Targeting the Notch-regulated non-coding RNA TUG1 for glioma treatment

Keisuke Katsushima1, Atsushi Natsume2, Fumiharu Ohka1,2, Keiko Shinjo1, Akira Hatanaka1, Norihisa Ichimura1, Shinya Sato3, Satoru Takahashi3, Hiroshi Kimura4, Yasushi Totoki5, Tatsuhiro Shibata5,6, Mitsuru Naito7, Hyun Jin Kim7, Kanjiro Miyata7,8, Kazunori Kataoka7,8,9 & Yutaka Kondo1,10

Targeting self-renewal is an important goal in cancer therapy and recent studies have focused on Notch signalling in the maintenance of stemness of glioma stem cells (GSCs). Understanding cancer-specific Notch regulation would improve specificity of targeting this pathway. In this study, we find that Notch1 activation in GSCs specifically induces expression of the lncRNA, TUG1. TUG1 coordinately promotes self-renewal by sponging miR-145 in the cytoplasm and recruiting polycomb to repress differentiation genes by locus-specific methylation of histone H3K27 via YY1-binding activity in the nucleus. Furthermore, intravenous treatment with antisense oligonucleotides targeting TUG1 coupled with a drug delivery system induces GSC differentiation and efficiently represses GSC growth in vivo. Our results highlight the importance of the Notch-lncRNA axis in regulating self-renewal of glioma cells and provide a strong rationale for targeting TUG1 as a specific and potent therapeutic approach to eliminate the GSC population.
glioblastomas (GBMs) show heterogeneous histological features. These distinct phenotypes are thought to be due to their derivation from glioma stem cells (GSCs), which are a highly tumorigenic and self-renewing sub-population of tumour cells that have different functional characteristics. As the self-renewing capability and multipotency may be closely associated with tumour progression and invasion, understanding the mechanisms underlying the regulation of stemness properties of tumour cells has been actively investigated.

In the neural stem cell, the Notch signalling pathway has a dominant role in inhibiting differentiation through the activities of its downstream effectors, such as Hairy and enhancer of split 1/5 (Hes1/5), which repress the implementation of neurogenic programs. In the context of oncogenesis, Notch signalling has been shown to promote GSC self-renewal and to suppress GSC differentiation. The mechanisms by which Notch regulates brain tumour stem cells appear to be similar to those governing regulation of neural stem cells during neural development. However, the mechanism by which Notch signalling and its downstream effectors maintain the stemness properties of GSCs through the function of a certain set of genes, such as SOX2, MYC and Nestin, remains unresolved.

It has been increasingly demonstrated that large parts of the human genome are transcribed into non-coding RNAs (ncRNAs). A recent study showed that Notch-triggered oncogenic activity can be due to not only its ability to regulate proteins, but also long ncRNAs (lncRNAs) in leukemia. To date, studies have shown that lncRNAs affect the chromatin structure, mRNA stability and miRNA-mediated gene regulation by acting as competing endogenous RNA or natural miRNA sponges.

Among these lncRNAs, Taurine Upregulated Gene 1 (TUG1) has been reported as cancer-related, and can bind to polycytoplasmic repressive complex 2 (PRC2) or PRC1 as well as repress gene expression. TUG1 was originally identified as a transcript upregulated by taurine, whose function is associated with retinal development. In the context of human malignancies, it is overexpressed in bladder cancer, gastric cancer and osteosarcoma, whereas it is downregulated in non-small cell lung cancer, suggesting context-dependent roles in different types of cancers. As the length of the TUG1 lncRNA is not short, ~7.1 kb, it is plausible that TUG1 has multiple functions, which remain unknown.

Here, we show that TUG1, the expression of which is regulated by the Notch signalling pathway, was highly expressed in GSCs and maintained the stemness features of glioma cells. Furthermore, we developed new antisense oligonucleotides (ASO) targeting TUG1 coupled with a potent drug delivery system (DDS), which can be used intravenously to provide efficient and selective delivery to glioma cells at sufficient concentrations to acquire antitumour effects. Our observations indicate that Notch-directed TUG1 is an effective epigenetic modulator that regulates the cancer stem cell population.

**Results**

**Identification of TUG1 as a Notch-regulated lncRNA.** The two GSC populations assessed (1228-GSC and 222-GSC) showed high levels of GSC markers, such as CD15 and SOX2, and GSC characteristics by functional analyses (in vivo limiting dilution assay) (Supplementary Fig. 1), which agree with the previously described model. To identify lncRNAs that are regulated by Notch signalling, we first performed RNA-sequencing (RNA-seq) analysis to investigate the lncRNA expression profile in these two GSC populations. In Notch signalling, the Notch intracellular domain (NICD) translocates into the nucleus and binds the transcription factor, RBPs. Therefore, we selected lncRNAs that contain the binding motifs of RBPs around their transcriptional start site (TSS, ~2 kb to +1 kb, indicates upstream of TSS and + indicates downstream of TSS) using the JASPAR CORE database (http://jaspar.rubin.ku.dk/). Commonly highly expressed lncRNAs with associated RBPs in both GSC populations were identified (Top 50, Fig. 1a).

To identify lncRNA potentially involved in Notch-induced GSC maintenance, we further performed lncRNA microarray analysis in GSCs with either treatment by small interfering RNA (siRNA) targeting Notch1 (si-Notch1), si-JAG1 (Notch ligand), or γ-secretase inhibitors (N-S-phenyl-glycine-t-butyl ester (DAPT) and RO4929097, which is undergoing clinical trial in glioma) (Supplementary Data 1). Notably, the use of DAPT or RO4929097 may not be very specific to Notch signalling inhibition but rather inhibition of γ-secretase. Therefore, we also examined lncRNA expression in si-JAG1 and si-Notch1 treated GSCs by RNA-seq analysis in addition to lncRNA microarray analysis and found that results of both analyses were quite concordant (R² = 0.9014 and 0.9152 in 1228- and 222-GSCs, respectively) (Supplementary Fig. 2 and Supplementary Data 2). These analyses revealed that six lncRNAs were commonly downregulated in the two GSC populations following inhibition of Notch signalling (Fig. 1b, Supplementary Fig. 3a–d and Supplementary Data 3). Among these, TUG1 (NR_002323.1) was the only lncRNA, which was also highly expressed as determined by RNA-seq analysis and possessed multiple RBPs motifs around its promoter region (Fig. 1a,c). Indeed, Notch1 bound to RBPs motifs within the upstream region of TUG1 TSS (Fig. 1d). We confirmed that level of TUG1 expression was efficiently reduced in GSCs following treatment with either si-Notch1, si-JAG1 or γ-secretase inhibitors (DAPT and RO4929097) (Fig. 1e–g and Supplementary Fig. 3a–d). Compellingly, downregulation of TUG1 expression by Notch signalling inhibition was rescued by ectopic NICD expression (Supplementary Fig. 3e–g). These data indicate that Notch signalling predominantly regulates TUG1 expression in glioma cells.

**TUG1 maintains stemness features of GSCs.** Inhibition of TUG1 by siRNA in GSCs reduced cell proliferation via induction of apoptosis as detected by FACS analysis with annexin V and 7-aminoactinomycin D staining (Fig. 2a,b). Notch signalling induces the promoter activity of Nestin, a well-characterized marker of stemness features. We analysed the effect of TUG1 depletion on GSC lines that stably expressed enhanced GFP (EGFP) under the regulation of the Nestin promoter (GSC-pE-Nes). In GSC-pE-Nes neurospheres, ~80% of cells were positive for Nestin-EGFP. The majority of Nestin-EGFP-expressing cells were positive for Notch1, whereas Nestin-EGFP-negative cells were positive for JAG1 and lacked Notch1 expression (Supplementary Fig. 4a). Although the viability of both EGFP-positive and EGFP-negative cells were comparable, Nestin-EGFP-positive cells were highly tumorigenic compared with Nestin-EGFP-negative cells (Supplementary Fig. 4b,c). TUG1 depletion in GSC lines reduced Nestin promoter activity together with impaired neurosphere formation in GSC-pE-Nes (Fig. 2c,d). These morphological and functional changes were also observed after Notch1 and JAG1 inhibition (Supplementary Fig. 4e,d). TUG1 depletion also effectively decreased the expression of stemness-associated genes (SOX2, MYC, Nestin and CD15) in two GSC lines (Fig. 2e,f). Taken together, it is evident that Notch-regulated TUG1 affects the stemness features of GSCs.

**TUG1 antagonizes miR-145 and regulates SOX2 and MYC.** Some lncRNAs are known to act as miRNA sponges in the
Figure 1 | Identification of TUG1 as a Notch downstream target in GSCs. (a) Top 50 commonly highly expressed lncRNAs with Notch/RBPJκ motifs in GSCs (1228 and 222) are shown. y-axis indicates expression levels of each lncRNA by FPKM value (log2). (b) lncRNA expression analysis of GSCs by microarray technology (1228 and 222) following use of either si-JAG1, si-Notch1 or Notch signal inhibitor (γ-secretase inhibitors, DAPT and RO4929097) treatment. Venn diagram depicts the numbers of downregulated lncRNAs identified by each treatment. (c,d) ChIP analysis of Notch1 in the upstream region of the TUG1 TSS. (e) Schematic diagram showing RBPJκ motifs around the TSS of TUG1. Open circles indicate RBPJκ motifs. (d) Enrichment of Notch1 in GSCs treated with either si-NC or si-Notch1. Regions examined by ChIP analysis are indicated as 5′-outside, R1, R2 and 3′-outside in (c). Enrichment of Notch1 is expressed as a percentage of input DNA. *P < 0.01. Student’s t-test. (e) Expression level of TUG1 in GSCs treated with siRNA against the indicated genes. Relative expression level to siRNA-negative control (si-NC) is indicated in the y-axis. *P < 0.01. Student’s t-test. (f,g) Expression level of TUG1 in GSCs treated with DAPT (f) or RO4929097 (g). Values are indicated relative to abundance in DMSO-treated cells. *P < 0.01. Kruskal–Wallis analysis. For all the experimental data, error bars indicate s.d. (n = 3).

Cytoplasm, where these lncRNAs bind to miRNAs and quench their activity12. As shown in previous reports, RNA-fluorescence in situ hybridization (FISH) analysis revealed that TUG1 localized to both the nucleus and the cytoplasm in GSCs (Supplementary Fig. 5a,b)13. We examined miRNA expression changes upon TUG1 inhibition in two GSC lines using a miRNA-based expression microarray approach. Twenty-one and 144 miRNAs were commonly upregulated and downregulated with a greater than twofold difference upon TUG1 inhibition, respectively (Supplementary Data 5). Among the upregulated miRNAs, miR-145 was most prominently increased by TUG1 inhibition (Fig. 3a–d). Intriguingly, the interaction between TUG1 and miR-
Figure 2 | TUG1 maintains stemness features of GSCs. (a) Expression level of TUG1 (left) and viable cell numbers (right) among GSCs treated with either si-NC or si-TUG1 (si-TUG1 #1 and #2). Viable cells were assessed by trypan blue staining. Values are indicated relative to abundance in si-NC-treated cells. *P<0.01, Student’s t-test. (b) The number of apoptotic cells in GSCs treated with si-NC or si-TUG1 (si-TUG1 #1 and #2) were counted by FACS analysis with 7-AAD and PE Annexin V staining. *P<0.01, Student’s t-test. (c,d) Effect of TUG1 depletion on Nestin activity. (c) GSC-pe-Nes-222 was treated with either si-NC or si-TUG1 (si-TUG1 #1 and #2). Expression level of TUG1 is indicated relative to abundance in si-NC-treated cells (left). Phase-contrast and Nestin-EGFP images. Scale bars, 100 μm (right). (d) Intensity of Nestin-EGFP (left) and number of viable cells (right) compared with si-NC control were quantified. Viable cells were assessed by trypan blue staining. *P<0.01, Student’s t-test. (e) Effect of TUG1 depletion on expression of the stemness-associated genes (SOX2, MYC, Nestin and CD15). y-axis indicates relative expression level compared with that seen in si-NC-treated cells. *P<0.01, Student’s t-test. (f) Protein expression levels of SOX2 and MYC in GSCs treated with either si-NC or si-TUG1 (si-TUG1 #1 and #2). For all the experimental data, error bars indicate s.d. (n = 3).

Using the TargetScan prediction algorithm (http://www.targetscan.org)29, we found predicted binding sites for miR-145 not only in TUG1 but also in SOX2 and MYC mRNAs, which encode for well-known stemness-associated transcription factors30. Ectopic expression of miR-145 impaired GSC growth and stemness properties (Supplementary Fig. 5e–h and 6a). This phenotypic change was analogous to that observed after TUG1 inhibition in GSCs (Fig. 2a,b). To further analyse the possible role of TUG1 as a miRNA sponge, we used partial TUG1 transcripts (1–2,132 nucleotides, corresponding to TUG1 exon 1), which contained the predicted binding sites for miR-145 (Fig. 3e). Ectopic expression of these partial TUG1 transcripts efficiently

145, which induced epithelial-to-mesenchymal transition through derepression of ZEB2, has been found in bladder cancer35. Consistently, RNA-FISH analysis revealed that numbers of TUG1 molecules were extremely abundant compared with those of miR-145 molecules in GSCs, whereas they were significantly increased by TUG1 inhibition (Fig. 3c,d, P<0.001). Notably, as determined by RNA immunoprecipitation (RIP) analysis, both TUG1 and miR-145 bound to wild-type AGO2 but did not bind to a mutant form of AGO2 devoid of the PAZ domain, the latter serving as a module for si/miRNA transfer in the RNA silencing pathway and as an anchoring site for the 3’ end of guide RNA within silencing effector complexes (Supplementary Fig. 5c,d)16,28.
impaired the repressive effect of miR-145 on SOX2 and MYC expression, whereas mutated TUG1 or seed sequence-deleted TUG1 did not (Fig. 3e,f and Supplementary Fig. 6b). In addition, disruption of neurosphere formation and inactivation of the Nestin promoter by miR-145 were rescued by ectopic TUG1 expression (Fig. 3g,h). These data indicate that TUG1 protects the transcripts of stemness-related genes from miR-145-mediated degradation and aids in maintaining stemness properties.

**Figure 3 | TUG1 antagonizes miR-145 and maintains expression of stemness-associated genes.** (a) Heatmap shows commonly upregulated miRNAs upon inhibition of TUG1 in GSCs (1228 and 222). Colour corresponds to expression level as indicated in the log2-transformed scale bar below the matrix. Red and blue reflect high and low levels, respectively. (b) Effect of TUG1 depletion on miR-145 expression in GSCs (1228 and 222). y-axis indicates relative miR-145 expression level compared with that in si-NC-treated cells. Expression levels were normalized to internal RNU6B. *P<0.01, Student’s t-test. (c) RNA-FISH analysis of TUG1 (red) and miR-145 (green) in GSCs treated with si-NC or si-TUG1. Nuclei are stained with DAPI. Scale bars, 10 μm. (d) The spot numbers relating to TUG1 and miR-145 detection were quantified per cell in GSCs treated with either si-NC or si-TUG1. *P<0.001, Student’s t-test. (e) Schematic of mutated (TUG1 MUT) or deleted (TUG1 DEL) TUG1. (f) Effect of exogenous miR-145 on TUG1, SOX2 and MYC expression in GSC-pE-Nes-222. Partial TUG1 transcripts (TUG1 WT, TUG1 MUT and TUG1 DEL as shown in (e) were added to GSC-pE-Nes-222 treated with the precursor molecule of miR-145. Expression levels of endogenous TUG1, SOX2 and MYC were measured. Values are indicated relative to abundance of negative control miRNA precursor (NC) treated cells. *P<0.01, Kruskal–Wallis analysis. (g) Phase-contrast and Nestin-EGFP images of miR-145+ TUG1 WT, + TUG1 MUT or + TUG1 DEL cells as shown in (f). Scale bars, 100 μm. (h) Intensity of Nestin-EGFP (left) and number of viable cells (right) were quantified. Viable cells were assessed by trypan blue staining. *P<0.01, Kruskal–Wallis test. For all the experimental data, error bars indicate s.d. (n = 3).
TUG1-PRC2 complex suppresses neuronal differentiation genes.

Studies have demonstrated that TUG1 in the nucleus interacts with PRC components such as Pc2 and EZH2 via its exon 2 and represses target gene expression in a trans manner. To clarify the functional roles of nuclear-localized TUG1 in GSCs, we first transfected TUG1 RNA labelled with 5′-bromo-uridine (BrU) (2,133–2,910 nucleotides, corresponding to the TUG1 exon 2) into GSCs. Immunoprecipitation analysis using an anti-BrU antibody revealed that TUG1 bound to PRC2 components (EZH2, SUZ12) and the YY1 transcription factor (Fig. 4a). Intriguingly, interaction between PRC2 and YY1 was disrupted by use of siRNA against TUG1 or RNase treatment, suggesting that TUG1 functions as a scaffold molecule between PRC2 and YY1 (Fig. 4b,c). We further determined the region within TUG1 that interacts with YY1 and EZH2 using a series of TUG1 deletion mutants. EZH2 and YY1 bound TUG1 at the regions of 2,316–2,555 and 2,746–2,910 nucleotides, respectively (Fig. 5a,b). Intriguingly, these two regions are well conserved in the genome. We then explored the association between TUG1 and the YY1 motif among the 1,714 genes that contain a YY1 motif around their TSS. Among 630 si-TUG1 target genes containing a YY1 motif (shown in Fig. 4d), 525 genes contain a YY1 motif. (Fig. 4e,f) Averaged expression value of 525 TUG1 target genes containing a YY1 motif (shown in Fig. 4d) upon inhibition of TUG1 (si-TUG1 #1 and #2). Colour corresponds to expression level as indicated in the log2-transformed scale bar below the matrix. Red and blue reflect high and low levels, respectively. (f) Averaged expression value of 525 genes shown in (e). Error bars indicate s.e.m. *P<0.001, Student’s t-test. (g) Enrichment of K27me3 in the BDNF, NGF and NTF3 promoter regions (left), and their mRNA expression levels (right) in GSCs treated with either si-NC or si-TUG1 were immunoprecipitated with anti-YY1 antibody and analysed by western blotting (RNase, left). Relative intensity values of EZH2 normalized to Input are indicated in (c). (d) Venn diagram shows relationship between TUG1 target genes (630 genes) and 1,714 genes that contain a YY1 motif around their TSS. Among 630 TUG1 target genes, 525 genes contain a YY1 motif. (e) Heatmap indicates expression changes of 525 TUG1 target genes containing a YY1 motif (shown in d) upon inhibition of TUG1 (si-TUG1 #1 and #2). Colour corresponds to expression level as indicated in the log2-transformed scale bar below the matrix. Red and blue reflect high and low levels, respectively. (f) Averaged expression value of 525 genes shown in (e). Error bars indicate s.e.m. *P<0.001, Student’s t-test. (g) Enrichment of K27me3 in the BDNF, NGF and NTF3 promoter regions (left), and their mRNA expression levels (right) in GSCs treated with either si-NC or si-TUG1 were immunoprecipitated with anti-YY1 antibody and analysed by western blotting (RNase, left). Relative intensity values of EZH2 normalized to Input are indicated in (c). (d) Venn diagram shows relationship between TUG1 target genes (630 genes) and 1,714 genes that contain a YY1 motif around their TSS. Among 630 TUG1 target genes, 525 genes contain a YY1 motif. (e) Heatmap indicates expression changes of 525 TUG1 target genes containing a YY1 motif (shown in d) upon inhibition of TUG1 (si-TUG1 #1 and #2). Colour corresponds to expression level as indicated in the log2-transformed scale bar below the matrix. Red and blue reflect high and low levels, respectively. (f) Averaged expression value of 525 genes shown in (e). Error bars indicate s.e.m. *P<0.001, Student’s t-test. (g) Enrichment of K27me3 in the BDNF, NGF and NTF3 promoter regions (left), and their mRNA expression levels (right) in GSCs treated with either si-NC or si-TUG1 were immunoprecipitated with anti-YY1 antibody and analysed by western blotting (RNase, left). Relative intensity values of EZH2 normalized to Input are indicated in (c). (d) Venn diagram shows relationship between TUG1 target genes (630 genes) and 1,714 genes that contain a YY1 motif around their TSS. Among 630 TUG1 target genes, 525 genes contain a YY1 motif. (e) Heatmap indicates expression changes of 525 TUG1 target genes containing a YY1 motif (shown in d) upon inhibition of TUG1 (si-TUG1 #1 and #2). Colour corresponds to expression level as indicated in the log2-transformed scale bar below the matrix. Red and blue reflect high and low levels, respectively. (f) Averaged expression value of 525 genes shown in (e). Error bars indicate s.e.m. *P<0.001, Student’s t-test. (g) Enrichment of K27me3 in the BDNF, NGF and NTF3 promoter regions (left), and their mRNA expression levels (right) in GSCs treated with either si-NC or si-TUG1 were immunoprecipitated with anti-YY1 antibody and analysed by western blotting (RNase, left). Relative intensity values of EZH2 normalized to Input are indicated in (c). (d) Venn diagram shows relationship between TUG1 target genes (630 genes) and 1,714 genes that contain a YY1 motif around their TSS. Among 630 TUG1 target genes, 525 genes contain a YY1 motif. (e) Heatmap indicates expression changes of 525 TUG1 target genes containing a YY1 motif (shown in d) upon inhibition of TUG1 (si-TUG1 #1 and #2). Colour corresponds to expression level as indicated in the log2-transformed scale bar below the matrix. Red and blue reflect high and low levels, respectively. (f) Averaged expression value of 525 genes shown in (e). Error bars indicate s.e.m. *P<0.001, Student’s t-test. (g) Enrichment of K27me3 in the BDNF, NGF and NTF3 promoter regions (left), and their mRNA expression levels (right) in GSCs treated with either si-NC or si-TUG1 were immunoprecipitated with anti-YY1 antibody and analysed by western blotting (RNase, left). Relative intensity values of EZH2 normalized to Input are indicated in (c). (d) Venn diagram shows relationship between TUG1 target genes (630 genes) and 1,714 genes that contain a YY1 motif around their TSS. Among 630 TUG1 target genes, 525 genes contain a YY1 motif. (e) Heatmap indicates expression changes of 525 TUG1 target genes containing a YY1 motif (shown in d) upon inhibition of TUG1 (si-TUG1 #1 and #2). Colour corresponds to expression level as indicated in the log2-transformed scale bar below the matrix. Red and blue reflect high and low levels, respectively. (f) Averaged expression value of 525 genes shown in (e). Error bars indicate s.e.m. *P<0.001, Student’s t-test.
between human and mouse (86.7% and 82.4% homologies, respectively).

Next, we performed a modified RNA pull-down assay coupled with promoter-microarray analysis using BrU-labelled TUG1-transfected cells. From this assessment, 3,183 and 2,333 genes were enriched with ectopic TUG1 in 1228- and 222-GSCs, respectively, and 630 genes were commonly identified as targets of TUG1 (Supplementary Data 6). Comparison between the TUG1 target genes (630 genes) and predicted YY1 target genes (1,714 genes) determined by Whitfield’s criterion31 revealed that 525 (83.3%) contained an YY1 motif (Fig. 4d). Gene expression microarray analysis revealed that the majority of those target genes (345/525 genes, 65.7%) were upregulated upon serum-differentiated GSC revealed that exon 2 transcript (2,133–2,910 nucleotides) and the TUG1 fragments with EZH2- and YY1-binding domains (2,316–2,910 nucleotides) could efficiently repress the expression of TUG1 target genes, whereas other deletion fragments could not (Fig. 5d). Taken together, these data suggest that TUG1 physically interacted with PRC2 and promoted locus-specific K27me3 via YY1 binding activity, which resulted in suppression of neuronal differentiation-associated genes.

Pivotal roles of the TUG1 exon 1 region in maintenance of GSC.

To examine what are the relative and contextual contributions of miR-145 and PRC2 to the TUG1 effects on stemness features, we overexpressed each exon of TUG1 in GSCs treated with gamma-secretase inhibitor (RO4929097) (Fig. 5a, Fig. 6a–c). Interestingly, the TUG1 exon 1 transcript (1–2,132 nucleotides) rescued those features. In addition, TUG1 exon 1 transcript efficiently rescued Nestin promoter activity together with impaired neurosphere formation in GSC-pE-Nes (Fig. 6d,e). Consistently, the effects of Notch1 and JAG1 inhibition by siRNA on the stemness features of GSCs were rescued by ectopic TUG1 exon 1 expression, but were not rescued by either exon 2 or exon 3 (Supplementary Fig. 7). Taken together, these data indicate the pivotal roles of the TUG1 exon 1 region in maintenance of stemness features in GSCs.
Expression analysis of TUG1 in human GBM tissues. To validate our findings in GSC analysis, we examined the interaction between Notch1 and TUG1 in clinical GBM samples. TUG1 expression in GBM tissues (n = 24) was significantly higher than in normal brain tissues (Fig. 7a, P = 6.419 × 10⁻⁴). RNA–FISH analysis in GBM samples showed that Notch1-positive cells were highly enriched around perivascular regions where JAG1-positive endothelial cells may have acted as niche cells to promote GSC...
self-renewal, as shown in previous studies. In addition, ~70% of Notch1-positive GBM cells showed high expression of TUG1 in the perivascular regions, whereas in areas distant from tumour vessels, the majority of GBM cells expressed neither Notch1 nor TUG1 (Fig. 7b–e). The positive correlation between TUG1 and Notch1 expression was further validated in an independent sample set using The Cancer Genome Atlas (TCGA) (Supplementary Fig. 8a).

Finally, we investigated the molecular effect of TUG1 inhibition on an intracranial xenograft mouse model. GSCs (GSC-pE-Nes) were inoculated into the brain of NOD-SCID mice. After 30 days of transplantation, TUG1 expression mediated by TUG1-DDS were even stronger than those observed with a γ-secretase inhibitor (RO4929097, oral administration) in our model (Fig. 7g,h, P = 9.237 × 10−3).

RNA-FISH analysis showed that neither TUG1 nor Notch1-positive cells were observed in residual tumours in mice with TUG1-DDS treatment, whereas in mice with CTRL-DDS coupled with a potent DDS using cyclic Arg–Gly–Asp (cRGD) peptide-conjugated polymeric micelle, which we call hereafter TUG1-DDS, was administered intravenously twice a week for 4 weeks (Fig. 7f). TUG1-DDS was specifically accumulated and retained at least 24 h in brain tumours (Supplementary Fig. 9c,d). Treatment with TUG1-DDS markedly reduced tumour growth compared with control-DDS (CTRL-DDS) together with the downregulation of TUG1 expression (Fig. 7g, P = 5.451 × 10−5).

Figure 7 | **TUG1 is a critical mediator of stemness and tumorigenicity in GSCs in vivo.** (a) Expression level of TUG1 in normal brain and GBM tissues (n = 24). The line inside the box represents the median, and the bottom and the top of the box are the first and the third quartiles, respectively. The whisks indicate the range of the data. P < 0.001, Student’s t-test. (b–e) Representation of RNA-FISH analysis of TUG1 and Notch1 in GBM tissues. (b) Hematoxylin and eosin (HE) staining of GBM section. Scale bars, 100 μm. (c,d) RNA-FISH analysis of TUG1 and Notch1 in tumour cells around area (perivascular region) and area (distant from blood vessel) in panel b. Nuclei are stained with DAPI. Scale bars, 10 μm. (e) Frequency of TUG1-positive cells in GBM specimens. Cell counts from multiple regions were averaged. Error bars indicate s.d. (f) GSC-pE-Nes-222 was transplanted intracranially in NOD/SCID mice. After 30 days of transplantation, CTRL-DDS or TUG1-DDS were intravenously injected twice a week for 4 weeks. RO4929097 was administered orally five times a week for 4 weeks. (g) Quantification of tumour volume at 4 weeks post treatment (left) and RNA expression levels of TUG1 in the tumour cells of mouse xenograft (right). Relative expression levels to that in CTRL-DDS-treated tumour are indicated on the y-axis (n = 4). Error bars indicate s.e.m. * P < 0.01, ** P < 0.001, Kruskal–Wallis analysis. (h) Representative HE-stained whole brain sections at 4 weeks post treatment. Tumour areas are surrounded by red dotted line. Scale bars, 1 mm.
treatment, TUG1 and Notch1 expression was predominantly observed in the perivascular regions (Fig. 8a). Consistently, Nestin-EGFP and CD15-positive cells were diminished in TUG1-DDS-treated xenografts, suggesting that inhibition of TUG1 effectively eliminated the GSC population in vivo (Fig. 8b). Furthermore, in TUG1-DDS-treated xenografts, miR-145 was significantly increased and MYC and SOX2 were decreased (Fig. 8c, \( P = 2.895 \times 10^{-3} \)), as was found in GSCs in vitro following siRNA treatment against TUG1 (Fig. 3c,d). Neuronal differentiation-associated genes (BDNF, NGF and NTF3) were reactivated in response to TUG1-DDS treatment (Fig. 8c,d). Taken together, these data indicate that both nuclear and cytoplasmic TUG1 cooperatively promote the stemness and tumorigenicity of GSCs in vivo.

Figure 8 | Molecular effects of TUG1 inhibition in mouse xenograft model. (a) RNA-FISH analysis of TUG1 (red) and Notch1 (green) in tumour cells of CTRL-DDS (upper panels) and TUG1-DDS-treated mice (bottom panels). (b) Immunostaining of CD15 (red) and Nestin-EGFP (green) in CTRL-DDS (upper panels) and TUG1-DDS-treated tumour (bottom panels). Asterisk indicates blood vessel in CTRL-DDS treated mice. Tumour areas are surrounded with the dashed line in TUG1-DDS-treated mice. Scale bars, 100 \( \mu \)m. (c) Expression levels of miR-145, SOX2, MYC and TUG1 target genes (BDNF, NGF and NTF3) were examined by qPCR in tumour cells derived from the mouse xenograft. Relative expression levels compared with that in the CTRL-DDS-treated tumour are indicated on the \( y \)-axis \((n=4)\). Error bars indicate s.e.m. * \( P<0.001 \), Student’s t-test. (d) Immunostaining of NGF in CTRL-DDS (left) or TUG1-DDS-treated tumour (right) at 4 weeks post treatment. Tumour areas are surrounded with the dashed line in TUG1-DDS-treated mice. Scale bars, 1 mm.
Discussion

An increasing number of studies have focused on the impact of Notch signalling in GSCs, which may have plasticity and respond to signals from their microenvironment. Because GSCs contribute to the growth, survival, invasion and recurrence of brain tumours, understanding the mechanisms that govern the specific regulation of a certain set of genes, which contribute to stemness properties, by the Notch signal pathway has been actively investigated. In the current study, we identified TUG1, which was found via an unbiased approach to be commonly upregulated in two independent GSC populations and downregulated following inhibition of Notch signalling. TUG1 promotes self-renewal by sponging miR-145 in the cytoplasm and recruiting polycomb via YY1-binding activity to repress differentiation genes in the nucleus. We also found that MALAT1 and NEAT1 were highly expressed in GSCs. Although these are well known important functional lncRNAs associated with glioma behaviour, they did not appear to be involved in the Notch-induced GSC maintenance system (Supplementary Fig. 4f-h).

Some lncRNAs contain miRNA-binding sites and may act as miRNA sponges, such as the abundant, cytoplasmic ncRNA TUG1. TUG1 modulates miR-145 levels through its function as a miRNA sponge to trap miR-145, which is known to regulate the expression of core stemness-associated factors. The miRNA sponge function of TUG1 is supported by three lines of evidence. First, we found that cytoplasmic TUG1 expression is extremely abundant compared with miR-145 in GSCs, whereas in TUG1-depleted GSCs, the level of miR-145 was found to be significantly increased. Second, downregulation of the stemness-associated factors by miR-145 was rescued by exogenous TUG1 expression, whereas TUG1 mutants that lacked specific miR-145 seed sequences could not. Third, both TUG1 and miR-145 were bound to wild-type AGO2 but not bound to a mutant form of AGO2 without the PAZ domain. This interaction between TUG1 and miR-145 is consistent with a previous elegant study showing that abundant and cytoplasmic linc-RoR functions as an endogenous miR-145 sponge, which negatively regulates mature miRNA expression levels through a posttranscriptional mechanism and avoids miR-145 increases in self-renewing hESCs.

Notably, TUG1 could prevent MYC mRNA from degradation by quenching miR-145. As MYC is an important downstream factor in Notch signalling, as well as a transcriptional activator of TUG1 (Supplementary Fig. 10), TUG1 and MYC may form a reinforcing activation loop leading to mutual stabilisation of their gene expression. In any case, the Jagged1-Notch1-TUG1 pathway can effectively enhance GSC self-renewal by antagonising miR-145 activity. Intriguingly, in addition to targeting stemness-associated factors, miR-145 is also known to act as a tumour suppressor by decreasing cell growth, apoptosis and angiogenesis in various cell types including those derived from GBM. These tumour-suppressive functions of miR-145 may also explain why inhibition of TUG1 efficiently induced apoptosis after partial neuronal differentiation.

In the nucleus, TUG1 selectively regulated the epigenetic status of neuronal differentiation-associated genes, such as BDNF, NGF and NTf3. Notably, BDNF acts as a tumour suppressor in gliogenesis. Theoretically, RNA such as TUG1 may freely diffuse and transmigrate between chromosomes, but some DNA elements may be required for efficient loading onto certain genomic loci. In this study, we illustrated that YY1 functional bridges facilitated the loading of regulatory TUG1 to its DNA targets. It is possible that YY1 functional bridges alone may not be sufficient to specify the target loci, as TUG1 was not simultaneously enriched at a large number of other YY1 sites. However, we found that TUG1 fragments with EZH2- and Y11-binding domains could efficiently repress the expression of the PRC2-TUG1-Y1 target genes, whereas other deletion fragments, which lack either EZH2 or Y11 binding domains (dominant-negative constructs), could not. Therefore, TUG1 in the nucleus appeared to have interacted with PRC2 and promoted locus-specific methylation of histone H3K27, at least in part, via Y11-binding activity. Of interest, both the regions where PRC2 and Y11 interact with TUG1 are evolutionarily conserved between mouse and human, suggesting the importance of these regions. Consistent with our findings, the concept of YY1 as docking protein has also been illustrated in an X chromosome inactivation model, in which YY1 tethers Xist RNA-PRC2 to the inactive X nucleation center.

These regional distinct functions of TUG1 may raise the question of what are the relative and contextual contributions of each region via miR-145 and PRC2 on stemness features. Importantly, attenuation of the Notch-TUG1 axis by siRNA or γ-secretase in regulating self-renewal of glioma cells was efficiently rescued by TUG1 exon 1 transcript where the miR-145-binding site locates indicating that there is a pivotal role for the exon 1 and miR-145 interaction in maintenance of stemness properties.

Many strategies to improve the delivery of drugs to GBMs are under investigation, as the blood–brain barrier and the blood–brain tumour barrier limit the enhanced permeability and retention effect, thereby resulting in poor efficacy due to inefficient drug delivery to GBM. Brain tumour vessels show overexpression of receptors that mediate ligand-dependent drug delivery. In particular, RGD peptides are promising ligand molecules for targeting αvβ3 and αvβ5 integrins, which are frequently overexpressed in GBM cells. In order to inhibit TUG1 expression in a mouse xenograft model, we used cRGD ligand-conjugated polymeric micelles for delivery. These targetable polymeric micelles retained ASO accumulation within tumours, which is associated with transcytosis-mediated penetration and enhanced gene silencing activity. Although further investigations are required, cRGD-mediated drug delivery is a powerful strategy for targeting GBMs through facilitated ASO delivery beyond the blood–brain tumour barrier.

Emerging insights into gliogenesis have revealed that GSCs have the potential to initiate and maintain the growth of gliomas, and may be crucial for their resistance to conventional therapies. Further, the existence of GSCs and plastic epigenetic regulation may be linked to the morphological and lineage heterogeneity that is observed in GBM. In the current study, we addressed the novel mechanism underlying the contribution of the Notch signalling pathway to the stemness features of GSCs in response to contact-dependent signals from adjacent cells expressing Jagged1. Further, we provide a new paradigm whereby targeting TUG1, especially coupled with a potent DDS, is an effective novel strategy for GBM treatment. One limitation of the current study is that both our GSC lines were classified as proneural type GBM by gene expression analysis (Supplementary Fig. 8b). Indeed, expression levels of TUG1 and Notch1 were prominently upregulated in proneural and classical GBM compared with two other subtypes (neural and mesenchymal) (Supplementary Fig. 8c). Therefore, it might be possible that targeting TUG1 is more promising for treatment of proneural or classical GBMs rather than other subtypes. However, given that inhibition of γ-secretase-mediated Notch cleavage is a primary focus for the development of targeted therapeutics in GBM (including application in clinical trials such as RO4929097), our data...
may provide a strong rationale that targeting TUG1 is a more specific and potent therapeutic approach to eliminate the GSC population.

Methods

GBM tissues, cell culture and drug treatment. GBM tissue samples were obtained from patients undergoing surgical treatment at Nagoya University hospital, Japan, after they provided written informed consent. Total RNA of normal brain was purchased from BioChain (Hayward, CA, USA). The procedures used for derivation of GSCs (1228-GSC and 222-GSC) were as described previously. In brief, dissociated tumour cells were cultured in Neurobasal Medium (Life Technologies, Carlsbad, CA) containing N2 and B27 supplements (Life Technologies) containing 10% fetal bovine serum. Serially transplanted GSCs were generated according to previously published methods. GSCs were routinely tested for mycoplasma contamination and used in the experiments. For blocking of Notch signalling in GSCs, the γ-secretase inhibitor DAPT (N-[(3,5-difluorophenacetyl)- l-alanyl]-S-phenyl glycine t-buty1 ester), Sigma-Aldrich, St Louis, MO, USA) was added, and the medium was changed every other day. For in vivo studies, RO4929097 (Selleck Chemicals, Houston, TX, USA) was used for derivation of GSCs (1228-GSC and 222-GSC) were as described previously. Total RNA was isolated from GSCs with TRIzol reagent (Life Technologies). The RNA-seq libraries were prepared using a paired-end RNA Sequencing Sample Prep Kit (Illumina, San Diego, CA, USA). Two microgram of total RNA was used as the starting material, and polyadenylated RNA was selected using Sera-Mag Magnetic Oligo (dT) Beads (Illumina). First-strand cDNA synthesis was performed using SuperScript II (Life Technologies), and second-strand CDNA synthesis was performed using DNA Pol I in the supplied GEX second-strand reaction buffer (Illumina). Paired-end adapters were ligated to the CDNA fragments. PCR (eight cycles) was performed with Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland). The PCR products were cleaned up with Agencourt AMPure XP magnetic beads (Beckman Coulter, Miami, FL, USA). cDNA was applied to the flow cell and paired-end 76 nucleotide-long reads were generated using Illumina GAIIx and the Sample Prep Kit (Illumina). First-strand cDNA synthesis was performed using SuperScript II (Life Technologies). Polyadenylated RNA was selected using Sera-Mag Magnetic Oligo (dT) Beads hybridized to SurePrint G3 Human GE 8x60K Microarrays (Bio-Rad, Richmond, CA, USA) and hybridized to microarrays at 55 °C with rotation at 20 rpm for 20 h. The arrays were scanned using an Agilent Microarray Scanner G2505B (Agilent Technologies). The DNA templates were amplified by PCR with the primer sets listed in Supplementary Data 4. In addition, PCR-generated fragments containing cDNA for NICD (not including the s-Notch1 target region), TUGI exon 1 (1–2,132 nucleotides), TUGI exon 2 (2,133–2,910 corresponding to exon 2 full-length, 2,316–2,910, 2,566–2,910, 2,746–2,910, 2,133–2,745, 2,133–2,555 and 2,133–2,315 nucleotides) using the CUGA7 in vitro Transcription Kit (Nippon Gene, Tokyo, Japan). The DNA templates were amplified by PCR with the primer sets listed in Supplementary Data 4. For the c-cap structure and 3'-polyA tail, the DNA template was ligated into the pT7TVector (Promega, Madison, WI, USA). The seed sequence of mir-145 (5'- AAC TGG A-3') within TUGI RNA exon 1 (1–2,132 nucleotides) was mutated or deleted using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with the primer sets listed in Supplementary Data 4. In addition, QPCR-generated fragments containing cDNA for NICD (not including the s-Notch1 target region), TUGI exon 1 (1–2,132 nucleotides), TUGI exon 2 (2,133–2,910 nucleotides), TUGI exon 3 (2,911–7,115 nucleotides) and luciferase were also ligated into a pDNA3.1 vector (Life Technologies), which were subsequently transfected into the glioma cells in order to overexpress those TUGI transcripts. Primer sequences for plasmid construction are summarized in Supplementary Data 4. Obtained plasmids were verified by conventional sequencing analysis.

Western blot analysis. For western blot analysis, anti-_SOX2 (3579, 1,100, Cell Signaling Technology), anti-YY1 (ab12132, Abcam) and anti-MYC (ab32, Abcam) antibodies. HRP-linked anti-mouse IgG (ab7076, 1,1000, Cell Signaling Technology) and HRP-linked anti-rabbit IgG (ab7074, 1,1000, Cell Signaling Technology) antibodies were used as secondary antibodies. Uncropped scans of the blots are provided in Supplementary Fig. 11.

Exogenous expression of TUG1. In vitro transcription of TUG1 RNA was performed with RNA templates that correspond to the different regions of the TUG1 RNA sequence (1–2,132 nucleotides, 2,133–2,910 corresponding to exon 2 full-length, 2,316–2,910, 2,566–2,910, 2,746–2,910, 2,133–2,745, 2,133–2,555 and 2,133–2,315 nucleotides) using the CUGA7 in vitro Transcription Kit (Nippon Gene, Tokyo, Japan). The DNA templates were amplified by PCR using the primer sets listed in Supplementary Data 4. To append the 5’-cap structure and 3’-polyA tail, the DNA template was ligated into the pT7T Vector (Promega, Madison, WI, USA). The seed sequence of mir-145 (5’- AAC TGG A-3') within TUGI RNA exon 1 (1–2,132 nucleotides) was mutated or deleted using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with the primer sets listed in Supplementary Data 4. In addition, PCR-generated fragments containing cDNA for NICD (not including the s-Notch1 target region), TUGI exon 1 (1–2,132 nucleotides), TUGI exon 2 (2,133–2,910 nucleotides), TUGI exon 3 (2,911–7,115 nucleotides) and luciferase were also ligated into a pDNA3.1 vector (Life Technologies), which were subsequently transfected into the glioma cells in order to overexpress those TUGI transcripts. Primer sequences for plasmid construction are summarized in Supplementary Data 4. Obtained plasmids were verified by conventional sequencing analysis.

mRNA microarray. The SurePrint G3 Human mRNA 8x60K Microarray (G4872A, Agilent Technologies) was used according to the manufacturer’s protocol. Total RNA was isolated from GSCs with TRIzol reagent (Life Technologies). In total, 100 ng of total RNA was labelled with Cy3 (Agilent Technologies) and 15 units of T4 RNA ligase (GE Healthcare, Little Chalfont, Buckinghamshire, UK) were added. The RNA was purified with RNeasy columns (Bio-Rad, Richmond, CA, USA) and hybridized to microarrays at 55 °C with rotation at 20 rpm for 20 h. The arrays were scanned using an Agilent Microarray Scanner (G2565b, Agilent Technologies). The scanned images were analysed using the Feature Extraction software, version 10.7.3.1 (Agilent Technologies) with background correction. Data analysis was performed with GeneSpring GX (version 12.6.0 (Silicon Genetics). Expression data were normalized to the 75th percentile using the GeneSpring normalisation option with no substantial difference in
RNA-FISH analysis. RNA was visualized in paraffin-embedded sections using the QuantiGene ViewRNA ISH Tissue Assay Kit (Affymetrix, Frederick, MD, USA). In brief, tissue sections were rehydrated and incubated with proteinase K. Subsequently, they were incubated with ViewRNA probe sets designed against human TUGI, miR-145 and Notch1 (Affymetrix).

Induction of miR-145 in GSCs. GSCs were transfected with a precursor molecule mimicking miR-145 (Pre-miR-145 precursor, final concentration of 30 nM, Applied Biosystems) or negative control miRNA (Pre-miR-Neg Control #1, 30 nM, Applied Biosystems) according to the manufacturer’s instructions. The expression level of miR-145 was measured by qPCR using the primers listed in Supplementary Data 4.

RIP analysis. RIP analysis was performed using RiboCluster Profiler RIP Assay Kit (RN1005, MBL International) according to the manufacturer’s protocols. RNA-protein complexes were immunoprecipitated with an anti-AGO2 antibody (RN0085M, MBL International), anti-Flag antibody (F18804, Sigma-Aldrich), and anti-IgG antibody as a negative control (PM035, MBL International).

Immunoprecipitated RNA was then analysed by qPCR using the primers listed in Supplementary Data 4. For analysis of the interaction between RNA and the AGO2 PAZ domain, GSCs were transfected with expression vectors encoding either Flag-AGO2 or Flag-AGO2 lacking the PAZ domain. The AGO2 expression vector was purchased from Addgene (Cambridge, MA, USA). The Flag-AGO2 variant lacking the PAZ domain was generated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) using the following primers—forward: 5’-CTGTCGCT TGGAAAACCTG-3’; and reverse: 5’-CCGAGCAAAGTGATTTAAGAAA 3’. The obtained vector was verified by conventional sequencing analysis.

Analysis of RNA-binding proteins by RNA pull-down assay. Nuclear extracts were obtained from GSCs (1 x 10^6) and incubated with 50 pmol of BrU-labeled TUGI RNA. RNA and nuclear extract conjugates were mixed with an anti-BrU antibody (MI-11-3, MBL International) for 2 h at 4°C. To collect the immunoprecipitated RNA-binding proteins, Dynabeads Protein G (LifeTechnologies) were added and incubated for 1 h at 4°C. These TUGI RNA-binding proteins were analysed by western blot analysis with anti-EZH2 antibody (#3147, Cell Signaling Technology), anti-LZ41 antibody (ab12132, Abcam, Cambridge, UK), and anti-IgG antibody as a negative control (PM035, MBL International). HRP-linked anti-mouse IgG (#7076, Cell Signaling Technology) and HRP-linked anti-rabbit IgG (#7074, Cell Signaling Technology) antibodies were used as secondary antibodies.

Analysis of TUG1 RNA-binding loci by ChIP assay in vivo. Ten nM BrU-labeled TUGI RNA (2,133–2,910 nucleotides) was transfected into GSCs using Lipofectamine 2000 (LifeTechnologies). After 72 h of transfection, GSCs were cross-linked with 0.5% formaldehyde, and chromatin fractions were extracted and homogenized. Chromatin and BrU-labeled TUGI RNA mixtures were immunoprecipitated using an anti-BrU antibody and Dynabeads Protein G. Purified chromatin was eluted to yield DNA, which was then subjected to microarray analysis or ChIP-qPCR analysis.

Equivalent amounts of DNA, which was extracted after immunoprecipitation for BrU-labeled TUGI RNA, and total input DNA were amplified in parallel using a random primer method with the GenomePlex Complete Whole Genome Amplification Kit (15 cycles), according to the manufacturer’s instructions (Sigma-Aldrich). Amplified products were labelled with Cy5 for immunoprecipitated DNA samples and Cy3 for input DNA using the BioPrime Array CGH Genomic Labeling System (Life Technologies). A total of 4 μg of labelled DNA was then hybridized to the human promoter ChIP-chip microarray (G4874A, Agilent Technologies) for 48 h at 65°C. Data were extracted from scanned images using the Feature Extraction software, version 10.7.3.1 (Agilent Technologies). The text files were then imported into Genomic Workbench, standard edition 5.0.14 (Agilent Technologies), for analysis.

ChIRP assay. ChIRP assays were performed as described.28 GS C were cross-linked with 1% glutaldehyde for 15 min at room temperature and then quenched with 125 mM glycine for 5 min. The cross-linked chromatin was then isolated and hybridized with the probes, followed by streptavidin bead-based capturing and wash/elution steps. The antisense DNA probes targeting TUG1 and EGFP-ORF sequences were designed using ChIRP Probe Designer version 4.2 (https://www.biosearchtech.com/chirp-designer) and divided into odds and even pools. The probes used for the ChIRP assays are listed in Supplementary Data 4. The ChIRP captured chromatin was then reverse cross-linked and analysed by qPCR using the primers listed in Supplementary Data 4.

Analysis of TCGA data. For analysis of gene expression (TUG1, Notch1, SOX2, MYC, BDNF, NGF and NTF3) in clinical GBM samples, we used the level-3 processed expression data of Affymetrix HT Human Genome U133A microarray platform from The TCGA (https://tcga-data.nci.nih.gov/).

Polymeric micelle preparation for administration in vivo. A cyclic Arg-Gly-Asp (cRGD) peptide-conjugated polymeric micelle29 was used for systemic delivery of ASOs in vivo. Sequences used were as follows (1) Firefly GL3 luciferase (CTRL): 5’-TCAAGATCTACCCGACTATTGT-3’; (2) TUGI: 5’-TGAATTCAATCCTT GAGAT-3’. For observation of ASO in vivo, ASOs were labelled with Alexa487.

Animal experiments. All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of Nagoya City University Graduate School of Medical Sciences. 222-GSC-pE-Nes were injected intracranially into 6-week-old female NOD/SCID mice (n = 12, SLC, Shizuoka, Japan). Four weeks after the injection of GSCs, CTRL-DDS (n = 4) or TUG1-DDS (n = 4; 1 mg/kg per day) were intravenously injected twice a week for 4 weeks. RO4929097 (n = 4; 20 mg/kg per day) was administered orally five times a week for 4 weeks. The accumulation of ASOs in tumour tissue was confirmed by an in vivo spectral imaging system (IVIS Lumina II, Xenogen, Alameda, CA, USA). Tumour volumes were calculated using the formula (width, height and length) x W x H x L/2.

Statistics. For the statistical analysis of clinical data sets from TCGA, we used R Statistical Software (version 2.14.0; R Foundation for Statistical Computing, Vienna, Austria). The statistical significance of the differences between two groups was analysed by paired Student’s t-test. Kruskal–Wallis analysis was used for to evaluate the extent of differences among more than three groups (StatView software version 5.0; Abacus Concepts, Berkeley, CA, USA). Microarray data analysis were performed by paired Student’s t-test. All reported P values were two-sided, with P < 0.01 considered statistically significant.

Data availability. The Gene Expression Omnibus accession numbers for the RNA-seq data, ChIP-chip microarray data, gene expression microarray, miRNA expression microarray for 1228- and 222-GSCs are GSE66620, GSE86348, GSE65910, GSE79897 and GSE79896, respectively.

References
1. Singh, S. K. et al. Identification of human brain tumour initiating cells. Nature 432, 396–401 (2004).
2. Suva, M. L. et al. Reconstructing and reprogramming the tumor-propagating potential of glioblastoma stem-like cells. Cell 157, 580–594 (2014).
3. Quintana, E. et al. Phenotypic heterogeneity among tumorigenic melanoma cells from patients that is reversible and not hierarchically organized. Cancer Cell 18, 510–523 (2010).
4. Cheng, L. et al. Glioblastoma stem cells generate vascular pericytes to support vessel function and tumor growth. Cell 153, 139–152 (2013).
5. Natsume, A. et al. Chromatin regulator PRC2 is a key regulator of epigenetic plasticity in glioblastoma. Cancer Res. 74, 4559–4570 (2010).
6. Louvi, A. & Artavanis-Tsakonas, S. Notch signalling in vertebrate neural development. Nat. Rev. Neurosci. 7, 93–102 (2006).
7. Fan, X. et al. NOTCH pathway blockade depletes CD133-positive glioblastoma cells and inhibits growth of tumour neuropheres and xenografts. Stem Cells 28, 5–16 (2010).
8. Pierfelice, T. J., Schreck, K. C., Eberhart, C. G. & Gianno, N. Notch, neural stem cells, and brain tumors. Cold Spring Harb. Symp. Quant. Biol. 73, 367–375 (2008).
9. Trimarchi, T. et al. Genome-wide mapping and characterization of Notch-regulated long noncoding RNAs in acute leukemia. Cell 158, 593–606 (2014).
10. Gupta, R. A. et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 464, 1071–1076 (2010).
11. Kretz, M. et al. Control of somatic tissue differentiation by the long non-coding RNA TINCR. Nature 493, 231–235 (2013).
12. Tay, Y., Rinn, J. & Pandolfi, P. P. The multilayered complexity of ceRNA crosstalk and competition. Nature 505, 344–352 (2014).
13. Khalil, A. M. et al. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc. Natl Acad. Sci. USA 106, 11687–11672 (2009).
14. Yang, L. et al. ncRNA- and Fc2 methylation-dependent gene relocation between nuclear structures mediates gene activation programs. Cell 147, 773–788 (2011).
15. Young, T. L., Matsuda, T. & Cepko, C. L. The noncoding RNA Taurine Ureapregulated Gene 1 is required for differentiation of the murine retina. Curr. Biol. 15, 501–512 (2005).
16. Tan, J., Qiu, K., Li, M. & Liang, Y. Double-negative feedback loop between long noncoding RNA TUG1 and miR-145 promotes epithelial to mesenchymal transition and radiosensitivity in human bladder cancer cells. FEBS Lett. 589, 3175–3181 (2015).
17. Cao, W. J., Wu, H. L., He, B. S., Zhang, Y. S. & Zhang, Z. Y. Analysis of long non-coding RNA expression profiles in gastric cancer. World J. Gastroenterol. 19, 3658–3664 (2006).
18. Zhang, Q. et al. Down-regulation of long non-coding RNA TUG1 inhibits osteosarcoma cell proliferation and promotes apoptosis. Asian Pac. J. Cancer Prev. 14, 2311–2315 (2013).
19. Zhang, E. B. et al. P53-regulated long non-coding RNA TUG1 affects cell proliferation in human non-small cell lung cancer, partly through epigenetically regulating DNMT1 expression. Cell Death Dis. 5, e1243 (2014).
20. Miura, Y. et al. Cyclic RGD-linked polymeric micelles for targeted delivery of platinum anticancer drugs to glioblastoma through the blood-brain tumor barrier. ACS nano 7, 8583–8592 (2013).
21. Magee, J. A., Piskounova, E. & Morrison, S. J. Cancer stem cells: impact, heterogeneity, and uncertainty. Cancer Cell 21, 283–296 (2012).
22. Natsume, A., Kondo, Y. & Wakabayashi, T. [IV. Epigenetic analysis for stem cell of brain cancer and treatment]. Gan To Kagaku Ryoho 40, 723–726 (2013).
23. Kopan, R. & Ilagan, M. X. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 137, 216–233 (2009).
24. Matheczek, A. M. & Wasserman, W. H. The next generation of transcription factor binding site prediction. PLoS Comput. Biol. 9, e1003214 (2013).
25. Zhu, T. S. et al. Endothelial cells create a stem cell niche in glioblastoma by providing NOTCH ligands that nurture self-renewal of cancer stem-like cells. Cancer Res. 71, 6061–6072 (2011).
26. Shih, A. H. & Holland, E. C. Notch signaling enhances nestin expression in gliomas. Neoplasia 8, 1072–1082 (2006).
27. Kawaguchi, A. et al. Nestin-EGFP transgenic mice: visualization of the self-renewal and multipotency of CNS stem cells. Mol. Cell Neurosci. 17, 259–273 (2001).
28. Maga, J. B., Ye, K. & Patel, D. J. Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. Mol. Cell 6, 299–309 (2013).
29. Shih, A. H. & Holland, E. C. Notch signaling enhances nestin expression in gliomas. Neoplasia 8, 1072–1082 (2006).
30. Wang, Y. et al. Endogenous miRNA sponge lincRNA-RoR regulates Oct4, Nanog, and Sox2 in human embryonic stem cell self-renewal. Dev. Cell 25, 69–80 (2013).
31. Whitfield, T. W. et al. Functional analysis of transcription factor binding sites in human promoters. Genome Biol. 13, R50 (2012).
32. Li, Z., Wang, H., Eyler, C. E., Hjelmeland, A. B. & Rich, J. N. Turning cancer stem cells inside out: an exploration of glioma stem cell signaling pathways. J. Biol. Chem. 284, 16705–16709 (2009).
33. Teodorczyk, M. & Schmidt, M. H. Notching on cancer’s door: Notch signaling in brain tumors. Front. Oncol. 4, 341 (2014).
34. Ma, K. et al. Long noncoding RNA MALAT1 associates with the malignant status and poor prognosis in glioma. Tumour Biol. 36, 3355–3359 (2015).
35. Zhen, L., Yun-Hui, L., Hong-Yu, D., Jun, M. & Yi-Long, Y. Long noncoding RNA NEAT1 promotes glioma pathogenesis by regulating miR-449b-5p/c-Met axis. Tumour Biol. 37, 673–683 (2016).
36. Quinn, J. I. & Chang, H. Y. Unique features of long non-coding RNA biogenesis and function. Nat. Rev. Genet. 17, 47–62 (2016).
37. Xu, N., Papagiannakopoulos, T., Pan, G., Thomson, J. A. & Kosik, K. S. MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. Cell 137, 647–658 (2009).
38. Palomero, T. et al. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. Proc. Natl Acad. Sci. USA 103, 18261–18266 (2006).
39. Lee, H. K. et al. MicroRNA-145 is downregulated in glial tumors and regulates glioma cell migration by targeting connective tissue growth factor. PLoS ONE 8, e54652 (2013).
40. Garofalo, S. et al. Enriched environment reduces glioma growth through immune and non-immune mechanisms in mice. Nat. Commun. 6, 6623 (2015).
41. Jeon, Y. & Lee, J. T. YY1 tethers Xist RNA to the inactive X nucleus center. Cell 146, 119–133 (2011).
42. Ulitsky, I. & Bartel, D. P. lincRNAs: genomics, evolution, and mechanisms. Cell 154, 26–46 (2013).
43. Liu, Y. & Lu, W. Recent advances in brain tumor-targeted nano-drug delivery systems. Expert Opin. Drug Deliv. 9, 671–686 (2012).
44. Ningaraj, N. S., Rao, M., Hashizume, K., Asotra, K. & Black, K. L. Regulation of blood-brain tumor barrier permeability by calcium-activated potassium channels. J. Pharmacol. Exp. Ther. 301, 838–851 (2002).
45. Desgrosellier, J. S. & Cheresh, D. A. Integrins in cancer: biological implications and therapeutic opportunities. Nat. Rev. Cancer 10, 9–22 (2010).
46. Lee, J. et al. Stem cell derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 9, 391–403 (2006).
47. Takebe, N., Harris, P. J., Warren, R. Q. & Ivy, S. P. Targeting cancer stem cells by inhibiting Wnt, Notch, and Hedgehog pathways. Nat. Rev. Clin. Oncol. 8, 97–106 (2011).
48. Wang, L. et al. Whole-exome sequencing of human pancreatic cancers and characterization of genomic instability caused by MLH1 haploinsufficiency and complete deficiency. Genome Res. 22, 208–219 (2012).
49. Hayashi-Takanaka, Y. et al. Tracking epigenetic histone modifications in single cells using Fab-based live endogenous modification labeling. Nucleic Acids Res. 39, 6475–6488 (2011).
50. Chu, C., Qu, K., Zhong, F. L., Artandi, S. E. & Chang, H. Y. Genomic maps of long noncoding RNA occupancy reveal principles of RNA-chromatin interactions. Mol. Cell 44, 667–678 (2011).

Acknowledgements
This research was supported by the Project for Development of Innovative Research on Cancer Therapeutics (P-DIRECT) and the Project for Cancer Research and Therapeutics Evolution (P-CREATE) from Japan Agency for Medical Research and development, AMED (Y. Kondo), by the PRESTO, JST (Y. Kondo), and by the Grant-in-Aid for Scientific Research, the Japan Society for the Promotion of Science (25290048, Y. Kondo).

Author contributions
Conception and design: K.K., Y.K.; development of methodology: K.K., F.O., H.K., Y.K.; acquisition of data: A.N., K.S., S.S., S.T., N.I.; analysis and interpretation of data: T.S., Y.T., K.K., A.H., Y.K.; writing, review and/or revision of the manuscript: K.K., Y.K.; administrative, technical, or material support: H.K., M.N., H.I.K., K.M., K.K., Y.K.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Katsushima, K. et al. Targeting the Notch-regulated non-coding RNA TUG1 for glioma treatment. Nat. Commun. 7, 13616 doi: 10.1038/ncomms13616 (2016).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.