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ABSTRACT

During chicken embryonic development, skeleton calcification mainly relies on the eggshell, whose minerals are progressively solubilized and transported to the embryo *via* the chorioallantoic membrane (**CAM**). However, the molecular components involved in this process remain undefined. We assessed eggshell demineralization and calcification of the embryo skeleton after 12 and 16 days of incubation, and analyzed the expression of several candidate genes in the CAM: carbonic anhydrases that are likely involved in secretion of protons for eggshell dissolution (CA2, CA4, CA9), ions transporters and regulators (CALB1, SLC4A1, ATP6V1B2, SGK1, SCGN, PKD2) and vitamin-D binding protein (GC).

Our results confirmed that eggshell weight, thickness and strength decreased during incubation, with a concomitant increase in calcification of embryonic skeletal system. In the CAM, the expression of CA2 increased during incubation while CA4 and CA9 were expressed at similar levels at both stages. SCL4A1 and SCGN were expressed, but not differentially, between the two stages, while the expression of ATP6V1B2 and PKD2 genes decreased. The expression of SGK1 and TRPV6 increased over time, although the expression of the latter gene was barely detectable. In parallel, we analyzed the expression of these candidate genes in the yolk sac **(YS)**, which mediates the transfer of yolk minerals to the embryo during the first half of incubation. In YS, CA2 expression increases during incubation, similar to the CAM, while the expression of the other candidate genes decreases. Moreover, CALB1 and GC genes were found to be expressed during incubation in the YS, in contrast to the CAM where no expression of either was detected.

This study demonstrates that the regulation of genes involved in the mobilization of egg minerals during embryonic development is different between the YS and CAM extraembryonic structures. Identification of the full suite of molecular components involved in the transfer of

eggshell calcium to the embryo via the CAM should help to better understand the role of this structure in bone mineralization.

Key words: chicken, eggshell, embryo, mineral, chorioallantoic membrane

INTRODUCTION

The nutrient reserve of the fertile egg consists of three distinct compartments that are progressively mobilized to support embryonic development: the yolk, the albumen and the shell (Romanoff, 1960; Romanoff and Romanoff, 1967; Bellairs and Osmond, 2014). During incubation, these nutrient reservoirs operate dynamically and serve at different stages of growth to meet embryo requirements for lipids, amino acids, carbohydrates, and minerals. When focusing on mineral ions, the shell is the main source of Ca, Mg, and Sr; the albumen is the major source of K and Na; and the yolk provides Cu, Fe, Mn, P, and Zn (Richards, 1997; Schaafsma et al., 2000; Yair and Uni, 2011; Hopcroft et al., 2019). Many studies indicate the importance of egg mineral ions for the development of the embryo but also for the skeletal health of chick and adult birds. In fact, a mineral deficiency has adverse repercussions on skeletal, immune and cardiovascular systems, reduces hatchability and increases mortality (Richards, 1997; Kidd, 2003; Angel, 2007; Dibner et al., 2007). The transfer of minerals from the yolk during the first half of incubation, and from the eggshell during the second half of incubation to the embryos (Romanoff, 1960), intrinsically depends on the functionality of extra-embryonic structures, namely the yolk sac (**YS**) for the yolk and the chorioallantoic membrane (**CAM**) for the eggshell.

During embryonic development, nutrients are transferred from the yolk contents to the embryo through the yolk sac membrane (**YSM**) and its surrounding vascular system (Uni et al., 2012). From embryonic day (**ED**) 19, the YS begins to be internalized into the abdominal cavity of the

65 embryo and residual yolk provides critical nutrients until hatched chicks have access to food
66 (Romanoff, 1960). It has been shown that the YSM expresses digestive enzymes and nutrient
67 transporters similarly to the intestine (Speake et al., 1998; Yadgary et al., 2011; Yair and Uni,
68 2011; Speier et al., 2012; Bauer et al., 2013; Yadgary et al., 2014). Yair and Uni (2011)
69 observed that the total calcium content of the yolk (30 mg at D0) decreases during the first half
70 of incubation to reach a plateau from ED11 (20 mg) until hatch. During the second half of
71 incubation, the eggshell becomes a major contributor of calcium for supporting skeletal
72 mineralization of the embryo (Yair and Uni, 2011). The solubilization of eggshell minerals and
73 the transfer of eggshell calcium to the embryo is ensured by the CAM, which is a highly
74 vascularized structure that lines the inner eggshell and develops from ED5 onwards (Romanoff,
75 1960). The CAM is complete by ED10-11, grows rapidly from ED11 to ED15-16, and starts to
76 degrade from day 19 onwards (Romanoff, 1960; Leeson and Leeson, 1963; Narbaitz and
77 Tellier, 1974; Makanya et al., 2016). The CAM is fully differentiated at ED15-16 and is
78 composed of three distinct cellular structures, namely the chorionic epithelium, the mesoderm
79 and the allantoic epithelium (Makanya et al., 2016), all of which are assumed to play different
80 but complementary roles. The chorionic epithelium participates in acid-base balance of the
81 embryo and mineral solubilization and transport from the eggshell (Gabrielli and Accili, 2010).
82 The mesoderm is the site of early development of the extraembryonic vascular system, which
83 serves the CAM epithelia and forms the chorionic capillary plexus to facilitate gaseous
84 exchange (Melkonian et al., 2002). The allantoic epithelium is involved in ion and H_2O
85 reabsorption from the allantoic fluid and maintains the acid-base balance of this fluid (proton
86 secretion and bicarbonate reabsorption) (Stewart and Terepka, 1969; Narbaitz, 1995). Hence,
87 the CAM is involved in the dissolution and transport of calcium from the eggshell to the
88 embryo, gaseous exchange, maintenance of acid-base balance, water and electrolyte
89 reabsorption from the allantoic cavity and innate immunity (Romanoff, 1960; Coleman and

Terepka, 1972a, b; Gabrielli and Accili, 2010; Hincke et al., 2019). Only a few candidate proteins in the CAM have been proposed to participate in eggshell solubilization and mineral ion transport to date: a calbindin-like protein, an anion exchanger (AE1), a H^+-ATPase, and soluble and membrane-bound isoforms of carbonic anhydrase (Tuan and Zrike, 1978; Rieder et al., 1980; Anderson et al., 1981; Narbaitz et al., 1981; Tuan, 1984; Tuan et al., 1986; Narbaitz et al., 1995; Gabrielli et al., 2001; Gabrielli, 2004; Gabrielli and Accili, 2010). Moreover, the identity of the associated genes remains ambiguous and depends on the annotated chicken genome assemblies used as a reference [Galgal4 (GCA_000002315.2) in 2013, Gallus_gallus-5.0 (GCA_000002315.3) in 2016, GRCg6a (GCA_000002315.5) in 2018) (Peona et al., 2018)].

In this work, we studied the expression of 10 candidate genes in the CAM and YS at ED12 (CAM is developed but not fully mature), and at ED16 (corresponding to a fully differentiated stage). The demineralization of the eggshell as well as the calcification of the embryo skeleton have been assessed in parallel to further appreciate the interrelationship between these two physiological processes. The data obtained in this article revisit some of the statements from the literature and show for the first time that the molecular components involved in mineral mobilization from the yolk and the eggshell are different.

MATERIALS AND METHODS

Incubation Procedures and Sample Collection

Sixty fertilized eggs were obtained from 29-wk old laying hens (Rhode Island Red, Novogen, France) and handled in the Poultry Experimental Facility (PEAT) UE1295 (INRAE, F-37380 Nouzilly, France, DOI: 10.15454/1.5572326250887292E12). Eggs were incubated under standard conditions (45% RH, 37.8°C, automatic turning every hour; Bekoto B64-S, Pont-Saint-Martin, France), after a 3-day storage at 16°C, 85% RH to favor synchronization of

developmental stages. For each embryonic day studied (ED12 and ED16), 30 eggs (64.1±1.8g) containing viable embryos were selected. At each stage, egg weight and eggshell strength were measured prior to tissue sampling (Digital Egg Tester 6000, Nabel, Kyoto, Japan). Eggs were opened at the air chamber end and the egg contents were poured into a Petri dish. Embryos were sacrificed by decapitation, and placed in a sterile flask containing 75 ml of 90% ethanol prior to staining (see below). The yolk sac and the chorioallantoic membrane were removed, washed several times with sterile saline solution (NaCl 0.9%), immersed in liquid nitrogen and stored at -80°C. Eggshell thickness was measured using a small piece of eggshell (Digital Egg Tester 6000, Nabel, Kyoto, Japan) and eggshell weight was obtained after drying for 2 hours at 110°C. Resulting eggshells were further stored at 4°C prior to analysis of mineral content. These experiments performed on embryos at day 12 and day 16 of development were conducted in compliance with the European legislation on the "Protection of Animals Used for Experimental and Other Scientific Purposes" (2010/63/UE) and under the supervision of an authorized scientist (S. Réhault-Godbert, Authorization no. 37-144). These experiments meet the guidelines approved by the institutional animal care and use committee (IACUC).

Alizarin Red S (Bone) and Alcian Blue (Cartilage) Staining of Chicken Embryos

Chicken embryo bone and cartilage staining was performed as described for mouse embryos (Scientific Protocols, 2014) with some small adjustments. After sampling, embryo bodies were fixed in 90% ethanol for 11 days at 4°C with renewal of the solution every 4 days. Skin, viscera, liver, kidney and gut were removed. Embryos were placed in an Alcian blue solution (0.1g/L) (Sigma, Saint-Louis, USA, ref. A5268) for 3 days, followed by rehydration with several baths of decreasing percentages of ethanol (from 70% to 0% in demineralized water; 70% during 2 hours/ 40% during one night/ 15% during 2 hours and finally, demineralized water for 4 hours). The clearing of embryos was achieved with 1% KOH solution for 2 days and the staining of

mineralized structures was performed during 3 days in Alizarin red / KOH solution at 0.01 g/L (Sigma, Saint-Louis, USA, ref. A5533). Stained embryos were rinsed in 1% KOH, followed by increasing solutions of glycerol (20 to 80%, over 5 days) / 1% KOH, before storage at 4°C in 100% glycerol. Stained ED12 and ED16 embryos were analyzed visually and photographed (Nikon apparatus D5100, Itteville, France). Identification of bones was based on the Atlas of Chick Development (plates 230 and 231, Bellairs and Osmond, 2014).

Eggshell Mineral Content

Eggshell mineral quantification was performed according to Park and Sohn (2018), with small adjustments. Eggshell fragments were washed, dried, weighed and then grinded using a Cryomill ball mill (Retsch, Haan, Germany). Eggshell powder (300 mg) was dissolved in 10 mL of 65% nitric acid, and then heated / digested in a microwave for 15 min at 200°C, 1800 W (Ethos Up, Milestone, Sorisole, Italy). Total P, Mg, K, Na and Ca were determined using an inductive coupled plasma atomic emission spectrometer (ICP OES ThermoscientificTM iCAPTM 7200; method 990.08; AOAC International, 2006). Standard solutions (P, Mg, K, Na and Ca) were prepared from a 1,000 mg/mL stock solution (Certipur® Merck, Darmstadt, Allemagne). All assays were performed in duplicates.

mRNA extraction and real-time quantitative PCR

All tissue samples (n=18 per stage, ED12 and Ed16) were homogenized in liquid nitrogen with a mechanical crusher A11 Basic (IKA, Staufen im Breisgau, Germany). For CAM samples, total RNA was extracted using the Nucleospin RNA kit according to the manufacturer's recommendations (Macherey-Nagel, Düren, Germany). For yolk sac samples, total RNA was

extracted using RNeasy® Lipid Tissue Mini Kit, according to the manufacturer's recommendations (Qiagen, Hilden, Germany).

To remove traces of genomic DNA, a second treatment with DNAse was performed on all samples (kit Turbo DNA-*free*TM, Life Technologies, Carlsbad, USA). Concentration and quality of the extracted RNA were assessed by spectrophotometry (Nanodrop 1000 spectrophotometer Nanodrop Technology, Wilmington, USA) and by migration of total RNA on a 1% agarose gel. Total RNA samples (1 µg) were reverse transcribed using RNase H-MMLV reverse transcriptase (Superscript^{TM} II RT, Invitrogen, Cergy Pontoise, France). Gene quantification was achieved by SYBR Green incorporation, using LightCycler 480 (Roche, Mannheim, Germany).

Candidate genes to be analyzed were selected according to the literature (Gabrielli, 2004; Gabrielli and Accili, 2010). Other studies related to eggshell calcification or intestinal calcium transporters in chickens provided additional candidates (Jonchère et al., 2012; Gloux et al., 2019; Gautron et al., 2021). The list of candidate genes is presented in Table 1.

Calculation of Relative Gene Expression

The relative normalized expression (*R*) of a candidate gene was calculated, based on the Efficiency (E) and the cycle threshold (Ct) deviation of cDNA samples (CAM or YS at ED12 and ED16 from individual embryos) *vs*. a calibrator. The calibrator corresponds to the pool of cDNA from all CAM samples (CAM analysis) or a pool of cDNA from all YS samples (YS analysis). Data were expressed relative to the normalization factor of housekeeping genes calculated by GeNorm (version 3.5) (Vandesompele et al., 2002). Briefly, normalized quantities were calculated using the following formula: gene efficiency^(Ctcalibrator –

Ctsample) / normalization factor of each sample (calculated based on geometric mean of housekeeping genes).

Five stable housekeeping genes could be utilized for CAM samples, while for the yolk sac, only one gene (ACTB) was shown to be invariant between stages and thus was selected for calculation of relative expression. The selected housekeeping genes were:

ACTB (Gene ID : 396526, Actin, Beta; Forward CTGGCACCTAGCACAATGA; Reverse CTGCTTGCTGATCCACATCT); PPIA (Gene ID : 776282, Peptidylpropyl isomerase A; Forward : CGCTGACAAGGTGCCCATAA; Reverse : GTCACCACCCTGACACATGA); STAG2 (Gene ID : 422360, Stromal antigen 2; Forward: GCACACACCAGTCATGATGC; Reverse: TGGTGTTCAGGCTGCATAGG) ; TBP (Gene ID : 395995, TATA-box binding protein; Forward: GCGTTTTGCTGCTGTTATTATGAG; Reverse: TCCTTGCTGCCAGTCTGGAC); YWHAZ (Gene ID : 425619, Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta; Forward: TGCTGCTGGAGATGACAAGA, Reverse: AGGCCTTCTCTGGGGAATTG).

Statistical Analyses

All statistical analyses were performed using R Software, version 4.0.2 (R Core Team, 2017, Vienna, Austria). Because the samples were not normally distributed (Shapiro test), statistical analyses were performed using a Wilcoxon test (P<5%).

RESULTS

Eggshell Physical Characteristics and Mineral Content

The effect of incubation on the eggshell quality parameters and mineral ion content (phosphorus (P), magnesium (Mg), potassium (K), sodium (Na) and calcium (Ca)) are shown in Table 2. The following parameters decreased the egg and eggshell weights, breaking strength and thickness all decrease significantly from ED12 to ED16 ($P=0.034$, $P=0.0133$, $P<0.0001$, $P<0.0001$, respectively). Concomitant to the decrease in eggshell weight, we observed a significant decrease in the total content of Mg, Na and Ca between ED12 and ED16 ($P=0.0112$, $P=0.0307$ and $P<0.01$, respectively).

Kinetic of skeleton Mineralization

The typical staining pattern of embryo skeletons with Alcian blue (a cationic dye that binds glycosaminoglycans and sulfated glycoproteins in cartilage) and Alizarin red (an anionic dye that binds cationic calcium and calcium deposits) at ED12 (A) and ED16 (B) is presented in Figure 1. The wing and leg bones are already partially mineralized at ED12 (humerus, radius, ulna, and femur, tibiotarsus, respectively), while calcification of the ribs is initiated at ED12 and complete around ED16. Some skeletal regions corresponding to the cervical vertebra, the ribs, the pelvic bones (ilium, ischium, pubis, caudal vertebra) and the digits of the legs exhibit a visually apparent increase in mineralization between these stages (Figure 1).

Relative Expression of Candidate Genes in the Yolk Sac and the CAM

The mRNA expression of eight candidate genes in both the CAM and the yolk sac is presented in Figures 2A and 3A. Three genes that are specifically expressed in the CAM but not the YS (TRPV6), and in the yolk sac but not in the CAM (CALB1 and GC), are presented in Figure 2B and 3B, respectively.

In the CAM, the expression of **CA2**, **SGK1** and **TRPV6** are higher at ED16 (about 2 fold, P<0.001) while the expression of **PKD2** and **ATP6V1B2** decreases (about 1.4 fold) between ED12 and ED16 (P<0.01 and P<0.05, respectively). The incubation stage has no effect on the mRNA expression of carbonic anhydrase 9 and 4 (**CA9** and **CA4**), **SLC4A1** or **SCGN**.

In the yolk sac, stage of development does not affect **PKD2** expression (Figure 3A) while the expression of **CA2** and **CALB1** increases when comparing ED16 to ED12 (15-20 fold, P<0.001) (Figure 3A and 3B, respectively). The relative expression of other candidate genes (**CA9**, **CA4**, **SLC4A1**, **ATP6V1B2**, **SGK1**, **SCGN**, and **GC**) decreases during incubation (up to a 20-fold decrease depending on the gene, P<0.001).

DISCUSSION

The Alteration in Eggshell Integrity Reflects Mineral Release

In birds, the egg contains all the protective and nutritive elements to ensure the development of the embryo until hatching. The eggshell is a physical barrier that protects the embryo from environmental changes and microbes. It also regulates gaseous exchange through its pores while limiting water loss, and provides most of the calcium that is necessary for mineralization of the embryonic skeleton (Nys et al., 2010). The yolk contains about 30 mg of calcium while up to 800 mg of eggshell calcium are resorbed from the day of lay to the day of hatch (Yair and Uni, 2011). Such amounts may slightly differ depending on initial egg weight. The decrease in eggshell weight is essentially observed during the second half of incubation when the embryo skeleton needs to be reinforced to support a 5-fold increase of the embryo body weight (from 5 g at ED11 to 25 g at ED18) (Makanya et al., 2016). In our experiment, between ED12 and ED16, eggshell loss is about 300 mg, corresponding to 120 mg of calcium (Table 2). The eggshell ultrastructure is complex, and is characterized (from inside to outside) by the

mammillary layer (where biomineralization is initiated), the palisade layer (responsible for most of the eggshell thickness and resistance to fracture), the vertical crystal layer and the cuticle. It has been reported that eggshell resorption mainly occurs from the calcium reserve body in the shell mammillary region (Tyler and Simkiss, 1959; Simons, 1971; Bond et al., 1988), and progressively induces the detachment of eggshell membranes together with erosion of the mammillary knobs (Simons, 1971; Bond et al., 1988; Chien et al., 2009). The loss of eggshell mineral and the weakening of the underlying support for the thick palisade layer likely explains the decrease in eggshell thickness and associated strength, as observed in Table 2. Such eggshell thinning may result in an increased susceptibility to penetration by environmental microbes, but concomitantly facilitates chick emergence (Hincke et al., 2019). Previous publications have reported that although the eggshell is 96% calcium carbonate, the distribution of minor mineral ions is heterogeneous: the Mg concentration is higher in the mammillary layer and at the outer palisade layer, while phosphorus (as inorganic phosphate or associated with phosphoproteins) is mainly incorporated during the eggshell termination process and is found in the outer palisade layer and cuticle (Cusack et al., 2003; Shen and Chen, 2003; Hincke et al., 2012). Calcium carbonate is deposited constantly throughout the process of eggshell formation (Waddel et al., 1989; Waddel et al., 1991; Shen and Chen, 2003; Gautron et al., 2021). Regulation of this process by the organic matrix results in the distinctive ultrastructure and microstructure of the eggshell (Dennis et al., 1996; Hincke et al., 2012; Rodriguez-Navarro et al., 2015; Gautron et al., 2021). The innermost layer that is in contact with the eggshell membranes is named the mammillary layer; each mammillary cone consists of a base plate that is the calcified foundation of the eggshell, a calcium reserve body, a cover and a crown. The calcium reserve body is described as the main source of calcium which is mobilized for skeletal mineralization during embryonic development (Dennis et al., 1996; Chien et al., 2008). A positive correlation is observed between the number of mammillary tips and calcium removal from the eggshell

(Karlsson and Lilja, 2008). In line with previously published data (Schaafsma et al., 2000), our results show that eggshell mineral (approximately 96% calcium carbonate) is composed of calcium (364 mg/g of eggshell) and magnesium (3.11 mg/g of eggshell) as its main cations, but also phosphate (1.08 mg/g of eggshell) (Table 2). In our experiment, the decrease in eggshell weight is associated with the decrease in calcium, magnesium and sodium but not in potassium and phosphorus (Table 2). These data suggest that potassium and phosphorus are concentrated in the outermost and intermediate layers of the eggshell that are not resorbed during incubation. Indeed, phosphate was described previously to regulate the termination of eggshell formation, which is consistent with its outer localization (Gautron et al., 1997; Cusack et al., 2003). These findings underline that eggshell phosphate is not required for bone mineralization, which supports the general statement that the phosphate reservoir for the embryo is the yolk, with about 180 mg at ED0 (Romanoff and Romanoff, 1967), and not the eggshell (Tuan and Ono, 1986; Chien et al., 2009).

The staining of the embryos collected at ED12 and ED16 with Alcian blue and Alizarin red (Figure 1) showed that long bones are already partially mineralized at ED12, which corroborates previous studies: the tibial calcium content begins to increase from 12 days of incubation to reach maximum values around ED19 (Kubota et al., 1981; Torres and Korver, 2018). This increase in bone calcification occurs in parallel with bone citrate decarboxylation and alkaline phosphatase activities, both reflecting osteoblast activity. These activities start to increase around ED10-12, reach a peak at ED19, and then decrease, and are strongly correlated with calcium-binding activity in the chorioallantoic membrane (Kubota et al., 1981). From embryonic stages ED14 to ED19, chicken long bones roughly double their length, thickness and total amount of bone mineral (Yair et al., 2012; Bellairs and Osmond, 2014), in order to support the rapid growth of the embryo. These structural modifications require massive transport of calcium from the eggshell and phosphorous from the yolk (Yair and Uni, 2011),

304 through the substantial vasculature of the CAM and of the YS. Kerschnitzki et al. (2016)
305 reported the presence of membrane-bound mineral particles (calcium and phosphorus) in blood
306 vessels during long bone development of chicken embryos, and it is generally observed that
307 osteogenesis is coupled with angiogenesis during this process (Kusumbe et al., 2014). Between
308 ED12 and ED16, a constant decrease in blood Ca^{2+} (measured via the allantoic vein) is
309 accompanied by an increase in tibial mineral calcium, which collectively corroborates the rapid
310 assimilation of circulating Ca^{2+} for bone mineralization (Everaert et al., 2008).

311 The transfer of calcium from the eggshell to the embryo in mediated by a three-layer structure,
312 namely the chorioallantoic membrane. The chorionic epithelium of the CAM lines the eggshell
313 membranes and is involved both in the solubilization of calcium from the inner eggshell, and
314 in the transfer of solubilized ions to the embryo via its capillary network.

315

316 ***The CAM expresses carbonic anhydrases and ion-binding proteins but not calbindin***
317 ***(CALB1) nor vitamin-D binding protein (GC)***

318 Distinct and specialized cell types characterize the mature chorionic epithelium: the villus
319 cavity (VC) cells and the capillary covering (CC) cells (Figure 4A). Previous publications have
320 shown that the major molecular components for extracellular acidification adjacent to the
321 eggshell are localized within the VC cells (Figure 4A). These are the AE1 anion exchanger
322 (AE1, possibly corresponding to SLC4A1), a cytoplasmic carbonic anhydrase 2 (CA2), a
323 membrane-bound carbonic anhydrase, and a H+ ATPase (Gabrielli and Accili, 2010); however,
324 except for CA2, the identity (i.e. gene ID) of most these major proteins remains undetermined.
325 In this context and based on information available in the literature, we investigated the
326 expression of 10 candidate genes in the CAM. Our results showed that the expression of CA2
327 increases over time as previously published (Tuan and Zrike, 1978), but we also showed for the

first time that CA4 and CA9 are constantly expressed between ED12 and ED16 (Figure 2). Indeed, carbonic anhydrases have been reported to play a major role in proton secretion for solubilization of the eggshell mineral calcite via VC cells (Tuan and Zrike, 1978; Rieder et al., 1980; Anderson et al., 1981; Narbaitz et al., 1981; Tuan, 1984; Tuan et al., 1986; Narbaitz et al., 1995; Gabrielli et al., 2001; Gabrielli and Accili, 2010). Noticeably, CA2 was also previously identified in mitochondria-rich cells (MRC) that are highly concentrated in the allantoic epithelium (Narbaitz et al., 1995; Gabrielli et al., 2001). This protein is assumed to participate in maintaining acid-base homeostasis in the allantoic fluid during embryonic development. Carbonic anhydrases catalyze the reversible hydration of carbon dioxide to produce protons and bicarbonate ions and, in parallel, are thought to regulate several bicarbonate transporter activities. SLC4A1 (AE1) is continuously expressed between the two stages and may transport bicarbonate ions into cells, to maintain an extracellular acidic environment, but also into erythrocytes, where bicarbonate ions accumulate between ED10 and ED16 of incubation (Everaert et al., 2008). Besides VC cells, both CA2 and SLC4A1 were reported to be expressed in erythrocytes to contribute to blood homeostasis. A vacuolar H+ ATPase was also previously described as participating in the extracellular proton flux. In our experiment, the subunit ATP6V1B2 was shown to be expressed in the CAM but its expression decreased modestly over time, which is not in accordance with the increased concentration of intracellular protons. However, the profile of expression of ATP6V1B2 between ED12 and ED16 resembles the activity profile of a proton pump that was described to be calcium-dependent (Tuan and Knowles, 1984). The activity of this protein followed a bimodal pattern with a decrease from day ED12 to ED16 followed by an increase essentially after ED16 of incubation to reach a maximum value around hatch (Tuan and Knowles, 1978).

We have shown above that ED16 is characterized by the loss of calcium, sodium and magnesium from the eggshell (Table 1). Ion transfers and exchanges are believed to be

353 mediated by the so-called capillary covering cells (CC cells, Figure 4A). Although we did not
354 explore magnesium-binding proteins and transporters, we identified SCGN whose expression
355 remains stable over time, and TRPV6 that is slightly overexpressed at ED16, as potential
356 calcium-binding proteins. Surprisingly, when looking at calcium-binding proteins, we found
357 that SCGN expression level remains stable between ED12 and ED16, while TRPV6 expression
358 is low and no expression of CALB1 could be detected. Altogether, these data suggest that other
359 not yet identified calcium-binding proteins are responsible for ion movements in the CAM. An
360 alternative hypothesis is that the calcium flux in the cells and subsequently in the blood, does
361 not involve calcium-binding proteins at this stage but maybe later during the time course of
362 development. Indeed, calcium-binding activity has been shown to be maximal at day 18 (6-fold
363 increase between ED15 and ED18) (Torres and Korver, 2018). The resulting high intracellular
364 calcium concentration is likely to downregulate PKD2 expression. Our hypothesis is that the
365 lower expression of PKD2 (a regulator of intracellular calcium signaling) may reflect the
366 necessity of CC cells to concentrate high intracellular calcium without triggering cellular
367 pathological signaling through an undesired PKD2 activation. The regulation of sodium flux
368 may involve SGK1, which is localized in the nucleus, but also in mitochondria and the plasma
369 membrane, and that is an important regulator of ion channels, including sodium channels (Lang
370 and Shumilina, 2013). This gene was shown to be overexpressed in the CAM during incubation
371 and may potentially be localized in CC cells but also in erythrocytes, as previously reported
372 (Maizels, 1954; Clarkson and Maizels, 1955). A schematic representation of the expression
373 pattern of candidate genes in the chorionic epithelium of the CAM is illustrated in Figure 4A.
374 Noticeably, the allantoic epithelium is also likely to be involved in the assimilation of calcium
375 and phosphate that accumulate in the allantoic fluid owing to the large increase in embryo
376 metabolism during the second half of incubation (Everaert et al., 2008). The absence of GC
377 (vitamin D transport protein) expression in the CAM at these two stages is consistent with the

fact that calcium transport and uptake by the CAM were not regulated by vitamin D (Packard et al., 1998).

The YS expresses CA2, calbindin (CALB1) and vitamin-D binding protein (GC)

In the YS, a variety of expression patterns were observed for the genes under study. Most of the candidate genes demonstrated a decrease in expression between ED12 and ED16; however, CA2 expression increased similarly to the CAM during incubation, PKD2 did not exhibit any difference in expression between the two stages, and TRPV6 was not expressed at either stage (Figure 4B). Carbonic anhydrases were demonstrated to be important in the formation of subembryonic fluid in early Japanese quail and turkey embryos and are localized in the endoderm of yolk sac during the early stages of incubation (Babiker and Baggott, 1995; Bakst and Holm, 2003). To our knowledge, the present study is the first to demonstrate the expression of carbonic anhydrase isoforms in the yolk sac. Calcium binding protein (CALB1) was reported to be essentially upregulated during the later stages of incubation, which we have corroborated, as seen in Figure 3 (Sechman et al., 1994; Yadgary et al., 2014). In addition, we observed a decrease in expression of GC between ED12 and ED16. These findings suggest that vitamin D uptake and calcium transport are mechanistically uncoupled. The vitamin D status of embryonic blood remains low up to hatching but may be concentrated in the bones (Nys et al., 1986). Indeed, vitamin D and a calcium-binding protein were shown to co-localize in dividing chondrocytes around hatch (Zhou et al., 1986). The uptake of vitamin D from the yolk during the first half of incubation and of eggshell calcium during the second half of incubation may correspond to highly orchestrated mechanisms that ultimately assist bone mineralization. In the yolk sac, we failed to detect TRPV6, a calcium selective channel that mediates Ca^{2+} uptake in various tissues, including intestine and uterus of laying hens (Yang et al., 2011; Yang et al., 2013). TRPV6 was reported to decrease from ED11 to ED13 followed by an increase up to

403 ED19 in the yolk sac (Yadgary et al., 2011; Wong and Uni, 2021), but remarkably, its
404 expression remains low between 10 and 15 days of incubation, and variability in later stages is
405 surprisingly high (Yadgary et al., 2011). In view of these results, the physiological role of this
406 calcium channel in calcium uptake from the eggshell and the yolk requires further study.

407

408 To conclude, the role of the yolk sac and the chorioallantoic membrane in transferring and
409 transporting ions from yolk (phosphorus) and eggshell (calcium, magnesium and sodium),
410 respectively, are complementary and involve distinct molecular components. These processes
411 are associated with specialized cell types and their expression is temporally regulated in a
412 coordinated manner. Both previous work and the novel results presented here highlight the need
413 to consider the entire period of embryonic development, in order to have a more comprehensive
414 picture of the relative functional roles of the CAM and the YS. None of the candidate genes
415 encoding calcium binding proteins were shown to be significantly regulated during incubation:
416 SCGN expression remains stable between the two developmental stages, CALB1 is not
417 expressed in the CAM and TRPV6 expression is barely detectable. Thus, to date, the CAM
418 proteins involved in the binding and transport of calcium from the eggshell to the embryo are
419 not known. This example demonstrates the limitations of a candidate-gene approach. We are
420 currently conducting a more systematic approach (RNA-Seq transcriptomics), which, combined
421 with the localization of highly expressed genes within the CAM, will help to decipher the exact
422 role of this multifunctional structure. We believe that the physiological and functional
423 characterization of the CAM needs to be revisited using modern high-throughput techniques,
424 similar to the strategy that has been used to explore the physiology of the yolk sac (Yadgary et
425 al., 2014).

426 In addition to its interest in understanding the physiology of the extraembryonic structures
427 which support embryonic development, this field of research may also have positive outputs

for the poultry industry. There is increasing evidence that intensive genetic selection of broiler breeders for meat production and layer hens for egg quality has precipitated the development of metabolic disorders including skeletal abnormalities (Thorp, 1994; Buzala et al., 2015; Eusemann et al., 2020). It is well known that skeletal integrity in chickens is affected by many factors including rapid growth rate, nutrition and genetics (Thorp, 1994). Fast growing broiler chicks exhibit impaired bone mechanical properties compared with slow-growing broiler chicks (Williams et al., 2000; Shim et al., 2012; Yair et al., 2017), while there is a very high prevalence of keel bone fractures in layer hens, regardless of the production system (Eusemann et al., 2020; Thøfner et al., 2021). Such bone pathologies compromise bird welfare and result in substantial economic losses for the poultry industry. The genetic determinants of bone and mineral metabolism are complex and involve multiple genetic loci (Mignon-Grasteau et al., 2016). The characterization of CAM and YS functions in skeletal mineralization of the embryo might help to identify *in ovo* markers as predictors of adult chicken bone health in modern poultry lines and lead to new selection tools.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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666 Table 1: Information related to candidate genes.

Symbol	Gene ID	Gene Name	Function	Subcellular location	Primer Sequence 5'- 3'	Amplicon size (bp)
CA2*	396257	Carbonic anhydrase 2	Bone resorption and osteoclast differentiation. Hydration of carbon dioxide and intracellular pH regulation	Cytosol/plasma membrane	Fw :ATCGTCAACAACGGGCACTCCTTC Rev :TGCACCAACCTGTAGACTCCATCC	101
CA4	417647	Carbonic anhydrase 4	Hydration of carbon dioxide, stimulation of the sodium/bicarbonate transporter activity of SLC4A4 (pH homeostasis)	Plasma membrane	Fw :GGAAGCAAACAGTCACCCATC Rev :GACTCCCCAGTGCAGATGAAA	225
CA9	770004	Carbonic anhydrase 9	Hydration of carbon dioxide, pH regulation	Plasma membrane	Fw :CCTGACAACCTGCACCTCTA Rev :GAGGTGGTTGTCGTCTGTCT	159
PKD2	422585	Polycistin 2, transient receptor potential cation channel	Component of a heteromeric calcium-permeable ion channel formed by PKD1 and PKD2	Plasma membrane	Fw :ACCTGAGAAGTGTTTTGCGG Rev :GAGCTGCGACATAACCCTCG	122
SLC4A1	396532	Solute carrier family 4 member 1	Mediation of chloride-bicarbonate exchange in the kidney, urine acidification	Plasma membrane	Fw :TGAGACCTTCGCCAAACTCG Rev :TTCAGCTTCTGCGTGTAGGT	291
ATP6V1B2	395497	ATPase H+ transporting V1 subunit B2	Acidification of intracellular compartments-organelles	Cytosol	Fw :CCCCACAATGAGATTGCAGC Rev :CATGGACCCATTTTCCTCAAAGTC	171
SGK1	395133	Serum/glucocorticoid regulated kinase 1	Regulation of various ion channels, renal Na+/K+ and intestinal Na+/H+ exchange and nutrient transport	Nucleus	Fw :GCCCAGTCCATCACAACAGA Rev :ATGCCGTGCAAGAAGAACCT	124
TRPV6	427502	Transient receptor potential cation channel, subfamily V, member 6	Mediation of Ca2+ uptake in various tissues, Ca2+ ion homeostasis (body and bones)	Plasma membrane	Fw :CACTCCTTCAAGCTGCCAAG Rev :CTGGTTCACTGCTGCAATGT	242
CALB1*	396519	Calbindin-1	Intracellular Ca2+ binding protein	Cytosol	Fw :CAGGGTGTCAAAATGTGTGC Rev :GCCAGTTCTGCTCGGTAAAG	215
GC	395696	GC vitamin D binding protein	Vitamin D transport and storage	Secreted	Fw :TAGCAACTCACGCCGAACAC Rev :CATGGCTCGGAAGTCATCCTT	95
SCGN	421001	Secretagogin, EF-hand calcium binding protein	Regulation of calcium ion concentration	Cytosol	Fw :GATGGACGTCTGGACCTGAA Rev :CCACTGATGCTGGGCTTGAC	221

667 * Primer sequences from Jonchère et al., 2012. Fw, forward; Rev, reverse

668 Table 2: Eggshell physical characteristics and mineral content for two stages of embryo
669 development (ED12 *vs* ED16) (n=30 per stage). P-values lesser than 0.05 were considered as
670 significant (in bold type) with arrows to describe the evolution between both stages.

		ED12	**ED16**	p-value
	Initial egg weight (g)	64.46 ± 1.85	63.95 ± 1.99	0.3669
	Egg weight at sampling (g)	60.4 ± 1.81	58.82 ± 2.09	**0.0034** ↓
Eggshell physical characteristics	Strength (N)	39.79 ± 4.69	34.01 ± 4.92	**<.0001** ↓
	Eggshell weight (g)	6.31 ± 0.47	6.01 ± 0.44	**0.0133** ↓
	Thickness (mm)	0.47 ± 00.3	0.43 ± 0.03	**<.0001** ↓
Eggshell mineral content (mg)*	P	6.78 ± 0.93	6.49 ± 0.88	0.1872
	Mg	19.59 ± 3.39	17.86 ± 2.90	**0.0112** ↓
	K	2.65 ± 0.35	2.54 ± 0.29	0.1124
	Na	6.43 ± 0.70	6.03 ± 0.65	**0.0307** ↓
	Ca	2298.49 ± 191.01	2171.66 ± 182.39	**<0.01** ↓

671 * For the eggshell mineral content, values correspond to those of the total shell

672

673

Figure legends

Figure 1: Staining of embryo skeletons with Alcian blue and Alizarin red at ED12 (A) and ED16 (B) (representative results, n=30 per stage). Blue color reveals the cartilaginous parts; in red/purple, the mineralized bones. Arrows indicate regions undergoing an increase in mineralization between the two stages of development.1, cervical vertebra; 2, ribs; 3, ilium; 4, caudal vertebra; 5, ischium; 6, digits of the legs.

Figure 2. RT-qPCR (n=18 per stage) of candidate genes in the CAM. A. Carbonic anhydrases (CA2, CA4, CA9), ion transporters and regulators (SLC4A1, ATP6V1B2, SGK1, SCGN, PKD2). B. TRPV6 (gene that is not expressed in the yolk sac). Experiments were conducted according to Materials and Methods. Normalized quantity was determined using five housekeeping genes as described in Materials and Methods.

Figure 3. RT-qPCR (n=18 per stage) of candidate genes in the YS. A. Carbonic anhydrases (CA2, CA4, CA9), ion transporters and regulators (SLC4A1, ATP6V1B2, SGK1, SCGN, PKD2). B. CALB1 and vitamin-D binding protein (GC). Experiments were conducted according to Materials and Methods. Normalized quantity was determined using only one housekeeping gene (ACTB) as described above due to the extreme variability of the other housekeeping gene candidates in the yolk sac at ED12 and ED16.

Figure 4. Hypothetical representation of the role of candidate genes in the chorionic epithelium of the CAM (A) and in the YS (B) during the second half of incubation, in mineral mobilization from the eggshell and the yolk, respectively. This model integrates the literature (detailed in the

discussion section) and the expression data obtained in the present study. A. The CAM is composed of three distinct layers (A) where the chorionic epithelium is assumed to be involved in the transepithelial ion transport (Gabrielli and Accili, 2010) from the blood (red rectangle) and the eggshell (grey rectangle). In this scheme inspired by Gabrielli and Accili, 2010, VC cells are specialized chorionic cells, which, via a vacuolar-type H+-ATPase present at the apical pole (ATP6V1V2), pump protons generated by cytoplasmic carbonic anhydrases (CA2, CA4 and CA9) towards the eggshell (step ❶). Proton secretion results in a local acidification (step ❷), thereby causing solubilization of the calcite mineral (step ❸). $HCO^{3-}$is proposed to be reabsorbed through VC cells via the anion exchanger SLC4A1, to maintain acid-base-balance within the CAM. Ca^{2+} and other ions including HCO^{3-}, Mg^{2+} and Na^{+} become available to be reabsorbed via by CC cells, for transport via the vasculature to the embryo. Our results suggest that ions transporters such as SCGN and PKD2 participate in Ca^{2+} binding and transport; however, CALB1expression was not detected at ED12 or ED16. The very low expression of TRPV6 in the CAM brings into question the role of this candidate gene in calcium uptake. SGK1 may be involved in Na^{+} transport while the transporter for Mg^{2+} is not yet known. As expected, since the eggshell does not contain vitamin D, the GC gene is not expressed in the CAM. B. In the yolk sac, carbonic anhydrases (CA2, CA4, CA9) and a proton-pumping ATPase (ATP6V1V2) contribute to the acid-base balance of YS cells and the yolk (step ❶). Minerals and vitamin D are absorbed from the yolk (yellow rectangle) to the blood (red arrows), via the transporters expressed by the YS (CALB1, SCGN, PKD2, SGK1, GC - step ❷). TRPV6 in not expressed in YS at ED12 or ED16 (~~TRPV6~~). In these proposed mechanisms (A and B), erythrocytes may also express CA2, SLC4A1 and SGK1.

Schematic representation of the CAM and the YS (left part) is inspired by Hincke et al. (2019) and Bauer et al. (2013). Some elements were obtained from Servier Medical Art (https://smart.servier.com), licensed under a Creative Commons Attribution 3.0 Unported

License. CC, capillary covering cells; VC, villous cavity cells. Grey arrows illustrate the transport from the blood to the eggshell via the CAM or the yolk via the yolk sac, and red arrows illustrate transport into the blood vessels.

It must be emphasized that the intact yolk sac and CAM tissues were analyzed: endoderm, mesoderm, and ectoderm for the yolk sac, and allantoic epithelium, mesoderm and chorionic epithelium for the CAM. Each candidate gene in this study may be differentially expressed in the various layers or cell types (Discussion section). Hence, the specific localization of all candidate genes and proteins in YS and CAM will require further experimental study.

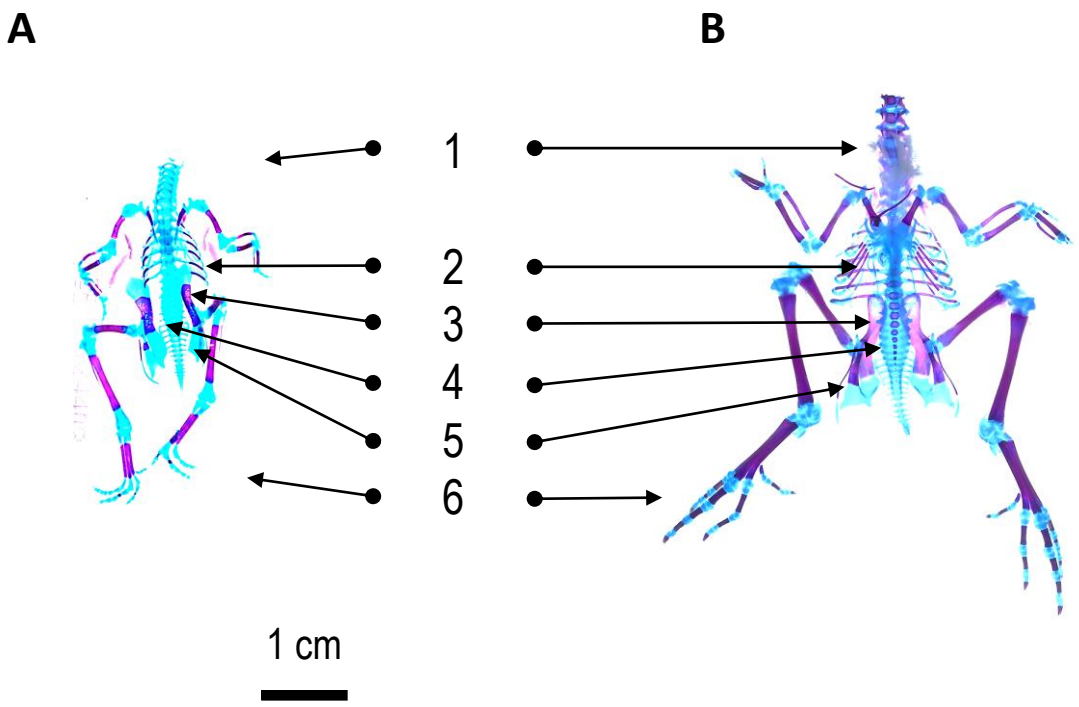
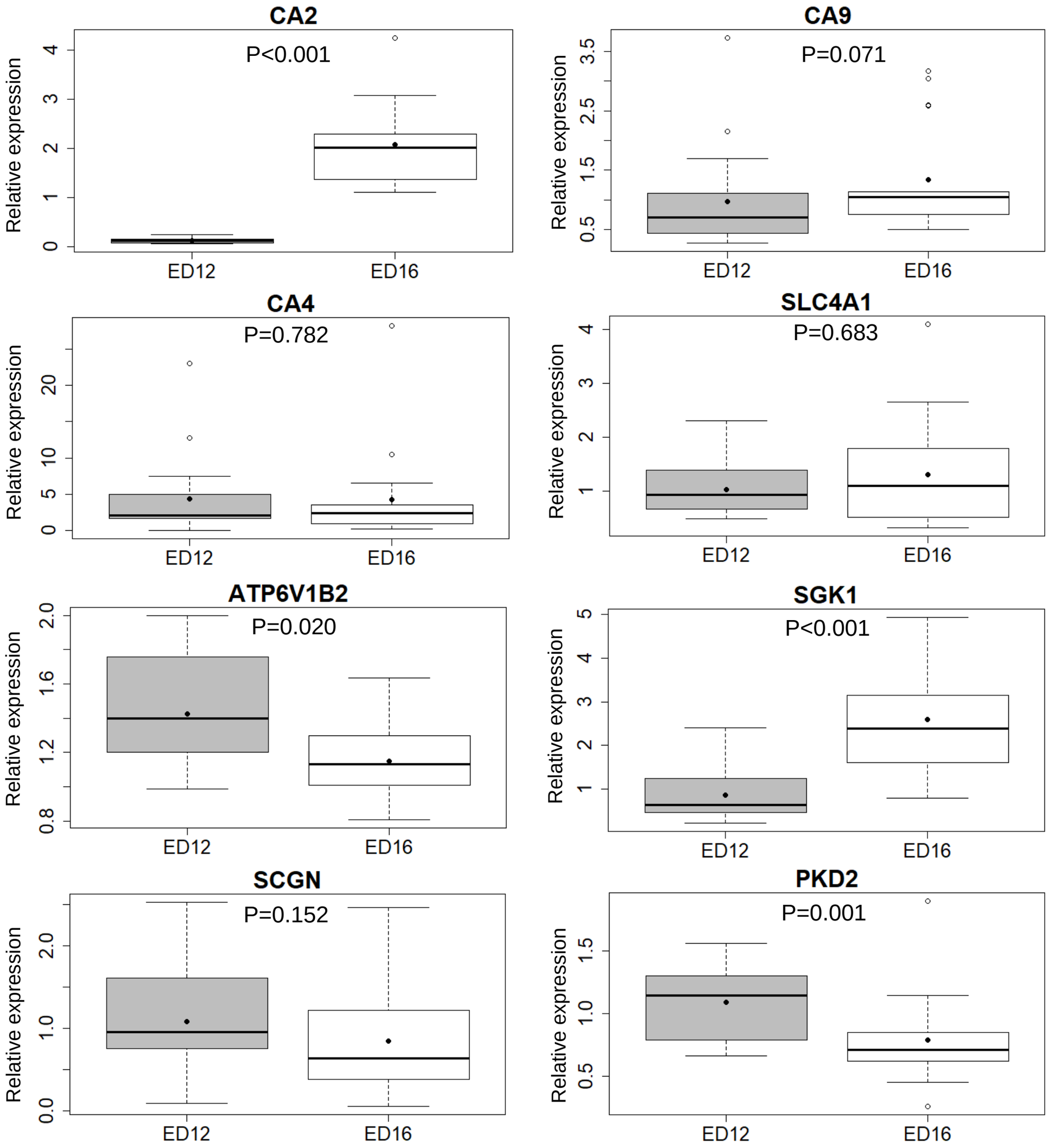


Figure 1

A


CA2
P<0.001
CA9
P=0.071
CA4
P=0.782
SLC4A1
P=0.683
ATP6V1B2
P=0.020
SGK1
P<0.001
SCGN
P=0.152
PKD2
P=0.001
Relative expression
ED12
ED16

B

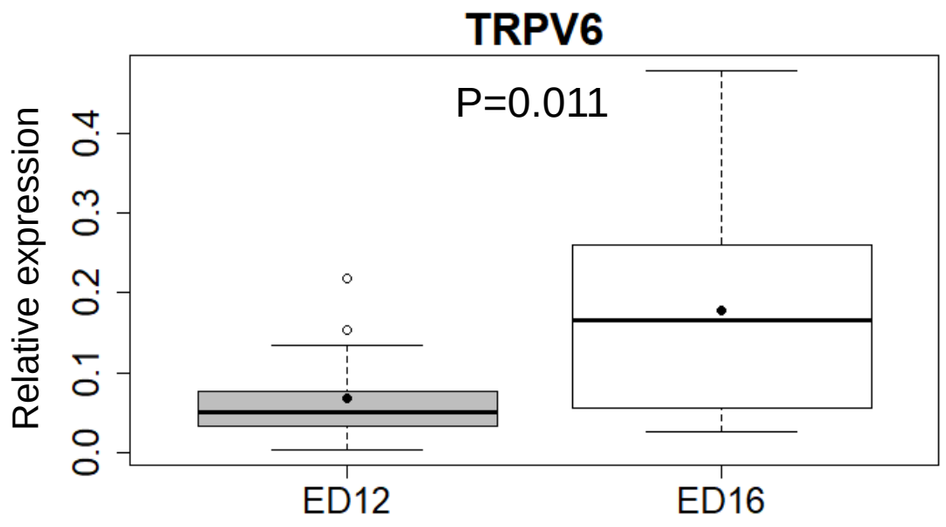
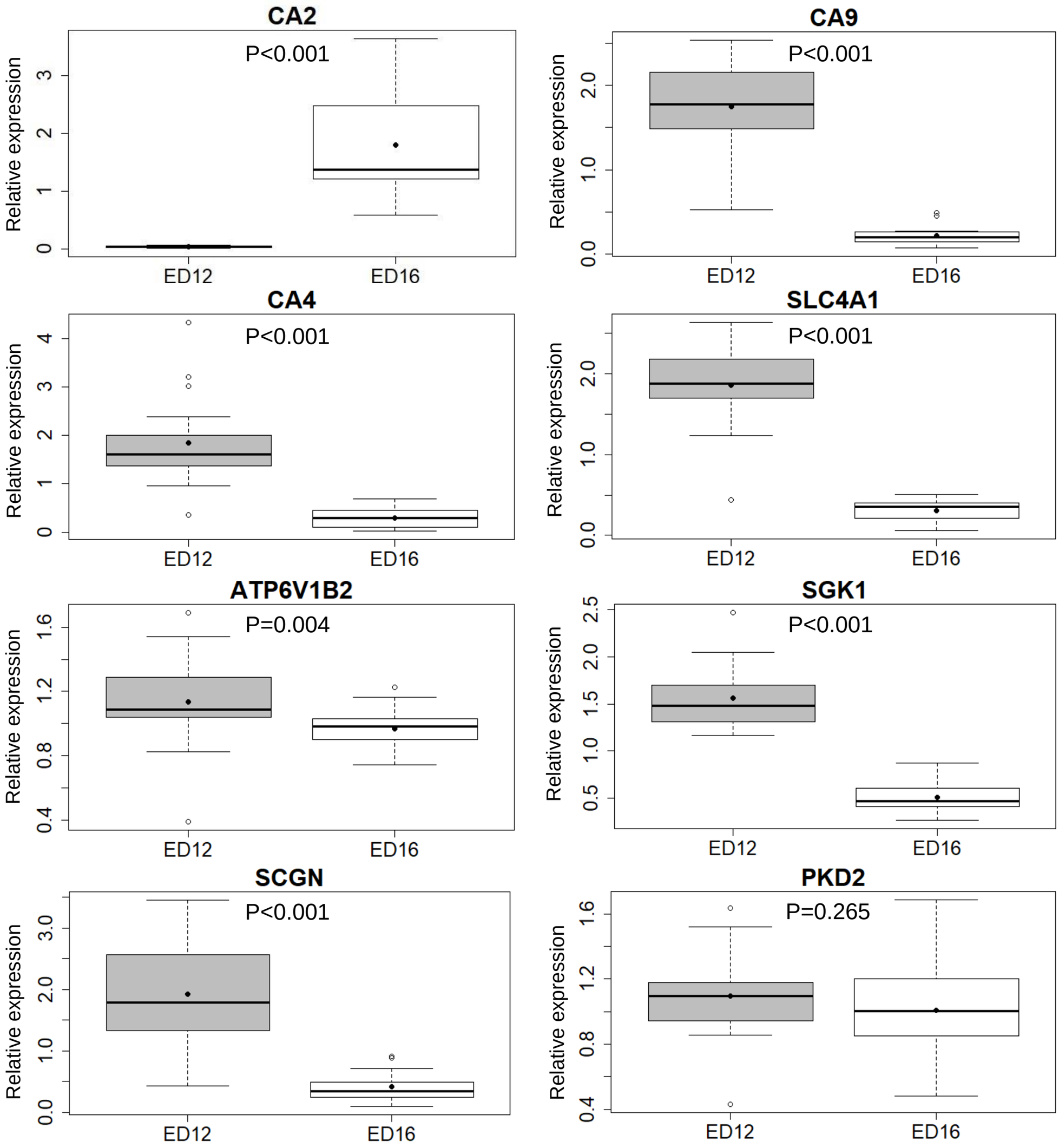

TRPV6
P=0.011
Relative expression
ED12
ED16

Figure 2

A


CA2
P<0.001
Relative expression
ED12
ED16
CA9
P<0.001
Relative expression
ED12
ED16
CA4
P<0.001
Relative expression
ED12
ED16
SLC4A1
P<0.001
Relative expression
ED12
ED16
ATP6V1B2
P=0.004
Relative expression
ED12
ED16
SGK1
P<0.001
Relative expression
ED12
ED16
SCGN
P<0.001
Relative expression
ED12
ED16
PKD2
P=0.265
Relative expression
ED12
ED16

B

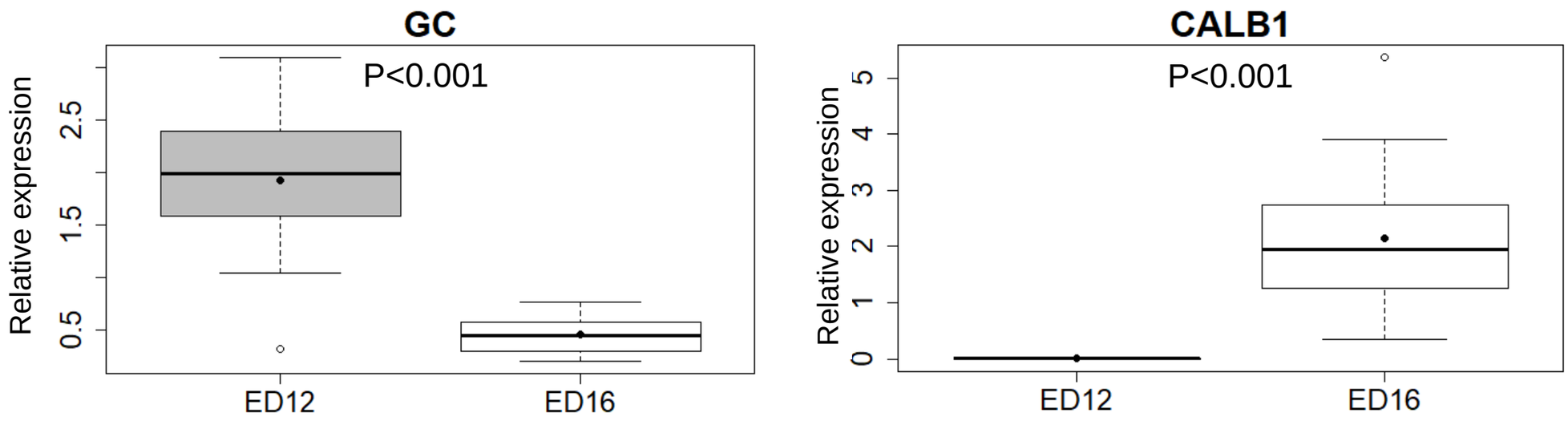
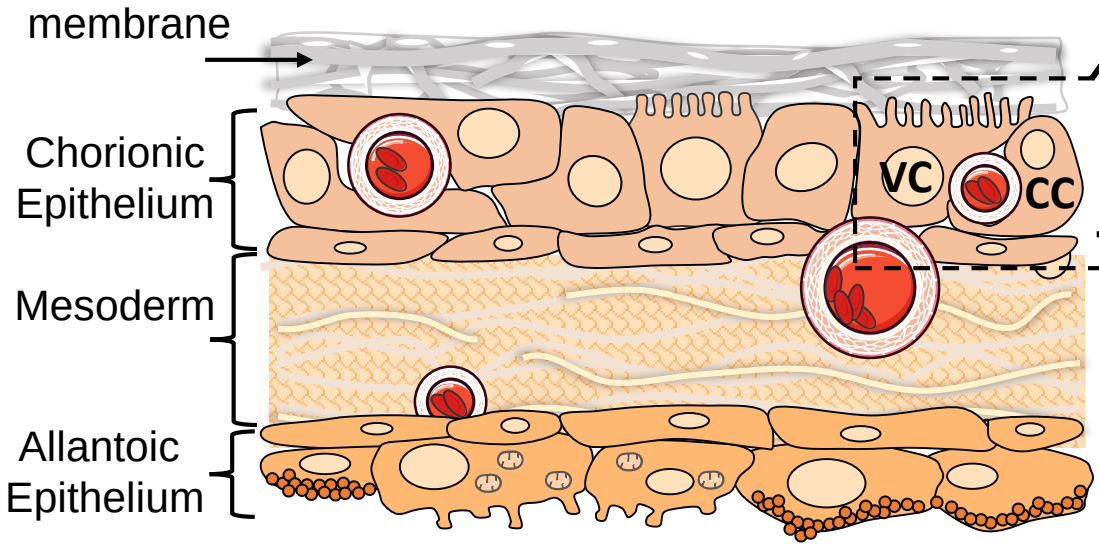
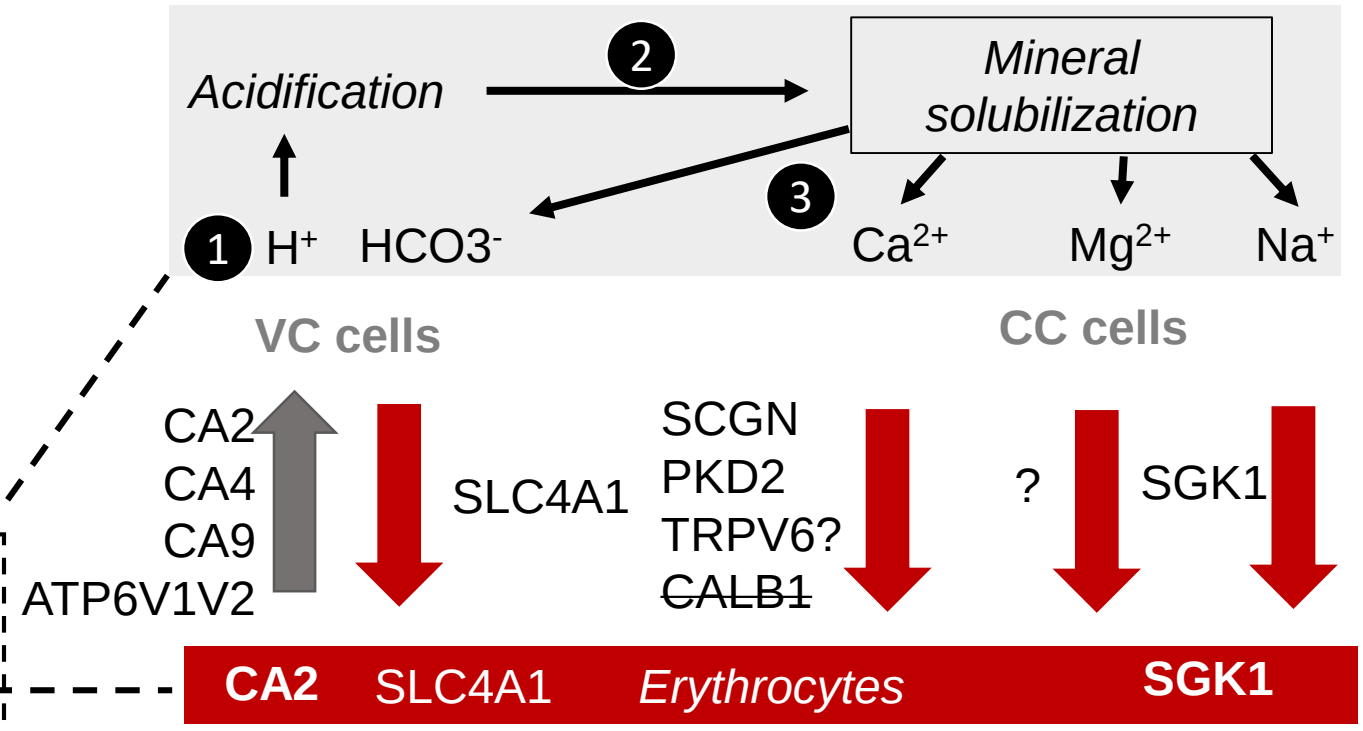

GC
P<0.001
Relative expression
ED12
ED16
CALB1
P<0.001
Relative expression
ED12
ED16

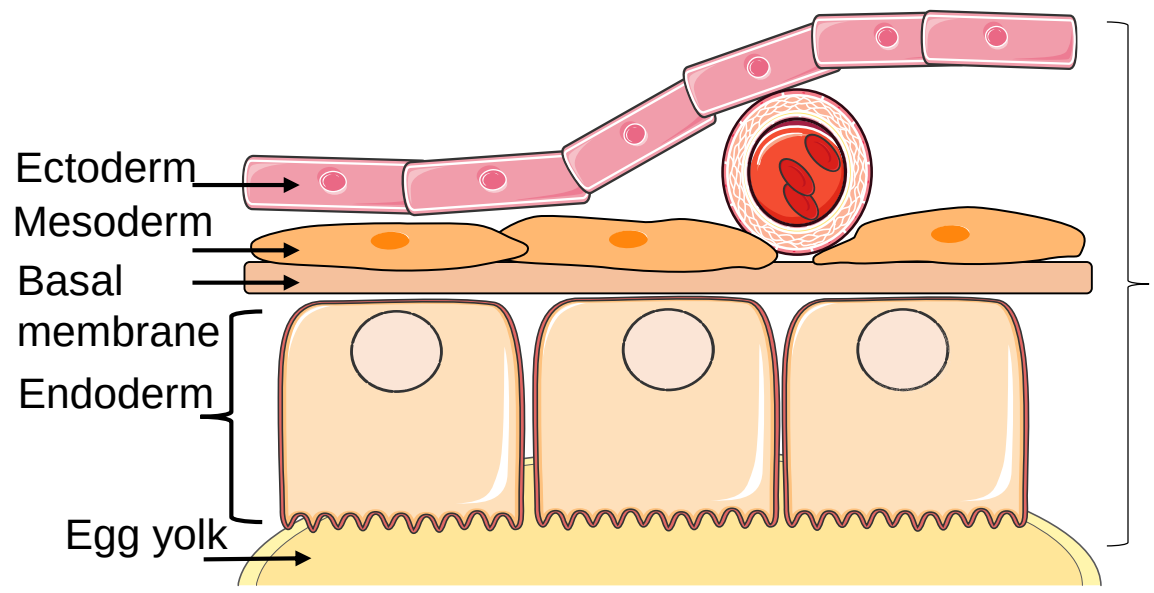
Figure 3

A Chorioallantoic membrane at ED16





B Yolk sac at ED16



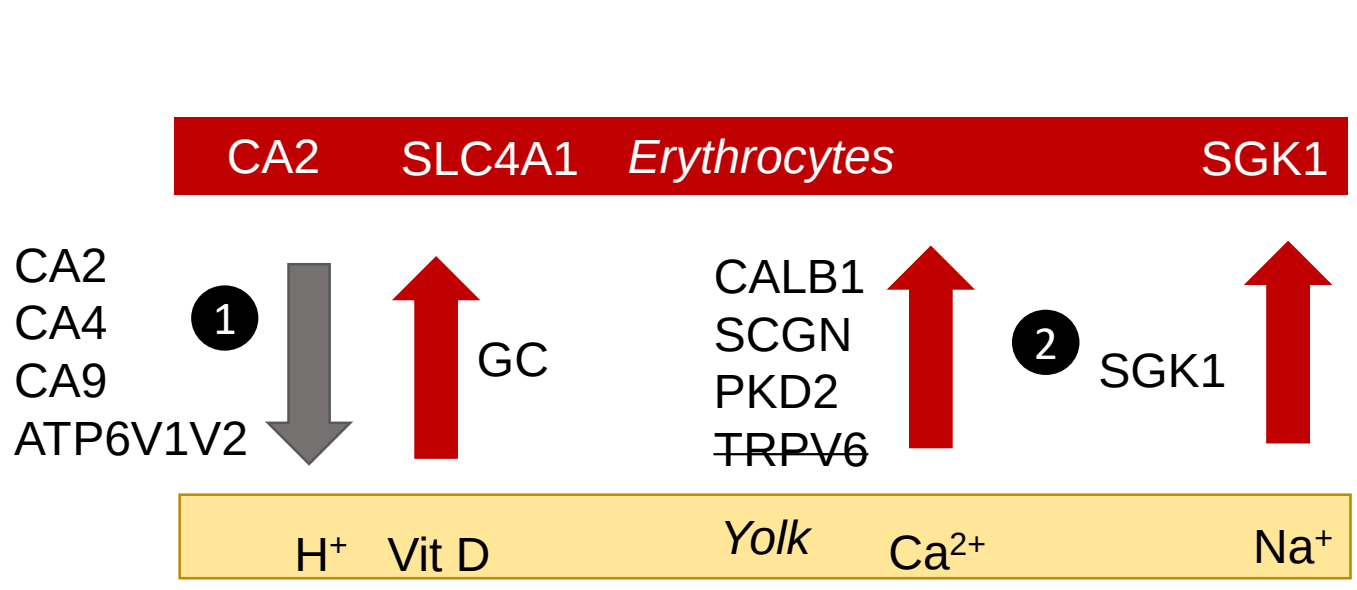


Figure 4