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Summary of Thesis

The role of evolutionarily conserved proteins BIR-1/Survivin and SKP-1 in the regulation of gene expression

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Contents

Abstrakt

SKIP a BIR/Survivin jsou evolučně zachovalé proteiny. SKIP je známý transkripční a sestřihový kofaktor a BIR-1/Survivin reguluje bunečné 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 cDNA *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 provedených metodou kvasinkového dvouhybridního 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

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* cDNA library using yeast two-hybrid screening 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.

Key words: BIR-1, gene expression, proteome, ribosomal stress, SKIP, Survivin

Introduction

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. Tissue and metabolic state specific transcription factors and coregulators direct proper gene expression to cope with particular developmental and metabolic needs on the level of cells, tissues and whole organisms. Further, downstream mechanisms then modulate gene expression on the level of RNA splicing and mRNA processing, nuclear export and mRNA translation into proteins. 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.

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* (1-3).

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 (4, 5) and later found in many species including *Dictyostellium discoideum* (6) and yeast (7). SKIP interacts with several transcription factors including nuclear receptors (8-11), Notch (12), Wnt/beta catenin (13), TGF beta and Smad protein complexes (14) and it was also identified as a component of the splicing machinery in yeast, mammals (15) and plants (16). It was identified in both transcription activating as well as transcription inhibiting complexes (17). 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 (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 (1), which is the homologue of the vertebrate protein Survivin. Survivin was first discovered as an antiapoptotic protein (18). It is expressed predominantly in fast dividing cells and is found upregulated in most if not all human cancers (19). The most obvious function of Survivin is its role in mitosis as a component of the chromosome passenger complex 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 (19). This function is conserved in the Survivin homologue, BIR-1 in *C. elegans* (20, 21). 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 (2).

Thesis statements: goals, hypotheses and synopsis

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 SKP-1 and BIR-1 interacting proteins using the yeast two-hybrid screening system.

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. This suggested that both studied

proteins may be components of a wide range of protein complexes that may have regulatory potential. For further validation of these results, we searched for the effect of a short time overexpression of BIR-1 on the proteome. To do this, we used a second unbiased approach – two dimensional comparative chromatographic analysis of complete proteomes of BIR-1 overexpressing and control synchronized L1 larvae.

The results confirmed that BIR-1 affects the chromatographic pattern of a large number of proteins. Both SKP-1 and BIR-1 interacted with ribosomal proteins. Several ribosomal proteins are key players in the regulation of the stress response and programmed cell death. Our work 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.

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.

Materials and methods

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) according to manufacturer specifications.

The *C. elegans* Bristol N2 strain was used whenever not specifically stated and was maintained as described (22). 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.

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. Lysates from synchronized L1 larvae were prepared and were subjected to two dimensional chromatography using the Beckman-Coulter ProteomeLab PF 2D system (Beckman Coulter, Inc., Fullerton, CA) following the protocol recommended by the manufacturer. 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 analyzed using 32Karat software. ProteoVue and DeltaVue software. 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 using LCQ^{DECA} ion trap mass spectrometer (ThermoQuest, San Jose, CA) 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 (23) using the following criteria: XCorr values were 1.7 for singly charged, 2.2 for doubly charged and 3.0 for triply charged peptides.

The bioinformatics analysis was done using NCBI bioinformatic tools BLAST (24), gene ontology tool DAVID (http://david.abcc.ncifcrf.gov/) (25, 26) and Wormbase WS242 (http://www.wormbase.org).

The following strategies were employed for further validation of results. Precipitation of 35S-methonine labelled proteins with GST-tagged proteins (using pGEX-2T vector (Amersham Pharmacia Biotech, Amsterdam, UK) and the TNT T7/T3 coupled reticulocyte lysate system (Promega,

Madison, Wi)). Analysis of *skp-1* reduction-of-function effect on cell division (by RNAi directed against *skp-1*) and comparison to known role of the potential interactant TAC-1), and staining with antibody detecting SPD-2 that localizes to centrosomes (denominated 9v5, LA, a kind gift of Dr. O'Connell) (27). For validation of results concerning the connection of BIR-1 and NMY-2, *bir-1* overexpressing larvae were stained with a commercial anti-NMY-2 antibody.

Analysis of BIR-1 effect on transcription of ribosomal proteins was done using *bir-1* inhibition by RNAi (published in (3)) and bioinformatics methods Affymetrix MAS 5.0 suite software and Robust Multichip Average method.

Results

SKIP-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.

List of SKP-1 interactors: eIF-2B, TAC-1, INF-1 (orthologous to mammalian eIF-4A), DPY-14, IFA-2 (intermediate filament), H2A.F HTZ-1, SORB-1, RPL-5, RACK-1, Mitochondrial protein T27A3.4, PAB-1, Mitochondrial transcription factor B1, RPL-11.1, CPR-6, Myosin Heavy Chain, PHO-11, Intestinal Acid Phosphatase Protein 4 member 2K223, NHR-92 (HNF4-like), W02D7.3 Similar to Ankyrin and KH repeat

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. 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.

List of BIR-1 interactors: MAI-1, Ubiquitin ligase ZK1240.9, NHR-6, COL-167, MCCC-1, PAL-1, CEY-2, Glycoside dehydrogenase D2096.3, CEY-1, RLA-0

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. 1**).

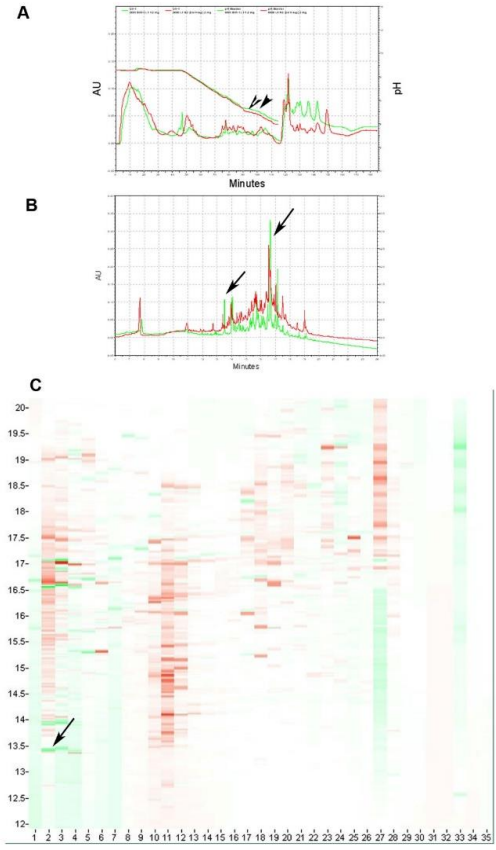

Fig. 1 Two dimensional comparative chromatography of complete proteomes of control and BIR-1 overexpressing L1 larvae. Panel A - First dimensional separation of protein lysates from wild type (N2) (red line) and *bir-1* overexpression samples (green line). Comparative analysis shows significant changes 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.

List of proteins identified only in BIR-1 hyperinduction fractions: 40 S ribosomal protein S3, Putative sideroflexin-like protein AH6.2, ATP synthase subunit alpha - mitochondrial precursor, 60S ribosomal protein L5, Myosin-4 (UNC-54), Nuclear Anchorage Protein1

List of proteins identified only in wild type (N2) fractions: Probable electron transfer flavoprotein subunit F27D4.1, Hit-like protein TAG-202, Triosephosphate isomerase *tpi-1*, Uncharacterized protein B0303.3, Probable 26S protease regulatory subunit S10 *rpt-4*, Probable ornithine aminotransferase, mitochondrial protein C16A3.10, Probable ornithine aminotransferase, mitochondrial protein C16A3.10, Probable malate dehydrogenase mitochondrial protein mdh-1, Probable Prefoldin subunit 5, Superoxide Dismutase [Cu-Zn] *sod-1*, Heat-shock Protein Hsp-12.2, Glyceraldehyde-3-phosphate Dehydrogenase 2 *gpd-2*

List of proteins identified differentially in both N2 and BIR-1 hyperinduction fractions: NHP2/L7aE family protein YEL026W homologue, Protein UNC-87, a calponin-related protein, Transthyretin-like protein T07C4.5 precursor, Tropomyosin isoforms a/b/d/f +c/e, Myosin, essential light chain, Elongation Factor 1-alpha, Fructose-bisphosphate aldolase 2, 40 S Ribosomal protein S8, 40 S Ribosomal protein S21

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.

Functional analysis of the connection between SKP-1 and TAC-1.

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 (28). Therefore, we studied the effects of SKP-1 inhibition on mitosis in detail **(Fig. 2)** 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 SPD-2 (27) showed that SKP-1 inhibition led to serious defects of mitoses including endomitoses and defects of centrosome migration in the G2 phase **(Fig. 2 F)**, similar to those previously shown following TAC-1 inhibition (29)**.**

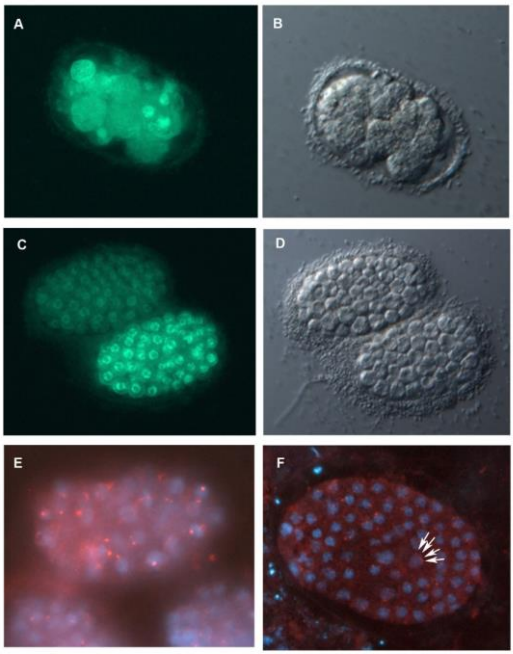

Fig. 2 Inhibition of SKP-1 induces cell division arrest and endomitoses. Panels A and C show Histone H2::GFP expressing embryos. Panels B and D are corresponding views in Nomarski optics. Panels A and B show mitotic defects of *skp-1* RNAi embryos. The nuclei lost their regular

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).

Validation for ribosomal proteins

Proteins that are 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 (30-33). 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. 3)**.

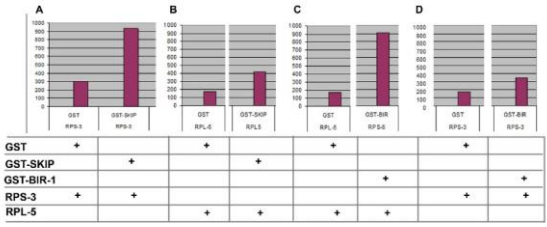

Fig. 3 SKP-1 and BIR-1 interact with RPS-3 and RPL-5. Panels A to D show interactions of SKP-1 (panels A and B) with RPS-3 and RPL-5 (panels A and B, respectively) and interactions of BIR-1 with RPL5 and RPS-3 (panels C and D).

Validation for myosin

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. 4 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. 4 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.

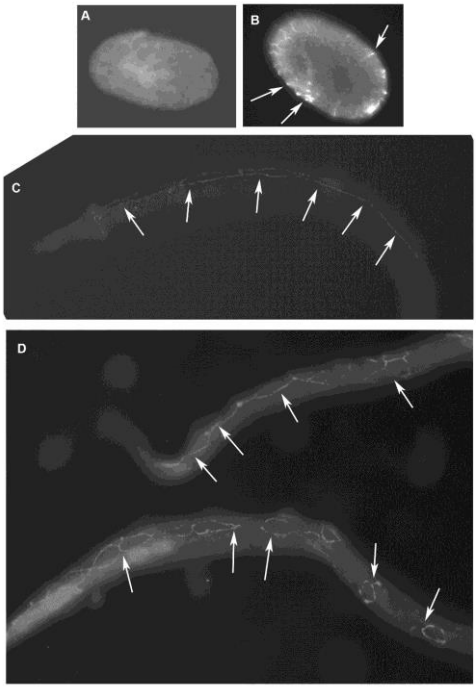

Fig. 4 The effect of short term overexpression of *bir-1* on non-muscle myosin localization in *C. elegans* embryos and development of seam cells. Panels A and B show *C. elegans* embryos stained for NMY-2. A) Control wild type (N2) embryo (containing control transgene consisting of empty vector), B) 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.

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

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 that showed a trend of slight decrease in the same analysis as above were 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).

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 are linked to the regulation of gene expression and development (1-3). Based on the results presented in this thesis I propose a concept of a direct regulatory connection between the structural state of the cell and the regulation of gene expression. In this concept, proteins constituting the free proteome (that is 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 and modulate gene expression accordingly. The fact that we detect such interactions for evolutionarily highly conserved proteins suggests that this regulatory pathway may be very ancient.

We searched for protein interactions that may transmit the specific cellular roles of SKP-1 and BIR-1 using unbiased high-throughput proteomic

methods based on 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 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.

A sudden overrepresentation of BIR-1 in non-dividing cells was achieved by a short time overexpression of BIR-1 in L1 stage and we analyzed the proteome using two dimensional comparative chromatography. This confirmed that BIR-1 overexpression is affecting a large number of specific proteins on the proteome level, independently of gene transcription.

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

We reasoned that if the proteins in focus are indeed forming complexes with multiple other proteins then a sudden forced overexpression of any of them should induce differences detectable on the level of the whole proteome. We designed such an experiment for BIR-1. Proteins in the cell are assembled in cellular structures or are present in structure independent form.

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 but rather present in protein complexes. Taking in account the variability of protein 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.

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

SKIP (SKI interacting protein, SNW) is a well established transcriptional and splicing cofactor (34). 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 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*) (20, 21, 35-39). 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. 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 (20, 21) between nematodes and vertebrates.

The transcriptional roles of SKIP are conserved between vertebrates and nematodes (1). Homologues of SKIP are found in Metazoa, yeast and in plants (40). 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 liberating some of them for additional regulatory tasks (41, 42). 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 and this has been confirmed (1-3).

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* (43, 44). MDM2 can reversely bind ribosomal proteins RPS3 (33), RPL5 (31, 45, 46), RPL11 (30, 32, 47, 48), and RPS28 (49). Additional proteins were shown to participate in the regulation of the p53 pathway, including RPL37, RPS15, and RPS20 (49). Various ribosomal proteins in the p53 pathway may function through multiple mechanisms, as was recently shown for ribosomal protein S26 (50). 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 (51). 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. The antiapoptotic function of Survivin through physical binding of caspases 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 further 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 (28). A possibility of direct centrosomal localization and function of SKIP is keeping with the centrosomal localization and mitotic function of SKI (52, 53), a protein which interacts physically and functionally with SKIP (54).

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 (55). These domains were often regarded as low-complexity proteins/protein domains in bioinformatic studies (56). 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 (57). Similarly, Mediator proteins use intrinsically disordered domains (58) 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 (59). 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.

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 very elegantly by a separation-of-function mutant (60).

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 (8, 9, 11). 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 (12). SKIP also directly binds the retinoblastoma tumor suppressor protein pRb and, in co-operation with Ski, overcomes the G1 arrest induced by pRb (61). SKIP is also involved in the regulation of splicing (15, 16, 62, 63). 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 (40, 64).

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 and contrary to mammals, which have approximately 2000 predicted pseudogenes of ribosomal proteins in their genome (65), 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 (66). SKP-1 and/or BIR-1 may be the sensor(s) in ribosomal protein transcription and in the ribosomal stress pathway.

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 (67-72). 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 (67). Similarly, p53 was recently shown to regulate gene expression on the translational level (73-75).

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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List of author's publications related to this thesis:

1. Kostrouch D, Kostrouchová M, Yilma P, Chughtai AA, Novotný JP, Novák P, Kostrouchová V, Kostrouchová M, Kostrouch Z.: SKIP and BIR-1/Survivin have potential to integrate proteome status with gene expression. J Proteomics. 2014 Oct 14;110:93-106. doi: 10.1016/j.jprot.2014.07.023. IF(2014)=3.888

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