C1q–tumour necrosis factor-related protein 8 (CTRP8) is a novel interaction partner of relaxin receptor RXFP1 in human brain cancer cells

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Abstract

We report a novel ligand–receptor system composed of the leucine-rich G-protein-coupled relaxin receptor, RXFP1, and the C1q–tumour necrosis factor-related protein 8 (CTRP8) in human primary brain cancer, a tumour entity devoid of the classical RXFP1 ligands, RLN1–3. In structural homology studies and computational docking experiments we delineated the N-terminal region of the globular C1q region of CTRP8 and the leucine-rich repeat units 7 and 8 of RXFP1 to mediate this new ligand–receptor interaction. CTRP8 secreted from HEK293T cells, recombinant human (rh) CTRP8, and short synthetic peptides derived from the C1q globular domain of human CTRP8 caused the activation of RXFP1 as determined by elevated intracellular cAMP levels and the induction of a marked pro-migratory phenotype in established glioblastoma (GB) cell lines and primary cells from GB patients. Employing a small competitor peptide, we were able to disrupt the CTRP8–RXFP1–induced increased GB motility. The CTRP8–RXFP1-mediated migration in GB cells involves the activation of PI3K and specific protein kinase C pathways and the increased production/secretion of the potent lysosomal protease cathepsin B (cathB), a known prognostic marker of GB. Specific inhibition of CTRP8–induced cathB activity effectively blocked the ability of primary GB to invade laminin matrices. Finally, co-immunoprecipitation studies revealed the direct interaction of human CTRP8 with RXFP1. Our results support a therapeutic approach in GB aimed at targeting multiple steps of the CTRP8–RXFP1 signalling pathway by a combined inhibitor and peptide-based strategy to block GB dissemination within the brain.

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Introduction

The heterodimeric peptide hormone relaxin binds to the G-protein-coupled receptor RXFP1 and is up-regulated in cancer [1,2]. The main human relaxin isoform, RLN2, can promote tumour progression in a tumour-specific context by enhancing tumour cell proliferation, tissue invasion, and tumour angiogenesis [3–5], and inhibiting apoptosis [6]. RLN2-mediated RXFP1 activation contributes to tissue invasion and metastasis of tumour cells in several ways. This involves the up-regulation of the motility-enhancing calcium-binding protein S100A4, also known as metastatin or Mts1 [7], and proteolytic effector pathways, including
the matrix-metalloproteinases (MMPs) collagenase 1 (MMP1) and 3 (MMP13), gelatinase A (MMP2) and B (MMP9), stromelysin 1 (MMP3), MT1-MMP (MMP14), and tissue inhibitors of MMP (TIMP3) in human breast and thyroid cancer [8,9]. Among the family of lysosomal hydrolases of the cathepsin (cath) family, we identified cathB, cathL, and cathD as important relaxin targets that facilitate extracellular matrix (ECM) degradation by human thyroid cancer cells [1,3]. Targeted down-regulation of RXFP1 by siRNA-coated nanoparticles in xenograft tumours of the human prostate cancer cell line PC3 caused a dramatic reduction in growth, metastasis rate, and altered expression profiles of genes associated with tumourigenesis [10].

The currently known 15 members of the family of C1q-tumour necrosis factor-related proteins (CTRPs) share a common structure composed of an N-terminal signal peptide, variable region, collagen domain, and C-terminal C1q globular domain [11]. Accumulating evidence positively correlates CTRP members with carcinogenesis and/or signalling pathways associated with cancer development/progression and metastasis. CTRP3/cartducin can activate ERK1/2 signalling, which contributes to osteosarcoma tumour growth [12]. CTRP4 increases tumour cell resistance to apoptosis [13], whereas CTRP6 expressed in hepatocellular carcinoma induces AKT and ERK phosphorylation and accelerates tumour neovascularization [14]. CTRP1, an oligomerization partner of CTRP6, also causes AKT, ERK activation, and CTRP9 contributes to endothelial-dependent AMPK/AKT/eNOS phosphorylation and NO production [15,16]. CTRP8 is a recent and most elusive member of the CTRP family [17]. Highly conserved throughout evolution, CTRP8 is secreted as a homotrimer but can form heterooligomeric complexes with C1q-related factor (CRF).

Here, we provide evidence for a new role of human CTRP8 as a novel interaction partner of RXFP1 and identify an important role of CTRP8 in promoting invasiveness of human primary GB cells derived from GB patients. We identified CTRP8–RXFP1-induced downstream signalling pathways and the cathepsin B (cathB) lysosomal protease as an important executioner of ECM invasion in human GB. Our data provide a novel insight into the unique biological roles of RXFP1 in primary malignant brain cancer and allude to new therapeutic strategies to curb RXFP1-mediated GB tissue invasion.

Materials and methods

Human tissues and primary glioma cell isolation

This study was approved by the ethical committees at the University of Manitoba and the Department of Pathology, Faculty of Medicine (ethics approval # H2010:116). All patients gave written consent prior to the collection of glioblastoma (GB) brain cancer tissues by surgical resection for clinical indications. The isolation of primary GB cells is described in the Supplementary materials and methods.

Cell culture

Two human primary GB cell populations (designated PBS-1 and PBS-10) and human brain cancer cell lines LN-18, LN-229, U251, A-172, U373, U87MG, T98G, and the human cervical cancer cell line HeLa were propagated in DME/F12 medium plus 10% FBS (HyClone). Human primary astrocytes were cultured in astrocyte-specific medium (both Sciencell, CA, USA). HEK 293T parental cells, HEK293T-RXFP1-hemagglutinin (HA; generously provided by Drs A Kern and G Bryant-Greenwood, USA) [18], HEK293T-RXFP2-HA (generously provided by Dr AI Agoulnik, USA), and HEK 293T-CTRP8 transfectants were grown in IMDM medium (HyClone) and 10% FBS. Transient transfectants of HEK293T harbouring pcDNA3.1/mycHis empty plasmid control (mock) or pcDNA3.1/mycHis containing a construct encoding FLAG-tagged full-length human (h) CTRP8 were grown in IMDM/1% FBS. Transient transfections were performed as previously described [3]. Culture supernatants were collected 24 h later and split to be (i) used directly as conditioned media to stimulate RXFP1 activation for motility assays and the analysis of signalling pathway responses or (ii) lyophilized and concentrated five-fold for FLAG-hCTRP8 detection (see below). In addition, cellular protein lysates were harvested for western blot detection of FLAG-hCTRP8.

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

Isolation of total RNA, cDNA preparations, and RT-PCR were performed as described previously [3]. CTRP8 transcripts were amplified using the Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Ontario, Canada) plus 3% DMSOS as described previously [17] (Supplementary Table 1).

RNA silencing

Primary glioma cell lines at 60% confluence were transfected with 100 nM siRNA targeting RXFP1 (5’GGAGAUAUAAAGAUUGAACAtt3’) or non-silencing randomized si-control (5’AAATTCGCCAAGTGTCACTGT3’) (both Ambion, Ontario, Canada) using SiLentFect (BioRad, Ontario, Canada). After 24 h of incubation, RXFP1 knockdown was assessed by RT-PCR and western blot.

Immunohistochemistry

Immunohistochemistry was performed on deparaffinized human GB tissue sections. Details may be found in the Supplementary materials and methods.
Immunoprecipitation

HEK293T parental cells and HEK293T stable transfectants expressing HA-tagged RXFP1 (HEK293T-RXFP1-HA) were incubated for 60 min at room temperature with CTRP8-His recombinant protein (100 ng/ml) prior to immunoprecipitation (IP; see Supplementary materials and methods for the IP procedure).

Western blot analysis and peptides

Cells at 80% confluency were harvested in 100 µl of protein extraction buffer [1 mM Tris (pH 6.8), 2% SDS, 1% glycerol, bromophenol blue (Sigma, St Louis, MO, USA)] as described previously [8,19] and in the Supplementary materials and methods. Recombinant peptides (> 95% purity; EZ Biolabs, IN, USA) were reconstituted in DMSO prior to use. The amino acid (aa) sequences of P59 and P74 have been described elsewhere [20]. For other peptides see the Supplementary materials and methods.

Incubation with specific PKC inhibitors

Cells (80 000 cells per well) were seeded in six-well plates for protein extraction and in 96 wells at 5000 cells per well for WST assays. Cells were incubated with 1% FCS-containing medium before commencing inhibitor treatments. Cells were pre-incubated for 60 min with the PI3-kinase (PI3K) inhibitor wortmannin (80 nM), the general PKC inhibitor tamoxifen (1 µM), the PKCδ inhibitor rottlerin (2.5 µM), and the specific PKζ inhibitor PKCζ113–125 myristoylated peptide (2.5 µM) (Enzo Life Science, NY, USA) or the specific cathB inhibitor CA074 (10 µM; Sigma) prior to treatment with recombinant human rhRLN2 (100 ng/ml; Phoenix Pharmaceuticals, CA, USA), synthetic peptides P74 and P59 (both 3 µM), rhCTRP8 (100 ng/ml; Creative BioMart, NY, USA) or supernatant from transient HEK293T transfections with pcDNA3.1-hCTRP8. Toxicity of the inhibitors was analysed using WST (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate) assay (Roche, Ontario, Canada). WST is a colorimetric assay for the quantification of cell proliferation and cell viability based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells.

Cell motility and competitive laminin migration assays

Motility and laminin migration assays have been described previously [3,8]. Details may be found in the Supplementary materials and methods. Mean numbers of migrated cells were determined in five separate filter areas at ten-fold magnification by bright field microscopy. All experiments were performed at least in triplicates, with every experiment consisting of three filters.

cAMP assay

Measurements of intracellular cAMP were carried out as described previously [3,21] (see Supplementary materials and methods).

Computational modelling for RXFP1 and CTRP8 and docking studies of their complexes

High-resolution structures homologous for the leucine-rich repeat (LRR) of human RXFP1 and the C-terminal domain of human CTRP8 were calculated.
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Figure 2. Immunohistochemical detection of (A) immunoreactive RXFP1 and (C) CTRP8 in human GB tissues. Specific cellular immunostaining was observed for both, RXFP1 and CTRP8, suggesting an autocrine/paracrine ligand–receptor system in GB cells. (B, D) When the primary antibody was replaced by the respective rabbit (B) and goat (D) isotype control IgG used as a negative control, no immunostaining was observed. Original magnifications: [A–D] × 630.

by SWISS-MODEL using the crystal structures of LRIM (pdb code: 3OJA; sequence identity: 35.8%) and the NC1 domain of collagen VIII (pdb code: 1O91; sequence identity: 35.2%) as templates [22,23]. Rigorous quality assessments and energy minimizations were performed as described earlier [24]. Docking of the human RXFP1 extracellular domain with globular human CTRP8 was performed using FTDOCK [25]. The lowest energy docked structures were subject to 500 cycles of unrestrained Powell minimization using CNS. Harmonic restraints were imposed on both the RXFP1 receptor and the CTRP8 ligand atoms (2 kcal/mol Å²) with increased weight (20 kcal/mol Å²). Final docking results were visualized using the program DINO.

Statistics
All experiments were repeated at least three times. For motility and migration assays, the mean value with standard error and independent two-tailed t-test was performed, with p < 0.05 being considered significant. For multiple experiment comparison, ANOVA table and Tukey’s HSD test were used, with p < 0.05 regarded as significant.

Results
Human GB cells express the novel RXFP1-interacting partner CTRP8
RXFP1 transcripts were detected by RT-PCR in 17 of 18 primary GB cases of our brain cancer cell repository and in four of seven human GB cell lines (Figure 1A). The expression of RXFP1 in primary GB cells showed no apparent correlation with clinical data. Normal human astrocytes were weakly positive for RXFP1 (Figure 1A). Established human GB cell lines, primary GB, and astrocytes were devoid of transcripts for the relaxin isoforms RLN1–3; two previously described RLN2 transcripts were weakly detected after 40 PCR cycles in three of seven GB cell lines but in none of the primary GB cells tested (Supplementary Figure 1) [26]. We detected the expression of CTRP8 in all but one (95%) primary GB, in all GB lines, and in normal astrocytes (Figure 1A). The CTRP8 hetero-oligomerization partner C1q-related factor (CRF) was not expressed in any of the cells investigated (data not shown) [17]. Immunoreactive RXFP1 (Figure 2A) and CTRP8 (Figure 2C) were detected in human GB tissues. The small peptides, P74 and P59, are homologous to the C1q globular domain of human CTRP8 and were previously shown
Figure 4. Representative results of motility assays with the human GB cell lines U87MG (A) and T98G (B) and RXFP1+ human GB cells isolated from a GB patient (C). These brain cancer cell types responded with significantly increased motility when exposed to secreted human (sh) CTRP8 from concentrated supernatants of HEK293T cells transiently transfected with an expression construct for human CTRP8 (400 ng/ml), the CTRP8-like peptides P59 and P74 (3 µM each), and rhRLN2 (100 ng/ml). Culture medium, concentrated supernatant of untransfected HEK293T, and the control peptide (10 nM) failed to cause any change in the motility of U87MG, T98G or primary GB cells. The presence of a functional RXFP1 was critical for the motility-enhancing effect of CTRP8 and RLN2. (C) Specific knockdown of RXFP1 in primary RXFP1+ human GB (shown in E) abolished the motility response to shCTRP8, P59/P74, and rhRLN2. (D) RXFP1− primary GB cells were non-responsive to the identical treatments. Independent two-tailed t-test was performed to determine significance: *p < 0.001; **p < 0.002; ***p < 0.004.

to cause increased cAMP levels in CHO-K1 transiently transfected with a human RXFP1 expression construct [20,27]. We transiently expressed a human CTRP8 expression construct in HEK293T cells and confirmed our previous result that HEK293T transfec-tants secreted FLAG-tagged CTRP8 into the culture medium (Figure 3A) [17]. Upon transfection, primary GB cells were also able to secrete FLAG-CTRP8 (data not shown). Similar to recombinant human (rh) RLN2 used as a positive control, secreted human CTRP8 (shCTRP8) and rhCTRP8 were biologically active and caused a significant increase in intracellular cAMP levels in RXFP1+ primary GB cells (Figure 3B). Similar to rhRLN2 used as a positive control, rhCTRP8 induced cAMP in RXFP1 transfectants of HEK293T cells (Supplementary Figure 2B) but failed to cause a cAMP induction in RXFP2 transfectants of HEK293T cells (Supplementary Figure 2C). HEK293T-RXFP2- HA transfectants responded with elevated cAMP levels to rhINSL3 used as a positive control, showing functionality of the RXFP2 receptor in these transfectants.

In contrast, parental HEK293T cells (Supplementary Figure 2A) and U251 cells (Supplementary Figure 2D), both negative for RXFP1 and RXFP2, did not respond to rhCTRP8 with elevated cAMP levels, demonstrating its specific action on RXFP1.

CTRP8 enhances the motility of GB cells

We performed transwell filter assays on U87MG and T98G RXFP1+ human GB cell lines and RXFP1+ human primary GB cells from two patients to determine the effect of CTRP8 and the linear peptides P59 and P74 on GB cell motility and compared these results with those obtained from RXFP1+ U251 GB cells (Figure 1A) and one primary RXFP1− GB cell line (Figure 1B) used as negative controls. HEK293T-derived shCTRP8 was as effective as P74/P59 and rhRLN2 in enhancing the motility of RXFP1+ U87MG (Figure 4A), T98G (Figure 4B), and human primary GB cells (Figure 4C). This motility-enhancing response was critically dependent on the presence...
using a affect GB cell motility. Significance of the experiments was tested (3 and 15 absence of increasing concentrations of the competitory peptide were stimulated with rhCTRP8 (100 ng/ml) in the presence and of motility competition assays with PBS1 primary GB cells. GB cells the CTRP8-induced motility increase. (C) Representative example

Figure 5. (A) Combined result of a dose-dependent motility increase of RXFP1-expressing primary GB cells (PBS1) in response to increasing concentrations of rhCTRP8 (1, 10, 100 ng/ml). The motility-enhancing effect of CTRP8 was significant at 10 ng/ml rhCTRP8 and abolished when RXFP1 transcripts were knocked down in the presence of siRXFP1. Similar results were obtained for primary GB PBS10. (B) The control peptide was unable to increase cell migration in RXFP1+ primary GB cells and did not prevent the CTRP8-induced motility increase. (C) Representative example of motility competition assays with PBS1 primary GB cells. GB cells were stimulated with rhCTRP8 (100 ng/ml) in the presence and absence of increasing concentrations of the competitor peptide (3 and 15 µM). At both concentrations employed, the competitor peptide abolished the motility-enhancing action of CTRP8 in a dose-dependent manner. Treatment of these GB cells with the competitor peptide alone at 3 and 15 µM or medium failed to affect GB cell motility. Significance of the experiments was tested using a t-test: *p < 0.01; **p < 0.001.

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Figure 6. (A) The significant increase in GB motility upon treatment with rhCTRP8, CTRP8-like peptide P74, and rhRLN2 was completely abolished in the presence of the PI3K inhibitor wortmannin (80 nM) and the general PKC inhibitor tamoxifen (1 µM). At the concentrations used in this assay, neither tamoxifen (B) nor wortmannin (C) was toxic to the primary GB cells, as determined by WST cell toxicity assays. Significance of the experiments was tested using a t-test: *p < 0.001.

of RXFP1. Specific siRNA-mediated knockdown of RXFP1 (Figure 4E) abolished the increase in GB motility, as observed for shCTRP8, rhRLN2, and peptides (Figure 4C). Primary GB cells devoid of RXFP1 were non-responsive to the treatment with shCTRP8, rhRLN2, and peptides, and failed to show increased motility (Figure 4D). When exposed to increasing concentrations of rhCTRP8, we observed a dose-dependent increase in motility which was absent upon specific RXFP1 knockdown in primary GB cells (Figure 5A). The control peptide used in this study neither increased cell motility nor inhibited rhCTRP8-induced cell motility (Figure 5B). The competitor peptide was unable to induce cell motility in RXFP1+ GB cells but prevented rhCTRP8-induced cell motility (Figure 5C), suggesting a new peptide-based approach to inhibit RXFP1-mediated cancer cell motility.

CTRP8 and RLN2 activate the PI3K–PKC pathway in GB

Both the phosphoinositol-3-kinase (PI3K) inhibitor wortmannin and the general protein kinase C (PKC) inhibitor tamoxifen were effective in suppressing GB cell migration in response to shCTRP8, P74, and rhRLN2, with the latter used as a positive control.
Figure 7. (A) Representative western blot analysis of total and phosphorylated (p) PKCδ and PKCζ/λ in RXFP1+ primary GB cells. Treatment with rhCTRP8 (100 ng/ml), P59 and P74 (3 μM each), and rhRLN2 (100 ng/ml) resulted in a marked increase in pPKCδ and pPKCζ/λ, which was absent when cells were treated with normal culture medium and the control peptide (3 μM). We failed to detect a change in phosphorylation status for PKCα/β (data not shown). Pretreatment of cells for 1 h with the PI3K inhibitor wortmannin (80 nM) resulted in a marked reduction in the levels of both pPKCδ and pPKCζ/λ whereas the PKCδ inhibitor rottlerin and the pPKCζ inhibitor PKCζ113-125 myristoylated peptide (both 2.5 μM) specifically blocked phosphorylation of their respective PKC isoforms only. Total cellular levels of PKCδ and PKCζ remained unchanged upon treatment with rhCTRP8, P59/P74, and rhRLN2. Beta-actin was used as a loading control. (B) When HEK293T-RXFP1-HA stable transfectants, but not HEK293T parental or HEK293T-RXFP2-HA transfectants, also responded to rhCTRP8 and rhRLN2 (100 ng/ml each) with strong phosphorylation of PKCζ without any effect on total cellular PKCζ levels (Figure 7B). These results suggested that under the specific conditions used, RXFP1 mediated signalling without affecting the total PKCζ and PKCδ protein level. Beta-actin was used as a loading control.
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Significance was tested using ANOVA with * assay experiments with three filter sets per experiment are shown. Results of three independent motility and migration and were non-toxic to the cells, as determined by WST assays (data not shown). Results of three independent motility and migration and were non-toxic to the cells, as determined by WST assays (data not shown).

Figure 8. Exposure of primary GB to rhCTRP8, P59/P74, and rhRLN2 caused a significant increase in GB cell motility, as shown here for PBS1. This increased GB motility was abolished in the presence of the PKCζ inhibitor rottlerin (A) and the pPKCζ inhibitor PKCζ113–125 myristoylated peptide (B) (both at 2.5 µM). These results and our previous data demonstrating the inhibitory effect of the PI3K inhibitor wortmannin on GB motility suggested that RXFP1 activation by CTRP8 and RLN2 resulted in the activation of PI3K–PKC signalling pathways in primary GB. The specific PKCζ and PKCζ inhibitors alone failed to have any effect on cell motility and were non-toxic to the cells, as determined by WST assays (data not shown). Results of three independent motility and migration assay experiments with three filter sets per experiment are shown. Significance was tested using ANOVA with * * $p < 0.001$, ** $p < 0.002$, and *** $p < 0.004$.

either PKCζ myristoylated peptide113–125 (Figure 8A) or rottlerin (Figure 8B) blocked the agonist-induced increase in motility of primary GB cells.

CTRP8 and RLN2 induce the production and secretion of cathepsin B (cathB)

RLN2 and the relaxin-like peptide INSL3 increase the expression/secretion of cathepsins to facilitate ECM invasion of human thyroid cancer cells [3,28]. Here, we identified the RXFP1-interacting molecules CTRP8, P59, P74, and RLN2 as strong inducers of cathB; demonstrated that this effect was dependent on the presence of a functional RXFP1 receptor in primary GB cells (Figures 9A and 9B) and in HeLa cells (Figures 9C and 9D) and showed that the presence of these RXFP1-interacting partners caused increased secretion of cathB in primary GB (Figure 10A). The CTRP8/RLN2-mediated increase in cathB production was also observed in HeLa cells with endogenous expression of RXFP1. When RXFP1 was knocked down with siRXFP1 and RXFP1 protein (Figure 9C) and transcripts (Figure 9D) were depleted, rhCTRP8 and rhRLN2 were unable to induce cathB protein above baseline levels in these cervical cancer cells. The RXFP1-mediated increased production and secretion of cathB in primary GB cells was dependent on a functional PI3K–PKC signalling cascade and blocked in the presence of the PI3K inhibitor wortmannin and the PKCζ and PKCζ inhibitors PKCζ myristoylated peptide113–125 and rottlerin, respectively (Figure 10A). To determine the functional consequences of the RXFP1 agonist-induced cathB secretion on the ability of GB cells to penetrate the extracellular matrix, we performed laminin migration assays. Laminins are major structural components of basement membranes (BM) brain tissue and cathB degrades laminin [29,30]. Laminin migration assays revealed significantly enhanced invasion of those primary GB cells exposed to rhCTRP8, P59 and P74, and rhRLN2 when compared with controls treated with either medium or control peptide. The laminin invasion was dependent on active cathB and blocked in the presence of the specific cathB inhibitor CA074 (Figure 10B).

RXFP1 interacts with CTRP8

The P74 peptide contains a duplication of the amino acid (aa) motif ‘RRYAAFSVG’ at its C-terminus that is absent in P59 [19]. This aa motif constitutes part of the N-terminal C1q globular domain of CTRP8 and, similar to previous reports [20,27], its duplication in P74 resulted in biological responses that were superior to P59 and similar to RLN2 in GB, whereas a control peptide was inactive. Employing homology modelling and algorithms for protein interaction simulations, our structural models of the human RXFP1 leucine-rich extracellular (LRR) region (Figure 11A) and C1q globular domain of CTRP8 (Figure 11B) predicted an interaction of the LRR 7–8...
tandem (Trp<sup>229</sup>) of RXFP1 with the conserved serine residue in the sequence motif ‘YAAFSVG’ of the C1q globular domain of CTRP8 (Figures 11C and 11D). In addition, the terminal tyrosine side chain is in van der Waals contacts with the edge LRR 11, providing additional contact points. Overall, the burying surface area between both molecules spans over 584Å<sup>2</sup>. Next, we employed co-immunoprecipitation (IP) studies to study the interaction between RXFP1 and CTRP8 in HEK-RXFP1-HA stable transfectants. Upon incubation of HEK-RXFP1 with recombinant His-tagged CTRP8 (Creative BioMart), HA-targeted co-IP and western blot analysis revealed the successful pull down of both HA-RXFP1 and CTRP8-His (Figure 12). Similar results were obtained when HEK293T-RXFP1-HA transfectants were transiently transfected with a construct encoding human FLAG-tagged CTRP8 followed by HA-targeted immunoprecipitation and western blot detection of HA and FLAG epitopes (data not shown). Predicted structural and co-IP data as well as the results from our signalling studies suggest a common mechanism of action for CTRP8, CTRP8-derived peptides P74/P59, and RLN2 in primary GB. In our proposed model, RXFP1 agonist-induced PI3K–PKC activation contributed to increased cathB secretion, which resulted in the enhanced migration of GB cells and ECM degradation. We identified the interaction between CTRP8 and RXFP1 as a novel signalling system in GB invasion (Figure 13).

**Discussion**

We demonstrate the presence of functional RXFP1 in established human brain cancer cell lines and in aggressive and incurable GB brain cancer cells isolated from GB patients. GB was devoid of the classical RXFP1 ligands RLN1–3 [31] but co-expressed a new member of the C1q-TNF-related peptide family, CTRP8 [17]. Algorithmic predictions had identified two linear peptides (P59 and P74) that interact with RXFP1 [17,20,27] and both peptides showed high sequence homology with the collagen and C1q domain of CTRP8 [17]. Both peptides and hCTRP8 were capable of activating RXFP1 in human GB cells, suggesting that CTRP8 may serve as a novel interaction partner of the RXFP1 in human GB cells. Successful co-IP of RXFP1 with CTRP8 identified (i) CTRP8 as a novel interacting partner of RXFP1 and (ii) RXFP1 as a first receptor for a member of the CTRP family. CTRP8 elicited signalling events in the presence of a functional RXFP1 in RXFP1-expressing human GB, HEK293T, and HeLa cells. RXFP2 failed to elicit a similar PKC response at concentrations of rhCTRP8 or
An independent two-tailed t-test was performed to test for significance: *p < 0.001, **p < 0.002. Equal loading of cell lysates was confirmed using beta-actin as a loading control. Supernatant protein concentrations were determined using the Bradford assay and 30 ng was loaded per lane.

Figure 10. (A) Representative western blot demonstrating increased production of single-chain cathB (30 kDa) and two-chain cathB (30 and 25 kDa) and the secretion of cathB into the culture medium upon treatment with rhCTRP8, P59/P74, and rhRLN2. The control peptide and normal culture medium had no effect on cathB production and/or secretion. However, in the presence of the PKCζ inhibitor rottlerin and the PKCζ inhibitor PKCζ113–125 myristoylated peptide, treatment of primary GB cells with the RXFP1 agonists failed to cause an increase in cathB secretion. Furthermore, the PI3K inhibitor wortmannin and the specific PKCζ and PKCζ inhibitors completely abolished the increase in cathB production in primary GB upon treatment with RXFP1 agonist. (B) We performed laminin-coated filter-based migration assays in the presence and absence of the specific cathB inhibitor CA074 (10 μM) to assess the potential impact of increased production and secretion of cathB for GB invasiveness. Consistent with the western blot results on cathB production/secrection, CA074 abolished the rhCTRP8, CTRP8-like peptide, and rhRLN2-induced invasion of GB cells through the laminin matrix. Thus, we identified cathB as an important mediator of tissue invasion by CTRP8 and RLN2 in primary GB cells through the laminin matrix. The CTRP8–RXFP1 interaction contributed to tumour cell migration in GB. RXFP1 and CTRP8 were co-expressed in the majority of human primary GB cells and established human brain cancer cell lines, implicating this novel ligand–receptor system in autocrine/paracrine functions as proposed previously for RLN2–RXFP1 in tumour tissues. This was also supported by our detection of CTRP8+ and RXFP1+ cell populations within GB tissues; the ability of cultured GB cells to secrete CTRP8; and the fact that, like RLN2, CTRP8 caused a significant and dose-dependent increase in the motility of primary GB cells at CTRP8 concentrations as low as 10 ng/ml.

CTRP members engage in homotrimer formation but also form hetero-oligomers with other CTRP family members [11,16,34–36]. We identified C1q-related factor (CRF) as the only currently known CTRP hetero-oligomerization partner [17]. However, HEK293T-CTRP8 transfectants and all human GB cells studied were devoid of CRF. Recombinant hCTRP8 was sufficient to interact with and activate RXFP1 expressed by GB cells. Glycosylating post-translational modifications were likely not essential for the interaction with RXFP1 since HEK-secreted shCTRP8 and E. coli-produced (no glycosylation) recombinant hCTRP8 were both equally capable of eliciting RXFP1 responses. Our modelling studies predicted an interaction of the extracellular human RXFP1 leucine-rich repeat (LRR) units 7 and 8 with the aa motif ‘YAAFSVG’ present in the C1q globular domain of CTRP8 and P74/P59. A peptide with alterations to this aa motif failed to elicit GB responses but, instead, successfully competed with and attenuated P74/P59-induced GB migration.

A hallmark of primary brain cancer is the extensive glioma cell dissemination within interstitial brain tissue [37–40]. Multimeric collagens (I, III, IV), laminins, proteoglycans, and nidogen/entactin are present in the extracellular matrix (ECM) of cerebral vasculature and basement membranes [37,40,41] and facilitate glioma cell motility and tissue invasion. GB cells actively modify the ECM composition by proteolysis in favour of pro-invasive cell–ECM interactions [42–44]. RXFP1 promotes an ECM remodelling pro-migratory phenotype in tumour cells. We and others have previously identified matrix metalloproteinase family members [8,45], metastasis-promoting S100A4 [7,46], and cathepsin lysosomal hydrolases [3,28] as important executioners of RXFP1-mediated migration. Intra-tumoural suppression of RXFP1 expression by siRNA-RXFP1-coated nanoparticles was shown to reduce tumour size and metastasis rates of human PC3 prostate cancer xenografts [10].

Similar to human RLN2 [28], CTRP8 and derived peptides caused the enhanced production and secretion...
A LRR domains of RXFP1

B Clq globular domain of CTRP8

C RXFP1:CTRP8 complex

D RXFP1:CTRP8 complex

Figure 11. (A) The putative binding regions of the human receptor RXFP1-LRR domain (boxed) and (B) the human CTRP8 C1q globular domain are shown in ribbon presentation with a spectrum colour scheme (beginning with the N-terminus). Both structures were modelled with the Protein model Portal software using high-resolution crystal structures as templates: for RXFP1-LRR, we used the LRIM1 (pdb code: 3OJA) [21] and for CTRP8, the collagen VIII NC1 domain (pdb code: 1091A) [22]. (C) The modelled interaction between RXFP1-LRR (surface presentation) and CTRP8 is shown. The docking algorithm used full-length versions of both proteins. However, convincing results could be obtained only for the RXFP1-LRR : CTRP8-C1Q fragments forming a hetero-complex. (D) Side view of the hetero-complex RXFP1 : CTRP8. The RXFP1-LRR fragments 7 and 8 (shown as ribbons) were found to form a close interaction network with the YAAFSG segment of human CTRP8.

of cathB and increased migration through laminin by human primary GB cells. The absence of RXFP1 in RXFP1−/− GB cells or upon specific RXFP1 knockdown resulted in muted responses to CTRP8 and P74. The motility-enhancing effect of CTRP8 and P74 was dose-dependent and increasing concentrations of competitor peptide silenced CTRP8 function in primary GB. Much like RLN2 exhibits anti-fibrotic properties in different organs including the airways [47–49], the anti-fibrotic and anti-inflammatory properties of P74 were found to reduce bleomycin-induced fibrotic lung injury and airway remodelling in mice [50]. Here we introduce a small peptide effectively blocking the motility-enhancing action of CTRP8/P74 in human GB which may serve as a novel therapeutic tool to inhibit glioma cell dissemination within the brain.

CTRP8-induced RXFP1 signalling in human GB involved cAMP elevation, the activation of PI3 kinase, and the increased phosphorylation of PKCζ and PKCδ. In different cell models including cancer cells, relaxin induced cAMP levels with activation of PKA and the \( \text{G}_{\alpha3} \text{–G} \beta \gamma \text{–PI3K–PKC} \zeta \) pathway [33,51,52]. In cardiac myocytes, relaxin engages PKCζ to alter myofilament function and protein phosphorylation [53]. Specific inhibition of PI3K, Akt, ERK1/2, PKCζ, and the transcription factors Elk-1 and c-Fos blocked the induction of MMP9 by relaxin in fibrocartilaginous cells [54]. Similar to RLN2 in other cancer cells [3,28], treatment of human GB cells with human CTRP8, P74/P59, and rhRLN2 caused increased production of the GB prognostic marker cathB, and RXFP1–PI3K–PKCζ/PKCδ cascades were important signalling pathways in CTRP8–RXFP1-activated cathB production and invasiveness in human GB. The CTRP8-induced increase in cathB production was dependent on the presence of RXFP1 and attenuated in the presence of the PI3K inhibitor wortmannin and the specific PKCζ and PKCδ inhibitors rotterlin and PKCζ113–125 myristoylated peptide, respectively. The general PKC inhibitor tamoxifen [55] and the PI3K inhibitor wortmannin were effective in blocking the migratory phenotype in primary GB when exposed to CTRP8, P74 or rhRLN2. Intriguingly, cathB is required for the formation of functional...
CTRP8 is a novel interaction partner of RXFP1 in human brain cancer cells

Figure 12. Co-immunoprecipitation of RXFP1-HA and rhCTRP8-His. HEK293T and HEK293T-RXFP1-HA were incubated with His-tagged rhCTRP8 (100 ng/ml) for 60 min. The HA tag of RXFP1 was used for immunoprecipitation and representative western blot detection of RXFP1-HA and rhCTRP8-His is shown. Antibodies specific for HA and His identified both co-immunoprecipitated RXFP-HA and rhCTRP8-His exclusively in HEK293T-RXFP1-HA treated with rhCTRP8, but not in untreated HEK293T-RXFP1-HA or HEK293T parental cells. Similar amounts of protein were used for the IPs.

β1 integrin–PKCζ/PKCδ complexes promoting glioma migration [56]. This may explain why inhibition of either PKCζ or PKCδ abolished cell motility, although different functions of PKCζ in glioma migration and PKCδ in glioma motility should also be considered, as was recently reported for eosinophils [57]. The importance of the cathB catalytic activity for effective laminin invasion by GB cells was emphasized by the use of a specific cathB inhibitor. Intracellular levels of cathL and cathD, an enzyme processing pro-cathB to active cathB [29], remained unaltered upon CTRP8, rhRLN2, and P74 treatment in primary and established GB cells (Supplementary Figure 3). The fact that the clinically established drugs tamoxifen and wortmannin were effective in preventing the CTRP8–RXFP1-mediated increase in GB invasion in vitro suggests that already available treatment options, including the use of tamoxifen for GB, may effectively mitigate GB invasiveness.

Our ongoing investigations address the binding kinetics and residues involved in the CTRP8–RXFP1 interaction and explore signalling pathways activated by CTRP8–RXFP1 in biological systems other than brain cancer. Our finding that both, CTRP8 and relaxin, are interaction partners of RXFP1 opens new and exciting avenues of relaxin/RXFP1 and CTRP research and may provide new clinical leads to block glioma tissue invasion.

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**Author contribution statement**

TK and SHK conceived the experiments, analysed the data, searched the literature, and guided this research. Experiments were done by AG, UK, JS, TP, TT, and JK. TT, MDB, and JK collected brain cancer tissues, and SHK and TT isolated primary GB cells. EW, GWW, MDB, JK, and CHV provided expert advice throughout the experimental process. All authors were involved in the writing of the manuscript and had given final approval of this submitted version.

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**SUPPORTING INFORMATION ON THE INTERNET**

The following supporting information may be found in the online version of this article.

Supplementary materials and methods.

**Figure S1.** Representative RT-PCR results for RXFP2 and RLN1 + 2 in established and primary human glioma cells.

**Figure S2.** CAMP assays were performed using HEK293T parental cells (RXFP1 and RXFP2 negative, A), HEK293T-RXFP1-HA transfectants (B), HEK293T-RXFP2-HA (C), and the U251glioma cell line (RXFP1 and RXFP2 negative, D).

**Figure S3.** Representative western blot analysis of protein extracts of RXFP1 primary GB exposed to rhRLN2 or rhCTRP8, both at 100 ng/ml, and the peptides P59, P74, and control peptide (3 µM each) for the detection of cathD and cathL.

**Table S1.** List of PCR primers.