

# The evolutionary appearance of signaling motifs in PGRMC1

Michael A. Cahill\*

School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, Australia.

## Summary

A complex PGRMC1-centred regulatory system controls multiple cell functions. Although PGRMC1 is phosphorylated at several positions, we do not understand the mechanisms regulating its function. PGRMC1 is the archetypal member of the membrane associated progesterone receptor (MAPR) family. Phylogenetic comparison of MAPR proteins suggests that the ancestral metazoan "PGRMC-like" MAPR gene resembled PGRMC1/PGRMC2, containing the equivalents of PGRMC1 Y139 and Y180 SH2 target motifs. It later acquired a CK2 site with phosphoacceptor at S181. Separate PGRMC1 and PGRMC2 genes with this "PGRMC-like" structure diverged after the separation of vertebrates from protochordates. Terrestrial tetrapods possess a novel proline-rich PGRMC1 SH3 target motif centred on P64 which in mammals is augmented by a phosphoacceptor at PGRMC1 S54, and in primates by an additional S57 CK2 site. All of these phosphoacceptors are phosphorylated *in vivo*. This study suggests that an increasingly sophisticated system of PGRMC1-modulated multicellular functional regulation has characterised animal evolution since Precambrian times.

**Keywords:** Phosphorylation, evolution, steroid signalling, kinases, metazoan

## 1. Introduction

Progesterone Receptor Membrane Component 1 (PGRMC1) is the archetypal protein of the Membrane Associated Progesterone Receptor (MAPR) family, which consists of proteins which share a basic cytochrome b<sub>5</sub> (Cytb<sub>5</sub>) domain fold, with the common insertion of a stretch of amino acids between helices 3 and 4 of the domain fold of cytochrome b<sub>5</sub> itself (1) (hereafter referred to as the MAPR-specific inter-helical insertion region, or MIHIR). Humans possess four MAPR proteins: PGRMC1, PGRMC2, Neudesin, and Neuferricin (2-4).

PGRMC1 is associated with an uncharacteristically large number of attested functions, including association with cytochrome P450 (CyP450) enzymes (activating steroidogenic CyP450s and repressing xenobiotic metabolizing CyP450s), regulation of sterol synthesis (conversion of lanosterol to cholesterol) and interaction with the INSIG1/SCAP/SREBP complex

that regulates synthesis of sterol precursors, conferring responsiveness to progesterone (pregn-4-ene-3,20-dione, hereafter: P4), activating vesicle trafficking, regulating entry into G<sub>0</sub> stage of cell cycle, association with Aurora kinase on the mitotic spindle kinetochore, participation in the protein complex containing the Sigma 2 Receptor, angiogenesis, invasive growth, motility, anchorage-independent growth, and hypoxic biology (reviewed by (5)). It is also localized to the outer mitochondrial membrane, where it interacts with Ferrochelatase (FECH), the final enzyme in the heme synthetic pathway (6). The yeast PGRMC1 homolog Dap1 is thought to be involved in either heme synthesis, or its cellular transport to Erg11p, the yeast sterologenic CyP450 enzyme (7). Based upon these observations, PGRMC1 has been proposed to be involved in the transport of heme (6) and perhaps other hydrophobic ligands (8) between subcellular locations.

The acquisition of multiple functions by PGRMC1 presumably occurred throughout evolution in a successive manner to produce a set of stratified influences. For instance PGRMC1 association with the mitotic spindle could represent a universal eukaryotic MAPR trait, or may represent a metazoan, vertebrate, or mammalian innovation. Sterol or heme synthesis are ancestral traits of all eukaryotes, and are indeed also widespread in bacteria (9,10). The involvement

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\*Address correspondence to:

Dr. Michael Cahill, School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW 2678, Australia.  
E-mail: mcahill@csu.edu.au

of PGRMC1 and its yeast homolog Dap1 in the conversion of lanosterol to cholesterol (animals) or ergosterol (yeast) (11) argues strongly for an ancestral eukaryotic role of MAPR proteins in sterol production, and perhaps also in heme synthesis based upon a role proposed by Ghosh *et al.* for yeast (7) and by Piel *et al.* in mammals (6).

On the other hand the presence of PGRMC1/MAPR proteins in the complex containing INSIG/SCAP/SREBP that regulates the mevalonate pathway leading to sterol precursor synthesis in animals (12) (reviewed in (13)), but not reported in yeast, argues that this should be an acquired higher organism PGRMC1-function. Similarly, steroid hormone signaling first evolved in metazoans. The Estrogen Receptor (ESR), the original classical steroid receptor, appeared in the lineage that gave rise to vertebrates, with subsequent classical steroid receptors such as the P4 receptor evolving later (14-16). Therefore, conferring P4 responsiveness onto cells is presumably an acquired secondary function (unless a PGRMC1-like P4-response predates the estrogen response).

Finally, both mammalian PGRMC1 and its nematode homologue VEM-1 fulfil conserved roles in animal embryogenesis by regulating the fidelity of nerve chord axonal guidance along the ventral midline of nematodes and the spinal chord of rats (17-19). While this function must be ancient in animals, axonal guidance must also be acquired by animals and absent from protists. I have previously proposed that this function relies on the vesicle trafficking properties of PGRMC1 to expose specific cell surface receptors required axonal guidance (5). If so, then vesicle trafficking may be another acquired metazoan PGRMC1 function.

I have previously proposed that PGRMC1 phosphorylation plays a paramount role in the regulation of its function (20,21). In this paper I have assessed the evolutionary appearance of PGRMC1 phosphorylation sites using BLASTp and CLUSTAL alignments by sampling MAPR proteins in some strategically selected species thought to be phylogenetically separated by differing evolutionary periods. This study creates a novel systematics and rationale in this field for the future experimental stratification and functional characterization of PGRMC1 and other MAPR protein functions.

## 2. Materials and Methods

### 2.1. Phosphosite post-translational modifications

The Phosphosite data base of post translational modifications (PTMs) detected by high throughput mass spectrometry analyses (22) was queried with UniProt IDs for human PGRMC1 (O00264), PGRMC2 (O15173), Neudesin (Q9UMX5) and Neferricin (Q8WUJ1). Any PTM scored by Phosphosite with a frequency of once or more was marked on the CLUSTAL multiple sequence alignment of Figure 1A.

### 2.2. BLAST and CLUSTAL analysis

For Figure 1, PGRMC1 (UniProt O00264) used as search sequence in the UniProt Protein Basic Local Alignment Search Tool (BLASTp) (<http://www.uniprot.org/blast/>). In the results, organisms were deliberately restricted to *Homo sapiens*, the flowering plant *Arabidopsis thaliana*, the fusion yeast *Schizosaccharomyces pombe*, and the budding yeast *Saccharomyces cerevisiae*. The results (4 human proteins, 4 *Arabidopsis* proteins, one protein for each yeast species) were aligned by inputting their UniProt IDs to the UniProt CLUSTAL O (1.2.3) (23) multiple sequence alignment tool (UniProt Align) (<http://www.uniprot.org/align/>). The CLUSTAL guide tree for Figure 1B was recreated and coloured in Powerpoint to accurate relative scale as the original. For Figure 1C-D, the human Neudesin UniProt ID (Q9UMX5) was used as BLASTp input, and selected phylogenetically diverse species were chosen.

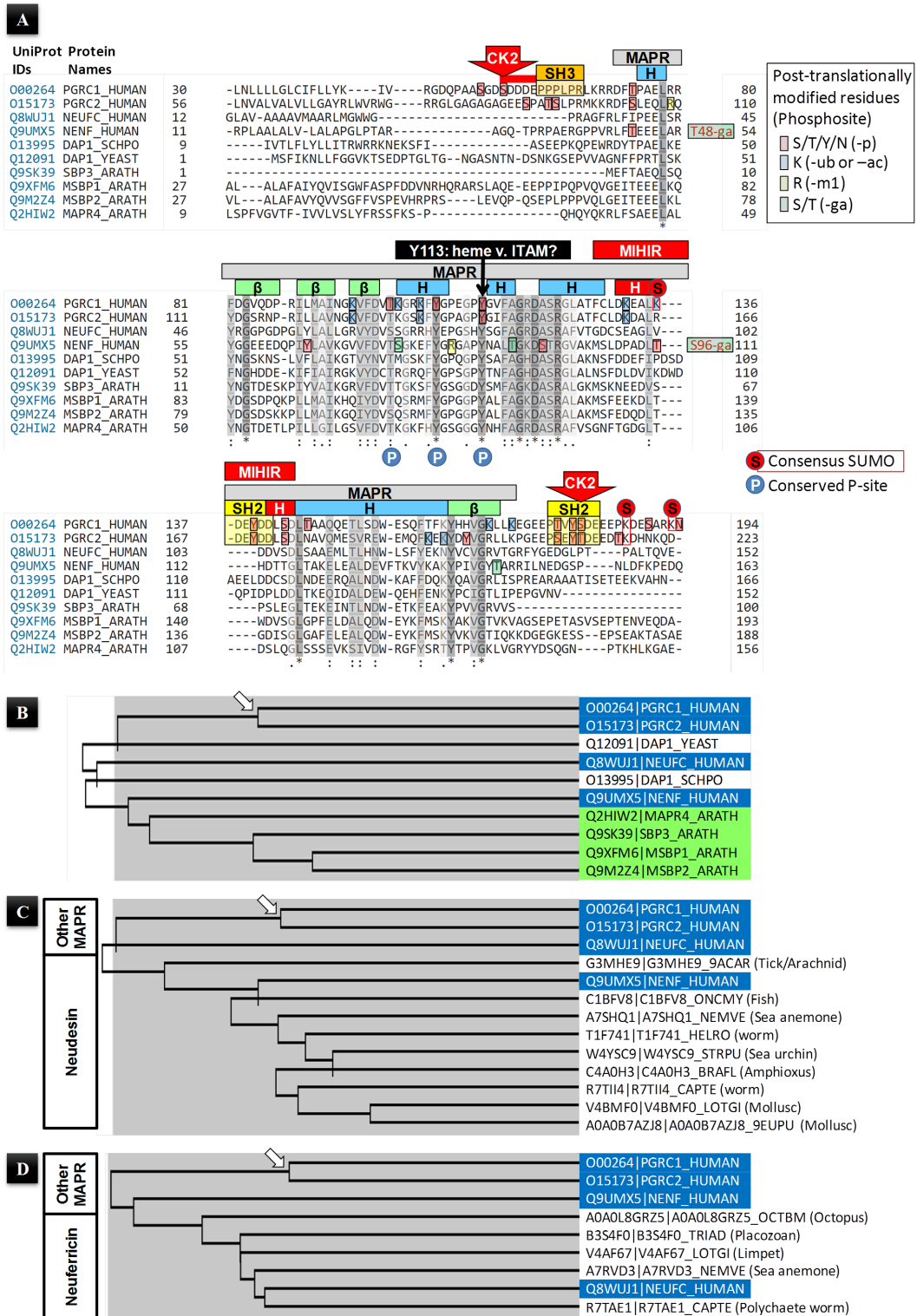
For Figure 2 and Table 1, the Uniprot FASTA sequences for the four human MAPR proteins were BLASTed against the indicated individual specific organisms using the NCBI BLASTp page (<https://blast.ncbi.nlm.nih.gov/>). The FASTA sequences of all identified MAPR proteins were then aligned with the Uniprot FASTA sequences for the four human MAPR proteins using the UniProt CLUSTAL multiple sequence alignment tool, as described above, and designated as Neudesin-like, Neferricin-like, PGRMC-like, PGRMC1-like or PGRMC2-like by closest alignment to human MAPR proteins in CLUSTAL tree topology.

Subsequent BLAST analyses were performed with NCBI BLASTp with query sequences and Organism taxid ID as described for each study. The top resulting BLASTp results were chosen, and FASTA sequence files added with NCBI identifiers to UniProt Align. The BLAST Query sequences were entered with UniProt Identifiers to color highlight them in UniProt Align results.

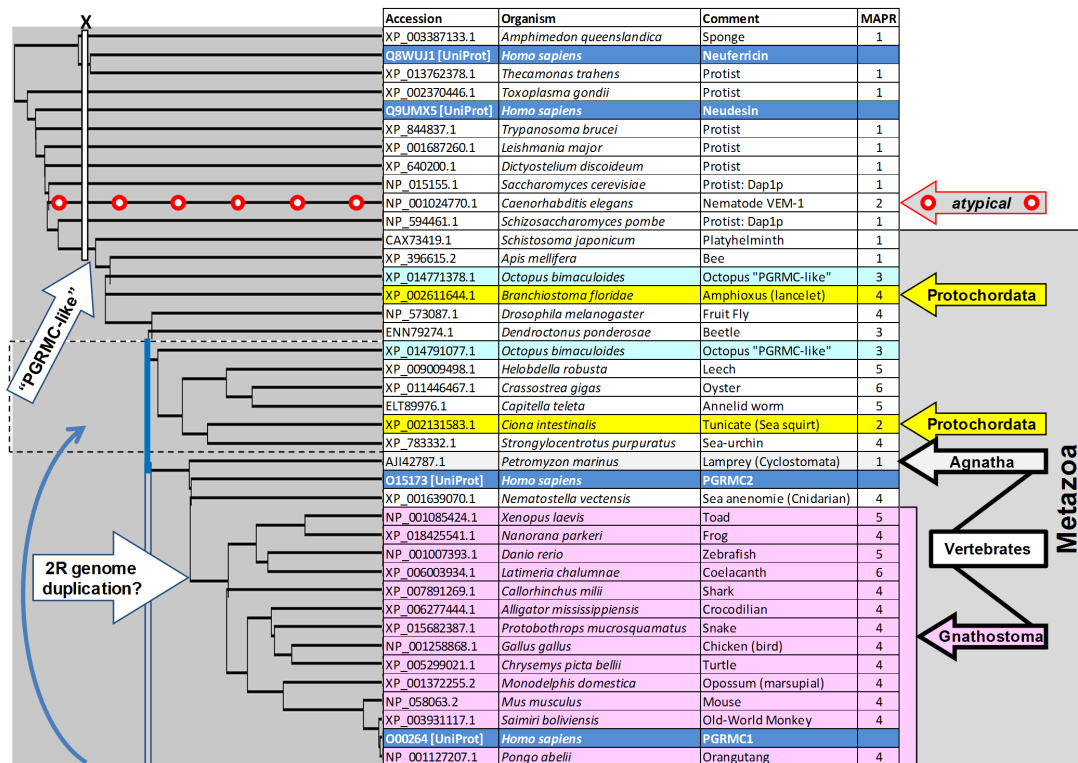
## 3. Results

### 3.1. Phosphorylation sites are variously conserved among MAPR proteins

PGRMC1 phosphorylations as documented in the Phosphosite data base (22) have been presented previously (21), as have PGRMC2 sites (8). Figure S1 shows the complete set of Phosphosite post-translational modifications (PTMs) documented for all four human MAPR proteins. Figure 1A maps the PTMs from Figure S1 on human MAPR proteins aligned against the sequences of two phylogenetically divergent yeast Dap1p MAPR family members, and four MAPR family members from the plant *Arabidopsis thaliana*. Phosphorylation of a Casein Kinase 2 (CK2) consensus motifs adjacent a C-terminal Src Homology



**Figure 1. Sequence alignment of selected phylogenetically disparate MAPR family members, showing known post-translational modifications of human proteins from Phosphosite. (A)** Post-translational modifications in the vicinity of the MAPR domain from Figure S1 are mapped to the amino acid sequence of the four human proteins, and aligned against 2 yeast and 4 plant MAPR proteins. The location of predicted SH2 target sequences centred on Y139 and Y180 (numbering refers to PGRMC1), and the SH3 target centred on P63 are marked. All four MAPR family proteins from human, four members from the flowering plant *A. thaliana*, and the single Dap1 family members from yeasts *S. pombe* and *S. cerevisiae* were aligned using the UniProt BLASTp tool, entering the UniProt identifiers (IDs) indicated in the figure. Similarity of amino acid properties are highlighted. The conserved cytochrome b5 domain of the MAPR family is marked by the grey box above the alignment. The positions of beta sheets ( $\beta$ ) or alpha helices (H) are indicated. The half inset red bar in the cytochrome b5 domain (PGRMC1 amino acids 128-146) represents the MIHIR insertion (see text) between cytochrome b5 domain helices which characterizes the MAPR family (1,13). Known post-translational modifications from the Phosphosite data base (Figure S1) are included for the human proteins as indicated in the key. Blue circled P depicts phosphorylation acceptor sites conserved across all species. Red circled S depict consensus Sumoylation sites in PGRMC1 (51,52). **(B)** The CLUSTAL similarity tree corresponding to **(A)**. **(C and D)** Divergence of PGRMC1-like proteins from other MAPR proteins predates the metazoan radiation. CLUSTAL alignment of MAPR proteins from phylogenetically diverse metazoans indicates that the PGRMC1/2 clade is distinct from the Neudesin **(C)** and the Neuffericin clades **(D)**. None of these families have been detected in unicellular eukaryotes (Table 1).



**Figure 2. The phylogenetic representation of PGRMC1-like MAPR proteins.** Either the sole MAPR family, or the most PGRMC1-like protein from each species (see Table 1) were grouped by similarity by CLUSTAL. A single exception is the octopus, where two PGRMC1-like (PGRMC-like: see Table 1) proteins are included to demonstrate that these are paralogous and not homologous to PGRMC1 and PGRMC2. *i.e.* octopus split from the vertebrate lineage before the divergence of PGRMC1 and PGRMC2. Therefore the octopus proteins represent a separate duplication of PGRMC-like proteins. It is not valid to speak of PGRMC1 and PGRMC2 for organisms which are not jawed vertebrates. PGRMC-like proteins appear to have arisen with metazoans. *C. elegans* VEMA protein is an atypical animal MAPR protein which has diverged from its metazoan ancestor (see Figure 3). PGRMC1 and PGRMC2 appear to have diverged correspondently with the emergence of jawed vertebrates, perhaps with the 2R genome duplication thought to have been associated with that lineage (53). For identities of MAPR proteins see Table 1. One region of the CLUSTAL alignment tree (dotted lines and curved arrow) was manually repositioned. Otherwise the CLUSTAL similarity topology has not been altered. The white bar marked "X" indicates phylogenetic separations thought by the author too deep for alignment to reasonably represent phylogeny.

2 (SH2) target motif is conserved between PGRMC1 and PGRMC2, as are various other tyrosine (Y) and serine/threonine (S/T) phosphorylation sites. The SH3-target motif centred on P64, along with phosphoacceptor residues at S54 and S57, are absent from PGRMC2, and none of these motifs are present in Neudesin or Neuferricin. Alignment with other MAPR family members from human, yeast, and plant (*A. thaliana*) show that some potential sites of phosphorylation (PGRMC1 T101, Y107, Y113) are conserved across these MAPR proteins, whereas PGRMC1 and PGRMC2 share multiple common sites (Figure 1A).

### 3.2. Posttranslational modifications of Neudesin and Neuferricin

PTMs are documented less frequently for Neudesin and Neuferricin than for PGRMC1 and PGRMC2. This may be because Neudesin and Neuferricin are present in the luminal compartment and secreted extracellularly (2,3,24-28), however the presence of some observed phosphorylation events suggests that the MAPR domain of these proteins may also be found in the cytoplasm. The detection of O-N-acetylgalactosamine (O-GalNAc)

glycosylation does not necessarily imply an extracellular location for Neudesin, since 14% of O-GalNAc proteins are annotated with nuclear Gene Ontology (GO) assignments (29). Indeed the Human Protein Atlas (30) lists the main subcellular location of Neudesin as nuclear based on two antibodies using immunofluorescence and confocal microscopy in human cells (<http://www.proteinatlas.org/ENSG00000117691-NENF/subcellular>). It is not unknown for proteins to exhibit dual orientation of membrane topology, such as human CD38 antigen which exists 90% with its C-terminus and active site in the extracellular/luminal compartment, with another 10% of protein molecules where these elements are cytoplasmic (31). Indeed the Cytb5 domain of PGRMC1 itself has been reported to be both cytoplasmic and extracellular (reviewed by (5)), such that dual membrane topology is conceivably a common feature of animal MAPR proteins.

### 3.3. The invertebrate divergence of Neudesin and Neuferricin predates that of PGRMC1/PGRMC2

Figure 1B depicts the Clustal guide tree corresponding to the alignment of Figure 1A. This is entirely based on

**Table 1. PGRMC1/2-like (PGRMC-like) proteins (which CLUSTAL groups with both PGRMC1 and PGRMC2) are found in metazoans higher than Platyhelminthes**

Accession	Organism	Comment	MAPR	NEUFC-like	NENF-like	"PGRMC"-like	PGRMC1	PGRMC2
XP_003387133.1	<i>Amphimedon queenslandica</i>	Sponge	1					
Q8WUJ1	<i>Homo sapiens</i>	Neuferricin						
XP_013762378.1	<i>Thecamonas trahens</i>	Protist	1					
XP_002370446.1	<i>Toxoplasma gondii</i>	Protist	1					
Q9UMX5	<i>Homo sapiens</i>	Neudesin						
XP_844837.1	<i>Trypanosoma brucei</i>	Protist	1					
XP_001687260.1	<i>Leishmania major</i>	Protist	1					
XP_640200.1	<i>Dictyostelium discoideum</i>	Protist	1					
NP_015155.1	<i>Saccharomyces cerevisiae</i>	Protist: Dap1p	1					
NP_001024770.1	<i>Caenorhabditis elegans</i>	Nematode VEM-1	2	1	NP_497868.1	0	1	NP_001024770.1
NP_594461.1	<i>Schizosaccharomyces pombe</i>	Protist: Dap1p	1					
CAX73419.1	<i>Schistosoma japonicum</i>	Platyhelminth	1	1		1	CAX73419.1	
XP_396615.2	<i>Apis mellifera</i>	Bee	1	1	XP_006566840.1	0	1	XP_396615.2
XP_014771378.1	<i>Octopus bimaculoides</i>	Octopus "PGRMC-like"	3	1	XP_014778686.1	0	2	XP_014791077.1, XP_014771378.1
XP_002611644.1	<i>Branchiostoma floridae</i>	Amphioxus (lancelet)	4	2	XP_002605720.1, XP_002605719.1	1	1	XP_002611644.1
NP_573087.1	<i>Drosophila melanogaster</i>	Fruit Fly	4	1	NP_572535.1		3	NP_723757.1, NP_609650.1, NP_573087.1
ENN79274.1	<i>Dendroctonus ponderosae</i>	Beetle	3	2	ERL94051.1, ENN78869.1		1	ENN79274.1
XP_014791077.1	<i>Octopus bimaculoides</i>	Octopus "PGRMC-like"	3	1	XP_014778686.1	0	2	XP_014791077.1, XP_014771378.1
XP_009009498.1	<i>Helobdella robusta</i>	Leech	5	1	XP_009019434.1	1	3	XP_009009498.1, XP_009012871.1, XP_009024185.1
XP_011446467.1	<i>Crassostrea gigas</i>	Oyster	6	3	EKC42144.1, XP_011418616.1, XP_011418617.1	1	2	XP_011446467.1, EKC28875.1
ELT89976.1	<i>Capitella teleta</i>	Annelid worm	5	1	ELT90457.1	1	3	ELT89976.1, ELU07585.1, ELU07586.1
XP_002131583.1	<i>Ciona intestinalis</i>	Tunicate (Sea squirt)	2	1	XP_002126672.1		1	XP_002131583.1
XP_783332.1	<i>Strongylocentrotus purpuratus</i>	Sea-urchin	4	2	XP_795139.1, XP_797342.1	1	1	XP_783332.1
AJI42787.1	<i>Petromyzon marinus</i>	Lamprey	1				1	AJI42787.1
O15173	<i>Homo sapiens</i>	PGRMC2						
XP_001639070.1	<i>Nematostella vectensis</i>	Sea anenome (Cnidarian)	4	2	XP_001636685.1, XP_001619521.1	1	1	XP_001639070.1, XP_001641507.1
NP_001085424.1	<i>Xenopus laevis</i>	Toad	5	1	XP_018102462.1	1	3	NP_001085424.1
XP_018425541.1	<i>Nanorana parkeri</i>	Frog	4	1	XP_018408871.1	1	2	XP_018425541.1
NP_001007393.1	<i>Danio rerio</i>	Zebrafish	5	1	NP_001096144.1	2	2	NP_001007393.1
XP_006003934.1	<i>Latimeria chalumnae</i>	Coelacanth	6	3	XP_006007296.1, XP_006007297.1, XP_006007298.1, XP_006007299.1	1	2	XP_006003934.1
XP_007891269.1	<i>Callorhynchus milii</i>	Shark	4	1	XP_007909304.1	1	2	XP_007891269.1
XP_006277444.1	<i>Alligator mississippiensis</i>	Crocodylian	4	1	XP_014463495.1	1	2	XP_006277444.1
XP_015682387.1	<i>Protobothrops mucrosquamatus</i>	Snake	4	1	XP_015676553.1	1	2	XP_015682387.1
NP_001258868.1	<i>Gallus gallus</i>	Chicken (bird)	4	1	XP_415743.1	1	2	NP_001258868.1
XP_005299021.1	<i>Chrysemys picta bellii</i>	Turtle	4	1	XP_007054253.1	1	2	XP_007060285.1
XP_001372255.2	<i>Monodelphis domestica</i>	Opossum (marsupial)	4	1	XP_001370477.1	1	3	XP_001372255.2
NP_058063.2	<i>Mus musculus</i>	Mouse	4	1	AAH86682.1	1	2	NP_058063.2
XP_003931117.1	<i>Saimiri boliviensis</i>	Old-World Monkey	4	1	XP_003933104.1	1	2	XP_003931117.1
O00264	<i>Homo sapiens</i>	PGRMC1						
NP_001127207.1	<i>Pongo abelii</i>	Orangutang	4	1	XP_002826904.1	1	2	NP_001127207.1

Neuferricin-like proteins are present in Cnidarians, nematodes, and higher animals. Neudesin-like proteins are present in worms, molluscs and chordates, but not in sampled insects. Distinct PGRMC1 and PGRMC2 proteins (as grouped by CLUSTAL) are present in jawed vertebrates (gnathostoma), but not agnathan vertebrates (lamprey) or protochordates (Amphioxus, tunicate/sea squirt).

the pairwise alignments, and should not be confused with an evolutionary phylogeny, which it vaguely approximates for more closely related species. However it quantitatively depicts the degree of similarity between proteins (23). The degree of similarity between Neudesin and Neuferricin is similar to that between the two yeast species, which are separated by a deep phylogenetic distance (32,33). All four MAPR family proteins from the plant species clustered together, consistent with their having shared a common ancestor after the monophyletic separation of plants from fungus and animals. This result suggested that the common ancestor of animal MAPR proteins was evolutionarily ancient. This was somewhat surprising, because Kimura *et al.* (26) reported that Neudesin was present in vertebrates, but not in the invertebrates *Caenorhabditis elegans* (a nematode roundworm) or *Drosophila melanogaster* (an insect), nor in the primitive chordate *Ciona intestinalis* (a sea squirt). That phylogenetic distribution would suggest that the gene for Neudesin originated in the chordate lineage leading to animals.

That interpretation seemed incompatible with the tree topology of Figure 1B. The UniProt Eumetazoan protein sequence data base was interrogated by BLASTp against human Neudesin (Figure 1C) and Neuferricin (Figure 1D) as query, revealing that sequences with greater similarity to both of these proteins are distributed across the animal kingdom, and therefore were present as separate proteins in the common ancestor of those organisms. Paradoxically, There were no BLASTp hits returned from *C. elegans* or *D. Melanogaster*, suggesting that insects and nematodes (but not arachnids or annelid worms) have lost their ancestral Neudesin genes. Therefore Kimura *et al.* (26) correctly reported Neudesin's absence in these species, but did not appreciate that the gene was probably secondarily lost in both lineages. Neuferricin was also widely distributed across the Eumetazoa (Figure 1D), including primitive groups such as Placozoans (B3S4F0), sea anemone (A7RVD3) Polychaete worm (R7TAE1) and molluscs (A0A0L8GRZ5, V4AF67), as well as a wide range of chordates and vertebrates but not insects (not shown), indicating that Neudesin- and Neuferricin-like MAPR proteins were present in an early metazoan ancestor.

#### 3.4. The ancestral Metazoan possessed a PGRMC-like MAPR protein

To examine the phylogenetic distribution of these MAPR families a series of single celled protists, and animals (metazoans) of various selected indicative phylogenetic affinities were BLASTed with NCBI BLASTp (which provides greater phylogenetic representation than UniProt) using all four human MAPR proteins together as input query sequence. The results are presented in Table 1, which indicates that all single cell level organisms screened possessed only one MAPR protein.

Platyhelminths (flat worms) possess only one PGRMC-like protein that aligns most closely with the PGRMC1/PGRMC2 group (subsequently "PGRMC-like", to denote proteins from organisms not descended from the earliest vertebrate ancestor in which PGRMC1 and PGRMC2 had diverged). Cnidarians (jellyfish and corals) possess Neuferricin-like and Neudesin-like proteins by CLUSTAL alignment. Loss of Neudesin-like proteins in several higher lineages is suggested since they are absent from nematodes, insects, and octopus (mollusc), although the Oyster possesses a Neudesin-like protein, indicating that the common mollusc progenitor possessed one. Neudesin must have been secondarily lost from Octopus, which interestingly may have accommodated the loss by evolving multiple PGRMC-like proteins (Table 1).

#### 3.5. The PGRMC-like ancestrally inherited metazoan protein diverged into PGRMC1 and PGRMC2 in the lineage leading to vertebrates

The jawless vertebrates Amphioxus and the lamprey have only one PGRMC-like protein. The lamprey has presumably secondarily lost its other ancestral complement of MAPR genes, since it has only a single PGRMC-like MAPR gene (Table 1). Jawed vertebrates exhibit at least two distinct PGRMC-like proteins which each clustered closer to either PGRMC1 or PGRMC2, indicating that the PGRMC1/PGRMC1 gene duplication event occurred in the lineage that gave rise to jawed vertebrates.

The same organisms as Table 1 are shown in Figure 2 along with the CLUSTAL alignment trees for the alignment of the most PGRMC1-like protein in each organism. Table 1 includes the single most PGRMC1-like protein per organism (with two exceptions, being human, where all four MAPR proteins are included, and octopus, to demonstrate that the two anciently diverged PGRMC-like proteins do not cluster more closely to either PGRMC1 or PGRMC1). The gene divergence that gave rise to PGRMC1 and PGRMC1 had not occurred in the organism that gave rise to molluscs and vertebrates. Therefore it is fallacious to refer to separate homologues of PGRMC1 and PGRMC2 for non-vertebrate organisms.

Figure 2 and Table 1 reveal that PGRMC-like proteins are shared by metazoans but not protists, and that PGRMC2 is most similar to lamprey, sea anemone and then the vertebrate PGRMC1 group (Figure 2), reinforcing that the PGRMC1/2 divergence occurred prior to the vertebrate radiation. Note that the *Caenorhabditis elegans* VEM-1 protein atypically clusters with protist MAPR proteins, which will be considered below.

#### 3.6. The ancestral animal PGRMC-like protein contained a tyrosine C-terminally to the MAPR domain corresponding to PGRMC1 Y180



Accession	Organism	Comment	Start AA	
O00264 [UniProt]	<i>Homo sapiens</i>	Human PGRMC1	52	ASGDSDDDEPPPLPRLKRRDFTPAEL
NP_001127207.1	<i>Pongo abelii</i>	Orangutang	52	ASGDSDDDEPPPLPRLKRRDFTPAEL
XP_003931117.1	<i>Saimiri boliviensis</i>	Old-World Monkey	52	ASGDSDDDEPPPLPRLKRRDFTPAEL
XP_014439595.1	<i>Tupaia chinensis</i>	Chinese tree shrew	51	AS-DSDDDEPPPLPRLKRRDFTPAEL
NP_058063.2	<i>Mus musculus</i>	Mouse	52	ASGDNDDDEPPPLPRLKRRDFTPAEL
XP_001372255.2	<i>Monodelphis domestica</i>	Opossum (marsupial)	51	GTAGAGDEEPPVLPPLKRRDFTLAQL
NP_001258868.1	<i>Gallus gallus</i>	Chicken (bird)	52	AQPGE--AGPPPLPKMKRRDFTLEQL
XP_006277444.1	<i>Alligator mississippiensis</i>	Crocodilian	46	P---AEPQGPPLPPLKRRDFTLEQL
XP_015682387.1	<i>Protobothrops mucrosquamatus</i>	Snake	57	AQPDGEEEAAPPLPKLKRDFTLAQL
XP_005299021.1	<i>Chrysemys picta bellii</i>	Turtle	47	RQPD---AEPPLPKLKRDFTLAQL
NP_001085424.1	<i>Xenopus laevis</i>	Toad	37	SNENT---EEQLPKMKRRDFTRAEL
XP_018425541.1	<i>Nanorana parkeri</i>	Frog	37	ESEDR---EEQLPKMKRRDFTMAQL
NP_001007393.1	<i>Danio rerio</i>	Zebrafish	43	DYGPV---EELPKLKRDFTLADL

\* :\*:\*\*\*\* :\*

**Figure 4. The PGRMC1 SH3 target motif centred on P64 (numbering refers to PGRMC1) with an adjacent CK2 site is present in the primate lineage.** The proline-rich SH3 target motif is present in all land vertebrates which evolved from amphibians. A phosphorylatable residue appears C-terminally at the S54 position in marsupials and mice, which is augmented by an adjacent CK2 consensus site (SDDDE) present in the primate lineage from shrews to apes. The phosphorylation site at T74 is highly conserved (Figure S1A), but of unknown function.

superposition of the S181 CK2 site motif upon the Y180 SH2 target motif (Figure 3A).

Annelid worms and molluscs, and other higher organism in Figure 3A additionally exhibit the adjacent T178 phosphorylation site in their PGRMC-like protein, which was variously lost in some lineages, but which became absolutely conserved in vertebrates, and therefore presumably performs some function enabling the complexity of PGRMC-like protein regulation required for vertebrate biology.

### 3.8. *C. elegans VEM-1 is an atypical PGRMC-like protein*

It becomes apparent why VEM-1 clusters away from other animal PGRMC-like proteins in Figure 2, because it lacks the Y139 and C-terminal negatively charged CK2-like motifs, which we can deduce were secondarily lost from the ancestral PGRMC-like condition in the nematode lineage. Alternatively, VEM-1 could be descended from a non-PGRMC1-like protein. To test this situation both *C. elegans* MAPR proteins were aligned with all four human MAPR proteins (Figure 3B). The alignment of VEM-1 with PGRMC1 and PGRMC2 clearly identifies it as a PGRMC-like protein which is distinct from the Neuferricin homologue (Figure 3B), but which has lost some hallmark PGRMC1-like features (Figure 3A). Note that *C. elegans* therefore does not have PGRMC1 and PGRMC2 homologues, but rather a PGRMC-like gene and a Neuferricin-like gene. It has also lost its ancestral Neudesin-like gene (Table 1).

### 3.9. The PGRMC1-specific SH3 target motif is shared by terrestrial tetrapods

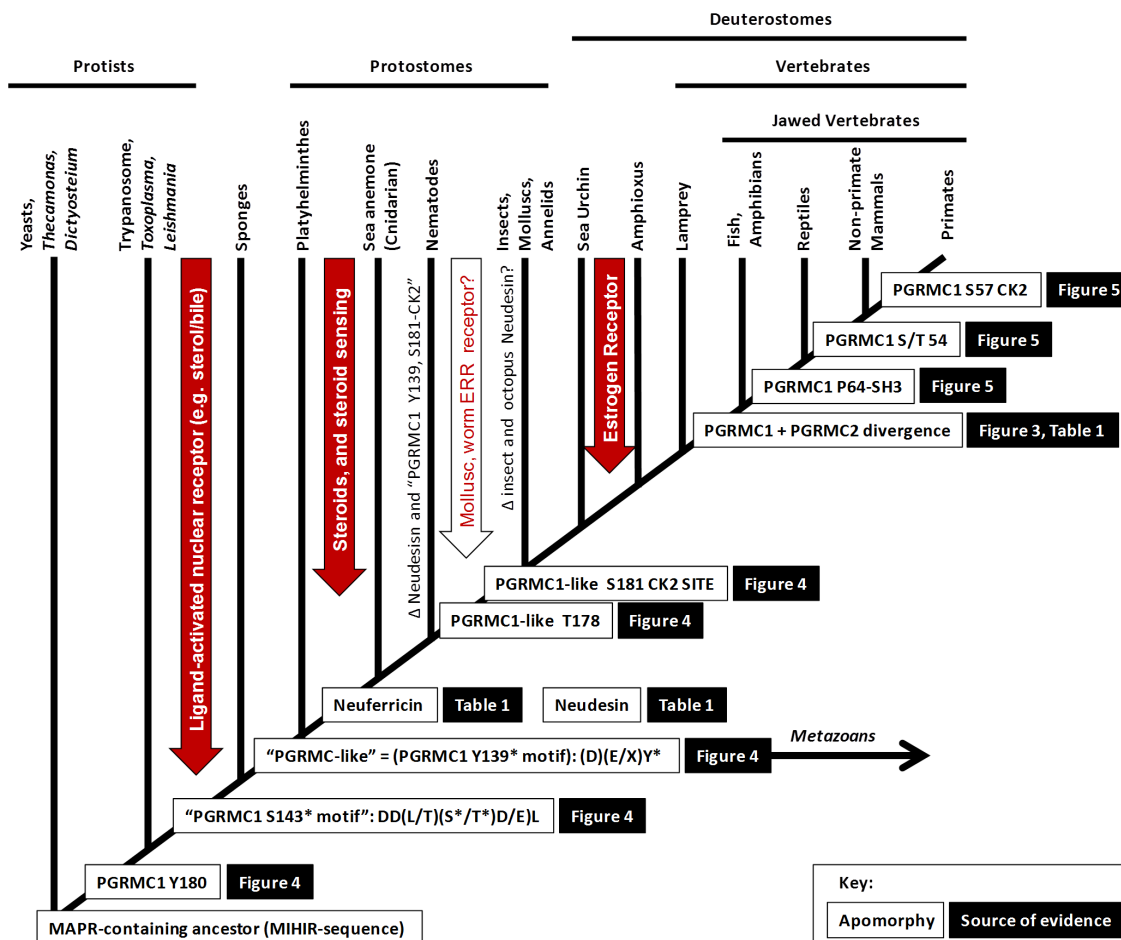
The N-terminal regulatory cytoplasmic region of PGRMC1 is not shared with PGRMC2, and therefore arose after their gene divergence. The conserved

PGRMC1 phosphorylation site at T74 in Figure 4 is shared with PGRMC2 (Figure 1A), however with the appearance of terrestrial tetrapods (reptiles including birds, and mammals, but not amphibians) a proline-rich sequence centred on P64 appeared. Placental and marsupial mammals, but not birds or reptiles, possess the phosphorylatable residue at T54, and all placental mammals have acquired the negatively charged DDDE motif immediately C-terminal to the proline-rich patch. The poly-negative charge probably influences the specificity of SH3 domain proteins that can interact with the adjacent proline-rich sequence. In the primate lineage this motif is further garlanded by the appearance of S57, to create the CK2 motif that is thought to sterically prevent interaction of SH3-domain proteins to the proline-rich SH3-target motif when phosphorylated (13,20,21), and provide new levels of finesse to the regulation of PGRMC1 function.

In summary, a new binding site for one or more SH3 domain proteins appeared on PGRMC1 as vertebrates colonised the land. In the mammalian lineage leading to humans this motif was equipped with the means for increasingly sophisticated regulation. It is currently unknown which biological processes are governed by such regulations. Possible functions which differ between the organisms involved include embryogenesis, skin ultrastructure including hair formation, properties of the ovaries/eggs, lactation, oestrus, and many more. Clearly an imperative research priority should be to develop reagents to assay for the state of phosphorylation of this and other PGRMC1 motifs to gain deeper understanding of its potential role in these foundational vertebrate processes.

PGRMC2 is fundamentally different to PGRMC1 in this region (Figure 1A). It should be equally important to determine which processes these two proteins can affect uniquely or in common, whether their effects on common processes are in the same directions, and the





**Figure 5.** A cladogram of eukaryotic organisms considered in this study based upon MAPR-family traits. The single celled eukaryotic ancestral organism to all organisms considered contained a MAPR family gene, called the the plesiomorphic condition. Apomorphies, or shared derived character states, are indicated on the cladogram stem with their source in black boxes. Amino acid numbers refer to motifs with reference to PGRMC1. The question mark (?) is present because a symmetrical segmented worm-like body plan is thought to have predated insect development, and because octopus and oysters are thought to be part of the same monophyletic evolutionary mollusc clade. This emphasizes the point that this is not a valid evolutionary tree, but a cladogram based solely upon inherited MAPR-related traits, and the appearance of steroids and steroid receptors (ERR, Estrogen related receptor; ESR estrogen receptor) following Baker (16) and Markov *et al.* (14) (vertical downward arrows) to separate some clades. Sea urchin is separated from protostomes by virtue of being a deuterostome (*i.e.* not by MAPR- or steroid-related traits).

molecular basis underlying any different functions.

3.10. Animal evolution topologically aligns with MAPR apomorphies

The above derived character traits of the MAPR family can be used to construct a cladogram based upon the appearance of new character states (apomorphies). Different organisms which inherit the same apomorphy share a synapomorphy which defines monophyletic groups of taxa (clades) which are descendants of the ancestral organism in in which the apomorphy first appeared (34). The cladogram of Figure 5 relatively accurately corresponds with known phylogeny of evolutionary divergence of these groups. Because of the association of PGRMC1 with P4 responsiveness, the cladogram has also been augmented by information about the evolutionary origins of ligand activated receptors,

steroid production and sensing, and the appearance of the Estrogen receptor in protochordates (Amphioxus in Figure 5), as referenced from Markov *et al.* (14). One interpretation of this cladogram is that the development of increasingly sophisticated regulation of PGRMC-like protein function throughout animal evolution has been intimately involved with the development of the key features which define these different lineages, right up to primates. In this model, PGRMC-like proteins provided some quintessential function to higher eukaryotic cell biology, and the tweaking and fine tuning of the MAPR/ PGRMC1 functional axis has been a powerful driving force throughout the diaspora of animal evolution. If so, it appears that the regulation of PGRMC-like proteins by phosphorylation (21) has been at the very heart of this evolutionary process.

This concept implies that animal evolution has involved regulated phosphorylation of unknown

signalling events based upon the Y180 motif of PGRMC-like proteins since our Precambrian ancestors. The motif was subject to increasingly sophisticated regulation during early animal evolution, particularly involving gain of the T178 site and its subsequent loss in several lineages. However, it appears to have been absolutely conserved in both PGRMC1 and PGRMC2 since their divergence prior to the origin of jawed vertebrates (Figure 3A, Figure 5), after which protein interactions with the PGRMC1 proline-rich SH3 motif appeared and became subject to increasingly complex regulatory possibilities (Figure 4, Figure 5).

#### 4. Discussion

##### 4.1. PGRMC or MAPR family?

McCallum *et al.* (35) referred to the MAPR family as the "PGRMC family". In this present paper I argue for the presence of PGRMC-like, Neuferricin-like, and Neudesin-like metazoan MAPR proteins, which are specific for animals (metazoan) but not other eukaryotes. "PGRMC-like" proteins (as defined here) are then a specific sub-clade of the MAPR family which does not include protist MAPR proteins. To avoid terminological confusion I refer to the MAPR family to denote the group of MIHIR-containing Cytb5 proteins. "PGRMC-like" better refers to those metazoan MAPR proteins which ancestrally contained the equivalent of PGRMC1 Y139, S143, and Y180. Neuferricin appears to have evolved by gene duplication of the PGRMC-like gene followed by loss of the Y180 homologous residue. Neudesin appears to have similarly later diverged prior to the divergence of Protostomes and Deuterostomes, from probably Neuferricin however that is uncertain (Figure 1B-D, Figure 3B, Figure 5, Table 1). All four MAPR proteins were present in the ancestor of annelids and molluscs, whereas ancestral Neudesin appears to have been lost from insects. Therefore use of the term "PGRMC family" (35) to denote the MAPR family appears to introduce room for potential systematic confusion.

##### 4.2. Progesterone responsiveness of PGRMC-like proteins is likely a vertebrate phenomenon

Participation of MAPR proteins in cholesterol synthesis is a function shared from protist to metazoan eukaryotes (11), which we can conclude was inherited from the earliest eukaryotic cell. Steroid hormones arose much later in evolution, presumably in the metazoan lineage. Both vertebrates and invertebrates possess ligand-activated classical/nuclear receptors for hydrophobic ligands such as terpenes, fatty acids, eicosanoids, sterols and bile acids, most of which are products of the mevalonate/isoprene pathway (15,36). From such a commonly inherited sterol sensing mechanism, separate clades are thought to have convergently evolved steroid

signalling mechanisms based upon cnidosteroids (cnidarians such as jellyfish and corals), lophosteroids (annelid worms, molluscs, *etc*), ecdysteroids (insects, other arthropods, and nematode worms), and vertebrosteroids (vertebrate lineage, commencing from an original ESR gene) (14). These steroid signalling systems evolved independently from one another in those clades in a striking example of parallel evolution.

The main steroidogenic CyP450 enzymes (37) as well as adrenal and sex steroid receptors (15) both recognizably arose in the protochordate lineage leading to vertebrates, with subsequent receptor gene duplications and functional diversification throughout vertebrate evolution (15). The estrogen receptor (ESR) was the first vertebrate steroid receptor (Figure 5), from which subsequent gene duplications eventually produced the classical progesterone receptor (14).

The enzyme P450 side-chain cleavage enzyme (P450<sub>scc</sub>, CYP11A1), which catalyzes the synthesis of the first vertebrate steroid hormone pregnenolone from cholesterol in mitochondria, has low sequence conservation (37), and therefore may not be recognizable in lower organisms. However, extensive BLAST analysis of lower organisms has not identified CYP11A1 in organisms lower than *Amphioxus* (14), and pregnenolone has not been convincingly reported in invertebrates to date (14).

From the above consideration, we can conclude by process of deductive logic that if sterol sensing arose in metazoans, and if steroid synthesis involving P4 appeared first in the vertebrate lineage, then any P4 or even progesterone sensing or signalling function of PGRMC1 is probably not an ancestral MAPR function. MAPR proteins do share an ancestrally inherited role in sterol synthesis (11). If an ancestral PGRMC-like protein participated in sterol sensing or sterol transport that involved low affinity interactions with cholesterol or derivatives, it is quite feasible that it could have secondarily acquired responsiveness to progesterone/progestagens once they appeared. (However whether PGRMC1 itself acts as a direct P4 receptor itself is still unclear (5).) Indeed, the ancestral metazoan nuclear steroid receptor is thought to have similarly originally been involved in sterol sensing, as well as being able to bind a broad range of metabolites including dietary sterols and xenobiotics. That activity had evolved into an ESR activity by the time the vertebrate estrogen synthetic pathway had evolved, and there is still some overlapping ligand affinity of certain modern steroid receptors for specific steroids and other hydrophobic ligands (14).

##### 4.3. Axonal migration probably does not involve PGRMC-like SH3 or SH2 target motifs

PGRMC1 is involved in directing the axonal migration of nerve cells in the embryologically developing nerve chord of nematodes and humans (17-19). Interestingly,

the lack of the Y139 motif and the S181 CK2 site from VEM-1 (Figure 3A) most probably mean that these motifs are not involved in that function exerted by PGRMC1, although we cannot be certain. VEM-1 interacts with members of Netrin-receptor family in *C. elegans* (18), and presumably in other animals in such a conserved process of body plan definition. The membrane trafficking function of PGRMC1 (reviewed by (5)) may be involved in regulating the cell surface expression of Netrin receptors to enable axon guidance in this process. Because they are the only major MAPR phosphorylation sites conserved between mammals and nematodes, presumably either Y113 (which contains a presumed membrane trafficking motif (5,21)) or Y107 are involved in this process. However that hypothesis requires experimental confirmation. This thought process demonstrates how systematic approaches such as this study can guide us to begin to functionally separate and stratify different pathways of the mutinodal signal integration web that is suspected to revolve around the fulcrum of PGRMC1 function (5). Indeed a central axis involving PGRMC1 Y180 is now revealed as running right back to single celled Precambrian biology.

Other intriguing PGRMC1 functions, in terms of cellular life as part of a multicellular organism, are controlled cell death, and hormonal signalling (reviewed by (5)). PGRMC1 confers resistance to death-inducing signals to some cells, and also confers responsiveness to progesterone (P4) and related progestogens. It is highly likely that these functions are separate to axonal migration guidance, and variously controlled by the different regulatory modules described here. It will be most interesting to see whether the observed co-evolution of a suite of mitochondrial genes with PGRMC1 (8) is related to any role of PGRMC1 in directing mitochondrial function, especially the changes in mitochondrial function that are associated with progression from single celled oocyte and zygote through embryogenesis towards a multicellular organism (38). These reflect the changes in mitochondrial function required in the evolutionary progression from protist eukaryotes to metazoans as considered in this paper.

#### 4.4. Is the PGRMC1/Sigma 2 Receptor function related to multicellularity?

The Sigma 2 Receptor (S2R) is an unidentified receptor activity that binds a large number of hydrophobic S2R ligands associated with neural disorders and cancer (39). Sigma receptors were originally described in 1976 as a subtype of the opiate receptor, based on the properties of ( $\pm$ )-SKF-10,047 (N-allylnormetazocine) and the structurally related analogues morphine and ketazocine. This led to the classification of three opiate receptor subtypes,  $\mu$  for morphine,  $\kappa$  for ketazocine, and  $\sigma$  for ( $\pm$ )-SKF-10,047. It was subsequently found that the (-) stereoisomer of SKF-10,047 bound to the  $\mu$  and  $\kappa$

opiate receptors. However the (+) isomer bound to an unknown non-opiate receptor, which became known as the  $\sigma$  (Sigma) receptor. Various ligands were found to bind this enigmatic receptor, some of which revealed that there were two distinct proteins with  $\sigma$  receptor activity, which became known as  $\sigma$ 1 (S1) and  $\sigma$ 2 (S2) receptors (reviewed by (39)). The sigma 1 receptor (S1R) was cloned in 1996, and found to have low homology with a sterol isomerase (40). It has been reported to be involved in lipid transport, the regulation of cholesterol-rich lipid raft microdomain formation at the plasma membrane, and the metabolism of cholesterol-containing cytoplasmic lipid droplets (41). S1R possesses protein chaperone function, where it provides an ER luminal hydrophobic binding site which binds to and stabilizes certain proteins, and which is located at cholesterol-rich ER regions, including mitochondria-associated ER membranes and the ER-cytoplasmic membrane interface (42). Like PGRMC1 (12), S1R is also associated with Insig-1, where S1R is implicated in the ER-associated degradation of proteins in a possibly sterol-dependent manner (42,43).

The identity of S2R remains unknown, however radio-ligand studies have shown that its activity was upregulated 10-fold in proliferating compared to quiescent cancer cells. In addition, certain S2R ligands bound their receptor at the cell surface, translocated to the mitochondrion, and killed tumor cells *via* both apoptotic and nonapoptotic mechanisms (39,44), which indicates an essential function for the S2R in some cancers. S2R ligands were internalized by both phenyl arsine oxide inhibitable receptor-mediated endocytosis, as well as *via* undefined non-inhibitable mechanism such as passive diffusion or non-receptor-mediated endocytosis (45).

In 2011 PGRMC1 was identified by Mach and colleagues as being part of a protein complex with S2R (46). A photoaffinity-labeled S2R ligand was cross linked to PGRMC1, indicating that PGRMC1 must be present with intimate proximity in an S2R-containing protein complex (46). In several cell types, but not all, PGRMC1 is required for S2R activity. This led to some confusion as to the possible identity of S2R and PGRMC1 (for review: (5)), which remains formally unresolved. However S2R is probably a separate 18 kDa protein unrelated to PGRMC1 (5).

S2R activity is vitally important for many cancer cells (39), and is associated with cancer stem cell properties related to proliferative status and survival (47-49). It is conceivable that this PGRMC1-S2R system, that regulates differentiation, proliferative, and survival decisions, reflects a remnant of the ancestral control of replication of the primitive single celled eukaryote. Thereby, the requirement of cancer and cancer stem cells for S2R activity may represent an ancestral S2R unicellular replication licensing function, which is modulated by PGRMC1-like proteins in metazoans that must impose strict restraints on proliferative activity.

If so, the PGRMC1 Y180 and/or the Y139 SH2 target motifs are probably involved since these originated with the appearance of multicellularity. The verification or falsification of this prediction must await the identification of the 18 kDa PGRMC1-associated protein which contains the S2R activity (for review: (5)).

## 5. Conclusions

Signalling and regulatory motifs on the PGRMC1 protein are shown here to have evolved along with animals during the metazoan radiation. This study portrays PGRMC1 as a cornerstone protein with functions central to eukaryotic biology and the origin of multicellular animals, that is potentially able to dramatically alter eukaryotic cell biology because of the deep evolutionary dependence of multiple cell functions upon MAPK proteins. If PGRMC1 regulates functions operating at a foundational cornerstone level of cell biology, then those alterations could have wide ranging pleiotropic effects relating to the strictures of multicellular life. Just as Archimedes thought to move the world with a fulcrum and a sufficiently large lever, so PGRMC1 phosphorylation could exert either tremendous metaphorical leverage, or occupy a cellular fulcrum with roots in the Precambrian. Obviously, such pleiotropic properties could be highly problematic for healthy biology when usurped by pathological processes such as cancer (5,20) or Alzheimer's disease (50). This raises the spectre that it is perhaps not the expression level of PGRMC1 which is most important to disease, but its state of modification. Since practically nothing is known about PGRMC1 modifying enzymes, this highlights an area which requires urgent investigation.

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## Supplemental Data

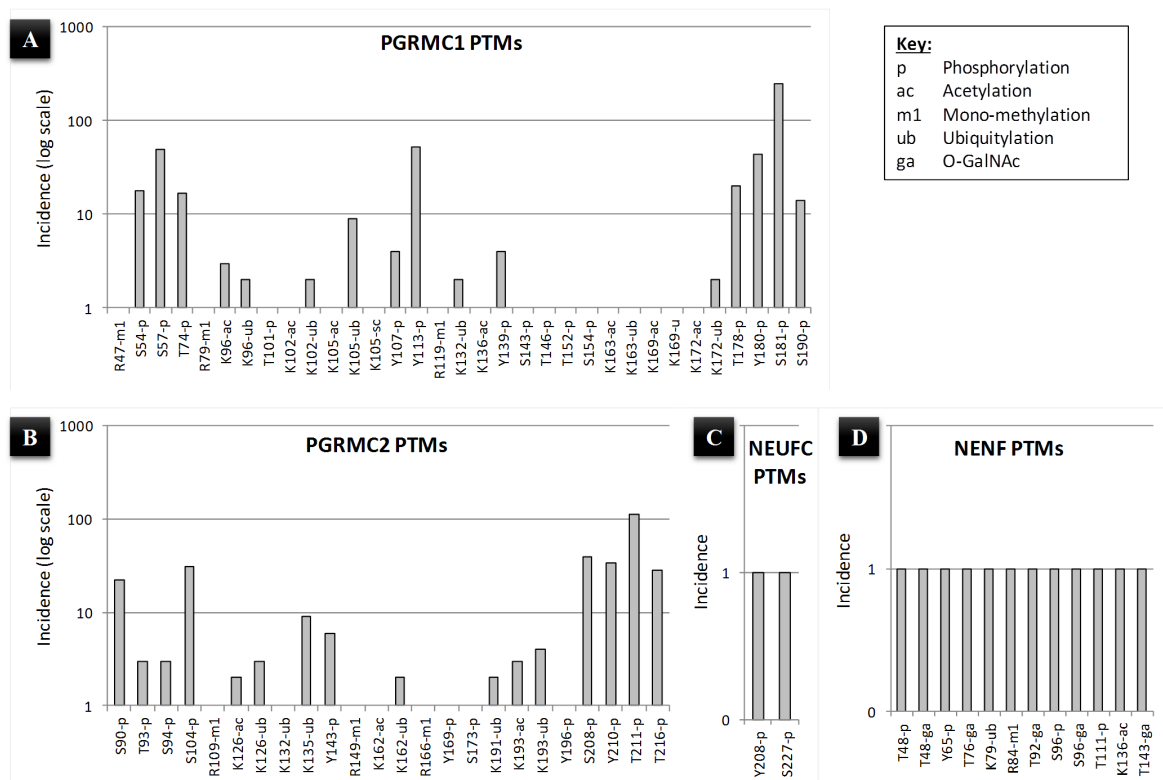


Figure S1. Post-translational modifications documented in the Phosphosite data base for human MAPR proteins PGRMC1 (A), PGRMC2 (B), Neuferricin (C) and Neudesin (D).