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ALANA EDUARDA DE CASTRO PANZENHAGEN

METILMERCÚRIO E NEURODESENVOLVIMENTO: UMA ABORDAGEM DE BIOLOGIA DE SISTEMAS

Porto Alegre
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Trabalho de Conclusão de curso apresentado como requisito parcial para obtenção do título de Bacharel em Ciências Biológicas na Universidade Federal do Rio Grande do Sul.

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“What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what’s going on.”

- *Jacques Yves Cousteau*

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INTRODUÇÃO

O mercúrio (Hg) é liberado no ambiente principalmente através de atividade vulcânica e erosão de solos (PIRRONE et al., 2010). Porém, processos antrópicos podem contribuir significativamente para a liberação de Hg inorgânico no ambiente (GROTTO et al., 2009; PEIXOTO et al., 2007). A exposição ao mercúrio é relativamente comum, uma vez que metais pesados não são criados nem destruídos, e sim, constantemente transformados (PIRRONE et al., 2010). Micro-organismos transformam Hg inorgânico em seu derivado orgânico, o metilmercúrio (MeHg), que representa grande perigo de contaminação ambiental, especialmente por sua característica bioacumulativa. Alguns de seus efeitos prejudiciais para organismos vivos são alterações no balanço oxidativo, danos a macromoléculas e prejuízo ao desenvolvimento cognitivo e neuronal (FARINA; ASCHNER; ROCHA, 2011). O MeHg pode causar dano grave e alguns dos principais sintomas da síndrome neurológica causada pelo envenenamento, chamada "Doença de Minamata", são: ataxia, perda da sensibilidade nas mãos e pés, musculatura fraca, diminuição do campo de visão e danos à fala e audição. Em casos extremos pode-se apresentar insanidade, paralisia, coma, e morte após algumas semanas do início dos sintomas (GROTTO et al., 2011; MYERS et al., 2005). As populações humanas são expostas ao MeHg principalmente através do consumo de peixe, afetando principalmente populações ribeirinhas. Dentre essas, crianças são as mais afetadas, apresentando prejuízo no processo de neurodesenvolvimento (FOSSATO DA SILVA et al., 2011; GROTTO et al., 2009).

A interação do MeHg com proteínas chave para os processos metabólicos pode elucidar mecanismos através dos quais esse composto age. A abordagem de biologia de sistemas abrange a modelagem matemática e computacional de sistemas biológicos complexos. Uma vez que a toxicidade do MeHg está relacionada com a interação de diversos fatores, a biologia de sistemas se encaixa perfeitamente nesse contexto. Uma abordagem típica da biologia de sistemas consiste na construção de redes de interação entre proteínas (PAPANIKOLAOU et al., 2015). Através de análises topológicas, como número de conexões e ligação de clusters, é possível inferir quais os nós mais importantes para a manutenção da estrutura da rede como um todo e, portanto, muito provavelmente, para a via de sinalização e interação dessas proteínas *in vivo*. Esse tipo de abordagem permite direcionar futuras pesquisas, destacando os principais alvos biológicos do composto.

O objetivo do estudo é aplicar abordagens de biologia de sistemas na análise de redes de interação proteína-proteína construídas a partir de dados da literatura. Além disso, encontrar proteínas alvo, associadas a processos específicos, cuja perturbação provavelmente causaria a

ruptura da rede. Uma rede instável significa que a conectividade do sistema biológico foi parcialmente perdida e sua funcionalidade pode estar prejudicada. Esperamos com esse trabalho direcionar o pesquisador às proteínas com maior importância na rede. Uma vez que os interatores são expandidos, podemos visualizar proteínas associadas àquelas comumente estudadas e futuramente estudar suas funções. Além disso, muitas destas proteínas podem ser de fato as causadoras dos efeitos encontrados nesses estudos, ainda que sua influência seja detectada através de proteínas relacionadas.

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METHYLMERCURY AND NEURODEVELOPMENT: A SYSTEMS BIOLOGY APPROACH

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Abstract

Bacteria metabolize mercury into its organic derivative, methylmercury (MeHg), which is toxic, bioaccumulative, and has the ability to cross the hematoencephalic and placental barriers. MeHg exposure is associated with neurodevelopmental impairments. Human contamination is a worldwide phenomenon and occurs mainly through fish comsumption. The interaction of MeHg with target proteins or metabolic processes might enlighten us as to the mechanisms through which MeHg acts. Our objective was to apply a systems biology approach, analyzing PPI networks constructed from literature data. We aimed at finding target proteins, associated with particular processes, whose perturbation would probably rupture the network. A systematic review was performed, in which 182 studies were found. A list of 169 proteins was extracted from those studies and its IDs were uploaded to the String-DB database in order to assemble a protein-protein interaction network. The network properties were evaluated through cluster, enrichment and topology analyses using Cytoscape v 3.7.0 and R. Our results suggest the most important process associated with MeHg *in utero* exposure and neurodevelopment is cellular signaling. In addition, not only this is the most prominent process in the main network, but also the target proteins found (RAC1, CDC42, AKT1, MAPK3, MAPK1) are included in this particular group. Our findings may help focusing research and elucidating the mechanisms of MeHg toxicity. Hopefully it might also contribute to the future development of new treatment or prophylactic measures to avoid neurodevelopmental damage following MeHg exposure.

Keywords

Methylmercury; neurodevelopment; systems biology; network; toxicity.

1. Introduction

Mercury (Hg) is released into the atmosphere mainly through natural processes (e.g., volcanic activity), but also human activity (e.g., industries waste and gold mining activities) [1]. Bacteria metabolize Hg into its organic derivative, methylmercury (MeHg), which is toxic, bioaccumulative, and has the ability to cross the hematoencephalic and placental barriers. Oceans and other aquifers are widely contaminated and it is already established that human exposure to MeHg derives primarily from fish consumption [2]. Therefore, seafood intake, which is highly recommended during pregnancy due to its high levels of omega-3 fatty acids [3], may actually harm the fetus to some extent. Few studies have warned of those effects, suggesting that pregnant women should only eat fish that are low in the food chain [4–6].

The first documented association of MeHg exposure with neurodevelopment dates from 1952. Children from a Swedish family presented intellectual and motor impairment without known cause. A thorough investigation was conducted and it was uncovered that the family had been using flour contaminated with MeHg [7]. In 1956, the first case of Minamata disease was diagnosed. This severe neurological syndrome affects both adults and fetuses and its symptoms include ataxia, numbness in hands and feet, general muscle weakness, and damage to the vision, hearing and speech. In extreme cases, the disease may also lead to death [8, 9]. Nevertheless, one could hypothesize that different MeHg doses may result in a spectrum of impairing symptoms and yet not be classified as Minamata disease. This has been shown to be true in some populations [10], where MeHg exposure was associated with neurological impairment, especially in children [11]. It is noteworthy that human contamination with MeHg is a worldwide phenomenon that occurs throughout the trophic chain [2]. Different populations are exposed to MeHg at several levels, wherein riverside communities are the most affected (e.g., Brazilian Amazon riverside population; [12, 13]).

Although MeHg toxicity has been extensively investigated [14–21], little is known regarding its molecular mechanisms. It is established that MeHg generates redox imbalance, mainly by decreasing GSH levels through the formation of GS-$HgCH_3$ complexes. Therefore, some of the consequences of MeHg contamination are macromolecule damage and cell death [22]. Indeed, studies have demonstrated elevated neuron loss due to exposure and the developing nervous system seems to be most susceptible to the MeHg toxic effects [11, 22–25]. Nevertheless, MeHg may entail redox imbalance due to its direct interaction with nucleophilic protein groups. Additionally, the disruption of protein pathways and networks might play the main role in MeHg neurodevelopment toxicity.

Systems biology approaches have proven to be of great value when analyzing large amounts of data [26–28]. Since MeHg toxicity is likely multifactorial [29], this kind of computational methodology may be fitting to investigate its biological mechanisms. A typical systems biology approach consists of protein-protein interaction (PPI) networks assembly. Network analysis deals with protein interaction at a systemic level by analyzing its emerging properties through methods derived from graph theory. Topological parameters enable us to infer which are the most important nodes for maintaining the network structure and connectivity [30]. The interaction of MeHg with target proteins or metabolic processes might enlighten us as to the mechanisms through which MeHg acts. In this sense, our objective was to apply a systems biology approach, analyzing PPI networks constructed from literature data. We aimed at finding target proteins, associated with particular processes, whose perturbation would probably rupture the network. An unstable network means that the biological system connectivity has been partially lost and its functionality is most likely impaired.

2. Material and methods

2.1. Systematic review and network assembly

In order to construct the PPI-network, a systematic review of the literature was performed. The search was conducted in the PubMed database under the syntax "(methylmercury OR MeHg) AND (neurodevelopment OR neurological development)". The studies collected from this search engine (n=182) provided an overview of the so far investigated proteins related to methylmercury and neurodevelopment. Every study that evaluated protein expression in mammals was included. A list of 169 proteins was extracted from those studies through manual textmining. There was no restriction on type of information extracted, such as proteomics, transcriptomics, physical interaction, and others. All 169 protein IDs were uploaded to the String-DB v.10.5 [31], the information retrieved was from *Homo sapiens* information, interactors number was turned up to 50, and the confidence level was set up to 0.7. The sources of known and predicted interaction used were "experimentally determined", "gene neighborhood", "curated databases", and "co-expression". Clusters not linked to the main network were excluded from the analysis.

2.2. Cluster analysis

Identifying clusters in a PPI-network allows the pinpointing of highly connected regions, which usually correspond to protein complexes or pathways. The cluster analysis was performed with the MCODE application [32] of Cytoscape v. 3.7.0 [30]. The parameters were set to default, in which networks are analyzed using scoring and parameters already optimized to an average network as described elsewhere [32].

2.3. Enrichment analysis

The functional annotation of the network leads to the comprehension of its biological background meaning. ClueGO and CluePedia are applications designed for cytoscape usage that perform the enrichment analysis from database information according to the genes encoding the proteins in a network [33, 34]. We aimed at identifying the cellular components, molecular functions, and biological processes associated with genes belonging to the network, according to Gene Ontology database [35]. Components and functions with at least 5 associated nodes, and processes with at least 20 associated nodes were included when p-values < 0.001 after Bonferroni step-down correction.

2.4. Centrality analyses

The topology of the network generated was later analyzed using Cytoscape v. 3.7.0. Topology parameters provide distinct metrics of nodes, edges or the network as a whole. For the purpose of our analysis, closeness centrality, betweenness centrality and degree of nodes were analyzed. These measures are classified by the European Bioinformatics Institute as follows: closeness centrality is a measure of how fast the flow of information travels through a given node to all nodes in the network; betweenness centrality is based on communication flow and measures how often a node occurs on all shortest paths between two nodes; finally, the degree parameter measures the number of edges connected to a node, in other words, a given node connectivity [30].

Degree and betweenness parameters were used to define hubs and bottlenecks as follows: hub-non-bottleneck (HNB) = p degree value > mean(network degree value); bottleneck-non-hub (BNH) = p betweenness value > mean(network betweenness value); hub-bottleneck (HB) = p degree value > mean(network degree value) AND p betweenness value > mean(network betweenness value); and non-hub-non-bottleneck (NHNB) = p degree value ≤ mean(network degree value) AND p betweenness value ≤ mean(network betweenness value, in which p = a given protein). The analysis of hubs and bottlenecks was performed in R, using stats package. Plots were constructed using both Cytoscape v 3.7.0 (cytoHubba application; [36]) and R package “ggplot2” [37]. A methodology workflow is provided in Fig. 1.

3. Results

String returned a PPI-network containing 178 nodes and 631 edges (interactions). The graph was reduced to 141 nodes and 540 interactions after the elimination of nodes and clusters disconnected from the main network (Fig. 2).

The clusterization revealed groups of proteins well related to each other. We were able to identify clusters of proteins associated with antioxidant and detoxifying activities (Fig. 3A); neurotransmitter receptors and transporters (Fig. 3B-E); cellular signaling cascades and cell cycle related proteins (Fig. 3F); proteins related to synaptic vesicle coupling, such as those belonging to the SNARE complex (Fig. 3G); and proteins related to other cellular response cascades (Fig. 3H)[38]. The enrichment analysis unraveled the cellular components (Fig. 4), molecular functions (Fig. 5), and biological processes (Fig. 6) with the largest number of associated genes. Tables containing the enrichment information per network node can be found in Online Resources 1-3.

In order to better understand which proteins are more likely maintaining the network structure, we analyzed its topology regarding some parameters. Proteins classified as hubs and/or bottlenecks are the ones most important to the network connectivity, between both nodes and clusters (Fig. 7). For the purpose of finding potential target proteins, we identified the ten proteins with the highest values of degree and closeness centrality, so that new subnetworks were assembled from these parameters (Fig. 8A-B). In order to refine the analysis, the intersection between the subnetworks was determined. The latter analysis revealed a group of five interacting proteins (Fig. 8C). Those five target proteins are associated with six main biological process groups, among which are: cell signaling/response, cell differentiation, MAPK cascade regulation and development. The enrichment analysis results based on biological processes are depicted in Fig. 9.

4. Discussion

4.1. Cluster analysis

Firstly, we investigated general parameters of the network through some graph theory methods. The cluster analysis revealed eight main groups of proteins (Fig. 3) that reflect their biological meaning, thus the network must be biologically meaningful, and not only randomly assembled.

4.1.1. Oxidative imbalance / detoxification process

Glutathione (GSH) is a tripeptide molecule initially described for its redox properties, due to the participation in the redox duality of glutathione disulfide (GSSG) and GSH [39]. Indeed, the GSH antioxidant properties have been associated with different metal poisoning agents, as copper, lead and iron [40–42], including mercury derivatives [22]. However, recent studies show that GSH plays many other roles in a cell [43, 44]. The distinct biological functions of GSH are dependent on the activity of certain enzymes, such as glutathione hydrolase proenzyme (GGT), glutathione S-transferase (GST), glutathione synthetase (GSS) and glutathione peroxidase (GPx) [45].

The first cluster (Fig. 3A) consists of proteins related to glutathione metabolism, such as subunits of GGT, GST, GSS, and GPx. This cluster is particularly interesting since methylmercury toxicity has been already associated with redox imbalance induction, mainly by decreasing GSH levels through the formation of GS-$HgCH_3$ complexes, associated with detoxification [22]. This process of detoxification causes GSH depletion, which in turn alters the activity of its interacting enzymes.

4.1.2. Neurotransmitters and receptors

Four clusters are composed of neurotransmitter related genes, mainly receptors, such as muscarinic acetylcholine receptors, alpha-2A adrenergic receptor, 5-hydroxytryptamine receptors, ephrin receptors, and the glutamate ionotropic receptor (Fig. 3B-E).

It has been already proposed that MeHg effects on specific neurotransmitter receptors may influence the susceptibility of granule cells. Results showed that MeHg inhibited $GABA_A$ receptors and stimulated muscarinic acetylcholine receptors [46]. Furthermore, a recent study evidenced lower neuron mortality by MeHg when co-exposed to dopamine. This protection was not accountable by dopamine metabolites, but dopamine receptors (D1 and D2) agonists also displayed protective effects [47]. Moreover, mainly protein members of the SNARE

complex compose one cluster (Fig 3G). This includes syntaxin, synaptotagmin, SNAP25 and other proteins related to vesicular anchoring and release. Therefore, it seems plausible that neurotransmitter related genes could play an important role in MeHg neurodevelopmental toxicity.

4.1.3. Cellular signaling / cycle

The last two clusters, depicted in Fig. 3F and 3H, are composed of proteins related to cell signaling and cycle. Studies have associated MeHg toxicity with signal transduction impairment among different pathways, such as AKT/CREB/BCL-2, MAPK, TRKA and PKA/CREB [48–51]. Furthermore, studies found cellular signaling pathways to be impaired in the cerebellum and hippocampus of developing rats exposed to MeHg *in utero* [15, 16]. Our analysis corroborates these results, suggesting signaling pathways may play the main role in developmental neurotoxicity induced by MeHg.

4.2. Network enrichment

Enrichment analysis of cellular components showed six different groups (Fig. 4). The largest group (group 6) corresponds to synaptic membrane related components, which was expected once we found several neurotransmitter related genes.

When molecular functions were analyzed, four different groups were created (Fig. 5). The largest group is of calcium related transport activity. Calcium channel activity has been extensively studied regarding MeHg exposure and studies demonstrate that there is an interaction that may influence toxicity [52–54].

The third enrichment analysis evaluated biological processes associated with the network (Fig. 6). The analysis revealed 12 associated groups, in which the largest (group 12) corresponds to secretion, exocytosis and cell signaling. Additionally, there are two other large groups associated with phosphatase activity and cellular apoptotic signaling (groups 11 and 10, respectively). This is interesting, although already expected, once several proteins of the network are associated with cell signaling and cycle. Moreover, these processes seem to be the most prominent in the network and may be especially important to the network structure maintenance.

4.3. Topology analyses

The topological parameters allowed the classification of proteins as HB, HNB, HNB and NHNB. The proteins whose names are depicted in Fig. 7 are those classified as HB. As

these nodes make more connections and are more important in maintaining communication between clusters, we hypothesize those could be target proteins to be investigated. The five proteins with higher degree and betweenness centrality values are RAC1, MAPK1, MAPK3, AKT, and CDC42.

A second approach was conducted, in which proteins with higher values (top 10) of closeness centrality and degree were picked. Although closeness centrality parameter is dependent on the degree value, it represents a different feature of the network, which gives a sense of communication flow speed. We found this parameter rather important and were interested in evaluating if its top 10 shared nodes with the degree top 10. In order to evaluate this, the intersection between top 10 subnetworks was extracted. We found that the intersection between parameters was composed of the very same top five HB proteins (Fig. 8).

A secondary enrichment analysis of biological processes was carried out including only the latter set of proteins (Fig. 9). The analysis demonstrated those proteins are mainly associated with cellular signaling pathways (Group 6 and 5 on Fig. 9C). This highly agrees with our previous findings regarding cluster analysis and enrichment of the whole network. Our results also corroborate previous studies, in which developing rats treated with MeHg *in utero* showed impairment of signaling pathways [15, 16].

4.4. Concluding remarks

In summary, our results suggest the most important process associated with MeHg *in utero* exposure and neurodevelopment is cellular signaling. In addition, not only this is the most prominent process in the main network, but also the target proteins found (RAC1, CDC42, AKT1, MAPK3, MAPK1) are included in this very same group. We propose future studies should prioritize the investigation of cell cycle and signaling proteins. Hopefully, our findings may help focusing research and developing new treatment or prophylactic measures to avoid neurodevelopmental damage following MeHg exposure. It is noteworthy that studies addressing MeHg exposure during development are scarce and new analyses would be beneficial should greater amounts of data be gathered.

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CONSIDERAÇÕES FINAIS

Esse é o primeiro estudo explorando a exposição à metilmercúrio através de uma abordagem de biologia de sistemas. Nossos resultados apontam para uma participação importante de proteínas relacionadas à sinalização celular na manutenção do sistema biológico. Dessa forma, recomendamos que estudos subsequentes foquem na investigação de tais proteínas e vias metabólicas a fim de elucidar os mecanismos através dos quais o MeHg age. Acreditamos que a partir da elucidação dos mecanismos envolvidos, esses resultados ajudarão no desenvolvimento de novos tratamentos e medidas profiláticas para evitar prejuízos causados pela intoxicação durante o desenvolvimento.

É importante destacar algumas limitações do estudo. Esse tipo de abordagem é dependente da quantidade e qualidade de informação depositada em bancos de dados de interação, bem como do número de estudos existentes na área.

Perspectivas

A partir dos resultados encontrados, identificamos que a sinalização celular é uma via chave na interação de proteínas relacionadas à toxicidade do MeHg e neurodesenvolvimento. A sinalização celular é um processo abrangente, em que essas proteínas interagem e regulam muitas outras. Nesse sentido, pequenas modificações no seu funcionamento (sutil inibição ou ativação, alteração na expressão ou metilação) podem se tornar alterações expressivas em uma cascata de ativação ou rede dentro da qual essas proteínas estão presentes. Pensando nisso, confirmaremos alguns dos nossos resultados *in vivo*. Ratos Wistar serão expostos a doses baixas de metilmercúrio durante o desenvolvimento. O tratamento será feito em ratas prenhas durante a gestação e amamentação dos filhotes. No vigésimo dia pós-natal os filhotes serão eutanasiados e os diferentes órgãos separados para testes específicos. Pretendemos avaliar a expressão e padrão de metilação dos genes citados em cortex, hipocampo e cerebelo. Dessa forma, podemos elucidar como a exposição a MeHg altera esses padrões quando administrado em doses correspondentes às ambientais durante o período de neurodesenvolvimento, mimetizando a exposição humana.

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ANEXO 1

Alana Castro Panzenhagen[1], Helen Tais da Rosa-Silva[1], Lucas Santos[1], Nauana Somensi[1], Daniel Pens Gelain[1], José Cláudio Fonseca Moreira[1]

FIGURES

METHYLMERCURY AND NEURODEVELOPMENT: A SYSTEMS BIOLOGY APPROACH

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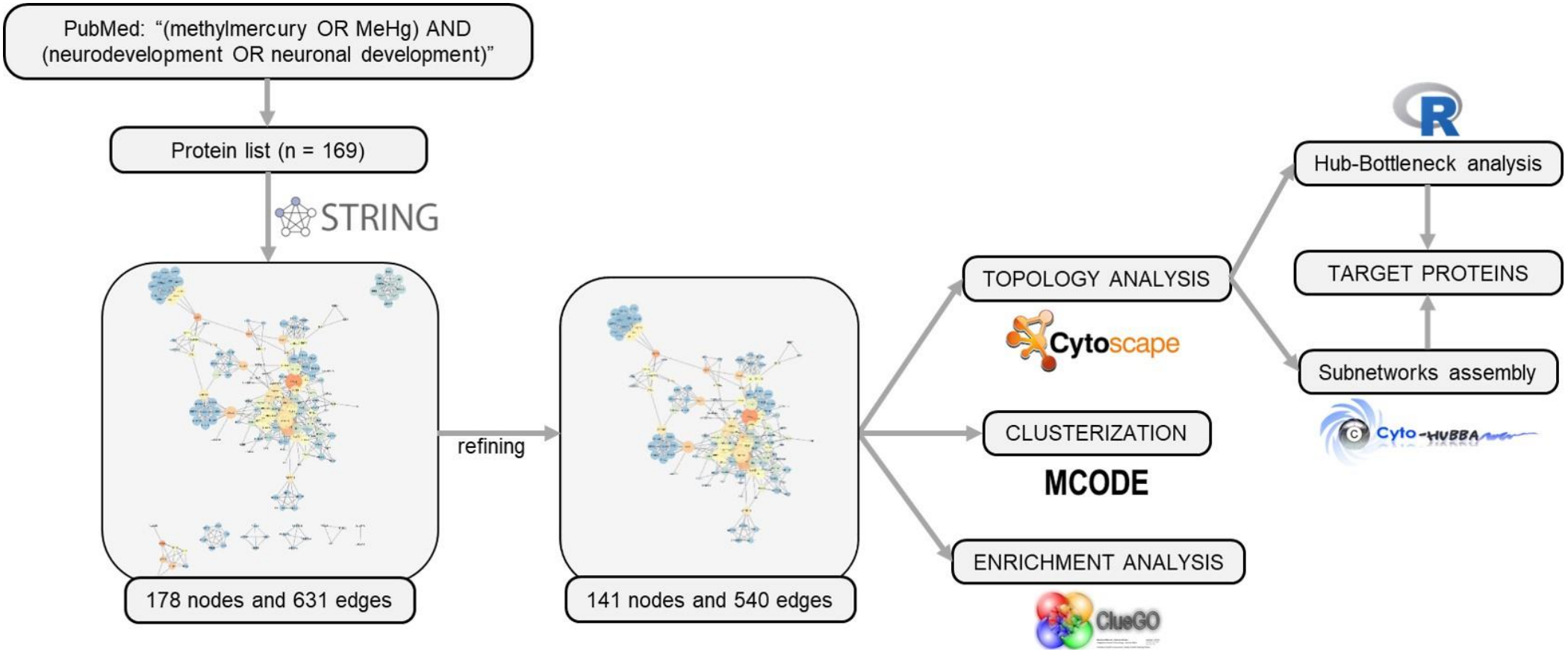


Fig. 1 Methodology workflow

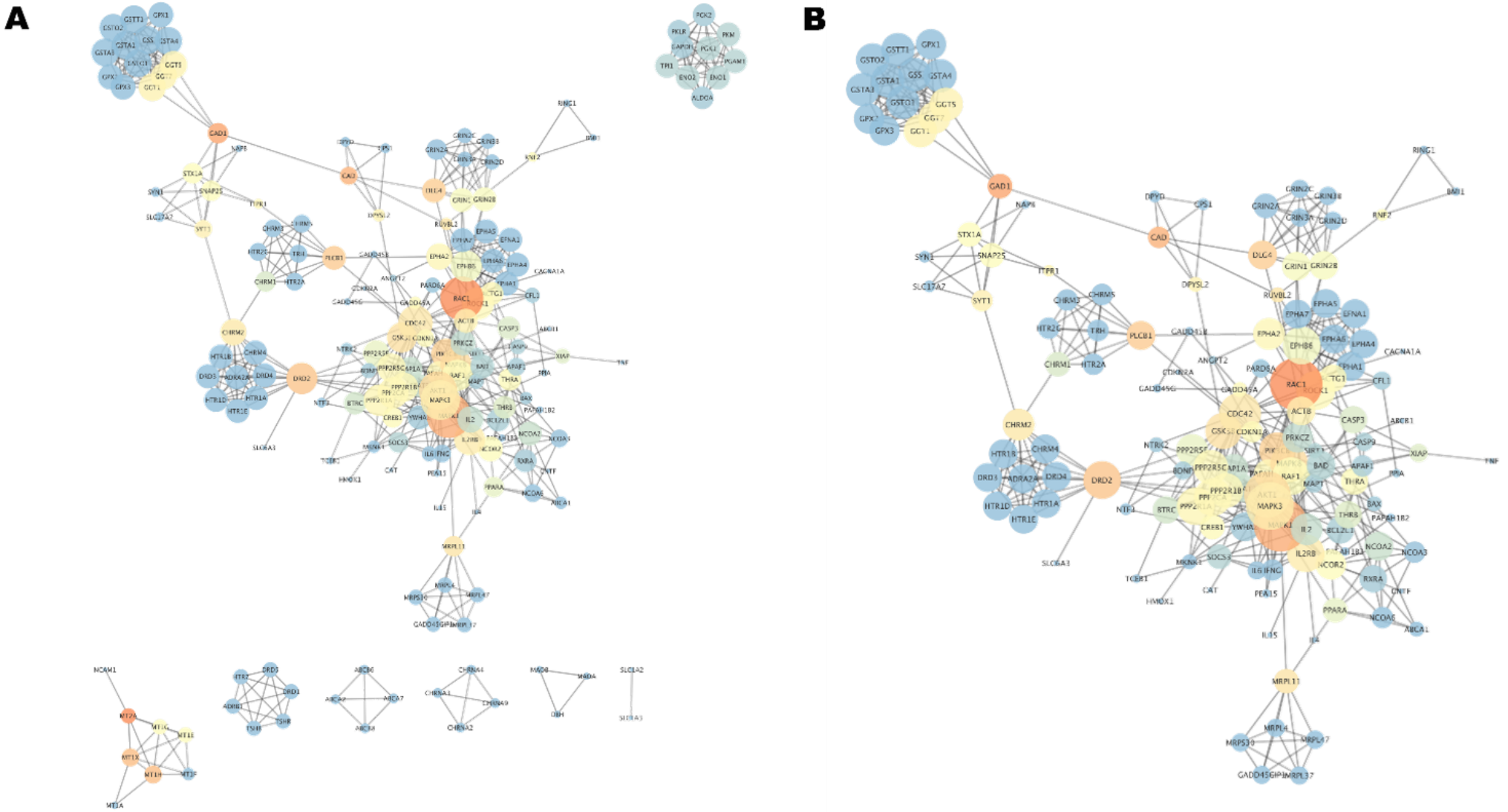


Fig. 2 Protein-protein interaction (PPI) network graphs A) Protein-protein interaction (PPI) network graph with 178 nodes and 631 interactions. B) Protein-protein interaction (PPI) network graph with 141 nodes and 540 interactions after the elimination of nodes and clusters disconnected from the main network. Node size represents the degree parameter (large nodes = high degree value). Colors represent the betweenness parameter (red = high betweenness centrality, blue = low betweenness centrality)

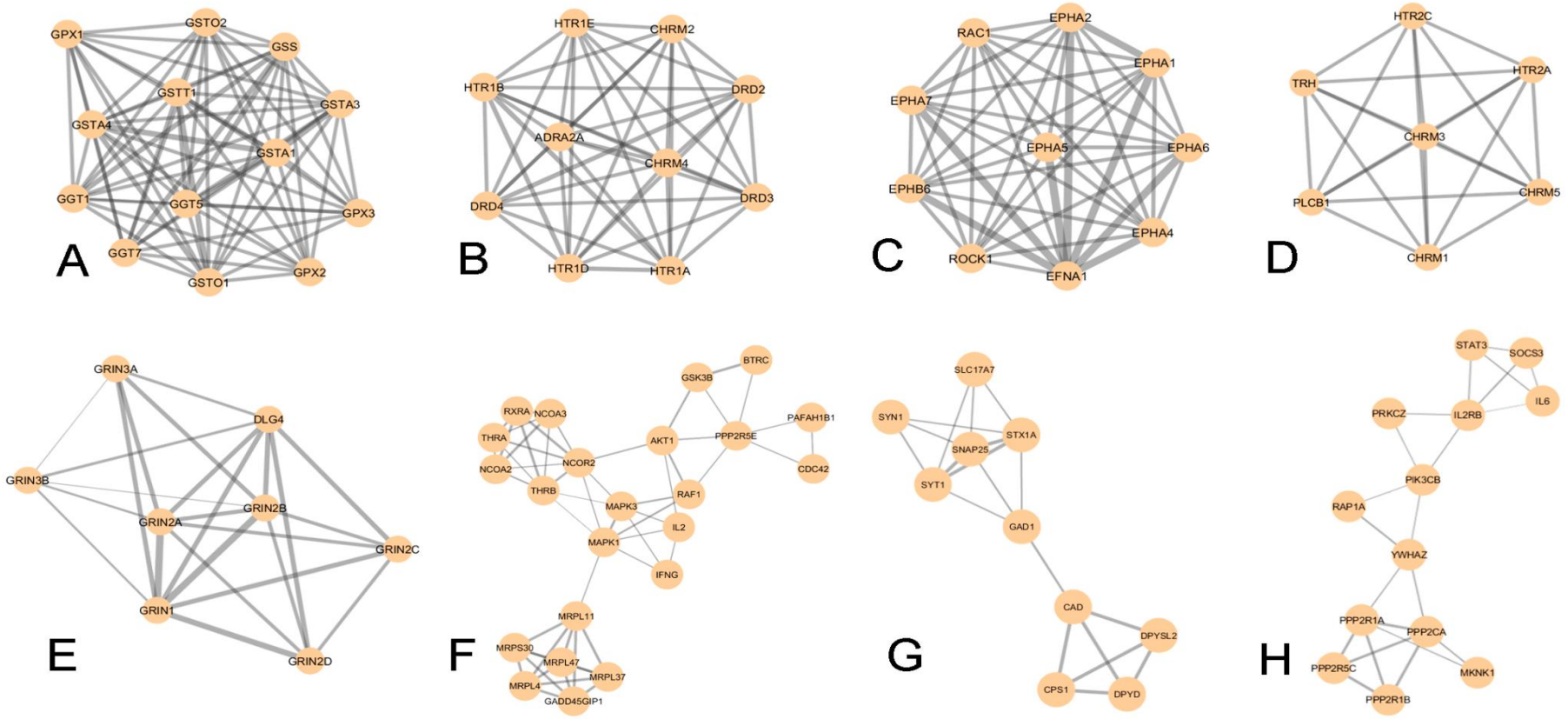


Fig. 3 Clusters of proteins identified by Cytoscape MCODE application. A) Cluster of proteins associated with antioxidant and detoxifying activities. B-E) Cluster of neurotransmitter receptors and transporters. F) Cellular signaling cascades and cell cycle related proteins. G) Cluster of proteins related to synaptic vesicle coupling, such as those belonging to the SNARE complex. H) Cluster of proteins related to other cellular response cascades

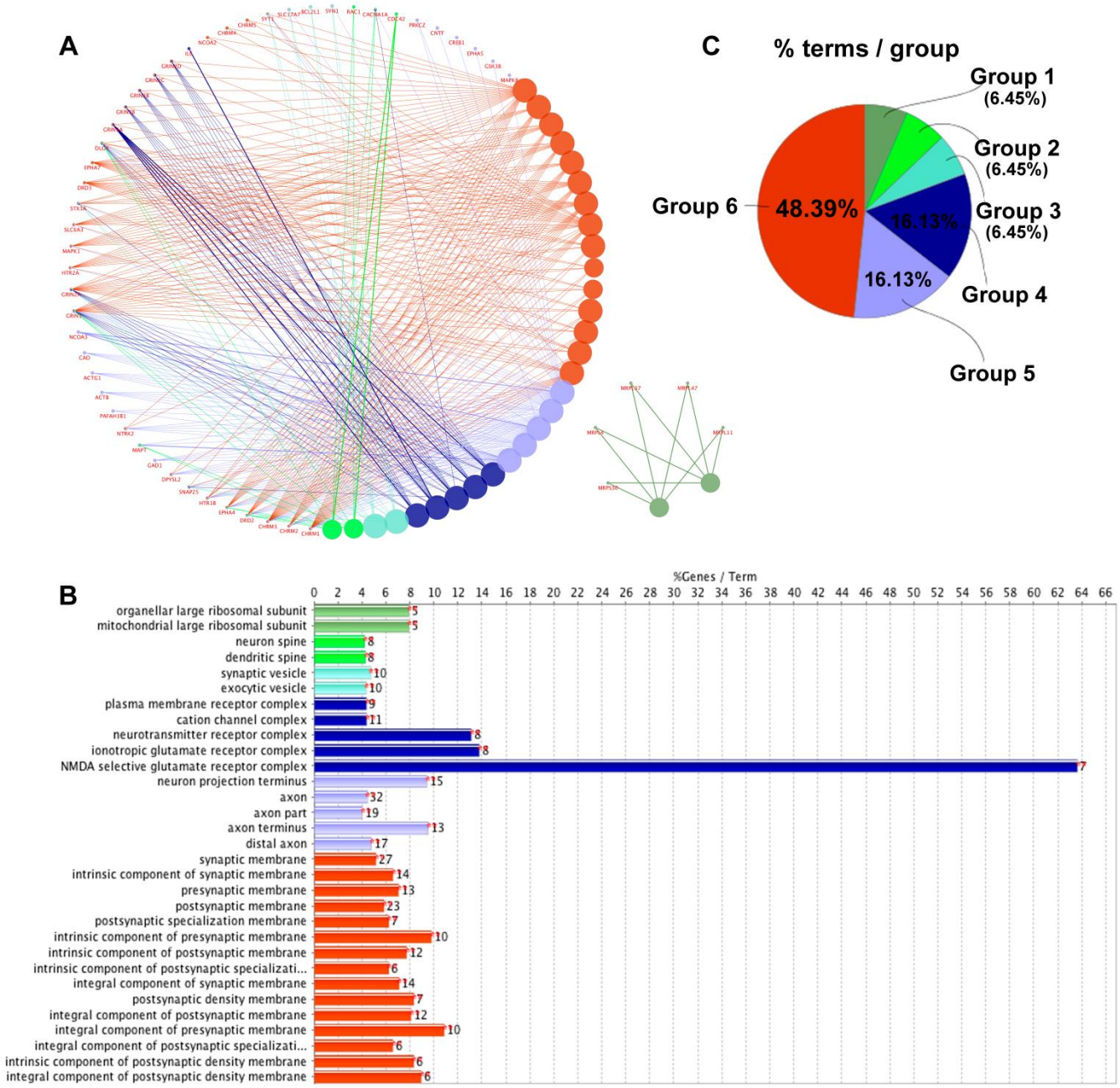


Fig. 4 Enrichment analysis of cellular components grouped using the Cytoscape ClueGO application. A) Network of enriched proteins and associated cellular components. B) Percentage of genes associated to each term. C) Percentage of terms per group. Colors = Gene ontology groups

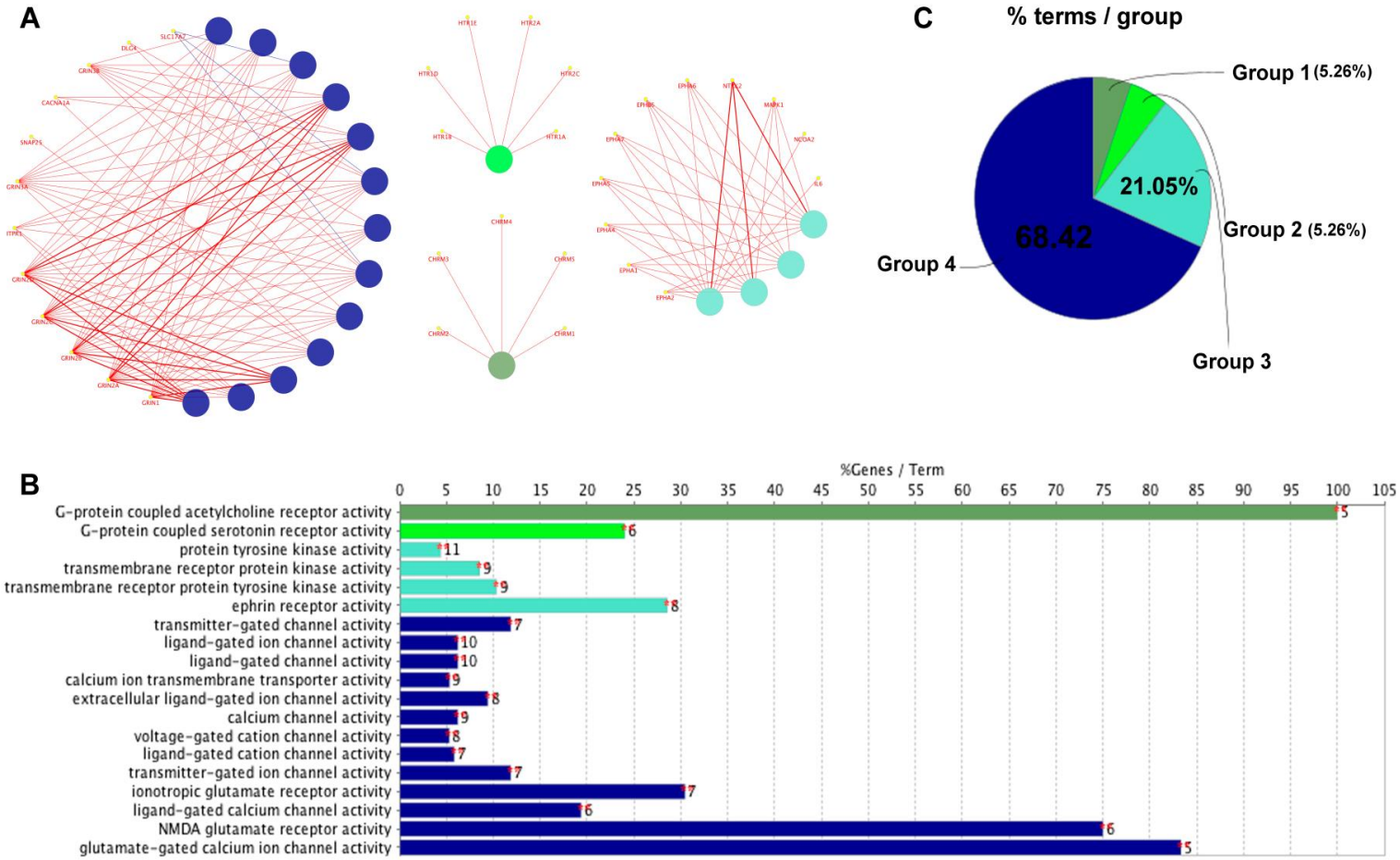
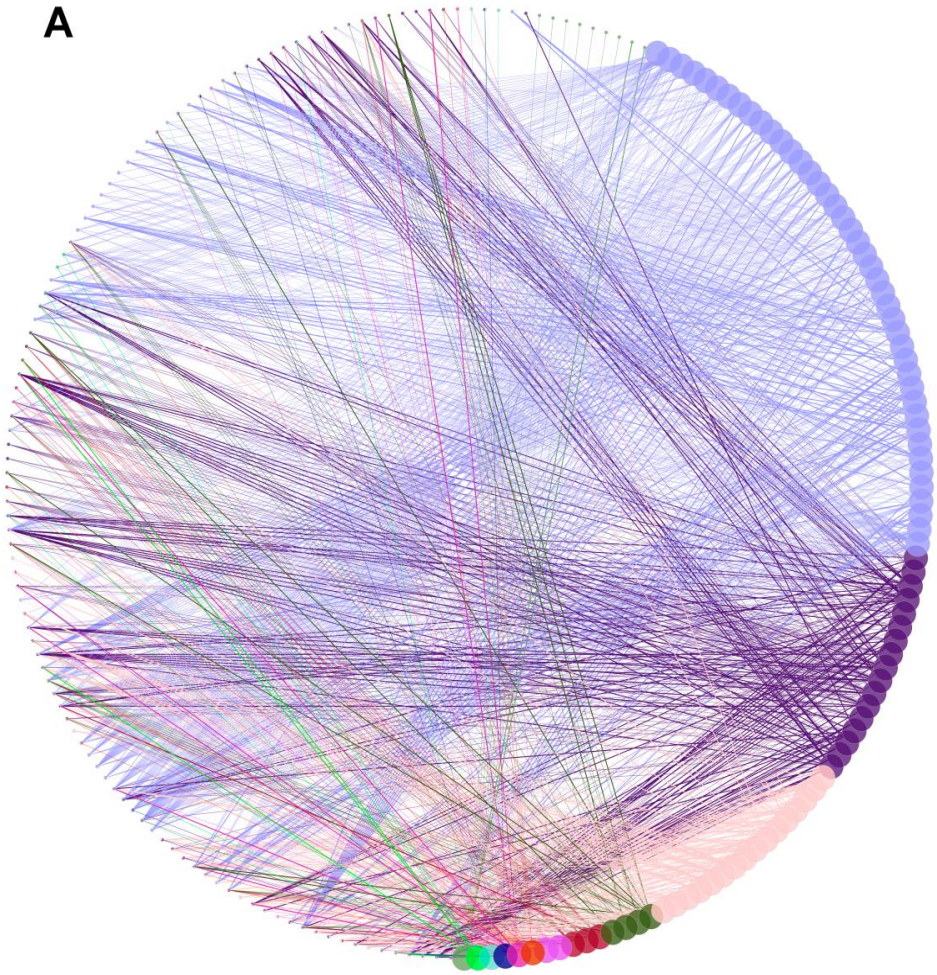
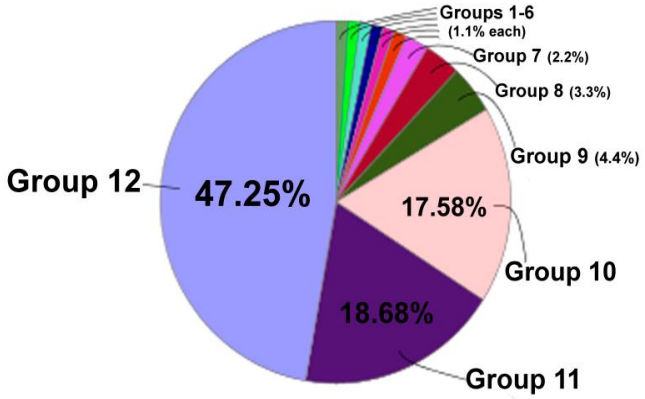


Fig. 5 Enrichment analysis of molecular functions grouped using the Cytoscape ClueGO application. A) Network of enriched proteins and associated molecular functions. B) Percentage of genes associated to each term. C) Percentage of terms per group. Colors = Gene ontology groups


A


B
% terms / group
Groups 1-6
(1.1% each)
Group 7 (2.2%)
Group 8 (3.3%)
Group 9 (4.4%)
Group 10
Group 11
Group 12
47.25%
17.58%
18.68%


C
%Genes / Term
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
glutathione metabolic process 11
regulation of endocytosis 14
mammary gland development 10
response to corticosteroid 10
negative regulation of phosphorylation 26
ephrin receptor signaling pathway 17
platelet activation 10
Fc receptor signaling pathway 10
negative regulation of neuron death 16
regulation of neuron apoptotic process 16
negative regulation of neuron apoptotic process 10
regulation of cell morphogenesis involved in differentiation 16
dendrite development 13
dendrite morphogenesis 10
regulation of axonogenesis 12
positive regulation of cellular protein localization 18
extrinsic apoptotic signaling pathway 17
positive regulation of neuron death 10
positive regulation of protein localization to membrane 10
regulation of mitochondrion organization 11
extrinsic apoptotic signaling pathway in absence of ligand 11
regulation of protein localization to cell periphery 10
regulation of protein localization to membrane 14
positive regulation of apoptotic signaling pathway 11
positive regulation of proteolysis 21
regulation of extrinsic apoptotic signaling pathway 11
positive regulation of peptidase activity 16
regulation of cysteine-type endopeptidase activity involv... 15
regulation of cysteine-type endopeptidase activity 18
positive regulation of endopeptidase activity 15
positive regulation of cysteine-type endopeptidase activity 12
inositol lipid-mediated signaling 13
positive regulation of leukocyte cell-cell adhesion 12
phosphatidylinositol-mediated signaling 13
positive regulation of DNA metabolic process 12
positive regulation of T cell activation 11
phosphatidylinositol 3-kinase signaling 13
regulation of phosphatidylinositol 3-kinase signaling 10
peptidyl-tyrosine modification 28
positive regulation of kinase activity 32
peptidyl-serine phosphorylation 17
peptidyl-tyrosine phosphorylation 28
positive regulation of protein kinase activity 28
regulation of peptidyl-tyrosine phosphorylation 20
positive regulation of MAP kinase activity 16
positive regulation of peptidyl-tyrosine phosphorylation 16
activation of protein kinase activity 18
positive regulation of protein serine/threonine kina... 20
signal release 24
memory 10
negative regulation of ion transport 10
regulation of trans-synaptic signaling 29
G-protein coupled receptor signaling pathway, coupled ... 14
glutamate receptor signaling pathway 11
second-messenger-mediated signaling 19
regulation of amine transport 11
hormone secretion 17
signal release from synapse 12
ammonium transport 10
regulation of exocytosis 16
regulation of hormone secretion 15
synaptic vesicle transport 13
establishment of synaptic vesicle localization 13
anterograde trans-synaptic signaling 42
adenylate cyclase-modulating G-protein coupled recept... 14
neurotransmitter secretion 12
calcium-mediated signaling 12
modulation of chemical synaptic transmission 29
positive regulation of synaptic transmission 14
catecholamine transport 11
regulation of blood vessel diameter 11
chemical synaptic transmission, postsynaptic 11
regulation of synaptic vesicle transport 10
regulation of synaptic plasticity 15
chemical synaptic transmission 42
peptide hormone secretion 15
regulation of neurotransmitter secretion 11
regulation of peptide hormone secretion 13
regulation of regulated secretory pathway 14
calcium ion regulated exocytosis 13
insulin secretion 13
adenylate cyclase-inhibiting G-protein coupled recept... 15
synaptic vesicle exocytosis 12
excitatory postsynaptic potential 10
regulation of calcium ion-dependent exocytosis 12
regulation of insulin secretion 12
regulation of synaptic vesicle exocytosis 10
calcium ion transmembrane transport 17
calcium ion transport into cytosol 12
cytosolic calcium ion transport 12
calcium ion transmembrane import into cytosol 12

Fig. 6 Enrichment analysis of biological processes grouped using the Cytoscape ClueGO application. A) Network of enriched proteins and associated biological processes. B) Percentage of genes associated to each term. C) Percentage of terms per group. Colors = Gene ontology groups

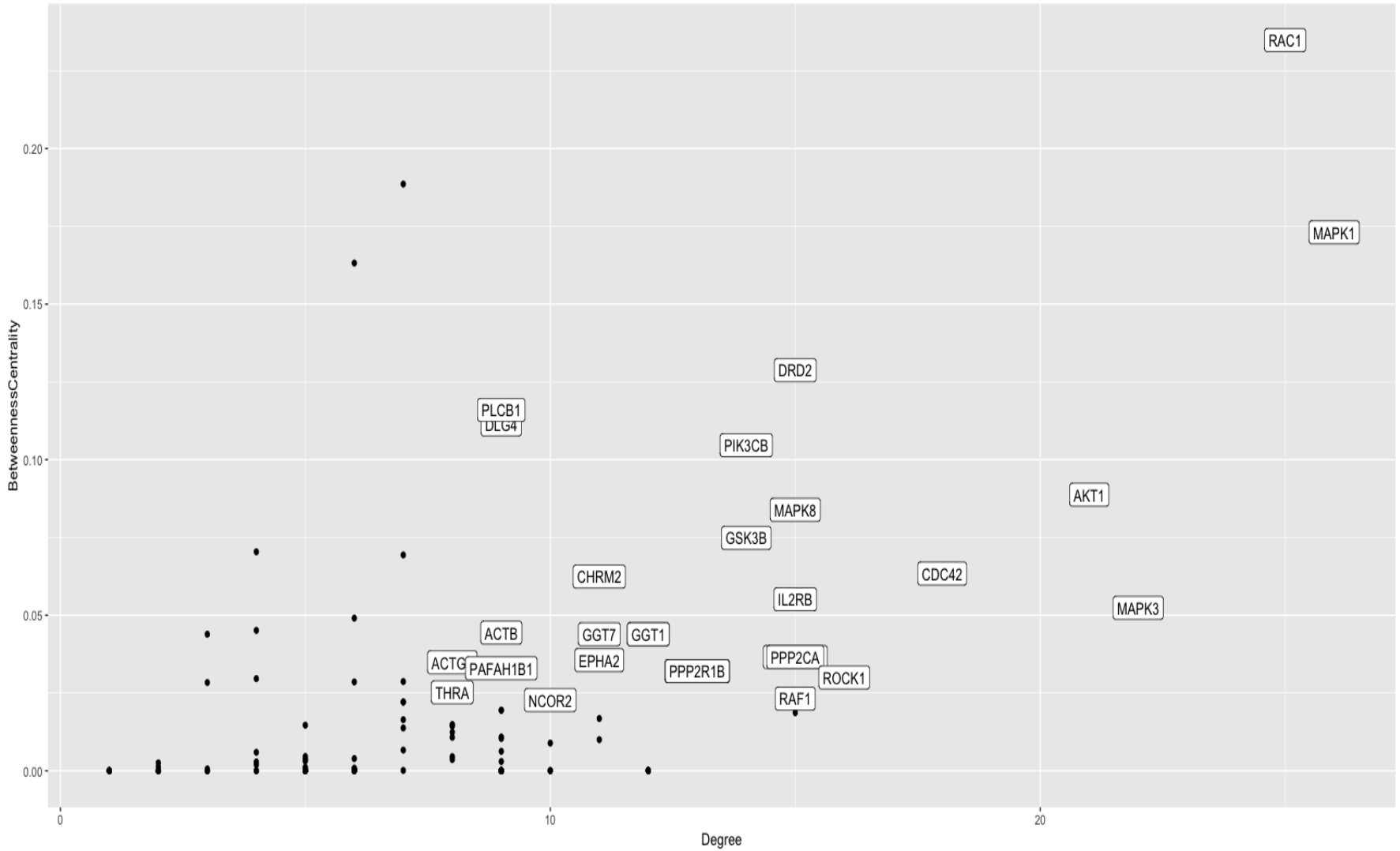


Fig. 7 Proteins classified by degree (x axis) and betweenness centrality (y axis). Proteins with names depicted in the graph are those with both degree and betweenness values above the network mean (hub-bottlenecks)

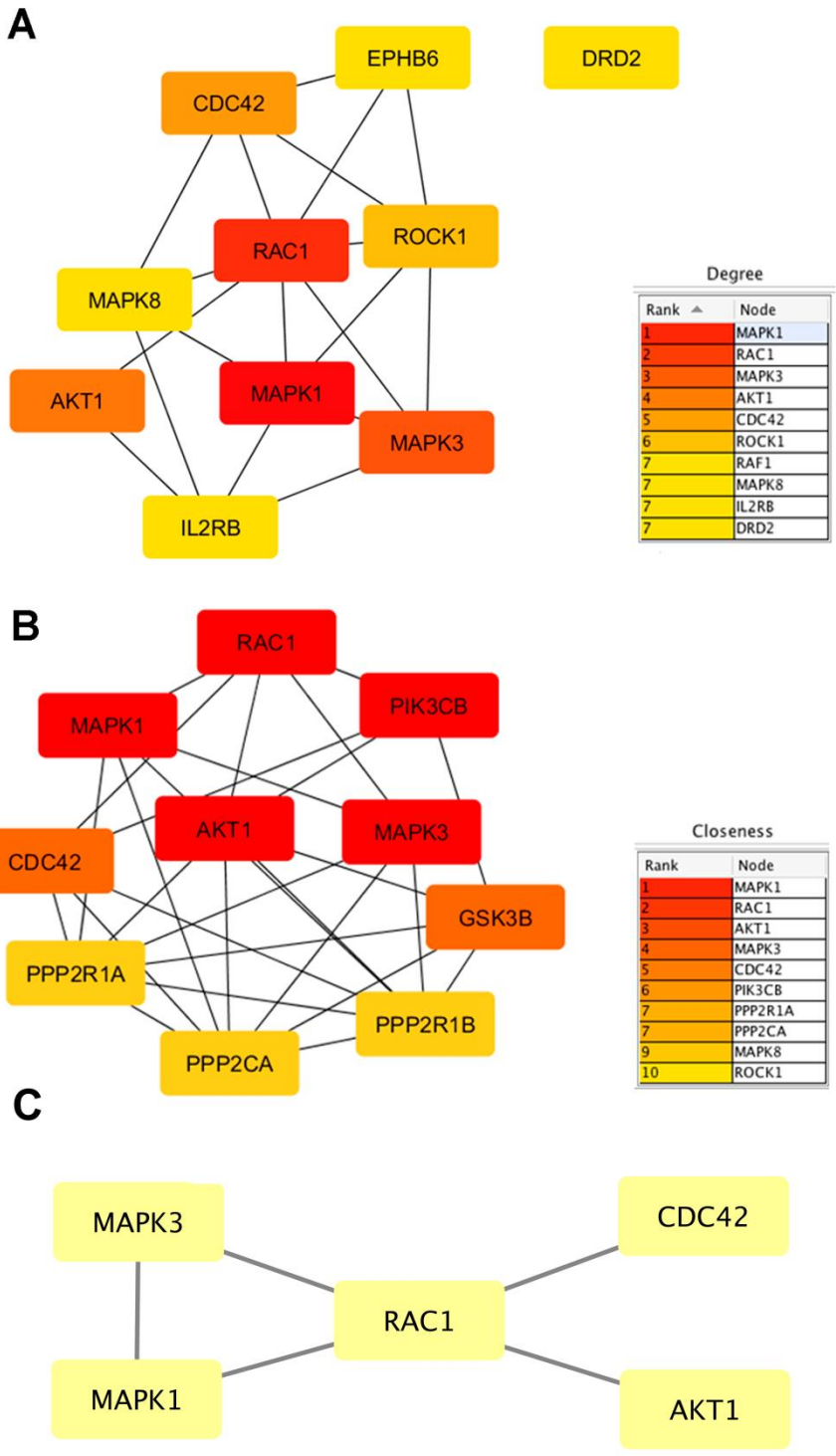


Fig. 8 CytoHubba analyses. A) The ten proteins with the highest values of degree represented in a subnetwork. Red = high degree value. B) The ten proteins with the highest values of closeness centrality represented in a subnetwork. Red = high closeness centrality value. C) The five interacting proteins in the intersection between the degree (A) and closeness centrality (B) subnetworks

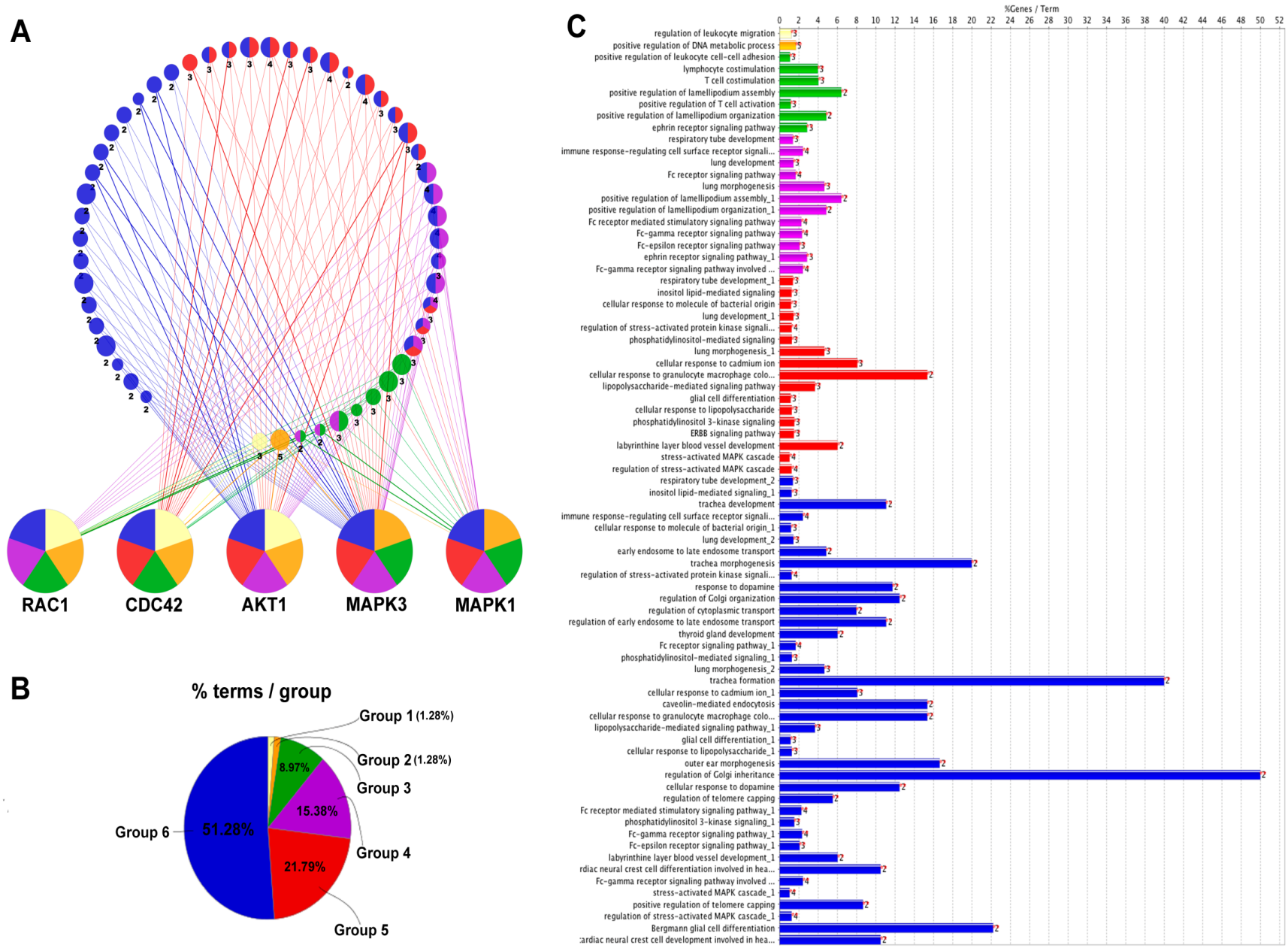


Fig. 9 Biological processes enrichment analysis of the five target proteins (RAC1, CDC42, AKT1, MAPK3 and MAPK1) grouped using the Cytoscape ClueGO application. A) Network of enriched proteins and associated biological processes. B) Percentage of terms per group. C) Percentage of genes associated to each term. Colors = Gene ontology groups