Molecular Phylogenetics and Evolution 148 (2020) 106814

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

# Early eukaryotic origins and metazoan elaboration of MAPR family proteins

Elisabeth Hehenberger[a,1], Michael Eitel[b], Sofia A.V. Fortunato[c], David J. Miller[c], Patrick J. Keeling[a], Michael A. Cahill[d,e,*]

[a] Department of Botany, University of British Columbia, 3529-6270 University Boulevard, Vancouver, BC V6T 1Z4, Canada
[b] Department of Earth and Environmental Sciences, Paleontology and Geobiology, Ludwig-Maximilians-Universität München, Munich, Germany
[c] ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, QLD 4811, Australia
[d] School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW 2678, Australia
[e] ACRF Department of Cancer Biology and Therapeutics, The John Curtin School of Medical Research, Canberra, ACT 2601, Australia

ARTICLE INFO

*Keywords:*
Membrane-associated progesterone receptor
Multicellularity
Phylogeny
Organizer
Opisthokont
Protein evolution
Tyrosine phosphorylation
Holozoa

ABSTRACT

The membrane-associated progesterone receptor (MAPR) family consists of heme-binding proteins containing a cytochrome $b_5$ ($cytb_5$) domain characterized by the presence of a MAPR-specific interhelical insert region (MIHIR) between helices 3 and 4 of the canonical cytb5-domain fold. Animals possess three MAPR genes (PGRMC-like, Neuferricin and Neudesin). Here we show that all three animal MAPR genes were already present in the common ancestor of the opisthokonts (comprising animals and fungi as well as related single-celled taxa). All three MAPR genes acquired extensions *C*-terminal to the $cytb_5$ domain, either before or with the evolution of animals. The archetypical MAPR protein, progesterone receptor membrane component 1 (PGRMC1), contains phosphorylated tyrosines Y139 and Y180. The combination of Y139/Y180 appeared in the common ancestor of cnidarians and bilaterians, along with an early embryological organizer and synapsed neurons, and is strongly conserved in all bilaterian animals. A predicted protein interaction motif in the PGRMC1 MIHIR is potentially regulated by Y139 phosphorylation. A multilayered model of animal MAPR function acquisition includes some pre-metazoan functions (e.g., heme binding and cytochrome P450 interactions) and some acquired animal-specific functions that involve regulation of strongly conserved protein interaction motifs acquired by animals (Metazoa). This study provides a conceptual framework for future studies, against which especially PGRMC1's multiple functions can perhaps be stratified and functionally dissected.

## 1. Introduction

### 1.1. The MAPR family

Progesterone receptor membrane component 1 (PGRMC1)[2] is the archetypal member of the membrane associated progesterone receptor (MAPR) family (Cahill, 2007; Mifsud and Bateman, 2002). Vertebrates including humans encode four MAPR proteins. PGRMC1 and the closely related PGRMC2 arose by gene duplication of an original 'PGRMC' gene during vertebrate evolution. We refer here to PGRMC1 and/or PGRMC2 for proteins from vertebrates that have inherited this gene duplication (Cahill, 2017; Ren et al., 2019), or otherwise to PGRMC. This study does not attempt to address functional differences between PGRMC1 and PGRMC2 but focuses on the evolution of PGRMC and the other two the MAPR proteins, represented by neuron-derived neurotrophic factor, commonly known as neudesin (NENF), and neuferricin (NEUFC), also sometimes called cytochrome $b_5$ domain-containing protein 2 (CYB5D2) (Cahill, 2017; Kimura et al., 2012; Ren et al., 2019; Ryu et al., 2017).

All MAPR proteins contain an insertion of an oligopeptide sequence between helices 3 and 4 of the canonical cytochrome $b_5$ ($cytb_5$) domain fold (as defined by human $cytb_5$). In place of a short loop at this position in classical $cytb_5$ domain proteins, MAPR proteins contain a MAPR interhelical insertion region (MIHIR) of variable length (Cahill, 2017;

* Corresponding author at: School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW 2678, Australia.
*E-mail address:* mcahill@csu.edu.au (M.A. Cahill).
[1] Current address: Ocean EcoSystems Biology Unit, RD3, GEOMAR | Helmholtz Centre for Ocean Research Kiel, 24105 Kiel, Germany.

[2] List of abbreviations. CK2: casein kinase 2; CNS: central nervous system; cyP450: cytochrome P450; cytb5: cytochrome b5; LEUMCA: last eumetazoan common ancestor; MAPR: membrane-associated progesterone receptor; MIHIR: membrane-associated progesterone receptor-specific interhelical insert region; NENF: Neudesin; NEUFC: Neuferricin; P4: progesterone; PGRMC: progesterone receptor membrane component; PGRMC1: progesterone receptor membrane component 1; SH2: Src homology 2; SH3: Src homology 3.

https://doi.org/10.1016/j.ympev.2020.106814
Received 4 September 2019; Received in revised form 24 March 2020; Accepted 1 April 2020
Available online 08 April 2020
1055-7903/ 

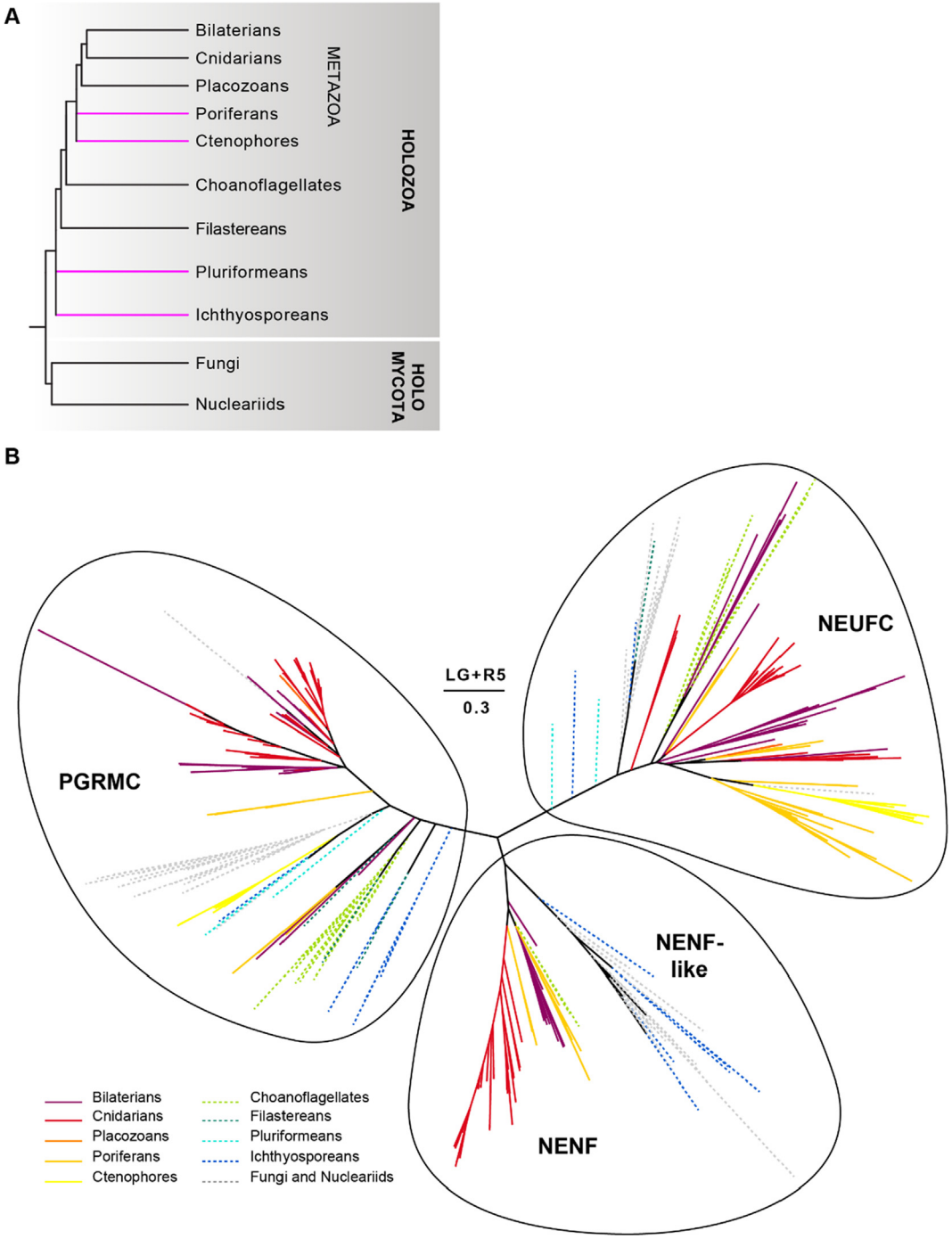


**Fig. 1.** Phylogenetic reconstruction of MAPR proteins in opisthokonts. (A) Schematic tree of opisthokont lineages analyzed in this work, with contentious branches presented as polytomies and colored in magenta. The branch topology of ctenophores and poriferans divergence is subject to strong ongoing debate, with both poriferans (Feuda et al., 2017; Simion et al., 2017) and ctenophores (Whelan et al., 2017) argued as forming sister groups to all other animals. Similarly, the pluriformeans (*Syssomonas* and *Corallochytrium*) were found to branch together with ichthyosporeans as sister to all other holozoans in alternative tree reconstructions (Lopez-Escardo et al., 2019; Torruella et al., 2015). (B) Phylogeny of 3 types of MAPR proteins in opisthokonts: progesterone receptor membrane component (PGRMC), neudesin (NENF and NENF-like) and neuferricin (NEUFC). Solid lines represent metazoan lineages, dashed lines represent non-metazoan lineages. Different lineages are indicated by color in the key. The scale bar and the number beneath it indicate the estimated number of substitutions per site, above the scale bar the model for tree reconstruction is indicated. For bootstrap support see Fig. A1, for a phylogeny containing also taxon information, see figshare repository doi: https://doi.org//10.6084/m9.figshare.9162164. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Mifsud and Bateman, 2002). The MIHIR of vertebrate PGRMC1 and PGRMC2, but not of NENF or NEUFC, contains a tyrosine (PGRMC1 Y139) that is strongly conserved in bilaterian animals (Cahill, 2017). By use of the term 'bilaterian' we refer to the triploblastic animal clade historically referred to as Bilateria, consisting of Xenacoelomorpha (triploblasts of primitive grade) and Nephrozoa (protostomes and deuterostomes). The bilaterians and cnidarians together constitute the eumetazoan clade (Cannon et al., 2016). We do not imply that the last eumetazoan common ancestor (LEUMCA) was not bilaterally symmetrical. Anthozoan cnidarians exhibit bilateral symmetry, and whether

bilateral symmetry was secondarily lost in other cnidarian lineages, or independently gained by Anthozoans, remains unresolved. In contrast, we define 'non-bilaterian animals' as those extant animals that branch as sister groups to bilaterians in the tree of Fig. 1A (cnidarians, placozoans, poriferans and ctenophores) (reviewed by Genikhovich and Technau, 2017).

### 1.2. MAPR biology

Reviews have been published on neural and cancer functions of MAPR (Cahill et al., 2016a; Kimura et al., 2012; Kimura et al., 2013; Petersen et al., 2013a, b), their interactions with cytochrome P450 proteins (Ryu et al., 2017), and as potential drug targets (Hasegawa et al., 2016). Under the synonym ventral midline antigen A (VEMA) PGRMC1 was identified as directing early embryonic nerve cord axon guidance (Runko and Kaprielian, 2002, 2004; Runko et al., 1999). Both NENF and NEUFC were identified as secreted neurotrophic factors with extracellular heme-binding MAPR domains (Kimura et al., 2010; Kimura et al., 2005), but can be post-translationally modified in a manner consistent with a cytoplasmic topology for the MAPR domain (Cahill and Medlock, 2017), and the NEUFC MAPR domain is cytoplasmic in HeLa cells (Bruce and Rybak, 2014). It should be noted that the PGRMC1 MAPR domain has been attested both intra- and extracellularly (reviewed by Cahill et al., 2016a)

NENF is part of circuits that control appetite (Byerly et al., 2013), anxiety (Novais et al., 2013), and it influences metabolism and the obesity axis (Ohta et al., 2015b). Preliminary results are consistent with a role in human diabetes-induced adiposity (Kratochvilova et al., 2019). Several studies have suggested cancer associations (Han et al., 2012; Koper-Lenkiewicz et al., 2019; Neubauer et al., 2006; Ortega-Bernal et al., 2018).

Comparatively far less work has been published on NEUFC. In addition to its neural functions NEUFC promotes survival against etoposide-induced death in HeLa cells (Xie et al., 2011), and has been associated with tumor suppressor activity in cervical cancer (Xie et al., 2016). Heme binding was required for NEUFC-mediated survival of chemotherapeutic treatments, which altogether was interpreted as NEUFC-modulation of CY450 activity (Bruce and Rybak, 2014). Like all vertebrate MAPR proteins, NEUFC is implicated with cytochrome P450 reactions, steroidogenesis, and neurobiology (Hasegawa et al., 2016; Petersen et al., 2013a, b; Ryu et al., 2017).

PGRMC1 is a multifunctional protein with a long list of seemingly disparate functions, ranging from involvement in steroid and heme synthesis, membrane trafficking, metabolic regulation, progesterone response in fertility and other situations, and conferral of progesterone-dependent anti-apoptosis, which have been reviewed elsewhere (Cahill, 2007; Cahill et al., 2016a; Cahill et al., 2016b; Cahill and Medlock, 2017; Kabe et al., 2018; Mueck et al., 2014; Peluso and Pru, 2014; Qin et al., 2015; Ryu et al., 2017). Reduction of PGRMC function in mice (Clark et al., 2017; McCallum et al., 2016; Peluso et al., 2018; Peluso et al., 2019) or fish (Aizen and Thomas, 2015; Wu et al., 2018; Wu et al., 2019; Wu and Zhu, 2020) reduces fertility, and especially oocyte maturation and function, which is thought to be related progesterone-dependent PGRMC1 regulation of TCF/LEF-driven genes (Peluso, 2013). In humans impaired PGRMC function is associated with premature ovarian failure and polycystic ovary syndrome (Li et al., 2016; Paulson et al., 2017; Schuster et al., 2010; Zheng et al., 2017).

Both PGRMC1 and PGRMC2 have been shown to influence cell entry to the G0 cell cycle stage (Griffin et al., 2014; Peluso et al., 2014; Peluso et al., 2019; Sueldo et al., 2015). PGRMC1 additionally has a role in traversing the G2/M checkpoint, and both PGRMC1 and PGRMC2 associate with the mitotic spindle (Peluso et al., 2014) and kinetochore chromosome attachment structures during meiosis and mitosis (Juhlen et al., 2018; Terzaghi et al., 2016). Progesterone stabilizes the mitotic spindle in a PGRMC1-dependent manner (Lodde and Peluso, 2011). A subpopulation of PGRMC1 is also localised in the granulosa cell interphase nucleolus (Terzaghi et al., 2018). The nuclear pore protein ALADDIN, which interacts with a PGRMC2 subpopulation in a punctate peripheral nuclear staining (Juhlen et al., 2016), is required for mitotic PGRMC1 spindle and kinetochore localization (Juhlen et al., 2018).

Heme-binding and cytochrome P450 (cyP450) modulation are PGRMC properties attested from mammalian and fungal MAPR proteins (Hand and Craven, 2003; Hughes et al., 2007; Kimura et al., 2012; Mallory et al., 2005). PGRMC1 is expressed in a variety of neurons of the central nervous system (CNS) (Bali et al., 2012; Bali et al., 2013; Olbrich et al., 2013; Zeng et al., 2016), and it is found in synapses where it affects membrane trafficking (Izzo et al., 2014). PGRMC1 therefore performs functions recognizably pre-metazoan in origin, and others that clearly arose in animals. While the functional similarities and differences between PGRMC1 and PGRMC2 are certainly of interest, they are beyond the scope of this study. Hereafter the reader should consider that both proteins are typical bilaterian PGRMC proteins. PGRMC amino acid numbers refer to PGRMC1 for simplicity.

### 1.3. PGRMC1 phosphorylation

Interest in PGRMC1 phosphorylation was sparked when it was found to exist in different phosphorylated forms in breast cancers that were positive or negative for estrogen receptor expression (Neubauer et al., 2008). Bioinformatics revealed the presence of two Src homology 2 (SH2) and one Src homology 3 (SH3) domain target motifs (short peptide sequences that would bind to a much larger SH2 or SH3 protein domain, respectively: hereafter SH3 and SH2 motifs), being a proline-rich SH3 motif centered on PGRMC1 P63, and SH2 motifs centered on Y139 and Y180 (Cahill, 2007; Peluso, 2006). Notably, the P63 SH3 motif and the Y180 SH2 motif were flanked by consensus casein kinase 2 (CK2) sites with phosphoacceptors at S57 and S181. CK2 is constitutively active in many cells, contributing to the order of 20% of the human phospho-proteome (Meggio and Pinna, 2003; Salvi et al., 2009). This suggested a model where CK2 phosphorylation could sterically prevent interactions of kinases or other interaction partners with the motifs at P63 and Y180, thereby negatively regulating PGRMC1 function (Cahill, 2007; Neubauer et al., 2008). Mutation of both of the CK2 sites, but not each individually, rendered MCF-7 breast cancer cells resistant to peroxide-induced cell death (Neubauer et al., 2008). However, a recent knockout of CK2 activity in C2C12 mouse muscle cells revealed marginally higher phosphorylation of S181 (and Y180), clearly showing that a kinase other than CK2 can target S181 (Franchin et al., 2018).

Phosphorylation of both residues at CK2 consensus sites and of the Y139 and Y180 SH2 motifs, as well as a variety of other residues, has been observed from high throughput proteomics studies (Cahill et al., 2016b). Phylogenetic analysis revealed that PGRMC1 acquired signaling and phosphorylation motifs during animal evolution: e.g., the PGRMC1 SH3 motif is absent from PGRMC2, and was gained by terrestrial tetrapods. The adjacent S57 phosphorylation site appeared during primate evolution (Cahill, 2017). It was previously incorrectly concluded that the ancestral metazoan appears to have possessed cognates of both the PGRMC1 Y139 and Y180 SH2 target motifs (Cahill, 2017). As we demonstrate here, this incorrect conclusion was due to mis-assignment of several non-bilaterian animal MAPR proteins as PGRMC orthologs.

A CK2 consensus site adjacent to Y180 was present in the common ancestor of bilaterians (Cahill, 2017), reflecting an embryological state before vertebrate body plan is determined. This is of particular interest to non-bilaterian animal evolution and embryology because, 1) ligands and receptors of the Wnt pathway evolved already in non-bilaterian animals (Srivastava et al., 2010; Suga et al., 2013), and PGRMC1 regulates this pathway in mammalian pluripotent stem cells (Kim et al., 2018); 2) Both nematode MAPR proteins are expressed through early nematode embryogenesis from the oocyte stage until the induction of germline segregation and neural differentiation (Hashimshony et al.,

2015); 3) PGRMC1 is implicated in essential progesterone (P4)/progestin responses in male (Correia et al., 2007; Losel et al., 2005; Thomas, 2008) and female (Peluso, 2006, 2007; Thomas, 2017) germline and reproductive cells; 4) PGRMC-like proteins direct ventral embryonic neural axon migration conserved between nematodes and mammals (Runko and Kaprielian, 2002, 2004); 5) PGRMC1 Y180 phosphorylation was observed only in synaptic fractions of mouse neurons (Munton et al., 2007); and 6) PGRMC1 is involved in synaptic membrane trafficking (Izzo et al., 2014), implicating a role of PGRMC1 phosphorylation in the synaptic signaling that serves a key organismic coordination role in all animals with a nervous system.

It remains unclear which function(s) may be regulated by PGRMC1 phosphorylation. We reasoned that the plethora of PGRMC1 functions should be separable into ancient eukaryotic roles (such as cyP450 interaction) and newly acquired metazoan roles, such as hypothesized tyrosine phosphorylation-mediated signaling in animals. In the present study we examine the nature of MAPR diversity in non-bilaterian animals as well as in unicellular lineages that represent the closest relatives of animals, with particular interest in the origins of PGRMC1 functional SH2 motifs in opisthokonts.

### 1.4. Animal origins and the opisthokonts

The opisthokonts are a eukaryotic supergroup forming two lineages, the Holozoa and the Holomycota (Adl et al., 2019). While the Holomycota include fungi and their relatives (such as nucleariids), the Holozoa consists of animals, together with their closely related unicellular sister lineages (choanoflagellates, filastereans, pluriformeans and ichthyosporeans) (Hehenberger et al., 2017; Lopez-Escardo et al., 2019) (Fig. 1A). Tyrosine kinases and SH2 domains (which bind to phosphorylated tyrosine residues) evolved in those holozoan unicellular animal relatives, as well as many other proteins normally associated with animals (Hunter, 2014; Suga et al., 2013; Suga et al., 2014; Tong et al., 2017), including new transcription factors, cell surface adhesion molecules, transposons, and extracellular matrix (Grau-Bove et al., 2017; Sebe-Pedros and Ruiz-Trillo, 2017).

A previous pilot study (Cahill, 2017) of the phylogenetic distribution of animal PGRMC1 suffered from taxonomic bias against non-bilaterian animals and their unicellular relatives due to insufficient taxon sampling, especially in critical transitions of non-bilaterian animal evolution, and from poor discrimination between MAPR family members in non-bilaterian animals. The guiding motivation of the present study was to identify members of the MAPR family in unicellular holozoans and non-bilaterian animals, aiming to understand the changes in MAPR proteins, identify evolutionarily conserved regions of importance, and particularly to better define the time of acquisition of PGRMC1 tyrosine phosphorylated residues. The underlying premise is that the evolutionary appearance and conservation of protein features is associated with protein function. Therefore the identification of new and conserved features during the evolution of the MAPR protein family should provide useful new insights into the function of MAPR proteins in human biology.

## 2. Materials and methods

### 2.1. Identification of MAPR proteins

The human sequences of PGRMC1, NENF and NEUFC were used in an initial BLASTP search against the NCBI non-redundant protein sequence database (using default NCBI BLASTP settings), retaining the best hit per organism. Additional sequences were identified by using the same queries in BLASTP searches (e-value 1e-25) against a custom database as described (Hehenberger et al., 2017), and further expanded using newly available unicellular opisthokont datasets (*Parvularia atlantis* and *Chromosphaera perkinsii*, available from http://multicellgenome.com) as well as additional fungal datasets (*Colletotrichum higginsianum, Cryptococcus depauperatus, Puccinia striiformis, Rhizoclosmatium globosum, Rhodotorula toruloides, Tilletia controversa;* downloaded from https://genome.jgi.doe.gov/mycocosm/home). The dataset was extended for the placozoan *Hoilungia hongkongensis*, three classes of poriferans, ctenophores, cnidarians and also choanoflagellates by BLAST searches against a set of choanoflagellate and non-bilaterian animal proteomes that have been previously established (Eitel et al., 2018; Simion et al., 2017). BLASTP searches were performed with specifying an e-value of 1e-10 and otherwise default settings using the human NENF, NEUFC and PGRMC1 as well as the initially identified placozoan *Trichoplax adhaerens* (Srivastava et al., 2008) NEUFC and PGRMC protein sequences. Specimens of the calcareous sponge *Pericharax orientalis* were collected from Dunk Island Mission beach in 2016 (under the authorization CMES59 provided by James Cook University). MAPR sequences of *Pericharax* were retrieved from a draft assembled transcriptome (Adamski et al. in prep). BLASTP searches using mammalian sequences as query allowed the identification of the *P. orientalis* homologs. The MAPR identity of all proteins used was verified by sequence alignment and confirmation of the presence of a MIHIR.

### 2.2. Phylogenetic reconstruction

All MAPR sequences described above were initially aligned with MAFFT v. 7.212 L-INS-i (Katoh and Standley, 2013). Ambiguously aligned positions were trimmed off with trimAL v. 1.2 (Capella-Gutierrez et al., 2009) using a gap threshold of 20% and a tree was calculated using FastTree v. 2.1.7 with default options (Price et al., 2010). The resulting phylogeny and the underlying alignment were manually inspected and obvious contaminations, duplicates and sequence variants (isoforms and/or allelic versions in transcriptomic datasets), that may also represent very recent paralogs, were removed. If multiple sequences were found in a specific taxon, we always kept only the shorter branching version of the sequence. The cleaned, unaligned sequences were then subjected to filtering with PREQUAL (Whelan et al., 2018) using the default options to remove non-homologous residues introduced by poor-quality sequences, followed by alignment with MAFFT G-INS-i using the VSM option (–unalignlevel 0.6) (Katoh and Standley, 2016) to control over-alignment. The alignments were subjected to Divvier (Ali et al., 2019) using the -divvygap option to improve homology inference before removing ambiguously aligned sites with trimAl v. 1.2 (-gt 0.01). We then extracted the region from position 46 to 139 (94 amino acid residues (aa), relative to human PGRMC1), that is conserved in all taxa of our alignment, from the trimmed alignment and performed the tree reconstruction based on this central conserved region only. Final trees were calculated with IQ-TREE v. 1.6.5 (Nguyen et al., 2015), using the -mset option to restrict model selection (to DAYHOFF, DCMUT, JTT, WAG, VT, BLOSUM62, LG, PMB, JTTDCMUT; model selected: LG + R5) for ModelFinder (Kalyaanamoorthy et al., 2017), while branch support was assessed with 1000 ultrafast bootstrap replicates (Hoang et al., 2018). We also prepared a conservative phylogeny without performing PREQUAL filtering prior to sequence alignment or Divvier analysis after alignment of the complete sequences, followed by stringent trimming with trimAl v. 1.2 (-gt 0.8), resulting in an alignment of 126 aa length. The tree was calculated using IQ-TREE and ModelFinder as described above (model selected: LG + R7), branch support was assessed with 1000 ultrafast bootstrap replicates. The raw tree files in newick, colored trees with taxon information (and accession numbers where available) in pdf format, and underlying trimmed alignments corresponding to both phylogenetic reconstructions have been deposited to a figshare repository under the DOI 10.6084/m9.figshare.9162164.

The alignment of selected sequences chosen to provide indicative phylogenetic representation of land vertebrates in Fig. 4 was made with the Computational Biology Research Consortium (CBRC) MAFFT platform (https://mafft.cbrc.jp/) using the L-INS-i strategy (Katoh et al.,

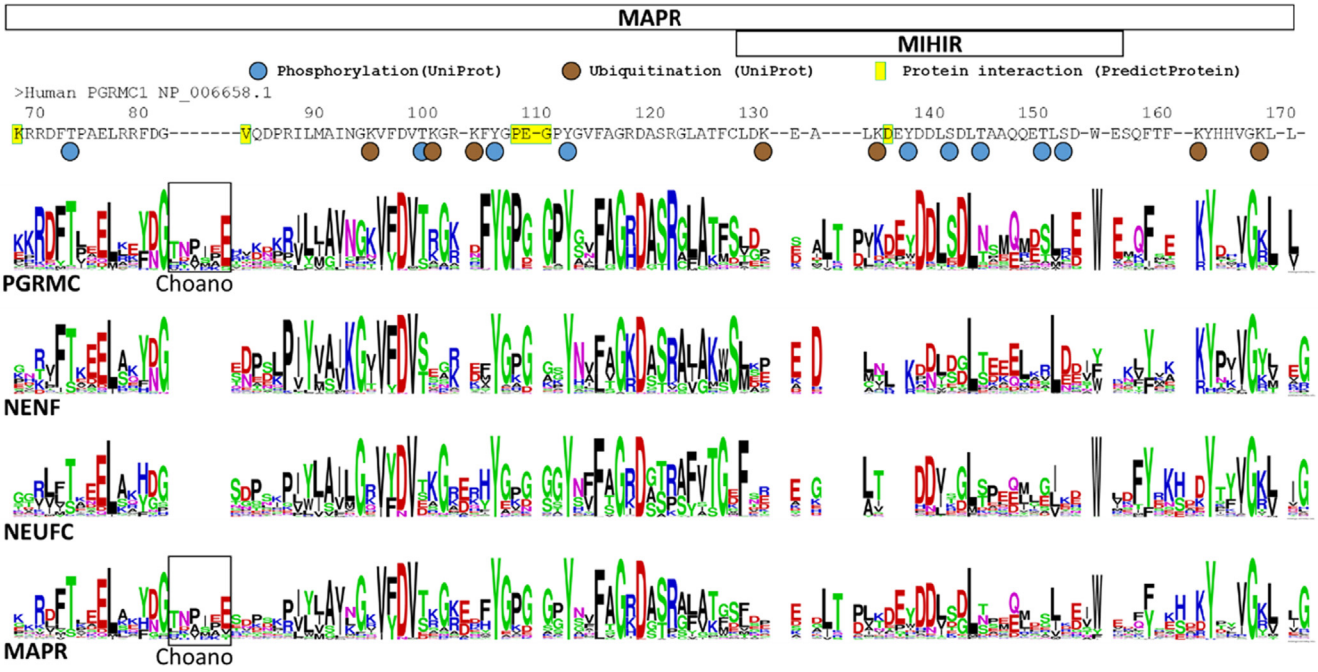


**Fig. 2.** Consensus MAPR and single protein Logo plots. Logo plots are presented for all orthologs of PGRMC, NENF, and NEUFC from Fig. 1. The consensus plot of all MAPR sequences in the lowest row highlights the overall MAPR domain sequence identity. Apomorphic sequence insertions from some individual sequences were deleted to facilitate presentation. The box represents an apomorphic insertion in choanoflagellates that is absent from PGRMC proteins of other species. The MAPR domain of human PGRMC1 is presented above the Logo plots for reference. Documented sites of phosphorylation, ubiquitination (UniProt) and predicted sites of interaction (ProteinPredict) are indicated for PGRMC1.

2019).

### 2.3. Other database platform queries

The same alignment used for tree reconstruction in Fig. 1 has been used to generate logo plots to identify conserved regions in groups of interest. The untrimmed 3-protein-alignment was split into three separate alignments for each protein (PGRMC, NENF and NEUFC), followed by removal of gap-only columns in each alignment. For the alignments in Figs. S1–S5, a choanoflagellate-specific insert indicated in Fig. 2 has been removed. The species corresponding to phylogenetic groups labelled in Figs. S2, S4, S6 and S8 were entered into Weblogo separately. Logo plot representation of consensus sequences were generated with the WebLogo platform (http://weblogo.berkeley.edu/) (Crooks et al., 2004), as described in the respective figure legends.

High and low stringency protein sequence BLAST of the PGRMC1 MIHIR region was performed using human PGRMC1 search string CLDKEALKDEYDDLSDLTAAQQETLSDWESQFTFKYHH with NCBI BLASTP (https://blast.ncbi.nlm.nih.gov). High stringency BLAST was performed using default BLASTP settings. Low stringency BLAST employed word size 3, expect threshold 1000, organism restricted to Homo sapiens (taxid:9606), 500 maximum target sequences, and with all other parameters set to default values. Subsequent BLAST queries targeted the organisms from Fig. 5A. Coiled-coil prediction (Lupas et al., 1991) was performed by the PRABI (Pôle Rhône-Alpes de Bioinformatique) server (https://npsa-prabi.ibcp.fr/). Sites of observed phosphorylation were obtained from Phosphosite (www.phosphosite.org) (Hornbeck et al., 2015) or UniProt (www.uniprot.org). Protein interaction sites were predicted with the PredictProtein server (www.predictprotein.org) (Yachdav et al., 2014). RGD protein interaction motif detection was by the ISIS (interaction sites identified from sequence) (Ofran and Rost, 2007) function of ProteinPredict.

## 3. Results

### 3.1. The origin of animal MAPR proteins predates opisthokonts

The relationships of the major opisthokont groups investigated in our analysis are depicted schematically in Fig. 1A based upon published phylogenies (Borowiec et al., 2015; Hehenberger et al., 2017), acknowledging that the tree of opisthokonts is not resolved (see the legend of Fig. 1A for details). We used two different approaches to generate the phylogenies presented here: in the first approach we reconstructed the phylogeny based on the central conserved domain present in all 3 MAPR proteins across all investigated lineages (Fig. 1B), while in the other approach we used the complete sequences in combination with more stringent trimming parameters to calculate the tree (deposited to figshare repository doi: https://doi.org//10.6084/m9.figshare.9162164). With both approaches we observed a similar topology, particularly the conspicuous split within the NENF clade: while choanoflagellates formed a well-supported holozoan clade with the metazoan NENF representatives, the holozoan lineage of ichthyosporeans grouped with high support with the Holomycota (fungi and nucleariids).

Since metazoan evolution has been associated with the evolution of many new membrane proteins relative to intracellular enzymes (Attwood et al., 2017), we were interested in the evolution of individual MAPR proteins, particularly in non-bilaterian animals. We obtained diverse MAPR proteins across a diverse selection of opisthokont taxa and investigated their phylogenetic relationships. The unrooted tree topology resulting from our MAPR analysis indicated the presence of three well-supported major branches of opisthokont MAPR proteins, representing PGRMC, NENF and NEUFC proteins (Kimura et al., 2012), with orthologs of each protein also present in holomycots (Fig. 1B and Fig. A1). Not all species sampled possessed representatives of each protein (e.g., both yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* contain only one MAPR family protein: Dap1, a PGRMC ortholog). However, this result clearly reveals that the three MAPR

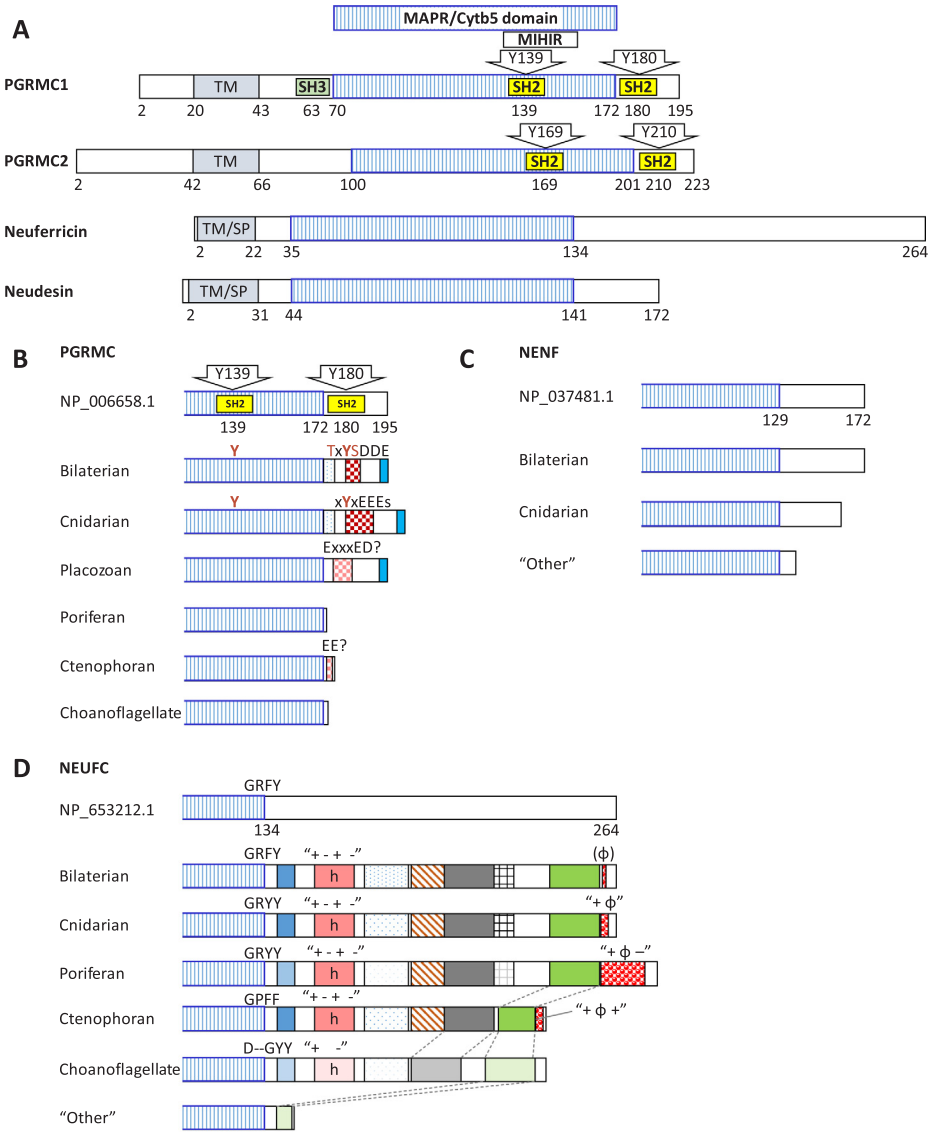


**Fig. 3.** Evolution of MAPR *C*-termini in the evolution of animals. (A) Schematic depiction of the four human MAPR genes, based upon consensus of proteins analysed in this study. Numbering refers to human proteins with accession numbers provided in subsequent panels. TM: transmembrane peptide of PGRMC1/2; TM/SP: Transmembrane/signal peptide of Neuferricin and Neudesin (Ren et al., 2019). (B) Schematic depiction of the evolution of the PGRMC *C*-terminus in the evolution from the common ancestor of choanoflagellates and metazoans. Human PGRMC1 from A is at the top for orientation. The cognate positions of Y139 and Y180 are shown to have appeared by the time of the LEUMCA but are absent from ctenophores, poriferans, and placozoans. Boxed regions show regions of amino acid similarity without identifiable known domains. These are not implied to possess specific functions. The PGRMC1 Y180 motif consisting of T178, Y180, S181 and adjacent negative D/E region appears to have evolved in a stepwise pattern. Logo plots and further details of the schematic diagrams can be found in Fig. A2. (C) The *C*-terminus of NENF proteins expanded in the descendents of the LEUMCA relative to other opisthokont sequences in the study. "Other" refers to poriferans, choanoflagellates, and NENF-like from Fig. A4, where logo plots and further details of the schematic diagrams can be found. (D) The *C*-terminus of NEUFC was expanded in the common ancestor of choanoflagellates and metazoans. Variously shaded boxes represent regions of presumed amino acid similarity by descent. No function is ascribed to any particular region. Consensus changes to the GFRY motif at the *C*-terminus of the human Neuferricin (NP_653212.1) are shown for each group. The boxes labelled h with "+ −" above represents regions of positive and negative charge that is predicted to be surface-exposed. Another region contains positively (+) or negatively (−) charged and/or aliphatic (ϕ) residues (see Fig. A7B-E). Logo plots and further details of the schematic diagrams can be found in Fig. A8. "Other" refers to non-choanoflagellate single-celled opisthokonts analyzed in the study.

proteins were already distinct in the common ancestor of opisthokonts. We initially also included non-opisthokont eukaryotes (such as amoebozoans and apusomonads) in our analysis, however the poor taxon sampling in some of these lineages in combination with the greater evolutionary distance did not allow us to place these sequences with confidence within one of the three MAPR clades. Below we therefore focus on describing the characteristics and the evolution of each MAPR protein within opisthokonts.

The consensus Logo plots of the MAPR domains for each individual protein from Fig. 1B, as well as for all MAPR sequences from all three proteins combined are shown in Fig. 2. Human PGRMC1 sequence is shown above the plots for orientation. The location of the MAPR domain in each of the human MAPR proteins is schematized in Fig. 3. Changes to each protein, particularly along the transition from unicellular to multicellular holozoans as well as from non-bilaterian animals to bilaterians, are discussed below. These include changes to the *C*-termini of each protein (Fig. 3).

### 3.2. The PGRMC proteins

Notably, PGRMC proteins feature prominent F106, P109 and P112 (with reference to human PGRMC1) around the heme-binding pocket of the MAPR domain (Fig. 2), suggesting unique ligand properties of this protein. Choanoflagellates, the closest unicellular sister group to animals, possess an apomorphic PGRMC-specific insertion between PGRMC1 G83 and V84 (Fig. 2).

#### 3.2.1. PGRMC N-terminus

There was no observable systematic change of PGRMC protein size *N*-terminally to the MAPR domain between single-celled and metazoan opisthokonts (not shown). PGRMC1 residues 47–49 encode a putative RGD protein interaction motif which has been present at least since the emergence of placental mammals (Fig. 4).

#### 3.2.2. PGRMC MAPR domain

The T74 and T101 phosphorylation sites of human PGRMC1 exhibit deep phylogenetic conservation (Fig. A2). In the transition from unicellular holozoans to cnidarians the PGRMC MAPR domain acquired a frequently represented *N*-terminal KKR consensus corresponding to PGRMC1 69–71. The two placozoan sequences, cnidarians and bilaterians all featured increasing frequency of K96 and K137, which are ubiquitinated in mammals. K96 is also acetylated (PhosphositePlus server (Hornbeck et al., 2015)). The frequency of tyrosine at the position of SH2 motif Y139 increases markedly in cnidarians and bilaterian animals (Fig. 3, Fig. A2).

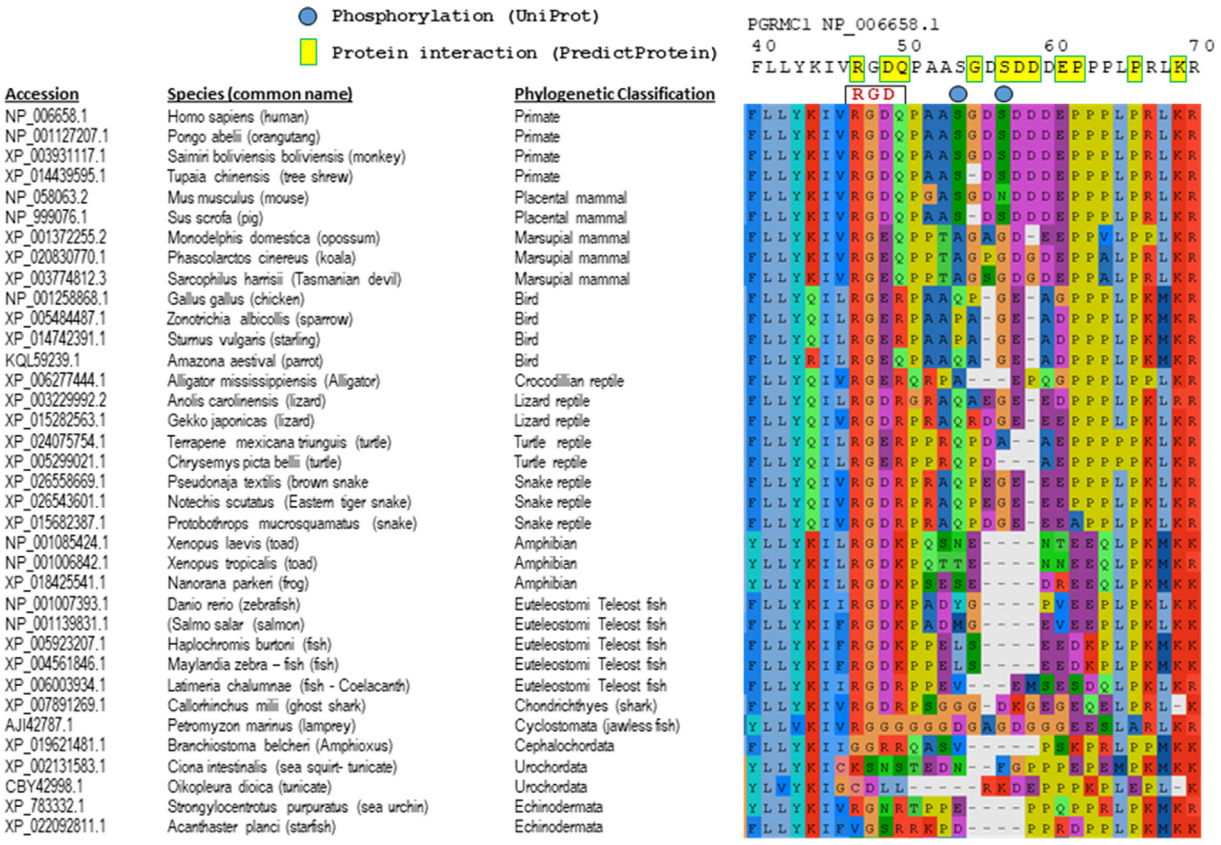


**Fig. 4.** Alignment of PGRMC1 39-74 region of selected chordates. This region spans part of the transmembrane helix (left) to the start of the MAPR domain (right). The indicated metazoan PGRMC sequences were aligned using MAFFT L-INS-i. The graphical presentation of the alignment was made using AliView (Larsson, 2014).

### 3.2.3. *C-terminal extension to the PGRMC MAPR domain*

Relative to unicellular holozoans, poriferans and ctenophores, a *C*-terminal extension beyond the PGRMC MAPR domain is present in placozoans, cnidarians and bilaterian animals, consistent with closer affiliation of placozoans to eumetazoans than to ctenophores and poriferans (Brunet and King, 2017; Laumer et al., 2018; Mills et al., 2018; Moroz and Kohn, 2016). A conspicuous conserved feature corresponds to the *C*-terminal 192–195 RKND motif of PGRMC1, which was already recognizable in both placozoan sequences available, and which featured strongly in eumetazoan proteins (Fig. A2).

The most prominent characteristic of the bilaterian PGRMC *C*-terminus corresponds to the PGRMC1 TxYSxDDE motif surrounding Y180, where phosphorylation of T and S is postulated to sterically impede Y phosphorylation and/or access of SH2 domain proteins to phosphorylated Y180. Although this has not been formally proven, no doubly phosphorylated peptides have been reported in the Phosphosite database (Hornbeck et al., 2015), suggesting mutually exclusive rather than cooperative phosphorylation at these sites. Cnidarians lack T178 but commonly possess an acidic stretch C-proximally to the cognate of Y180, also commonly including proximal *C*-terminal S and T potential sites of phosphorylation, resembling the CK2 consensus site of S181 (Fig. A2). We propose that a novel functional antecedent to the PGRMC1 Y180 motif evolved in the LEUMCA. We note that placozoans may also be descended from the LEUMCA (Laumer et al., 2018), in which case they must have secondarily lost the PGRMC1 Y139/Y180 combination, as well as the NENF *C*-terminal extension (Fig. 3). If so, these MAPR features could be associated with functions secondarily lost by placozoans.

K193 at the PGRMC1 *C*-terminus is a consensus SUMOylation site (Cahill, 2017), which predates the LEUMCA (Fig. A2). SUMOylated proteins are frequently nuclear (Ovaa and Vertegaal, 2018), perhaps hinting that the occasional nuclear localisation of PGRMC1 reflects an evolutionarily novel function acquired by non-bilaterian animals that is conserved in bilaterian animals.

### 3.2.4. *PGRMC1 Y139 and Y180 appeared in the LEUMCA*

Synapses are present in both cnidarians and ctenophores (Ovsepian, 2017), however, the groups are thought to have independently evolved neurons (Haen Whitmer, 2018). Although ctenophores possess tentacles, a gut, polarized epithelia and nerves, these are developed using different genetic pathways than in other animals, so that the ctenophores are thought to represent a sister group to other metazoans (Belahbib et al., 2018; Brunet and King, 2017; Fierro-Constain et al., 2017; Jensen et al., 2016). Cnidarian and bilaterian synapses are thought to have evolved from a common ancestor (Moroz and Kohn, 2016). The LEUMCA was the first animal to acquire the combination of the cognates of PGRMC1 Y139 and Y180, and these are strongly conserved across the bilaterians, indicating synapomorphic evolutionary appearance in the LEUMCA. This combination corresponds closely to the presence of ubiquitinated K96 and K137 and consensus SUMOylation site K193 mentioned above. Sabbir has recently demonstrated the inducible presence of PGRMC1 phosphorylation as well as SUMOylation and ubiquitination that affected PGRMC1 stability as well as Tcf/LEF transcriptional activation in human HEK293 cells (Sabbir, 2019). By employing PGRMC sequences of poriferans, ctenophores and placozoans as well as 88 cnidarian PGRMC sequences we can conclude with high certainty that Y180 arose in the LEUMCA. The position of Y139 was commonly a W in many unicellular holozoans, with occasional examples of substitution for Y in choanoflagellates, ctenophores and placozoans, however the Y139/Y180 combination provided new animal-specific functionality to PGRMC1 in descendants of the LEUMCA because it is strongly conserved (Fig. A2). Y139 is the less strongly conserved of these two residues, suggesting its function is not as critical to animal biology.

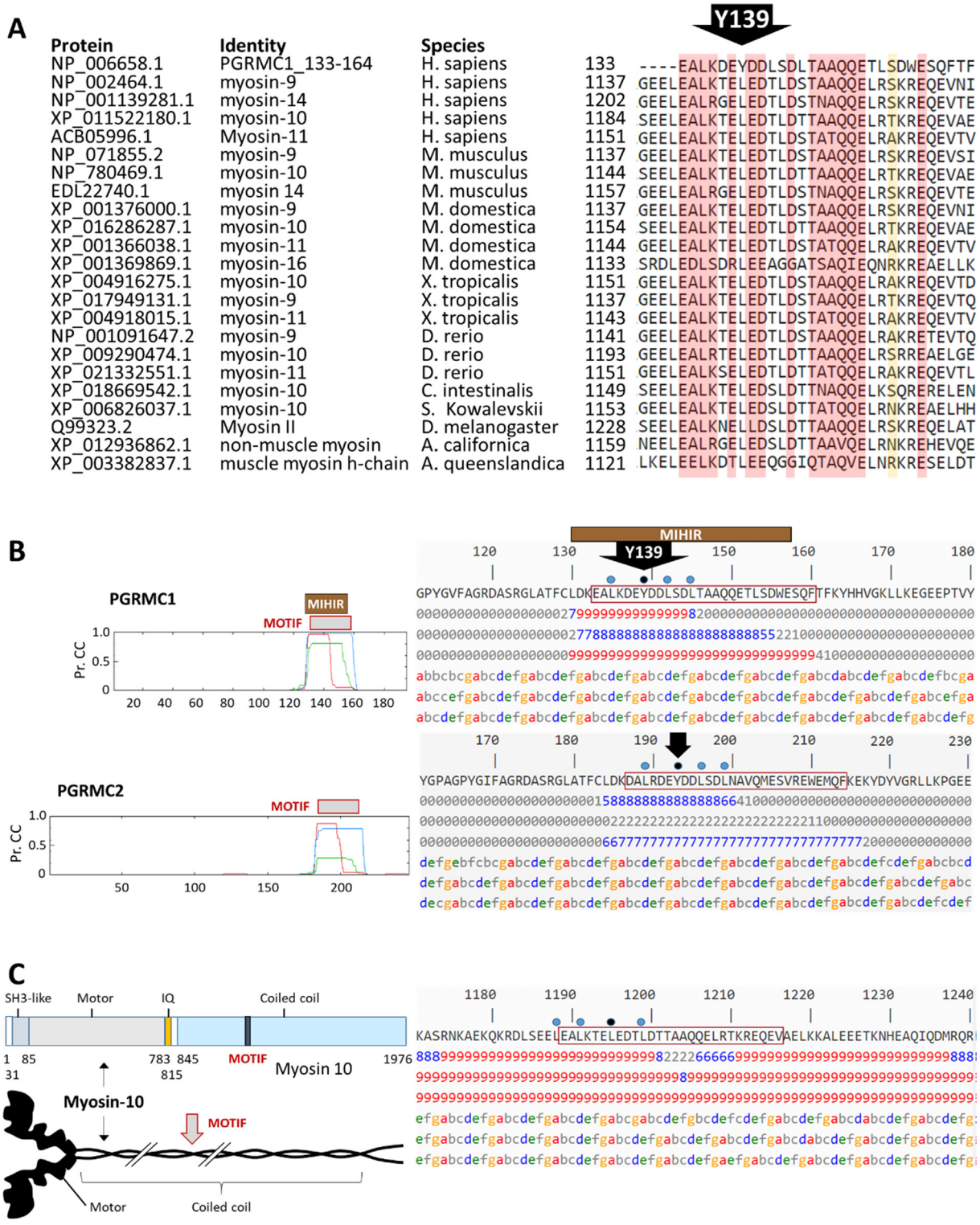


**Fig. 5.** The PGRMC1 MIHIR has predicted coiled-coil character shared with some myosins. (A) Alignment of PGRMC1 MIHIR residues 133–164 with selected myosin proteins detected by low stringency BLAST. (B) The PGRMC1 and PGRMC2 MIHIR regions contain predicted high propensity to form coiled coil. The images to the left depict the probability for a particular residue to form coiled-coil based upon calculation for surrounding windows of 14 (red), 21 (blue) and 28 (green) residues, generated by the PRABI server. Panels to the right present the numerical depiction of the same result. Numbers under the sequence are the probabilities for coiled-coil formation abbreviated to first digit for windows of 14, 21 and 28 residues (i.e. 9 represents $p \geq 0.9$). Letters a-g represent the corresponding coiled coil heptad register. The positions of predicted heptad hydrophobic coiled-coil core residues including PGRMC1 Y139 are indicated. (C) The motif from A is present in the coiled-coil region of human Myosin 10. The left side shows the position of the motif in the primary and tertiary structure of the protein. The right side format follows the conventions of B. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

#### 3.2.5. Animal PGRMC MIHIR regions are predicted to form a coiled-coil protein interaction region

In order to investigate possible functions of the SH2 motif Y139-containing PGRMC1 MIHIR region we performed low stringency BLAST using the human PGRMC1 MIHIR sequence as the search string. We reasoned that evolutionarily conserved residues would be conserved due to functional requirements. Standard BLAST parameters returned only PGRMC1 and PGRMC2 with 100% and 63% similarity and BLAST scores of 82 and 53. However, low stringency BLAST also recognized Neudesin as the next top hit with 48% similarity and a score of 28, followed by a long list of proteins including multiple myosins with BLAST scores above 25. The region of best alignment (PGRMC1 133-164) was then BLASTed against animal species, revealing similar myosin-like motifs from poriferans, insects and chordates (Fig. 5a). This region was found to have partial predicted coiled-coil character in both PGRMC1 and PGRMC2 (Fig. 5b). α-Helical coiled-coils are protein-folding and -interaction motifs in which two or more α-helical chains from different proteins intertwine, typically involving aliphatic amino acids that exhibit heptad repeats to align on the protein-interaction side of each α-helix. As such, coiled-coils are associated with protein-protein interactions (Woolfson, 2017; Woolfson et al., 2012). The corresponding motif of Myosin 10 was in the coiled-coil region of the protein (Fig. 5c). For both PGRMC and myosin motifs, predicted coiled-coil probability was higher in the *N*-terminal portion of the motif, and reduced in the *C*-terminal residues.

We analyzed the probability for coiled-coil formation of this MIHIR motif among selected MAPR proteins by summing the single digit coiled-coil probability scores for each residue in the homologous motif (Fig. A3). The motif showed higher propensity for coiled-coil in PGRMC and NENF, with negligible levels in NEUFC species. However, coiled-coil probability was not a consistently conserved feature of PGRMC or NENF proteins across species (Fig. A3). These data are suggestive of protein-protein interactions occurring through the MIHIR region, possibly via coiled-coil interactions in vertebrate PGRMC proteins. Lack of coiled-coil formation does not argue against mediation of protein interactions by the MIHIR. There is no requirement for coiled-coil formation to enable functional protein interactions, as long as the respective interaction surfaces co-evolved compatibly in any given species.

### 3.3. The NENF family

#### 3.3.1. NENF N-terminus

The NENF MAPR domain is quite proximal to its transmembrane domain/signal peptide, with no evident systematic patterns observed between transmembrane helix and MAPR domain during opisthokont evolution (File B2).

#### 3.3.2. The NENF MAPR domain

In our phylogenetic reconstruction, holomycots plus ichthyosporeans formed a well-supported clade separate from the remaining holozoans. Therefore we have denoted all sequences from this clade as “NENF-like”. The major differences between NENF-like and NENF MAPR proteins are color-coded in Fig. A4. With regards to accepted rooted phylogeny of the groups concerned (Fig. 1B), NENF-like proteins appear to represent an ancestral/plesiomorphic state for the NENF supergroup. The NENF-like state was present in the common ancestor of opisthokonts, as exemplified by fungi, nucleariids and ichthyosporeans. Therefore, the animal-like NENF MAPR domain status represents a synapomorphy that appeared after the common ancestor of ichthyosporeans and metazoans (Fig. A4, File B3) from where it was inherited by animals. The common ancestor of choanoflagellates and metazoans possessed the synapomorphic NENF state. Because we do not have pluriformean or filasterean NENF-like or NENF sequences in the analysis, we cannot identify the origin of the NENF state more accurately.

Two sites of observed phosphorylation in human NENF (T48 and Y64) are strongly conserved across opisthokonts. However, there is only one report (Tsai et al., 2015) of phosphorylation at both sites in the Phosphosite database, and therefore no evidence that amino acid conservation is related to phosphorylation.

#### 3.3.3. The NENF C-terminus

Eumetazoan NENF proteins have acquired a *C*-terminal extension relative to choanoflagellates, poriferans, placozoans and ctenophores (Fig. 3, Fig. A4). The ProteinPredict server predicts a protein interaction region between residues 145–150 (Fig. A5). There is no sequence similarity between the *C*-terminal extensions of PGRMC and NENF proteins, indicating probable pronounced functional divergence and specialization of these proteins during early metazoan evolution.

### 3.4. The NEUFC proteins

#### 3.4.1. The NEUFC MAPR domain

There are several conspicuous developments during the evolution of NEUFC. Ctenophores, cnidarians and bilaterians acquired a common histidine at H72 (human NEUFC numbering) in the vicinity of the heme binding pocket, suggesting altered ligand-binding. Animals but not single-celled holozoans have a greater probability of aspartate at D103, and relative to choanoflagellates, animals possess a two-residue extension at the *C*-terminus of the MAPR domain including highly conserved G135 (Fig. A6). This region corresponds to a site of predicted protein interaction (Fig. A7).

#### 3.4.2. The NEUFC C-terminus

Like all MAPR proteins, NEUFC acquired a *C*-terminal extension during opisthokont evolution. It is the largest conserved *C*-terminus of the animal MAPR family. Unlike in PGRMC and NENF, the NEUFC *C*-terminus is elongated already in choanoflagellates (Fig. 3), relative to other single-celled opisthokonts in the study. Elements of similar amino acid composition in the evolutionarily newly acquired NEUFC *C*-terminus are strongly conserved between choanoflagellates and all animals surveyed (Fig. A8), implying that this region plays a necessary role in the organismal biology of metazoans. However, since nothing is known concerning the biology of this protein region, we cannot speculate about functions associated with the conserved amino acids.

The three sites of protein phosphorylation and ubiquitination observed for human NEUFC are not strongly conserved in bilaterians (Fig. A8). Each modification has been reported only once, from three separate PhosphoSite curation sets. Therefore generalizations cannot be made. We have not explored whether they may be strongly conserved in e.g. vertebrate or primate evolution.

Various sites of protein interaction were predicted in the *C*-terminus by ProteinPredict, as well as a predicted solvent-exposed helix from approximately residues 150–170 (Fig. A7A–B). That helical region exhibited a high probability of coiled-coil formation in some but not all NEUFC species sampled (Fig. A7C–E). The overall conservation of those residues seems to be more associated with charged residues rather than heptad hydrophobic residues required for coiled-coils. Choanoflagellates exhibit what appears to be a classical evolutionary intermediary stage between the state of other single-celled opisthokonts on the one hand, and that of animals on the other (Fig. A8). In summary it is highly likely that the NEUFC *C*-terminus is involved in protein interactions through solvent-accessible residues, however further studies will be required to determine the nature of such interactions, and shed light on the function of NEUFC.

## 4. Discussion

### 4.1. Evolution of the MAPR family proteins

The first major finding of this study is that all three animal MAPR family proteins had already diverged in the last common ancestor of the

opisthokonts. While unicellular opisthokonts with all three genes are rare, one such lineage must have proliferated from the ancestral opisthokont to give rise to choanoflagellates and animals.

The second major finding is that, strikingly, the extension of the *C*-termini for all three MAPR proteins appeared either before the choanoflagellate/animal divergence, or at some point during non-bilaterian animal evolution. For NEUFC the origins of this extension occurred prior to the emergence of the choanoflagellates, the sister group to animals. The PGRMC *C*-terminus gained an extension before the divergence of placozoans and cnidarians, while for NENF this occurred with the LEUMCA. There is no sequence similarity between these *C*-terminal extensions, so this phenomenon represents a further functional divergence between the three MAPR proteins of non-bilaterian animals, associated with increased organismal complexity.

### 4.2. PGRMC1/2 tyrosine phosphorylation motifs and the organizer

Our third and perhaps the most important new finding is that the combination of SH2 motif PGRMC1 phosphoacceptor residues Y139 and Y180 (which are also conserved in PGRMC2) first appeared in the LEUMCA, being absent from poriferans, ctenophores and placozoans (Fig. A2), which was not apparent from a previous pilot study (Cahill, 2017). The LEUMCA was the first animal in the direct lineage leading to vertebrates to possess a gut, and a nervous system with bilaterian-like synapses (Arnellos and Keijzer, 2019; Moroz and Kohn, 2016). While cnidarians and bilaterians also possess innervated striated muscles, these arose independently in the two groups (Steinmetz et al., 2012). PGRMC1 is Y-phosphorylated in synapses (Munton et al., 2007), and affects synaptic function (Izzo et al., 2014).

The LEUMCA, which was probably bilaterally symmetrical, evolved a gastrulation organizer capable of inducing differentiation of surrounding cells to define embryological body axes and induce tissue identities after gastrulation at an animal pole. The body plan in LEUMCA descendants arises from an orchestrated set of post-gastrulation events involving the induced expression of conserved transcription factors such as *brachyury*, *goosecoid* and *foxa* (Genikhovich and Technau, 2017). This is reflected by organizers recognized, for instance, in cnidarians (Hayward et al., 2015; Kraus et al., 2016), and bilaterians including planarian flatworms (Sureda-Gomez and Adell, 2019), arthropods (Oda et al., 2019), spiralian protostomes (Henry et al., 2017), and of course the deuterostome/chordate embryological entity known as the Spemann-Mangold organizer in *Xenopus*, which develops from an epidermal region known as the primitive streak (Lapraz et al., 2015; Nielsen et al., 2018).

All of the bilaterians *sensu stricto* possess the Y180 motif with adjacent T178 and S181 residues (Fig. A2), all of which can be phosphorylated in mammals (Cahill et al., 2016b). Because of the evolutionary appearance of this motif at the same time that the rules governing vertebrate embryological cell-type and tissue differentiation became established, we predict that inappropriate alterations in the phosphorylation status of Y180 in PGRMC1 (or PGRMC2) could impose profound effects on vertebrate cell differentiation state, and therefore could be of potentially monumental clinical importance in humans. Tyrosine phosphorylation is typically caused by induced signal transduction pathways. The signal systems surrounding the regulation of Y180 are likely to feature prominently in human disease.

Cnidarians such as *Hydra* possess a Wnt-dependent head organizer which drives axis specification through a protein gradient (Bode, 2012; Hobmayer et al., 2000; Lengfeld et al., 2009). The Spemann-Mangold organizer also specifies vertebrate dorso-ventral axis via Wnt-signaling (Reid et al., 2012). PGRMC1 is involved in the maintenance of human embryonic stem cell pluripotency via regulation of the Wnt pathway (Kim et al., 2018). Based upon the strongly conserved coincident presence of the Y139 and Y180 motifs shared between LEUMCA descendants we hypothesize that PGRMC phosphorylation might also play an important role in the cnidarian organizer.

The descendants of the LEUMCA embryologically develop a cell collective of multiple states of differentiated morphologies within one organism that was enabling for the subsequent evolution of bilaterian body plans (Newman, 2019). Here, the ability to phosphorylate Y139/Y180 could have been associated with the evolutionary origin of organizer function and tissue differentiation that led first to the formation of the differentiated gut epithelium following gastrulation. PGRMC1 is known to be SUMOYlated which affects TCF/LEF-driven transcription (Peluso et al., 2012; Sabbir, 2019). TCF/LEF is probably the exclusive conduit for Wnt signalling (Schuijers et al., 2014). Taken together, PGRMC1 is strongly implicated in the evolution of animal organizer function with profound potential to influence animal cell differentiation status and its plasticity (e.g. cancer) (Cahill et al., 2016a; Sabbir, 2019; Shih et al., 2019).

Elsewhere (Thejer et al., 2020a, 2020b), we show that mutational manipulation of the phosphorylation status of the Y180 motif dramatically affects cultured cancer cell morphology, PI3K/Akt signaling activity, mitochondrial form and function, glycolytic/energy metabolism, and ability to form tumours in MIA PaCa-2 pancreatic cancer cells. This is reflected in altered cell metabolites, genomic stability, and dramatic changes in genomic CpG methylation, which are all consistent with PGRMC1 involvement in organizer biology.

### 4.3. MAPR involvement in neurology

In addition to the combination of PGRMC1 Y139/Y180 discussed above, the *C*-terminal extension of NENF was also acquired in the LEUMCA, coincident with the appearance of neurons with synapses. NENF neurotrophism (Kimura et al., 2005), NEUFC neurodifferentaiton (Kimura et al., 2010) and PGRMC1 axonal guidance (Runko and Kaprielian, 2002, 2004) are at least superficially similar functions.

For reasons such as these, we were expecting to find that animals inherited a single MAPR gene from their unicellular ancestors, which diverged into three proteins during metazoan evolution, with e.g. ancestral neural-related functions having undergone functional specialization following gene duplication. While this manuscript was in preparation Ren et al. (2019) concluded that choanoflagellates and the first metazoans contained only PGRMC and NEUFC genes. Our findings that all three MAPR proteins were already distinct in the ancestral opisthokont argues against these previous suggestions. However, the observation remains that PGRMC1 and NENF both acquired new conserved functional features at the emergence of the LEUMCA, correlating with the evolution of the gastrulation organizer, despite having been inherited into animals as separate genes from a unicellular ancestor. There seems to have been some feature of MAPR biology which was important in the ancestors of animals and which favored the subsequent evolution towards a LEUMCA body organization which likely included gastrulation and the establishment of nerves with synapses. See Genikhovich and Technau (2017) for reconstruction of the properties of the LEUMCA. We hypothesize that at least PGRMC1 Y180 was crucial in the latter.

### 4.4. Contribution of this study to NENF and NEUFC fields

Beyond knowledge of functions in neurology and cancer (see above), there is little known about what sequence motifs in NEUFC and NENF proteins are important for mediating effects, except that mutations which abrogate heme-binding also impair several effects mediated by NEUFC and NENF (see introduction). This present study for the first time identifies functionally conserved regions of amino acid sequence in these proteins that were evolutionarily acquired before multicellularity, or during the divergence of non-bilaterian animals. The involvement of these proteins in neurobiology, and the evolutionary acquisition of new conserved protein regions at or before the appearance of nerves, strongly implicates the *C*-termini of all three MAPR families as having enabled the evolution of the animal-specific

processes of neurodifferentiation and neurotropism (Kimura et al., 2010; Kimura et al., 2012; Kimura et al., 2013; Ohta et al., 2015a). This novel insight points the way for future investigations. Guidepost analyses such as this study are desirable to newly identify protein regions of functional importance. Characterization of newly identified conserved regions may lead to future drug development if the proteins that interact with them are found to be involved in clinical pathologies.

### 4.5. Is the MIHIR a protein-interaction motif?

Our fourth novel finding is the identification of a motif with probable coiled-coil characteristics in the MIHIR sequence of both PGRMC1 and PGRMC2, as well as similar sequences in some myosins. The motif was predicted with high probability to form a short coiled-coil at its *N*-terminus, and lower probability at its *C*-terminus. Of particular interest was the observation that Y139 formed part of the hydrophobic heptad repeat required for interaction of adjacent helices in the coiled-coil. In unicellular holozoans and some non-bilaterian animals the Y139 position was commonly a tryptophan, another large bulky hydrophobic residue. The putative involvement of Y139 in a coiled-coil interaction presents immediate connotations when the residue is phosphorylated. Not only would it be unable to interact with coiled-coil interaction partners, but it could then interact with a new set of SH2 domain-containing proteins in the tyrosine-phosphorylated form. This suggests the acquisition of a regulatory switch in MIHIR functionality by PGRMC genes in the LEUMCA.

It should be noted that this region of PGRMC1 in the 4X8Y crystal structure exhibited a high B or scatter factor, indicating relatively poor mapping of electron density to amino acid sequence (Kabe et al., 2016), as indicative of a region of low structural stability in the crystal. We therefore conclude that the MIHIR region should be able to rapidly sample many conformations in solution, which is compatible with availability to form protein-protein interactions.

In myosins, the analogous motif is found within the rod-like coiled-coil region, and represents an area where the probability of coiled coil interaction is diminished, as shown for myosin 10 in Fig. 5b. Such coiled-coil regions with weaker stability, often including disruption of the requisite heptad repeat coiled-coil motif, have been proposed to form sites of binding to potential target interacting proteins (Sunitha et al., 2012). A phylogenetic survey of some MAPR proteins from opisthokonts (Fig. A3) showed that high predicted probability of coiled-coil formation was conserved in animals (except the sea anemone *Aiptasia pallida*), as well as in the choanoflagellate *Monosiga brevicollis*. The degree of coiled-coil propensity was weaker in unicellular opisthokonts other than choanoflagellates, being for example minimally present in the yeast *S. pombe* (Fig. A3). We have not systematically analyzed this in non-opisthokont eukaryotic groups in this study. We predict that the MAPR MIHIR sequence, which has recognizable sequence conservation across all three MAPR proteins (Fig. 2) and was presumably inherited from the last common eukaryotic ancestor, enables protein interactions not shared with other $cytb_5$ domain proteins. These may or may not involve coiled-coil formation. Association with the actin/myosin cytoskeleton through the MIHIR motif may be related to the membrane-trafficking functions of PGRMC1. We have recently demonstrated that PGRMC1 co-immunoprecipitates in protein complexes containing multiple proteins associated with the actin cytoskeleton, and that these interactions are sensitive to the small molecule antagonist of PGRMC1 function AG-205 (Teakel et al., 2020).

### 4.6. A PGRMC1 RGD integrin-interaction motif?

The fifth new insight of this study is the discovery, in what is conventionally considered the cytoplasmic region of PGRMC1, of a potential RGD motif in some vertebrates, being absent in the turtles, birds and marsupials studied (Fig. 4). This is not present in PGRMC2 where the corresponding residues (human) are RWG (not shown). This assignment is tenuous because RGD motifs are predicted to be involved with extracellular integrin interactions (Nieberler et al., 2017). Integrins are important in many processes, including the regulation of synaptic plasticity and memory (Park and Goda, 2016). The RGD motif would need to be extracellular to interact with integrins. However, the conventionally cytoplasmic *C*-terminal region of PGRMC1 is extracellular in several attested situations, including synapses, where PGRMC1 is involved in a mechanism that affects synaptic plasticity (Izzo et al., 2014), in pluripotent stem cells (Kim et al., 2019), where PGRMC1 is associated with the maintenance of pluripotency involving the Wnt/beta-catenin pathway (Kim et al., 2018), and in cancer cells, where PGRMC1 may be secreted by the exosome pathway (Mir et al., 2012). PGRMC1 is documented as an exosomal protein in the Exocarta database (http://www.exocarta.org/) (Keerthikumar et al., 2016), however it remains unclear whether it may be translated with an alternative membrane topology as a transmembrane protein (Cahill et al., 2016a; Cahill and Medlock, 2017).

We surmise that the likelihood of a conserved functional RGD integrin-interaction motif in vertebrates would be much greater if the protein exhibits alternative translational topology as a transmembrane protein, rather than as a secreted protein. In view of the association of PGRMC1 with fertility (Engmann et al., 2006; Peluso, 2007, 2011), it is conceivable that this motif is involved in the evolution of post-fertilization vertebrate embryology, perhaps involving the relationship between amniotic sac and eggshell, or other major features related to differences in vertebrate embryology and oocyte/egg biology between these groups.

Positively charged residues cytoplasmically proximal to transmembrane signal peptide helices are critical in determining the orientation of transmembrane proteins (Hartmann et al., 1989). There is potential to alter membrane topology of PGRMC1 by post-translational modification of K44 or R47 during translation. It is known that R47 can be methylated (Phosphosite data base). This may be relevant to the observation of extracellular PGRMC1 *C*-terminus in the synaptic extracellular space of neurons (Izzo et al., 2014), potentially via regulated non-conventional orientation during translation. However we stress that in the absence of validated integrin interaction data, the presence of a functional RGD motif in vertebrate PGRMC1 must be considered cautiously.

## 5. Conclusions

We initiated this study to try to provide a systematic platform to understand the evolution and reported multifunctionality of PGRMC1. Surprisingly, we discovered that all three animal MAPR proteins had originated prior to the origin of opisthokonts. Our study did not systematically address the presence of MAPR proteins in non-opisthokont eukaryotes. Although many single-celled opisthokont species have lost one or more MAPR genes, the common ancestor of each major opisthokont branch leading to animals must have possessed all three MAPR proteins, evidencing a degree of conservation which indicates that each protein performs separate unicellular functions that were essential in the evolution of the holozoan and holomycotan subfamilies of opisthokonts.

We propose that the attested PGRMC1 membrane-trafficking function may be related to the eukaryotic acquisition of the MAPR-defining MIHIR region. In a separate study (submitted) we show that the MIHIR is a eukaryotic development, and propose that the ancestral MAPR protein may have been one of the pivotal proteins involved in the development of the first truly eukaryotic cell, with roles in the evolution of steroid biology.

Mitochondria require cholesterol to increase membrane potential across the inner membrane, and many of PGRMC1's functions involve steroid biology (Cahill and Medlock, 2017). Interaction of the eukaryotic MIHIR with the actin cytoskeleton may have been involved in the origin of membrane trafficking that was necessary to target

cytoplasmically-synthesized steroids to the proto-mitochondrion before the endosymbiont could lose genes to the nucleus. Additional or parallel to such ancient roles, we hypothesize that the early evolutionary diaspora of eukaryotic diversity involved functional specialization of at least three opisthokont MAPR genes.

The ancestor of opisthokonts already contained the tripartite MAPR repertoire, although multiple unicellular species have either variously lost MAPR genes or we were not able to find them. The transition from the common ancestor of choanoflagellates and animals to animal multicellularity involved enlargement of the *C*-terminus in all three MAPR proteins, commencing with the common ancestors of bilaterians and placozoans (PGRMC), cnidarians (NENF), and choanoflagellates (NEUFC), respectively (Fig. 3). A major new finding of this study is that the PGRMC gene acquired the combination of Y139 and Y180 at the stage of evolution of the LEUMCA, concomitantly with the appearance of the gastrulation organizer and its derived gut and synapsed nerves, and prior to the evolution of bilaterians *sensu stricto* and their associated new mechanisms for embryological body pattern formation and tissue differentiation.

Our study suggests a stratified acquisition of PGRMC1 functions, and points towards potentially dramatic effects of cell differentiation status if this ancient axis of PGRMC1 tyrosine phosphorylation is perturbed in disease processes. Results presented in accompanying papers strongly support this hypothesis (Thejer et al., 2020a, 2020b). These combined findings signpost the direction for productive future studies, which was our aim.

## CRediT authorship contribution statement

**Elisabeth Hehenberger:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing - original draft. **Michael Eitel:** Formal analysis, Investigation, Resources, Writing - review & editing. **Sofia A.V. Fortunato:** Resources, Writing - review & editing. **David J. Miller:** Resources, Supervision, Writing - review & editing. **Patrick J. Keeling:** Funding acquisition, Methodology, Resources, Supervision, Writing - review & editing. **Michael A. Cahill:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing - original draft.

## Declaration of Competing Interest

M.A.C. is scientific advisor to and minor shareholder of Cognition Therapeutics, a company developing sigma-2 receptor ligands against Alzheimer's disease. This work was performed independently of and without input from the company. The authors declare that they have no other competing interests.

## Acknowledgements

This work was supported by Charles Sturt University (CSU) School of Biomedical Sciences (SBMS) Compact grant A541-900-xxx-40513, SBMS support A534-900-xxx-41066, and CSU Competitive grant A102-900-xxx-40002, all to MAC. M.E. acknowledges financial support through the LMU Munich's Institutional Strategy LMUexcellent within the framework of the German Excellence Initiative (granted to Gert Wörheide, LMU Munich).

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2020.106814.

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