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The ADP-ribosyl cyclases - the current evolutionary state of the ARCs

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1. ABSTRACT

The major ADP-ribosylating enzyme families are the focus of this special issue of *Frontiers in Bioscience*. However, there is room for another family of enzymes with the capacity to utilize nicotinamide adenine dinucleotide (NAD): the ADP-ribosyl cyclases (ARCs). These unique enzymes catalyse the cyclization of NAD to cyclic ADP ribose (cADPR), a widely distributed second messenger. However, the ARCs are versatile enzymes that can manipulate NAD, NAD phosphate (NADP) and other substrates to generate various bioactive molecules including nicotinic acid adenine dinucleotide diphosphate (NAADP) and ADP ribose (ADPR). This review will focus on the group of well-characterized ARCs identified in invertebrate and vertebrate animals, whose common gene structure allows us to trace their origin to the ancestor of bilaterian animals. Behind a façade of gene and protein homology lies a family with a disparate functional repertoire dictated by the animal model and the physical trait under investigation. Here we present a phylogenetic view of the ARCs to better understand the evolution of function in this family.

2. INTRODUCTION

"In the past 150 years, many have worked to explain the cellular and molecular mechanisms leading to egg activation in sea urchins and starfish, frogs, mice and other oocytes and eggs, and because of the apparent morphogenesis of the fertilization envelope, and the ease of observing and manipulating fertilization, the sea urchin served (and remains today!) as a rich source for experimentation."

G. M. Wessel et al., The Biology of Cortical Granules (1)

Eggs, this "rich source for experimentation" have been a cornucopia for the ARCs. It was in the egg of the sea urchin *Lytechinus pictus* that cADPR was first identified as a mediator of stimulus-induced calcium release (2). It was another invertebrate egg, that of the sea slug *Aplysia californica*, that gave us the first enzyme capable of cADPR biosynthesis (3). Here is the story in brief.

The excitement began just over 25 years ago when it first emerged that NAD, NADP and then cADPR could trigger Ca^{2+} release in eggs of the sea urchin *Lytechinus pictus* (4), on a par with inositol-1,4,5-trisphosphate (InsP3) (5), until then one of the most potent intracellular messengers. Although the capacity to synthesize cADPR and mobilize Ca^{2+} was shown to be quite broadly distributed in animal tissues (6), the enzyme responsible for converting NAD to cADPR remained unknown. By a classic

stroke of serendipity, the enzyme was detected by scientists studying ADP-ribosylation of G-proteins by the bacterial cholera and diptheria exotoxins in the marine gastropod mollusc *Aplysia californica* (7). ADP-ribosylation occurred in all examined tissues, except in the ovotestis. The unexpected finding was intelligently pursued until the 'inhibitory factor' was characterized as a NAD-splitting protein capable of producing cADPR, similar to the enzymatic activity first identified in sea urchin eggs. This first ARC was molecularly cloned (8) and called 'ADP-ribosyl cyclase' in 1991 by Lee and Aarhus to avoid confusion with other NAD glycohydrolases (EC 3.2.2.5) (3). As will be discussed below, the antagonism between the mono-ADP-ribosyltransferases (mART) and the ARCs revealed in molluscan eggs is probably just the tip of the iceberg of this phenomenon. In the meantime, the availability of an amino acid sequence enabled the sequence homology-based identification of ARCs in other species, first amongst these, human CD38 (9).

3. THE ADP-RIBOSYL CYCLASES IN BILATERIAN SPECIES

To examine the ARCs from an evolutionary perspective requires experimentally validated proteins that allow comparison in form, localization and function. Therefore, we focus here on the structurally and biochemically well-characterized ARCs (Figure 1).

The identification of a human counterpart of the *Aplysia* ARC in the shape of CD38 was soon followed by the cloning of a third member of the family, human Bst1 (alias CD157). The vertebrate ARC tally increased with the identification of Bst1- and CD38-related proteins in other mammals. Bovine erythrocyte NADase was an important model for the study of the connection between NAD and second messenger signalling (10) and when the NADase was isolated from spleen, it was identified as a CD38 orthologue (11). Non-mammalian ARC sequences was reported for the domestic chicken *Gallus gallus* (NBCI ADQ89191; ADQ89192) and more extensively for the African clawed frog *Xenopus laevis* (12, 13). In invertebrates, the firstborn ARC from *Aplysia* was joined by a relative from the pathogenic blood fluke *Schistosoma mansoni* (14) and various groups working in the sea urchin have led to a harvest of four ARCs from *Strongylocentrotus purpuratus* (15-18). Note that *S. purpuratus* is the sea urchin of choice for molecular studies and has had its genome completely sequenced (19) while the *L. pictus* sea urchin has more transparent eggs making it a better model for fluorescent imaging studies (20).

This distribution of ARCs among vertebrate and invertebrate species leads to the inevitable conclusion that an ARC gene must have been present in the last common ancestor of bilaterian animals (Figure 1). This hypothetical ancestor, commonly referred to as the urbilaterian, is featured as a triploblastic embryo with antero/posterior and dorso/ventral axes of symmetry (21) The urbilaterian has been reconstructed as a small marine animal resembling a flatworm, whose inner simplicity or complexity is debated; however, its reproductive system may have had germ cells of mesodermal origin (22) and we can at least speculate that this is where the ARC/egg connection began. However the urbilaterian reproduced, aided or not by the primordial ARC, it must have been very efficient given the wonderful diversity of animal life that descended from it.

4. DESCENT OF THE ADP-RIBOSYL CYCLASE GENES FROM THE URBILATERIAN ANCESTOR

The gene structures of few ARCs have been described in the literature. In vertebrates, *Bst1* and *CD38* genes have been described in human (23-25); Bst1 was also reported in mouse, under the name *Bp3* (26). Among invertebrates, only the ARC gene of the Japanese sea slug *Aplysia kurodai* (a closely related species to *A. californica*) was isolated (27). The genes show great conservation of their intron-exon structure (Figure 2a), suggesting descent from a very similar gene in the urbilaterian. With minor modifications such as an intron insertion or loss, it is possible to obtain the three slightly different gene configurations found in human and *Aplysia* ARC genes (Figure 2a). Chromosomal localization and conservation of synteny, for which data is available for the vertebrate ARCs, yields further information: from the human to the *Xenopus* genome, *Bst1* and *CD38* are adjacent to one another indicating their origin by tandem duplication of an ancestral vertebrate ARC gene (Figure 2b).

5. THE ADP-RIBOSYL CYCLASE FAMILY OF HOMOLOGOUS PROTEINS

The biochemically characterized ARC proteins are glycoproteins whose mass ranges from 29 to 45 kDa in their monomeric form. The ARCs are single-domain proteins that contain the *ribosyl_hydrolase* domain (http://pfam.sanger.ac.uk/Pfam02667). This domain consists of around 250 residues; its boundaries approximately coincide with the first and the tenth cysteine residue of the ARC-defining 10-cysteine array (Figure 3A). These highly conserved cysteines form the five disulfides that fold the ARCs into similar three-dimensional structures (Figure 3B). Between the highly conserved Cys and substrate-binding residues, the ARC primary sequences are quite divergent. The N- and C- termini are highly divergent, so that amino acid conservation between invertebrate and vertebrate whole-protein ARC sequences can be as low as 20% over the length of the whole sequence (Table 1). As a rule of thumb, proteins with amino acid sequence identity greater or equal to 40% tend to share function, under 25% they tend to differ (28).

While protein sequence comparison and 3D structure overlap leave little doubt that the ARCs are homologous proteins, we will describe below that the ARC repertoire in bilaterian animals consists of proteins that show a complete lack of uniformity in topology and enzymatic activity. The result is that in the small cohort of biochemically characterized ARCs, almost every protein has a unique combination of these two properties.

6. A BRIEF HISTORY OF cADPR AND NAADP

"*An ADP-ribose cyclized head-to-tail is such a novel and beautiful structure that I knew at the very moment of realization that it had never been described before*"

Hon Cheung Lee, Cyclic ADP-ribose and NAADP (29)

The most common physiological substrates of the ARCs are believed to be NAD and, perhaps, its phosphorylated form (NADP). A phylogenetically ancient and ubiquitous small molecule, NAD features prominently in every biochemistry textbook as a coenzyme of redox reactions, a sort of catalytic bridesmaid playing a supporting role for hundreds of enzymes involved in cell metabolism. In a first paradigm shift, NAD started to emerge from the redox shadows to a starring role in cell signalling about 50 years ago with the discovery that it could be split and its ADP-ribose moiety transferred to a target protein, causing a change of function in the protein in question by the now-familiar process defined as ADP-ribosylation (30). Subsequently, the world of NAD-consuming enzymes opened up, leading to the description and characterization of three major, functionally distinct, families: the mono-ADP-ribose transferases, the poly(ADP)ribose polymerases and the sirtuins.

Another paradigm shift in the life of NAD came with the discovery of its role in calcium signalling. For a number of years, the undisputed protagonist of stimulus-dependent calcium mobilization was InsP3: upon interaction with specific receptors in the endo/sarcoplasmic reticulum, InsP3 triggers intracellular Ca^{2+} release, a mechanism that is ubiquitous both in animal tissues and animal taxonomy. However, this mechanism is not the only one that is operational. An observation by Epel in 1964 described a dramatic increase in pyridine nucleotide concentration in sea urchin eggs minutes after fertilization occurred (31). Following up on this finding, in 1987 the laboratory of H.C. Lee (University of Minnesota) tested the effects of NAD and NADP using crude egg homogenates from the sea urchin *L. pictus*. For the first time, the pyridine nucleotides demonstrated their ability to release calcium from intracellular stores (4). However, there were temporal differences in their calcium-mobilizing effects: NAD-induced calcium efflux did not occur immediately after addition, but after a brief time lapse of a few minutes, hinting at a modification of NAD to its active form. It took years of hard work to obtain the structure of this elusive molecule - small wonder Lee was so lyrical in describing the structure of cADPR. On the other hand, addition of NADP required no time-lapse for catalytic conversion because commercial sources contained trace amounts of the active metabolite NAADP. Another stroke of serendipity for the prepared mind.

6.1. Distinct signalling pathways mediated by cADPR and NAADP

The molecular structures of cADPR and NAADP are shown (Figure 4). Both molecules fit the description of second messengers, defined as molecules that are present endogenously and whose intracellular levels increasing following activation by the first messenger (such as egg activation by sperm in the sea urchin, acetylcholine release in *Aplysia* neurons). Both cADPR and NAADP function by modulating release of Ca^{2+} from intracellular vesicular systems. At this point, the similarities end as cADPR and NAADP have been recruited to activate different calcium signalling pathways, recently reviewed in (32). Cyclic ADPR activates the ryanodine receptor (RyR) leading to calcium release from the endo/sarcoplasmic reticulum, while NAADP targets the two-pore channels (TPC) to modulate release of Ca^{2+} from intracellular acidic stores (reserve granules, lysosomes). There is a greater complexity that belies this perhaps too neat dichotomy: for example, the cADPR/RyR interaction requires additional proteins such as calmodulin and FK506-binding protein (32). The ARCs also contribute to the ADPR pool by breakdown of NAD and cADPR, and ADPR is known to activate TRPM2 (transient potential receptor melastatin-2), a plasma membrane cation channel (33). Cyclic ADPR and NAADP also influence TRPM2 function through synergistic facilitation (33). In metazoan phylogeny, the origins of the RyRs and TPCs date back at least to the urbilaterian (34), so that the signalling kit consisting of messengers, their biosynthetic enzymes and their calcium-releasing receptors, is found throughout invertebrate and vertebrate species, as is the InsP3-dependent signalling mechanism.

Finally, there is the question of topology (35), an important issue for membrane proteins (36) and a particularly relevant one for the ARCs because, with the notable exception of the *Aplysia* homolog, they are all membrane-bound (Figure 5). However, being both membrane-bound *and* NAD-splitting enzymes raises the further issues of where the proteins are expressed in relation to availability of the substrate (NAD) and how the bioactive products of catalysis (cADPR and NAADP) reach their targets.

7. CONNECTIONS 1: BETWEEN STRUCTURE, SUBCELLULAR LOCALIZATION AND BIOCHEMISTRY OF THE ARCS

Understanding the evolution of protein function represents a major challenge in modern ARC-ology. A vital consideration is that ARC function must bring together the essential protein determinants of topology, subcellular localization and enzymatics within the context of species. For this reason, we discuss these latter issues in phylogenetic order.

7.1. The invertebrate ADP-ribosyl cyclases

The overwhelming majority (97 percent) of animal species are invertebrates yet only three invertebrate species with characterized ARCs are currently available.

7.1.1. *Aplysia californica*. The *Aplysia* ARC is the only member of the family to date that is not membrane-bound: the protein was purified from the aqueous-soluble fraction of ovotestis, and protein sequencing established that a signal peptide is cleaved off to form the native protein. This protein must enter the secretory pathway as the protein is concentrated in 5 - 10 micron egg granules or vesicles in the cytoplasm of immature oocytes (higher expression) and mature oocytes or eggs (8). Unfortunately, little is known of the role of this ARC in fertilization and *Aplysia* is not famous for its use as a model of reproduction (although to the non-expert they are rather interesting simultaneous hermaphrodites with alternating male and female behavior). *Aplysia's* claim to fame of course depends on its 20,000 enormous neurons which paved the way decades ago for the study of behavioral neurobiology (37). *Aplysia* ARC has been detected in the presynaptic neuron of a cholinergic synapse in the buccal ganglion (38). Subsequent studies using abdominal, pedal and pleural ganglia further clarified that the *Aplysia* ARC was localized in the cytosol of the neuronal soma and tranlocates to the nucleus during neuronal depolarization (39).

We have already mentioned that the enzyme from *Aplysia* oocytes is distinctive among the bilaterian ARCs because it is not membrane-bound. *Aplysia* ARC has another, currently unique, characteristic: at physiological pH, the enzymatic reaction it catalyses almost exclusively synthesizes cADPR from NAD (3) (Figure 6). As for the biosynthesis of NAADP, this can be accomplished at acidic pH by base exhange of nicotinic acid in lieu of nicotinamide in NADP (40). The *Aplysia* ARC is capable of this reaction, making it a truly remarkable enzyme that can convert two different substrates into two chemically distinct messengers. Based on the expression and enzymatic characteristics of the *Aplysia* ARC, we speculate that it might be bifunctional, participating through cADPR production in synaptic transmission in neurons, and acting through NAADP production in oocytes granules, with the enzyme product determined by local pH.

One may have anticipated that these amazing enzymatic properties of the family archetype would represent the general activities common to all members of the family and resolve forever the question of who syntesizes cADPR and NAADP. This is patently not so. Indeed, as we procede with a systematic examination of the ARCs, it becomes clearer that this handful of enzymes share their affinity for NAD but no two ARCs appear to do the same thing with it.

7.1.2. *Strongylocentrotus purpuratus*. This animal appears to have its own miniature ARC molecular evolutionary workshop.To date, the ARC repertoire of *S. purpuratus* is the most numerous with four highly diversified proteins (Table 1) and three different topologies (type I, type II and GPI-anchored) (Fig. 4). Oocyte expression is a common theme linking ARC expression in *Aplysia* and sea urchin: oocyte expression was observed for at least three of the sea urchin ARCs. Unlike *Aplysia*, the sea urchin egg is a wonderful model for the study of fertilization. In this highly coordinated process, contact between sperm and oocyte leads to oocyte activation. The oocyte cortex, the peripheral region of the oocyte beneath the plasma membrane, is filled with small vesicles called cortical granules. Upon sperm entry, the cortical granules fuse with the egg membrane and spill out their contents, modifying the egg surface in order to prevent polyspermia. Since the process of fertilization is powered by calcium mobilization, it does not take a big leap of imagination to assign an important functional role to the ARCs in oocyte activation as: (i) ARC2/alpha, one of the two GPI-anchored proteins, is expressed ectocellularly on the egg plasma membrane; (ii) ARC1/beta, a type I membrane protein, is expressed in egg cortical granules; (iii) ARC3/gamma, a type II protein, is also expressed in egg cortical granules (15). The fourth, ARC4 (GPI-anchored) locates to the plasma membrane upon heterologous transfection but its native pattern of expression is unknown (16). So at least three of the four sea urchin ARCs are expressed in oocytes. Are they catalytically active? Biochemical analysis of ARC1/beta (cortical granules) and ARC2/alpha (plasma membrane) shows they prevalently convert NAD to NAADP (15). SpARC4 instead resembles the *Aplysia* ARC in mostly producing cADPR (16). In addition, ARC1 and ARC2 proved to be more efficient catalytically *in vitro* at acidic pH (15), as would befit enzymes working inside cortical granules. For excellent reviews about the role of the ARCs and calcium movement in echinoderm fertilization, see (41, 42).

7.1.3. *Schistosoma mansoni*. The blood-dwelling fluke *S. mansoni* is one of at least eight species of the genus *Schistosoma*, parasites with a complex life cycle. Human infection is a scourge for millions of people, and begins when the parasite is acquired transcutaneously, travels through the blood and terminates its peregrination in the portal system (in the case of *S. mansoni*) where adult worms (male and female forms) mature, mate and release eggs. Chronic human disease is caused by the accumulation of eggs trapped in the liver, and the ensuing immune response of the host. The ARC of *S. mansoni* is GPI-anchored and has a very precise developmental and sexual pattern of expression, limited to the adult female worm where the ARC is localized exclusively on the tegument surface covering the worm. The tegument surface consists of a normal plasma membrane that is coated by a secreted membranocalyx. This structure represents the parasite's first line of defence located at the interface with the host immune system, and this is where the schistosome ARC is very highly expressed along with other ectoenzymes such as carbonic anhydrase and alkaline phosphatase (43). What of the enzymatic activity of this ARC? It is so reluctant to convert NAD to cADPR that the authors working on the biochemistry of this protein referred to it as NACE, for NAD-catabolizing enzyme. An enzyme subjected to detailed in vitro studies, NACE almost exclusively hydrolyses NAD to produce ADPR (14). However, this protein bears a very interesting mutation (H103W) in which a highly conserved tryptophan residue (in the NAD binding pocket) is mutated to histidine (44). Production of a modified protein that reverted to W103 showed some cADPR synthetase activity (45). Thus this single-residue mutation in NACE has a powerful effect on catalysis. The presence of the same mutation in the African (*S. mansoni*) and Asian (*S. japonicum*) schistosomes that diverged from 70-148 million years ago (46) suggests this mutation may have been positively selected for and that the tegumental ARC of the schistosomes plays an

immunomodulatory role that utilizes its NAD-splitting activity. In view of the immunosuppressive role of adenosine (see below), it would be interesting to see if the enzymatic machinery for adenosine production via ARC is operational at the tegument surface. Last but not least, the drug praziquantel, the only recommended treatment for schistosomiasis, acts on the parasite's calcium ion channels although its mechanisms of action remain unclear (47). However, a second effect of praziquantel is to block adenosine uptake by the schistosome (48). Could there be a connection?

7.2. The vertebrate ADP-ribosyl cyclases

While we can approximate the origins of the extant ARCs to their most recent common ancestor, the urbilaterian, we can be a bit more precise in timing the origin of two members of the family: Bst1 (alias CD157) and CD38. The current evidence suggests that an ARC gene present in the tetrapod ancestor underwent a local duplication generating tandem sibling genes, an event that occurred prior to the divergence of amphibians and reptiles *circa* 340 million years ago (timetree). From *Xenopus* on, the Bst1 and CD38 genes have remained connected in tandem in orthologous chromosomal positions, as described above. Although we cannot establish which is the parent gene, which the daughter, what counts is that both genes were retained: in these terms, the siblings Bst1 and CD38 are equal as neither was thrown away by nature. Following duplication, the two genes went their separate ways and diverged in structure and sequence under the influence of mutation, selection and drift. Chronologically, CD38 came first in cloning, with the cDNA sequence of human CD38 obtained in 1990 (49); the cloning of the *Aplysia* ARC was reported in 1991 (3), and the sequence homology between the two detected in 1992 (9). Bst1 didn't become a member of the family until 1994 (50), and has been trying to catch up ever since with its more glamouous sib CD38.

7.2.1. Bst1. Bst1 orthologues have been isolated and partly characterized in tetrapod models from human through macaque, rodents, chicken and *Xenopus* (sources are indicated in Figure 1). As is predicted for a family of orthologous proteins, amino acid sequence homology is high, with the most distant orthologues (human/xenopus) showing 51% amino acid identity in the *ribosyl_hydrolase* domain. At this level of conservation, proteins are predicted to maintain function (28). As for the invertebrate ARCs, understanding what the function of Bst1 is requires integrating protein localization and enzymatics with tissue expression in a specie-specific context.

Bst1 proteins have been consistently verified to be GPI-anchored glycoproteins. A common feature of these proteins is their localization in lipid rafts, plasma membrane microdomains enriched with signalling elements (51). Of the vertebrate orthologues, human Bst1 has been the most studied in terms of association with other proteins within rafts. First identified as a myeloid differentiation antigen (52), Bst1 was found to have an important function in neutrophil and monocyte trafficking, reviewed in (53). In myeloid cells, Bst1 clustering by specific antibody ligation causes beta 1/CD29 and beta 2/CD18 integrins to translocate into lipid rafts and activates downstream signalling pathways (54). This dynamic interplay between Bst1 and integrins is instrumental in controlling leukocyte adhesion to extracellular matrix proteins, migration and diapedesis. The link between Bst1 and cell mobility extends beyond and has been found to regulate tumor cell progression and transition to a mesenchymal phenotype with increased mobility in ovarian epithelial cancer cells (55). The current evidence is that the functions of Bst1 in cell motility are receptorial and catalysis-independent.

According to *in vitro* analyses, mammalian Bst1 is capable of splitting NAD prevalently to ADPR, with weak cADPR synthetase activity which is increased by metal ions (Zn^{2+} and Mn^{2+}) (56). In *Xenopus*, however, a recent report on the analysis of the Bst1 protein failed to detect any enzymatic activity at all (13). In addition, none of the Bst1 orthologues have been credited with NAADP production to our knowledge. Be that as it may, the capacity of Bst1 to generate cADPR has recently been described to play a central role in connecting calorie restriction with increased function in the mouse intestinal stem cell niche. Calorie restriction acts via mechanistic target of rapamycin complex 1(mTORC1) to upregulate Bst1 in Paneth cells and increase stem cell function via cADPR (57). It has been suggested that the stromal cell role of Bst1 may also depend upon its weak activity as a cADPR-generating enzyme, acting at hormonal concentrations and being transported inside stem cells by nucleoside transporters (58). Bst1 is also part of a group of 'stemness' genes in human epithelial cells (59) and in human corneal stem and stromal cells (60).

Following up on an observation that Bst1 was expressed at discrete moments of T and B cell development (61), a knockout mouse model was generated to analyse the immunological role of Bst1. The principal defects were observed in the B cell lineage; among them, alteration of peritoneal B-1 cell development, and decreased serum and mucosal antibody production (62). Further data pointing to an important role for Bst1 in B lymphocyte development and migration is that the Bst1 gene has been identified as a target of the transcription factor Pax5, the B-cell identity factor, in murine pro-B cells (63).

With its dual receptor/ectoenzyme roles, the functional repertoire of Bst1is gradually getting more interesting as it expands from immunity to cancer and metabolism, with a common thread of cell migration and 'stemness' connecting the functions.

7.2.2. CD38. In all its vertebrate representations, CD38 is always predicted to be a type II (N_{cyto}/C_{exo}) protein. However, at least in the case of human orthologue, CD38 is a most intriguing example of a membrane protein with dual topology: it has both type II and type III (transmembrane anchor but N_{exo}/C_{cyto} orientation) configurations (64). This represents two different ways of anchoring the same protein sequence to the cell membrane with the difference that the type II protein has the active domain in

the extracellular milieu while the type III has the active domain inside the cell. The type III form of CD38 offers a neat solution to the topological dilemma associated with this protein, representing a small but potentially relevant amount (*circa* 10%) of the total CD38 expressed. Dimeric and other multimeric forms of human CD38 have also been described, as reviewed in (65), and a novel tetrameric signalling form associated with lipid rafts was reported recently (66)

Plasma membrane attachment is a complex but undisputed issue with CD38. However, CD38 appears to be bound not only to the plasma membrane but also to nuclear [see (67) and references within], mitochondrial (68) and intracellular vesicular membranes (69) in cells from standard mammalian models. In keeping with the invertebrate (sea urchin, *Aplysia*) tradition of associating ARCs with intracellular vesicles, there are fascinating reports on the presence of CD38 in membrane vesicles of endosomal origin, or exosomes, and plasma membrane origin, or microvesicles. Exosomal (and enzymatically active) CD38 was present in exosomes shed from human lymphoblastoid B cells (70) and from an HIV-1-infected T lymphocyte cell line (71). Microvesicles released from prostate epithelial cells also contain CD38 (72) and, more excitingly, prostasomes transfer CD38 and other components of the calcium signalling machinery to sperm, a basis for progesterone-induced sperm motility (73).

The expression profile of CD38 is broad in the customary mammalian species, with high expression in the hemato-lymphoid compartment (blood, bone marrow, lymph, lymph node, spleen, thymus and tonsil) and in the major organ systems (brain, heart, liver, muscle, pancreas). CD38 expression in mouse brain (hypothalamus and neurohypophysis) has opened a stream of research on its role in the regulation of oxytocin release and social behavior (74). The EST-based expression profile of bovine CD38 shows an intriguingly elevated expression in the pineal gland (NCBI UniGene EST profile Bt.248), where the circadian clock-regulator melatonin is expressed. CD38 expression in frog (*Xenopus laevis*) is reported as ubiquitous (12, 13) and detected in the embryo, unlike Bst1 expression which is PCR-negative (12). This leads us to the enzymatic activity of CD38, and the numerous biochemical studies carried out on wild type and mutant human CD38 that identified the active site glutamate residues and the single or double amino acid residue mutations that can dramatically alter the catalytic activity (32, 75). In general, the CD38 orthologues prevalently catalyse NAD hydrolysis to ADPR, with a side production of cADPR (about 2-3%), reviewed in (32).

Like the sea urchin egg for fertilization, *Xenopus laevis*, the African claw-toed frog, is a favourite model for early embryonic development and has provided the first evidence of an essential role for an ARC in development. Disruption of CD38 expression in *X. laevis* embryos provoked alterations in differentiation that led to embryonic death (12). This is a most interesting result and is perhaps the first true double Bst1/CD38 knockout as the frog embryo does not espress Bst1, and even if expression could be activated by a compensatory mechanism, frog Bst1 has been reported to enzymatically silent (13). CD38 knockout mice have been produced with different genetic backgrounds, but leaving the sibling Bst1 intact thus we cannot exclude that functional redundancy between paralogs (76) may be limiting the knockout phenotype. Indeed, CD38 KO mice suffer a decrease but not a complete loss of cADPR, whereas NAD levels increase (77). Of course, there may be other as yet unidentified enzymes at play that generate cADPR. The disruption of murine CD38 also does not abolish the capacity to produce NAADP (78). For sure, the knockout mice have yielded a cornucopia of information, and we prefer here to summarize the data in table 2, noting that it represents just the results obtained since 2011. For further knockout data and an extensive review of CD38 in human health and medicine, see (65). Ever the leukocyte marker, CD38 remains an excellent target for various forms blood cancer and there has been great progress in the therapeutic use of anti-CD38 antibodies (79, 80).

8. CONNECTIONS No. 2: ARCs AND OTHER NAD-CONSUMING ENZYMES

The need for NAD. Obviously no protein acts alone and the ARCs are part of highly complex and interdependent pathways which must supply the substrates for ARC enzymatic activity, among other things. NAD is supplied through primary biosynthesis or salvage pathways (81). Being a life-essential molecule, not surprisingly almost all species in the three kingdoms of life have one or another means to synthesize or procure NAD, and a phylogenetic analysis detailing the distribution of NAD biosynthetic enzymes has been recently reported (82). A correlation was noted between the phylogenetic appearance of the ARCs and nicotinamide phosphoribosyltransferase (Nampt), the rate-limiting enzyme of the NAD salvage pathway (82). A further and more functional connection between Nampt and CD38 has been recently reported: blocking Nampt in human pancreatic cell lines lead to decreased growth and survival, an effect dependent on high CD38 expression (83).

On the other side of the NAD synthesis/degradation equilibrium is an army of illustrious and less illustrious NAD-consuming enzymes flanking the ARCs: among the former are the sirtuins and the ADP-ribosyltranferases/glycohydrolases (PARPs, mARTs and PARGs); less broadly described are the tRNA 2'-phosphotransferases (84).

ADP ribosylation. As mentioned in section 1, it was the simultaneous presence of an ARC and an ART in a test tube that ultimately led to the first identification of ADP-ribosyl cyclase which proved to be the NAD-splitting factor in *Aplysia* ovotestis extracts that inhibited bacterial exotoxins. This was the first indication that ARCs could control ADP-ribosylation-mediated cell signalling by restricting the availability of NAD. On the other hand, evidence of ART/ARC interaction on the ectocellular surface emerged using less artificial models such as murine T lymphocytes or pancreatic islets cells (85-87). This stream of research led to a murine model of NAD-induced cell death, described in T lymphocytes (88) and astrocytes (89), that is

orchestrated by ART2 and P2X7 purinergic receptor. However, as in the ovotestis extract, it is the ARC (CD38 in this case) that applies the brakes by restricting substrate availability (90).

A new role for an ARC has recently emerged in the course of studies on the mechanism of ADP-ribosylation perpetrated by yet another pivotal ADP-ribosylating toxin, this time of fungal origin: brefeldin A. In cell assays, brefeldin disrupts the Golgi complex, in part by ADP-ribosylating a protein called BARS (brefeldin A ADP-ribosylated substrate) which is necessary for vesicle fission (91). However, in a rat brain model, brefeldin did not directly act on BARS but was first endowed with an ADP-ribose moiety provided by CD38 (92), opening an exciting new avenue of research into the possible therapeutic uses of brefeldin in $CD38^{hi}$ tumor cells.

Finally, for the sake of completeness, let us mention here the capacity of CD38 (murine) to auto-ADP-ribosylate and ADP-ribosylate other proteins (93), although the physiological impact of this capacity is unknown.

Sirtuins, PARPs and ARCs. These three families of NAD-consuming enzymes have evolved to carry out distinct tasks: sirtuins to deacetylate histone proteins and regulate transcription, PARPs to attach ADPR polymers onto DNA in order to maintain chromatin structure. The end result it that they all compete for NAD and this competition affects NAD homeostasis: PARP activation is reported to decrease intracellular NAD levels by 10-20% within minutes while the link between sirtuins and NAD levels is not so clear (81). Other authors have suggested that CD38 is the principal regulator of NAD degradation while sirtuins, PARPs and mARTs play a secondary role (83). In view of the role of these enzyme families in NAD homeostasis, metabolism, ageing, and cancer (94) the interaction between NAD-consuming families will continue to be a hot topic for years to come.

CD38/CD203a/CD73. There is increasing interest in the role of extracellular nucleotides (ATP, NAD) and how they link metabolism, innate immunity and inflammation (95). While extracellular ATP and NAD have been recruited as proinflammatory danger signals, the ATP metabolite adenosine has mainly anti-inflammatory properties. The canonical route from ATP to adenosine is via consecutive modification by CD39/NTPDase (ecto-nucleoside triphosphate diphosphohydrolase) and CD73/5'-NT (5'-nucleotidase). However, a novel alternative pathway for the generation of adenosine from ATP and NAD involves CD38, CD203a (ecto-nucleotide pyrophosphatase/phosphodiesterase) and CD73 (96). Evaluation of this novel route to adenosine production and analysis of co-ordinated expression of the CD38/CD203a/CD73 ectoenzymes is showing promising results in tumor biology (96).

9. CONCLUSIONS

The goal of this paper was to review the current state of the ARCs, and to view this family with its functional quirks and enzymatic foibles, using a phylogenetic approach. We are currently wading through a plethora of genomes in search of a more complete history of the ARCs but whatever comes out of the computer will require a lengthy process of validation in the laboratory so let this group of bilaterian ADP-ribosyl cyclases set the standard for future comparisons.

"That's how enduring knowledge of the natural world grows: little by little with the help of enthusiasts"

Richard Fortey, '*Dry Store Room No. 1*' (97)

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Abbreviations: ARC, ADP-ribosyl cyclase; NAD, nicotinamide adenine dinucleotide; cADPR, cyclic ADP ribose; NAADP, nicotinic acid adenine dinucleotide phosphate; InsP3, inositol-1,4,5-trisphosphate; GPI, glycosyl-phosphatidylinositol; ADPR, ADP-ribose; mono-ADP-ribosyltransferases, mART; RyR, ryanodine receptor; TPC, two-pore channel; TRPM2, transient potential receptor melastatin-2; NACE, NAD-catabolizing enzyme.

Key words: Protein family; evolution; NAD-consuming enzymes; ADP-ribosyl cyclases; cADPR and NAADP.

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FIGURE LEGENDS

Figure 1. A simplified view of bilaterian phylogeny illustrating the distribution of the 20 experimentally-defined vertebrate and invertebrate ARCs reviewed in this paper. The divergence times are based on TimeTree (www.timetree.org); mya, millions of years ago. The ARCs from human (*Homo sapiens*), cynomolgus monkey (*Macaca fascicularis*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), bovine (*Bos taurus*), chicken (*Gallus gallus*), frog (*Xenopus laevis*), sea urchin (*Strongylocentrotus purpuratus*), sea slug (*Aplysia californica*, *A. kurodai*), and the blood fluke (*Schistosoma mansoni*) are indicated together with their NCBI sequence accession number (http://www.ncbi.nlm.nih.gov/). ARC taxonomy is indicated by phylum and vertebrate/invertebrate distribution. Based on early embryological development, bilaterian animals are divided into two major clades, protostomes and deuterostomes, as indicated. The urbilaterian represents the archetypal last common ancestor of protostomes and deuterostomes.

Figure 2. Evolution and conserved synteny of the ARC genes. Panel A. Descent with modification from the hypothetical urbilaterian ARC gene to the extant human and molluscan ARC genes. A parsimonious scenario requiring two intron insertions (intron a, intron b) and one duplication is shown. Intron/exon organization: conserved exons (blue shaded boxes), modified exons (orange shaded boxes), separated by introns (thin purple boxes). Panel B. Conserved synteny of Bst1, CD38 and neighbouring genes in vertebrate genomes. Orthologous genes maintain box color in different species; color also maintained in CC2D2A frog paralogs. Genes are: C1QTNF7, C1q and tumor necrosis factor related protein 7; CC2D2A, coiled-coil and C2 domain containing 2A; FBXL5, F-box and leucine-rich repeat protein 5; FAM200B, family with sequence similarity 200, member B; Bst1; CD38; FGFBP1, fibroblast growth factor binding protein 1; FGFBP2, fibroblast growth factor binding protein 2; PROM1, prominin 1. Arrows indicate 5' - 3' gene orientation.

Figure 3. Sequence and structural conservation of the ARC proteins. Panel A. Logo of the multiple sequence alignment of 20 bilaterian ARCs based on the central 250 amino acids, and excluding the N- and C-terminal tracts. The alignment was made in MAFFT (http://mafft.cbrc.jp/alignment/server//), auto strategy, Blosum45 matrix. The Logo graphic was generated with Seq2logo (98). The 10 conserved Cys correspond to Logo residues 12, 38, 58, 124, 142, 152, 236, 257, 269 and 278. Active site Glu, residue 203. Panel B. 3D-structures of *A. californica* and human ARCs (Bst1, CD38) showing variable and conserved residues, obtained with ConSurf (99). Protein data bank (PDB) accesion codes: 1LBE (*Aplysia*), 1ISI (human Bst1), 1YH3 (human CD38). The proteins are represented as a spacefill model in PyMOL, where the residue conservation scores are color-coded according to ConSurf colors. The color-coding bar shows the coloring scheme: conserved amino acids are colored bordeaux, residues of average conservation are white, and variable amino acids are turquoise.

Figure 4. Topology of ARC proteins. Classification of the membrane-bound ARC proteins according to topologies, from type I, type II, type III to GPI. The non-anchored *Aplysia* ARC is also shown. The lipid bilayer may represent plasma membrane or intracellular membranes. Human CD38 has dual type II/type III topologies.

Figure 5. Stucture of cADPR and NAADP. Stick model of the molecules rendered in PyMOL and colored by element: carbon/green, oxygen/red, nitrogen/blue, phosphorus/ orange, hydrogen/white. Panel A: molecular structure of cADPR. Panel B: molecular structure of NAADP.

Figure 6. Enzymatic activity of the ARCs. Panel A. Cyclase activity. ARCs can convert NAD to cADPR. This is the major activity of *Aplysia* ARC and sea urchin *Sp*ARC4, but represents a minor part of CD38 output. Panel B. NAD glycohydrolase activity. Panel C. Base exchange reaction with NADP. This seems to be the main activity of sea urchin *Sp*ARC1 and *Sp*ARC2, and can be performed by other ARCs in appropriate conditions of pH and substrate availability (see text). Enzymatic pocket is represented as spacefill model generated in PyMOL. Substrates and products are represented as stick models and colored by element.

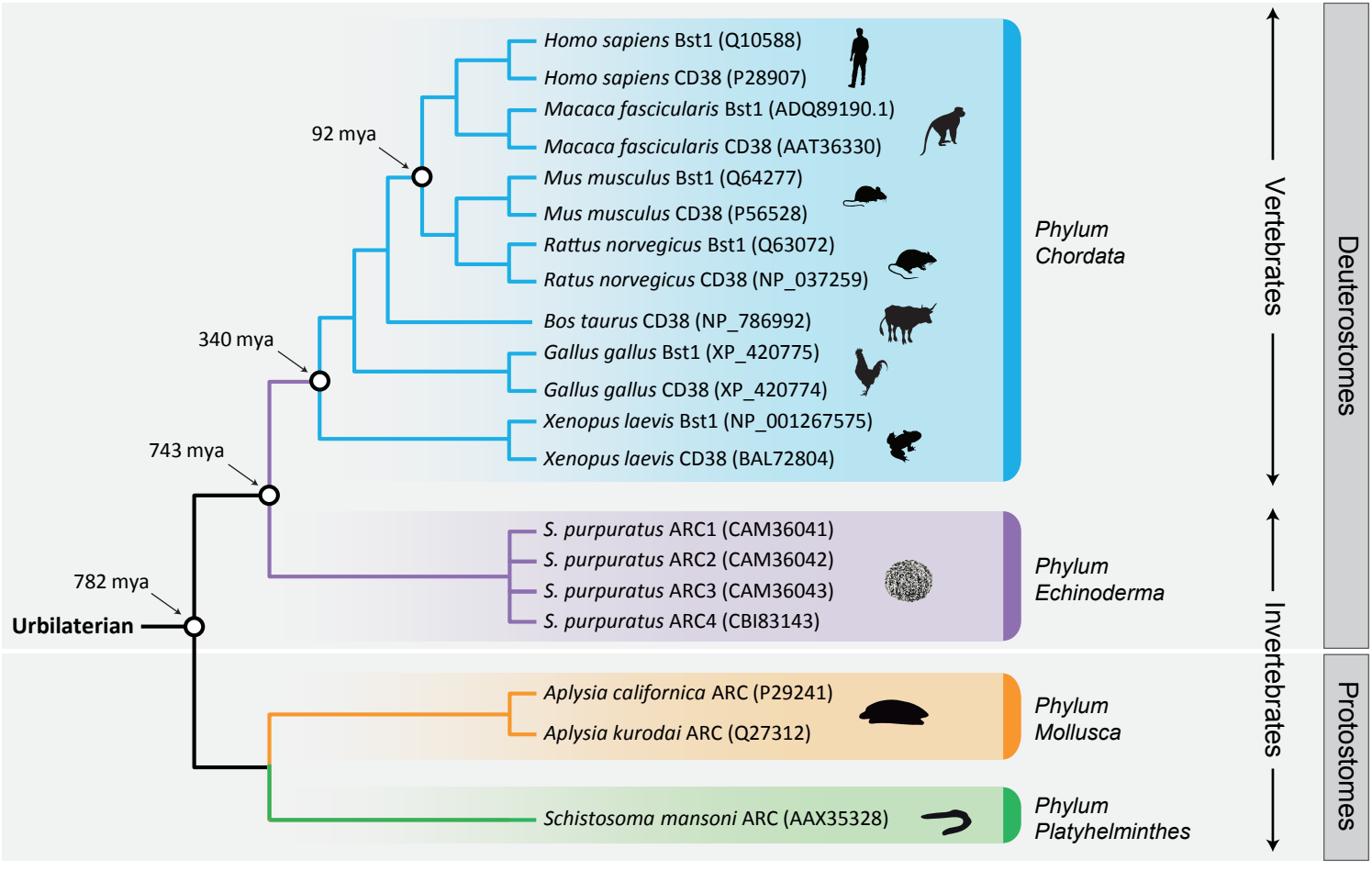
TABLES

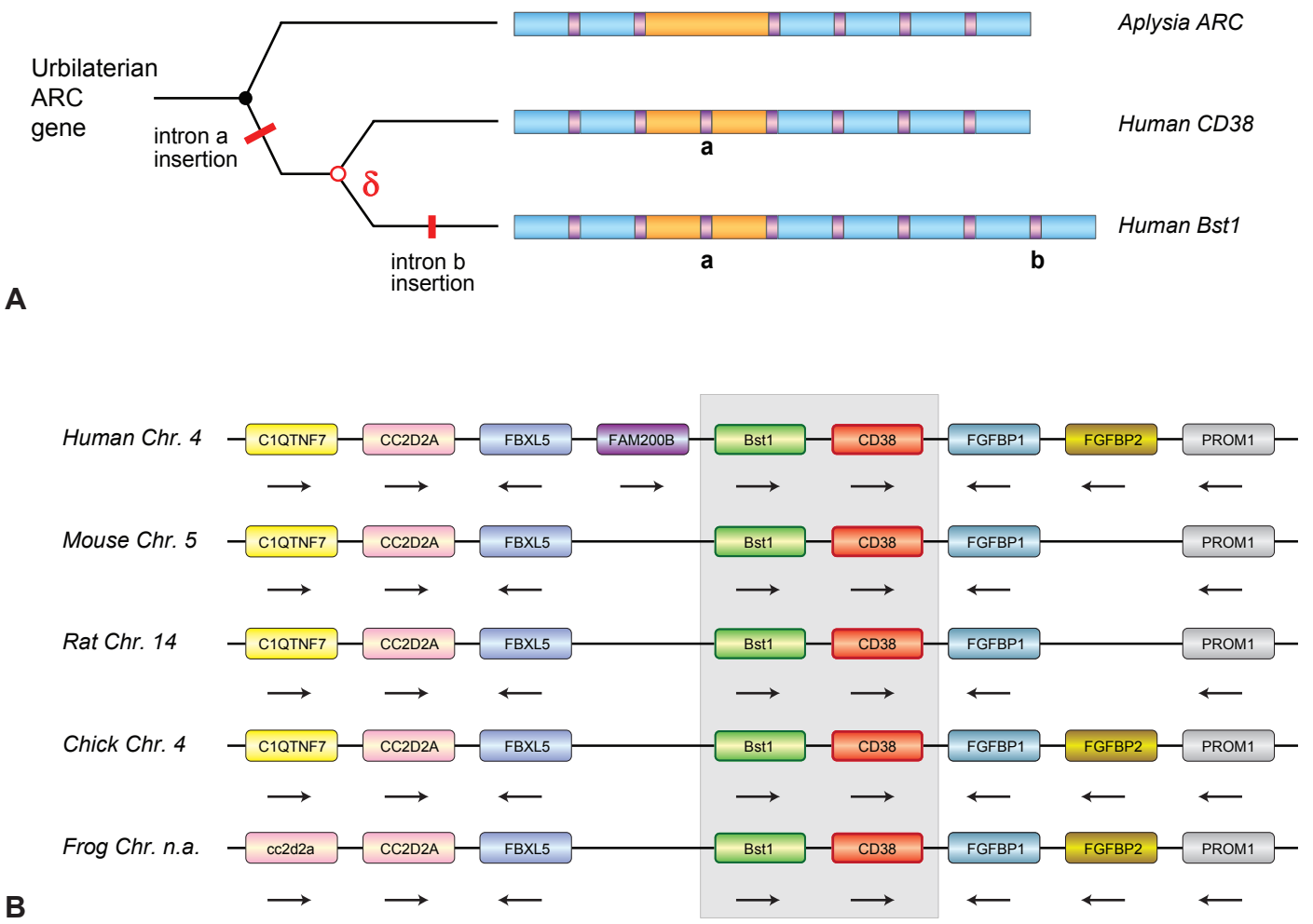
Table 1. Percent identity and similarity between whole-length ARC proteins

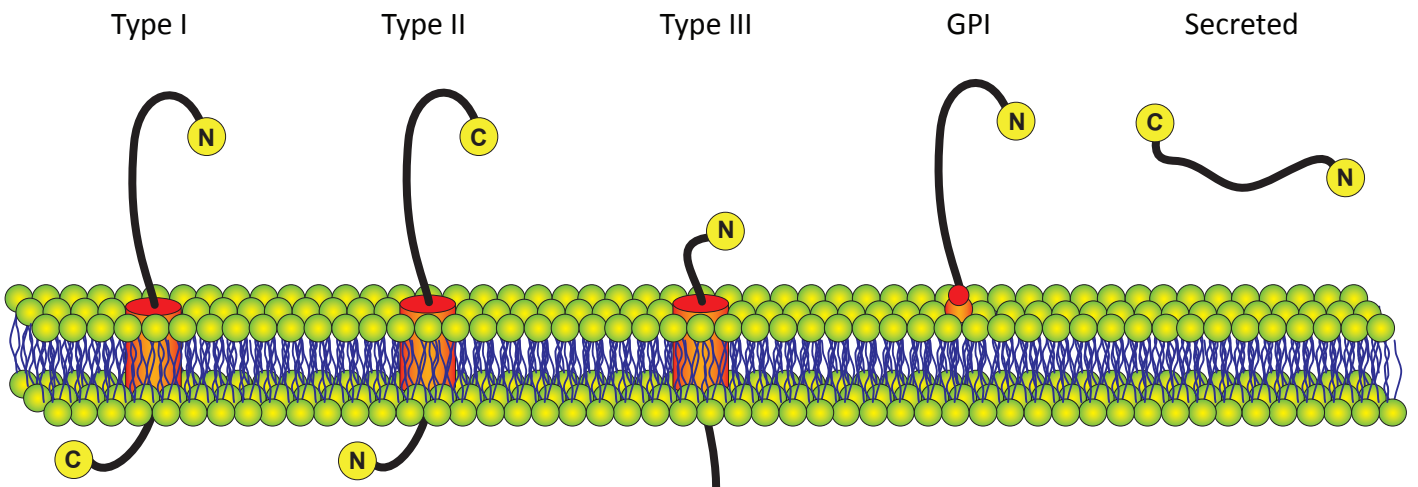
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
	86	24	26	26	24	26	27	29	29	27	26	30	26	30	27	27	27	28	27	1. *Aplysia cal.* ARC
		24	28	26	25	27	27	29	28	27	26	29	26	29	27	28	27	29	27	2. *Aplysia kur.* ARC
			23	19	21	23	20	22	21	24	21	21	21	21	23	21	20	20	21	3. *S. mansoni* ARC
				24	51	24	23	28	26	24	22	22	25	22	27	21	28	20	27	4. *Strpu* ARC1/beta
					24	26	23	28	28	28	22	21	23	21	22	22	25	22	26	5. *Strpu* ARC3/gamma
						25	23	27	24	25	22	23	24	23	26	23	26	22	26	6. *Strpu* ARC2/alpha
							26	23	27	24	22	25	24	26	25	25	22	25	24	7. *Strpu* ARC4
								36	43	31	35	39	29	42	30	36	27	36	28	8. Frog CD38
									38	50	30	30	43	30	42	27	43	28	42	9. Frog Bst1
										32	38	44	32	44	34	41	36	42	35	10. Chick CD38
											25	27	48	27	47	27	47	26	47	11. Chick Bst1
												42	26	41	26	40	26	41	26	12. Bovine CD38
													29	88	29	59	30	58	30	13. Rat CD38
														29	88	28	72	28	72	14. Rat Bst1
															30	58	31	58	32	15. Mouse CD38
																28	71	29	71	16. Mouse Bst1
																	31	92	30	17. Macaque CD38
																		31	91	18. Macaque Bst1
																			31	19. Human CD38
																				20. Human Bst1

Table 2. Phenotypes reported in CD38 knockout mice since 2011

Activity affected	**Phenotype**	References
Behavior	Altered paternal behavior in pup retrieval	(100)
	Oxytoxin signalling defect and autism	(101)
Immunity and infection	Increased susceptibility to *Listeria* infection	(102)
	CD38 interaction with CD19/CD81	(103)
	Reduced Fc gamma R-induced phagocytosis	(104)
	Increased susceptibility to *Entamoeba* infection	(105)
Inflammation	Increased post-ischaemic inflammation	(106)
Metabolism	Altered glucose and insulin homeostasis	(107)
	Altered glucose uptake in adipocytes	(108)
	Altered CD38 internalization and glucose-induced calcium signalling	(109)
	Altered superoxide production arterial myocytes	(110)
	Effective NAADP degradation	(111)
	Altered behavior and metabolic circadian rhythms	(112)
	Increased testosterone and enhanced cardiac function	(113)
	Defective ATP release in visceral smooth muscle	(114)
Renal physiology	Regulation of renin release	(115)
Lung disease	Asthma	(116)
Tumor immunity	Attenuated glioma progression	(117)
Autoimmunity	Attenuated collagen type II-induced arthritis	(118)
Haemostasis	Altered platelet activity	(119)


Homo sapiens Bst1 (Q10588)
Homo sapiens CD38 (P28907)
Macaca fascicularis Bst1 (ADQ89190.1)
Macaca fascicularis CD38 (AAT36330)
Mus musculus Bst1 (Q64277)
Mus musculus CD38 (P56528)
Rattus norvegicus Bst1 (Q63072)
Ratus norvegicus CD38 (NP_037259)
Bos taurus CD38 (NP_786992)
Gallus gallus Bst1 (XP_420775)
Gallus gallus CD38 (XP_420774)
Xenopus laevis Bst1 (NP_001267575)
Xenopus laevis CD38 (BAL72804)
S. purpuratus ARC1 (CAM36041)
S. purpuratus ARC2 (CAM36042)
S. purpuratus ARC3 (CAM36043)
S. purpuratus ARC4 (CBI83143)
Aplysia californica ARC (P29241)
Aplysia kurodai ARC (Q27312)
Schistosoma mansoni ARC (AAX35328)
92 mya
340 mya
743 mya
782 mya
Urbilaterian
Phylum Chordata
Phylum Echinoderma
Phylum Mollusca
Phylum Platyhelminthes
Vertebrates
Invertebrates
Deuterostomes
Protostomes


Urbilaterian
ARC
gene
intron a
insertion
δ
intron b
insertion
Aplysia ARC
Human CD38
Human Bst1
a
a
b
A
Human Chr. 4
Mouse Chr. 5
Rat Chr. 14
Chick Chr. 4
Frog Chr. n.a.
C1QTNF7
cc2d2a
CC2D2A
FBXL5
FAM200B
Bst1
CD38
FGFBP1
FGFBP2
PROM1
B



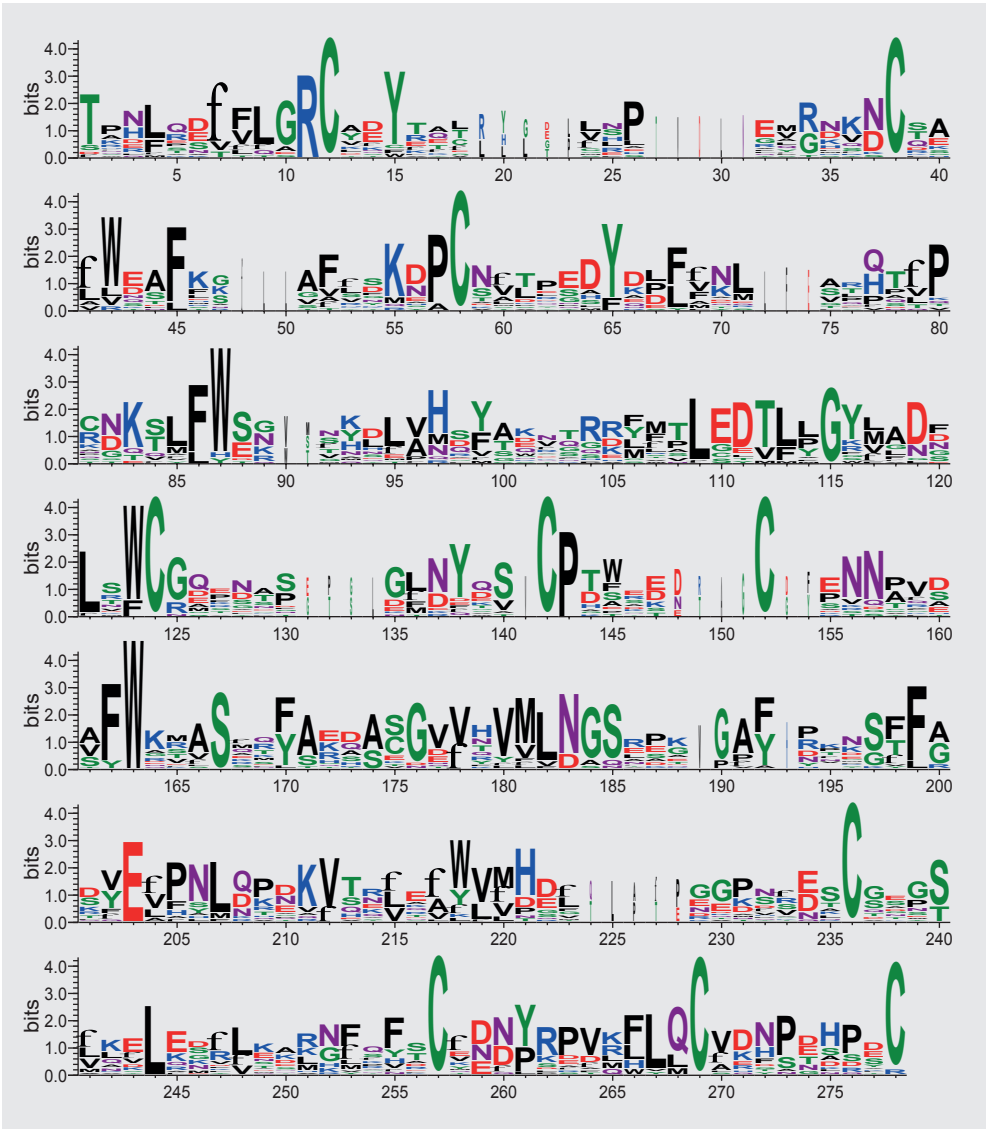
*Sp*ARC1

Human CD38
Macaque CD38
Mouse CD38
Rat CD38
Chick CD38
Frog CD38
*Sp*ARC3

Human CD38

Human Bst1
Macaque Bst1
Mouse Bst1
Rat Bst1
Chick Bst1
Frog Bst1
*Sp*ARC2
*Sp*ARC4
S.mansoni ARC

Aplysia ARC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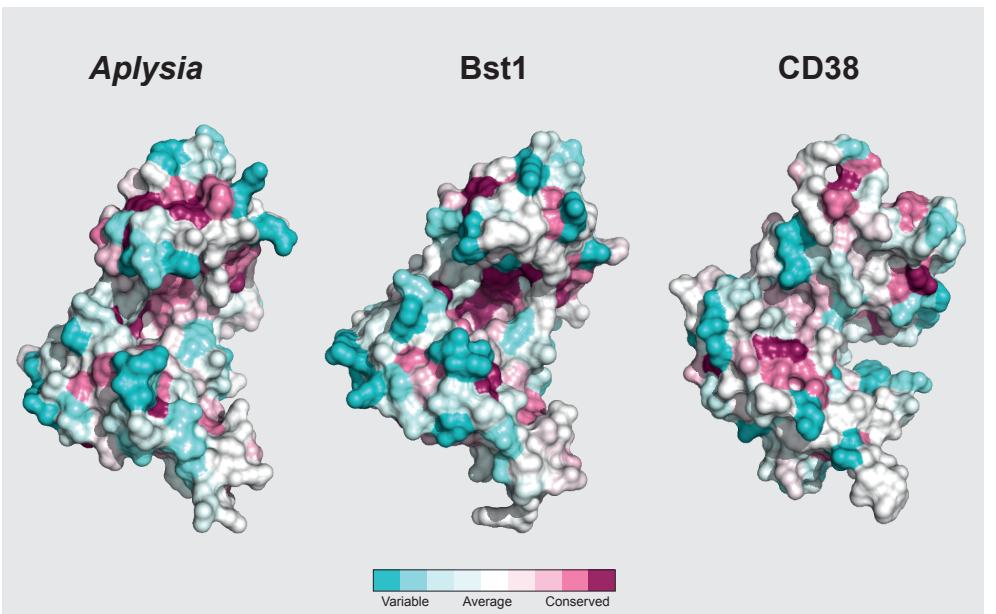
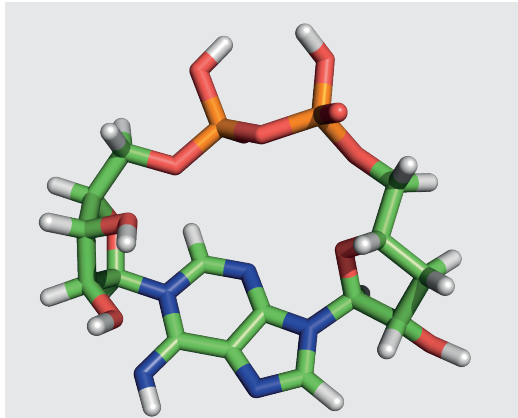
A


Aplysia
Bst1
CD38
Variable
Average
Conserved

B



cADPR

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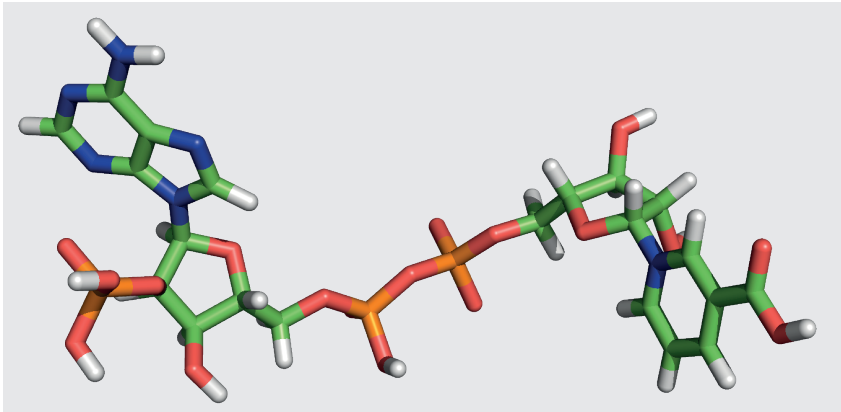
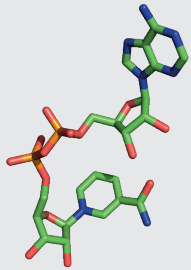


NAADP

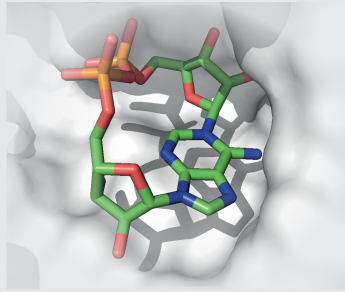
B



NAD

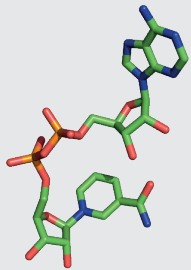
Aplysia ARC
*Sp*ARC4
CD38

ADP-ribosyl cyclase



cADPR

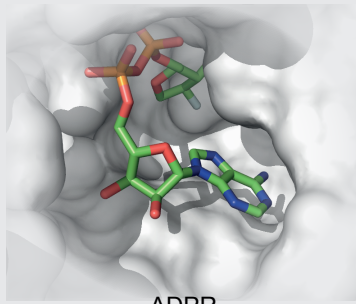
A



NAD

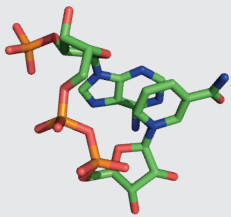
CD38
*Sm*ARC
Bst1

NAD glycohydrolase



ADPR

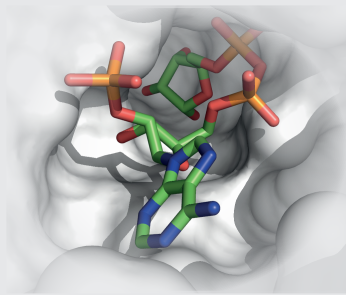
B



NADP

CD38
*Sp*ARC1
*Sp*ARC2

Base exchange



NAADP

C