Charles University in Prague

First Faculty of Medicine

Study program: Doctoral program in biomedicine

Study field: Biology and pathology of the cell

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Úloha evolučně konzervovaných proteinů BIR-1/Survivin a SKP-1
v regulaci genové exprese

The role of evolutionarily conserved proteins BIR-1/Survivin and SKP-1

in the regulation of gene expression

PhD Thesis

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Prague, 2016

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V Praze, 29.03.2016

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Podpis

Identifikační záznam:

KOSTROUCH, David. *Úloha evolučně konzervovaných proteinů BIR-1/Survivin a SKIP v regulaci genové exprese. [The role of evolutionarily conserved proteins BIR-1/ Survivin and SKIP in the regulation of gene expression]*. Praha, 2016. 141s. Disertační práce. Univerzita Karlova v Praze, 1. lékařská fakulta. Vedoucí práce Zdeněk Kostrouch, MD., PhD.

Acknowledgements

Contents:

1. Abbreviations

Apaf1 - Apoptotic protease activating factor-1

ATP - Adenosine triphosphate

Bcl - B-cell lymphoma

BH - BCL-2 Homology

BIR - Baculovirus IAP repeat

bp – base pair

BRE - TFIIB Recognition Element

CAR - Constitutive androstane receptor (vertebrate NR1I3)

CoA – Coenzyme A

CTD - C-terminal domain

DBD – DNA binding domain

DCE - Downstream Core element

DHR3 – Drosophila hormone receptor 3 (insect NR1F)

DISC - Death inducing signaling complex

DNA - Deoxyribonucleic acid

DPE - Downstream Promoter Element

dsRNA – double stranded RNA

eRNA - enhancer RNA

FXR - Farnesoid X receptor (vertebrate NR1H4)

HAT - Histone acetyltransferase

HDAC – Histone deacetylase

HP1 - Heterochromatin protein 1

IAP - Inhibitor of apoptosis protein

lncRNA – long non-coding RNA

Inr - Initiator

ISWI - Imitation SWI

LBD – Ligand binding domain

LXR - Liver X receptor (vertebrate NR1H2/3)

Mbp - Mega base pair

MDM2- Mouse double minute 2 homologue

MED1 - Mediator complex subunit 1

Mi-2/NuRD - Nucleosome remodeling and deacetylase

mRNA - Messenger RNA

miRNA – Micro RNA

MTE - Motif ten element

NCBI - National Center for Biotechnology Information

NCOR - Nuclear receptor co-repressor

NHR – Nuclear hormone receptor

NES - Nuclear export signal

NLS - Nuclear localization signal

NR – Nuclear receptor

PIC - Preinitiation complex

piRNA – Piwi-interacting RNA

PPAR - Peroxisome proliferator-activated receptor (vertebrate NR1C)

pre-miRNA - precursor miRNA

pri-miRNA - primary miRNA

PXR - Pregnane X receptor (vertebrate NR1I2)

RAR – Retinoic acid receptor, NR1B

RISC - RNA-induced silencing complex

rRNA – ribosomal RNA

tRNA – transfer RNA

RNA - Ribonucleic acid

RNAi – RNA interference

RNA PolII – DNA dependent RNA polymerase II

ROR - RAR-related orphan receptor (vertebrate NR1F)

siRNA - Small interfering RNA

SKIP - SKI-interacting protein, SNW

SKP-1 - *C. elegans* SKIP

SL1 - Splice leader 1

SMAC/DIABLO - Second mitochondria-derived activator of caspases / Direct IAP binding protein with low pI

SMRT - Silencing mediator of retinoid and thyroid receptors

snRNA – small nuclear RNA

snRNP - small nuclear ribonuclear protein

SRC - Steroid receptor coactivator

STAT - Signal transducers and activators of transcription

SWI/SNF - SWitch/Sucrose non-fermentable

TAF - TATA-binding protein associated factor

TF – Transcription factor

TGF - Transforming growth factor

TNF – Tumor necrosis factor

UTR - Untranslated region

XCPE1 - X core promoter element 1

XIAP - X-linked inhibitor of apoptosis protein

2. Abstract

SKIP and BIR/Survivin are evolutionarily conserved proteins. SKIP is a known transcription and splicing cofactor while BIR-1/Survivin regulates cell division, gene expression and development. Loss of function of *C. elegans* SKIP (SKP-1) and BIR-1 induces overlapping developmental phenotypes. In order to uncover the possible interactions of SKP-1 and BIR-1 on the protein level, we screened the complete *C. elegans* mRNA library using the yeast two-hybrid system. These experiments identified partially overlapping categories of proteins as SKP-1 and BIR-1 interactors. The interacting proteins included ribosomal proteins, transcription factors, translation factors and cytoskeletal and motor proteins suggesting involvement of the two studied proteins in multiple protein complexes. To visualize the effect of BIR-1 on the proteome of *C. elegans* we induced a short time pulse BIR-1 overexpression in synchronized L1 larvae. This led to a dramatic alteration of the whole proteome pattern indicating that BIR-1 alone has the capacity to alter the chromatographic profile of many target proteins including proteins found to be interactors in yeast two hybrid screens. The results were validated for ribosomal proteins RPS-3, RPL-5, non-muscle myosin and TAC-1, a transcription cofactor and a centrosome associated protein. Together, these results suggest that SKP-1 and BIR-1 are multifunctional proteins that form multiple protein complexes in both shared and distinct pathways and have the potential to connect proteome signals with the regulation of gene expression.

Souhrn

SKIP a BIR/Survivin jsou evolučně zachovalé proteiny. SKIP je známý transkripční a sestřihový kofaktor a BIR-1/Survivin reguluje buněčné dělení, genovou expresi a vývoj. Inaktivace SKP-1 a BIR-1 indukuje podobné vývojové fenotypy. K odhalení možných interakcí SKP-1 a BIR-1 jsme použili kvasinkový dvojhybridní systém a knihovnu kompletní mRNA *C. elegans*. Tyto experimenty identifikovaly částečně se překrývající kategorie proteinů jako interaktory proteinů SKP-1 a BIR-1. Identifikované interagující proteiny zahrnovaly ribozomální proteiny, transkripční faktory, translační faktory, cytoskeletální a motorové proteiny. Tyto výsledky naznačují jejich možnou účast v mnohočetných proteinových komplexech. Pomocí krátkodobé nadměrné exprese BIR-1 jsme sledovali účinek BIR-1 na proteom *C. elegans* v larválním stádiu L1. To způsobilo dramatickou změnu v celém proteomu což naznačuje, že BIR-1 má schopnost změnit chromatografický profil mnohočetných cílových proteinů včetně těch, které jsme již dříve identifikovali jako interagující proteiny v experimentech s kvasinkovým dvouhybridním systému. Výsledky jsme následně potvrdili pro RPS-3, RPL-5, myosin (non-muscle myosin) a TAC-1 (transkripční kofaktor a protein asociovaný s centrosomy). Tyto výsledky naznačují, že SKP-1 a BIR-1 jsou multifunkční proteiny, které jsou schopné vytvářet mnohočetné proteinové komplexy ve sdílených a samostatných regulačních cestách a mají potenciál spojovat signály z proteomu s regulací genové exprese.

Klíčová slova: BIR-1, genové exprese, proteom, ribosomální stres, SKIP, Survivin

3. Introduction

3.1 Thesis statements and synopsis: goals, hypotheses

Genes and their regulated expression are the hallmark of the existence of living organisms and their evolution. Central to the regulation of gene expression on the transcription level are transcription factors and proteins that connect transcription factors with the basal transcription machinery. Nuclear receptors are very potent and important transcription factors specific for Metazoa that are able to specifically regulate a large number of genes in response to developmental, metabolic and external stimuli. While in the human genome there are 48 genes coding for nuclear receptors, other species contain from 18 (insects) to 284 (*Caenorhabditis elegans*) genes coding for nuclear receptors (Kostrouchova & Kostrouch 2015). *Caenorhabditis elegans* offers a very versatile and powerful system that allows efficient analyses of regulatory roles of specific genes and proteins transcribed from them. This can be achieved by searches for similar phenotypic changes and further studies by means of advanced genetic and genomic approaches (Brenner 1974; Schrimpf & Hengartner 2010; Volovik et al. 2014). NHR-23, a nuclear receptor from the NR2 subfamily related to *Drosophila melanogaster* DHR3 (Koelle et al. 1992; Lam et al. 1997) and mammalian ROR (Becker-Andre et al. 1993; Becker-Andre et al. 1994) nuclear receptors is a master regulator of nematode developmental transitions and larval stages, similarly as thyroid hormone receptor and retinoid receptors in vertebrates (Kostrouchova et al. 1998; Kostrouchova et al. 2001; Kouns et al. 2011). The regulatory roles of NHR-23 overlap with an evolutionarily conserved transcriptional cofactor and splicing factor SKIP (SKP-1) and another evolutionarily conserved protein BIR-1 (a homologue of vertebrate Survivin), that is in the *C. elegans* genome expressed from the same operon as SKP-1 (Kostrouchova et al. 2002; Kostrouchova et al. 2003).

The goal of the experimental work related to this thesis was to identify the protein network that may be responsible for the overlapping and cooperative regulation of transcription or in a wide sense gene expression linked to *C. elegans* development involving NHR-23, SKP-1 and BIR-1. Additionally, our goal was to

test the hypothesis that the overlapping phenotypes of NHR-23, SKP-1 and BIR-1 loss of function may be derived from protein complexes in which these three proteins in question may participate.

To do this, we chose to use unbiased and high-throughput methods. We decided to search for NHR-23, SKP-1 and BIR-1 interacting proteins using yeast two-hybrid screens.

The first screen focused on NHR-23 indicated that NHR-23 is a strongly autoactivating protein in the yeast two hybrid screens suggesting that it has a direct and very strong affinity for some components of basal transcriptional machinery. In contrast, yeast two hybrid screens for SKP-1 and BIR-1 indicated that these two proteins have a potential to interact with relatively diverse proteins often from the same protein families.

Interestingly, SKP-1 interacted with motor and cytoskeletal structural proteins and BIR-1, which was expected to interact with cytoskeletal proteins, interacted with transcription factors and coregulators. Using functional studies, we showed that the two studied proteins are involved in the regulation of gene expression on the proteome level.

Our work suggests that structural proteins may have the potential to transmit signals reflecting the structural state of the cell towards gene expression by interacting with the two studied, evolutionarily conserved proteins, SKIP-1 and BIR-1.

Especially interesting was the finding that both SKP-1 and BIR-1 interact with ribosomal proteins. Several ribosomal proteins are key players in the regulation of stress response (ribosomal stress) and programmed cell death. This brought additional support for SKP-1 as a regulator of the stress response and programmed cell death.

Our work also suggests that the involvement of BIR-1 in the regulation of programmed cell death through proteome signals and ribosomal stress may be older than its role in the regulation of programmed cell death through the interaction with caspases. This also indicates that BIR-1 has the potential to act as an anti-apoptotic protein also in nematodes.

3.2 Overview of the literature and background of the field

Regulation of gene expression is the basis of the proper function of organisms, their development and metabolism. This regulation is complex and is executed on multiple levels. However, the regulation of gene expression on the level of chromatin by transcription factors, which recognize and bind specific regions in promoters of genes, and in cooperation with transcription coregulators attract and activate Polymerase II complex proteins is one of the most important. Further, downstream mechanisms then modulate gene expression on the level of RNA splicing and mRNA processing, nuclear export and translation into proteins. Tissue and metabolic state specific transcription factors and coregulators that are expressed in response to specific developmental and metabolic stimuli then direct proper gene expression to cope with particular developmental and metabolic needs on the level of cells, tissues and whole organisms. This basic regulatory network is likely to include additional mechanisms that sense the functional and structural cellular states and link them with gene expression regulation to achieve a fast and precise regulatory response.

Chromatin and its effect on gene expression

Chromatin is a complex of macromolecules found in the nuclei of eukaryotic cells. It consists of DNA, proteins and RNA. The main protein component of chromatin are histones. Histones are responsible for the packaging of DNA into nucleosomes and also play a role in regulating gene expression by making DNA more or less accessible to the transcription machinery. Histone tails are subject to diverse posttranslational modifications that alter their interaction with DNA. These modifications include methylation, acetylation, ubiquitination, phosphorylation, sumoylation and others (Strahl & Allis 2000).

A great number of histone modifications have been described. These modifications are thought to represent a histone code, where specific histone modifications influence the transcription of specific genes (Jenuwein & Allis 2001).

Histone acetylation, or more precisely acetylation of lysine residues at the N-termini of histones, is a process which removes the positive charge on histones and

decreases the interaction between histones and negatively charged phosphate groups of DNA. This causes a decondensation of chromatin and makes the DNA more accessible to the transcription machinery. Thus acetylation and deacetylation of histones play an important part in the regulation of gene expression. Histone acetyltransferases are enzymes that acetylate lysines on histone tails by transferring an acetyl group from acetyl-CoA to the ε-amino group of lysine side chains. Histone deacetylases catalyze the opposite reaction, the removal of acetyl groups from ε-N-acetyl lysine on histones. This causes the histones to wrap around the DNA more tightly and represses transcription (Bannister & Kouzarides 2011).

Methylation of histones occurs mostly on the side chains of lysine and arginine and unlike acetylation, methylation does not change the charge of the histone. Lysines may be mono-, di-or tri-methylated by numerous methyltransferases (Bannister & Kouzarides 2011).

Trimethylation of H3 lysine 4 (H3K4Me3) and trimethylation of H3 lysine 36 (H3K36Me3) are two modifications that are particularly associated with actively transcribed genes. Lysine 4 methylation occurs at the promoters of active genes and corresponds with the transcriptional activity of the gene (Krogan et al. 2003). It is catalysed by the Set1 subunit of the COMPASS complex. Lysine 36 methylation is performed by Set2 methyltransferase and occurs during the elongation phase of transcription (Shilatifard 2006). Both of these modification are more likely to be consequences of active transcription than causes of transcription (Dong & Weng 2013).

Several histone modifications are associated with repressed genes. Trimethylation of H3 lysine 27 is catalyzed by the polycomb complex PRC2 and is an important marker of gene repression (Cao et al. 2002). Di- and tri-methylation of H3 lysine 9 is associated with heterochromatin and is bound specifically by HP1 (Heterochromatin protein 1). Through the association with multiple other proteins like HDACs and RNAs, HP1 is believed to compact chromatin into heterochromatin which represses transcription (Stewart et al. 2005).

Chromatin remodeling complexes

The condensation of eukaryotic DNA in the nucleus allows an enormous amount of information in the form of DNA to be packaged according to the needs of the cell. Chromatin can be found in a variety of more or less compacted forms. The basic structure of chromatin is the nucleosome, a 147 base pair segment of DNA wrapped around an octamer of core histones. With the addition of the linker histone H1, this chromatin takes the shape of a 30 nm fiber. Highly compacted forms of chromatin are represented by heterochromatin and metaphase chromosomes. While less compacted forms of chromatin are easily accessible to transcription, replication and DNA repair machinery, compacted chromatin is not. Thus chromatin can be divided into transcriptionally active and repressed regions based upon the level of compaction (Tang et al. 2010; Aydin et al. 2014; Biegel et al. 2014; Kapoor & Shen 2014; Kadoch & Crabtree 2015; Kadoch et al. 2016).

Until recently chromatin was thought to be fairly static material. However, with the discovery of chromatin remodeling enzymes and covalent histone-modifying complexes, molecular machines that catalyze a wide range of chromatin changes, it has become clear that chromatin not only has the potential to change chromatin structure, but can actively influence numerous cellular processes such as gene expression (Narlikar et al. 2013). It has to be noted that the field of chromatin structure, despite the wealth of obtained data is still not sufficiently understood. The existence of certain forms of chromatin, e.g. the 30 nm chromatin fiber is only observed under some specific conditions (Staynov 2008; Maeshima et al. 2010).

Several chromatin remodeling complexes have been identified in eukaryotes. These molecular machines function by binding DNA or nucleosomes and destabilizing histone-DNA bonds in an ATP-dependent manner (Johnson et al. 2005). The best studied chromatin remodeling complexes include members of the SWI/SNF (SWitch/Sucrose Non-Fermentable) family that are found in both prokaryotes and eukaryotes and are capable of altering the position of nucleosomes along DNA (Whitehouse et al. 1999). It is presumed that SWI/SNF complexes function by rotating DNA along its axis, generating supercoils, which then lead to the disruption of the histone octamer and relocation of histones to different DNA locations.

ISWI or Imitation SWI of *Drosophila melanogaster* has a high similarity to the SWI/SNF chromatin remodeling group in the ATPase domain. These complexes function by creating loops and bulges in DNA that propagate along the DNA molecule to the octamer surface and lead to the destabilization of histone-DNA complexes and to nucleosome remodeling (Ogbourne & Antalis 1998; Whitehouse et al. 1999; Strahl & Allis 2000; Jenuwein & Allis 2001; Cao et al. 2002; Kassabov et al. 2002; Krogan et al. 2003; Johnson et al. 2005; Stewart et al. 2005; Maston et al. 2006; Shilatifard 2006; Bannister & Kouzarides 2011; Kolovos et al. 2012; Dong & Weng 2013). The Mi-2/NuRD (Nucleosome remodeling and deacetylase) complex is unique because it couples histone deacetylation and chromatin remodeling ATPase activity in the same complex. NuRD can both activate and repress transcription and is involved in DNA damage repair (Denslow & Wade 2007; Alqarni et al. 2014).

DNA motifs – enhancers, silencers, insulators

The human genome contains hundreds of thousands of enhancer sequences, which are cis-acting, short regions of DNA (50-1500 bp), that can be bound by transcription factors/activators to activate transcription of a gene. They can be located up to 1 Mbp upstream or downstream from the gene transcription start site and their function is not dependent on their orientation. Even though enhancer sequences can be located far away from the gene they regulate in terms of base pairs, the folding of chromatin and formation of loops allows for a spatial conformation that brings the regulatory sequence close to the promoter of the regulated gene, allowing it to interact with general transcription factors and RNA polymerase II (Cook 2003).

Enhancer sequences can also be transcribed into eRNAs (enhancer RNA) that do not code for proteins and stabilize enhancer-promoter interactions (Melamed et al. 2016).

Silencers are the opposite of enhancers. They are also cis-acting sequences, bound by transcription factors (repressors), that negatively affect transcription of a specific gene. Silencers are most commonly found 20 – 2000 base pairs upstream of the promoter. But they can also be found downstream of the promoter, within exon or intron sequences of the regulated gene. There are two basic types of silencers. The

classic silencer represses gene expression by interfering with general transcription factor assembly. They can also target helicase sites in the DNA, thus interfering with the unwinding of DNA. Non-classical negative regulatory elements passively repress gene expression/transcription by inhibiting other elements upstream of the regulated gene (Ogbourne & Antalis 1998; Maston et al. 2006; Kolovos et al. 2012).

Insulators are genetic elements that insulate or protect genes from inappropriate interactions. They function in two main ways. Insulators can shield genes from distant enhancers if the insulator is located between the enhancer and the promoter. This prevents the enhancer from activating an adjacent gene. Their other function is to set the boundaries of repressive chromatin. Some enhancers can have both functions (West et al. 2002; Barkess & West 2012).

RNA polymerase

Transcription is the first step in gene expression. It is the production of mRNA and is mediated by RNA polymerases. In Metazoa, there are three distinct DNA dependent RNA polymerases in the nucleus that are similar in structure but transcribe different groups of genes. RNA polymerase I transcribes 18S, 28S and 5.8S rRNAs, RNA polymerase II transcribes precursors of mRNA, snRNA, microRNA and RNA polymerase III transcribes tRNA, 5S rRNA and other small RNAs (Willis 1993; Grummt 1999; Lee et al. 2004). RNA polymerases IV and V have been identified in plants (Herr et al. 2005; Wierzbicki et al. 2009).

The core promoter and basal transcription machinery

In the textbook simplification, transcription begins when RNA polymerase binds to DNA in the promoter region which is located upstream of the transcription start site. Using the most basic definition the promoter is a region of DNA found upstream of the transcription site, that includes binding sites that are necessary for transcription initiation. In spite of the complexity of the genome, the multitude of transcription factors and coregulators, it is accepted that transcription initiation can indeed be specific and regulated and that individual sequences in promoter regions act in unison with the basal transcription machinery and regulatory proteins to make this event possible.

Transcription initiation can be divided into focused and dispersed initiation. Focused initiation starts from a single nucleotide or within a cluster of several nucleotides while dispersed initiation starts from one of several weak transcription sites that are scattered over a region of 50-100 nucleotides. In vertebrates, the majority (2/3) of genes have dispersed promoters, these tend to be constitutive genes. Regulated genes on the other hand tend to have focused promoters, which regulate biologically important genes.

Through the analysis of large numbers of eukaryotic promoters consensus sequences of numerous binding motifs have been defined and several core promoter elements have been identified, which are essential for proper promoter function and assembly of the transcription preinitiation complex. These elements include the TATA box, BREu and BREd (upstream and downstream TFIIB Recognition Element), Inr (Initiator), MTE (Motif Ten Element), DPE (Downstream Promoter Element), DCE (Downstream Core element), and XCPE1 (X Core Promoter Element 1). However, dispersed promotors usually do not have TATA, BRE, DPE, and MTE motifs (Smale & Kadonaga 2003; Sandelin et al. 2007; Juven-Gershon & Kadonaga 2010).

RNA polymerase II requires the presence of a core promoter region and accessory factors for site-specific initiation of transcription. These factors are known as general or basal transcription factors. They are commonly abbreviated as TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH. Together with RNA polymerase II they make up the basal transcription machinery – the pre-initiation complex, which is both necessary and sufficient to begin activator-dependent/regulated transcription (Thomas & Chiang 2006). The core promoter is a region of DNA that is located about 35 base pairs upstream and/or downstream of the transcription initiation site and interacts directly with components of the basal transcription machinery. It is essential in the initiation of transcription of protein-coding genes for the formation of the pre-initiation complex.

In the majority of core promoters pre-initiation complex formation begins with TFIID binding to either the TATA box, Initiator and/or DPE, which are found in most core promoters. This is followed by the assembly of other general transcription factors.

The Initiator (Inr) is the most common core promoter motif and includes the transcription start site. TFIID binds to the Initiator motif and its consensus sequence is YYANWYY in humans (Y − pyrimidine C/T, N − nucleobase, any of the four bases, W − weak A/T). The Initiator functions synergistically with DPE and MTE core promoter motifs (Juven-Gershon & Kadonaga 2010).

The TATA box was among the first eukaryotic core promoters identified and is present in about 1/3 of protein coding gene promoters. In metazoans its consensus sequence is TATAWAAR and it is usually located 31 or 30 base pairs upstream of the A+1 position of the Inr. The TATA box is bound by the TBP (TATA binding protein), which is a subunit of TFIID. TATA-driven core promoters require the presence of RNA pol II and TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Smale & Kadonaga 2003).

Two BRE (TFIIB Recognition element) sequences have been identified upstream and downstream of the TATA box that bind TFIIB, BREu and BREd. They function together with the TATA box in fine-tuning basal transcription by both increasing and decreasing transcription (Juven-Gershon et al. 1998; Smale & Kadonaga 2003; Juven-Gershon & Kadonaga 2010).

As noted previously only about 1/3 of promoters contain the TATA-box. Some TATA-less promoters contain DPE and Inr core promoter elements. DPE (Downstream promoter element) acts synergistically with Inr and is located 28-32 base pairs downstream of the A+1 nucleotide in the Inr motif (Kutach & Kadonaga 2000). TFIID binds to both the Inr and DPE elements, but different factors are needed to initiate transcription from DPE-driven promoters. The difference between TATA and DPE dependent transcription can be shown on a simple circuit where TBP (TATA binding protein) promotes TATA dependent transcription and represses DPE dependent transcription. DPE dependent transcription requires the presence of NC2 (Negative cofactor 2) and Mot1 (ATPase that removes TBP from DNA) which block TBP function and promotes DPE dependent transcription (Hsu et al. 2008; van Werven et al. 2008).

Mediator and its connection to multiple regulatory pathways

Mediator is a 1,2 MDa, 26 subunit protein complex specific to eukaryotic organisms (Thompson et al. 1993). Mediator is a large multiprotein complex that interacts extensively with RNA polymerase II and most general transcription factors and regulates gene expression in a gene specific but also general manner (Cantin et al. 2003; Black et al. 2006; Chen et al. 2006; Taatjes 2010). Its sequences contain almost no predicted functional motifs and an unusually high amount of intrinsically disordered regions within Mediator subunits. This likely contributes to its structural plasticity and large potential for protein-protein interactions (Toth-Petroczy et al. 2008). Intrinsically disordered proteins are recently strongly acquiring importance in many fields of protein-protein interactions. It is expected that in some way, large proportion of proteins contain domains that can be characterized as intrinsically disordered and are likely to contribute to the broad spectrum of protein-protein interactions (Permyakov et al. 2015; Uversky 2015; Uversky 2016).

Mediator was first considered to be a co-activator of transcription. It plays essential roles in activator-dependent transcription, but it can also stimulate basal, activator-independent, transcription (Fondell et al. 1996; Mittler et al. 2001). Thus Mediator in fact functions more like a general transcription factor.

Mediator has diverse functions. These include general regulatory roles like Pol II recruitment and activation, coordination of the pre-initiation complex assembly, control of TFIIH dependent RNA Polymerase II CTD (C-terminal domain) phosphorylation within the PIC, physical or functional interactions with HAT complex STAGA (SPT3-TAF(II)31-GCN5L acetylase) (Cantin et al. 2003; Black et al. 2006). Mediator can function as a scaffold around which the basal transcription machinery assembles. The Mediator also has gene-selective roles: MED1 (Mediator complex subunit 1) can be phosphorylated by ERK which stabilizes MED1 within the Mediator. MED1 phosphorylated Mediators have an enhanced ability to activate transcription at thyroid hormone receptor regulated promoters (Chen et al. 2006).

Transcription Factors

Beside the basal transcription machinery, there is a large and diverse group of proteins that bind to specific sequences of DNA and modulate transcription on the level of transcription initiation. The accumulated knowledge in this field has led to several major changes of understanding the mechanistic processes that lead to transcription initiation and subsequent events projecting finally to the formation of pre-mRNA and its splicing and further processing. RNA Polymerase II (DNA dependent RNA Polymerase II) is phosphorylated at the C-terminal domain in the process of building the core complex by TFIID.

It has been accepted, that active phosphorylated RNA Polymerase II is thus recruited to active promoters. However, new technical developments allowing high-throughput analyses of genome regions occupied by active (phosphorylated) RNA Polymerase II and the correlation with transcriptomes analyzed by high-throughput sequencing have confirmed that the activated polymerase does not overlap with actively transcribed genes. These contradictory findings again open the question of the precise mechanisms that project to specific gene expression. Regardless of this, it is a fact, that cell and metabolic specific transcription depends on tissue and metabolism specific transcription factors and coregulators.

Numerous specific functions of transcription factors have been characterized to a great detail. Large numbers of sequences and DNA motifs that are specifically bound by transcription factors have been uncovered and reported in the literature. The extent of collected data far exceeds the capacity of classical scientific literature. Beside the classical reports documenting particular features of the transcription factors, their binding sites and additional functional data, the acquired knowledge is also being deposited in databases and analyzed by search engines that allow further informatics investigation of the newly acquired data, e.g. TRANSFAC (Wingender 2008) (http://www.gene-regulation.com/pub/databases.html).

As a general rule, transcription factors (TFs) selectively bind to short sequences of DNA or motifs (usually 6-8 bp in length) in a sequence dependent manner. The sequences to which transcription factors bind are called response elements and the binding is dependent on biophysical interactions between the protein structure of the transcription factor and DNA. Often, molecules of H_2O are

intercalated between the bases and amino acid residues that specifically transmit the interaction between transcription factors and the response elements (reviewed in (Claessens & Gewirth 2004)). DNA accessibility (chromatin structure) also plays an important part, as well as additional proteins that participate in transcription complexes but do not bind DNA (Latchman 1997; Nebert 2002; Spitz & Furlong 2012; de Mendoza et al. 2013). This will be specified later.

Again, as a general rule that has more exceptions than regularities, transcription factors bind to the promoter regions of DNA that are near the transcription start site of the gene they regulate. This is certainly true in the majority of well-studied genes in *Caenorhabditis elegans*, which has a relatively small and dense genome with promoters often shorter than DNA wrapped on as little as two or three nucleosomes (Blackwell & Walker 2006). The core promoter has been discussed previously.

Some transcription factors affect gene expression by binding more remote DNA motifs. Enhancers are bound by activators (TFs) to activate the transcription of a gene. Transcription factors function by a variety of mechanisms. Their basic function is to up or downregulate transcription by recruiting RNA polymerase or inhibit its recruitment to DNA. They can also affect the acetylation or deacetylation of histones directly or in cooperation with other proteins (HATs). Altogether, nine superclasses of transcription factors have been identified, comprising 40 classes and 111 families. In the human genome, 1558 transcription factors have been classified so far. This number increases to >2900 different TFs when including their isoforms that are generated by alternative splicing or protein processing events (Wingender et al. 2013).

A very characteristic feature of TFs is their modular structure. All transcription factors have a DNA binding domain (DBD) through which the TF binds to enhancer or promoter sequences. The trans-activating domain contains binding sites for coregulators. Some transcription factors contain a signal-sensing domain (ligand binding domain) which senses external signals (Mangelsdorf et al. 1995).

Transcription factors can be regulated by many different mechanisms. They are regulated by intercellular as well as intracellular signaling cascades, causing the upregulation or downregulation of genes in the recipient cell. Transcription factors

can themselves be regulated by other TFs thus forming a whole network that receives positive as well as negative feedback loops. Many TFs are autoregulatory in these regulatory loops. Transcription factors can also be regulated through their intracellular localization by regulated nuclear import or nuclear export. In a large number of cases, this transport is dependent on specific protein sequences localized on the surface of TF molecules called nuclear localization signals (NLS) or nuclear export signals (NES) (Jans & Hassan 1998; Stevens & Mann 2007).

Many TFs have been shown to be able to both activate and inhibit RNA transcription. The fundamental mechanism of gene activation and repression was elucidated on model systems including nuclear receptors such as thyroid hormone receptors, the vitamin D receptor or glucocorticoid receptor. Especially thyroid hormone receptor and vitamin D receptor, receptors that are bound to the response elements regardless of their occupation by the particular hormonal molecule (ligand) offered an elegant way for a mechanistic visualization of transcription activation and repression. It was shown that the conformational change induced by hormone binding can be projected to specific binding of additional proteins that are regulating transcription by posttranslational modification of nucleosome histones, acetylation and deacetylation in the first place (Jenuwein & Allis 2001).

Transcription factors themselves can be activated or deactivated through their signal-sensing domain. Ligand binding and post-translational modifications (such as phosphorylation) can affect the activity of transcription factors (e.g. STAT proteins must be phosphorylated to be activated) (Nadeau et al. 1999). Transcription factors usually function in cooperation with other TFs and transcription usually requires the presence and binding of multiple transcription factors to regulatory DNA sequences. The particular binding sites that were proven in detailed studies indicate that the consensus sequences do not need to be completely conserved. This led to the discovery of very diverse sets of response elements with specific and often antagonistic regulatory potentials. For example, specific arrangement of the binding sites for thyroid hormone receptor may allow inhibition of gene expression by liganded receptors (Nakano et al. 2004). The binding sites of TFs are now deposited to several databases and can be used for further bioinformatics analyses of potential

promoters (accessible through NCBI, http://www.ncbi.nlm.nih.gov and ExPASy, http://www.expasy.org/ and other databases).

There are several systems by which TFs are classified, e.g. according to their DBD (Yusuf et al. 2012; Wingender et al. 2013).

Nuclear receptors

Nuclear receptors are a subgroup of transcription factors. They are intracellular proteins that often bind small, hydrophobic signal molecules that diffuse directly across the plasma membrane of target cells such as steroid hormones, thyroid hormones, retinoids and vitamin D. The binding of a ligand causes these receptors to be activated, bind to DNA and regulate the transcription of specific genes. Nuclear receptors have a very wide range of functions from regulating development, to regulating homeostasis and metabolism. Inactive receptors are usually bound to inhibitory protein complexes. In these cases ligand binding alters the conformation of the receptor, causing the inhibitory complex to dissociate and the receptor to bind coactivator proteins that induce gene transcription. Because nuclear receptors have the ability to directly bind DNA and regulate the expression of specific genes, they are classified as transcription factors (Evans 1988).

Nuclear receptors contain characteristic structural and functional domains: an N-terminal regulatory domain, a DNA-binding domain made up of two zinc-fingers that binds to specific sequences of DNA, a flexible hinge region, a ligand-binding domain and a C-terminal domain (Parker 1990; Kumar & Thompson 1999; Thompson & Kumar 2003).

Nuclear receptors can be divided into four classes according to their mechanism of action.

Type I receptors are found in the cytosol, where the binding of a ligand results in the dissociation of heat shock proteins, homo-dimerization of the receptors and their translocation into the nucleus. There they bind to specific DNA sequences known as hormone response elements. Estrogen receptors, androgen receptors, progesterone receptors and glucocorticoid receptors are type I receptors (Mangelsdorf et al. 1995).

Type II receptors are found in the nucleus in the form of a heterodimer (with RXR) and bind DNA even in the absence of a ligand. Without ligand, type II receptors are generally bound to a corepressor. The binding of a ligand to the receptor causes dissociation of the corepressor and binding of coactivator proteins. Retinoic acid receptor, retinoid X receptor and thyroid hormone receptor are type II receptors (Klinge et al. 1997).

Type III receptors bind DNA as homodimers and function like type I receptors. Instead of binding direct repeats they bind inverted repeats (Mangelsdorf et al. 1995).

Type IV nuclear receptors bind DNA as monomers. The majority of orphan receptors belong to type III and type IV nuclear receptors and until recently, most type IV nuclear receptors were thought to be orphan receptors. Their importance has been difficult to test because of their over-lapping functions (Mangelsdorf et al. 1995; Sever & Glass 2013).

Ever since research began on nuclear receptors, it has been clear that there are numerous receptors that have no known ligand. These receptors are designated as orphan receptors. Research into orphan receptors has brought about very interesting discoveries not only about the ligands themselves - endogenous ligands for PPAR, cholesterol metabolites for LXR, but has shown how the chemical structure of the ligand can be a sign of the intrinsic function of the receptor and its physiological role: fatty acid metabolism – PPAR, sterol homeostasis – LXR, bile acid homeostasis – FXR, and endobiotic/xenobiotic metabolism for pregnane X and constitutive androstane receptors – (PXR and CAR) (Evans & Mangelsdorf 2014).

Transcription coregulators

In prokaryotes, sequence specific DNA-binding factors (such as the lambda phage cI protein) recruit RNA polymerase to the promoter through direct contact and initiate transcription (Hochschild & Lewis 2009). In eukaryotes, the situation is much more complex and the regulatory network has to integrate an enormous diversity of molecular signals that have to be interpreted by the transcriptional machinery. This role is played by specialized adapters (coregulators – coactivators and corepressors) that interact with different classes of transcriptional regulators and

the basal transcriptional machinery and modulate their function (Kato et al. 2011; Lonard & O'Malley 2012; Dasgupta et al. 2014; Dasgupta & O'Malley 2014; Giudici et al. 2015).

Transcription coregulators can roughly be divided into 5 classes. The first class consists of activators and repressors that are inherent to or intimately associated with the basal transcription machinery. This includes the TAFs (TATA-binding protein Associated Factors) of TFIID or the TFIIA subunit. The second class includes cofactors that are principally associated with activator or repressor molecules, those that modulate DNA binding, target other coregulators or the basal transcription machinery. Examples include Notch, OCA-B, HCF. The third class includes large multi-subunit coactivators – Mediator and its metazoan counterparts. The fourth class includes chromatin-modifying molecules that covalently modify nucleosomes. They include histone acetylases CBP/p300 and deacetylases HDAC-1, HDAC-2. The fifth class includes ATP-dependent chromatin remodeling complexes – SWI/SNF-related, ISWI-containing chromatin remodeling complexes (Lemon & Tjian 2000).

Transcription coregulators are of great importance in the field of nuclear receptors, where they act as essential regulators of gene expression by modulating the activity of most if not all nuclear receptors. Experiments conducted more than 25 years ago first showed that in some cases ligand-bound nuclear receptors were not sufficient by themselves to interact with the basal transcription machinery and to activate transcription. They are frequently referred to as master regulators in the literature. Currently more than 400 coregulators have been identified (reviewed in (Dasgupta et al. 2014; Dasgupta & O'Malley 2014)).

Nuclear receptor coactivators

Nuclear receptor coactivators are molecules that activate transcription in association with ligand-bound (agonist-bound) nuclear receptors. SRC-1, steroid receptor coactivator-1, was the first nuclear receptor coactivator to be discovered and along with SRC-2 and SRC-3 is also one of the best characterized. When overexpressed, SRC-1 enhances ligand-induced transcriptional activation by numerous receptors - namely progesterone receptor, estrogen receptor α,

glucocorticoid receptor, thyroid receptor, and retinoid X receptor (Johnson & O'Malley 2012).

Nuclear receptor coactivators function by bridging nuclear receptors to the basal transcription machinery. In addition, nuclear receptor coactivators can also modify chromatin structure in the promoter and enhancer regions. They may also recruit other proteins called co-coactivators that can modify chromatin in a way to make DNA more accessible to other enhancer regulatory proteins and general transcription factors mainly through histone acetylation and histone methylation. Unlike nuclear receptors, nuclear receptor coregulators do not have a conserved structural domain. However, several coactivators, for example those of the SRC family, have a common motif through which they bind to the LBD of NR. It is called the NR box or the LXXLL motif (L=leucine, X=any amino acid) (Johnson & O'Malley 2012).

Nuclear receptor corepressor

Nuclear receptor corepressors function by altering the structure of chromatin in promoters to an inactive state. SMRT (Silencing Mediator of Retinoid and Thyroid receptors) (Chen & Evans 1995; Chen et al. 1996) and NCOR(Nuclear receptor CORepressor) (Horlein et al. 1995; Kurokawa et al. 1995; Zamir et al. 1996) are examples of NR corepressors and function by recruiting histone deacetylases, which alter the chromatin structure to a repressed state. The first nuclear receptor corepressors to be characterized were those that interact with nuclear receptors of the second class – such as thyroid hormone receptor, PPAR and LXR (Note: class means functional class not subfamily of nuclear receptors). These function by binding DNA as a heterodimer with RXR and without ligand are bound by corepressors and actively repress transcription. Only after binding of ligand are the corepressors replaced by coactivators. Certain coregulators can also have dual functions, they can act as coactivators and in other situation as corepressors (Meyer et al. 1989; Klein-Hitpass et al. 1990; Onate et al. 1995; Johnson & Barton 2007). Currently the generally accepted classification of nuclear receptors is based on their sequential and structural similarity. Nuclear receptors are in this classification divided into 7 subfamilies (Germain et al. 2006).
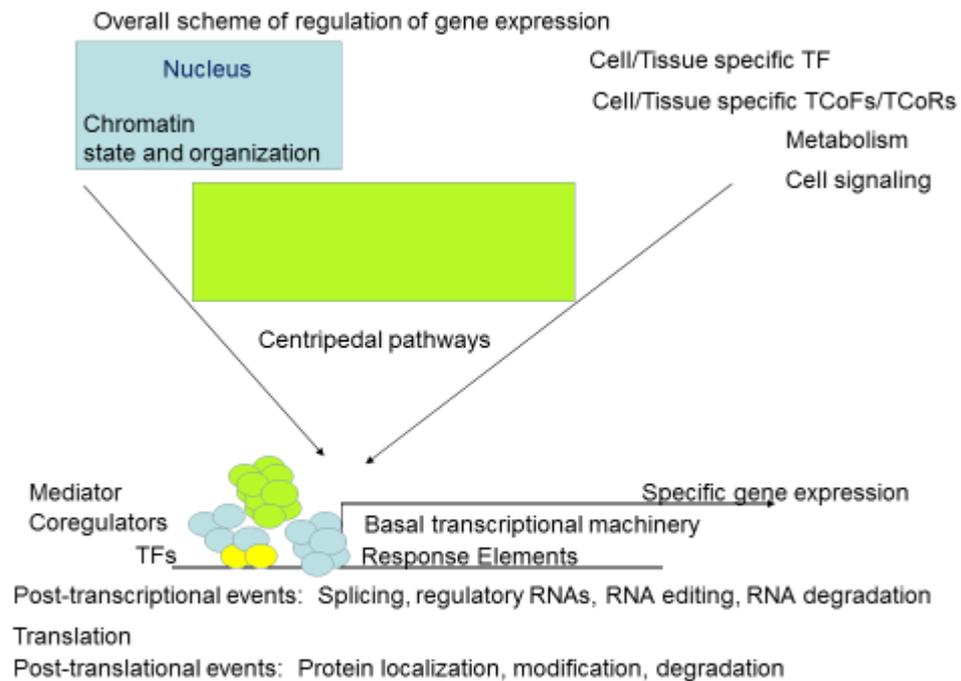
Fig. 1 Schematic representation of the regulatory flow of gene expression regulation.

Thus, the contemporary view of the regulation of gene expression includes transcription factors and their interacting cofactors that interact in a complex way with the basal transcription machinery through modulatory interactions with Mediator complex. This large number of regulatory proteins is able to further integrate regulation via signaling cascades (Fig. 1).

The regulation of gene expression is executed on additional levels downstream of transcription initiation

Following transcription initiation, gene expression is regulated at additional transcriptional events. The rate of elongation is not the same for all genes and appears to be regulated. RNA polymerase proceeds with elongation in fragmented steps with interruptions called RNA polymerase pausing (Gaertner & Zeitlinger 2014).

Besides coding regions, most eukaryotic genes also contain non-coding sequences called introns that are removed after they are transcribed by a mechanism called splicing. There are four types of introns, i. introns that are removed by the spliceosome (spliceosomal introns), ii. introns in nuclear and archaeal transfer RNA genes that are removed by specific proteins (tRNA introns), iii. self-splicing introns that are divided into two groups (group I and II introns) that are removed by RNA catalysis. Group III introns form a fifth family that is still not sufficiently characterized (Saldanha et al. 1993; Mattick 1994; Fedorova & Fedorov 2003; Irimia & Roy 2014; Yoshihisa 2014).

Beside this, different mRNA molecules may be formed from a single gene by alternative splicing. In this process, individual or multiple introns and exons are removed based on the presence of cis-acting regulatory sites (intronic and exonic splicing enhancers, intronic and exonic splicing silencers) and splicing factors that bind to these motifs (SR proteins, heterogeneous nuclear ribonucleoproteins). Alternative splicing enables cells to increase the variability of proteins originating from a particular gene (Yang et al. 1995).

Several transcription factors and cofactors were found to also act at the level of splicing (Zhang et al. 2003a; Bres et al. 2005; Wang et al. 2012).

Trans-splicing is a form of RNA processing that has been characterized in certain eukaryotes, where exons from two different primary transcripts are joined as opposed to cis-splicing which processes a single molecule. In *C. elegans* up to 70 % of mRNAs are trans-spliced to 22 nucleotide splice leaders SL1 and SL2, which are not associated with the gene. In this process the splice leader, which is donated by a 100 nucleotide snRNP (small nuclear ribonuclear protein), replaces the 5´ end of a transcript by splicing (Allen et al. 2011).

Regulation of gene expression by non-coding RNAs

Research in molecular biology and genetics had long been consumed with only protein-coding sequences of eukaryotic genomes and the vast non-coding sequences had been overlooked as mostly "junk" DNA. This situation started to change in 1993 with the discovery of RNA interference (Napoli et al. 1990; Fire et

al. 1991; Romano & Macino 1992; Guo & Kemphues 1995; Fire et al. 1998) and siRNA (Zamore et al. 2000; Elbashir et al. 2001a; Elbashir et al. 2001b) and gradually evidence suggesting important roles for non-coding RNA has accumulated. Additionally, high-throughput analysis of transcriptomes and genomes hint that up to 90% of the genome may be transcribed and most of this transcribed material accounts for non-coding RNA. What concrete biological significance these non-coding transcripts have remains to be seen (Bertone et al. 2004; Kaikkonen et al. 2011).

This non-coding RNA can be generally divided into two groups. Constitutively expressed non-coding RNAs that code for ribosomal, transfer, small nuclear, small nucleolar RNAs belong to one group while regulatory RNAs that code for microRNA (miRNA), piwi-interacting RNA (piRNA), small interfering RNA (siRNA), and long non-coding RNA (lncRNA) (plus promoter associated RNAs, enhancer RNAs) belong to the second group. There is still much research going on in this field, but the regulation of gene expression by numerous non-coding RNAs in several model organisms has been described. This regulation is mostly inhibitory (Carthew & Sontheimer 2009).

There are three main classes of small regulatory RNAs – miRNA, piRNA and siRNA (Lee et al. 1993). The boundaries between individual classes of RNAs are becoming less clear but certain characteristics do exist.

Micro RNAs are small non-coding RNA molecules 20-24 nucleotides in length and function in the post-transcriptional regulation of gene expression and RNA silencing. They were first discovered in *C. elegans*, where it was shown that two miRNAs (lin-4, let-7) regulate the timing of nematode development. It is presumed that miRNAs may regulate up to 50% of genes at the post-transcriptional level.

MicroRNAs are produced by several mechanisms. They can be transcribed from polycistronic units, introns of their host genes or from their own genes. They are usually transcribed by RNA polymerase II, but in some instances can also be transcribed by RNA polymerase III and go through several processing steps. At first a hairpin loop/stemloop structure is created. This transcript, which is called pri-miRNA (primary miRNA), is then capped and polyadenylated and spliced and can

be the precursor for several miRNAs. These pri-miRNAs are then processed by Pasha and Drosha enzymes and form pre-miRNAs (precursor miRNA). About 10% of pre-miRNAs are altered by RNA editing and are then exported from the nucleus. In the cytoplasm pre-miRNAs are further processed by Dicer, an RNase III, creating a duplex of about 22 nucleotides in length. One strand is then brought to the RISC complex where the miRNA and the mRNA target interact usually with the 3' UTR. Depending on the level of complementarity between these two sequences this process can induce cleavage of the mRNA, translational repression or mRNA degradation (deadenylation, decapping, exonucleolytic degradation) (Wightman et al. 1993; Carthew & Sontheimer 2009; Guo et al. 2010).

Short interfering RNAs are similar to miRNAs and share certain features such as double stranded precursors and are both associated with Argonaut proteins. siRNAs are 20-25 nucleotide double stranded molecules of RNA that have multiple precursors. The canonical precursor of siRNA is a long, linear molecule of dsRNA that is imported into the cytoplasm and this siRNA pathway is a natural defense mechanism that uses exogenous dsRNA (double-stranded RNA) to produce siRNA and protect the organism from viruses. This role of RNA interference is conserved across kingdoms (Gitlin et al. 2002; Susi et al. 2004; Wilkins et al. 2005).

Endogenous sources of siRNAs have been uncovered and include transcripts from transposons, centromeres, transgenes, convergent transcripts, and others. These dsRNAs are processed by Dicer to form double stranded siRNAs. This RNA is then loaded onto the RISC complex, where it is divided into a guide and passenger strand. The guide strand is selected according to the thermodynamic properties of its 5´terminus and the passenger strand is discarded. In the model RNAi (RNA interference) pathway, the guide strand locates the RISC to perfectly matching complementary RNA sequences which are degraded (cleaved by endo and then exonucleases). Imperfectly paired targets are silenced at the post-transcriptional level (similar to miRNA function) by exonucleolytic degradation and translational repression (Fire et al. 1998; Carthew & Sontheimer 2009).

Piwi-interacting RNAs are small non-coding RNAs (24-31 nucleotides in length) that function by interacting with and forming complexes with Piwi proteins (Argonaut family of proteins). Their main role is to suppress the activity of

transposons in germ line development in *Drosophila melanogaster* (Sarot et al. 2004) and they have also been identified in *Caenorhabditis elegans* (Ishizu et al. 2012).

Long non-coding RNAs are RNA molecules more than 200 nucleotides in length that do not code for proteins. Short RNAs can be derived from primary transcripts of these sequences.

Long non-coding RNAs can be classified according to their genomic localization. Stand-alone lncRNAs are found in intergenic regions. Many of these sequences are transcribed by RNA polymerase II, are polyadenylated and spliced and have a length of about 1 kb. Examples include Xist. Natural antisense transcripts are transcribed from the opposite strand of DNA that code for proteins. They may overlap partly or fully with the antisense strand. lncRNAs can also be transcribed from pseudogenes and many long transcripts have been found in introns of protein-coding genes but few have been studied in detail (He et al. 2008; Cabili et al. 2011).

Even though the transcription levels of many lncRNAs correlate with developmental processes or disease states only a few have been studied in detail. One of the best studied examples include Xist, which is responsible for X chromosome inactivation. Xist itself is regulated by lncRNAs such as Tsix. lncRNA have been shown to function also in imprinting and development (Kung et al. 2013).

Regulation of gene expression on the level of translation

Translation is the process of protein synthesis by ribosomes. It is one of the fundamental points of the Central dogma of molecular biology and genetics. Here the product of transcription, mRNA, is decoded by ribosomes, which then produce a specific amino acid chain (Crick 1970).

Translation, especially translation initiation, is a highly regulated process described to great detail (Asano et al. 2001; Sonenberg & Hinnebusch 2007; Sonenberg & Hinnebusch 2009; Hinnebusch 2011; Hinnebusch & Lorsch 2012; Hinnebusch 2014). Besides this, a specific regulatory code for regulation of gene expression on the level of translation was demonstrated on cells whose translation control was high jacked by Herpes simplex virus type 1 through the viral protein US11 (Diaz et al. 1996; Greco et al. 2001; Catez et al. 2002).

Fig. 2 summarizes the major regulatory levels of gene expression.

Control of eukaryotic gene expression
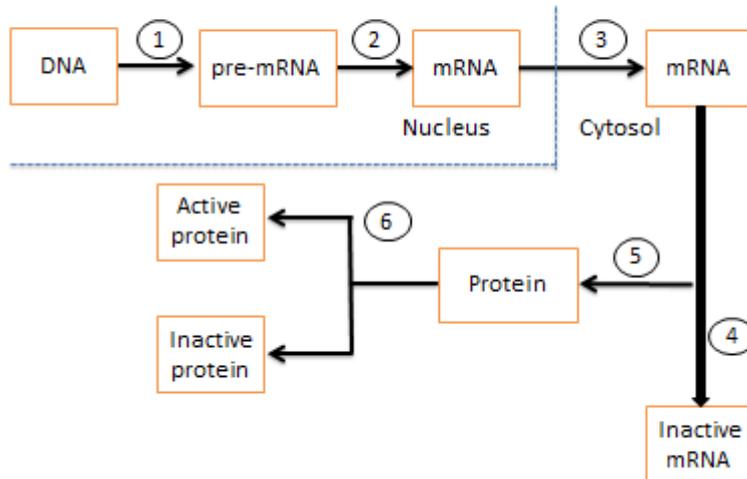
Fig. 2 Control of eukaryotic gene expression on multiple levels

1. Transcriptional control

2. RNA processing

3. RNA transport, RNA localization control

4. mRNA degradation

5. Control of translation

6. Protein activity control

Key proteins in this thesis

The two proteins that are central to the research presented in this thesis, SKIP (SKP-1) and BIR-1 were identified as proteins functionally connected with an evolutionarily conserved nuclear receptor NHR-23 in *C. elegans* (Kostrouchova et al. 2002; Kostrouchova et al. 2003; Liby et al. 2006b).

SKIP

SKIP is an ancient transcription cofactor found in all multicellular organisms as well as in yeast. It was originally identified as BX42, a *Drosophila* nuclear protein associated with active transcription (puffs) on polytene chromosomes (Saumweber et al. 1990; Wieland et al. 1992) and later found in many species including

Dictyostellium discoideum (Folk et al. 1996) and yeast (Martinkova et al. 2002). SKIP interacts with several transcription factors including nuclear receptors (Baudino et al. 1998; Barry et al. 2003; Fantappie et al. 2008; Abankwa et al. 2013), Notch (Zhou et al. 2000), Wnt/beta catenin (Wang et al. 2010), TGF beta and Smad protein complexes (Leong et al. 2001) and it was also identified as a component of the splicing machinery in yeast, mammals (Zhang et al. 2003a) and plants (Wang et al. 2012). It was identified in both transcription activating as well as transcription inhibiting complexes (Leong et al. 2004). In *C. elegans*, SKP-1 is indispensable for normal development and its inhibition results in multiple phenotypes including defects of larval transition and molting that is dependent on NHR-23 (Kostrouchova et al. 2002).

BIR-1

In *C. elegans*, the gene coding for SKIP (SKP-1) is organized in an operon together with the gene coding for the mitotic and microtubule organizing protein BIR-1 (Kostrouchova et al. 2002), which is the homologue of the vertebrate protein Survivin. Survivin was first discovered as an antiapoptotic protein (Ambrosini et al. 1997). It is expressed predominantly in fast dividing cells and is found upregulated in most if not all human cancers (Li et al. 1998). It was realized very early after its discovery that the main function of Survivin is its role in mitosis, to ensure the proper segregation of chromatids. This function is mediated by the chromosome passenger complex that is made up of AuroraB, Incenp, Borealin and Survivin. The role of Survivin, beside its requirement for the formation of this complex is to destabilize the attachment of microtubules to kinetochores thus providing more time for the attachment machinery to sense and control the attachment of both chromatids to opposing poles of the mitotic spindle (Li et al. 1998). This function is conserved in the Survivin homologue, BIR-1 in *C. elegans* (Fraser et al. 1999; Speliotes et al. 2000). Since operons ensure that co-organized genes are co-expressed, at least on the transcriptional level, it was hypothesized that these two proteins may be linked functionally. In *C. elegans*, it was previously shown that both BIR-1 and SKIP are involved in the regulation of gene expression and development. Further, in a heterologous transfection system with thyroid receptor/triiodothyronine, these factors

were shown to act cooperatively in activating gene expression (Kostrouchova et al. 2003).

The model organism *Caenorhabditis elegans*

The model organism used in the experiments constituting this thesis was the nematode *Caenorhabditis elegans*.

C. elegans is a transparent nematode approximately 1 mm in length that lives in the soil in most temperate environments. *C. elegans* feeds on bacteria and is not parasitic. It has a short life cycle and is easy to keep at laboratory conditions. Nematodes are grown on Petri dishes with nematode growth medium and are fed with *Escherichia coli*. *C. elegans* has a constant number of somatic cells. Adult hermaphrodites have 959 somatic cells and adult males have 1031 cells. The complete cell lineage of *C. elegans* has been described. Because of its unique features and simplicity it has become a very useful model organism in genetic and developmental studies (Brenner 1974).

C. elegans has two sexes, males (XO) and hermaphrodites (XX). Males produce sperm and can fertilize hermaphrodites. Hermaphrodites can produce both sperm and oocytes and have the ability to self-fertilize, but they cannot fertilize each other. Males are rare (1:500) and arise spontaneously by non-disjunction of sex chromosomes.

Hermaphrodites lay eggs which hatch into larvae in about 12 hours. *C. elegans* develops through four larval stages into adults in about three days and lives for about three weeks. When environmental conditions are unfavorable, *C. elegans* develops into dauer larvae instead of L3 larvae. Dauer larvae can survive for several months without feeding, and when food becomes available, molt into normal L4 larvae (Hu 2007).

C. elegans embryogenesis can be divided into two stages (Sulston et al. 1983). The first stage, proliferation, involves cell divisions from a single cell into 558 cells. Initial cell divisions generate six founder cells – AB, E, MS, C, D, P4 (AB – epidermis, neurons, muscles; E – intestinal cells; MS – gonads, muscle; C – epidermis, neurons, muscles; D – body wall muscle cells; P4 – germ line). These

cells then give rise to a predetermined number of cells by a series of symmetrical and synchronous divisions (Deppe et al. 1978).

In the second stage of embryogenesis, organogenesis/morphogenesis occurs and cells differentiate without additional cell divisions. The embryo elongates and has fully differentiated tissues. At the end of embryogenesis, the main body plan of the animal is established. In postembryonic development, the germ-line proliferates and mature gonad is formed in the L4 stage. In this stage the number of somatic cells increases to 959 in hermaphrodites and 1031 in males.

C. elegans genetics

The *C. elegans* genome of about 10^8 base pairs is relatively small and consists of six chromosomes and a mitochondrial genome. *C. elegans* hermaphrodites contain five pairs of autosomes and a pair of X chromosomes while males have five pairs of autosomes and a single X chromosome. Recombination occurs in the sperm of males and in both the sperm and oocytes of hermaphrodites (Brenner 1974).

In 1998 *C. elegans* became the first multicellular organism whose nearly whole genome was sequenced. By 2002 the sequencing of the whole genome was completed.

C. elegans along with some other nematodes and a few eukaryotes (trypanosomes, flatworms (Turbellaria), the chordate tunicate − *Oikopleura dioica*) have operons. Operons consist of several structural genes, which are arranged under a common promoter. In *C. elegans*, about 15% of genes are organized in operons and these operons usually contain 2-8 genes (Blumenthal et al. 2002).

The *C. elegans* genome contains about 20,000 protein-coding genes which are spread over the whole genome (Hillier et al. 2005). About 35 % of *C. elegans* genes have human homologues. Most *C. elegans* genes are about 3 kb in length, exons and introns are also usually short (exons – median 123 bp, introns – median 47 bp). Alternative splicing occurs in *C.* elegans, but the number of splicing isoforms per gene is low and most genes have only one or two isoforms. 18S, 5.8S, 26S rRNA genes are found on chromosome I and form large tandem repeats. 5S rRNA genes are found on chromosome V (consisting of 100 tandem repeats).

The number of non-coding RNA genes in the *C. elegans* genome has increased dramatically during the last decade. While in 2005 the number of non-coding RNA genes was though to number around 1300 (Stricklin et al. 2005), currently Worm base lists more than 16,000 RNA genes in the *C. elegans* genome (Ruby et al. 2006; Wang & Ruvinsky 2012).

Apoptosis / Programed cell death

Apoptosis is a highly regulated process of programmed cell death that occurs in multicellular organisms. The major apoptosis pathways are carried out by caspases, cysteine-dependent aspartate-directed proteases. Apoptosis can be initiated by numerous mechanisms, but there are two main and best described pathways - the intrinsic and extrinsic pathway. Both pathways activate initiator caspases (caspase-2, -8, -9), which are soluble inactive monomers, and these in turn activate executioner caspases (inactive dimers, caspase-3, -6, -7), which then start a proteolytic cascade that cleaves thousands of proteins (McIlwain et al. 2013; McIlwain et al. 2015).

In the extrinsic pathway an extracellular signal protein (TNF, FAS ligand) binds to cell-surface death receptors (TNF-receptor, FAS receptor) and this results in the formation of the Death inducing signaling complex (DISC). DISC then activates executioner caspases (Elmore 2007).

Mitochondria are central to the intrinsic apoptotic pathway, where cellular stress (DNA damage) or developmental signals cause the release of mitochondrial proteins into the cytosol and initiate the programmed cell death pathway. Cytochrome c, a component of the electron-transfer chain, is a key player in the best studied forms of the intrinsic pathway. When cytochrome c is released into the cytoplasm, it binds to Apaf1 (apoptotic protease activating factor-1) which then oligomerizes into a heptamer called the apoptosome. Caspase-9 proteins are then recruited and activated by the apoptosome. The activated caspase-9 molecules then activate executioner caspases and induce apoptosis (Elmore 2007).

Morphologically, apoptosis is an organized degradation of the cell and its cellular organelles. Unlike necrosis it does not trigger inflammation. The main morphological signs include cell shrinkage and rounding, pyknosis (condensation of chromatin into compact patches), karyorrhexis (fragmentation of the nucleus),

blebing of the cell membrane. Eventually the cell breaks into apoptotic bodies (vesicles), which are phagocytosed. Dying cells are marked for phagocytosis by displaying phagocytotic molecules on their surface (phosphatidylserine). Phosphatidylserine is found on the inner side of the plasma membrane in living cells and is redistributed to the extracellular side during apoptosis (Elmore 2007).

Apoptosis is very tightly regulated in all organisms because once it is initiated it cannot be reversed. There are multiple mechanisms to ensure that caspases are only activated in the correct situation.

Members of the Bcl-2 (B-cell lymphoma 2) family of proteins are important regulators of the intrinsic pathway of apoptosis. In mammals, members of this family can be both pro- and anti-apoptotic. Bcl-2 itself and Bcl-xL are anti-apoptotic. Pro-apoptotic members include effector Bcl-2 family proteins Bax and Bak and BH3-only proteins. Acting on apoptotic signals, Bax and Bak aggregate to form oligomers in the outer mitochondrial membrane and cause the release of cytochrome c. Anti-apoptotic Bcl-2 proteins function by binding and inhibiting pro-apoptotic Bcl-2 members in the cytosol or on the mitochondrial membrane. BH3-only proteins bind and inhibit anti-apoptotic Bcl-2 family proteins and stimulate the aggregation of Bax and Bak. p53 activates the transcription of genes that code for BH3-only proteins (Portt et al. 2011; Shamas-Din et al. 2013).

Another regulatory mechanism involves proteins belonging to the inhibitor of apoptosis protein (IAP) family. These proteins were first identified in baculoviruses (insect viruses), which use IAP proteins to prevent the host cells from killing themselves when infected by the virus. IAP proteins have been identified in most animal cells and function by binding and inhibiting caspases through their BIR (Baculovirus IAP repeat) domain. IAPs can also mark caspases for destruction by proteosomes by polyubiquitinating them. IAPs can negatively regulate both main pathways and set an inhibitory threshold that caspases must overcome to trigger apoptosis. Among the best studied of human IAPs are XIAP (X-linked inhibitor of apoptosis protein) and Survivin. The anti-apoptotic functions of IAP can be neutralized by anti-IAP proteins such as SMAC/DIABLO (second mitochondria-derived activator of caspases / direct IAP binding protein with low pI) which inhibits XIAP (Elmore 2007; de Almagro & Vucic 2012; de Almagro et al. 2015; de

Almagro & Vucic 2015). Contrary to invertebrates, the function of IAP and anti-IAP proteins in mammals is not so clear. While in *Drosophila* IAP also function in development, mice develop normally when missing the major mammalian IAP, XIAP. Nematodes do not contain a caspase inhibiting IAP protein (Fraser et al. 1999; Speliotes et al. 2000).

p53

p53 (tumor protein p53) is a crucial tumor suppressor protein and the most frequently mutated protein in human cancer (in more than 50%). p53 is a transcription factor which has many additional functions in apoptosis, genomic stability, DNA repair and regulation of the cell cycle. It can be activated in response to different stress signals such as DNA damage, oncogene activation, hypoxia. When activated, p53 elicits different cellular responses such as apoptosis, cell cycle arrest, senescence in a context-dependent manner by activating or repressing important genes (Yee & Vousden 2005; Riley et al. 2008; Green & Kroemer 2009; Zilfou & Lowe 2009).

Unstressed cells maintain a low level of p53. This is accomplished by a negative feedback loop where p53 positively regulates the transcription of the MDM2 gene (Haupt et al. 1997; Honda et al. 1997; Freedman & Levine 1998).

MDM2

MDM2, Mouse double minute 2 homologue, is a protein that is also known as E3 ubiquitin-protein ligase. MDM2 is a key negative regulator of the p53 tumor suppressor protein. MDM2 regulates p53 in several ways. As an E3 ubiquitin ligase, MDM2 polyubiquitinates p53 and labels it for degradation by the proteasome. But MDM2 also functions on the level of transcription by binding essential transcription factors and p53 specific co-activators (CBP, p300). It also specifically localizes to chromatin, to the p53 responsive promoter regions, where it directly inhibits p53 transcription (Shi & Gu 2012; Shi et al. 2014).

Ribosomal stress

Cell proliferation and cell growth are closely linked. The increase in cell mass which accompanies cell growth requires substantial protein synthesis and especially a large number of ribosomes. Because ribosome biogenesis is an energetically very demanding process it is very closely regulated. Intrinsic and extrinsic cellular signals converge in the nucleolus, which is a central regulatory point of the ribosome biogenesis program. This pathway is also linked to p53. Dysregulation of ribosome synthesis is present in cancer (through the dysregulation of c-myc, pI3K – mTOR pathway) and recently it has been hypothesized that this dysregulation is not merely required for cancer, but a driving force of cancer (Golomb et al. 2014).

Because ribosome biosynthesis is such a highly demanding process in terms of energy and resources, many types of cellular stress result in a shutdown of rRNA transcription, which is termed nucleolar stress (Ellis 2014; James et al. 2014). Morphologically this process is represented by the condensation of the nucleolus and its segregation into nucleolar caps (Holmberg Olausson et al. 2012; Ellis 2014; James et al. 2014). A signaling pathway involving RPL 11 (Ribosomal protein L 11) and RPL 5, 5S rRNA can transfer this stress message to MDM2/p53 (Golomb et al. 2014).

MDM2 is the main negative regulator of p53 as mentioned above. The interaction of MDM2 and p53 can be mediated by post-translational modifications of both proteins. Especially p53 is the target of numerous post-translational modifications including phosphorylation, acetylation, sumoylation, methylation. Ubiquitination has already been mentioned previously. These post-translational modifications can influence the interaction of p53 with MDM2, enhance p53 tetramerization and enhance the binding of p53 to DNA response elements (Gu & Roeder 1997; Meek 1998; Ashcroft et al. 1999a; Ashcroft et al. 1999b; Ashcroft & Vousden 1999; Maya et al. 2001; Pereg et al. 2005; Brooks & Gu).

Protein interactions can also alter the interaction of p53 with MDM2. ARF (p14^{ARF}), a tumor suppressor protein, which is induced in response to mitogenic stimulation from c-myc (c-myc overexpression) can bind and sequester MDM2 in the nucleolus. This sequestration prevents the negative feedback regulation of p53.

ARF can also block MDM2 function by binding the acidic domain of MDM2 and blocking its E3 ligase activity (Zindy et al. 1998; Weber et al. 1999).

Ribosomal proteins (RPs) can also bind MDM2 and activate p53. Fourteen ribosomal proteins have been shown to bind MDM2 (L5, L11, L23, L26, S3, S7, S14, S15, S20, S25, S26, S27, S27L), from this group RPL 5 and RPL 11 are among the best studied in this regard. It is not clear why so many ribosomal proteins bind to MDM2. It is theorized that the binding of multiple RPs on MDM2 can have an additive effect, different RPs can be present in the nucleoplasm under different conditions of ribosomal stress, or a complex of RPs may be needed to regulate MDM2 (Zhang & Lu 2009; Manfredi 2010; Golomb et al. 2014; Kim et al. 2014).

RPL 5, RPL 11 and RPL 23 can regulate p53 much like ARF and overexpression of these ribosomal proteins reduces MDM2 ubiquitin ligase activity and stabilizes p53.

There are several reasons for regarding RPL 5 and RPL 11 as important mediators of ribosomal stress. The knockdown of these proteins reduces the activation of p53 in response to ribosome biogenesis stress signals. Following ribosomal stress endogenous RPL 5 and RPL 11 accumulate in non-ribosomal fractions, where they can bind MDM2. The source of p53 activating ribosomal proteins is still under debate. It was first postulated that ribosomal proteins are released from the nucleolus into the nucleoplasm following ribosomal stress due to nucleolar disruption. But some ribosomal biogenesis stressors do not cause nucleolar damage (no evidence of nucleolar disruption of integrity) but still elicit the activation of p53 in an RPL11/MDM2 dependent manner. Protein synthesis is a possible source of p53 activating RP. While other RP are degraded by the proteasome, RPL 5 and 11 are protected from degradation and imported into the nucleus (Horn & Vousden 2008a; Zhang & Lu 2009; Manfredi 2010; Kim et al. 2014).

3.3 Study strategy and reasoning

Previous work indicated that three structurally dissimilar proteins are functionally interconnected: development regulating nuclear receptor NHR-23, transcriptional and splicing cofactor SKIP and the mitosis regulating protein BIR-1.

This study focused on the possible involvement of SKP-1 and BIR-1 in a protein regulatory network and this was done by searching for their interacting proteins using yeast two-hybrid screens. Surprisingly this strategy indicated that SKP-1 and BIR-1 interact with a wide variety of partially overlapping categories of proteins but not directly with each other. The regulatory potential of BIR-1 was visualized using a short time overproduction of BIR-1 in synchronized *C. elegans* larvae and by a whole proteome differential display. This confirmed that elevated levels of BIR-1 project to immediate whole proteome changes. The results were validated for ribosomal proteins RPS-3 and RPL-5, non-muscle myosin and TAC-1, a transcription cofactor and a centrosome associated protein implicated in cancer. Our results show that SKP-1 and BIR-1 are linked more than previously thought. They have potential to link the proteome status with major cellular regulatory pathways including gene expression, ribosomal stress pathway, apoptosis and cell division. SKP-1 and BIR-1 may be regarded as proteome sensors.

4. Materials and methods

4.1 Experimental design

Screening for interacting proteins of BIR-1 and SKP-1 was performed using the ProQuest Two-Hybrid System with Gateway Technology purchased from Invitrogen (Carlsbad, California, USA). Potential direct interactions between BIR-1 and SKP-1 were analyzed using the same system. The effect of a short-time forced expression of BIR-1 on the near-complete proteome of non-dividing cells of *C. elegans* L1 larvae was visualized by two dimensional comparative chromatography using the Proteome fractionation system from Beckman Coulter (Brea, CA, USA) and fractions with differential protein content were visualized by DeltaVue software and were further examined by mass spectrometry. Selected proteins identified as BIR-1 and SKP-1 interacting proteins or proteomic targets of BIR-1 were analyzed in pull-down experiments using BIR-1 and SKP-1 GST-fusion proteins. Analyzed target proteins were expressed in vitro using the reticulocyte TNT system from Promega (Fitchburg, WI, USA) and labeled by ^{35}S-methionine (Institute of Isotopes Co., Budapest, Hungary). Bound interacting proteins were detected by Liquid scintillation analyzer Tri-Carb 1600TR Packard (Meriden, CT, USA). The effect of BIR-1 short-time overexpression on selected candidate interacting proteins was visualized using immunohistochemistry or by functional studies of cell cycle and apoptosis (employing immunohistochemistry and lines carrying integrated GFP fusion transgenes).

Strains used in the study

The *C. elegans* Bristol N2 strain was used whenever not specifically stated and was maintained as described (Brenner 1974). For visualization of chromatin structure, the line AZ212 expressing Histone H2B::GFP was used.

BIR-1 overexpressing worms were created as lines expressing *bir-1* mRNA from heat-shock regulated promoter and were prepared by amplifying *bir-1* cDNA from wild-type mRNA. Sub-cloned and sequence verified constructs were cloned into the heat-shock promoter vector pPD49.83. 100ng/µl of plasmid DNA was microinjected along with a marker plasmid pPRF4, *rol-6 (su10060)* using an

Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) equipped with a PC-10 Narishige Microinjection System (Narishige, Tokyo, Japan).

4.2 Yeast two-hybrid system

To identify BIR-1 and SKP-1 interacting proteins, we used the ProQuest Two-Hybrid System with Gateway Technology purchased from Invitrogen. The *C. elegans* mixed stages (Bristol N2) library (originally made by Monique A. Kreutzer and Sander van den Heuvel) was purchased from Invitrogen (Cat. No. 11288-016). *bir-1* and *skp-1* cDNAs were amplified from N2 mixed stages cDNA using primers having flanking sequences ATT and ligated into the pENTRY vector. After cloning the cDNAs, the inserts containing complete coding regions were transferred into pDEST^{TM}32 vector using Clonase leading to DBX constructs (bait vectors) creating BIR-1 and SKP-1 fused to GAL4 DNA binding domain. These vectors were used for screening of the *C. elegans* cDNA library after testing for self-activation of both *bir-1* and *skp-1* bait vectors.

The vector pDEST 32 contains the GAL4 DNA binding domain, the ARS/CEN6 sequence for replication and maintenance of low copy numbers in yeast, the LEU2 gene for selection in yeast on medium lacking leucine, the constitutive moderate-strength promoter and transcription terminator of the yeast Alcohol Dehydrogenase gene (ADH1) to drive expression of the GAL4 DBD bait fusion, the dominant CYH 2 S allele that confers sensitivity to cycloheximide in yeast for plasmid shuffling, a pUC-based replication origin and gentamicin resistance gene (Gmr) for replication and maintenance in *E. coli*.

To analyze the binding of BIR-1 to SKP-1 the pDEST™22 vector containing a GAL4 Activation Domain (GAL4 AD) containing Gateway ® Destination Vector was used.

Similarly, the constructs for SKP-1 were prepared using the same vectors. To reduce false positive interactants, the "Three Reporter Genes" system was used (HIS3, URA3 and lacZ stably integrated in the yeast genome). Additional controls included yeast control strains A to E, a self-activation test of the bait constructs, and a test of growth on histidine deficient media. For both BIR-1 and SKP-1 interacting proteins a total of 250 µl of competent cells containing more than 10^6 transformants

was acquired. Screening yielded 54 colonies for SKP-1 and approximately 30 for BIR-1 that were prepared as yeast minipreps, screened by PCR using primers 5036 and 5037 (derived from pPC86 vector) and sequenced. All sequences were controlled for proper frame ligation of the insert by sequencing.

4.3 Two-dimensional comparative chromatography

Two-dimensional chromatographic separation of worm lysates was performed on the ProteomeLab PF 2D Protein Fractionation System (Beckman Coulter, Inc., Fullerton, CA) as recommended by the manufacturer. The chromatograms were analyzed using computer software provided by the manufacturer.

In order to detect differences in proteomes of mutant and wild-type larvae we prepared total protein from synchronized, bleached N2 L1 worms and homozygous *bir-1* animals. Proteomes were then analyzed using PF2D. In the first dimension all proteins were separated into 37 fractions by chromatofocusing, according to their isoelectric point, and eluted by a pH gradient. Proteins with an isoelectric point below pH 4.0 were then eluted by a rising concentration of NaCl (by rising ionic strength). Each of the 37 fractions was then further separated into an additional 35 fractions in the second dimension according to hydrophobicity by reversed-phase chromatography.

Preparation of protein lysates

In order to prepare larvae overexpressing *bir-1* in a short time period, we prepared embryos from transgenic hermaphrodites carrying *bir-1* gene regulated by heat-shock responding promoter. Synchronized L1 larvae were prepared by food deprivation. Control larvae were prepared in the same way. Larvae were then exposed to 34°C for 30 minutes, left for 60 minutes at room temperature to recover. Larvae were than pelleted by centrifugation and frozen in aliquots. In order to obtain sufficient amount of material, these experiments had to be repeated 20 times over the period of 3 months. Control larvae were prepared in parallel in the same number of individual experiments. For preparation of protein lysates, frozen samples were melted on wet ice, pooled in 0.2 ml of 50 mM Tris-HCL (pH 8.0) and vortexed. The

samples were then mixed with 1.6 ml of lysis buffer (7.5 M urea, 2.5 M thiourea, 12.5 % glycerol, 50 mM Tris, 2.5 % n-octylglucoside, 6.25 mM Tris-(carboxyethyl) phosphine hydrochloride containing 1x Protease Inhibitor Cocktail (Boehringer Mannheim, Mannheim, Germany)). The suspensions were incubated on ice for 10 minutes and sonicated in five cycles, each consisting of four times 10s sonication /10s interruption (20 kHz, amplitude 20 μm, 60 W) (Ultrasonic Processor (Cole-Parmers Instruments, Vernon Hills, IL)) using the internal sonication rod. The suspension was cleared from the non-soluble material by centrifugation at 20,000 x g for 60 minutes at 4°C and the supernatants were harvested. For subsequent first and second dimension chromatographic separations, the Beckman ProteomeLab PF 2D kit (part No. 380977) (Beckman Coulter, Inc., Fullerton, CA) was used. The sample was supplemented with Start Buffer to a final volume of 2.5 ml. The lysis buffer was then exchanged for the Start Buffer supplied by Beckman using the PD10 column (Amersham Pharmacia Biosciences, Uppsala, Sweden) equilibrated with Start Buffer for the first dimension separation (pH 8.5±0.1, pH adjusted with iminodiacetic acid and ammonium hydroxide). The samples were loaded onto the PD10 columns, the first eluents were discarded. Start Buffer was used to elute the proteins that were collected in the first 3.5 ml fractions. The protein content was estimated using a BCA kit (Pierce, Rockford, IL) (C=0.62 μg/μl for *bir-1* overexpression and C=1.06 μg/μl for controls). 1.2 mg of total protein were loaded into the First Dimension Module in a total volume of 2 ml (the volume for control protein lysate was increased to 2 ml using 1x Start Buffer).

First dimension separation - Chromatofocusing HPLC (HPCF)

For the chromatofocusing separation, the first module of the Beckman Coulter ProteomeLab PF 2D system was used (Beckman Coulter, Inc., Fullerton, CA). The HPCF column was equilibrated with 25 column volumes of Start Buffer. The pH gradient was based on the buffers supplied by the manufacturer, the Start Buffer and the Elution Buffer (Beckman Coulter, Inc; pH adjusted with iminodiacetic acid and ammonium hydroxide). The upper limit of the pH gradient was set by the Start Buffer (pH 8.5) and the lower limit was set by the Elution Buffer (pH 4.0). The pH was monitored using a flow-through on-line probe. Following the

pH gradient elution, the proteins remaining in the column were eluted by increasing ionic strength gradient (based on 1 M and 5 M NaCl). Protein content in eluates was determined by UV absorbance at 280 nm. Fractions were collected in 96 well plates (2 ml well capacity). The chromatofocusing fractionation was based first on time for the first 9 fractions (5 minute intervals), then on pH (in the range of pH 8.5 to 4.0, 17 fractions, steps of pH approximately 0.27) and finally on ionic strength (1 M NaCl to 5 M NaCl) for the last 14 fractions. The fractions were collected by Beckman Coulter FC/I Module (Fraction collector/Injector). The reproducibility of the first (and the second dimension) separation was tested using control material assayed by Western blots for selected nuclear hormone receptors and was satisfactory for both dimensions. A ProteomeLab PF 2D kit containing new buffers and columns was used for the first dimension separation of control proteome and BIR-1 overexpression proteome and both analyses were done in the same day after careful wash and equilibration of the first dimension chromatofocusing HPLC (HPCF) system in an air-conditioned laboratory at 23°C.

Second dimension separation – Reversed Phase HPLC (HPRP – High Performance Reversed Phase Chromatography)

The second module of Beckman Coulter ProteomeLab PF 2D system was used for the separation of proteins according to the surface hydrophobicity with two solvents. Solvent A was 0.1 % trifluoroacetic acid (TFA) in water and solvent B was 0.08 % TFA in acetonitrile. The aliquots of first dimension fractions were separated on HPRP columns packed with nonporous silica beads at 50°C. The module was equilibrated with solvent A for 10 minutes. The gradient was run from 0 to 100 % of solvent B in 35 minutes, followed by an elution with solvent B for 5 minutes to elute the remaining proteins from the column. The fractions were collected at 1 minute intervals (at the flow rate 0.2 ml/min) into 96 well plates (2 ml well capacity) using Fraction collector FC 204 (Gilson, Inc., Middleton, WI, USA). The module was then washed and equilibrated with solvent A for 10 minutes and prepared for second dimension separation of another first dimension separation fraction. Fractions were frozen before following mass spectrometry analysis. A total of 1260 fractions was

collected for each control proteome and the proteome of BIR-1 overexpressing larvae.

Chromatograms from corresponding paired fractions were then analysed using 32Karat software. ProteoVue and DeltaVue software enabled us to represent differentially the entire proteome and also individual fractions. Some paired samples required manual compensation for a higher baseline.

The 98 paired fractions that showed prominent differences in major chromatographic peaks were selected for further analysis by mass spectrometry to identify their protein components. Chromatographic fractions that corresponded to identified peaks of paired fractions were prepared and analyzed using liquid chromatography-tandem mass spectrometry (LC/MS/MS) to identify present proteins by peptide microsequencing to derive sequences of individual proteins.

Specific fractions that were chosen for further analysis by mass spectrometry were prepared in the following manner – fractions were dried down into pellets (by SpeedVac – N-Biotek Inc.), these pellets were then dissolved in 15 μl of cleavage buffer which contained 0.01% 2-mercaptoethanol (Sigma Aldrich, St. Louis, MO, USA), 0.05 M 4-ethylmorpholine acetate pH 8.1(Fluka (Sigma Aldrich)), 5% MeCN (Merck, Whitehouse Station, NJ, USA), and 10 ng/μl of sequencing grade trypsin (Promega). Digestion was carried out at 37°C overnight and the resultant peptides were subjected to analysis by mass spectrometry.

Five microliters of the mixture was applied on a Magic-C18 column, (0.180 × 150 mm, 200 Å, 5 μm -Michrom Bioresources, Auburn, CA) and separated by gradient elution. The column was connected to a LCQ^{DECA} ion trap mass spectrometer (ThermoQuest, San Jose, CA) and equipped with a nanoelectrospray ion source. Spectrum analysis was done using SEQUEST^{TM} software against the SwissProt database. SEQUEST results were processed with BioWorks Browser software (Tabb et al. 2002) using the following criteria: XCorr values were 1.7 for singly charged, 2.2 for doubly charged and 3.0 for triply charged peptides (Pohludka et al. 2008).

4.4 Bioinformatics analysis

The bioinformatics analysis was done using NCBI bioinformatic tools BLAST (Altschul et al. 1990), gene ontology tool DAVID (http://david.abcc.ncifcrf.gov/) (Huang et al. 2009b; Huang et al. 2009a) and Wormbase WS242 (http://www.wormbase.org). GO terms with the enrichment factor bigger than 2 were considered as significant. Curated GO terms keeping with known functions of either BIR-1 of SKP-1 were considered as criteria of shared functions.

4.5 Pull-down experiments for selected proteins

The complete cDNA of BIR-1 and SKP-1 (not including the first methionine codon) was amplified by PCR and cloned into the pGEX-2T vector (Amersham Pharmacia Biotech, Amsterdam, UK) and sequenced. The GST (Glutathione-S-transferase) fusion proteins were expressed in the BL-21 strain of *Escherichia coli*. Empty pGEX-2T vectors expressing the protein domain of GST were used for control experiments. Cultures of transformed bacteria that were obtained from single bacterial colonies were grown overnight at 37°C in 400 ml of Luria-Broth medium with 100 μg/ml of Ampicillin. Cultures were grown to an O.D. (600 nm) of 0.8 and subsequently induced by 1 mM isopropylthiogalactopyranoside (IPTG), incubated at 20° C for 5 hours and centrifuged to pellets at 3300 xg at 4° C for 10 minutes. The pellets were washed twice in LB medium and then resuspended in 6 ml of phosphate-buffered saline (PBS). Cell lysis of bacteria was performed in 6 ml of Lysis buffer (Biorad - 2x Native lysis buffer, CA), that was supplemented with protease inhibitors (1x Complete, Roche, Penzberg, Germany). The samples were incubated on ice for 10 minutes with intermittent vortexing and sonication (4x for 10 seconds at 80 % intensity) – (Sonicator UP 100 H, Hielscher, Teltow, Germany). The lysates were centrifuged at 10 000 RPM for 5 min. at 4° C. The supernatant was removed and filtered by ROTH 0.22μm filter (Carl Roth GmbH, Karlsruhe, Germany). Glutathione-agarose (Sigma-Aldrich, St. Louis, Mo) was used for the binding of GST, GST-SKIP and GST-BIR and was prepared by swelling 0.01 g of beads in 1 ml of PBS, which were then collected by sedimentation and then resuspended in 100μl of PBS. Purification of fusion proteins and control was done in 100μl of slurry,

300μl of bacterial lysates that were incubated for 30 minutes at 4°C, and mixed intermittently (every 4 minutes). Next the beads were washed 4 times in 1 ml of PBS Triton X-100 (1%). Beads were collected by sedimentation and resuspended in 500 μl of PBS. Elution was done in 10mM reduced glutathione and 50mM TRIS-HCl, pH 9.5 (all chemicals were obtained from Sigma-Aldrich).

The TNT T7/T3 coupled reticulocyte lysate system (Promega) was used together with 1.48 MBq of ^{35}S-Methionine (37 TBq/mmol) to prepare ^{35}S-radiolabeled in the final volume of 50μl (Institute of Isotopes, Budpest, Hungary). Binding was done at 22°C for 30 minutes using 10μl of the TNT product (mixed every 4 minutes) and then the samples were washed 3x in 1 ml of PBS and resuspended in 40 μl of PBS. Afterwards 5μl of 2x Laemmli Buffer and 1μl of β-mercaptoethanol were added. The samples were boiled for 5 minutes and 35μl of the sample was used for polyacrylamide gel electrophoresis and autoradiography. 10μl of supernatant was analyzed using the Liquid Scintillation Analyzer Tri-Carb 1600 TR (Packard , Meriden, CT) and Ultima-Gold scintillation cocktail (Perkin-Elmer, Watham, MA). For determination of input in binding experiments, 2μl of in-vitro transcribed – translated product was resolved using polyacrylamide gel electrophoresis, transferred on Whitman 3M paper, dried and radioactivity determined in cut strips containing the translated proteins but not the unincorporated ^{35}S-Methionine.

4.6 *skp-1* reduction-of-function effect

The knockdown of *skp-1* was induced by injecting *skp-1* specific dsRNA directly into the gonads of adult wild type N2 hermaphrodites. The progeny was harvested and stained with DAPI and by antibody staining against SPD-2 that localizes to centrosomes (denominated 9v5, LA, a kind gift of Dr. O'Connell) (Kemp et al. 2004) and used diluted 1:100. We searched for phenotypic changes described earlier (Kostrouchova et al. 2002) and we added screening for cell cycle arrest phenotypes.

4.7 Antibody staining

Antibody experiments (for NMY-2) were done on transgenic embryos and larvae (expressing *bir-1* from heat shock regulated promoter) that were bleached, heated for 30 minutes at 34°C and allowed 1 hour for recovery at room temperature. Controls were wild type N2 embryos and larvae prepared and heated in parallel to experimental embryos. Embryos and larvae were put on poly-L-lysine-coated slides and fixed by adding10μl of 5 % paraformaldehyde to embryos and larvae that were in 5μl of water, incubated for 30 minutes in a wet chamber at room temperature and frozen on a chilled aluminum block for 5 minutes. After freeze cracking the samples were placed in cold methanol (-20°C) and cold acetone (-20°C) for ten minutes. Rehydration was done in a series of rehydration buffers in ethanol (10 minutes in 90% cold ethanol, 60 % cold ethanol, 30% ethanol at room temperature, and 1 hour in TTBS - Tris-Tween-buffered saline). The NMY-2 antibody was then applied in a 1:50 dilution and the slides kept in a wet chamber overnight at 4°C. The next day the slides were washed 3x in TTBS and a secondary antibody (goat anti-rabbit IgG AF 488 antibody, goat anti-mouse IgM AF 488 antibody; diluted 1:100) was added. The slides were incubated for 2 hours at room temperature, washed 3x in TTBS and mounted in 10μl of mounting medium.

4.8 Downregulation of *bir-1* expression

Large populations of mixed stages *C. elegans* cultures were prepared as described (Brenner 1974). Nematodes were collected in PBS, washed in deionized water (with intermediate centrifugations for 5 min at 4°C and centrifugal force CF_{max} 400 xg.

Eggs were left to hatch in medium lacking any food (M9 solution) and synchronized populations of L1 larvae were plated with two sets of HT115 bacteria transformed with control empty plasmid or the plasmid based on L4440 vector with a sequence targeting 400 bp fragment of *bir-1*. Part of the sequence of *bir-1* mRNA was omitted from the RNAi construct and used for validation of *bir-1* mRNA downregulation that was confirmed in selected cultures and in aliquots of experimental cultures removed from samples before their further processing for

subsequent experiments including microarrays. Production of dsRNA was induced by 4 mM IPTG (Isopropyl-beta-D-thiogalactoside) in both control and experimental cultures. Worms were kept on 2% agarose plates for 21 h at 20 °C, collected, and approximately 200 µl of worms resuspended in PBS were subsequently used for individual experiments. Nematodes were disintegrated using a Mixer-Mill (Miller-Mill 300) apparatus and total RNA was isolated by the RNeasy Mini Kit (Qiagen, Germantown, MD).

4.9 Analysis of microarray results

C. elegans whole genome expression microarrays (Affymetrix, Santa Clara, CA) were used to profile whole genome expression from three independent experiments for both experimental and control samples. The method is based on the primary technique introduced by (Schena et al. 1995). The three paired independent experiments were done subsequently one pair at a time in three weeks and samples were frozen at -80º C. Then RNA extraction, reverse transcription and microarray experiments were done simultaneously in order to minimize the experimental errors. Microarray chip data was collected and analyzed by both Affymetrix MAS 5.0 suite software with a threshold ≥1.6-fold change in mRNA expression and Robust Multichip Average (RMA) threshold ≥1.2-fold change in mRNA expression as set by the Partek genomics suite software package. For assuming a statistical significance, the p-value less than or equal to 0.05 was used. For direct comparison of values generated by Affymetrix software, the reasoning of comparison of reading values over the background values in each individual experiment was used as described (Elo et al. 2005). The indexes of expressional values in microarrays refer to statistical values of signals of a set of true probes across the transcript and background readings from mismatch probes which are further dependent on the total signal recorded by the particular experiment; only paired values of the same probe sets from experimental sets and controls with the same total readings were compared. The values obtained allowed direct comparison of the three control and two RNAi experimental sets. The third replicate of *bir-1* RNAi set was not used for direct comparison, but was included in the evaluation by Affymetrix MAS 5.0 suite

software and Robust Multichip Average method in which the total reading is not affecting the results.

5. Results:

5.1 Identification of BIR-1 and SKP-1 interactors by yeast two hybrid screens

Previous suggestions of functional connections between SKP-1 and BIR-1 (Kostrouchova et al. 2002; Kostrouchova et al. 2003; Liby et al. 2006b) led us to investigate if these two proteins may interact on the protein level, directly or indirectly. The commercially available *C. elegans* cDNA library (Invitrogen) was used to screen for SKP-1 and BIR-1 interacting proteins using yeast two-hybrid screens.

5.1.1 SKP-1 interacting proteins

The search for SKP-1 interacting proteins identified proteins involved in translation, translation initiation factors 2B and 4A, polyadenylate binding protein PAB-1, ribosomal protein RPL-5 and RPL-11, and transcription cofactor TAC-1, NHR-92 and Myosin Heavy Chain protein **(Table 1).**

Table 1. Proteins identified as SKP-1 interactors in a yeast two-hybrid screen

No	Sequence	Gene/Protein type
1	F11A3.2/C50F4; NM_073051.1	eIF-2B
2	Y54E2A.3	TAC-1
3	F57B9.6a	INF-1 (orthologous to mammalian eIF-4A)
4	K02F2 (WBGene00001075)	DPY-14
5	W10G6.3	IFA-2 (intermediate filament)

6	4F2011/ R08C7.3	H2A.F HTZ-1
7	Y45F10D.13, F56F12.1	SORB-1
8	F54C9.5	RPL-5
9	4K941 K04D7.1	RACK-1
10	C10G11.9, T27A3.4	Mitochondrial protein
11	Y106G6H	PAB-1
12	1E420, T03F1.7	Mitochondrial transcription factor B1
13	T22F3.4 gi|17563233|ref|NM_071607.1|	RPL-11.1
14	gi|671714|gb|L39894.1|CELCPR6A	CPR-6
15	3L413 T07A5	Myosin Heavy Chain
16	C05C10.4	PHO-11, Intestinal Acid Phosphatase Protein 4 member 2K223
17	4C397 Y41D4B.8	NHR-92 (HNF4-like)
18	W02D7.3	Similar to Ankyrin and KH repeat

5.1.2 BIR-1 interacting proteins

BIR-1 interaction studies yielded NHR-6, acid ribosomal protein RLA-0 and PAL-1, and two Y-box containing cold shock proteins, CEY-1 and CEY-2, that are homologues of vertebrate proteins that regulate gene expression on the level of transcription as well as mRNA in the cytoplasm (**Table 2**). Neither screen identified a direct interaction between SKP-1 and BIR-1. We also directly tested their potential interaction using the yeast two-hybrid system by cloning BIR-1 in one vector and SKP-1 in the other vector. This system did not show a direct interaction between BIR-1 and SKP-1 either.

Table 2. Proteins identified as BIR-1 interactors in a yeast two-hybrid screen

No	Sequence	Gene/Protein type
1	B0546.1	MAI-1
2	ZK1240.9	Ubiquitin ligase
3	C48D5.1	NHR-6
4	T10E10.2	COL-167
5	F32B6.2	MCCC-1
6	C38D4.6	PAL-1
7	F46F11.2	CEY-2
8	D2096.3	Glycoside dehydrogenase
9	F33A8.3	CEY-1
10	F25H2.10	RLA-0

5.2 Analysis of BIR-1 regulatory potential in a short time forced expression and whole proteome comparative display

Since our yeast-two hybrid experiments indicated that SKP-1 and BIR-1 may influence gene expression through shared pathways, but did not show a direct interaction, we attempted to visualize the effect of BIR-1 short-time forced overexpression on the whole proteome using a proteome differential display of *C. elegans* synchronized L1 larvae.
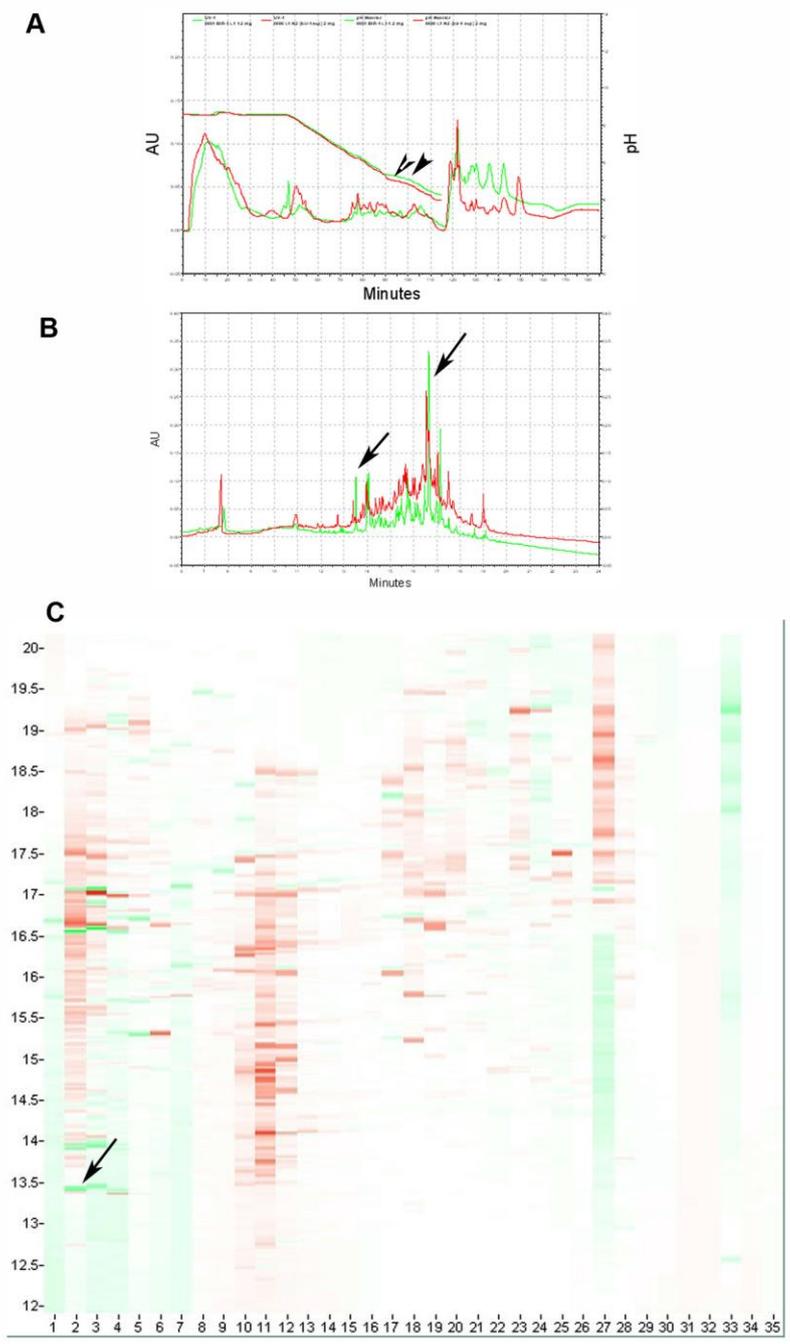
Fig. 3 Two dimensional comparative chromatography of complete proteomes of control and BIR-1 overexpressing L1 larvae. Panel A - First dimensional separation of protein lysates from wild type (N2) (red line) and *bir-1* overexpression samples (green line). Comparative analysis shows significant changes in the whole pI spectrum. The pH changes from pH 8.5 to pH 4 (arrowheads). In the last third of the chromatogram, proteins are eluted at pH 4 with an increasing concentration of NaCl

to elute acidic proteins. Panel B – Second dimension separation. A representative chromatogram of second dimension separation (fraction A2). The arrows indicate an elevated absorbance (A_{214}) indicating higher protein content in the eluate in BIR-1 overexpressing larvae (green line) during the particular chromatographic time (arrows). Panel C – Graphical representation of the differential proteome using the DeltaVue computer program. The protein content in particular chromatographic fractions is indicated in a gel-like pattern by red (for control proteome) and green colors (proteome of BIR-1 overexpressing larvae). The protein difference is indicated by the intensity of the color. Proteins constituting ninety-eight paired fractions that showed a prominent difference in protein content were used for analysis by mass spectrometry. The arrow in panel C indicates a fraction containing elevated amount of protein in BIR-1 overexpressing larvae corresponding to the peak visible in the second dimension chromatogram (panel B, left arrow). There are clearly visible dramatic differences in the differential display of both proteomes across the pH spectrum.

We hypothesized that this experimental setup may help us visualize the involvement of BIR-1 with proteins functionally shared with SKP-1, which cannot be easily targeted in other ways.

To determine the time course of maximal expression, heat shock induced BIR-1 was monitored on the mRNA level by quantitative RT-PCR. This showed that at the time of harvesting larvae for the proteome study, the mRNA level of BIR-1 was approximately 20 times higher than in control animals. Experiments detecting possible adverse effects of forced expression of BIR-1 were also conducted. As in previous experiments, we did not observe any developmental phenotype or defects of mitoses in larvae expressing increased levels of *bir-1* even after prolonged exposures.

For the comparative near-whole proteome analysis, we used the Proteome Lab Protein fractionation system (Beckman-Coulter, Brea, CA, USA). Protein lysates from synchronized *C. elegans* larvae with heat shock induced BIR-1 and wild type controls were obtained and small proteins eliminated together with salts on

PD10 columns (with the fractionation range Mr 5000) thus eliminating proteins smaller than approximately 45 amino acids. The complete proteomes were separated using pH/NaCl gradient in the first dimension and stored in 40 fractions for each proteome **(Supplementary table S1)**.

Chromatographic profiles obtained from these samples clearly differed in specific regions between the proteomes of BIR-1 overproducing larvae and control proteomes **(Fig.3 A).** In the second dimension chromatographic analysis (protein separation by hydrophobicity) approximately 1200 fractions were obtained from each control and experimental proteome. As shown on a representative chromatogram, BIR-1 hyperinduction leads to specific increases and decreases of protein content in fractions collected during the chromatographic elution. A differential display of the control and BIR-1 induction proteomes was obtained using DeltaVue software provided by the manufacturer **(Fig. 3B).** The two dimensional comparative chromatography showed, to our surprise, that short time-forced expression of *bir-1* led to complex proteome changes in approximately 100 chromatographic fractions. 98 fractions were selected for further analysis by mass spectrometry. Spectrum analysis by SEQUEST^{TM} software against the SwissProt database identified numerous *C. elegans* proteins together with proteins assigned to other species including bacteria and vertebrates. Filtering against confidence criteria (score, number of peptides) and selecting only *C. elegans* proteins yielded 24 proteins that were detected in 8 fractions (**Supplementary table S2 - List of chromatographic fractions containing differently represented proteins in BIR-1 overexpressing and control larvae**). Seventeen proteins showed clear differences between larvae expressing large levels of BIR-1 and controls (**Table 3 A and B**). These proteins were detected by mass spectrometry with high confidence only in fractions from BIR-1 overexpressing larvae (**Table 3A**) or only in the paired fraction from control larvae (**Table 3B** and **Supplementary tables S2 to S10**). Nine proteins were detected with a high confidence in both paired fractions (**Table 3 C**) including myosin and tropomyosin in acidic fraction (fraction 27) and elongation factor EF1 alpha. Interestingly, these proteins are likely to be shifted in BIR-1 overproducing larvae to more acidic fractions (**Fig. 3 C**, first dimension fraction No. 33) (but were not confirmed by mass spectrometry). These proteins were considered as candidate

differential proteins. Their likely shift to more acidic fractions (especially fraction No. 33) can be seen in the chromatograms shown in **Supplementary figures S1 to S8**. In addition to proteins with high confidence score, mass spectrometry detected many proteins with lower confidence scores. These proteins were not included in further analyses.

Table 3. Proteins detected by MS in fractions with chromatographically altered pattern in two-dimensional comparative chromatography

A. Proteins identified only in BIR-1 hyperinduction fractions		
Protein	**Gene**	**GO** (WormBase WS243)
40 S ribosomal protein S3	*rps-3*	apoptotic process, lifespan, embryo dev., molting, larval dev., reproduction, translation
Putative sideroflexin-like protein AH6.2	**AH6.2**	cation transport
ATP synthase subunit alpha, mitochondrial precursor	**H28O16.1**	ATP binding, rotational mech.
60S ribosomal protein L5	*rpl-5*	body morphogen., embryo and larval dev., reproduction, translation, (apoptosis in vertebrates)
Myosin-4 (UNC-54)	*unc-54*	morphogen., locomotion, myosin assembly
Nuclear Anchorage Protein1	*anc-1*	cytoskeleton organization, pronuclear migration

B. Proteins identified only in wild type (N2) fractions		
Protein	**Gene**	**GO** (WormBase WS243)
Probable electron transfer flavoprotein subunit	**F27D4.1**	embryo and larval dev., mitochondrial
Hit-like protein TAG-202	*tag-202*	catalytic (tumor suppressor in vertebrates)
Triosephosphate isomerase	*tpi-1*	catalytic
Uncharacterized protein B0303.3	**B0303.3**	metabolic, mitochondrion
Probable 26S protease regulatory subunit S10	*rpt-4*	morphogenesis, proteasome regulation
Probable ornithine aminotransferase, mitochondrial protein	**C16A3.10**	catalytic, transaminase activity
Probable malate dehydrogenase mitochondrial protein	*mdh-1*	catalytic
Probable Prefoldin subunit 5	**R151.9** *pfd-5*	embryo dev., pronuclear migration, protein folding, locomotion
Superoxide Dismutase [Cu-Zn]	*sod-1*	metabolic, germ cell dev., striated muscle myosin thick filament assembly
Heat-shock Protein Hsp-12.2	*hsp-12.2*	reproduction, hsp binding
Glyceraldehyde-3-phosphate Dehydrogenase 2	*gpd-2*	metabolic, development

C. Proteins identified differentially in both N2 and BIR-1 hyperinduction fractions

Protein	Gene	GO (WormBase WS243)
NHP2/L7aE family protein YEL026W homologue	**M28.5**	morphogenesis, development
Protein UNC-87, a calponin-related protein	*unc-87*	actin bundle assembly, morphogenesis
Transthyretin-like protein T07C4.5 precursor	**T07C4.5**	extracellular (enriched in muscle)
Tropomyosin isoforms a/b/d/f +c/e	*lev-11*	morphogenesis, development, cytokinesis, molting, negative regulation of actin filament depolymerization
Myosin, essential light chain	*mlc-3*	locomotion, oviposition
Elongation Factor 1-alpha	*eft-3*	apoptosis, development, growth, translational elongation
Fructose-bisphosphate aldolase 2	**F01F1.12**	catalytic, embryo dev., reproduction
40 S Ribosomal protein S8	*rps-8*	apoptosis, development, translation
40 S Ribosomal protein S21	*rps-21*	molting, development, translation

Gene ontology analysis of proteins identified as differentially expressed in BIR-1 overexpressing larvae compared to control N2 larvae using David Ontology Tool indicated BIR-1 involvement in the regulation of growth, embryonic development, molting cycle and cuticle formation, larval morphogenesis, locomotion, larval development, translational elongation and translation and gamete generation.

The set of proteins clearly affected by BIR-1 induction included ribosomal proteins RPS-3 and RPL-5 and myosin. These proteins were further analyzed

functionally for a possible connection with BIR-1 and SKP-1 together with interacting proteins identified by yeast two-hybrid screens which were indicating shared involvement of BIR-1 and SKP-1 in the ribosomal stress pathway, in apoptosis and in the regulation of cytoskeleton during mitosis.

5.3 Validation of SKP-1 and BIR-1 protein interactors by functional analyses

The protein interactions identified in yeast two hybrid screens indicated that SKP-1 and BIR-1 may be part of functionally linked protein complexes. The variability and expected cellular localizations of identified protein interactors led us to conclude that the interactions are likely to occur in separate cellular compartments and under specific conditions. We have chosen selected proteins for additional studies for validation of the proteomic data.
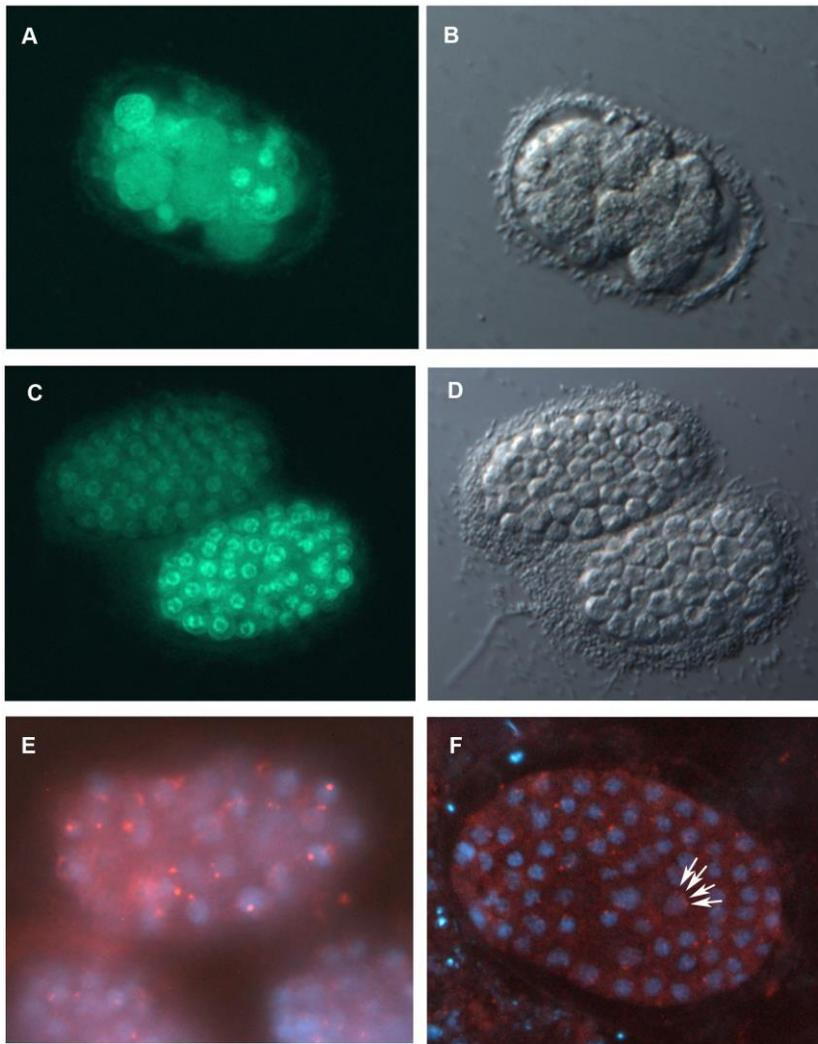
Fig. 4 Inhibition of SKP-1 induces cell division arrest and endomitoses. Panels A and C show Histone H2B::GFP expressing embryos. Panels B and D are corresponding views in Nomarski optics. Panels A and B show mitotic defects of *skp-1* RNAi embryos. The nuclei lost their regular architecture and the embryo arrested at approximately the 20 cell stage of development. Panel E shows a control embryo stained for DAPI and centrosomes. An embryo treated with *skp-1* RNAi (panel F) stained in the same way is arrested in development and contains cells that underwent endomitotic divisions with twice duplicated centrosomes with defective migration (arrows).

Because the yeast two-hybrid screens demonstrated that TAC-1 interacts with SKP-1, we wondered if these two proteins are related functionally. TAC-1, a transforming coiled coil protein, is a known cofactor of nuclear receptors and is indispensable for normal centrosomal functions, centrosome migration and mitosis (Bellanger et al. 2007). Therefore, we studied the effects of SKP-1 inhibition on mitosis in detail **(Fig. 4)** specifically assaying for the characteristic TAC-1 reduction-of-function phenotypes in centrosome migration during the G2 phase of the cell cycle. Staining of SKP-1 inhibited embryos with an antibody against *C.elegans* centrosomal protein SPD-2 (Kemp et al. 2004) showed that SKP-1 inhibition led to serious defects of mitoses including endomitoses and defects of centrosome migration in the G2 phase **(Fig. 4 F)**, similar to those previously shown following TAC-1 inhibition (Srayko et al. 2003)**.**

A protein category that is clearly represented in our yeast two-hybrid screen for the SKP-1 interactome, as well as after BIR-1 hyperinduction, are ribosomal proteins. Interestingly, three specific proteins found in our study are ribosomal proteins involved in the ribosomal stress pathway (Zhang et al. 2003b; Dai et al. 2006; Horn & Vousden 2008b; Yadavilli et al. 2009). We have therefore searched if the proteins that were identified in our experiments may interact with BIR-1 and SKP-1 in a GST fusion system. We prepared GST-fusion proteins and precipitated in vitro transcribed ribosomal proteins labeled with ^{35}S-methionine. Both GST-BIR-1 and GST-SKP-1, but not GST alone, showed binding to RPS-3 and RPL-5 **(Fig. 5)**.

Since three myosin-related proteins were identified as proteins with an altered chromatographic pattern in BIR-1 hyperinduced larvae compared to controls, we searched if BIR-1 overproduction alters the immunocytochemical pattern of non-muscle myosin. As shown on **Fig. 6 B**, forced expression of *bir-1* leads to more prominent staining of NMY-2 at the cellular peripheries.

We also searched if a short exposure of *C. elegans* larvae to high levels of BIR-1 may affect organization of intermediate filaments in epidermis using a monoclonal antibody MH27 that specifically recognizes the MH-27 protein, which is similar to human trichohalin, and is likely to be involved in organizing intermediate filaments in the hypodermis. As shown in **Fig. 6 D**, larvae that developed in the presence of high expression of *bir-1* had higher levels of MH-27 localized at cellular

borders of seam cells compared to controls. This supports the relevance of cytoskeletal and motor proteins detected as targets of BIR-1 on the protein level.

	A		B		C		D	
GST	+		+		+		+	
GST-SKIP		+		+				
GST-BIR-1						+		+
RPS-3	+	+					+	+
RPL-5			+	+	+	+		
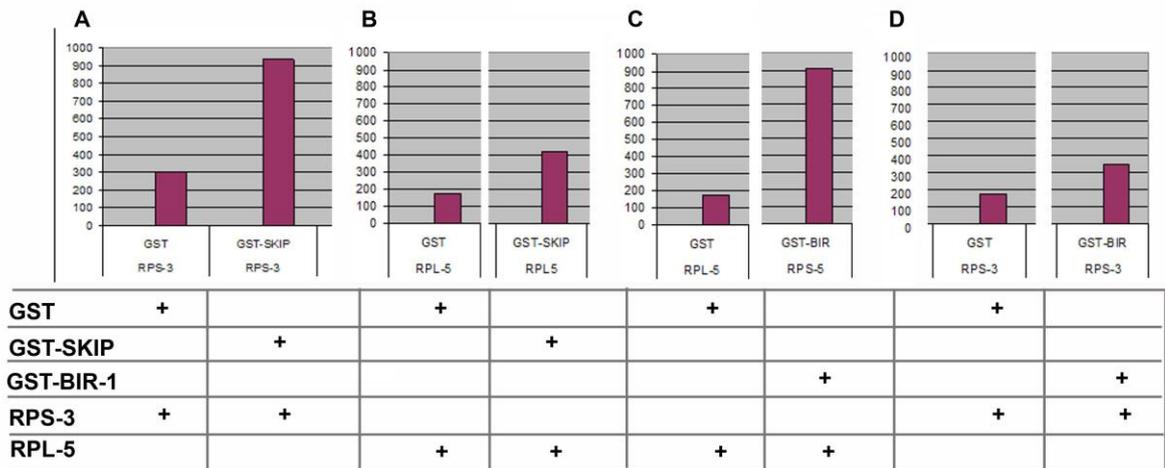
Fig. 5 SKP-1 and BIR-1 interact with RPS-3 and RPL-5. Panels A to D show interactions of SKP-1 (panels A and B) with RPS-3 and RPL-5 (panels A and B, respectively) and interactions of BIR-1 with RPL5 and RPS-3 (panels C and D).
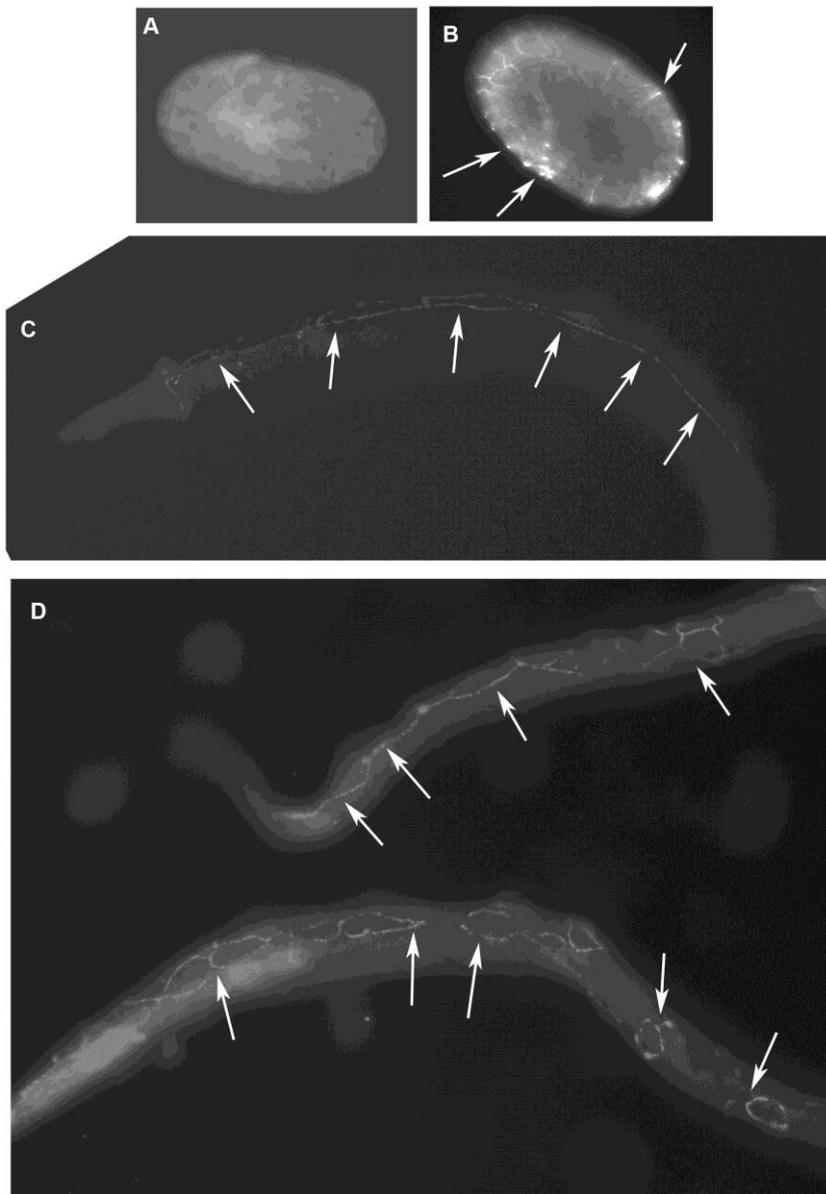
Fig. 6 The effect of short term overexpression of *bir-1* on non-muscle myosin localization in *C. elegans* embryos and development of seam cells. Panels A and B show *C. elegans* embryos stained for NMY-2. Panel A - Control wild type (N2) embryo (containing control transgene consisting of empty vector). Panel B - Embryo overexpressing *bir-1* from a transgene regulated by heat shock promoter. Arrows indicate accumulation of NMY-2 at the cell borders. Panels C and D show L1 larvae stained for MH27 antigen. Panel C shows a control larva with regularly developed seam cells forming a ribbon of rectangular cells along the length and side of the

animal. Panel D shows a L1 larva that developed from embryos affected by short term *bir-1* overexpression. Arrows indicate seam cells that are bigger than in wild type controls, not properly connected to each other, and that often have an irregular shape.

5.4 Analysis of BIR-1 transcriptional role on the expression of ribosomal proteins in the whole genome transcriptome

Synchronized larval cultures of *C. elegans* offer a laboratory model that allows analysis of transcriptional roles of multifunctional proteins, especially of proteins directly affecting cell division which have a profound effect on gene expression per se. This is the case of BIR-1 (Survivin). Only several cell divisions are happening during the larval transition from L1 to L2 and the gene expression pattern is not yet affected by growth of the germline. The resulting microarrays showed clear involvement of BIR-1 in the regulation of expression of collagen genes connected with the growth of the organism (Liby et al. 2006b).

Bioinformatics analysis focused on ribosomal proteins using the Affymetrix algorithm were used to identify genes with a statistically significant change in the number of expressed copies, and subsequently analyzed using WormMart (http://www.wormbase.org) and David Ontology bioinformatics tools (https://david.ncifcrf.gov/). This identified 72 proteins decreased by *bir-1* inhibition (**Supplementary Table S11A**) that included four genes coding for ribosomal proteins (rps-1, rps-2, rps-21 and rpl-31), and represents 12 times enrichment compared to the null hypothesis (even distribution in upregulated genes). (The total number of *C. elegans* ribosomal proteins is 32 rps genes and 50 rpl genes http://ribosome.med.miyazaki-u.ac.jp/rpg.cgi?mode=orglist&org=Caenorhabditis%20elegans). The set of genes recognized as decreased in response to *bir-1* downregulation included 12 collagen genes, which is an 11,6 x enrichment compared to the proportion of collagen genes in the *C. elegans* genome. Inversely, 214 genes were increased in response to *bir-1* inhibition (**Supplementary Table S11B**). Gene ontology analysis of genes decreased in *bir-1* inhibited animals showed that BIR-1 loss of function negatively affects the

expression of proliferative and developmental genes (**Supplementary Table S12**). Interestingly, the set of expressionally increased genes in *bir-1* inhibited larvae were mostly metabolic genes (**Supplementary Table S13**) and did not contain any ribosomal protein coding genes. Analysis of raw number of indexes calculated by the Affymetrix program (http://www.osa.sunysb.edu/udmf/Affy-Platform-Comparison-Tech-Note.pdf, processing of microarray data Nature Biotechnology 22, 656 - 658 (2004)doi:10.1038/nbt0604-656b) Affymetrix MAS 5.0 suite software with a threshold ≥1.6-fold change in mRNA expression and Robust Multichip Average (RMA) with threshold ≥1.2-fold change in mRNA expression allowed comparison of values obtained by the same chip set probes in paired control and experimental Microarray experiments with similar total readings and background values.

This showed that the trend of a decrease in the expression of ribosomal protein coding genes in *bir-1* inhibited animals is visible for a large number of ribosomal proteins. The set of ribosomal proteins that were decreased by *bir-1* RNA included genes coding for RPLs (11.2; 24.1; 16, 21; 17; 4; 36; 11.2; 2; 35; 9; 15; 22; 12; 31; 32; 37; 24.1; 26; 20; 33). Genes coding for RPS proteins also showed a trend of slight decrease in the same analysis as above (28; 12; 6; 24; 9; 8; 5; 19; 21; 14; 9; 2; 26; 3; 4; 23; 16; 25; 3; 1; 0). No genes coding for ribosomal proteins were found increased in *bir-1* downregulated samples, but some were not changed (RPS 18; 30; 20; 10; 22).

6. Discussion

In this thesis I present the results of proteomic and functional analyses aimed at elucidating possible links between SKP-1 and BIR-1. These proteins are coexpressed from an operon and their loss of function phenotypes were shown to be linked to the regulation of gene expression and development (Kostrouchova et al. 2002; Kostrouchova et al. 2003; Liby et al. 2006a). Based on the results presented in this thesis I propose a concept which may explain the connection of the structural state of the cell with the regulation of gene expression. In this concept, proteins constituting the free proteome (proteins not restricted in cellular structures) are proposed to interact with proteins regulating gene expression. We detected this signaling potential in evolutionarily conserved proteins SKP-1 and BIR-1 (Survivin). I propose that such proteome connections are likely to be part of a wider system which enables the cell to sense its structural state. The fact that we detect such interactions for evolutionarily highly conserved proteins suggests that this regulatory pathway may be very ancient.

Both SKP-1 and BIR-1 (Survivin) are evolutionarily old and highly conserved proteins that may be expected to be important for fundamental regulatory events. We searched for immediate protein interactions that may transmit the specific cellular roles of SKP-1 and BIR-1 and hoped to uncover the mechanistic basis of these interactions. We searched for direct interacting proteins of SKP-1 and BIR-1 using unbiased high-throughput proteomic methods employing yeast two-hybrid screens. Our screens identified proteins with overlapping and complementary functions as SKP-1 and BIR-1 interactors. We also searched for a direct interaction between SKP-1 and BIR-1 in screens with clones engineered for BIR-1 and SKP-1. This, however, did not support a direct interaction between these two proteins. The wide spectrum of processes in which SKP-1 and BIR-1 are involved, that is the regulation of gene expression and cell division makes their analysis challenging. Mitosis itself has profound effects on the proteome and these effects have to be distinguished from proteome states caused by specific developmental or metabolic situations.

The model system of *C. elegans* allowed us to bypass these difficulties. It is a suitable system in which functional analyses and very powerful genetic techniques that may be linked to proteomic studies focused at proteins that function in interphase as well as in mitosis.

In *C. elegans*, cell divisions occur in embryonic and larval stages in a precisely timed way and it is possible to obtain synchronized larval cultures that contain almost exclusively non-dividing cells. During larval stages (L1, L2) only a few cells divide and the growing gonad is small and does not affect significantly the complete proteome. This opens a wealth of possibilities for experimental functional analyses for proteins with multiple roles.

In the second approach, we attempted to visualize the direct effect of sudden overrepresentation of one protein in focus, BIR-1, on the proteome of non-dividing cells. This was achieved by a short time overexpression of BIR-1 (2 hours in total, consisting of one hour of heat shock induced transgene expression and one hour of protein synthesis period) and we analyzed the status of BIR-1 overexpressing proteome using a comparative whole proteome unbiased analysis employing two dimensional comparative chromatography. This showed that BIR-1 overexpression is affecting a large number of specific proteins on the proteome level, independently of gene transcription. This complemented our previous studies that showed that BIR-1 affects gene transcription by inducing changes connected to cellular proliferation (Liby et al. 2006a).

6.1 Our results confirmed that SKP-1 and BIR-1 are functionally connected on the proteome level

Proteomic analyses are very important for understanding cellular processes. Although the life of organisms is dependent on expression of structural and effector proteins from the particular genome of an organism, the life constituting processes are executed on the level of the proteome, that is restricted to cellular structures or present in cellular and organismal compartments independently of the defined cellular structures. Proteins in these compartments, especially in the cytosol, are regarded in this thesis as the free protein proteome.

The proteins in the free proteome are unlikely to be individually distributed in cellular compartments but rather present in protein complexes. Taking in account the variability of primary protein structures, their post-translational modifications and their final secondary and tertiary structures, it can be expected that the involvement of proteins in actual protein complexes is extremely variable. Our analyses, which focused only on two proteins, support the complex projections of single proteins towards these proteomic interactions.

6.2 SKP-1 and BIR-1 are involved in critical regulatory pathways

SKIP (SKI interacting protein, SNW) is a well established transcriptional and splicing cofactor (Folk et al. 2004). BIR-1, the nematode orthologue of Survivin, is a member of the chromosome passenger complex, and through this complex is involved in the regulation of certain mitotic events, such as chromosome segregation. The chromosome passenger complex consists of nematode orthologues of Incenp, Aurora B, Survivin and Borealin (ICP-1, AIR-2, BIR-1 and CSC-1 in *C. elegans*) (Speliotes et al. 2000; Adams et al. 2001; Wheatley et al. 2001; Wheatley et al. 2004, Fraser, 1999 #105; Ruchaud et al. 2007; Carmena et al. 2012). Survivin was originally identified as a protein overexpressed in most cancers. It contains the Baculovirus Inhibition of Apoptosis Repeat domain (termed BIR domain) and because of this attention was focused to its role in the inhibition of apoptosis, a known role for the Baculovirus Inhibition of Apoptosis (IAP) proteins. Viruses from the viral family *Baculoviridae* that infect many invertebrate species contain in their genomes two types of antiapoptotic proteins, one acting as a metalloproteinase and a second (P35/P49 homologues) that inhibit apoptosis by direct binding and inhibition of caspases (Clem & Miller 1994; Tamm et al. 1998; Pei et al. 2002). However, functional studies indicated that BIR-1 does not function in the regulation of physiological apoptosis in *C. elegans* but its mitotic function is conserved (Fraser et al. 1999; Speliotes et al. 2000). Survivin homologues are found in all metazoan species and in yeast. The organization of *bir-1* with *skp-1* in the same operon contradicted the expectation that *bir-1* should not be expressed in non-dividing cells since *skp-1* is widely expressed during development. The strong expression of *bir-1*

in all larval stages (containing non-significant number of dividing cells) was later experimentally confirmed (Kostrouchova et al. 2003; Liby et al. 2006a).

The transcriptional roles of SKIP are conserved between vertebrates and nematodes (Kostrouchova et al. 2002). Homologues of SKIP are found in Metazoa, yeast and also in plants (Zhang et al. 2013). Operons that are formed in nematodes often include genes whose protein products are not obviously functionally linked, provided that the simultaneous expression of coregulated genes is tolerated by the organism and fits its evolutionary history. Generally, the formation of operons in nematodes is viewed as a strategy to save the regulatory potential which may be advantageous by allowing the organism to perform the regulatory tasks with a smaller number of regulatory molecules and liberating some of them for additional regulatory roles (Blumenthal et al. 2002; Blumenthal 2012). The fact that BIR-1 is expressed together with transcription and splicing regulating SKP-1 strongly suggests that it has additional functions unrelated to cell division and apoptosis. Functional studies confirmed the role of BIR-1 in the regulation of transcription and identified a functional connection between SKP-1 and BIR-1 (Kostrouchova et al. 2002; Kostrouchova et al. 2003; Liby et al. 2006a).

This raised an obvious question, how is the cooperation of these two proteins executed on the molecular level. We have chosen strategies that are able to uncover molecular interactions and functional connections using unbiased methods: yeast two-hybrid screens, differential proteome profiling and whole genome transcriptome mapping. Each of these approaches has advantages and limitations. Yeast two-hybrid screens in the variant that we have chosen to employ reveal the potential of two proteins to interact if these proteins actually meet on the molecular level at physiological or pathological states of the cell. We have performed yeast two-hybrid screens for both BIR-1 and SKP-1. We also attempted to visualize the direct effect of BIR-1 on cellular proteins using high-throughput methods. To do this, we have chosen to follow the immediate, short time effect of *bir-1* overexpression on the whole proteome of synchronized larvae in L1/L2 stages where only a small number of dividing cells are present and it is unlikely that they affect the result coming from the vast majority of nondividing cells. In the search for BIR-1's role in the regulation of gene expression in nondividing cells, whole genome microarrays are preferable.

For these experiments, we decided to use downregulation of *bir-1* by RNAi in L1/L2 stages and apply RNAi at settings that did not trigger developmental changes of nematodes. We reasoned that if BIR-1 is coregulatory in transcription, we may be able to visualize its effect on the transcriptome in a system that contains only a small minority of dividing cells (Liby et al. 2006a). Visualizing the transcriptional effect in these stages would importantly strengthen the concept of a wider role of BIR-1 as a transcription regulating protein.

All three unbiased high throughput approaches support the concept that BIR-1 and SKP-1 are functionally linked, although the yeast two-hybrid screens did not reveal a direct interaction of these two proteins in neither of the two screens nor in a screen in which the interaction of BIR-1 and SKP-1 was assayed directly by cloning the two proteins into the bait and prey constructs.

Our screens identified interactions of both SKP-1 and BIR-1 in complexes that are indicating involvement of SKIP and BIR-1 in regulatory complexes in the cytoplasm (e.g. in cytokinesis, microtubule attachment during mitosis) and their capacity to be involved in similar complexes acting on the level of the regulation of translation and transcription.

This suggests that the interacting capacity of key regulatory proteins to form complexes involved in cell structural regulation is also likely to be used in the regulation of gene expression.

The yeast two-hybrid system is a very powerful method, which can be used for the identification of the protein binding potential of all proteins expressed from mRNA present in tissues or in entire organisms. Beside some technical limitations, such as missing post-translational modifications of potentially interacting proteins, or false positive results. The false positive results often arise from auto-activating proteins that have an affinity for the basal transcriptional machinery and thus do not require the DNA binding potential of the bait constructs. To increase stringency, the system that was used in presented experiments included double positive and one negative selection. The positive selection was based on the elimination of two amino acids from the media and requirement of positive clones to synthesize these two amino acids through a system regulated positively by prey constructs. The negative selection system was based on URA3 (Walhout & Vidal 1999), which if activated by

autoactivating prey constructs converts 5-fluoroorotic acid by Orotidine 5'-phosphate decarboxylase (5FOA) into the highly toxic 5-fluorouracil. All clones were additionally checked by cloning and sequencing that confirmed the proper frame and integrity of identified candidate binding proteins. Additionally, we performed two independent yeast two-hybrid screens simultaneously and it can be expected that auto-activating constructs would be detected in both screens. The results were finally validated by functional analyses of identified interactors that confirmed the capacity of identified interactors to act in processes that involve studied proteins SKIP and BIR-1.

6.2.1. SKP-1 and BIR-1 are likely to be connected through participation in overlapping complexes

The identification of ribosomal proteins as both SKP-1 and BIR-1 interactors and as targets of BIR-1 hyperinduction was unexpected but it further supports the functional connections between these two factors. The direct binding of SKP-1 and BIR-1 to RPS-3 and RPL-5 was confirmed by pull-down experiments. The physical interaction between SKP-1 and BIR-1 with ribosomal proteins that are known to participate in the ribosomal stress pathway opens a possibility that both SKP-1 and BIR-1 may be or their evolutionary ancestors were involved in ribosomal stress and apoptosis. Although *C. elegans* doesn't have a known MDM2 ortholog, it is likely that a protein that is still not identified in the *C. elegans* genome supports this function. MDM2-p53 is a very ancient regulatory pathway that is already functional in a basal Metazoan - *Trichoplax adhaerens* (Lane et al. 2010; von der Chevallerie et al. 2014). MDM2 can reversely bind ribosomal proteins RPS3 (Yadavilli et al. 2009), RPL5 (Marechal et al. 1994; Dai & Lu 2004; Horn & Vousden 2008b), RPL11 (Lohrum et al. 2003; Zhang et al. 2003b; Dai et al. 2006; Morgado-Palacin et al. 2012), and RPS28 (Daftuar et al. 2013). Additional proteins were shown to participate in the regulation of the p53 pathway, including RPL37, RPS15, and RPS20 (Daftuar et al. 2013). Various ribosomal proteins in the p53 pathway may function through multiple mechanisms, as was recently shown for ribosomal protein S26 (Cui et al. 2013). SKP-1 and BIR-1 are thus likely to be functionally linked on

multiple levels in the regulation of apoptosis, stress pathways and gene expression. Keeping with this, SKP-1 counteracts p53-regulated apoptosis through regulation of p21Cip1 mRNA splicing (Chen et al. 2011). It seems likely, that the role of SKP-1 and BIR-1 in the regulation of apoptosis through interaction with ribosomal proteins may be more ancient than the role of Survivin in inhibition of apoptosis through the direct binding and inactivation of caspases. In *C. elegans*, BIR-1 doesn't regulate apoptosis through inactivation of caspases but its role in apoptosis induced by ribosomal stress has not yet been tested. This function of Survivin may have evolved later in evolution on the basis of the ability of BIR (Survivin) to physically interact with variable proteins.

There are additional lines of evidence indicating that SKP-1 and BIR-1 may be functionally linked on the proteome level. BIR-1 is a regulator of microtubule attachment to chromosomes in anaphase and progression of mitosis. TAC-1 that was found as SKP-1 interactor has a critical role in mitosis, specifically in the relocation of ZYG-9 to centrosomes. TAC-1 is found localized on centrosomes as well as in the nucleus where it plays critical roles in gene expression regulation. Interestingly, SKP-1 inhibition results in the same cellular event, that is G2 arrest and failure of centrosome migration as is known for TAC-1 (Bellanger et al. 2007). A possibility of direct centrosomal localization and function of SKIP is keeping with the centrosomal localization and mitotic function of SKI (Marcelain & Hayman 2005; Mosquera et al. 2011), a protein which interacts physically and functionally with SKIP (Prathapam et al. 2001).

Proteome interactions detected by yeast two-hybrid screens are likely to represent only a small fraction of actual proteomic interactions on the whole proteome scale. Many weak interactions are eliminated by the double negative selection that was used in the presented results.

The extent of possible protein-protein interactions is magnified by proteins that contain domains that have multiple conformational states. Proteins have domains that are structurally very characteristic and may have limited conformational states. Such structures are defined as zinc-finger motifs, helix-loop-helix and other structures. On the other hand, many proteins contain regions lacking unique 3-D structure. These protein domains are usually visualized in structural studies as

domains that do not produce satisfactory 3-D images. In some cases, these proteins can be found in two or more conformational states. In many cases the protein conformational states are so numerous that these domains are not visible in structural studies at all. These domains are termed intrinsically disordered domains (Uversky 2015). These domains were often regarded as low-complexity proteins/protein domains in bioinformatic studies (Banerjee 2016). Proteins with this characterization are very numerous and it is expected that at least one third of all proteins contain intrinsically disordered domains. It may be surprising that ribosomal proteins, which are part of one of the most conserved cellular structures, have a number of intrinsically disordered domains that are necessary for their functions (Uversky 2014). Similarly, Mediator proteins use intrinsically disordered domains (Toth-Petroczy et al. 2008) in combination with protein-protein interaction motifs for association with multiple proteins using a so called fuzzy protein interface, which allows interactions in variable orientations (Warfield et al. 2014). Both types of variable conformations are likely to add another level of combinatorics that is likely to be inherent to proteome interactions and their projections toward the gene expression regulating machinery. Intrinsically disordered proteins have one additional feature, they tend to aggregate (Breydo & Uversky 2011). It can be hypothesized that aggregation of proteins made in excess would be a very potent negative regulatory mechanism if one molecule of the protein aggregate would be bound to components of the transcriptional or translational machinery. This hypothetical situation may answer a decades long search for direct mechanisms that may connect protein overproduction directly with negative regulation of gene expression especially on the translational level.

6.2.2. BIR-1 affects the proteomic pattern on the whole proteome scale

In our case, we studied the effect of short time BIR-1 hyperinduction on the *C. elegans* proteome in non-dividing cells. This approach identified several proteins that were found by yeast two-hybrid screens as SKP-1 and BIR-1 interactors to be also targets of BIR-1 hyperinduction on the proteomic level. The wide range of proteins identified as SKP-1 and BIR-1 interactors by both approaches included cytoskeletal and motor proteins, ribosomal proteins known to be active in the

ribosomal stress pathway and transcription and translation regulating proteins. BIR-1 hyperinduction had a profound effect on the composition of the whole proteome in non-dividing cells. This indicated that BIR-1 hyperinduction may influence a wide spectrum of target proteins and/or regulates proteins that affect other proteins. Some proteins found by our screens fulfill these criteria: protein involved in the proteasome pathway, enzymes, transcription and translation regulators.

Selected proteins that were studied functionally support the concept that incorporation of BIR-1 and SKP-1 in cellular mechanistic events may be linked to their regulatory roles in major cellular events: cell cycle progression and mitosis, ribosomal stress, (and apoptosis) and gene expression. Some connections were expected from known functions of BIR-1 or its vertebrate homologue Survivin. The connection between BIR-1 and non-muscle myosin is in agreement with the role of Survivin in cytokinesis that was revealed by a separation-of-function mutant (Szafer-Glusman et al. 2011).

6.3 SKP-1 and BIR-1 are evolutionarily conserved proteins involved in regulation of major cellular events: cell division, ribosomal stress, apoptosis and gene expression

Our results suggest that BIR-1 and SKP-1 are part of a larger network that is likely to participate not only on the same mechanistic events but that this network also has a potential to connect proteome signals with the regulation of gene expression on multiple levels. Several lines of evidence indicate that this network is real and functionally important. For example, SKIP is known to be a multifunctional protein involved in the regulation of transcription and is a co-activator for nuclear receptors (Baudino et al. 1998; Barry et al. 2003; Abankwa et al. 2013). SKIP also interacts with nuclear receptor co-repressor SMRT and functions in the Notch pathway through binding of Notch IC that is required for Notch biological activity (Zhou et al. 2000). SKIP also directly binds the retinoblastoma tumor suppressor protein pRb and, in co-operation with Ski, overcomes the G1 arrest induced by pRb (Prathapam et al. 2002). SKIP is also involved in the regulation of splicing (Zhang et al. 2003a; Figueroa & Hayman 2004; Bres et al. 2005; Wang et al. 2012). Thus, SKIP has a well-documented role in the regulation of transcription and the cell cycle.

It may be hypothesized that the pleiotropic protein interactions that we have identified for SKP-1 and BIR-1 are part of a proteome regulatory network with the capacity to project proteomic states towards gene expression regulation. Our data further link functionally SKP-1 and BIR-1. Both proteins bind proteins of the ribosomal stress pathway and possibly other stress pathways. SKIP was shown to be affecting stress related genes in plants. In rice and in *Arabidopsis*, it regulates stress related genes (Hou et al. 2009; Zhang et al. 2013).

C. elegans is a suitable model for the analysis of ribosomal proteins. The complement of *C. elegans* ribosomal proteins contains the homologues of the majority of vertebrate ribosomal proteins **(Supplementary Table S14)** and contrary to mammals, which have approximately 2000 predicted pseudogenes of ribosomal proteins in their genome (Tonner et al. 2012), the search for pseudogenes in the *C. elegans* genome yields only three possible candidates (not shown).

The ribosomal stress pathway thus may represent a special case of cytoplasmic proteomic signaling towards gene expression. If such proteomic signaling would be proved as a more general mechanism by which proteome composition projects directly towards gene expression, it may be considered as a proteome code. Such regulatory loops should include proteins that are localized in specific cellular structures and when liberated or synthesized in excess of cellular needs assume their additional regulatory roles. In fact such inhibition of gene expression was shown to be the autoregulatory mechanism for RPL-12, which was shown to affect its own splicing most likely through a sensor affecting transcription (Mitrovich & Anderson 2000). SKP-1 and/or BIR-1 may be the sensor(s) in ribosomal protein transcription and in the ribosomal stress pathway. The proposed view of participation of free structural proteins in the regulation of gene expression is shown in **Fig. 7**.

Our results identified the involvement of SKP-1 and BIR-1 on both transcriptional and translational levels. Such a dual role may not be surprising and has been well documented for Y box proteins (Swamynathan et al. 1997; Dhalla et al. 1998; Nambiar et al. 1998; Swamynathan et al. 1998; Swamynathan et al. 2000; Swamynathan et al. 2002). Interestingly, a disruption of only one allele of the Y-box protein gene, Chk-YB-1b, results in major defects in the cell cycle. This may indicate

a tight connection between the expressional level of Chk-YB-1b and cell cycle regulation (Swamynathan et al. 2002). Similarly, p53 was recently shown to regulate gene expression on the translational level (Terrier et al. 2011; Marcel et al. 2013; Marcel et al. 2015).
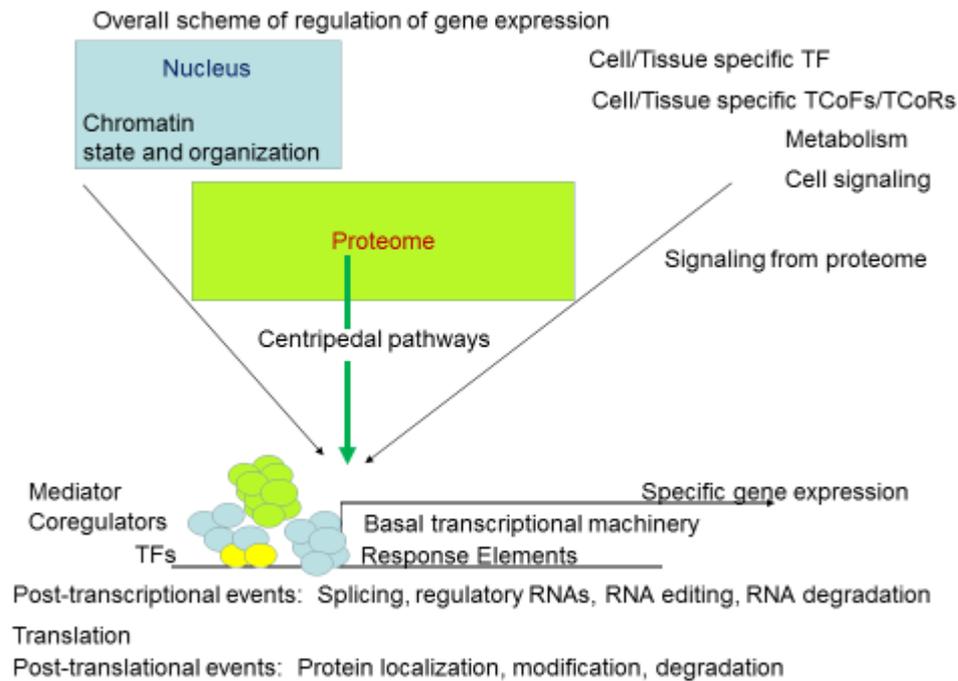
Fig. 7 Proposed involvement of free proteome signaling towards gene expression regulation.

7. Conclusions:

Our proteomic analyses further support the functional links between SKP-1 and BIR-1 with connected and overlapping roles in the ribosomal stress pathway and regulation of gene expression on transcriptional and translational levels.

We propose that SKP-1 and BIR-1 (Survivin) are components of complexes connecting cellular structural states with the regulation of gene expression on the level of transcription and translation.

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9. Supplementary material

List of supplementary files and tables

1. Supplementary Table S1. List of chromatographic paired fractions examined by mass spectrometry

2. Supplementary Table S2. List of chromatographic fractions containing proteins differently represented in BIR-1 overexpressing and control larvae

3. Supplementary Tables S3 to S10. The supplementary tables list specifications of proteins identified by mass spectrometry with high confidence (HighC) in the analyzed second dimension chromatographic separation fractions. Coordinates included in the titles [first dimension fraction number – first dimension fraction denomination in the system collector – injector unit – time of collection of the second dimension separation]; example: Table-S3-HighC-5-A2-16-17, second dimension fraction number = 5, position of the first dimension fraction in Collector-injector = A2, time of acquisition of the second dimension fraction = 16 to 17 min.

Table-S3-HighC-5-A2-16-17

Table-S4-HighC-9-A3-17-18

Table-S5-HighC-11-A3-16-17

Table-S6-HighC-12-A3-13-14

Table-S7-HighC-13-A4-18-19

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Table-S9-HighC-94-C3-17-18

Table-S10-HighC-95-E3-16-17

4. Analysis of BIR-1 induced transcriptome

List of Supplementary figures

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Figure-S2-A3-17-18-Fr9

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Figure-S7-C3-17-18-Fr94

Figure-S8-E3-16-17-Fr95

Legend to supplementary figures. Chromatograms on the left side represent N2 control fractions (displayed in red color). Chromatograms on the right side represent fractions from BIR-1 overproducing larvae (displayed in green color). Absorbance A_{214} for peaks marked by numbers in chromatograms is shown in tables shown above chromatograms.

5. Supplementary tables 11-14. Analysis of transcriptome of *bir-1* inhibited larvae

Supplementary table 11 A. Genes decreased in *bir-1* inhibited larvae
Supplementary table 11 B. Genes increased in *bir-1* inhibited larvae
Supplementary table 12. Functional annotation of genes decreased in *bir-1* inhibited larvae
Supplementary table 13. Functional annotation of genes upregulated in *bir-1* RNAi experiments
Supplementary table 14. The complement of *C. elegans* ribosomal proteins

1. **Supplementary Table S1 – List of chromatographic paired fractions examined by mass spectrometry**

Supplementary Table S1. Summary and coordinates of the paired control and BIR-1 overproduction first dimension separation fractions

Fraction No	Fraction Name	Elution time Control	Elution time BIR-1 overexpression	pH/Ionic gradient - Control	pH/Ionic gradient – BIR-1 overexpression
1	A1	0 – 5	0 – 5	8.6	8.6
2	A2	5 – 10	5 – 10	8.6	8.6
3	A3	10 – 15	10 – 15	8.6	8.6
4	A4	15 – 20	15 – 20	8.6	8.6
5	A5	20 – 25	20 – 25	8.6	8.6
6	A6	25 – 30	25 – 30	8.6	8.6
7	A7	30 – 35	30 – 35	8.6	8.6
8	A8	35 – 40	35 – 40	8.6	8.6
9	A9	40 – 45	40 – 45	8.6	8.6
10	A10	45 – 50	45 – 50	**8.6 – 8.35**	**8.6 – 8.35**
11	A11	50 – 53.30	50 – 53.30	**8.35 – 8.0**	**8.35 – 8.0**
12	A12	53.30 – 57.30	53.30 – 57.30	**8.0 – 7.7**	**8.0 – 7.7**
13	B12	57.30 – 62	57.30 – 62	**7.7 – 7.4**	**7.7 – 7.4**
14	B11	62 – 65	62 – 65	**7.4 – 7.1**	**7.4 – 7.1**
15	B10	65 – 69.30	65 – 69.30	**7.1 – 6.8**	**7.1 – 6.8**
16	B9	69.30 – 73.30	69.30 – 73.30	**6.8 – 8.5**	**6.8 – 8.5**
17	B8	73.30 – 78	73.30 – 78	**6.5 – 6.28**	**6.5 – 6.25**

18	B7	78 – 82	78 – 82	**6.28 - 5.9**	**6.25 -5.9**
19	B6	82 – 85	82 – 85	**5.9 – 5.65**	**5.9 – 5.7**
20	B5	85 – 88	85 – 88	**5.7 – 5.35**	**5.7 – 5.4**
21	B4	88– 93	88 – 93	**5.35 – 5.0**	**5.4 – 5.1**
22	B3	93 – 97.30	93 – 97.30	**5.0 – 4.9**	**5.1 – 5.0**
23	B2	97.30 – 103	97.30 – 103	**4.9 – 4.6**	**5.0 – 4.7**
24	B1	103 – 107	103 – 107	**4.6 – 4.3**	**4.7 – 4.4**
25	C1	107 – 112	107 – 112	**4.3 – 4.0**	**4.4 – 4.2**
26	C2	112– 117	112– 117	**4.0**	**4.2 – 4.1**
27	C3	117 – 122	117 – 122	Ionic elution	Ionic elution
28	C4	122 – 127	122 – 127	Ionic elution	Ionic elution
29	C5	127 – 132	127 – 132	Ionic elution	Ionic elution
30	C6	132 – 137	132 – 137	Ionic elution	Ionic elution
31	C7	137 – 142	137 – 142	Ionic elution	Ionic elution
32	C8	142 - 147	142 - 147	Ionic elution	Ionic elution
33	C9	147– 152	147– 152	Ionic elution	Ionic elution
34	C10	152 – 157	152 – 157	Ionic elution	Ionic elution
35	C11	157– 162	157– 162	Ionic elution	Ionic elution
36	C12	162 – 167	162 – 167	Ionic elution	Ionic elution
37	D12	167 – 172	167 – 172	Ionic elution	Ionic elution
38	D11	172 – 177	172 – 177	Ionic elution	Ionic elution
39	D10	177 – 182	177 – 182	Ionic elution	Ionic elution
40	D9	182 – 185	182 – 185	Ionic elution	Ionic elution

2. **Supplementary Table S2 – List of chromatographic fractions containing proteins differently represented in BIR-1 overexpressing and control larvae**

Supplementary Table S2. Coordinates of fractions that included differentially represented proteins in mass spectrometry

2nd dimension fraction No.	Fraction coordinates First dimension position and elution time of 2nd dimension	pH and actual elution time of the 1st dimension Control	pH and actual elution time of the 1st dimension BIR-1 overexpression
5	A2 (16 – 17)	8.6 (5 – 10 min)	8.6 (5 – 10 min)
9	A3 (17 – 18)	8.6 (10 – 15min)	8.6 (10 – 15min)
11	A3 (16 – 17)	8.6 (10 – 15min)	8.6 (10 – 15min)
12	A3 (13 – 14)	8.6 (10 – 15min)	8.6 (10 – 15min)
13	A4 (18 – 19)	8.6 (15 – 20 min)	8.6 (15 – 20 min)
14	A4 (17 – 18)	8.6 (15 – 20 min)	8.6 (15 – 20 min)
94	C3 (17 – 18)	4.0 (117 – 122 min)	4.1 (117 – 122 min)
95	C3 (16 – 17)	4.0 (117 – 122 min)	4.1 (117 – 122 min)

3. **Supplementary Tables S3 – S10. Characterization of proteins identified as differentially expressed in comparative chromatography and mass spectrometry**

The supplementary tables list specifications of proteins identified by mass spectrometry with high confidence (HighC) in the analyzed second dimension chromatographic separation fractions. Coordinates included in the titles [first dimension fraction number – first dimension fraction denomination in the system collector – injector unit – time of collection of the second dimension separation]; example: Table-S3-HighC-5-A2-16-17, second dimension fraction number = 5, position of the first dimension fraction in Collector-injector = A2, time of acquisition of the second dimension fraction = 16 to 17 min.

Supplementary Table-S3-HighC-5-A2-16-17

	Reference File, Scan(s)	Peptide	MH+	z	P (pro) P (pep)	Score XC Cn	Coverage Delta Cn	MW Sp	Accession RSp	Peptide (Hits) Ions	Count
3	gi|1706582|sp|P53013|EF1A_CAEEL Elongation factor 1-alpha (EF-1-alpha)				1,00E +00	50,22		506 68,0	17065 82	7 (7 0 0 0 0)	
	Kost_BIR1_5, 744	R.FTEITNEVSGFIK.K	1485,6296	2	0,00E +00	4,4 0	-	192 3,7	1	21/24	
	Kost_N2_5, 710	R.FTEITNEVSGFIK.K	1485,6296	2	0,00E +00	4,4 4	-	135 1,2	1	20/24	
	Kost_N2_5, 675	K.PGMVVTFAPQNVTTEV K.S	1819,1511	2	0,00E +00	3,2 8	-	641,2	38	16/32	4
	Kost_N2_5, 830	K.TLLEALDSIIPPQRPTDR. P	2036,31812	3	0,00E +00	3,2 5	-	110 4,0	16	30/68	
	Kost_BIR1_5, 732	R.VETGIIKPGMVVTFAPQ NVTTEVK.S	2560,0659	2	0,00E +00	3,7 4	-	989,8	1	22/46	
	Kost_N2_5, 662	R.VETGIIKPGM*VVTFAPQ NVTTEVK.S	2576,0651	3	0,00E +00	4,3 8	-	102 1,1	20	37/92	
	Kost_BIR1_5, 689	R.VETGIIKPGM*VVTFAPQ NVTTEVK.S	2576,0651	3	0,00E +00	3,4 9	-	765,1	53	34/92	
5	gi|3183074|sp|O02640|MDHM_CAEEL Probable malate dehydrogenase, mitochondrial pr				1,00E +00	40,24		351 19,3	31830 74	4 (4 0 0 0 0)	
	Kost_N2_5, 650	R.DDLFNTNAGIVR.D	1335,4775	2	0,00E +00	3,9 1	-	152 4,7	1	18/22	
	Kost_N2_5, 874	K.VALLGAAGGIGQPLGLL LK.Q	1762,17273	2	0,00E +00	4,7 4	-	150 6,4	1	24/36	

	Reference (File, Scan(s) / Peptide)	MH+	z	XCorr	DeltaCn	Sp	RSp	Ions	Count
	Kost_N2_5, 690	K.GVEYFSTPVELGPNGVEK.I	1923,1 1206	2	3,1 9	0,00E+00 -	818,3	1	21/34
	Kost_N2_5, 856	K.ALIAIITNPVNSTVPIASEVLK.K	2264,6 9043	2	4,6 5	0,00E+00 -	124 3,3	1	25/42
9	gi|1168412|sp|P46563|ALF2_CAEEL Fructose-bisphosphate aldolase 2 (Aldolase CE-2			20,20	1,00E+00	388 45,9	11684 12	3 (3 0 0 0 0)	
	Kost_N2_5, 622	K.ASHEAIGLATVTALR.R	1510,7 2046	2	3,2 9	0,00E+00 -	158 3,1	1	21/28
	Kost_N2_5, 786	K.LDLGVVPLAGTIGEGTTQGLDK.L	2155,4 3457	2	4,0 0	0,00E+00 -	744,7	1	23/42
	Kost_BIR1_5, 797	K.LDLGVVPLAGTIGEGTTQGLDK.L	2155,4 3457	2	3,5 8	0,00E+00 -	786,2	1	23/42
10	gi|12644467|sp|Q93615|ETFA_CAEEL Probable electron transfer flavoprotein subuni			20,20	1,00E+00	344 54,3	12644 467	2 (2 0 0 0 0)	
	Kost_N2_5, 626	K.LGNEVSVLVTGANATK.V	1573,7 7332	2	3,9 5	0,00E+00 -	124 0,6	1	20/30
	Kost_N2_5, 670	K.LDVSSISDVTEVHSADSFTR.T	2166,2 8809	3	3,6 8	0,00E+00 -	355 2,5	1	46/76
16	gi|1724019|sp|P53795|YHIT_CAEEL HIT-like protein tag-202			10,20	1,00E+00	142 42,4	17240 19	1 (1 0 0 0 0)	
	Kost_N2_5, 687	R.IDM*LENAVDSDAALIGK.L	1792,0 0227	2	4,0 5	0,00E+00 -	195 2,5	1	22/32
20	gi|1350989|sp|P48152|RS3_CAEEL 40S ribosomal protein S3			10,18	1,00E+00	273 13,5	13509 89	1 (1 0 0 0 0)	
	Kost_BIR1_5, 665	R.NALPDHVQIVEPQEEVLPK.E	2156,4 2432	3	3,5 2	0,00E+00 -	145 4,1	179	31/72

Supplementary Table-S4-HighC-9-A3-17-18

	Reference (File, Scan(s) / Peptide)	MH+	z	Score XCn / DeltaCn	Coverage	MW Sp	Accession RSp	Peptide (Hits) Ions	Count
8	gi|21542472|sp|Q10657|TPIS_CAEEL Triosephosphate isomerase (TIM) (Triose-phosp			20,19		2657 4,2	215424 72	3 (3 0 0 0 0)	
	Kost_N2_9_zt, 590	R.IIYGGSVTADNAAELGK.K	1679,85 278	2	3,8 5	-	1634,0	1	25/32
	Kost_N2_9_zt, 586	R.IIYGGSVTADNAAELGK.K	1679,85 278	3	3,3 6	-	2529,6	2	37/64
	Kost_N2_9_zt, 747	K.KPDIDGFLVGGASLKPDFVK.I	2104,43 408	3	3,7 8	-	3013,2	3	37/76
9	gi|1168412|sp|P46563|ALF2_CAEEL Fructose-bisphosphate aldolase 2 (Aldolase CE-			20,19		3884 5,9	116841 2	2 (2 0 0 0 0)	

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	Reference / Scan	Peptide	Score		Coverage		MW	Accession	Peptide (Hits)
	Kost_N2_9_zt, 632	K.ASHEAIGLATVTALR.R	1510,72 046	2	2,58	-	1296,4	1	16/28
	Kost_N2_9_zt, 814	K.LDLGVVPLAGTIGEGTTQGLDK.L	2155,43 457	2	3,83	-	758,0	1	23/42
11	gi|2500347|sp|Q21568|NHPX_CAEEL NHP2/L7aE family protein YEL026W homolog				20,17		1400 1,3	250034 7	2 (2 0 0 0)
	Kost_BIR1_9_zt, 727	K.LMDLVQQAMNYK.Q	1454,74 060	2	3,17	-	2000,2	1	20/22
	Kost_N2_9_zt, 598	K.LM*DLVQQAM*NYK.Q	1486,74 059	2	3,34	-	1343,2	1	18/22
12	gi|465684|sp|P34255|YKA3_CAEEL Uncharacterized protein B0303.3				20,14		4787 4,2	465684	2 (2 0 0 0)
	Kost_N2_9_zt, 682	R.TPFVVSGTVFK.D	1182,39 319	2	2,50	-	642,0	71	14/20
	Kost_N2_9_zt, 791	K.FTDVVPVFLDGK.K	1337,54 517	2	2,90	-	994,6	3	19/22
14	gi|1706582|sp|P53013|EF1A_CAEEL Elongation factor 1-alpha (EF-1-alpha)				10,21		5066 8,0	170658 2	2 (2 0 0 0)
	Kost_N2_9_zt, 735	R.FTEITNEVSGFIK.K	1485,66 296	2	4,12	-	1731,8	1	22/24
	Kost_BIR1_9_zt, 698	R.FTEITNEVSGFIK.K	1485,66 296	2	3,48	-	1783,1	1	22/24
17	gi|21264496|sp|O17071|PRS10_CAEEL Probable 26S protease regulatory subunit S10				10,19		4585 8,7	212644 96	1 (1 0 0 0)
	Kost_N2_9_zt, 663	K.M*SHEDPGNISYSDVGGLAEQIR.E	2392,54 557	3	3,77	-	1504,2	18	33/84
26	gi|1176642|sp|Q09201|YP22_CAEEL Putative sideroflexin-like protein AH6.2				10,13		3675 4,6	117664 2	1 (1 0 0 0)
	Kost_BIR1_9_zt, 806	R.IAM*AM*PYMVMTPIIM*NR.I	2032,57 000	2	2,61	-	722,7	95	16/32
34	gi|75029335|sp|Q9XXK1|ATPA_CAEEL ATP synthase subunit alpha, mitochondrial pre				10,12		5778 7,2	750293 35	1 (1 0 0 0)
	Kost_BIR1_9_zt, 592	R.ILGTETGINLEETGK.V	1575,74 292	2	2,40	-	995,3	2	18/28
41	gi|21264470|sp|Q18040|OAT_CAEEL Probable ornithine aminotransferase, mitochond				10,11		4645 3,5	212644 70	1 (1 0 0 0)
	Kost_N2_9_zt, 624	R.SIAAISASTDPDSFAR.F	1609,71 936	2	2,24	-	899,4	7	17/30

Supplementary Table-S5-HighC-11-A3-16-17

Reference	Score	Coverage	MW	Accession	Peptide (Hits)

	File, Scan(s)	Peptide	MH+	z	XC	DeltaCn	Sp	RSp	Ions	Count
4	gi|1706582|sp|P53013|EF1A_CAEEL Elongation factor 1-alpha (EF-1-alpha)				30,24		5066 8,0	170658 2	5 (5 0 0 0)	
	Kost_BIR1_11_zt, 726	R.FTEITNEVSGFIK.K	1485,66 296	2	4,28	-	1901, 4	1	22/24	
	Kost_N2_11_zt, 726	R.FTEITNEVSGFIK.K	1485,66 296	2	4,83	-	1863, 6	1	22/24	
	Kost_N2_11_zt, 720	R.VETGIIKPGMVVTFAPQNVTTEVK.S	2560,00 659	2	3,03	-	512,4	6	17/46	
	Kost_BIR1_11_zt, 716	R.VETGIIKPGMVVTFAPQNVTTEVK.S	2560,00 659	2	2,91	-	668,2	1	18/46	
	Kost_N2_11_zt, 675	R.VETGIIKPGM*VVTFAPQNVTTEVK.S	2576,00 651	2	2,26	-	567,3	1	19/46	
7	gi|3183074|sp|O02640|MDHM_CAEEL Probable malate dehydrogenase, mitochondrial p				20,19		3511 9,3	318307 4	2 (2 0 0 0)	
	Kost_N2_11_zt, 715	K.GVEYFSTPVELGPNGVEK.I	1923,11 206	2	2,75	-	587,1	5	17/34	
	Kost_N2_11_zt, 891 - 892	K.ALIAIITNPVNSTVPIASEVLK.K	2264,69 043	2	3,88	-	716,7	1	20/42	
17	gi|1168412|sp|P46563|ALF2_CAEEL Fructose-bisphosphate aldolase 2 (Aldolase CE-2				10,17		3884 5,9	116841 2	1 (1 0 0 0)	
	Kost_N2_11_zt, 816	K.LDLGVVPLAGTIGEGTTQGLDK.L	2155,43 457	2	3,38	-	762,2	1	23/42	
25	gi|12230441|sp|Q21993|PFD5_CAEEL Probable prefoldin subunit 5				10,13		1715 1,4	122304 41	1 (1 0 0 0)	
	Kost_N2_11_zt, 728	K.IATAGHTALIPLSESLYIR.A	2027,35 291	2	2,35	-	955,2	2	18/36	

Supplementary Table-S6-HighC-12-A3-13-14

	Reference				Score	Coverage	MW	Accession	Peptide (Hits)	
	File, Scan(s)	Peptide	MH+	z	XC	DeltaCn	Sp	RSp	Ions	Count
1	gi|114152904|sp|P37806|UNC87_CAEEL Protein unc-87 (Uncoordinated protein 87)				40,25		62727 ,4	1141529 04	4 (4 0 0 0)	
	Kost_N2_12_zt, 634	R.VAQNPADLAELPEEK.I	1624,773 93	2	4,28	-	1589, 4	1	22/28	
	Kost_N2_12_zt, 668	K.SENLQEIPEDIANR.T	1628,722 78	2	3,08	-	732,1	16	17/26	
	Kost_BIR1_12zt, 682	R.NTTFEAEGGELPYEAMK.V	1888,044 80	2	3,74	-	1688, 4	1	23/32	

#	File, Scan(s) / Reference	Peptide	MH+	z	XC / Score	DeltaCn	Sp / MW	RSp / Accession	Ions / Peptide (Hits)	Count
	Kost_N2_12_zt, 630	R.NTTFEAEGGELPYEAM*K.V	1904,04480	2	4,94	-	1678,9	1	25/32	
6	gi|1350962|sp|P49197|RS21_CAEEL 40S ribosomal protein S21				20,28		9674,9	1350962	4 (4 0 0 0 0)	
	Kost_N2_12_zt, 524	R.M*GESDDAILR.L	1123,22022	2	2,90	-	1212,6	1	17/18	1
	Kost_N2_12_zt, 726	K.DHASVQIDFVDVDPETGR.M	2001,09973	3	4,23	-	1461,2	12	31/68	
	Kost_N2_12_zt, 727	K.DHASVQIDFVDVDPETGR.M	2001,09973	2	5,15	-	2060,7	1	24/34	
	Kost_BIR1_12zt, 714	K.DHASVQIDFVDVDPETGR.M	2001,09973	2	5,66	-	1985,5	1	24/34	
7	gi|21542313|sp|Q22288|YNS5_CAEEL Transthyretin-like protein T07C4.5 precursor				20,21		14495,7	21542313	2 (2 0 0 0)	
	Kost_BIR1_12zt, 900	K.IGGTYDMTYVTLDILSAK.D	1962,25305	2	3,01	-	1199,4	1	20/34	
	Kost_N2_12_zt, 822	K.IGGTYDM*TYVTLDILSAK.D	1978,25309	2	4,14	-	1887,2	1	21/34	
8	gi|464769|sp|P34697|SODC_CAEEL Superoxide dismutase [Cu-Zn]				20,17		16237,1	464769	2 (2 0 0 0)	
	Kost_N2_12_zt, 583	K.SENDQAVIEGEIK.G	1432,51538	2	2,99	-	1671,7	1	18/24	
	Kost_N2_12_zt, 542	R.HVGDLGNVEAGADGVAK.I	1609,72266	3	3,45	-	1841,1	8	31/64	
16	gi|1350756|sp|P49405|RL5_CAEEL 60S ribosomal protein L5				10,19		33385,8	1350756	1 (1 0 0 0)	
	Kost_BIR1_12zt, 744	K.FLAAGLNADNLVATYQK.V	1810,04321	2	3,71	-	1488,3	1	21/32	
25	gi|465781|sp|P34328|HSP10_CAEEL Heat shock protein Hsp-12.2				10,14		12264,7	465781	1 (1 0 0 0)	
	Kost_N2_12_zt, 566	K.LPDDVDVSTVK.S	1188,30969	2	2,84	-	1139,1	1	18/20	

Supplementary Table-S7-HighC-13-A4-18-19

#	Reference / File, Scan(s)	Peptide	MH+	z	Score XC / DeltaCn	Coverage	MW / Sp	Accession / RSp	Peptide (Hits) / Ions	Count
17	gi|75029335|sp|Q9XXK1|ATPA_CAEEL ATP synthase subunit alpha, mitochondrial pre				10,21		57787,2	75029335	1 (1 0 0 0)	

	Reference / File, Scan(s)	Peptide	MH+	z	XC	Delta Cn	Sp	RSp	Ions	Count
	Kost_BIR1_13B_zt, 867	R.EVAAFAQFGSDLDASTQQLLNR.G	2382,57 153	3	4,27	-	2548,3	4	36/84	
1 9	gi|1706582|sp|P53013|EF1A_CAEEL Elongation factor 1-alpha (EF-1-alpha)				10,19		50668,0	170658 2	1 (1 0 0 0)	
	Kost_N2_13B_zt, 715	R.FTEITNEVSGFIK.K	1485,66 296	2	3,76	-	1712,2	1	22/24	
2 4	gi|120646|sp|P17329|G3P2_CAEEL Glyceraldehyde-3-phosphate dehydrogenase 2 (GAP				10,18		36498,2	120646	1 (1 0 0 0)	
	Kost_N2_13B_zt, 850	K.VINDNFGIIEGLM*TTVHAVTATQK.T	2589,95 014	3	3,55	-	1205,4	7	40/92	5

Supplementary Table-S8-HighC-14-A4-17-19

	Reference / File, Scan(s)	Peptide	MH+	z	Score XC	Coverage Delta Cn	MW Sp	Accession RSp	Peptide (Hits) Ions	Count
3	gi|1706582|sp|P53013|EF1A_CAEEL Elongation factor 1-alpha (EF-1-alpha)				30,25		50668,0	170658 2	4 (4 0 0 0)	
	Kost_BIR1_14_zt, 742	R.FTEITNEVSGFIK.K	1485,66 296	2	4,78	-	1847,4	1	21/24	
	Kost_BIR1_14_zt, 730	R.VETGIIKPGMVVTFAPQNV TTEVK.S	2560,00 659	3	3,96	-	1427,6	13	38/92	
	Kost_N2_14_zt, 699	R.VETGIIKPGM*VVTFAPQN VTTEVK.S	2576,00 651	3	5,01	-	1426,7	1	40/92	
	Kost_BIR1_14_zt, 690	R.VETGIIKPGM*VVTFAPQN VTTEVK.S	2576,00 651	3	3,96	-	1244,2	3	39/92	
9	gi|1350962|sp|P49197|RS21_CAEEL 40S ribosomal protein S21	<mark>　</mark>			10,22		9674,9	135096 2	1 (1 0 0 0)	
	Kost_N2_14_zt, 718	K.DHASVQIDFVDVDPETGR. M	2001,09 973	3	4,47	-	1884,3	7	33/68	
1 1	gi|1168412|sp|P46563|ALF2_CAEEL Fructose-bisphosphate aldolase 2 (Aldolase CE-2				10,20		38845,9	116841 2	2 (2 0 0 0)	
	Kost_N2_14_zt, 828	K.LDLGVVPLAGTIGEGTTQ GLDK.L	2155,43 457	2	3,30	-	744,1	1	22/42	
	Kost_BIR1_14_zt, 818	K.LDLGVVPLAGTIGEGTTQ GLDK.L	2155,43 457	2	4,04	-	870,9	1	25/42	
1 6	gi|21542313|sp|Q22288|YNS5_CAEEL Transthyretin-like protein T07C4.5 precursor				10,17		14495,7	215423 13	1 (1 0 0 0)	
	Kost_N2_14_zt, 815	K.IGGTYDM*TYVTLDILSAK. D	1978,25 309	2	3,40	-	1504,5	1	20/34	

#	Reference / File, Scan(s)	Peptide	Score / MH+	z	XC	Coverage / DeltaCn	MW / Sp	Accession / RSp	Peptide (Hits) / Ions	Count
19	gi|114152904|sp|P37806|UNC87_CAEEL Protein unc-87 (Uncoordinated protein 87)		10,16			3,2	62727,4	114152904	1 (1 0 0 0)	
	Kost_N2_14_zt, 634	R.VAQNPADLAELPEEK.I	1624,77393	2	1	-	1129,1	2	19/28	
21	gi|120646|sp|P17329|G3P2_CAEEL Glyceraldehyde-3-phosphate dehydrogenase 2 (GAPD		10,14			2,3	36498,2	120646	1 (1 0 0 0)	
	Kost_BIR1_14_zt, 795	R.VVDLISYIATK.A	1222,45557	2	3	-	1435,5	1	16/20	3
22	gi|48429003|sp|P61889|MDH_ECOLI Malate dehydrogenase		10,14				32337,0	48429003	1 (1 0 0 0)	
25	gi|75029335|sp|Q9XXK1|ATPA_CAEEL ATP synthase subunit alpha, mitochondrial pre		10,11			2,2	57787,2	75029335	1 (1 0 0 0)	
	Kost_N2_14_zt, 760	R.TGAIVDVPVGDGLLGR.V	1539,75854	2	5	-	528,4	143	15/30	

Supplementary Table-S9-HighC-94-C3-17-18

Reference / File, Scan(s)	Peptide	Score / MH+	z	XC	Coverage / DeltaCn	MW / Sp	Accession / RSp	Peptide (Hits) / Ions	Count

No peptides with high confidence detected

Supplementary Table-S10-HighC-95-E3-16-17

#	Reference / File, Scan(s)	Peptide	Score / MH+	z	XC	Coverage DeltaCn	MW / Sp	Accession / RSp	Peptide (Hits) / Ions	Count
1	gi|42559735|sp|Q22866|TPM1_CAEEL Tropomyosin isoforms a/b/d/f (Levamisole resi		80,21				33003,4	42559735	11 (11 0 0 0 0)	
	Kost_BIR1_95_zt, 610	R.ANTVEAQLK.E	974,09351	2	2,48	-	1222,2	2	15/16	
	Kost_BIR1_95_zt, 674	K.EAQLLAEEADR.K	1245,32141	2	3,47	-	926,8	3	17/20	4
	Kost_BIR1_95_zt, 822	R.MTLLEEELER.A	1263,44324	2	3,34	-	1109,2	1	16/18	4

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#	Sample		Peptide								
	Kost_N2_95_zt, 718		R.MTLLEEELER.A	1263,44 324	2	2,8 3	-	1180,6	4	16/18	4
	Kost_N2_95_zt, 636		K.TVQEAEAEVASLNR.R	1517,62 354	2	3,8 2	-	2135,5	1	20/26	
	Kost_BIR1_95_zt, 734		K.TVQEAEAEVASLNR.R	1517,62 354	2	3,8 7	-	2176,2	1	22/26	
	Kost_BIR1_95_zt, 570		K.LEEATHNVDESER.V	1529,54 797	3	3,7 5	-	1117,2	17	31/48	1
	Kost_BIR1_95_zt, 1050		K.TISEELDSTFQELSGY	1819,90 063	2	4,2 4	-	1784,4	1	19/30	
	Kost_N2_95_zt, 626		K.MTQTGDDLDKAQEDLSAATSK.L	2226,36 206	3	3,6 7	-	2086,1	1	38/80	
	Kost_BIR1_95_zt, 726		K.MTQTGDDLDKAQEDLSAATSK.L	2226,36 206	3	3,7 0	-	1548,2	2	35/80	
	Kost_BIR1_95_zt, 719		K.M*TQTGDDLDKAQEDLSAATSK.L	2242,36 207	3	3,6 9	-	3722,9	1	43/80	
2	gi|127743|sp|P02566|MYO4_CAEEL Myosin-4 (Myosin heavy chain B) (MHC B) (Uncoor			50, 26		2,8		22512 3,9	127743	5 (5 0 0 0 0)	
	Kost_BIR1_95_zt, 694		K.DLTDQLGEGGR.S	1161,20 447	2	2,8 5	-	1680,0	1	18/20	2
	Kost_BIR1_95_zt, 806		K.INELQEALDAANSK.N	1516,63 550	2	5,1 1	-	2264,8	1	21/26	
	Kost_BIR1_95_zt, 735		R.LVGDLDDAQVDVER.A	1544,64 575	2	4,5 6	-	2427,8	1	22/26	
	Kost_BIR1_95_zt, 926		K.NAQEELAEVVEGLR.R	1557,68 762	2	4,2 9	-	2383,2	1	20/26	
	Kost_BIR1_95_zt, 774		R.ATLLQSEKEELLVANEAAER.A	2215,44 678	3	3,5 8	-	2003,4	97	31/76	
3	gi|42559736|sp|Q27249|TPM3_CAEEL Tropomyosin isoforms c/e (Levamisole resistan			50, 17		2,3 6		29631, 6	425597 36	7 (7 0 0 0 0)	
	Kost_N2_95_zt, 655		R.LEDELLLEK.E	1102,26 001	2	2,3 8	-	617,0	215	13/16	1
	Kost_BIR1_95_zt, 758		R.IVLVEEDLER.T	1215,37 830	2	2,8 2	-	1215,0	1	16/18	1
	Kost_N2_95_zt, 650		R.IVLVEEDLER.T	1215,37 830	2	3,1 4	-	1278,0	2	16/18	1
	Kost_BIR1_95_zt, 698		R.EDAEAEVAALNR.R	1288,34 631	2	3,1 2	-	1446,0	2	17/22	1
	Kost_BIR1_95_zt, 738		R.ANFLETQVDEAK.V	1365,47 058	2	3,4 4	-	1288,9	6	18/22	1
	Kost_N2_95_zt, 746		K.EGAQQTSLLDVLK.K	1402,57 556	2	2,9 5	-	948,6	6	17/24	1
	Kost_BIR1_95_zt, 854		K.EGAQQTSLLDVLK.K	1402,57 556	2	2,8 8	-	1361,5	1	20/24	1
4	gi|1709055|sp|P53014|MLE_CAEEL Myosin, essential light chain (Myosin light cha			40, 18				17144, 3	170905 5	4 (4 0 0 0 0)	

	Kost_BIR1_95_zt, 756	R.LSADEADELLK.G	1204,30 920	2	0	-	1461,4	3	17/20	
	Kost_BIR1_95_zt, 858	K.EIFNLYDEELDGK.I	1585,69 312	2	0	-	826,4	3	18/24	
	Kost_N2_95_zt, 902	R.LTFEEWLPMYEQLAK.E	1899,19 873	3	7	-	1960,2	4	32/56	
	Kost_N2_95_zt, 762	K.EIFNLYDEELDGKIDGTQVGDVAR.A	2697,89 209	3	5	-	1334,6	2	36/92	
17	gi|29428019|sp|Q9N4M4|ANC1_CAEEL Nuclear anchorage protein 1 (Anchorage 1 prot		10,18				95649 2,7	294280 19	1 (1 0 0 0 0)	
	Kost_BIR1_95_zt, 814	K.AVAPSSLISHDDLVVGLPEK.V	2048,32 568	3	7	-	2056,4	2	40/76	5

List of Supplementary figures

Supplementary Figure-S1-A2-16-17-Fr5
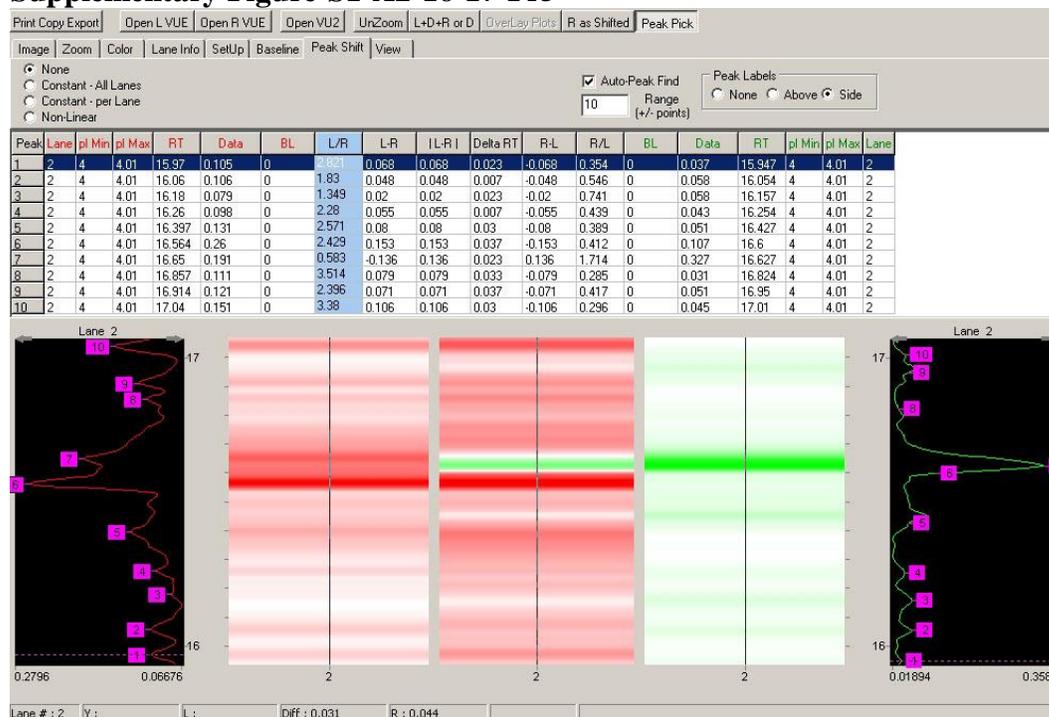
Supplementary Figure-S2-A3-17-18-Fr9
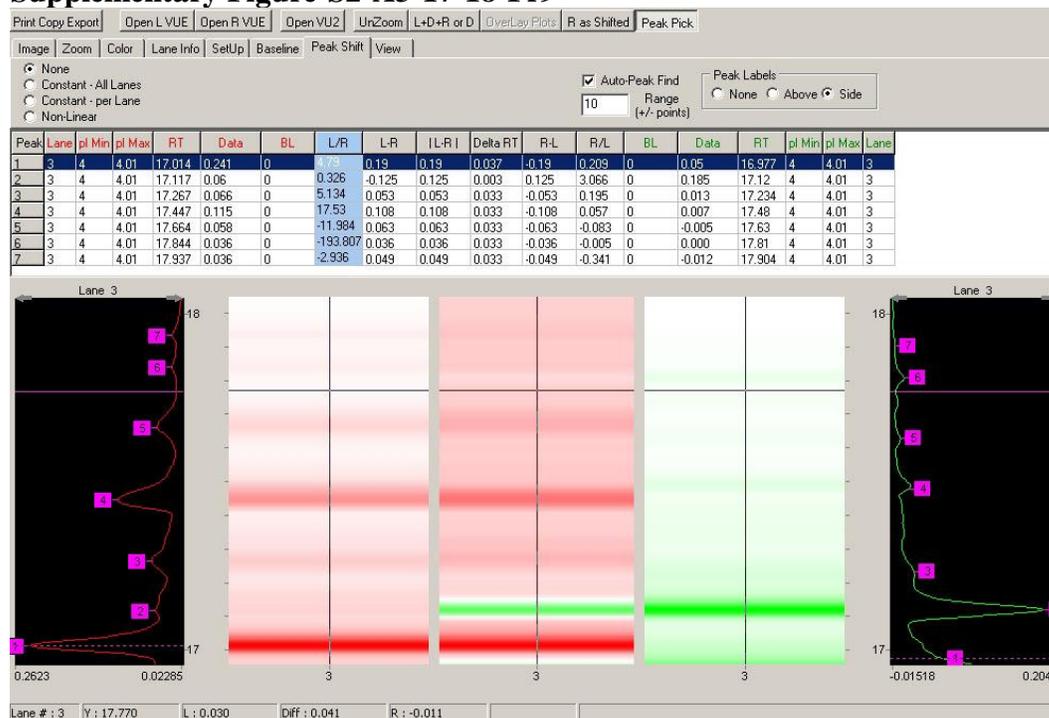
Supplementary Figure-S3-A3-16-17-Fr11

Peak	Lane	pl Min	pl Max	RT	Data	BL	L/R	L-R	|L-R|	Delta RT	R-L	R/L	BL	Data	RT	pl Min	pl Max	Lane
1	3	4	4.01	16.024	0.07	0	4.048	0.052	0.052	0.03	-0.052	0.247	0	0.017	16.054	4	4.01	3
2	3	4	4.01	16.1	0.087	0	2.815	0.056	0.056	0.03	-0.056	0.355	0	0.031	16.07	4	4.01	3
3	3	4	4.01	16.207	0.066	0	1.698	0.027	0.027	0.03	-0.027	0.589	0	0.039	16.177	4	4.01	3
4	3	4	4.01	16.3	0.05	0	2.196	0.027	0.027	0.03	-0.027	0.455	0	0.023	16.27	4	4.01	3
5	3	4	4.01	16.414	0.087	0	4.459	0.067	0.067	0.01	-0.067	0.224	0	0.019	16.424	4	4.01	3
6	3	4	4.01	16.43	0.079	0	1.606	0.03	0.03	0.037	-0.03	0.623	0	0.049	16.467	4	4.01	3
7	3	4	4.01	16.62	0.229	0	1.027	0.006	0.006	0.023	-0.006	0.974	0	0.223	16.644	4	4.01	3
8	3	4	4.01	16.87	0.132	0	8.038	0.116	0.116	0.03	-0.116	0.124	0	0.016	16.9	4	4.01	3
9	3	4	4.01	17.014	0.241	0	5.788	0.199	0.199	0.03	-0.199	0.173	0	0.042	16.984	4	4.01	3

Supplementary Figure-S4-A3-13-14-Fr12

Peak	Lane	pl Min	pl Max	RT	Data	BL	L/R	L-R	|L-R|	Delta RT	R-L	R/L	BL	Data	RT	pl Min	pl Max	Lane
1	3	4	4.01	13.444	0.23	0	3.875	0.171	0.171	0.033	-0.171	0.258	0	0.059	13.477	4	4.01	3
2	3	4	4.01	13.47	0.114	0	0.429	-0.152	0.152	0.033	0.152	2.332	0	0.266	13.504	4	4.01	3
3	3	4	4.01	13.734	0.03	0	-98.482	0.031	0.031	0.017	-0.031	-0.01	0	0.000	13.717	4	4.01	3
4	3	4	4.01	13.874	0.041	0	112.152	0.041	0.041	0.037	-0.041	0.009	0	0.000	13.91	4	4.01	3
5	3	4	4.01	13.994	0.079	0	1.087	0.006	0.006	0.003	-0.006	0.92	0	0.072	13.99	4	4.01	3

Supplementary Figure-S5-A4-18-19-Fr13
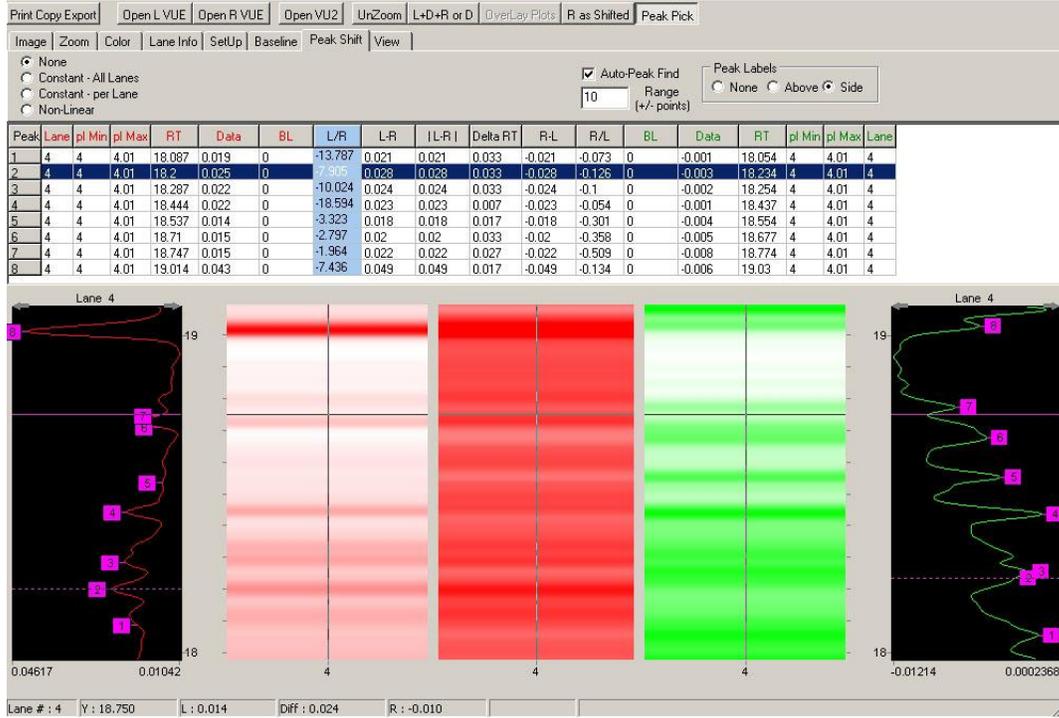
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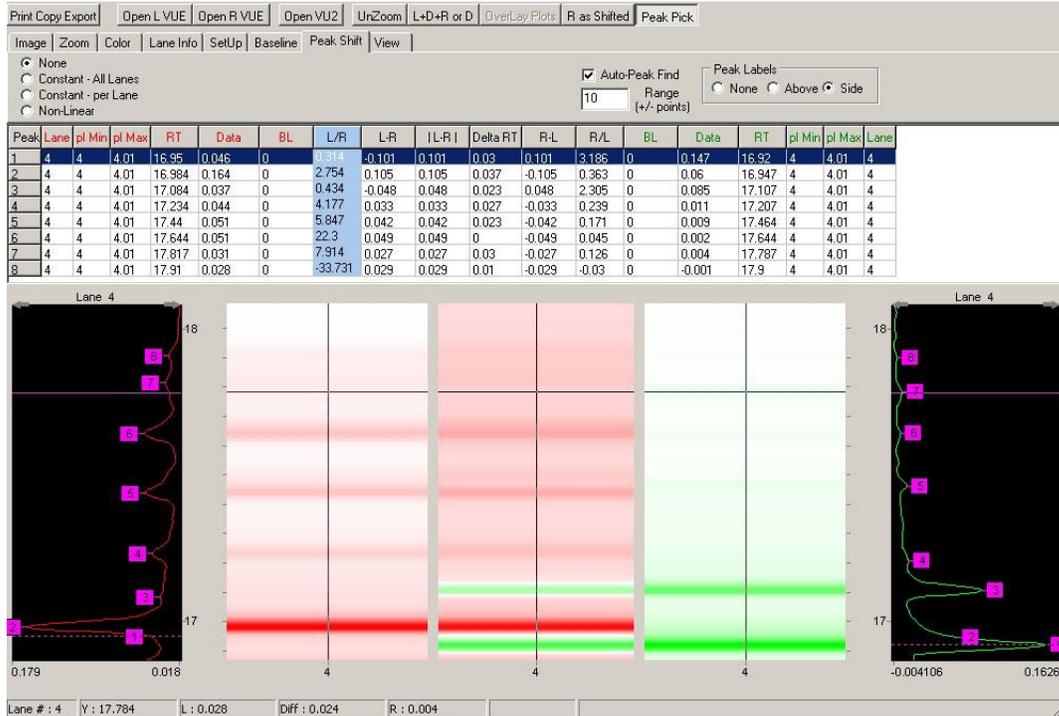
Supplementary Figure-S7-C3-17-18-Fr94
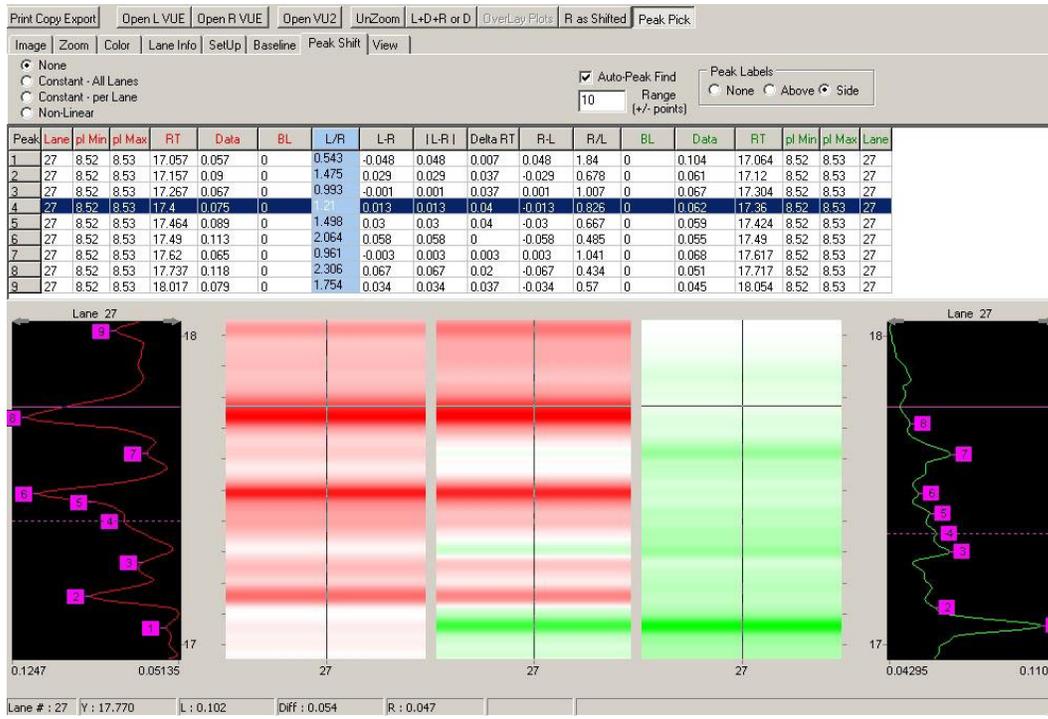
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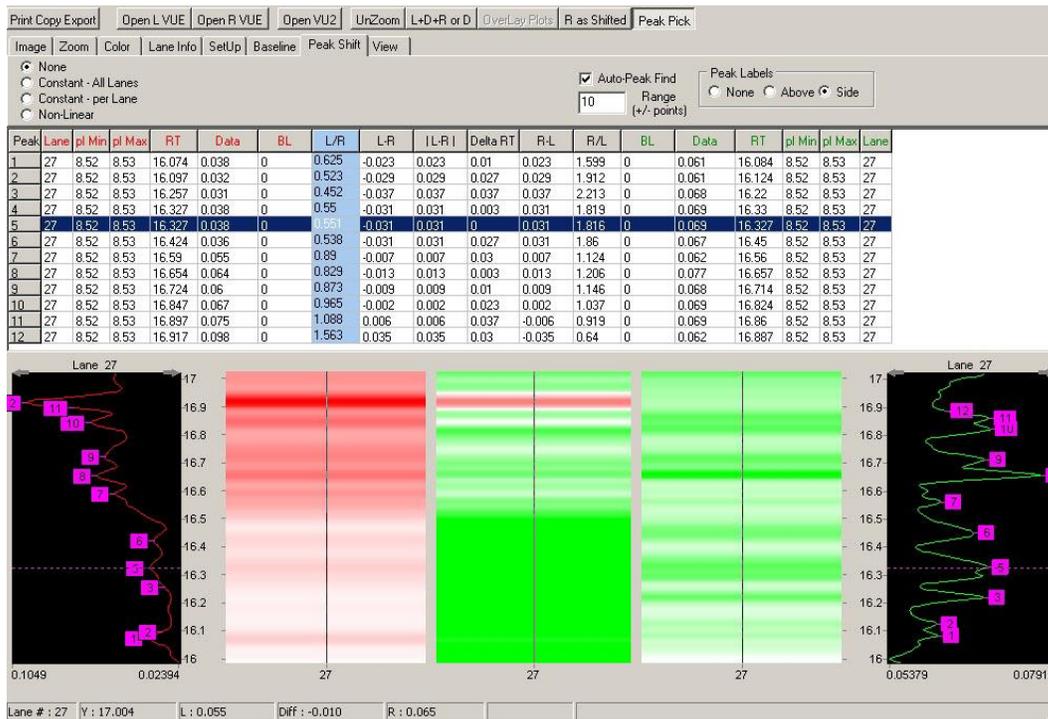
Legend to supplementary figures. Chromatograms on the left side represent N2 control fractions (displayed in red color). Chromatograms on the right side represent fractions from BIR-1 overproducing larvae (displayed in green color). Absorbance A_{214} for peaks marked by numbers in chromatograms is shown in tables shown above chromatograms.

Supplementary table 11 – Analysis of transcriptome of *bir-1* inhibited larvae

Supplementary table 11 A: Genes decreased in *bir-1* inhibited larvae

WBGene00000741	col-168
WBGene00000740	col-167
WBGene00000739	col-166
WBGene00001922	his-48
WBGene00001881	his-7
WBGene00009621	fipr-21
WBGene00000691	col-117
WBGene00004445	rpl-31
WBGene00001932	his-58
WBGene00004490	rps-21
WBGene00012256	lpr-5
WBGene00022024	Y64H9A.2
WBGene00013673	Y105E8A.13
WBGene00021850	Y54F10AM.6
WBGene00020083	R57.2
WBGene00012032	T26C5.2
WBGene00012255	lpr-6
WBGene00009108	F25D1.3
WBGene00008964	mltn-9
WBGene00010835	M03B6.3
WBGene00012123	T28D6.3
WBGene00008743	F13D12.9
WBGene00009888	F49E2.5
WBGene00000928	dao-2
WBGene00077490	M03A1.8
WBGene00009816	F47B10.5
WBGene00012635	Y38H8A.1
WBGene00020550	T17H7.1
WBGene00007867	C32H11.4
WBGene00009926	noah-2
WBGene00013225	Y56A3A.2

WBGene00012692	Y39B6A.33
WBGene00016422	noah-1
WBGene00006956	wrt-10
WBGene00016429	C35A11.2
WBGene00019520	K08B12.1
WBGene00010033	F54B11.10
WBGene00017658	F21C10.9
WBGene00012591	nspe-1
WBGene00010321	F59B10.5
WBGene00015163	B0361.9
WBGene00001072	dpy-10
WBGene00019212	H19M22.3
WBGene00000282	cah-4
WBGene00009342	fasn-1
WBGene00008292	C54C8.4
WBGene00001071	dpy-9
WBGene00001070	dpy-8
WBGene00020808	T25F10.6
WBGene00001769	gst-21
WBGene00017535	F17A9.3
WBGene00013074	hmit-1.2
WBGene00001069	dpy-7
WBGene00004471	rps-2
WBGene00004470	rps-1
WBGene00006370	sym-5
WBGene00006366	sym-1
WBGene00001430	fkb-5
WBGene00012186	mlt-11
WBGene00000065	act-3
WBGene00001684	gpd-2

Supplementary table 11 B: Genes increased in *bir-1* inhibited larvae

Gene WB ID	Gene Public Name
WBGene00017756	F23F12.12
WBGene00000090	age-1
WBGene00001955	hlh-11
WBGene00019957	R08E3.1
WBGene00006476	rhgf-2
WBGene00000802	crt-1
WBGene00009692	F44E5.5
WBGene00000027	abu-4
WBGene00044521	Y5H2A.4
WBGene00022164	Y71H2AL.1
WBGene00004123	pqn-36

WBGene00004170	pqn-90
WBGene00018500	F46F5.9
WBGene00004160	pqn-79
WBGene00004158	pqn-76
WBGene00020456	fbxa-60
WBGene00016225	fbxa-58
WBGene00000028	abu-5
WBGene00016193	C28H8.2
WBGene00019020	F57H12.5
WBGene00002020	hsp-16.49
WBGene00001026	dnj-8
WBGene00018349	F42C5.9
WBGene00022500	lfi-1
WBGene00019481	cogc-6
WBGene00001229	eif-3.F
WBGene00021771	Y51F10.10
WBGene00009120	F25H2.6
WBGene00000998	dig-1
WBGene00002222	klp-11
WBGene00002043	hyl-1
WBGene00000532	clh-5
WBGene00000034	abu-11
WBGene00007592	C14H10.2
WBGene00003497	mup-4
WBGene00004894	sms-3
WBGene00011283	R90.1
WBGene00012128	nra-1
WBGene00011441	T04F8.2
WBGene00022668	ZK154.6
WBGene00009819	F47B10.8
WBGene00011803	T16G12.1
WBGene00004008	pgp-14
WBGene00006591	toh-1
WBGene00010113	F55D12.5
WBGene00008341	ttr-44
WBGene00017438	F13H8.5
WBGene00004036	plc-1
WBGene00007943	C34F6.9
WBGene00009957	F53B2.8
WBGene00019465	acl-14
WBGene00003610	nhr-11
WBGene00019365	K03E6.7
WBGene00008803	lips-10
WBGene00020586	T19F4.1
WBGene00017967	ada-2
WBGene00019237	H24G06.1
WBGene00003772	nlr-1
WBGene00018604	F48E3.3

WBGene00001410	feh-1
WBGene00020567	T19D2.1
WBGene00013033	Y49E10.10
WBGene00000672	col-97
WBGene00008652	F10D11.6
WBGene00021396	cutl-24
WBGene00007911	C34B7.1
WBGene00004183	prk-2
WBGene00014215	obr-3
WBGene00007460	C08F11.13
WBGene00011642	T09B9.1
WBGene00016378	imp-3
WBGene00019987	R09F10.3
WBGene00016585	oac-59
WBGene00001577	gem-4
WBGene00021507	Y41D4A.5
WBGene00007368	C06B8.2
WBGene00003031	lin-46
WBGene00002274	lec-11
WBGene00000072	add-1
WBGene00003779	nob-1
WBGene00000516	cki-1
WBGene00000955	des-2
WBGene00021339	Y34F4.4
WBGene00017041	D2007.1
WBGene00015519	C06E1.3
WBGene00021977	Y58A7A.3
WBGene00021979	Y58A7A.5
WBGene00022326	fbxa-14
WBGene00021978	Y58A7A.4
WBGene00020774	T24E12.5
WBGene00022412	Y102A11A.2
WBGene00018031	F35B3.4
WBGene00044642	clec-69
WBGene00015381	C03B1.13
WBGene00015356	tag-278
WBGene00000666	col-91
WBGene00000024	abu-1
WBGene00007344	C05E7.2
WBGene00011535	T06E4.8
WBGene00014136	ZK896.5
WBGene00010660	K08D8.6
WBGene00008944	F19B2.5
WBGene00008530	F02E9.5
WBGene00008602	oac-14
WBGene00007804	C29F3.3
WBGene00010977	R02D5.3
WBGene00004153	pqn-71

WBGene00007903	lgc-21
WBGene00007506	C10C5.2
WBGene00009916	F52A8.3
WBGene00007716	fbxa-82
WBGene00012101	T27F2.4
WBGene00010019	F54B8.4
WBGene00015859	C16D9.4
WBGene00006310	sul-3
WBGene00017703	F22E5.1
WBGene00009432	F35E12.8
WBGene00000033	abu-10
WBGene00020240	T05B4.9
WBGene00009433	F35E12.9
WBGene00018787	cutl-20
WBGene00020254	T05C3.2
WBGene00017839	F26G1.3
WBGene00019754	M03E7.2
WBGene00077593	C49G7.12
WBGene00020280	T06A1.5
WBGene00020995	W03F8.6
WBGene00001713	grl-4
WBGene00021075	W07E6.3
WBGene00022736	ZK418.7
WBGene00011176	R09E10.6
WBGene00007833	oac-6
WBGene00020760	T24C4.4
WBGene00010464	K01D12.5
WBGene00010111	F55D12.2
WBGene00018618	F48G7.7
WBGene00008926	F17H10.2
WBGene00004148	pqn-65
WBGene00004139	pqn-54
WBGene00019615	K10B4.3
WBGene00016218	C29F9.3
WBGene00007453	fbxa-98
WBGene00008726	F13A7.11
WBGene00018282	F41D9.2
WBGene00018616	F48G7.5
WBGene00000711	col-138
WBGene00019404	K05B2.4
WBGene00017667	ttr-37
WBGene00015574	irg-1
WBGene00020413	T10E9.3
WBGene00019717	trx-3
WBGene00019564	K09D9.1
WBGene00018641	F49E10.4
WBGene00019660	K11H12.4
WBGene00012794	Y43E12A.2

WBGene00015933	C17H12.8
WBGene00016788	C49G7.10
WBGene00020720	T23B12.5
WBGene00021590	Y46D2A.2
WBGene00195076	Y46D2A.5
WBGene00020421	T10E10.4
WBGene00016502	C37C3.10
WBGene00020241	T05B4.10
WBGene00008058	C41G6.12
WBGene00020242	phat-5
WBGene00017091	ttr-40
WBGene00023485	ttr-49
WBGene00022117	Y71F9AL.12
WBGene00017218	dct-5
WBGene00015428	C04E7.1
WBGene00016004	C18H9.6
WBGene00014245	ZK1307.2
WBGene00004218	ptr-3
WBGene00001695	grd-6
WBGene00004237	ptr-23
WBGene00005324	T27A1.1
WBGene00015048	faah-2
WBGene00017312	F09G2.3
WBGene00010661	tyr-2
WBGene00000683	col-109
WBGene00022743	mlt-7
WBGene00000625	col-48
WBGene00003999	pgp-5
WBGene00002188	kgb-2
WBGene00004052	pme-4
WBGene00015930	C17H12.4
WBGene00000678	col-104
WBGene00003544	nas-25
WBGene00006954	wrt-8
WBGene00012168	W01A8.6
WBGene00019515	ugt-19
WBGene00000190	arf-1.1
WBGene00003526	nas-7
WBGene00007072	ugt-1
WBGene00011006	ugt-47
WBGene00008486	ugt-44
WBGene00007946	ugt-33
WBGene00014989	ZK675.3
WBGene00006718	ubc-23
WBGene00009257	F29G6.1
WBGene00012144	T28H10.3
WBGene00000480	cgp-1
WBGene00011404	T03F7.7

WBGene00006409	tag-19
WBGene00006677	twk-24
WBGene00003995	pgp-1
WBGene00000249	bir-1
WBGene00015544	C06E8.5
WBGene00013496	Y70D2A.1
WBGene00007048	tag-182
WBGene00001644	gly-19
WBGene00007932	zip-5
WBGene00014148	ZK909.3
WBGene00000031	abu-8

Supplementary table 12 - Functional annotation of genes decreased in *bir-1* inhibited larvae

Functional annotation of genes downregulated in *bir-1* RNAi experiments

Category	Term	RT	Genes	Count	%	P-Value	Benjamini
GOTERM_MF_FAT	structural molecule activity	RT		12	20.0	1.9E-7	1.1E-5
GOTERM_MF_FAT	structural constituent of cuticle	RT		8	13.3	7.8E-7	2.3E-5
INTERPRO	Collagen triple helix repeat	RT		8	13.3	2.0E-6	1.9E-4
INTERPRO	Nematode cuticle collagen, N-terminal	RT		7	11.7	8.2E-6	3.9E-4
SP_PIR_KEYWORDS	collagen	RT		6	10.0	1.1E-5	5.3E-4
SMART	PAN_AP	RT		4	6.7	1.2E-5	2.0E-4
INTERPRO	Apple-like	RT		4	6.7	5.0E-5	1.6E-3
INTERPRO	PAN-1 domain	RT		4	6.7	5.0E-5	1.6E-3
GOTERM_BP_FAT	body morphogenesi	RT		11	18.3	1.2E-4	1.2E-2

Category	Term	RT	Genes	Count	%	P-Value	Benjamini
	s						
KEGG_PATHWAY	Ribosome	RT		4	6.7	2.7E-3	1.1E-2
SP_PIR_KEYWORDS	ribosomal protein	RT		4	6.7	3.4E-3	8.1E-2
GOTERM_BP_FAT	molting cycle, collagen and cuticulin-based cuticle	RT		6	10.0	4.0E-3	1.8E-1
GOTERM_BP_FAT	molting cycle, protein-based cuticle	RT		6	10.0	4.0E-3	1.8E-1
GOTERM_BP_FAT	molting cycle	RT		6	10.0	4.1E-3	1.3E-1
GOTERM_BP_FAT	post-embryonic development	RT		16	26.7	4.5E-3	1.1E-1
SP_PIR_KEYWORDS	ribonucleoprotein	RT		4	6.7	5.7E-3	9.2E-2
GOTERM_CC_FAT	intracellular non-membrane-bounded organelle	RT		7	11.7	8.1E-3	1.9E-1
GOTERM_CC_FAT	non-membrane-bounded organelle	RT		7	11.7	8.1E-3	1.9E-1
GOTERM_BP_FAT	growth	RT		13	21.7	9.4E-3	1.7E-1
GOTERM_BP_FAT	nematode larval development	RT		15	25.0	1.1E-2	1.7E-1
GOTERM_BP_FAT	larval development	RT		15	25.0	1.1E-2	1.5E-1
GOTERM_MF_FAT	structural constituent of ribosome	RT		4	6.7	1.5E-2	2.5E-1
GOTERM_CC_FAT	ribosome	RT		4	6.7	1.6E-2	1.8E-1

Category	Term	RT	Genes	Count	%	P-Value	Benjamini
GOTERM_BP_FAT	positive regulation of growth	**RT**		15	25.0	2.2E-2	2.5E-1
GOTERM_BP_FAT	positive regulation of growth rate	**RT**		14	23.3	2.3E-2	2.3E-1
GOTERM_BP_FAT	regulation of growth rate	**RT**		14	23.3	2.3E-2	2.1E-1
GOTERM_BP_FAT	regulation of growth	**RT**		15	25.0	2.9E-2	2.4E-1
GOTERM_CC_FAT	ribonucleoprotein complex	**RT**		4	6.7	3.1E-2	2.4E-1
UP_SEQ_FEATURE	cross-link:Glycyl lysine isopeptide (Lys-Gly) (interchain with G-Cter in ubiquitin)	**RT**		2	3.3	3.2E-2	6.4E-1
SMART	LRR_TYP	**RT**		2	3.3	5.1E-2	3.6E-1
SP_PIR_KEYWORDS	isopeptide bond	**RT**		2	3.3	5.3E-2	4.9E-1
SP_PIR_KEYWORDS	nucleosome core	**RT**		2	3.3	5.3E-2	4.9E-1
GOTERM_BP_FAT	collagen and cuticulin-based cuticle development	**RT**		3	5.0	5.7E-2	3.9E-1
GOTERM_BP_FAT	cuticle development	**RT**		3	5.0	5.9E-2	3.8E-1
GOTERM_BP_FAT	protein-based cuticle development	**RT**		3	5.0	5.9E-2	3.8E-1
INTERPRO	Histone core	**RT**		2	3.3	6.1E-2	7.8E-1
SP_PIR_KEYWORDS	ubl conjugation	**RT**		2	3.3	7.2E-2	5.3E-1
INTERPRO	Protein of	**R**		2	3.3	7.3E	7.7E-1

Category	Term	RT	Genes	Count	%	P-Value	Benjamini
	unknown function DB	T				-2	
INTERPRO	Leucine-rich repeat, typical subtype	RT		2	3.3	7.6E-2	7.2E-1
GOTERM_BP_FAT	translation	RT		4	6.7	9.2E-2	5.1E-1
INTERPRO	Histone-fold	RT		2	3.3	9.5E-2	7.4E-1
GOTERM_CC_FAT	nucleosome	RT		2	3.3	9.8E-2	4.9E-1
SMART	ZP	RT		2	3.3	9.9E-2	4.5E-1
SP_PIR_KEYWORDS	cuticle	RT		2	3.3	1.0E-1	5.8E-1

Supplementary table 13: Functional annotation of genes upregulated in *bir-1* RNAi experiments

Category	Term	RT	Genes	Count	%	P-Value	Benjamini
INTERPRO	Protein of unknown function DUF1096	RT		11	5.2	2.7E-14	7.2E-12
INTERPRO	Cysteine rich repeat, tripleX	RT		10	4.7	1.6E-11	2.2E-9
PIR_SUPERFAMILY	PIRSF002689:gliadin	RT		6	2.8	1.1E-8	4.9E-7
INTERPRO	Metridin-like ShK toxin	RT		8	3.8	4.3E-4	3.7E-2
SMART	ShKT	RT		8	3.8	7.7E-4	5.6E-2
GOTERM_BP_	lipid modification	R		5	2	3.2	5.6E-1

Category	Term	RT	Genes	Count	%	P-Value	Benjamini
FAT		T			.3 2	E-3	
INTERPRO	CUB-like region	RT		5	2 . 3 3	3.3 E-3	1.9E-1
INTERPRO	EGF-like region, conserved site	RT		8	3 . 8	5.9 E-3	2.7E-1
INTERPRO	Transthyretin-like	RT		5	2 . 3 3	7.3 E-3	2.7E-1
PIR_SUPERFAMILY	PIRSF005678:glucuronosyltransferase	RT		4	1 . 9	8.0 E-3	1.7E-1
GOTERM_MF_FAT	carbohydrate binding	RT		8	3 . 8	8.0 E-3	7.5E-1
GOTERM_BP_FAT	lipid glycosylation	RT		4	1 . 9	1.0 E-2	7.3E-1
INTERPRO	Pleckstrin homology	RT		5	2 . 3	1.1 E-2	3.4E-1
INTERPRO	Protein of unknown function DUF1647	RT		3	1 . 4	1.2 E-2	3.2E-1
SMART	PH	RT		5	2 . 3	1.6 E-2	4.5E-1
INTERPRO	UDP-glucuronosyl/UDP-glucosyltransferase	RT		5	2 . 3	1.7 E-2	3.9E-1
INTERPRO	C2 calcium-dependent membrane targeting	RT		4	1 . 9	1.9 E-2	3.9E-1
INTERPRO	von Willebrand factor, type A	RT		4	1 . 9	2.5 E-2	4.5E-1
SMART	C2	RT		4	1 .	2.5 E-2	4.7E-1

Category	Term	RT	Genes	Count	%	P-Value	Benjamini
SP_PIR_KEYWORDS	egf-like domain	**RT**		5	92.3	2.6E-2	8.9E-1
INTERPRO	Protein of unknown function DB	**RT**		3	1.4	3.1E-2	5.0E-1
SMART	VWA	**RT**		4	1.9	3.3E-2	4.6E-1
GOTERM_BP_FAT	phospholipid metabolic process	**RT**		4	1.9	3.3E-2	9.5E-1
SP_PIR_KEYWORDS	hydrolase	**RT**		12	5.6	3.4E-2	7.6E-1
GOTERM_CC_FAT	internal side of plasma membrane	**RT**		3	1.4	3.4E-2	7.5E-1
INTERPRO	Lipase, GDXG, active site	**RT**		2	0.9	3.6E-2	5.2E-1
INTERPRO	EGF-like	**RT**		5	2.3	3.8E-2	5.1E-1
SP_PIR_KEYWORDS	metalloprotease	**RT**		4	1.9	3.8E-2	6.6E-1
GOTERM_BP_FAT	organophosphate metabolic process	**RT**		4	1.9	4.2E-2	9.4E-1
SP_PIR_KEYWORDS	Protease	**RT**		6	2.8	4.5E-2	6.2E-1
SP_PIR_KEYWORDS	disulfide bond	**RT**		7	3.3	4.8E-2	5.7E-1
GOTERM_BP_FAT	sphingomyelin biosynthetic process	**RT**		2	0.9	5.0E-2	9.3E-1

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Category	Term	RT	Genes	Count	%	P-Value	Benjamini
INTERPRO	C2 membrane targeting protein	**RT**		3	1.4	5.0E-2	5.9E-1
INTERPRO	ABC transporter, transmembrane region	**RT**		3	1.4	5.3E-2	5.9E-1
SMART	EGF	**RT**		5	2.3	5.3E-2	5.6E-1
PIR_SUPERFAMILY	PIRSF038285:cuticle collagen	**RT**		4	1.9	5.5E-2	5.8E-1
INTERPRO	EGF-type aspartate/asparagine hydroxylation conserved site	**RT**		3	1.4	6.0E-2	6.1E-1
INTERPRO	Pleckstrin homology-type	**RT**		4	1.9	6.2E-2	6.0E-1
SP_PIR_KEYWORDS	sphingolipid metabolism	**RT**		2	0.9	6.2E-2	5.9E-1
COG_ONTOLOGY	Defense mechanisms	**RT**		3	1.4	6.6E-2	5.0E-1
INTERPRO	Collagen triple helix repeat	**RT**		6	2.8	6.9E-2	6.3E-1
INTERPRO	ABC transporter integral membrane type 1	**RT**		3	1.4	7.3E-2	6.3E-1
GOTERM_MF_FAT	metallopeptidase activity	**RT**		6	2.8	7.4E-2	1.0E0
GOTERM_CC_FAT	extracellular region part	**RT**		3	1.4	7.8E-2	7.9E-1
UP_SEQ_FEATURE	disulfide bond	**RT**		6	2.8	7.8E-2	1.0E0

Category	Term	RT	Genes	Count	%	P-Value	Benjamini
GOTERM_BP_FAT	ceramide biosynthetic process	RT		2	0.9	7.9E-2	9.7E-1
GOTERM_BP_FAT	sphingoid biosynthetic process	RT		2	0.9	7.9E-2	9.7E-1
KEGG_PATHWAY	Natural killer cell mediated cytotoxicity	RT		2	0.9	7.9E-2	7.3E-1
INTERPRO	Copine	RT		2	0.9	8.1E-2	6.5E-1
GOTERM_BP_FAT	proteolysis	RT		10	4.7	8.2E-2	9.6E-1
INTERPRO	Peptidase M12A, astacin	RT		3	1.4	8.3E-2	6.5E-1
SP_PIR_KEYWORDS	collagen	RT		4	1.9	8.4E-2	6.5E-1
GOTERM_BP_FAT	sphingomyelin metabolic process	RT		2	0.9	8.8E-2	9.5E-1
GOTERM_BP_FAT	sphingolipid biosynthetic process	RT		2	0.9	8.8E-2	9.5E-1
GOTERM_BP_FAT	membrane lipid biosynthetic process	RT		2	0.9	8.8E-2	9.5E-1
INTERPRO	Concanavalin A-like lectin/glucanase, subgroup	RT		3	1.4	9.1E-2	6.6E-1
PIR_SUPERFAMILY	PIRSF015803:Caenorhabditis elegans hypothetical protein F48G7.8	RT		2	0.9	9.1E-2	6.7E-1

Legend to Functional annotation: DAVID Bioinformatics Resources 6.7 from National Institute of Allergy and Infectious Diseases (NIAID), NIH was used on January 3, 2016

(https://david.ncifcrf.gov). The grey part of table are results with p>0.05.

Supplementary table 14. The complement of *C. elegans* ribosomal proteins

The complement of *C. elegans* ribosomal proteins

Small Subunit		Large Subunit	
SA	rps-0	L3	rpl-3
S2	rps-2	L4	rpl-4
S3	rps-3	L5	rpl-5
S3A	rps-1	L6	rpl-6
S4	rps-4	L7	rpl-7
S5	rps-5	L7A	rpl-7A
S6	rps-6	L8	rpl-2
S7	rps-7	L9	rpl-9
S8	rps-8	L10	rpl-10
S9	rps-9	L10A	rpl-1
S10	rps-10	L11	rpl-11.2
S11	rps-11		rpl-11.1
S12	rps-12	L12	rpl-12
S13	rps-13	L13	rpl-13
S14	rps-14	L13A	rpl-16
S15	rps-15	L14	rpl-14

S15A	rps-22	L15	rpl-15
S16	rps-16	L17	rpl-17
S17	rps-17	L18	rpl-18
S18	rps-18	L18A	rpl-20
S19	rps-19	L19	rpl-19
S20	rps-20	L21	rpl-21
S21	rps-21	L22	rpl-22
S23	rps-23	L23	rpl-23
S24	rps-24	L25	rpl-25.1
S25	rps-25	L25	rpl-25.2
S26	rps-26	L24	rpl-24.1
S27	rps-27	L26	rpl-26
S27A	ubl-1	L27	rpl-27
S28	rps-28	L27A	Y37E3.8a
S29	rps-29	L28	
S30	rps-30	L29	rpl-29
		L30	
		L31	rpl-31
		L32	rpl-32
		L34	rpl-34
		L35	rpl-35
		L35A	rpl-33
		L36	rpl-36
		L36A	rpl-41
		L37	rpl-37

	W01D2.1
L37A	rpl-43
L38	rpl-38
L39	rpl-39
L40	rpl-40.1
L41	rpl-41.1
	rpl-41.2
LP0	rpa-0
LP1	rla-1
LP2	rla-2
LP3	