-3-Tag-1A (Addgene, Cambridge, MA). Human EWS1 cDNA (1,971 bp) and MAZ cDNA (1,482 bp) were provided by Dr. Ralf Janknecht or amplified from NB tissues with primers (Appendix Table S2), and their truncations were subcloned into pcMV-N-Myc or pcMV-3-Tag-1A (Addgene). Mutation of circ-CUX1 or EWS1 was prepared with GeneTailorTM Site-Directed Mutagenesis System (Invitrogen) and primers (Appendix Table S2). Oligonucleotides specific for shRNAs (Appendix Table S3) were inserted into GV298 (GeneChem Co., Ltd, Shanghai, China). Stable cells were screened by neomycin or puromycin (Invitrogen).

Rescue of target gene expression

To rescue circ-CUX1 knockdown-altered target gene expression, EWS1 or MAZ was transfected into stable cell lines. To restore target gene expression altered by circ-CUX1, shRNAs against EWS1 or MAZ (Appendix Table S3) were transfected into tumor cells using GeneSilencer Transfection Reagent (Genlantis, San Diego, CA).

Lentivirus packaging

Lentiviral vectors were co-transfected with packaging plasmids psPAX2 and pMD2G (Addgene) into HEK293T cells. Infectious lentivirus was harvested at 36 and 60 h after transfection, and filtered through 0.45 µm PVDF filters. Recombinant lentivirus was concentrated 100-fold by ultracentrifugation (2 h at 120,000 g), dissolved in phosphate-buffered saline (PBS), and injected into mice within 48 h.
RNA-seq assay

Total RNA of tumor cells (1 × 10⁶) was extracted using TRIzol® reagent (Life Technologies, Inc.). Library preparation and transcriptome sequencing on an Illumina HiSeq X Ten platform were carried out at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). Fragments per kilobase of transcript per million fragments mapped (FPKM) of each gene were calculated.

Dual-luciferase reporter assay

Complementary oligonucleotides containing four canonical binding sites of CUX1 or MAZ, and promoter fragments of ENO1 (−1,880/+301), GPI (−1,854/+247), PGK1 (−882/+246), or p200 CUX1 (−2,084/+106) amplified from genomic DNA (Appendix Table S2) were subcloned into pGL3-Basic (Promega). Mutation of MAZ-binding site was performed with GeneTailor™ Site-Directed Mutagenesis System (Invitrogen) and primers (Appendix Table S2). To test specificity of shRNA, target sequences of circ-CUX1 and CUX1 were amplified using primers (Appendix Table S2) and subcloned into 3′-untranslated region of Renilla luciferase within psiCHECK2 (Promega). Dual-luciferase assay was performed as previously described (Zhang et al., 2012; Zhao et al., 2016; Li et al., 2018b).

RNA pull-down and mass spectrometry

Biotin-labeled oligonucleotide probes targeting junction sites of circRNAs were synthesized (Invitrogen). Linear circ-CUX1 was in vitro transcribed using Biotin RNA Labeling Mix (Roche) and T7 RNA polymerase, incubated with guide oligonucleotides (Appendix Table S2), circularized using T4 RNA ligase I, treated with RNase R, and purified with RNeasy Mini Kit (Qiagen Inc.). RNA pull-down was performed as previously described (Li et al., 2018b). Retrieved protein was detected by Western blot or mass spectrometry (Wuhan Institute of Biotechnology, Wuhan, China), while recovered transcripts were measured by RT–PCR using primers (Appendix Table S1). In ChIRP assay, cells were harvested, cross-linked, sonicated, hybridized with probes, and mixed with streptavidin magnetic beads (Chu & Chang, 2016). The retrieved DNA was detected by PCR using primers (Appendix Table S1).

RNA-FISH

Biotin-labeled antisense or sense probe for circ-CUX1 junction was synthesized (Appendix Table S2). The probes for GAPDH and U1 were generated by in vitro transcription of PCR products (Appendix Table S1) using DIG Labeling Kit (MyLab Corporation, Beijing, China). Cells were incubated with 40 nM FISH probe in hybridization buffer (100 mg ml⁻¹ dextran sulfate, 10% formamide in 2 × SSC) at 37°C for 16 h, with or without RNase R (3 U µg⁻¹) treatment. The signals of circ-CUX1 were detected by Fluorescent In Situ Hybridization Kit (RiboBio, Guangzhou, China), with nuclei staining by 4′,6-diamidino-2-phenylindole (DAPI).

Fluorescence immunocytochemical staining

Tumor cells were grown on coverslips, incubated with antibodies specific for EWSR1 (ab93837; Abcam Inc.; 1:100 dilution) at 4°C overnight, and treated with Alexa Fluor 594 goat anti-rabbit IgG (1:1,000 dilution) and DAPI (300 nM) staining. The images were photographed under a Nikon A1Si Laser Scanning Confocal Microscope and applied for 3D reconstruction using NIS-Elements Viewer (Nikon Instruments Inc., Japan).

Co-immunoprecipitation (co-IP)

Co-IP was performed as previously described (Jiao et al., 2018; Li et al., 2018b), with antibodies (1:200 dilution) specific for EWSR1 (sc-28327, Santa Cruz Biotechnology), MAZ (ab85725), Flag (ab18230), or Myc (ab9106, Abcam Inc.). Bead-bound proteins were released and analyzed by Western blot.

Bimolecular fluorescence complementation system (BiFC)

Human EWSR1 cDNA (1,971 bp) and MAZ cDNA (1,482 bp) were subcloned into BiFC vectors pBifC-VN173 and pBifC-VC155 (Addgene), and co-transfected into tumor cells for 24 h. The fluorescence emission was observed under a confocal microscope, with excitation and emission wavelengths of 488 and 500 nm, respectively (Kerppola, 2008).

Chromatin immunoprecipitation (ChiP)

ChiP assay was undertaken using EZ-ChIP kit (Upstate Biotechnology, Temecula, CA) (Zhao et al., 2016; Li et al., 2018b), with antibodies (1:100 dilution) specific for CUX1 (#81557, Cell Signaling Technology, Inc., Danvers, MA) or MAZ (ab85725, Abcam Inc.) and primers targeting gene promoters (Appendix Table S1).

Cross-linking RNA immunoprecipitation (RIP)

Cells (1 × 10⁶) were ultraviolet light cross-linked at 254 nm (200 J cm⁻²). RIP assay was performed using Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore) (Zhao et al., 2016; Li et al., 2018b), with antibodies (1:100 dilution) specific for AGO2 (ab186733, Abcam Inc.), EWSR1 (#11910, Cell Signaling Technology, Inc.), GST (sc-33614, Santa Cruz Biotechnology), or AMPKβ1 (ab32112, Abcam Inc.). Co-precipitated RNAs were detected by RT–PCR or real-time qRT–PCR with specific primers (Appendix Table S1).

In vitro binding assay

A series of EWSR1 truncations were amplified with primers (Appendix Table S2), subcloned into pGEX-6P-1 (Addgene), and transformed into E. coli to produce GST-tagged EWSR1 protein (Zhao et al., 2016; Li et al., 2018b). The EWSR1-circRNA complexes were pulled down using GST beads (Sigma). Protein was detected by SDS–PAGE and Western blot, while circRNA was measured by RT–PCR with specific primers (Appendix Table S1).

RNA EMSA

Biotin-labeled circ-CUX1 probe was synthesized as described above. RNA EMSA using nuclear extracts was performed using LightShift Chemiluminescent RNA EMSA Kit (Thermo Fisher Scientific, Inc., Waltham, MA).
Design and synthesis of inhibitory peptides

Based on interacting region of EWSR1 revealed by mutagenesis and in vitro binding assays, wild-type and mutant inhibitory peptides blocking circ-CUX1 and EWSR1 interaction were designed and synthesized by linking with biotin-labeled 11 amino acid cell-penetrating peptide (YGRKKRRQRRR) from Tat protein transduction domain at the N-terminus and conjugating with fluorescein isothiocyanate (FITC) at the C-terminus (ChinaPeptides Co. Ltd, Shanghai, China), with purity larger than 95%.

Biotin-labeled peptide pull-down

Total RNA was isolated using RNeasy Mini Kit (Qiagen Inc.) and incubated with biotin-labeled peptide at 4°C overnight. Then, incubation of RNA-peptide complex with streptavidin-agarose was undertaken at 4°C for 2 h. Beads were extensively washed, and circRNAs pulled down were measured by real-time qRT-PCR.

Aerobic glycolysis and seahorse extracellular flux assays

Cellular glucose uptake, lactate production, and ATP levels were detected as previously described (Ma et al., 2014). ECAR and OCR were measured in XF media under basal conditions and in response to glucose (10 mM), oligomycin (2 μM), and 2-deoxylcyloleucine (50 mM) using a Seahorse Biosciences XFe24 Flux Analyzer (North Billerica, MA).

In vitro cell viability, growth, and invasion assays

The 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) colorimetric (Li et al., 2015a, 2018b), soft agar (Zhang et al., 2012; Zhao et al., 2016; Li et al., 2018a,b), and Matrigel invasion (Zhang et al., 2012; Zhao et al., 2016; Li et al., 2018a,b) assays were undertaken to measure in vitro viability, growth, and invasive capabilities of tumor cells.

In vivo growth, metastasis, and therapeutic assays

All animal experiments were carried out in accordance with NIH Guidelines for the Care and Use of Laboratory Animals, and approved by the Animal Care Committee of Tongji Medical College (approval number: Y20080290). For in vivo tumor growth and experimental metastasis studies, tumor cells (1 × 10⁶ or 0.4 × 10⁶) were injected into dorsal flanks or tail vein of blindly randomized 4-week-old female BALB/c nude mice (National Rodent Seeds Center, Shanghai, China) breeding at specific pathogen free (SPF) condition (n = 5 per group) (Zhang et al., 2012; Zhao et al., 2016; Li et al., 2018a,b). For in vivo therapeutic studies, tumor cells (1 × 10⁶ or 0.4 × 10⁶) were injected into dorsal flanks or tail vein of nude mice, respectively. One week later, mice were blindly randomized and treated by tail vein injection of synthesized cell-penetrating peptide (ChinaPeptides, Shanghai, China) or lentivirus (1 × 10⁸ plaque-forming units) as indicated. The In Vivo Optical Imaging System (In-Vivo FX PRO, Bruker Corporation, Billerica, MA) was applied to acquire fluorescent images of xenograft tumors in nude mice.

Statistical analysis

All data were shown as mean ± standard error of the mean (SEM). Cutoff of gene expression was defined by average values. Two-sided unpaired Student’s t-test and one-way ANOVA were used to
compare difference. Pearson’s correlation coefficient assay was used to analyze expression correlation. Log-rank test and Cox regression models were used to assess survival difference and hazard ratio. All statistical tests were considered significant when $P < 0.05$. Randomization and blinding strategies were used whenever possible. Experimental sample size was determined on the basis of power analysis assuming a significance level (alpha) of 0.05 and a power of 80%. Animal cohort sizes were determined on the basis of similar studies. The exact $P$-values and number of replicates were indicated in Appendix Table S4.

**Data availability**

RNA-seq data have been deposited in Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo), under accession number GSE136135.

**Expanded View** for this article is available online.

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**Author contributions**

Conception and design: QT and LZ; Methodology and resources: HL, FY, XW, EF, and DL; Acquisition of data: HL, FY, AH, XW, EF, YC, DL, HS, JW, YG, YL, and HJL; Supervision: QT, LZ, and KH; Writing the manuscript: HL, FY, QT, and LZ.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**For more information**

Publically available datasets can be found

(i) GEO database (GSE16476, GSE62564, GSE41258, GSE8851, GSE6956, https://www.ncbi.nlm.nih.gov/geo).

(ii) The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov).

(iii) Kaplan–Meier plotter (http://kmplot.com).

(iv) Oncogenicomics (https://pob.abcc.ncifcrf.gov/cgi-bin/k). 

(v) cBioPortal for Cancer Genomics (http://cbioportal.org).

(vi) Tumor alterations relevant for genomics-driven therapy (TARGET, https://software.broadinstitute.org/cancer/cga/target).

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