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REVIEW

The Rh protein family: gene evolution, membrane biology, and disease association

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Abstract The Rh (Rhesus) genes encode a family of conserved proteins that share a structural fold of 12 transmembrane helices with members of the major facilitator superfamily. Interest in this family has arisen from the discovery of Rh factor's involvement in hemolytic disease in the fetus and newborn, and of its homologs widely expressed in epithelial tissues. The Rh factor and Rh-associated glycoprotein (RhAG), with epithelial cousins RhBG and RhCG, form four subgroups conferring upon vertebrates a genealogical commonality. The past decade has heralded significant advances in understanding the phylogenetics, allelic diversity, crystal structure, and biological function of Rh proteins. This review describes recent progress on this family and the molecular insights gleaned from its gene evolution, membrane biology, and disease association. The focus is on its long evolutionary history and surprising structural conservation from prokaryotes to humans, pointing to the importance of its functional role, related to but distinct from ammonium transport proteins.

Keywords Rh protein family · Plasma membrane · Epithelia · Erythrocytes · Channels and transporters · Systemic pH · Gene evolution · Molecular genetics · Disease association

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Introduction

Most of us become aware of the "Rh (Rhesus) factor" at a blood bank or sense its importance in a hospital where we see "Rh" along with "ABO" as labels on all bags of blood to be transfused into patients. At other times we may hear the words "Rh-positive" and "Rh-negative" or learn touching stories from friends or relatives about pregnant women who needed clinical care owing to a mismatch with their baby's Rh blood type. All these now routine medical practices rest on the genotype-phenotype principles of human genetics, as illuminated by Landsteiner, Levine, and Weiner in their seminal discoveries of the ABO and Rh blood group substances [1–4]. Indeed, the Rh and ABO antigens are still the clinically most significant [5] and genetically most polymorphic of all human blood group systems to date [6]. However, ABO are carbohydrate antigens [7] depending on the enzymatic activity and specificity of allelic glycosyltransferases [8], whereas Rh antigens are protein motifs [9, 10], whose surface expression entails an interaction of two genetic loci [11, 12]. The protein nature endows Rh antigens, particularly the more recently evolved D antigen, with the inherent ability to mount potent alloimmune reactions to counteract such conflicting situations as fetal-maternal incompatibility.

As a model system that has been well studied for seven decades, Rh proteins have generated many exciting moments of discovery in the disciplines of hematology, biochemistry, and human genetics. The foundation of Rh phenotypic variation and population genetics was laid out in the first 35 years [13], culminating in the development of prophylaxis therapy for hemolytic disease of the fetus and newborn (HDFN) in the 1960s [14, 15]. A series of studies in the 1980s established Rh antigens and Rh-associated glycoprotein (RhAG) as integral membrane proteins [7, 8, 16].

Such endeavors led to the partially determined protein sequences [17–19] that paved the way for cloning of Rh30 (*RHCE* and *RHD*) and RhAG (*RHAG*) in the early 1990s [20–24]. Ensuing studies resulted in definition of the common D and CcEe antigens in molecular terms [25] and compilation of a thorough compendium of human Rh allelic diversification [6, 9, 26–30]. The nature of RhAG as a genetic regulator for Rh antigen expression was also verified via the identification at the *RHAG* locus of mutations that cause Rh-deficiency syndrome [31–37].

Despite their folding into 12 transmembrane helices (TMH) common to members of the major facilitator superfamily (MFS) [38], the red cell Rh proteins have been notoriously refractory to functional definition [39, 40], let alone specification of their elusive substrate. The cloning of epithelial RhBG and RhCG cousins and their remote relatives has extended the erythroid paradigm and opened new avenues of research in nonerythroid tissues and model organisms [41–43]. The functional importance of such nonerythroid homologs in their natural settings has been established in the unicellular green alga *C. reinhardtii* [44–46], the worm *C. elegans* [47, 48], and the mouse as a mammal [49]. Now Rh30, RhAG, RhBG, and RhCG together define the four subgroups of the family and grant vertebrates a genealogical commonality [50–52]. The past decade has heralded a new era for our understanding of the Rh family in terms of its genetic diversity, protein evolution, three-dimensional (3D) structure, and biological function. Despite the debate on their substrate specificities as CO_2 and/or ammonia, Rh proteins have emerged as a key class of plasma-membrane proteins playing important roles in maintaining systemic pH at the organismal level. This review provides an update of recent findings on the Rh family and focuses on multifaceted insights newly gained from its molecular evolution, structural conservation, membrane biology, and disease association.

Genetic structure and molecular evolution of the Rh protein family

Origin and taxonomic distribution

The Rh family is now believed to have arisen from a common ancestor of prokaryotic origins, based on our increasing awareness of the presence of its homologs in bacteria [52] (Table 1). Despite their absence in sequenced archaeal genomes [46], Rh homologs are present as single-copy loci in certain bacterial taxa dwelling in soils, waters or subsurface and showing unique metabolic features, slower cell cycles, or more elaborate intracellular partitions [53–61]. Of the ammonia-oxidizing and CO_2-fixing bacteria, three are aerobic lithoautotrophs [53, 54] and one is an anaerobic anammox [55]. Four are rod-shaped free-living *Clostridia* species that are obligate anaerobes capable of sporulating and acetate-oxidation or sulfate-reduction along with CO_2-fixation [58, 59]. *Geobacter* sp. M21 belongs to clade 1 of the four *Geobacteraceae* clades sampled from Fe(III)-reducing subsurface regions [60], whereas the Ellin345 isolate of *A. bacteria* is a highly capsulated aerobic heterotroph widely found in soils [61]. The open reading frames (ORF) of these bacterial genes encode Rh proteins with a notable sequence identity to one another and to human proteins, particularly the RhAG, RhBG, and RhCG glycoproteins (Table 1). The common features dictate an orthologous relationship between bacterial and human Rh proteins implicating a conserved function across enormously distant phylogenies.

In contrast to their prokaryotic rarity, members of the Rh family show a much broader distribution in eukaryotes (Fig. 1). It appears that Rh genes were first dispersed among unicellular eukaryotes and then became ubiquitous in the animal kingdom from primitive metazoans to invertebrates [52]. Ultimately, the Rh family genesis arrived at its prominence and steady state in vertebrates, with definitive subgroups emerging from fish to mammals, as typified by the four loci in humans (Table 1). Of unicellular eukaryotes, most Rh-harboring species are free-living microbes in soils and/or waters, such as social amoebae slime molds (myxomycetes), water molds (oomycetes), or marine diatoms (stramenophiles). Rh genes are also present in the green alga *C. reinhardtii* [44], but so far not in other nonvascular plants, i.e., the Bryophyta (mosses), Marchantiophyta (liverworts), Anthocerotophyta (hornworts), or vascular (seed) plants. Strikingly Rh genes are absent or lost all at once in the morphologically similar but phylogenetically dissimilar fungi and in certain parasites such as the obligate parasitic protists, Apicomplexa and Kinetoplastida (Fig. 1).

Rh gene gains and losses in eukaryotic evolution

Gene duplication is a critical selection force that gives rise to raw materials for neofunctionalization and subfunctionalization of protein families [62]. As to this novelty, several rounds of gene duplication involving the Rh family have occurred and may account for its presence in extant organisms [50]. A careful inspection of copy number, exon–intron junction, and physical location of *RH* loci discloses hallmarks that recapitulate their expansion and contraction in the eukaryotic life domain (Fig. 2). Together, the evolutionary events have shaped the conservation and diversification of the Rh family in transitions from single-copy to multi-copies, from intronless to multi-introns, from unicellularity to multicellularity, and from epithelial cells (nonerythroid) to non-epithelial (erythroid)

Table 1 Comparison between bacterial and human Rh proteins

Taxonomic name	Bacterial Rh homolog		Human Rh protein family				
	ORF (aa)	% Id/Sm (aa align)	% Id/Sm (aa align)				
			RhAG 409	RhBG 458	RhCG 479	RhCE 417	RhD 417
Proteobacteria β							
Nitrosomonas europaea	425	100/100 (425)	37/58 (354)	36/51 (407)	34/53 (387)	29/48 (318)	31/49 (277)
Nitrosomonas sp. AL212	457	70/83 (401)	38/58 (357)	39/55 (407)	33/50 (430)	29/48 (314)	33/51 (283)
Nitrosomonas multiformis	407	73/84 (407)	36/57 (355)	41/59 (340)	36/55 (313)	28/47 (313)	29/50 (317)
Proteobacteria δ							
Geobacter sp. M21	403	51/66 (362)	36/55 (342)	37/55 (301)	33/51 (336)	29/48 (284)	29/48 (286)
Planctomycetes							
Kuenenia stuttgartiensis	585	50/67 (360)	33/55 (368)	39/59 (313)	34/57 (296)	25/46 (321)	25/46 (338)
Firmicutes-Clostridia							
Clostridium carboxidivorans	404	50/70 (345)	35/55 (376)	36/56 (346)	34/55 (313)	26/49 (311)	28/52 (277)
Clostridium cellulovorans	401	50/70 (345)	36/58 (345)	36/60 (319)	30/50 (384)	27/47 (318)	28/52 (309)
Clostridium papyrosolvens	391	63/78 (356)	37/57 (352)	38/58 (306)	32/53 (379)	27/48 (340)	28/49 (335)
Desulfotomaculum acetoxidans	400	51/68 (346)	38/57 (341)	35/56 (347)	34/55 (292)	27/50 (304)	30/53 (273)
Acidobacteria							
Acidobacteria bacterium	390	61/75 (388)	36/56 (345)	36/54 (342)	34/54 (312)	27/46 (332)	29/49 (290)
Archaea							
Archaeoglobus fulgidus Amt1	391	26/44 (222)	27/44 (240)	28/43 (216)	28/43 (153)	41/48 (97)	28/44 (225)

% Id/Sm Percent identity/similarity based on pairwise alignment of protein sequences. *N. europaea* Rh (*top*) and *A. fulgidus* Amt1 (*bottom*) are used for comparison since their 3D structures are known. % Id/Sm between each bacterial Rh and human Rh proteins (RhAG to RhD) is shown (left to right). The number *in parenthesis* is the total amino acid sites aligned. Note that three β-proteobacteria have Rh only, whereas the remaining bacteria retain both Rh and Amt genes

ORF Open reading frame in total amino acids (aa). The length of bacterial and human Rh proteins falls in the range from 390–585 to 409–479 residues, respectively

cells. Thus the Rh family is an excellent model system, on a large taxonomic scale, to look into the pattern of gene gain and gene loss throughout molecular evolution (Fig. 2).

Unicellular eukaryotic microbes may carry one (common) to three genes (rare). Such Rh duplicates seem to have arisen by adaptation, as their intraspecific identity generally exceeds their interspecific identity [50, 52]. Metazoan Rh members are also duplicates of a common ancestor but display more complex gene-gain and gene-loss patterns (Fig. 2). Supporting evidence includes a single-copy gene in the bona fide outgroup *M. brevicollis* (a unicellular choanoflagellate) [63]; one to three in invertebrates (water flea *D. duplex* is special, harboring six); six in teleost fish; and four in frogs, birds, and mammals [50]. While gene duplication apparently occurred twice in invertebrates, the doubling of genes from three in lancelet [64] or tunicate [65] to six in teleost fish [50] likely resulted from whole-genome duplication through tetraploidization [66]. This genome-wide duplication had reset the stage to place Rh genes on different chromosomes. The subsequent loss of two genes finalized four loci, i.e., RhAG, RhBG, RhCG, and Rh30, which together define the four distinct subgroups and confer upon vertebrate animals a genealogical commonality [52]. In recent primate to human evolution, the division of *RH* into *RHCE* and *RHD* via tandem gene duplication reformed the Rh blood group system [11, 12]. Gene duplications in metazoans went through chaotic changes in exon–intron organization of Rh genes in the wide variety of phylogenies. Although Rh genes are intronless in bacteria and certain eukaryotes, they keep multiple introns in all metazoans (Fig. 2). Depending on species, these introns invade the protein-coding sequence at different positions and thus break exons with changing numbers (Fig. 2); yet, Rh proteins still maintain their conserved features, thereby sharing astounding structural homologies.

Negative versus positive selection on subgroups

Congruent with species orders, Rh homologs cluster in subgroups [50] suggesting that natural selection has acted differently on these subgroups. The Rh family as a whole is highly conserved, but its subgroups vary in divergence rate and sites thereby relating functional specification to species

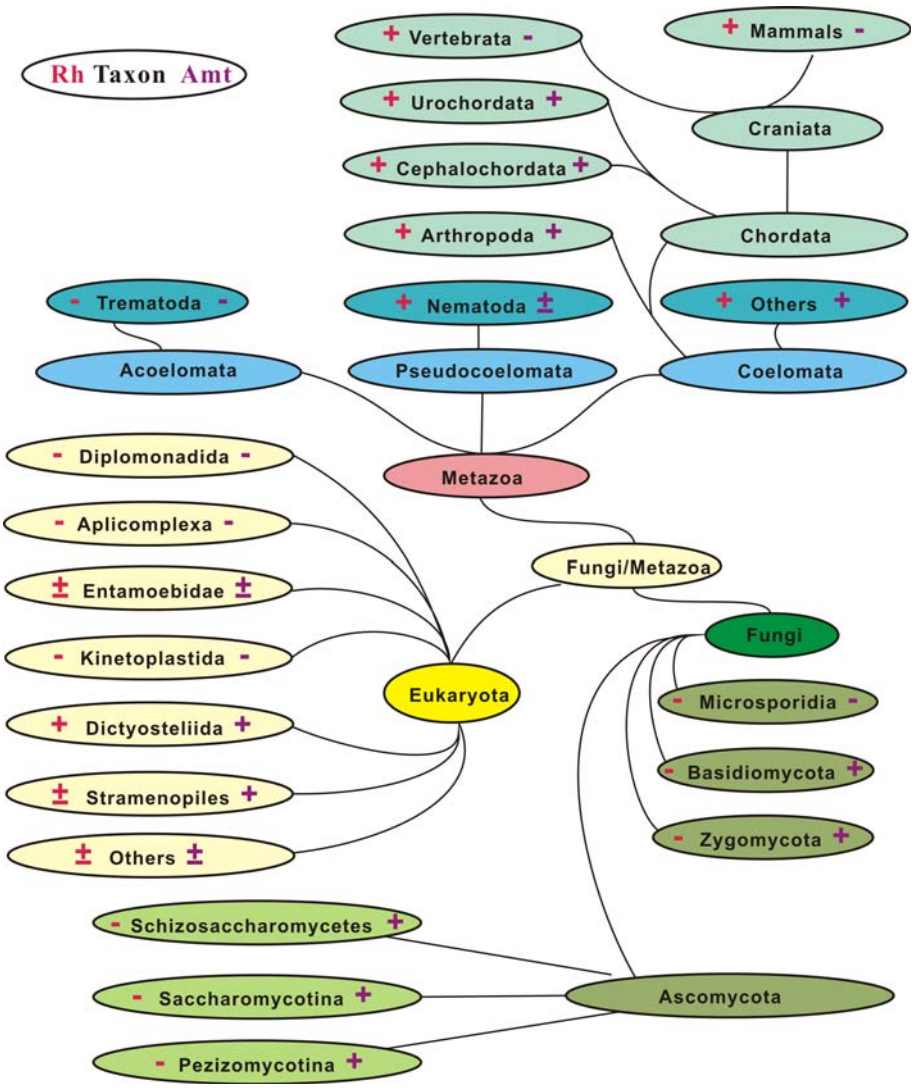

Fig. 1 Distribution of Rh genes and their coexistence with Amt genes in eukaryotes. *Plus* and *minus* indicate presence and absence, respectively, of Rh (*left*) or Amt genes (*right*). The results were obtained from tblastn/blastp search using Rh or Amt protein queries against genome databases. Taxonomic divisions are modified from the eukaryotic tree (www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi). Certain taxa lack both Rh and Amt

adaptation [52]. One key advance from unicellularity to multicellularity is the origin of epithelia; the duplication of Rh gene from one in sponge [67] to two in the Placozoa [68], the basal metazoans with epithelia [69, 70], coincides with this morphological innovation (Fig. 2). Invertebrate Rh proteins comprise a large group having endured a long period of negative selection and a similar degree of sequence identity to the individual vertebrate subgroups, RhBG > RhCG > RhAG > Rh30 [52]. Hence the Rh ancestors born with epithelia must have branched to engender those homologs now expressed in invertebrate hemocytes and vertebrate red cells. In vertebrates, negative selection continues on epithelial homologs, but positive selection occurs to erythroid Rh proteins. The Rh30 subgroup has diverged rapidly and steadily after its origin [71–74], whereas the RhAG subgroup has experienced two phases of selection, negatively in lower vertebrates but positively in mammals parallel to Rh30 selection [51]. The fast co-evolution of Rh30/RhAG matches with the timeline of morphological transition of red cells from elliptical to biconcave through enucleation.

Distant relatedness of Rh to Amt

Ammonia transporters (Amt) are thought to be members of the MFS club [38]; they and Rh proteins form the only known related families [46, 75]. Our knowledge of their differences and similarities in organism distribution, molecular evolution, and protein structure has dramatically increased since the report linking human erythroid Rh proteins to Amt proteins [75]. The two families not only show an opposite pattern of distribution but coexist in a great variety of organisms (Fig. 1). Apparently Rh ancestors had already branched off from Amt ancestors in prokaryotes and the two had gone through their respective evolutionary pathways after separation [50, 51] (Fig. 3a). In addition, the Rh proteins and Amt proteins in those organisms from bacteria to invertebrates that have both

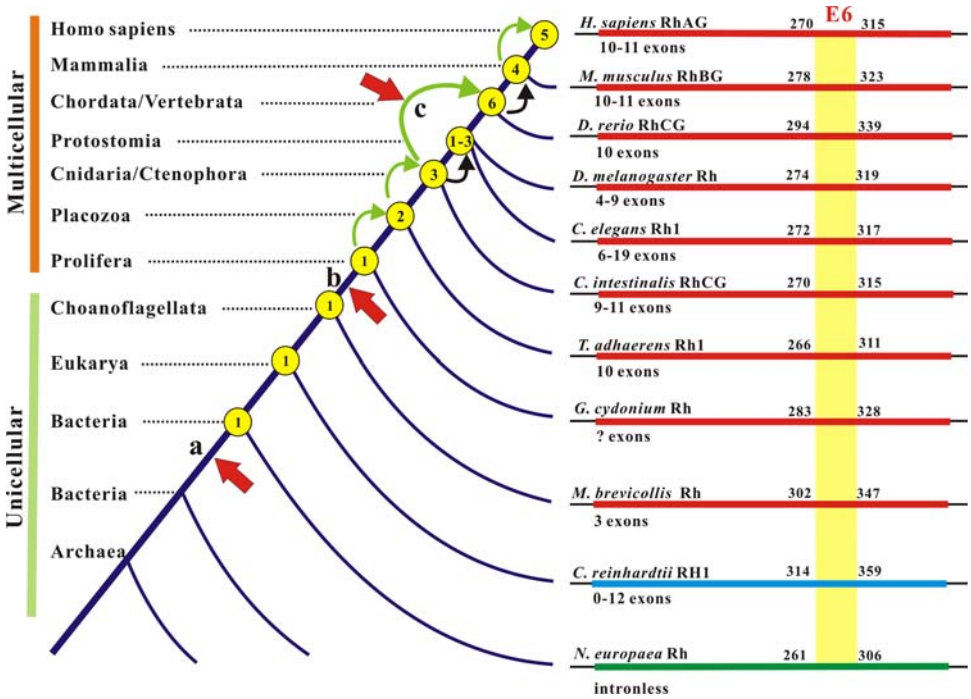

Fig. 2 Gene gain and gene loss in the genesis and evolution of the Rh protein family. *Left* Gene gain and loss are denoted by copy numbers (*circled*) on the tree trunk (not to scale). *Bending arrows* indicate gene duplications (*green*) or gene contractions (*black*). The increase from three to six genes may arise by a genome-wide duplication. *Vertical arrows* to the truck point to evolutionary events with which Rh gene duplications coincide: *a* origin of an ancient Rh gene and its branch off Amt genes in Bacteria and Archaea below the arrow; *b* origin of epithelia; *c* origin of erythrocyte. *Right* Exon remodeling in Rh family genes from representative taxa is shown. E6 (exon 6), the most conserved exon encoding 46 amino acids in metazoans, is used as a reference

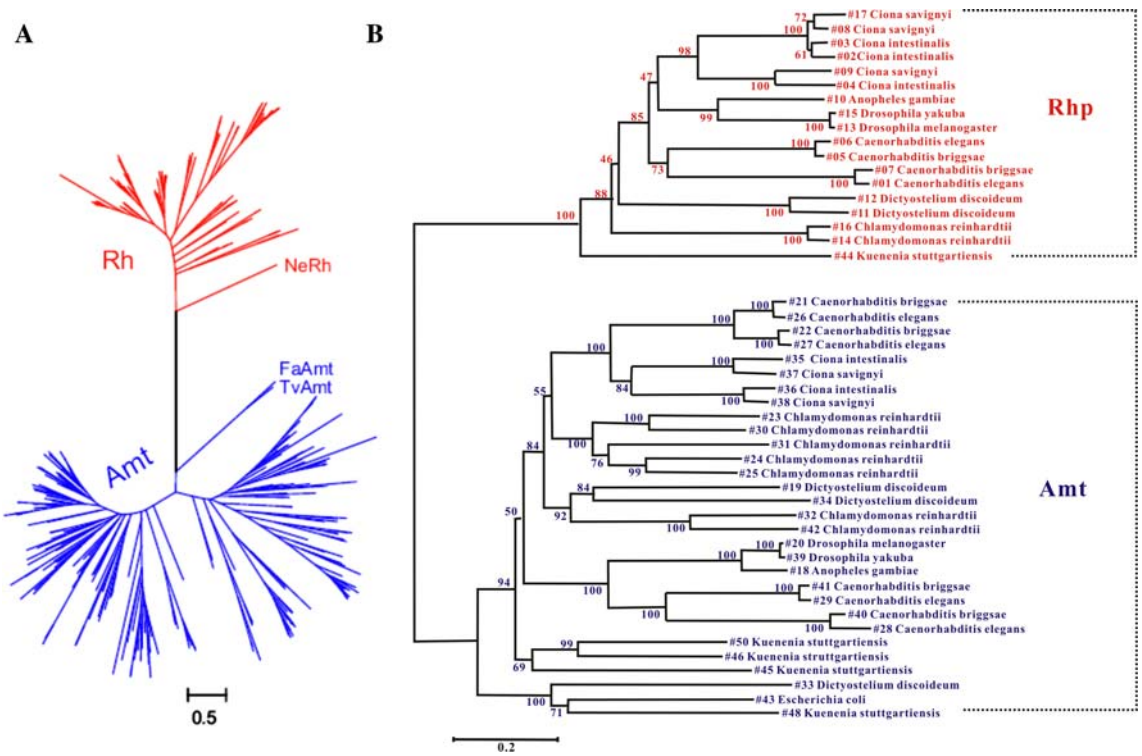

Fig. 3 Rh and Amt are distantly related families but have gone through divergent and independent evolution. **a** The maximum likelihood (ML) joint tree of 111 Rh proteins (*red*) and 260 Amt (*blue*) proteins. Bacterial NeRh and archaeal FaAmt and TvAmt are at the base of the respective families. **b** The ML joint protein tree of 18 Rh and 30 Amt in species that harbor both types of genes. The values *at nodes* are the bootstrap proportion from ML. This expanded analysis covers species from bacteria to invertebrates

types of genes also cluster independently and are separated by an unpredictable distance because of the long period of divergent evolution (Fig. 3b). These findings pinpoint a distant and paralogous relatedness arguing for a functional distinction between the Rh family and the Amt family [46, 52].

Primary structure, transembrane fold, and 3D structure of Rh proteins

Biochemical features of primary structures

With the primary structures of Rh proteins known in human red cells [20–24], epithelial cells, and other species [41, 42], a consensus has emerged as to the index of conservation and diversification for the Rh family [50, 51]. Analysis of selected members from bacteria to humans reinforces such common features (Table 2). The total length of Rh proteins differs considerably depending on species, e.g., it is 390 aa for the *A. bacteria* Rh protein and 958 aa for the Rh2 homolog of *N. gruberi* (a soil/water amoeboflagellate), but the vast majority have a size of 400–500 aa (Table 2). The primary sequences that define TM helices are most conserved and thus hydrophobic residues account for two-thirds or more of total residues. For the TM helices, the ratio of hydrophobic/polar residues is constant (3:1) and so is the Gly/Pro ratio (4–5:1) and strict His occurrence (Table 2). The patterns of charge and size are distinct among intracellular loops (ICL), extracellular loops (ECL), and *C*-tails. *C*-tail is most diverged in sequence and size between subgroups [43]. Paradoxical to this variation is the invariant retention of charged residues in *N* and *C*-termini, although not at the same sites. Negative charges show a greater presence in the *C*-tail of RhAG, RhBG, or RhCG and positive ones in the *N*-terminus of Rh30, manifesting a bi-modularity pI profile as acidic and basic, respectively (Table 2). As little variability occurs in

Table 2 Primary composition and biochemical features of Rh proteins

Name	ORF (aa)	pI	TM%	Hydro/polar		TM		ECL (aa)	ICL (aa)	Total (aa) (Nt/Ct)
				Overall	Tm	G/P	H			
Human										
RhAG	409	6.66	64.8	182/96	159/52	31/7	5	68	42	25 (4/21)
RhBG	458	6.70	57.9	203/99	151/53	31/7	8	96	43	55 (13/42)
RhCG	479	6.20	55.3	209/108	150/63	27/7	5	100	42	72 (10/62)
RhCE	417	9.41	63.5	202/98	164/57	22/5	2	69	44	39 (11/28)
RhD	417	8.51	63.5	201/98	161/59	23/5	2	69	44	39 (11/28)
Eukaryotes										
DmRh	449	5.74	59.9	203/105	164/56	25/6	3	103	43	38 (9/29)
CeRh1	463	6.21	58.1	198/114	155/58	23/7	7	96	41	61 (10/51)
TaRh1	445	5.36	59.6	204/108	155/59	22/6	4	88	41	52 (7/45)
TaRh2	459	5.73	57.7	213/116	158/65	20/4	6	102	41	54 (8/46)
GcRh	523	4.79	51.4	225/129	153/58	28/7	3	129	40	87 (6/81)
MbRh	480	5.51	55.2	210/119	155/56	29/6	6	115	48	48 (5/43)
Bacteria										
NeRh	425	6.33	62.4	214/89	163/52	28/7	3	61	39	60 (4/56)
NiRh	457	5.85	58.0	209/103	160/49	30/8	6	60	39	93 (4/89)
NmRh	407	5.60	65.1	200/89	167/47	27/7	4	61	39	41 (4/37)
GeRh	403	4.77	65.8	200/83	158/54	24/6	3	57	38	43 (4/39)
KsRh	585	6.85	45.3	248/134	157/56	24/6	4	260	38	22 (3/19)
CcRh	404	8.03	65.6	192/96	160/53	23/9	3	75	38	26 (4/22)
ClRh	401	5.22	66.1	193/90	157/56	26/8	3	60	39	23 (4/19)
CpRh	391	6.34	67.8	197/79	160/54	28/6	4	75	38	27 (4/23)
DaRh	400	7.25	66.2	187/96	158/57	25/8	3	75	38	22 (4/18)
AaRh	390	6.85	67.9	186/87	157/56	27/7	3	60	42	23 (4/19)
Archaea										
AfAmt1[a]	391	5.90	66.0	208/71	171/43	25/4	3	63	43	28 (3/25)

TM % Percent of TMH residues to total residues of a full-length protein, *Hydro/Polar* hydrophobic vs. polar residues in full-length protein (*overall*) or in TMH (*Tm*), *G/P and H* Gly/Pro and His residues assigned to transmembrane segments, *ECL and ICL* total size of extracellular and intracellular loops in residue numbers, *Nt/Ct* the total and respective size of *N*- and *C*-terminal regions in residue numbers. The data were from structure-based sequence alignments and are available upon request

[a] AfAmt1 lacks TMH0 or signal peptide and thus has 11 TM helices. This Amt is chosen for comparison because it gives rise to a better alignment with Rh proteins than EcAmtB

TMH and ICL, the *C*-tail contributes a net negative charge to Rh glycoproteins, a feature also pertaining to most Amt proteins [51]. Hence, the variable *C*-tail and the tightly packed TM helices consolidate Rh function and regulation in a single adaptable modular design in the membrane.

Fold of transmembrane helices

Rh proteins are predicted to have 12 transmembrane helices [40, 43], a condition which has now been observed in the 3D structure of NeRh, the *N. europaea* Rh, and human RhCG [76–78]. Figure 4 shows a 2D topology of NeRh with its short *N*-terminus and long *C*-tail facing the cytoplasm. This model and Table 2 illuminate some identifying features, both general and particular to Rh proteins. The two 6-TMH halves (TMH0-5 and 6–11) grossly appear as "self-images" given their patched identities and shared motifs. For Rh glycoprotein homologs TMH1, 3, 5, 6, 8, and 10 are conserved with key amino acids, e.g., twin-His and twin-Phe. TMH0 is peripheral [78] as suggested [79, 80], but TMH1:6, 2:7, 3:8, 4:9, and 5:10 each form a pair of TM domains packed close in the membrane [76–78]. The surface-charge across the lipid bilayer is asymmetric because ECL and ICL are coated by net negative and net positive charges, respectively (Fig. 4). ICL are short and invariant in size (Table 2); of these, ICL3 between TMH5 and 6 is the longest allowing 6-TM halves limited mobility, and others are just long enough for helix turns. ECL changes are largely confined to elongation or truncation of ECL0 and ECL5. The strict total length of ICL as a feature common to Rh and Amt (Table 2) makes them unique within the MFS club. It is tempting to speculate that oscillation of TMH5 plays a crucial role in triggering the Rh channel function as in the AmtB protein [81].

Of note is TMH0, an integral part of Rh proteins [82, 83] but a cleavable signal peptide (SP) either present [84–86] or absent in Amt proteins [87]. Thus unlike the Rh proteins, membrane-bound Amt proteins lack the TMH0 but have the *N*-terminal end from part of ECL0 float in the periplasmic space. In the heterologous *E. coli* cell NeRh-TMH0 was likely recognized as SP and removed [76, 77]; but whether it is so processed in the native host *N. europaea* is unclear [88, 89]. Perhaps different from Amt Rh-TMH0 is a hybrid motif of SP and TMH given its possible origin from an SP-like segment. As a key issue related to intracellular routing and membrane assembly, the dual role of Rh-TMH0 in both prokaryotes and eukaryotes needs to be verified. The absence of TMH0 in NeRh crystals [76, 77] but presence in human Rh proteins [82, 83] also raises the question of what effects TMH0 has on protein oligomerization and intramembrane folding [79, 80].

3D structure of NeRh protein and its implications for function

The high-resolution 3D structures of *E. coli* AmtB (EcAmtB) and *A. fulgidus* Amt1 (AfAmt1) have provided fresh insights into the mechanism of ammonia conduction [85–87]. EcAmtB and AfAmt1 both fold as homotrimers with each monomer forming a vestibule for NH_4^+/NH_3 binding and a long narrow hydrophobic channel for ammonia transport. Mechanistically, both Amt proteins appear to operate as a gas channel that mediates the passage of neutral species of ammonia, but the conduit of proton transfer/movement as a prerequisite for gas conduction is only partially understood [90–92]. Given the known relatedness, the 3D structures of Amt proteins had been used as a template for homology modeling of human Rh proteins to gain insight into their function [79, 80, 93, 94]. The studies suggest that Rh glycoproteins and Rh30 proteins both adopt a 3D fold similar to the two Amt proteins, but Rh30 lacks the conserved His and/or Phe residues important for substrate conduction.

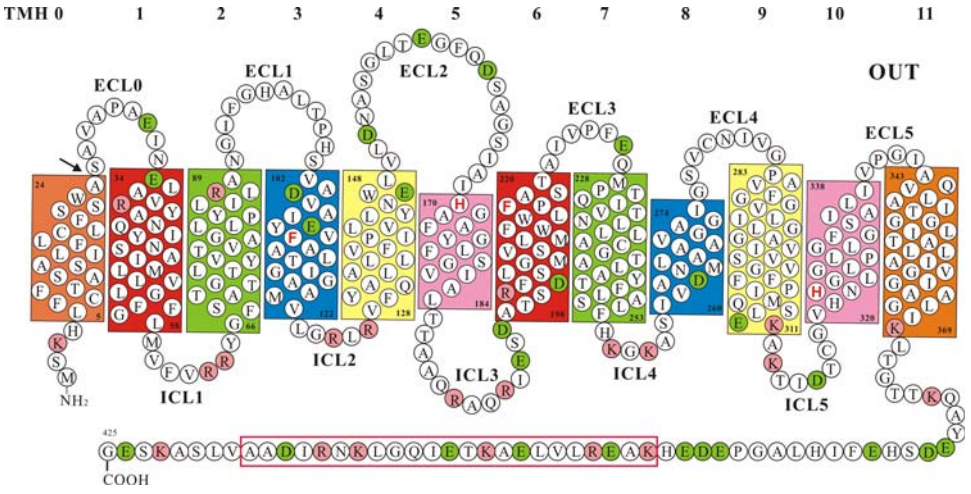

Fig. 4 Membrane topology of *N. europaea* Rh protein as a model for the Rh family. TMH0 is absent in the crystals and is based on hydropathy plot, whereas TMH 1-11 are from the 3D structure. Closely packed TMH are colored the same except TMH0. The residues positioned at the bilayer leaflets are numbered. Twin-His H170/H324 and twin-Phe F110/F218 are bold (*red*). Positive K/R charges are colored *pink* and negative D/E charges *green*. The α-helical bundle-forming sequence in the *C*-tail is *boxed*

The diffraction of NeRh crystals has laid a solid foundation to relate the 3D structure to the function of Rh family proteins [76, 77]. As compared to the Amt structures [85–87], the overall fold and membrane topology of NeRh are similar. Due to TMH0 removal each subunit is composed of 11 TMH with pseudo two-fold symmetry enclosing a central pore presumably mediating gas transport (Fig. 5a). The NeRh structure is also an α_3-homotrimer generated by a crystallographic three-fold passing through the center of the three monomers (Fig. 5b). A cartoon shows the extracellular vestibule, central channel, and other notable features in one subunit (Fig. 5c). Besides the general folding pattern, a prominent conservation is the twin-His site, namely His170/His324 in NeRh [76, 77], His170/His320 in EcAmtB [85, 86], and His157/His305 in AfAmt1 protein [87]. Although controversial, this twin-His site is thought to mediate NH_4^+ ion deprotonation [85] and is crucial for function [95]. In both Rh and Amt structures, the twin-His site lies just below the twin-Phe barrier (Fig. 5c); the two His adopt an unusual coplanar orientation and situate in a fairly hydrophobic environment. The coplanar orientation may enable the two His residues to stabilize a proton between them, and the hydrophobic milieu may shield them from approaching water molecules and thereby foster anhydrous proton migration. Together with twin-Phe, twin-His may also add in selectivity by blocking the passage of small cations such as Na^+ and K^+, while allowing the passage of neutral gases such as CO_2 or NH_3 [76, 77]. Notably, the twin-His site is conserved in Rh glycoproteins, but one or both residues are mutated in Rh30 homologs [50, 79, 80], supporting that the Rh30 group has evolved to gain a different function [11, 12, 27, 30].

Structural comparison also reveals critical differences between Rh and Amt crystals. (1) Most notably, NeRh has a *C*-tail α-helix directed along the three-fold axis but away from the trimer proper [76, 77] (Fig. 5a). The three helices come close to form a left-handed three-helix bundle, a fairly mobile part with much higher average residue B factor (76 $Å^2$) than the rest of the protein (20 $Å^2$). (2) NeRh has a CO_2-binding pocket, a unique structure formed by fairly conserved residues when native crystals were pressurized under CO_2 and then flash cooled in liquid nitrogen [76]. This pocket is located within a deep cavity near the channel exit to the cytoplasm (Fig. 5d); its role is presently unclear but could be to promote CO_2 movement in and out of the pore as a secondary site. (3) NeRh has a higher presence of prolines in TMH like its homologs (Table 2). Such internal prolines can lead to distortions or kinks in TM helices [96] and thereby impact helix packing or protein function (e.g., dictating the hinge sites for a conformational change). (4) NeRh lacks the extracellular vestibule π-cation binding site formed by three aromatic

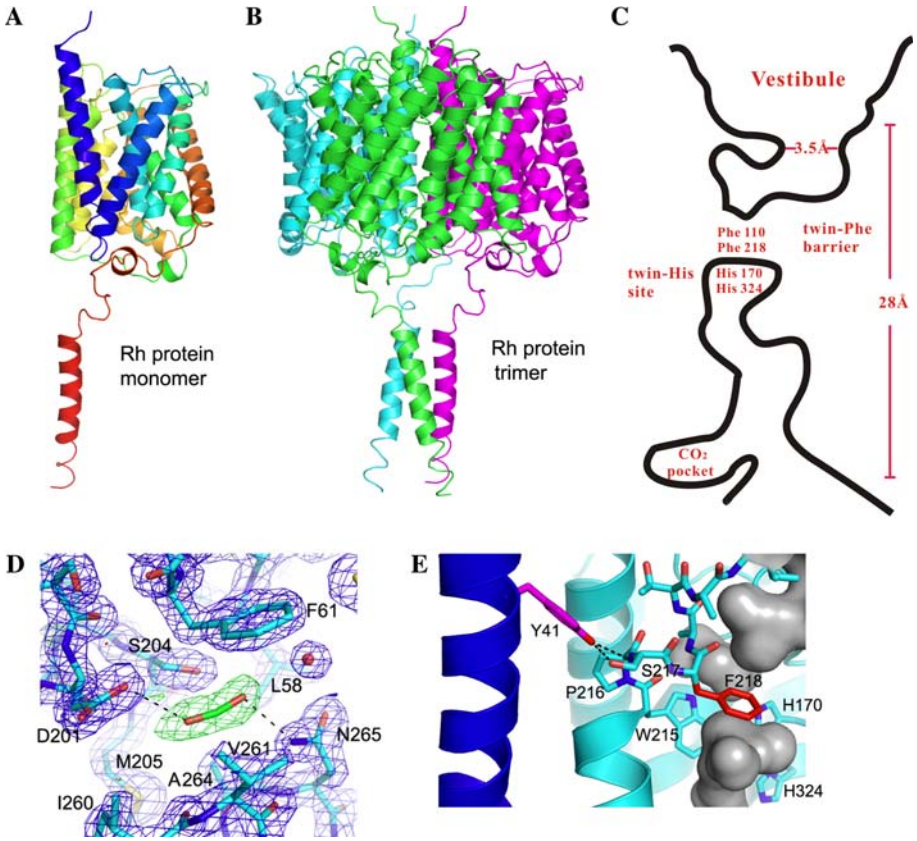

Fig. 5 3D structure of the *N. europaea* Rh protein. Each drawing is oriented such that the periplasm is above and cytoplasm is below. **a** Ribbon diagram of a monomer. **b** Ribbon diagram of a homotrimer. **c** Diagram of the putative central channel in a monomer. The constriction of extracellular vestibule (3.5 Å) and membrane-crossing (28 Å) are denoted. Twin-Phe barrier, twin-His site, and CO_2-binding site are illustrated. **d** Atomic details of CO_2-binding residues in the pocket. **e** Interactions between Tyr41 and TMH1. Potential hydrogen bonds between Tyr41 and Ser217 are shown as *black dotted lines*

residues thought to be key to NH_4^+/NH_3 recruitment in EcAmtB [84]. (5) The twin-Phe are conserved in NeRh (Phe110/Phe218) (Fig. 5c), EcAmtB (Phe107/Phe215), or AfAmt1 (Phe96/Phe204) but show altered orientation. In Amt, the two Phe adopt positions that block substrate passage through the channel [85–87] as important functional sites [97]. In NeRh Phe218 appears to be the major barrier, since Phe110 adopts a different orientation [76, 77]. (6) Although the role of twin-His may be similar, one difference in potential functional impact is that in NeRh the Nε2 atoms of His170 and His324 form hydrogen bonds to neighboring water molecules. In contrast, His320 of EcAmtB or His305 of AfAmt1 has only one hydrogen-bonding partner. The structural differences between Rh and Amt have implications for function and suggest important targets for future studies.

A putative gating mechanism for opening of Rh channel proteins

The α-helical bundle found in NeRh *C*-tail suggests that it may mediate a functionally relevant protein–protein interaction [76, 77]. The 3D structures of AmtB-GlnK protein complexes yield clues to this view in that GlnK inhibits ammonia uptake via binding to the ICL face [98, 99]. If the *C*-tail of NeRh binds to a protein, it could similarly block substrate passage by linking such an interaction to the putative CO_2 binding site in two ways [76]: (1) substrate transfer is facilitated by the binding partner; (2) substrate-induced protein recruitment is executed as a sensing mechanism, as has been proposed for *A. brasilense* AmtB [100]. Currently, the solved 3D structures of Amt and Rh proteins are all in a channel-closed configuration [76, 77, 85–87], where twin-Phe separates the channel into two parts and thereby shuts off transport (Fig. 5c). To open the channel passage through NeRh, a *C*-tail-mediated partner binding must disengage this twin-Phe block, for example, by altering its orientation.

There are three key structural elements that support this potential form of regulation. (1) The *C*-tail α-helix has a glutamate that is salt bridged to Arg63 and Arg64 on ICL1 between TMH1 and TMH2 (Fig. 4). (2) Tyr41 on TMH1 is hydrogen bonded to the main-chain NH and side-chain-OH of Ser217 on TMH6 of an adjacent subunit (Fig. 5e). (3) Ser217 lies in between the internal Pro216 that induces a helical kink in the TMH6 helix (Fig. 4) and the Phe218 that serves as a major steric barrier for transport (Fig. 5c). Thus changes in the interactions between Tyr41 and Ser217 could affect the magnitude of TMH6 tilting, which in turn impacts the movement of Phe218 to enable channel opening or closing [76, 77].

The mechanism envisaged in which pore opening of the Rh protein is dictated by *C*-tail-mediated interactions begs for binding partners. Potential candidates include carbonic anhydrase (CAH) or RuBisCO if Rh proteins conduct CO_2 or ammonia monoxygenase (AMO) and glutamine synthetase if Rh proteins were Amt equivalents. Of special note is the α-CAH enzyme, for its distribution is totally correlated with that of Rh proteins in all organisms whose genome sequences have been determined [50]. Given this phylogenetic coexistence, it is of great interest to test out whether α-CAH could be docked onto NeRh trimer and, if so, whether the interaction occurs in biochemical and functional forms in vivo [101]. It is also of interest to determine how *C*-tail-mediated regulations cope with the great *C*-tail sequence diversity in functional adaptations of Rh subgroups.

Substrate specificity

Rh proteins were inferred to participate in ammonia transport by sequence relatedness to Amt proteins [75], a view evidenced by human RhAG expression in a yeast *mep* mutant showing growth on ammonia as the only nitrogen source [102]. Follow-up studies in oocyte, yeast, or other cell types indicated that Rh proteins mediated the passage of both charged and uncharged species [103–114]. NeRh protein also seemed to have a role in NH_3 transport [88, 89], as [^{14}C]-methylamine uptake was competitively inhibited by NH_3 and NeRh expression improved growth of the yeast *mep* mutant on limited ammonium. However, one challenge to the view of Rh proteins being ammonia channels is that AMO, the chief site of ammonia oxidation in *N. europaea*, is an integral membrane protein. Although acetylene did not affect ammonia passage [88], it inhibits the active site but not the substrate channel of AMO that might facilitate NH_3 transport. These observations would make ammonia transport into the cell less important.

There is also evidence supporting an alternate view for the function of Rh proteins as a CO_2 channel. Studies of Rh1 in *C. reinhardtii* showed its strong up-regulation upon high [CO_2] induction [44]. Further studies of the phenotypic properties of *C. reinhardtii* strains depleted of Rh1 expression by RNAi provide evidence for its role in CO_2 transport [45]. Under a high [CO_2] condition, the *rh1*-deficient mutants grow more slowly than wild-type cells. Moreover, arguing against its role in ammonia transport, these *rh1* mutants accumulate [^{14}C]-methylamine normally when grown with either arginine (high accumulation) or ammonia (low accumulation) as the nitrogen source [45]. Recently it was found that the rate of CO_2 transport is much lower in Rh_{null} cells lacking the Rh proteins than in normal red cells [115, 116]. Together these data corroborate the view that Rh proteins have a distinct functional role in mediating CO_2 passage as compared to Amt proteins [46].

Expression of Rh genes and proteins: location and induction

Tissue and cell-specific expression

Rh antigens are abundant non-glycosylated proteins of the red cell membrane [9, 10] with $\sim 1 \times 10^5$ copies per cell [40] and their glycosylated partner RhAG may be of similar quantity [16, 19]. In humans and mice, Rh30 and RhAG promoters contain *cis*-acting regulatory elements to direct erythroid-specific expression [32, 117–120]. RhAG is also expressed in human esophageal epithelia [121] and in mouse brain [122] independently of Rh30. RhCG and RhBG are highly expressed in kidney, testis, liver, brain, or skin and variably in many other tissues [41, 42] with RhCG being a most abundant renal transcript based on quantitative RNA profiling [123]. The 5′-regions of RhCG and RhBG are highly C/G-rich, thus acting as general promoters to govern their broad epithelial expression [41, 42]. In the case of *C. reinhardtii* [44] and *C. elegans* [47], the level of Rh expression is also high. In *C. elegans*, the Rh1 gene is driven by a strong promoter and is highly expressed in multiple sites mimicking the pattern of mammals. The high-level expression of Rh proteins and their slow evolution may be correlated with the demand for precise protein folding [124].

Location and induction of expression

As in humans, Rh proteins from *C. elegans* and *C. reinhardtii* are also routed to the plasma membrane [47, 125]. The only exception is RhgA from *D. discoideum*, which resides in contractile vacuoles [126], organelles that play a key role in osmoregulation [127]. A stretch of negatively charged residues clustered in the *C*-tail of RhgA appears crucial for this targeting [128]. In human red cells, Rh30 and RhAG are vertically linked to the cytoskeleton through their *C*-tails. Whereas Rh30 is present in both ankyrin R and protein 4.1R-based complexes [129], in an ankyrin-based assembly RhAG associates Rh30 and band three as a macrocomplex, which is thought to be an integral gas exchange metabolon for CO_2 transport [129–131]. In contrast, RhCG and RhBG are found in nearly all types of epithelia and are targeted to distinct membrane domains: RhCG is mainly apical and RhBG mainly basolateral. For RhBG, its *C*-tail tyrosine-sorting signal appears crucial for basolateral location and its *C*-tail may attach to the ankyrin G-based network in MDCK cells [132]. However, RhCG and RhBG may not always occur in the same cell type or the same tubular structure. In mice, RhBG is found in periveinous hepatocytes [133] but coexpressed with RhCG in gastrointestinal tracts [134]. In rats, Rhbg is ubiquitously expressed in the alimentary tract, i.e., esophagus, stomach, duodenum, jejunum, ileum, and colon, and Rhcg is coexpressed with Rhbg in these tissues except the stomach and colon [135]. In human RhCG is widely found in esophageal epithelia [121] and various renal tubules [136, 137], but RhBG is barely detected in kidneys [138]. The differences may reflect organ-specific physiology or species-specific adaptation.

The plasma-membrane homing means Rh proteins face external challenges directly in unicellular eukaryotes and indirectly in multicellular animals. Thus Rh genes are prone to induction by developmental cues and environmental perturbations. In *C. reinhardtii*, the expression of Rh1 gene or protein is specifically induced by high CO_2 [44]. In *C. elegans*, Rh genes show increased, differential expression in a stage-specific manner as required for embryonic and adult development [47]. In mammals, Rh genes in epithelial tissues and erythroid cells show an elevated expression in a stage-specific pattern [43, 118]. In whole animals such as rodents, epithelial Rh expression also responds to changes in internal milieu and food sources. In rats the expression of Rhbg and Rhcg responds to metabolic acidosis [139]. In mice, depleting potassium from food alters Rh expression in the renal collecting duct, resulting in Rhcg up-regulation and Rhbg down-regulation, respectively [140].

The Rh gene family and human disease associations

The genes of the human Rh family are located on three chromosomes (Fig. 6a); they share synteny with mouse homologs in both exon–intron structure and the linkage map that characterize the subgroups [11, 43]. The association of erythroid Rh proteins with red cell disorders has been well established [11, 12, 27]. Rh antigens, particularly the D antigen, play a major part in HDFN due to the incompatibility between Rh-negative and Rh-positive blood [3, 4] (Fig. 6a, left). Hence accurate *RH* genotyping forms a core of HDFN management and transfusion therapy in clinical settings [141–143]. The complete absence of red-cell Rh antigens caused by complex mutations in the *RH* locus defines the amorph type of Rh_{null} syndrome with mild phenotypes [144, 145] (Fig. 6b, upper). In contrast, genetic mutations of *RHAG* (Fig. 6b, lower) cause recessive Rh deficiency syndrome (regulator type Rh_{null} or Rh_{mod}) [31–37, 146], or dominant over-hydrated hereditary stomatocytosis (OHSt) showing increased permeability to nomovalent cations [147]. The two genetic disorders show mild chronic hemolytic anemia and share some phenotypic features with other forms of hereditary stomatocytosis [148]. Such changes indicate that ablation or disruption of the RhAG function also affects red cell integrity.

On the other hand, negative selection has placed epithelial RhBG and RhCG under highly conserved evolution

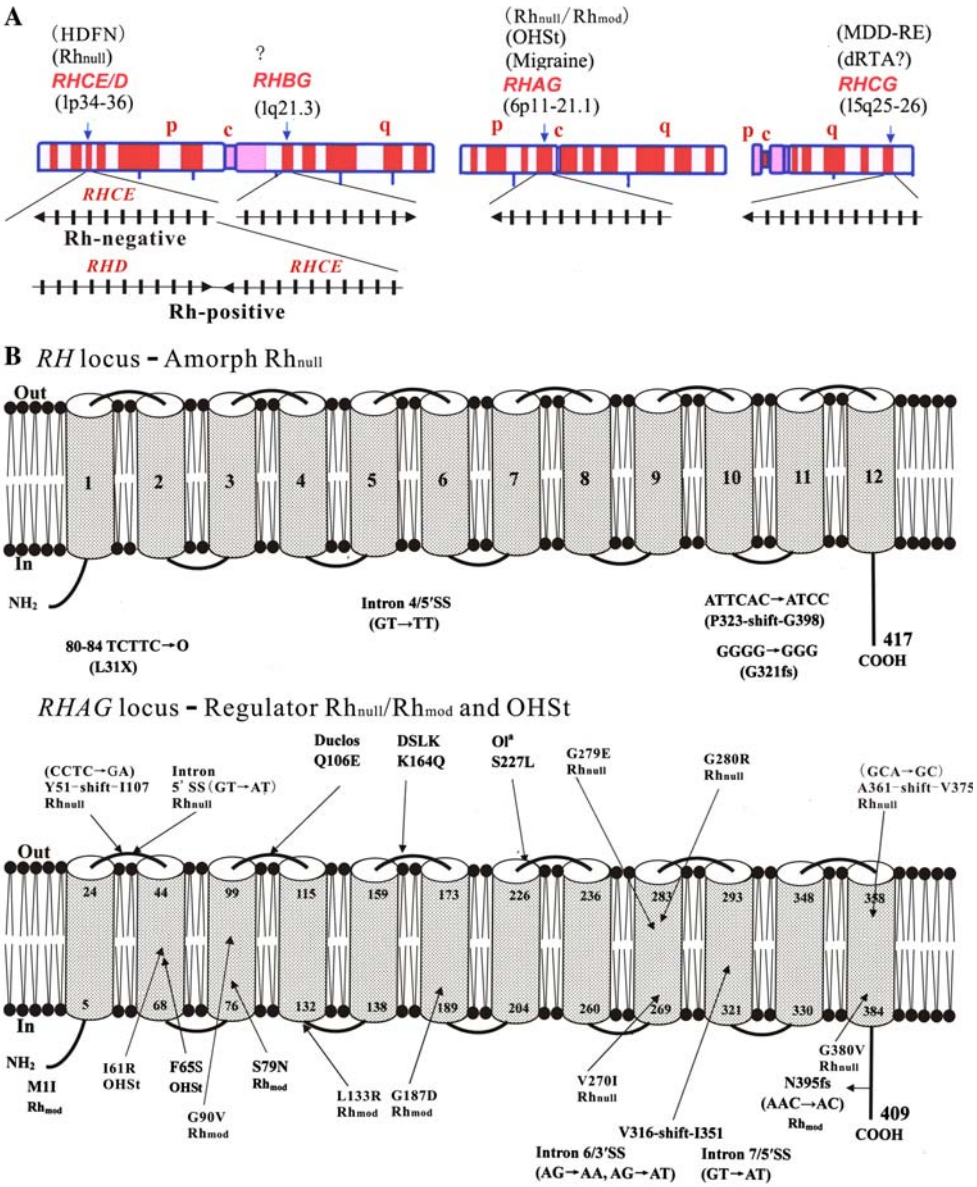

Fig. 6 Chromosomal location and disease association of human Rh family genes. **a** *RH*, *RHBG*, *RHAG*, and *RHCG* reside in chromosomes 1, 6, and 15. The exon–intron structure and orientation of each gene as well as Rh-negative and Rh-positive haplotypes are shown. *c* Centromere, *p* short arm, *q* long arm, *HDFN* hemolytic disease of the fetus and newborn, *OHSt* over-hydrated hereditary stomatocytosis, *MDD-RE* recurrent early-onset major depressive disorders, *dRTA* distal renal tubular acidosis. *Question mark* denotes unknown. **b** Diagram of mutations of *RH* and *RHAG* genes. Amorph type Rh_{null} (*upper*); regulator Rh_{null}, Rh_{mod}, and OHSt (*lower*). The 12-TMH of Rh and RhAG is based on NeRh (Fig. 4). Duclos, DSLK, and Ol^a located on the ECLs are point changes of RhAG likely to be neutral antigenic polymorphisms

[50], accounting for the current lack of their genetic mutations in humans. However, their physiologic importance has been shown by the knockdown phenotypes of orthologs from primitive species such as *C. reinhardtii* and *C. elegans* [45, 47, 48]. RhBG and RhCG have been implied as potential tumor suppressors given their sharp down-regulation in human esophageal squamous epithelial cancers [121] and mouse brain tumors [149]. Lately the physiological importance of mammalian Rh proteins has been shown in *Rhcg* knockout mice, which have pH perturbations in urine and epididymal fluids [49], indicating a deficit in pH balance that is primary to renal NH_4^+ excretion. In contrast to the negative data observed in *Rhbg* knockout mice [150], this work implies that the human RhCG gene, when mutated, may result in distal renal tubular acidosis and male infertility [49]. Notably human *RHCG* has also been identified as a candidate gene for early-onset major depressive disorder [151], and human *RHAG* is linked to a subtype of migraine [152] (Fig. 6a). It is hoped that these studies will stimulate future efforts to decipher the role of Rh glycoprotein genes in human diseases.

Conclusion and outlook

The last decade has seen much progress in research on the Rh family while a debate goes on regarding its substrate specificity. Current evidence suggests a role for Rh proteins as dual channels for CO_2 and ammonia, whether in neutral

forms or as charged species (CO_2/HCO_3^- vs. NH_3/NH_4^+). This review highlights their long history of evolution and their surprising structural conservation from prokaryotes to humans and elicits a challenging question as to their biological function: why would nature give rise to two proteins, Rh and Amt, to coexist and fulfill the same function across a wide range of organisms? The evidence accumulated is compelling that the functional role of Rh proteins is related to but distinct from Amt proteins. A mechanism is required to reconcile the substrate duality given the differences and similarities of CO_2 versus NH_3 in physicochemical properties. Of note is that pH is an intimate factor linked to the two molecules, but its role in transport may be overlooked in studies of Rh proteins. Thorough studies of the pH effect on the transport process of Rh and Amt may hold a key to our understanding of their function.

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