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Title: **The evolution of the GPCR signalling system in eukaryotes: modularity, conservation and the transition to metazoan multicellularity**

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Abstract

The G Protein Coupled Receptor (GPCR) signalling system is one of the main signalling pathways in eukaryotes. Here we analyse the evolutionary history of all its components, from receptors to regulators, to gain a broad picture of its system-level evolution. Using eukaryotic genomes covering most lineages sampled to date, we find that the various components of the GPCR signalling pathway evolved independently, highlighting the modular nature of this system. Our data show that some GPCR families, G proteins and Regulators of G proteins (RGS) diversified through lineage-specific diversifications and recurrent domain shuffling. Moreover, most of the gene families involved in the GPCR signalling system were already present in the Last Common Ancestor of Eukaryotes (LECA). Furthermore, we show that the unicellular ancestor of Metazoa already had most of the cytoplasmic components of the GPCR signalling system, including, remarkably, all of the G protein alpha subunits, which are typical of metazoans. Thus, we show how the transition to multicellularity involved conservation of the signalling transduction machinery, as well as a burst of receptor diversification to cope with the new multicellular necessities.

Keywords: Arrestin, Phosducin, Ric8, GRK, Heterotrimeric G protein complex

Introduction

A molecular system to receive and transduce signals from the environment or from other cells is key to multicellular organisms (Gerhart 1999; Pires-daSilva and Sommer 2003), although molecular signalling pathways are not only required within a multicellular context. Unicellular species face similar signalling needs as multicellular organisms, dealing with a changing environment and, in some cases, coordinating different cells (e.g. density sensing) (Crespi 2001; King 2004; Rokas 2008).

Both animals (metazoans) and plants have evolved complex signalling pathways to govern their embryonic development, and, according to current genomic data, some of these pathways appear to be specific to either metazoans or plants. This is the case of the metazoan-specific WNT and Hedgehog signalling pathways (Ingham et al. 2011; Niehrs 2012) and the land plant-specific Auxin and Cytokinin (Rensing et al. 2008). Other signalling pathways, such as the metazoan Notch pathway, have instead been assembled from various, more ancient components by domain-shuffling (Gazave et al. 2009). Finally, other signalling pathways were already present in the unicellular ancestors and were subsequently co-opted for multicellular functions. A good example are the receptor tyrosine kinases, which emerged and expanded in unicellular holozoans (i.e., choanoflagellates and filastereans), and were later recruited for developmental control in metazoans (King et al. 2008; Manning et al. 2008; Suga et al. 2012). The re-use of previously assembled signalling systems is indeed an important mechanism of signalling pathway co-option in multicellular lineages (King et al. 2008).

One of the major eukaryotic signalling pathways is the G Protein Coupled Receptors (GPCRs) and their associated signalling modules (Fritz-Laylin et al. 2010; Anantharaman et al. 2011; Krishnan et al. 2012), which are conserved from excavates to

animals. GPCRs are involved in many processes apart from developmental control, such as cell growth, migration, density sensing or neurotransmission (Bockaert and Pin 1999; Pierce et al. 2002; Rosenbaum et al. 2009). GPCRs are able to sense a wide diversity of signals, including proteins, nucleotides, ions and photons. Structurally, GPCRs have a 7 transmembrane domain (they are also known as 7TM receptors), which forms a ligand-binding pocket in the extracellular region, and a cytoplasmic G-protein-interacting domain (Pierce et al. 2002; Lagerström and Schiöth 2008), which binds to G-proteins to mediate intracellular signalling. G proteins form a heterotrimeric complex that is disassembled when activated by the GPCR, which acts as a Guanidine Exchange Factor (GEF), and transduce the signal into downstream effectors (Oldham and Hamm 2008). The G protein heterotrimeric complex has three different subunits of distinct evolutionary origin, *alpha*, *beta* and *gamma*. G protein heterotrimeric signalling is, in turn, regulated by various proteins families, including RGS and GoLoco-motif-containing proteins (Pierce et al. 2002; Siderovski and Willard 2005; Wilkie and Kinch 2005). The combination of GPCR, G proteins and their regulators results in many diverse signalling outputs.

Besides the classic GPCR-G protein signalling system described above, there are alternative upstream and downstream molecules (Figure 1). For instance, seven transmembrane receptors associated to RGS antagonize "self-activated" G *alpha* proteins in some lineages, acting as GTPase-accelerating proteins (GAP) receptors (Urano et al. 2012; Bradford et al. 2013). In plants, a single pass transmembrane receptor has been recently characterized to interact with G alpha proteins (Bommert et al. 2013). Moreover, monomeric G protein *alpha* activation by Ric 8 (Resistance to inhibitors of cholinesterase 8) is also GPCR-independent (Wilkie and Kinch 2005; Hinrichs et al. 2012), and Beta-Gamma heterodimers are regulated via Phosducins

(Willardson and Howlett 2007). Complementarily, GPCRs can perform downstream signalling independently of G proteins by G protein-coupled Receptor Kinases (GRK), Arrestins and Arrestin Domain-Containing proteins (ARDCs) (Gurevich and Gurevich 2006; Reiter and Lefkowitz 2006; DeWire et al. 2007; Liggett 2011; Shenoy and Lefkowitz 2011).

Most of the proteins involved in the GPCR signalling pathway have previously been analysed as single units in various phylogenetic contexts (Blaauw et al. 2003; Fredriksson and Schiöth 2005; Alvarez 2008; Oka et al. 2009; Anantharaman et al. 2011; Krishnan et al. 2012; Mushegian et al. 2012; Bradford et al. 2013). However, not much attention has been paid to the system-level evolution of the entire pathway, and given the modularity of the system, it is important to investigate its evolution from a global point of view. In this paper, we provide an update on the evolutionary histories of all components of the GPCR signalling system using a genomic survey that includes representatives of all eukaryote supergroups. We analyse the modular structure of the signalling pathway and show how different parts of the system co-evolved in complementary or independent patterns. We also reconstruct the GPCR signalling system in the Last Common Ancestor of Eukaryotes (LECA) and track its evolution in various lineages. Finally, we analyse the evolution of the system in the transition from unicellular ancestors to metazoans. We observe strong conservation in the pathway components associated with cytoplasmic signalling transduction, while receptors radiated extensively in metazoans, becoming one of the largest gene families in metazoan genomes (Fredriksson and Schiöth 2005). The dissimilarity between the pattern of evolution in pre-adapted signalling transduction machinery and active diversification of receptors provides clues on how key innovations in metazoan complexity could have evolved from pre-existing machineries.

Results

GPCR families: ancient origins and architecture diversifications

A widely-accepted classification of the metazoan GPCR complement is the GRAFS system, which is based on both phylogeny and structural similarity (Fredriksson et al. 2003; Fredriksson and Schiöth 2005; Lagerström and Schiöth 2008; but see Pierce et al. for an alternative classification). The GRAFS system divides GPCRs into 5 different families, Glutamate (also known as Class C), Rhodopsin (Class A), Adhesion (Class B), Secretin (class B), and Frizzled (Class F). This system can be extended to GPCR types described in non-metazoans, including the cAMP (Class E), ITR-like and GPR-108-like families, as well as several lineage-specific receptor families such as insect odorant receptors, nematode chemoreceptors or vertebrate vomeronasal receptors (Nordström et al. 2011). Fungi also have well defined GPCR families such as Ste2 and Ste3 (both included in Class D), and Git3 and plant Absicic acid receptors are also thought to be GPCRs (Plakidou-Dymock et al. 1998; Tuteja 2009; Krishnan et al. 2012). Most GPCR families are associated with a characteristic PFAM domain (Fredriksson et al. 2003; Fredriksson and Schiöth 2005; Lagerström and Schiöth 2008).

First, we assessed the presence and abundance of GPCR family domains in diverse eukaryotic genomes (see Figure 2 for a complete taxon sampling). Our data show that the distribution of GPCR families in eukaryotes follows two distinct evolutionary patterns. Some families are pan-eukaryotic, while others are biased towards amorpheans (unikonts). For instance, GRAFS are more abundant in amorpheans, especially in

metazoans, although some (Glutamate, Adhesion/Secretin and Rhodopsin) are also observed in some bikonts. Other families, such as cAMP receptors, Git3, ITR-like, GPR-108-like and Absicic acid Receptors are found in similar abundance among eukaryotes. Interestingly, non-GRAFS GPCR families are never expanded in any species (<10 members in all genomes). We also surveyed the taxonomically restricted metazoan families, and, although we found chemosensory receptors (7tm_7) and the Serpentine type chemoreceptors Srw and Srx in some previously unreported metazoan genomes, none were observed in non-metazoan eukaryotes (supplementary figure S1 and S2), with exception of OA1 (Ocular Albinism receptor), which is specific to metazoans and *Capsaspora owczarzaki*. These results indicate that most GPCR families have ancient origins in the last eukaryotic common ancestor.

Diversification of ancient GPCR families is usually accompanied by architectural diversification of the N-terminal protein domain (Lagerström and Schiöth 2008). Thus we analysed the architectural diversity of each GPCR family in each genome, and observed two types of GPCRs in terms of N-terminal domain diversity (diversifying versus non-diversifying in supplementary figure S2). Some, such as Glutamate, Adhesion/secretin, and, to a lesser extent, Rhodopsin are susceptible to the recruitment of new domains in the N-terminal region, especially in Metazoa, while others, such as like cAMP, Git3, OA1, Absicic Acid receptors, GPR108-like and ITR-like, have substantially lower diversity of protein domains at the N-terminal. This result suggests that some GPCR families have functional constraints while others are prone to diversify through recruitment of concurrent domains.

To gain further insights into domain diversification, we searched for evolutionary conservation of specific protein domain architectures (Figure 3), and found that some architectures are highly conserved across lineages. For example, Glutamate receptors

(7tm_3) have protein domain configurations that are conserved in distant eukaryotic lineages, including those with Venus Flytrap module (ANF_receptor), OpuAC or Bmp domains (Figure 3). Additionally, several non-metazoan species have diversified their own species-specific configurations of glutamate receptors (supplementary figure S3). The Adhesion family is also quite structurally diverse, especially in metazoans and, to a lesser extent, unicellular holozoans (supplementary figure S3). Similarly, the Rhodopsin family is architecturally diversified, mainly in metazoans. Finally Fz-Frizzled, RpkA (cAMP-PIP5K domain architecture) and Git3-Git3_C protein domain architectures could be identified in several eukaryotic genomes (supplementary figure S4), expanding the previous distribution of those architectures at LECA or at the root of Amorphea/Unikonta. Remarkably most of the GPCR complex architectures belong to GRAFS families and are mostly diversified and conserved within metazoans.

Heterotrimeric G protein Complex

GPCRs typically signal through G proteins. In an inactive state, the three G protein subunits (*alpha*, *beta* and *gamma*) form a heterotrimeric complex (Pierce et al. 2002; Oldham and Hamm 2008) (Figure 1). When a ligand activates a GPCR it acts as a Guanidine Exchange Factor (GEF), promoting GDP to GTP exchange in the G *alpha* subunit. This exchange alters G *alpha* subunit conformation and promotes the disaggregation of the heterotrimeric complex. The active G *alpha* subunit and an active dimer of *beta* and *gamma* subunits mediate further downstream signalling through various effectors (Milligan and Kostenis 2006; Oldham and Hamm 2008). G *alpha* is a low-efficiency GTPase, while G beta has various WD-40 repeats (PF00400) and G

gamma is a small protein containing a conserved domain (Milligan and Kostenis 2006; Anantharaman et al. 2011).

Using the signature domains of each G protein, we surveyed our dataset to find their general distribution patterns, and found that the abundance of each subunit varies markedly across eukaryotes, and that some taxa have lost these three subunits entirely (Anantharaman et al. 2011). G protein *alpha* is the most susceptible to diversification, and, interestingly, *beta* and *gamma* subunits have multiple copies in G *alpha* rich species. Although combination of the three elements is important for signalling plasticity, G *alpha* is the most evolutionarily dynamic of the three G proteins.

To gain further insights into the evolution of G *alpha* proteins, we performed phylogenetic analyses using our eukaryotic dataset (Figure 4), and the resulting tree shows that several groups have lineage-specific diversifications, such as those in *Naegleria gruberi*, *Bigelowiella natans*, and *Emiliania huxleyi*. The opisthokonts have a diverse but conserved repertoire of G *alpha* proteins. Fungi have four distinct paralogs (GPA-1 to 4) present in Ascomycota, Basidiomycota, Mucoromycotina and Chytridiomycetes (families reviewed in Li, Wright, Krystofova, Park, & Borkovich, 2007), and therefore were most likely present in the fungal ancestor. Holozoa also have four ancient paralogs, Gαs, Gαq/12/13, Gαi/o and Gαv (described for Metazoa in Oka, Saraiva, Kwan, & Korsching, 2009). It is worth mentioning that all of the metazoan G *alpha* families are conserved in the unicellular relatives of Metazoa, indicating that they originated prior to the diversification of metazoans from the rest of holozoans.

We also identified a new and divergent family of holozoan G *alpha* subunits that branches out from the Opisthokonta clade, comprising *Nematostella vectensis*, *Lottia*

gigantea and other holozoans (Figure 4). Additionally we observed a cluster of conserved G *alpha* subunits in several distant eukaryotic lineages (what we call conserved-eukaryotic group I): Ichthyosporea, *Allomyces macrogynus* and dictyostelids within the Amorphea, and *B. natans* and *Ectocarpus siliculosus* within the Bikonts. It is likely that this particular family originated in the LECA and was lost many times during eukaryotic evolution.

We also performed a phylogenetic analysis of eukaryotic *beta*-subunits, in order to compare the evolutionary histories of *alpha* and *beta* (supplementary figure S5). Our tree shows that holozoans have a particular ancient duplication, G β 1-4 and G β 5, with the more derived G β 5 known to interact with G *gamma*-like subunits, such as RGS7 (Sondek and Siderovski 2001; Anderson et al. 2009), a multi-domain protein that contains a G *gamma* domain. We identified RGS7 in both chytrid fungi and holozoans (Figure 3 and supplementary figure 6), and therefore the ancient duplication of G protein beta as well as its partner, RGS7, are ancient features of holozoans.

Regulatory proteins: RGS and GoLoco

Regulation of G-proteins is a key step in GPCR signalling that involves two main protein families, RGS (Regulators of G protein Signalling) and GoLoco motif-containing proteins (Siderovski and Willard 2005; Wilkie and Kinch 2005). RGS proteins act as GTPase-accelerating proteins (GAP), turning GTP into GDP and thereby promoting the formation of the G protein heterotrimeric complex and completing G alpha signalling (Siderovski and Willard 2005). Nevertheless, not all RGS domains act as GAP proteins in G protein signalling, and some have lost their GAP activity and have developed scaffolding functions (Anantharaman et al. 2011). GoLoco-motif-containing

proteins (also known as G Protein Regulators) act as guanine dissociation antagonists, inhibiting the dissociation of the heterotrimeric complex by binding to G *alpha*-GDP and blocking downstream signal transduction (Siderovski and Willard 2005).

We traced the distribution and abundance of RGS and GoLoco motif proteins in eukaryotes, and found that RGS is present in many different eukaryotes, mainly coinciding with the presence of heterotrimeric subunits (Figure 1). The number of RGS varies from one single copy in some taxa to numerous copies in other lineages. For example, some eukaryotes such as *Naegleria gruberi* (229), *Bigelowiella natans* (39), *Ectocarpus siliculosus* (47) or the ichthyosporeans (22 to 119) have more RGS proteins than *Homo sapiens* (34), while other multicellular lineages such as plants possess only one copy. In contrast, the GoLoco motif appears to be exclusive to metazoans and choanoflagellates (Figure 2 and Figure 3), and while its copy number may vary, it is less abundant than RGS. Therefore, our data show that the eukaryotic RGS system underwent independent radiations in lineages including amoebozoans, ichthyosporeans, heteroloboseans and rhizarians, while GoLoco is a later development that originated prior to the divergence of choanoflagellates and metazoans.

We then examined the architectural configurations of RGS proteins, since they are known to combine with many other protein domains (Siderovski and Willard 2005; Anantharaman et al. 2011). Our survey shows that species with distant phylogenetic relationships to each other evolved their own architectural repertoires, and generally have unique configurations that are not found elsewhere (supplementary figure S6). Moreover, many configurations evolved independently, recruiting the same domain in different configurations. For example DEP, cNMP binding, Kinases, Rho GTPase, Leucine Rich Repeat (LRR), START, and Ankyrin repeats are all present in various combinations in RGS genes from divergent taxa (shown in red in supplementary figure

S6). However some complex multi-domain architectures are evolutionary conserved (Figure 3 and supplementary figure S6). For example opisthokonts share some common RGS architectures, namely Sorting Nexins (SNX13/14/25) and the previously mentioned RGS7. Additionally, the RGS-like domain, typical of PDZ-RhoGEF, is an innovation of Holozoa (Figure 2), while RGS12 and Axin are metazoan innovations (supplementary figure S6). Our results emphasize that metazoans and their unicellular relatives have conserved elements of RGS complement, which is quite susceptible to diversification through domain re-arrangements.

Of specific interest are RGS proteins with transmembrane (TM) domains (Anantharaman et al. 2011; Urano et al. 2012; Bradford et al. 2013), as they localize to the cell membrane next to heterotrimeric G proteins. We found that in most lineages RGS is fused to at least one TM domain (supplementary figure S7) but in apusozoans, amoebozoans and haptophytes. In plants and other eukaryotes RGS domains have been observed together with 7TM organizations, somehow resembling a GPCR but with the opposite effect on G proteins (Urano et al. 2012; Bradford et al. 2013). Many bikonts possess 7TM-RGS architectures, but we found that chytrid fungi, filastereans and ichthyosporeans also have this type of receptors, while metazoans do not, suggesting that metazoans dispensed with GAP transmembrane signalling and restricted on typical GPCR signalling.

GoLoco motif-containing proteins are also part of multi-domain proteins. Our results show that choanoflagellates have a unique configuration (SH2-GoLoco) and a shared architecture with metazoans, G-protein-signaling modulator/Rapsynoid (Figure 3 and supplementary figure S6). Metazoans have some additional conserved architectures, such as RGS12/RGS14 and Rap1GAP (supplementary figure S6).

Upstream alternative regulators: Ric8 and Phosducin

Ric8 is a long domain that acts as a GEF (Guanidine Exchange Factor), activating G *alpha* subunits in the absence of GPCR signalling, or as a chaperone to stabilize G *alpha* (Hinrichs et al. 2012; Chan et al. 2013). Ric8-mediated activation of monomeric G *alpha* is involved in development and signalling in metazoans, fungi and *Dictyostelium* (Hinrichs et al. 2012; Kataria et al. 2013). While we found Ric8 in almost all amorpheans, suggesting it was secondarily lost in some species (Microsporidia, *T. trahens* and *E. histolytica*) (Figure 2), it is rare in bikonts, and found only in a small number of Heterokonta. The presence of Ric8 in only a few heterokonts could be explained by horizontal gene transfer, although our phylogenetic analysis does not support this hypothesis (supplementary figure S8), but suggests instead that Ric8 was present in the LECA, and secondarily lost in many eukaryotic lineages.

Phosducins belong to a small and ancient gene family, Phosducin-like (Blaauw et al. 2003; Willardson and Howlett 2007), and act as co-chaperones of the G beta/gamma dimers, allowing normal dimer configuration and transiently inhibiting their junction with G *alpha* (Willardson and Howlett 2007). We performed a phylogenetic analysis of Phosducin-like proteins, and the resulting tree shows three great clades: Phosducin I, Phosducin II/III, and orphan phosducin (supplementary figure S9). The only one known to interact with G protein beta subunits is the Phosducin-I or Phosducin/PhLP1 clade (Blaauw et al. 2003), and this is further reinforced by the fact that most species that have Phosducin I proteins also possess the heterotrimeric beta subunit. Conversely, the phosducin-II/III clade includes chlorophyte sequences, a group that lacks G protein

signalling. This suggests that proteins belonging to the phosducin-II/III clade have substrates other than G proteins (Willardson and Howlett 2007).

Alternative signalling inputs: GRK, Arrestins and Arrestin Domain-Containing Proteins

GPCRs can also signal independently of G-proteins, which is mainly achieved through interactions with G protein coupled Receptor Kinases (GRKs) and Arrestins, where Arrestins can either antagonize G protein signalling or connect GPCRs to other signalling modules (Gurevich and Gurevich 2006; Reiter and Lefkowitz 2006; DeWire et al. 2007; Shenoy and Lefkowitz 2011). GRKs have an active kinase domain and an inactive RGS domain, which allows it to scaffold with GPCRs. Like other kinases (e.g. PKC and PKA) GRKs phosphorylate active GPCR receptors in a process called desensitization, inhibiting the GPCR and allowing Arrestin binding. Arrestin binding promotes receptor internalization by endocytosis, which can result in ubiquitination or recycling of the GPCR (Pierce et al. 2002; Gurevich and Gurevich 2006; DeWire et al. 2007). Additionally, Arrestins can also act as adaptors for other signal transduction pathways such as MAPK or Akt (DeWire et al. 2007). Thus, understanding the evolutionary dynamics of Arrestin/GRK signalling is key to building a complete picture of GPCR signalling.

We found that GRK-like proteins are present in a reduced subset of eukaryotes, including Holozoa, Dictyostelida, Heterokonta and Haptophyta (Mushegian et al. 2012)(supplementary figure S10 and S11). Our phylogenetic analysis supports the duplication of GRKa and GRKb paralog groups at the root of Holozoa, as some sequences belonging to filastereans and ichthyosporeans branch within the GRKa clade

(supplementary figure S10). Nevertheless, using the kinase domain to unravel the evolutionary history of GRKs, some RGS-kinase architectures seem to be convergent, choanoflagellate and dictyostelid RGS are fused to a Tyrosine Kinase Like, instead they are fused to an AGC kinase (supplementary figure S11). While the absence of GRK in many GPCR rich genomes is not surprising, since other kinases can replicate this function, holozoans retained two paralogs of this specialized kinase.

While GRKs are rather scarce in eukaryotes, Arrestins domain-containing proteins (ARDCs) are broadly distributed, and our survey shows that most eukaryotes have a variable number of ARDCs (Figure 2). To gain insights into the evolutionary history of Arrestins and ARDCs, we performed a phylogenetic analysis and identified three major clades, though with low nodal support (supplementary figure S12). One clade includes metazoan Arrestins, as well as several sequences from unicellular holozoans, making Arrestins a pre-metazoan invention. The tree also shows a large lineage-specific expansion of ARDCs in Ciliophorans, fungal clades dominated by Mucoromycotina sequences, and the metazoans *Caenorhabditis elegans*, *Drosophila melanogaster* and *Trichoplax adhaerens*. Interestingly both Arrestins and ARDCs are known to interact with GPCR (Alvarez 2008), and therefore their presence and expansion suggests a complementary system to G protein signalling.

GPCR signalling system

After addressing the evolutionary histories of the various components of GPCRs and their signalling modules, we analysed them at system level by reducing the diversity of molecules into the main functional categories and analysing their co-evolution (Figure

5). Our data show that holozoans, fungi, amoebozoans, heterokonts/stramenopiles, haptophytes, rhizarians and heteroloboseans have most of the components of the GPCR signalling system, while others, such as *G. lamblia* and the miscrosporidians, are completely reduced. Other lineages have retained only a subset of the components involved in GPCR signalling, which challenges general views on the basic mechanics of the system. First, Absicic acid receptors (PF12430) and GPR-108-like (PF06814) are present in genomes where most of the GPCR signalling system has been lost (such as *Cyanidioschyzion merolae* and *Leishmania major*, see Figure 2), which implies that their role as GPCRs is doubtful, as previously suggested (Maeda et al. 2008; Anantharaman et al. 2011).

Furthermore, there are other taxa in which some GPCRs are present, even though the heterotrimeric complex is absent (or partially absent). For example, the apusozoan *Thecamonas trahens,* which lacks heterotrimeric subunits, has four cAMP receptors and one Adhesion receptor, all of which are canonical GPCRs. Similarly, ciliophorans, which only have the G protein subunit beta, have members of Rhodopsin, Adhesion, cAMP and ITR-like receptors. Interestingly both *T. trahens* and ciliophorans have ARDCs, in high numbers in the latter group, suggesting that ARDCs might provide an alternative link between GPCRs and other signal transduction pathways in those lineages. This is not the case in *Guillardia theta*, however, which has cAMP and ITR-like GPCRs but neither G proteins nor ARDCs. All of these data suggest that GPCRs might be connected to alternative signalling modules other than G proteins.

The modularity of the GPCR signalling system is further supported by the fact that various G protein subunits can be found independently of the other subunits. For example, the G *alpha* subunit, but not the G *beta* and *gamma* subunits, is present in *Trichomonas vaginalis* and *Cyanophora paradoxa*. The former has 7TM-RGS proteins,

which, in the absence of GPCR and two of the components of the heterotrimeric complex, may be interacting with other signalling pathways (Bradford et al. 2013), but no RGS is detected in *C. paradoxa*. Ciliophorans only have the G beta subunit, but have several Phosducin-like genes, which may also imply that ciliophorans have co-opted Phosducin and G protein beta into a distinct function. Additionally *T. trahens* has an RGS protein with no obvious function due to the absence of G *alpha* subunits. Thus, the evolutionary conservation of some components in simplified genomes underpins the modular plasticity of the GPCR signalling system.

We also performed a Principal Components Analysis of our eukaryote dataset with the aim of elucidating different evolutionary tendencies (Supplementary figure 13). We observed at least three clusters among eukaryotes that illustrate different patterns of evolution: expansion, simplification and conservation of the GPCR signalling system. Principal Component 1 (PC1) is principally loaded by the core functional categories of the GPCR signalling system, clustering the most simplified taxa together, including strict parasites such as microsporidians, *G. lamblia*, trypanosomatids, *Perkinsus marinus* or apicomplexans. Interestingly, many autotrophic lineages, such as Archaeplastida and Cryptophyta, also have a considerably reduced complement of GPCRs. On the other hand, PC2 differentiates between the two kinds of diversification of the GPCR signalling system. In a cluster characterized by the loading of G *alpha* and *beta* subunits, RGS, and cAMP receptors we find some ichthyosporeans (*A. whisleri*, *P. gemmata* and *A. parasiticum*), *N. gruberi*, *B. natans* and *Allomyces macrogynus*. Metazoans are differentiated in PC2 by the presence of 7tm1, 7tm2, GoLoco and Frizzled. Therefore our data indicate that the composition of the GPCR signalling system evolved repeatedly towards a more complex pathway in various eukaryotic

lineages. In particular, metazoans developed a more complex system through the expansion of GPCR signalling components.

Reconstruction of GPCR signalling components in LECA

We reconstructed the evolutionary stories of the various modules throughout the eukaryotic branch of the tree of life (Figure 6) using the amorphea-bikont root for eukaryotes (Derelle and Lang 2012) and taking into account the topology from the most recent phylogenomic studies (Brown et al. 2012; Burki et al. 2012; Torruella et al. 2012). Our data show that most GPCR families are ancient, and that some of the specific architectures of each family can be traced back to the eukaryotic ancestor. Therefore, the LECA already had a complex GPCR signalling system, as well as many other diversified gene families (Derelle et al. 2007; Fritz-Laylin et al. 2010; Wickstead et al. 2010; Grau-Bové et al. 2013). Most interestingly, some complex GPCR architectures are conserved in bikonts (being *B. natans* the major example), contradicting the hypothesis that claims that canonical GPCR signalling through G-proteins evolved in amorpheans (Bradford et al. 2013).

Discussion

Our genomic survey and evolutionary reconstruction show that the LECA had a complex repertoire of GPCRs (Figure 6). Independent expansions of the GPCR signalling system occurred in some eukaryotic lineages, and, interestingly, most of the

species that have these expansions are unicellular or colonial, such as *B. natans*, *N. gruberi* and ichthyosporeans (Supplementary figure 13). This supports the view that unicellular lifestyles also require complex signalling machineries (Crespi 2001). In fact multicellular fungi such as the Basidiomycota *Coprinus cinereus* and the Ascomycota *Tuber melanosporum* have rather simpler complements of GPCRs than other fungal lineages, including chytrids and Mucoromycotina. Similarly, embryophytes possess a reduced GPCR signalling system. Of course, other signalling pathways are also present in eukaryotes, such as Histidine kinases, Serine/Threonine kinases or Tyrosine Kinases (Anantharaman et al. 2007; Schaller et al. 2011; Suga et al. 2012), and these can have more important roles in the taxa where GPCR signalling is simplified.

An important conclusion from our work is the modularity of the system. We find that some species have GPCRs without G proteins and vice versa, and we also show how different parts of the GPCR signalling system evolved independently so that different functional categories involved in the pathway can become simplified without altering the others, as has been hinted at in other studies (Wilkie and Kinch 2005; Anantharaman et al. 2011). In addition, some parts of the pathway have diversified, both in terms of gene number and domain architecture, while other elements remain conservative. All of this evidence suggests that the system is plastic, and that drastic rearrangements can occur without complete loss of functionality. This robustness of eukaryotic signalling systems has been compared to the simpler and more direct signalling systems of prokaryotes (Anantharaman et al. 2007), and indeed modularity is a key feature of eukaryotic signalling pathways, which show great diversity of signalling machineries across different lineages (Anantharaman et al. 2007; Schaller et al. 2011).

Modularity is not only observed in how the various elements of the GPCR signalling pathway evolve, but also at the level of protein domain architectures. Overall, our

results on domain architectures clearly show that domain shuffling is a major mechanism of signalling system evolution. Indeed, pervasive convergent evolution of domain arrangements is a major feature of both GPCR receptors and RGS proteins (Nordström et al. 2009; Anantharaman et al. 2011; Krishnan et al. 2012). However, since not all GPCR families are equally susceptible to acquiring new domains, functional constraints might also exist that prevent this evolutionary mechanism of innovation.

A recent functional study in a subset of different G *alpha* subunits of various eukaryotes suggests that canonical GPCR signalling is restricted to amorpheans (Bradford et al. 2013). But our results suggest some inconsistencies under that perspective. For example, the presence of Ric8 in heterokonts (including *E. siliculosus* tested in the study) may imply that in that lineage there is GEF activation of G protein *alpha* subunits and not only "self-activation". Also, the presence of both 7TM-RGS and canonical GPCRs in opisthokonts (filastereans, ichthyosporeans and early-branching fungi) blurs the distinction between GAP and GEF receptor based G protein signalling, as they coexist in some lineages. Furthermore the monophyly of lineage specific G *alpha* protein clades implies that each of those lineages had diversified their own repertoires. Thus, there is not a conserved "self-activation" subfamily. Instead "self-activation" could had evolved as a convergent character of G *alpha* subunits. Since only the activity of a single paralogue of G *alpha* subunit has been tested for most lineages, it would be interesting to test more paralogues to clarify whether self-activation is the only mechanism in bikonts (Bradford et al. 2013). Finally, the presence of many GPCR types with functionally known amorphean domain architectures and rich heterotrimeric protein complements in bikonts, such as in *P. infestans* and *B. natans,* suggest that they may have had a canonical GPCR signalling. Those species should be ideal to test

different G *alpha* subunits experimentally. Overall our results suggest that GPCR-G protein canonical signalling is older than previously hypothesized, most likely already being functional at the LECA.

Irrespectively if the canonical GPCR signalling evolved in the root of amorpheans or before, regarding the origin of metazoans our results show a bimodal pattern of evolution of the elements of the GPCR signalling system. Cytoplasmic transduction elements, such as G proteins, Ric-8, GoLoco motif, Arrestins and RGS families, are largely conserved between unicellular holozoans and metazoans, both in terms of gene families and protein domain architectures (Figure 7). In contrast, receptors underwent a dramatic expansion in metazoans compared to their closest unicellular relatives, and a similar pattern has also been observed for tyrosine kinases, Hippo signalling and Notch signalling elements (Gazave et al. 2009; Sebé-Pedrós et al. 2012; Suga et al. 2012). The signalling output of GPCRs depend on the combinatory of heterotrimeric G proteins and their regulators, and, remarkably, the combination that originated in ancient holozoans was already sufficient for transducing the huge amount of GPCR signalling inputs present in metazoans. The expansion of receptors is probably driven by metazoans' multicellularity, which co-opted the GPCR signalling system for many new functions, such as cell-cell communication, developmental control, and most importantly in the case of GPCR, complex environmental sensing, from light sensing to odour and taste. We suggest that the shift from a universal eukaryotic signalling system to a dramatic expansion and refinement in metazoans played a key role in the acquisition of complex multicellularity.

Materials and Methods

Taxon sampling, data gathering

The 75 publicly available genomes used in this study were downloaded from databases at NCBI, The Joint Genome Institute, and The Broad Institute. Data from some unicellular holozoan species come from RNAseq sequenced in-house (*Pirum gemmata*, *Abeoforma whisleri* and *Corallochytrium limacisporum*) or from The Broad Institute "Origin of Multicellularity Database" (*Ministeria vibrans* and *Amoebidium parasiticum*). The RNAseq data was translated 6-frames.

All the protein domains that are components of the GPCR signalling machinery were selected from the literature and the PFAM database (Punta et al. 2012). All proteomes were scanned using PfamScan with PFAM A version 26 as query and selecting gathering threshold option. Gathering threshold is important in the case of GPCRs because it helps to disambiguate between different GPCR families by selecting the most significant hit. Additionally, PfamScan gathering threshold avoids the spurious partial hits typical of transmembrane proteins and is a conservative approach to minimize false positives that may arise with other more sensitive methods (Punta et al. 2012). General distribution patterns were obtained by counting proteins with at least one domain belonging to the GPCR signalling machinery present in the PfamScan proteomic outputs. The same files were used to obtain multi domain architectures, with the exception of the Transmembrane domains analysed in RGS proteins, which were obtained using the TMHMM software (Krogh et al. 2001). In the case of G protein *gamma* subunits additional tBLASTn searches against reference genomes where performed to avoid false negatives using bikont and opisthokont sequences as query. Gene loss is very difficult to assess due to the different degrees of incompleteness of the

available genomes. To overcome this problem we used, when possible, more than one taxa for each eukaryotic clade. Transcriptome data does not account for gene loss, as genes can be missed due to low expression, but in our dataset most species with transcriptomic data have sister species with genome sequence available.

Heatmaps, PCA and parsimony reconstruction

Heatmaps were built using R heatmap.2 function, from the gplots package. Principal component analysis (PCA) was carried out using the built-in R prcomp function, with scaling and a covariance matrix, and were plotted using the R bpca package. We assumed Dollo parsimony to infer ancestral gains and secondary loss reconstructions in Figure 6 using Mesquite (Maddison and Maddison 2011).

Phylogenetic analyses

Arrestins/ARDCs, Ric8, G *alpha* subunit, G *beta* subunit, Phosducin, Kinase and RGS domains were used for phylogenetic analyses. The alignments were obtained using MAFFT with the L-INS-i option (Katoh and Standley 2013), and these alignments were manually trimmed to avoid ambiguous regions. Seed alignments are available upon request and deposited at Dryad repository. The amino acid model of evolution used for phylogenetic inference was LG, with a discrete gamma distribution of among-site variation rates (four categories) and a proportion of invariable sites.

Maximum Likelihood analyses were performed using RAxML version 7.2.6. (Stamatakis 2006). The best-tree topology depicted in the figures was obtained by selecting the best tree out of 100 replicates. Bootstrap support was obtained using 100 bootstrap replicates of the same alignment. Bayesian inference trees were inferred using

PhyloBayes v3.3 (Lartillot et al. 2009). The resulting tree and posterior probabilities were obtained when two parallel runs converged (tracecomp standard values), after surpassing at least 500.000 generations. The runs were sampled every 100 generations, and the burn-in was established using a bpcomp maxdiff < 0.3.

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Figure Legends

Figure 1. Schematic representation of the GPCR signalling pathway. Protein families belonging to similar functional categories are grouped as specified in the colour legend.

Figure 2. Distribution and abundance of GPCR signalling components in 78 eukaryotic genomes. Numbers and abundance of domain containing proteins are depicted according to the colour legend in the upper-left, being black absence of the given domain in a given species. Yellower colours indicate smaller amounts, while the scale to purple indicates more abundance. The various domains are grouped into functional modules specified in Figure 1, as shown in the schema at the bottom-right. Species marked with an asterisk are only covered by RNA-seq data, therefore gene absence is not definitive. The original numbers of the heatmap are available at Supplementary Table 1.

Figure 3. Conservation of the domain architecture of different GPCR signalling components across eukaryotic genomes. A black dot indicates the presence of a given domain architecture. A white dot refers to similar domain architecture, Tyrosine Kinase instead of Serine/Threonine kinase in the case of Choanoflagellate GRK-like genes. For simplicity, only the most common architectures are shown. The percentage of genes found with a given architecture within a family is indicated at the bottom part of the table, as well as the total number of genes within the family. GPM in the first column of

GoLoco motif containing proteins stands for G Protein Modulator/Rapsynoid. The complete domain architectures of the GPCR signalling system components are found in supplementary figures 3, 4 and 6.

Figure 4. Maximum likelihood (ML) phylogenetic tree inferred by the G protein alpha subunit. Different eukaryotic lineages are represented by a colour code depicted in the legend. Within the gene family clades, the specific taxonomic groups which comprise eukaryotic lineages represented in that clade (i.e. eumetazoans, placozoans) are shown on the right. Nodal supports indicate 100-replicate ML bootstrap support and Bayesian Posterior Probability (BPP). Supports are only shown for nodes recovered by both ML and Bayesian inference, with BPP>0.9.

Figure 5. Schematic representation of the functional modules in eukaryotic lineages that were analysed in the study. Green boxes indicate the presence, white the absence and half-filled squares the presence with some simplification or uncertain affiliation. Asterisks in Arrestin and Phosducin rows indicate the presence of orthologs of a subfamily (B-arrestins and Phosducin-I clade), as discussed in the main text. In the upper part of the table, red dots indicate full reduction of GPCR signalling and green dots indicate severe simplifications but with some conserved functional modules.

Figure 6. Cladogram representing the major patterns of evolution of GPCR signalling components in a eukaryotic phylogeny. Coloured boxes with white text indicate specific components defined by a domain, while coloured boxes with black text refer to specific gene family acquisitions. Green and red boxes depict gain and loss of domains, blue boxes depict significant enrichments of the component shown, according to a Wilcoxon rank-sum test, with p-value threshold of <0.01. Additionally, a selected set of conserved

GPCR architectures placed where they must have appeared according to Dollo Parsimony.

Figure 7. Graphic depicts the median number of GPCR signalling components in opisthokont lineages. Total numbers of G protein *alpha*, *beta* and *gamma* subunits are comprised in the Heterotrimeric G proteins category, RGS and Go-Loco-motif containing proteins are comprised in Regulators of G proteins category, and GPCR types presented in Figure 2 are comprised in GPCR category. Median values were obtained using all taxa of a given clade as shown in the Supplementary Table 1.

Supplementary Figures

Supplementary figure 1. Distribution and abundance of metazoa-specific GPCR types. Domain numbers and abundance are depicted according to the colour code legend. Black boxes indicate absence of the domain in a given species' genome. No hits were found in non-metazoan genomes.

Supplementary figure 2. Abundance of diverse domain architectures of a given domain in Eukaryotic genomes. Pale green indicates a single domain architecture, i.e. the core-domain. Black indicates absence of the domain. Among GPCRs, 7tm_1, 7tm_2 and 7tm_3 are the richest in terms of domain architecture, and RGS also very often has diverse domain arrangements.

Supplementary figure 3. Distribution of specific protein domain architectures of Rhodopsin (7tm_1), Adhesion/Secretin (7tm_2) and Glutamate (7tm_3) GPCR families

across eukaryotic genomes. Domain architectures are named according to PFAM nomenclature (Punta et al. 2012).

Supplementary figure 4. Distribution of specific protein domain architectures of cAMP (Dicty_CAR), Git3 and Frizzled GPCR families across eukaryotic genomes. Domain architectures are named according to PFAM nomenclature (Punta et al. 2012).

Supplementary figure 5. ML phylogenetic tree of G protein *beta* subunit. Different eukaryotic lineages are represented by the colour code depicted in the legend. Nodal supports indicate 100-replicate ML bootstrap support and Bayesian Posterior Probability (BPP). Supports are only shown for nodes recovered by both ML and Bayesian inference and with BPP>0.7.

Supplementary figure 6. Distribution of domain architectures of RGS and GoLoco families across eukaryotic genomes. Domain architectures are named according to PFAM nomenclature (REF PFAM). Red text indicates domain architectures that use the same domains but have convergent origins. The name of the gene family to which the domain architecture belongs is shown in bold.

Supplementary figure 7. Table showing the number of transmembrane domains present in RGS proteins. The percentage of RGS proteins that have an associated transmembrane domain are shown on the right. The acronym of each organism consists of the first letter of the generic name and the first 3 letters of the species name (e.g., Hsap= *Homo sapiens*).

Supplementary figure 8. ML phylogenetic tree of the domain Ric8. The various eukaryotic lineages are represented by the colour code depicted in the legend. Nodal supports indicate 100-replicate ML bootstrap support and Bayesian Posterior

Probability (BPP). Supports are only shown for nodes recovered by both ML and Bayesian inference and with BPP>0.7.

Supplementary figure 9. ML phylogenetic tree of Phosducin domain proteins. Different eukaryotic lineages are represented by a colour code depicted in the legend. Nodal supports indicate 100-replicate ML bootstrap support and Bayesian Posterior Probability (BPP). Supports are only shown for nodes recovered by both ML and Bayesian inference and with BPP>0.7.

Supplementary figure 10. ML phylogenetic tree of RGS domain including SNX13/14/25 protein as the closest out-group to GRK. The various eukaryotic lineages are represented by the colour code depicted in the legend. Nodal supports indicate 100-replicate ML bootstrap support and Bayesian Posterior Probability (BPP). Supports are only shown for nodes recovered by both ML and Bayesian inference and with BPP>0.7.

Supplementary figure 11. ML phylogenetic tree of Kinase domain including most eukaryotic Serine/Threonine/Tyrosine Kinase families. The eukaryotic lineages that present GRK-like genes are represented by the colour code depicted in the legend. Best tree obtained from 100 independent runs in RAxML. Nodal supports indicate 100-replicate ML bootstrap support and Bayesian Posterior Probability (BPP). Supports are only shown for nodes recovered by both ML and Bayesian inference and with BPP>0.7.

Supplementary figure 12. ML phylogenetic tree of Arrestin and Arrestin Domain-Containing proteins (Arrestin_N and Arrestin_C domains used to construct the alignment). The various eukaryotic lineages are represented by the colour code depicted in the legend. Nodal supports indicate 100-replicate ML bootstrap support and Bayesian

Posterior Probability (BPP). Supports are only shown for nodes recovered by both ML and Bayesian inference and with BPP>0.7.

Supplementary figure 13. Principal component analysis showing the clustering of eukaryotic genomes according to GPCR signalling components. The two principal components displayed account for 26.4% (PC1, Principal Component 1) and 12.65% (PC2, Principal Component 2) of variation. Colour coding of dots according to taxonomic grouping is represented in the colour legend on the right. The acronym of each organism consists of the first letter of the generic name and the first 3 letters of the species name (e.g., Hsap= *Homo sapiens*).

G protein heterotrimeric complex
G protein regulators
Alternative upstream to G proteins
Alternative dowstream to GPCR

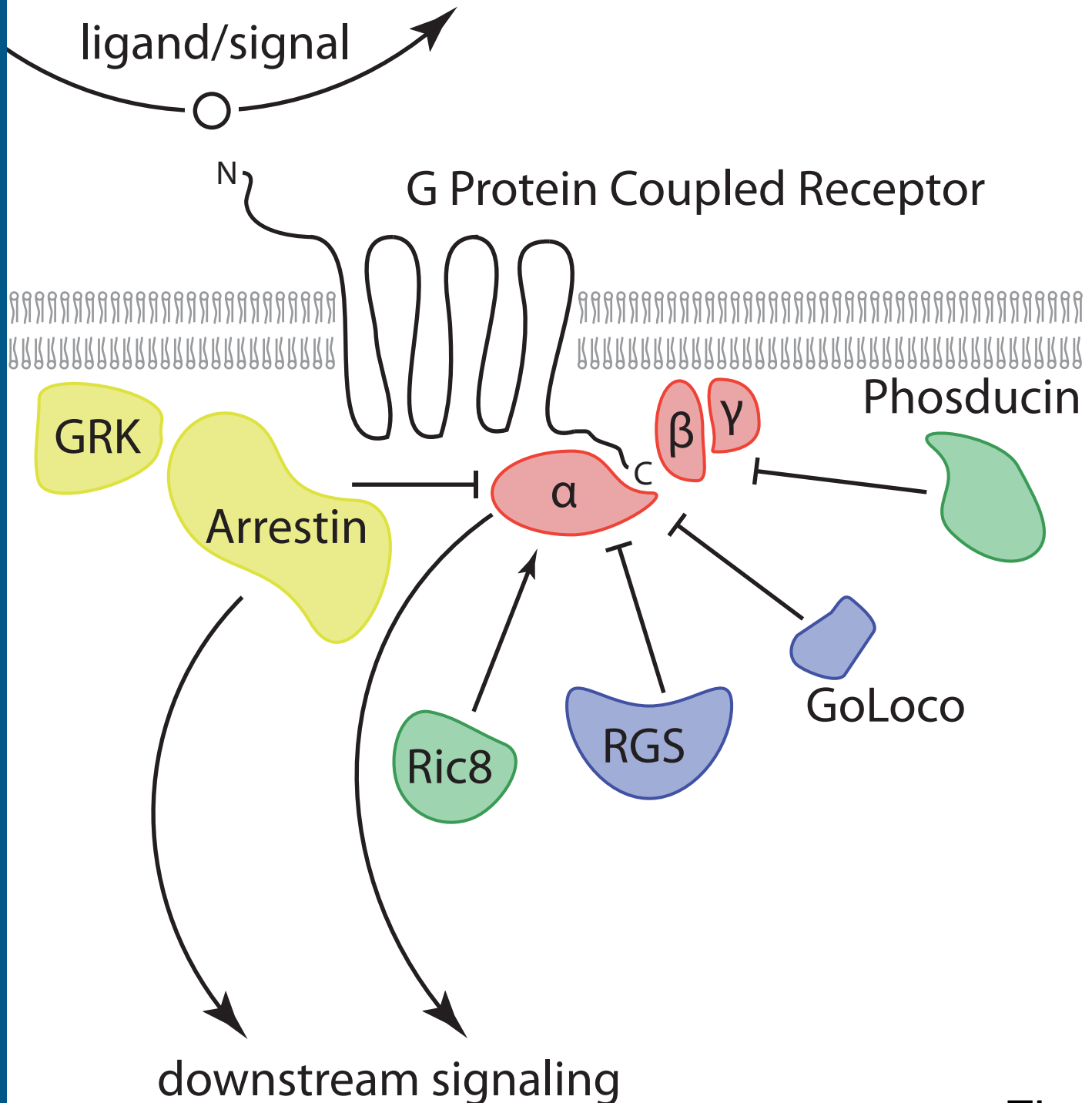


Figure 1

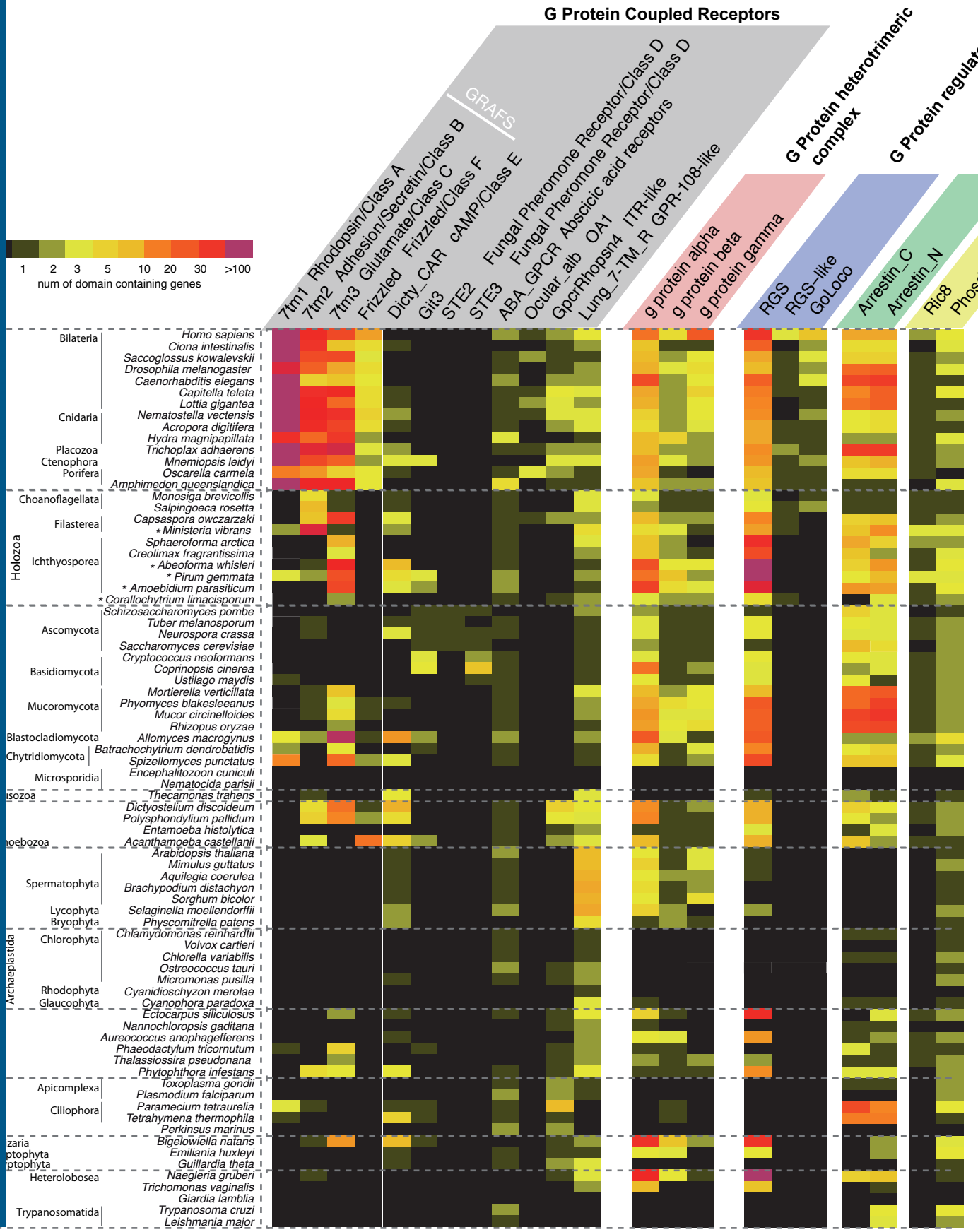
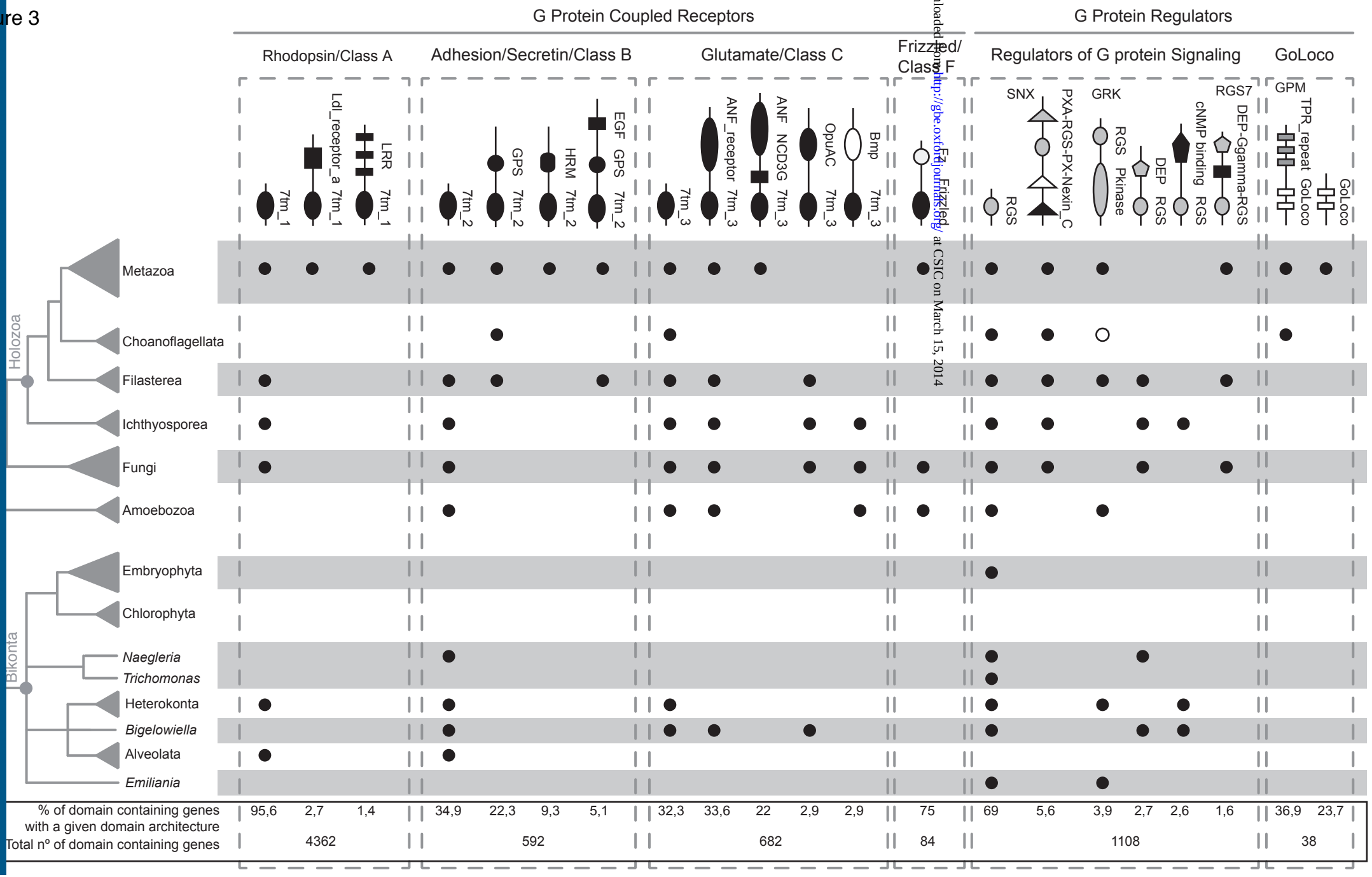


Figure 2

Figure 3



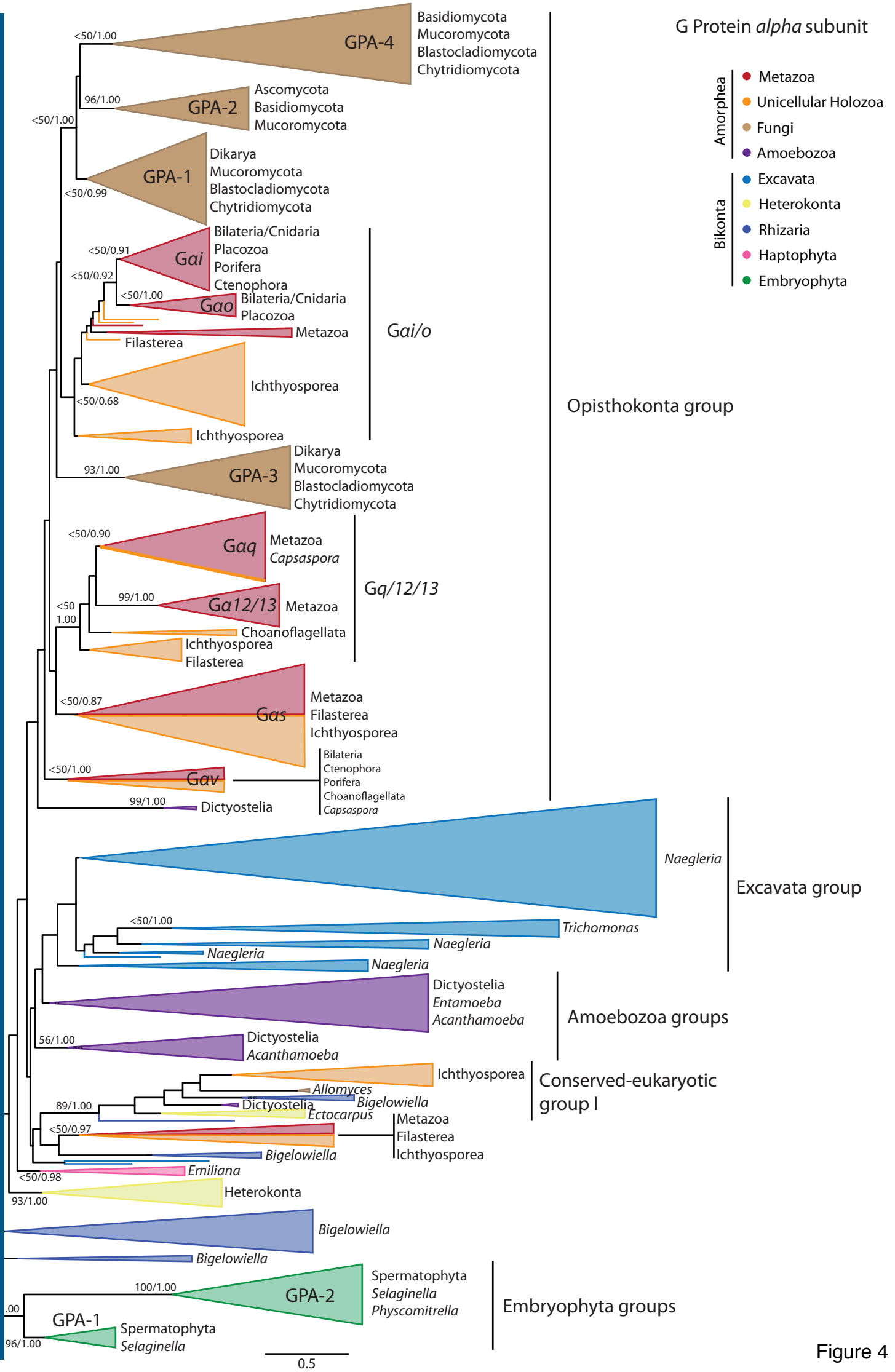


Figure 4

Figure 5

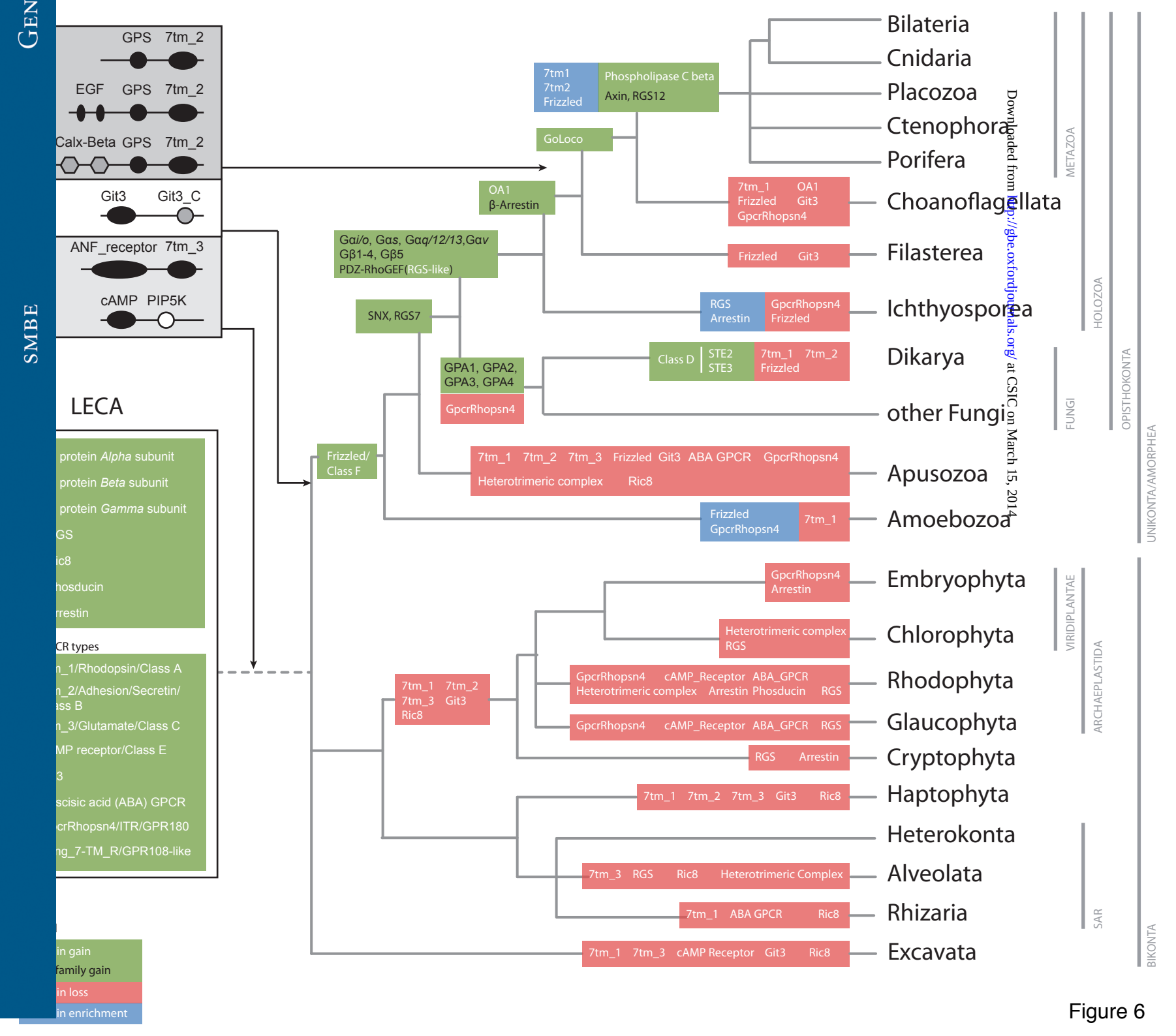

GPS 7tm_2
EGF GPS 7tm_2
Calx-Beta GPS 7tm_2
Git3 Git3_C
ANF_receptor 7tm_3
cAMP PIP5K
LECA
protein Alpha subunit
protein Beta subunit
protein Gamma subunit
GS
ic8
hosducin
rrestin
CR types
n_1/Rhodopsin/Class A
n_2/Adhesion/Secretin/
ss B
n_3/Glutamate/Class C
MP receptor/Class E
3
scisic acid (ABA) GPCR
crRhopsn4/ITR/GPR180
ng_7-TM_R/GPR108-like
in gain
family gain
in loss
in enrichment
7tm1 7tm2 Frizzled
Phospholipase C beta
Axin, RGS12
GoLoco
OA1 β-Arrestin
Gαi/o, Gαs, Gαq/12/13,Gαv
Gβ1-4, Gβ5
PDZ-RhoGEF(RGS-like)
SNX, RGS7
GPA1, GPA2, GPA3, GPA4
GpcrRhopsn4
Frizzled/ Class F
7tm_1 7tm_2 7tm_3 Frizzled Git3 ABA GPCR GpcrRhopsn4
Heterotrimeric complex Ric8
Frizzled GpcrRhopsn4 7tm_1
7tm_1 7tm_2 7tm_3 Git3 Ric8
7tm_1 OA1 Frizzled Git3 GpcrRhopsn4
Frizzled Git3
RGS Arrestin
GpcrRhopsn4 Frizzled
Class D
STE2 STE3
7tm_1 7tm_2 Frizzled
GpcrRhopsn4 Arrestin
Heterotrimeric complex RGS
GpcrRhopsn4 cAMP_Receptor ABA_GPCR Heterotrimeric complex Arrestin Phosducin RGS
GpcrRhopsn4 cAMP_Receptor ABA_GPCR RGS
RGS Arrestin
7tm_1 7tm_2 7tm_3 Git3 Ric8
7tm_3 RGS Ric8 Heterotrimeric Complex
7tm_1 ABA GPCR Ric8
7tm_1 7tm_3 cAMP Receptor Git3 Ric8
Bilateria
Cnidaria
Placozoa
Ctenophora
Porifera
Choanoflagellata
Filasterea
Ichthyosporea
Dikarya
other Fungi
Apusozoa
Amoebozoa
Embryophyta
Chlorophyta
Rhodophyta
Glaucophyta
Cryptophyta
Haptophyta
Heterokonta
Alveolata
Rhizaria
Excavata
METAZOA
HOLOZOA
FUNGI
OPISTHOKONTA
UNIKONTA/AMORPHEA
VIRIDIPLANTAE
ARCHAEPLASTIDA
SAR
BIKONTA
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Figure 6

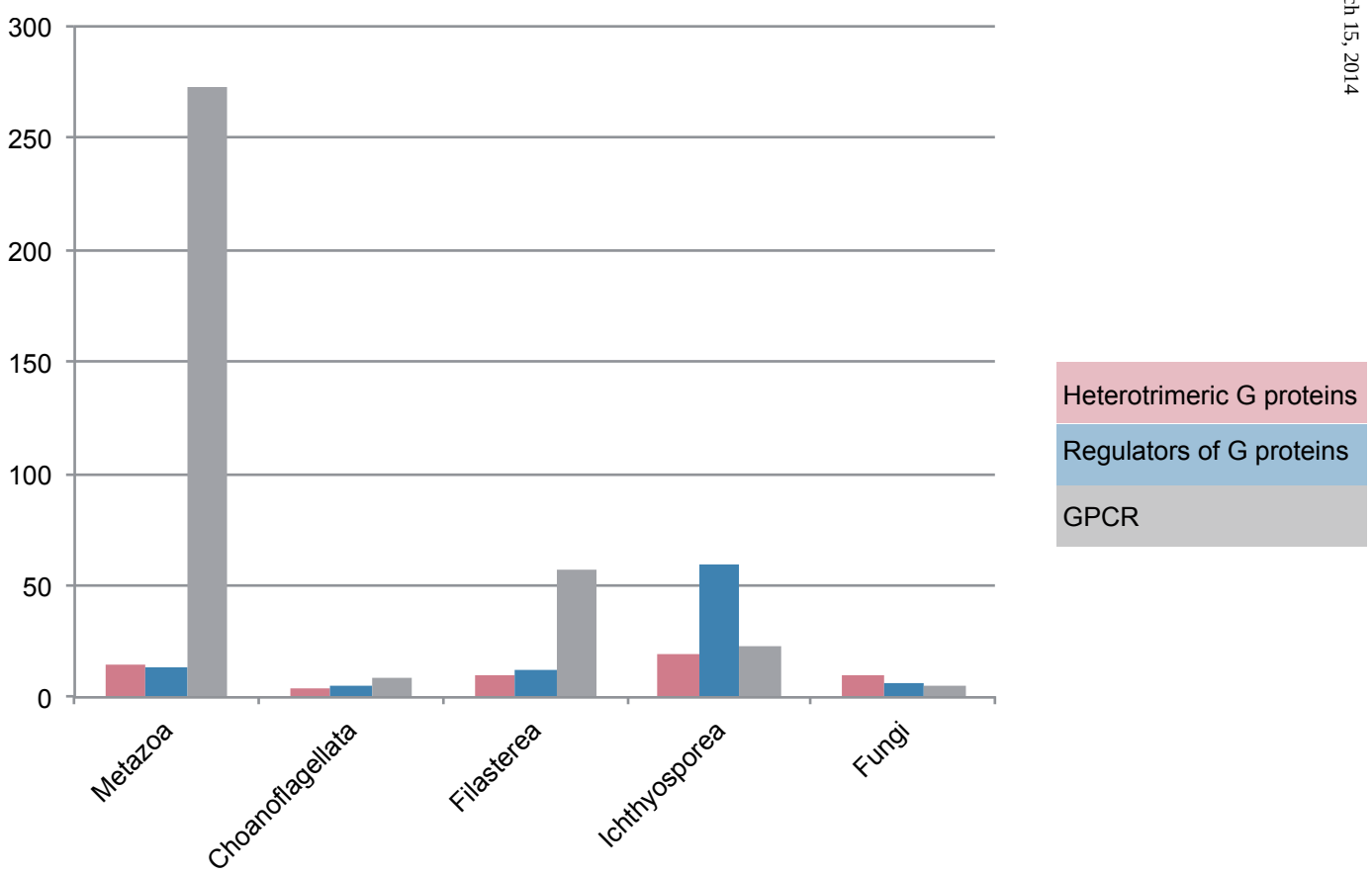
300
250
200
150
100
50
0
Metazoa
Choanoflagellata
Filasterea
Ichthyosporea
Fungi
Heterotrimeric G proteins
Regulators of G proteins
GPCR

Figure 7