

MODULATING ENDOTHELIAL PERMEABILITY TO IMPROVE DRUG DELIVERY IN
GLIOBLASTOMA

By

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B.A., UNIVERSITY OF CALIFORNIA, BERKELEY, 2017

Thesis

Submitted in partial fulfillment of the requirements for the Degree of Master of Science in the
Graduate Program of Biotechnology at Brown University

PROVIDENCE, RHODE ISLAND

MAY 2023

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Acknowledgements

I would like to thank my PI, Dr. Sean Lawler, for taking me into his lab given my limited time for thesis research and being supportive throughout my time here. He always makes himself available to mentor and has incredibly wonderful personality to work with. Many unpublished data included in this thesis are at the courtesy of Dr. Jorge Luis Jimenez-Macias. He has trained me and provided great amount of guidance on the project. I cannot thank him enough for his mentorship and the scintillating discussions we have had. He is an exemplar scientist with curiosity and dedication. I would also like to acknowledge all the current/past members of the Lawler Lab including Andy, Noe, Billy, Jasmine and Fernando, who have cultivated a welcoming and collaborative lab environment, and Jason and Michael for helping with the ongoing immunofluorescence work. Additionally, I want to thank the Genomics Core (especially Dr. Christoph Schorl) and the Bioimaging Core for providing invaluable equipment support, as well as the El-Deiry Lab and Bartnikas Lab for sharing their IVIS and ChemiDoc machines. I am thankful for the department leadership, Dr. Jacquelyn Schell, who has been extremely accommodating and supportive throughout my academic journey at Brown. I would also like to thank Dr. Rosalyn Sayaman who has been my long-term mentor since my undergraduate research at Dr. Mina Bissell's lab and provided me with bioinformatics training through her ongoing I-SPY2 clinical trial working group. I want to appreciate all the people I have interacted with during my previous employment at UCSF, especially my PI Dr. Mark Schumacher, the late and beloved Dr. Manohar Sharma and all my coworkers also friends in the Department of Anesthesia. I have gained so much research experience and grown as a scientist there. Last but not the least, thank you mom and dad for being supportive in my scientific pursuit!

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Abstract of MODULATING ENDOTHELIAL PERMEABILITY TO IMPROVE DRUG DELIVERY IN GLIOBLASTOMA, by Zheyun Xu, ScM, Brown University, May 2023.

Objective: Hindered drug delivery due to the presence of the blood-tumor barrier (BTB) is one of the limiting factors in effective glioblastoma therapy. We intend to investigate regulatory mediators involved in the brain endothelial integrity and vascular permeability in glioblastoma, using a small molecule 6-bromoindirubin acetoxime (BIA) as a model compound.

Methods: RNA-sequencing data from cultured human cerebral microvascular endothelial (hCMEC/D3) cells treated with BIA or vehicle control were analyzed and genes were prioritized based on fold-change and known functions. Expression levels of the lead candidates, including the junctional protein VE-cadherin (CDH5) and transcription factors ETV1 and ERG, were assessed by RT-qPCR, Western blot and immunofluorescence on cultured hCMEC/D3 and primary human brain microvascular endothelial (HBMEC) cells, as well as *in vivo* treated murine glioblastoma xenograft tissue samples. The specificity and enhancement of drug penetration at the BTB following BIA treatment *in vivo* were validated by TRITC-dextran and sodium fluorescein tracers. Barrier formation of hCMEC/D3 cells upon ETV1 or ERG inhibition was tested using the electric cell-substrate impedance sensing (ECIS) assay. A human phospho-kinase array was used to screen for possible kinase targets involved in BIA's mechanism of action.

Results: BIA-induced reduction of VE-cadherin expression was consistently observed. ETV1 and ERG expression levels were downregulated after BIA treatment. siRNA of ETV1 seemed to

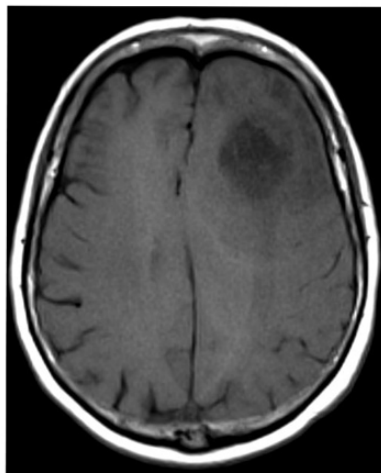
partially inhibit endothelial barrier formation. BIA appeared to inactivate a broad spectrum of kinases, including Yes, p38 α , STAT2 and STAT5 α /STAT5 β .

Conclusions: BIA reduces endothelial permeability at the BTB likely via modulation of kinases (i.e. p38 α) and transcription factors (i.e. ETV1) that control VE-cadherin expression.

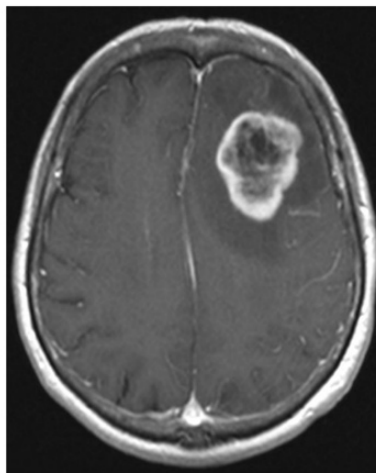
1. Introduction

1.1 Overview of glioblastoma (GBM) and the blood-tumor-barrier (BTB)

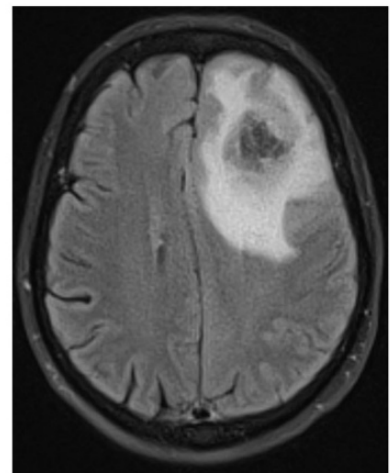
Glioblastoma (GBM) is the most common form of primary malignant tumors of the central nervous system (CNS) with an incidence rate of 3-5 per 100,000 people every year in the United States^{1,2}. **Figure 1** shows representative magnetic resonance imaging (MRI) images of GBM causing necrosis and lesions in the brain². GBM tumors are highly heterogeneous. Many biomarkers such as IDH, MGMT, EGFR, p53 and PI3K, have been identified and pursued in clinical drug development pipelines^{2,3}. The current standard of care is maximal safe surgical resection, followed by 6-weeks of radiotherapy (60 Gy) with adjunct chemotherapy of temozolomide^{1,2}. Unfortunately, recurrence is common, and prognosis of GBM remains poor⁴. The 5-year survival rate for GBM is approximately 5%, with median survival at 15 months¹.



Axial T1-weighted MRI



Axial T1-weighted MRI after gadolinium contrast enhancement - the central irregular hypo-enhancement is characteristic and reflects necrosis



Axial T2-weighted FLAIR MRI - signal hyperintensity typically extends far beyond the enhancing margins of the lesion and can represent edema or tumor infiltration

Figure 1: MRI Images of GBM showing necrotic core of the tumor and tumor infiltration into surrounding tissue ²

Typical MRI images of GBM showing the tumor (first image, without contrast agent), the necrotic core of the tumor (second image, with gadolinium contrast enhancement) and tumor infiltrating into surrounding tissue (third image, with fluid-attenuated inversion recovery contrast).

A major challenge in effective GBM therapy is the presence of the blood-tumor-barrier (BTB), the interface between the tumor and the blood-brain-barrier (BBB). The BBB is a tightly sealed monolayer of highly polarized endothelial cells supported by surrounding pericytes, astrocytes, vascular smooth muscle cells, neurons, microglia, oligodendrocytes, perivascular macrophages, and the extracellular matrix composed of laminins, collagens, nidogens and proteoglycans ⁵⁻⁸. Such well-organized architecture crucial for the homeostasis in the brain, is disrupted when a tumor develops (**Figure 2**) via processes of vasculogenesis, sprouting angiogenesis, vascular mimicry, or vascular co-option⁹. The interaction between the tumor and the BBB generates a special tumor microenvironment that is permissive for tumor growth and invasion, but remains challenging for therapeutic penetration⁵.

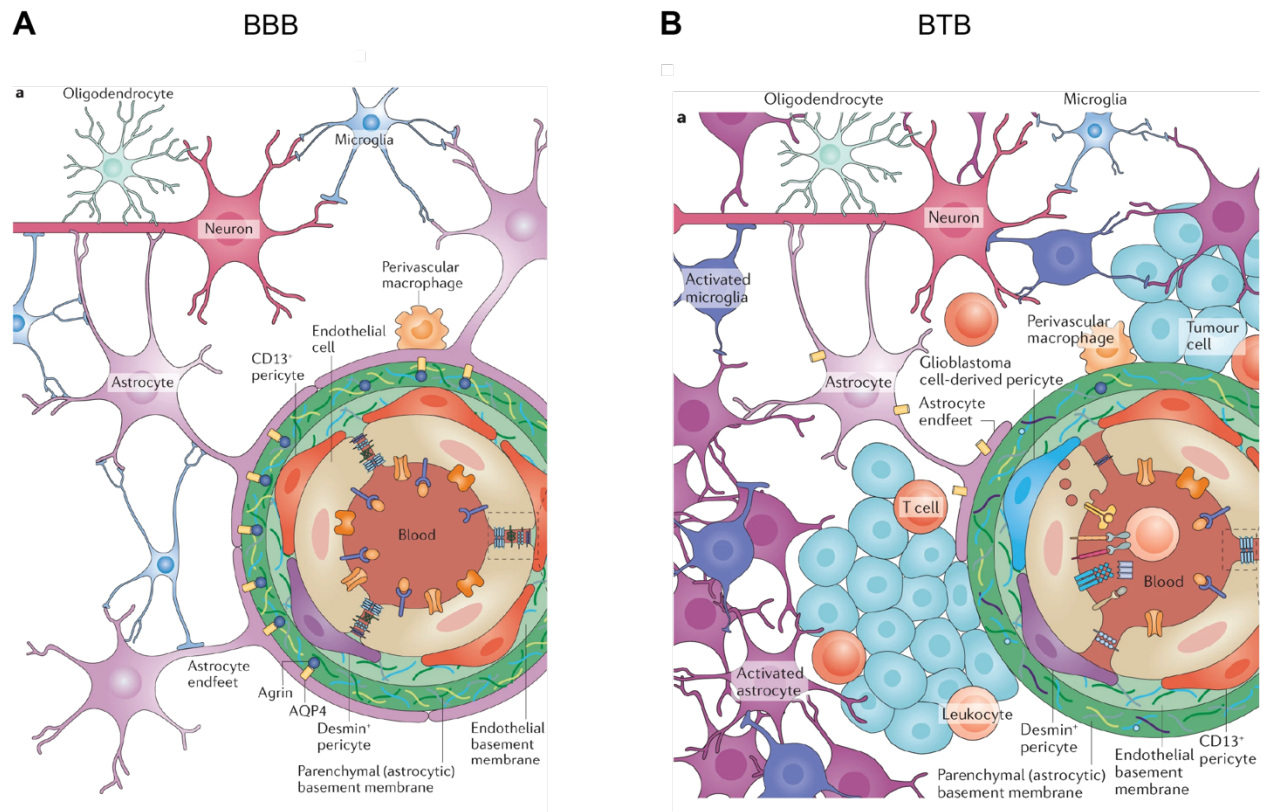


Figure 2: Anatomy of the blood-brain-barrier (BBB) vs. blood-tumor-barrier (BTB)⁵

(A) Architecture of the BBB including endothelial cells with well-established junctional protein complexes to limit paracellular leakage, and surrounding pericytes, astrocytes, neurons, microglia, oligodendrocytes and extracellular matrices (endothelial and parenchymal); (B) Architecture of the BTB including endothelial cells with altered junctional protein complexes, and neuroinflammatory microenvironment composed of tumor cells, activated astrocytes and microglia, infiltrated immune cells (T cells, leukocytes and macrophages), apoptotic neurons, oligodendrocytes and basement membrane (endothelial and parenchymal) to promote tumorigenesis and angiogenesis.

1.2 BIA disrupts endothelial barrier formation and improves drug uptake in tumors

6-bromoindirubin acetoxime (BIA) is a derivative of indirubin, an active ingredient in the Chinese herbal medicine Dang Gui Long Hui Wan¹⁰. BIA has demonstrated some potential in GBM therapy by improving the survival of xenografted animals, inhibiting tumor migration, and reducing angiogenesis¹¹. Recently, we have discovered that BIA at 1 μ M concentration can significantly inhibit endothelial cells from forming a barrier, by measuring the transepithelial/transendothelial electrical resistance (TEER) in the electric cell-substrate impedance sensing (ECIS) assay (**Figure 3A-C**, Jimenez-Macias unpublished data)¹². High electrical impedance is indicative of a well-formed endothelial monolayer. We confirmed that BIA did not affect barrier formation through inducing apoptosis using the CellTiter-Glo 3D viability assay as previously described¹³. The Lethal Concentration 50 (LC50) of BIA at 72hr treatment time on human cerebral microvascular endothelial (hCMEC/D3) cells was approximately 8.8 μ M (**Figure 3D**, Jimenez-Macias unpublished data).

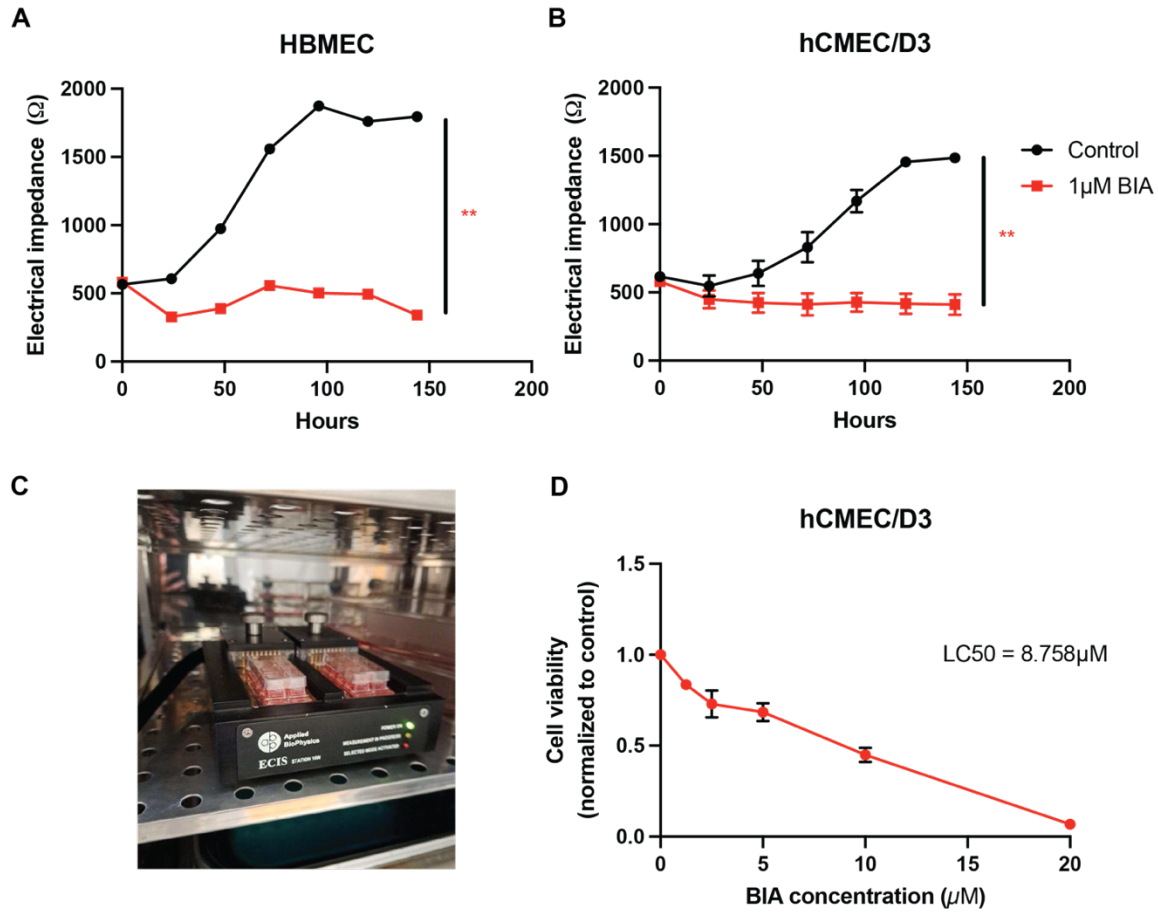


Figure 3: Suppression of endothelial barrier formation by BIA and LC50 of BIA

(A) 1μM BIA significantly inhibited HBMEC cell barrier formation in the ECIS assay (N = 1, unpaired t-test ** p-value = 0.0023); (B) 1μM BIA inhibited hCMEC/D3 cell barrier formation in the ECIS assay (N = 2, unpaired t-test ** p-value = 0.0057); (C) ECIS assay setup utilizing a special tissue culture chamber with 40 active electrodes at the bottom of each well to measure real-time electrical impedance; (D) CellTiter-Glo assay indicated low cytotoxicity of BIA at 1μM concentration for hCMEC/D3 cells (N = 3).

When BIA was administered intravenously (i.v.) via lateral tail vein injection to nude female mice bearing either G9-pCDH or G34-pCDH tumors at 20mg/kg on day 9 or 10 post-tumor

implantation, we detected a stronger accumulation of cisplatin (5mg/kg, intraperitoneal injection – i.p., 5hr prior to sample collection) in the tumor by inductively-coupled plasma mass spectrometry (ICP-MS) on the following day (**Figure 4**, Jimenez-Macias unpublished data)¹³. This effect was not observed in the contralateral healthy brain, indicating the possibility of BIA to modulate endothelial permeability in the BTB specifically, and not in the BBB.

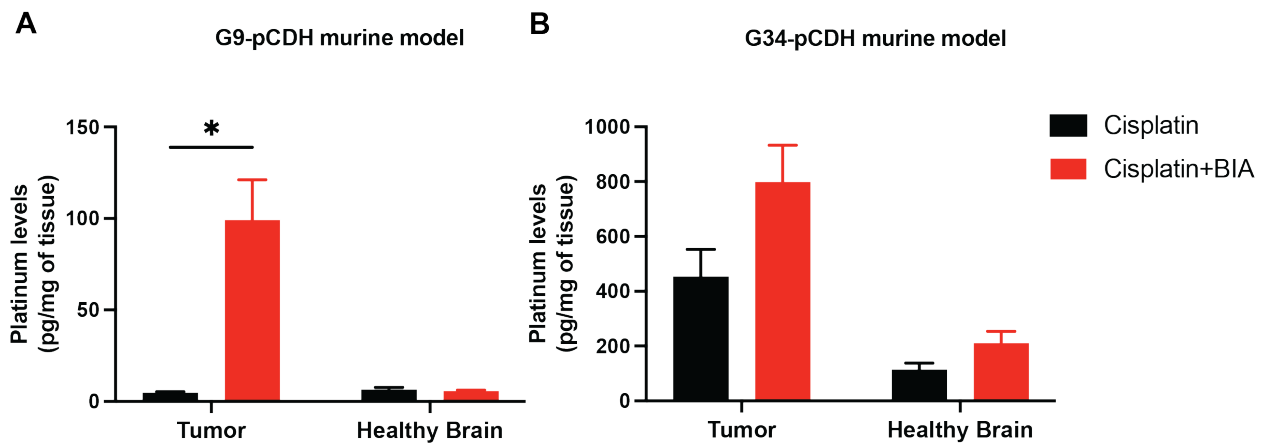


Figure 4: BIA increased cisplatin uptake in tumor but not in healthy brain

(A) Platinum levels in tumor vs. contralateral healthy brain harvested from G9-pCDH xenograft mice on day 10 or 11 post-tumor implantation. Measurement was performed by ICP-MS. Animals were treated with either BIA (20mg/kg, i.p.) or vehicle control on day 9 or 10. Cisplatin (5mg/kg, i.p.) was administered 5 hours prior to sample harvesting. Multiple unpaired t-test with Holm-Šídák correction (tumor, N=3 * adj p-value = 0.025694; healthy brain, N= 6, ns = not significant); (B) Same experimental procedure was performed for G34-pCDH xenograft mice and platinum levels were quantified by ICP-MS. Multiple unpaired t-test with Holm-Šídák correction (N=5, ns = not significant).

1.3 BIA as a model compound for the identification of endothelial permeability modulators at the BTB

Integral to the barrier function of the BBB are the junctional complexes between the endothelial cells, comprised of tight junctions (TJ), adherens junctions (AJ) and gap junctions (GJ)^{6-8,14}. In particular, TJ and AJ seal the interendothelial cleft and regulate paracellular diffusion which is the main pathway mediating the transport of cells and macromolecules larger than 3nm in size, such as oncology therapeutics including platinum agents and temozolomide^{5,7,14}. The main components of the junctional complexes are illustrated in **Figure 5**. Claudin 5 has been identified as essential for TJ formation and barrier function in genetic models, while occludin and JAM 1/2/3 have a more regulatory role^{6,8}. Scaffolding proteins in the form of heterodimers of ZO-1 with ZO-2/ZO-3 (Zonula Occludens) facilitate TJ assembly and TJ anchoring to actin cytoskeleton^{6,8}. The major transmembrane AJ protein VE-cadherin is exclusively expressed in vessels and interacts with ZO-1 and the actin cytoskeleton as a complex with p120-catenin and β -catenin/plakoglobin (γ -catenin) via an actin binding protein α -catenin^{6,14,15}. Alterations in the expression, post-translational modification (e.g. phosphorylation) and the localization of junctional proteins can lead to changes in endothelial permeability^{6,8,15}.

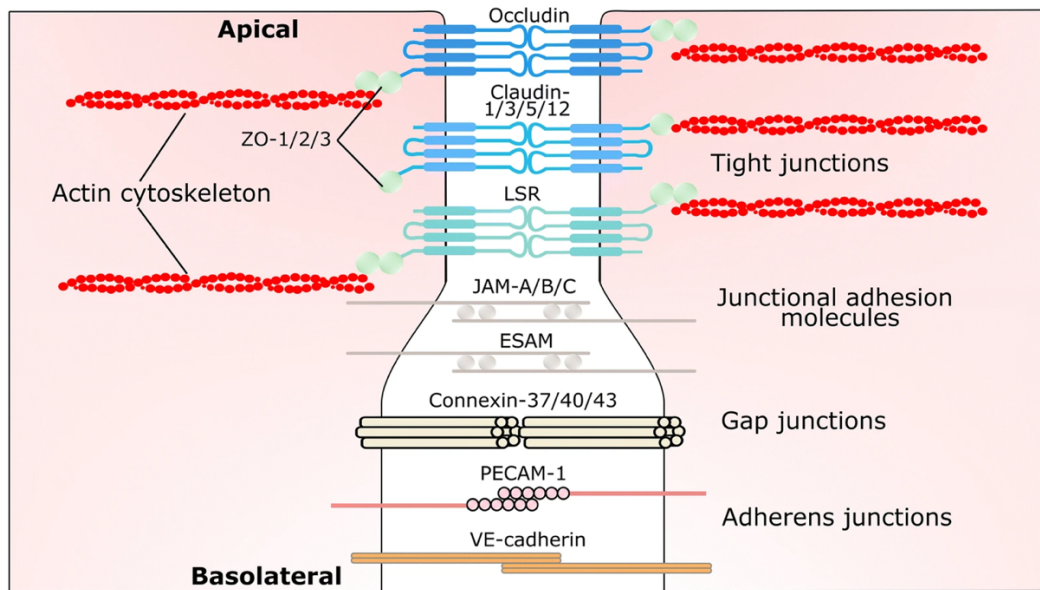


Figure 5: Architecture of the Brain Endothelial Junctional Complex¹⁶

Three classes of junctional proteins in the brain vasculature are tight junctions (TJ), gap junctions (GJ) and adherens junctions (AJ). TJ include occludin, claudins and junctional adhesion molecules (JAM-A/B/C, ESAM). GJ are connexin molecules, involved in solute exchanges between endothelial cells. VE-cadherin is the major AJ and exclusively expressed in the endothelium. Under pathological conditions, VE-cadherin expression can also be found in GBM cells as they adopt vasculogenic mimicry¹⁷. The relative localization of each junctional protein in this illustration is not representative.

Indirubin and its derivatives have been found to inhibit cyclin-dependent kinase (CDK)1/cyclin B, CKD2/cyclin A, CDK2/cyclin E, GSK3 β , CDK5/p25¹⁸. For CDK enzymes specifically, indirubin has been demonstrated to act through competitively inhibiting ATP binding in the catalytic domain¹⁸. BIA, a potent GSK3 inhibitor, is likely to interact with other kinases in a similar manner¹¹. Based on our prior observation that BIA can disrupt endothelial barrier

formation and selectively increase drug uptake in GBM xenografts (Jimenez-Macias unpublished data), understanding its mechanism of action can elucidate critical endothelial permeability modulators, specifically junctional proteins and associated pathways, at the BTB. We hypothesized that BIA downregulates select junctional protein(s) through inhibiting kinases that either phosphorylate transcription factors responsible for the gene expression of these junctional proteins and/or phosphorylate these junctional proteins directly to alter their stability, and consequently disrupts junctional complex stability, increases paracellular diffusion, and promotes barrier permeability.

2. Methods

2.1 Animals

Athymic nude female mice between age 8-12 weeks were obtained from Taconic Biosciences or Envigo and housed within the Brown University animal care facility in a climate-controlled room on a 12hr light/dark cycle. Lab diet was available *ad libitum*, except when the mice were under surgery.

2.2 Cell culture

Human cerebral microvascular endothelial (hCMEC/D3) cells (Millipore Sigma #SCC066) and primary human brain microvascular endothelial (HBMEC) cells (ScienCell #1000) were maintained in Endothelial Cell Medium (ScienCell #1001) supplemented with 5% fetal bovine serum, 1% endothelial cell growth supplement and 1% penicillin-streptomycin. Both endothelial cell lines were used for experiments before passage 20.

GBM cell lines G9-pCDH, G34-pCDH and G30-LRP (Chiocca Lab, The Ohio State University) were cultured as neurospheres in stem cell conditions in Neurobasal Medium (Gibco #21103049) supplemented with 20µg/mL of human recombinant EGF (Peprotech), 20µg/mL of FGF-B (Peprotech), 2% B-27 supplement (Thermo). GBM neurosphere dissociation was done using StemPro Accutase Cell Dissociation Reagent (Gibco #A1110501) to obtain single cell suspensions.

All cell lines were maintained in humidified 5% CO₂ incubator at 37°C.

2.3 *In vivo* tumor implantation

Experimental protocols were approved by Brown University, Institutional Animal Care and Use Committee (IACUC) and adhered to the NIH Guide for the Care and Use of Laboratory Animals.

GBM neurospheres were grown to 70% confluency before dissociation into single cell on the day of surgery. Experimental mice were anesthetized with Ketamine: Xylazine (100mg/kg:10mg/kg in normal saline, i.p.), and provided with pre-operative analgesic Meloxicam (5mg/kg in normal saline, i.p.). Each animal was placed into the stereotactic apparatus with the head stabilized by ear bars and the front teeth latched onto the anterior clamp. Eye ointment was applied to prevent excessive drying. Surgical area was cleaned with 10% povidone iodine. An anterior-to-posterior incision slightly to the right of the midline was made by sterile micro scissors to expose the skull. 10% hydrogen peroxide was applied to help visualize the bregma. Injection site was identified by the following stereotactic coordinates: x = 2mm (to the right of the bregma), y = 1mm (towards the anterior), z = 3mm (downward). 50,000 cells in 3 μ L sterile DPBS were injected intracranially into the striatum of experimental mice via a 10 μ L Hamilton syringe. Experimental animals were also treated with post-operative analgesic Buprenorphine-SR (0.6mg/kg, subcutaneous injection - s.c.) immediately after surgery, and with Meloxicam (5mg/kg, i.p.) for the following three days. Tumors were left to grow for 8-9 days and visualized in Xenogen *in vivo* imaging system (IVIS) for tumor growth confirmation before treatment. G9-pCDH and G34-pCDH have constitutive GFP expression. G30-LRP will emit luminescence when 100 μ L D-luciferin (Promega # P1041, 150mg/kg, i.p.) is administered. Animals were randomized to treatment groups.

2.4 Drugs

BIA (Sigma-Aldrich #SML0531) was administered i.p. at 20mg/kg in 10% DMSO, 2% Tween-20, 1% PEG 400 in DPBS. For *in vitro* experiments, BIA was used at 1 μ M concentration at maximal 0.02% DMSO, unless otherwise noted. ETV1 inhibitor BRD32048 (Sigma-Aldrich, #531057) was resuspended in DMSO at 10mM stock concentration. Cells were treated with BRD32048 at a final DMSO concentration not exceeding 0.5%.

2.5 Measurement of fluorescent tracer penetration

Animals were injected with 100 μ L of 70kDa TRITC-dextran (Thermo Fisher #D1818, 50mg/mL, i.p.). 2hr after administration, animals were euthanized, and brain samples were harvested for immunofluorescence.

Sodium fluorescein (NaFl) (Sigma-Aldrich #F6377) is another compound commonly used for BBB study^{7,19}. Experimental animals were injected i.v. via lateral tail vein with NaFl at 20mg/kg in normal saline. 30min after administration when peak fluorescence was reached in the brain¹⁹, mice were euthanized for immediate tissue harvest. Fresh brain and liver samples were visualized in IVIS. Quantification of pixel intensities from acquired images was performed in Fiji ImageJ. Tumor and healthy brain tissue samples (~100mg) were dissected from the harvested whole brain and weighed. Samples were then homogenized in 1mL 60% trichloroacetic acid in DPBS and measured at 488nm in Molecular Devices SpectraMax M2 plate reader. A standard curve of known NaFl amount in ng was generated. Fluorescence reading from each sample was blanked and normalized according to the standard curve, and the absolute amount of NaFl (ng per mg tissue) was calculated.

2.6 Quantitative real-time PCR (RT-qPCR)

Total RNA was isolated from culture cells using QIAzol Lysis Reagent (Qiagen #79306) following manufacturer's protocol. RNA extraction from harvested tumor and contralateral healthy brain tissues was performed under homogenization using 23G and 26G needles in QIAzol. Sample RNA quality was assessed by NanoDrop One Spectrophotometer (Thermo Scientific) (**Appendix 1**). First strand cDNA was prepared using iScript cDNA Synthesis Kit (Bio-Rad #1708891). mRNA expression levels were quantified using iTaq Universal SYBR Green SuperMix (Board #1725122) on QuantStudio 6 Pro System (Applied Biosciences), normalized by housekeeping gene GAPDH expression and represented as relative expression using comparative $\Delta\Delta C_T$ method.

All primers were designed using NCBI Primer-Blast tool, except for ETV1²⁰. Primers used in study have been tested for amplification efficiency and passed melt curve analysis. Detailed information on primer sequence can be found in **Table 1**.

Target	Amplicon (bp)	Forward Primer (5' -> 3')	Length	Reverse Primer (5' -> 3')	Length	Efficiency	Adjusted R ²
hGAPDH	104	CCA GCA AGA GCA CAA GAG GA	20	ACA TGG CAA CTG TGA GGA GG	20	89.3%	0.9995
hETV1	135	CCT GAG GAA ATC TCC AGC TA	20	ACA ATT TCT CCC ACG CTG AC	20	99.2%	0.9865
hERG	137	GAT CGC ATT ATG GCC AGC AC	20	TGT CCA TAG TCG CTG GAG GA	20	97.7%	0.994
hCDH5	119	GGT CGA TGC AGA GAC AGG AG	20	GAG TCT CCA GGT TTT CGC CA	20	108%	0.9863
hTJP1	91	CTG GAG AAA TCC CGG AAA AAT GA	23	TTG CTG CCA AAC TAT CTT GAG A	22	95%	0.9921

Table 1: List of primers used for RT-qPCR

2.7 Western blot

Cell lysates were collected in ice-cold RIPA Lysis and Extraction Buffer (Thermo Fisher #89900) supplemented with 1x protease/phosphatase inhibitor cocktail (Cell Signaling #5872S).

Lysate collection from tissue samples (~ 30mg) was performed under homogenization using 23G and 26G needles. Total protein concentration was measured using Pierce 660nm Protein Assay Reagent (Thermo Scientific #22660) at 660nm absorbance in Molecular Devices SpectraMax M2 plate reader.

Samples of equivalent protein amount (~10 μ g) were incubated in 1x Laemmli sample buffer (Bio-Rad #1610747) at 95°C for 5min before loading onto 10% Mini-PROTEAN TGX precast protein gel (Bio-Rad #4561036). PageRuler Plus Prestained Protein Ladder (Thermo Scientific #26619) was used as ladder control.

Nitrocellulose membranes containing samples were blocked in 5% milk in 1x Tris-buffered saline with 0.5% Tween-20 (TBST) (Thermo Fisher #J60448.K2) for 1hr at RT under gentle rocking. 1x TBST was also used for all washing steps. Incubation with respective primary antibodies (**Table 2**) diluted in 5% BSA in DPBS/1x TBST was performed at 4°C overnight under gentle rocking. Appropriate secondary antibodies, Goat anti-mouse-HRP (Sigma-Aldrich #12-349) or Goat anti-rabbit-HRP (Sigma-Aldrich #401353) were used at 1:5,000 dilution in 5% milk in 1x TBST.

Antibody	Company	Cat #	Clone	Host	Specificity (Hu)	Specificity (Ms)	MW	Concentration	Dilution
Histone-H3	Proteintech	17168-1-AP	polyclonal	Rb	Yes	Yes	15-17 kDa	0.6mg/mL	1:5000
GAPDH	Proteintech	10494-1-AP	polyclonal	Rb	Yes	Yes	36 kDa	50 μ g/150 μ L	1:5000
ETV1	Invitrogen	PA5-77975	polyclonal	Rb	Yes	Yes	55-60 kDa	0.21mg/mL	1:1000
ERG	Invitrogen	MA5-32036	SP06-04	Rb	Yes	Yes	51,54kDa	1mg/mL	1:1000
VE-Cadherin	Invitrogen	14-1449-82	16B1	Ms	Yes	Yes (published)	110-140kDa	0.5mg/mL	1:1000
ZO-1	Invitrogen	33-9100	ZO1-1A12	Ms	Yes	Yes	225 kDa, 130 kDa (faint)	0.5mg/mL	1:1000
CD31	Invitrogen	MA3100	HEC7	Ms	Yes	Yes (published)	130 kDa	1mg/mL	1:1000

Table 2: List of antibodies used for Western blot

Immunoblots were visualized using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific #34577) with ChemiDoc Imaging System (Bio-Rad).

2.8 Immunofluorescence and fluorescence microscopy

After euthanasia with CO₂ and intracardiac DPBS perfusion, mouse brain tissues were collected and fixed in 10% formalin at 4°C on a tube rotator for 2 days. Tissues were transferred to 30% sucrose containing 0.01% sodium azide at 4°C on a tube rotator for at least 24hr. Samples were embedded in Optimal Cutting Temperature (OCT) compound, frozen and serial sliced into 20µm sections using Leica cryostat. Tissue samples were incubated in 0.1% Triton X-100 in blocking buffer (1% normal donkey serum in 0.025% Tween-20 in DPBS) at room temperature for 1hr.

hCMEC/D3 cells cultured on 8-well chamber slide (Lab-Tek #154534) were fixed in 10% formalin for 10min at room temperature. Samples were permeabilized in 0.1% Triton X-100 in DPBS for 30min, and then blocked in blocking buffer for 1hr at room temperature.

Slides were stained with primary antibodies diluted in blocking buffer at 4°C overnight, washed in DPBS, and incubated with species-matched fluorophore-coupled secondary antibodies for 1hr (cells) or 3hr (cryosections) at room temperature (**Table 3**). Hoechst 33342 (Thermo Scientific #62249) was used for nuclear staining at 1:1000 dilution. Slides were mounted with VectaMount Permanent Mounting Medium (Vector Laboratories #H-5000) and cover slipped.

Primary Antibody	Company	Cat #	Clone	Host	Specificity (Hu)	Specificity (Ms)	Concentration	Dilution
VE-Cadherin	Invitrogen	14-1449-82	16B1	Ms	Yes	Yes (published)	0.5mg/mL	1:200
ZO-1	Invitrogen	33-9100	ZO1-1A12	Ms	Yes	Yes	0.5mg/mL	1:100
CD31	R&D Systems	AF3628-SP	polyclonal	Gt	No	Yes	1mg/mL	1:200

Secondary Antibody	Company	Cat #	Use Dilutions
Dk α Ms Alexa Fluor 594	Invitrogen	A21203	1:500
Dk α Gt Alexa Fluor Plus 405	Invitrogen	A48259	1:500
Dk α Gt Alexa Fluor 647	Invitrogen	A21447	1:500
Gt α Ms Alexa Fluor Plus 647	Invitrogen	A32728	1:500

Table 3: List of antibodies used for immunofluorescence

Zeiss LSM 880 Confocal Laser Scanning Microscope or Nikon Ti2-E Fluorescence Microscope was used for image capture.

2.9 Electric cell-substrate impedance sensing (ECIS) analysis

Approximately 50,000 hCMEC/D3 cells were plated on 8W10E+ PET 8-well arrays (Applied Biophysics #NC1637226) which had all electrodes pre-equilibrated in ECIS Z-Theta Instrument through ECIS Software (Applied Biophysics). Impedance measurement was taken at 3000 Hz every 30min. Data were exported to Excel and transferred to Prism (GraphPad) to generate the plots and conduct statistical analysis.

2.10 siRNA

hCMEC/D3 cells were cultured to approximately 50% confluency and transfected using Lipofectamine RNAiMax Transfection Reagent (Invitrogen #13778150) for 2 days. All experimental steps followed manufacturer's protocol with the modification of using 20 μ M siRNA in the working stock and 4.2 μ L Lipofectamine to minimize toxicity. MISSION siRNA universal negative control (Sigma-Aldrich #SIC001) was used as siControl. Silencer Select siETV1 (#s4855) and Silencer siERG (#116761) were acquired from Invitrogen.

2.11 Human phospho-kinase array

hCMEC/D3 cells were treated with either 1 μ M BIA or vehicle DMSO control for 48hr. Cell lysates were collected in manufacturer provided Lysis Buffer 6 supplemented with 10 μ g/mL Aprotinin (Tocris #4139), 10 μ g/mL Leupeptin hemisulfate (Tocris #1167) and 10 μ g/mL Pepstatin A (Tocris #1190). 50 μ g of lysate from each sample was loaded to each of the membrane. All experiment procedure was performed with Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems, #ARY003C) following manufacturer's protocol.

2.12 RNA-seq data analysis

hCMEC/D3 cells treated with 1 μ M BIA or vehicle DMSO control for 24hr (N=3) were collected for RNA as described above with the additional step of RNeasy Mini Kit (Qiagen) and on-column DNase digestion (Qiagen) to remove potential DNA contaminants. At least 500ng of RNA was submitted for bulk RNA-sequencing at GeneWiz (Azenta Life Sciences). Library was prepared with Poly(A) selection and sequencing was performed using Illumina HiSeq.

Differential gene expression was analyzed from the RNA-seq raw FASTQ files by Azenta Life Sciences using DESeq2 aligning to human transcriptome. Data QC was verified. Log₂ fold change (Log₂FC) was calculated by Log₂ (BIA group mean normalized counts divided by Control group mean normalized counts). The Wald test p-value and Benjamini-Hochberg adjusted p-value were calculated. A heatmap of top adjusted p-value differentially expressed genes (DEGs) in ensemble ID annotation biclustering to treatment conditions was generated.

Manual curation of the provided heatmap was performed to represent top adjusted p-value DEGs in gene symbols. Gene symbols in the provided human alignment significant DEGs list were verified in R and updated to the current HGNC build. Discrepancy in the gene symbols identified by HGNCHELPER package was corrected to reflect the latest information on NCBI Gene database. DEGs were mapped to KEGG pathway database in R. Junctional protein complex genes were pulled from Gene Set Enrichment Analysis (GSEA) Molecular Signatures Database (MSigDB) curated gene sets (C2) canonical pathways (CP) KEGG subset. Pathways included for indexing were “Adherens Junction”, “Gap Junction”, “Tight Junction”, “Cell Adhesion Molecules CAMS”, “Focal Adhesion” and “ECM Receptor Interaction.” R packages HGNCHELPER (version 0.8.1), msigdbR (version 7.5.1), KEGGREST (version 1.38.0) and org.Hs.eg.db (version 3.16.0) were used with R studio (version 1.4.1717) on R version 4.2.2.

EnrichR²¹⁻²³ was used to perform functional enrichment analysis based the Gene Ontology Biological Process 2021 database. Clustergram was generated to represent the enriched terms and their top associated genes ranked by p-values. Enrichment Analysis Visualization Appyter²⁴ was used to visualize similarly clustered Gene Ontology (GO) Biological Process 2021 terms from enrichment analysis data in scatter plot format. Similar gene sets are clustered together using Leiden algorithm, and the darker and larger dots represent more significantly enriched GO terms with smaller p-value. The most significant terms are black-lined dots.

STRING database²⁵ was used to visualize protein interactions in network format. When applicable, top 10 interactors were plotted to represent the strongest interactors.

2.13 Statistical analysis

Analysis was performed using Prism 9 (GraphPad). Data were expressed as mean $\pm$ SEM.

Ordinary one-way or RM two-way ANOVA with Tukey *post hoc* test was performed for experiments with three or more groups, and multiple unpaired t-test with Holm-Šídák correction for two groups, as described in figure legends. * p-value <0.05 , ** p-value <0.01 , *** p-value <0.001 , **** p-value <0.0001 . Comparisons without statistical significance (ns = not significant) were not displayed. Figures were compiled in Adobe Illustrator.

3. Results

3.1 RNA-seq revealed alteration in endothelial programs and junctional proteins, especially VE-cadherin

Cultured hCMEC/D3, a common *in vitro* model for the BBB, can also loosely represent the developing tumor vasculature at the BTB, as healthy endothelium of the BBB generally remains quiescent²⁶⁻²⁸. RNA-sequencing of hCMEC/D3 cells treated with 1 μ M BIA vs. DMSO vehicle control for 24hr was performed to identify principal components of the endothelial barrier that regulate permeability. VE-cadherin (gene name: CDH5) appeared as a top differentially expressed gene (DEG) with a log2 fold change of -3.06 and an adjusted p-value of 9.75×10^{-252} , indicating significant downregulation in its gene expression after BIA treatment (**Figure 6A**).

Gene ontology analysis was performed using STRING²⁵. This further indicated a significant dysregulation (mainly downregulation) of endothelial-related genes by BIA (**Figure 6B**). CDH5 forms a network with other altered genes which have functions in endothelial cell biology.

According to NCBI Gene Database, KDR (also known as VEGFR2) encodes one of the two receptors of the vascular endothelial growth factor (VEGF), a major growth factor for endothelial cells, mediating VEGF-induced endothelial sprouting, migration, development, proliferation and survival (log2FC = -2.21, adj p-value = 2.06×10^{-125}); MMRN2 encodes an ECM glycoprotein multimurin 2, which is involved in tumor angiogenesis and growth via inhibiting TGF- β and VEGF-A/VEGFR2 pathway (log2FC = -2.99, adj p-value = 2.61×10^{-156}); adhesion G protein-coupled receptor G1, ADGRG1 (as known as GPR56) regulates brain cortical patterning and

tumor progression ($\log_2FC = -2.60$, adj p-value = 1.91×10^{-168}); CD93 participates in intercellular adhesion by linking transmembrane proteins to the cytoskeleton, and in cytoskeleton remodeling ($\log_2FC = -1.86$, adj p-value = 9.51×10^{-129}).

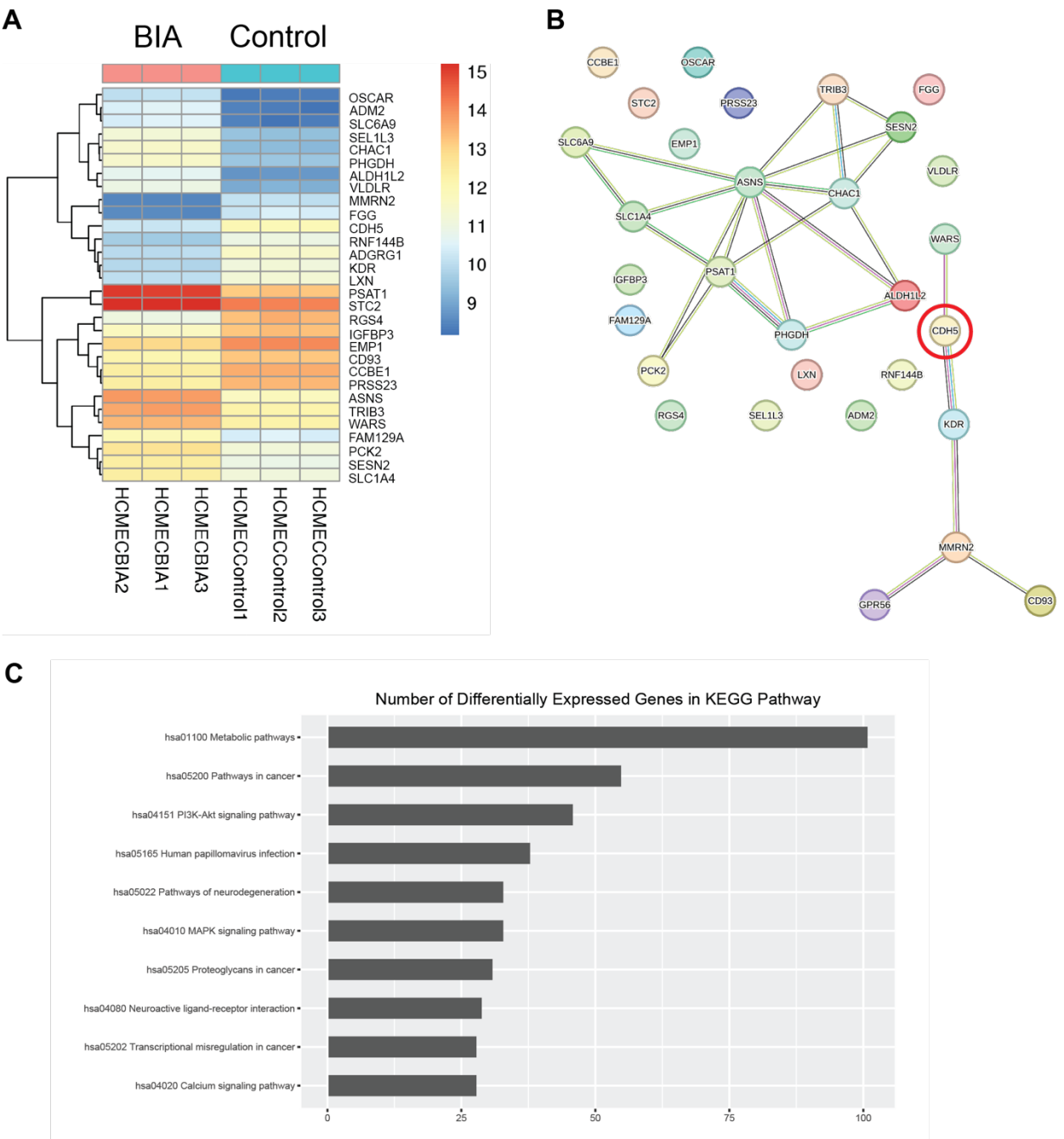


Figure 6: VE-cadherin, PI3K/AKT signaling pathway and MAPK signaling pathway are likely involved in BIA-mediated vascular permeability

(A) Biclustering heatmap of top differentially expressed genes (DEGs) by adjusted p-value in BIA (1 μ M, 24hr) vs. control treated hCMEC/D3 (N=3) RNA-seq dataset ; (B) STRING network of top DEGs showing an endothelial-related gene (including CDH5 – VE-cadherin, circled in red) cluster and a metabolic cluster; (C) Top 10 KEGG pathways associated with DEGs include PI3K/AKT signaling pathway and MAPK signaling pathway.

KEGG pathway analysis (**Table 4**) further illustrated the broad-spectrum disruption in endothelial junctional complexes by BIA treatment. For instance, ZO-2 (TJP2), claudin-7 (CLDN7), claudin-11 (CLDN11), endothelial cell adhesion molecule (ESAM), integrin β 3 (ITGB3), platelet and endothelial cell adhesion molecule 1 (PECAM1) were all significantly downregulated. **Figure 6C** shows the top 10 KEGG pathways associated with all DEGs in the BIA RNA-seq dataset. Among them, PI3K/AKT signaling pathway and MAPK signaling pathway, which have extensive implications in cancer and angiogenesis, might provide insight into the selective modulation of BTB permeability through VE-cadherin by BIA²⁹⁻³⁷.

ID	log2_Fold_Change	pvalue	padj	HCMCEB1A1	HCMCEB1A2	HCMCEB1A3	HCMCEControl	HCMCEControl2	HCMCEControl3	Gene.name
ENSG000000179776	-3.063472954	2.81E-255	9.75E-252	641.4826252	716.1230775	708.581549	6061.959644	5632.910549	5564.925457	CDH5
ENSG000000164692	-2.3513995459	2.25E-121	1.22E-118	484.444207	425.0994921	440.5923734	2341.573748	2418.466047	2155.458291	COL1A2
ENSG000000128052	-2.214988898	3.33E-128	2.06E-125	649.8955121	658.5483059	607.5178345	2840.146719	3153.383141	2910.704142	KDR
ENSG00000015914	-2.050491511	0.001366858	0.005028269	5.608591259	0.788681893	7.94881479	25.78825714	17.44610313	15.0380811	HR23B
ENSG000000174661	-1.773355284	1.44E-88	4.54E-86	724.2093463	660.1266695	689.277444	2332.977662	2339.958583	2416.118364	PIK3R3
ENSG000000108821	-1.769544826	2.06E-82	5.38E-80	3729.847491	3193.372622	3327.153788	12252.86057	10931.07399	10069.6662	COL1A1
ENSG000000105974	-1.705506699	8.42E-81	2.11E-78	9094.330726	9999.696586	8992.399498	28356.76755	33182.48816	30661.95957	CAV1
ENSG00000013721	-1.660721111	1.39E-35	8.71E-34	197.001768	187.7062692	177.1453873	675.6523369	560.4650631	553.9026539	PDGFRB
ENSG000000150630	-1.609402317	1.63E-62	2.38E-60	434.665826	428.2542193	426.9658052	1356.462325	1275.746291	1308.313056	VEGFC
ENSG000000102755	-1.598024106	3.34E-57	4.23E-55	500.5667688	466.1109458	483.7431729	1354.743108	1534.166694	1498.795416	FLT1
ENSG000000178011	-1.592293298	1.03E-100	4.26E-98	22496.76061	24688.10449	23366.15791	70933.18008	72381.70133	69416.61781	THBS1
ENSG000000174059	-1.567845087	1.84E-09	2.01E-08	26.6408048	48.10959041	43.15079946	116.9067657	130.8457735	99.41842506	CD34
ENSG000000112769	-1.5524832437	1.93E-76	4.18E-74	4851.431439	5108.292041	4571.713648	14130.24569	15024.36594	13480.80426	LAMA4
ENSG000000204762	-1.510499264	8.56E-69	1.44E-66	1743.570808	1817.122875	1698.778842	4855.06921	5362.49595	4771.248954	COL5A2
ENSG000000142798	-1.465230563	2.24E-21	4.45E-20	4456.025755	3813.27652	4019.871634	14974.38131	10153.63202	8820.670015	HSFG2
ENSG000000169439	-1.46046712	1.08E-09	1.23E-08	49.7765442	41.80013558	37.47306269	122.0644171	138.4784436	99.41842506	SPC2
ENSG000000149177	-1.38594353	3.62E-71	6.41E-69	3128.191775	3164.191395	3109.128656	8604.681797	7825.667636	8149.804508	PTPRJ
ENSG000000166949	-1.310795822	2.13E-52	2.36E-50	7062.618543	6789.737382	6595.299033	17427.70471	15382.01105	16685.58643	SNAD3
ENSG000000074211	-1.2891381335	1.00E-28	4.46E-27	373.6723926	387.2427655	400.848216	914.6235197	860.3109607	1055.172024	PPR2C
ENSG000000181885	-1.275996741	8.59E-36	5.56E-34	508.9796567	465.322264	473.523467	1162.190788	1110.008312	1234.793548	CDN7
ENSG000000013297	-1.257175937	1.26E-66	2.02E-64	7382.755749	7074.475777	7457.139475	17897.09045	17006.67941	17238.65364	CDN11
ENSG000000110799	-1.212104353	8.51E-17	1.83E-15	323.1950713	264.997086	244.1476812	747.8594569	632.4212385	564.7634903	VWF
ENSG00000013104	-1.201391386	3.80E-29	1.79E-27	2980.781389	3495.497936	2826.373865	6883.745438	7940.157688	6461.36218	TPR2
ENSG000000149564	-1.193399928	7.26E-36	4.72E-34	1133.636508	1011.878754	1054.923492	2645.875182	2336.687438	2349.282448	ESAM
ENSG000000167552	-1.183368622	3.63E-47	3.53E-45	2022.598223	2005.051916	2071.238374	4957.34537	4493.461938	4504.740739	TUBA1A
ENSG000000152861	-1.152745234	1.30E-29	6.35E-28	3920.40529	4571.988415	3841.556699	8200.665769	9769.817754	9448.092177	GIA1
ENSG000000161638	-1.13044899	2.59E-52	2.84E-50	33458.91609	35231.20484	35414.11534	81764.24807	762.38.3803	74299.81693	ITGA5
ENSG000000115414	-1.124123014	2.09E-25	7.98E-24	149596.5525	162132.4731	133092.9632	364142.2253	318849.3424	286604.9396	FN1
ENSG000000105971	-1.115341301	1.52E-25	5.92E-24	3930.921988	4651.645777	3992.944697	8295.227212	10264.85093	8681.15004	CAV2
ENSG000000065534	-1.089841858	2.04E-34	1.23E-32	2847.762122	2828.212947	2788.904302	6589.759307	5894.602096	5549.051927	MYLK
ENSG000000261371	-1.086601452	1.18E-32	1.19E-30	1198.836482	1181.445442	1173.020417	2317.504708	2699.78446	2527.22038	PCAM1
ENSG000000259207	-1.031714883	4.27E-31	2.26E-29	2072.37447	2276.135685	2087.136037	4411.511187	4093.291947	4652.615203	ITGB3
ENSG000000143127	-1.031234877	2.72E-24	9.71E-23	715.7964594	644.353034	776.143903	1500.876565	1438.213127	1423.605011	ITGA10
ENSG000000154229	-1.030652405	2.74E-40	2.12E-38	2421.509276	2433.872045	2322.194339	5054.498398	4793.316835	4826.388585	PRKCA
ENSG000000119139	-1.027402319	5.54E-46	5.25E-44	3752.147552	3775.419793	3676.902333	7562.836209	7690.460337	7587.547365	TIP2
ENSG000000164742	-1.0698276	0.00118715	0.0095514	78.9224244	61.51718067	78.3527644	42.9804256	26.169157	35.0888559	ADCV1
ENSG000000160223	-1.116995509	0.00668438	0.019765955	63.09665166	29.96990633	46.55744152	36.10359999	11.9941959	19.21532585	ICOSLG
ENSG000000099866	-1.134476839	4.22E-05	0.000222478	77.81920371	65.46038969	78.3527644	27.50747428	33.80182482	37.59520275	MADCAM1
ENSG000000058335	-1.224531664	1.80E-11	2.59E-10	207.5178766	252.3781771	208.9407132	101.4338114	81.77860843	103.5956698	RASGRF1
ENSG000000171791	-1.371584451	8.59E-09	8.59E-08	123.3890077	169.5665877	129.4523984	37.82277713	64.3325053	57.64597756	BCL2
ENSG000000173175	-1.443936341	0.00936385	0.027728479	40.64181641	9.464181641	31.7952192	8.590085712	8.723051566	9.189938451	ADCV5
ENSG000000189056	-1.54866753	0.000294126	0.001275526	40.6622863	61.51718067	36.33751533	27.50747428	6.542788674	15.87353005	RELN
ENSG000000112715	-1.561699372	3.19E-99	1.20E-96	11722.6568	11364.11611	11693.86665	3993.741422	3797.798874	3991.775084	VEGFA
ENSG000000109063	-1.619331144	2.41E-27	1.03E-25	386.2917729	394.3409017	391.763872	128.9412857	110.128526	141.1908726	VH93
ENSG000000139219	-1.768203696	0.009368674	0.02629165	14.72255205	16.5623147	12.4910209	6.76868569	5.451907229	1.6708979	COL2A1
ENSG000000101680	-1.945980619	9.33E-18	2.13E-16	179.4749203	164.8344969	158.976296	60.17259998	35.98258771	39.26610065	LAMA1
ENSG000000184144	-2.003175469	0.00152509	0.000707741	35.75476927	30.75859033	23.84649444	13.75373714	8.723051566	2.50634661	CNTA2
ENSG000000067606	-2.073327045	1.45E-06	1.02E-05	35.75476927	44.16618099	54.506273	10.31530285	15.26534024	6.683591601	PRKZ
ENSG00000012124	-2.14157486	0.003022238	0.00980161	20.3314331	14.9677246	12.4910209	6.9788569	4.36135783	0.83544895	CD22
ENSG00000081059	-2.474083525	3.95E-86	1.16E-83	1623.687169	1449.597155	1789.62763	336.966599	267.1434542	304.1034178	TCF7
ENSG000000174175	-2.47030494	0.00147676	0.000687355	26.6408048	20.50572689	14.7621156	5.157651427	3.271144337	3.3417958	SELP
ENSG000000050555	-2.698793998	1.18E-09	58.18913431	48.89827181	40.01525211	42.01525211	5.157651427	8.723051566	8.354489501	LAMA3
ENSG000000169896	-2.759376055	6.61E-38	4.61E-36	289.5435237	299.6990833	299.7845015	63.61103427	27.25953614	45.11424331	ITGAM

Table 4: Differentially expressed genes relating to junctional complex in KEGG pathway ordered from most downregulated (blue) to most upregulated (red) in BIA

Enrichment analysis of RNA-seq data using Gene Ontology (GO) Biological Process 2021 database (**Figure 7**) revealed significant alteration in angiogenesis, cell motility/migration and proliferation, which are all hallmarks of vasculature at the BTB rather than the BBB. A comparative analysis (**Figure 8**) between all the downregulated DEGs and all the upregulated DEGs in KEGG Pathway and GO Biological Process libraries, indicated that BIA-induced transcriptional downregulation likely drives the observed endothelial phenotype. The clustering of gene sets from enriched GO terms in scatter plot was driven by downregulated genes (data not shown), as similar enriched GO terms were obtained with all the downregulated DEGs by p-value ranking (**Figure 8C**): regulation of cell migration, positive regulation of cell migration, positive regulation of cell motility, regulation of endothelial cell proliferation, regulation of endothelial apoptotic process, regulation of angiogenesis, extracellular structure organization, negative regulation of endothelial proliferation, glomerulus vasculature development and external encapsulating structure organization. This finding of significant downregulation of those genes involved in cell motility and migration supported the prior observation that BIA reduced angiogenesis and endothelial migration¹¹. On the contrary, upregulated genes by BIA were mostly implicated in metabolic processes (**Figure 8B, 8D**). **Figure 8E-F** shows a distinct list of genes associated with the top enriched GO terms for either downregulated DEGs or upregulated DEGs. Many of the genes involved in the junctional complex such as VE-cadherin (**Table 4**) were downregulated DEGs and appeared in **Figure 8E** as genes strongly associated with the top enriched GO processes.

The importance of VE-cadherin in the BTB was also highlighted in the publicly available data from human GBM samples (**Figure 9**). GBM samples expressed higher levels of CDH5 mRNA

as compared to non-GBM samples (TCGA database), and VE-cadherin proteins was also detected extensively in GBM pathology samples (Human Protein Atlas), correlating to active angiogenesis and retained junctional integrity at the BTB.

Based on the RNA-sequencing findings, a closer investigation on the role of VE-cadherin in endothelial permeability modulation and possible contributing mechanisms was conducted.

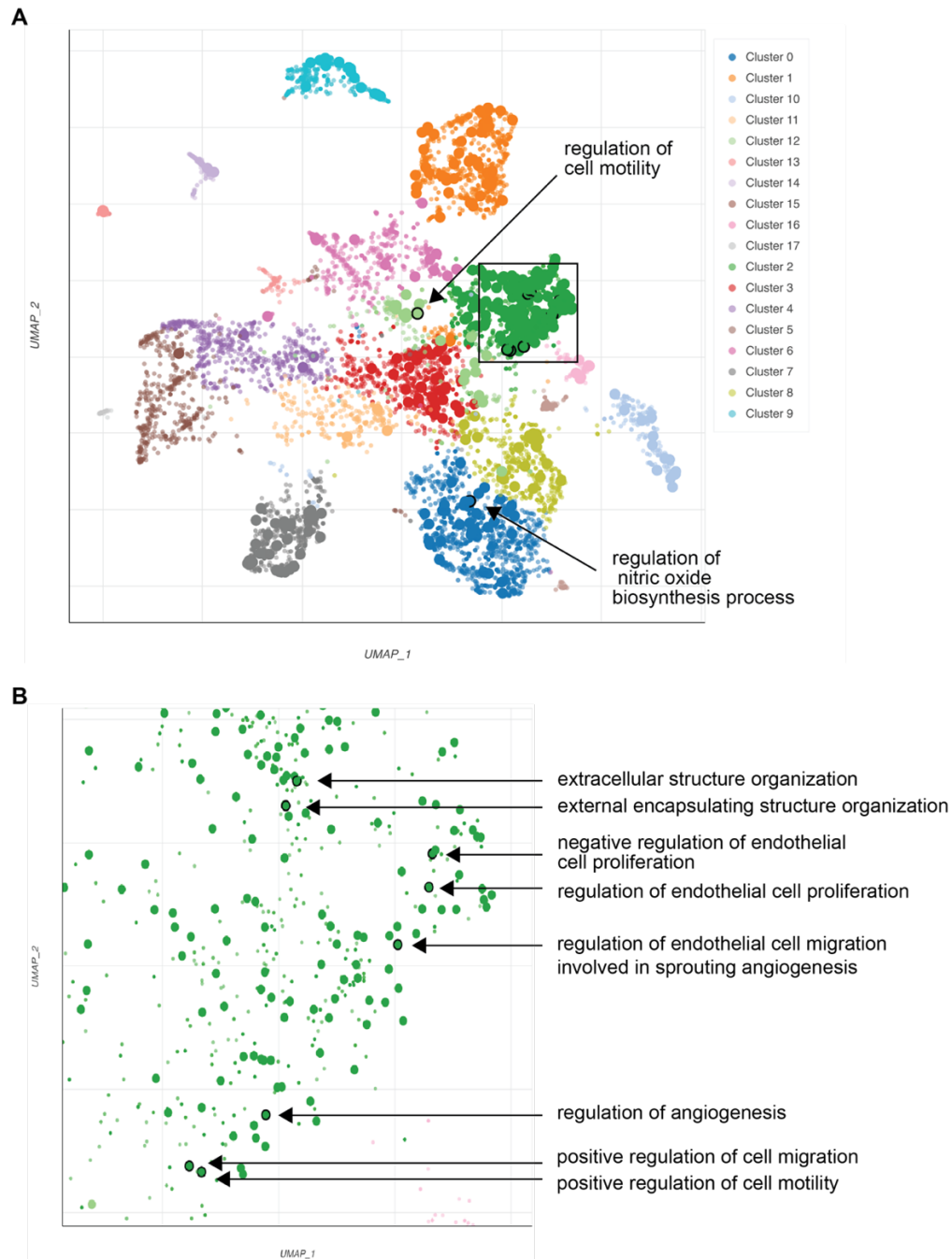


Figure 7: Enrichment Analysis Visualization Appyter for Gene Ontology Biological Process

2021 library confirmed endothelial motility, migration and proliferation as major ontologies affected by BIA

(A) Scatter plot of all DEGs organized in similar gene set clusters by enrichment analysis – larger and darker colored dots as more significant GO terms, and black circled dots indicating most significantly enriched terms by adjusted p-value; (B) Zoom-in view of cluster 2 containing the majority of the top significant GO terms.

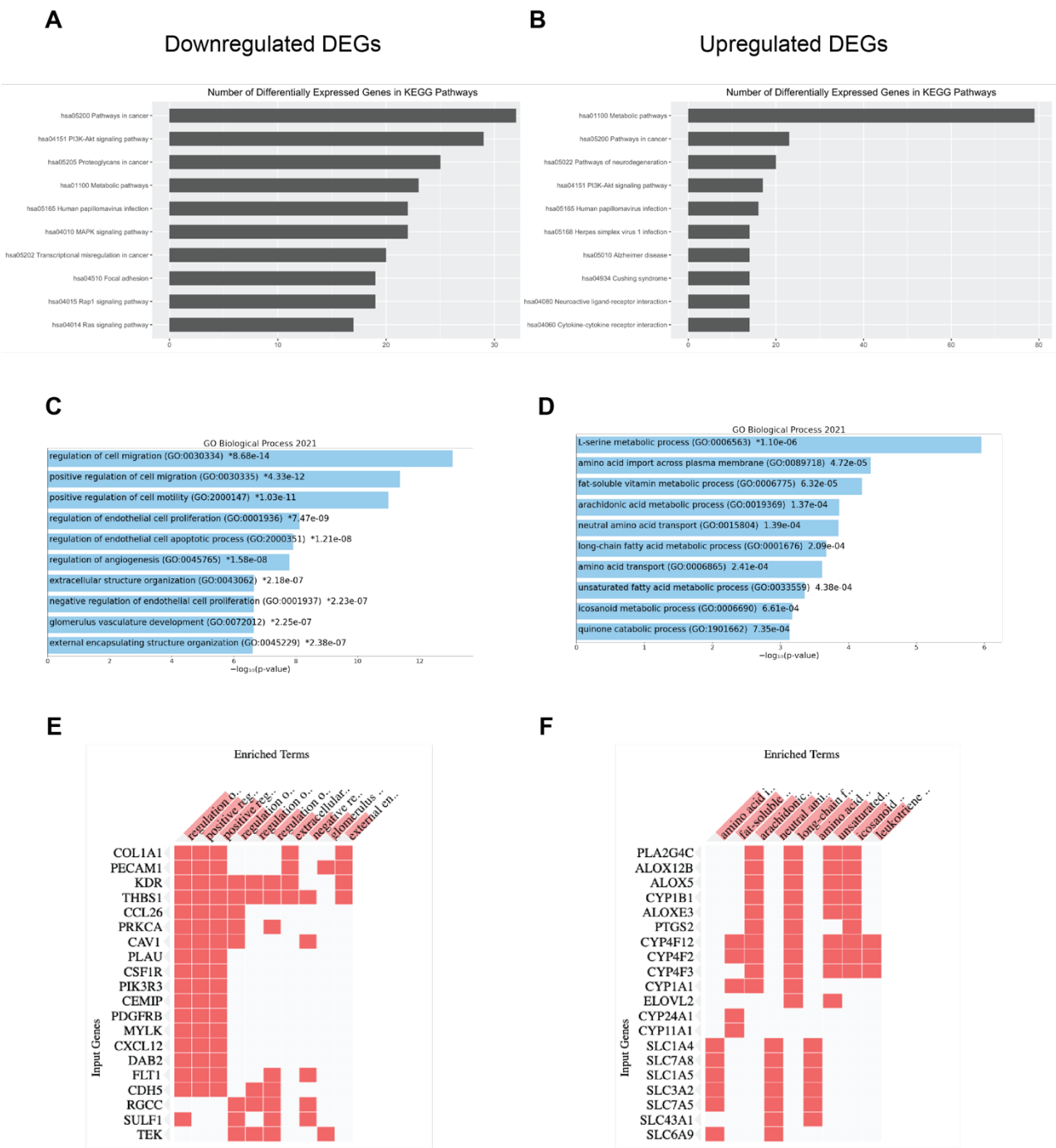


Figure 8: Comparison between downregulated DEGs and upregulated DEGs in KEGG pathway and GO Biological Process.

(A-B) KEGG pathway analysis; (C-D) Gene Ontology Biological Process 2021 analysis; (E-F) Clustergrams of most significant genes (by p-value) associated with each of the top GO Biological Process 2021 analysis enriched terms. A, C, E contained all downregulated DEGs in BIA; B, D, F contained all upregulated DEGs.

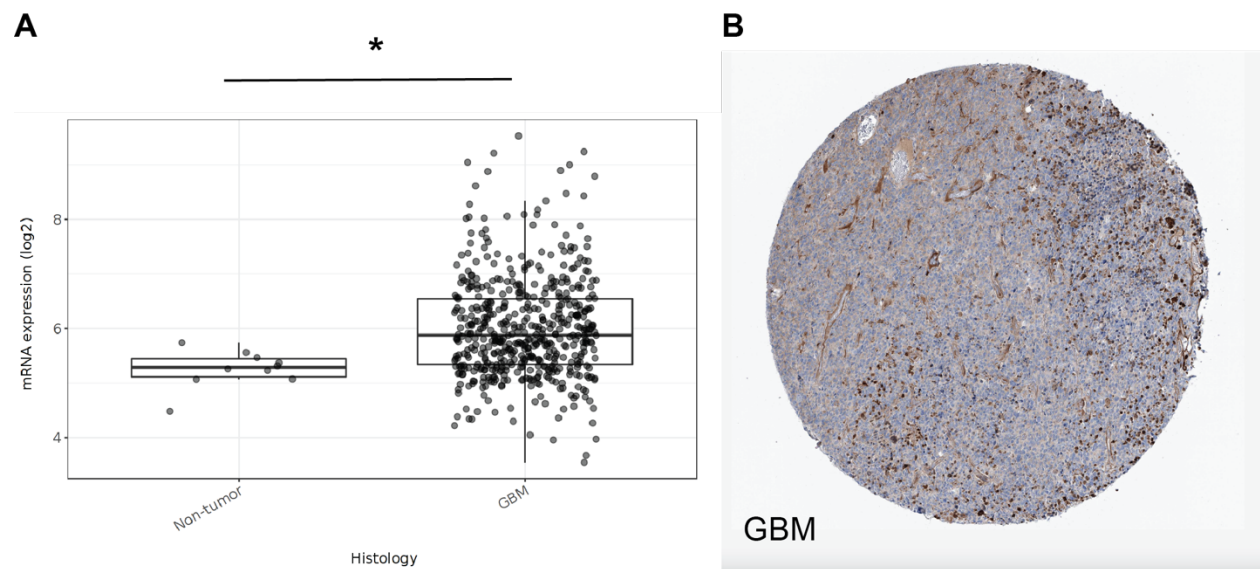


Figure 9: mRNA levels of CDH5 (GlioVis: TCGA_GBM) and CDH5 GBM pathology (Human Protein Atlas)

(A) mRNA levels of CDH5 (VE-cadherin) in TCGA_GBM. Tukey's Honest Significant Difference (HSD) test, * adj p-value = 0.01³⁸; (B) Human Protein Atlas GBM pathology showing extensive CDH5 (VE-cadherin, indicated in brown) staining³⁹.

3.2 Kinetic analysis of gene transcription revealed sustained downregulation of VE-cadherin and suppression of two ETS family transcriptional factors ETV1 and ERG

KEGG pathway analysis of the RNA-sequencing data suggested the involvement of PI3K/AKT signaling pathway and MAPK signaling pathway in BIA-induced modulation of endothelial permeability (**Figure 6C, 8A**). For instance, PI3K/AKT and MAPK pathways are thought to participate in angiopoietin-1 (Ang-1)-induced endothelial cell survival, proliferation, migration, and adhesion⁴⁰. Transcription factors downstream of these two signaling pathways could be a potential mechanism in which the transcription of endothelial junctional proteins, particularly VE-cadherin, was hampered upon BIA treatment, leading to vascular permeability. Two members of the ETS family transcription factors, ETS translocation variant 1 (ETV1) and ETS-related gene (ERG), appeared as possible candidates, with a log2FC of -2.22 (adj p-value = 1.12×10^{-30}) and a log2FC of -2.00 (adj p-value = 5.74×10^{-79}) respectively in the RNA-seq dataset (**Figure 10A**).

ETS family transcription factors are important in endothelial development, angiogenesis, contact inhibition and other endothelial functions⁴¹⁻⁴⁴. The RAS-RAF-MEK-MAPK pathway (**Figure 10D**) is considered as the main mechanism for the activation of the ETS family transcription factors, which all contain common binding domains (**Figure 10C**) including several MAPK interaction motifs^{45,46}. ERG has been demonstrated to bind ETS binding sites on the VE-cadherin promoter (**Figure 10E**) and is also a transcriptional activator of several other endothelial cell-specific genes including endoglin, von Willebrand factor (VWF), intercellular adhesion molecule (ICAM)-2 and claudin-5 to regulate vascular permeability⁴⁷⁻⁴⁹. ERG can promote vascular

stability, in part via PI3K/AKT-dependent Ang-1/Tie-2 signaling⁵⁰. The overexpression of ETS family transcription factors is also implicated in the tumorigenesis of many cancer types⁴⁶. For instance, in prostate cancer, chromosomal rearrangement involving the fusion of the androgen receptor (AR) response gene TMPRSS2 and ETS transcription factors and cooperation with AR during transcription activation, result in ETS transcription factor gene overexpression and lead to prostate neoplastic development^{40,46}.

A kinetic analysis of the gene transcription of ETV1, ERG, VE-cadherin (gene name: CDH5) and another junctional protein ZO-1 (gene name: TJP1) was conducted to validate the RNA-sequencing results and provide insights into ETV1 and ERG function.

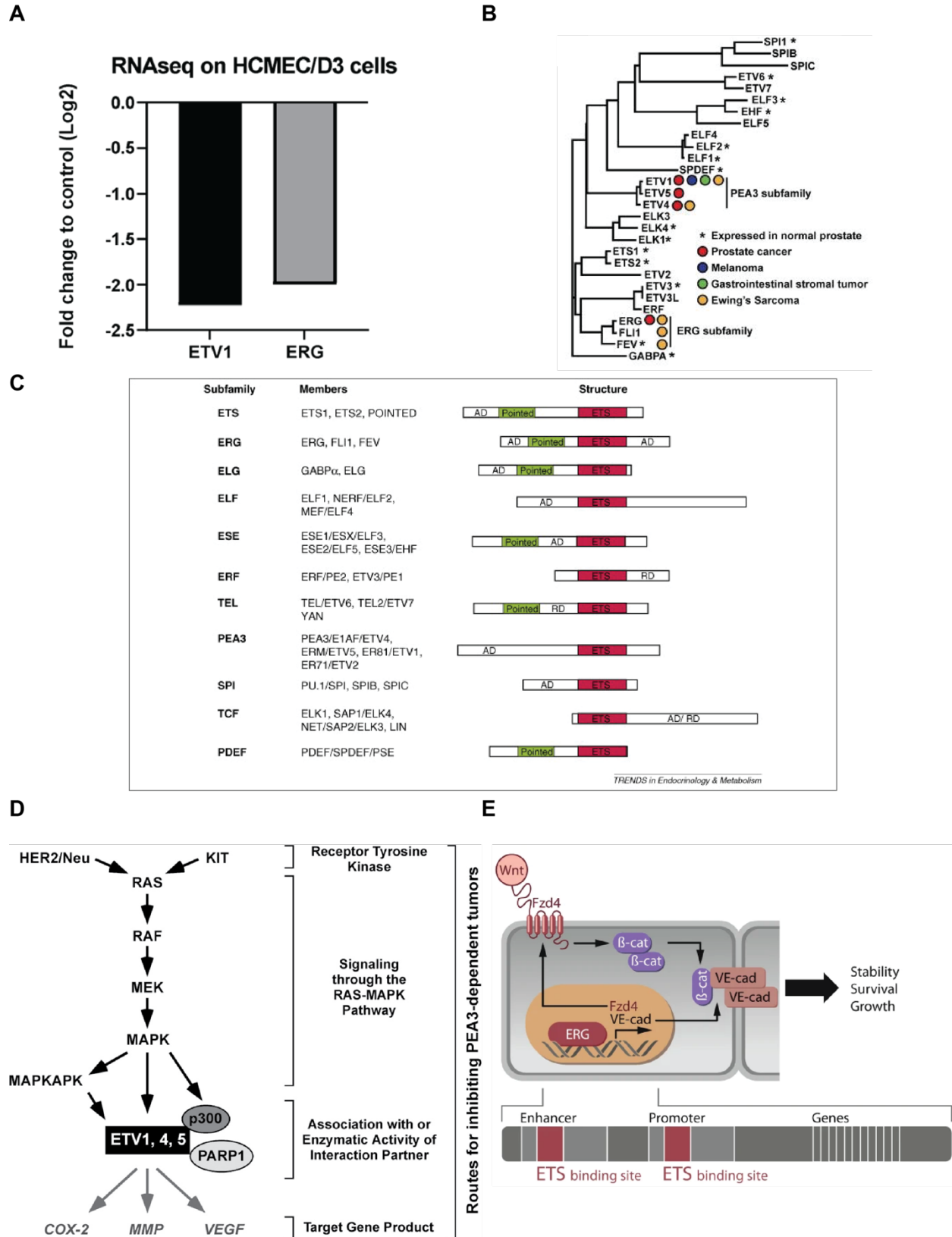


Figure 10: ETV1 and ERG downregulation by BIA, their sequence and signaling pathway.

(A) RNA-sequencing revealed significant reduction in ETV1 and ERG gene transcription, $\log_2FC \sim 2$ ($N=3$); (B) Phylogeny trees of ETS family transcription factors and their involvement in human diseases⁵¹; (C) Conserved binding domains shared across ETS family members⁵²; (D) Activation of ETV1, 4, 5 and ETV1, 4, 5-mediated gene transcription via MAPK pathway⁴⁶; (E) ERG can initiate VE-cadherin transcription by binding to its ETS binding sites on the promoter⁴⁷.

Figure 11 shows the relative mRNA expression of ETV1, ERG, CDH5 and TJP1 in cultured hCMEC/D3 cells, the same cell type used for the RNA-sequencing experiment. There was a pronounced reduction in the number of CDH5 transcripts, starting as early as only 3hr post 1 μ M BIA treatment. The inhibition of VE-cadherin gene transcription was significant and time-dependent, as longer incubation with BIA resulted in greater reduction. 24hr BIA treatment reduced VE-cadherin transcription to only 10% of the level in vehicle DMSO control (**** adj p-value < 0.0001). Such reduction was maintained at similar level when hCMEC/D3 cells were treated for an additional 24hr (**** adj p-value < 0.0001). In contrast to VE-cadherin, gene expression of ZO-1 was not time-dependently altered by BIA, despite of a 30-70% reduction. Only at 24hr treatment, downregulation in ZO-1 to 30% of the control level, was detected as significant (** adj p-value = 0.004). Because ZO-1 is a scaffolding protein which interacts with many junctional complex proteins, is not considered as indispensable in junctional complex formation in literature, and was not a DEG in RNA-seq, other junctional proteins such as claudin-5 might be better candidates for assessing junctional complex integrity, in addition to VE-cadherin.

As shown in **Figure 11**, suppression of ETV1 gene expression reached the maximum (80% reduction) when hCMEC/D3 cells were treated with BIA for 12hr (**** adj p-value < 0.0001). Interestingly, a gradual recovery of ETV1 gene expression was observed afterwards. 48hr BIA

treatment resulted in 60% downregulation of ETV1 gene expression (***) adj p-value = 0.0001).

ERG, on the other hand, showed a reduction in its transcriptional level upon BIA in a time-dependent manner, such that longer BIA treatment resulted in greater downregulation in ERG transcription. 48hr BIA treatment reduced ERG gene transcription to 20% of the control level (**** adj p-value < 0.0001).

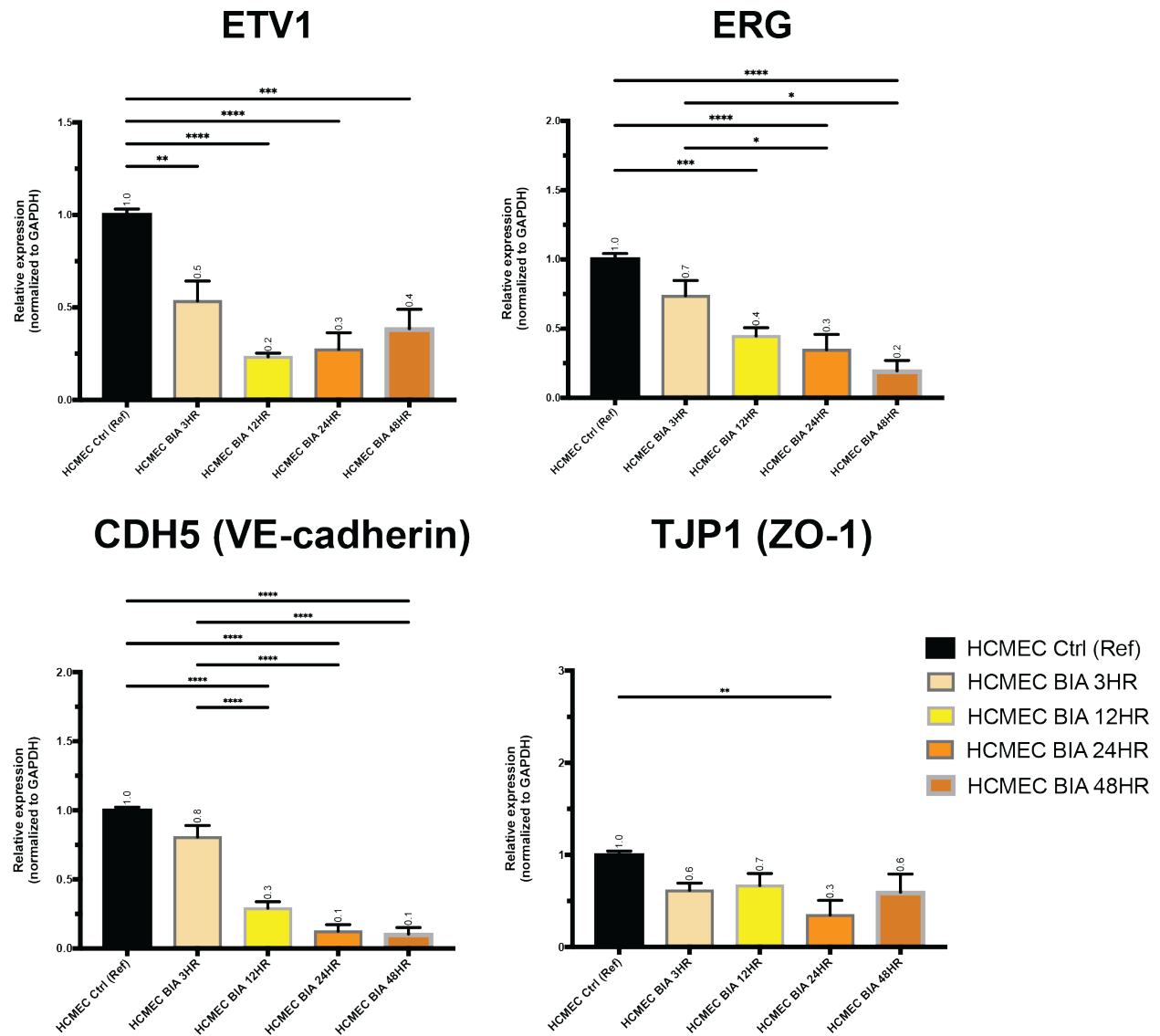


Figure 11: RT-qPCR of ETV1, ERG, CDH5 and TJP1 gene expression by 3hr, 12hr, 24hr or 48hr 1μM BIA treatment in hCMEC/D3 cells.

Ordinary one-way ANOVA with Tukey *post hoc* test (N = 3 culture replicates from two independent experiments).

Another cell culture model HBMEC, was also used to further validate our findings. There appeared to be a cell line difference in terms of the sensitivity to transcriptional disturbance induced by BIA, and/or possibly a cell-line specific transcriptome profile. Overall, BIA treatment generated similar patterns of VE-cadherin, ZO-1, ETV1 and ERG transcriptional inhibition in HBMEC cells (**Figure 12**). Time-dependent reduction in VE-cadherin expression was observed, although to a lesser degree, with only 60% reduction after 48hr BIA treatment (ns = not significant). Downregulation in ZO-1 expression was also detected at a non-significant level. A surprising induction of ZO-1 gene expression (* adj p-value = 0.0116) occurred at 24hr of BIA treatment time point and was consistently observed between two independent experiments, and therefore unlikely to be an artifact. However, its significance is unclear until proven otherwise by screening at additional time points of BIA treatment or other proteins involved in ZO-1 gene transcription.

As shown in **Figure 12**, BIA treatment for 3hr, 12hr or 48hr all induced a significant 50% reduction in ETV1 gene expression (3hr ** adj p-value = 0.0022; 12hr ** adj p-value = 0.0017; 48hr * adj p-value = 0.0117). Similar to ZO-1, a recovery of mRNA transcript level at 24hr was also observed for ETV1. Consistent with the findings from hCMEC/D3 cells (**Figure 11**), a time-dependent decrease in ERG transcription was noted, but this difference did not reach statistical significance.

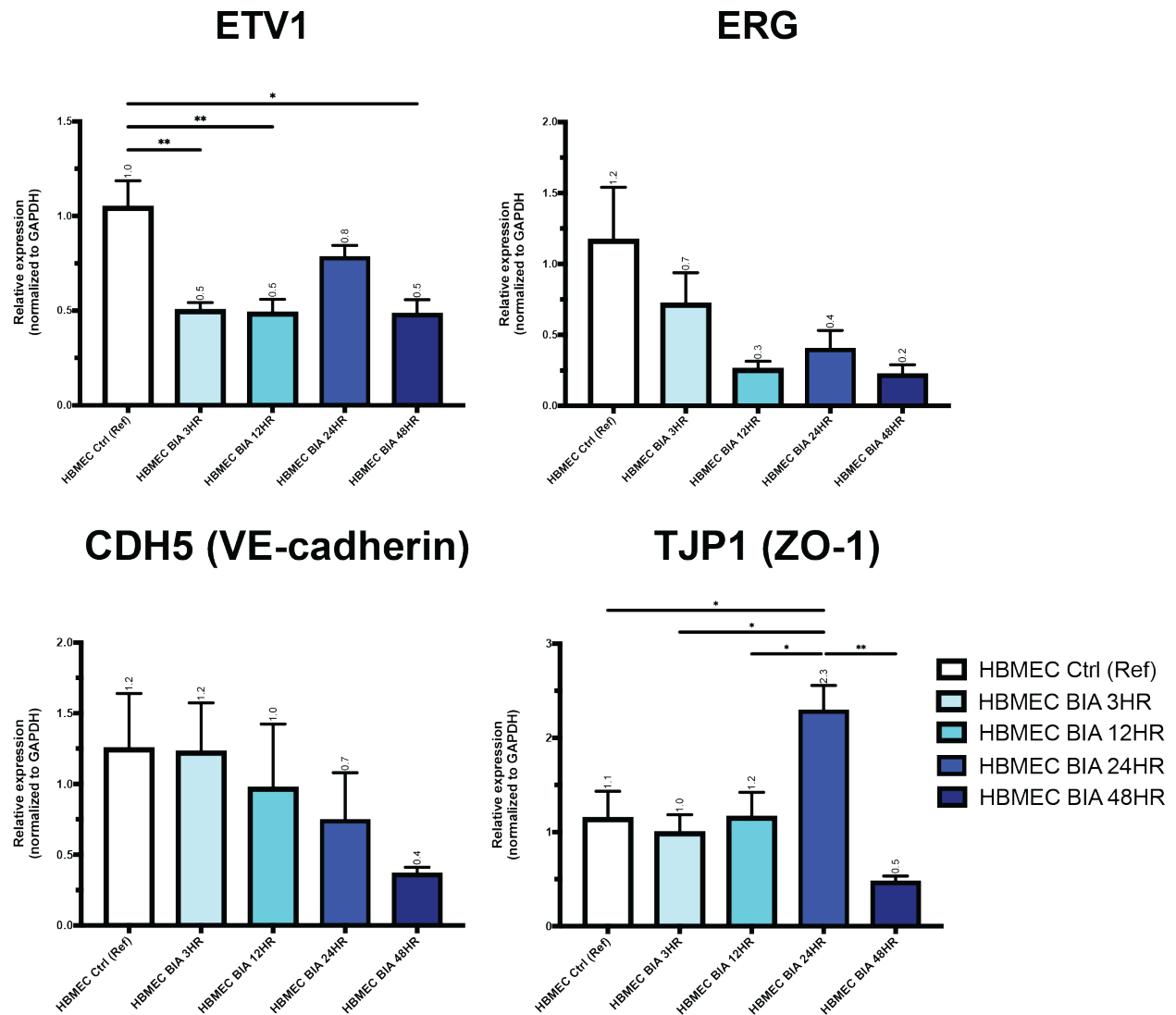


Figure 12: RT-qPCR of *ETV1*, *ERG*, *CDH5* and *TJP1* gene expression by 3hr, 12hr, 24hr or 48hr 1 μ M BIA treatment in HBMEC cells.

Ordinary one-way ANOVA with Tukey *post hoc* test (N = 3 culture replicates from two independent experiments).

3.3 Detection of BIA-induced VE-cadherin, ETV1 and ERG protein reduction

Detection of protein-level alteration of endothelial junctional proteins such as VE-cadherin is necessary to deduce functional inhibition of endothelial barrier formation. **Figure 13A** shows Western blot of junctional proteins ZO-1, CD31 and VE-cadherin, as well as the loading control Histone-H3. Nude female mice with G34-pCDH tumor were either treated with BIA (20mg/kg, i.p.) or vehicle control on day 9 or 10 post-tumor implantation and sacrificed for tumor (identified by GFP fluorescence and/or the anatomical coordinates of implantation) and the contralateral healthy brain sample collection 24hr later. G34-pCDH tumor cells were GFP-labeled. BIA treatment almost completely abolished the expression of VE-cadherin, ZO-1 and CD31 at the protein level, except for BIA mouse #3 (on the far right) which carried a huge tumor with prominent blood clots. BIA#3 was likely a non-responder to BIA treatment. Considering the tumor size, a higher dose might be required to produce any therapeutic effect on BIA#3. Comparing tumor with healthy brain from the same mouse, VE-cadherin seemed to be overexpressed in tumor, consistent with human biopsy results from TCGA GBM data repository (**Figure 9A**). The expression pattern of ZO-1 and CD31 in tumor vs. healthy brain was less clear. This in part, was attributed to the uneven loading and/or transfer of lysate samples, as evident in the varying amount of loading control Histone-H3 across samples. This experiment needs to be repeated when Western blot technical issues can be resolved. Further investigation is also required to understand the specificity of BIA on modulating endothelial permeability at the BTB vs. the BBB. The downregulation of junctional proteins due to BIA seemed to occur in both tumor (BTB) and healthy brain (BBB). Naïve mice treated with BIA or vehicle control should be included for baseline comparison in future studies, especially considering that the

contralateral healthy brain regions might already have an altered phenotype induced by tumor signaling.

Cultured hCMEC/D3 cells treated with 1 μ M BIA or vehicle control for 24hr displayed a strong reduction of VE-cadherin, ETV1 and ERG protein levels (**Figure 13B**), consistent with their gene level reduction upon BIA treatment. *In vivo* samples should be used to validate this finding.

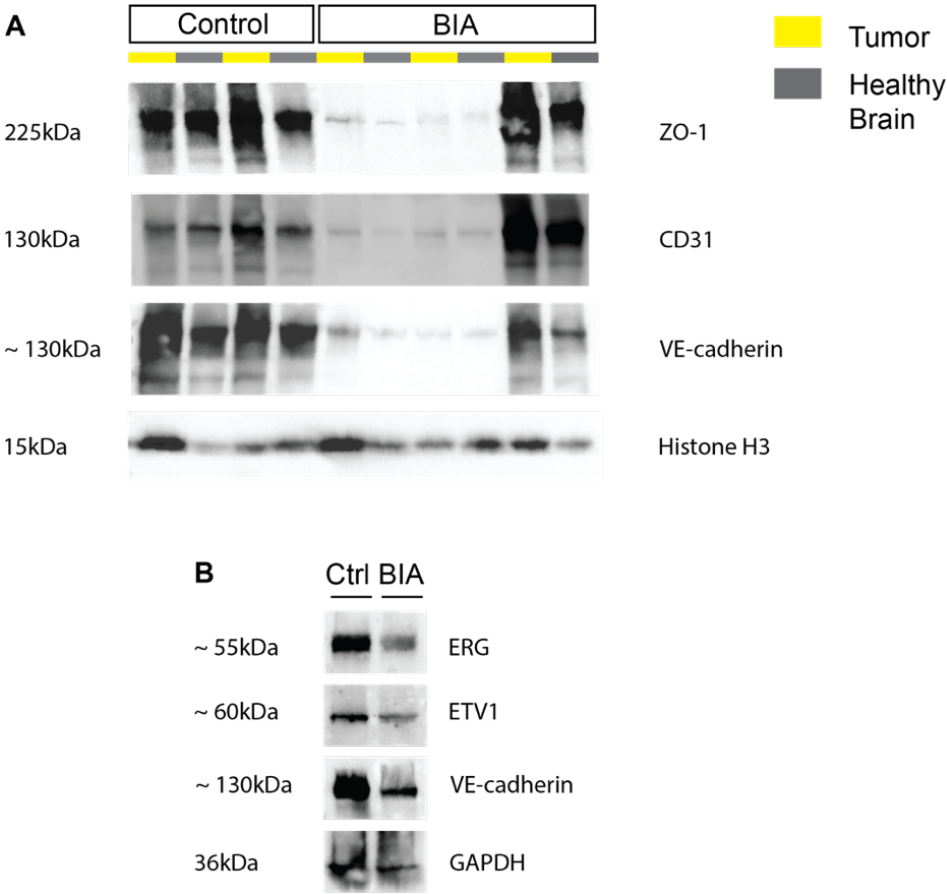


Figure 13: Western blot detected BIA-induced reduction of junctional proteins (VE-cadherin, ZO-1, CD31 – also an endothelial marker), ETV1 and ERG at the protein level

(A) G34-pCDH xenograft murine tissue samples from tumor vs. contralateral healthy brain (the adjacent two samples were from the same mouse) harvested at 24hr after BIA treatment

(20mg/kg, i.p.) or vehicle control; (B) lysates from hCMEC/D3 cells treated with either 1 μ M BIA or vehicle control for 48hr.

Alteration of VE-cadherin and ZO-1 protein levels after BIA treatment was further supported by immunofluorescence staining of fixed hCMEC/D3 cells (treated with either 2 μ M BIA or vehicle control for 48hr) on glass chamber slides and G34-pCDH xenograft murine brain cryosections (treated with either 20mg/kg BIA or vehicle control, i.p.).

BIA-treated hCMEC/D3 cells showed no intercellular expression of VE-cadherin (**Figure 14**). Administration of BIA *in vivo* resulted in a similar loss of VE-cadherin protein expression in the brain endothelium (**Figure 15**). The common endothelial marker CD31 was used to visualize the endothelium. Although CD31 expression was downregulated by BIA (**Figure 13A**), there was still a detectable amount, sufficient for endothelium visualization. As shown in **Figure 15**, individual blood vessels were clearly visualized by CD31 staining (displayed in blue). White arrows displayed in **Figure 15C, F**, indicate areas of co-localization for CD31 and VE-cadherin (magenta). A significant decrease in VE-cadherin signal in the tumor periphery and intratumorally was observed in BIA treated samples. ZO-1 protein expression in the brain endothelium (**Figure 16**) was also downregulated in BIA treatment samples, consistent with the Western blot result from *in vivo* samples (**Figure 13A**). ETV1 and ERG visualization in *in vivo* samples treated with BIA or vehicle control is underway to inform about their role in BIA-induced vascular permeability.

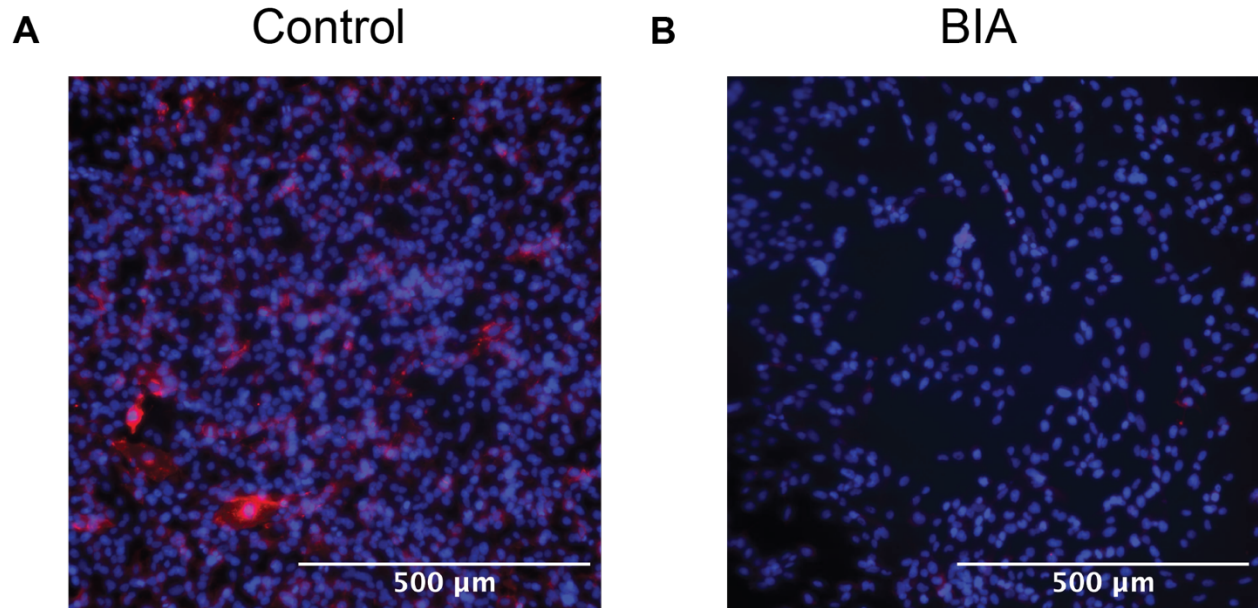


Figure 14: *BIA diminished VE-cadherin protein expression at hCMEC/D3 cell-cell junction*

hCMEC/D3 cells were treated with 2 μ M BIA or vehicle control for 48hr before fixation and staining. Nuclei stained by Hoechst 33342 (blue) and VE-cadherin-AF649 (red). Scale bar = 500 μ m. VE-cadherin was minimally detected in BIA treated sample.

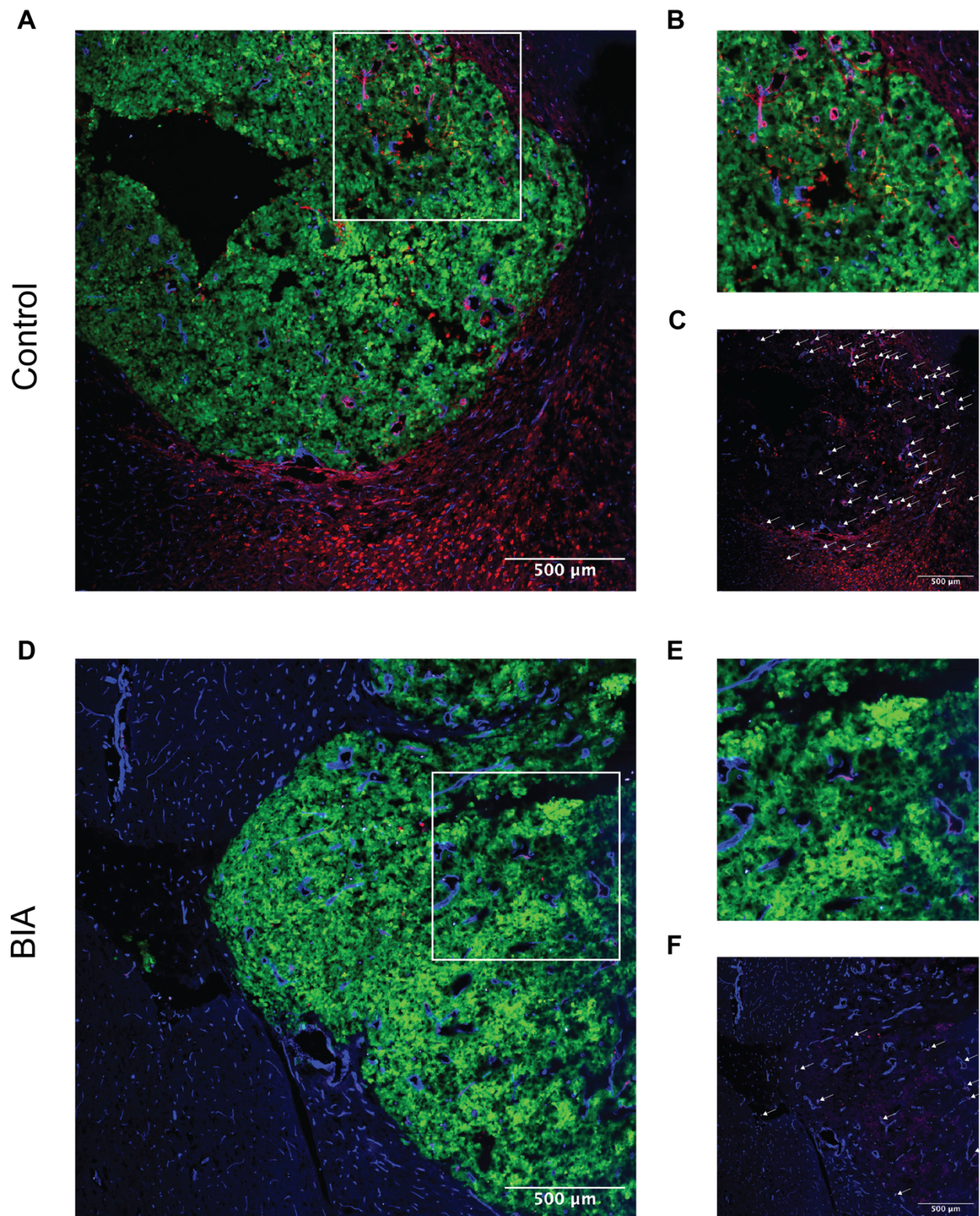


Figure 15: G34-pCDH xenograft murine brain samples showed reduced VE-cadherin expression on endothelial cells 24hr after BIA treatment (20mg/kg, i.p.)

(A-C) Vehicle control treated murine brain cryosection stained for endothelial marker CD31-AF405 (blue) and VE-cadherin-AF594 (red); (D-F) BIA (20mg/kg, i.p.) treated murine brain cryosection stained for the same proteins. Samples all harvested at 24hr post-drug treatment. G34-pCDH tumor cell were GFP-labeled (green). (A, E) complete tiled image taken with 20x objective; (B, E) zoom-in view of the white-squared area in A and E respectively; (C, F) complete tiled image take at 20x objective showing only CD31 and VE-cadherin. White arrows indicated areas of co-localization for CD31 and VE-cadherin (magenta). Scale bar = 500 μ m.

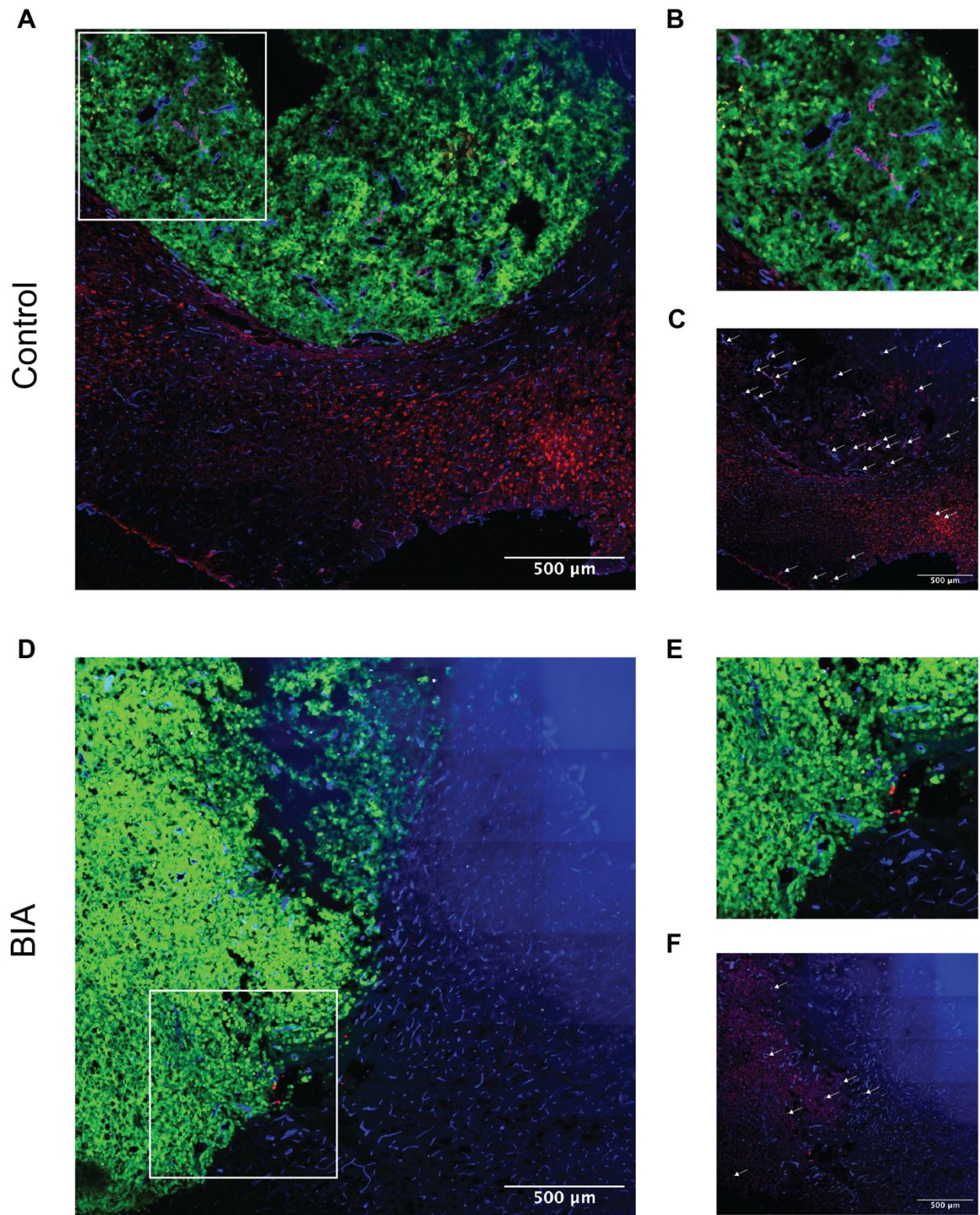


Figure 16: G34-pCDH xenograft murine brain samples showed reduced ZO-1 expression on endothelial cells 24hr after BIA treatment (20mg/kg, i.p.)

(A-C) Vehicle control treated murine brain cryosection stained for endothelial marker CD31-AF405 (blue) and ZO-1-AF594 (red); (D-F) BIA (20mg/kg, i.p.) treated murine brain cryosection stained for the same proteins. Samples all harvested at 24hr post-drug treatment. G34-pCDH tumor cell were GFP-labeled (green). (A, E) complete tiled image taken with 20x objective; (B, E) zoom-in view of the white-squared area in A and E respectively; (C, F) complete tiled image take at 20x objective showing only CD31 and ZO-1. White arrows indicated areas of co-localization for CD31 and ZO-1 (magenta). Scale bar = 500 μ m.

3.4 Validation of increased endothelial permeability by BIA treatment using TRITC-dextran and sodium fluorescein

As described in the background section, quantification of cisplatin uptake was used to assess vascular permeability at the BBB vs. BTB. A second method to measure vascular permeability *in vivo* could further support the hypothesis of differential modulation on the tumor vasculature vs. normal BBB by BIA treatment. A common BBB permeability tracer used for the measurement of paracellular leakage is dextran, available at different molecular weight⁷. We first used 70kDa TRITC-dextran, a version of dextran conjugated to a TRITC fluorophore. **Figure 17** shows visualization of TRITC-dextran in the brain from either BIA- (20mg/kg, i.p.) or vehicle control-treated mice bearing G9-pCDH tumor. Very little TRITC-dextran was detected by fluorescence microscopy as indicated by white arrows. There appears to be more TRITC-dextran in BIA-treated tumor/brain sample (# of white arrows = 20), as compared to control (# of white arrows = 14). However, some yellow dots appeared in clusters, making it very difficult to objectively quantify the amount of dextran. In addition, the sample from the BIA-treated mouse had a much smaller tumor as compared with control (**Figure 17A, D**). Varying tumor size can also confound the comparison.

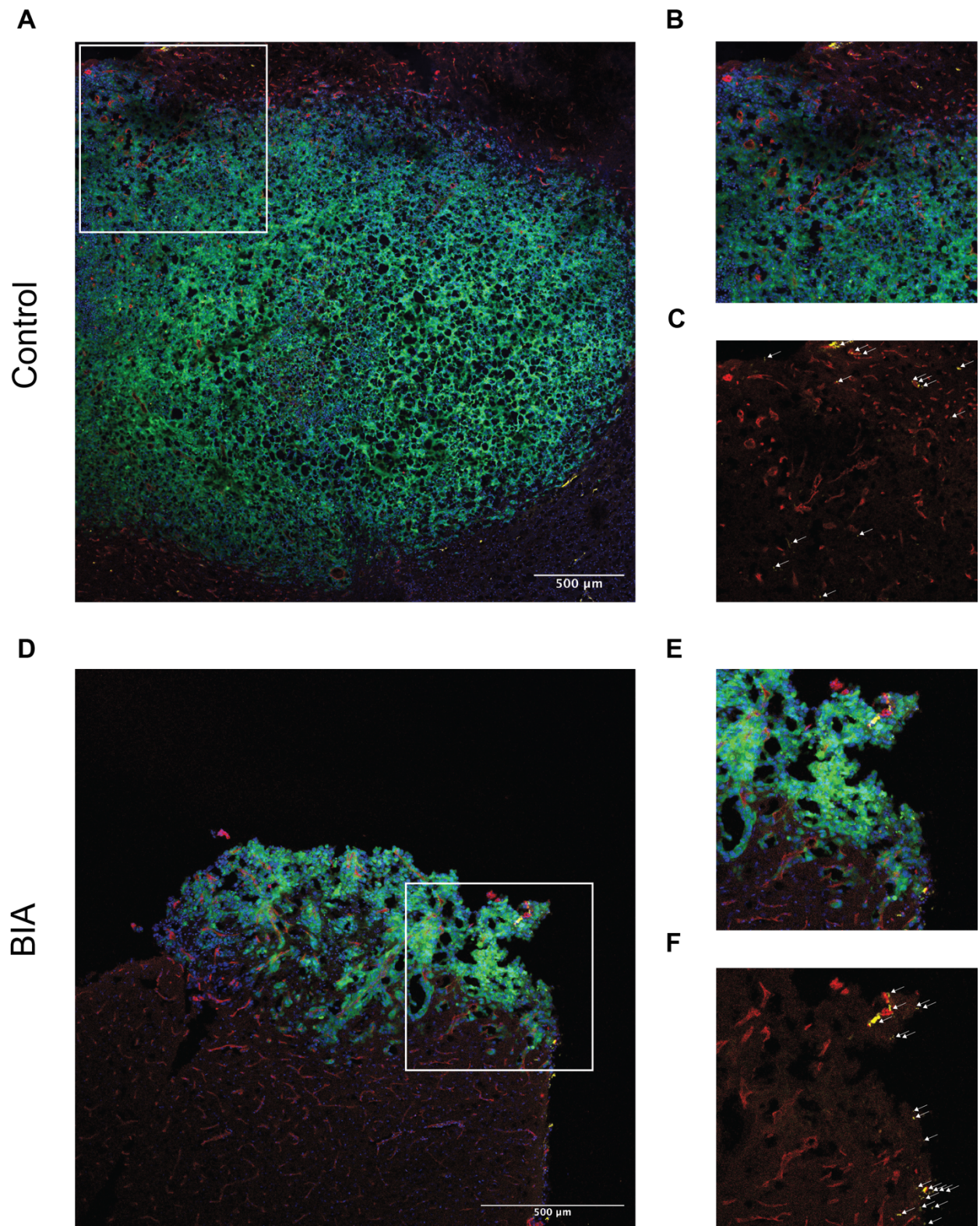


Figure 17: Immunofluorescence visualization of TRITC-dextran on G9-pCDH xenograft murine brain tissues

(A-C) Vehicle control treated murine brain cryosection stained for nuclei-Hoechst33342 (blue) and endothelial marker CD31-AF647 (red); (D-F) BIA (20mg/kg, i.p.) treated murine brain cryosection stained for the same proteins. Samples all harvested at 24hr post-drug treatment. G9-pCDH tumor cell were GFP-labeled (green). TRITC-dextran was displayed in yellow color. (A, E) complete tiled image taken with 20x objective; (B, E) zoom-in view of the white-squared area in A and E respectively; (C, F) zoom-in view of the white-squared area in A and E respectively, showing only TRITC-dextran and CD31. White arrows indicated areas of TRITC-dextran penetration through the BTB/BBB (yellow). Scale bar = 500 μ m.

Because of the difficulty in TRITC-dextran visualization, we sought an alternative tracer for barrier integrity measurement - sodium fluorescein (NaFl) (MW = 0.4kDa)^{7,19}. Pilot experiment with NaFl in the G30-LRP xenograft murine model supported its potential utility (**Figure 18**). G30-LRP tumor cells were implanted instead of G9-pCDH or G34-pCDH because they were not labeled with GFP. The maximal absorbance wavelength of NaFl is at 479nm and emission peak at 538nm; NaFl can also generate fluorescence in the GFP spectrum⁵³. Maximal intensity of NaFl in the brain can be detected 30min after its administration (20mg/kg, i.v.)¹⁹. Based on prior literature, we injected 20mg/kg NaFl i.v. on G30-LRP xenograft mice 24hr after BIA (20mg/kg, i.p.) or vehicle control treatment, and 30min later, collected entire brain and liver as a control. **Figure 18A** shows a significant increase in NaFl uptake in tumor following BIA treatment (** adj p-value = 0.007317), whereas no difference was observed in healthy brain regions or liver control, using pixel quantification of IVIS captured images in the GFP spectrum. The absolute amount of NaFl in tumor vs. healthy brain (ng per mg) was also quantified (**Figure 18C**). There was a trend for an increase in NaFl accumulation in BIA-treated tumor samples and a reduction

in NaFl quantity in BIA-treated healthy brain samples, as compared to control (ns = not significant). Additional healthy brain and liver samples were collected from naïve mice at 1hr post-NaFl administration time-point. The average ng of NaFl per mg of tissue in the healthy brain was the same for BIA-treated mice and control mice, despite of a non-significant increase in NaFl penetration in the BIA-treated liver (**Figure 18D**). The difference in NaFl levels between healthy brain of naïve mice (ng NaFl / mg tissue = 0.4, harvested at 1hr post NaFl) and healthy brain of xenograft mice (ng NaFl / mg tissue = 0.6 – 0.7, harvested at 30min post NaFl) might simply be a result of the pharmacokinetics of NaFl. Based on prior literature, the peak intensity of NaFl was detected at 30min post-administration followed by a gradual decrease in signal¹⁹. Further experiments should be performed to validate these findings, and analysis of NaFl signal by spectrometry method should be optimized and repeated. Although IVIS analysis suggested statistical significance in NaFl uptake in tumor samples after BIA treatment, IVIS quantification relies on one-dimensional image capture and is rather relative. Nevertheless, taken together, detection of vascular permeability by measuring NaFl uptake appears to be promising. More importantly, BIA has consistently demonstrated its ability to potentiate the penetration of compounds into GBM tumors.

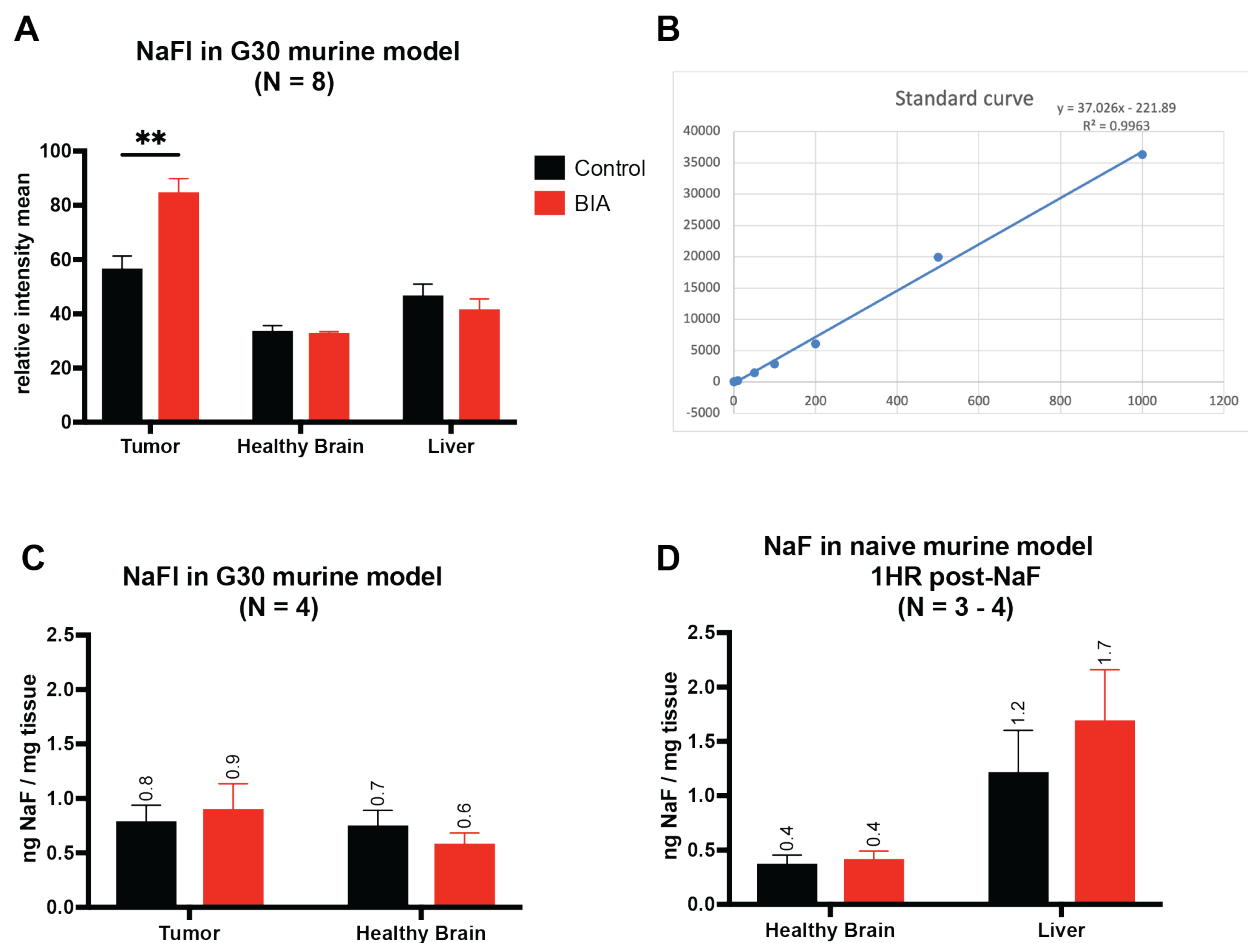


Figure 18: Sodium fluorescein (NaFl) uptake quantification by IVIS and spectrometry

(A) Nude mice with G30-LRP xenograft were treated with either BIA (20mg/kg, i.p.) or vehicle control on day 9 or 10. 24hr following drug treatment, experimental animals were given 20mg/kg NaFl i.v., and 30min later, sacrificed for organ collection. Relative pixel quantification from IVIS captured images using Fiji ImageJ (N = 8); (B) standard curve of NaFl (C) Normalized ng NaFl per mg tissue measured by fluorescence spectrometry (N = 4); (D) Exact same experimental protocol, except that mice were naïve (no tumor implantation) (N = 3 in control and N = 4 in BIA). Multiple unpaired t-test with Holm-Šídák correction.

3.5 Functional investigation of ETS transcription factors as mediators of BIA-induced endothelial permeability

To clarify the role of ETS transcription factors ETV1 and ERG in BIA-mediated endothelial permeability demonstrated by a loss of the expression of VE-cadherin and possibly other junctional proteins, pilot experiments with these two transcription factors inhibited either pharmacologically or using siRNA were performed.

A commercially available small molecule inhibitor for ETV1 is BRD32048 (**Figure 19A**). BRD32048 acts by inhibiting p300-facilitated acetylation which is supposed to increase ETV1 stability in conjunction with MAPK-initiated phosphorylation, leading to faster ETV1 degradation and reduction in ETV1 transcriptional activity^{46,54}. As shown in **Figure 19B**, BRD32048 was tested at a 1 μ M, 10 μ M and 50 μ M in hCMEC/D3 barrier formation ECIS assay. Positive control BIA at 1 μ M showed a statistically significant inhibition of barrier formation (**** adj p-value < 0.0001). However, BRD32048 at 1 μ M or 10 μ M was unable to produce a physiological effect. The significant disruption of barrier formation at 50 μ M of BRD32048 was likely attributed to its cytotoxicity, as cells appeared to be less healthy. No reduction in ETV1 protein level was observed for any of the tested BRD32048 concentration (1 μ M, 10 μ M or 30 μ M) at 24hr or 48hr treatment time point (**Figure 19C**). The difference in protein amount between 24hr- and 48hr-treated samples, as demonstrated by Histone-H3 abundance, was likely a technical issue resulting from inconsistent transfer quality between different Western blot transfer chambers. The blot for 48hr samples contained unevenly loaded samples rendering any interpretation difficult. Regardless of its inability to affect ETV1 protein level, BRD032048

appeared to reduce CD31 protein level at a dose-dependent manner, and likely ZO-1 protein level at 30 μ M concentration (24hr time point) as well. Immunoblotting for VE-cadherin was also performed but unfortunately samples were lost due to membrane cutting at inadequate band size prior to antibody incubation. It is possible that BRD32048 inhibited other ETS family transcription factors which share many common binding domains with ETV1 (**Figure 10C**), resulting in a downregulation of endothelial cell junctional protein CD31 and possibly ZO-1 at higher concentration.

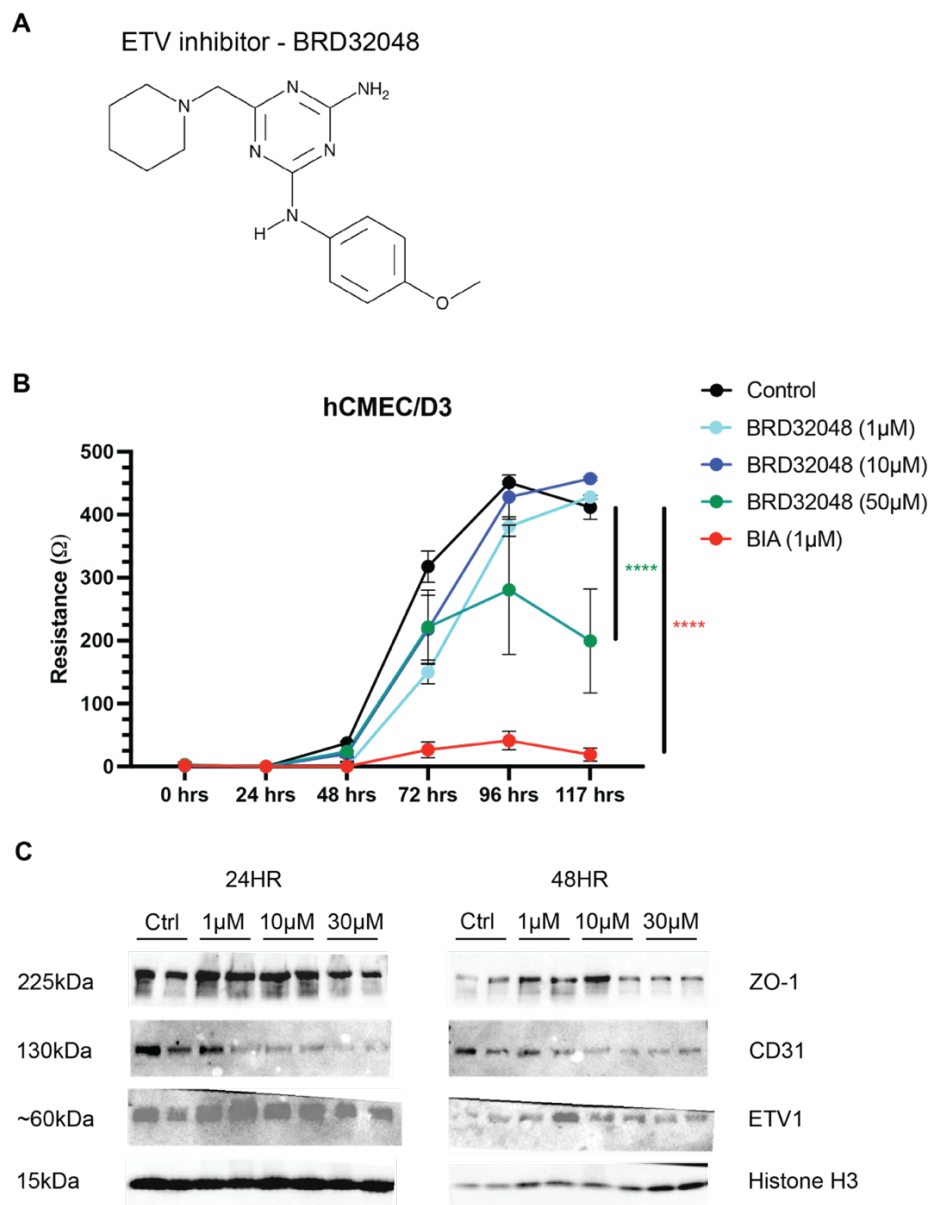


Figure 19: ECIS analysis of ETV1 inhibitor BRD32048 on hCMEC/D3 endothelial permeability and Western blot verification

(A) Chemical structure of BRD32048; (B) ECIS assay showing consistent inhibition of $1\mu\text{M}$ BIA (**** adj p-value < 0.0001) and no effect from BRD32048 treatment at $1\mu\text{M}$ or $10\mu\text{M}$ on cultured hCMEC/D3 cells. $50\mu\text{M}$ BRD32048 had a significant effect (**** adj p-value < 0.0001) and cells had unhealthy morphology. RM Two-way ANOVA with Tukey *post hoc* test. Significance symbols indicated for the last time point - 117hr (N = 2); (CA) Western blot of ZO-1, CD31 and ETV1 of lysates from cultured hCMEC/D3 cells treated with BRD32048 for 24hr or 48hr at $1\mu\text{M}$, $10\mu\text{M}$, $30\mu\text{M}$ or vehicle control.

Considering the lack of specificity of BRD32048 and the unavailability of commercial ERG antagonists, we turned to an siRNA approach to inhibit ETV1 and ERG protein expression. hCMEC/D3 cells were transfected with siRNA against ETV1, ERG or scramble siControl for 48hr and collected for lysates to detect any protein level changes of target protein ETV1 or ERG. hCMEC/D3 cells plated on ECIS arrays were also transfected with siRNA against ETV1, ERG, scramble siControl, with the addition of ETV+ERG combo, to test barrier properties. **Figure 20A-B** shows that siERG was unable to knockdown ERG protein expression, nor inhibit barrier formation. **Figure 20C-D** shows a partially disrupted vascular barrier (ns = not significant) and a possible reduction in ETV1 protein level by siETV1. The possible reduction in the electrical impedance in the siETV1+siERG combo condition, was likely driven by siETV1, as the change in resistance followed the siETV1 curve and siERG did not perturb ERG expression. The lysates in **Figure 20D** were run on the same Western blot, but the image was cropped and stitched back together to remove other irrelevant samples in-between the siControl and siETV1 samples to

avoid confusion. There continued to be technical difficulties with Western blot. The HRP-conjugated secondary antibodies seemed to be falling and resulted in low signal in chemiluminescence visualization. VE-cadherin protein level seemed to be unaffected by ETV1 knockdown, while ZO-1 expression appeared to decrease.

Overall, attempts to inhibit ETV1 and ERG transcriptional activity have not yet yielded conclusive results. Additional experiments with siRNA or shRNA, as well as Western blot and immunofluorescence staining on *in vivo* BIA treated samples should be pursued.

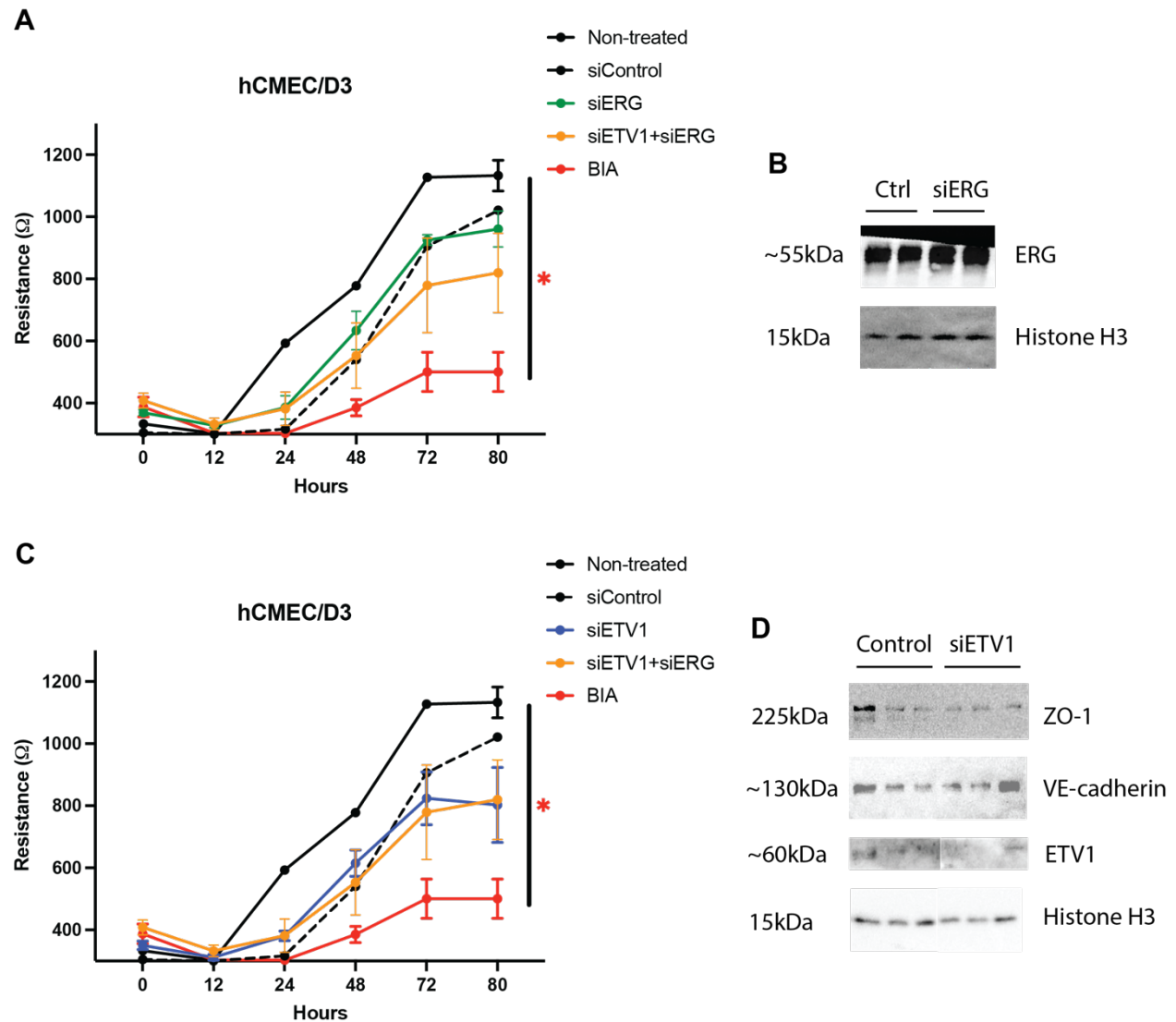


Figure 20: ECIS analysis of siETV1 and siERG on hCMEC/D3 endothelial permeability and Western blot verification

(A, C) ECIS. RM Two-way ANOVA with Tukey *post hoc* test. Significance symbols indicated for the last time point – 80hr (N=2); (B) Western blot of lysates from hCMEC/D3 cells transferred with ERG siRNA (N=2); (C) Western blot of lysates from hCMEC/D3 cells transferred with ETV1 siRNA (N=3).

3.6 Phospho-kinase array revealed Yes, p38 α , STAT2 and STAT5 α/β as potential candidates mediating endothelial permeability

The human phospho-kinase array has been used as a screening tool to identify kinases and signaling pathways involved in human diseases and therapeutic response⁵⁵⁻⁵⁹. The indirubin derivative BIA, a known inhibitor for GSK3, is speculated to be a non-specific kinase inhibitor with binding interactions in a similar manner to indirubin¹⁸. **Figure 21** indicates a broad-spectrum change in the phosphorylation status with BIA treatment. 1 μ M BIA treatment on hCMEC/D3 cells for 48hr resulted in a reduced level of phospho-proteins for most of the targets, indicating a general inhibition of kinase signaling. **Table 5** shows a summary of all included protein targets, with literature reviewed annotation on their class (kinase or substrate), major signaling pathway membership (src, MAPK, PI3K/AKT/mTOR, cell cycle/p53 or JAK-STAT), and the function of the specified phosphorylation sites (activation or inhibition). Lower levels of phosphorylated GSK3 α /GSK3 β proteins at S9 and S21 residues were detected in BIA condition. This effect is likely to be an indirect result from BIA inactivating other kinases that can phosphorylate GSK3 at these sites and inhibit GSK3 signaling.

Statistically significant reductions in phosphorylated STAT2 (**** adj p-value < 0.0001), Yes (** adj p-value = 0.002103), P38 α (also known as p38MAPK or MAPK14) (** adj p-value = 0.004632) and STAT5 α /STAT5 β (** adj p-value = 0.006686) were observed (**Figure 21B**).

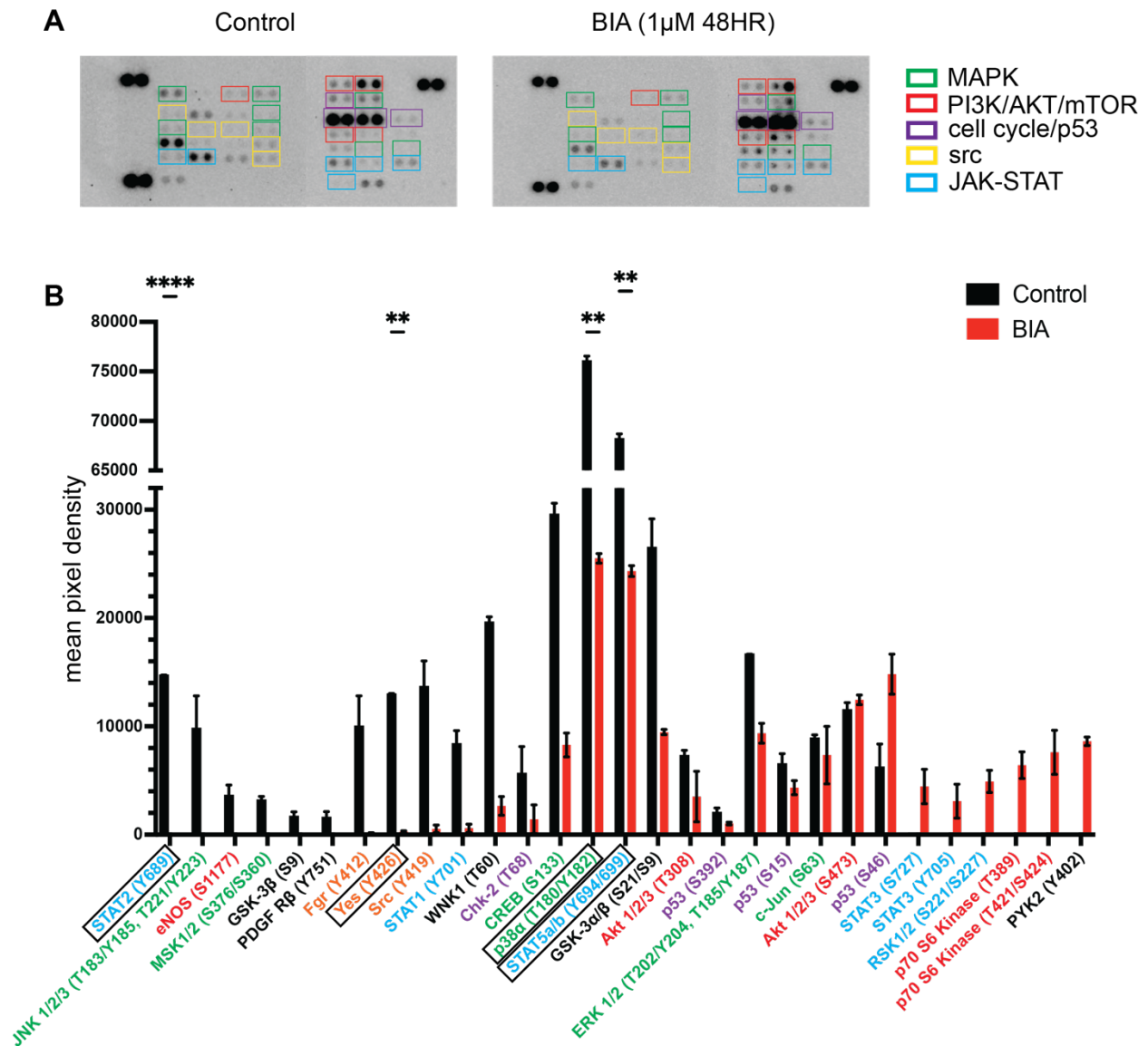


Figure 21: Detection of broad kinase activity perturbation by BIA in hCMEC/D3 cells

(A) Images of antibody array for hCMEC/D3 cells treated with 1 μ M BIA or vehicle control for 48hr. Expression of each phospho-antibody was shown in duplicates (N = 2); (B) Relative phospho-protein expression quantified by pixel intensity measurement of the antibody array images. Undetected proteins were not displayed. Phospho-proteins with statistically significant alteration by BIA were highlighted by a black box on the x-axis and statistical symbols on the bar plot. Data were displayed in the order of proteins with highest downregulation in

phosphorylation on the far left to proteins with highest upregulation in phosphorylation on the far right, upon BIA treatment (red bars). Multiple unpaired t-test with Holm-Šídák correction (N=2). Protein membership was indicated by color of the boxes in the antibody array or x-labels in the bar plot: yellow – src; green – MAPK; red – PI3K/AKT/mTOR; purple – cell cycle/p53; blue – JAK-STAT.

Membrane/Coordinate	Target/Control Reference Spot	Gene Symbol	Gene ID	Gene Name	Protein Name (Preferred)	Phosphorylation Site	Class	Function on Signaling / Transcription
A-A1, A2		NA	NA	NA	NA	NA	NA	NA
B-A11, A12	AK1 I/2/3	AKT1, AKT2, AKT3	207, 208, 10000	AKT serine/threonine kinase 1, AKT serine/threonine kinase 2, AKT serine/threonine kinase 3	RAC-alpha serine/threonine-protein kinase, RAC-beta serine/threonine-protein kinase, RAC-gamma serine/threonine-protein kinase	T308	kinase	Activating
B-A13, A14	AK1 I/2/3	AKT1, AKT2, AKT3	207, 208, 10000	AKT serine/threonine kinase 1, AKT serine/threonine kinase 2, AKT serine/threonine kinase 3	RAC-alpha serine/threonine-protein kinase, RAC-beta serine/threonine-protein kinase, RAC-gamma serine/threonine-protein kinase	S473	kinase	Activating
B-A17, A18	Reference Spot	NA	NA	NA	NA	NA	NA	NA
A-B3, B4	CREB	CREB1	1385	CAMP responsive element binding protein 1	cyclic AMP-responsive element-binding protein 1	S133	substrate	Activating
A-B5, B6	EGF R	EGFR	1956	epidermal growth factor receptor	epidermal growth factor receptor	Y1086	kinase	Activating
A-B7, B8	eNOS	NOS3	4846	nitric oxide synthase 3	nitric oxide synthase, endothelial	S1177	substrate	Activating
A-B9, B10	ERK1/2	MAPK3, MAPK1	5595, 5594	mitogen-activated protein kinase 3, mitogen-activated protein kinase 1	mitogen-activated protein kinase 3, mitogen-activated protein kinase 1	T202/Y204, T185/Y187	kinase	Activating
B-B11, B12	Chk-2	CHK2	11200	checkpoint kinase 2	serine/threonine-protein kinase Chk2	T68	kinase	Activating
B-B13, B14	e-Jun	JUN	3725	jun proto-oncogene, AP-1 transcription factor subunit	transcription factor Jun	S63	substrate	Activating
A-C3, C4	Fgr	FGFR	2268	FGFR proto-oncogene, Src family tyrosine kinase	tyrosine-protein kinase Fgr	Y412	kinase	Activating (suspected)
A-C5, C6	GSK3 α/β	GSK3A, GSK3B	2931, 2932	glycogen synthase kinase 3 alpha, glycogen synthase kinase 3 beta	glycogen synthase kinase-3 alpha, glycogen synthase kinase-3 beta	S21/S9	kinase	inhibitory
A-C7, C8	GSK3 β	GSK3B	2932	glycogen synthase kinase 3 beta	glycogen synthase kinase-3 beta	S9	kinase	inhibitory
A-C9, C10	HSP27	HSPB1	3315	heat shock protein family B (small) member 1	heat shock protein beta-1	S78/S82	substrate	Activating
B-C11, C12	p53	TP53	7157	tumor protein p53	cellular tumor antigen p53	S15	substrate	Activating
B-C13, C14	p53	TP53	7157	tumor protein p53	cellular tumor antigen p53	S46	substrate	Activating
B-C15, C16	p53	TP53	7157	tumor protein p53	cellular tumor antigen p53	S392	substrate	Activating
A-D3, D4	JNK I/2/3	MAPK8, MAPK9, MAPK10	5599, 5601, 5602	mitogen-activated protein kinase 8, mitogen-activated protein kinase 9, mitogen-activated protein kinase 10	mitogen-activated protein kinase 8, mitogen-activated protein kinase 9, mitogen-activated protein kinase 10	T183/Y185, T221/Y223	kinase	Activating
A-D5, D6	Lck	LCK	3932	LCK proto-oncogene, Src family tyrosine kinase	tyrosine-protein kinase Lck	Y394	kinase	Activating
A-D7, D8	Lyn	LYN	4067	LYN proto-oncogene, Src family tyrosine kinase	tyrosine-protein kinase Lyn	Y397	kinase	Activating
A-D9, D10	MSK1/2	RPS6KA5, RPS6KA4	9252	ribosomal protein S6 kinase A5, ribosomal protein S6 kinase A4	ribosomal protein S6 kinase alpha-5, ribosomal protein S6 kinase alpha-4	S376/S360	kinase	Activating
B-D11, D12	p70 S6 Kinase	RPS6KB2	6199	ribosomal protein S6 kinase B2	ribosomal protein S6 kinase beta-2	T389	kinase	Activating
B-D13, D14	p70 S6 Kinase	RPS6KB2	6199	ribosomal protein S6 kinase B2	ribosomal protein S6 kinase beta-2	T421/S424	kinase	Activating
B-D15, D16	PRAS40	AKT1S1	84335	AKT substrate 1	proline-rich AKT1 substrate 1	T246	substrate	Activating
A-E3, E4	p38 α	MAPK14	1432	mitogen-activated protein kinase 14	mitogen-activated protein kinase 14	T180/Y182	kinase	Activating
A-E5, E6	PDGFR β	PDGFRB	5159	platelet derived growth factor receptor beta	1-phosphatidylinositol 4,5 bisphosphate phosphodiesterase	Y751	kinase	Activating
A-E7, E8	PLC- γ	PLCG1	5335	phospholipase C gamma 1	gamma-1	Y783	kinase	Activating
A-E9, E10	Src	SRC	6714	SRC proto-oncogene, non-receptor tyrosine kinase	proto-oncogene tyrosine-protein kinase Src	Y419	kinase	Activating
B-E11, E12	PyK2	PTK2B	2185	protein tyrosine kinase 2 beta	protein-tyrosine kinase 2-beta	Y402	kinase	Activating
B-E13, E14	RSK1/2	RPS6KA1, RPS6KA3	6195, 6197	ribosomal protein S6 kinase A1, ribosomal protein S6 kinase A3	ribosomal protein S6 kinase alpha-1, ribosomal protein S6 kinase alpha-3	S321/S327	kinase	Activating
B-E15, E16	RSK1/2/3	RPS6KA1, RPS6KA3, RPS6KA4	6195, 6197, 6192	ribosomal protein S6 kinase A1, ribosomal protein S6 kinase A2, ribosomal protein S6 kinase A3	ribosomal protein S6 kinase alpha-1, ribosomal protein S6 kinase alpha-2, ribosomal protein S6 kinase alpha-3	S380/S386/S377	kinase	Activating
A-F3, F4	STAT2	STAT2	6773	signal transducer and activator of transcription 2	signal transducer and activator of transcription 2	Y689	substrate	Activating
A-F5, F6	STAT5a/b	STAT5A, STAT5B	6776, 6777	signal transducer and activator of transcription 5A, signal transducer and activator of transcription 5B	signal transducer and activator of transcription 5A, signal transducer and activator of transcription 5B	Y694/Y699	substrate	Activating
A-F7, F8	Wnk1	WNK1	65125	WNK lysine deficient protein kinase 1	serine/threonine-protein kinase WNK1	T60	kinase	Activating
A-F9, F10	Yes	YES1	7525	YES proto-oncogene 1, Src family tyrosine kinase	tyrosine-protein kinase Yes	Y426	kinase	Activating
B-F11, F12	STAT1	STAT1	6772	signal transducer and activator of transcription 1	signal transducer and activator of transcription 1-alpha/beta	Y701	substrate	Activating
B-F13, F14	STAT3	STAT3	6774	signal transducer and activator of transcription 3	signal transducer and activator of transcription 3	Y705	substrate	Activating
B-F15, F16	STAT3	STAT3	6774	signal transducer and activator of transcription 3	signal transducer and activator of transcription 3	S727	substrate	Activating
A-G1, G2	Reference Spot	NA	NA	NA	NA	NA	NA	NA
A-G3, G4	β -Catenin	NA	NA	NA	NA	NA	NA	NA
A-G9, G10	PHS (Negative Control)	NA	NA	NA	NA	NA	NA	NA
B-G11, G12	STAT6	STAT6	6778	signal transducer and activator of transcription 6	signal transducer and activator of transcription 6	Y641	substrate	Activating
B-G13, G14	HSP90	NA	NA	NA	NA	NA	NA	NA
B-G17, G18	PHS (Negative Control)	NA	NA	NA	NA	NA	NA	NA

src (oncogene)
MAPK
PI3K/AKT/mTOR
cell cycle/p53
JAK-STAT

Table 5: Human Phospho-Kinase Protein Array Annotation

The function of each phosphorylation site was annotated according to literature search⁶⁰⁻⁹⁶. All phosphorylation sites in the kit were reported to activate protein function and/or signaling pathways, except for GSK3 α /GSK3 β . Proteins were classified as either a kinase or a substrate of kinase. Cells were highlighted to indicate membership: yellow – src; green – MAPK; red – PI3K/AKT/mTOR; purple – cell cycle/p53; blue – JAK-STAT.

As shown in **Figure 21B**, BIA completely suppressed phosphorylation of STAT2 at Y689, thereby deactivating signaling downstream of STAT2. Activated STAT2 by Y689 phosphorylation can mediate immune signaling by interacting with phosphorylated STAT1 and IRF9 to form the ISGF3 transcriptional complex and initiating transcription of IFN-stimulated response elements (ISREs)^{89,97}. BIA treated hCMEC/D3 cells expressed 60-70% less phospho-STAT5 α /STAT5 β (mean pixel intensity = 24336.5), as compared to control (mean pixel intensity = 68198). STAT5 plays an important role in angiogenesis^{98,99}. Phosphorylation of STAT5 α at Y694 and STAT5 β at Y699 is usually catalyzed by receptor associated Janus kinases (JAKs), predominantly by JAK2 following prolactin stimulation⁹⁰. Transcription of STAT5 α /STAT5 β target genes was limited by the reduced amount of activated STAT5. Whether this effect was mediated by BIA directly inhibiting STAT5 or indirectly through interfering with the activation of JAKs was unclear.

The active form of the Yes kinase was mostly absent in BIA treated hCMEC/D3 cells (**Figure 21B**). The pixel intensity of phospho-Yes (Y426) was reduced to a mean value of 266.5 in BIA

as compared to 12964.5 in control. p38 α (as known as p38MAPK or MAPK14) was also significantly affected by BIA treatment (**Figure 21B**). Control treated hCMEC/D3 cells showed p38 α expression at a mean pixel intensity of 76048, while BIA treated cells at 25511.

Phosphorylation of p38 α at T180/Y182 also leads to activation of its kinase activity and downstream signaling cascade, and therefore p38 α -initiated signaling was attenuated upon BIA treatment⁸⁰.

Figure 22 shows STRING network of protein-protein interactions, including junctional complex-related proteins CD31 (PECAM1), VE-cadherin (CDH5), VEGFR2 (KDR) and integrin β 3 (ITGB3); transcription factors ETV1, ERG, and androgen receptor (AR) which often co-occur in cancers; kinases Yes (YES1), Src (SRC) and p38 α (MAPK14). Colored circles indicated proteins of interests, either DEGs from RNA-seq dataset or candidates from phospho-kinase array.

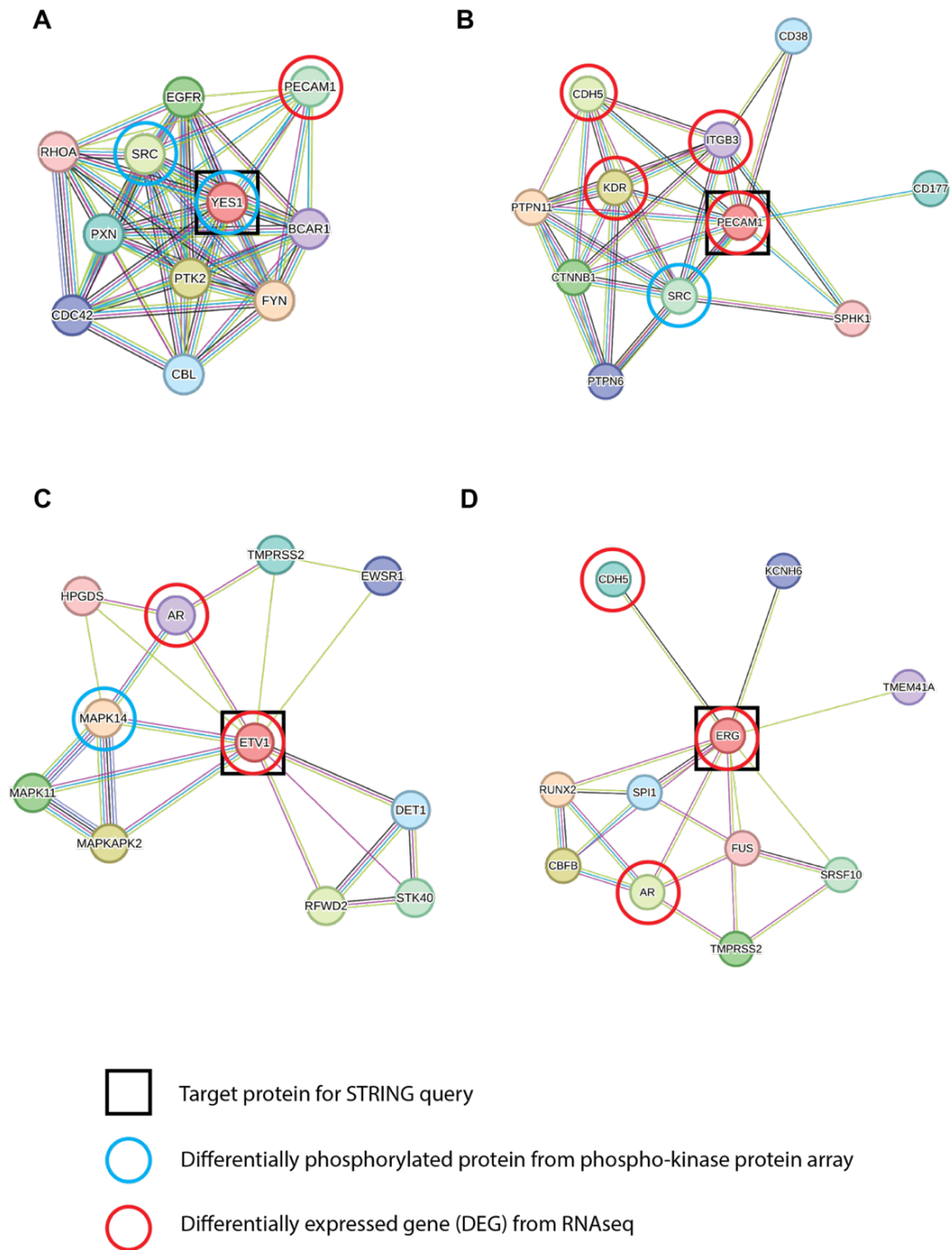


Figure 22: STRING network analysis of significant proteins in the human phospho-kinase array and proteins of interest from BIA hCMEC/D3 RNA-seq dataset

Only top 10 interactors with strongest evidence of protein interaction displayed.

4. Discussion

4.1 VE-cadherin is a candidate mediator of the effects of BIA on endothelial permeability

Using RNA-seq, we identified AJ protein VE-cadherin as a candidate protein participating in BIA-induced vascular permeability at the BTB. VE-cadherin is the major AJ protein expressed in the brain endothelium, mediating cell adhesion via homophilic, Ca^{2+} -dependent interactions through its extracellular domain and out-in signaling through its cytoplasmic connections with various scaffolding proteins and the actin cytoskeleton^{6,14,15}. VE-cadherin has been shown to regulate gene expression of the essential TJ protein claudin-5, through inhibiting the activity of claudin-5 transcriptional repressor FoxO1 and sequestering the FoxO1 stabilizer β -catenin in the AJ complex⁸. It also mediates contact inhibition during confluency by sequestering VEGFR2 in the AJ complex containing β -catenin and inhibiting the VEGF/VEGFR2 signaling-stimulated cell proliferation^{8,100}. VE-cadherin phosphorylation on Y658, S665, Y685, Y731 residues can also affect VE-cadherin stability and increase barrier permeability^{8,9,15}. In particular, the vascular endothelial growth factor (VEGF), a major angiogenic factor during morphogenesis but also secreted from tumor cells to induce angiogenesis, can lead to phosphorylation of junctional proteins such as VE-cadherin in conjunction with activated VEGFR2 and the cytosolic tyrosine kinase c-src (constitutively associated with VE-cadherin) and induce vascular permeability^{8,9,15}. Transforming growth factor TGF- β (a proinflammatory cytokine) and activation of PYK2 (proline rich kinase 2) have also been implicated in the phosphorylation of VE-cadherin and BBB permeability^{6,8}.

We confirmed the reduction of VE-cadherin expression in BIA-treated hCMEC/D3 and HBMEC cells (at both mRNA and protein levels), and in xenograft murine models (at the protein level). VE-cadherin protein expression at the brain endothelium was reduced to a minimal level after BIA treatment (**Figure 14-15**), indicating the significant absence of VE-cadherin in the junctional protein complex and possibly disruption in junctional complex assembly and paracellular contact. VE-cadherin protein destabilization by induced Tyr-phosphorylation downstream of intracellular signaling events might also be present; however, considering the significant and sustained reduction in VE-cadherin gene expression after BIA treatment (**Figure 11-12**), downregulation of VE-cadherin gene expression is likely to be the main mechanism of action behind BIA-induced vascular permeability.

4.2 ETS family transcription factors are potential mediators of the effects of BIA on endothelial permeability

We hypothesized that BIA-induced downregulation of VE-cadherin gene expression was mediated by ETS family transcription factors, based on their involvement in endothelial functions⁴¹⁻⁴⁴. In particular, ETV1 and ERG, with gene expression also significantly downregulated in the BIA RNA-seq dataset, were speculated to activate the transcription of junctional protein genes such as VE-cadherin and thus selected for preliminary experimentation. ETV1 and ERG expression was suppressed by BIA at both the transcription and translation levels, indicated by a reduction in the mRNA transcript abundance and a decreased protein quantity in cell lysates.

We observed a U-shaped pattern of ETV1 gene expression (**Figure 11**) following different treatment durations of BIA. Maximal reduction (80%) on ETV1 gene expression was achieved when hCMEC/D3 cells were treated with BIA for 12hr, but longer BIA treatment time failed to maintain the transcriptional suppression of ETV1 (60% reduction at 48hr). Additional time points of BIA treatment, e.g. 72hr and 96hr, could help clarify the transcriptional patterns of ETV1. Negative feedback loops involving transcription factors/cofactors, for example as compensation mechanisms of ETS family members to maintain homeostasis, might help explain the observed phenomenon. In the HBMEC cell model, BIA attenuated the transcription level of ETV1 by approximately 50% (**Figure 12**). It might be possible that a 50-60% reduction of ETV1 transcription is sufficient to result in the pronounced downregulation of VE-cadherin, if ETV1 is a transcriptional activator for VE-cadherin. siRNA of ETV1 appeared to interfere with ETV1 protein expression and produce a partial inhibition of endothelial barrier formation in hCMEC/D3 cells (**Figure 20C**).

In human GBM samples, the overexpression of ETV1 mRNA was detected (***) adj p-value < 0.001), but ERG was found to be non-significant (data not shown, Gliovis: TCGA GBM, Tukey's Honest Significant Difference (HSD) test). The time-dependent decrease in ERG transcription in BIA treated primary HBMEC cells was also not significant (**Figure 12**). This in part, was due to the minimal level of ERG expression in HBMEC cells. ERG transcripts were detected at Ct ~ 35 in HBMEC cells, compared with Ct values in the range of 20-25 in hCMEC/D3 cells, indicating a difference of more than 1,000-fold difference in abundance. A difference of 3 cycles is approximately a 10-fold difference in transcript levels. There was even no detectable level of ERG transcripts in one of the two independent experiments. Combined

with the prior observation that BIA inhibited barrier formation in HBMEC cells in the ECIS assay (**Figure 3A**), ERG is likely not the major transcriptional regulator for endothelial junction complex genes and vascular permeability at the BTB. Further investigation should focus on ETV1.

Other ETS members important for endothelial functions, although not identified as DEGs in RNA-seq, might also participate in BIA-mediated endothelial permeability in some other ways, for instance, at the post-translational level, downstream of MAPK signaling. For example, ETS1, absent in mature adult endothelium, can become expressed during tumor-induced angiogenesis and drive VE-cadherin expression through binding to ETS-binding sites on VE-cadherin promoter⁴³. ETV2 is considered as a master regulator in endothelial cell development and implicated in the endo-transdifferentiation of GBM stem cells and GBM malignancy in a VEGF-A independent mechanism^{101,102}.

Quantifying the expression of ETS family transcription factors in *in vivo* model and conducting additional *in vitro* knockdown/knockout experiments are necessary next steps to clarify their involvement in transcriptional activation of junctional proteins such as VE-cadherin.

4.3 p38 α is a candidate mediator of the effects of BIA on endothelial permeability

Because BIA is a known kinase inhibitor, a top-down approach was also utilized to probe for kinase interactors of BIA capable of initiating signaling cascades. BIA appeared to inactivate a

broad spectrum of kinases and substrates in the human phospho-kinase array, including all probed src family kinases such as Yes and all MAPK family kinases such as p38 α .

Yes is a member of the src family kinases (SFK) which mediate vascular permeability in response to inflammatory signals and growth factors, and can also promote cellular survival and proliferation via phosphorylating/activating the transcriptional activator Yes Associated Protein (YAP)^{92,103}. Activation of Yes via phosphorylation at the Y426 residue has been implicated in tumor invasion/metastasis via activating tumor-promoting transcription factors (myc, STAT3 and STAT6), as well as drug resistance in HER2+ breast cancer^{92,96,104}. Interestingly, Yes can be transcriptionally activated by direct ETV2 binding to the Yes promoter region to promote cell proliferation during development¹⁰³. Activated Yes is essential in the constitutive VE-cadherin turnover, as Yes can phosphorylate all major phosphorylation sites on VE-cadherin and induce VE-cadherin endocytosis¹⁰⁵. Yes can also induce VE-cadherin endocytosis by phosphorylating β -catenin at Y142 and reducing its binding with VE-cadherin and thus VE-cadherin stability¹⁰⁶. Yes dysfunction leads to clustering of VE-cadherin and increased macromolecular leakage, as well as reduced cell motility¹⁰⁵. Whether inactivation of Yes is a mechanism for BIA-induced endothelial permeability warrants further investigation; however, loss of intercellular VE-cadherin expression was observed rather than VE-cadherin clustering.

p38 α is involved in α -catenin (the actin binding protein connecting VE-cadherin/p120-catenin/ β -catenin complex to the actin cytoskeleton) phosphorylation, cytoskeleton remodeling and angiogenesis¹⁰⁷⁻¹⁰⁹. Specifically on endothelial barrier function, p38 α signaling modulates extracellular matrix (ECM) adhesion downstream of TGF- β signaling and is associated with the

paracellular permeability (characterized by a loss of TJ protein occludin) in Alzheimer's Disease^{14,110}. KEGG pathway analysis of the RNA-seq data also suggests the involvement of P38 α in MAPK (top RNA-seq DEGs mapped KEGG pathway, **Figure 6C**), FoxO and VEGF signaling (**Appendix 2**). As mentioned before, members of the ETS family all contain several MAPK interaction motifs and can be activated downstream of MAPK signaling^{45,46}. For instance, phosphorylation by MAPK or MAPKAPK, such as RSK1 and MSK1, at S94, T139, T142, S146, S191, S216 can activate ETV1 transcriptional activity⁴⁶. Specifically, p38 α has been found to have kinase activity against ETV1 and strong specificity for additional ETS family transcription factors such as ETV3 in *in vitro* kinase assay⁴⁵.

Combining all data and literature findings, p38 α is a promising candidate to mediate vascular permeability via inactivation of its MAPK signaling involving downstream target proteins such as the transcription factor ETV1 following BIA treatment, and ultimately transcriptional inactivation of junctional proteins, particularly VE-cadherin. Dysregulation in the phosphorylation of VE-cadherin as a result of MAPK family kinase inactivation by BIA, might also be involved in the observed vascular leakage. Further investigation is required to elucidate the role of p38 α .

4.4 Limitation of the study

Most of the data shown are preliminary and have not proved or disproved the hypothesis. Experiments need to be repeated, especially target protein detection by Western blot and immunofluorescence of *in vivo* treated animal samples, as well as siRNA/shRNA of ETV1.

Knockout and overexpression experiments are necessary to evaluate the possible causal relationship between candidate mediators (e.g. VE-cadherin, ETV1, p38 α) and BIA-induced endothelial permeability at the BTB.

A major limitation of the phospho-kinase array is that it only detects the phospho-proteins. If BIA transcriptionally inhibit the target protein, instead of enzymatically inhibiting kinase activity (either upstream or directly in the case of autoregulation), a reduction in phospho-protein level would also be observed. Validation experiments to immunoblot total protein levels in comparison with the phosphorylated protein levels, especially for the most significant proteins (Yes, p38 α , STAT2 and STAT5 α /STAT5 β), are of priority.

hCMEC/D3 and HBMEC cells are models of the BBB, and not representative of the BTB.

Biomechanical properties of the blood vessels (e.g. fluid flow), cell-cell interactions (e.g. with astrocytes and pericytes) and signaling molecules from GBM are absent in our *in vitro* model. *In vivo* models should be considered whenever possible. hCMEC/D3 and HBMEC cells can also be cultured with conditioned medium from GBM cells to simulate the BTB environment.

4.5 Future directions

In summary, BIA reduces endothelial permeability at the BTB likely by modulating kinases and transcription factors that control VE-cadherin expression. To further test our hypothesis, barrier formation of hCMEC/D3 cells with VE-cadherin overexpression, treated with BIA or vehicle control, will be assessed using the ECIS assay. Immunofluorescence visualization of ETV1 and

ERG on GBM xenograft murine tissues and additional siRNA/shRNA experiment of ETV1 and ERG should be immediately pursued to elucidate their role in VE-cadherin regulation and endothelial barrier function. Validation of select proteins from the phospho-kinase array such as p38 α by total protein detection (Western blot) will be performed to identify critical kinases mediating the effects of BIA on endothelial cells. If funding permits, spatial transcriptomics or RNA-sequencing of *in vivo* samples (tumor vs. brain, with or without BIA treatment), possibly in combination with proteomics, would provide critical insights into the specific signaling mediators of endothelial permeability (i.e. kinases, transcription factors and gene targets) at the BTB vs. the BBB following BIA treatment. Ultimately, this research is expected to aid the development of therapeutics that can modulate of BTB-specific endothelial permeability and improve drug delivery in GBM.

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6. Appendix

Appendix 1 RNA sample quality

Date of Exp	Sample	Concentration (ng/ μ L)	260/280	260/230
6/22/22	HBMEC DMSO ctrl	203.4	1.89	1.58
6/22/22	HBMEC BIA 1 μ M 24HR	281.4	1.88	1.97
1/31/23	HBMEC DMSO ctrl 1	213.946	2.123	1.576
1/31/23	HBMEC DMSO ctrl 2	112.421	1.913	1.856
1/31/23	HBMEC DMSO ctrl 3	91.889	1.981	1.815
1/31/23	HBMEC BIA 1 μ M 3HR-1	71.112	1.734	1.202
1/31/23	HBMEC BIA 1 μ M 3HR-2	52.955	1.671	1.178
1/31/23	HBMEC BIA 1 μ M 3HR-3	94.696	1.698	1.355
1/31/23	HBMEC BIA 1 μ M 12HR-1	36.172	2.055	1.518
1/31/23	HBMEC BIA 1 μ M 12HR-2	42.061	2.034	1.592
1/31/23	HBMEC BIA 1 μ M 12HR-3	102.209	1.812	1.235
1/31/23	HBMEC BIA 1 μ M 24HR-1	53.504	1.868	1.55
1/31/23	HBMEC BIA 1 μ M 24HR-2	60.864	2.082	1.751
1/31/23	HBMEC BIA 1 μ M 24HR-3	86.62	2.105	2.092
1/31/23	HBMEC BIA 1 μ M 48HR-1	154.079	2.119	1.978
1/31/23	HBMEC BIA 1 μ M 48HR-2	173.782	2.125	1.886
1/31/23	HBMEC BIA 1 μ M 48HR-3	152.03	2.11	1.977
2/13/23	HBMEC DMSO ctrl 1	111.61	1.998	1.95
2/13/23	HBMEC DMSO ctrl 2	163.823	1.991	2.022
2/13/23	HBMEC DMSO ctrl 3	154.784	1.953	2.009
2/13/23	HBMEC BIA 1 μ M 3HR-1	104.676	1.884	2.032
2/13/23	HBMEC BIA 1 μ M 3HR-2	102.392	1.847	1.671
2/13/23	HBMEC BIA 1 μ M 3HR-3	83.807	1.937	1.922
2/13/23	HBMEC BIA 1 μ M 12HR-1	107.039	2.033	1.972
2/13/23	HBMEC BIA 1 μ M 12HR-2	100.717	2.002	1.958
2/13/23	HBMEC BIA 1 μ M 12HR-3	129.123	2.011	1.977
2/13/23	HBMEC BIA 1 μ M 24HR-1	108.483	1.976	2.005
2/13/23	HBMEC BIA 1 μ M 24HR-2	139.234	1.965	2.078
2/13/23	HBMEC BIA 1 μ M 24HR-3	140.201	2.006	2.049

Date of Exp	Sample	Concentration (ng/ μ L)	260/280	260/230
7/9/22	hCMEC/D3 DMSO ctrl	145.7	1.7	1.95
7/9/22	hCMEC/D3 BIA 1 μ M 24HR	299.8	1.82	2.08
1/31/23	hCMEC/D3 DMSO ctrl 1	255.516	2.131	2.154
1/31/23	hCMEC/D3 DMSO ctrl 2	206.925	2.121	1.702
1/31/23	hCMEC/D3 DMSO ctrl 3	177.081	2.087	1.755
1/31/23	hCMEC/D3 BIA 1 μ M 12HR-2	54.164	1.93	2.038
1/31/23	hCMEC/D3 BIA 1 μ M 24HR-2	32.064	1.716	1.693
1/31/23	hCMEC/D3 BIA 1 μ M 24HR-3	30.68	1.828	1.803
1/31/23	hCMEC/D3 BIA 1 μ M 48HR-1	208.496	2.113	1.683
1/31/23	hCMEC/D3 BIA 1 μ M 48HR-2	42.208	1.68	2.446
1/31/23	hCMEC/D3 BIA 1 μ M 48HR-3	206.272	2.116	1.646
2/13/23	hCMEC/D3 DMSO ctrl 1	111.322	2.055	1.984
2/13/23	hCMEC/D3 DMSO ctrl 2	139.71	2.08	1.875
2/13/23	hCMEC/D3 DMSO ctrl 3	129.812	2.082	1.894
2/13/23	hCMEC/D3 BIA 1 μ M 3HR-1	135.454	2.088	1.849
2/13/23	hCMEC/D3 BIA 1 μ M 3HR-2	157.207	2.059	1.958
2/13/23	hCMEC/D3 BIA 1 μ M 3HR-3	163.492	2.063	1.975
2/13/23	hCMEC/D3 BIA 1 μ M 12HR-1	224.992	2.094	1.634
2/13/23	hCMEC/D3 BIA 1 μ M 12HR-2	177.3	2.02	1.88
2/13/23	hCMEC/D3 BIA 1 μ M 12HR-3	137.6	2.03	1.93
2/13/23	hCMEC/D3 BIA 1 μ M 24HR-1	119.2	2.03	2.046
2/13/23	hCMEC/D3 BIA 1 μ M 24HR-2	189.523	2.109	1.532
2/13/23	hCMEC/D3 BIA 1 μ M 24HR-3	237.081	2.112	1.583

Appendix 2 Phospho-kinase array mapped to junctional complex, signaling pathways and cancer KEGG pathways

Targets of significantly altered phosphorylation status in BIA treatment highlighted in yellow.

gene_symbol	gene_ID	pathway_description	pathway_ID	pathway_group
AKT1	207	Tight junction	hsa04530	junctional complex
AKT1	207	Focal adhesion	hsa04510	junctional complex
AKT1	207	MAPK signaling pathway	hsa04010	signaling pathways
AKT1	207	ErbB signaling pathway	hsa04012	signaling pathways
AKT1	207	Ras signaling pathway	hsa04014	signaling pathways
AKT1	207	Rap1 signaling pathway	hsa04015	signaling pathways
AKT1	207	cGMP-PKG signaling pathway	hsa04022	signaling pathways
AKT1	207	cAMP signaling pathway	hsa04024	signaling pathways
AKT1	207	Chemokine signaling pathway	hsa04062	signaling pathways
AKT1	207	HIF-1 signaling pathway	hsa04066	signaling pathways
AKT1	207	FoxO signaling pathway	hsa04068	signaling pathways
AKT1	207	Sphingolipid signaling pathway	hsa04071	signaling pathways
AKT1	207	Phospholipase D signaling pathway	hsa04072	signaling pathways
AKT1	207	mTOR signaling pathway	hsa04150	signaling pathways
AKT1	207	PI3K-Akt signaling pathway	hsa04151	signaling pathways
AKT1	207	AMPK signaling pathway	hsa04152	signaling pathways
AKT1	207	VEGF signaling pathway	hsa04370	signaling pathways
AKT1	207	Apelin signaling pathway	hsa04371	signaling pathways
AKT1	207	Toll-like receptor signaling pathway	hsa04620	signaling pathways
AKT1	207	C-type lectin receptor signaling pathway	hsa04625	signaling pathways
AKT1	207	JAK-STAT signaling pathway	hsa04630	signaling pathways
AKT1	207	T cell receptor signaling pathway	hsa04660	signaling pathways
AKT1	207	B cell receptor signaling pathway	hsa04662	signaling pathways
AKT1	207	Fc epsilon RI signaling pathway	hsa04664	signaling pathways
AKT1	207	TNF signaling pathway	hsa04668	signaling pathways
AKT1	207	Neurotrophin signaling pathway	hsa04722	signaling pathways
AKT1	207	Insulin signaling pathway	hsa04910	signaling pathways
AKT1	207	Estrogen signaling pathway	hsa04915	signaling pathways
AKT1	207	Prolactin signaling pathway	hsa04917	signaling pathways
AKT1	207	Thyroid hormone signaling pathway	hsa04919	signaling pathways
AKT1	207	Adipocytokine signaling pathway	hsa04920	signaling pathways
AKT1	207	Glucagon signaling pathway	hsa04922	signaling pathways
AKT1	207	Relaxin signaling pathway	hsa04926	signaling pathways
AKT1	207	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
AKT1	207	Pathways in cancer	hsa05200	cancer
AKT1	207	Proteoglycans in cancer	hsa05205	cancer
AKT1	207	Colorectal cancer	hsa05210	cancer
AKT1	207	Pancreatic cancer	hsa05212	cancer
AKT1	207	Endometrial cancer	hsa05213	cancer
AKT1	207	Prostate cancer	hsa05215	cancer
AKT1	207	Small cell lung cancer	hsa05222	cancer
AKT1	207	Non-small cell lung cancer	hsa05223	cancer
AKT1	207	Breast cancer	hsa05224	cancer
AKT1	207	Gastric cancer	hsa05226	cancer
AKT1	207	Central carbon metabolism in cancer	hsa05230	cancer
AKT1	207	Choline metabolism in cancer	hsa05231	cancer
AKT1	207	PD-L1 expression and PD-1 checkpoint pat	hsa05235	cancer

AKT1S1	84335	mTOR signaling pathway	hsa04150	signaling pathways
AKT1S1	84335	AMPK signaling pathway	hsa04152	signaling pathways
AKT2	208	Tight junction	hsa04530	junctional complex
AKT2	208	Focal adhesion	hsa04510	junctional complex
AKT2	208	MAPK signaling pathway	hsa04010	signaling pathways
AKT2	208	ErbB signaling pathway	hsa04012	signaling pathways
AKT2	208	Ras signaling pathway	hsa04014	signaling pathways
AKT2	208	Rap1 signaling pathway	hsa04015	signaling pathways
AKT2	208	cGMP-PKG signaling pathway	hsa04022	signaling pathways
AKT2	208	cAMP signaling pathway	hsa04024	signaling pathways
AKT2	208	Chemokine signaling pathway	hsa04062	signaling pathways
AKT2	208	HIF-1 signaling pathway	hsa04066	signaling pathways
AKT2	208	FoxO signaling pathway	hsa04068	signaling pathways
AKT2	208	Sphingolipid signaling pathway	hsa04071	signaling pathways
AKT2	208	Phospholipase D signaling pathway	hsa04072	signaling pathways
AKT2	208	mTOR signaling pathway	hsa04150	signaling pathways
AKT2	208	PI3K-Akt signaling pathway	hsa04151	signaling pathways
AKT2	208	AMPK signaling pathway	hsa04152	signaling pathways
AKT2	208	VEGF signaling pathway	hsa04370	signaling pathways
AKT2	208	Apelin signaling pathway	hsa04371	signaling pathways
AKT2	208	Toll-like receptor signaling pathway	hsa04620	signaling pathways
AKT2	208	C-type lectin receptor signaling pathway	hsa04625	signaling pathways
AKT2	208	JAK-STAT signaling pathway	hsa04630	signaling pathways
AKT2	208	T cell receptor signaling pathway	hsa04660	signaling pathways
AKT2	208	B cell receptor signaling pathway	hsa04662	signaling pathways
AKT2	208	Fc epsilon RI signaling pathway	hsa04664	signaling pathways
AKT2	208	TNF signaling pathway	hsa04668	signaling pathways
AKT2	208	Neurotrophin signaling pathway	hsa04722	signaling pathways
AKT2	208	Insulin signaling pathway	hsa04910	signaling pathways
AKT2	208	Estrogen signaling pathway	hsa04915	signaling pathways
AKT2	208	Prolactin signaling pathway	hsa04917	signaling pathways
AKT2	208	Thyroid hormone signaling pathway	hsa04919	signaling pathways
AKT2	208	Adipocytokine signaling pathway	hsa04920	signaling pathways
AKT2	208	Glucagon signaling pathway	hsa04922	signaling pathways
AKT2	208	Relaxin signaling pathway	hsa04926	signaling pathways
AKT2	208	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
AKT2	208	Pathways in cancer	hsa05200	cancer
AKT2	208	Proteoglycans in cancer	hsa05205	cancer
AKT2	208	Colorectal cancer	hsa05210	cancer
AKT2	208	Pancreatic cancer	hsa05212	cancer
AKT2	208	Endometrial cancer	hsa05213	cancer
AKT2	208	Prostate cancer	hsa05215	cancer
AKT2	208	Small cell lung cancer	hsa05222	cancer
AKT2	208	Non-small cell lung cancer	hsa05223	cancer
AKT2	208	Breast cancer	hsa05224	cancer
AKT2	208	Gastric cancer	hsa05226	cancer
AKT2	208	Central carbon metabolism in cancer	hsa05230	cancer
AKT2	208	Choline metabolism in cancer	hsa05231	cancer

AKT2	208	PD-L1 expression and PD-1 checkpoint pat	hsa05235	cancer
AKT3	10000	Tight junction	hsa04530	junctional complex
AKT3	10000	Tight junction	hsa04530	junctional complex
AKT3	10000	Focal adhesion	hsa04510	junctional complex
AKT3	10000	Focal adhesion	hsa04510	junctional complex
AKT3	10000	MAPK signaling pathway	hsa04010	signaling pathways
AKT3	10000	ErbB signaling pathway	hsa04012	signaling pathways
AKT3	10000	Ras signaling pathway	hsa04014	signaling pathways
AKT3	10000	Rap1 signaling pathway	hsa04015	signaling pathways
AKT3	10000	cGMP-PKG signaling pathway	hsa04022	signaling pathways
AKT3	10000	cAMP signaling pathway	hsa04024	signaling pathways
AKT3	10000	Chemokine signaling pathway	hsa04062	signaling pathways
AKT3	10000	HIF-1 signaling pathway	hsa04066	signaling pathways
AKT3	10000	FoxO signaling pathway	hsa04068	signaling pathways
AKT3	10000	Sphingolipid signaling pathway	hsa04071	signaling pathways
AKT3	10000	Phospholipase D signaling pathway	hsa04072	signaling pathways
AKT3	10000	mTOR signaling pathway	hsa04150	signaling pathways
AKT3	10000	PI3K-Akt signaling pathway	hsa04151	signaling pathways
AKT3	10000	AMPK signaling pathway	hsa04152	signaling pathways
AKT3	10000	VEGF signaling pathway	hsa04370	signaling pathways
AKT3	10000	Apelin signaling pathway	hsa04371	signaling pathways
AKT3	10000	Toll-like receptor signaling pathway	hsa04620	signaling pathways
AKT3	10000	C-type lectin receptor signaling pathway	hsa04625	signaling pathways
AKT3	10000	JAK-STAT signaling pathway	hsa04630	signaling pathways
AKT3	10000	T cell receptor signaling pathway	hsa04660	signaling pathways
AKT3	10000	B cell receptor signaling pathway	hsa04662	signaling pathways
AKT3	10000	Fc epsilon RI signaling pathway	hsa04664	signaling pathways
AKT3	10000	TNF signaling pathway	hsa04668	signaling pathways
AKT3	10000	Neurotrophin signaling pathway	hsa04722	signaling pathways
AKT3	10000	Insulin signaling pathway	hsa04910	signaling pathways
AKT3	10000	Estrogen signaling pathway	hsa04915	signaling pathways
AKT3	10000	Prolactin signaling pathway	hsa04917	signaling pathways
AKT3	10000	Thyroid hormone signaling pathway	hsa04919	signaling pathways
AKT3	10000	Adipocytokine signaling pathway	hsa04920	signaling pathways
AKT3	10000	Glucagon signaling pathway	hsa04922	signaling pathways
AKT3	10000	Relaxin signaling pathway	hsa04926	signaling pathways
AKT3	10000	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
AKT3	10000	Pathways in cancer	hsa05200	cancer
AKT3	10000	Proteoglycans in cancer	hsa05205	cancer
AKT3	10000	Colorectal cancer	hsa05210	cancer
AKT3	10000	Pancreatic cancer	hsa05212	cancer
AKT3	10000	Endometrial cancer	hsa05213	cancer
AKT3	10000	Prostate cancer	hsa05215	cancer
AKT3	10000	Small cell lung cancer	hsa05222	cancer
AKT3	10000	Non-small cell lung cancer	hsa05223	cancer
AKT3	10000	Breast cancer	hsa05224	cancer
AKT3	10000	Gastric cancer	hsa05226	cancer
AKT3	10000	Central carbon metabolism in cancer	hsa05230	cancer

AKT3	10000	Choline metabolism in cancer	hsa05231	cancer
AKT3	10000	PD-L1 expression and PD-1 checkpoint pat	hsa05235	cancer
CHEK2	11200	p53 signaling pathway	hsa04115	signaling pathways
CREB1	1385	cGMP-PKG signaling pathway	hsa04022	signaling pathways
CREB1	1385	cAMP signaling pathway	hsa04024	signaling pathways
CREB1	1385	PI3K-Akt signaling pathway	hsa04151	signaling pathways
CREB1	1385	AMPK signaling pathway	hsa04152	signaling pathways
CREB1	1385	TNF signaling pathway	hsa04668	signaling pathways
CREB1	1385	Estrogen signaling pathway	hsa04915	signaling pathways
CREB1	1385	Glucagon signaling pathway	hsa04922	signaling pathways
CREB1	1385	Relaxin signaling pathway	hsa04926	signaling pathways
CREB1	1385	Prostate cancer	hsa05215	cancer
EGFR	1956	Adherens junction	hsa04520	junctional complex
EGFR	1956	Gap junction	hsa04540	junctional complex
EGFR	1956	Focal adhesion	hsa04510	junctional complex
EGFR	1956	MAPK signaling pathway	hsa04010	signaling pathways
EGFR	1956	ErbB signaling pathway	hsa04012	signaling pathways
EGFR	1956	Ras signaling pathway	hsa04014	signaling pathways
EGFR	1956	Rap1 signaling pathway	hsa04015	signaling pathways
EGFR	1956	Calcium signaling pathway	hsa04020	signaling pathways
EGFR	1956	HIF-1 signaling pathway	hsa04066	signaling pathways
EGFR	1956	FoxO signaling pathway	hsa04068	signaling pathways
EGFR	1956	Phospholipase D signaling pathway	hsa04072	signaling pathways
EGFR	1956	PI3K-Akt signaling pathway	hsa04151	signaling pathways
EGFR	1956	JAK-STAT signaling pathway	hsa04630	signaling pathways
EGFR	1956	GnRH signaling pathway	hsa04912	signaling pathways
EGFR	1956	Estrogen signaling pathway	hsa04915	signaling pathways
EGFR	1956	Oxytocin signaling pathway	hsa04921	signaling pathways
EGFR	1956	Relaxin signaling pathway	hsa04926	signaling pathways
EGFR	1956	Pathways in cancer	hsa05200	cancer
EGFR	1956	Proteoglycans in cancer	hsa05205	cancer
EGFR	1956	MicroRNAs in cancer	hsa05206	cancer
EGFR	1956	Colorectal cancer	hsa05210	cancer
EGFR	1956	Pancreatic cancer	hsa05212	cancer
EGFR	1956	Endometrial cancer	hsa05213	cancer
EGFR	1956	Prostate cancer	hsa05215	cancer
EGFR	1956	Bladder cancer	hsa05219	cancer
EGFR	1956	Non-small cell lung cancer	hsa05223	cancer
EGFR	1956	Breast cancer	hsa05224	cancer
EGFR	1956	Gastric cancer	hsa05226	cancer
EGFR	1956	Central carbon metabolism in cancer	hsa05230	cancer
EGFR	1956	Choline metabolism in cancer	hsa05231	cancer
EGFR	1956	PD-L1 expression and PD-1 checkpoint pat	hsa05235	cancer
FGR	2268	Chemokine signaling pathway	hsa04062	signaling pathways
GSK3A	2931	Chemokine signaling pathway	hsa04062	signaling pathways
GSK3B	2932	Focal adhesion	hsa04510	junctional complex
GSK3B	2932	ErbB signaling pathway	hsa04012	signaling pathways
GSK3B	2932	Chemokine signaling pathway	hsa04062	signaling pathways

GSK3B	2932	mTOR signaling pathway	hsa04150	signaling pathways
GSK3B	2932	PI3K-Akt signaling pathway	hsa04151	signaling pathways
GSK3B	2932	Wnt signaling pathway	hsa04310	signaling pathways
GSK3B	2932	Hedgehog signaling pathway	hsa04340	signaling pathways
GSK3B	2932	Hippo signaling pathway	hsa04390	signaling pathways
GSK3B	2932	IL-17 signaling pathway	hsa04657	signaling pathways
GSK3B	2932	T cell receptor signaling pathway	hsa04660	signaling pathways
GSK3B	2932	B cell receptor signaling pathway	hsa04662	signaling pathways
GSK3B	2932	Neurotrophin signaling pathway	hsa04722	signaling pathways
GSK3B	2932	Insulin signaling pathway	hsa04910	signaling pathways
GSK3B	2932	Prolactin signaling pathway	hsa04917	signaling pathways
GSK3B	2932	Thyroid hormone signaling pathway	hsa04919	signaling pathways
GSK3B	2932	Pathways in cancer	hsa05200	cancer
GSK3B	2932	Colorectal cancer	hsa05210	cancer
GSK3B	2932	Endometrial cancer	hsa05213	cancer
GSK3B	2932	Prostate cancer	hsa05215	cancer
GSK3B	2932	Breast cancer	hsa05224	cancer
GSK3B	2932	Gastric cancer	hsa05226	cancer
HSPB1	3315	MAPK signaling pathway	hsa04010	signaling pathways
HSPB1	3315	VEGF signaling pathway	hsa04370	signaling pathways
JUN	3725	Focal adhesion	hsa04510	junctional complex
JUN	3725	MAPK signaling pathway	hsa04010	signaling pathways
JUN	3725	ErbB signaling pathway	hsa04012	signaling pathways
JUN	3725	cAMP signaling pathway	hsa04024	signaling pathways
JUN	3725	Wnt signaling pathway	hsa04310	signaling pathways
JUN	3725	Toll-like receptor signaling pathway	hsa04620	signaling pathways
JUN	3725	NOD-like receptor signaling pathway	hsa04621	signaling pathways
JUN	3725	C-type lectin receptor signaling pathway	hsa04625	signaling pathways
JUN	3725	IL-17 signaling pathway	hsa04657	signaling pathways
JUN	3725	T cell receptor signaling pathway	hsa04660	signaling pathways
JUN	3725	B cell receptor signaling pathway	hsa04662	signaling pathways
JUN	3725	TNF signaling pathway	hsa04668	signaling pathways
JUN	3725	Neurotrophin signaling pathway	hsa04722	signaling pathways
JUN	3725	GnRH signaling pathway	hsa04912	signaling pathways
JUN	3725	Estrogen signaling pathway	hsa04915	signaling pathways
JUN	3725	Oxytocin signaling pathway	hsa04921	signaling pathways
JUN	3725	Relaxin signaling pathway	hsa04926	signaling pathways
JUN	3725	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
JUN	3725	Pathways in cancer	hsa05200	cancer
JUN	3725	Colorectal cancer	hsa05210	cancer
JUN	3725	Breast cancer	hsa05224	cancer
JUN	3725	Choline metabolism in cancer	hsa05231	cancer
JUN	3725	PD-L1 expression and PD-1 checkpoint pat	hsa05235	cancer
LCK	3932	NF-kappa B signaling pathway	hsa04064	signaling pathways
LCK	3932	T cell receptor signaling pathway	hsa04660	signaling pathways
LCK	3932	PD-L1 expression and PD-1 checkpoint pat	hsa05235	cancer
LYN	4067	Chemokine signaling pathway	hsa04062	signaling pathways
LYN	4067	NF-kappa B signaling pathway	hsa04064	signaling pathways

LYN	4067	B cell receptor signaling pathway	hsa04662	signaling pathways
LYN	4067	Fc epsilon RI signaling pathway	hsa04664	signaling pathways
MAPK1	5594	Adherens junction	hsa04520	junctional complex
MAPK1	5594	Gap junction	hsa04540	junctional complex
MAPK1	5594	Focal adhesion	hsa04510	junctional complex
MAPK1	5594	MAPK signaling pathway	hsa04010	signaling pathways
MAPK1	5594	ErbB signaling pathway	hsa04012	signaling pathways
MAPK1	5594	Ras signaling pathway	hsa04014	signaling pathways
MAPK1	5594	Rap1 signaling pathway	hsa04015	signaling pathways
MAPK1	5594	cGMP-PKG signaling pathway	hsa04022	signaling pathways
MAPK1	5594	cAMP signaling pathway	hsa04024	signaling pathways
MAPK1	5594	Chemokine signaling pathway	hsa04062	signaling pathways
MAPK1	5594	HIF-1 signaling pathway	hsa04066	signaling pathways
MAPK1	5594	FoxO signaling pathway	hsa04068	signaling pathways
MAPK1	5594	Sphingolipid signaling pathway	hsa04071	signaling pathways
MAPK1	5594	Phospholipase D signaling pathway	hsa04072	signaling pathways
MAPK1	5594	mTOR signaling pathway	hsa04150	signaling pathways
MAPK1	5594	PI3K-Akt signaling pathway	hsa04151	signaling pathways
MAPK1	5594	TGF-beta signaling pathway	hsa04350	signaling pathways
MAPK1	5594	VEGF signaling pathway	hsa04370	signaling pathways
MAPK1	5594	Apelin signaling pathway	hsa04371	signaling pathways
MAPK1	5594	Toll-like receptor signaling pathway	hsa04620	signaling pathways
MAPK1	5594	NOD-like receptor signaling pathway	hsa04621	signaling pathways
MAPK1	5594	C-type lectin receptor signaling pathway	hsa04625	signaling pathways
MAPK1	5594	IL-17 signaling pathway	hsa04657	signaling pathways
MAPK1	5594	T cell receptor signaling pathway	hsa04660	signaling pathways
MAPK1	5594	B cell receptor signaling pathway	hsa04662	signaling pathways
MAPK1	5594	Fc epsilon RI signaling pathway	hsa04664	signaling pathways
MAPK1	5594	TNF signaling pathway	hsa04668	signaling pathways
MAPK1	5594	Neurotrophin signaling pathway	hsa04722	signaling pathways
MAPK1	5594	Insulin signaling pathway	hsa04910	signaling pathways
MAPK1	5594	GnRH signaling pathway	hsa04912	signaling pathways
MAPK1	5594	Estrogen signaling pathway	hsa04915	signaling pathways
MAPK1	5594	Prolactin signaling pathway	hsa04917	signaling pathways
MAPK1	5594	Thyroid hormone signaling pathway	hsa04919	signaling pathways
MAPK1	5594	Oxytocin signaling pathway	hsa04921	signaling pathways
MAPK1	5594	Relaxin signaling pathway	hsa04926	signaling pathways
MAPK1	5594	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
MAPK1	5594	Pathways in cancer	hsa05200	cancer
MAPK1	5594	Proteoglycans in cancer	hsa05205	cancer
MAPK1	5594	MicroRNAs in cancer	hsa05206	cancer
MAPK1	5594	Colorectal cancer	hsa05210	cancer
MAPK1	5594	Pancreatic cancer	hsa05212	cancer
MAPK1	5594	Endometrial cancer	hsa05213	cancer
MAPK1	5594	Prostate cancer	hsa05215	cancer
MAPK1	5594	Thyroid cancer	hsa05216	cancer
MAPK1	5594	Bladder cancer	hsa05219	cancer
MAPK1	5594	Non-small cell lung cancer	hsa05223	cancer

MAPK1	5594	Breast cancer	hsa05224	cancer
MAPK1	5594	Gastric cancer	hsa05226	cancer
MAPK1	5594	Central carbon metabolism in cancer	hsa05230	cancer
MAPK1	5594	Choline metabolism in cancer	hsa05231	cancer
MAPK1	5594	PD-L1 expression and PD-1 checkpoint path	hsa05235	cancer
MAPK10	5602	Focal adhesion	hsa04510	junctional complex
MAPK10	5602	MAPK signaling pathway	hsa04010	signaling pathways
MAPK10	5602	ErbB signaling pathway	hsa04012	signaling pathways
MAPK10	5602	Ras signaling pathway	hsa04014	signaling pathways
MAPK10	5602	cAMP signaling pathway	hsa04024	signaling pathways
MAPK10	5602	FoxO signaling pathway	hsa04068	signaling pathways
MAPK10	5602	Sphingolipid signaling pathway	hsa04071	signaling pathways
MAPK10	5602	Wnt signaling pathway	hsa04310	signaling pathways
MAPK10	5602	Toll-like receptor signaling pathway	hsa04620	signaling pathways
MAPK10	5602	NOD-like receptor signaling pathway	hsa04621	signaling pathways
MAPK10	5602	RIG-I-like receptor signaling pathway	hsa04622	signaling pathways
MAPK10	5602	C-type lectin receptor signaling pathway	hsa04625	signaling pathways
MAPK10	5602	IL-17 signaling pathway	hsa04657	signaling pathways
MAPK10	5602	T cell receptor signaling pathway	hsa04660	signaling pathways
MAPK10	5602	Fc epsilon RI signaling pathway	hsa04664	signaling pathways
MAPK10	5602	TNF signaling pathway	hsa04668	signaling pathways
MAPK10	5602	Neurotrophin signaling pathway	hsa04722	signaling pathways
MAPK10	5602	Insulin signaling pathway	hsa04910	signaling pathways
MAPK10	5602	GnRH signaling pathway	hsa04912	signaling pathways
MAPK10	5602	Prolactin signaling pathway	hsa04917	signaling pathways
MAPK10	5602	Adipocytokine signaling pathway	hsa04920	signaling pathways
MAPK10	5602	Relaxin signaling pathway	hsa04926	signaling pathways
MAPK10	5602	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
MAPK10	5602	Pathways in cancer	hsa05200	cancer
MAPK10	5602	Colorectal cancer	hsa05210	cancer
MAPK10	5602	Pancreatic cancer	hsa05212	cancer
MAPK10	5602	Choline metabolism in cancer	hsa05231	cancer
MAPK14	1432	MAPK signaling pathway	hsa04010	signaling pathways
MAPK14	1432	Rap1 signaling pathway	hsa04015	signaling pathways
MAPK14	1432	FoxO signaling pathway	hsa04068	signaling pathways
MAPK14	1432	Sphingolipid signaling pathway	hsa04071	signaling pathways
MAPK14	1432	VEGF signaling pathway	hsa04370	signaling pathways
MAPK14	1432	Toll-like receptor signaling pathway	hsa04620	signaling pathways
MAPK14	1432	NOD-like receptor signaling pathway	hsa04621	signaling pathways
MAPK14	1432	RIG-I-like receptor signaling pathway	hsa04622	signaling pathways
MAPK14	1432	C-type lectin receptor signaling pathway	hsa04625	signaling pathways
MAPK14	1432	IL-17 signaling pathway	hsa04657	signaling pathways
MAPK14	1432	T cell receptor signaling pathway	hsa04660	signaling pathways
MAPK14	1432	Fc epsilon RI signaling pathway	hsa04664	signaling pathways
MAPK14	1432	TNF signaling pathway	hsa04668	signaling pathways
MAPK14	1432	Neurotrophin signaling pathway	hsa04722	signaling pathways
MAPK14	1432	GnRH signaling pathway	hsa04912	signaling pathways
MAPK14	1432	Prolactin signaling pathway	hsa04917	signaling pathways

MAPK14	1432	Relaxin signaling pathway	hsa04926	signaling pathways
MAPK14	1432	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
MAPK14	1432	Proteoglycans in cancer	hsa05205	cancer
MAPK14	1432	PD-L1 expression and PD-1 checkpoint pat	hsa05235	cancer
MAPK3	5595	Adherens junction	hsa04520	junctional complex
MAPK3	5595	Gap junction	hsa04540	junctional complex
MAPK3	5595	Focal adhesion	hsa04510	junctional complex
MAPK3	5595	MAPK signaling pathway	hsa04010	signaling pathways
MAPK3	5595	ErbB signaling pathway	hsa04012	signaling pathways
MAPK3	5595	Ras signaling pathway	hsa04014	signaling pathways
MAPK3	5595	Rap1 signaling pathway	hsa04015	signaling pathways
MAPK3	5595	cGMP-PKG signaling pathway	hsa04022	signaling pathways
MAPK3	5595	cAMP signaling pathway	hsa04024	signaling pathways
MAPK3	5595	Chemokine signaling pathway	hsa04062	signaling pathways
MAPK3	5595	HIF-1 signaling pathway	hsa04066	signaling pathways
MAPK3	5595	FoxO signaling pathway	hsa04068	signaling pathways
MAPK3	5595	Sphingolipid signaling pathway	hsa04071	signaling pathways
MAPK3	5595	Phospholipase D signaling pathway	hsa04072	signaling pathways
MAPK3	5595	mTOR signaling pathway	hsa04150	signaling pathways
MAPK3	5595	PI3K-Akt signaling pathway	hsa04151	signaling pathways
MAPK3	5595	TGF-beta signaling pathway	hsa04350	signaling pathways
MAPK3	5595	VEGF signaling pathway	hsa04370	signaling pathways
MAPK3	5595	Apelin signaling pathway	hsa04371	signaling pathways
MAPK3	5595	Toll-like receptor signaling pathway	hsa04620	signaling pathways
MAPK3	5595	NOD-like receptor signaling pathway	hsa04621	signaling pathways
MAPK3	5595	C-type lectin receptor signaling pathway	hsa04625	signaling pathways
MAPK3	5595	IL-17 signaling pathway	hsa04657	signaling pathways
MAPK3	5595	T cell receptor signaling pathway	hsa04660	signaling pathways
MAPK3	5595	B cell receptor signaling pathway	hsa04662	signaling pathways
MAPK3	5595	Fc epsilon RI signaling pathway	hsa04664	signaling pathways
MAPK3	5595	TNF signaling pathway	hsa04668	signaling pathways
MAPK3	5595	Neurotrophin signaling pathway	hsa04722	signaling pathways
MAPK3	5595	Insulin signaling pathway	hsa04910	signaling pathways
MAPK3	5595	GnRH signaling pathway	hsa04912	signaling pathways
MAPK3	5595	Estrogen signaling pathway	hsa04915	signaling pathways
MAPK3	5595	Prolactin signaling pathway	hsa04917	signaling pathways
MAPK3	5595	Thyroid hormone signaling pathway	hsa04919	signaling pathways
MAPK3	5595	Oxytocin signaling pathway	hsa04921	signaling pathways
MAPK3	5595	Relaxin signaling pathway	hsa04926	signaling pathways
MAPK3	5595	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
MAPK3	5595	Pathways in cancer	hsa05200	cancer
MAPK3	5595	Proteoglycans in cancer	hsa05205	cancer
MAPK3	5595	MicroRNAs in cancer	hsa05206	cancer
MAPK3	5595	Colorectal cancer	hsa05210	cancer
MAPK3	5595	Pancreatic cancer	hsa05212	cancer
MAPK3	5595	Endometrial cancer	hsa05213	cancer
MAPK3	5595	Prostate cancer	hsa05215	cancer
MAPK3	5595	Thyroid cancer	hsa05216	cancer

MAPK3	5595	Bladder cancer	hsa05219	cancer
MAPK3	5595	Non-small cell lung cancer	hsa05223	cancer
MAPK3	5595	Breast cancer	hsa05224	cancer
MAPK3	5595	Gastric cancer	hsa05226	cancer
MAPK3	5595	Central carbon metabolism in cancer	hsa05230	cancer
MAPK3	5595	Choline metabolism in cancer	hsa05231	cancer
MAPK3	5595	PD-L1 expression and PD-1 checkpoint path	hsa05235	cancer
MAPK8	5599	Focal adhesion	hsa04510	junctional complex
MAPK8	5599	MAPK signaling pathway	hsa04010	signaling pathways
MAPK8	5599	ErbB signaling pathway	hsa04012	signaling pathways
MAPK8	5599	Ras signaling pathway	hsa04014	signaling pathways
MAPK8	5599	cAMP signaling pathway	hsa04024	signaling pathways
MAPK8	5599	FoxO signaling pathway	hsa04068	signaling pathways
MAPK8	5599	Sphingolipid signaling pathway	hsa04071	signaling pathways
MAPK8	5599	Wnt signaling pathway	hsa04310	signaling pathways
MAPK8	5599	Toll-like receptor signaling pathway	hsa04620	signaling pathways
MAPK8	5599	NOD-like receptor signaling pathway	hsa04621	signaling pathways
MAPK8	5599	RIG-I-like receptor signaling pathway	hsa04622	signaling pathways
MAPK8	5599	C-type lectin receptor signaling pathway	hsa04625	signaling pathways
MAPK8	5599	IL-17 signaling pathway	hsa04657	signaling pathways
MAPK8	5599	T cell receptor signaling pathway	hsa04660	signaling pathways
MAPK8	5599	Fc epsilon RI signaling pathway	hsa04664	signaling pathways
MAPK8	5599	TNF signaling pathway	hsa04668	signaling pathways
MAPK8	5599	Neurotrophin signaling pathway	hsa04722	signaling pathways
MAPK8	5599	Insulin signaling pathway	hsa04910	signaling pathways
MAPK8	5599	GnRH signaling pathway	hsa04912	signaling pathways
MAPK8	5599	Prolactin signaling pathway	hsa04917	signaling pathways
MAPK8	5599	Adipocytokine signaling pathway	hsa04920	signaling pathways
MAPK8	5599	Relaxin signaling pathway	hsa04926	signaling pathways
MAPK8	5599	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
MAPK8	5599	Pathways in cancer	hsa05200	cancer
MAPK8	5599	Colorectal cancer	hsa05210	cancer
MAPK8	5599	Pancreatic cancer	hsa05212	cancer
MAPK8	5599	Choline metabolism in cancer	hsa05231	cancer
MAPK9	5601	Focal adhesion	hsa04510	junctional complex
MAPK9	5601	MAPK signaling pathway	hsa04010	signaling pathways
MAPK9	5601	ErbB signaling pathway	hsa04012	signaling pathways
MAPK9	5601	Ras signaling pathway	hsa04014	signaling pathways
MAPK9	5601	cAMP signaling pathway	hsa04024	signaling pathways
MAPK9	5601	FoxO signaling pathway	hsa04068	signaling pathways
MAPK9	5601	Sphingolipid signaling pathway	hsa04071	signaling pathways
MAPK9	5601	Wnt signaling pathway	hsa04310	signaling pathways
MAPK9	5601	Toll-like receptor signaling pathway	hsa04620	signaling pathways
MAPK9	5601	NOD-like receptor signaling pathway	hsa04621	signaling pathways
MAPK9	5601	RIG-I-like receptor signaling pathway	hsa04622	signaling pathways
MAPK9	5601	C-type lectin receptor signaling pathway	hsa04625	signaling pathways
MAPK9	5601	IL-17 signaling pathway	hsa04657	signaling pathways
MAPK9	5601	T cell receptor signaling pathway	hsa04660	signaling pathways

MAPK9	5601	Fc epsilon RI signaling pathway	hsa04664	signaling pathways
MAPK9	5601	TNF signaling pathway	hsa04668	signaling pathways
MAPK9	5601	Neurotrophin signaling pathway	hsa04722	signaling pathways
MAPK9	5601	Insulin signaling pathway	hsa04910	signaling pathways
MAPK9	5601	GnRH signaling pathway	hsa04912	signaling pathways
MAPK9	5601	Prolactin signaling pathway	hsa04917	signaling pathways
MAPK9	5601	Adipocytokine signaling pathway	hsa04920	signaling pathways
MAPK9	5601	Relaxin signaling pathway	hsa04926	signaling pathways
MAPK9	5601	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
MAPK9	5601	Pathways in cancer	hsa05200	cancer
MAPK9	5601	Colorectal cancer	hsa05210	cancer
MAPK9	5601	Pancreatic cancer	hsa05212	cancer
MAPK9	5601	Choline metabolism in cancer	hsa05231	cancer
NOS3	4846	Calcium signaling pathway	hsa04020	signaling pathways
NOS3	4846	cGMP-PKG signaling pathway	hsa04022	signaling pathways
NOS3	4846	HIF-1 signaling pathway	hsa04066	signaling pathways
NOS3	4846	Sphingolipid signaling pathway	hsa04071	signaling pathways
NOS3	4846	PI3K-Akt signaling pathway	hsa04151	signaling pathways
NOS3	4846	VEGF signaling pathway	hsa04370	signaling pathways
NOS3	4846	Apelin signaling pathway	hsa04371	signaling pathways
NOS3	4846	Estrogen signaling pathway	hsa04915	signaling pathways
NOS3	4846	Oxytocin signaling pathway	hsa04921	signaling pathways
NOS3	4846	Relaxin signaling pathway	hsa04926	signaling pathways
NOS3	4846	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
PDGFRB	5159	Gap junction	hsa04540	junctional complex
PDGFRB	5159	Focal adhesion	hsa04510	junctional complex
PDGFRB	5159	MAPK signaling pathway	hsa04010	signaling pathways
PDGFRB	5159	Ras signaling pathway	hsa04014	signaling pathways
PDGFRB	5159	Rap1 signaling pathway	hsa04015	signaling pathways
PDGFRB	5159	Calcium signaling pathway	hsa04020	signaling pathways
PDGFRB	5159	Phospholipase D signaling pathway	hsa04072	signaling pathways
PDGFRB	5159	PI3K-Akt signaling pathway	hsa04151	signaling pathways
PDGFRB	5159	JAK-STAT signaling pathway	hsa04630	signaling pathways
PDGFRB	5159	Pathways in cancer	hsa05200	cancer
PDGFRB	5159	MicroRNAs in cancer	hsa05206	cancer
PDGFRB	5159	Prostate cancer	hsa05215	cancer
PDGFRB	5159	Central carbon metabolism in cancer	hsa05230	cancer
PDGFRB	5159	Choline metabolism in cancer	hsa05231	cancer
PLCG1	5335	ErbB signaling pathway	hsa04012	signaling pathways
PLCG1	5335	Ras signaling pathway	hsa04014	signaling pathways
PLCG1	5335	Rap1 signaling pathway	hsa04015	signaling pathways
PLCG1	5335	Calcium signaling pathway	hsa04020	signaling pathways
PLCG1	5335	Chemokine signaling pathway	hsa04062	signaling pathways
PLCG1	5335	NF-kappa B signaling pathway	hsa04064	signaling pathways
PLCG1	5335	HIF-1 signaling pathway	hsa04066	signaling pathways
PLCG1	5335	Phospholipase D signaling pathway	hsa04072	signaling pathways
PLCG1	5335	VEGF signaling pathway	hsa04370	signaling pathways
PLCG1	5335	T cell receptor signaling pathway	hsa04660	signaling pathways

PLCG1	5335	Fc epsilon RI signaling pathway	hsa04664	signaling pathways
PLCG1	5335	Neurotrophin signaling pathway	hsa04722	signaling pathways
PLCG1	5335	Thyroid hormone signaling pathway	hsa04919	signaling pathways
PLCG1	5335	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
PLCG1	5335	Pathways in cancer	hsa05200	cancer
PLCG1	5335	Proteoglycans in cancer	hsa05205	cancer
PLCG1	5335	MicroRNAs in cancer	hsa05206	cancer
PLCG1	5335	Non-small cell lung cancer	hsa05223	cancer
PLCG1	5335	Choline metabolism in cancer	hsa05231	cancer
PLCG1	5335	PD-L1 expression and PD-1 checkpoint pat	hsa05235	cancer
PTK2B	2185	Calcium signaling pathway	hsa04020	signaling pathways
PTK2B	2185	Chemokine signaling pathway	hsa04062	signaling pathways
PTK2B	2185	Phospholipase D signaling pathway	hsa04072	signaling pathways
PTK2B	2185	GnRH signaling pathway	hsa04912	signaling pathways
RPS6KA1	6195	MAPK signaling pathway	hsa04010	signaling pathways
RPS6KA1	6195	mTOR signaling pathway	hsa04150	signaling pathways
RPS6KA1	6195	Neurotrophin signaling pathway	hsa04722	signaling pathways
RPS6KA2	6196	MAPK signaling pathway	hsa04010	signaling pathways
RPS6KA2	6196	mTOR signaling pathway	hsa04150	signaling pathways
RPS6KA2	6196	Neurotrophin signaling pathway	hsa04722	signaling pathways
RPS6KA3	6197	MAPK signaling pathway	hsa04010	signaling pathways
RPS6KA3	6197	mTOR signaling pathway	hsa04150	signaling pathways
RPS6KA3	6197	Neurotrophin signaling pathway	hsa04722	signaling pathways
RPS6KA4	8986	MAPK signaling pathway	hsa04010	signaling pathways
RPS6KA4	8986	TNF signaling pathway	hsa04668	signaling pathways
RPS6KA5	9252	MAPK signaling pathway	hsa04010	signaling pathways
RPS6KA5	9252	TNF signaling pathway	hsa04668	signaling pathways
RPS6KA5	9252	Neurotrophin signaling pathway	hsa04722	signaling pathways
RPS6KA5	9252	Pathways in cancer	hsa05200	cancer
RPS6KA5	9252	MicroRNAs in cancer	hsa05206	cancer
RPS6KA5	9252	Bladder cancer	hsa05219	cancer
RPS6KB2	6199	ErbB signaling pathway	hsa04012	signaling pathways
RPS6KB2	6199	HIF-1 signaling pathway	hsa04066	signaling pathways
RPS6KB2	6199	mTOR signaling pathway	hsa04150	signaling pathways
RPS6KB2	6199	PI3K-Akt signaling pathway	hsa04151	signaling pathways
RPS6KB2	6199	AMPK signaling pathway	hsa04152	signaling pathways
RPS6KB2	6199	TGF-beta signaling pathway	hsa04350	signaling pathways
RPS6KB2	6199	Apelin signaling pathway	hsa04371	signaling pathways
RPS6KB2	6199	Insulin signaling pathway	hsa04910	signaling pathways
RPS6KB2	6199	Pathways in cancer	hsa05200	cancer
RPS6KB2	6199	Proteoglycans in cancer	hsa05205	cancer
RPS6KB2	6199	Colorectal cancer	hsa05210	cancer
RPS6KB2	6199	Pancreatic cancer	hsa05212	cancer
RPS6KB2	6199	Breast cancer	hsa05224	cancer
RPS6KB2	6199	Gastric cancer	hsa05226	cancer
RPS6KB2	6199	Choline metabolism in cancer	hsa05231	cancer
RPS6KB2	6199	PD-L1 expression and PD-1 checkpoint pat	hsa05235	cancer
SRC	6714	Adherens junction	hsa04520	junctional complex

SRC	6714	Gap junction	hsa04540	junctional complex
SRC	6714	Tight junction	hsa04530	junctional complex
SRC	6714	Focal adhesion	hsa04510	junctional complex
SRC	6714	ErbB signaling pathway	hsa04012	signaling pathways
SRC	6714	Rap1 signaling pathway	hsa04015	signaling pathways
SRC	6714	Chemokine signaling pathway	hsa04062	signaling pathways
SRC	6714	VEGF signaling pathway	hsa04370	signaling pathways
SRC	6714	C-type lectin receptor signaling pathway	hsa04625	signaling pathways
SRC	6714	GnRH signaling pathway	hsa04912	signaling pathways
SRC	6714	Estrogen signaling pathway	hsa04915	signaling pathways
SRC	6714	Prolactin signaling pathway	hsa04917	signaling pathways
SRC	6714	Thyroid hormone signaling pathway	hsa04919	signaling pathways
SRC	6714	Oxytocin signaling pathway	hsa04921	signaling pathways
SRC	6714	Relaxin signaling pathway	hsa04926	signaling pathways
SRC	6714	Proteoglycans in cancer	hsa05205	cancer
SRC	6714	Bladder cancer	hsa05219	cancer
STAT1	6772	Chemokine signaling pathway	hsa04062	signaling pathways
STAT1	6772	Toll-like receptor signaling pathway	hsa04620	signaling pathways
STAT1	6772	NOD-like receptor signaling pathway	hsa04621	signaling pathways
STAT1	6772	C-type lectin receptor signaling pathway	hsa04625	signaling pathways
STAT1	6772	JAK-STAT signaling pathway	hsa04630	signaling pathways
STAT1	6772	Prolactin signaling pathway	hsa04917	signaling pathways
STAT1	6772	Thyroid hormone signaling pathway	hsa04919	signaling pathways
STAT1	6772	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
STAT1	6772	Pathways in cancer	hsa05200	cancer
STAT1	6772	Pancreatic cancer	hsa05212	cancer
STAT1	6772	PD-L1 expression and PD-1 checkpoint pat	hsa05235	cancer
STAT2	6773	Chemokine signaling pathway	hsa04062	signaling pathways
STAT2	6773	NOD-like receptor signaling pathway	hsa04621	signaling pathways
STAT2	6773	C-type lectin receptor signaling pathway	hsa04625	signaling pathways
STAT2	6773	JAK-STAT signaling pathway	hsa04630	signaling pathways
STAT2	6773	Pathways in cancer	hsa05200	cancer
STAT3	6774	Chemokine signaling pathway	hsa04062	signaling pathways
STAT3	6774	HIF-1 signaling pathway	hsa04066	signaling pathways
STAT3	6774	FoxO signaling pathway	hsa04068	signaling pathways
STAT3	6774	JAK-STAT signaling pathway	hsa04630	signaling pathways
STAT3	6774	Prolactin signaling pathway	hsa04917	signaling pathways
STAT3	6774	Adipocytokine signaling pathway	hsa04920	signaling pathways
STAT3	6774	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
STAT3	6774	Pathways in cancer	hsa05200	cancer
STAT3	6774	Proteoglycans in cancer	hsa05205	cancer
STAT3	6774	MicroRNAs in cancer	hsa05206	cancer
STAT3	6774	Pancreatic cancer	hsa05212	cancer
STAT3	6774	Non-small cell lung cancer	hsa05223	cancer
STAT3	6774	PD-L1 expression and PD-1 checkpoint pat	hsa05235	cancer
STAT5A	6776	ErbB signaling pathway	hsa04012	signaling pathways
STAT5A	6776	JAK-STAT signaling pathway	hsa04630	signaling pathways
STAT5A	6776	Prolactin signaling pathway	hsa04917	signaling pathways

STAT5A	6776	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
STAT5A	6776	Pathways in cancer	hsa05200	cancer
STAT5A	6776	Non-small cell lung cancer	hsa05223	cancer
STAT5B	6777	ErbB signaling pathway	hsa04012	signaling pathways
STAT5B	6777	Chemokine signaling pathway	hsa04062	signaling pathways
STAT5B	6777	JAK-STAT signaling pathway	hsa04630	signaling pathways
STAT5B	6777	Prolactin signaling pathway	hsa04917	signaling pathways
STAT5B	6777	AGE-RAGE signaling pathway in diabetic c	hsa04933	signaling pathways
STAT5B	6777	Pathways in cancer	hsa05200	cancer
STAT5B	6777	Non-small cell lung cancer	hsa05223	cancer
STAT6	6778	JAK-STAT signaling pathway	hsa04630	signaling pathways
STAT6	6778	Pathways in cancer	hsa05200	cancer
TP53	7157	MAPK signaling pathway	hsa04010	signaling pathways
TP53	7157	Sphingolipid signaling pathway	hsa04071	signaling pathways
TP53	7157	p53 signaling pathway	hsa04115	signaling pathways
TP53	7157	PI3K-Akt signaling pathway	hsa04151	signaling pathways
TP53	7157	Wnt signaling pathway	hsa04310	signaling pathways
TP53	7157	Neurotrophin signaling pathway	hsa04722	signaling pathways
TP53	7157	Thyroid hormone signaling pathway	hsa04919	signaling pathways
TP53	7157	Pathways in cancer	hsa05200	cancer
TP53	7157	Transcriptional misregulation in cancer	hsa05202	cancer
TP53	7157	Proteoglycans in cancer	hsa05205	cancer
TP53	7157	MicroRNAs in cancer	hsa05206	cancer
TP53	7157	Colorectal cancer	hsa05210	cancer
TP53	7157	Pancreatic cancer	hsa05212	cancer
TP53	7157	Endometrial cancer	hsa05213	cancer
TP53	7157	Prostate cancer	hsa05215	cancer
TP53	7157	Thyroid cancer	hsa05216	cancer
TP53	7157	Bladder cancer	hsa05219	cancer
TP53	7157	Small cell lung cancer	hsa05222	cancer
TP53	7157	Non-small cell lung cancer	hsa05223	cancer
TP53	7157	Breast cancer	hsa05224	cancer
TP53	7157	Gastric cancer	hsa05226	cancer
TP53	7157	Central carbon metabolism in cancer	hsa05230	cancer
YES1	7525	Adherens junction	hsa04520	junctional complex
YES1	7525	Tight junction	hsa04530	junctional complex