UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE BIOCIÊNCIAS

COMISSÃO DE GRADUAÇÃO DO CURSO DE BIOTECNOLOGIA

TRABALHO DE CONCLUSÃO DE CURSO

Análises comparativas *in silico* de possíveis funções *moonlighting* da enzima glicolítica enolase em interatomas de quatro espécies

Gabriela Prado Paludo

Porto Alegre, julho de 2014

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE BIOCIÊNCIAS
COMISSÃO DE GRADUAÇÃO DO CURSO DE BIOTECNOLOGIA
TRABALHO DE CONCLUSÃO DE CURSO

Análises comparativas *in silico* de possíveis funções *moonlighting* da enzima glicolítica enolase em interatomas de quatro espécies

Gabriela Prado Paludo

Trabalho de Conclusão de Curso submetido à COMGRAD do curso de Biotecnologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do título de Bacharel em Biotecnologia com Habilitação em Bioinformática.

Orientador: Dr. Henrique B. Ferreira

Porto Alegre, junho de 2014

Este trabalho foi realizado no Laboratório de Genômica Funcional e Estrutural do Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul e contou com o apoio financeiro da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Agradecimentos

Ao Dr. Henrique Bunselmeyer Ferreira, pela oportunidade de realizar a minha iniciação científica no laboratório de Genômica Funcional e Estrutural, pela excelente orientação, pela amizade e confiaça depositada em mim para a realização deste trabalho.

Ao Dr. Diego Bonatto, pela minha iniciação na biologia de sistemas e orientação neste trabalho.

À MSc Karina Rodrigues Lorenzatto, por todos os ensinamentos, incentivos e amizade nestes últimos 4 anos. E pelos conselhos feitos para que este trabalho se desenvolvesse da melhor forma possível.

A todos os colegas dos laboratórios 210, 206 e 204 pela amizade e apoio.

Aos meus colegas de curso e amigos Carolina, Fábio e Monique pelos cafés e longas discussões sobre a Biotecnologia.

À minha família, pelo apoio e incentivo em todos os momentos.

Ao Guilherme pelo apoio e entusiasmo que sempre demonstrou pelo meu trabalho (mesmo sem saber exatamente o que era).

A todos os professores que tive durante a graduação pelos ensinamentos.

Sumário

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

EgEno1: enolase 1 de *E. granulosus*

ES: excreção-secreção

FDR (de *false discovery rate*): frequência de descobertas falsas

GO (de *gene ontology*): ontologia gênica

KOG (de *eukaryotic orthologous groups*): grupos de ortólogos eucarióticos

LC-ESI-MS/MS (de liquid chromatography-electrospray ionization/multi-stage mass spectrometry): cromatografia líquida acoplada a uma fonte de ionização por *Electrospray tandem* espesctroscopia de Massas

NCBI: *National Center for Biotechnology Information*

PPI (de *protein-protein interaction*): interação proteína-proteína

LISTA DE FIGURAS

CAPÍTULO 1

LISTA DE TABELAS

CAPÍTULO 1

CAPÍTULO 2

RESUMO

Enzimas glicolíticas, como a enolase, têm sido descritas como proteínas multifuncionais complexas, podendo desempenhar funções não glicolíticas, ditas *moonlighting*. Porém, pouco se sabe sobre estas funções, especialmente em parasitos. Em *Echinococcus granulosus*, o agente causador da hidatidose cística, uma isoforma da enolase (EgEno1) está entre as proteínas intracelulares detectadas nos produtos de excreção/secreção e entre componentes de interface parasito-hospedeiro. Estas localizações ectópicas são indicativas de que a EgEno1 poderia estar desempenhando funções *moonlighting*, tornando esta proteína um atraente alvo para estudos. Em *Homo sapiens*, a enolase α é a isoforma predominantemente expressa em todo o organismo e é a ortóloga da EgEno1. Além de sua atividade catalítica clássica, a enolase α tem sido descrita em outros processos funcionais como modulação imunológica e na sensibilidade de células tumorais à drogas. Embora a multifuncionalidade da enolase seja cada vez mais evidente, ainda não está claro se estas funções são evolutivamente conservadas ou não, sendo esse o alvo deste estudo. Uma das estratégias que tem sido utilizada para investigar funções *moonlighting* é o uso de ferramentas de biologia de sistemas, as quais permitem a predição de funções/interações de proteínas através do estudo de redes biológicas. Assim, redes de interação proteína-proteína (PPI) foram projetadas com a ferramenta STRING para *H. sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans* e *Saccharomyces cerevisiae*. As análises das redes foram realizadas no *software* Cytoscape (versão 2.8.2) através dos *plug-ins*: AllegroMCODE, para análises de modularidade; CentiScaPe, para análises de centralidade; e BiNGO, para o enriquecimento funcional. As proteínas de interação com a enolase, comuns a todas as redes projetadas, foram identificadas e classificadas funcionalmente conforme os termos do banco de dados Gene Ontology usando a ferramenta Blast2GO. Anotações do Eukaryotic Orthologous Groups (KOG) foram também atribuídas às proteínas identificadas com base em buscas por similaridade. Adicionalmente, dados de proteínas de interação com a EgEno1 em *E. granulosus* foram comparados aos dados das redes de PPI. Foram identificadas 14 proteínas de interação com a EgEno1 em *E. granulosus*, das quais apenas duas preditas nas redes de PPI. As demais, sem predição in silico, sugerem funções *moonlighting* específicas da EgEno1 que podem estar associadas a interações parasito-hospedeiro.

ABSTRACT

Glycolytic enzymes, such as enolase, have been described as complex multifunctional proteins that may perform non-glycolytic moonlighting functions. However, little is known about such functions, specially in parasites. In *Echinococcus granulosus*, the causative agent of cystic hydatid disease, an enolase isoform (EgEno1) is among the intracellular proteins also detected as excretory-secretory products and in components of parasite-host interface. These ectopic localizations are indicative of EgEno1 possible moonlighting functions, making this protein an attractive target for study. In *Homo sapiens*, α-enolase is the predominantly expressed isoform throughout the body and is the orthologue of EgEno1. In addition to its classic catalytic activity, α-enolase roles have been described in other processes, such as immune modulation and tumor cell sensitivity to drugs. Although enolase multifunctionality is increasingly evident, it is still unclear whether these functions are evolutionarily conserved or not, which is the aim of this study. One of the strategies that have been used to investigate moonlighting functions is the use of systems biology tools, which allow the prediction of protein functions/interactions based on the study of biological networks. Thus, protein-protein interaction (PPI) networks have been designed with the STRING tool for: *H. sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae* Network analyses were performed by Cytoscape software (version 2.8.2), by its plugins: plugins AllegroMCODE, modularity analyses; CentiScaPe, centrality analyses; and BiNGO, functional enrichment. The proteins that interact with enolase, common to all designed networks, were identified and functionally classified according to the terms of the Gene Ontology database using the tool Blast2GO. Annotations of Eukaryotic Orthologous Groups (KOG) were also attributed to proteins identified based on similarity searches. Additionally, data from EgEno1 interacting proteins in *E. granulosus* were compared to data of PPI networks. We identified 14 *E. granulosus* proteins that interact with EgEno1, with only two of them predicted in the PPI networks. The other, with no *in silico* prediction, are suggestive of EgEno1 specific moonlighting functions that may be associated with host-parasite interactions.

1. INTRODUÇÃO

1.1. O *Echinococcus granulosus* e a hidatidose cística

A fase larval (cisto hidático ou metacestódeo) de *Echinococcus granulosus* (Platyhelminthes, Cestoda) é o agente etiológico da hidatidose (ou equinococcose) cística, uma parasitose endêmica ou hiperendêmica para seres humanos e ungulados domésticos (especialmente bovinos e ovinos) (Moro & Schantz, 2009). O cisto hidático desenvolve-se predominantemente no fígado ou pulmões dos hospedeiros intermediários. Ao longo da infecção, o cisto cresce, ocasionando lesões císticas crônicas que podem persistir muitos anos, causando sintomas variados, dependendo de seu tamanho e localização.

A hidatidose cística representa um problema sério de saúde pública humana e animal em todo mundo, com significativo impacto sócio-econômico. Ela é considerada pela Organização Mundial da Saúde como uma doença negligenciada (http://www.who.int/neglected_diseases/diseases/en/) (Torgerson, 2003; Moro *et al.*, 2011), apesar do reconhecimento de seu caráter de zoonose cosmopolita emergente/re-emergente em nível mundial (Moro & Schantz, 2009; Mandal & Mandal, 2012). Levantamentos recentes demonstram que a hidatidose cística já causa prejuízos anuais da ordem de US$ 760 milhões, em gastos no tratamento de pacientes humanos, e de mais de US$ 2 bilhões, associados à diminuição de rendimento na pecuária (Budke, 2006, Battelli, 2009, Benner *et al.,* 2010). Na América do Sul, a hidatidose é endêmica ou hiperendêmica no Cone Sul e na região andina (de la Rue, 2008; de la Rue *et* al., 2011; Moro *et al*., 2011).

A inespecificidade dos sintomas e dos métodos físicos usualmente disponíveis para a detecção de cistos hidáticos (radiologia e ultrassonografia) dificulta o diagnóstico clínico da hidatidose cística humana (Mandal & Mandal, 2012). Também a caracterização limitada do repertório antigênico do parasito limita o desenvolvimento de vacinas, embora algumas proteínas com potencial para utilização em vacinação contra a hidatidose cística ou contra a hidatidose

alveolar (causada por *Echinococcus multilocularis*) já tenham sido identificadas (Dang *et al*., 2009; Jabbar *et al*., 2011).

Poucas proteínas de *E. granulosus* têm sido caracterizadas além da determinação de seus potenciais antigênico ou imunogênico. Dentre as proteínas do parasito que já tiveram pelo menos algum grau adicional de caracterização funcional podem ser citadas a malato-desidrogenase citosólica (Ferreira & Zaha, 1994), uma proteína de fragmentação de filamentos de actina (AFFP) da família da gelsolina (Cortez-Herrera *et al*., 2001; Grimm *et al*., 2006) e proteínas 14-3-3 (Siles-Lucas *et al*., 2001; Nunes *et al*., 2004). Há, contudo, uma carência de mais estudos para que estas e outras proteínas tenham seus papéis na fisiologia do parasito melhor esclarecidos, especialmente no que diz respeito ao potencial envolvimento em processos de interação parasito-hospedeiro. Neste sentido, nosso grupo de pesquisa vem investindo na caracterização do repertório de proteínas expressas pela fase larval patogênica do *E. granulosus* (cisto hidático) (Monteiro *et al*., 2010), com ênfase na identificação de proteínas de superfície ou presentes entre os produtos de ES do parasito no líquido hidático. Com isso, já foram identificadas diversas proteínas interessantes para investigação funcional mais aprofundada, dentre as quais se destacam, enzimas glicolíticas com possíveis funções *moonlighting*.

1.2. Funções *moonlighting*

Proteínas *moonlighting* exibem atividades funcionais, dentro ou fora da célula, não relacionadas à sua função metabólica descrita (Copley, 2012). Alguns dos primeiros exemplos de proteínas *moonlighting* a serem descritos foram os de certas cristalinas, proteínas estruturais presentes nos cristalinos dos olhos de vertebrados, que são, na verdade, enzimas bem conhecidas. Por exemplo, a ε-cristalina de patos é uma lactato-desidrogenase (Hendriks *et al*., 1988) e a τ-cristalina de tartarugas é a enzima glicolítica α-enolase (Wistow *et al*., 1988). Desde então, muitas outras proteínas *moonlighting* têm sido descobertas (Huberts *et al*., 2010b; Copley, 2012).

As proteínas *moonlighting* conhecidas utilizam diversos mecanismos para troca de funções (Jeffery, 2009). Algumas proteínas podem ter funções diferentes quando expressas em diferentes locais no interior da célula. Por exemplo, algumas proteínas citoplasmáticas são também encontradas na superfície celular, onde podem funcionar como receptores (Luo *et al*., 2009; Shevade *et al*., 2013; Gómez-Arreaza *et al*., 2014), enquanto outras podem ser encontradas no núcleo, onde podem atuar como fatores de transcrição (Gómez-Arreaza *et al*., 2014). Outras proteínas *moonlighting* trocam de função quando são secretadas (Niinaka *et al*., 1998; Gómez-Arreaza *et al*., 2014). Além disso, modificações pós-traducionais (Pal-Bhowmick *et al*., 2007; Shevade *et al*., 2013), formação de complexos multiproteicos (Zheng *et al.*, 2003) e formação de estruturas quaternárias alternativas (Saccoccia *et al*., 2012) podem ser determinantes de funções alternativas.

Muitas das proteínas multifuncionais (*moonlighting*) conhecidas são enzimas altamente conservadas, como, por exemplo, enzimas envolvidas na glicólise (Huberts *et al*., 2010b). Há evidências de que 7 das 10 enzimas da via glicolítica exibem atividades *moonlighting* (Sriram *et al*., 2005). Dependendo do organismo, as funções alternativas de enzimas glicolíticas podem variar desde funções estruturais e regulatórias (Starnes *et al*., 2009; Gómez-Arreaza *et al*., 2014) até, em organismos parasitos, funções de interação e invasão do hospedeiro (Luo *et al*., 2009; Nogueira *et al*., 2010; Gómez-Arreaza *et al*., 2014).

Dentre as proteínas já descritas como tendo funções *moonlighting* em organismos parasitas, destacam-se enzimas da via glicolítica, as quais têm sido comumente identificadas no tegumento e nos produtos de ES de diversos parasitos, onde apresentam propriedades antigênicas e de interação com o hospedeiro (Bernal *et al*., 2004; Marcilla *et al*., 2007; Huang *et al.*, 2009; Gómez-Arreaza *et al*., 2014). Tanto em espécies parasitas como nas de vida livre, enzimas glicolíticas, como a enolase, podem ter funções não glicolíticas na regulação do citoesqueleto, no transporte celular, na transcrição ou na apoptose (Pancholi, 2001; Kim & Dang, 2005; Sherawat *et al*., 2008; Gómez-

Arreaza *et al*., 2014). Mas, especificamente em parasitos, elas podem desempenhar papeis importantes em processos-chave para o modo de vida parasitário, como motilidade, adesão, invasão, diferenciação e desenvolvimento, entre outros (Jewett & Sibley, 2003; Labbé *et al*., 2006; Pal-Bhowmick *et al*., 2007; Pomel *et al*., 2008). A enolase também já teve descrita sua capacidade de ligação a macromoléculas do hospedeiro, como o plasminogênio (Bernal *et al*., 2004; Marcilla *et al*., 2007; Gómez-Arreaza *et al*., 2014), em processos de degradação de componentes de matriz extracelular importantes para a disseminação de patógenos em tecidos de seus hospedeiros (Nogueira *et al*., 2010). No caso de helmintos parasitas, a ligação de proteínas glicolíticas ao plasminogênio pode impedir a formação de coágulos ao redor do parasito, contribuindo para o estabelecimento da infecção no hospedeiro (Marcilla *et al.*, 2007; Ramajo-Hernández *et al*., 2007).

1.3 Biologia de sistemas

Um dos maiores desafios da biologia pós-genômica é o entendimento de como os genes, proteínas e outras moléculas interagem para formar sistemas celulares (Zhang *et al*., 2007). Com o rápido avanço da biologia e o desenvolvimento de altas tecnologias para o estudo de interações de moléculas biológicas, como microarranjo, ensaios de duplo-híbrido, coimunoprecipitação, entre outras, foi possibilitada a criação de bancos de dados de interação proteína-proteína (PPI) de organismos modelo. Com a utilização desses bancos de dados, é possível a criação de redes de PPI que possibilitam o entendimento de como os eventos celulares são coordenados a nível molecular, um dos principais objetivos da biologia de sistemas (Gunsalus & Rhrissorrakrai, 2011).

Entre os mecanismos associados à troca de função de proteínas *moonlighting* estão as PPIs. Deste modo, uma das estratégias que tem sido utilizada para investigar funções *moonlighting* é o uso de ferramentas de biologia de sistemas, as quais permitem a predição de funções/interações de proteínas através do estudo de redes biológicas. O princípio das redes de PPI sugere que estas possuem a informação necessária para inferir a

multifuncionalidade de uma proteína (Gómez *et al*., 2011). Dessa forma os bancos de dados de PPI podem ser considerados uma ferramenta valiosa para o estudo de funções *moonlighting*. Portanto, as ferramentas de biologia de sistemas têm sido utilizadas para predizer funções de proteínas, através da utilização de redes de PPI (Janga *et al*., 2011;).Por exemplo, esta estratégia foi bem sucedida na descrição das propriedades funcionais das redes de pequenas GTPases (Delprato, 2012).

Outra vantagem que a biologia de sistemas possibilita é o estudo de redes de PPI de forma comparativa. Como os dados de PPI ainda são limitados e disponíveis especialmente para organismos-modelo, o estudo comparativo de redes de interação pode revelar aspectos funcionais conservados ao longo da escala evolutiva e que, possivelmente podem ser extendidos para organismos cujos bancos de PPI são reduzidos ou até mesmo inexistentes. Por exemplo, para o parasito *Plasmodium falciparum* foi descrita a criação de uma rede de PPI para elucidar como ocorrem as relações entre as funções metabólicas necessárias para a manutenção da doença (Ramaprasad *et al*., 2012). Como não estão disponíveis bancos de dados de *P. falciparum* para a criação de redes de PPI, foram utilizados bancos de dados de organismos modelo e resultados de ensaios de duplo-hibrido de levedura para gerar a rede interação modelo para este organismo.

A caracterização de funções não glicolíticas da enolase tanto em organismos parasitas como não parasitas tem tornado cada vez mais evidente a multifuncionalidade desta enzima (Bernal *et al*., 2004; Sriram *et al*., 2005; Marcilla *et al*., 2007;Nogueira *et al*., 2010), porém ainda não está claro se algumas destas funções são conservadas evolutivamente. Dessa forma, a biologia de sistemas é uma abordagem interessante para a investigação de possíveis funções *moonlighting* da enolase evolutivamente conservadas. Uma vez que processos funcionais conservados da enolase sejam revelados, eles poderão ser extendidos para organismos como *E. granulosus*, para o qual um banco de PPI é inexistente, e servirão como base para o direcionamento de

estudos de validação funcional da multifuncionalidade da enolase neste parasito.

1.4. Justificativas e objetivos

Uma forma de compreender a função de uma proteína em uma célula é perguntar onde exatamente esta proteína está na célula, e com quais proteínas ela está interagindo. Estas são algumas das perguntas que a análise de redes biológicas de PPI pode responder.

A enolase 1 de *E. granulosus* (EgEno1) tem se mostrado uma enzima potencialmente *moonlighting* devido à localização em compartimentos celulares não usuais (Lorenzatto et al. 2012). Além disso, estudos preliminares de interação proteína-proteína também são sugestivos da multifuncionalidade desta enzima em *E. granulosus*. Uma abordagem que ainda não foi utilizada para avaliar a multifuncionalidade da enolase é o estudo comparativo de redes de PPI. Como bancos de dados de PPI para *E. granulosus* ainda não estão disponíveis, informações disponíveis para organismos modelo podem ser utilizados para projetar as redes de PPI de proteínas ortólogas da EgEno1. Dessa forma, espera-se evidenciar processos funcionais da enolase que sejam conservados evolutivamente, e que também possam ser verdadeiros para a enolase de *E. granulosus*.

O trabalho aqui apresentado dá seguimento ao trabalho de caracterização funcional da EgEno1, com ênfase na busca de evidências de funções *moonlighting*. Do ponto de vista de pesquisa básica, os estudos propostos deverão identificar possíveis proteínas e interações proteicas conservados em organismos modelo e que poderão evidenciar mecanismos moleculares e processos importantes para a sobrevivência e desenvolvimento do cisto hidático durante a infecção crônica do hospedeiro intermediário.

1.4.1 Objetivos

1.4.1.1. Objetivo geral

O objetivo desse trabalho é a obtenção de evidências de funções *moonlighting* evolutivamente conservadas das proteínas enolase de eucariotos e, especificamente, da proteína EgEno1.

1.4.1.2. Objetivos específicos

i. Recuperação dos dados de PPI e projeção das redes de PPI para enolases de *H. sapiens*, *D. melanogaster*, *C. elegans* e *S. cerevisiae*;

ii. Identificação dos módulos de proteínas altamante conectadas das redes de PPI geradas;

iii. Identificação das proteínas centrais das redes;

iv. Identificação das proteínas ortólogas presentes nas diferentes redes de PPI;

v. Identificação das principais funções biológicas desempenhadas pelas proteínas das redes de PPI;

vi. Identificação das proteínas de interação da EgEno1 em protoescólices.

2. CAPÍTULO 1 - *Systems biology approach reveals possible evolutionarily conserved moonlighting functions for enolase*

2.1. Apresentação

O manuscrito que constitui esta seção foi escrito seguindo as orientações da revista *Molecular Biosystems* (www.rsc.org/molecularbiosystems). Todos os experimentos descritos no manuscrito, assim como a sua redação, foram realizados pela aluna Gabriela Prado Paludo, sendo os demais autores responsáveis pela sua orientação.

Systems biology approach reveals possible evolutionarily conserved moonlighting functions for enolase

Gabriela Prado Paludo [1], Karina Rodrigues Lorenzatto [1,3], Diego Bonatto [2,3,4] and Henrique Bunselmeyer Ferreira [1,3,4]

[1] Laboratório de Genômica Estrutural e Funcional, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS – Brazil.

[2] Laboratório de Radiobiologia Molecular Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS – Brazil.

[3] Programa de Pós-Graduação em Biologia Celular e Molecular, Centro de Biotecnologia, UFRGS, Porto Alegre, RS – Brazil.

[4] Departamento de Biologia Molecular e Biotecnologia, Instituto de Biociências, UFRGS, Porto Alegre, RS – Brazil.

Short title: Enolase interactions and moonlighting functions

[*] **To whom correspondence should be sent:**

Dr. Henrique Bunselmeyer Ferreira

Laboratório de Genômica Estrutural e Funcional

Centro de Biotecnologia

Universidade Federal do Rio Grande do SulAv. Bento Gonçalves, 9500 - Prédio 43-421, Sala 210

Caixa Postal 15005

91501-970 Porto Alegre, RS

BRAZIL

Phone: (+55 51) 3308-7768

Contract/grant sponsor: CNPq, CAPES, and FAPERGS.

2.2. Abstract

Glycolytic enzymes, such as enolase, have been described as multifunctional complex proteins that also display non-glycolytic activities, termed moonlighting functions. Although enolase multifunctionality has been described for several organisms, the conservation of enolase alternative functions through different phyla has not been explored in more details. One useful strategy to investigate moonlighting functions is the use of systems biology tools, which allow the prediction of protein functions/interactions by graph design and analysis. In this work, available information from protein-protein interactions (PPI) databases were used to design enolase PPI networks for four eukaryotic organisms, covering a wide spectrum of this domain of life, *Homo sapiens, Drosophila melanogaster*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae*. Modularity and centrality analyses, and functional enrichment for all generated networks were performed. PPI networks with number of nodes ranging from 140 to 411 and up to 15,855 connections were generated. In addition, we obtained subnetworks, clusters, related to different biological processes, like development, stress response, and apoptosis, among others. The performed analyses showed that enolase is a central node within the networks, and that, in addition to its canonical interactions with proteins related to glycolysis and energetic metabolism, it is also part of conserved proteins clusters related to different biological processes like transcription and development. Our results indicate that enolase can be involved in regulation of these different processes, through patterns that are repeated in some of the organisms studied here. Finally, this work gives an overview of the possible conserved non-glycolytic processes in which enolase may be involved.

Key-words: enolase, moonlighting functions, systems biology, protein-protein interaction network

2.3. Introduction

One of the major challenges of post-genomic biology is to understand how genes, proteins and other molecules interact to compose cellular systems[1]. In recent years, the idea of “one gene, one protein, one function” has been replaced by the knowledge that many proteins display multiple functions.[2] For example, moonlighting proteins could display two or more different functions within a single polypeptide chain.[3] The functions of a moonlighting protein depends on cellular localization, cell type, oligomeric state, and/or the cellular concentration of a ligand, substrate, cofactor or product.[4] These different mechanisms are not mutually exclusive and, in many cases, the functions of a protein depends on the physiological status of the cell. Although the identification of a multifunctional protein from PPI databases can be a difficult task, systems biology approaches have been successfully applied for this purpose.[5,6,7,8]

The development of high-throughput methods for the study of biological molecules interactions, such as protein microarrays,[9] two-hybrid assays,[10] co-immunoprecipitation[11] and peptide phage display,[12] allowed the generation of robust databases of protein-protein interaction (PPI) for four eukaryotic organisms. To understand how cellular events are coordinated at the molecular level, the information contained in these databases can be arranged in a graph context to create PPI networks.[13,14] The properties of PPI networks can be explored to identify interaction possibilities whose effectiveness depend on physical binding events.[15] The cumulative effect of such events results in a distribution of protein complexes that ultimately determines cellular behavior.

Enolase, also known as phosphopyruvate hydratase, is a glycolytic enzyme that catalyzes the dehydratation of 2-phosphoglycerate to phosphoenolpyruvate. This protein is present in all three domains of life and is one of the most abundantly expressed cytosolic proteins in many organisms.[16] In addition to its classical involvement in glycolysis, other functional roles have been described for enolase. For example, enolase plays an important role in mouse mast cell differentiation.[17] Knockdown of enolase expression in different

tumor cell lines causes a dramatic increase in their sensitivity to microtubule targeting drugs, suggesting that enolase expression levels can affect the sensitivity of tumor cell lines to anti-tubulin drugs.[18] Moreover, enolase (and other glycolityc enzymes) has been involved in an specific apoptosis mechanism, in which it is externalized and participate in immune modulation, holding promise for understanding and addressing causes of autoimmune and inflammatory pathology.[19] Although the multifunctionality of enolase has been increasingly evident, it is not yet clear whether its non-glycolytic functions are evolutionarily conserved or not.

In this work, a systems biology approach was used to investigate the moonlighting functions of enolase in different eukaryotic organisms. This approach allowed the comparison of possible enolase interactions and functions in *Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae*. Our results showed that enolase is a central node in all designed networks and is involved in conserved interactions with proteins related to different cellular functions. Besides its canonical interactions with proteins related to glycolysis and energetic metabolism, enolase was also found as part of conserved proteins clusters associated with stress response, development, among others biological processes. The importance of enolase moonlighting functions in these evolutionarily conserved networks is discussed.

2.4. Material and methods

Protein-protein interaction network design and global topological analysis

Enolase PPI networks of model organisms were design using the metasearch tool STRING 8.2 (http://www.string-db.org). In this sense, the following parameters were used: active prediction methods all enabled except text mining; no more than 50 interactions; and high confidence score (0.700). To calculate the confidence score of a specific connection, various major sources of interaction/association data were benchmarked independently by STRING,[20] where a combined score is computed which indicates higher confidence when more than one type of information supports a given association. This means

that interactions of high reliability are those which are supported by more than one source. Taking these parameters into account, a network was generated for *H. sapiens*, *D. melanogaster*, *C. elegans* and *S. cerevisiae*. The results gathered from these search engines were subsequently analyzed using Cytoscape 2.6.3 (http://www.cytoscape.org).[21] The networks were analysed in terms of global topology (subnetworks) using AllegroMCODE, which is a Cytoscape plugin freely available at http://www.allegroviva.com/allegromcode.[22] The parameters used in MCODE to generate subnetworks were: loops included; degree cutoff 2, node score cutoff 0.2, *K*-Core 2, and maximum depth of network 100. Each subnetwork obtained from this analysis was evaluated in terms of gene ontologies (GOs).

Gene ontology analyses

The protein subnetworks generated previously were analyzed in order to identify major associated biological processes. For this purpose, we used the Biological Networks Gene Ontology (BiNGO) software (version 2.44), a Cytoscape plugin available in http://www.cytoscape.org.[23] The degree of functional enrichment for a given cluster and category (*P*-value) was calculated using hypergeometric distribution, and multiple test correction was also valued by the false discovery rate (FDR) algorithm, which was fully implemented through BiNGO software with a significance level of $P<0.05$.

Protein orthology evaluation

The degree of evolutionary network conservation among different organisms was evaluated by analyzing the type and number of orthologous proteins found in each network. For this purpose, we compared orthologous proteins between each two networks, considering all possible pairs, and, finally, among all networks. Amino acid sequences from probable orthologous proteins were submitted to multiple alignments using the Clustal W2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2).[24] Only the proteins with similarity higher than 50% were considered orthologous. The functional category of the orthologous were identified using the KOG tool

(http://www.ncbi.nlm.nih.gov/COG/grace/kognitor.html).[25] To compare the proteins present in the networks, the identity of proteins were verified in specific databases for each organisms (*H. sapiens*: http://www.genecards.org; *D. melanogaster*: http://flybase.org; *C. elegans*: http://www.wormbase.org; *S. cerevisiae*: http://www.yeastgenome.org).

Network centralities analyses

Networks centralities (node degree and betweenness) were calculated by CentiScaPe 1.21 plug-in.[26] Nodes with high degree (highly connected) are called hubs and were defined as all nodes with node degree value higher than the threshold generated by the software for the total network. Bottleneck nodes were defined as all nodes with high betweenness value, higher than the network threshold.

2.5. Results and discussion

Eukaryotic enolase protein-protein interaction network design and global topological analysis

For this study we used a sample of 4 eukaryotic species with sufficient volume of data and covering much of eukaryotic biodiversity, from yeast to mammalian undergoing helminths and arthropods. With this sample we believe cover possible similarities evolutionarily conserved in this domain of life, comparison of these networks will show similarities and differences that can be observed in these so different organisms PPI.

The networks were generated from data of protein-protein interactions available in the organisms databases. So, were chosen organisms that possess robust databases with large amounts of PPI information. For the used data could be trusted, we selected only interactions with a high degree of confidence (above 0.700), this high degree of confidence indicates that the interaction was confirmed by more than one way. [20] Thus, in the generated networks, the number of enolase interaction proteins (nodes) recovered from databases varied from 140 to 411, the number of connections (edges) varied widely among

the networks, and the number of proteins directly connected to enolase or that are its first neighbors, varied from 20 to 39 proteins. This variation among the networks as the number of nodes and connections, depends on available data in databases and as each organism was studied. Despite the differences we observed some similarities, as the number of proteins directly linked to enolase had a small variation (between 20 and 39 proteins).

To show the results obtained from the projection of PPI networks, the **Figure 2.1** shows the information of the total number of proteins, total number of connections and the number of enolase first neighbors for all four organisms. In addition to modularity results (see below), clusters that perform the same biological function are in the same color.

The *H. sapiens* network contains 411 proteins and 4,643 connections. Among these proteins, 20 are directly connected to human α-enolase. Likewise, we designed the *Drosophila*-associated network, with 390 proteins, 15,855 connections and 39 enolase first neighbors; the *Caenorhabditis elegans*-associated network, with 140 proteins, 3,147 connections and 24 enolase first neighbors; and the *Saccharomyces cerevisiae*-associated network, with 397 proteins, 3,367 connections and 31 enolase first neighbors (**Figure 2.1**).

Networks are built from data with more than one confirmation, ie high confidence, that way, you can trust that these interactions are occurring. In addition, the filter used to select only high confidence interactions not prevented a recovered enough data to this study. Even the worm network (140 nodes) was not considered too small, since other studies show that it is possible to obtain conclusions still working whit small networks.[27,28,29]

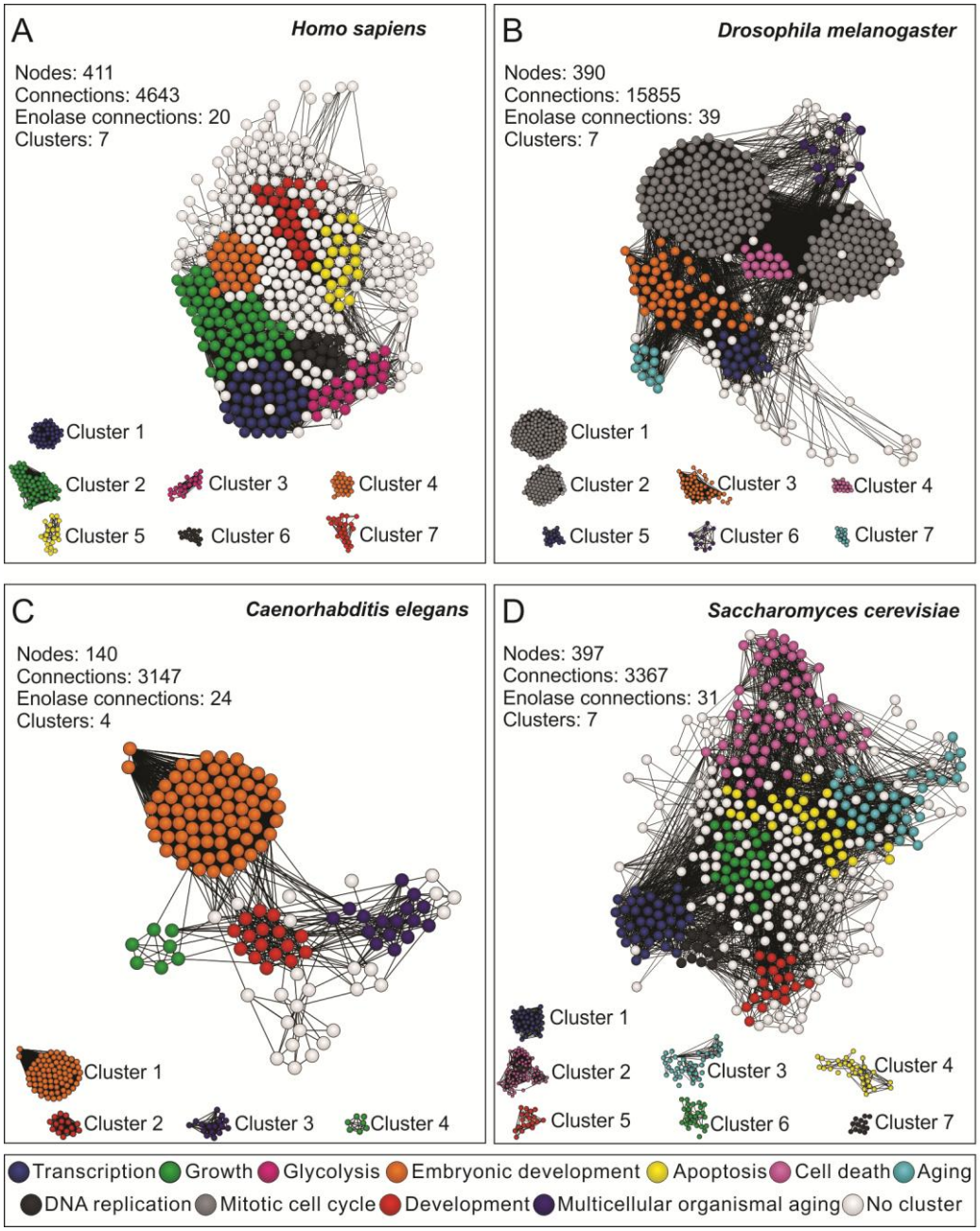

Figure 2.1. Enolase PPI networks. Networks of (A) *H. sapiens*, (B) *D. melanogaster*, (C) *C. elegans* and (D) *S. cerevisiae*. Clusters are colored according to their functional category (at the bottom of the figure). The total number of nodes, connections, the enolase first neighbors number (Enolase connections) and the number of cluster are described for each network.

In order to identify the presence of orthologous proteins in PPIs of 4 species , we searched for orthologous proteins present in more than one network. Several orthologous were present in more than one network (**Figure 2.2, S-Table 2.1**). Nine of these orthologous are present in all four networks (**Figure 2.2**).

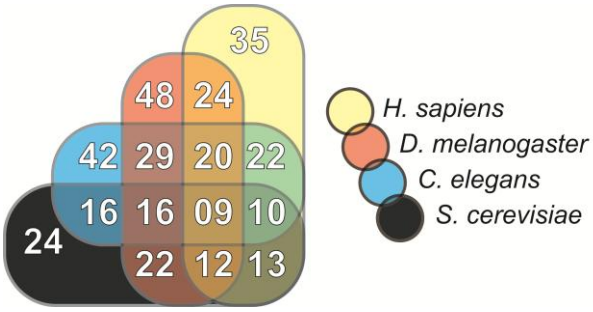

Figure 2.2. Venn diagrams showing the distribution of the orthologous shared among the organisms.

The identification of orthologous shared among the networks is important to infer some possible enolase moonlighting functions that are evolutionarily conserved. In order to get a general idea of these possible functions, eukaryotic orthologous groups (KOG) annotations were assigned for the 9 orthologous present in all four networks (**Figure 2.3A, S-Table 2.1**), based on sequence similarity searches against annotated proteins in KOG (http://www.ncbi.nlm.nih.gov/COG/grace/kognitor.html)[25] As a result, 6 out of 9 belong for group G (carbohydrate metabolism and transport), which means that some of these orthologous identified are related to other than carbohydrate metabolism and transport functional class. This result rise the question why these proteins are in the enolase PPI networks. One of the unusual functional class is transcription (group K), with 1 orthologous identified. This result suggest that, enolase can be acting in the process of transcription, that corroborates with the described data that the formation of complexes with enolase in the nuclei can modulate the expression of the MYC gene.[30] Gene Ontology (GO) terms were also applied to 55 orthologous proteins **(S-Table 2.1)** using Blast2GO software (http://www.blast2go.com/b2ghome) .[31] The overall result obtained from Blast2GO analysis is shown in graphics with this data, including molecular functions, biologic process and cellular component (**Figure 2.3B, C, D**). When it comes to the results for the Blast2GO, however, have some further data. The cellular component results of Blast2GO showed some unexpected components, as glycolysis occurs in the cell cytoplasm. Thus, the presence of extracellular proteins in the enolase PPI networks is a curious fact, since the difference in location should prevent this interaction.

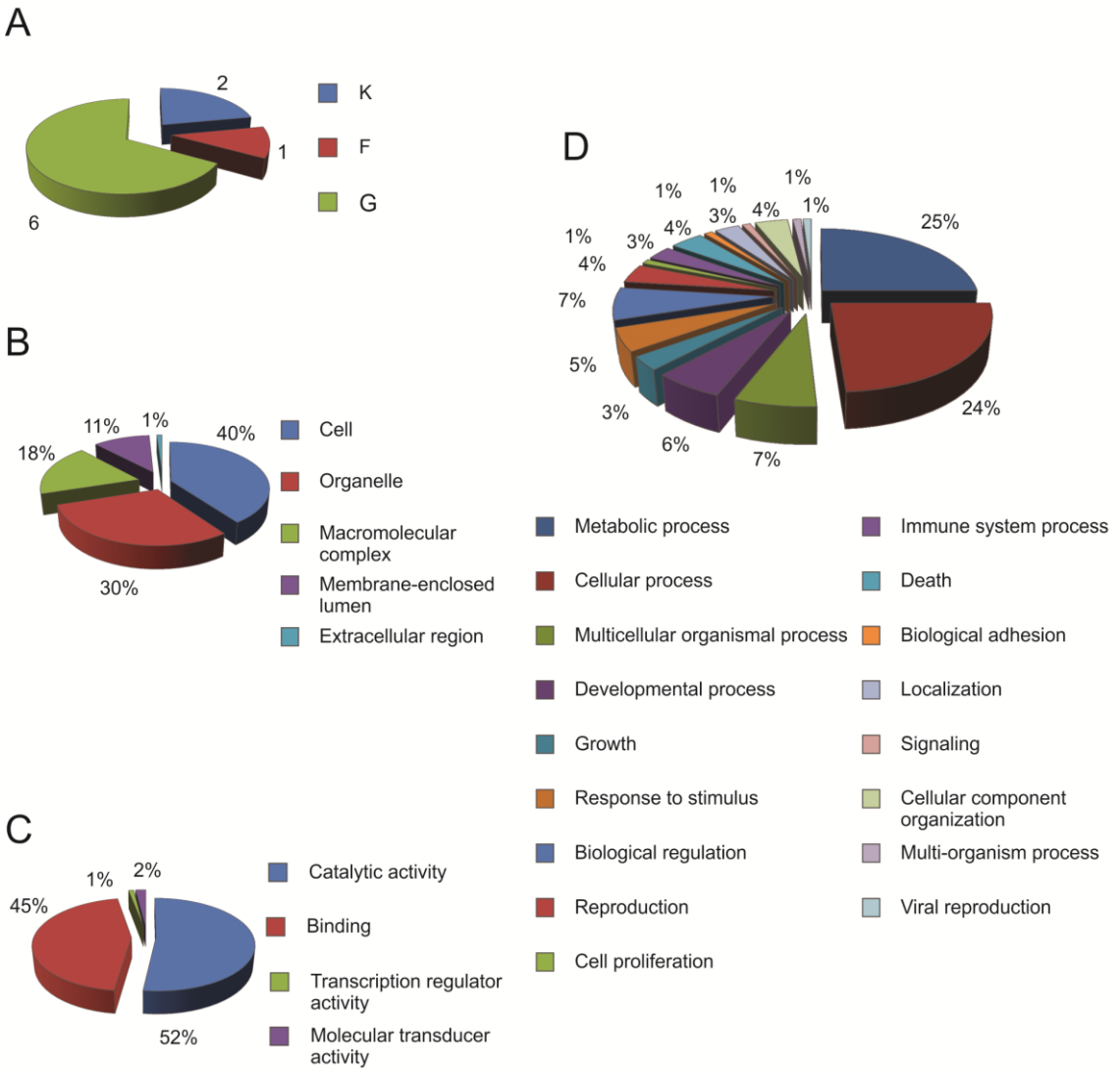

Figure 2.3. Functional enrichment applied to the orthologous identified in common with the four networks. (A) Functional classification according to KOG annotations of the 9 orthologous present in all four networks; G (carbohydrate metabolism and transport), F (nucleotide metabolism and transport) e K (transcription). The GO terms were applied to all 55 the orthologous proteins and their functional classification is shown according to (B) cellular component, (C) molecular function and (D) biological process.

Considering molecular function, nearly half of the orthologous have non-catalytic activities, as transcription regulatory activity, molecular transducer activity and binding (**Figure 2.3B**). The biological process analysis also shows interesting results with proteins related to unexpected process, like immune system process or developmental process (**Figure 2. 3D**). Finally, the enolase interaction with glycolytic proteins is naturally expected. However, a large

number of interactions with proteins not related to glycolysis process or energy obtainment was present in the interactome analysis (**Figure 2.3C**). The fact that several non glycolytic proteins have been found in enolase networks is taken as indicative of enolase multifunctionality.

Network centralities and local topological analyses

In the centralities analysis, we can evaluate each network node according to the node degree and betweenness to select the most central nodes within the network.[26] Thus, this analysis helps to detect which proteins could be a relevant target for the search for enolase moonlighting functions. Betweenness indicates the number of shortest paths that go through each node. Thus, nodes with higher betweenness scores, when compared to the average betweenness score of the network, are responsible for controlling the flow of information through the network, thus characterizing the so-called bottleneck nodes. Finally, node degree is a measure that indicates the number of connections that involve a specific node. Nodes with a high node degree are called hubs[54] and have key regulatory functions in the cell.

With this method, it was possible to observe that enolase is a bottleneck node in all networks, indicating it is an important protein connecting others and also clusters.The enolase is a hub only in *S. cerevisiae* (**Figure 2.4**), which may indicate a high regulatory relevance of the enolase in this network.

We selected proteins that act as hub-bottleneck of all networks, which combine the bottleneck and the hub property into a scale-free biological network.[32] So, we compared these hub-bottleneck proteins with the orthologous table from the different organisms (**S-Table 2.1**) and create a new table containing just the hub-bottleneck proteins that have orthologous from the other networks (**Table 2.1**). Most orthologous are shared between two networks. Only the orthologous for nucleoside diphosphate kinase, RNA polymerase II subunit and transketolase were shared among three organisms (*H. sapiens*, *D. melanogaster* and *S. cerevisiae*).

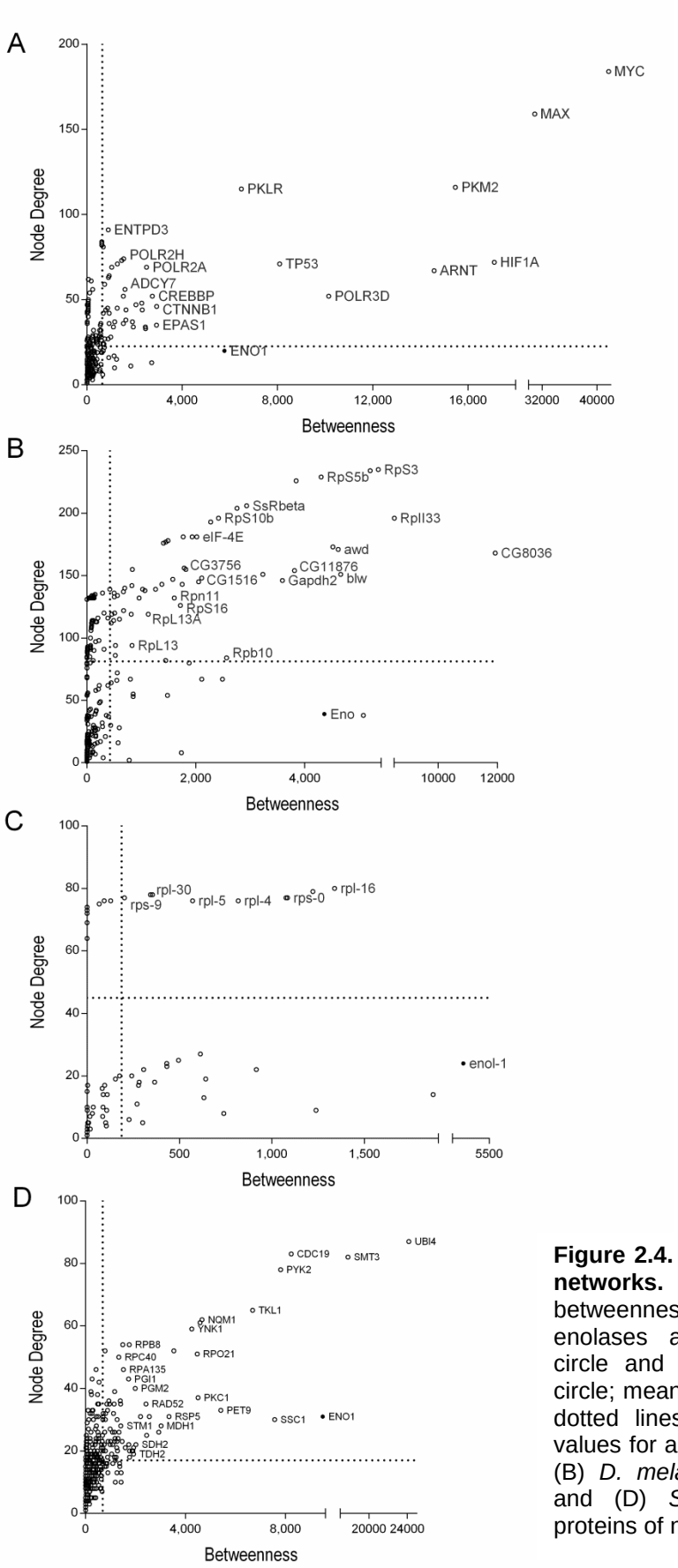

Figure 2.4. Centrality analysis of the networks. Node degree and betweenness values were plotted; enolases are represented by filled circle and other proteins by unfilled circle; mean values are represented by dotted lines. Each graph shows the values for an organism: (A) *H. sapiens*, (B) *D. melanogaster*, (C) *C. elegans* and (D) *S. cerevisiae*. The major proteins of networks are named.

Table 2.1. Hub-bottleneck orthologous proteins shared by the organisms

Protein name	*H. sapiens*	*D. melanogaster*	*C. elegans*	*S. cerevisiae*
Fructose-bisphosphate aldolase	ALDOA			Fba1
Glucose-6-phosphate isomerase	GPI			Pgi1
Nucleoside diphosphate kinase	NME1	awd		Ynk1
RNA polymerase II subunit	POLR2L	Rpb10		Rpb2
Transaldolase	TALDO1			Tal1
DNApolymerase delta		FBpp0075277		Pol3
Pyruvate carboxylase		CG1516		Pyc2
Transketolase	TKT	CG8036		Tkl1
Succinate dehydrogenase		SdhB		Sdh2
Glyceraldehyde 3-phosphate dehydrogenase		Gapdh2		Tdh2
Ubiquitin	UBC			Ubi4
Elongation fator γ		Ef1gamma		Tef4
Ribosomal protein, small subunit		RpS23	rps-23	

It was observed in cluster 7 of *H. sapiens* associated enolase network (**Figure 2.1A**), the connection of the bottleneck protein ESR1 (estrogen receptor), that can act in multicellular organismal development with EP300 (E1A binding protein p300), a hub-bottleneck protein, which functions as histone acetyltransferase that regulates transcription via chromatin remodeling and is important in the processes of cell proliferation and differentiation,[33] and also acts in cardiac maturation in mouse.[34] Another protein found in cluster 7 is ARNT (aryl hydrocarbon receptor nuclear translocator), a hub-bottleneck, connected to EP300, which acts in response to oxidative stress.[35] The HNF1A (HNF1 homeobox A), that was found in the cluster 7, is a transcriptional activator that regulates the tissue specific expression of multiple genes and can act in reproduction.

In the *D. melanogaster* hub-bottleneck proteins set (**Table 2.1**) there are : RpS23 (ribosomal protein, small subunit), that is a structural constituent of ribosome,[36] and belongs to cluster 2; Rpb10 (RNA polymerase II subunit), that have DNA-directed RNA polymerase activity,[37] and belongs to cluster 3; and all other proteins belong to cluster 1. The fly hub-bottleneck proteins from cluster 1

form a click, which means that each protein has connection to all other. The awd (nucleoside diphosphate kinase) is a fly hub-bottleneck protein, it is a NME (human protein) orthologous, and several studies have shown proteins from NME/Awd families involved in tumor metastasis.[38, 39]

The only *C. elegans* hub-bottleneck protein that has orthologous in other networks is the rps-23 (ribosomal protein, small subunit). The rps-23 is from cluster 1 (embryonic development) and most proteins which are connected to it also are t from cluster 1. In *C. elegans*, the rps-23 activity is required for germline development, vulval morphogenesis, and the overall health of the animal.[40]

In the *S. cerevisiae* network there are twelve hub-bottleneck proteins that have orthologous in other networks. The Ubi4 (ubiquitin) is a hub-bottleneck protein whose abundance increases in response to DNA replication stress.[41] The Ubi4 is directly connected to two other hub-bottleneck proteins: the Tkl1 (transketolase) and the Rpb2 (RNA polymerase II subunit).

Modularity within the eukaryotic enolase networks

High aggregation is an essential characteristic of biological networks, and it reflects high modularization of networks.[42] Therefore, it is expected that the PPI network of an organism be very complex, and consists of large number of sub-networks, modules. Functional modules can be identified from complex protein interaction networks. It follows that the investigation of functional clusters will generate a better understanding of cellular organization, processes and functions.

Although clusters are identified on the basis of the topology, the assumption underlying this approach is that clusters will identify groups of proteins that similar functions.[43] In this sense, seven clusters of highly connected proteins were found in *H. sapiens*, *D. melanogaster* and *S. cerevisiae* networks, while four clusters were found in *C. elegans* network (see **Figure 2.1**).

Using gene ontology (GO) analyses, the functional enrichment indicates functional processes in which the proteins from these clusters may be involved. The most representative biological processes observed in the networks were highlighted in Figure 1. Thus, for each identified cluster, a main function was assigned.

Among the functional processes assigned to the network proteins, we found that several of these processes are shared among different networks (**Figure 2.1 and S-Table 2.2**). To analyze the results obtained from the modularization, the sizes of the clusters were compared with the same biological function in different organisms (see **S-Table 2.3**). In this study it is observed that, although most exhibit great variation in number of proteins, someones showed similar sizes. This is the case of the transcription clusters of human and yeast, 37 and 35 proteins, respectively. Other cases of clusters with similar sizes are the clusters related to apoptosis, DNA replication and development. In other cases there are a large variation among the number of proteins of each cluster. The variation may be an indication that some databases may be less information available of this biological function.

Using the hub-bottleneck proteins (**Table 2.1**) it was possible to compare the clusters which they belong. An interesting fact was that *H. sapiens* proteins related to glycolysis (human cluster 3) fructose-bisphosphate aldolase, glucose-6-phosphate isomerase, transaldolase and transketolase, have their orthologues in yeast belonging to the cell death cluster (Cluster 2 yeast). Another curious fact was that the pyruvate carboxylase, glyceraldehyde 3-phosphate dehydrogenase and elongation factor γ fly proteins have their yeast orthologs belonging to the apoptosis cluster. The nucleoside diphosphate kinase protein belongs to the transcription cluster for both human and yeast, but it is part of the mitotic cell cycle cluster in fly. Another similar case is for the PolR2L protein that belongs to transcrition cluster in human and yeast, but belongs to embrionary development cluster in fly.

In addition to the hub-bottleneck proteins, other important proteins were found in the clusters. Further analysis allowed the comparison of the biological

functions. One of them is embryonic development. For *H. sapiens*, 4 proteins from cluster 4 were related to embryonic development, namely (i) SMAD2 and SMAD4, signal transducers and transcriptional modulators that mediate multiple signaling pathways;[44] (ii) SKI, that may play a role in neural tube development and muscle differentiation;[45] and (iii) MAPK1, which is involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development.[46] The functional enrichment indicates that these proteins are associated with embryonic development ending in birth or egg hatching. Additionally, the connection between enolase and these proteins is made by MYC in the *H. sapiens* interactome. MYC is able to activate the transcription of growth-related genes, where a significant proportion of genes (10% to 15%) in both the human and *Drosophila* genomes are regulated by MYC.[47,48] Furthermore, it was demonstrated that the expression induction of c-myc gene contributes to tumorigenesis,[49] and the enolase association to other proteins in the nucleus can modulate c-myc expression.[30]

Development is another functional process found in clusters of *H. sapiens*, *C. elegans* and *S. cerevisiae*. Analyzing the *H. sapiens* development cluster, we found proteins as AES and ITGB1, which are related to multicellular development and, in *H. sapiens,* both proteins are also involved with maternal process in female pregnancy. Some transcription factors, such as HNF1A, SP1 and TCF7L2, were also found in development clusters. In addition, the *H. sapiens* cluster also shows an estrogen receptor, ESR1. Interestingly, it has been described that enolase secretion from stromal cells to extracellular space is dependent of an estrogen receptor and the stromal-derived secreted enolase acts in a paracrine manner to promote plasminogen activation and prostate cancer. [50]

In *S. cerevisiae* development-associated cluster, it was observed proteins as Sgs1, Mdy2 and Ubi4. Ubi4 is directly connected to enolase and it is required for sporulation and resistance to stress conditions.[51] Ubi4 orthologous were identified in *H. sapiens* and *D. melanogaster* networks (**S-Table 2.1**). The Mdy2 protein, which contains a ubiquitin-like domain, act in cell morphogenesis and is

involved in conjugation with cellular fusion.[52] Mdy2 is connected to Ssc1, an ATPase of the Hsp70 family, directly connected to enolase. A similar case occurs for Sgs1, related in replicative cell aging,[53] that connect to Smt3, a ubiquitin-like protein of the SUMO family, which is connected to enolase.

For *C. elegans*, most of proteins identified related to nematode development were RNA polymerases and a nucleoside diphosphate kinase (F25H2.5). The F25H2.5 protein connects to two pyruvate kinases, pyk-1 and pyk-2, that connect to enolase. The same is observed in *H. sapiens*, where the nucleoside diphosphate kinase NME1, connect to a pyruvate kinase PKLR, which is directly connected to enolase. Interestingly, in addition to the nucleoside diphosphate kinase activity, mammalian Nme proteins exhibit 30-50 exonuclease activity[54] and endonuclease nicking activity.[55] Nme proteins are indeed involved in several cellular functions, including DNA repair,[56] caspase-independent cell death,[57] as well as cell migration/metastasis.[58] The activation of F25H2.5 gene during *C. elegans* embryogenesis may result in aberrant development.[59] Although these proteins are also present in *D. melanogaster* interactome, the functional enrichment was not able to identify them as related to development (**Figure 2.1**). In the fly network, the awd protein, nucleoside diphosphate kinase, connect to PyK, pyruvate kinase, and both connect to blw, a protein related to growth and spermatid development, the blw is connected to enolase.

Nucleoside diphosphate kinase have been shown to be involved in major cellular events such as proliferation, differentiation, development, apoptosis, and metastasis dissemination, through mechanisms still largely unknown.[60] This pattern identified in these model organisms demonstrates the importance of conducting comparison studies, whereas the functional enrichment, conducted with data from individual studies, identify these proteins related to different processes: in *C. elegans* these proteins were identified in cluster 2 (development) and in *H. sapiens* the proteins are present in cluster 1 (transcription). This case is indicative that although some proteins are recruited

to be multifunctional, the additional functions can vary depending on the organism.

DNA replication clusters were identified only in *H. sapiens* and *S. cerevisiae* networks. In yeast, the pyruvate kinase Cdc19 is directly connected to enolase. Cdc19 is important for high-temperature tolerance in *S. cerevisiae*.[61] However, the *H. sapiens* PKM2 protein, orthologous to Cdc19 in yeast, was identified in the transcription cluster (**S-Table 2.4** and **Figure 2.1**). Interestingly, PKM2 and enolase were identified as up-regulated proteins in lung cancer and chronic obstructive pulmonary disease.[62] Furthermore, studies showed the role of PKM2 in the production of lactic acid through glycolysis rather than producing energy through mitochondrial oxidative phosphorylation.[63,64] This may help tumor cells to survive in low glucose and low oxygen environments, and facilitate tumour invasion. Thus, this interaction could be another interesting study target to predict enolases moonlighting functions.

The triose-phosphate isomerase (TPI) protein is present in cluster 6 of *D. melanogaster* (directly connected to enolase) and its orthologous, tpi-1, in cluster 3 of *C. elegans* (**S-Table 2.4**). The *C. elegans* tpi-1 protein is related to worm lifespan and aging.[65] Additionally, the *S. cerevisiae* Tpi, enolase, pyruvate kinase, among other proteins, were identified as extracellular proteins secreted by a unconventional pathway.[66] Yeast cells with reduced activity of Tpi1 exhibit an increased resistance to the thiol-oxidizing reagent diamide.[67] This phenotype is conserved in *C. elegans* and the underlying mechanism is based on a redirection of the metabolic flux from glycolysis to the pentose phosphate pathway, altering the redox equilibrium of the cytoplasmic NADP(H) pool.[68] This evolutionarily conserved function of triose-phosphate isomerase in yeast and worm is an example of moonlighting function of glycolytic enzymes.

Figure 5 shows the connection between the different clusters and enolase, as well as existing connections only between two clusters. Furthermore, the enolase position in the networks, relative to the clusters, was examined. The *H. sapiens* network has seven identified clusters, of which 4 are directly connected to enolase (**Figure 2.5A**). These clusters are: (i) cluster 1

that is related to transcription, (ii) cluster 2, related to growth, (iii) cluster 3, associated with glycolysis, and (iv) cluster 7, related to development.

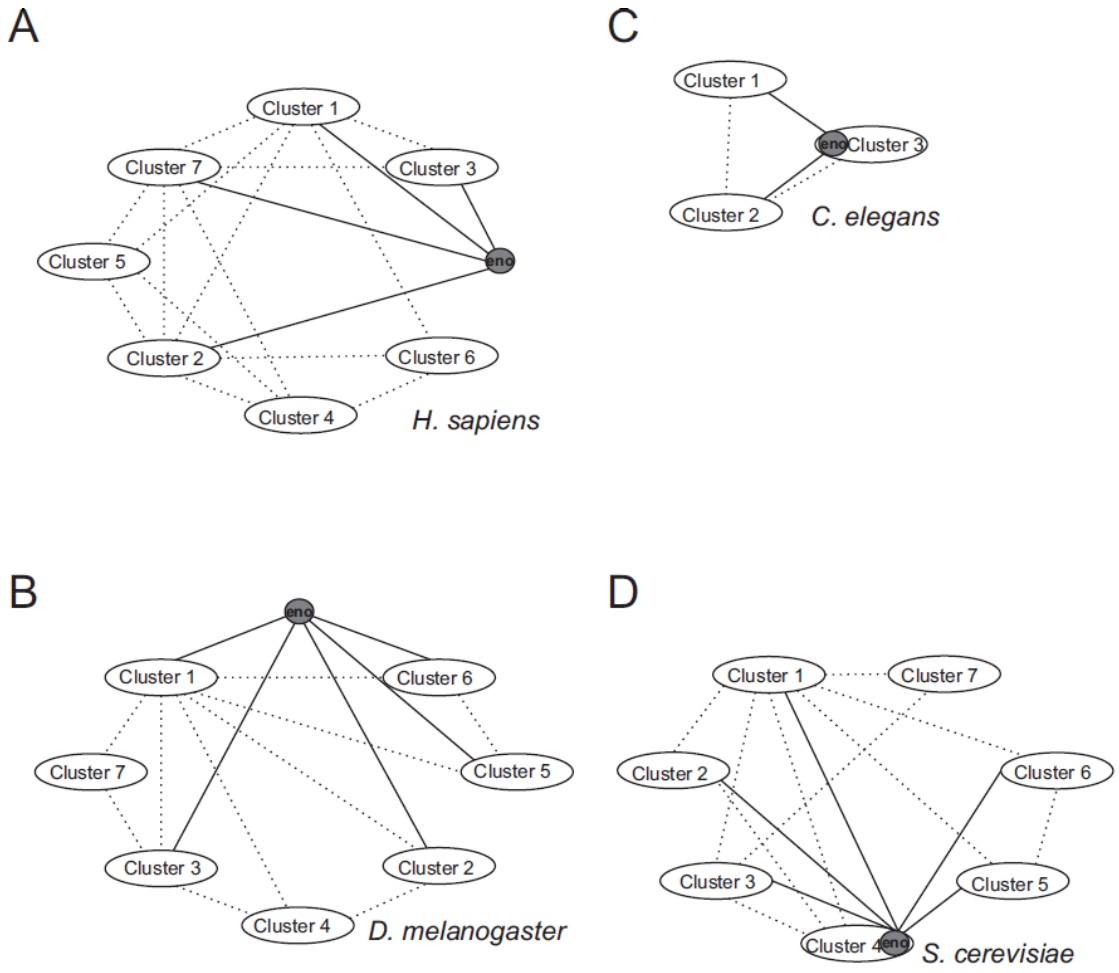

Figure 2.5. Connection among clusters and the enolases for each organisms. The clusters are represented by elipses and enolases are represented br gray circles. For *S. cerevisie* and *C. elegans*, the enolase belongs to a specific cluster (brackets), so the gray circles are within the ellipses. Enolases connections are represented by lines; connections between two clusters are represented by dotted lines.

In *D. melanogaster* there is a large number of connections among the different clusters (**Figure 2.5B**), and enolase has connection with the clusters 1, 2, 3, 5 e 6. It was not observed a connection of *Drosophila*-associated enolase with clusters 4, related to cell death, and 7, related to aging (**Figure 2.5** and **Figure 2.1**). Additionally, clusters 4 and 7 are connected to proteins related to mitotic cell cycle and embryonic development (**Figure 2.5** and **Figure 2.1**).

Although enolase is not directly connected to the aging cluster in *Drosophila*, it is directly connected to cluster 6, associated to multicellular organismal aging (**Figure 2.5** and **Figure 2.1**). Furthermore, it was observed that the fly enolase is connected to the transcription cluster in a similar way as observed for human enolase (**Figure 2.5** and **Figure 2.1**).

In *C. elegans* network, enolase is connected to all clusters (**Figure 2.5C**). This case is indicative that enolase have a role among the different processes in worm, which includes embryonic development, development, growth and multicellular organismal aging (**Figure 2.1C**).

Moreover, for *S. cerevisiae* network, the enolase is connected to all clusters (**Figure 2.5D**), except to the cluster 7, related to DNA replication. The only other network that has a cluster related to DNA replication is the human network, for which the enolase also is not connected . It was observed in yeast that enolase is connected to clusters related to transcription, growth, aging, development, cell death, and apoptosis.

2.6. Conclusion

Several studies have shown that enolase is a multifunctional protein.[16,17,18,69] The use of enolase (and other proteins) for additional tasks in the cell seems to be recurrent. However, it was not so clear whether these functions were conserved among different organisms. It is known that protein-protein interactions are among the mechanisms that can determine enolase additional functions. Here, we successfully used a systems biology approach to shed some light on protein-protein interactions that define enolase moonlighting functions in distantly related eukaryotic species.

We demonstrate the enolase ability to bind some orthologous proteins is conserved along different networks, like nucleoside diphosphate kinase and pyruvate kinase, providing good candidates for functional studies in the organisms studied here. Although the presence of conserved orthologous revealed that enolase may have binding sites which favor its interaction with some kinds of proteins, insights in the functional processes in which enolase is

involved were only highlighted when the networks were explored in a higher level of complexity. In the cluster level, it was possible to detect common functional processes among the interactomes, like development and growth, indicating that some enolase moonlighting processes are evolutionarily conserved.

This work indicates that different organisms have recruited this protein to serve different moonlighting functions and also that enolase is serving several moonlighting functions within the same organism. The evolutionarily conserved protein interaction patterns identified in this study also serves as the starting point for developing of studies for other non-model organisms for which protein-protein interaction data are poor, making the use of a system biology approach impossible. Because moonlighting functions can play important roles in disease processes, an improved understanding of moonlighting proteins will provide new opportunities for pharmacological manipulations that specifically target a function involved in pathology.

Acknowledgements

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

2.7. References

1. S. Zhang, G. Jin, X.-S. Zhang, and L. Chen, *Proteomics*, 2007, **7**, 2856–69.
2. C. J. Jeffery, *Molecular bioSystems*, 2009, **5**, 345–50.
3. S. D. Copley, *Bioessays*, 2012, **34,** 578-88.
4. C. J. Jeffery, *Trends Biochem Sci*, 1999, **24**, 8-11.
5. A. Gómez, S. Hernández, I. Amela, J. Piñol, J. Cedano, and E. Querol, *Molecular bioSystems*, 2011, **7**, 2379–82.
6. A. Delprato, *PloS one*, 2012, **7**, e44882.
7. A. Zhang, H. Sun, W. Sun, Y. Ye, and X. Wang, *Applied biochemistry and biotechnology*, 2012.
8. S. Campanaro, S. Picelli, R. Torregrossa, L. Colluto, M. Ceol, D. Del Prete, A. D'Angelo, G. Valle, and F. Anglani, *BMC genomics*, 2007, **8**, 383.
9. R. B. Jones, A. Gordus, J. A. Krall, and G. MacBeath, *Nature*, 2006, **439**, 168–74.
10. H. Yu, P. Braun, M. A. Yildirim, I. Lemmens, K. Venkatesan, J. Sahalie, T. Hirozane-Kishikawa, F. Gebreab, N. Li, N. Simonis, T. Hao, J.-F. Rual, A. Dricot, A. Vazquez, R. R. Murray, C. Simon, L. Tardivo, S. Tam, N. Svrzikapa, C. Fan, A.-S. de Smet, A. Motyl, M. E. Hudson, J. Park, X. Xin, M. E. Cusick, T. Moore, C. Boone, M. Snyder, F. P. Roth, A.-L. Barabási, J. Tavernier, D. E. Hill, and M. Vidal, *Science (New York, N.Y.)*, 2008, **322**, 104–10.
11. A. Hegele, A. Kamburov, A. Grossmann, C. Sourlis, S. Wowro, M. Weimann, C. L. Will, V. Pena, R. Lührmann, and U. Stelzl, *Molecular cell*, 2012, **45**, 567–80.

12. M. Carducci, L. Perfetto, L. Briganti, S. Paoluzi, S. Costa, J. Zerweck, M. Schutkowski, L. Castagnoli, and G. Cesareni, *Biotechnology advances*, 2012, **30**, 4–15.
13. N. Simonis, J. Rual, A. Carvunis, M. Tasan, I. Lemmens, T. Hirozane-Kishikawa, T. Hao, J. M. Sahalie, K. Venkatesan, F. Gebreab, S. Cevik, N. Klitgord, C. Fan, P. Braun, N. Li, N. Ayivi-Guedehoussou, E. Dann, N. Bertin, D. Szeto, A. Dricot, M. A. Yildirim, C. Lin, A.-S. de Smet, H.-L. Kao, C. Simon, A. Smolyar, J. S. Ahn, M. Tewari, M. Boxem, S. Milstein, H. Yu, M. Dreze, J. Vandenhaute, K. C. Gunsalus, M. E. Cusick, D. E. Hill, J. Tavernier, F. P. Roth, and M. Vidal, *Nature methods*, 2009, **6**, 47–54.
14. T. Milenković and N. Przulj, *Cancer informatics*, 2008, **6**, 257–73.
15. E. J. Deeds, J. Krivine, J. Feret, V. Danos, and W. Fontana, *PloS one*, 2012, **7**, e32032.
16. M. Piast, I. Kustrzeba-Wójcicka, M. Matusiewicz, and T. Banaś, *Acta biochimica Polonica*, 2005, **52**, 507–13.
17. S. Y. Ryu, G. U. Hong, D. Y. Kim, and J. Y. Ro, *Cellular signalling*, 2012, **24**, 60–70.
18. E. Georges, A.-M. Bonneau, and P. Prinos, *International journal of biochemistry and molecular biology*, 2011, **2**, 303–8.
19. D. S. Ucker, M. R. Jain, G. Pattabiraman, K. Palasiewicz, R. B. Birge, and H. Li, *The Journal of biological chemistry*, 2012, **287**, 10325–43.
20. D. Szklarczyk, A. Franceschini, M. Kuhn, M. Simonovic, A. Roth, P. Minguez, T. Doerks, M. Stark, J. Muller, P. Bork, L. J. Jensen, and C. von Mering, *Nucleic acids research*, 2011, **39**, D561–8.
21. P. Shannon, A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski, and T. Ideker, 2003, 2498–2504.
22. D. F. Gudbjartsson, K. Jonasson, M. L. Frigge, and A. Kong, *Nature genetics*, 2000, **25**, 12–3.
23. S. Maere, K. Heymans, and M. Kuiper, *Bioinformatics (Oxford, England)*, 2005, **21**, 3448–9.
24. M. A. Larkin, G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson and D. G. Higgins, *Bioinformatics*, 2007, **23,** 2947-2948.
25. S. Götz, R. Arnold, P. Sebastián-León, S. Martín-Rodríguez, P. Tischler, M.-A. Jehl, J. Dopazo, T. Rattei, and A. Conesa, *Bioinformatics (Oxford, England)*, 2011, **27**, 919–24.
26. G. Scardoni, M. Petterlini, and C. Laudanna, *Bioinformatics (Oxford, England)*, 2009, **25**, 2857–9.
27. J. Zhang, M. Jiang, F. Yuan, K. Y. Feng, Y. D. Cai, X. Xu, and L. Chen, *Biomed Res Int*, 2013, 523415.
28. N. S. Abul-Husn, S. P. Annangudi, A. Ma'ayan, D. L. Ramos-Ortolaza, S. D. Stockton Jr, I. Gomes, J. V. Sweedler, and L. A. Devi, *PLoS One*, 2011, **6**, e25535.
29. T. G. Lilburn, H. Cai, Z. Zhou, and Y. Wang, *BMC Genomics*, 2011, **12**, Suppl 5:S9
30. K.-W. Hsu, R.-H. Hsieh, Y.-H. W. Lee, C.-H. Chao, K.-J. Wu, M.-J. Tseng, and T.-S. Yeh, *Molecular and cellular biology*, 2008, **28**, 4829–42.
31. E. V Koonin, N. D. Fedorova, J. D. Jackson, A. R. Jacobs, D. M. Krylov, K. S. Makarova, R. Mazumder, S. L. Mekhedov, A. N. Nikolskaya, B. S. Rao, I. B. Rogozin, S. Smirnov, A. V Sorokin, A. V Sverdlov, S. Vasudevan, Y. I. Wolf, J. J. Yin, and D. A. Natale, *Genome biology*, 2004, **5**, R7.
32. H. Yu, P. M. Kim, E. Sprecher, V. Trifonov, and M. Gerstein, *PLoS computational biology*, 2007, **3**, e59.
33. S. P. Panicker, B. Raychaudhuri, P. Sharma, R. Tipps, T. Mazumdar, A. K. Mal, J. M. Palomo, M. A. Vogelbaum, and S. J. Haque, *Oncotarget*, 2010, **1**, 289-303.
34. M. Schueler, Q. Zhang, J. Schlesinger, M. Tönjes, and S. R. Sperling, *Mol Biosyst*, **8**, 495-503.
35. C. Gu, J. Gonzalez, T. Zhang, S. Kamel-Reid, R. A. Wells RA, *Leuk Res*, 2013, **37**, 1750-1756.
36. J. Alonso, J. F. Santaren, *Journal of Proteome Research*, 2006, **5**, 2025-2032.
37. N. Aoyagi, D. A. Wassarman, *Journal of Cell Biology*, 2000, **150,** F45-F49.
38. P. S. Steeg, M. Zollo, and T. Wieland, *Naunyn Schmiedebergs Arch Pharmacol*, 2011, **384**,331-339.
39. T. Hsu, *Naunyn Schmiedebergs Arch Pharmacol*, 2011, **384**, 363-372.
40. R. S. Kamath, A. G. Fraser, Y. Dong, G. Poulin, R. Durbin, M. Gotta, A. Kanapin, N. Le Bot, S. Moreno, M. Sohrmann, D. P. Welchman, P. Ziperlen, and J. Ahringer, *Nature*, 2003, **421**, 231-237.

41. J. M. Tkach, A. Yimit, A. Y. Lee, M. Riffle, M. Costanzo, D. Jaschob, J. A. Hendry, J. Ou, J. Moffat, C. Boone, T. N. Davis, C. Nislow, and G. W. Brown, *Nature Cell Biology*, 2012, **14**, 966-976.
42. G. L. Li, X. H. Xu, B. A. Wang, Y. M. Yao, Y. Qin, S.R. Bai, J. Rong, T. Deng, Y. H. Hu, *Eur J Med Res*. 2014, **19** :15.
43. G. C. K. W. Koh, P. Porras, B. Aranda, H. Hermjakob, and S. E. Orchard, *Journal of proteome research*, 2012, **11**, 2014–31.
44. V. J. Hall, *Reproduction, fertility, and development*, 2012, **25**, 94–102.
45. M. Berk, S. Y. Desai, H. C. Heyman, and C. Colmenares, *Genes & development*, 1997, **11**, 2029–39.
46. O. Halevy and L. C. Cantley, *Experimental cell research*, 2004, **297**, 224–34.
47. A. Orian, B. van Steensel, J. Delrow, H. J. Bussemaker, L. Li, T. Sawado, E. Williams, L. W. M. Loo, S. M. Cowley, C. Yost, S. Pierce, B. A. Edgar, S. M. Parkhurst, and R. N. Eisenman, *Genes & development*, 2003, **17**, 1101–14.
48. P. C. Fernandez, S. R. Frank, L. Wang, M. Schroeder, S. Liu, J. Greene, A. Cocito, and B. Amati, *Genes & development*, 2003, **17**, 1115–29.
49. S. Adhikary and M. Eilers, *Nature reviews. Molecular cell biology*, 2005, **6**, 635–45.
50. L. Yu, J. Shi, S. Cheng, Y. Zhu, X. Zhao, K. Yang, X. Du, H. Klocker, X. Yang, and J. Zhang, *Molecular endocrinology (Baltimore, Md.)*, 2012, **26**, 1521–30.
51. M. Hochstrasser, *Annual review of genetics*, 1996, **30**, 405–39.
52. Z. Hu, B. Potthoff, C. P. Hollenberg, and M. Ramezani-Rad, *Journal of cell science*, 2006, **119**, 326–38.
53. D. A. Sinclair, K. Mills, and L. Guarente, *Science (New York, N.Y.)*, 1997, **277**, 1313–6.
54. D. Ma, J. R. McCorkle, and D. M. Kaetzel, *The Journal of biological chemistry*, 2004, **279**, 18073–84.
55. Z. Fan, P. J. Beresford, D. Y. Oh, D. Zhang, and J. Lieberman, *Cell*, 2003, **112**, 659–72.
56. D. M. Kaetzel, Q. Zhang, M. Yang, J. R. McCorkle, D. Ma, and R. J. Craven, *Journal of bioenergetics and biomembranes*, 2006, **38**, 163–7.
57. D. Chowdhury, P. J. Beresford, P. Zhu, D. Zhang, J.-S. Sung, B. Demple, F. W. Perrino, and J. Lieberman, *Molecular cell*, 2006, **23**, 133–42.
58. L. Garzia, C. Roma, N. Tata, D. Pagnozzi, P. Pucci, and M. Zollo, *Journal of bioenergetics and biomembranes*, 2006, **38**, 205–13.
59. F. Napolitano, F. D'Angelo, M. Bimonte, V. Perrina, C. D'Ambrosio, A. Scaloni, T. Russo, and N. Zambrano, *Neurochemical research*, 2008, **33**, 2547–55.
60. M. Boissan, S. Dabernat, E. Peuchant, U. Schlattner, I. Lascu, and M.-L. Lacombe, *Molecular and cellular biochemistry*, 2009, **329**, 51–62.
61. S. Benjaphokee, P. Koedrith, C. Auesukaree, T. Asvarak, M. Sugiyama, Y. Kaneko, C. Boonchird, and S. Harashima, *New biotechnology*, 2012, **29**, 166–76.
62. M. D. Pastor, A. Nogal, S. Molina-Pinelo, R. Meléndez, A. Salinas, M. González De la Peña, J. Martín-Juan, J. Corral, R. García-Carbonero, A. Carnero, and L. Paz-Ares, *Journal of proteomics*, 2013, **89**, 227–37.
63. E. Eigenbrodt, F. Kallinowski, M. Ott, S. Mazurek, and P. Vaupel, *Anticancer research*, **18**, 3267–74.
64. H. R. Christofk, M. G. Vander Heiden, M. H. Harris, A. Ramanathan, R. E. Gerszten, R. Wei, M. D. Fleming, S. L. Schreiber, and L. C. Cantley, *Nature*, 2008, **452**, 230–3.
65. J. P. Gnerer, R. A. Kreber, and B. Ganetzky, *Proceedings of the National Academy of Sciences of the United States of America*, 2006, **103**, 14987–93.
66. N. Miura, A. Kirino, S. Endo, H. Morisaka, K. Kuroda, M. Takagi, and M. Ueda, *Eukaryotic cell*, 2012, **11**, 1075–82.
67. M. Ralser, G. Heeren, M. Breitenbach, H. Lehrach, and S. Krobitsch, *PloS one*, 2006, **1**, e30.
68. M. Ralser, M. M. Wamelink, A. Kowald, B. Gerisch, G. Heeren, E. A. Struys, E. Klipp, C. Jakobs, M. Breitenbach, H. Lehrach, and S. Krobitsch, *Journal of biology*, 2007, **6**, 10.69. K. R. Lorenzatto, K. M. Monteiro, R. Paredes, G. P. Paludo, M. M. da Fonsêca, N. Galanti, A. Zaha, H. B. Ferreira. *Gene*. 2012, **506,** 76-84.

2.1.10. Supplementary material

S-Table 2.1. Pairs of orthologous proteins.

	C. elegans X *S. cerevisiae*		*C. elegans* X *D. melanogaster*		*D. melanogaster* X *S. cerevisiae*		*D. melanogaster* X *H. sapiens*		*H. sapiens* X *S. cerevisiae*		*H. sapens* X *C. elegans*	
Protein	*C. elegans*	*S. cerevisiae*	*C. elegans*	*D. melanogaster*	*D. melanogaster*	*S. cerevisiae*	*D. melanogaster*	*H. sapiens*	*H. sapiens*	*S. cerevisiae*	*H. sapiens*	*C. elegans*
Actin					Arp87C	ACT1	Arp87C	ACTB	ACTB	ACT1		
Adenylate kinase					Adk3	ADK2	Adk2	AK2	AK2	ADK1		
Aldehyde dehydrogenase									ALDH9A1	ALD3		
ATP synthase			atp-2	FBpp0088250								
CTP synthase	W06H3.3	URA7	W06H3.3	CG6854	CG6854	URA7						
Cytochrome C					Cyt-c-p	CYC1						
Deoxyribose-phosphate aldolase			F09E5.3	CG8525							DERA	F09E5.3
DNA helicase							rept	CHD8				
DNA-polymerase-delta					FBpp0075277	POL3			POLD1	POL3		
DNA primase									PRIM1	PRI1		
Enolase	enol-1	ENO1	enol-1	Eno	Eno	ENO1	Eno	ENO1	ENO3	ENO1	ENO1	enol-1
Elongation factor alpha					FBpp0087143	TEF1						
Elongation factor gamma					Ef1gamma	TEF4						
Fructose-bisphosphate aldolase			aldo-1	Ald			Ald	ALDOA			ALDOA	aldo-1
Glucose-6-phosphate dehydrogenase	gspd-1	ZWF1	gspd-1	Zw	Zw	ZWF1						
Glucose-6-Phosphate Isomerase	gpi-1	PGI1	gpi-1	Pgi			Pgi	GPI	GPI	PGI1	GPI	gpi-1
Glyceraldehyde 3-phosphate dehydrogenase	gpd-1	TDH1	gpd-2	Gapdh2	Gapdh2	TDH1	Gapdh1	GAPDH	GAPDH	TDH3	GAPDH	gpd-2
Guanylate kinase					CG11811	GUK1	CG11811	GUK1				
Heat shock protein					Hsc70-4	SSA1			HSPD1	HSP60		
Initiation factor							eIF4E-4	EIF4E				
Lactate dehydrogenase							ImpL3	LDHB				
Malic enzyme							Menl-2	ME1				
Nucleoside diphosphate kinase	F25H2.5	YNK1	F25H2.5	awd	awd	YNK1	awd	NME1				
Phosphoenolpyruvate carboxykinase			R11A5.4	CG10924			Pepck	PCK1			PCK2	R11A5.4
Phosphofructokinase					Pfk	PFK2	Pfk	PFKM				
Phosphoglucolsomerase					Pgi	PGI1						
Phosphoglucomutase									PGM1	PGM2		
Phosphoglycerate kinase	pgk-1	PGK-1	pgk-1	Pgk	Pgk	PGK1	Pgk	PGK1	PGK1	PGK1		
Phosphoglycerate mutase					Pglym78	GPM1	Pglym78	PGAM2				
Phosphomannose isomerase					CG8417	PMI40						
Polyribonucleotide nucleotidyltransferase							CG11337	PNPT1				
Porin					porin	POR1						
Proteasome regulatory particle	rpt-4	RPT1	rpt-4	Pros45								
Proteasome alpha subunit			pas-4	Pros28.1								
Pyruvate carboxylase					CG1516	PYC1	CG1516	PC	PC	PYC1		
Pyruvate dehydrogenase					CG11876	PDB1						
Pyruvate kinase	pyk-2	PYK2	pyk-2	PyK	PyK	PYK2	PyK	PKM2	PKM2	PYK2	PKM2	pyk-2
Receptor of activated C kinase			rack-1	Rack1								
Ribokinase					CG13369	RBK1						
Ribose 5-phosphate isomerase	rpia-1	RKI1			CG30410	RKI1						
Ribosomal protein, large subunit	rpl-3	RPL3	rpl-38	RpL38								
Ribosomal protein, small subunit			rps-23	RpS23								
Ribulose 5-phosphate epimerase	F08F8.7	RPE1	F08F8.7	CG30499	CG30499	RPE1						
Ribonucleoside-diphosphate reductase alpha subunit									RRM1	RNR1		
Ribonucleoside-diphosphate reductase beta subunit									RRM2	RNR2		
RNA polymerase I subunit							RpI135	POLR1B	POLR1B	RPA135		
RNA polymerase II subunit	rpb-2	RPB2	rpb-2	RpII140			Rpb10	POLR2L	POLR2L	RPB10	POLR2L	Y37E3.3
RNA polymerase III subunit	rpc-1	RPO31	rpc-1	CG17209	RpIII128	RET1	CG33785	POLR3K	POLR3A	RPO31	POLR3A	rpc-1
Small ubiquitin-related modifier									SUMO1	SMT3		
Succinate dehydrogenase					SdhB	SDH2						
Thioredoxin peroxidase					Jafrac1	TSA1			PRDX3	TSA1		
Transaldolase	Y24D9A.8	TAL1							TALDO1	TAL1	TALDO1	Y24D9A.8
Transketolase			tkt-1	CG8036	CG8036	TKL1	CG8036	TKT			TKT	tkt-1
Triose phosphate isomerase	tpi-1	TPI1	tpi-1	Tpi								
Ubiquitin							Ubi-p63E	UBC	UBB	UBI4		

S-Table 2.2. Specific gene ontology (GO) classes derived from PPI networks.

Organism	Cluster	Description	GO number	p-value	Corrected p-value	k	f	Proteins
H. sapiens	1	Transcription initiation from RNA polymerase II promoter	6367	4,52E-16	5,89E-14	12	67	POLR2H|POLR2G|POLR2F|POLR2E|POLR2L|POLR2K|POLR2J|POLR2I|POLR2D|POLR2C|POLR2B|POLR2A
	1	RNA splicing	8380	2,76E-08	3,99E-07	12	287	POLR2H|POLR2G|POLR2F|POLR2E|POLR2L|POLR2K|POLR2J|POLR2I|POLR2D|POLR2C|POLR2B|POLR2A
	2	transcription	6350	1,86E-18	1,47E-15	22	345	POLR3G|KAT2A|TAF1|SUPT3H|TAF4|MLL|POLR3K|POLR1D|TAF7|TP53|POLR1A|TAF2E|HCFC1|TBP|TAF2G|POLR1B|POLR3C|POLR3B|MAX|TAF10|TAF12|PELP1
	2	regulation of histone modification	31056	6,65E-01	4,81E+00	3	22	MLL|TAF7|TP53
	2	anterior/posterior pattern formation	9952	1,98E+01	1,06E+02	4	147	KAT2A|MLL|RNF2|TP53
	2	negative regulation of apoptosis	43066	2,33E+01	1,19E+02	6	376	NME5|CHD8|TP53|NMELV|TAF2G|NME6
	2	multicellular organism growth	35264	3,12E+01	1,52E+02	2	23	KAT2A|TP53
	2	negative regulation of cell size	45792	7,44E+01	3,05E+02	3	110	TP53|NPR1|NME6
	2	regulation of apoptosis	42981	1,12E+02	4,10E+02	8	852	KAT2A|NME5|CHD8|TP53|NMELV|TAF2G|ADCY10|NME6
	2	regulation of organelle organization	33043	1,15E+02	4,18E+02	4	242	MLL|TAF7|TP53|NME6
	3	glycolysis	6096	3,28E-25	2,41E-23	12	42	LDHC|ALDOA|GPI|LDHB|LDHAL6B|LDHAL6A|ALDOC|ALDOB|PDHA2|BPGM|PDHA1|PDHB
	4	regulation of transcription	45449	8,01E-06	1,16E-03	15	2622	E2F1|E2F2|E2F3|CTBP1|E2F4|RBL1|SMAD4|SMAD2|SKI|RB1|MAPK1|CDKN1B|MAPK3|TFDP2|TFDP1
	4	interphase of mitotic cell cycle	51329	9,65E-04	2,91E-02	5	102	E2F1|CDKN1A|CDKN1B|E2F4|RB1
	4	negative regulation of cell proliferation	8285	1,34E-03	3,73E-02	7	379	CDKN1A|CTBP1|CDKN1B|SMAD4|SKI|SMAD2|RB1
	4	negative regulation of growth	45926	1,25E-01	1,56E+00	4	126	CDKN1A|CDKN1B|SMAD4|RB1
	4	multicellular organismal development	7275	6,73E+00	4,19E+01	10	2971	E2F1|MAPK1|CDKN1A|E2F4|CCND2|MAPK3|SMAD4|SKI|SMAD2|RB1
	4	embryonic development ending in birth or egg hatching	9792	7,55E+00	4,47E+01	4	364	MAPK1|SMAD4|SKI|SMAD2
	4	positive regulation of apoptosis	43065	1,44E+02	3,88E+02	3	442	MAPK1|CDKN1A|CDKN1B
	4	organ growth	35265	2,12E+02	4,93E+02	1	18	SMAD2
	5	regulation of cell size	8361	2,14E+00	8,44E+01	4	219	AKT1|EDN1|RBBP7|IL2
	5	regulation of apoptosis	42981	8,25E+00	1,34E+02	6	852	AKT1|IL2RB|SIN3A|IL2RA|LCK|IL2

	5	regulation of growth	40008	1,37E+01	1,61E+02	4	359	AKT1|RBBP7|STAT3|IL2
	5	negative regulation of programmed cell death	43069	1,71E+01	1,80E+02	4	381	AKT1|IL2RB|SIN3A|IL2
	5	in utero embryonic development	1701	3,20E+01	2,46E+02	3	216	AKT1|SIN3A|EDN1
	6	cellular response to stress	33554	1,25E-04	9,77E-04	8	619	POLD3|POLD4|POLE2|POLD1|POLD2|POLE|POLA1|RRM2B
	6	S phase of mitotic cell cycle	84	1,60E-01	7,76E-01	2	7	POLD1|POLA1
	6	interphase of mitotic cell cycle	51329	9,56E-01	4,08E+00	3	102	POLD1|POLE|POLA1
	7	organ development	48513	2,33E-06	5,65E-04	15	1792	HNF1A|EPAS1|ESR1|TLE1|TCF7L2|ITGB1|SIRT1|TCF7L1|ARNT|EP300|AES|SP1|ITGA6|JUN|COL1A2
	7	multicellular organismal development	7275	2,97E-03	9,60E-02	15	2971	HNF1A|EPAS1|ESR1|TLE1|TCF7L2|ITGB1|SIRT1|TCF7L1|ARNT|EP300|AES|SP1|ITGA6|JUN|COL1A2
	7	cell differentiation	30154	3,12E-03	9,75E-02	12	1668	EP300|ITGA6|SP1|EPAS1|JUN|CREBBP|ESR1|SIRT1|ITGB1|TCF7L2|TCF7L1|ARNT
	7	response to oxidative stress	6979	4,42E-02	8,93E-01	5	184	EP300|HNF1A|EPAS1|JUN|ARNT
	7	embryonic development	9790	1,07E-01	1,68E+00	7	601	EP300|HNF1A|SP1|EPAS1|TCF7L2|TCF7L1|ARNT
	7	regulation of apoptosis	42981	8,26E+00	4,85E+01	6	852	JUN|ESR1|SIRT1|TCF7L2|TERT|BRCA1
	7	reproduction	3	4,35E+01	1,59E+02	5	809	HNF1A|SP1|ESR1|SIRT1|TCF7L2
	7	development of primary female sexual characteristics	46545	5,23E+01	1,81E+02	2	78	ESR1|SIRT1
	7	negative regulation of developmental process	51093	7,21E+01	2,16E+02	3	289	CREBBP|SIRT1|TCF7L2
	7	cell development	48468	1,04E+02	2,83E+02	4	632	EPAS1|CREBBP|ESR1|TCF7L2
D. melanogaster	1	cytoskeleton organization	7010	1,74E-11	4,05E-09	32	478	EB1|CG7033|14-3-3ZETA|CG10932|ACT57B|ARP87C|EIF-2BETA|ACT5C|CG11963|CCTGAMMA|CG8036|HEL25E|CG3731|EIF-4E|RAN|CG5525|TCP-1ETA|CG8142|EIF-4A|RPA-70|PROS29|EF2B|STA|CCT5|T-CP1|CG11876|CG8258|TSR|ACT42A|KHC|TER94|TCP-1ZETA
	1	mitotic spindle organization	7052	1,97E-08	1,67E-06	19	191	EB1|HEL25E|EIF-4E|CG7033|CG5525|TCP-1ETA|EIF-4A|CG10932|RPA-70|PROS29|EF2B|EIF-2BETA|STA|T-CP1|CCT5|CG11963|CCTGAMMA|CG8258|TCP-1ZETA
	1	aerobic respiration	9060	2,93E-05	1,05E-03	8	31	NC73EF|SDHB|CG11963|KDN|CG5214|CG6439|CG5028|L(1)G0156
	1	cell cycle	7049	6,81E-04	1,87E-02	25	598	EB1|CG7033|14-3-3ZETA|CG10932|EIF-2BETA|AWD|CG11963|CCTGA

								MMA|HEL25E|EIF-4E|RAN|CG5525|TCP-1ETA|EIF-4A|RPA-70|PROS29|EF2B|STA|CCT5|RFC40|T-CP1|MAD2|CG8258|TSR|TCP-1ZETA
	1	oocyte microtubule cytoskeleton polarization	8103	1,60E+01	1,69E+02	3	19	14-3-3ZETA**|KHC**|TER94
	1	esponse to DNA damage stimulus	6974	1,91E+01	2,00E+02	8	181	PROS45|RFC40|PROSALPHA7|TRXR-1|EIF-4A|PROS28.1|JAFRAC1|RPS3
	1	chromosome segregation	7059	4,91E+01	4,63E+02	6	126	EB1|TOP1|RFC40|EIF-4E|RAN|14-3-3ZETA
	2	mitotic spindle elongation	22	5,31E-64	3,25E-62	39	74	RPL18|RPL17|RPL36A|RPL10AB|RPL19|RPL14|RPL13|RPL35|RPL39|RPS4|RPL30|RPS5A|RPL7|RPL32|RPS3A|RPL6|RPL31|RPL9|RPL3|RPL11|RPL7A|RPL12|EIF-1A|RPL26|RPL27|RPS9|QM|RPL24|RPL28|RPS18|RPL18A|RPL23|RPS16|RPL22|RPL21|RPS15|RPS30|RPS13|RPS11
	2	microtubule cytoskeleton organization	226	7,77E-36	1,36E-34	39	309	RPL18|RPL17|RPL36A|RPL10AB|RPL19|RPL14|RPL13|RPL35|RPL39|RPS4|RPL30|RPS5A|RPL7|RPL32|RPS3A|RPL6|RPL31|RPL9|RPL3|RPL11|RPL7A|RPL12|EIF-1A|RPL26|RPL27|RPS9|QM|RPL24|RPL28|RPS18|RPL18A|RPL23|RPS16|RPL22|RPL21|RPS15|RPS30|RPS13|RPS11
	2	negative regulation of autophagy	10507	2,92E+01	2,31E+02	2	11	SEC61BETA|SRP54K
	3	transcription from RNA polymerase II promoter	6366	4,33E-08	8,42E-07	9	93	RPB11|RPII15|RPII18|RPB10|RPB8|RPB5|RPB7|RPII215|RPII140
	3	microtubule cytoskeleton organization	226	6,65E-06	1,06E-04	11	309	DEBB|TSU|STC|CG13298|CG11985|DIM1|SMD3|MAGO|NOI|SMB|SNRNP70K
	3	regulation of RNA splicing	43484	4,01E-01	3,90E+00	4	65	CG10418|SNF|HRB87F|SNRNP70K
	3	oocyte anterior/posterior axis specification	7314	1,74E+01	1,11E+02	3	80	TSU|STC|MAGO
	4	autophagic cell death	48102	6,39E+01	2,51E+02	2	73	EIF4E-5|CG9769
	5	transcription from RNA polymerase III promoter	6383	3,30E-05	3,49E-04	4	12	CG17209|CG5380|RPIII128|CG7339
	6	multicellular organismal aging	10259	2,55E+02	4,12E+02	2	100	TPI|LEVY
	6	determination of adult lifespan	8340	2,55E+02	4,12E+02	2	100	TPI|LEVY
	7	transcription initiation from RNA polymerase II promoter	6367	3,11E-07	7,71E-06	6	64	TAF10|TAF10B|TAF13|TFIIEBETA|TFIIA-S|TFIIB
	7	RNA elongation from RNA polymerase II promoter	6368	1,79E-01	8,86E-01	2	6	CG8117|TFIIS

S. cerevisiae	1	RNA biosynthetic process	32774	7,11E-33	1,03E-30	29	230	RPC40|RPA190|RET1|RPA34|RPC25|RPA14|RPO31|RPA12|RPC53|RPB9|RPA135|RPO21|RPB8|RPB5|RPO26|RPB4|RPB7|RPA49|RPB2|RPB11|RPB3|RPB10|RPC19|RPC34|RPC82|RPA43|RPC31|RPC10|RPC11
	1	gene expression	10467	2,89E-06	4,21E-05	29	1921	RPC40|RPA190|RET1|RPA34|RPC25|RPA14|RPO31|RPA12|RPC53|RPB9|RPA135|RPO21|RPB8|RPB5|RPO26|RPB4|RPB7|RPA49|RPB2|RPB11|RPB3|RPB10|RPC19|RPC34|RPC82|RPA43|RPC31|RPC10|RPC11
	1	transcription initiation	6352	2,01E-01	2,25E+00	5	61	RPC25|RPB9|RPC31|RPB4|RPB7
	1	transcription termination	6353	2,39E-01	2,58E+00	4	31	RPB11|RPB3|RPA12|RPC11
	2	cell death	8219	1,02E-06	1,74E-05	10	52	QCR2|PET9|COX6|MIR1|COX4|COR1|SDH4|COX5A|CYT1|RIP1
	2	cellular response to oxidative stress	34599	8,43E+01	3,36E+02	4	76	CTT1|GRE3|YJR096W|HSP12
	3	protein import into mitochondrial matrix	30150	8,96E-17	3,21E-14	10	20	PAM18|PAM17|PAM16|TIM21|MGE1|TIM23|TIM50|TIM44|TIM17|SSC1
	3	organelle organization	6996	1,12E+00	1,50E+01	13	1235	TIM50|STM1|PAM18|PAM17|PAM16|TIM21|MGE1|RPL3|GRS1|TIM23|TIM17|TIM44|SSC1
	3	age-dependent general metabolic decline involved in replicative cell aging	1321	3,71E+01	2,95E+02	1	1	HSP104
	3	regulation of biological quality	65008	5,76E+01	4,39E+02	6	465	PRS3|DED81|ASC1|SSC1|RPL31B|STM1
	4	posttranscriptional regulation of gene expression	10608	1,00E+00	5,32E+00	7	206	SSB2|RPL13A|TEF4|YEF3|EFT1|RPL7B|WRS1
	4	DNA-dependent DNA replication	6261	1,43E+01	5,67E+01	4	91	POL12|POL1|PRI2|PRI1
	4	regulation of vacuole fusion, non-autophagic	32889	2,19E+01	8,32E+01	2	13	ENO2|ENO1
	4	regulation of vacuole organization	44088	2,19E+01	8,32E+01	2	13	ENO2|ENO1
	4	apoptosis	6915	6,28E+01	2,21E+02	2	22	TDH3|TDH2
	5	response to stress	6950	9,80E-04	1,01E-01	12	704	SLX5|WSS1|HSC82|SGS1|AOS1|SGT2|STI1|RAD52|SSA1|SLX8|HSP82|UBI4
	5	response to stimulus	50896	3,12E-03	2,40E-01	13	965	WSS1|AOS1|MDY2|STI1|SSA1|RAD52|HSP82|UBI4|SLX5|HSC82|SGS1|SGT2|SLX8
	5	protein insertion into membrane	51205	5,75E+00	8,05E+01	2	12	MDY2|YOR164C
	5	regulation of organelle organization	33043	5,83E+01	3,77E+02	3	123	HSC82|ULP2|HSP82
	5	negative regulation of cell cycle process	10948	7,78E+01	4,36E+02	2	44	SGS1|ULP2

	5	reproductive developmental process	3006	9,72E+01	4,99E+02	3	148	SGS1|MDY2|UBI4
	6	cellular cell wall organization	7047	5,08E-04	2,14E-02	7	254	TOR2|RHO1|PKC1|SLT2|GAS1|SWI4|SPA2
	6	actin cytoskeleton organization	30036	2,33E-01	4,39E+00	4	105	TOR2|RHO1|PKC1|SPA2
	6	actin filament organization	7015	1,28E+00	1,53E+01	3	59	TOR2|PKC1|SPA2
	6	autophagy	6914	1,31E+00	1,53E+01	4	163	MKK1|MKK2|PKC1|SLT2
	6	reproduction	3	4,91E+00	4,50E+01	5	427	RHO1|PKC1|SLT2|SWI4|SPA2
	6	growth	40007	1,59E+01	9,24E+01	3	139	RHO1|GAS1|SPA2
	6	actin filament reorganization involved in cell cycle	30037	7,07E+01	2,89E+02	1	4	TOR2
	6	asexual reproduction	19954	9,62E+01	3,67E+02	2	86	RHO1|SPA2
	6	chromatin silencing	6342	1,24E+02	4,19E+02	2	98	SIR2|GAS1
	7	DNA replication	6260	1,11E-03	2,74E-02	6	143	RNR1|POL30|RNR2|POL32|RNR3|RNR4
	7	postreplication repair	6301	5,75E+00	5,29E+01	2	19	POL30|POL32
C. elegans	1	growth	40007	1,04E-36	1,45E-35	62	1334	RPL-18|RPL-19|RPL-16|RPL-17|RPL-14|RPL-15|RPL-13|RPS-30|RPL-22|RPL-21|RPL-20|RPL-11.2|RPL-27|RPL-28|RPL-23|RPL-26|RACK-1|RPS-7|RPL-31|RPL-24.1|RPL-30|RPS-5|RPL-33|RPL-32|RPS-3|RPS-2|RPS-1|RPS-0|RPL-36|RPS-8|RPS-11|RPS-12|RPS-9|RPL-34|RPL-25.1|RPS-13|RPL-35|RPS-14|RPS-15|RPS-16|RPL-38|RPS-17|RPL-39|RPS-10|RPL-43|RPL-41|RPS-24|RPL-1|RPS-22|RPS-23|RPL-5|RPL-6|RPS-29|RPS-26|RPL-3|RPS-27|RPL-9|RPL-7|RPS-20|RPS-21|RPL-10|RPS-19
	1	larval development	2164	5,54E-33	6,05E-32	64	1712	RPL-18|RPL-19|RPL-16|RPL-17|RPL-14|RPL-15|RPL-12|RPL-13|RPS-30|RPL-22|RPL-21|RPL-20|RPL-11.2|RPL-27|RPL-28|RPL-23|RPL-26|RACK-1|RPS-7|RPL-31|RPL-24.1|RPS-6|RPL-30|RPS-5|RPL-33|RPL-32|RPS-3|RPS-2|RPS-1|RPS-0|RPL-36|RPS-8|RPS-11|RPS-12|RPS-9|RPL-34|RPL-25.1|RPS-13|RPL-35|RPS-14|RPS-15|RPS-16|RPL-38|RPS-17|RPL-39|RPS-10|RPL-43|RPL-41|RPL-1|RPS-24|RPS-22|RPS-23|RPL-5|RPL-6|RPS-29|RPS-26|RPL-3|RPS-27|RPL-9|RPL-7|RPS-20|RPS-21|RPL-10|RPS-19

	1	reproduction	3	1,83E-25	1,65E-24	63	2146	RPL-18|RPL-19|RPL-17|RPL-14|RPL-15|RPL-12|RPL-13|RPS-30|RPL-22|RPL-21|RPL-20|RPL-11.2|RPL-27|RPL-23|RPL-26|RACK-1|RPS-7|RPL-31|RPL-24.1|RPS-6|RPL-30|RPS-5|RPL-33|RPL-32|RPS-3|RPS-2|RPS-1|RPS-0|RPL-36|RPS-8|RPS-11|RPS-12|RPL-25.2|RPS-9|RPL-25.1|RPS-13|RPL-35|RPS-14|RPS-15|RPS-16|RPL-38|RPS-17|RPS-18|RPL-39|RPS-10|RPL-41|RPS-24|RPL-1|RPS-22|RPS-23|RPL-5|RPL-6|RPS-29|RPS-26|RPL-3|RPS-27|RPL-4|RPL-9|RPL-7|RPS-20|RPS-21|RPL-10|RPS-19
	1	multicellular organismal development	7275	9,65E-21	7,77E-20	70	3604	RPL-18|RPL-19|RPL-16|Y37E3.8|RPL-17|RPL-14|RPL-15|RPL-12|RPL-13|RPS-30|RPL-22|RPL-21|RPL-20|RPL-11.2|RPL-27|RPL-28|RPL-23|RPL-26|RACK-1|RPS-7|RPL-31|RPL-24.1|RPS-6|RPL-30|RPL-33|RPS-5|RPL-32|RPS-4|RPS-3|RPS-2|RPS-1|RPS-0|RPL-36|RPS-8|RPS-11|RPS-12|RPL-25.2|RPS-9|RPL-34|RPL-25.1|RPS-13|RPL-35|RPS-14|RPS-15|RPS-16|RPL-38|RPS-17|RPL-39|RPS-10|RPL-43|RPL-41|RPL-1|RPS-24|RPS-25|RPS-22|RPS-23|RPL-5|RPL-6|RPS-29|RPS-26|RPL-3|RPS-27|RPL-4|RPL-9|RLA-2|RPL-7|RPS-20|RPS-21|RPL-10|RPS-19
	1	embryonic development ending in birth or egg hatching	9792	1,31E-20	1,00E-19	63	2581	RPL-18|RPL-19|RPL-16|Y37E3.8|RPL-17|RPL-14|RPL-15|RPL-12|RPL-13|RPL-22|RPL-21|RPL-20|RPL-27|RPL-28|RPL-23|RPL-26|RACK-1|RPS-7|RPL-31|RPL-24.1|RPL-30|RPS-5|RPL-33|RPS-4|RPS-3|RPS-2|RPS-1|RPS-0|RPL-36|RPS-11|RPS-12|RPL-25.2|RPS-9|RPL-34|RPL-25.1|RPS-13|RPL-35|RPS-14|RPS-15|RPS-16|RPS-17|RPL-39|RPS-10|RPL-43|RPL-41|RPL-1|RPS-25|RPS-22|RPS-23|RPL-5|RPL-6|RPS-29|RPS-26|RPL-3|RPS-27|RPL-4|RPL-9|RLA-2|RPL-7|RPS-20|RPS-21|RPL-10|RPS-19
	1	embryonic development	9790	4,25E-20	3,09E-19	63	2632	RPL-18|RPL-19|RPL-16|Y37E3.8|RPL-17|RPL-14|RPL-15|RPL-12|RPL-13|RPL-22|RPL-21|RPL-20|RPL-27|RPL-28|RPL-23|RPL-26|RACK-1|RPS-7|RPL-31|RPL-24.1|RPL-30|RPS-5|RPL-33|RPS-4|RPS-3|RPS-2|RPS-1|RPS-0|RPL-36|RPS-11|RPS-12|RPL-25.2|RPS-9|RPL-34|RPL-

								25.1|RPS-13|RPL-35|RPS-14|RPS-15|RPS-16|RPS-17|RPL-39|RPS-10|RPL-43|RPL-41|RPL-1|RPS-25|RPS-22|RPS-23|RPL-5|RPL-6|RPS-29|RPS-26|RPL-3|RPS-27|RPL-4|RPL-9|RLA-2|RPL-7|RPS-20|RPS-21|RPL-10|RPS-19
	1	developmental process	32502	1,43E-19	9,93E-19	70	3747	RPL-18|RPL-19|RPL-16|Y37E3.8|RPL-17|RPL-14|RPL-15|RPL-12|RPL-13|RPS-30|RPL-22|RPL-21|RPL-20|RPL-11.2|RPL-27|RPL-28|RPL-23|RPL-26|RACK-1|RPS-7|RPL-31|RPL-24.1|RPS-6|RPL-30|RPL-33|RPS-5|RPL-32|RPS-4|RPS-3|RPS-2|RPS-1|RPS-0|RPL-36|RPS-8|RPS-11|RPS-12|RPL-25.2|RPS-9|RPL-34|RPL-25.1|RPS-13|RPL-35|RPS-14|RPS-15|RPS-16|RPL-38|RPS-17|RPL-39|RPS-10|RPL-43|RPL-41|RPL-1|RPS-24|RPS-25|RPS-22|RPS-23|RPL-5|RPL-6|RPS-29|RPS-26|RPL-3|RPS-27|RPL-4|RPL-9|RLA-2|RPL-7|RPS-20|RPS-21|RPL-10|RPS-19
	1	molting cycle, collagen and cuticulin-based cuticle	18996	2,56E-11	1,51E-10	20	240	RPL-18|RPL-27|RPS-11|RPS-22|RPS-23|RPS-14|RPL-14|RPL-23|RPS-16|RPL-15|RPS-21|RPS-10|RPL-31|RPS-6|RPS-19|RPL-32|RPS-3|RPS-1|RPL-41|RPS-0
	1	positive regulation of growth	45927	5,92E-06	3,24E-05	39	1831	RPS-12|RPL-25.2|RPS-9|RPL-34|RPL-25.1|RPL-17|RPS-14|RPL-14|RPS-15|RPL-15|RPL-12|RPL-38|RPS-17|RPL-39|RPS-30|RPL-22|RPL-20|RPL-11.2|RPL-43|RPL-41|RPL-1|RPS-25|RPS-22|RPL-5|RPL-6|RPS-29|RPL-3|RPS-27|RPL-26|RLA-2|RPL-7|RPS-20|RPS-21|RACK-1|RPL-10|RPL-24.1|RPL-30|RPL-33|RPS-0
	1	multicellular organismal aging	10259	2,18E-02	9,80E-02	18	626	RPS-8|RPS-11|RPL-19|RPS-9|RPL-34|RPS-22|RPS-23|RPS-15|RPL-6|RPS-26|RPS-27|RPL-4|RPL-9|RPS-10|RPL-30|RPS-6|RPS-5|RPS-3
	1	determination of adult lifespan	8340	2,18E-02	9,80E-02	18	626	RPS-8|RPS-11|RPL-19|RPS-9|RPL-34|RPS-22|RPS-23|RPS-15|RPL-6|RPS-26|RPS-27|RPL-4|RPL-9|RPS-10|RPL-30|RPS-6|RPS-5|RPS-3
	1	response to temperature stimulus	9266	1,57E+01	6,51E+01	4	61	RPS-22|RPS-15|RPL-9|RPS-6
	1	positive regulation of multicellular organism growth	40018	2,54E+01	9,96E+01	8	289	RPL-25.1|RPL-15|RPS-17|RPL-39|RPL-22|RPL-30|RPS-20|RPL-41
	2	embryonic development	9790	1,75E-02	1,70E-01	13	2632	W01G7.3|AMA-1|Y37E3.3|W06H3.3|RPB-

							8|H27M09.2|RPB-7|RPB-6|RPB-4|RPB-3|RPB-2|RPB-12|F25H2.5
2	gastrulation	7369	4,61E+00	2,37E+01	3	98	RPB-7|AMA-1|RPB-3
2	nematode larval development	2119	2,12E+01	8,99E+01	8	1711	RPB-8|RPB-7|W01G7.3|RPB-3|RPB-2|W06H3.3|RPB-12|F25H2.5
2	growth	40007	2,49E+01	1,00E+02	7	1334	RPB-8|RPB-7|W01G7.3|RPB-3|W06H3.3|RPB-12|F25H2.5
2	reproductive developmental process	3006	6,62E+01	1,95E+02	5	806	H27M09.2|RPB-8|W01G7.3|RPB-12|F25H2.5
2	organ development	48513	1,24E+02	3,40E+02	5	935	H27M09.2|RPB-8|W01G7.3|RPB-12|F25H2.5
2	regulation of growth	40008	1,82E+02	4,68E+02	7	1890	RPB-8|W01G7.3|AMA-1|RPB-3|RPB-2|W06H3.3|RPB-12
3	multicellular organismal aging	10259	4,57E-01	2,84E+00	6	626	GPI-1|ENOL-1|TPI-1|F57B10.3|GPD-2|GPD-3
3	positive regulation of multicellular organism growth	40018	4,90E+01	2,19E+02	3	289	GPD-1|ENOL-1|GPD-4
3	multicellular organismal development	7275	7,75E+01	2,49E+02	9	3604	Y43F4B.5|GPD-1|GPI-1|ENOL-1|TPI-1|F57B10.3|GPD-4|GPD-2|GPD-3
3	regulation of multicellular organism growth	40014	1,00E+02	3,14E+02	3	374	GPD-1|ENOL-1|GPD-4
3	embryonic development ending in birth or egg hatching	9792	2,02E+02	4,56E+02	7	2581	Y43F4B.5|GPD-1|GPI-1|ENOL-1|F57B10.3|GPD-4|GPD-3
4	growth	40007	4,40E+00	1,06E+01	5	1334	H28O16.1|Y82E9BR.3|ATP-2|ATP-3|F58F12.1
4	determination of adult lifespan	8340	6,28E+01	1,23E+02	3	626	H28O16.1|ATP-2|ATP-3
4	aging	7568	6,34E+01	1,23E+02	3	628	H28O16.1|ATP-2|ATP-3

S-Table 2.3. Clusters sizes for each biological function in the four analyzed species.

Function	*H. sapiens*	*D. melanogaster*	*C. elegans*	*S. cerevisiae*
Transcription	37	-	-	35
Growth	56	-	6	11
Glycolysis	16	-	-	-
Embryonic development	17	35	75	-
Apoptosis	20	-	-	34
Cell death	-	16	-	66
Aging	-	-	-	23
DNA replication	13	-	-	12
Mitotic cell cycle	-	132	-	-
Mitotic cell cycle	-	79	-	-
Development	21	-	16	19
Multicellular organismal aging	-	-	15	-

3. CAPÍTULO 2 - Busca por funções *moonlighting* da Enolase 1 de *E. granulosus*

3.1. Apresentação

O capítulo 2 tem como objetivo relacionar os resultados obtidos no Capítulo 1 com a análise das interações de proteínas ocorridas no *E. granulosus*. Desta forma, esta seção tem como objetivo identificar funções *moonlighting* da EgEno1 conservadas em eucariotos e espécie-específica do parasito.

3.2. Materiais e métodos

3.2.1. Alinhamento múltiplo de sequências ortólogas de enolases para futuras análises de interação *in sílico*

O alinhamento múltiplo das sequências das enolases de *H. sapiens*, *D. melanogaster, C. elegans e S. cerevisiae,* juntamente com a EgEno1. A realização do alinhamento foi através do Clustal2.1 (http://www.ebi.ac.uk/Tools/msa/clustalw2) utilizando a matriz de Gonnet.

3.2.2. Ensaios de *cross-linking* para identificação de proteínas de interação com a EgEno1

As proteínas foram identificadas através da realização de ensaios de *cross-linking* utilizando Sulfo-SBED (Pierce, Thermo Scientific). Onde a proteína EgEno1 recombinante, disponívl no laboratório (Lorenzatto *et al*., 2012), foi ligada a uma das extremidades do Sulfo-SBED. Posteriormente, o extrato proteico do parasito em sua fase pré-adulta (protoescólices) foi incubado com o complexo proteína recombinante-Sulfo-SBED e expostos a luz ultravioleta para ativação do grupamento azida-arila na outra extremidade do crosslinker, para que ele possa interagir com proteínas ligantes associadas. Após a interação, o complexo teve sua ligação dissulfeto reduzida e as proteínas ligadas à proteína recombinante ficaram marcadas com biotina, podendo então ser recuperadas por cromatografia de afinidade em coluna de avidina (Monomeric Avidin Resin, Pierce, Thermo Scientific). As proteínas de

interação eluídas da coluna de afinidade foram identificadas por espectrometria de massas (LC-ESI-MS/MS).

3.3. Resultados

3.3.1. Identificação das proteínas ortólogas da EgEno1

A identificação das proteínas ortólogas à EgEno1 nos organismos *H. sapiens*, *D. melanogaster, C. elegans e S. cerevisiae*n, foi realizada através de alinhamento múltiplo das sequências proteicas recuperadas no banco de dados de proteínas do NCBI (http://www.ncbi.nlm.nih.gov/protein/). O resultado do alinhamento múltiplo está descrito através de uma matriz de identidade na Tabela 3.1. Desta forma foram selecionadas a enolase α de *H. sapiens*; enolase, isoforma A, de *D. melanogaster*; enolase1, isoforma A, de *C. elegans*; e a enolase de *S. cerevisiae*.

Tabela 3.1. Matriz de percentual de identidade criada pelo Clustal2.1. A matriz demonstra os valores de identidade gerado pelo alinhamento entre cada par de sequências. A sequência 9 corresponde à EgEno1, e os melhores resultados de identidade para cada um dos outros organismos estão destacados em negrito.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1: Enolase1,isoforma B (*C. elegans*)	**-**	100.00	100.00	73.21	72.02	74.11	72.32	72.32	72.92	65.88
2: Enolase1, isoforma C (*C. elegans*)	100.00	**-**	100.00	73.67	73.21	75.29	73.90	70.26	74.83	65.67
3: Enolase1, isoforma A (*C. elegans*)	100.00	100.00	**-**	73.67	73.21	75.29	73.90	73.90	74.83	65.67
4: Enolase γ (*H. sapiens*)	73.21	73.67	73.67	**-**	83.41	83.41	72.69	72.69	71.53	60.83
5: Enolase β (*H. sapiens*)	72.02	73.21	73.21	83.41	**-**	83.41	72.92	72.92	72.69	62.67
6: Enolase α (*H. sapiens*)	74.11	75.29	75.29	83.41	83.41	**-**	72.92	72.92	74.77	62.21
7: Enolase,isoforma A (*D.melanogaster*)	72.32	73.90	73.90	72.69	72.92	72.92	**-**	100.00	71.36	63.74
8: Enolase,isoforma B (*D. melanogaster*)	72.32	70.26	73.90	72.69	72.92	72.92	100.00	**-**	71.36	63.74
9: Enolase1 (*E. granulosus*)	72.92	74.83	**74.83**	71.53	72.69	**74.77**	**71.36**	71.36	**-**	**66.28**
10: Enolase (*S. cerevisiae*)	65.88	65.67	65.67	60.83	62.67	62.21	63.74	63.74	66.28	**-**

1: gi|25815065|emb|CAD57704.1| Protein ENOL-1, isoform b [Caenorhabditis elegans]

2: gi|51011814|emb|CAH10783.1| Protein ENOL-1, isoform c [Caenorhabditis elegans]

3: gi|3879986|emb|CAA92692.1| Protein ENOL-1, isoform a [Caenorhabditis elegans]

4: gi|5803011|ref|NP_001966.1| gamma-enolase [Homo sapiens]

5: gi|301897469|ref|NP_001967.3| beta-enolase isoform 1 [Homo sapiens]

6: gi|1167843|emb|CAA34360.1| alpha-enolase [Homo sapiens]

7: gi|22945470|gb|AAN10458.1| enolase, isoform A [Drosophila melanogaster]

8: gi|22945466|gb|AAN10455.1| enolase, isoform B [Drosophila melanogaster]

9: gi|262192839|gb|ACY30465.1| enolase [Echinococcus granulosus]

10: gi|171457|gb|AAA88713.1| enolase [Saccharomyces cerevisiae]

3.3.2. Identificação de proteínas de interação com a EgEno1 por LC-ESI-MS/MS

Para a investigação de proteínas que interagem com a EgEno1 no próprio parasito, foi realizado um experimento de *cross-linking* da EgEno1 com o extrato proteico de protoescólices. Assim, foi possível a identificação de algumas proteínas de interação que estão descritas na Tabela 3.1.

Tabela 3.2. Proteínas de interação com a EgEno1 em protoescólices

Proteína	Código*	GO**
Adenosil-homocisteinase	EgrG_000076900	one-carbon metabolic process
Carbonil-redutase 1	EgrG_000113500	-
Fator de alongamento 1 α	EgrG_000982200	Tradução
Ndr	EgrG_001065500	-
Peróxido-redutase tioredoxina-dependente	EgrG_000913300	oxidação-redução
Proteína de choque térmico (membro HSP 3)	EgrG_000249600	resposta ao estresse
Proteína do complexo T	EgrG_000683800	enovelamento de proteínas
Transaldolase	EgrG_000092800	processo metabólico carboidrato
Catepsina D	EgrG_000970500	proteólise
Isocitrato-desidrogenase	EgrG_000344100	oxidação-redução
Malato-desidrogenase	EgrG_001185000	oxidação-redução
Profilina	EgrG_000122100	organização do citoesqueleto de actina
Aldeído-desidrogenase (membro A3 da família 1)	EgrG_000389100	oxidação-redução
Proteína de membrana basal (heparana sulfato)	EgrG_000575900	-

* Código das proteínas acessados no endereço http://www.uniprot.org/

** Ontologia do GO gerada através de anotação automática. Proteínas sem função determinada estão marcadas com um traço.

4. DISCUSSÃO

A enolase catalisa a transformação reversível entre 2-fosfoglicerato e fosfoenolpiruvato. Em vertebrados, existem três subunidades de enolase, α, β e γ, sendo cada uma codificada por um gene. Esta enzima exerce sua atividade enzimática geralmente na forma de homodímeros (dímeros de enolase αα, ββ ou γγ). A enolase αα existe na maioria dos tecidos, a enolase ββ é específica de tecidos musculares e a enolase γγ é específica de tecidos neuronais (Gan *et al*., 2010). A enolase pode também estar envolvida na regulação da transcrição gênica em uma ampla variedade de organismos (Kim *et al*., 2005). Além disso, ela foi identificada como uma proteína estrutural do cristalino de vertebrados (Wistow *et al*., 1988), e pode regular a morfologia celular e o tráfego de moléculas através de interações com o citoesqueleto, em células musculares murinas (Keller *et al*., 2007).

Em organismos parasitas, algumas funções alternativas desempenhadas por esta proteína já foram descritas. A enolase pode estar presente no núcleo, regulando a transcrição de genes relacionados à proliferação e diferenciação celular de protozoários parasitas (Ferguson *et al*., 2002; Holmes *et al*.. 2010; Gómez-Arreaza *et al*., 2014). Em *Streptococcus sobrinus* a enolase é descrita como uma proteína com funções glicolíticas dentro da célula e um fator imunossupressor fora da célula do organismo, e a enolase secretada pelo helminto parasita *Steinernema glaseri* pode suprimir a resposta imune de insetos (Veiga-Malta *et al*., 2004; Liu *et al*., 2012)

Estudos proteômicos anteriores realizados em por nosso grupo de pesquisa mostrou proteínas secretadas pelo *E. granulosus* em sua fase larval, cisto hidático. Através destes estudos, foi possível identificar a EgEno1 como uma proteína no componente extracelular do cisto, líquido hidático, indicando que esta proteína pode estar sendo secretada (Monteiro *et al*., 2010). Mais recentemente, a EgEno1 foi identificada no tegumento do parasito em sua fase pré-adulta, protoescólices (Lorenzatto et al., 2012).

Com o intuito de identificar interações conservadas da enolase com proteínas não glicolíticas, foi realizada uma análise de biologia de sistemas. Através da geração de redes de PPI é possível unir informações de interação de diferentes estudos contidas em banco de dados. Dessa forma, um fator limitante para a geração de uma rede de PPI robusta é a quantidade de dados disponíveis. Como o *E. granulosus* não possui um banco de dados com grande quantidade de informações de PPI, a geração de uma rede de PPI para este organismo torna-se inacessível.

As funções *moonlighting* podem ser funções específicas de um organismo, porém, também é possível que estas funções sejam evolutivamente conservadas (Huberts *et al*., 2010a). Assim, é possível que alguma função *moonlighting* da EgEno1 seja conservada evolutivamente. Com isso, foram escolhidos quatro organismos eucarióticos: *H. sapiens*, *D. melanogaster*, *C. elegans* e *S. cerevisiae*, que possuem bancos de dados com quantidade suficiente de informações para a construção de redes de PPI robustas e confiáveis. A comparação destas redes podem indicar possíveis processos conservados evolutivamente e, dessa forma, funções *moonlighting* da EgEno1.

Para que o resultado da análise de biologia de sistemas pudesse ser comparável a processos que possam estar ocorrendo em *E. granulosus,* foram selecionadas as isoformas da enolase de cada organismo, mais compatíveis com a EgEno1. Os resultados e discussão da análise das quatro redes de PPI estão descritos na sessão 2 (Capítulo 1).

Experimentos preliminares realizados em nosso laboratório apontam as proteínas de interação da EgEno1 (Tabela 3.2) (Lorenzatto *et al*., dados não publicados). Entre as proteínas de interação com a EgEno1, é possível observar a interação desta com a proteína fator de alongamento 1 α. Interessantemente, esta interação foi observada na rede de PPI de *S. cerevisiae*, onde a proteína enolase α está diretamente conectada à proteína TEF2. Em seres humanos, tanto a enolase como a proteína fator de alongamento 1 α são descritos como possíveis proteínas secretadas por exossomos (Yu *et al*., 2006).

Um resultado mais interessante é a interação entre a enolase e a transaldolase. Esta interação é observada em todas as redes de PPI geradas neste trabalho, além de se repetir no parasito *E. granulosus*, ver Tabela 3.2. Na bactéria *Bifidobacterium bifidum*, parasita humano, foi descrito que tanto a enolase como a transaldolase são secretadas pela bactéria e atuam na facilitação da adesão desta, no intestino humano (González-Rodríguez *et al*., 2012).

As funções moleculares das proteínas de interação com a EgEno1 foram recuperadas do GO. Este resultado mostrou que as proteínas de interação com a enolase do parasito não possuem as principais funções observadas nas redes de PPI, com exceção da proteína de choque térmico que está relacionada à resposta ao estresse,

Os resultados do experimento de interação de proteínas com a EgEno1 ainda são considerados preliminares. Por tanto, outros experimentos serão realizados a fim de identificar outras possíveis proteínas de interação. Além disso, a utilização de novas metodologias de experimentos de PPI possibilitará avaliar a reprodutibilidade dos resultados obtidos, e posteior comparação com as redes de PPI modelo.

Apesar do experimento não ter sido realizado em organismos parasitas, é possível observar que há semelhanças entre as proteínas de interação em *E. granulosus* e os demais organismos. Apesar de estas similaridades serem poucas, algumas diferenças também podem ser consideradas alvos importantes para o estudo de funções *moonlighting* específicas do parasito. Além disso, diferenças entre os organismos estudados neste trabalho são esperadas, uma vez que se trata de organismos com considerável distância evolutiva.

Por fim, as semelhanças identificadas entre as redes de PPI e o *E. granulosus* podem ser consideradas interações evolutivamente conservadas, podendo estas estarem relacionadas a funções *moonlighting* da enolase. Além disso, a identificação de interações da EgEno1 com proteínas não relacionadas

à glicólise e não identificadas nas redes de PPI podem possuir relevância em aspectos espécie-específicos ou mesmo relacionadas ao parasitismo.

REFERÊNCIAS BIBLIOGRÁFICAS

Battelli, G. (2009). Echinococcosis: costs, losses and social consequences of a neglected zoonosis. Vet. Res. Commun. Suppl 1:47-52.

Benner, C., Carabin, H., Sánchez-Serrano, L. P., Budke, C. M., Carmena, D. (2010). Analysis of the economic impact of cystic echinococcosis in Spain. Bull World Health Organ. Erratum in: Bull World Health Organ. 236, 49-57.

Bernal, D., de la Rubia, J. E., Carrasco-Abad, A. M., Toledo, R., Mas-Coma, S., Marcilla, A. (2004). Identification of enolase as a plasminogen-binding protein in excretory-secretory products of Fasciola hepatica. FEBS Lett. 563, 203-206.

Budke, C. M. (2006). Global socioeconomic impact of cystic echinococcosis. Emerg. Infect. Dis. 12, 296-303.

Copley, S. D. (2012). Moonlighting is mainstream: Paradigm adjustment required. Bioessays. 34, 578-88.

Cortez-Herrera, E., Yamamoto, R. R., Rodrigues, J. S. S., Farias, S. E., Ferreira, H. B., Zaha, A. (2001). Echinococcus granulosus: Cloning and functional in vitro characterization of an actin filament fragmenting protein. Experimental Parasitology. 97, 215-225.

Dang, Z., Yagi, K., Oku, Y., Kouguchi, H., Kajino, K., Watanabe, J., Matsumoto, J., Nakao, R., Wakaguri, H., Toyoda, A., Sugimoto, C. (2009). Evaluation of Echinococcus multilocularis tetraspanins as vaccine candidates against primary alveolar echinococcosis. Vaccine. 52, 7339-7345.

de la Rue, M. L. (2008). Cystic echinococcosis in Southern Brazil. Rev Inst Med Trop. 50, 53-62.

de la Rue, M. L., Takano, K., Brochado, J. F., Costa, C. V., Soares, A. G., Yamano, K., Yagi, K., Katoh, Y., Takahashi, K. (2011). Infection of humans and animals with Echinococcus granulosus (G1 and G3 strains) and E. ortleppi in Southern Brazil. Vet Parasitol. 177, 97-103.

Delprato, A. (2012). Topological and functional properties of the small GTPases protein interaction network. PLoS One. 7, e44882.

Farias, L.N., Malgor, R., Cassaravilla, C., Bragança, C., de la Rue, M. L. (2004). Echinococcosis in Southern Brazil: efforts toward implementation of a control program in Santana do Livramento, Rio Grande do Sul. Rev. Inst. Med. Trop. 46, 153-156.

Ferreira, H.B., Zaha, A. (1994). Expression and analysis of the diagnostic value of an Echinococcus granulosus antigen gene clone. Int J Parasitol. 24, 863-870.

Ferguson, D. J. P., Parmley, S. F., and Tomavo, S. (2002). Evidence for nuclear localisation of two stage-specific isoenzymes of enolase in Toxoplasma gondii correlates with active parasite replication. International Journal for Parasitology 32, 1399-410.

Gan, W., Zhao, G., Xu, H., Wu, W., Du, W., Huang, J., Yu, X., and Hu, X. (2010). Reverse vaccinology approach identify an Echinococcus granulosus tegumental membrane protein enolase as vaccine candidate. Parasitology Research 106, 873-82.

Gunsalus, K. C., Rhrissorrakrai, K. (2011). Networks in Caenorhabditis elegans. Curr Opin Genet Dev. 21, 787-798.

Gómez, A., Hernández, S., Amela, I., Piñol, J., Cedano, J., Querol, E. (2011). Do protein-protein interaction databases identify moonlighting proteins? Molecular bioSystems 7, 2379-2382.

Gómez-Arreaza, A., Acosta, H., Quiñones, W., Concepción, J. L., Michels, P. A., Avilán, L. (2014). Extracellular functions of glycolytic enzymes of parasites: unpredicted use of ancient proteins. Mol Biochem Parasitol. 193, 75-81.

González-Rodríguez, I., Sánchez, B., Ruiz, L., Turroni, F., Ventura, M., Ruas-Madiedo, P., Gueimonde, M., Margolles, A. (2012). Role of extracellular transaldolase from Bifidobacterium bifidum in mucin adhesion and aggregation. Appl Environ Microbiol. 78, 3992-3998.

Grimm, E. D., Portugal, R. V., Neto, M. D., Martins, N. H., Polikarpov, I., Zaha, A., Ferreira, H. B. (2006). Structural analysis of an Echinococcus granulosus actin-fragmenting protein by small-angle X-ray scattering studies and molecular modeling. Biophysical Journal. 90, 3216-3223.

Hendriks, W., Mulders, J. W. M., Bibby, M., Slingsby, C., Jong Bloemendal, H., W. W. (1988). Duck lens ε-crystallin and lactate dehydrogenase B4 are identical: A single copy gene.

Proceedings of the National Academy of Sciences of the United States of America. 85, 7114-7118.
Holmes, M., Liwak, U., Pricop, I., Wang, X., Tomavo, S., and Ananvoranich, S. (2010). Silencing of tachyzoite enolase 2 alters nuclear targeting of bradyzoite enolase 1 in Toxoplasma gondii. Microbes and Infection 12, 19-27.
Huang, J., Huang, Y., Wu, X., Du, W., Yu, X., Hu, X. (2009). Identification, expression, characterization, and immunolocalization of lactate dehydrogenase from Taenia asiatica. Parasitol. Res. 104, 287-293.
Huberts, D. H., van der Klei, I. J. (2010a). Moonlighting proteins: an intriguing mode of multitasking. Biochim Biophys Acta. 1803, 520-525.
Huberts, D. H., Venselaar, H., Vriend, G., Veenhuis, M., van der Klei, I. J. (2010b). The moonlighting function of pyruvate carboxylase resides in the non-catalytic end of the TIM barrel. Biochim Biophys Acta. 1803, 1038-1042.
Jabbar, A., Jenkins, D. J., Crawford, S., Walduck, A. K., Gauci, C. G., Lightowlers, M. W. (2011). Oncospheral penetration glands are the source of the EG95 vaccine antigen against cystic hydatid disease. Parasitology. 138, 89-99.
Janga, S. C., Díaz-Mejía, J. J., Moreno-Hagelsieb, G. (2011). Network-based function prediction and interactomics: the case for metabolic enzymes. Metab Eng. 13, 1-10.
Jeffery, C. J. (2009). Moonlighting proteins-an update. Mol. Biosyst. 5, 345-350.
Jewett, T. J., Sibley, L. D. (2003). Aldolase forms a bridge between cell surface adhesins and the actin cytoskeleton in apicomplexan parasites. Mol. Cell. 11, 885-894.
Kim, J. W., Dang, C. V. (2005). Multifaceted roles of glycolytic enzymes. Trends Biochem. Sci. 30, 142-150.
Keller, a, Peltzer, J., Carpentier, G., Horváth, I., Oláh, J., Duchesnay, a, Orosz, F., and Ovádi, J. (2007). Interactions of enolase isoforms with tubulin and microtubules during myogenesis. Biochimica et Biophysica Acta 1770, 919-26.
Labbé, M., Péroval, M., Bourdieu, C., Girard-Misguich, F., Péry, P. (2006). Eimeria tenella enolase and pyruvate kinase: a likely role in glycolysis and in others functions. Int. J. Parasitol. 36, 1443-1452.
Liu, H., Zeng, H., Yao, Q., Yuan, J., Zhang, Y., Qiu, D., Yang, X., Yang, H., Liu, Z. (2012). Steinernema glaseri surface enolase: molecular cloning, biological characterization, and role in host immune suppression. Mol Biochem Parasitol. 185, 89–98.
Lorenzatto, K. R., Monteiro, K. M, Paredes, R., Paludo, G. P., da Fonsêca. M. M., Galanti, N., Zaha, A., Ferreira, H. B. (2012). Fructose-bisphosphate aldolase and enolase from Echinococcus granulosus: Genes, expression patterns and protein interactions of two potential moonlighting proteins. Gene. 506, 76-84.
Luo, S., Poltermann, S., Kunert, A., Rupp, S., Zipfel, P. F. (2009). Immune evasion of the human pathogenic yeast Candida albicans: Pra1 is a Factor H, FHL-1 and plasminogen binding surface protein. Molecular Immunology. 47, 541-550.
Mandal, S., Mandal, M. D. (2012). Human cystic echinococcosis: epidemiologic, zoonotic, clinical, diagnostic and therapeutic aspects. Asian Pac J Trop Med. 5, 253-260.
Marcilla, A., Pérez-García, A., Espert, A., Bernal, D., Muñoz-Antolí, C., Esteban, J. G., Toledo, R. (2007). Echinostoma caproni: identification of enolase in excretory/secretory products, molecular cloning, and functional expression. Exp. Parasitol. 117, 57-64.
Monteiro, K. M., de Carvalho, M. O., Zaha, A., Ferreira, H. B. (2010). Proteomic analysis of the Echinococcus granulosus metacestode during infection of its intermediate host. Proteomics. 10, 1985-1999.
Moro, P., Schantz, P. M. (2009). Echinococcosis: a review. Int. J. Infect. Dis. 13, 125-133.
Moro, P. L., Budke, C. M., Schantz, P. M., Vasquez, J., Santivañez, S. J., Villavicencio, J. (2011). Economic impact of cystic echinococcosis in peru. PLoS Negl Trop Dis. 5: 5-1179.
Niinaka, Y., Paku, S., Haga, A., Watanabe, H., Raz, A. (1998). Expression and secretion of neuroleukin/phosphohexose isomerase/maturation factor as autocrine motility factor by tumor cells. Cancer Research. 58, 2667-2674.
Nogueira, S. V., Fonseca, F. L., Rodrigues, M. L., Mundodi, V., Abi-Chacra, E. A., Winters, M. S., Alderete, J. F., de Almeida Soares, C. M. (2010). Paracoccidioides brasiliensis enolase is a surface protein that binds plasminogen and mediates interaction of yeast forms with host cells. Infection and immunity. 78, 4040-4050.

Nunes, C. P., Zaha, A., Gottstein, B., Muller, N., Siles-Lucas, M. D. (2004). 14-3-3 gene characterization and description of a second 14-3-3 isoform in both Echinococcus granulosus and E-multilocularis. Parasitology Research. 93, 403-409.

Pal-Bhowmick, I., Vora, H. K., Jarori, G. K. (2007). Sub-cellular localization and post-translational modifications of the Plasmodium yoelii enolase suggest moonlighting functions. Malar. J. 6, 45.

Pancholi, V. (2001). Multifunctional alpha-enolase: its role in diseases. Cell. Mol. Life Sci. 58, 902-920.

Pomel, S., Luk, F. C. Y., Beckers, C. J. M. (2008). Host cell egress and invasion induce marked relocations of glycolytic enzymes in Toxoplasma gondii tachyzoites. PLoS Pathogens. 4: e1000188.

Ramajo-Hernández, A., Oleaga, A., Ramajo-Martín, V., Pérez-Sánchez, R. (2007). Carbohydrate profiling and protein identification of tegumental and excreted/secreted glycoproteins of adult Schistosoma bovis worms. Veterinary Parasitology. 144, 45-60.

Ramaprasad, A., Pain, A., Ravasi, T. (2012). Defining the protein interaction network of human malaria parasite Plasmodium falciparum. Genomics. 99, 69-75.

Saccoccia, F., di Micco, P., Boumis, G., Brunori, M., Koutris, I., Miele, A. E., Morea, V., Sriratana, P., Williams, D. L., Bellelli, A., Angelucci, F. (2012). Moonlighting by Different Stressors: Crystal Structure of the Chaperone Species of a 2-Cys Peroxiredoxin. Structure. 20, 429-439.

Sherawat, M., Tolan, D. R., Allen, K. N. (2008). Structure of a rabbit muscle fructose-1,6-bisphosphate aldolase A dimer variant. Acta Crystallogr. D Biol. Crystallogr. 64, 543-550.

Shevade, S., Jindal, N., Dutta, S., Jarori, G. K. (2013). Food vacuole associated enolase in plasmodium undergoes multiple post-translational modifications: evidence for atypical ubiquitination.

Siles-Lucas, M., Nunes, C. P., Zaha, A. (2001). Comparative analysis of the 14-3-3 gene and its expression in Echinococcus granulosus and Echinococcus multilocularis metacestodes. Parasitology. 122, 281-287.

Sriram, G., Martinez, J. A., McCabe, E. R., Liao, J. C., Dipple, K. M. (2005). Single-gene disorders: what role could moonlighting enzymes play? Am J Hum Genet. 76, 911-924.

Starnes, G. L., Coincon, M., Sygusch, J., Sibley, L. D. (2009). Aldolase is essential for energy production and bridging adhesin-actin cytoskeletal interactions during parasite invasion of host cells. Cell Host & Microbe. 5, 353-364.

Torgerson, P. R., Carmona, C., Bonifacino, R. (2000). Estimating the economic effects of cystic echinococcosis: Uruguay, a developing country with upper-middle income. Ann. Trop. Med. Parasitol. 94, 703-713.

Torgerson, P. R. (2003). Economic effects of echinococcosis. Acta Trop. 85, 113-118.

Veiga-Malta, I., Duarte, M., Dinis, M., Tavares, D., Videira, A., Ferreira, P. (2004). Enolase from Streptococcus sobrinus is an immunosuppressive protein. Cell Microbiol. 6, 79-88.

Wistow, G. J., Lietman, T., Williams, L. A., Stapel, S. O., Jong, W. W., Horwitz, J., Piatigorsky, J. (1988). τ-Crystallin/α-Enolase: One Gene Encodes Both an Enzyme and a Lens Structural Protein. The Journal of Cell Biology. 107, 2729-2736.

Yu, X., Harris, S.L., Levine, A.J. (2006). The regulation of exosome secretion: a novel function of the p53 protein. Cancer Res. 66, 4795-4801.

Zhang, S., Jin, G., Zhang, X. S., Chen, L. (2007). Discovering functions and revealing mechanisms at molecular level from biological networks. Proteomics. 7, 2856-2869.

Zheng, L., Roeder, R. G. Luo, Y. (2003). S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component. Cell. 114, 255-266.