CARBONIC ANHYDRASES IN METAZOAN MODEL ORGANISMS: MOLECULES, MECHANISMS, AND PHYSIOLOGY

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CLINICAL HIGHLIGHTS

Carbonic anhydrases are ubiquitous enzymes that are present in all living organisms from archaea and bacteria to mammals.

Genetic defects in human carbonic anhydrase genes result in clinically significant consequences.

Several animal models have been created to recapitulate the genetic defects associated with carbonic anhydrases.

Several carbonic anhydrase inhibitors are clinically used or are being evaluated in trials as treatments for relevant diseases such as brain edema, glaucoma, epilepsy, cancer, and acute mountain sickness.

Carbonic anhydrase inhibitors used in animal models have provided invaluable information about the functional mechanisms and roles of carbonic anhydrases in physiology.

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ABSTRACT

During the past three decades, mice, zebrafish, fruit flies, and *Caenorhabditis elegans* have been the primary model organisms used for the study of various biological phenomena. These models have also been adopted and developed to investigate the physiological roles of carbonic anhydrases (CAs) and carbonic anhydrase-related proteins (CARPs). These proteins belong to eight CA families and are identified by Greek letters: α, β, γ, δ, ζ, η, θ, and ι. Studies using model organisms have focused on two CA families, α-CAs and β-CAs, which are expressed in both prokaryotic and eukaryotic organisms with species-specific distribution patterns and unique functions. This review covers the biological roles of CAs and CARPs in light of investigations performed in model organisms. Functional studies demonstrate that CAs are not only linked to the regulation of pH homeostasis, the classical role of CAs but also contribute to a plethora of previously undescribed functions.

Keywords: Carbonic anhydrase, Mouse, Zebrafish, *Danio rerio*, Fruit fly, *Drosophila melanogaster*, *Caenorhabditis elegans*, Model organism, Phenotype, Physiology, pH regulation, Ion transport

1. INTRODUCTION TO CARBONIC ANHYDRASES

1.1. Carbonic Anhydrase Enzyme Families

Carbonic anhydrases (CAs, EC 4.2.1.1) are metalloenzymes that catalyze the reversible hydration of carbon dioxide in the $CO_2 + H_2O \leftrightharpoons HCO_3^- + H^+$ reaction (1). The active site of a CA most often contains a zinc ion, which is required for CA catalytic activity. Different classes of CAs may also contain other metal ions instead of zinc, for example, cadmium in the case of ζ-CAs and iron or cobalt in γ-CAs (2, 3). The CA-catalyzed reaction is fundamental for the regulation of acid-base homeostasis in all living organisms from archaea and bacteria to fungi, protists, plants, and animals. Additionally, CAs are involved in many other processes, including various biosynthetic pathways (1).

CAs are grouped into eight evolutionarily distinct families: α-, β-, γ-, δ-, ζ-, η-, θ-, and ι-CAs (4), the members of which have unique expression patterns and functions (Figure 1).

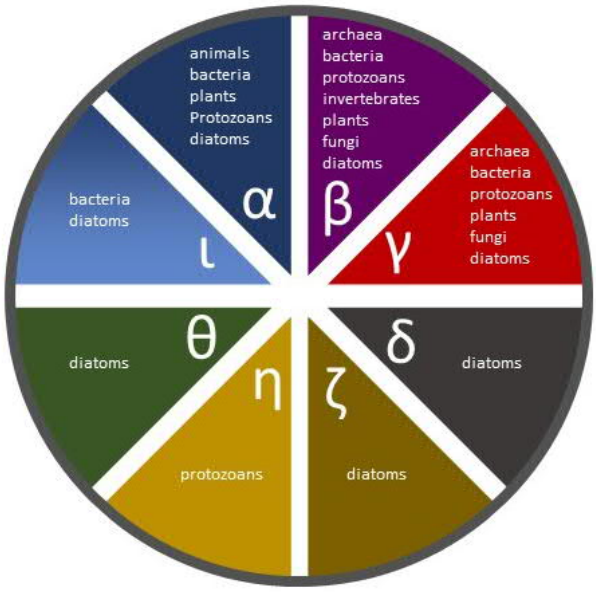

Figure 1. Distribution of CA enzyme families among different species groupings (4-13).

Members of the best characterized enzyme family, the α-CAs, are expressed in many prokaryotic and eukaryotic organisms. In mammals, 13 enzymatically active α-CA isozymes have been discovered (14) with different subcellular localizations (Figure 2). Table 1 describes the

nomenclature of human and mouse *α-CA* gene nomenclature in parallel with the corresponding zebrafish *α-ca* gene names.

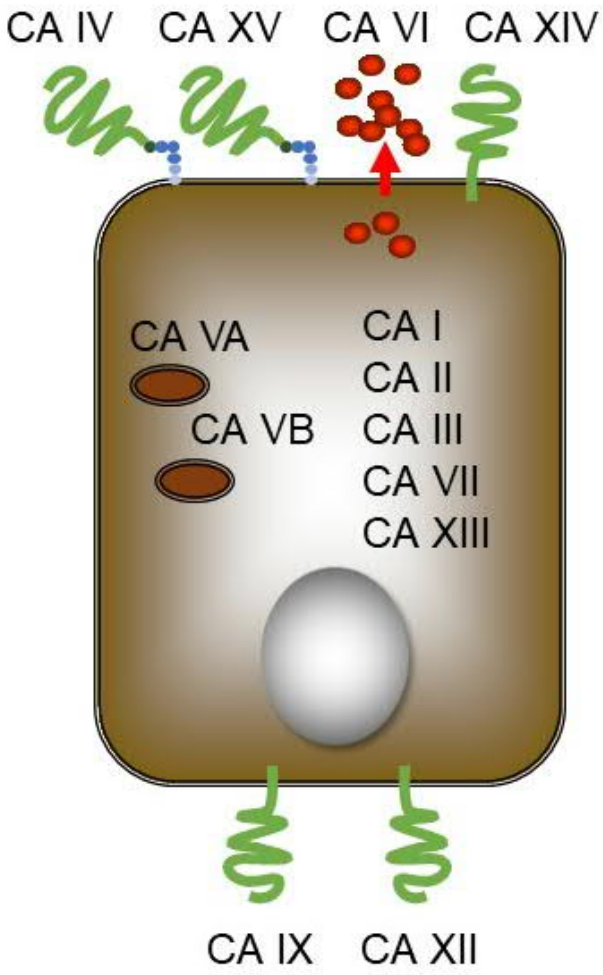

Figure 2. Schematic presentation of the subcellular localization of enzymatically active mammalian α-CAs. CA I, II, III, VII, and XIII are cytosolic enzymes. CA VA and VB are located in the mitochondrial matrix. CA VI is transported via the secretory pathway into saliva and milk. CA IV and XV are bound to the plasma membrane through a glycosylphosphatidylinositol anchor, whereas CA IX, XII, and XIV are single-pass type I membrane proteins.

Table 1. Nomenclature of human, mouse, and zebrafish *α-CA* genes.

	Human	Mouse	Zebrafish	
Cytosolic			ZFIN genes	suggested*
■	*CA1*	*Car1*		
■	*CA2*	*Car2*	*ca2*	*ca17a*
■	*CA3*	*Car3*	*cahz*	*ca17b*
■	*CA13*	*Car13*		
■	*CA7*	*Car7*	*ca7*	*ca7*
□	*CA8*	*Car8*	*ca8*	*ca8*
Mitochondrial				
■	*CA5A*	*Car5a*	*ca5a*	*ca5*
■	*CA5B*	*Car5b*		
Secreted				
■	*CA6*	*Car6*	*ca6*	*ca6*
□	*CA10*	*Car10*	*ca10a*	*ca10a*
□			*ca10b*	*ca10b*
□	*CA11*	*Car11*	n/a	
TM helix-anchored				
■	*CA9*	*Car9*	*ca9*	*ca9*
■	*CA12*	*Car12*	*ca12*	*ca12*
■	*CA14*	*Car14*	*ca14*	*ca14*
GPI-anchored				
■	*CA4*	*Car4*	*ca4a*	*ca4a*
■			*ca4b*	*ca4b*
■			*ca4c*	*ca4c*
■	n/a	*Car15*	*car15*	*ca15*
■	n/a	n/a	*si:ch211-173d10.4*	*ca20a*
■	n/a	n/a	*ca15b*	*ca20b*
■	n/a	n/a	*zgc:153760*	*ca20c*
■	n/a	n/a	*ca15a*	*ca20d*
■	n/a	n/a	*ca15c*	*ca20e*

■ Enzymatically active

□ Inactive, CA-related proteins

ZFIN, Zebrafish Information Network; TM, transmembrane; GPI, glycosylphosphatidylinositol; *, here for clarity

β-CAs are widely distributed among different organisms and are expressed in both prokaryotes and eukaryotes, including fungi, algae, plants, protozoans, arthropods, and nematodes (7, 13, 15-17). Previously, it was believed that animals possess only α-CAs, but during the past decade, it was documented that β-CAs are also widely expressed in the animal kingdom, specifically in

invertebrates (7, 13). Of the different CA enzyme families, β-CAs have garnered particular interest for use in clinical applications because these enzymes are found in many parasitic organisms but not in humans or other vertebrates (18, 19). γ-CAs are found in plants, archaea and some bacteria, and δ- and ζ-CAs are expressed in many species of marine phytoplankton (15). The recently discovered η-CA class has been found in parasites of the genus *Plasmodium*, which are malarial vectors (8). The most recently discovered CA enzyme families are θ- and ι-CAs, which were first described in the marine diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (4, 10).

1.2. Overview of Carbonic Anhydrase Expression in Human and Mouse Tissues

Identification of various tissues and cell types expressing a particular CA augments our understanding of the potential contributions of CAs to physiology. Most of the work on CAs has been performed during the past four decades using various techniques, such as immunohistochemistry, *in situ* hybridization, western and northern blotting, and reverse transcriptase PCR. Most recently, RNA-Seq analysis has become a standard practice, and large publicly available datasets on tissue-specific gene expression are now available. In Figures 3 and 4, we demonstrate the distribution of different α-CAs in both humans and mice across several primary tissues with major biological relevance on the basis of available data in publicly available gold standard datasets.

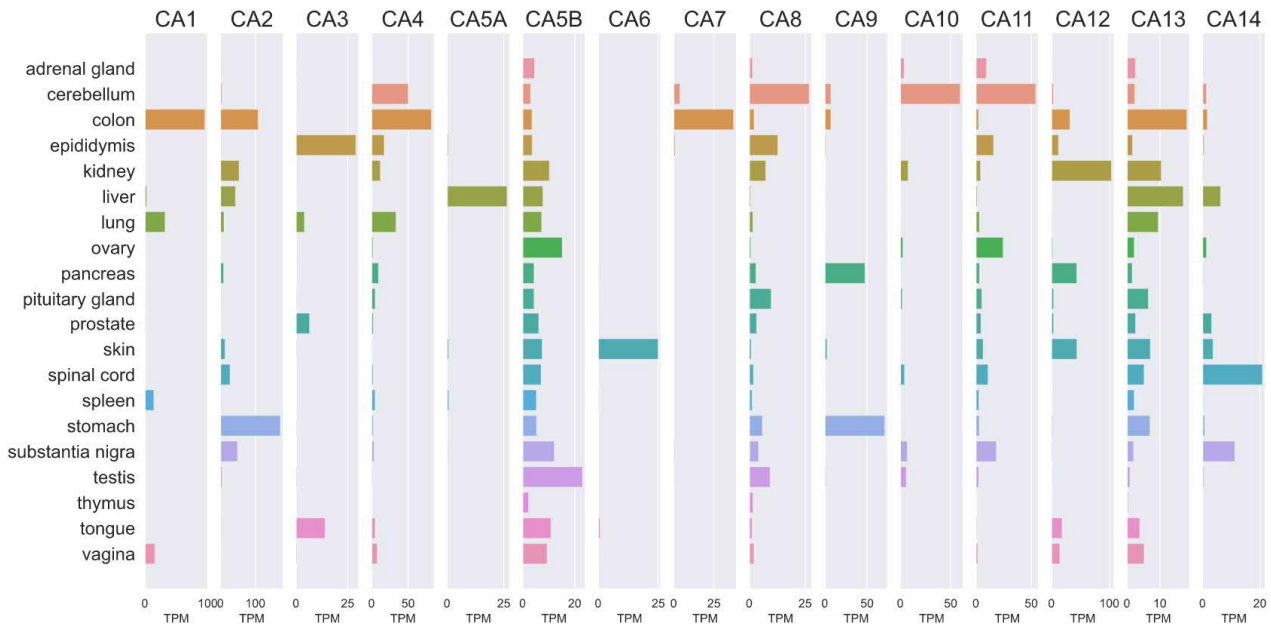

Figure 3. Human CA RNA-Seq expression data. Expression data for all genes were extracted from the Human Protein Atlas (HPA) database that had been obtained as consensus values from the Genotype-Tissue Expression (GTEx) project (20), and Functional Annotation Meeting (FANTOM5) project (21), as well as the HPA project itself (http://www.proteinatlas.org) (22). The expression value for each gene in each of the 62 tissues cataloged represents the highest value found among the three RNA-Seq datasets. From these data, the expression values for the 15 human CAs in 20 selected tissues were extracted and plotted using Python libraries Seaborn (23) and Matplotlib (24). Expression values are presented as transcripts per million (TPM).

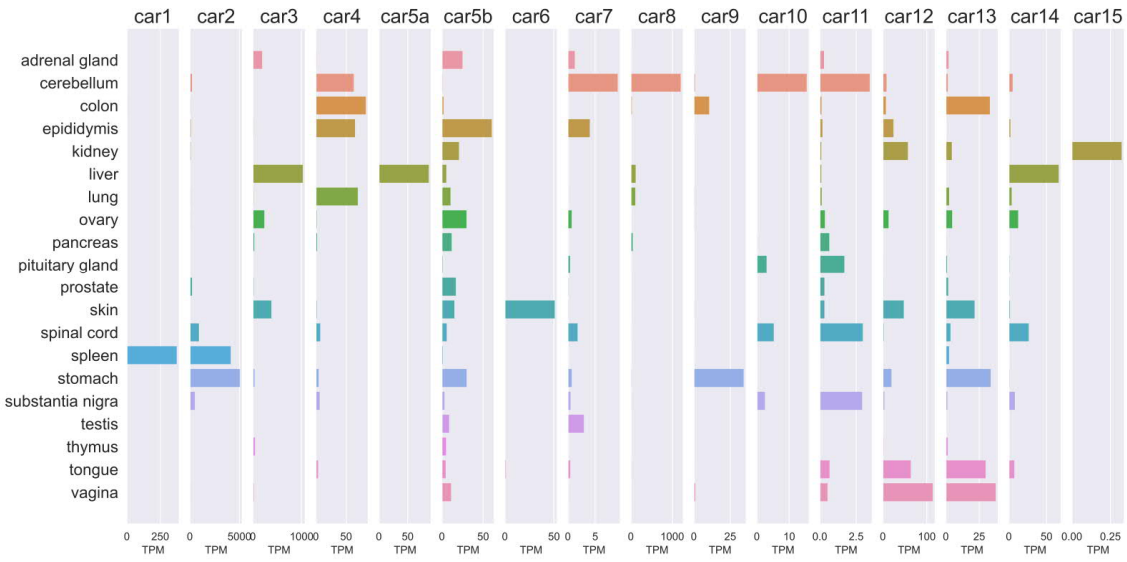

Figure 4. Mouse CA RNA-Seq expression data. The expression data for all genes were extracted from the functional annotation meeting (FANTOM5) project (21). The expression values for the 16 mouse CAs in 20 selected tissues were extracted from this dataset and plotted using the Python libraries Seaborn (23) and Matplotlib (24). The expression values are presented as transcripts per million (TPM) transcripts.

2. QUESTIONS TO BE ANSWERED USING MODEL ORGANISMS

2.1. Genetic Diseases with Carbonic Anhydrase Gene Mutations or Deficiencies

Table 2 shows a list of the general characteristics of human *CA* genes, which may be helpful for determining the locations of mutations in each gene described later in the text.

To date, there is only sporadic or no information available on clinical phenotypes associated with defects in the *CA3*, *CA7*, *CA9*, *CA10*, *CA11*, *CA13*, or *CA14* gene. Public data obtained from The Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php) reveals a single missense mutation of the *CA10* gene (leading to an S42R substitution in the CARP X protein) that was found in patients with schizophrenia (25). Two studies suggest an association between *CA10* gene alterations and bone physiology. An intronic variant of the *CA10* gene has been associated with osteoporosis in women (26), and a copy number deletion has been linked to a lower frequency of osteoarthritis (27).

Table 2. Human *CA* transcripts, pseudogenes, and predicted protein lengths. The data were extracted from the Ensembl database (www.ensembl.org).

Gene name	Transcript id	Chr	Genomic length (bp)	Protein (aa)
CA1	ENST00000431316	8	21672	261
CA1	ENST00000517590	8	48282	175
CA1	ENST00000517618	8	13121	251
CA1	ENST00000519129	8	39693	22
CA1	ENST00000519991	8	49519	137
CA1	ENST00000520663	8	48357	87
CA1	ENST00000521679	8	9806	178
CA1	ENST00000521846	8	44581	149
CA1	ENST00000522389	8	13220	127
CA1	ENST00000522579	8	44585	149
CA1	ENST00000522662	8	41168	118
CA1	ENST00000522814	8	44583	148
CA1	ENST00000523022	8	50505	261
CA1	ENST00000523858	8	41111	99
CA1	ENST00000523953	8	51406	261
CA1	ENST00000524324	8	49548	194
CA1	ENST00000542576	8	14614	261
CA1	ENST00000626824	8	51406	127
CA2	ENST00000285379	8	17486	260
CA3	ENST00000285381	8	10181	260
CA3	ENST00000520921	8	68694	19
CA4	ENST00000300900	17	9573	312
CA4	ENST00000587265	17	856	99
CA4	ENST00000590203	17	1814	184

CA4	ENST00000591725	17	7600	38
CA5A	ENST00000648177	16	55031	191
CA5A	ENST00000649158	16	55034	300
CA5A	ENST00000649794	16	48516	305
CA5A (pseudogene)	ENST00000550637	16	17428	0
CA5A (pseudogene)	ENST00000616011	16	6936	0
CA5A (pseudogene)	ENST00000568175	16	17543	0
CA5B	ENST00000318636	X	50141	317
CA5B	ENST00000454127	X	34565	317
CA5B	ENST00000479740	X	22660	136
CA5B	ENST00000498004	X	75835	76
CA5B (pseudogene)	ENST00000605701	13	303	0
CA5B (pseudogene)	ENST00000380331	X	21166	0
CA5B (pseudogene)	ENST00000380333	X	27634	0
CA5B (pseudogene)	ENST00000380334	X	28178	0
CA5B (pseudogene)	ENST00000380336	X	22819	0
CA5B (pseudogene)	ENST00000435806	X	22139	0
CA5B (pseudogene)	ENST00000448692	X	16268	0
CA6	ENST00000377436	1	28557	313
CA6	ENST00000377442	1	28817	248
CA6	ENST00000377443	1	29224	308
CA6	ENST00000480186	1	6769	179
CA6	ENST00000549778	1	21878	187
CA7	ENST00000338437	16	9733	264
CA7	ENST00000394069	16	9221	208
CA8	ENST00000317995	8	95988	290
CA9	ENST00000378357	9	7231	459
CA9	ENST00000617161	9	7237	356
CA10	ENST00000285273	17	529032	328
CA10	ENST00000442502	17	529704	328
CA10	ENST00000451037	17	528394	328
CA10	ENST00000570565	17	529689	253
CA10	ENST00000575097	17	86195	56
CA10	ENST00000575181	17	526371	276
CA11	ENST00000084798	19	8241	328
CA11	ENST00000596080	19	1437	113
CA12	ENST00000178638	15	60468	354
CA12	ENST00000344366	15	57147	343
CA12	ENST00000422263	15	57139	283
CA13	ENST00000321764	8	38615	262
CA14	ENST00000369111	1	7304	337
CA14	ENST00000607082	1	1847	130
CA14	ENST00000647854	1	7798	337
CA14 (pseudogene)	ENST00000400982	1	11218	0
CA14 (pseudogene)	ENST00000453762	1	1227	0

Chr, chromosome; id, identifier; bp, base pair; aa, amino acids

2.1.1. Carbonic anhydrase I deficiency

The first case of deficiency to be identified, CA I deficiency, described the absence of a particular CA due to an inherited defect. This finding was based on an extensive search of CA I-deficient individuals among 23,000 subjects in several different populations. By the end of the study, three members of one family from the Greek island of Icaria were found to exhibit a nearly complete absence of CA I (28). The absence of CA I in these individuals was confirmed by electrophoresis of hemolysates as well as radioimmunoassay methods and, in addition, two presumed heterozygotes were identified. In this study, no obvious hematological defects were attributed to CA I deficiency, nor did a documented CA I-deficient homozygote show any abnormality in renal acidification when metabolic acidosis was induced by ammonium chloride ingestion. During the time of the investigation, many testing methods used today were unavailable or unreliable, and the actual genetic defect was not determined until 14 years later (1). Individuals with CA I deficiency carry a missense mutation in the *CA1* gene, leading to an R246H amino acid change. It has been suggested that this mutation may lead to an unstable form of the CA I molecule. The availability of a CA I-deficient mouse and thorough investigation of this model would shed more light on the functional significance of CA I. Furthermore, we still know only a little about this isozyme that was discovered many decades ago and remains the second most abundant protein in erythrocytes after hemoglobin (29).

2.1.2. Carbonic anhydrase II deficiency

Early studies in the 1970s and 1980s demonstrated that CA II is widely expressed in mammalian tissues and pointed to its important physiological functions. Therefore, it is natural that much CA research has focused on this high-activity enzyme. The first report on genetic defects in CA II was published by Sly and coworkers in 1983 (30); they reported on three siblings of one family affected with an autosomal recessive syndrome characterized by renal tubular acidosis, osteopetrosis, and cerebral calcification. This finding caused considerable excitement because it provided clear genetic and functional evidence for the pivotal role of CA II at the organismal level. The phenotypic consequences of CA II deficiency had been largely explained by previous CA expression and inhibition studies. CA II had also been previously reported in several segments of the nephron and

collecting duct (31), and CA activity found in osteoclasts was later attributed to CA II (32, 33). The role of CA II in cerebral calcification was perhaps the most surprising finding since, in the brain, CA II had previously been localized only to oligodendrocytes, not to capillary walls (34). Other features later reported included developmental delay, mental retardation, short stature, and multiple bone fractures before patients reached adolescence (35).

In 2004, Shah and coworkers reported 11 novel mutations in the human *CA2* gene; therefore, 23 mutations have been discovered to date (35). Individuals with *CA2* mutations represent 17 different nationalities. These *CA2* gene mutations were scattered throughout the genomic sequence from exon 2 to exon 7 and led to various missense mutations, frameshifts, splicing mutations, and early stop codons. Notably, all 11 novel mutations caused renal tubular acidosis and osteopetrosis. Developmental delay was observed in all patients, except in one patient who was not analyzed. Prior to the study by Shah and coworkers, it had been generally understood that certain missense mutations result in mild symptoms due to residual CA II activity. The results from Shah and coworkers indicated, however, that symptom heterogeneity is a result of complicated biology. As an example, a woman carrying a truncation mutation expected to lead to a complete absence of CA II expression and, presumably, severe disease, graduated from high school and worked as an office clerk. This finding led the authors to point out that there are unanswered questions in genotype/phenotype correlations in CA II deficiency, especially in the degree of cognitive changes resulting from a null mutation.

Since the report by Shah and coworkers, a few novel mutations have been discovered in the *CA2* gene. A novel T85C missense mutation leading to S29P amino acid change was reported in a 12-year-old Indian boy with osteopetrosis, renal tubular acidosis, basal ganglia calcification, poor cognition, short stature, and overcrowded teeth (36). Shivaprasad et al. (37) described a 24-year-old patient with a novel deletion resulting in a frameshift and early stop codon corresponding to position 90 in the amino acid sequence; this patient also presented with typical signs of CA II deficiency, including osteopetrosis, renal tubular acidosis, and cerebral calcification with mental retardation. In addition, the patient had low height gain, a history of recurrent bone fractures, and overcrowded and misaligned teeth. In 2015, Pang and coworkers reported two novel mutations of the *CA2* gene in two Chinese patients (38). The mutations included a change in the splicing site and a nonsense mutation targeting position 127 in the primary amino acid sequence. In addition to osteopetrosis, renal tubular acidosis, and cerebral calcifications, these patients presented with

other clinical manifestations, such as short stature, increased frequency of bone fractures, dental abnormalities, cranial nerve compression, and developmental delay. Recently, Alsharidi et al. (39) described a 19-year-old girl from Saudi Arabia who carried two compound heterozygous mutations: a common Arab splice site mutation in intron 2 and a novel deletion in exon 5 that led to a frameshift, leading to premature truncation of 35 amino acid residues downstream of position 162. This patient had a number of clinical abnormalities, including renal tubular acidosis, osteopetrosis, short stature, recurrent bone fractures, renal stones, facial deformations, crowded teeth, and intracranial calcifications. Most recently, Yang et al. (40) reported a nonsense mutation in which 368G in the *CA2* gene was substituted with 368A, leading to early termination of translation. The patient, a 21-year-old male, presented with multiple symptoms and signs, including recurrent limb weakness, hypokalemia, hyperchloremia, metabolic acidosis, slow nail growth, cognitive defects, chronic rhinitis, cerebral calcifications, and osteopetrosis.

The common features of CA II-deficiency syndrome can be classified into two categories. The first category includes classical symptoms such as renal tubular acidosis, osteopetrosis, and cerebral calcification. In the second class, the signs and symptoms vary but are common among patients. They include hypokalemia, hyperchloremia, recurrent fractures, developmental delay, cognitive defects, dental abnormalities, and short stature (38). Several unanswered questions related to the mechanisms of these symptoms and signs remain. It is also well documented that CA II is highly expressed in certain organs and tissues, such as the gastrointestinal and reproductive tracts (41-43), where no physiological consequences of CA II deficiency have been reported. We believe that a more detailed investigation of the mouse homolog *Car2*$^{-/-}$ phenotype in these tissues may shed more light on the role of CA II.

2.1.3. Carbonic anhydrase 4 gene mutations

Retinitis pigmentosa constitutes a group of rare eye diseases that eventually lead to profound vision loss, involving loss of night vision, loss of peripheral or central vision, defective color vision, and even total blindness. The type of symptoms and their rates of onset vary from person to person. It has been described as a highly heterogeneous disease exhibiting 1) genetic heterogeneity, in which similar phenotypes are caused by mutations in different genes; 2) allelic heterogeneity, in which several disease-causing mutations are in the same gene; 3) phenotypic heterogeneity, in which different mutations in the same gene produce different phenotypes; and

4) clinical heterogeneity, in which the same mutation in different patients may manifest different clinical phenotypes (44). Mutations in more than 50 genes have been associated with retinitis pigmentosa, and more than 3,000 different mutations have been reported in these genes (44). One of the genes associated with the autosomal dominant form of retinitis pigmentosa (RP-17) encodes the CA IV protein (45). Recently, de Bruijn et al. (46) described eight complex structural variants which also caused autosomal dominant retinitis pigmentosa at the RP-17 locus in over 300 affected individuals. This may represent another example of the genetic heterogeneity of the disease as mentioned above.

In 1991, Hageman and colleagues reported CA IV expression in the choriocapillaris of the eye (47). Notably, the authors concluded the abstract with the following statement: “Defining the physiological role of this ocular isozyme remains a challenge”. Thirteen years later, Rebello et al. (45) reported a signal sequence mutation, R14W, which causes RP-17. In COS-7 cells, this mutation reduced the enzymatic activity of CA IV by 28% due to both defective synthesis and accelerated protein turnover. The mutation induced the unfolded protein response, endoplasmic reticulum stress, and cell apoptosis. These findings showed that apoptosis of the endothelial cells in the choriocapillaris leads to retinal ischemia and ultimately to the emergence of symptomatic RP-17.

Recent studies have described other novel mutations in the *CA4* gene and different molecular mechanisms that may link CA IV to clinical RP-17. Yang and coworkers identified two mutations that may cause retinal degeneration (48). One missense mutation, R219S, encodes a protein with no CA activity. In transfected HEK-293 cells, coexpression of Na^+/HCO_3^- cotransporter 1 (NBC1) and wild-type (WT) CA IV increased the rates of intracellular pH recovery after acid loading compared with cells expressing only NBC1. Interestingly, mutant CA IV proteins (either R14W or R219S) failed to improve pH recovery. In addition, Yang´s team showed that both R14W and R219S mutant proteins appeared on the cell surface in almost similar amounts, excluding a defect in the intracellular secretory pathway. Considering several lines of evidence, they suggested that impaired pH regulation by the CA IV-NBC1 complex, due to either the R14W or R219S mutation, may cause the retinal phenotype exhibited by RP-17 patients. They further suggested that the enzymatically active CA IV R14W mutant fails to bind to NBC1, whereas the enzymatic inactivity caused by R219S is another mechanism leading to the same phenotypic outcome.

A total of 13 mutations were found when Alvarez and colleagues screened the *CA4* gene in 96 RP-17 patients of Chinese ethnicity (49). Among these mutations, a novel R69H alteration was

identified that was not present in any of the 432 ethnically matched control chromosomes. Similar to the findings described above, the R69H amino acid substitution impaired NBC1-mediated pH recovery after acid loading in cotransfected HEK293 cells. In addition, coimmunoprecipitation and GST pull-down assays showed that WT CA IV binds to NBC1, whereas no physical interaction was observed between the CA IV R69H mutant and NBC1.

Another study focusing on screening of Chinese patients with sporadic RP included a cohort of 116 patients and 263 controls (50). A total of ten different sequence alterations were found in the patient group. Two heterozygous variations were novel, whereas all the other alterations represented previously known benign polymorphisms. The A12T signal sequence mutation was detected in an 11-year-old girl with RP and panretinal dysfunction of photoreceptors. No significant difference in apoptosis rates was observed in transfected HEK293 cells expressing either WT CA IV or A12T mutant enzyme, nor was any significant difference detected at the protein expression level.

Additional *CA4* gene variants associated with RP are located outside the coding sequence (48, 50). Interestingly, these variants have been shown to lead to a 30–50% reduction in *CA4* mRNA expression levels in peripheral lymphocytes. These findings are questionable, because *CA4* mRNA expression is negligible in human lymphocytes, according to data in the Human Protein Atlas (https://www.proteinatlas.org/).

Taken together, several mutations in the *CA4* gene appear to cause RP-17 disease. The published data suggest that these mutations may cause RP through different mechanisms. The R14W mutation has been linked to the unfolded protein response, endoplasmic reticulum stress, and induction of apoptosis (45). The other mutations may affect the interaction between CA IV and NBC1 and thus impair the normal pH regulation of the retina and choriocapillaris (48, 49). There is also some evidence that *CA4* gene mutations may lead to reduced CA IV expression (48, 50).

2.1.4. Carbonic anhydrase VA deficiency

Mitochondrial CA VA deficiency was the third genetic disease found to be linked to *CA* gene alterations and leads to significant phenotype variation in humans. In the early 1980s, certain metabolic processes, such as gluconeogenesis, ureagenesis, and fatty acid synthesis, were found to require bicarbonate, which is produced by a CA-catalyzed reaction within the mitochondrial

matrix (51, 52). In 2014, Karnebeek and coworkers described four children from three families who presented with three different *CA5A* gene alterations (53). The ethnic backgrounds of the children included Russian, Belgian-Scottish (two cases), and Pakistani. These children had infantile hyperammonemic encephalopathy, hypoglycemia, hyperlactatemia, elevated ketone bodies in urine, metabolic acidosis, and excretion of carboxylase substrates and related metabolites into urine. The patients in this study carried genetic alterations unique to each family. One was a S233P mutation that resulted in an 85–95% reduction in CA enzymatic activity. The second and third alterations included deletions in exon 4 and exon 6, resulting in deletions at positions 154–186 and 207–258 of the primary sequence, respectively. Both deletions affected critical sites of the CA VA enzyme and, thus, were predicted to impair enzymatic activity and possibly cause misfolding and proteasomal degradation of the protein.

Further investigations on *CA5A* gene defects involved a cohort of 96 patients of various ethnic backgrounds with neonatal-onset hyperammonemia (54). These patients showed no signs of other urea cycle disorders or organic aciduria. *CA5A* mutations were detected in ten of the patients, while no mutations were found in the *CA5B* gene. The ethnic backgrounds of these patients were Turkish, Indian (two cases), Pakistani (six), and Bangladeshi. The laboratory parameters of all ten patients showed hyperammonemia, elevated lactate, and elevated ketone bodies in urine. In this cohort, the deletion corresponding to amino acid positions 207–258 was the most common alteration and was detected in five patients. The E241K mutation was found in two patients. One mutation resulted in a truncation at the W41 position, and two patients had mutations in predicted splicing sites. The effects of the observed S233P and E241K mutations and exon 4 and exon 6 deletions were studied using recombinant proteins. The *CA5A* S233P mutant showed 49.9% enzymatic activity compared to the WT enzyme. E241K substitution resulted in residual activity of 26.5%. The recombinant proteins with either exon 4 or exon 6 deletion showed no CA activity. In summary, *CA5A* gene mutations or deletions can produce a clinical syndrome associated with hyperammonemia, hyperlactatemia, elevated urine ketone bodies, and, occasionally, other metabolic manifestations.

2.1.5. Carbonic anhydrase 6 gene polymorphism

The roles of the salivary CA VI enzyme have been linked to four potential functions in the mouth: 1) modulation of taste reception, 2) protection from caries, 3) modulation of oral microbial flora, and 4) growth of fungiform papillae of the tongue (55-58). There has been some speculation about its role in the regulation of salivary pH and/or buffer capacity, but these functions are probably relevant only in certain microenvironments of the mouth, such as those of dental plaque or enamel pellicle (56, 59, 60). Notably, there has been no report on the human genetic deficiency of CA VI, while several studies have focused on polymorphisms of the *CA6* gene with potential physiological relevance (61-63).

In the first study involving *CA6* gene polymorphisms, three changes in exons 2 (rs2274327 and rs2274328) and exon 3 (rs2274333) were investigated, and they were found to lead to changes in the primary amino acid sequence. The genotypes were correlated with salivary buffer capacity, decayed/missing/filled teeth (DMFT-index), and variation in dental plaque pH (61). No association was found between genotype and caries experience. A significant association was reported between buffer capacity and the rs2274327 polymorphism. In a subsequent study, the effects of these three polymorphisms on the enzymatic activity and concentration of CA VI in saliva were examined (64). No association was observed between genotype and CA VI activity. The polymorphisms rs2274327 and rs2274333 showed associations with CA VI concentrations in saliva. Li et al. (63) investigated the association of seven *CA6* gene polymorphisms with dental caries susceptibility in 164 patients with high caries prevalence and 191 patients with low caries experience. They showed that the rs17032907 genotype was associated with an increased risk of dental caries. In a systematic review, Lips et al. (65) described four studies in which *CA6* gene polymorphisms were investigated in relation to caries susceptibility and/or salivary parameters, such as pH and buffer capacity. In another recent study, Hatipoglu and Saydam also identified four articles that met the criteria for inclusion in a meta-analysis (66). Because of the largely contradictory results of the original studies highlighted in this meta-analysis, no firm conclusions were drawn about the effect of *CA6* polymorphisms in physiology or cariogenesis. Interestingly, Esberg and coworkers recently reported the effects of 27 single-nucleotide polymorphisms of the *CA6* gene on the oral microbiota in 154 Swedish adolescents (57). The results suggested that *CA6* gene polymorphisms of rs10864376, rs3737665, and rs12138897 are associated with *Streptococcus mutans* colonization, overall microbiota composition, and dental caries. These results clearly open new avenues to better understand the mechanisms by which CA VI may

contribute to oral physiology and health. There is clearly a great need for systematic experimental studies using model organisms, such as CA VI-deficient mouse models (67).

CA6 gene polymorphisms may be associated with taste function. Padiglia and colleagues investigated the effect of the rs2274333 genotype on 6-n-propylthiouracil (PROP) taste responses (62). They reported that the genotype affected the bitter taste function. In two subsequent studies, the same researchers reported that the PROP taste phenotype is based on the combined effect of the bitter receptor *TAS2R38* and *CA6* genotypes (58, 68). They further demonstrated that both *CA6* and *TAS2R38* genotypes were associated with fungiform papillae density on the tongue, and the *CA6* genotype was also associated with the size and shape variation of these papillae (58). Although several studies have suggested that polymorphisms in the *CA6* gene may affect bitter taste function, we acknowledge that the role of CA VI in taste reception remains controversial. Feeney and Hayes showed no association between 12 single-nucleotide polymorphisms and bitter taste perception, nor did they observe any effect on fungiform papillae density (69). Notably, these polymorphisms included rs2274333, which was previously associated with the bitter taste response. Interestingly, Feeney and Hayes reported a statistically significant association of two polymorphisms (rs3737665 and rs3765964) with perceived saltiness (NaCl) taste intensity, which complicates the overall picture on the role of CA VI.

2.1.6. Carbonic anhydrase 8 gene mutations

CARP VIII is a cytosolic protein with no CA catalytic activity. It was first described in cerebellar Purkinje cells (70) and later in several other tissues, including the mouse submandibular gland, liver, stomach, and lung (71). Human RNA-Seq expression data indicated the strongest signals in the cerebellum, epididymis, kidney, pituitary gland, stomach, and testis (Figure 3). CARP VIII shows high structural similarity with catalytically active CAs (72). Its inactivity has been attributed to the substitution of the first of the three histidine residues (by arginine in vertebrates) that are required for the coordination of zinc atom in the active site. Notably, the highly conserved CARP VIII has been found in all vertebrates and in many deuterostome invertebrate species (72).

The first defect in the human *CA8* gene was reported in members of an Iraqi family (73). The mutation leads to an S100P alteration in the amino acid sequence of CARP VIII, which presumably disrupts the normal folding of the protein (72). The affected individuals showed mild mental

retardation and congenital ataxia that was accompanied by quadrupedal gait (73). Another variation in the *CA8* gene, leading to a G162R amino acid substitution, was reported in members of a Saudi Arabian family (74). The affected individuals presented with variable cerebellar ataxia and mild cognitive impairment but no quadrupedal gait. Homozygosity for the G162R variation cosegregated with the disease phenotype, and the mutation was not detected in 200 unrelated healthy controls with the same ethnic origin, indicating that the G162R variation is likely pathogenic. Brain magnetic resonance imaging showed a loss of cerebellar volume in the Saudi Arabian group of patients (74).

Recently, Richmond and coworkers reported a 9-year-old boy of Caucasian parents who presented with generalized hypotonia of early childhood, poor fine motor skills, cerebellar ataxia, delayed speech, dysarthria, poor oral-motor coordination, but no intellectual disability (75). Magnetic resonance imaging performed when the patient was seven years old indicated cerebellar vermian and hemispheric atrophy. Whole-exome sequencing revealed a novel homozygous nonsense mutation of the *CA8* gene, leading to truncation at position 78 of the amino acid sequence. The authors pointed out that the 9-year-old patient, despite carrying a severe defect in the *CA8* gene, had achieved limited bipedal gait with intensive support and physical therapy.

2.1.7. Carbonic anhydrase 12 gene mutations

CA XII was discovered almost simultaneously by two research groups and reported in 1998 (76, 77). It is a membrane-associated enzyme that is most abundantly expressed in the human kidney, endometrium, colon, pancreas, and skin (Figure 3) (78-81). It is overexpressed in several malignant tumors, such as renal, breast, and brain cancers (76, 81-84).

In 2010, Feldshtein and colleagues reported for the first time that CA XII is linked to autosomal recessive hyperchlorhidrosis (85). They identified members of an Israeli Bedouin family, some of whom were referred for sweat testing due to hyponatremic dehydration and suspected cystic fibrosis. Screening identified seven family members with abnormally high sweat chloride levels. All affected individuals had normal height and weight, their psychomotor development and cognition were normal, and physical examination revealed no other abnormalities. The results of the following laboratory tests were normal: serum and urinary electrolyte, serum amylase, and fecal elastase level determination; renal function tests; renin-aldosterone axis function tests; blood and

urinary pH level determination; and pulmonary function tests (FEV1). The gene test for cystic fibrosis transmembrane conductance regulator (CFTR) showed no indication of classical cystic fibrosis disease. Further genetic analysis revealed a homozygous missense mutation of the *CA12* gene in the affected individuals, which led to the E143K alteration in the amino acid sequence. A few months later, another research group reported three affected children from a different clan of Bedouins (86); these patients had slightly variable phenotypes, but all showed hyponatremia and hyperkalemia in infancy. No chronic renal, respiratory or gastrointestinal abnormalities were reported in any of these patients. Positional cloning identified the missense mutation leading to the same E143K alteration in the CA XII amino acid sequence that had been previously reported. The E143K alteration is located in close proximity to the highly conserved H145 residue, which is the third zinc-binding histidine. The mutant enzyme showed approximately 70% CA activity compared to the WT CA XII enzyme (85). This finding was confirmed in a subsequent study, which indicated that the E143K mutant enzyme retained 76% residual activity (87). It is plausible that this much residual activity is enough to protect the affected individuals from acquiring a more-severe phenotype.

More recently, *CA12* variants were identified in two unrelated pedigrees: A Caucasian American and Omani siblings exhibiting cystic fibrosis-like signs (87). The American patient had shown hyponatremic dehydration, a high sweat chloride concentration, airway obstruction, and failure to thrive in infancy or childhood. At the age of 19–22 years, this patient exhibited pulmonary exacerbations and was treated with bronchodilators, antibiotics, and steroids. This patient had two variant *CA12* mRNA transcripts, both of which were predicted to generate aberrant CA XII protein. Indeed, experimental results showed that the reported mutations cause instability of the protein. The Omani patients, a brother and sister, also showed hyponatremic dehydration and elevated sweat chloride levels. A novel homozygous missense mutation was identified in exon 4 that resulted in an H121Q substitution. The H121Q mutation appeared to be more deleterious to the function of CA XII than the E143K mutation. The reduction in activity was greater than 99% compared to that of the WT enzyme. The Omani patients were young (6 and 11 years), which may explain the relatively mild phenotypes that they presented.

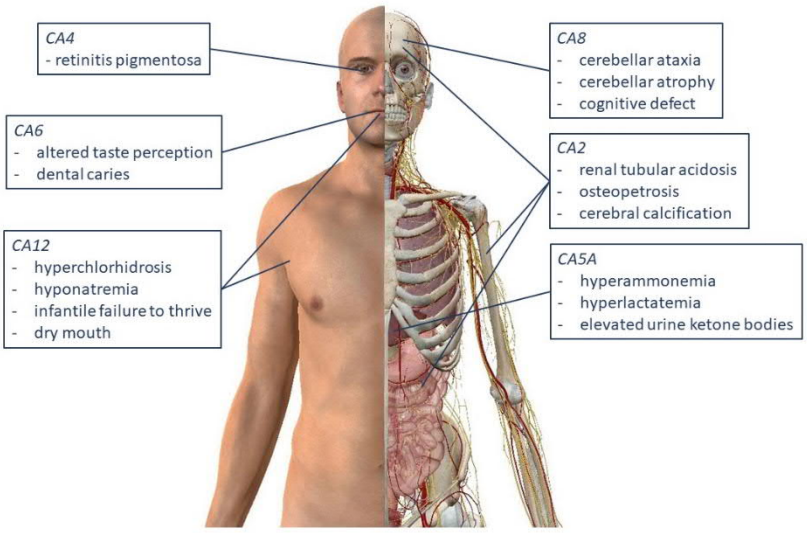

Figure 5. Human health-related effects of variations in *CA* genes. CA I deficiency has not been attributed to any diseases and is thus omitted from the figure. The figure was created using a Visible Body application (Wolters Kluwer).

2.2. Carbonic Anhydrase Inhibitors in Model Organisms

Studies using CA inhibitors in model organisms can be divided into two main types: i) the use of model organisms in preclinical safety and efficacy testing of novel CA inhibitors and ii) the use of CA inhibitors in model organisms for studying physiological phenomena. Most of the studies have been performed using either mouse or zebrafish models.

2.2.1. Preclinical studies of CA inhibitors using mouse models

CA enzymes are important therapeutic targets for the treatment of various diseases, including acute high-altitude illness, glaucoma, epilepsy, brain edema, pseudotumor cerebri, and cancer. A number of proven CA inhibitors are clinically used, and several novel compounds are in the development pipelines to treat clinically relevant diseases (88). Clinically approved drugs with CA inhibitory properties include such as acetazolamide, methazolamide, ethoxzolamide, dorzolamide, brinzolamide, dichlorphenamide, topiramate, zonisamide, lacosamide, imatinib, and statins (89-92).

CA II has often been considered a relevant treatment target because of its high enzymatic activity, wide distribution in tissues, and physiological significance. Notably, however, tissues often express several CA isozymes concurrently, and CA inhibitors can affect any of these isoforms according to their characteristic binding properties. The cancer-associated isozymes CA IX and XII, which are considered promising targets for anticancer drug development, have been the foci of intensive research (89). In addition, recent studies have introduced novel classes of compounds that have been designed to inhibit enzymes belonging to CA families other than the mammalian α-CAs. We describe here some case examples of preclinical studies where model organisms have been instrumental in finding better CA inhibitors for clinical use.

Mice have been widely used as preclinical animal models in the drug development process. The effects of classical sulfonamide CA inhibitors were first tested in mice in the 1950s (93). In the 1970s and early 1980s, a few studies were performed to investigate the suspected teratogenic effects of known CA inhibitors (94-97). The use of mouse models has substantially increased since the 1990s, when a connection was found between CAs and cancer. Indeed, many seminal observations have paved the way for testing various CA inhibitors with potential anticancer properties in tumor xenograft mouse models. First, solid tumors exhibit a fundamentally different microenvironment than corresponding normal tissues. The combination of increased tumor metabolism and poorly formed tumor vasculature results in tumor hypoxia, high interstitial fluid pressure, and low extracellular pH (98). In tumors, intracellular pH is typically in a physiological range of 7.0–7.4, similar to normal cells, whereas extracellular pH is slightly acidic, ranging between 6.4–7.0, although values as low as 5.6 have been reported (99). Second, this extracellular acidity, due to efficient lactate and proton export, is linked to many tumorigenic processes, such as genomic instability, delayed DNA repair, vascularization, metabolic changes, altered cell adhesion, extracellular matrix degradation and remodeling, invasion, immune escape, and enhanced survival of tumor cells (99). Third, CA isozymes, particularly CA IX and XII expressed on the outer surface of tumor cells, are key players in the regulation of intratumoral pH (100). Fourth, it has also been shown that acetazolamide, the classical CA inhibitor, inhibits the invasion capacity of renal cancer cells expressing CA II, CA IX, and CA XII (101).

Indisulam, E7070 (N-(3-chloro-7-indolyl)-1,4-benzenedisulfonamide), was the first promising CA inhibitor that entered clinical trials for the treatment of cancer. It was defined as a multifunctional compound that arrests the cell cycle in the G1 phase and inhibits CA activity (102, 103). Indisulam

has been shown to inhibit human CA I, II, IV, IX, and XII activity with inhibition constants in the range of 3–65 nM (104, 105). In the original 1999 report, Indisulam showed significant antitumor activity against HCT116 human colon carcinoma both *in vitro* and *in vivo* (103). In nude mice inoculated with tumor cells, the compound produced both tumor growth suppression and a reduction in tumor size. To date, several phase I and phase II clinical trials have been conducted using Indisulam in either single or combination therapies for the treatment of malignancies, such as breast, gastric, colorectal, and renal cancer, leukemia, and melanoma (https://clinicaltrials.gov/ct2/results?recrs=&cond=&term=Indisulam&cntry=&state=&city=&dist=) .

In 2009, two acetazolamide derivatives were described as having either a charged fluorophore- or an albumin-binding moiety (106). These compounds preferentially targeted CA IX and XII on the surface of SK-RC-52 renal cell cancer cells, as confirmed by the localization of fluorophore-bound acetazolamide. The growth of xenograft tumors in nude mice was delayed when they were treated with modified acetazolamide at a 28-day follow-up. These data provided clear proof of principle for sulfonamide-based antitumor effects *in vivo*. However, acetazolamide lacks specificity for CA IX and XII, and therefore, many efforts have been made to find novel inhibitors with specificity against these tumor-associated enzymes (15, 107).

CAI17 is one of the earliest discovered promising CA IX inhibitors used both *in vitro* and *in vivo*. It is a sulfonamide-type fluorescent inhibitor tested in BALB/c mouse models inoculated with 4T1 mouse breast cancer cells expressing CA IX (108). The results showed that CAI17 significantly inhibited tumor growth in these model mice. Another CA IX inhibitor, ureido-sulfonamide U-104, was tested using a highly metastatic variant of the MDA-MB-231 cell line in mice. This inhibitor resulted in a significant reduction in metastasis formation. In the same set of experiments, two CA IX-selective inhibitors in the coumarin class, GC-204 and GC-205, significantly reduced the formation of tumor metastases. In another study, U-104 and GC-205 were tested in mice inoculated with the MDA-MB-231 LM2-4 Luc^{+} human breast cancer cell line (109). Tumor xenografts showed significant growth reduction when the mice were treated with either U-104 or GC-205. Interestingly, the cancer stem cell population, determined by epithelial cell adhesion molecule (EpCAM) and aldehyde dehydrogenase 1A3 (ALDH1A3) expression, was reduced in the tumors that were treated with CA inhibitors. Thus, it was concluded that the inhibition of CA IX activity may retard tumor growth by targeting cancer stem cells.

The pioneering investigations described above revealed that coumarin-class CA inhibitors were promising candidate compounds with specificity against CA IX and XII (15, 108). Thus, a series of 7-glycosylated 4-methyl coumarins were prepared and studied for the inhibition of CA I, CA II, CA IX, and CA XII (110). Several of these novel compounds were found to be nanomolar inhibitors of CA IX and XII, with good specificity. The most efficient inhibitor of CA IX, 7-mannosyl-4-methylumbelliferone, significantly inhibited primary tumor growth in a mouse model inoculated with 4T1 breast cancer cells.

Sulfamates constitute another potential class of CA inhibitors with antitumoral activity. Some of these compounds have shown high specificity with nanomolar affinity for the tumor-associated isozymes CA IX and XII. The effect of 4-(3´-(3”,5”-dimethylphenyl)ureido)phenyl sulfamate (S4) was determined using an orthotopic MDA-MB-231 mouse breast cancer model (111). S4 treatment reduced metastatic tumor burden in the lung but failed to decrease primary tumor growth. In another study, a novel ureido-sulfamate, designated FC9398A, was also tested in MDA-MB-231 xenografts (112). This compound significantly reduced tumor volume; however, it did not reduce the number of viable tumor cells. Nevertheless, these results suggested that sulfamate class CA inhibitors are potential lead molecules for designing novel anticancer drugs, particularly for the treatment of breast cancer.

Sensitization of drug-resistant cancer cells to improve the efficacy of combination therapies by blocking CA IX and XII enzymatic activity appears to be a rational strategy for fighting hard-to-treat cancers. The repositioning of acetazolamide as a cancer therapy is an attractive option because it is widely used and found to be tolerated well by patients with other diseases. Indeed, acetazolamide has been tested in combination with known anticancer drugs in xenograft models, including bronchial carcinoid tumors (113), bladder cancer (114), neuroblastoma (115), and gliomas (116). Notably, a few ongoing clinical trials for neoplastic diseases have included acetazolamide as an adjuvant drug, which is encouraging. The current targets are malignant astrocytoma treated with temozolomide and ionizing radiation (https://clinicaltrials.gov/ct2/show/NCT03011671) and small cell lung cancer treated with platinum, etoposide, and radiochemotherapy (https://clinicaltrials.gov/ct2/show/NCT03467360).

The compound 4-(2-aminoethyl)benzene sulfonamide, which shows high affinity for CA IX (117), can be considered a potential drug candidate or lead molecule for radiation sensitization in renal

cancer. Treatment of nude mice with xenografts composed of 786-O renal cancer cells with this compound clearly sensitized tumors to radiation (118).

SLC-0111 is a promising ureido-benzenesulfonamide that efficiently inhibits both CA IX and XII activity. Preclinical studies with mice have shown that it sensitizes glioblastoma cells to traditional cytotoxic drugs (119). SLC-0111 has entered clinical trials enrolling patients with advanced solid tumors (https://clinicaltrials.gov/ct2/show/NCT02215850). Recruitment is ongoing for a phase Ib study of a combination treatment of SLC-0111 and gemcitabine for metastatic pancreatic ductal cancer (https://clinicaltrials.gov/ct2/show/NCT03450018).

Coumarins have long been considered the primary group of natural compounds with CA inhibitory properties. Recently, a natural primary sulfonamide, psammaplin C, was identified as a nanomolar inhibitor of CA XII (120). It may be effective when used in combination with known chemotherapies, such as temozolomide, in patients with glioblastoma. Using a glioblastoma xenograft model, psammaplin C resensitized drug-resistant glioblastoma to temozolomide and extended the overall survival of treated mice.

Although several CA inhibitors have shown promise as adjuncts to enhance the effectiveness of cancer treatments, the complexity of cancers and off-target effects of drugs must be considered before these novel compounds are combined with standard treatments. For example, Kuijk and coworkers recently observed that the sulfamate small-molecule inhibitor S4 surprisingly reduced the efficacy of doxorubicin in an MDA-MB-231 tumor model (121). In contrast, Bryant and colleagues demonstrated a positive outcome using the same compound in small cell lung cancer xenografts (122). Administered alone or in combination with cisplatin it reduced the hypoxic areas of tumors and inhibited xenograft tumor growth. These results highlight the need for careful preclinical assessment of drugs before they are entered into clinical trials.

During the past few years, several additional CA inhibitor compounds have been tested in mouse xenograft models. For example, 2,4,6-trimethyl-1-(3-sulfamoyl-phenyl)-pyridinium perchlorate salt 6 induces significant cytotoxicity in osteosarcoma cells and reduces tumor growth by inducing cell necrosis (123). Recently, Andring and coworkers characterized a series of “STX” sulfamoylated drugs that, in many cases, were initially derived from the classic steroid template (124). They found three sulfamate compounds, classified as “nonsteroidal”, to be highly selective towards CA IX (the CA IX K_i/CA II K_i selectivity ratios were 104–202). Some of these molecules have shown

promising antiangiogenic effects and growth inhibition in xenograft models (125). These compounds have been suggested to be potential drug development targets for triple-negative breast cancer.

2.2.2. Preclinical studies of CA inhibitors using zebrafish models

The zebrafish has recently emerged as a useful model organism for the evaluation of toxicity and phenotypic effects of various chemical compounds (126-128). It has several properties that make it a versatile preclinical model (129). An important advantage is fast breeding, as one zebrafish female can lay batches of 200–300 eggs for *ex vivo* development. The model is particularly practical because the eggs and developing embryos require no external feeding for up to a week, and the embryos are transparent, making them suitable for microscopy (130). Furthermore, the compounds to be studied can be directly administered into the water, where they can enter the organism by diffusion through the chorion and after hatching through the skin, gills, and mouth of larvae. The experiments do not necessarily require high amounts of chemical compounds, some of which can be expensive and/or difficult to produce (130, 131). The critical steps of zebrafish organogenesis occur 2–5 days postfertilization (dpf) (132), and therefore, toxic compounds administered during the sensitive period of embryonic development induce observable phenotypic defects in zebrafish larvae (130).

A few preclinical toxicity studies of CA inhibitors have recently been performed with zebrafish. The first investigation was focused on fluorinated benzenesulfonamide anticancer inhibitors (133). The toxicity of two compounds (VD12-09 and VD11-4-2) was tested using embryos 1–5 dpf, and the results were compared to parallel analyses performed with a classical CA inhibitor, ethoxzolamide. The LC_{50} values of VD12-09, VD11-4-2, and ethoxzolamide were 13 µM, 120 µM, and 9 µM, respectively. In the phenotypic analysis, pericardial edema was observed in the embryos treated with 125 µM VD11-4-2. VD12-09 produced fewer phenotypic changes than the other drugs. In fact, ethoxzolamide, a classical sulfonamide, induced pericardial edema and abnormal appearance of the body even at a 7 µM concentration. These results suggested that the described fluorinated benzenesulfonamides are promising lead compounds for further development of anticancer drugs.

Nitroimidazoles are CA inhibitors that have been tested for toxicity in a zebrafish model (134). The two nitroimidazole molecules DTP338 and DTP348, incorporating either sulfamate or sulfamide

moieties, respectively, have been defined by *in vitro* assays as specific, subnanomolar inhibitors of CA IX (135). In zebrafish embryos, DTP338 and DTP348 at 300 µM or lower concentrations showed no toxicity. Surprisingly, inhibition of human CA IX *in vivo* required low micromolar concentrations of these compounds, although they were efficient inhibitors *in vitro* at low nanomolar concentrations. The discrepancy between the *in vitro* and *in vivo* results may be due to several different factors. It is possible that other proteins in the vicinity of CA IX may interfere with inhibitor binding *in vivo*, which may alter the pharmacokinetics of the inhibitors. The chemical stability of these inhibitor compounds may also have been affected *in vivo* by various biological and chemical factors.

The toxicity of three other nitroimidazole compounds was investigated in a recent study using the analogous zebrafish model as described above (136): 4-(2-(3-(2-(2-Methyl-5-nitro-1H-imidazol-1-yl)ethyl)thioureido)ethyl)phenyl sulfamide (designated compound 3), 4-(2-(3-(2–(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl) thioureido)ethyl)phenyl sulfamate (compound 4), and 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethane-1-sulfonamide (compound 10). When tested against human CA I, CA II, and CA IX, compound 4 showed selectivity towards CA II (K_i 58.6 nM), while the affinity of compound 10 was highest for CA IX (K_i 147.3 nM). LC_{50} values at the end of 5 dpf were 3 mM for compound 3 and 2.6 mM for both compounds 4 and 10. Phenotypic analyses with zebrafish showed no adverse changes when these compounds were administered at submillimolar concentrations.

Mycobacterium tuberculosis β-CAs have emerged as promising target enzymes for designing novel antituberculosis drugs. The *M. tuberculosis* genome has three β-CA genes, Rv1284 (*β-CA1*), Rv3588c (*β-CA2*) and Rv3273 *(β-CA3*), the protein products of which can be successfully inhibited with various compounds (137). Mycobacterial β-CAs are important for the growth and survival of bacteria under starvation conditions (138-141). Interestingly, CA activity is also essential for the transport of extracellular DNA (eDNA) into the bacterial biofilm matrix (142), a characteristic process for the generation of mycobacterial biofilm that may also be involved in the development of tolerance to antibiotics (143). Many classes of CA inhibitors can be developed to target mycobacterial β-CAs. Recently, a series of 14 monothiocarbamates and dithiocarbamates were evaluated *in vitro* to determine their inhibitory properties and *in vivo* to determine their level of toxicity in zebrafish embryos (144). Eleven compounds administered at nanomolar or submicromolar concentrations inhibited *M. tuberculosis* β-CA3 activity, with K_i values in the range

of 2.4–812 nM. Among 14 compounds, 9 showed no adverse phenotypic changes in embryos 5 dpf. Based on both the *in vitro* inhibitory and toxicological data, the authors concluded that five of these compounds are promising drug targets and should undergo further preclinical characterization.

2.2.3. Physiological studies in model organisms using CA inhibitors

Although this review concentrates on studies with mice, zebrafish, *D. melanogaster*, and *C. elegans*, we acknowledge that many valuable physiological studies were initially carried out using larger vertebrate models, such as dogs, rabbits, and rats. The change in opinion in society and the scientific community concerning animal experimentation, tightened legislation, and increased costs have led to a gradual change towards the use of smaller and simpler animal models.

Several decades of intensive CA research have improved our understanding of the physiology and clinical relevance of CA isozymes. Indeed, a plethora of studies have been conducted in model organisms using CA inhibitors to study various physiological aspects in different organs, tissues, and cells. Because of the vast number of publications in this research field, it is not feasible to cover all aspects and studies in a single review. Instead, we chose to highlight some examples of CA inhibitor studies that have markedly broadened the view of the physiological roles of CAs. The selected cases were focused on studies on neuronal, gastrointestinal, and reproductive physiology.

Comprehensive mRNA expression profiles of all enzymatically active CAs along the mouse gastrointestinal tract showed that several CAs are expressed in each segment of the tract (145). Hence, it is plausible that these enzymes play multiple roles in gastrointestinal physiology. In fact, some of the key roles of CAs in the regulation of gastrointestinal functions have been known for several decades. In 1958, Janowitz described the role of CA activity in gastric and pancreatic secretion in dogs using the enzyme inhibitor acetazolamide (146). In 1981, Kivilaakso and Silen demonstrated that the CA-catalyzed production of bicarbonate is essential for the protection of gastric mucosa (147). CA activity is also involved in bicarbonate production in other segments of the gastrointestinal tract, such as the duodenum and pancreas. The bicarbonate present in both pancreatic and duodenal fluids neutralizes gastric acid entering the duodenum and provides an optimal pH environment for digestive enzymes to function properly in the small intestine (148). The mechanism of apical bicarbonate transport in epithelial cells has long been debated. A key

finding was the evidence that, in addition to classical anion exchange (AE) proteins, cystic fibrosis transmembrane conductance regulator (CFTR) is an important bicarbonate transport protein in several organs, including the duodenum and pancreas (149-152). These findings were instrumental for better understanding the physiological mechanisms of bicarbonate secretion in the gastrointestinal tract, the processes that had been linked to CAs in the 1950s by using classical CA inhibitors in animal models. Interestingly, it has been shown that high levels of CA II activity may not be as important as predicted for the actual provision of bicarbonate secreted from enterocytes into the mouse duodenal lumen (153). In fact, CA II is instrumental for sensing luminal acidity, which then leads to the subsequent stimulation of bicarbonate secretion. It remains to be investigated how the CA isozymes present in different segments of the gut mutually contribute to the maintenance of the optimal pH environment for digestion, absorption, and microbiome composition.

The nervous system has been at the epicenter of CA research because of the wide expression and key roles of CA isozymes in virtually all cell types in the brain. The localization of CAs in both neuronal and nonneuronal cells is presented in Table 3. In the nervous system, CAs play multiple roles, e.g., neuronal signal transduction (154, 155), fluid and ion compartmentalization (156), formation of cerebrospinal fluid (CSF) (157), the respiratory response to carbon dioxide (158), the generation of bicarbonate for biosynthetic reactions (159), and seizure activity (160).

Table 3. Localization of CA isoforms in the central nervous system.

Localization	Isoforms
Neurons	II, V, VII, VIII, XI, XIV (70, 71, 154, 161-165)
Astrocytes	II, V, VIII, XI (159, 163, 165-171)
Oligodendrocytes	II, XIII (34, 161, 172-177)
Myelin sheath	II (173, 176)
Choroid plexus	II, III, VIII, XII, XIV (161, 162, 165, 176, 178-180)
Microglial cells	III (181)
Endothelial cells	IV (182)

In vivo CA inhibitor studies focusing on the nervous system have mainly involved research on the modulation of neuronal signal transduction (183, 184), fear memory (185, 186), pain recognition (187-193), and epileptic seizures (194-197). Some of these studies have mainly concentrated on basic neurophysiological research, while others have had strong translational connections to clinical research and therapies. The modulation of pH in the nervous system, the generation of activity-dependent pH changes (both intracellular and extracellular), and their functional consequences have been previously introduced in several excellent review articles (184, 198, 199). We briefly outline some examples of studies in which CA inhibitors have been successfully implemented in research using mice as experimental models.

Rapid changes in extracellular (interstitial) pH occur during neuronal activity. The magnitude and speed of extracellular pH changes have been linked to the interstitial buffering capacity that is maintained via the bicarbonate buffering system (184). Even though the reversible hydration of carbon dioxide occurs spontaneously, the extremely rapid changes require extracellular catalysis by CAs in the neuronal microenvironment, as suggested in 1983 by Kraig and colleagues (200). This phenomenon was difficult to understand until the discovery of membrane-associated CA IV and XIV. Initially, extracellular CA activity was linked to CA IV (201). The generation of CA IV- and CA XIV-null mice enabled the roles of these isozymes in buffering alkaline pH shifts to be determined *in vivo* (183). The results of these studies demonstrated that either isozyme can modulate buffering in the absence of the other. In the case of double knockouts, the blocking of the buffering effect was almost as effective as that observed in WT mice administered the CA inhibitor benzolamide.

Notably, CAs can modulate neuronal signal transduction through several mechanisms. Recently, Bertone and coworkers showed that CA activity may be involved in the recycling of synaptic vesicles (202). Initially, they found no effect of acetazolamide on acetylcholine release at mouse neuromuscular junctions except for a small reduction in transmitter release at low stimulation frequency. More significantly, they demonstrated that acetazolamide accelerated vesicle endocytosis in synapses via cytosolic acidification and activation of myosin light chain kinase. This effect may contribute to the therapeutic effects induced by the CA inhibition observed in certain neurological diseases.

CAs play important roles in reproductive physiology. The bicarbonate ion is a key stimulator of sperm motility that is activated by bicarbonate-sensitive adenylyl cyclase in sperm cells (203, 204)

(Figure 6). It has been suggested that a soluble form of adenylyl cyclase, present in spermatozoa, can functionally associate with CAs and act as a sensor of pH and CO_2 (205). The bicarbonate in human semen primarily originates from the seminal vesicle, ductus deferens, and ampulla of the ductus deferens, all of which express high levels of CA II (41). External bicarbonate can then enter sperm cells via ion transport proteins, such as Na^+/HCO_3^- cotransporters or HCO_3^-/Cl^- exchangers (206, 207), activate bicarbonate-sensitive adenylyl cyclase, modulate plasma membrane potential, and largely affect sperm capacitation (208). Diffusion of CO_2 through the plasma membrane may represent another mechanism in which intracellular bicarbonate is generated via the function of CA II and CA XIII present in sperm cells (42, 177, 209). In mice, CA II activity has been found to account for one-half of the total CA activity in capacitated cells (209). It has also been shown that spermatozoa express CA IV, which is transferred onto the plasma membrane as the sperm pass through the epididymis (210). In the presence of bicarbonate-containing buffer, the CA inhibitors acetazolamide and ethoxzolamide inhibited the flagellar beat frequency of spermatozoa. The key role of CA IV was recognized through the finding that the sperm cells of CA IV-knockout mice had a reduced flagellar beat in response to CO_2 than that of the control mice.

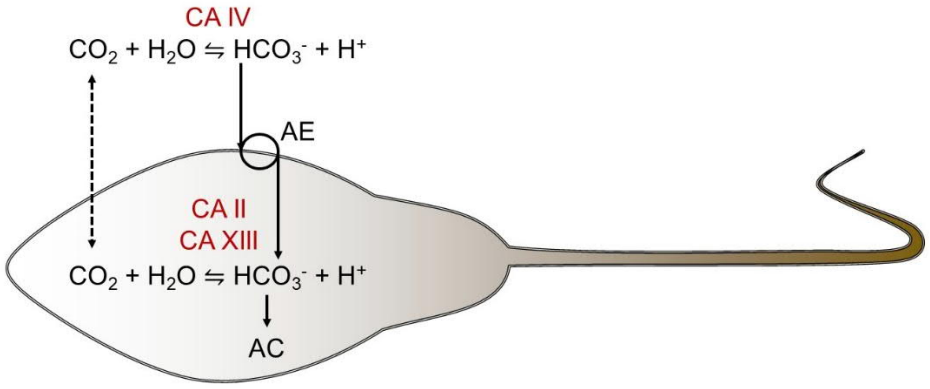

Figure 6. Schematic model of CA function in sperm cells. CA IV, bound to the plasma membrane, regulates the extracellular CO_2/HCO_3^- balance. CO_2 can freely diffuse through the membrane. Intracellular CO_2 and H_2O can be converted to HCO_3^- and H^+ by CA II and/or CA XIII. Extracellular HCO_3^- can also be transported via anion exchangers (AE) to the intracellular space. Intracellular HCO_3^- is utilized by adenylyl cyclase (AC) to facilitate sperm motility.

The roles of CAs in reproductive physiology is not limited to the male reproductive tract, as female tissues distinctly express several CAs. It is generally known that the physiological conditions of the female reproductive tract markedly change during the menstrual cycle. For example, the volume of cervical mucus secretion, the chemical composition and physical characteristics of the mucus change cyclically throughout the menstrual cycle (211). The luminal pH in the reproductive tract is one of the important characteristics that changes during the menstrual cycle (212, 213) and changes momentarily during sexual intercourse (214). Several CA isozymes are expressed in the female reproductive tract. Based on current knowledge, the most significant enzymes in the cervix and endometrium are CA XII and CA XIII (78, 177), and in the mouse endometrium, CA II shows high expression, but it is absent in humans (177). Although the exact roles of CAs in the female reproductive tract are not fully understood, it is conceivable that they contribute to the maintenance of an optimal localized pH environment. In the mouse uterus, CA II and CA XII show the highest expression levels at estrus, and the uterine surface pH values are also higher at estrus than they are at diestrus (213). This pH change was significantly reduced by acetazolamide administration to mice. Moreover, Hu and Spencer demonstrated that treatment of mice with subcutaneous acetazolamide between postnatal days 3 and 18 decreased the number of endometrial glands without affecting the differentiation of the stroma or myometrium (215). This finding suggested a specific role for CAs during postnatal uterine morphogenesis.

3. MOUSE MODELS OF CARBONIC ANHYDRASE DEFICIENCIES

3.1. The Mouse as a Model Organism

Transgenic mouse technologies have represented a huge leap towards a better understanding of physiology in mammals. Different transgenic mice have been classically produced by two alternative approaches: 1) targeted genomic manipulation and 2) random integration of mutation into the genome (216). While the targeted genomic manipulation technique is more precise than random integration, it is also more time-consuming and expensive. The first studies in which specific murine genes were targeted for homologous recombination in embryonic stem cells were reported in the late 1980s (217). In the 1990s, the number of knockout mouse strains increased rapidly as more reliable and efficient technologies became available. It also became possible to introduce conditional knockouts possessing mutations that can be activated at specific time points or in specific cells or organs both during development and in the adult animal. In 2007, Mario R.

Capecchi, Martin J. Evans, and Oliver Smithies were awarded the Nobel prize for their discoveries of “principles for introducing specific gene modifications in mice by the use of embryonic stem cells” (https://www.nobelprize.org/prizes/medicine/2007/summary/). As a result of this monumental development, thousands of murine genes have been targeted. Three major mouse knockout programs — the Knockout Mouse Project (KOMP), European Conditional Mouse Mutagenesis Program (EUCOMM), and North American Conditional Mouse Mutagenesis Project (NorCOMM) — were established with the goal of mutating all protein-encoding genes of the mouse (218, 219). Recently, the International Knockout Mouse Consortium (IKMC), consisting of KOMP, EUCOMM, NorCOMM, Tools for Functional Annotation of the Mouse Genome (EUCOMMTOOLS), and Texas A&M Institute for Genomic Medicine (TIGM), has made substantial progress towards the ambitious goal of knocking out and phenotypically analyzing all the genes of the mouse genome.

Genome editing by targetable nucleases has become a widely used tool to generate knockout models with reasonable accuracy. In 2020, Emmanuelle Charpentier and Jennifer A. Doudna jointly received the Nobel Prize “for the development of a method for genome editing”, highlighting the revolutionary significance of CRISPR/Cas9 technologies in functional genomics research (https://www.nobelprize.org/prizes/chemistry/2020/summary/). Initially, CRISPR/Cas9 technology was considered the gene editing tool of choice in both fundamental and clinical research contexts. More recently, it has been shown that even this new technology may face severe specificity issues, including large deletions and more complex genomic rearrangements at targeted sites, as well as distal lesions and crossover events (220).

Even though transgenic technologies are indispensable for modern research on comparative physiology, these methods have several other drawbacks: approximately 15% of gene knockouts are developmentally lethal, limiting phenotypic analyses. In many instances, knockout mice have failed to produce an observable phenotype, or the phenotype has been significantly milder than that acquired by humans in which the same gene is inactivated. These drawbacks, among others, have somewhat tempered the enthusiasm for transgenic models, but various model organisms are still actively being developed and analyzed. Additionally, technical improvements made to improve the efficiency and specificity of gene targeting are actively being developed and assessed (221). Figure 7, showing the number of PubMed hits returned when the term “knockout mouse” is searched, indicates that, although a plateau phase in publication activity within this field peaked

after 2010, there has been no marked reduction in the number of published articles, except in 2020. The COVID-19 pandemic has globally hampered experimental work with mice, and because the most recent data may be incomplete, the decline in publication numbers in 2020 may be easily explained.

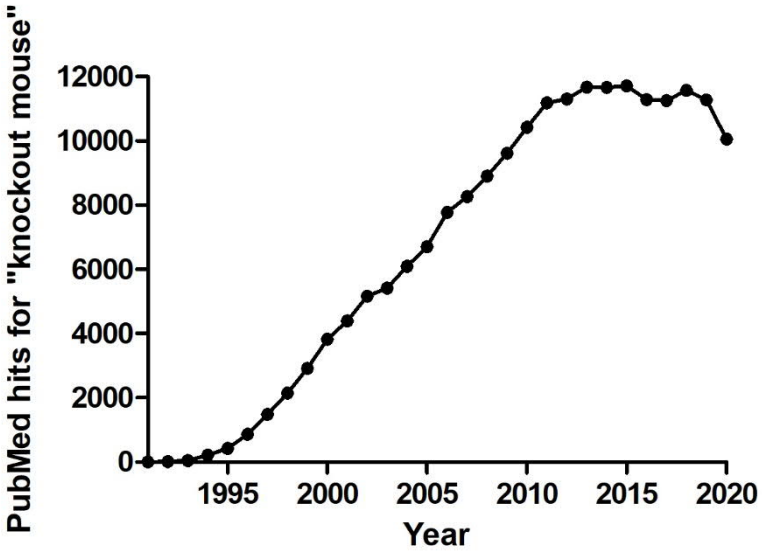

Figure 7. PubMed hits returned when the term “knockout mouse” was searched in articles published in the period 1991–2020. The data were acquired on June 17, 2021.

3.2. CA II-Deficient Mice

Before gene-targeting technologies became available, researchers identified many naturally occurring or chemically induced mutations in mice, which opened avenues of study into the physiological roles of the affected proteins. One of the early examples of this type of study involved CA II-deficient mice, which were obtained by chemical mutagenesis using N-ethyl-N-nitrosourea (222). The first report indicated that CA II-deficient mice were runted and showed renal tubular acidosis. Functional defects of the kidney were predictable since Sly and coworkers had previously demonstrated similar findings in human patients with CA II deficiency, including renal tubular acidosis, as well as osteopetrosis and cerebral calcification (30). Later, histopathological analyses showed vascular calcification of small arteries in several organs of homozygous *Car2*$^{-/-}$ mice (223). It has also been demonstrated that CA II is involved in ectopic

calcification: when pieces of glutaraldehyde-fixed bovine pericardium were subcutaneously implanted in mice, the specimens became significantly more calcified in the *Car2*$^{-/-}$ mice than in the control animals (224).

In contrast to reports showing cerebral calcification in CA II-deficient mice, ultrastructural studies indicated no phenotypic abnormalities in the brain (225). Even oligodendrocytes, which express high levels of CA II, exhibited no signs of degeneration or other abnormalities. On the other hand, functional studies indicated an important role for CA II in the brain: it may modulate susceptibility to seizures induced by either flurothyl or audiogenic stimulation (226). Both CA deficiency and CA inhibition exhibited similar anticonvulsant functions in mice.

Renal tubular acidosis is a hallmark of CA II deficiency in both humans and mice. Notably, CA II contributes to both bicarbonate reabsorption and proton secretion processes. Breton and coworkers demonstrated that the intercalated cells of collecting ducts were severely depleted and replaced by principal cells in *Car2*$^{-/-}$ mice (227). In the kidney, CA II deficiency decreased the gene and protein expression of the *Slc26* gene family, including Slc26a4 (pendrin) and Slc26a7 (228), which are important bicarbonate transport proteins of intercalated cells. CA II may also contribute to innate immune responses in the kidney. It has been shown that *Car2*$^{-/-}$ mice have increased kidney and bladder bacterial burdens, decreased bacterial clearance in the kidney, and decreased inflammatory response after transurethral inoculation of uropathogenic *Escherichia coli* (229).

CA II has been shown to function in conjunction with various transport proteins, such as the electrogenic Na^+/HCO_3^- cotransporter (230, 231) and anion exchanger AE1 (232). It has also been shown that CA II both functionally and physically interacts with the downregulated adenoma (DRA) chloride/bicarbonate transporter, but this interaction is weaker than that between CA II and AE1 (233). In the heart muscle, CA II and NHE1 form a transport metabolon that was exacerbated in obese type 2 diabetic mice (*ob*$^{-/-}$) (234). Notably, CA II deficiency produced physiological cardiac hypertrophy without any decrease in cardiac function (235). This phenomenon was suggested to be linked to the role played by CA II as a provider of protons and bicarbonate ions for NHE1 and AE3. Monocarboxylate transporters (MCTs) represent another class of membrane-associated transport proteins that seem to cooperate with CA II. MCT1 transport activity is enhanced by the presence of CA II in cells (236). CA II probably forms a “proton antenna” for the MCT transporter. Interestingly, a similar increase in MCT transport activity was observed when six histidine residues (without CA II) were integrated into the C-terminal tail of MCT4 (237). It is conceivable that CA II

deficiency can lead to functional disturbances that may be attributed to defective metabolon functions, even though experimental evidence of these mechanisms is currently lacking.

CA II is highly expressed in the gastrointestinal tract and plays a role in proton secretion from parietal cells into gastric juice and in bicarbonate production to neutralize excess acidity and protect mucosal surfaces (238). An interesting question remains: does CA II deficiency lead to a gastrointestinal phenotype when no symptoms or physiological changes have been reported in CA II-deficient patients or observed in *Car2*$^{-/-}$ mice? To explore this question further, Leppilampi and coworkers decided to focus on the duodenal bicarbonate secretory response to prostaglandin E2 exposure, which is relatively easy to monitor in mice (239). The results indicated that the duodenal bicarbonate secretion response to prostaglandin E2 exposure was completely absent in *Car2*$^{-/-}$ mice, thus suggesting a role for CA II in the protection of duodenal mucosa from excess acidity. Recently, Sjöblom et al. (153) proposed that CA II is involved in sensing duodenal acidity rather than directly in bicarbonate secretion.

Bicarbonate is an important regulator of sperm motility. Reproductive tract organs and even spermatozoa express CA isozymes, which are involved in the regulation of pH and bicarbonate concentrations to ensure that they are optimal for fertility (41, 42, 240, 241). Recent studies on both *Car2*$^{-/-}$ and *Car4*$^{-/-}$ mice revealed that deficiency of either enzyme led to imbalanced bicarbonate homeostasis, resulting in reduced sperm motility, swimming speed, and bicarbonate-enhanced beat frequency (210).

3.3. CA III-Deficient Mice

CA III is a cytosolic enzyme and exhibits the lowest catalytic activity among all CAs. It is expressed in many organs, with the highest levels in skeletal muscle and adipocytes, composing up to 24% of the soluble proteins in adipocytes (242, 243). Although CA III is abundantly expressed, its function is poorly understood in these tissues. Before the *Car3*$^{-/-}$ mice became available, it was suggested that CA III participates in embryogenesis (244), augmentation of fatty acid metabolism (244), and the oxidative stress response (245, 246). The availability of CA III-deficient mice has aided in the investigation of the role of CA III in normal physiology. The very first studies with *Car3*$^{-/-}$ mice were somewhat disappointing since the mice were completely viable and fertile, had normal life spans and showed no morphological phenotype (247). Both the amount and distribution of fat were

normal, and they also showed the same response to a hyperoxic challenge as their WT siblings. Based on these data, it was concluded that CA III is dispensable for mice living under standard laboratory husbandry conditions.

Later studies have indicated certain, although relatively mild, phenotypic alterations in *Car3*$^{-/-}$ mice. The first observed significant difference between the *Car3*$^{-/-}$ and control mice was related to skeletal muscle function. Notably, the results may have varied by skeletal muscle type because CA III is expressed in a muscle fiber type-specific manner. By the 1980s, it had been shown that CA III is predominantly present in type I slow-twitch muscle fibers (248). Using a CA III-deficient mouse model, Liu et al. (249) demonstrated that two minutes of intense stimulation of the gastrocnemius muscle caused phosphocreatine, ATP, and pH to fall and ADP and P_i to rise, and these changes were significantly larger (except ATP) in *Car3*$^{-/-}$ mice than in WT mice. In the mutant mice, the phosphocreatine and P_i recovery rates were delayed in the first minute after stimulation. Based on these observations, it was concluded that CA III deficiency impairs mitochondrial ATP synthesis. In a more recent study, CA III was also implicated in muscle function. Feng and Jin (250) demonstrated that the loss of CA III did not change muscle mass in the soleus and tibialis anterior muscles, nor did it change the sarcomere protein isoform content. The baseline twitch and tetanic contractility remained unchanged compared with age-matched WT mice. However, the tibialis anterior muscle in the CA III-deficient mice showed faster force reduction upon initiation of electrical stimulation but higher resistance at the end of the stimulation period, followed by slower post-fatigue recovery compared to the WT mice.

Using embryonic fibroblasts from *Car3*$^{-/-}$ mice, Mitterberger and coworkers demonstrated induction of fatty acid-binding protein 4 (FABP4) and increased triglyceride levels (251). They suggested that CA III regulates adipogenic differentiation, which occurs at the level of peroxisome proliferator-activated receptor-γ2 (*PPARγ2*) gene expression. In another study, the role of CA III in fatty acid metabolism was recently investigated in CA III-deficient mice (252). To determine whether CA III affects body weight or composition, *Car3*$^{-/-}$ and control mice were fed a high-fat diet or standard chow starting at five weeks of age and measured once per week for ten weeks. The body weights of the WT and *Car3*$^{-/-}$ mice on either diet did not differ significantly at any age, nor did the mice show any difference in food consumption. The knockout and WT mice showed no significant differences in total energy expenditure, energy substrate utilization, or high-fat-diet-induced insulin resistance. The *Car3*$^{-/-}$ mice had normal lipid/fatty acid biomarker levels in both

serum and tissue, and they also showed normal thermoregulation. Due to the absence of phenotypic change in their study, Renner and coworkers concluded that the physiological role of CA III remains to be determined.

3.4. CA IV-Deficient Mice

CA IV is a membrane-associated, glycosylphosphatidylinositol (GPI)-linked isozyme first described in the 1980s; it was purified from bovine lung microsomes (253), human kidney (254), human lung and kidney (255), and rat lung (256). In early studies, the protein was found by immunohistochemistry to be located on the plasma membranes of the proximal convoluted tubule and thick ascending limb of Henle in the rat kidney (256), in the endothelial cells of the choriocapillaris of the human eye (47), in the endothelial cells of rat brain blood vessels (182), in the enterocytes of the human and rat distal small intestine and large intestine (257), in the human epididymis and ductus deferens (241), and in the human gallbladder (258).

CA IV-deficient mice were created by targeted mutagenesis, and the first phenotypic analysis was reported simultaneously with CA XIV-deficient mice (183). Surprisingly, the number of mice homozygous for the *Car4* deletion was only 38% of the number predicted on the basis of Mendelian ratios. Moreover, only 31% of the homozygotes were female. Regardless of sex, the surviving *Car4*$^{-/-}$ mice appeared healthy, thrived, and were fertile, as evidenced when crossed with WT mice. However, mated male and female homozygous knockout mice produced small litters, and the pups did not survive. Homozygous *Car14*$^{-/-}$ mice appeared healthy, thrived, and were fertile, but in contrast to the *Car4*$^{-/-}$ mice, the number and gender ratios were nearly the same as those predicted on the basis of Mendelian ratios. The study, from which the first article describing a major phenotypic analysis of knockout mice was published, was focused on the roles of both CA IV and CA XIV in neural function in the hippocampus. The biophysical data obtained suggested that both enzymes contribute to extracellular buffering in the mouse brain. Notably, each enzyme was sufficient to compensate when the other was eliminated.

Both CA IV and CA XIV are abundant in renal tubules, where they can play roles in the regulation of metabolic pH homeostasis (256, 259). Analyses of the acid-base status of *Car4*$^{-/-}$/*Car14*$^{-/-}$ double-knockout mice indicated, however, that these animals exhibited no renal acidosis (260), in contrast to CA II-deficient mice, which readily develop metabolic acidosis and have high urine pH

(227). Renal tubules contain at least three additional CA enzymes: membrane-associated CA XII (261) and CA XV (262), as well as cytosolic CA II (31). The unexpected finding in which *Car4*$^{-/-}$/*Car14*$^{-/-}$ double knockouts showed no signs of acidosis may have been the result of mutual compensation of defective enzymes with other CAs expressed in the kidney.

CA IV expression has been demonstrated in rodent skeletal and heart muscle cells. Through immunoelectron microscopy, positive immunoreactions were detected in the capillary endothelium, sarcolemma, and sarcoplasmic reticulum of rat soleus muscle (263). CA IV has been reported to be expressed in both the sarcoplasmic reticulum and sarcolemma of the mouse heart muscle (264). Physiological studies using the *Car4*$^{-/-}$ mouse model showed that the muscle functions in the CA IV-deficient mice were unaffected compared to the fibers in the WT control mice. However, the muscle function of *Car4*$^{-/-}$/*Car14*$^{-/-}$ double-knockout mice unexpectedly showed a decrease in rise and relaxation time and in the force of single twitches. The whole spectrum of CA function in muscle cells is rather complicated because CA IV and CA XIV are not the only isozymes involved in muscle cell function. CA IX seems to be another membrane-associated CA that contributes substantially to the total CA activity in the sarcoplasmic reticulum (260). Similar to the intricate functions of CA isozymes in the kidney, different CAs may mutually compensate for each other to maintain an optimal microenvironment for muscle function in the absence of one isozyme.

As indicated above, CA IV is highly expressed in the male reproductive tract. Studies on human tissue specimens indicated that CA IV is expressed in the microvilli and apical plasma membrane of the epithelium lining the epididymal duct, ductus deferens, and ampulla of the ductus deferens (241). In the rat epididymis, CA IV is located at the apical plasma membrane of epithelial cells in the distal caput, corpus, and proximal cauda epididymides, but it is absent in the epithelium of the ductus deferens, seminal vesicle, and ventral prostate (240). Based on these results, it was proposed that this enzyme may be functionally linked to the acidification of the epididymal fluid that prevents premature sperm activation. Using *Car4*$^{-/-}$ mice, Wandernoth and coworkers investigated the specific role of CA IV in fertility (265). First, they investigated the expression pattern of CA IV in tissue specimens using real-time PCR, immunohistochemistry and western blotting. A positive signal for the CA IV protein was detected in the corpus and cauda segments of the epididymis and ductus deference. This group surprisingly found that CA IV is expressed not only in the lining epithelium but also in spermatozoa. The signal first appeared in the corpus

segment, and then, signals were also observed in more-distal segments. Immunohistochemical staining indicated that the positive signal was strongest in the plasma membrane of the sperm tail region. Functional experiments revealed that the number of motile sperm and the quantity of fast progressive sperm cells were significantly reduced in *Car4*$^{-/-}$ mice compared to WT controls. A subsequent follow-up study by Wandernoth and coworkers confirmed the corpus segment of the mouse epididymis as the main site of CA IV expression (210). The signal for CA IV obtained by mass spectrometry was higher in this segment than in the kidney, which was used as a positive control. In addition, Wandernoth and coworkers demonstrated that both CA II and CA IV appeared in the epididymis with the onset of puberty. CA II- and CA IV-deficient mice displayed imbalanced HCO_3^- homeostasis, resulting in poorer sperm motility and reduced HCO_3^--stimulated beat frequency. The residual CA activity in the sperm cells of *Car2*$^{-/-}$ and *Car4*$^{-/-}$ mice was 35% and 68% that of the WT mice, respectively.

3.5. CA VA- and VB-Deficient Mice

Mitochondrial CA activity was originally demonstrated in the early 1980s (52) and first described as a single isozyme, named CA V (266). Later, two mitochondrial forms were identified in humans and mice, CA VA and VB, which were both encoded in the nuclear genome (267-269). CA VA is highly expressed in the liver, to a lesser degree in skeletal muscle, and in the kidney (based on mRNA data) (269). CA VB is ubiquitously expressed in most tissues, except for the human liver; in contrast, it seems to be present in the mouse liver (267, 269). Mitochondrial CAs contribute to several metabolic processes, including gluconeogenesis, ureagenesis, and lipogenesis, by providing bicarbonate for carboxylation reactions (266, 270, 271). Bicarbonate is required for the function of four hepatic mitochondrial enzymes involved in essential metabolic pathways: carbamoyl phosphate synthetase 1 (which functions in the first step of the urea cycle), pyruvate carboxylase (which is involved in gluconeogenesis), propionyl-CoA carboxylase (which is involved in methylmalonyl-CoA formation), and 3-methylcrotonyl-CoA carboxylase (which is involved in branched-chain amino acid catabolism) (54) (Figure 8). Using CA inhibitors, it was shown that mitochondrial CAs indeed play functional roles in metabolic pathways, with the most significant contributions linked to pyruvate, fatty acid, and succinate metabolism (272). Recently, Bernardino´s team identified three CAs, CA VB, CA IX, and CA XII, in human Sertoli cells and

showed that inhibition of CA activity in these cells by acetazolamide treatment may lead to impaired mitochondrial biogenesis (273), which may be attributed to a reduction in CA VB activity.

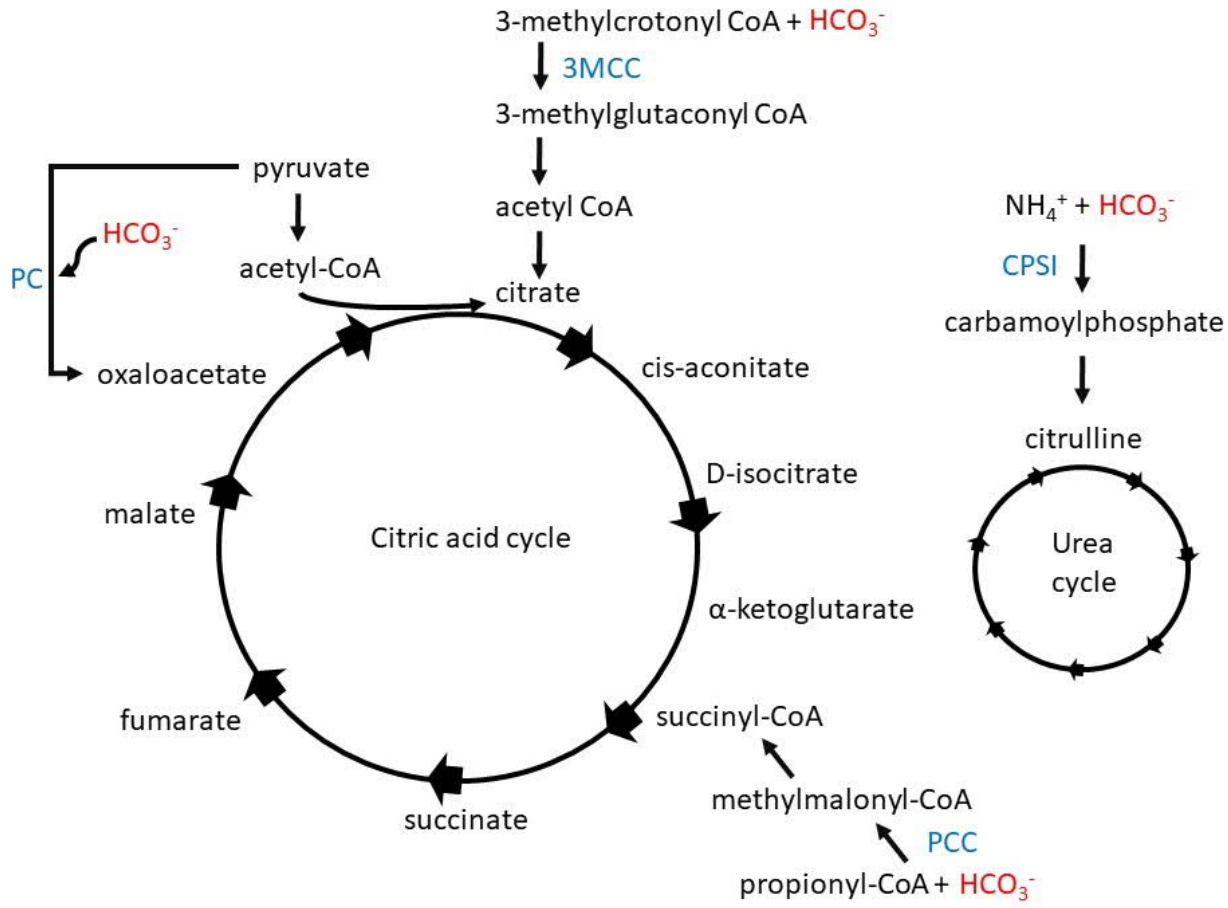

Figure 8. Biochemical pathways in hepatocyte mitochondria initiated by bicarbonate produced by carbonic anhydrase VA. CPSI, carbamoyl phosphate synthetase 1; PC, pyruvate carboxylase; PCC, propionyl-CoA carboxylase; and 3MCC, 3-methylcrotonyl-CoA carboxylase

A major breakthrough in CA V research was achieved when Shah and coworkers reported the creation and characterization of *Car5A*$^{-/-}$ *and 5B*$^{-/-}$ mouse models (274). The *Car5A*-null mice clearly showed a more severe phenotype than the *Car5B*$^{-/-}$ mice. The *Car5A*$^{-/-}$ mice were smaller than their WT littermates, and their blood ammonia levels were elevated. Interestingly, they bred poorly under normal conditions, but their fertility was restored when given access to sodium-potassium citrate-supplemented water. Even though the *Car5B*-null mutation alone produced no observable phenotype, it played at least some complementary physiological role. Indeed, *Car5A*$^{-/-}$

and *5B*$^{-/-}$ double-knockout mice showed additional phenotypic changes. Both growth impairment and hyperammonemia were more profound in the double-knockout mice than in the single *Car5A*$^{-/-}$ mice. Survival of the double-knockout mice after weaning was reduced. Fasting blood glucose levels were significantly lower than those of the WT mice. These findings confirmed that mitochondrial CAs contribute to ammonia detoxification and blood glucose level regulation, key processes in ureagenesis and gluconeogenesis.

Soon after the publication describing CA VA- and CA VB-deficient mouse models, van Karnebeek et al. (53) reported the first four cases of human patients with CA VA deficiency. In the initial report, three different *CA5A* gene defects were identified. As discussed earlier in this review, typical symptoms and laboratory findings include lethargy, hyperlactatemia, and hyperammonemia during the neonatal period and early childhood. In addition, several sporadic changes were reported in laboratory parameters linked to defective functions of bicarbonate-dependent enzymes carbamoyl phosphate synthetase 1, pyruvate carboxylase, propionyl-CoA carboxylase, and 3-methylcrotonyl-CoA carboxylase.

3.6. CA VI-Deficient Mice

CA VI is the only secretory isozyme of the mammalian α-CA family. It was first described in 1975 as a novel salivary protein, gustin (275), which was shown much later by other experiments to be CA VI (276). Ross Fernley from the University of Melbourne identified CA VI in the sheep parotid gland and saliva (277), and subsequently, CA VI was isolated from rat (278) and human saliva (279). CA VI is highly expressed in the serous acinar cells of the parotid and submandibular glands (280, 281), where it is secreted in high concentrations to saliva. Although CA VI shows a very limited distribution pattern, its expression is not restricted to only salivary glands. In some reports, CA VI was immunohistochemically identified in the lacrimal gland (282). However, our recent analysis with mass spectrometry failed to identify CA VI in human tear fluid (unpublished observation). In addition to saliva, milk contains high levels of CA VI, which is produced by the epithelial cells of the mammary glands (283). Colostrum contains, on average, eight-fold greater concentrations of CA VI than mature milk or saliva.

The exact physiological roles of CA VI are unclear, although the enzyme was described over 40 years ago. CA VI may contribute to the maintenance of optimal pH homeostasis of the upper

alimentary tract (56, 238). The role of salivary CA VI may be of special importance on tooth surfaces, and indeed, a clinical study suggested that it may play a protective role against cariogenesis (284). Due to its abundant presence in both milk and colostrum, it may also be involved in the developmental processes of the gastrointestinal canal during the postnatal period (283).

The development of a CA VI-knockout mouse model has already helped us better understand the physiological role of CA VI (67). The first report on that model indicated that *Car6*$^{-/-}$ mice were viable, fertile, and had a normal life span. More lymphoid follicles were counted in the small intestinal Peyer's patches of *Car6*$^{-/-}$ mice compared to control mice, suggesting an immunological phenotype for CA VI deficiency (67). This result was corroborated by functional clustering of differentially expressed genes in which genes related to the “immune system process” were enriched in the duodenum of knockout mice. The second report using an *in vivo* cariogenesis model surprisingly revealed that *Car6*$^{-/-}$ mice exhibited a lower rate of cariogenesis and oral colonization of *Streptococcus mutans* than WT controls (285). This result indicated that the original hypothesis on the role of CA VI as a protective factor against cariogenesis may not be feasible, at least in this murine model.

Another article on a *Car6*$^{-/-}$ mouse model described transcriptional changes in the respiratory tract caused by CA VI deficiency (286). A total of 44 genes in the trachea and two genes in the lung were either up- or downregulated. Functional clustering of the results revealed several altered biological processes, such as antigen transport by M-cells, potassium transport, muscle contraction, and thyroid hormone synthesis. No morphological changes were reported in the trachea or lungs of *Car6*$^{-/-}$ mice.

In 1981, gustin (CA VI) was linked to the regulation of the taste function (55). Decades later, a link was reported between the bitter taste modality and CA VI through polymorphism in the human *CA6* gene (rs2274333 (A/G)), which contributed to 6-n-propylthiouracil taster status (62). A *Car6*$^{-/-}$ mouse model was recently utilized to further investigate the role of CA VI in taste function (287). The results from IntelliCage™ behavioral monitoring confirmed that CA VI deficiency leads to an abnormal bitter taste perception. *Car6*$^{-/-}$ mice preferred drinking water treated with a low concentration of the bitter-tasting substance 3 µM quinine over pure water. Based on this observation, it was concluded that CA VI may be one of the factors that can contribute to avoidance of bitter, potentially toxic substances.

CA VI is expressed as another stress-induced intracellular form called CA VI-b (288). It is expressed after activation of the endoplasmic reticulum unfolded protein stress response pathway mediated by the CCAAT/enhancer-binding protein homologous protein (CHOP). Studies with *Car6*$^{-/-}$ mice demonstrated that CA VI-b is a necessary component of a larger adaptive signaling pathway downstream of CHOP (289). Six years after the first article on *Car6*$^{-/-}$ mice (67), Xu and coworkers reported another CA VI-deficient mouse model, but their model was created by CRISPR/Cas9 technology to allow specific knockout of *Car6-b* in immune cells (290). *Car6*$^{-/-}$ mice were more susceptible to *Listeria monocytogenes* infection and exhibited lower levels of interleukin-12 production. Importantly, the survival rate of the *Car6*$^{-/-}$ mice was significantly lower than that of the WT controls after *L. monocytogenes* infection. There was no difference in either the cytoplasmic or nuclear pH of peritoneal macrophages with or without infection. Moreover, the catalytically inactive mutant CA VI-b rescued interleukin-12 production, suggesting that CA VI-b promotes interleukin-12 expression independent of its CA catalytic activity. Finally, it was demonstrated that CA VI-b induces interleukin-12 production by interacting with protein arginine N-methyltransferase 5 (PRMT5), which links CA VI-b to epigenetic regulation of interleukin-12 in innate immunity.

3.7 Waddles Mice with the *Car8* Gene Deletion

Carbonic anhydrase VIII is also known as carbonic anhydrase-related protein VIII (CARP VIII) and is one of the catalytically inactive CA isoforms (70, 71, 291, 292). Among the catalytically inactive CA isoforms, CA VIII was the first to be reported (293, 294). Subsequently, several studies were carried out mainly related to its expression in both mice and humans (71, 165, 295, 296). These studies showed that CA VIII is mainly expressed in cerebellar Purkinje cells during embryonic development in both humans and mice, suggesting a role in cerebellar development and motor coordination. More direct evidence for the involvement of CA VIII in motor coordination came from waddle (wdl) mice with a 19-bp deletion in the *Car8* gene and from members of Iraqi and Saudi Arabian families with *CA8* gene mutations (73, 74, 297). The wdl mice showed wobbly side-to-side ataxic movement throughout their life span. The members of Iraqi and Saudi Arabian families with a mutation in the *CA8* gene showed a reduction in cerebellar volume with cerebellar ataxia and cognitive impairment, and the members of an Iraqi family showed quadrupedal gait.

3.8. CA IX-Deficient Mice

CA IX differs from all other mammalian CA isozymes in that it shows very limited distribution in normal tissues, whereas it is widely expressed in various malignancies. It is present in normal gastric, intestinal and biliary mucosa (298). When investigated in greater detail, CA IX was found to be highly expressed in the enterocytes of the human duodenum, jejunum, ileum and proximal colon, while the signal diminished towards distal segments of the gut (299). The expression was more positive in the proliferating cryptal enterocytes than in the mature epithelial cells of the mucosa. Based on these results, CA IX was suggested to represent a marker of cell proliferation in the normal gastrointestinal tract (298, 299). CA IX is also located in the epithelial cells of the skin, pancreas and male excurrent ducts (80, 300, 301), suggesting that it may function in ion transport and concentration processes in these organs. It has also been visualized in fetal extraocular fibrous tissues (302). In the nervous system, CA IX is expressed at low levels in ventricle-lining cells and the choroid plexus (303). In the esophagus, only weak CA IX expression is detectable in the basal cells of squamous epithelium, and this level is increased in dysplastic cells (304). CA IX is expressed in mouse skeletal muscle at a special location; it is not localized in the plasma membrane but in the region of the t-tubular/terminal sarcoplasmic reticulum membrane (260). Takacova and coworkers recently published an *in silico* analysis of mouse *Car9* mRNA distribution based on public GENEVESTIGATOR data (305). The results indicated a weak positive signal in a number of murine tissues. The most abundant expression was detected in the stomach, intestine, pancreas, hematopoietic stem/progenitor cells, dendritic cells, undefined skeletal system cells, undefined female reproductive system cells, and kidney. Takacova and colleagues also demonstrated that — similar to the human enzyme — mouse CA IX expression is regulated by several transcription factors, such as HIF-1, SP1, and AP1.

CA IX is typically overexpressed in hypoxic tumors (82) and is transcriptionally regulated by HIF-1 alpha (306). It is a marker for poor prognosis in various hypoxic cancers, such as gliomas (307), breast ductal carcinoma (308), oral squamous cell carcinoma (309), and lung adenocarcinoma (310).

When mice with targeted disruption of the *Car9* gene were generated, the first study results indicated only mild phenotypic changes, most prominently gastric hyperplasia and some cystic

changes within the gastric mucosa (311). Gastric acid secretion remained unchanged, indicating that other CA isozymes mainly contribute to normal gastric pH homeostasis.

To further elucidate the role of CA IX in the gastric mucosa, C57/BL6 and BALB/c mice were fed either a standard or high-salt diet for 20 weeks (312). The initial hypothesis was that a high-salt diet may induce some dysplastic changes in the gastric mucosa in *Car9*$^{-/-}$ mice because the high salt content of the food is recognized as a procarcinogenic factor (313), and it also causes gastric hyperplasia and parietal cell loss (314). The results confirmed previous findings (311) that CA IX-deficient mice with either a BALB/c or C57/BL6 genetic background and a standard diet showed gastric pit cell hyperplasia and glandular atrophy of the gastric mucosa (312). In C57/BL6 mice, CA IX deficiency was associated with gastric submucosal inflammation. In contrast to the initial hypothesis, excess dietary salt had no significant effect on the severity of pit cell hyperplasia, and no dysplasia was detected in any of the groups. The findings suggested that although CA IX deficiency does not promote tumor formation or dysplastic changes per se, it does induce glandular atrophy of the gastric mucosa, a lesion that is considered preneoplastic in the stomach.

Preliminary studies also suggested mild behavioral changes and a morphological disruption of brain histology in 1.5-year-old CA IX-deficient mice. In a 1-year follow-up study, morphological analysis showed vacuolar degenerative changes in the brains of *Car9*$^{-/-}$ mice (315). The changes were first visible when the mice were from eight to ten months old. These mice also exhibited abnormal locomotor activity and poor performance in a memory test. In genome-wide cDNA microarray analyses of brain specimens, a number of genes were significantly up- or downregulated in CA IX-deficient mice compared to WT mice. Through functional annotation, these genes were linked to various important processes, such as the regulation of ion homeostasis and behavior.

3.9. CA XII-Deficient Mice

CA XII is another transmembrane, cancer-associated CA isozyme. It differs substantially from CA IX: 1) CA XII does not contain a proteoglycan-like domain (82); 2) it shows a wider distribution pattern in normal tissues than CA IX (82, 303); and 3) CA XII has significantly lower CA enzymatic activity than CA IX (316). Among many normal human tissues, CA XII is highly expressed in the colorectal epithelium (79), pancreas (80), esophagus (317), kidney (81), endometrium (78), efferent ducts

and sporadic cells of epididymal ducts (300), and skin (301). In addition, *CA12* mRNA is among the most highly expressed mRNA species in the human corneal endothelium (318). In addition to many normal tissues, CA XII is often present and, in some cases, overexpressed in tumors (82).

Due to the wide distribution of CA XII, the functional consequences of *Car12* deletion in mice are of great interest for future investigation. Indeed, CA XII-deficient mice were generated using the *Sleeping Beauty* transposon method (319). Unfortunately, this knockout mouse colony became extinct soon after the first report, and thus, no detailed functional studies are available. The only reported change indicated that heterozygous carriers of *Car12* mutation produced homozygous litters in a non-Mendelian pattern.

Although no comprehensive studies on the role of CA XII have been performed in knockout animals, this enzyme may have important physiological roles in tissues where it is expressed. Mutations in the human *CA12* gene have been associated with several clinical symptoms and findings, such as hyponatremic dehydration, hyperkalemia, high sweat chloride concentration, and failure to thrive (85, 86). These findings confirmed the important function of CA XII in the body water-ion balance.

3.10. CA XIV-Deficient Mice

CA XIV is a transmembrane CA isozyme that was originally reported by Fujikawa-Adachi et al. (320) in 1999. Northern blot experiments revealed the expression of *CA14* mRNA in the human heart, brain, liver, and skeletal muscle. In addition to these tissues, an RNA dot blot analysis indicated positive CA XIV signals in the spinal cord, kidney, urinary bladder, small intestine and colon. Immunohistochemical studies later confirmed CA XIV expression in human and mouse neurons and the choroid plexus (162), rat and mouse kidney (259), mouse hepatocytes (321), mouse retina (322), rat epididymis (323), mouse skeletal muscle (324), and mouse heart (264). The CA XIV-knockout mouse model was published by Shah et al. (183) in conjunction with the CA IV-deficient mouse model. The CA XIV-deficient mice appeared healthy, grew normally and were fertile. When CA IV/CA XIV double-knockout mice were produced, the number of double mutants was lower than predicted on the basis of Mendelian ratios, and the sex ratio also showed male dominance (74%). A phenotypic analysis showed that the double-knockout mice were significantly smaller than the WT controls, many of the female double mutants died early, and those that survived

were infertile. Electrophysiological data indicated that both CA IV and CA XIV contribute to pH regulation in the extracellular space of the mouse hippocampus. Comparison of the effects further suggested that CA IV had a somewhat greater effect on pH regulation than CA XIV in this region of the brain.

Another report on CA XIV-deficient mice focused on muscle physiology (324). It was first demonstrated that these mice exhibit no metabolic acidosis, although the enzyme is prominently expressed in the kidney. Under normal conditions, CA XIV accounts for approximately 50% and 66% of the total CA activity in sarcoplasmic reticulum and sarcolemmal fractions, respectively. Electrophysiological measurements of the fast-twitch extensor digitorum longus and slow-twitch soleus muscles demonstrated that CA XIV deficiency alone did not affect maximum force, rise and relaxation times or fatigue behavior. It is thus plausible that other CA isozymes, normally contributing to 44–50% of membrane-associated CA activity, compensate for the lack of CA XIV in this mouse model.

4. CARBONIC ANHYDRASE KNOCKDOWN AND KNOCKOUT ZEBRAFISH MODELS

4.1. The Zebrafish as a Model Organism

The zebrafish (*Danio rerio*) has attracted the attention of researchers who need an easy yet sufficiently complex vertebrate model to study the function of conserved genes (325). The widespread use of zebrafish as a model is largely attributed to Dr. George Streisinger from the University of Oregon, who understood the advantages of using zebrafish as a model organism (326). He initiated one of the first genetic screens to identify spontaneous zebrafish mutations and used gamma-ray to induce random mutations in zebrafish DNA to identify offspring with rare phenotypes, such as defects in pigmentation. The zebrafish, as a model organism for biological and medical research, gained momentum during the 1990s when genetic mutants were created by gamma irradiation and chemical mutagenesis, and novel phenotypes were then described (327-329).

The zebrafish is a tropical fish that lives in the Ganges River in northern India. Its anatomical structures and physiology resemble the respective features of mammals, which makes it an excellent disease model (330, 331). The zebrafish has a high fertility rate, and an adult female produces approximately 200 eggs per week. Important features of zebrafish embryos include

transparency and rapid development (24 hrs) outside the mother, allowing exceptionally detailed observations of developmental processes down to the subcellular level (332). The maintenance of large-scale colonies of zebrafish is affordable in a relatively small laboratory space. In addition to all these advantages, the zebrafish genome is well known and can be modified using a broad range of straightforward manipulation methods. The consequences of genetic manipulation can be followed over time through various techniques, such as *in vivo* microscopy, thus allowing the collection of data directly from a living vertebrate organism (333-335). Fully sequenced genomes are available for several strains of zebrafish, and importantly, 71% of human genes have at least one ortholog in zebrafish genomes, and 69% of zebrafish genes have at least one human ortholog (330). To support the role of zebrafish as a useful model organism for human genetic diseases, 82% of the known disease-causing genes in humans have a zebrafish ortholog (330). Many of these zebrafish genes have previously been shown to recapitulate human disease when affected (336).

Several techniques have been developed to create transgenic and knockout zebrafish models. Although the first transgenic zebrafish were created through injection of naked, linearized DNA, more efficient systems of genomic incorporation are now used (336-339). An expanded molecular toolbox for genetic manipulation has allowed the zebrafish research community to create a large number of transgenic, knockdown, and knockout models that have been characterized and maintained for functional studies. To study zebrafish gene function, molecular tools include TILLING (340), morpholino oligonucleotides (339), zinc-finger nucleases (ZFNs) (341), TALENs (342), CRISPR/Cas (338, 343), and Tol2 transposons combined with bacterial artificial chromosomes (344). These resources have facilitated gene manipulation studies, with a sharp increase in research publications related to zebrafish-knockdown and zebrafish-knockout models since 2000 (Figure 9).

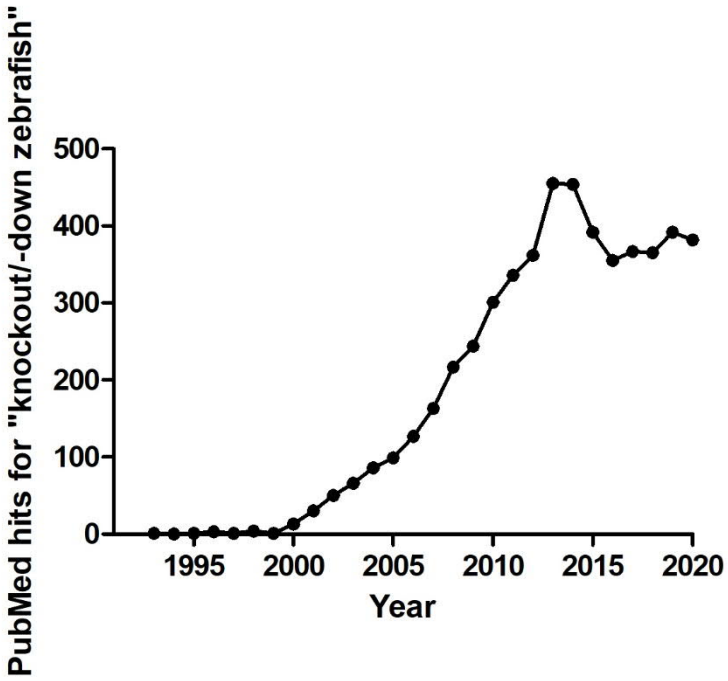

Figure 9. Number of publications on studies involving knockout or knockdown zebrafish models published since 1993. The data were obtained from the PubMed database on June 17, 2021, using the search phrase “zebrafish knockdown” OR “zebrafish knockout”.

Recently, using morpholino oligonucleotides and CRISPR/Cas technologies, several zebrafish knockdown and knockout models have been generated to study the physiological roles of CAs, both during embryonic development and in adult fish (Table 4). The availability of different zebrafish strains, fluorescent reporter lines and powerful imaging techniques, such as *in situ* hybridization and confocal microscopy, has facilitated investigation into the physiological roles of CAs at the molecular level. Clearly, the mechanisms of pH regulation in teleost fishes are profoundly different than those in mammals, and the *CA* gene repertoire also differs. Notably, fishes almost entirely rely on metabolic compensation, which involves the exchange of acid-base equivalents from the external environment: H^+ and HCO_3^- are directly exchanged for Na^+ and Cl^-, respectively (345). The cytosolic CAs present in fish, especially in the gills and kidney, are the major players in this metabolic compensation process because they provide protons and bicarbonate ions that are exported into water (346). In zebrafish, the key mechanisms of acid-base regulation involve contributions from solute carrier family 26 (SLC26), which is dependent on cytosolic CA (347).

Table 4. Knockout/knockdown zebrafish models currently available to study the functions of CAs.

Zebrafish CAs	Modification tool	Reference
CA2 (CA17a)	MOs and CRISPR/Cas9	(348, 349)
CA6	MOs[a] and CRISPR/Cas9[b]	[a](350)
CA8	MO	(351)
CA10a	MOs and CRISPR/Cas9	(352)
CA10b	MOs and CRISPR/Cas9	(352)
CA14	MOs and CRISPR/Cas9	(353)

[b]The adult zebrafish *ca6*-knockout model obtained through CRISPR/Cas9 technology is currently available in our laboratory (unpublished).

4.2. Zebrafish Model of *ca2* (*ca17a*) Knockdown

The physiological importance of high-activity cytosolic CA was originally determined by analyzing the phenotypes of both humans and mice with CA II enzyme deficiency as described in the previous chapters. Among the many isoforms of CAs present in zebrafish, there seem to be two closely related cytosolic isoforms: One isoform originally known as CA II-like b, CAb, Cahz, or CA2b is considered a predominant cytosolic isoform expressed in red blood cells (345, 349). The other isoform, previously termed CA II-like a, CAc, CA2a, or Ca2, is more widely distributed in tissues, including the gills and kidney, but it may be weakly expressed or absent in red blood cells (345, 346, 354). The distribution of these enzymes in various fish tissues has not yet been fully clarified, as Lin and colleagues showed by RT-PCR that both mRNAs are ubiquitously expressed (349). The CA II-like b, CAb, Cahz, or CA2b and CA II-like a, CAc, CA2a, or Ca2 isoforms have been recently renamed CA17b and CA17a, respectively (345). In zebrafish, Ca2 (CA17a) has been shown to localize to a subtype of ion-transporting cells (ionocytes) that express apical H^+-ATPase, Na^+/H^+ exchanger 3b (Nhe3b), and Rhcg1 ammonia transporter (354). Ionocytes are important sites of ammonium-dependent Na^+ uptake that is mediated by Nhe3b (355). Therefore, it has been suggested that cytosolic CA might play an important role in Na^+ uptake by ionocytes by providing H^+ to Nhe3b and H^+-ATPase via hydration of CO_2 (356). Two studies have confirmed that knockdown/knockout of the *ca2* (*ca17a*) gene using morpholinos and CRISPR/Cas9 resulted in an

increase in Na^+ uptake in zebrafish larvae (348, 349). Knockout of the *ca2* (*ca17a*) gene stimulated Na^+ uptake and reduced Cl^-. In addition, both *ca17a*$^{-/-}$ knockout and treatment with ethoxzolamide in zebrafish showed that the Ca2 (CA17a) enzyme is essential for physiological Cl^- uptake. Importantly, the complete lack of Ca2 (CA17a) was lethal for zebrafish, in contrast to the effect of CA II deficiency in humans and mice (30, 222, 348). One hundred percent mortality was observed in *ca17a*$^{-/-}$ zebrafish larvae by 19 dpf (348). Homozygous mutant zebrafish larvae exhibited a characteristic bimodal swim bladder phenotype; that is, the bladder is either over- or underinflated compared to that in the heterozygous or WT larvae. In addition, by 9 dpf, the homozygous larvae exhibited a shorter body length than the heterozygous or WT larvae.

4.3. Zebrafish Model of *ca6* Knockdown

Although several studies have suggested that the CA VI enzyme may function in taste perception and immunological modulation in mammals (55, 58, 67, 286, 287), its precise physiological roles are still inadequately understood. When the presence of a *ca6* gene was reported in zebrafish, the corresponding protein was predicted to contain an additional pentraxin (PTX) domain at the C-terminal end (350). The discovery of *ca6* with a C-terminal pentraxin domain was a novel finding in the *CA* gene family and among pentraxins. The recombinant CA6-PTX enzyme contained 530 residues and exhibited high CA enzymatic activity. Light scattering studies indicated that, similar to several other pentraxins, the CA6-PTX protein exists as a pentamer in solution (350).

CA6-PTX is expressed in several organs of zebrafish, including the skin, heart, gills, and swim bladder (350). The immunohistochemical signal was strongest on cell surfaces, suggesting plasma membrane-bound localization. Analysis of *ca6* mRNA expression using qRT-PCR showed that the gene is prominently expressed in the fins/tail and brain, while a weaker signal was reported in the gills, kidney, teeth, skin, and spleen. Interestingly, the swim bladder in morphant embryos was either absent or deflated 4 dpf, but no other morphological changes were observed. The swim pattern analysis of morphant larvae 4 dpf revealed decreased buoyancy and lower swimming activity compared to the control group larvae. When *ca6* mRNA expression was restored in larvae 5 dpf, the swimming pattern of the morphant larvae returned to a nearly normal pattern. Together, these results suggested that CA6-PTX is required for swim bladder development and/or function in zebrafish. The distribution of CA6 in both fish and mammals suggested a role in physical barriers against the external environment (gut, skin, and gills in zebrafish; skin, saliva,

milk, and respiratory tract in mammals), consistent with its putative function in primary immune defense.

4.4. Zebrafish Model of *ca8* Knockdown

Bioinformatic and phylogenetic studies have shown high similarity between vertebrate and invertebrate CARP VIII/CA8 protein sequences (71). The zebrafish *ca8* gene contains 9 exons that encode 281 amino acids (351). A comparison of the amino acid sequence of zebrafish CA8 with the human CA VIII sequence indicated 79% identity, and the shared amino acid identity in the CA domain was 84%. The high degree of homology between the sequences suggested a conserved and essential function.

CARP VIII/CA8 may functionally contribute to intracellular regulation of calcium concentration. This enzyme interacts with inositol 1,4,5-trisphosphate receptor type 1 (ITPR1), an ion channel protein that regulates internal Ca^{2+} ion release (357). Although it was previously believed that nearly the entire CA domain of CARP VIII was required for ITPR1 binding and regulation of intracellular calcium concentration (292, 357), Upadhyay et al. (358) recently showed that even a truncated 22-kDa polypeptide of human CARP VIII contributed to ITPR1-dependent calcium release similar to that of the 33-kDa full-length polypeptide. A coevolutionary analysis of the *CA8* and *itpr1a* genes suggested that these two proteins evolved together (292). Zebrafish *ca8* mRNA is highly expressed in the central nervous system, especially in cerebellar Purkinje cells, similar to that of human CARP VIII (351, 357). The similar expression patterns suggested that the function of CA8 is conserved in zebrafish and that the protein is required for the development of cerebellum and motor coordination (351). A developmental expression analysis showed the presence of *ca8* mRNA 0 hpf, suggesting maternal origin and involvement in zebrafish brain development during early embryogenesis. High expression of *ca8* mRNA was also found in other tissues, including the heart, kidney, eye, and skin. Interestingly, the expression pattern of *itpr1a* mRNA during zebrafish development was fairly similar to that of *ca8*, corroborating the interaction and functional role of ITPR1 and CA8 in early embryogenesis (351).

Knockdown of the *ca8* gene using gene-specific translation and splice site-blocking morpholinos produced abnormal changes in the head of morphant zebrafish embryos as early as 9 hpf (351). In addition, the *ca8* morphant larvae exhibited a fragile body, curved tail, small eyes, and pericardial

edema. As development progressed, defects in the morphant larvae became more prominent, manifesting as a shortened tail, curved body axis, and absence of swim bladder and otolith vesicles. A terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay showed apoptosis of the cells in the head region of the *ca8*-morphant larvae. Transmission electron microscopy confirmed an increased rate of neuronal cell death. Apoptotic changes include a condensed nucleus, fragmented mitochondrial profiles, and debris of dead cells. The *ca8* morphants showed an ataxic movement pattern analogous to humans with *CA8* mutations, confirming the role of this gene in motor coordination function.

4.5. Zebrafish Models of *ca10a* and *ca10b* Knockdown

CARP X/CA10 and CARP XI/CA11 are the other catalytically inactive CA isoforms in which catalytic inactivity is due to the absence of two and three of the critical histidine residues, respectively, that are required for the enzymatic activity of CAs (359). The presence of *CA10* and *CA11* genes was first mentioned by Hewett-Emmett and Tashian in 1996 based on expressed sequence tags (ESTs) (294). Later, the sequence of *CA10* was discovered by screening a human brain cDNA library (360), and the sequence of *CA11* was identified during the construction of a physical map for cone-rod retinal dystrophy (361). An RT-PCR expression analysis of *CA10* mRNA indicated a positive signal in the salivary glands, kidney, testis, and brain (360). An RNA dot blot revealed widespread distribution in the human brain, while immunohistochemical staining revealed that the CARP X protein was mainly located in the myelin sheaths of the human brain, while the cerebral neural cells, astrocytes, and oligodendroglia remained unstained (165). Human CARP XI is also mainly expressed in the brain. An RNA dot blot expression analysis showed that *CA11* mRNA in all parts of the brain analyzed, including the amygdala, caudate nucleus, cerebellum, cerebral cortex, hippocampus, and medulla oblongata, as well as the spinal cord (360). A RT-PCR analysis revealed positive amplicons in the human pancreas, liver, kidney, salivary gland, and spinal cord (362). Immunohistochemical staining showed the presence of CA XI protein in human neurons, astrocytes, choroid plexus, and pia arachnoid, whereas the oligodendroglia remained negative for CA XI staining (165).

A phylogenetic analysis of *CA10* and *CA11* sequences suggested that the *CA11* gene emerged in tetrapods through a gene duplication of *CA10* after the divergence of the fish and tetrapod

lineages (71). Due to a separate duplication event in fishes, zebrafish and other ray-finned fishes have two *CA10* orthologs, *ca10a* and *ca10b*. As a result, the protein encoded by the *ca10a* gene is very similar to mammalian CARP X (90% identity to human CARP X at the protein level), whereas the protein encoded by *ca10b* diverges slightly, with 75% identity to human CARP X (352). An expression analysis of *ca10a* and *ca10b* genes using real-time quantitative PCR in zebrafish showed that these genes are highly expressed in the adult nervous system and developing embryos. *ca10a* mRNA was found to be highly expressed in the brain, heart, and eye, while the highest expression of *ca10b* was found in the ovary, brain, and swim bladder, and a moderate signal was detected in the testis, spleen, and eye. An analysis of developing embryos showed that the *ca10b* mRNA was of maternal origin with the highest signal at 0 hpf, decreasing to very low levels 4–48 hpf, increasing to high levels 96 hpf, and then remaining relatively constant until 168 hpf. The expression pattern of the *ca10b* gene suggested that this gene plays an important role in early embryogenesis and is also required during the later developmental period. *ca10a* mRNA was detected throughout the developmental period of zebrafish embryos, but the levels were very low at 0–48 hpf. The highest expression levels of *ca10a* mRNA were observed between 96 and168 hpf, when the signals remained nearly constant. This expression pattern suggested that the *ca10a* gene plays an important role during embryonic development, especially after 72 hpf. The evolutionary conservation of *CA10*-like genes, their relatively wide distribution in several tissues, and the high mRNA levels expressed during embryonic development suggested a crucial role for CARP X-like proteins in vertebrates (292, 352).

Zebrafish larvae with a morphant *ca10a* gene had abnormal body shape and defects in the head, including small eyes (352). These abnormalities became more prominent during development, with growing zebrafish showing a long curved body, curved tail, pericardial edema, and absence of both swim bladder and otolith sacs. The *ca10b* morphant larvae displayed more severe phenotypic defects than the *ca10a* morphants, and changes were observed as early as 12 hpf. The *ca10b* morphant larvae had a short and abnormally shaped body 24 hpf; the body was fragile, and the mortality rate was high. The *ca10b* morphant embryos had difficulty hatching and displayed curved tails, small heads and eyes, mild pericardial edema, absence of otolith sacs, and unutilized yolk sacs. They did not survive beyond 3 dpf. A TUNEL assay on sections of *ca10a* morphant zebrafish larvae 5 dpf revealed apoptotic cells, especially in the head and eye regions. Similarly, large areas of apoptotic cells were observed in the head region of *ca10b* morphant larvae 5 dpf,

and a weaker signal was observed in the tail region. Both *ca10a* and *ca10b* morphant embryos showed abnormal movement patterns, suggesting an association of these genes in the motor coordination function in zebrafish (352). The partial rescue of *ca10a* and *ca10b* morphant embryos with the injection of corresponding human mRNAs also confirmed the specificity of the *ca10a* and *ca10b* antisense morpholinos used in the study. The phenotypes of the zebrafish larvae obtained by morpholino-based knockdown of the ca*10a* and *ca10b* genes were also confirmed by silencing these genes using CRISPR/Cas9 genome-editing technology. Similar to the morpholino-injected larvae, these CRISPR/Cas9-generated *ca10b* mutant larvae possessed a severe phenotype with high mortality 1 dpf, and no larvae survived beyond 2 dpf. The *ca10a*-mutant larvae showed a less severe phenotype with a lower mortality rate 1 dpf than the *ca10b*-mutant larvae.

4.6. Zebrafish Model of *ca14* Knockdown

Microphthalmia-associated transcription factor (MITF) is a master regulator of melanocyte biology and is involved in a number of key cellular functions, such as differentiation, survival, senescence, invasion, lysosome biogenesis, proliferation, metabolism, and DNA damage repair (363). Hoek and coworkers were the first to report that MITF may regulate the expression of CA XIV in human melanoma cells (364). Recently, Ayyappa Raja and colleagues provided evidence that MITF activation directly induces *ca14* gene expression in zebrafish and that the CA14 protein is essential for melanocyte maturation (353). In zebrafish, pigment-producing cells are termed melanophores and are considered functionally similar to the melanocytes of higher vertebrates, with conserved gene networks (353). At 2 dpf, the *ca14* morphant zebrafish showed light pigmentation compared to the control group larvae. The significant reduction in pigmented melanophores suggested that CA14 plays an important role in the process of melanogenesis, a crucial event associated with melanocyte maturation. During this process, the pigmentation genes *tyr, dct,* and *tyrp1b* are normally upregulated. In the *ca14* morphant fish, these genes exhibited reduced transcription levels, suggesting that the CA14 protein interferes with melanocyte maturation by altering the expression of key genes involved in pigmentation. As CA14 is regulated by MITF and considering its ability to control intracellular pH and sustain the melanin content of zebrafish melanophores, it is a likely a mediator of melanocyte maturation.

To confirm the role of CA14 in pigmentation in zebrafish, the coding region of the *ca14* gene was targeted using the CRISPR/Cas9 system (353). A frameshift mutation in the third codon of the *ca14* gene was introduced by deletion of two bases. The *ca14*-mutant fish had small eyes, an enlarged heart, and decreased pigmentation. An analysis of genes differentially expressed 36 hpf, the time period of pigment cell migration and maturation, indicated reduced levels of *tyr, tyrp1b*, and *dct* expression in the mutant embryos. The downregulation of these genes confirmed that the cells were in an immature, less-pigmented state and that pigmentation-promoting gene expression was severely reduced in the absence of CA14. Thus, the authors concluded that CA14 is an activator of the MITF regulatory network and amplifies the expression of melanocyte maturation genes by altering histone acetylation *via* programmed intracellular pH changes.

5. *DROSOPHILA MELANOGASTER* AND *CAENORHABDITIS ELEGANS* AS MODELS TO STUDY CARBONIC ANHYDRASE FUNCTION

5.1. *Drosophila melanogaster* as a Model Organism

Drosophila melanogaster, or fruit fly, is a dipteran invertebrate species. It has been widely used as a model organism in research since the beginning of the 1900s, when Thomas Hunt Morgan started using flies in his studies on heredity. The complete *D. melanogaster* genome sequence was obtained in 2000, and it was found to be relatively small, approximately 1/20th the size of the human and mouse genomes (365, 366). *D. melanogaster* has four sets of chromosomes: the sex chromosomes, X and Y; two autosomal chromosomes, 2 and 3; and a very small chromosome, 4 (367). The number of *D. melanogaster* genes is approximately 15,000 (365, 366), and their products are less functionally redundant than products of human genes. Despite these significant genomic differences, 75% of the known disease-causing genes in humans have a homolog in the fruit fly (368).

D. melanogaster has a fast life cycle: a fertile mating pair can produce hundreds of progeny in approximately 10 days at 25°C (369). *Drosophila* developmental stages, from egg to adult, can be utilized for modeling different phenomena. For example, the embryo can be used to study fundamental aspects of developmental biology, e.g., pattern formation, organogenesis, and cell fate determination. Larvae and pupae can be used to study many developmental and physiological processes and cell cycle. The *Drosophila* adult is a complex organism that has many organs

equivalent to mammals in terms of biology, such as the brain, heart, lung, kidney, gut, and reproductive tract. Therefore, even complex behaviors, such as waking and sleeping, learning and memory, feeding, and aggressive behavior, can be studied in *Drosophila* adults (369, 370).

Many genetic tools have been developed during the history of *Drosophila* use as a model organism, which makes *Drosophila* ideal for many gene function studies. One invaluable tool is balancer chromosomes, which are used to maintain lethal and sterile mutations in the fly stock in an extremely stable way. These types of tools are not available for use with other model organisms, such as mice (371). There are many strategies to study genes of interest in *Drosophila*. The forward genetic approach, where mutations are introduced randomly and in which flies are screened for a chosen phenotype, is considered the classical approach (372-374). In reverse genetics, RNA interference (RNAi)-mediated silencing of the genes of interest can be carried out *in vitro* in *Drosophila* cultured cells or *in vivo* (375). For *in vivo* studies, the most commonly used system is the UAS-GAL4 system that has been adapted from the yeast system (376), which can either silence or overexpress genes of interest. Targeted mutations of genes can be generated, e.g., using the CRISPR/Cas9 system (377). Great online resources for fly work are available, the most comprehensive of which is FlyBase (http://flybase.org) (370), which provides information about available fly lines (GAL4 driver lines, RNAi lines, mutants, etc.), sequences and their human homologs, expression data, and more.

5.2. Roles of Carbonic Anhydrases in *Drosophila melanogaster*

The number of studies concerning *Drosophila* CAs and invertebrate CAs, in general, is limited. Most *Drosophila* CAs belong to the α-class. In total, one β-CA and nine α-CAs have been identified. There are also at least five CARPs (378), whose functions are currently unknown.

Of the *Drosophila* α-CAs, CAH1 and CAH2 are the best-characterized isozymes (Table 5). A phylogenetic analysis showed that *D. melanogaster* CAH1 and mammalian cytoplasmic CA isozymes share a common ancestor (379). In addition, CAH2 shares an ancestor with mammalian extracellular CA isozymes. High-throughput expression studies reported in FlyBase revealed that CAH1 has the highest level of expression and the widest distribution of all *D. melanogaster* CAs. The highest expression was found in the salivary gland, midgut, and hindgut in both larvae and adults. High CAH1 expression has also been found in larval trachea. CAH2 is most highly expressed

in the larval hindgut, midgut, trachea, adult salivary gland, and Malpighian tubules. Both CAH1 and CAH2 are highly catalytically active (379). CAH1 possesses a k_{cat} of 6.4 x 10^5 s^{-1} and k_{cat}/K_m of 1.2 x 10^8 $M^{-1}s^{-1}$; for CAH2, these values are 6.0 x 10^5 s^{-1} and 1.0 x 10^8 $M^{-1}s^{-1}$, respectively.

In a recent study, the effect of CA inhibitors was tested in the *Aedes aegypti* mosquito, which causes yellow fever, and *D. melanogaster* larvae (380). The CA inhibitor dichlorphenamide caused biphasic elevation and depression of central nervous system firing in *D. melanogaster* larvae. The effects were enhanced by lowering the buffer concentration of the insect saline solution. The buffer dependence of these effects supports the hypothesis that CAs have physiological actions in the regulation of nerve and muscle functions in *Drosophila*. However, the specific CA that mediates this effect has not been determined.

Many animals, including humans, generate low pH in the gastrointestinal canal, the purpose of which is to accelerate protein digestion and absorption of nutrients and to destroy pathogens and parasites. *Drosophila* are no exception, and CAs participate in acidification of the fly gut (381). A recent study increased our understanding of this role of CAs. Overend et al. (382) showed by semiquantitative analysis that knockdown of CAH1 markedly increased pH in the acidic part of the *Drosophila* gut. It was also observed that disruption of pH leads to changes in the bacterial flora of the *Drosophila* gut, thus affecting immune defense. Similar to that in humans, the low pH region of the *Drosophila* gut protects against pathogenic bacterial colonization and regulates the colonization of nonpathogenic bacteria in the gut.

For a long time, it was thought that β-CAs are not present in any animal species. However, Fasseas et al. (383) first described two *β-CA* genes in the nematode *Caenorhabditis elegans*. One of the two enzymes was shown to be active. A few months later, we identified and biochemically characterized a novel β-CA enzyme in another invertebrate animal, *D. melanogaster* (7). *D. melanogaster* β-CA is a dimeric mitochondrial enzyme. It is highly catalytically active, with k_{cat} of 9.5 x 10^5 s^{-1} and k_{cat}/K_m of 1.1 x 10^8 $M^{-1}s^{-1}$ (Table 5). To the best of our knowledge, this β-CA is the only mitochondrial CA in *Drosophila*, while all other CAs, namely, α-CAs, reside in other cell compartments. Our study suggested that at least one *β-CA* gene can be found in all *Animalia* species, excluding those in the *Chordata* phylum. This finding is important because many pathogenic parasites and disease-carrying vectors are invertebrates and thus express β-CAs. The difference in active site structure between mammalian α-CA and parasite β-CA enzymes has

opened new avenues for developing parasite-specific medications with minimal harmful side effects on vertebrates, including humans.

The physiological role of β-CA in *D. melanogaster* was investigated by knocking down the expression of β-CA using RNAi (384). The RNAi constructs were controlled both temporally and spatially using the UAS-GAL4 system. Interestingly, β-CA function turned out to be crucial for the fertility of *D. melanogaster* females. Morphological studies demonstrated that partial loss of β-CA function leads to disturbances in border cell migration during oogenesis (Figure 10). This may explain the reduced fertility in knockdown female *Drosophila* adults, since defective border cell migration leads to sterility due to impaired morphogenesis of the so-called "micropyle" (385), a structure needed for normal fertilization. RNAi-knockdown flies typically laid no eggs, even though virgin female flies were expected to ovulate, although at a slow rate (approximately 1 egg/day) (386). The mechanism by which oocytes are released from *Drosophila* ovaries is largely unknown. The loss of β-CA function might affect conditions in the ovulatory tract, which would then hinder ovulation. The highest β-CA expression levels in *Drosophila* have been found in spermatheca (an organ for sperm storage in females), fat body, and heart (http://flyatlas.org/atlas.cgi?name=cg11967-ra). Although direct experimental evidence is lacking, the sterility of female *β-CA* knockdown flies may also be due to abnormal function of spermatheca or disturbed ovulation.

It is currently unclear how β-CA affects border cell migration in *D. melanogaster*. Among human CAs, isozymes IX and XII have been linked to tumor invasiveness and migration (82, 101). It was suggested that CAs facilitate cancer cell migration by lowering the pH of the extracellular matrix, which in turn activates matrix metalloproteinases. Human CAs IX and XII are membrane-bound enzymes, whereas *Drosophila* β-CA is mitochondrial. HCO_3^- does not easily diffuse through cell membranes, and thus, it is unlikely that β-CA can affect the pH of the extracellular space. A more plausible explanation is that mitochondrial β-CA is involved in biosynthetic reactions, and the impairment of these functions may affect border cell migration. Since the enzyme is mitochondrial, the effect of RNAi knockdown on the mitochondrial respiratory chain was investigated, as well as the effect on fly survival (384). It was found that neither the respiratory chain nor survival was affected, at least not with the gene-silencing levels reached in the reported study.

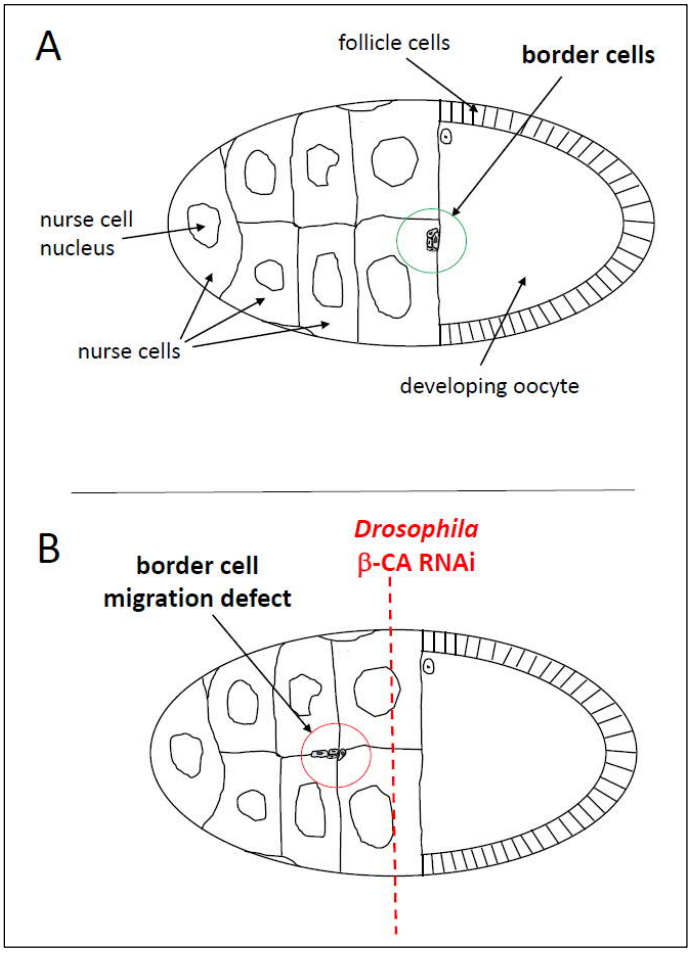

Figure 10. A schematic diagram of early stage 10 *Drosophila melanogaster* oocyte development. Border cells detach from the epithelium at the distal end of the egg chamber and migrate between the nurse cells towards the oocyte. A) In WT *D. melanogaster*, the border cells end up on the border of nurse cells and oocytes. B) When *D. melanogaster* β-CA expression is ubiquitously silenced using RNAi, border cell migration is impaired (modified from (384)).

5.3. *Caenorhabditis elegans* as a Model Organism

Caenorhabditis elegans is a very small, free-living nematode that can be found in environments worldwide. *C. elegans* worms exist primarily as self-fertilizing hermaphrodites, although males emerge at a very low rate. *C. elegans* has four larval stages, L1, L2, L3, and L4, and adult worms can produce up to 300 progeny in their life span. The *C. elegans* life cycle is dependent on temperature: at 25°C, the life cycle is fast, resulting in a new generation, from eggs to adults, in 2.5 days. In the laboratory, *C. elegans* worms are generally grown on agar plates containing a lawn of *Escherichia coli* bacteria, and when large quantities of worms are needed, they can be cultured in

liquid medium (387). Newly hatched larvae are 0.25-mm long, and adults are 1-mm long (388). Under stressful conditions, such as crowding during growth, food restriction, or high temperature, the worms can enter the Dauer stage, where their development arrests at the second molt (389). This can be useful in maintaining the worms, since they can be kept at the Dauer stage for several months at 11°C or 16°C (387). At the right developmental stage, worms can be frozen and kept in liquid nitrogen at -196°C virtually indefinitely (387, 390). Because of their small size, the movement, eating, mating, and egg-laying behavior of *C. elegans* is usually observed with a dissecting microscope. Compound or confocal microscopes, allowing higher magnification, are often used for performing experiments. *C. elegans* worms are transparent, and therefore, individual cells and subcellular details can be visualized using Nomarski (also called differential interference contrast, DIC) optics (388). *C. elegans* is also very well suited for use in studies where fluorescent proteins are expressed (391, 392).

Because of its many useful features, *C. elegans* has been a powerful model of choice for eukaryotic genetic studies for decades. A famous example is the pattern of cell divisions and resulting cell lineages, which have been thoroughly investigated in this nematode model. In *C. elegans*, strictly determined cell lineages from one to eight sequential divisions give rise to a fixed number of progeny cells with distinct fates, including cells of the neuronal, digestive and muscular systems (393). Moreover, the development of the nervous system has been exhaustively studied in *C. elegans*: the ~7,000 synapses of the whole-worm nervous system have been mapped using serial-section electron micrographs through an effort that took more than ten years for completion (394). Therefore, the most complete wiring of any nervous system has been identified in *C. elegans* (388). Because of the invariant cell lineage and the relatively simple neural circuit, mutation in these systems gives rise to easily detectable developmental or behavioral phenotypes that can be utilized in genetic screening. Moreover, *C. elegans* was the first multicellular organism for which the genome was sequenced (395), making it a highly useful model for identifying novel key genes in fundamental biological processes (388).

5.4. α-Carbonic Anhydrases in *C. elegans*

The genome of *C. elegans* encodes seven α-CAs, namely, CAH-1, CAH-2, CAH-3, CAH-4a, CAH-4b, CAH-5, and CAH-6 (383). According to a bioinformatics analysis, many nematode α-CAs contain

signal peptides that target them to the secretory pathway (396). However, this pattern may not apply to *C. elegans* α-CAs. CAH-3, CAH-4b and CAH-5 are cytoplasmic enzymes (397), and CAH-4a is directed to the cell nucleus, suggesting a role in pH regulation therein. Fasseas and coworkers concluded that only CAH-3, CAH-4, and CAH-5 possess the three conserved zinc-binding histidine residues characteristic of enzymatically active human CAs. From these three CAH enzymes, CAH-3 and CAH-4 have been proven to be enzymatically active. The other three enzymes (CAH-1, CAH-2, and CAH-6) were presented as CARPs that lack CA activity. It was also demonstrated that silencing of the *cah-3* and *cah-4* genes seemed to affect the lifespan of *C. elegans*. However, the reduction in worm maximum lifespan was small, even though the *cah-3* and *cah-4* genes had the highest expression levels of the studied α-CAs.

There are limited kinetic data concerning *C. elegans* α-CAs. CAH-4b has been shown to be functional with a K_{cat} of 7.2×10^5 s^{-1} and K_{cat}/K_m of 5.4×10^7 $M^{-1}s^{-1}$ (398) (Table 5). It has also been shown that a CA inhibitor, acetazolamide, did not penetrate the nematode cuticle. *C. elegans* is highly resistant to pH changes, with more than 90% surviving in pH conditions ranging from pH 3 to 10, a property that is probably, at least partially, related to its CA activity. Studies have suggested that a CA in *Ostertagia ostertagi* (a parasitic nematode) may play a critical role in the immediate early developmental events that follow exsheathment initiation, which in turn is the first step in the initiation of infection (399).

Given that one-half of the *C. elegans α-CA* genes seem to encode acatalytic CAs (CARPs), it is likely that these proteins are involved in other important processes independent of CO_2 metabolism. Similar to acatalytic CARPs in humans, acatalytic isoforms of CAs in *C. elegans* are mainly expressed in neurons (397). Notably, adult *C. elegans* show an immediate avoidance response upon exposure to carbon dioxide by discontinuing forward movement and starting backward movement (400). This reaction might be linked to CA activity. These observations indicate potentially important roles for α-CAs in the physiology and pathogenesis of nematodes.

5.5. β-Carbonic Anhydrases in *C. elegans*

In 2009, Fasseas et al. (383) characterized *C. elegans* β-CA, the first characterized invertebrate β-CA. They discovered two β-CA genes in *C. elegans*, named *BCA-1* and *Y116A8C.28*. The protein encoded by the latter gene showed low catalytic activity with a k_{cat} of 2.77×10^4 s^{-1} and a k_{cat}/K_m of

6.383×10^5 M^{-1} s^{-1}. The other β-CA they discovered seemed to lack catalytic activity. However, the sequence initially found for *BCA-1* was incorrect; in the original analysis, the coding sequence of the preceding gene, *MTP18*, which was fused to the β-*CA* reading frame, was erroneously included. The sequence annotation was later corrected, and it is likely that both β-CAs in *C. elegans* are active enzymes (7). RNAi studies of *C. elegans* β-CA did not reveal any visible phenotype.

Although research on nematode β-CAs and other invertebrate β-CAs is limited, β-CAs are considered attractive targets for antiparasitic drugs because β-CAs are missing in humans and other vertebrates. Inhibitors specific for these enzymes can be designed, which assumedly would induce minimal side effects via inhibition of mammalian α-CAs.

Table 5. Characterized CAs in *D. melanogaster* and *C. elegans*. Subcellular locations are either predicted or studied using GFP tagging. In addition to the CAs listed, both *D. melanogaster* and *C. elegans* express several CAs and CARPs that have yet to be characterized (378).

Organism	Enzyme	CA class	subcellular localization	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})	Physiological function
D. melanogaster	CAH1	α	cytoplasmic	1.2×10^8	6.4×10^6	not known
D. melanogaster	CAH2	α	membrane-attached	1.0×10^8	6.0×10^6	not known
D. melanogaster	DmBCA	β	mitochondrial	1.1×10^8	9.5×10^5	border cell migration/fertility of female flies
C. elegans	CAH-3	α	cytoplasmic	active *in vivo*	active *in vivo*	not known
C. elegans	CAH-4a	α	nuclear	6.68×10^4	6.85×10^5	not known
C. elegans	CAH-4b	α	cytoplasmic/excretory	5.4×10^7	7.2×10^5	not known
C. elegans	Y116A8C.28	β	not known	6.38×10^5	2.77×10^4	not known

6. CONCLUSIONS AND FUTURE DIRECTIONS

Professor Robert E. Forster, a renowned physiologist and CA researcher from the University of Pennsylvania, made a far-seeing statement during the International Conference of Carbonic Anhydrases in Spoleto, Italy, in 1990: “With so many questions about its functions, some about new problems and some about old problems freshly seen, research on carbonic anhydrase has a bright future.” Since then, we have witnessed several major scientific breakthroughs in the field of

CAs. Eight structurally divergent but functionally convergent CA gene families have been established. Several new members of the mammalian α-CA gene family have been discovered, existing now as thirteen enzymatically active and three CA-related members. More efficient techniques have been introduced to create knockout and knockdown models not only with mice but also with lower vertebrate and invertebrate organisms. Several unique animal models have been generated to study the physiological role of each isoform. Research in the postgenomic era has generated an unprecedented amount of genomic data that have been utilized to better understand the evolution of these ancient enzymes and to define genetic variations with physiological and clinical significance. During the past two decades, the development and testing of CA inhibitors have been the most intensively studied areas of CA research. Several inhibitors are in clinical use, and several others are undergoing or entering clinical trials. CA inhibitors will have great potential to bolster, for example, the anticancer and antimicrobial armamentarium. Considering these facts and prospects for new findings, we strongly believe that research on CAs has a future brighter than ever before.

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AUTHOR CONTRIBUTIONS

S.P. and S.V. prepared figures, S.P., H.B., A.A., L.S., and S.V. drafted manuscript, All authors edited and revised manuscript. All authors approved final version of manuscript.

REFERENCES

1. Sly WS, and Hu PY. Human carbonic anhydrases and carbonic anhydrase deficiencies. *Annu Rev Biochem* 64: 375-401, 1995.
2. Ferry JG. The gamma class of carbonic anhydrases. *Biochim Biophys Acta* 1804: 374-381, 2010.
3. Lane TW, Saito MA, George GN, Pickering IJ, Prince RC, and Morel FM. Biochemistry: a cadmium enzyme from a marine diatom. *Nature* 435: 42, 2005.
4. Jensen EL, Clement R, Kosta A, Maberly SC, and Gontero B. A new widespread subclass of carbonic anhydrase in marine phytoplankton. *ISME J* 13: 2094-2106, 2019.
5. Akocak S, and Supuran CT. Activation of alpha-, beta-, gamma- delta-, zeta- and eta- class of carbonic anhydrases with amines and amino acids: a review. *J Enzyme Inhib Med Chem* 34: 1652-1659, 2019.
6. Cainzos M, Marchetti F, Popovich C, Leonardi P, Pagnussat G, and Zabaleta E. Gamma carbonic anhydrases are subunits of the mitochondrial complex I of diatoms. *Mol Microbiol* 2021.
7. Syrjanen L, Tolvanen M, Hilvo M, Olatubosun A, Innocenti A, Scozzafava A, Leppiniemi J, Niederhauser B, Hytonen VP, Gorr TA, Parkkila S, and Supuran CT. Characterization of the first beta-class carbonic anhydrase from an arthropod (Drosophila melanogaster) and phylogenetic analysis of beta-class carbonic anhydrases in invertebrates. *BMC Biochem* 11: 28, 2010.
8. Del Prete S, Vullo D, Fisher GM, Andrews KT, Poulsen SA, Capasso C, and Supuran CT. Discovery of a new family of carbonic anhydrases in the malaria pathogen Plasmodium falciparum--the eta-carbonic anhydrases. *Bioorg Med Chem Lett* 24: 4389-4396, 2014.
9. Del Prete S, Nocentini A, Supuran CT, and Capasso C. Bacterial iota-carbonic anhydrase: a new active class of carbonic anhydrase identified in the genome of the Gram-negative bacterium Burkholderia territorii. *J Enzyme Inhib Med Chem* 35: 1060-1068, 2020.
10. Kikutani S, Nakajima K, Nagasato C, Tsuji Y, Miyatake A, and Matsuda Y. Thylakoid luminal theta-carbonic anhydrase critical for growth and photosynthesis in the marine diatom Phaeodactylum tricornutum. *Proc Natl Acad Sci U S A* 113: 9828-9833, 2016.
11. Hopkinson BM, Dupont CL, and Matsuda Y. The physiology and genetics of CO2 concentrating mechanisms in model diatoms. *Curr Opin Plant Biol* 31: 51-57, 2016.
12. Zimmerman SA, and Ferry JG. The beta and gamma classes of carbonic anhydrase. *Curr Pharm Des* 14: 716-721, 2008.
13. Zolfaghari Emameh R, Barker H, Tolvanen ME, Ortutay C, and Parkkila S. Bioinformatic analysis of beta carbonic anhydrase sequences from protozoans and metazoans. *Parasit Vectors* 7: 38, 2014.
14. Hilvo M, Tolvanen M, Clark A, Shen B, Shah GN, Waheed A, Halmi P, Hanninen M, Hamalainen JM, Vihinen M, Sly WS, and Parkkila S. Characterization of CA XV, a new GPI-anchored form of carbonic anhydrase. *Biochem J* 392: 83-92, 2005.
15. Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nat Rev Drug Discov* 7: 168-181, 2008.
16. Zolfaghari Emameh R, Barker H, Hytonen VP, Tolvanen ME, and Parkkila S. Beta carbonic anhydrases: novel targets for pesticides and anti-parasitic agents in agriculture and livestock husbandry. *Parasit Vectors* 7: 403, 2014.
17. Zolfaghari Emameh R, Syrjanen L, Barker H, Supuran CT, and Parkkila S. Drosophila melanogaster: a model organism for controlling Dipteran vectors and pests. *J Enzyme Inhib Med Chem* 30: 505-513, 2015.

18. Syrjanen L, Kuuslahti M, Tolvanen M, Vullo D, Parkkila S, and Supuran CT. The beta-carbonic anhydrase from the malaria mosquito Anopheles gambiae is highly inhibited by sulfonamides. *Bioorg Med Chem* 23: 2303-2309, 2015.
19. Syrjanen L, Vermelho AB, Rodrigues Ide A, Corte-Real S, Salonen T, Pan P, Vullo D, Parkkila S, Capasso C, and Supuran CT. Cloning, characterization, and inhibition studies of a beta-carbonic anhydrase from Leishmania donovani chagasi, the protozoan parasite responsible for leishmaniasis. *J Med Chem* 56: 7372-7381, 2013.
20. Consortium GT. Human genomics. The Genotype-Tissue Expression (GTEx) pilot analysis: multitissue gene regulation in humans. *Science* 348: 648-660, 2015.
21. Consortium F, the RP, Clst, Forrest AR, Kawaji H, Rehli M, Baillie JK, de Hoon MJ, Haberle V, Lassmann T, Kulakovskiy IV, Lizio M, Itoh M, Andersson R, Mungall CJ, Meehan TF, Schmeier S, Bertin N, Jorgensen M, Dimont E, Arner E, Schmidl C, Schaefer U, Medvedeva YA, Plessy C, Vitezic M, Severin J, Semple C, Ishizu Y, Young RS, Francescatto M, Alam I, Albanese D, Altschuler GM, Arakawa T, Archer JA, Arner P, Babina M, Rennie S, Balwierz PJ, Beckhouse AG, Pradhan-Bhatt S, Blake JA, Blumenthal A, Bodega B, Bonetti A, Briggs J, Brombacher F, Burroughs AM, Califano A, Cannistraci CV, Carbajo D, Chen Y, Chierici M, Ciani Y, Clevers HC, Dalla E, Davis CA, Detmar M, Diehl AD, Dohi T, Drablos F, Edge AS, Edinger M, Ekwall K, Endoh M, Enomoto H, Fagiolini M, Fairbairn L, Fang H, Farach-Carson MC, Faulkner GJ, Favorov AV, Fisher ME, Frith MC, Fujita R, Fukuda S, Furlanello C, Furino M, Furusawa J, Geijtenbeek TB, Gibson AP, Gingeras T, Goldowitz D, Gough J, Guhl S, Guler R, Gustincich S, Ha TJ, Hamaguchi M, Hara M, Harbers M, Harshbarger J, Hasegawa A, Hasegawa Y, Hashimoto T, Herlyn M, Hitchens KJ, Ho Sui SJ, Hofmann OM, Hoof I, Hori F, Huminiecki L, Iida K, Ikawa T, Jankovic BR, Jia H, Joshi A, Jurman G, Kaczkowski B, Kai C, Kaida K, Kaiho A, Kajiyama K, Kanamori-Katayama M, Kasianov AS, Kasukawa T, Katayama S, Kato S, Kawaguchi S, Kawamoto H, Kawamura YI, Kawashima T, Kempfle JS, Kenna TJ, Kere J, Khachigian LM, Kitamura T, Klinken SP, Knox AJ, Kojima M, Kojima S, Kondo N, Koseki H, Koyasu S, Krampitz S, Kubosaki A, Kwon AT, Laros JF, Lee W, Lennartsson A, Li K, Lilje B, Lipovich L, Mackay-Sim A, Manabe R, Mar JC, Marchand B, Mathelier A, Mejhert N, Meynert A, Mizuno Y, de Lima Morais DA, Morikawa H, Morimoto M, Moro K, Motakis E, Motohashi H, Mummery CL, Murata M, Nagao-Sato S, Nakachi Y, Nakahara F, Nakamura T, Nakamura Y, Nakazato K, van Nimwegen E, Ninomiya N, Nishiyori H, Noma S, Noma S, Noazaki T, Ogishima S, Ohkura N, Ohimiya H, Ohno H, Ohshima M, Okada-Hatakeyama M, Okazaki Y, Orlando V, Ovchinnikov DA, Pain A, Passier R, Patrikakis M, Persson H, Piazza S, Prendergast JG, Rackham OJ, Ramilowski JA, Rashid M, Ravasi T, Rizzu P, Roncador M, Roy S, Rye MB, Saijyo E, Sajantila A, Saka A, Sakaguchi S, Sakai M, Sato H, Savvi S, Saxena A, Schneider C, Schultes EA, Schulze-Tanzil GG, Schwegmann A, Sengstag T, Sheng G, Shimoji H, Shimoni Y, Shin JW, Simon C, Sugiyama D, Sugiyama T, Suzuki M, Suzuki N, Swoboda RK, t Hoen PA, Tagami M, Takahashi N, Takai J, Tanaka H, Tatsukawa H, Tatum Z, Thompson M, Toyodo H, Toyoda T, Valen E, van de Wetering M, van den Berg LM, Verado R, Vijayan D, Vorontsov IE, Wasserman WW, Watanabe S, Wells CA, Winteringham LN, Wolvetang E, Wood EJ, Yamaguchi Y, Yamamoto M, Yoneda M, Yonekura Y, Yoshida S, Zabierowski SE, Zhang PG, Zhao X, Zucchelli S, Summers KM, Suzuki H, Daub CO, Kawai J, Heutink P, Hide W, Freeman TC, Lenhard B, Bajic VB, Taylor MS, Makeev VJ, Sandelin A, Hume DA, Carninci P, and Hayashizaki Y. A promoter-level mammalian expression atlas. *Nature* 507: 462-470, 2014.
22. Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson A, Kampf C, Sjostedt E, Asplund A, Olsson I, Edlund K, Lundberg E, Navani S, Szigyarto CA, Odeberg J, Djureinovic D, Takanen JO, Hober S, Alm T, Edqvist PH, Berling H, Tegel H, Mulder J, Rockberg J, Nilsson P, Schwenk JM, Hamsten M, von Feilitzen K, Forsberg M, Persson L, Johansson F, Zwahlen M, von Heijne G, Nielsen J, and Ponten F. Proteomics. Tissue-based map of the human proteome. *Science* 347: 1260419, 2015.
23. Waskom M, Botwinnik O, Ostblom J, Lukauskas S, Hobson P, MaozGelbart, Gemperline DC, Augspurger T, Halchenko Y, and Cole JB. Mwaskom/Seaborn: V0.10.0 (January 2020). *Zenodo* 2020.
24. Hunter JD. Matplotlib: A 2D Graphics Environment. *Computing in Science & Engineering* 9: 90-95, 2007.
25. Fromer M, Pocklington AJ, Kavanagh DH, Williams HJ, Dwyer S, Gormley P, Georgieva L, Rees E, Palta P, Ruderfer DM, Carrera N, Humphreys I, Johnson JS, Roussos P, Barker DD, Banks E,

Milanova V, Grant SG, Hannon E, Rose SA, Chambert K, Mahajan M, Scolnick EM, Moran JL, Kirov G, Palotie A, McCarroll SA, Holmans P, Sklar P, Owen MJ, Purcell SM, and O'Donovan MC. De novo mutations in schizophrenia implicate synaptic networks. *Nature* 506: 179-184, 2014.
26. Mori S, Kou I, Sato H, Emi M, Ito H, Hosoi T, and Ikegawa S. Nucleotide variations in genes encoding carbonic anhydrase 8 and 10 associated with femoral bone mineral density in Japanese female with osteoporosis. *J Bone Miner Metab* 27: 213-216, 2009.
27. Moon S, Keam B, Hwang MY, Lee Y, Park S, Oh JH, Kim YJ, Lee HS, Kim NH, Kim YJ, Kim DH, Han BG, Kim BJ, and Lee J. A genome-wide association study of copy-number variation identifies putative loci associated with osteoarthritis in Koreans. *BMC Musculoskelet Disord* 16: 76, 2015.
28. Kendall AG, and Tashian RE. Erythrocyte carbonic anhydrase I: inherited deficiency in humans. *Science* 197: 471-472, 1977.
29. Villeval JL, Testa U, Vinci G, Tonthat H, Bettaieb A, Titeux M, Cramer P, Edelman L, Rochant H, Breton-Gorius J, and et al. Carbonic anhydrase I is an early specific marker of normal human erythroid differentiation. *Blood* 66: 1162-1170, 1985.
30. Sly WS, Hewett-Emmett D, Whyte MP, Yu YS, and Tashian RE. Carbonic anhydrase II deficiency identified as the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. *Proc Natl Acad Sci U S A* 80: 2752-2756, 1983.
31. Spicer SS, Sens MA, and Tashian RE. Immunocytochemical demonstration of carbonic anhydrase in human epithelial cells. *J Histochem Cytochem* 30: 864-873, 1982.
32. Gay CV, and Mueller WJ. Carbonic anhydrase and osteoclasts: localization by labeled inhibitor autoradiography. *Science* 183: 432-434, 1974.
33. Vaananen HK, and Parvinen EK. High active isoenzyme of carbonic anhydrase in rat calvaria osteoclasts. Immunohistochemical study. *Histochemistry* 78: 481-485, 1983.
34. Ghandour MS, Langley OK, Vincendon G, Gombos G, Filippi D, Limozin N, Dalmasso D, and Laurent G. Immunochemical and immunohistochemical study of carbonic anhydrase II in adult rat cerebellum: a marker for oligodendrocytes. *Neuroscience* 5: 559-571, 1980.
35. Shah GN, Bonapace G, Hu PY, Strisciuglio P, and Sly WS. Carbonic anhydrase II deficiency syndrome (osteopetrosis with renal tubular acidosis and brain calcification): novel mutations in CA2 identified by direct sequencing expand the opportunity for genotype-phenotype correlation. *Hum Mutat* 24: 272, 2004.
36. Nampoothiri S, and Anikster Y. Carbonic anhydrase II deficiency a novel mutation. *Indian Pediatr* 46: 532-534, 2009.
37. Shivaprasad C, Paliwal P, Khadgawat R, and Sharma A. Identification of a novel mutation in an Indian patient with CAII deficiency syndrome. *J Postgrad Med* 56: 290-292, 2010.
38. Pang Q, Qi X, Jiang Y, Wang O, Li M, Xing X, Dong J, and Xia W. Two novel CAII mutations causing carbonic anhydrase II deficiency syndrome in two unrelated Chinese families. *Metab Brain Dis* 30: 989-997, 2015.
39. Alsharidi A, Al-Hamed M, and Alsuwaida A. Carbonic anhydrase II deficiency: report of a novel mutation. *CEN Case Rep* 5: 108-112, 2016.
40. Yang Y, Tang N, Zhu Y, Zhang L, Cao X, Liu L, Xia W, Li P, and Yang Y. A novel homozygous nonsense mutation in the CA2 gene (c.368G>A, p.W123X) linked to carbonic anhydrase II deficiency syndrome in a Chinese family. *Metab Brain Dis* 36: 589-599, 2021.
41. Kaunisto K, Parkkila S, Tammela T, Ronnberg L, and Rajaniemi H. Immunohistochemical localization of carbonic anhydrase isoenzymes in the human male reproductive tract. *Histochemistry* 94: 381-386, 1990.
42. Parkkila S, Kaunisto K, Kellokumpu S, and Rajaniemi H. A high activity carbonic anhydrase isoenzyme (CA II) is present in mammalian spermatozoa. *Histochemistry* 95: 477-482, 1991.
43. Parkkila S, Parkkila AK, Juvonen T, and Rajaniemi H. Distribution of the carbonic anhydrase isoenzymes I, II, and VI in the human alimentary tract. *Gut* 35: 646-650, 1994.
44. Daiger SP, Sullivan LS, and Bowne SJ. Genes and mutations causing retinitis pigmentosa. *Clin Genet* 84: 132-141, 2013.

45. Rebello G, Ramesar R, Vorster A, Roberts L, Ehrenreich L, Oppon E, Gama D, Bardien S, Greenberg J, Bonapace G, Waheed A, Shah GN, and Sly WS. Apoptosis-inducing signal sequence mutation in carbonic anhydrase IV identified in patients with the RP17 form of retinitis pigmentosa. *Proc Natl Acad Sci U S A* 101: 6617-6622, 2004.
46. de Bruijn SE, Fiorentino A, Ottaviani D, Fanucchi S, Melo US, Corral-Serrano JC, Mulders T, Georgiou M, Rivolta C, Pontikos N, Arno G, Roberts L, Greenberg J, Albert S, Gilissen C, Aben M, Rebello G, Mead S, Raymond FL, Corominas J, Smith CEL, Kremer H, Downes S, Black GC, Webster AR, Inglehearn CF, van den Born LI, Koenekoop RK, Michaelides M, Ramesar RS, Hoyng CB, Mundlos S, Mhlanga MM, Cremers FPM, Cheetham ME, Roosing S, and Hardcastle AJ. Structural Variants Create New Topological-Associated Domains and Ectopic Retinal Enhancer-Gene Contact in Dominant Retinitis Pigmentosa. *Am J Hum Genet* 107: 802-814, 2020.
47. Hageman GS, Zhu XL, Waheed A, and Sly WS. Localization of carbonic anhydrase IV in a specific capillary bed of the human eye. *Proc Natl Acad Sci U S A* 88: 2716-2720, 1991.
48. Yang Z, Alvarez BV, Chakarova C, Jiang L, Karan G, Frederick JM, Zhao Y, Sauve Y, Li X, Zrenner E, Wissinger B, Hollander AI, Katz B, Baehr W, Cremers FP, Casey JR, Bhattacharya SS, and Zhang K. Mutant carbonic anhydrase 4 impairs pH regulation and causes retinal photoreceptor degeneration. *Hum Mol Genet* 14: 255-265, 2005.
49. Alvarez BV, Vithana EN, Yang Z, Koh AH, Yeung K, Yong V, Shandro HJ, Chen Y, Kolatkar P, Palasingam P, Zhang K, Aung T, and Casey JR. Identification and characterization of a novel mutation in the carbonic anhydrase IV gene that causes retinitis pigmentosa. *Invest Ophthalmol Vis Sci* 48: 3459-3468, 2007.
50. Tian Y, Tang L, Cui J, and Zhu X. Screening for the carbonic anhydrase IV gene mutations in Chinese retinitis pigmentosa patients. *Curr Eye Res* 35: 440-444, 2010.
51. Dodgson SJ, Forster RE, 2nd, and Storey BT. The role of carbonic anhydrase in hepatocyte metabolism. *Ann N Y Acad Sci* 429: 516-524, 1984.
52. Dodgson SJ, Forster RE, 2nd, Storey BT, and Mela L. Mitochondrial carbonic anhydrase. *Proc Natl Acad Sci U S A* 77: 5562-5566, 1980.
53. van Karnebeek CD, Sly WS, Ross CJ, Salvarinova R, Yaplito-Lee J, Santra S, Shyr C, Horvath GA, Eydoux P, Lehman AM, Bernard V, Newlove T, Ukpeh H, Chakrapani A, Preece MA, Ball S, Pitt J, Vallance HD, Coulter-Mackie M, Nguyen H, Zhang LH, Bhavsar AP, Sinclair G, Waheed A, Wasserman WW, and Stockler-Ipsiroglu S. Mitochondrial carbonic anhydrase VA deficiency resulting from CA5A alterations presents with hyperammonemia in early childhood. *Am J Hum Genet* 94: 453-461, 2014.
54. Diez-Fernandez C, Rufenacht V, Santra S, Lund AM, Santer R, Lindner M, Tangeraas T, Unsinn C, de Lonlay P, Burlina A, van Karnebeek CD, and Haberle J. Defective hepatic bicarbonate production due to carbonic anhydrase VA deficiency leads to early-onset life-threatening metabolic crisis. *Genet Med* 18: 991-1000, 2016.
55. Shatzman AR, and Henkin RI. Gustin concentration changes relative to salivary zinc and taste in humans. *Proc Natl Acad Sci U S A* 78: 3867-3871, 1981.
56. Kivela J, Parkkila S, Parkkila AK, Leinonen J, and Rajaniemi H. Salivary carbonic anhydrase isoenzyme VI. *J Physiol* 520 Pt 2: 315-320, 1999.
57. Esberg A, Haworth S, Brunius C, Lif Holgerson P, and Johansson I. Carbonic Anhydrase 6 Gene Variation influences Oral Microbiota Composition and Caries Risk in Swedish adolescents. *Sci Rep* 9: 452, 2019.
58. Melis M, Atzori E, Cabras S, Zonza A, Calo C, Muroni P, Nieddu M, Padiglia A, Sogos V, Tepper BJ, and Tomassini Barbarossa I. The gustin (CA6) gene polymorphism, rs2274333 (A/G), as a mechanistic link between PROP tasting and fungiform taste papilla density and maintenance. *PLoS One* 8: e74151, 2013.
59. Kivela J, Parkkila S, Metteri J, Parkkila AK, Toivanen A, and Rajaniemi H. Salivary carbonic anhydrase VI concentration and its relation to basic characteristics of saliva in young men. *Acta Physiol Scand* 161: 221-225, 1997.
60. Leinonen J, Kivela J, Parkkila S, Parkkila AK, and Rajaniemi H. Salivary carbonic anhydrase isoenzyme VI is located in the human enamel pellicle. *Caries Res* 33: 185-190, 1999.

61. Peres RC, Camargo G, Mofatto LS, Cortellazzi KL, Santos MC, Nobre-dos-Santos M, Bergamaschi CC, and Line SR. Association of polymorphisms in the carbonic anhydrase 6 gene with salivary buffer capacity, dental plaque pH, and caries index in children aged 7-9 years. *Pharmacogenomics J* 10: 114-119, 2010.
62. Padiglia A, Zonza A, Atzori E, Chillotti C, Calo C, Tepper BJ, and Barbarossa IT. Sensitivity to 6-n-propylthiouracil is associated with gustin (carbonic anhydrase VI) gene polymorphism, salivary zinc, and body mass index in humans. *Am J Clin Nutr* 92: 539-545, 2010.
63. Li ZQ, Hu XP, Zhou JY, Xie XD, and Zhang JM. Genetic polymorphisms in the carbonic anhydrase VI gene and dental caries susceptibility. *Genet Mol Res* 14: 5986-5993, 2015.
64. Aidar M, Marques R, Valjakka J, Mononen N, Lehtimaki T, Parkkila S, de Souza AP, and Line SR. Effect of genetic polymorphisms in CA6 gene on the expression and catalytic activity of human salivary carbonic anhydrase VI. *Caries Res* 47: 414-420, 2013.
65. Lips A, Antunes LS, Antunes LA, Pintor AVB, Santos D, Bachinski R, Kuchler EC, and Alves GG. Salivary protein polymorphisms and risk of dental caries: a systematic review. *Braz Oral Res* 31: e41, 2017.
66. Hatipoglu O, and Saydam F. Effects of the carbonic anhydrase VI gene polymorphisms on dental caries: A meta-analysis. *Dent Med Probl* 56: 395-400, 2019.
67. Pan PW, Kayra K, Leinonen J, Nissinen M, Parkkila S, and Rajaniemi H. Gene expression profiling in the submandibular gland, stomach, and duodenum of CAVI-deficient mice. *Transgenic Res* 20: 675-698, 2011.
68. Calo C, Padiglia A, Zonza A, Corrias L, Contu P, Tepper BJ, and Barbarossa IT. Polymorphisms in TAS2R38 and the taste bud trophic factor, gustin gene co-operate in modulating PROP taste phenotype. *Physiol Behav* 104: 1065-1071, 2011.
69. Feeney EL, and Hayes JE. Exploring associations between taste perception, oral anatomy and polymorphisms in the carbonic anhydrase (gustin) gene CA6. *Physiol Behav* 128: 148-154, 2014.
70. Kato K. Sequence of a novel carbonic anhydrase-related polypeptide and its exclusive presence in Purkinje cells. *FEBS Lett* 271: 137-140, 1990.
71. Aspatwar A, Tolvanen ME, and Parkkila S. Phylogeny and expression of carbonic anhydrase-related proteins. *BMC Mol Biol* 11: 25, 2010.
72. Aspatwar A, Tolvanen ME, and Parkkila S. An update on carbonic anhydrase-related proteins VIII, X and XI. *J Enzyme Inhib Med Chem* 28: 1129-1142, 2013.
73. Turkmen S, Guo G, Garshasbi M, Hoffmann K, Alshalah AJ, Mischung C, Kuss A, Humphrey N, Mundlos S, and Robinson PN. CA8 mutations cause a novel syndrome characterized by ataxia and mild mental retardation with predisposition to quadrupedal gait. *PLoS Genet* 5: e1000487, 2009.
74. Kaya N, Aldhalaan H, Al-Younes B, Colak D, Shuaib T, Al-Mohaileb F, Al-Sugair A, Nester M, Al-Yamani S, Al-Bakheet A, Al-Hashmi N, Al-Sayed M, Meyer B, Jungbluth H, and Al-Owain M. Phenotypical spectrum of cerebellar ataxia associated with a novel mutation in the CA8 gene, encoding carbonic anhydrase (CA) VIII. *Am J Med Genet B Neuropsychiatr Genet* 156B: 826-834, 2011.
75. Richmond CM, Leventer R, Ryan MM, and Delatycki MB. Cerebellar ataxia with normal intellect associated with a homozygous truncating variant in CA8. *Clin Genet* 97: 516-520, 2020.
76. Ivanov SV, Kuzmin I, Wei MH, Pack S, Geil L, Johnson BE, Stanbridge EJ, and Lerman MI. Down-regulation of transmembrane carbonic anhydrases in renal cell carcinoma cell lines by wild-type von Hippel-Lindau transgenes. *Proc Natl Acad Sci U S A* 95: 12596-12601, 1998.
77. Tureci O, Sahin U, Vollmar E, Siemer S, Gottert E, Seitz G, Parkkila AK, Shah GN, Grubb JH, Pfreundschuh M, and Sly WS. Human carbonic anhydrase XII: cDNA cloning, expression, and chromosomal localization of a carbonic anhydrase gene that is overexpressed in some renal cell cancers. *Proc Natl Acad Sci U S A* 95: 7608-7613, 1998.
78. Karhumaa P, Parkkila S, Tureci O, Waheed A, Grubb JH, Shah G, Parkkila A, Kaunisto K, Tapanainen J, Sly WS, and Rajaniemi H. Identification of carbonic anhydrase XII as the membrane isozyme expressed in the normal human endometrial epithelium. *Mol Hum Reprod* 6: 68-74, 2000.

79. Kivela A, Parkkila S, Saarnio J, Karttunen TJ, Kivela J, Parkkila AK, Waheed A, Sly WS, Grubb JH, Shah G, Tureci O, and Rajaniemi H. Expression of a novel transmembrane carbonic anhydrase isozyme XII in normal human gut and colorectal tumors. *Am J Pathol* 156: 577-584, 2000.
80. Kivela AJ, Parkkila S, Saarnio J, Karttunen TJ, Kivela J, Parkkila AK, Pastorekova S, Pastorek J, Waheed A, Sly WS, and Rajaniemi H. Expression of transmembrane carbonic anhydrase isoenzymes IX and XII in normal human pancreas and pancreatic tumours. *Histochem Cell Biol* 114: 197-204, 2000.
81. Parkkila S, Parkkila AK, Saarnio J, Kivela J, Karttunen TJ, Kaunisto K, Waheed A, Sly WS, Tureci O, Virtanen I, and Rajaniemi H. Expression of the membrane-associated carbonic anhydrase isozyme XII in the human kidney and renal tumors. *J Histochem Cytochem* 48: 1601-1608, 2000.
82. Pastorekova S, Parkkila S, and Zavada J. Tumor-associated carbonic anhydrases and their clinical significance. *Adv Clin Chem* 42: 167-216, 2006.
83. Haapasalo J, Hilvo M, Nordfors K, Haapasalo H, Parkkila S, Hyrskyluoto A, Rantala I, Waheed A, Sly WS, Pastorekova S, Pastorek J, and Parkkila AK. Identification of an alternatively spliced isoform of carbonic anhydrase XII in diffusely infiltrating astrocytic gliomas. *Neuro Oncol* 10: 131-138, 2008.
84. Watson PH, Chia SK, Wykoff CC, Han C, Leek RD, Sly WS, Gatter KC, Ratcliffe P, and Harris AL. Carbonic anhydrase XII is a marker of good prognosis in invasive breast carcinoma. *Br J Cancer* 88: 1065-1070, 2003.
85. Feldshtein M, Elkrinawi S, Yerushalmi B, Marcus B, Vullo D, Romi H, Ofir R, Landau D, Sivan S, Supuran CT, and Birk OS. Hyperchlorhidrosis caused by homozygous mutation in CA12, encoding carbonic anhydrase XII. *Am J Hum Genet* 87: 713-720, 2010.
86. Muhammad E, Leventhal N, Parvari G, Hanukoglu A, Hanukoglu I, Chalifa-Caspi V, Feinstein Y, Weinbrand J, Jacoby H, Manor E, Nagar T, Beck JC, Sheffield VC, Hershkovitz E, and Parvari R. Autosomal recessive hyponatremia due to isolated salt wasting in sweat associated with a mutation in the active site of Carbonic Anhydrase 12. *Hum Genet* 129: 397-405, 2011.
87. Lee M, Vecchio-Pagan B, Sharma N, Waheed A, Li X, Raraigh KS, Robbins S, Han ST, Franca AL, Pellicore MJ, Evans TA, Arcara KM, Nguyen H, Luan S, Belchis D, Hertecant J, Zabner J, Sly WS, and Cutting GR. Loss of carbonic anhydrase XII function in individuals with elevated sweat chloride concentration and pulmonary airway disease. *Hum Mol Genet* 25: 1923-1933, 2016.
88. Supuran CT. Emerging role of carbonic anhydrase inhibitors. *Clin Sci* in press, 2021.
89. Pastorekova S, Parkkila S, Pastorek J, and Supuran CT. Carbonic anhydrases: current state of the art, therapeutic applications and future prospects. *J Enzyme Inhib Med Chem* 19: 199-229, 2004.
90. Temperini C, Innocenti A, Scozzafava A, Parkkila S, and Supuran CT. The coumarin-binding site in carbonic anhydrase accommodates structurally diverse inhibitors: the antiepileptic lacosamide as an example and lead molecule for novel classes of carbonic anhydrase inhibitors. *J Med Chem* 53: 850-854, 2010.
91. Parkkila S, Vullo D, Maresca A, Carta F, Scozzafava A, and Supuran CT. Serendipitous fragment-based drug discovery: ketogenic diet metabolites and statins effectively inhibit several carbonic anhydrases. *Chem Commun (Camb)* 48: 3551-3553, 2012.
92. Parkkila S, Innocenti A, Kallio H, Hilvo M, Scozzafava A, and Supuran CT. The protein tyrosine kinase inhibitors imatinib and nilotinib strongly inhibit several mammalian alpha-carbonic anhydrase isoforms. *Bioorg Med Chem Lett* 19: 4102-4106, 2009.
93. Gray WD, Lipchuck LM, and Ronsberg MA. The prolonged anticonvulsant action of methazolamide in acidotic mice: the role of carbonic anhydrase. *J Pharmacol Exp Ther* 126: 24-29, 1959.
94. Green MC, Azar CA, and Maren TH. Strain differences in susceptibility to the teratogenic effect of acetazolamide in mice. *Teratology* 8: 143-145, 1973.
95. Castro-Correia J, Sousa-Nunes A, and Silva-Bacelar A. Teratogenic action of acetazolamide in mice. *Teratology* 10: 221-225, 1974.
96. Tellone CI, Baldwin JK, and Sofia RD. Teratogenic activity in the mouse after oral administration of acetazolamide. *Drug Chem Toxicol* 3: 83-98, 1980.
97. Hirsch KS, Wilson JG, Scott WJ, and O'Flaherty EJ. Acetazolamide teratology and its association with carbonic anhydrase inhibition in the mouse. *Teratog Carcinog Mutagen* 3: 133-144, 1983.

98. Cairns R, Papandreou I, and Denko N. Overcoming physiologic barriers to cancer treatment by molecularly targeting the tumor microenvironment. *Mol Cancer Res* 4: 61-70, 2006.
99. Boedtkjer E, and Pedersen SF. The Acidic Tumor Microenvironment as a Driver of Cancer. *Annu Rev Physiol* 82: 103-126, 2020.
100. Parkkila S. Significance of pH regulation and carbonic anhydrases in tumour progression and implications for diagnostic and therapeutic approaches. *BJU Int* 101 Suppl 4: 16-21, 2008.
101. Parkkila S, Rajaniemi H, Parkkila AK, Kivela J, Waheed A, Pastorekova S, Pastorek J, and Sly WS. Carbonic anhydrase inhibitor suppresses invasion of renal cancer cells in vitro. *Proc Natl Acad Sci U S A* 97: 2220-2224, 2000.
102. Supuran CT. Indisulam: an anticancer sulfonamide in clinical development. *Expert Opin Investig Drugs* 12: 283-287, 2003.
103. Owa T, Yoshino H, Okauchi T, Yoshimatsu K, Ozawa Y, Sugi NH, Nagasu T, Koyanagi N, and Kitoh K. Discovery of novel antitumor sulfonamides targeting G1 phase of the cell cycle. *J Med Chem* 42: 3789-3799, 1999.
104. Vullo D, Innocenti A, Nishimori I, Pastorek J, Scozzafava A, Pastorekova S, and Supuran CT. Carbonic anhydrase inhibitors. Inhibition of the transmembrane isozyme XII with sulfonamides-a new target for the design of antitumor and antiglaucoma drugs? *Bioorg Med Chem Lett* 15: 963-969, 2005.
105. Abbate F, Casini A, Owa T, Scozzafava A, and Supuran CT. Carbonic anhydrase inhibitors: E7070, a sulfonamide anticancer agent, potently inhibits cytosolic isozymes I and II, and transmembrane, tumor-associated isozyme IX. *Bioorg Med Chem Lett* 14: 217-223, 2004.
106. Ahlskog JK, Dumelin CE, Trussel S, Marlind J, and Neri D. In vivo targeting of tumor-associated carbonic anhydrases using acetazolamide derivatives. *Bioorg Med Chem Lett* 19: 4851-4856, 2009.
107. Thiry A, Dogne JM, Masereel B, and Supuran CT. Targeting tumor-associated carbonic anhydrase IX in cancer therapy. *Trends Pharmacol Sci* 27: 566-573, 2006.
108. Lou Y, McDonald PC, Oloumi A, Chia S, Ostlund C, Ahmadi A, Kyle A, Auf dem Keller U, Leung S, Huntsman D, Clarke B, Sutherland BW, Waterhouse D, Bally M, Roskelley C, Overall CM, Minchinton A, Pacchiano F, Carta F, Scozzafava A, Touisni N, Winum JY, Supuran CT, and Dedhar S. Targeting tumor hypoxia: suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. *Cancer Res* 71: 3364-3376, 2011.
109. Lock FE, McDonald PC, Lou Y, Serrano I, Chafe SC, Ostlund C, Aparicio S, Winum JY, Supuran CT, and Dedhar S. Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche. *Oncogene* 32: 5210-5219, 2013.
110. Touisni N, Maresca A, McDonald PC, Lou Y, Scozzafava A, Dedhar S, Winum JY, and Supuran CT. Glycosyl coumarin carbonic anhydrase IX and XII inhibitors strongly attenuate the growth of primary breast tumors. *J Med Chem* 54: 8271-8277, 2011.
111. Gieling RG, Babur M, Mamnani L, Burrows N, Telfer BA, Carta F, Winum JY, Scozzafava A, Supuran CT, and Williams KJ. Antimetastatic effect of sulfamate carbonic anhydrase IX inhibitors in breast carcinoma xenografts. *J Med Chem* 55: 5591-5600, 2012.
112. Ward C, Meehan J, Mullen P, Supuran C, Dixon JM, Thomas JS, Winum JY, Lambin P, Dubois L, Pavathaneni NK, Jarman EJ, Renshaw L, Um IH, Kay C, Harrison DJ, Kunkler IH, and Langdon SP. Evaluation of carbonic anhydrase IX as a therapeutic target for inhibition of breast cancer invasion and metastasis using a series of in vitro breast cancer models. *Oncotarget* 6: 24856-24870, 2015.
113. Mokhtari RB, Kumar S, Islam SS, Yazdanpanah M, Adeli K, Cutz E, and Yeger H. Combination of carbonic anhydrase inhibitor, acetazolamide, and sulforaphane, reduces the viability and growth of bronchial carcinoid cell lines. *BMC Cancer* 13: 378, 2013.
114. Islam SS, Mokhtari RB, Akbari P, Hatina J, Yeger H, and Farhat WA. Simultaneous Targeting of Bladder Tumor Growth, Survival, and Epithelial-to-Mesenchymal Transition with a Novel Therapeutic Combination of Acetazolamide (AZ) and Sulforaphane (SFN). *Target Oncol* 11: 209-227, 2016.
115. Bayat Mokhtari R, Baluch N, Ka Hon Tsui M, Kumar S, T SH, Aitken K, Das B, Baruchel S, and Yeger H. Acetazolamide potentiates the anti-tumor potential of HDACi, MS-275, in neuroblastoma. *BMC Cancer* 17: 156, 2017.

116. Wu L, Bernal GM, Cahill KE, Pytel P, Fitzpatrick CA, Mashek H, Weichselbaum RR, and Yamini B. BCL3 expression promotes resistance to alkylating chemotherapy in gliomas. *Sci Transl Med* 10: 2018.
117. Akurathi V, Dubois L, Lieuwes NG, Chitneni SK, Cleynhens BJ, Vullo D, Supuran CT, Verbruggen AM, Lambin P, and Bormans GM. Synthesis and biological evaluation of a 99mTc-labelled sulfonamide conjugate for in vivo visualization of carbonic anhydrase IX expression in tumor hypoxia. *Nucl Med Biol* 37: 557-564, 2010.
118. Duivenvoorden WC, Hopmans SN, Gallino D, Farrell T, Gerdes C, Glennie D, Lukka H, and Pinthus JH. Inhibition of carbonic anhydrase IX (CA9) sensitizes renal cell carcinoma to ionizing radiation. *Oncol Rep* 34: 1968-1976, 2015.
119. Boyd NH, Walker K, Fried J, Hackney JR, McDonald PC, Benavides GA, Spina R, Audia A, Scott SE, Libby CJ, Tran AN, Bevensee MO, Griguer C, Nozell S, Gillespie GY, Nabors B, Bhat KP, Bar EE, Darley-Usmar V, Xu B, Gordon E, Cooper SJ, Dedhar S, and Hjelmeland AB. Addition of carbonic anhydrase 9 inhibitor SLC-0111 to temozolomide treatment delays glioblastoma growth in vivo. *JCI Insight* 2: 2017.
120. Mujumdar P, Kopecka J, Bua S, Supuran CT, Riganti C, and Poulsen SA. Carbonic Anhydrase XII Inhibitors Overcome Temozolomide Resistance in Glioblastoma. *J Med Chem* 62: 4174-4192, 2019.
121. van Kuijk SJ, Gieling RG, Niemans R, Lieuwes NG, Biemans R, Telfer BA, Haenen GR, Yaromina A, Lambin P, Dubois LJ, and Williams KJ. The Sulfamate Small Molecule CAIX Inhibitor S4 Modulates Doxorubicin Efficacy. *PLoS One* 11: e0161040, 2016.
122. Bryant JL, Gieling RG, Meredith SL, Allen TJ, Walker L, Telfer BA, Supuran CT, Williams KJ, and White A. Novel carbonic anhydrase IX-targeted therapy enhances the anti-tumour effects of cisplatin in small cell lung cancer. *Int J Cancer* 142: 191-201, 2018.
123. Perut F, Carta F, Bonuccelli G, Grisendi G, Di Pompo G, Avnet S, Sbrana FV, Hosogi S, Dominici M, Kusuzaki K, Supuran CT, and Baldini N. Carbonic anhydrase IX inhibition is an effective strategy for osteosarcoma treatment. *Expert Opin Ther Targets* 19: 1593-1605, 2015.
124. Andring JT, Dohle W, Tu C, Potter BVL, and McKenna R. 3,17beta-Bis-sulfamoyloxy-2-methoxyestra-1,3,5(10)-triene and Nonsteroidal Sulfamate Derivatives Inhibit Carbonic Anhydrase IX: Structure-Activity Optimization for Isoform Selectivity. *J Med Chem* 62: 2202-2212, 2019.
125. Leese MP, Leblond B, Smith A, Newman SP, Di Fiore A, De Simone G, Supuran CT, Purohit A, Reed MJ, and Potter BV. 2-substituted estradiol bis-sulfamates, multitargeted antitumor agents: synthesis, in vitro SAR, protein crystallography, and in vivo activity. *J Med Chem* 49: 7683-7696, 2006.
126. Selderslaghs IW, Van Rompay AR, De Coen W, and Witters HE. Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. *Reprod Toxicol* 28: 308-320, 2009.
127. Brannen KC, Panzica-Kelly JM, Danberry TL, and Augustine-Rauch KA. Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. *Birth Defects Res B Dev Reprod Toxicol* 89: 66-77, 2010.
128. Aspatwar A, Hammaren M, Parikka M, and Parkkila S. Rapid evaluation of toxicity of chemical compounds using zebrafish embryos. *JoVE* 1-7, 2019.
129. Kanungo J, Cuevas E, Ali SF, and Paule MG. Zebrafish model in drug safety assessment. *Curr Pharm Des* 20: 5416-5429, 2014.
130. Truong L, and Tanguay RL. Evaluation of Embryotoxicity Using the Zebrafish Model. *Methods Mol Biol* 1641: 325-333, 2017.
131. Peterson RT, Link BA, Dowling JE, and Schreiber SL. Small molecule developmental screens reveal the logic and timing of vertebrate development. *Proc Natl Acad Sci U S A* 97: 12965-12969, 2000.
132. Stainier DY, and Fishman MC. The zebrafish as a model system to study cardiovascular development. *Trends Cardiovasc Med* 4: 207-212, 1994.
133. Kazokaite J, Aspatwar A, Kairys V, Parkkila S, and Matulis D. Fluorinated benzenesulfonamide anticancer inhibitors of carbonic anhydrase IX exhibit lower toxic effects on zebrafish embryonic development than ethoxzolamide. *Drug Chem Toxicol* 40: 309-319, 2017.
134. Aspatwar A, Becker HM, Parvathaneni NK, Hammaren M, Svorjova A, Barker H, Supuran CT, Dubois L, Lambin P, Parikka M, Parkkila S, and Winum JY. Nitroimidazole-based inhibitors DTP338 and

DTP348 are safe for zebrafish embryos and efficiently inhibit the activity of human CA IX in Xenopus oocytes. *J Enzyme Inhib Med Chem* 33: 1064-1073, 2018.
135. Rami M, Dubois L, Parvathaneni NK, Alterio V, van Kuijk SJ, Monti SM, Lambin P, De Simone G, Supuran CT, and Winum JY. Hypoxia-targeting carbonic anhydrase IX inhibitors by a new series of nitroimidazole-sulfonamides/sulfamides/sulfamates. *J Med Chem* 56: 8512-8520, 2013.
136. Aspatwar A, Parvathaneni NK, Barker H, Anduran E, Supuran CT, Dubois L, Lambin P, Parkkila S, and Winum JY. Design, synthesis, in vitro inhibition and toxicological evaluation of human carbonic anhydrases I, II and IX inhibitors in 5-nitroimidazole series. *J Enzyme Inhib Med Chem* 35: 109-117, 2020.
137. Aspatwar A, Winum JY, Carta F, Supuran CT, Hammaren M, Parikka M, and Parkkila S. Carbonic Anhydrase Inhibitors as Novel Drugs against Mycobacterial beta-Carbonic Anhydrases: An Update on In Vitro and In Vivo Studies. *Molecules* 23: 2018.
138. Suarez Covarrubias A, Larsson AM, Hogbom M, Lindberg J, Bergfors T, Bjorkelid C, Mowbray SL, Unge T, and Jones TA. Structure and function of carbonic anhydrases from Mycobacterium tuberculosis. *J Biol Chem* 280: 18782-18789, 2005.
139. Covarrubias AS, Bergfors T, Jones TA, and Hogbom M. Structural mechanics of the pH-dependent activity of beta-carbonic anhydrase from Mycobacterium tuberculosis. *J Biol Chem* 281: 4993-4999, 2006.
140. Sassetti CM, Boyd DH, and Rubin EJ. Genes required for mycobacterial growth defined by high density mutagenesis. *Mol Microbiol* 48: 77-84, 2003.
141. Sassetti CM, and Rubin EJ. Genetic requirements for mycobacterial survival during infection. *Proc Natl Acad Sci U S A* 100: 12989-12994, 2003.
142. Rose SJ, and Bermudez LE. Identification of Bicarbonate as a Trigger and Genes Involved with Extracellular DNA Export in Mycobacterial Biofilms. *MBio* 7: 2016.
143. Rose SJ, Babrak LM, and Bermudez LE. Mycobacterium avium Possesses Extracellular DNA that Contributes to Biofilm Formation, Structural Integrity, and Tolerance to Antibiotics. *PLoS One* 10: e0128772, 2015.
144. Aspatwar A, Hammaren M, Parikka M, Parkkila S, Carta F, Bozdag M, Vullo D, and Supuran CT. In vitro inhibition of Mycobacterium tuberculosis beta-carbonic anhydrase 3 with Mono- and dithiocarbamates and evaluation of their toxicity using zebrafish developing embryos. *J Enzyme Inhib Med Chem* 35: 65-71, 2020.
145. Pan PW, Rodriguez A, and Parkkila S. A systematic quantification of carbonic anhydrase transcripts in the mouse digestive system. *BMC Mol Biol* 8: 22, 2007.
146. Janowitz HD. Control of gastric and pancreatic secretion by inhibition of carbonic anhydrase. *Lancet* 1: 1353-1355, 1958.
147. Kivilaakso E, and Silen W. The role of mucosal carbonic anhydrase in the protection of gastric mucosa against luminal H+. *Scand J Gastroenterol Suppl* 67: 219-221, 1981.
148. Park HW, and Lee MG. Transepithelial bicarbonate secretion: lessons from the pancreas. *Cold Spring Harb Perspect Med* 2: 2012.
149. Poulsen JH, Fischer H, Illek B, and Machen TE. Bicarbonate conductance and pH regulatory capability of cystic fibrosis transmembrane conductance regulator. *Proc Natl Acad Sci U S A* 91: 5340-5344, 1994.
150. Hogan DL, Crombie DL, Isenberg JI, Svendsen P, Schaffalitzky de Muckadell OB, and Ainsworth MA. CFTR mediates cAMP- and Ca2+-activated duodenal epithelial HCO3- secretion. *Am J Physiol* 272: G872-878, 1997.
151. Hogan DL, Crombie DL, Isenberg JI, Svendsen P, Schaffalitzky de Muckadell OB, and Ainsworth MA. Acid-stimulated duodenal bicarbonate secretion involves a CFTR-mediated transport pathway in mice. *Gastroenterology* 113: 533-541, 1997.
152. Lee MG, Choi JY, Luo X, Strickland E, Thomas PJ, and Muallem S. Cystic fibrosis transmembrane conductance regulator regulates luminal Cl-/HCO3- exchange in mouse submandibular and pancreatic ducts. *J Biol Chem* 274: 14670-14677, 1999.

153. Sjoblom M, Singh AK, Zheng W, Wang J, Tuo BG, Krabbenhoft A, Riederer B, Gros G, and Seidler U. Duodenal acidity "sensing" but not epithelial HCO3- supply is critically dependent on carbonic anhydrase II expression. *Proc Natl Acad Sci U S A* 106: 13094-13099, 2009.
154. Ruusuvuori E, Li H, Huttu K, Palva JM, Smirnov S, Rivera C, Kaila K, and Voipio J. Carbonic anhydrase isoform VII acts as a molecular switch in the development of synchronous gamma-frequency firing of hippocampal CA1 pyramidal cells. *J Neurosci* 24: 2699-2707, 2004.
155. Makani S, Chen HY, Esquenazi S, Shah GN, Waheed A, Sly WS, and Chesler M. NMDA receptor-dependent afterdepolarizations are curtailed by carbonic anhydrase 14: regulation of a short-term postsynaptic potentiation. *J Neurosci* 32: 16754-16762, 2012.
156. Bourke RS, and Kimelberg HG. The effect of HCO-3 on the swelling and ion uptake of monkey cerebral cortex under conditions of raised extracellular potassium. *J Neurochem* 25: 323-328, 1975.
157. Maren TH. Carbonic anhydrase: chemistry, physiology, and inhibition. *Physiol Rev* 47: 595-781, 1967.
158. Ridderstrale Y, and Hanson M. Histochemical study of the distribution of carbonic anhydrase in the cat brain. *Acta Physiol Scand* 124: 557-564, 1985.
159. Cammer W. Immunostaining of carbamoylphosphate synthase II and fatty acid synthase in glial cells in rat, mouse, and hamster brains suggests roles for carbonic anhydrase in biosynthetic processes. *Neurosci Lett* 129: 247-250, 1991.
160. Anderson RE, Engstrom FL, and Woodbury DM. Localization of carbonic anhydrase in the cerebrum and cerebellum of normal and audiogenic seizure mice. *Ann N Y Acad Sci* 429: 502-504, 1984.
161. Kida E, Palminiello S, Golabek AA, Walus M, Wierzba-Bobrowicz T, Rabe A, Albertini G, and Wisniewski KE. Carbonic anhydrase II in the developing and adult human brain. *J Neuropathol Exp Neurol* 65: 664-674, 2006.
162. Parkkila S, Parkkila AK, Rajaniemi H, Shah GN, Grubb JH, Waheed A, and Sly WS. Expression of membrane-associated carbonic anhydrase XIV on neurons and axons in mouse and human brain. *Proc Natl Acad Sci U S A* 98: 1918-1923, 2001.
163. Ghandour MS, Parkkila AK, Parkkila S, Waheed A, and Sly WS. Mitochondrial carbonic anhydrase in the nervous system: expression in neuronal and glial cells. *J Neurochem* 75: 2212-2220, 2000.
164. Hsieh M, Chang WH, Hsu CF, Nishimori I, Kuo CL, and Minakuchi T. Altered expression of carbonic anhydrase-related protein XI in neuronal cells expressing mutant ataxin-3. *Cerebellum* 12: 338-349, 2013.
165. Taniuchi K, Nishimori I, Takeuchi T, Fujikawa-Adachi K, Ohtsuki Y, and Onishi S. Developmental expression of carbonic anhydrase-related proteins VIII, X, and XI in the human brain. *Neuroscience* 112: 93-99, 2002.
166. Kimelberg HK, Stieg PE, and Mazurkiewicz JE. Immunocytochemical and biochemical analysis of carbonic anhydrase in primary astrocyte cultures from rat brain. *J Neurochem* 39: 734-742, 1982.
167. Snyder DS, Zimmerman TR, Jr., Farooq M, Norton WT, and Cammer W. Carbonic anhydrase, 5'-nucleotidase, and 2',3'-cyclic nucleotide-3'-phosphodiesterase activities in oligodendrocytes, astrocytes, and neurons isolated from the brains of developing rats. *J Neurochem* 40: 120-127, 1983.
168. Cammer W, and Tansey FA. The astrocyte as a locus of carbonic anhydrase in the brains of normal and dysmyelinating mutant mice. *J Comp Neurol* 275: 65-75, 1988.
169. Cammer W, and Tansey FA. Carbonic anhydrase immunostaining in astrocytes in the rat cerebral cortex. *J Neurochem* 50: 319-322, 1988.
170. Cammer W, and Zhang H. Carbonic anhydrase in distinct precursors of astrocytes and oligodendrocytes in the forebrains of neonatal and young rats. *Brain Res Dev Brain Res* 67: 257-263, 1992.
171. Jeffrey M, Wells GA, and Bridges AW. Carbonic anhydrase II expression in fibrous astrocytes of the sheep. *J Comp Pathol* 104: 337-343, 1991.
172. Ghandour MS, Langley OK, Vincendon G, and Gombos G. Double labeling immunohistochemical technique provides evidence of the specificity of glial cell markers. *J Histochem Cytochem* 27: 1634-1637, 1979.

173. Roussel G, Delaunoy JP, Nussbaum JL, and Mandel P. Demonstration of a specific localization of carbonic anhydrase C in the glial cells of rat CNS by an immunohistochemical method. *Brain Res* 160: 47-55, 1979.
174. Langley OK, Ghandour MS, Vincendon G, and Gombos G. Carbonic anhydrase: an ultrastructural study in rat cerebellum. *Histochem J* 12: 473-483, 1980.
175. Kumpulainen T, Dahl D, Korhonen LK, and Nystrom SH. Immunolabeling of carbonic anhydrase isoenzyme C and glial fibrillary acidic protein in paraffin-embedded tissue sections of human brain and retina. *J Histochem Cytochem* 31: 879-886, 1983.
176. Kumpulainen T, and Korhonen LK. Immunohistochemical localization of carbonic anhydrase isoenzyme C in the central and peripheral nervous system of the mouse. *J Histochem Cytochem* 30: 283-292, 1982.
177. Lehtonen J, Shen B, Vihinen M, Casini A, Scozzafava A, Supuran CT, Parkkila AK, Saarnio J, Kivela AJ, Waheed A, Sly WS, and Parkkila S. Characterization of CA XIII, a novel member of the carbonic anhydrase isozyme family. *J Biol Chem* 279: 2719-2727, 2004.
178. Nogradi A, Kelly C, and Carter ND. Localization of acetazolamide-resistant carbonic anhydrase III in human and rat choroid plexus by immunocytochemistry and in situ hybridisation. *Neurosci Lett* 151: 162-165, 1993.
179. Halmi P, Parkkila S, and Honkaniemi J. Expression of carbonic anhydrases II, IV, VII, VIII and XII in rat brain after kainic acid induced status epilepticus. *Neurochem Int* 48: 24-30, 2006.
180. Kallio H, Pastorekova S, Pastorek J, Waheed A, Sly WS, Mannisto S, Heikinheimo M, and Parkkila S. Expression of carbonic anhydrases IX and XII during mouse embryonic development. *BMC Dev Biol* 6: 22, 2006.
181. Nogradi A. Differential expression of carbonic anhydrase isozymes in microglial cell types. *Glia* 8: 133-142, 1993.
182. Ghandour MS, Langley OK, Zhu XL, Waheed A, and Sly WS. Carbonic anhydrase IV on brain capillary endothelial cells: a marker associated with the blood-brain barrier. *Proc Natl Acad Sci U S A* 89: 6823-6827, 1992.
183. Shah GN, Ulmasov B, Waheed A, Becker T, Makani S, Svichar N, Chesler M, and Sly WS. Carbonic anhydrase IV and XIV knockout mice: roles of the respective carbonic anhydrases in buffering the extracellular space in brain. *Proc Natl Acad Sci U S A* 102: 16771-16776, 2005.
184. Chesler M. Regulation and modulation of pH in the brain. *Physiol Rev* 83: 1183-1221, 2003.
185. Schmidt SD, Costa A, Rani B, Godfried Nachtigall E, Passani MB, Carta F, Nocentini A, de Carvalho Myskiw J, Furini CRG, Supuran CT, Izquierdo I, Blandina P, and Provensi G. The role of carbonic anhydrases in extinction of contextual fear memory. *Proc Natl Acad Sci U S A* 117: 16000-16008, 2020.
186. Yang MT, Chien WL, Lu DH, Liou HC, and Fu WM. Acetazolamide impairs fear memory consolidation in rodents. *Neuropharmacology* 67: 412-418, 2013.
187. Kalisha Vali Y, Gundla R, Singh OV, Tamboli Y, Di Cesare Manelli L, Ghelardini C, Al-Tamimi AS, Carta F, Angeli A, and Supuran CT. Spirocyclic sulfonamides with carbonic anhydrase inhibitory and anti-neuropathic pain activity. *Bioorg Chem* 92: 103210, 2019.
188. Bozdag M, Poli G, Angeli A, Lucarini E, Tuccinardi T, Di Cesare Mannelli L, Selleri S, Ghelardini C, Winum JY, Carta F, and Supuran CT. N-aryl-N'-ureido-O-sulfamates: Potent and selective inhibitors of the human Carbonic Anhydrase VII isoform with neuropathic pain relieving properties. *Bioorg Chem* 89: 103033, 2019.
189. Angeli A, Di Cesare Mannelli L, Ghelardini C, Peat TS, Bartolucci G, Menicatti M, Carta F, and Supuran CT. Benzensulfonamides bearing spyrohydantoin moieties act as potent inhibitors of human carbonic anhydrases II and VII and show neuropathic pain attenuating effects. *Eur J Med Chem* 177: 188-197, 2019.
190. Angeli A, di Cesare Mannelli L, Lucarini E, Peat TS, Ghelardini C, and Supuran CT. Design, synthesis and X-ray crystallography of selenides bearing benzenesulfonamide moiety with neuropathic pain modulating effects. *Eur J Med Chem* 154: 210-219, 2018.

191. Carta F, Di Cesare Mannelli L, Pinard M, Ghelardini C, Scozzafava A, McKenna R, and Supuran CT. A class of sulfonamide carbonic anhydrase inhibitors with neuropathic pain modulating effects. *Bioorg Med Chem* 23: 1828-1840, 2015.
192. Asiedu MN, Mejia GL, Hubner CA, Kaila K, and Price TJ. Inhibition of carbonic anhydrase augments GABAA receptor-mediated analgesia via a spinal mechanism of action. *J Pain* 15: 395-406, 2014.
193. Sun YJ, Chen Y, Pang C, Wu N, and Li J. Acetazolamide attenuates chemical-stimulated but not thermal-stimulated acute pain in mice. *Acta Pharmacol Sin* 35: 41-47, 2014.
194. Mishra CB, Kumari S, Angeli A, Bua S, Buonanno M, Monti SM, Tiwari M, and Supuran CT. Discovery of potent anti-convulsant carbonic anhydrase inhibitors: Design, synthesis, in vitro and in vivo appraisal. *Eur J Med Chem* 156: 430-443, 2018.
195. Mishra CB, Kumari S, Angeli A, Monti SM, Buonanno M, Tiwari M, and Supuran CT. Discovery of Benzenesulfonamides with Potent Human Carbonic Anhydrase Inhibitory and Effective Anticonvulsant Action: Design, Synthesis, and Pharmacological Assessment. *J Med Chem* 60: 2456-2469, 2017.
196. Bruno E, Buemi MR, De Luca L, Ferro S, Monforte AM, Supuran CT, Vullo D, De Sarro G, Russo E, and Gitto R. In Vivo Evaluation of Selective Carbonic Anhydrase Inhibitors as Potential Anticonvulsant Agents. *ChemMedChem* 11: 1812-1818, 2016.
197. Thiry A, Rolin S, Vullo D, Frankart A, Scozzafava A, Dogne JM, Wouters J, Supuran CT, and Masereel B. Indanesulfonamides as carbonic anhydrase inhibitors and anticonvulsant agents: structure-activity relationship and pharmacological evaluation. *Eur J Med Chem* 43: 2853-2860, 2008.
198. Chesler M. The regulation and modulation of pH in the nervous system. *Prog Neurobiol* 34: 401-427, 1990.
199. Chesler M, and Kaila K. Modulation of pH by neuronal activity. *Trends Neurosci* 15: 396-402, 1992.
200. Kraig RP, Ferreira-Filho CR, and Nicholson C. Alkaline and acid transients in cerebellar microenvironment. *J Neurophysiol* 49: 831-850, 1983.
201. Tong CK, Brion LP, Suarez C, and Chesler M. Interstitial carbonic anhydrase (CA) activity in brain is attributable to membrane-bound CA type IV. *J Neurosci* 20: 8247-8253, 2000.
202. Bertone NI, Groisman AI, Mazzone GL, Cano R, Tabares L, and Uchitel OD. Carbonic anhydrase inhibitor acetazolamide shifts synaptic vesicle recycling to a fast mode at the mouse neuromuscular junction. *Synapse* 71: 2017.
203. Dessole S, Cherchi PL, Buscarinu G, Riggio V, Posadino PM, and Cossu A. Postcoital test after vaginal washing with NaHCO3. *Clin Exp Obstet Gynecol* 12: 33-35, 1985.
204. Okamura N, Tajima Y, Soejima A, Masuda H, and Sugita Y. Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. *J Biol Chem* 260: 9699-9705, 1985.
205. Tresguerres M, Barott KL, Barron ME, and Roa JN. Established and potential physiological roles of bicarbonate-sensing soluble adenylyl cyclase (sAC) in aquatic animals. *J Exp Biol* 217: 663-672, 2014.
206. Demarco IA, Espinosa F, Edwards J, Sosnik J, De La Vega-Beltran JL, Hockensmith JW, Kopf GS, Darszon A, and Visconti PE. Involvement of a Na+/HCO-3 cotransporter in mouse sperm capacitation. *J Biol Chem* 278: 7001-7009, 2003.
207. Parkkila S, Rajaniemi H, and Kellokumpu S. Polarized expression of a band 3-related protein in mammalian sperm cells. *Biol Reprod* 49: 326-331, 1993.
208. Harrison RA, Ashworth PJ, and Miller NG. Bicarbonate/CO2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Mol Reprod Dev* 45: 378-391, 1996.
209. Jose O, Torres-Rodriguez P, Forero-Quintero LS, Chavez JC, De la Vega-Beltran JL, Carta F, Supuran CT, Deitmer JW, and Trevino CL. Carbonic anhydrases and their functional differences in human and mouse sperm physiology. *Biochem Biophys Res Commun* 468: 713-718, 2015.

210. Wandernoth PM, Mannowetz N, Szczyrba J, Grannemann L, Wolf A, Becker HM, Sly WS, and Wennemuth G. Normal Fertility Requires the Expression of Carbonic Anhydrases II and IV in Sperm. *J Biol Chem* 290: 29202-29216, 2015.
211. Grande G, Milardi D, Vincenzoni F, Pompa G, Biscione A, Astorri AL, Fruscella E, De Luca A, Messana I, Castagnola M, and Marana R. Proteomic characterization of the qualitative and quantitative differences in cervical mucus composition during the menstrual cycle. *Molecular BioSystems* 11: 1717-1725, 2015.
212. Wagner G, and Ottesen B. Vaginal physiology during menstruation. *Ann Intern Med* 96: 921-923, 1982.
213. He Q, Chen H, Wong CH, Tsang LL, and Chan HC. Regulatory mechanism underlying cyclic changes in mouse uterine bicarbonate secretion: role of estrogen. *Reproduction* 140: 903-910, 2010.
214. Fox CA, Meldrum SJ, and Watson BW. Continuous measurement by radio-telemetry of vaginal pH during human coitus. *J Reprod Fertil* 33: 69-75, 1973.
215. Hu J, and Spencer TE. Carbonic anhydrase regulate endometrial gland development in the neonatal uterus. *Biol Reprod* 73: 131-138, 2005.
216. Scott GJ, and Gruzdev A. Genome Editing in Mouse Embryos with CRISPR/Cas9. *Methods Mol Biol* 1960: 23-40, 2019.
217. Capecchi MR. The new mouse genetics: altering the genome by gene targeting. *Trends Genet* 5: 70-76, 1989.
218. International Mouse Knockout C, Collins FS, Rossant J, and Wurst W. A mouse for all reasons. *Cell* 128: 9-13, 2007.
219. Austin CP, Battey JF, Bradley A, Bucan M, Capecchi M, Collins FS, Dove WF, Duyk G, Dymecki S, Eppig JT, Grieder FB, Heintz N, Hicks G, Insel TR, Joyner A, Koller BH, Lloyd KC, Magnuson T, Moore MW, Nagy A, Pollock JD, Roses AD, Sands AT, Seed B, Skarnes WC, Snoddy J, Soriano P, Stewart DJ, Stewart F, Stillman B, Varmus H, Varticovski L, Verma IM, Vogt TF, von Melchner H, Witkowski J, Woychik RP, Wurst W, Yancopoulos GD, Young SG, and Zambrowicz B. The knockout mouse project. *Nat Genet* 36: 921-924, 2004.
220. Kosicki M, Tomberg K, and Bradley A. Erratum: Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nat Biotechnol* 36: 899, 2018.
221. Gertsenstein M, Mianne J, Teboul L, and Nutter LMJ. Targeted Mutations in the Mouse via Embryonic Stem Cells. *Methods Mol Biol* 2066: 59-82, 2020.
222. Lewis SE, Erickson RP, Barnett LB, Venta PJ, and Tashian RE. N-ethyl-N-nitrosourea-induced null mutation at the mouse Car-2 locus: an animal model for human carbonic anhydrase II deficiency syndrome. *Proc Natl Acad Sci U S A* 85: 1962-1966, 1988.
223. Spicer SS, Lewis SE, Tashian RE, and Schulte BA. Mice carrying a CAR-2 null allele lack carbonic anhydrase II immunohistochemically and show vascular calcification. *Am J Pathol* 134: 947-954, 1989.
224. Rajachar RM, Tung E, Truong AQ, Look A, and Giachelli CM. Role of carbonic anhydrase II in ectopic calcification. *Cardiovasc Pathol* 18: 77-82, 2009.
225. Ghandour MS, Skoff RP, Venta PJ, and Tashian RE. Oligodendrocytes express a normal phenotype in carbonic anhydrase II-deficient mice. *J Neurosci Res* 23: 180-190, 1989.
226. Velisek L, Moshe SL, Xu SG, and Cammer W. Reduced susceptibility to seizures in carbonic anhydrase II deficient mutant mice. *Epilepsy Res* 14: 115-121, 1993.
227. Breton S, Alper SL, Gluck SL, Sly WS, Barker JE, and Brown D. Depletion of intercalated cells from collecting ducts of carbonic anhydrase II-deficient (CAR2 null) mice. *Am J Physiol* 269: F761-774, 1995.
228. Sun X, Soleimani M, and Petrovic S. Decreased expression of Slc26a4 (Pendrin) and Slc26a7 in the kidneys of carbonic anhydrase II-deficient mice. *Cell Physiol Biochem* 21: 95-108, 2008.
229. Hains DS, Chen X, Saxena V, Barr-Beare E, Flemming W, Easterling R, Becknell B, Schwartz GJ, and Schwaderer AL. Carbonic anhydrase 2 deficiency leads to increased pyelonephritis susceptibility. *Am J Physiol Renal Physiol* 307: F869-880, 2014.
230. Becker HM, and Deitmer JW. Carbonic anhydrase II increases the activity of the human electrogenic Na+/HCO3- cotransporter. *J Biol Chem* 282: 13508-13521, 2007.

231. Pushkin A, Abuladze N, Gross E, Newman D, Tatishchev S, Lee I, Fedotoff O, Bondar G, Azimov R, Ngyuen M, and Kurtz I. Molecular mechanism of kNBC1-carbonic anhydrase II interaction in proximal tubule cells. *J Physiol* 559: 55-65, 2004.
232. Sterling D, Reithmeier RA, and Casey JR. A transport metabolon. Functional interaction of carbonic anhydrase II and chloride/bicarbonate exchangers. *J Biol Chem* 276: 47886-47894, 2001.
233. Sterling D, Brown NJ, Supuran CT, and Casey JR. The functional and physical relationship between the DRA bicarbonate transporter and carbonic anhydrase II. *Am J Physiol Cell Physiol* 283: C1522-1529, 2002.
234. Jaquenod De Giusti C, Blanco PG, Lamas PA, Carrizo Velasquez F, Lofeudo JM, Portiansky EL, and Alvarez BV. Carbonic anhydrase II/sodium-proton exchanger 1 metabolon complex in cardiomyopathy of ob(-/-) type 2 diabetic mice. *J Mol Cell Cardiol* 136: 53-63, 2019.
235. Brown BF, Quon A, Dyck JR, and Casey JR. Carbonic anhydrase II promotes cardiomyocyte hypertrophy. *Can J Physiol Pharmacol* 90: 1599-1610, 2012.
236. Becker HM, Hirnet D, Fecher-Trost C, Sultemeyer D, and Deitmer JW. Transport activity of MCT1 expressed in Xenopus oocytes is increased by interaction with carbonic anhydrase. *J Biol Chem* 280: 39882-39889, 2005.
237. Noor SI, Pouyssegur J, Deitmer JW, and Becker HM. Integration of a 'proton antenna' facilitates transport activity of the monocarboxylate transporter MCT4. *FEBS J* 284: 149-162, 2017.
238. Parkkila S, and Parkkila AK. Carbonic anhydrase in the alimentary tract. Roles of the different isozymes and salivary factors in the maintenance of optimal conditions in the gastrointestinal canal. *Scand J Gastroenterol* 31: 305-317, 1996.
239. Leppilampi M, Parkkila S, Karttunen T, Gut MO, Gros G, and Sjoblom M. Carbonic anhydrase isozyme-II-deficient mice lack the duodenal bicarbonate secretory response to prostaglandin E2. *Proc Natl Acad Sci U S A* 102: 15247-15252, 2005.
240. Kaunisto K, Parkkila S, Parkkila AK, Waheed A, Sly WS, and Rajaniemi H. Expression of carbonic anhydrase isoenzymes IV and II in rat epididymal duct. *Biol Reprod* 52: 1350-1357, 1995.
241. Parkkila S, Parkkila AK, Kaunisto K, Waheed A, Sly WS, and Rajaniemi H. Location of a membrane-bound carbonic anhydrase isoenzyme (CA IV) in the human male reproductive tract. *J Histochem Cytochem* 41: 751-757, 1993.
242. Spicer SS, Ge ZH, Tashian RE, Hazen-Martin DJ, and Schulte BA. Comparative distribution of carbonic anhydrase isozymes III and II in rodent tissues. *Am J Anat* 187: 55-64, 1990.
243. Lynch CJ, Brennan WA, Jr., Vary TC, Carter N, and Dodgson SJ. Carbonic anhydrase III in obese Zucker rats. *Am J Physiol* 264: E621-630, 1993.
244. Lyons GE, Buckingham ME, Tweedie S, and Edwards YH. Carbonic anhydrase III, an early mesodermal marker, is expressed in embryonic mouse skeletal muscle and notochord. *Development* 111: 233-244, 1991.
245. Raisanen SR, Lehenkari P, Tasanen M, Rahkila P, Harkonen PL, and Vaananen HK. Carbonic anhydrase III protects cells from hydrogen peroxide-induced apoptosis. *FASEB J* 13: 513-522, 1999.
246. Zimmerman UJ, Wang P, Zhang X, Bogdanovich S, and Forster R. Anti-oxidative response of carbonic anhydrase III in skeletal muscle. *IUBMB Life* 56: 343-347, 2004.
247. Kim G, Lee TH, Wetzel P, Geers C, Robinson MA, Myers TG, Owens JW, Wehr NB, Eckhaus MW, Gros G, Wynshaw-Boris A, and Levine RL. Carbonic anhydrase III is not required in the mouse for normal growth, development, and life span. *Mol Cell Biol* 24: 9942-9947, 2004.
248. Vaananen HK, Paloniemi M, and Vuori J. Purification and localization of human carbonic anhydrase. III. Typing of skeletal muscle fibers in paraffin embedded sections. *Histochemistry* 83: 231-235, 1985.
249. Liu M, Walter GA, Pathare NC, Forster RE, and Vandenborne K. A quantitative study of bioenergetics in skeletal muscle lacking carbonic anhydrase III using 31P magnetic resonance spectroscopy. *Proc Natl Acad Sci U S A* 104: 371-376, 2007.
250. Feng HZ, and Jin JP. Carbonic Anhydrase III Is Expressed in Mouse Skeletal Muscles Independent of Fiber Type-Specific Myofilament Protein Isoforms and Plays a Role in Fatigue Resistance. *Front Physiol* 7: 597, 2016.

251. Mitterberger MC, Kim G, Rostek U, Levine RL, and Zwerschke W. Carbonic anhydrase III regulates peroxisome proliferator-activated receptor-gamma2. *Exp Cell Res* 318: 877-886, 2012.
252. Renner SW, Walker LM, Forsberg LJ, Sexton JZ, and Brenman JE. Carbonic anhydrase III (Car3) is not required for fatty acid synthesis and does not protect against high-fat diet induced obesity in mice. *PLoS One* 12: e0176502, 2017.
253. Whitney PL, and Briggle TV. Membrane-associated carbonic anhydrase purified from bovine lung. *J Biol Chem* 257: 12056-12059, 1982.
254. Wistrand PJ, and Knuuttila KG. Renal membrane-bound carbonic anhydrase. Purification and properties. *Kidney Int* 35: 851-859, 1989.
255. Zhu XL, and Sly WS. Carbonic anhydrase IV from human lung. Purification, characterization, and comparison with membrane carbonic anhydrase from human kidney. *J Biol Chem* 265: 8795-8801, 1990.
256. Brown D, Zhu XL, and Sly WS. Localization of membrane-associated carbonic anhydrase type IV in kidney epithelial cells. *Proc Natl Acad Sci U S A* 87: 7457-7461, 1990.
257. Fleming RE, Parkkila S, Parkkila AK, Rajaniemi H, Waheed A, and Sly WS. Carbonic anhydrase IV expression in rat and human gastrointestinal tract regional, cellular, and subcellular localization. *J Clin Invest* 96: 2907-2913, 1995.
258. Parkkila S, Parkkila AK, Juvonen T, Waheed A, Sly WS, Saarnio J, Kaunisto K, Kellokumpu S, and Rajaniemi H. Membrane-bound carbonic anhydrase IV is expressed in the luminal plasma membrane of the human gallbladder epithelium. *Hepatology* 24: 1104-1108, 1996.
259. Kaunisto K, Parkkila S, Rajaniemi H, Waheed A, Grubb J, and Sly WS. Carbonic anhydrase XIV: luminal expression suggests key role in renal acidification. *Kidney Int* 61: 2111-2118, 2002.
260. Scheibe RJ, Mundhenk K, Becker T, Hallerdei J, Waheed A, Shah GN, Sly WS, Gros G, and Wetzel P. Carbonic anhydrases IV and IX: subcellular localization and functional role in mouse skeletal muscle. *Am J Physiol Cell Physiol* 294: C402-412, 2008.
261. Kyllonen MS, Parkkila S, Rajaniemi H, Waheed A, Grubb JH, Shah GN, Sly WS, and Kaunisto K. Localization of carbonic anhydrase XII to the basolateral membrane of H+-secreting cells of mouse and rat kidney. *J Histochem Cytochem* 51: 1217-1224, 2003.
262. Saari S, Hilvo M, Pan P, Gros G, Hanke N, Waheed A, Sly WS, and Parkkila S. The most recently discovered carbonic anhydrase, CA XV, is expressed in the thick ascending limb of Henle and in the collecting ducts of mouse kidney. *PLoS One* 5: e9624, 2010.
263. Decker B, Sender S, and Gros G. Membrane-associated carbonic anhydrase IV in skeletal muscle: subcellular localization. *Histochem Cell Biol* 106: 405-411, 1996.
264. Scheibe RJ, Gros G, Parkkila S, Waheed A, Grubb JH, Shah GN, Sly WS, and Wetzel P. Expression of membrane-bound carbonic anhydrases IV, IX, and XIV in the mouse heart. *J Histochem Cytochem* 54: 1379-1391, 2006.
265. Wandernoth PM, Raubuch M, Mannowetz N, Becker HM, Deitmer JW, Sly WS, and Wennemuth G. Role of carbonic anhydrase IV in the bicarbonate-mediated activation of murine and human sperm. *PLoS One* 5: e15061, 2010.
266. Dodgson SJ, and Forster RE, 2nd. Inhibition of CA V decreases glucose synthesis from pyruvate. *Arch Biochem Biophys* 251: 198-204, 1986.
267. Fujikawa-Adachi K, Nishimori I, Taguchi T, and Onishi S. Human mitochondrial carbonic anhydrase VB. cDNA cloning, mRNA expression, subcellular localization, and mapping to chromosome x. *J Biol Chem* 274: 21228-21233, 1999.
268. Nagao Y, Platero JS, Waheed A, and Sly WS. Human mitochondrial carbonic anhydrase: cDNA cloning, expression, subcellular localization, and mapping to chromosome 16. *Proc Natl Acad Sci U S A* 90: 7623-7627, 1993.
269. Shah GN, Hewett-Emmett D, Grubb JH, Migas MC, Fleming RE, Waheed A, and Sly WS. Mitochondrial carbonic anhydrase CA VB: differences in tissue distribution and pattern of evolution from those of CA VA suggest distinct physiological roles. *Proc Natl Acad Sci U S A* 97: 1677-1682, 2000.
270. Dodgson SJ, and Forster RE, 2nd. Carbonic anhydrase: inhibition results in decreased urea production by hepatocytes. *J Appl Physiol (1985)* 60: 646-652, 1986.

271. Lynch CJ, Fox H, Hazen SA, Stanley BA, Dodgson S, and Lanoue KF. Role of hepatic carbonic anhydrase in de novo lipogenesis. *Biochem J* 310 (Pt 1): 197-202, 1995.
272. Arechederra RL, Waheed A, Sly WS, Supuran CT, and Minteer SD. Effect of sulfonamides as carbonic anhydrase VA and VB inhibitors on mitochondrial metabolic energy conversion. *Bioorg Med Chem* 21: 1544-1548, 2013.
273. Bernardino RL, Dias TR, Moreira BP, Cunha M, Barros A, Oliveira E, Sousa M, Alves MG, and Oliveira PF. Carbonic anhydrases are involved in mitochondrial biogenesis and control the production of lactate by human Sertoli cells. *FEBS J* 286: 1393-1406, 2019.
274. Shah GN, Rubbelke TS, Hendin J, Nguyen H, Waheed A, Shoemaker JD, and Sly WS. Targeted mutagenesis of mitochondrial carbonic anhydrases VA and VB implicates both enzymes in ammonia detoxification and glucose metabolism. *Proc Natl Acad Sci U S A* 110: 7423-7428, 2013.
275. Henkin RI, Lippoldt RE, Bilstad J, and Edelhoch H. A zinc protein isolated from human parotid saliva. *Proceedings of the National Academy of Sciences of the United States of America* 72: 488-492, 1975.
276. Thatcher BJ, Doherty AE, Orvisky E, Martin BM, and Henkin RI. Gustin from human parotid saliva is carbonic anhydrase VI. *Biochemical and biophysical research communications* 250: 635-641, 1998.
277. Fernley RT, Wright RD, and Coghlan JP. A novel carbonic anhydrase from the ovine parotid gland. *FEBS Lett* 105: 299-302, 1979.
278. Feldstein JB, and Silverman DN. Purification and characterization of carbonic anhydrase from the saliva of the rat. *The Journal of biological chemistry* 259: 5447-5453, 1984.
279. Murakami H, and Sly WS. Purification and characterization of human salivary carbonic anhydrase. *The Journal of biological chemistry* 262: 1382-1388, 1987.
280. Kadoya Y, Kuwahara H, Shimazaki M, Ogawa Y, and Yagi T. Isolation of a novel carbonic anhydrase from human saliva and immunohistochemical demonstration of its related isozymes in salivary gland. *Osaka city medical journal* 33: 99-109, 1987.
281. Parkkila S, Kaunisto K, Rajaniemi L, Kumpulainen T, Jokinen K, and Rajaniemi H. Immunohistochemical localization of carbonic anhydrase isoenzymes VI, II, and I in human parotid and submandibular glands. *J Histochem Cytochem* 38: 941-947, 1990.
282. Ogawa Y, Matsumoto K, Maeda T, Tamai R, Suzuki T, Sasano H, and Fernley RT. Characterization of lacrimal gland carbonic anhydrase VI. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* 50: 821-827, 2002.
283. Karhumaa P, Leinonen J, Parkkila S, Kaunisto K, Tapanainen J, and Rajaniemi H. The identification of secreted carbonic anhydrase VI as a constitutive glycoprotein of human and rat milk. *Proc Natl Acad Sci U S A* 98: 11604-11608, 2001.
284. Kivela J, Parkkila S, Parkkila AK, and Rajaniemi H. A low concentration of carbonic anhydrase isoenzyme VI in whole saliva is associated with caries prevalence. *Caries Res* 33: 178-184, 1999.
285. Culp DJ, Robinson B, Parkkila S, Pan PW, Cash MN, Truong HN, Hussey TW, and Gullett SL. Oral colonization by Streptococcus mutans and caries development is reduced upon deletion of carbonic anhydrase VI expression in saliva. *Biochim Biophys Acta* 1812: 1567-1576, 2011.
286. Patrikainen MS, Pan P, Barker HR, and Parkkila S. Altered gene expression in the lower respiratory tract of Car6 (-/-) mice. *Transgenic Res* 25: 649-664, 2016.
287. Patrikainen M, Pan P, Kulesskaya N, Voikar V, and Parkkila S. The role of carbonic anhydrase VI in bitter taste perception: evidence from the Car6(-)/(-) mouse model. *J Biomed Sci* 21: 82, 2014.
288. Sok J, Wang XZ, Batchvarova N, Kuroda M, Harding H, and Ron D. CHOP-Dependent stress-inducible expression of a novel form of carbonic anhydrase VI. *Mol Cell Biol* 19: 495-504, 1999.
289. Matthews TA, Abel A, Demme C, Sherman T, Pan PW, Halterman MW, Parkkila S, and Nehrke K. Expression of the CHOP-inducible carbonic anhydrase CAVI-b is required for BDNF-mediated protection from hypoxia. *Brain Res* 1543: 28-37, 2014.
290. Xu J, Xu X, Wang B, Ma Y, Zhang L, Xu H, Hu Y, Wu J, and Cao X. Nuclear carbonic anhydrase 6B associates with PRMT5 to epigenetically promote IL-12 expression in innate response. *Proc Natl Acad Sci U S A* 114: 8620-8625, 2017.

291. Aspatwar A, Tolvanen ME, Ortutay C, and Parkkila S. Carbonic anhydrase related protein VIII and its role in neurodegeneration and cancer. *Curr Pharm Des* 16: 3264-3276, 2010.
292. Aspatwar A, Tolvanen ME, Ortutay C, and Parkkila S. Carbonic anhydrase related proteins: molecular biology and evolution. *Subcell Biochem* 75: 135-156, 2014.
293. Kato K. A Collection of cDNA Clones with Specific Expression Patterns in Mouse Brain. *Eur J Neurosci* 2: 704-711, 1990.
294. Hewett-Emmett D, and Tashian RE. Functional diversity, conservation, and convergence in the evolution of the alpha-, beta-, and gamma-carbonic anhydrase gene families. *Mol Phylogenet Evol* 5: 50-77, 1996.
295. Lakkis MM, Bergenhem NC, O'Shea KS, and Tashian RE. Expression of the acatalytic carbonic anhydrase VIII gene, Car8, during mouse embryonic development. *Histochem J* 29: 135-141, 1997.
296. Taniuchi K, Nishimori I, Takeuchi T, Ohtsuki Y, and Onishi S. cDNA cloning and developmental expression of murine carbonic anhydrase-related proteins VIII, X, and XI. *Brain Res Mol Brain Res* 109: 207-215, 2002.
297. Jiao Y, Yan J, Zhao Y, Donahue LR, Beamer WG, Li X, Roe BA, Ledoux MS, and Gu W. Carbonic anhydrase-related protein VIII deficiency is associated with a distinctive lifelong gait disorder in waddles mice. *Genetics* 171: 1239-1246, 2005.
298. Pastorekova S, Parkkila S, Parkkila AK, Opavsky R, Zelnik V, Saarnio J, and Pastorek J. Carbonic anhydrase IX, MN/CA IX: analysis of stomach complementary DNA sequence and expression in human and rat alimentary tracts. *Gastroenterology* 112: 398-408, 1997.
299. Saarnio J, Parkkila S, Parkkila AK, Waheed A, Casey MC, Zhou XY, Pastorekova S, Pastorek J, Karttunen T, Haukipuro K, Kairaluoma MI, and Sly WS. Immunohistochemistry of carbonic anhydrase isozyme IX (MN/CA IX) in human gut reveals polarized expression in the epithelial cells with the highest proliferative capacity. *J Histochem Cytochem* 46: 497-504, 1998.
300. Karhumaa P, Kaunisto K, Parkkila S, Waheed A, Pastorekova S, Pastorek J, Sly WS, and Rajaniemi H. Expression of the transmembrane carbonic anhydrases, CA IX and CA XII, in the human male excurrent ducts. *Mol Hum Reprod* 7: 611-616, 2001.
301. Syrjanen L, Luukkaala T, Leppilampi M, Kallioinen M, Pastorekova S, Pastorek J, Waheed A, Sly WS, Parkkila S, and Karttunen T. Expression of cancer-related carbonic anhydrases IX and XII in normal skin and skin neoplasms. *APMIS* 122: 880-889, 2014.
302. Abe S, Nakao T, Yoshimoto T, Parkkila S, Murakami G, and Cho BH. Expression of carbonic anhydrase in the fetal eye and extra-ocular tissues. *Okajimas Folia Anat Jpn* 90: 59-68, 2013.
303. Ivanov S, Liao SY, Ivanova A, Danilkovitch-Miagkova A, Tarasova N, Weirich G, Merrill MJ, Proescholdt MA, Oldfield EH, Lee J, Zavada J, Waheed A, Sly W, Lerman MI, and Stanbridge EJ. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. *Am J Pathol* 158: 905-919, 2001.
304. Turner JR, Odze RD, Crum CP, and Resnick MB. MN antigen expression in normal, preneoplastic, and neoplastic esophagus: a clinicopathological study of a new cancer-associated biomarker. *Hum Pathol* 28: 740-744, 1997.
305. Takacova M, Barathova M, Zatovicova M, Golias T, Kajanova I, Jelenska L, Sedlakova O, Svastova E, Kopacek J, and Pastorekova S. Carbonic Anhydrase IX-Mouse versus Human. *Int J Mol Sci* 21: 2019.
306. Wykoff CC, Beasley NJ, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talks KL, Maxwell PH, Pugh CW, Ratcliffe PJ, and Harris AL. Hypoxia-inducible expression of tumor-associated carbonic anhydrases. *Cancer Res* 60: 7075-7083, 2000.
307. Haapasalo JA, Nordfors KM, Hilvo M, Rantala IJ, Soini Y, Parkkila AK, Pastorekova S, Pastorek J, Parkkila SM, and Haapasalo HK. Expression of carbonic anhydrase IX in astrocytic tumors predicts poor prognosis. *Clin Cancer Res* 12: 473-477, 2006.
308. Chia SK, Wykoff CC, Watson PH, Han C, Leek RD, Pastorek J, Gatter KC, Ratcliffe P, and Harris AL. Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase IX, in invasive breast carcinoma. *J Clin Oncol* 19: 3660-3668, 2001.

309. Choi SW, Kim JY, Park JY, Cha IH, Kim J, and Lee S. Expression of carbonic anhydrase IX is associated with postoperative recurrence and poor prognosis in surgically treated oral squamous cell carcinoma. *Hum Pathol* 39: 1317-1322, 2008.
310. Giatromanolaki A, Koukourakis MI, Sivridis E, Pastorek J, Wykoff CC, Gatter KC, and Harris AL. Expression of hypoxia-inducible carbonic anhydrase-9 relates to angiogenic pathways and independently to poor outcome in non-small cell lung cancer. *Cancer Res* 61: 7992-7998, 2001.
311. Gut MO, Parkkila S, Vernerova Z, Rohde E, Zavada J, Hocker M, Pastorek J, Karttunen T, Gibadulinova A, Zavadova Z, Knobeloch KP, Wiedenmann B, Svoboda J, Horak I, and Pastorekova S. Gastric hyperplasia in mice with targeted disruption of the carbonic anhydrase gene Car9. *Gastroenterology* 123: 1889-1903, 2002.
312. Leppilampi M, Karttunen TJ, Kivela J, Gut MO, Pastorekova S, Pastorek J, and Parkkila S. Gastric pit cell hyperplasia and glandular atrophy in carbonic anhydrase IX knockout mice: studies on two strains C57/BL6 and BALB/C. *Transgenic Res* 14: 655-663, 2005.
313. Lee SA, Kang D, Shim KN, Choe JW, Hong WS, and Choi H. Effect of diet and Helicobacter pylori infection to the risk of early gastric cancer. *J Epidemiol* 13: 162-168, 2003.
314. Fox JG, Dangler CA, Taylor NS, King A, Koh TJ, and Wang TC. High-salt diet induces gastric epithelial hyperplasia and parietal cell loss, and enhances Helicobacter pylori colonization in C57BL/6 mice. *Cancer Res* 59: 4823-4828, 1999.
315. Pan PW, Parkkila AK, Autio S, Hilvo M, Sormunen R, Pastorekova S, Pastorek J, Haapasalo H, and Parkkila S. Brain phenotype of carbonic anhydrase IX-deficient mice. *Transgenic Res* 21: 163-176, 2012.
316. Hilvo M, Innocenti A, Monti SM, De Simone G, Supuran CT, and Parkkila S. Recent advances in research on the most novel carbonic anhydrases, CA XIII and XV. *Curr Pharm Des* 14: 672-678, 2008.
317. Nortunen M, Huhta H, Helminen O, Parkkila S, Kauppila JH, Karttunen TJ, and Saarnio J. Carbonic anhydrases II, IX, and XII in Barrett's esophagus and adenocarcinoma. *Virchows Arch* 473: 567-575, 2018.
318. Gottsch JD, Seitzman GD, Margulies EH, Bowers AL, Michels AJ, Saha S, Jun AS, Stark WJ, and Liu SH. Gene expression in donor corneal endothelium. *Arch Ophthalmol* 121: 252-258, 2003.
319. Geurts AM, Wilber A, Carlson CM, Lobitz PD, Clark KJ, Hackett PB, McIvor RS, and Largaespada DA. Conditional gene expression in the mouse using a Sleeping Beauty gene-trap transposon. *BMC Biotechnol* 6: 30, 2006.
320. Fujikawa-Adachi K, Nishimori I, Taguchi T, and Onishi S. Human carbonic anhydrase XIV (CA14): cDNA cloning, mRNA expression, and mapping to chromosome 1. *Genomics* 61: 74-81, 1999.
321. Parkkila S, Kivela AJ, Kaunisto K, Parkkila AK, Hakkola J, Rajaniemi H, Waheed A, and Sly WS. The plasma membrane carbonic anhydrase in murine hepatocytes identified as isozyme XIV. *BMC Gastroenterol* 2: 13, 2002.
322. Nagelhus EA, Mathiisen TM, Bateman AC, Haug FM, Ottersen OP, Grubb JH, Waheed A, and Sly WS. Carbonic anhydrase XIV is enriched in specific membrane domains of retinal pigment epithelium, Muller cells, and astrocytes. *Proc Natl Acad Sci U S A* 102: 8030-8035, 2005.
323. Hermo L, Chong DL, Moffatt P, Sly WS, Waheed A, and Smith CE. Region- and cell-specific differences in the distribution of carbonic anhydrases II, III, XII, and XIV in the adult rat epididymis. *J Histochem Cytochem* 53: 699-713, 2005.
324. Wetzel P, Scheibe RJ, Hellmann B, Hallerdei J, Shah GN, Waheed A, Gros G, and Sly WS. Carbonic anhydrase XIV in skeletal muscle: subcellular localization and function from wild-type and knockout mice. *Am J Physiol Cell Physiol* 293: C358-366, 2007.
325. Streisinger G, Okada Y, Emrich J, Newton J, Tsugita A, Terzaghi E, and Inouye M. Frameshift mutations and the genetic code. This paper is dedicated to Professor Theodosius Dobzhansky on the occasion of his 66th birthday. *Cold Spring Harbor symposia on quantitative biology* 31: 77-84, 1966.
326. Streisinger G, Walker C, Dower N, Knauber D, and Singer F. Production of clones of homozygous diploid zebra fish (Brachydanio rerio). *Nature* 291: 293-296, 1981.

327. Schulte-Merker S, Hammerschmidt M, Beuchle D, Cho KW, De Robertis EM, and Nusslein-Volhard C. Expression of zebrafish goosecoid and no tail gene products in wild-type and mutant no tail embryos. *Development (Cambridge, England)* 120: 843-852, 1994.
328. Weinstein BM, Stemple DL, Driever W, and Fishman MC. Gridlock, a localized heritable vascular patterning defect in the zebrafish. *Nature medicine* 1: 1143-1147, 1995.
329. Halpern ME, Ho RK, Walker C, and Kimmel CB. Induction of muscle pioneers and floor plate is distinguished by the zebrafish no tail mutation. *Cell* 75: 99-111, 1993.
330. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, McLaren S, Sealy I, Caccamo M, Churcher C, Scott C, Barrett JC, Koch R, Rauch GJ, White S, Chow W, Kilian B, Quintais LT, Guerra-Assuncao JA, Zhou Y, Gu Y, Yen J, Vogel JH, Eyre T, Redmond S, Banerjee R, Chi J, Fu B, Langley E, Maguire SF, Laird GK, Lloyd D, Kenyon E, Donaldson S, Sehra H, Almeida-King J, Loveland J, Trevanion S, Jones M, Quail M, Willey D, Hunt A, Burton J, Sims S, McLay K, Plumb B, Davis J, Clee C, Oliver K, Clark R, Riddle C, Elliot D, Threadgold G, Harden G, Ware D, Begum S, Mortimore B, Kerry G, Heath P, Phillimore B, Tracey A, Corby N, Dunn M, Johnson C, Wood J, Clark S, Pelan S, Griffiths G, Smith M, Glithero R, Howden P, Barker N, Lloyd C, Stevens C, Harley J, Holt K, Panagiotidis G, Lovell J, Beasley H, Henderson C, Gordon D, Auger K, Wright D, Collins J, Raisen C, Dyer L, Leung K, Robertson L, Ambridge K, Leongamornlert D, McGuire S, Gilderthorp R, Griffiths C, Manthravadi D, Nichol S, Barker G, Whitehead S, Kay M, Brown J, Murnane C, Gray E, Humphries M, Sycamore N, Barker D, Saunders D, Wallis J, Babbage A, Hammond S, Mashreghi-Mohammadi M, Barr L, Martin S, Wray P, Ellington A, Matthews N, Ellwood M, Woodmansey R, Clark G, Cooper J, Tromans A, Grafham D, Skuce C, Pandian R, Andrews R, Harrison E, Kimberley A, Garnett J, Fosker N, Hall R, Garner P, Kelly D, Bird C, Palmer S, Gehring I, Berger A, Dooley CM, Ersan-Urun Z, Eser C, Geiger H, Geisler M, Karotki L, Kirn A, Konantz J, Konantz M, Oberlander M, Rudolph-Geiger S, Teucke M, Lanz C, Raddatz G, Osoegawa K, Zhu B, Rapp A, Widaa S, Langford C, Yang F, Schuster SC, Carter NP, Harrow J, Ning Z, Herrero J, Searle SM, Enright A, Geisler R, Plasterk RH, Lee C, Westerfield M, de Jong PJ, Zon LI, Postlethwait JH, Nusslein-Volhard C, Hubbard TJ, Roest Crollius H, Rogers J, and Stemple DL. The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496: 498-503, 2013.
331. Kalueff AV, Echevarria DJ, and Stewart AM. Gaining translational momentum: more zebrafish models for neuroscience research. *Progress in neuro-psychopharmacology & biological psychiatry* 55: 1-6, 2014.
332. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, and Schilling TF. Stages of embryonic development of the zebrafish. *Developmental dynamics : an official publication of the American Association of Anatomists* 203: 253-310, 1995.
333. Cha YR, and Weinstein BM. Visualization and experimental analysis of blood vessel formation using transgenic zebrafish. *Birth defects research Part C, Embryo today : reviews* 81: 286-296, 2007.
334. Hocking JC, Distel M, and Koster RW. Studying cellular and subcellular dynamics in the developing zebrafish nervous system. *Experimental neurology* 242: 1-10, 2013.
335. Rieger S, Wang F, and Sagasti A. Time-lapse imaging of neural development: zebrafish lead the way into the fourth dimension. *Genesis (New York, NY : 2000)* 49: 534-545, 2011.
336. Baeten JT, and de Jong JLO. Genetic Models of Leukemia in Zebrafish. *Front Cell Dev Biol* 6: 115, 2018.
337. Stuart GW, McMurray JV, and Westerfield M. Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. *Development* 103: 403-412, 1988.
338. Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, and Joung JK. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* 31: 227-229, 2013.
339. Nasevicius A, and Ekker SC. Effective targeted gene 'knockdown' in zebrafish. *Nat Genet* 26: 216-220, 2000.
340. Wienholds E, van Eeden F, Kosters M, Mudde J, Plasterk RH, and Cuppen E. Efficient target-selected mutagenesis in zebrafish. *Genome research* 13: 2700-2707, 2003.

341. Doyon Y, McCammon JM, Miller JC, Faraji F, Ngo C, Katibah GE, Amora R, Hocking TD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, and Amacher SL. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nature biotechnology* 26: 702-708, 2008.
342. Huang P, Xiao A, Zhou M, Zhu Z, Lin S, and Zhang B. Heritable gene targeting in zebrafish using customized TALENs. *Nature biotechnology* 29: 699-700, 2011.
343. Irion U, Krauss J, and Nusslein-Volhard C. Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. *Development (Cambridge, England)* 141: 4827-4830, 2014.
344. Bussmann J, and Schulte-Merker S. Rapid BAC selection for tol2-mediated transgenesis in zebrafish. *Development (Cambridge, England)* 138: 4327-4332, 2011.
345. Ferreira-Martins D, McCormick SD, Campos A, Lopes-Marques M, Osorio H, Coimbra J, Castro LF, and Wilson JM. A cytosolic carbonic anhydrase molecular switch occurs in the gills of metamorphic sea lamprey. *Sci Rep* 6: 33954, 2016.
346. Gilmour KM, and Perry SF. Carbonic anhydrase and acid-base regulation in fish. *J Exp Biol* 212: 1647-1661, 2009.
347. Bayaa M, Vulesevic B, Esbaugh A, Braun M, Ekker ME, Grosell M, and Perry SF. The involvement of SLC26 anion transporters in chloride uptake in zebrafish (Danio rerio) larvae. *J Exp Biol* 212: 3283-3295, 2009.
348. Zimmer AM, Mandic M, Yew HM, Kunert E, Pan YK, Ha J, Kwong RWM, Gilmour KM, and Perry SF. Use of a carbonic anhydrase Ca17a knockout to investigate mechanisms of ion uptake in zebrafish (Danio rerio). *Am J Physiol Regul Integr Comp Physiol* 320: R55-R68, 2021.
349. Lin TY, Liao BK, Horng JL, Yan JJ, Hsiao CD, and Hwang PP. Carbonic anhydrase 2-like a and 15a are involved in acid-base regulation and Na+ uptake in zebrafish H+-ATPase-rich cells. *Am J Physiol Cell Physiol* 294: C1250-1260, 2008.
350. Patrikainen MS, Tolvanen MEE, Aspatwar A, Barker HR, Ortutay C, Janis J, Laitaoja M, Hytonen VP, Azizi L, Manandhar P, Jager E, Vullo D, Kukkurainen S, Hilvo M, Supuran CT, and Parkkila S. Identification and characterization of a novel zebrafish (Danio rerio) pentraxin-carbonic anhydrase. *PeerJ* 5: e4128, 2017.
351. Aspatwar A, Tolvanen ME, Jokitalo E, Parikka M, Ortutay C, Harjula SK, Ramet M, Vihinen M, and Parkkila S. Abnormal cerebellar development and ataxia in CARP VIII morphant zebrafish. *Hum Mol Genet* 22: 417-432, 2013.
352. Aspatwar A, Tolvanen ME, Ojanen MJ, Barker HR, Saralahti AK, Bauerlein CA, Ortutay C, Pan P, Kuuslahti M, Parikka M, Ramet M, and Parkkila S. Inactivation of ca10a and ca10b Genes Leads to Abnormal Embryonic Development and Alters Movement Pattern in Zebrafish. *PLoS One* 10: e0134263, 2015.
353. Raja DA, Gotherwal V, Burse SA, Subramaniam YJ, Sultan F, Vats A, Gautam H, Sharma B, Sharma S, Singh A, Sivasubbu S, Gokhale RS, and Natarajan VT. pH-controlled histone acetylation amplifies melanocyte differentiation downstream of MITF. *EMBO Rep* 21: e48333, 2020.
354. Ito Y, Kobayashi S, Nakamura N, Miyagi H, Esaki M, Hoshijima K, and Hirose S. Close Association of Carbonic Anhydrase (CA2a and CA15a), Na(+)/H(+) Exchanger (Nhe3b), and Ammonia Transporter Rhcg1 in Zebrafish Ionocytes Responsible for Na(+) Uptake. *Front Physiol* 4: 59, 2013.
355. Shih TH, Horng JL, Liu ST, Hwang PP, and Lin LY. Rhcg1 and NHE3b are involved in ammonium-dependent sodium uptake by zebrafish larvae acclimated to low-sodium water. *Am J Physiol Regul Integr Comp Physiol* 302: R84-93, 2012.
356. Kumai Y, and Perry SF. Mechanisms and regulation of Na(+) uptake by freshwater fish. *Respir Physiol Neurobiol* 184: 249-256, 2012.
357. Hirota J, Ando H, Hamada K, and Mikoshiba K. Carbonic anhydrase-related protein is a novel binding protein for inositol 1,4,5-trisphosphate receptor type 1. *Biochem J* 372: 435-441, 2003.
358. Upadhyay U, Zhuang GZ, Diatchenko L, Parisien M, Kang Y, Sarantopoulos KD, Martin ER, Smith SB, Maixner W, and Levitt RC. Reversion mutation of cDNA CA8-204 minigene construct produces a truncated functional peptide that regulates calcium release in vitro and produces profound analgesia in vivo. *Mamm Genome* 31: 287-294, 2020.

359. Lovejoy DA, Hewett-Emmett D, Porter CA, Cepoi D, Sheffield A, Vale WW, and Tashian RE. Evolutionarily conserved, "acatalytic" carbonic anhydrase-related protein XI contains a sequence motif present in the neuropeptide sauvagine: the human CA-RP XI gene (CA11) is embedded between the secretor gene cluster and the DBP gene at 19q13.3. *Genomics* 54: 484-493, 1998.
360. Okamoto N, Fujikawa-Adachi K, Nishimori I, Taniuchi K, and Onishi S. cDNA sequence of human carbonic anhydrase-related protein, CA-RP X: mRNA expressions of CA-RP X and XI in human brain. *Biochim Biophys Acta* 1518: 311-316, 2001.
361. Bellingham J, Gregory-Evans K, and Gregory-Evans CY. Sequence and tissue expression of a novel human carbonic anhydrase-related protein, CARP-2, mapping to chromosome 19q13.3. *Biochem Biophys Res Commun* 253: 364-367, 1998.
362. Fujikawa-Adachi K, Nishimori I, Taguchi T, Yuri K, and Onishi S. cDNA sequence, mRNA expression, and chromosomal localization of human carbonic anhydrase-related protein, CA-RP XI. *Biochim Biophys Acta* 1431: 518-524, 1999.
363. Goding CR, and Arnheiter H. MITF-the first 25 years. *Genes Dev* 33: 983-1007, 2019.
364. Hoek KS, Schlegel NC, Eichhoff OM, Widmer DS, Praetorius C, Einarsson SO, Valgeirsdottir S, Bergsteinsdottir K, Schepsky A, Dummer R, and Steingrimsson E. Novel MITF targets identified using a two-step DNA microarray strategy. *Pigment cell & melanoma research* 21: 665-676, 2008.
365. Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, George RA, Lewis SE, Richards S, Ashburner M, Henderson SN, Sutton GG, Wortman JR, Yandell MD, Zhang Q, Chen LX, Brandon RC, Rogers YH, Blazej RG, Champe M, Pfeiffer BD, Wan KH, Doyle C, Baxter EG, Helt G, Nelson CR, Gabor GL, Abril JF, Agbayani A, An HJ, Andrews-Pfannkoch C, Baldwin D, Ballew RM, Basu A, Baxendale J, Bayraktaroglu L, Beasley EM, Beeson KY, Benos PV, Berman BP, Bhandari D, Bolshakov S, Borkova D, Botchan MR, Bouck J, Brokstein P, Brottier P, Burtis KC, Busam DA, Butler H, Cadieu E, Center A, Chandra I, Cherry JM, Cawley S, Dahlke C, Davenport LB, Davies P, de Pablos B, Delcher A, Deng Z, Mays AD, Dew I, Dietz SM, Dodson K, Doup LE, Downes M, Dugan-Rocha S, Dunkov BC, Dunn P, Durbin KJ, Evangelista CC, Ferraz C, Ferriera S, Fleischmann W, Fosler C, Gabrielian AE, Garg NS, Gelbart WM, Glasser K, Glodek A, Gong F, Gorrell JH, Gu Z, Guan P, Harris M, Harris NL, Harvey D, Heiman TJ, Hernandez JR, Houck J, Hostin D, Houston KA, Howland TJ, Wei MH, Ibegwam C, Jalali M, Kalush F, Karpen GH, Ke Z, Kennison JA, Ketchum KA, Kimmel BE, Kodira CD, Kraft C, Kravitz S, Kulp D, Lai Z, Lasko P, Lei Y, Levitsky AA, Li J, Li Z, Liang Y, Lin X, Liu X, Mattei B, McIntosh TC, McLeod MP, McPherson D, Merkulov G, Milshina NV, Mobarry C, Morris J, Moshrefi A, Mount SM, Moy M, Murphy B, Murphy L, Muzny DM, Nelson DL, Nelson DR, Nelson KA, Nixon K, Nusskern DR, Pacleb JM, Palazzolo M, Pittman GS, Pan S, Pollard J, Puri V, Reese MG, Reinert K, Remington K, Saunders RD, Scheeler F, Shen H, Shue BC, Siden-Kiamos I, Simpson M, Skupski MP, Smith T, Spier E, Spradling AC, Stapleton M, Strong R, Sun E, Svirskas R, Tector C, Turner R, Venter E, Wang AH, Wang X, Wang ZY, Wassarman DA, Weinstock GM, Weissenbach J, Williams SM, WoodageT, Worley KC, Wu D, Yang S, Yao QA, Ye J, Yeh RF, Zaveri JS, Zhan M, Zhang G, Zhao Q, Zheng L, Zheng XH, Zhong FN, Zhong W, Zhou X, Zhu S, Zhu X, Smith HO, Gibbs RA, Myers EW, Rubin GM, and Venter JC. The genome sequence of Drosophila melanogaster. *Science* 287: 2185-2195, 2000.
366. Graveley BR, Brooks AN, Carlson JW, Duff MO, Landolin JM, Yang L, Artieri CG, van Baren MJ, Boley N, Booth BW, Brown JB, Cherbas L, Davis CA, Dobin A, Li R, Lin W, Malone JH, Mattiuzzo NR, Miller D, Sturgill D, Tuch BB, Zaleski C, Zhang D, Blanchette M, Dudoit S, Eads B, Green RE, Hammonds A, Jiang L, Kapranov P, Langton L, Perrimon N, Sandler JE, Wan KH, Willingham A, Zhang Y, Zou Y, Andrews J, Bickel PJ, Brenner SE, Brent MR, Cherbas P, Gingeras TR, Hoskins RA, Kaufman TC, Oliver B, and Celniker SE. The developmental transcriptome of Drosophila melanogaster. *Nature* 471: 473-479, 2011.
367. Deng Q, Zeng Q, Qian Y, Li C, and Yang Y. Research on the karyotype and evolution of Drosophila melanogaster species group. *J Genet Genomics* 34: 196-213, 2007.
368. Rubin GM, Yandell MD, Wortman JR, Gabor Miklos GL, Nelson CR, Hariharan IK, Fortini ME, Li PW, Apweiler R, Fleischmann W, Cherry JM, Henikoff S, Skupski MP, Misra S, Ashburner M, Birney E, Boguski MS, Brody T, Brokstein P, Celniker SE, Chervitz SA, Coates D, Cravchik A, Gabrielian A, Galle RF, Gelbart WM, George RA, Goldstein LS, Gong F, Guan P, Harris NL, Hay BA, Hoskins RA, Li J, Li Z, Hynes RO, Jones SJ, Kuehl PM, Lemaitre B, Littleton JT, Morrison DK, Mungall C, O'Farrell PH, Pickeral OK, Shue C,

Vosshall LB, Zhang J, Zhao Q, Zheng XH, and Lewis S. Comparative genomics of the eukaryotes. *Science* 287: 2204-2215, 2000.
369. Yamaguchi M, and Yoshida H. Drosophila as a Model Organism. *Adv Exp Med Biol* 1076: 1-10, 2018.
370. Pandey UB, and Nichols CD. Human disease models in Drosophila melanogaster and the role of the fly in therapeutic drug discovery. *Pharmacol Rev* 63: 411-436, 2011.
371. Kaufman TC. A Short History and Description of Drosophila melanogaster Classical Genetics: Chromosome Aberrations, Forward Genetic Screens, and the Nature of Mutations. *Genetics* 206: 665-689, 2017.
372. Venken KJ, and Bellen HJ. Chemical mutagens, transposons, and transgenes to interrogate gene function in Drosophila melanogaster. *Methods* 68: 15-28, 2014.
373. Ida H, Suzusho N, Suyari O, Yoshida H, Ohno K, Hirose F, Itoh M, and Yamaguchi M. Genetic screening for modifiers of the DREF pathway in Drosophila melanogaster: identification and characterization of HP6 as a novel target of DREF. *Nucleic Acids Res* 37: 1423-1437, 2009.
374. Cook RK, Christensen SJ, Deal JA, Coburn RA, Deal ME, Gresens JM, Kaufman TC, and Cook KR. The generation of chromosomal deletions to provide extensive coverage and subdivision of the Drosophila melanogaster genome. *Genome Biol* 13: R21, 2012.
375. Valanne S. Functional genomic analysis of the Drosophila immune response. *Dev Comp Immunol* 42: 93-101, 2014.
376. Brand AH, and Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118: 401-415, 1993.
377. Port F, and Bullock SL. Creating Heritable Mutations in Drosophila with CRISPR-Cas9. *Methods Mol Biol* 1478: 145-160, 2016.
378. Ortutay C, Olatubosun A, Parkkila S, Vihinen M, and Tolvanen ME. An evolutionary analysis of insect carbonic anhydrases. In: *Adv Med Biol*, edited by Berhardt LV. Hauppauge: Nova Science Publishers, 2010, p. 145-168.
379. Syrjanen L, Tolvanen ME, Hilvo M, Vullo D, Carta F, Supuran CT, and Parkkila S. Characterization, bioinformatic analysis and dithiocarbamate inhibition studies of two new alpha-carbonic anhydrases, CAH1 and CAH2, from the fruit fly Drosophila melanogaster. *Bioorg Med Chem* 21: 1516-1521, 2013.
380. Francis SA, Taylor-Wells J, Gross AD, and Bloomquist JR. Toxicity and Physiological Actions of Carbonic Anhydrase Inhibitors to Aedes aegypti and Drosophila melanogaster. *Insects* 8: 2016.
381. Shanbhag S, and Tripathi S. Epithelial ultrastructure and cellular mechanisms of acid and base transport in the Drosophila midgut. *J Exp Biol* 212: 1731-1744, 2009.
382. Overend G, Luo Y, Henderson L, Douglas AE, Davies SA, and Dow JA. Molecular mechanism and functional significance of acid generation in the Drosophila midgut. *Sci Rep* 6: 27242, 2016.
383. Fasseas MK, Tsikou D, Flemetakis E, and Katinakis P. Molecular and biochemical analysis of the beta class carbonic anhydrases in Caenorhabditis elegans. *Mol Biol Rep* 37: 2941-2950, 2010.
384. Syrjanen L, Valanne S, Kuuslahti M, Tuomela T, Sriram A, Sanz A, Jacobs HT, Ramet M, and Parkkila S. beta carbonic anhydrase is required for female fertility in Drosophila melanogaster. *Front Zool* 12: 19, 2015.
385. Montell DJ, Rorth P, and Spradling AC. slow border cells, a locus required for a developmentally regulated cell migration during oogenesis, encodes Drosophila C/EBP. *Cell* 71: 51-62, 1992.
386. Heifetz Y, Lung O, Frongillo EA, Jr., and Wolfner MF. The Drosophila seminal fluid protein Acp26Aa stimulates release of oocytes by the ovary. *Curr Biol* 10: 99-102, 2000.
387. Stiernagle T. Maintenance of C. elegans. *WormBook* 1-11, 2006.
388. Corsi AK, Wightman B, and Chalfie M. A Transparent Window into Biology: A Primer on Caenorhabditis elegans. *Genetics* 200: 387-407, 2015.
389. Hu PJ. Dauer. *WormBook* 1-19, 2007.
390. Brenner S. The genetics of Caenorhabditis elegans. *Genetics* 77: 71-94, 1974.
391. Chalfie M, Tu Y, Euskirchen G, Ward WW, and Prasher DC. Green fluorescent protein as a marker for gene expression. *Science* 263: 802-805, 1994.

392. Boulin T, Etchberger JF, and Hobert O. Reporter gene fusions. *WormBook* 1-23, 2006.
393. Sulston JE, and Horvitz HR. Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. *Dev Biol* 56: 110-156, 1977.
394. White JG, Southgate E, Thomson JN, and Brenner S. The structure of the nervous system of the nematode Caenorhabditis elegans. *Philos Trans R Soc Lond B Biol Sci* 314: 1-340, 1986.
395. Consortium CeS. Genome sequence of the nematode C. elegans: a platform for investigating biology. *Science* 282: 2012-2018, 1998.
396. Zolfaghari Emameh R, Barker HR, Syrjanen L, Urbanski L, Supuran CT, and Parkkila S. Identification and inhibition of carbonic anhydrases from nematodes. *J Enzyme Inhib Med Chem* 31: 176-184, 2016.
397. Sherman TA, Rongali SC, Matthews TA, Pfeiffer J, and Nehrke K. Identification of a nuclear carbonic anhydrase in Caenorhabditis elegans. *Biochim Biophys Acta* 1823: 808-817, 2012.
398. Hall RA, Vullo D, Innocenti A, Scozzafava A, Supuran CT, Klappa P, and Muhlschlegel FA. External pH influences the transcriptional profile of the carbonic anhydrase, CAH-4b in Caenorhabditis elegans. *Mol Biochem Parasitol* 161: 140-149, 2008.
399. Moore J, Tetley L, and Devaney E. Identification of abundant mRNAs from the third stage larvae of the parasitic nematode, ostertagia ostertagi. *Biochem J* 347 Pt 3: 763-770, 2000.
400. Hallem EA, and Sternberg PW. Acute carbon dioxide avoidance in Caenorhabditis elegans. *Proc Natl Acad Sci U S A* 105: 8038-8043, 2008.