Abcg2a is the functional homolog of human ABCG2 expressed at the zebrafish blood-brain barrier

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Abstract

A principal protective component of the mammalian blood-brain barrier (BBB) is the high expression of the multidrug efflux transporters P-glycoprotein (P-gp, encoded by *ABCB1*) and ABCG2 (encoded by *ABCG2*) on the luminal surface of endothelial cells. The zebrafish P-gp homolog Abcb4 is expressed at the BBB and phenocopies P-gp. Comparatively little is known about the four zebrafish homologs of the human *ABCG2* gene: *abcg2a*, *abcg2b*, *abcg2c*, and *abcg2d*. Here we report the functional characterization and brain tissue distribution of zebrafish ABCG2 homologs. To determine substrates of the transporters, we stably expressed each in HEK-293 cells and performed cytotoxicity and fluorescent efflux assays with known ABCG2 substrates. We found Abcg2a had the greatest substrate overlap with ABCG2, and Abcg2d appeared to be the least functionally similar. Using RNAscope *in situ* hybridization we identified *abcg2a* as the only homolog expressed at the adult and larval zebrafish BBB, based on its localization to claudin-5 positive brain vasculature. These results demonstrate the conserved function of zebrafish Abcg2a and suggest that zebrafish may be an appropriate model organism for studying the role of ABCG2 at the BBB.

Introduction

The blood-brain barrier (BBB) refers to the specialized protective adaptations of the brain microvasculature, which pose a significant impediment to the delivery of therapeutics to the brain [1]. Brain endothelial cells play a prominent role at the BBB in that they form tight junctions that prevent paracellular transport constituting a physical barrier to compounds, but they also express ATP-binding cassette transporters that form a chemical barrier and actively transport compounds back into the blood supply, thus limiting brain penetration [2]. P-glycoprotein (P-gp, encoded by the *ABCB1* gene) and ABCG2 (encoded by the *ABCG2* gene) are the most highly expressed ABC transporters at the mammalian BBB. Knockout mouse models have consistently demonstrated the important role played by P-gp and ABCG2 at the BBB in limiting the brain penetration of therapeutic agents [3]. For example, eight hours after oral administration of 10 mg/kg of the janus kinase (JAK) 1 and 2 inhibitor momelotinib, the brain concentration was 3.1-fold, 6.5-fold, and 48.4-fold higher in mice deficient in Abcb1a/b, Abcg2 or Abcb1a/b;Abcg2 compared to wild-type mice [4]. Several targeted therapies are known to be restricted from the brain due to ABC transporter overexpression as demonstrated by knockout mouse models [5], however, mouse models have certain limitations.

Given its small size and amenability to higher throughput assays, the zebrafish has been suggested as a suitable *in vivo* model of the BBB. However, little is known about the role of ABC transporters at the zebrafish BBB. Zebrafish express 2 homologs of

human *ABCB1*, termed *abcb4* and *abcb5* [6]. While early studies with the P-glycoprotein antibody C219 suggested that a zebrafish homolog of P-gp was expressed in brain capillaries [7, 8], the epitope for the C219 antibody is present in both homologs of P-gp in the zebrafish [8]. We subsequently showed that Abcb4 is the sole P-gp homolog expressed at the zebrafish BBB and found that the substrate specificity of Abcb4 completely overlaps with that of P-gp, while Abcb5 confers resistance to a slightly narrower range of substrates [9]. Much less work has been done to characterize the zebrafish homologs of *ABCG2*.

Zebrafish have 4 direct homologs of the human *ABCG2* gene—*abcg2a*, *abcg2b*, *abcg2c* and *abcg2d*—yet relatively little is known about the function or expression of the transporters [10]. Tsinkalovsky and colleagues hypothesized that an *ABCG2*-related gene was expressed in cells extracted from zebrafish kidney marrow, which serves a function similar to that of bone marrow in mammals, based on the discovery of a side-population of cells that accumulated low levels of Hoechst 33342, a fluorescent ABCG2 substrate [11]. This side population of cells could be reduced by the addition of reserpine or fumitremorgin C (FTC) both of which are known to inhibit ABCG2-mediated transport [11]. These observations were extended by Kobayashi et al., who isolated side population cells and found expression of both *abcg2a* and *abcg2c* [12]. To determine which was responsible for transport of Hoechst 33342, they transfected cells with either gene and found that *abcg2a* overexpression resulted in decreased Hoechst 33342 retention [12]. Additionally, expression of *abcg2a* and *abcg2b* was found to be high in the intestine, while *abcg2c* expression was high in the head kidney, body kidney,

spleen, intestine and gill; *abcg2d* was not detected in any of the tissues examined [12]. Given the paucity of data, a thorough investigation of the zebrafish homologs of human ABCG2 is needed. While we hypothesize that an ABCG2 homolog is present at the zebrafish BBB, this has not been proven. We therefore sought to determine which ABCG2 homologs are expressed at the zebrafish BBB and to compare the substrate specificity of the various homologs to that of human ABCG2.

Materials and Methods

Chemicals

Mitoxantrone was obtained from Sigma-Aldrich (St. Louis, MO). Pheophorbide a was from Frontier Specialty Chemicals (Logan, UT). MLN-7243 and MLN-4924 were purchased from ChemieTek (Indianapolis, IN). THZ531 and Ko143 were obtained from Cayman Chemical (AnnArbor, MI). PF-3758309 and Gedatolisib were from ApexBio Technology (Houston, TX). CUDC-101, KS176 and tariquidar were from MedChem Express (Monmouth Junction, NJ). Elacridar was from Selleck chemicals (Houston, TX).

Zebrafish housing and husbandry

Zebrafish (*Danio rerio*) wild-type strain TAB5 were housed with a 14-hour/10-hour light/dark cycle with a water temperature of 28.5°C in a recirculating aquatic system. Zebrafish husbandry and embryonic staging was conducted as previously described [13]. Zebrafish embryos were raised in an incubator at 28.5 °C in Instant Ocean (Blacksburg, VA) (60 mg/L DI water) and were euthanized at 7 days post-fertilization.

All animal studies were compliant with the National Cancer Institute-Bethesda Animal Care and Use Committee approved study protocol (LCB-033-2).

Cell lines

HEK-293 cells (ATCC, Manassas, VA) were transfected with empty pcDNA3.1 vector or with vector containing full-length *abcg2a* (NM_001042775), *abcg2b* (NM_001039066), *abcg2c* (XM_005156523) or *abcg2d* (NM_001042772) (all from Genscript, Piscataway, NJ), flanked by a FLAG tag sequence. The R-5 cell line was generated as previously described from HEK-293 cells transfected with full-length *ABCG2* [14]. Transfected cells were cultured at 37 °C 5% CO_2 in EMEM (Quality Biological, Gaithersburg, MD) supplemented with penicillin/streptomycin (Quality Biological) and 2 mg/ml G418 (Mediatech, Manassas, VA).

Flow Cytometry

Flow cytometry efflux assays with fluorescent human ABCG2 substrates were based on those previously described [15]. Briefly, trypsinized cells were plated and incubated with fluorescent substrates (5 µM pheophorbide a or 5 µM mitoxantrone) in the presence or absence of ABCG2 inhibitors (10 µM KS-176, 10 µM Ko 143, 10 µM elacridar, 10 µM tariquidar) at 37 °C for 30 minutes. The medium was removed, and cells were incubated in media in the presence or absence of inhibitors for 1 h. Cells were then washed and re-suspended in ice-cold PBS prior to analysis using a FACSCanto II flow cytometer (BD Biosciences San Jose, CA) and data were then analyzed with FlowJo software (v 10.8.1, Tree Star, Inc, Ashland, OR). Samples incubated with fluorescent substrates

without inhibitors are defined as the ‘efflux’ histogram, and unstained cell samples are the control cell autofluorescence histogram.

Cytotoxicity Assays

Trypsinized cells were plated at 5,000 cells/well in white opaque 96-well plates and allowed to adhere overnight. Drugs were then added, with each concentration in triplicate, and cells were incubated for 72 hours. Cell growth was assessed using the CellTiter-Glo (Promega, Madison, WI) reagent, according to the manufacturer’s instructions. Luminescence was measured on a Tecan Infinite M200 Pro microplate reader (Tecan Group, Morrisville, NC). 50% growth inhibition (GI_{50}) values were calculated at the drug concentration at which 50% luminescence was observed in comparison to untreated cells. Relative resistance (RR) values are the ratio of the GI_{50} values of the transporter transfected versus the empty vector transfected HEK-293 cells.

Immunofluorescence and RNAScope probes

Adult zebrafish were euthanized by immersion in a lethal dose of Tricane Methanesulfonate for 30 minutes, prior to fixation in 10% neutral buffered formalin (NBF) for 24 hours at room temperature. Larvae were anesthetized using Tricane Methanesulfonate and euthanized via rapid chilling (hypothermic shock), prior to fixation in 10% NBF for 24 hours at room temperature. Zebrafish were incubated in 0.5 M EDTA (pH 8) at room temperature for 7 days with gentle agitation. Before embedding and processing, zebrafish were washed twice with nuclease-free PBS. Paraffin-embedded

samples were processed as previously described [9]. Briefly, coronal and transverse sections were cut from the paraffin block at 5 µm and placed on positively charged slides for RNA in situ hybridization (ISH) (RNAscope) and immunohistochemistry (IHC).

The expression of *Danio rerio abcg2a*, *abcg2b*, *abcg2c*, *abcg2d*, and *abcb4* genes was detected by staining 5 µm FFPE sections with the RNAscope® 2.5 LS Probe-Dr-abcg2a (Advanced Cell Diagnostics (ACD) (Newark, CA), Cat# 493571), Dr-abcg2b-C2 (ACD, Cat# 516878-C2), Dr-abcg2c-C2 (ACD, Cat# 516888-C2), Dr-abcg2d-C2 (ACD, Cat# 516898-C2), or Dr-abcb4-C2 (ACD, Cat# 493558-C2) with the RNAscope LS Multiplex Fluorescent Assay (ACD, Cat# 322800) using the Bond RX auto-stainer (Leica Biosystems, Deer Park, IL) with a tissue pretreatment of 15 minutes at 95°C with Bond Epitope Retrieval Solution 2 (Leica Biosystems), 15 minutes of Protease III (ACD) at 40°C and 1:750 dilution of OPAL570 or OPAL690 reagents (Akoya Biosciences, Marlborough, MA). Immunohistochemistry for claudin-5 co-localization was performed after RNAScope staining, using an anti-claudin-5 antibody (Invitrogen, Grand Rapids, NY, cat #35-2500) at a 1:50 dilution for 30 minutes using the Bond Polymer Refine Kit (Leica Biosystems, cat# DS9800) minus the DAB and Hematoxylin with a 1:150 dilution of OPAL520 reagent for 10 min. The RNAscope 3-plex LS Multiplex Negative Control Probe [Bacillus subtilis dihydrodipicolinate reductase (dapB) gene) in channels C1, C2, and C3, ACD, Cat# 320878] followed by IHC with no primary antibody was used as an ISH and IHC negative control. Slides were digitally imaged using an Aperio ScanScope FL Scanner (Leica Biosystems).

Results

Characterization of HEK-293 cells stably expressing zebrafish Abcg2a-d

Human embryonic kidney (HEK-293) cells were stably transfected to express an empty vector, or a vector containing human *ABCG2*, zebrafish *abcg2a*, *abcg2b*, *abcg2c*, or *abcg2d*. Zebrafish gene constructs included a 5' FLAG tag sequence to enable protein immunodetection, as there are no commercially available antibodies that cross-react with any of the zebrafish ABCG2 homologs. Positive FLAG staining was observed in pellets of Abcg2a-d expressing cells (Fig 1.A). RNAscope probe positive signal was only observed in the corresponding transfected cell line, demonstrating specific detection of *abcg2a-d* homologs (Fig 1.B). The level of RNAscope probe staining was comparable among samples, indicating a similar level of mRNA expression among the corresponding cell lines. The typical punctate staining pattern seen in RNAscope probe images is not observed here due to the high density of signal.

Zebrafish Abcg2 homologs confer resistance to ABCG2 substrates

Cytotoxicity assays were performed using known cytotoxic substrates of ABCG2 [16-21], to determine if zebrafish Abcg2a-d were able to confer resistance, quantified by the drug concentration that produces 50% cell growth inhibition (GI_{50}). Abcg2a conferred the highest level of resistance of the zebrafish Abcg2 homologs to MLN7243 (a ubiquitin-activating enzyme inhibitor), MLN4924 (NEDD8 activating enzyme inhibitor), and CUDC-101 (histone deacetylase and receptor tyrosine kinase inhibitor) (Fig 2, Table S1). Abcg2d conferred the least resistance to THZ 531 (CDK12/13 inhibitor), CUDC-101, and Gedatolisib (an inhibitor of phosphatidylinositol-3-kinase (PI3K) and

mammalian target of rapamycin (mTOR)). All zebrafish Abcg2 homologs conferred resistance to PF-3758309 (p21-activated kinase 4 inhibitor). Abcg2b and Abcg2c often conferred similar levels of resistance to the same substates, as evidenced by MLN7243, MLN4924, THZ 531, CUDC-101 and Gedatolisib.

Zebrafish Abcg2 homologs can efflux fluorescent ABCG2 substrates

We assessed the ability of Abcg2a-d homolog-expressing cells to efflux the fluorescent ABCG2 substrates mitoxantrone and pheophorbide a. Cells were incubated with 5 µM mitoxantrone or pheophorbide a in the absence or presence of 10 µM of the ABCG2 inhibitors KS176, Ko 143, elacridar or tariquidar, and flow cytometry was used to measure intracellular fluorescence. When co-incubated with inhibitors, ABCG2 expressing cells, exhibit an increase in intracellular fluorescence evidenced by a shift to the right of the inhibited peaks compared to the uninhibited efflux peak, with little overlap between the peaks (Fig 3). There was a rightward inhibited peak shift for Abcg2a with mitoxantrone, and to a more pronounced shift with pheophorbide a, indicating Abcg2a can efflux both substrates. Pheophorbide a also appears to be a substrate for Abcg2b and Abcg2c. Neither mitoxantrone nor pheophorbide a were transported by Abcg2d, indicated by the overlay of the efflux and inhibited histograms. In addition to substrate specificities, these data also show all 4 ABCG2 inhibitors were able to inhibit Abcg2a, Abcg2b and Abg2c.

***abcg2a* is expressed at the adult blood-brain barrier**

Since ABCG2 is highly expressed at the mammalian BBB, we hypothesized that one or more of the ABCG2 homologs would localize to the adult zebrafish brain. Positive staining for *abcg2a* RNA was present in the adult brain, evidenced by the punctate signal seen in cross-sections of the telencephalon (forebrain), which localized to the vasculature (Fig 4). No positive staining was observed for *abcg2b, abcg2c* or *abcg2d* in the brain. To determine if the vasculature where *abcg2a* localized had BBB properties, we co-stained with the endothelial tight junction protein claudin-5, with an antibody that cross-reacts with mammalian and zebrafish claudin-5 homologs, which is a canonical marker of the BBB [22]. The positive signal for *abcg2a* co-localized with claudin-5 positive vasculature, indicating it is expressed at the adult zebrafish BBB (Fig 5). The zebrafish P-gp homolog *abcb4*, which we previously determined is expressed at the zebrafish BBB [9], also localized to the same claudin-5 positive vessels as *abcg2a*.

***abcg2a* is expressed at the developing larval blood-brain barrier**

The zebrafish BBB begins to form between 1- and 3-days post fertilization (dpf) and becomes progressively restrictive until 10 dpf [8, 23]. We therefore sought to determine if any *abcg2* homologs localized to the developing larval BBB. At 7 dpf we detected *abcg2a* which localized to claudin-5 positive brain vasculature (Fig 6). In concordance with our adult staining, *abcg2b, abcg2c* and *abcg2d* do not localize to the larval brain.

Discussion

Here we report our characterization of the function and brain distribution of the zebrafish homologs of ABCG2. We used stably transfected HEK-293 cells as a model to study the

function of each of the zebrafish Abcg2 homologs in isolation, and a panel of 8 substrates and 4 inhibitors of ABCG2 in cytotoxicity and fluorescent efflux assays. We found that Abcg2a had the greatest substrate overlap with ABCG2 and was able to transport all assayed ABCG2 substrates. Abcg2d appeared to be the most divergent from ABCG2 and had the lowest GI_{50} for 4 out of the 6 cytotoxic substrates and did not efflux either fluorescent substrate. We expected Abcg2a to be the most similar to ABCG2, given that it has the highest amino acid homology (61.4%, Table S2), is known to efflux the ABCG2 substrate Hoechst 33342, and is inhibited by the ABCG2 inhibitor verapamil [12]. It was surprising to us that Abcg2d appeared to be functionally the least similar to ABCG2, as it has the second highest amino acid homology (57.4%) to ABCG2 and was predicted based on synteny to be the most direct homolog of ABCG2 [10]. Thus, we found that all 4 zebrafish homologs were able to transport select ABCG2 substrates to varying degrees and that the substrate profile of Abcg2a is most similar to that of human ABCG2. The only homolog that localized to the adult and larval zebrafish BBB was *abcg2a*.

Overall, the ABCG2 substrates we assayed seem to be weaker substrates for the zebrafish homologs, evidenced by the lower GI_{50} values in cytotoxicity assays compared to ABCG2 (Fig 2, Table S1), and the less pronounced peak shift between the uninhibited and inhibited conditions in fluorescent efflux assays (Fig 3). The only exception to this was in the case of Gedatolisib, which was transported slightly more efficiently by Abcg2b (GI_{50} 4.06±1.46 μM) than ABCG2 (GI_{50} 3.84±1.53μM). The ABCG2 inhibitors KS176, Ko 143, elacridar and tariquidar were all able to inhibit

Abcg2a, -b and -c, at the same concentration used with ABCG2 (10 μM). It is unknown if they are efficacious against Abcg2d, and this will need to be assessed with additional fluorescent substrates, or in cytotoxicity assays with identified Abcg2d substrates. Some human P-gp inhibitors are less effective inhibitors of the zebrafish homologs Abcb4 and Abcb5 in the case of select substrates. For example, the P-gp (and ABCG2) inhibitor elacridar was less effective than the P-gp inhibitor valspodar at inhibiting Abcb4 and Abcb5 rhodamine 123 transport [9]. It is not possible to determine if this phenomenon exists for the zebrafish Abcg2 isoforms, although the overlay of the inhibited peaks suggests all 4 inhibitors are similarly effective at inhibiting the Abcg2a-c transport of pheophorbide a. A wider range of ABCG2 substrates and varying inhibitor concentrations are needed to assess substrate-specific inhibitor efficacy for zebrafish Abcg2.

Expression of zebrafish ABC transporters in mammalian cells is a tractable method to assess their function in isolation; however, it comes with the caveat that zebrafish proteins may not function in these heterologous expression systems as they do *in vivo* in zebrafish. Fluorescent ABCG2 substrates could be administered *in vivo* to zebrafish larvae in the presence or absence of inhibitors in conjunction with fluorescent microscopy to confirm if they are Abcg2 substrates, as has been done for zebrafish Abcb4 with rhodamine 123 and calcein-AM [6].

Zebrafish *abcg2a* was the only homolog expressed at the BBB, evidenced by its localization to claudin-5 positive brain vasculature in adult and larval zebrafish. *abcg2a*

and *abcb4* localized to the same vessels in the adult brain, and likely represent the relevant ABCG2 and P-gp homologs at the BBB. This suggests they may function in concert to protect the zebrafish brain parenchyma, as they do in mammals. It will be interesting to determine at what developmental stage *abcg2a* and *abcb4* localize to the BBB, as this will inform the utility of larval zebrafish for BBB studies. Kobayashi and colleagues detected *abcg2a* transcripts in the adult zebrafish kidney, spleen, intestine and gills; *abcg2b* in the spleen, liver and intestine; *abcg2c* in the kidney, spleen, liver, intestine and gills; and *abcg2d* was not detected in any of the assessed tissues [12]. ABCG2 is also expressed in these tissues in mammals, and zebrafish Abcg2a-c may have conserved functions in these organs. The function of Abcg2d remains elusive.

Farrell and colleagues recently published an online resource called Daniocell, which presents single-cell whole-animal RNAseq data from wild-type zebrafish embryos and larvae from 3.3-120 hours post fertilization (hpf), with data compiled from their 2 published datasets [24-26]. Their data confirm the presence of *abcg2a* at the BBB, with high transcript expression detected from 24 hpf onwards in the ‘hema.28 vasculature – blood-brain barrier’ subcluster; suggesting *abcg2a* localizes to the early BBB during angiogenesis. Interestingly, *abcb4* is also expressed from 24 hpf in this subcluster. Consistent with the literature and our data, *abcg2a* is predominantly expressed in the ‘endoderm’ and ‘hematopoietic/vasculature’ cell groups. *abcg2b* was only detected in the ‘endoderm’ and ‘pronephros’; and *abcg2c* is ubiquitously expressed at low levels across cell groups. *abcg2d* was detected at low levels except for in the ‘eye.22 retinal pigmented epithelium - early/developing’ subcluster of the ‘eye’ cell group.

In conclusion, we have shown zebrafish ABCG2 homologs show functional conservation, with Abcg2a appearing to be most similar to ABCG2. The localization of *abcg2a* to the BBB, and its substrate and inhibitor overlap with ABCG2, indicates that the zebrafish has potential utility for the study of Abcg2 activity at the BBB. Our data, and the literature, demonstrate functional similarities between Abcg2a and ABCG2, and Abcb4 and P-gp, giving credence to the idea that the zebrafish could be a relevant model system to assess the brain penetration of substrate drugs [6, 9, 12].

Abbreviations

ABC, ATP-binding cassette; BBB, blood-brain barrier; dpf, days post-fertilization; ABCG2, human protein; *ABCG2* human gene; Abcg2a-d, zebrafish proteins; *abcg2a-d*, zebrafish genes; ISH, *in situ hybridization*; IHC, immunohistochemistry.

Declarations

Ethics approval and consent to participate:

Zebrafish studies were performed under a protocol approved by the National Cancer Institute Animal Care and Use Committee (IACUC Number LCB-033).

Consent for publication:

Not applicable

Availability of data and materials:

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests:

The authors have no competing interests to declare.

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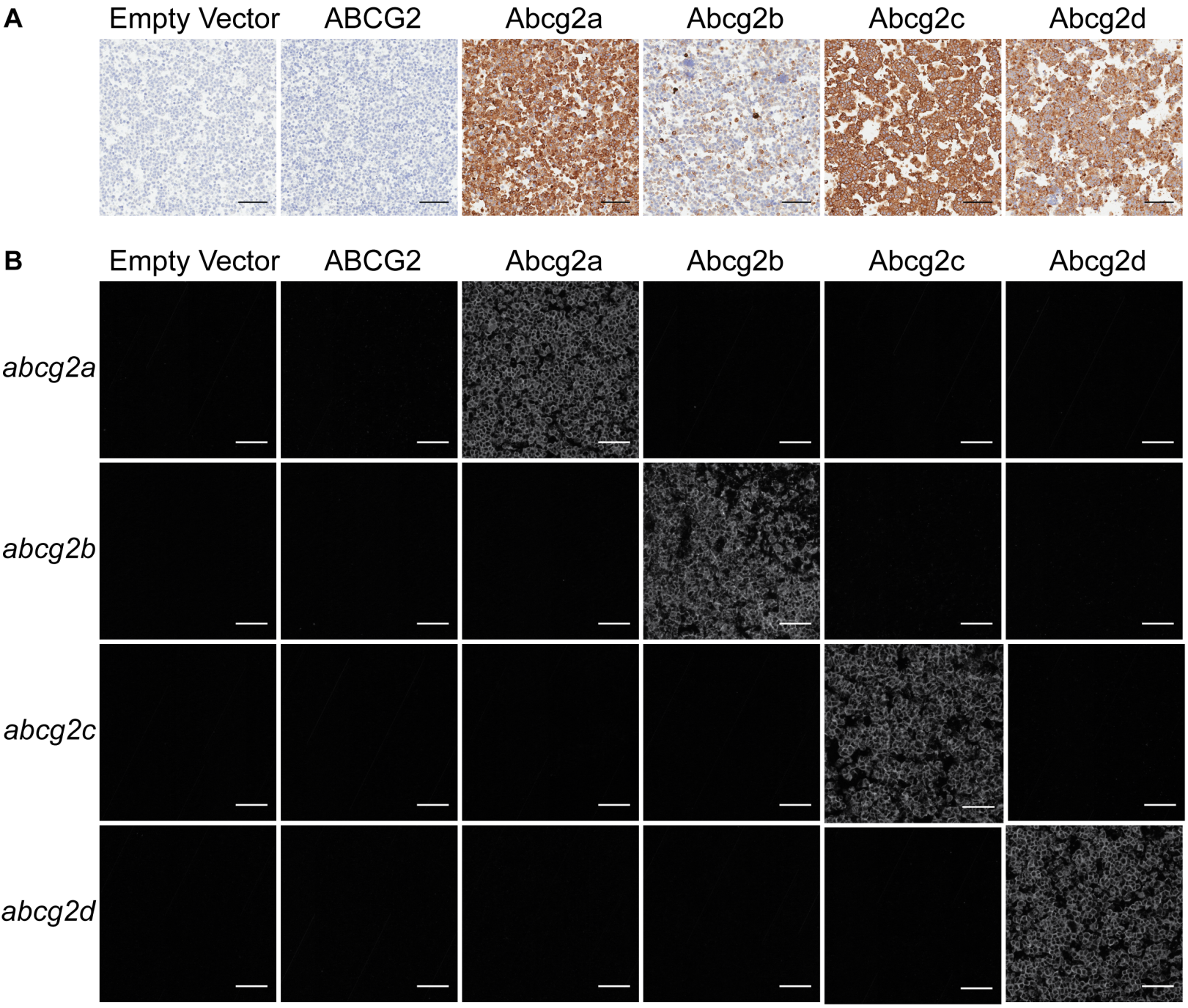

Figure 1: Characterization of HEK-293 cells transfected to express an empty vector, human ABCG2, zebrafish Abcg2a, Abcg2b, Abcg2c, or Abcg2d. Paraffin-embedded cells were probed with (A) an anti-FLAG tag antibody. Staining appears lower in Abcg2b expressing cells; however, in other replicates of this staining, the levels have been comparable to Abcg2a, Abcg2c and Abcg2d expressing cells. (B) RNAscope probes to detect *abcg2a, abcg2b, abcg2c or abcg2d* mRNA.Scale bar = 100 µm.

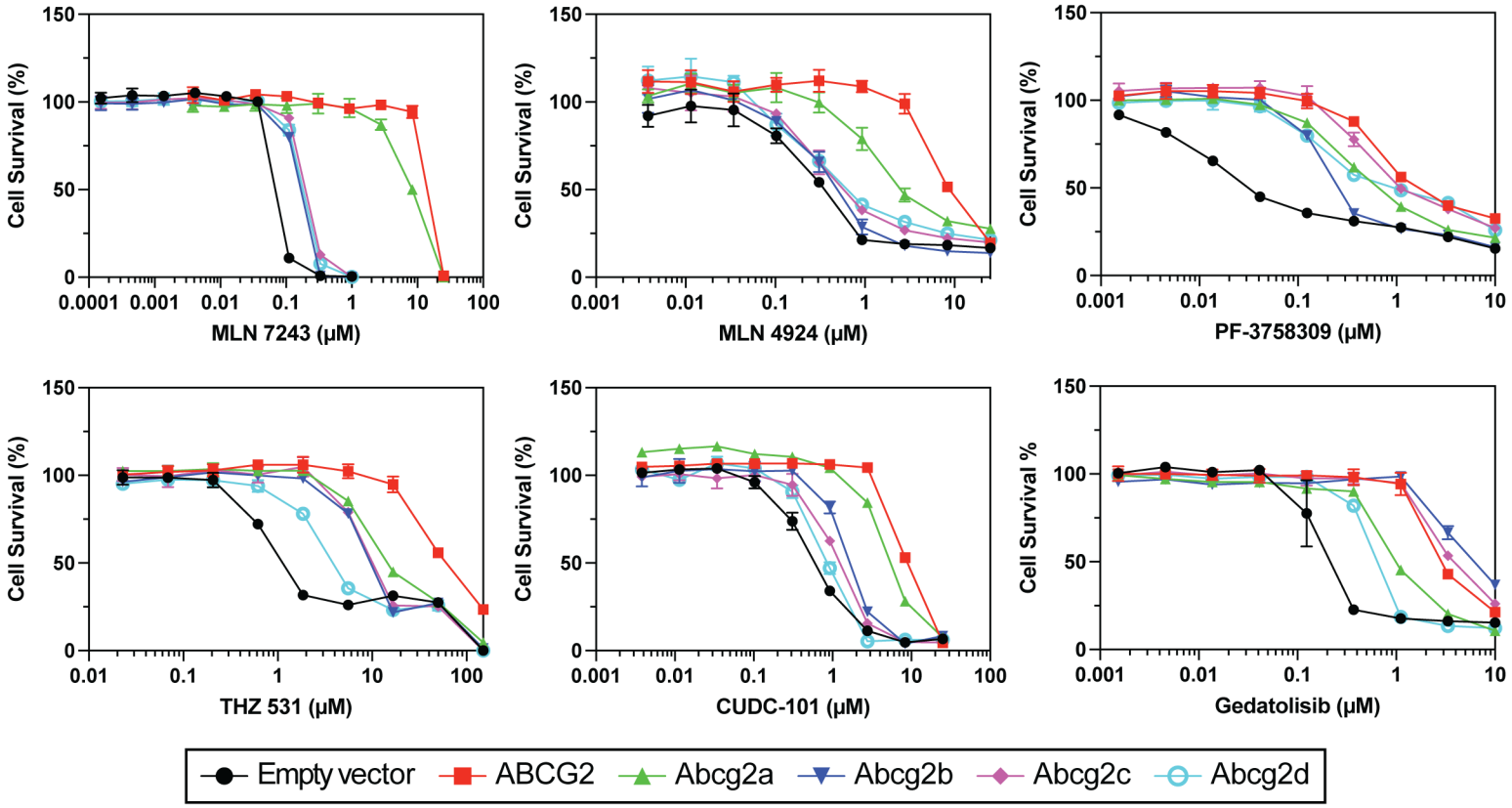

Figure 2: Zebrafish ABCG2 homologs confer resistance to cytotoxic ABCG2 substrates. HEK-293 cells stably expressing empty vector, ABCG2 or Abcg2a-d were treated with known cytotoxic ABCG2 substrates for 3 days. GI_{50} and relative resistance values are summarized in supplemental table 1.

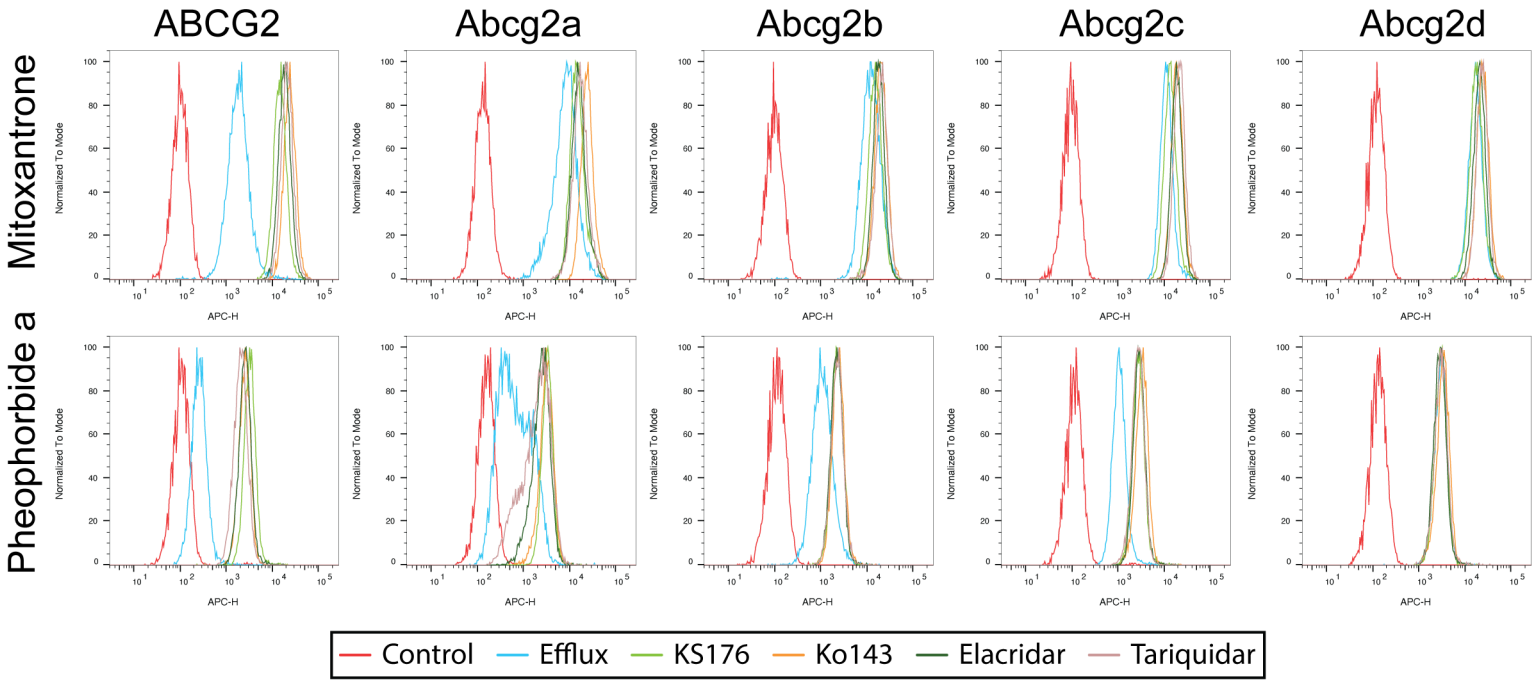

Figure 3: Zebrafish ABCG2 homologs differentially efflux fluorescent ABCG2 substrates. HEK293 cells overexpressing empty vector (EV), ABCG2 or Abcg2a-d were treated the fluorescent ABCG2 substrates mitoxantrone and pheophorbide a in the absence or presence of the ABCG2 inhibitors KS176, Ko 143, elacridar and tariquidar. Representative histograms from at least 3 biological replicates of intracellular fluorescence measured by flow cytometry. The control condition (red) of untreated cells shows cell autofluorescence, and the efflux condition (blue) is cells treated with substrate without inhibitor.

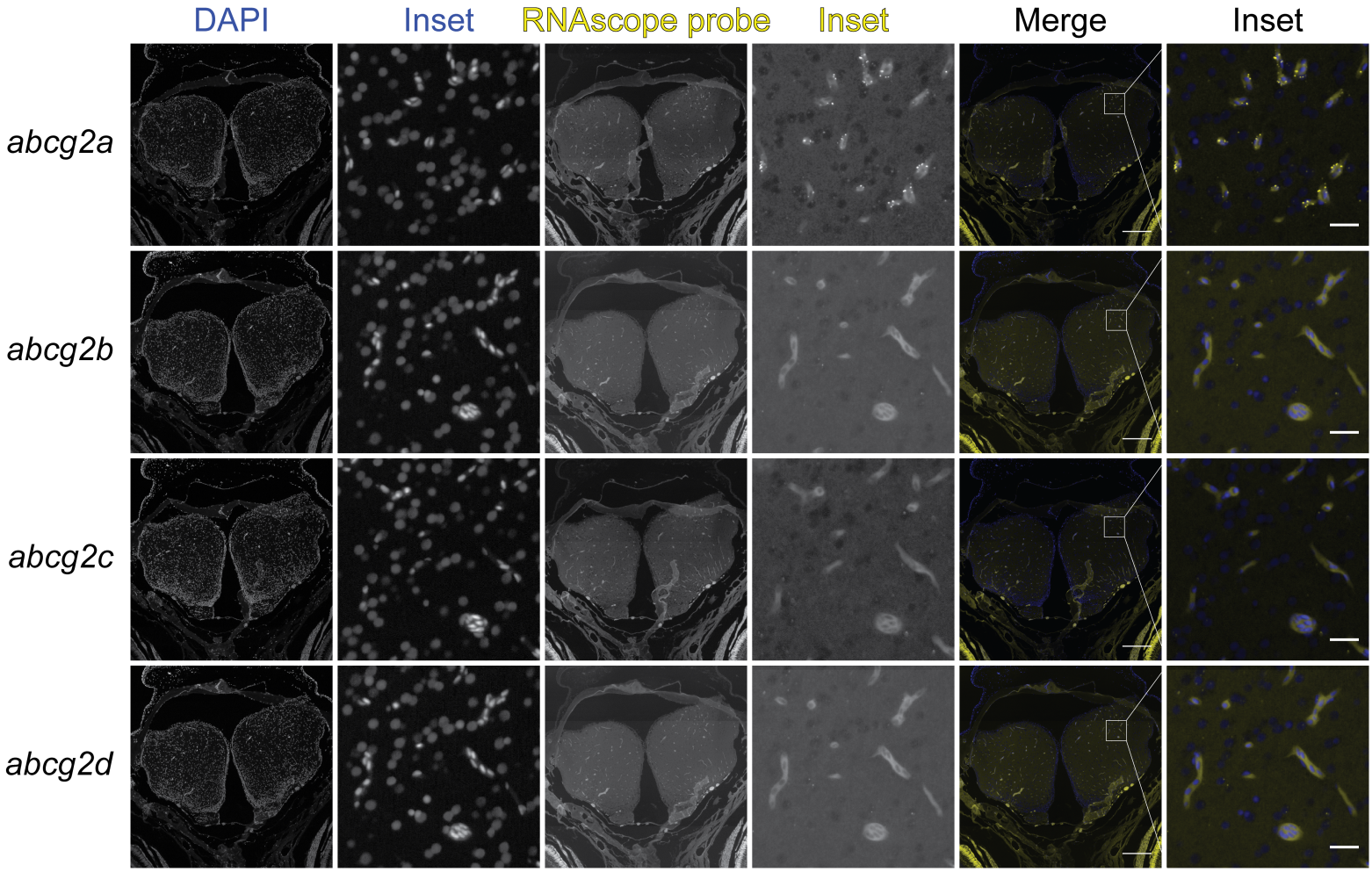

Figure 4: *abcg2a* is the only ABCG2 homolog detected in adult zebrafish brains. Paraffin-embedded adult zebrafish sections were probed with RNAscope probes (yellow) to detect *abcg2a, abcg2b, abcg2c* or *abcg2d* RNA and DAPI (blue). Each punctate dot signal represents amplification of a single target RNA molecule. Sections shown are crossections of the telencephalon. Scale bar = 200 µm, inset scale bar = 20 µm.

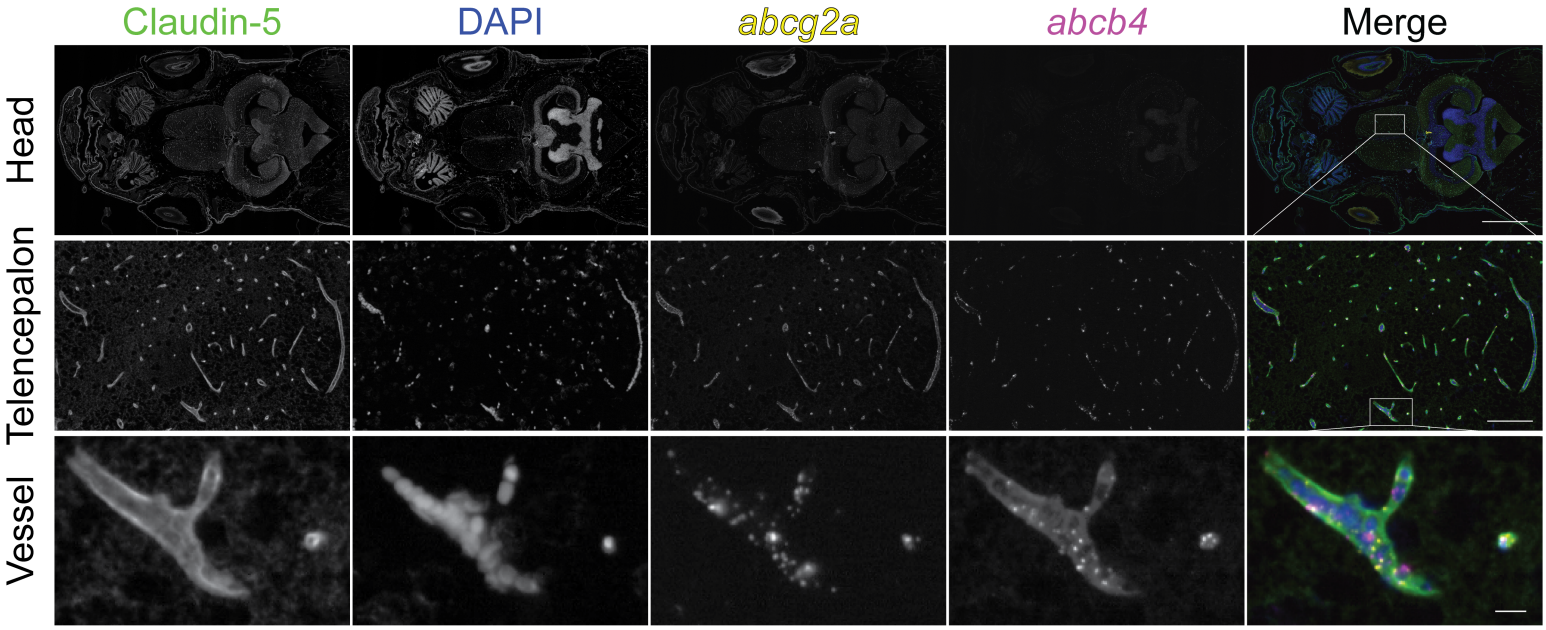

Figure 5: *abcg2a* and *abcb4* are expressed in claudin-5 positive adult brain vasculature.Paraffin-embedded adult zebrafish sections were probed with RNAscope probes (yellow) to detect *abcg2a* and *abcb4* mRNA, an antibody against claudin-5 (green), and DAPI (blue). Head scale bar = 1 mm, telencephalon scale bar = 100 µm, vessel scale bar = 10 µm

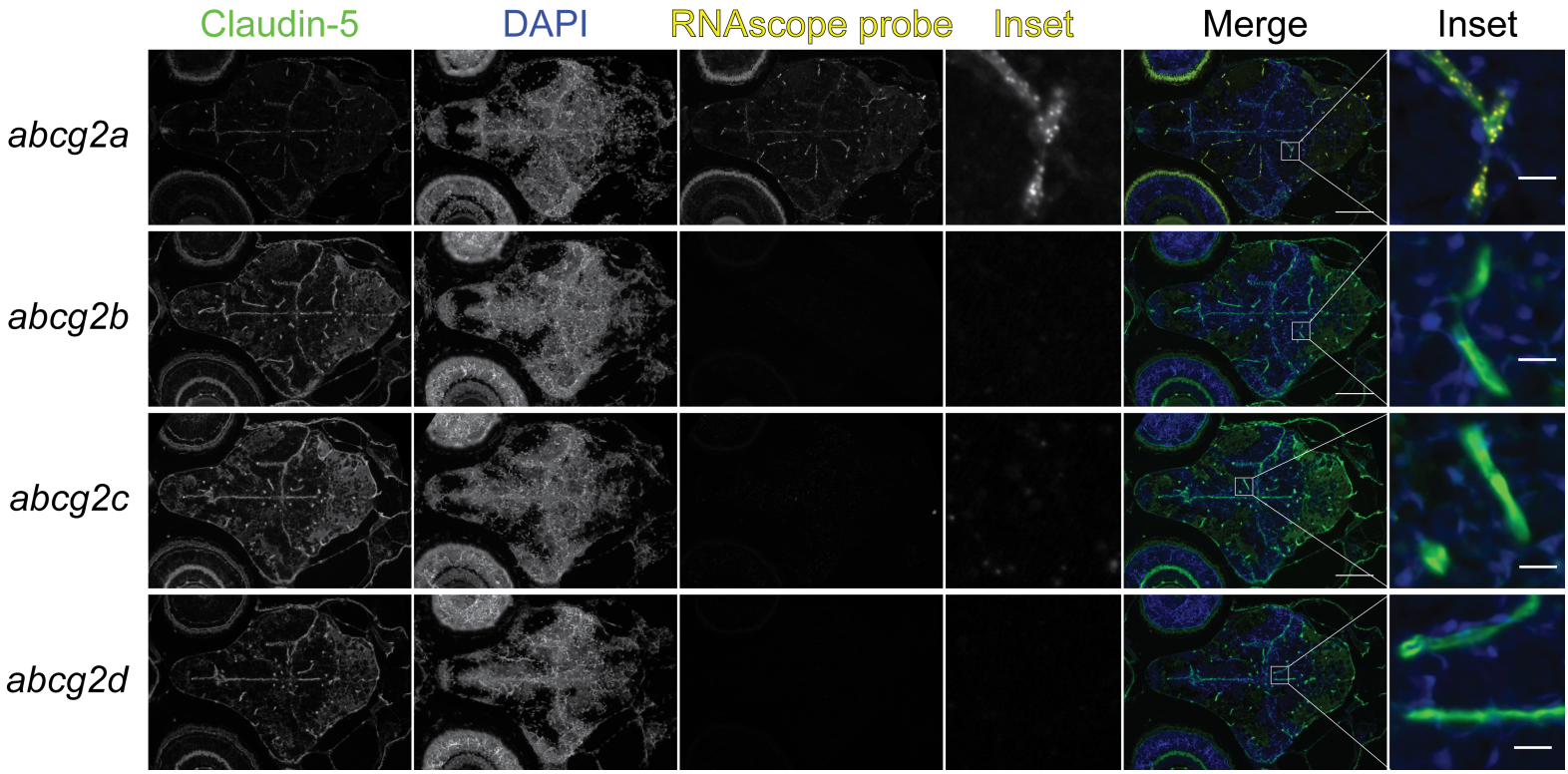

Figure 6: *abcg2a* is expressed in claudin-5 positive larval brain vasculature.Paraffin-embedded 7 dpf larval zebrafish sections were probed with RNAscope probes (yellow) to detect *abcg2a* mRNA, an antibody against claudin-5 (green) and DAPI (blue). Scale bar = 100 µm, inset scale bar = 10 µm