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Autoreferát disertační práce

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Gene expression regulation by nuclear receptors in a specific metabolic context – evolutionary perspective

Regulace genové exprese jaderními receptory ve specifickém metabolickém kontextu – evoluční perspektiva

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištené podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

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Abstract

In animals, some of the most critical regulators of gene expression are nuclear hormone receptors (NRs) and their coregulators, specifically the Mediator complex. Of particular interest are the NRs implicated in metabolic and developmental regulation and in carcinogenesis: thyroid hormone receptors (TRs) and retinoid X receptors (RXRs). In this work, I venture to elucidate some aspects of gene expression regulation by these NRs: the degree of evolutionary conservation of their signalling pathways; the mechanisms of negative regulation by NRs; and possible implications of these findings for clinical medicine. State-of-the-art bioinformatical, genome editing and microscopic techniques are applied at three levels of animal evolution to study NRs and Mediator. Reverse genomics in human patients suffering from the syndrome of resistance to thyroid hormones type β are used to infer the structure and function of TRβ subdomains. Alignments, binding studies and *in vivo* experiments in *Trichoplax adhaerens* allowed identification of a close orthologue of human RXR at the basis of metazoan evolution. Employing database queries, genome editing and microscopy, we describe a correct orthologue of the Mediator subunit 28 in *Caenorhabditis elegans*, indicating a complete homology of the Mediator complex between nematodes and human. Analysing the results between species, we provide further indications that regulation by the NR-Mediator axis is conserved throughout *metazoans,* and we propose a hypothetical working model of the negative regulation by NRs.

Keywords

Caenorhabditis elegans, gene expression regulation, Mediator subunit 28 (MED28), negative regulation by TRs, nuclear receptors, retinoid X receptors (RXRs), syndrome of resistance to thyroid hormones – type β (RTHβ), thyroid hormone receptors (TRs), *Trichoplax adhaerens*.

Abstrakt

Mezi nejdůležitější regulátory genové exprese u zvířat patří jaderné receptory (NRs) a jejich koregulátory, zejména Mediátorový komplex. Zvláštní zájem vzbuzují NRs zúčastňující se na metabolické a vývojové regulaci a na karcinogenesi: receptory hormonů štítné žlázy (TRs) a retinoidové X receptory (RXRs). Ve své práci se podjímám úkolu objasnit některé aspekty regulace genové exprese těmito NRs: míru evoluční konzervace jejich signalizačních drah; mechanismy negativní regulace jadernými receptory; a možné aplikace těchto objevů v klinické medicíně. Použil jsem bioinformatické a mikroskopické metody, včetně metod genové editace, a to na třech úrovních evoluce zvířat ke studiu vtahu NRs a Mediátoru. Reversní genomická analýza u lidských pacientů trpících syndromem rezistence k thyroidním hormonům je využita k posouzení struktury a funkce subdomén TRβ. Porovnání sekvencí, vázné studie a *in vivo* experimenty u *Trichoplax adhaerens* vedly k identifikaci blízkého ortologu lidského RXR na počátku evoluce zvířat. Použitím analýzy databází, editace genomu a mikroskopie jsme identifikovali skutečný ortolog mediátorové podjednotky 28 u *Caenorhabditis elegans*, což poukazuje na zásadní homologii Mediátorového komplexu mezi nematody a člověkem. Analýza vztahu mezi druhy posiluje koncept konzervace regulační osy NR-Mediator u všech *metazoa.* Na tomto základě navrhuji hypotetický funkční model negativní regulace NRs a diskutuji možnou roli TR, RXR a MED s dopady na pochopení karcinogenese a možné aplikace v molekulární a klinické onkologii.

Klíčové pojmy

Caenorhabditis elegans, jaderné receptory (NRs), mediatorová podjednotka 28 (MED28), negativní regulace prostřednictvím TRs, regulace genové exprese, retinoidní X receptory (RXRs), syndrom resistence k thyroidním hormonum β (RTHβ), thyroidní receptory (TRs), *Trichoplax adhaerens*.

1. Introduction

Intercellular communication defines multicellular life. Its molecular mechanisms must allow processing of incoming signals from environment, as well as from other cells of the organism, their interpretation and response. The basic decision-making is based on a process called signal integration. In the very centre of signal integration is transcriptional regulation by two central sets of protein complexes: (1) specific transcription factors (**sTFs**) that detect incoming signals and localise them to specific DNA sequences; and (2) **coregulators** that integrate all incoming signals together with current chromatin structure and transmit the ultimate effect to the transcription machinery [1].

In the category of **sTFs**, nuclear receptors (**NRs**) are among the evolutionarily most ancient. NRs are responsible for gene expression regulation in metabolism and development of virtually all animals, as well as in carcinogenesis in vertebrates [2]. A relatively uniform structure of NRs includes a regulatory N-terminal domain, a conserved DNA-binding domain (DBD), a variable hinge domain, a polyfunctional ligand-binding domain (LBD) and an inconstant C-terminal domain. LBDs of most NRs are very similar, consisting of 12 α-helices and 2-4 β-sheets. Functionally, LBD mediates ligand binding via its ligand-binding pouch (LBP), dimerization, coactivator binding via the Activated Function 2 (AF2) surface and corepressor binding [3].

Based on sequence similarity and function, NRs are classified into 8 subfamilies. Subfamily 1 contains the most typical NRs, such as thyroid hormone receptors (TRs), vitamin D receptors (VDR), retinoic acid receptors (RARs) and Peroxisome proliferator-activated receptors (PPARs). Central, yet enigmatic subfamily 2 includes metabolic receptors such as retinoid X receptor (RXR), hepatocyte nuclear factor 4 (HNF4) and COUP-TF. Steroid

receptors such as oestrogen receptor (ER), progesterone receptor (PR), glucocorticoid receptor (GR), *etc.* belong to the subfamily 3, which is functionally and structurally distinct from the typical NRs [4].

DBDs of most NRs from subfamilies 1-3 bind to identic or similar nucleotide sequences and the binding specificity is uniquely procured by dimerization on DNA response elements consisting of two 6-nucleotide-long half-sites. These half-sites are variously organised, which allows the specific binding of the correct NR dimer. Steroid receptors of subfamily 3 form homodimers on palindromic response elements. Subfamily 1 receptors heterodimerize with RXR on variously organised sites, directing correct NRs to correct gene promoters and enhancers [5].

Depending on presence or absence of ligands, NRs bind **coactivators** or **corepressors**. These large complexes integrate signals from multiple sTFs and other signalling pathways and activate or supress transcription of genes by the RNA polymerase II (Pol II). Two proteins called Nuclear receptor CoRepressor 1 (**NCoR**1) and Silencing Mediator of Retinoid and Thyroid hormone receptors (NCoR2/SMRT) are the central corepressors [6]. Multiple coactivator complexes are known, of which the most important is the Pol II transcription mediator complex (Mediator) [7].

Mediator integrates incoming signals from many sTFs and other pathways and directly interacts with Pol II to promote transcription initiation [8]. It is a fundamental regulatory complex indispensable for transcriptional regulation and for life in general in all known eukaryotes. The higher structure of Mediator is well conserved throughout eukaryotic life, consisting of 25 protein subunits in yeast and 30 in human. In multicellular *metazoa* from invertebrates

to human, all subunits are structurally and functionally homologous. These proteins are organised in four main modules: head, middle, tail and cyclin-dependent kinase 8 (CDK8) module. The head and middle modules are directly interacting with Pol II and bind most NRs and other sTFs [8], [9].

Of particular interest to this thesis is the central metabolic signalling based on **TRs**. In human, two TR paralogues TRα and TRβ are mediating all effects of the thyroid hormone, 3,5,3'-triiodothyronine (T_3). It exerts its functions in cooperation with one of three **RXR** paralogues, forming a non-permissive heterodimer on thyroid hormone response elements [10].

In the conventional positive signalling model, in absence of T_3, RXR/TR dimers bind to corepressors and repress gene expression to a subnative level. Upon T_3-binding, the dimer dissociates corepressors and binds coactivators, including Mediator, via the interaction of TR and RXR AF2 modules with a consensus coactivator motif LxxLL, activating transcription [10].

However, some genes are **regulated negatively** by TRs, i.e. their transcription is activated in absence of T_3 and supressed in its presence [11]. The exact mechanism is currently not clear, but it depends on TRβ and one model proposes that in this case corepressors activate and coactivators inhibit gene expression (Figure 1).

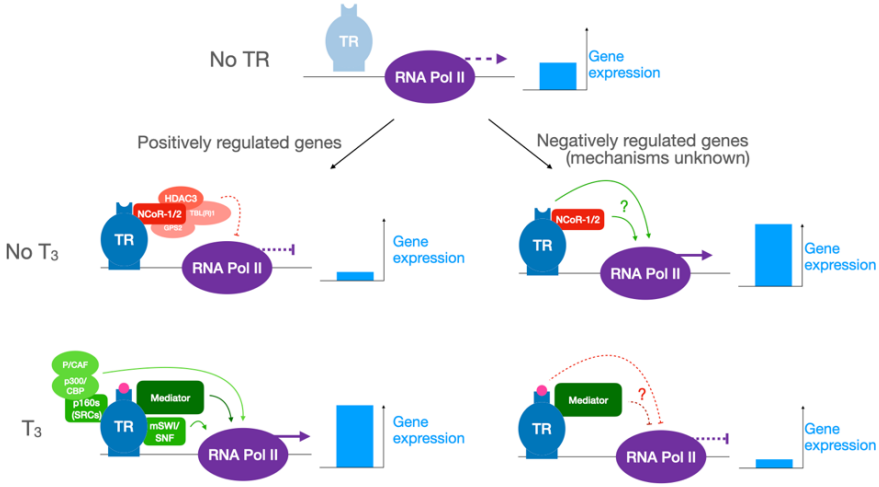

Figure 1. *Mechanisms of TR regulation on positively and negatively regulated genes. The mechanism of negative regulation is not known, the figure shows the most likely of proposed models.TR – thyroid hormone receptor, T_3 – thyroid hormone.*

2. Premises and aims of thesis

Gene expression regulation by NRs and Mediator is of crucial significance in physiological conditions and in cancer. Direct structural and mechanical studies of these large complexes are not feasible with current methods. Therefore, indirect evolutionary and reverse genetic studies need to be applied to gain insight into molecular mechanism of NR/Mediator action.

I set out to clarify some aspects of the gene expression regulation by NRs, especially RXR/TRs and the Mediator in following steps:

1. Elucidate the molecular mechanisms of negative regulation by TRs by studying phenotype of its mutations in patients with syndrome of resistance to thyroid hormones - type β (RTHβ).

2. Analyse the evolutionary conservation of the most ubiquitous of NRs, the RXR, in the simplest metazoan *Trichoplax adhaerens*.

3. Dissect the role of a Mediator subunit that seemed to be the only one to be functionally not conserved in a model organism *Caenorhabditis elegans*, MED28.

4. Compare the structural and functional conservation of TR, RXR and Mediator to estimate possible molecular interactions of these proteins in gene expression regulation.

3. Methods

In order to analyse the functional dedication of individual subdomains of the **TRβ** LBD, we ventured to analyse naturally occurring mutation in the *THRB* gene present in patients with the syndrome of resistance to thyroid hormones β (RTHβ). In cooperation with clinicians, we recruited patients diagnosed with the typical biochemical phenotype of elevated serum T_3 levels with non-supressed thyroid stimulating hormone (TSH). Genomic DNA from their peripheral blood samples was isolated, the four terminal exons of the *THRB* gene corresponding to the LBD were amplified and sequenced.

Discovered mutations were assessed for integral importance by assessing their evolutionary conservation and mapped onto the known crystal structures of TRβ LBD. We performed an *in silico* analysis of these residues to deduce their function. We associated the function of these residues with the biochemical and clinical presentation of RTHβ to infer which function of TRβ LBD is responsible for the negative regulation.

Next we studied the binding partner of TR, the **RXR**, in *T. adhaerens*. A candidate orthologue gene was identified by database searches and alignments. The coding sequence of the TaRXR gene

was amplified from total *Trichoplax* cDNA, cloned into pGEX-2T plasmid vector and expressed in a dedicated *E. coli* strain BL21. We incubated the *in vitro* expressed protein with radioactive ^{3}H-labelled 9-*cis*-RA and ^{3}H-labelled ATRA and determined binding as a ratio of bound radioactivity and total radioactivity (bound and washed out).

We probed the functional conservation of TaRXR by exposing a *Trichoplax* culture to 9-*cis*-RA or vehicle alone and observing relative change in reporter gene expression by qPCR and ddPCR. To assess the functional dedication of the TaRXR, we assessed the effect of 9-*cis*-RA on the animals in cultures with various food compositions.

Finally, we provided experimental evidence to disprove the annotation of a transcript W01A8.1 as an orthologue of **MED28** in *C. elegans*. Database searches and multi-species alignments allowed the identification of signature motifs of MED28 homologues and of the W01A8.1 to find that it shared superior homology with Perilipins than with MED28.

A newly discovered MED28 orthologue candidate gene F28F8.5 and two Mediator subunits known to interact with MED28 in human, MDT-6 and MDT-23, were amplified from nematode cDNA and cloned into protein expression vectors pGEX-2T, pTNT and pET28a(+) respectively. GST::F28F8.5 and MDT-23::FLAG were expressed in BL21 and ^{35}S-Met-MDT-6 was expressed in a reticulocyte lysate TNT system. Binding was determined after gel electrophoresis by autoradiography and anti-FLAG antibody Western Blot.

We studied both genes (W01A8.1 and F28F8.5) by several transgenic and genetic techniques. We inhibited their expression by RNA interference (RNAi). We employed CRISPR/Cas9-

dependent restriction and recombination to prepare animals expressing the two genes bound to fluorescent proteins, as well as lines with disruption in both genes. We also crossed the W01A8.1$^{-/-}$ line with a line expressing autophagy marker LGG-1::GFP and a line deficient in the effector lipase HOSL-1$^{-/-}$ to study function of W01A8.1. The animals were imaged using modern microscopic methods: confocal microscopy, fluorescence lifetime imaging microscopy (FLIM) and coherent antistokes Raman scattering (CARS) microscopy.

4. Results

We recruited 17 patients with suspected **RTHβ**. In 9, various heterozygous mutations in the *THRB* gene were found. We found following mutations: K443E (lysine to glutamic acid in the position 443), F459L, Y321C and finally, a previously undescribed change of threonine to arginine in the codon 273 (T273R) [B].

Evolutionary analysis showed complete conservation of the threonine 273 in vertebrates. This residue was mapped to the TRβ LBD helix 2, interacting with the loop connecting helices 11 and 12, the functional core of the AF2 subdomain. Therefore, the T273R mutation is likely to impair coactivator binding by AF2, but not ligand-binding. Patients with this mutation have slightly increased T_3 and T_4 levels with non-supressed TSH levels, mild neuro-developmental deficits and mild signs of connective tissue hypothyroidism but no signs of hyperthyroidism [B].

Thereafter, we turned our attention to **RXR**, employing an evolutionary approach. A candidate RXR orthologue gene in *T. adhaerens* was identified. The TaRXR protein shares a remarkable 66% overall identity and complete similarity to human RXRs within the DBD and LBD. It has a 100% identical dimerization

motif and highly similar LBP. An *in vitro* binding study showed specific binding of the 9-*cis*-RA, an RXR- specific ligand [C].

We also proved its conserved function by showing a gene expression activation of a metabolic reporter gene *in vivo* upon exposition of animals to 9-*cis*-RA. At the same time, we analysed the expression change of all four known and newly identified NR orthologues upon treatment with 9-*cis*-RA, revealing an increase in expression of RXR itself and of oestrogen related receptor (ERR) but decrease in COUP-TF expression. Finally, we showed that the proliferation of *T. adhaerens* culture has been accelerated up to ten times upon adding red algae to the green algal *milieu*. Effect of 9-*cis*-RA on culture proliferation was modulated by the presence of these red algae, suggesting that RXR was implicated in alimentary sensing in *T. adhaerens* [C].

We followed by analysing the most important coregulator of NRs, the Mediator, in a well-established model system, *C. elegans*. Our subunit of interest, **MED28** had an orthologue gene described in *C. elegans*: the transcript W01A8.1 had been annotated as *mdt-28*.

A bioinformatical analysis revealed that the W01A8.1 protein is rather a homologue of Perilipins, a group of lipolysis-regulating proteins localised to lipid droplet (LDs), thought to be absent from *C. elegans* [E]. Our experimental data showed that W01A8.1 localises to LDs and not to the nucleus, as would be expected for a Mediator subunit [E]. Its knock-down by RNAi and knock-out by CRISPR/Cas9 resulted in embryonic lipid accumulation and deficient lipolysis with activation of a secondary lipolytic pathway, lipophagy, but no developmental defects [A], [E].

An *ab initio* bioinformatical analysis of MED28 in various species was performed, discovering a candidate MED28 orthologue protein F28F8.5. We confirmed that this protein localises

predominantly to the cell nucleus and our binding studies revealed a specific biding of two other Mediator subunits MDT-6 and MDT-23, orthologues of both known to bind MED28 in mammals. F28F8.5-defficient animals had pronounced phenotype of developmental defects and were completely sterile, suggesting that F28F8.5, like MED28, is a vital gene implicated in development and tissue differentiation [D].

5. Discussion

In accord with my aims, my colleagues and I provided new evidence corroborating that the basic principles, proteins and their interactions in the gene expression regulation by NRs are conserved throughout the *metazoan* evolution. In our research, we concentrated on metabolic and developmental signalling by TR, RXR and Mediator.

To study RXR, we chose a distant *metazoan* with presumably little evolutionary divergence from a common ancestor with human, *T. adhaerens*. We showed that even in this simplest known animal, RXR is structurally and functionally conserved.

Concerning the study of Mediator, we observed that animal-specific subunits are well conserved throughout the kingdom. However, we came across a surprising irregularity in an important model organism, *C. elegans*. The Mediator subunit 28 in this nematode seemed functionally unrelated to the same subunit in other animals. This subunit is of key interest to our work for two reasons: (1) it is one of only two known subunits to regulate gene expression negatively; and (2) it counters tissue differentiation and is implicated in various cancers. Therefore, we investigated why the W01A8.1 gene annotated as *mdt-28* was unrelated to MED28 and found that in fact, this was an alignment error. We provided

evidence that W01A8.1 is actually an orthologue of a Perilipin and we identified another gene F28F8.5 as the correct MED28 orthologue. The corrected MDT-28 protein and the whole *C. elegans* Mediator complex is now considered functionally homologous.

Having proven homology of RXR and MDT-28 in these two organisms is important to our work not only because it permits studying NR pathways in simple and convenient model systems but also because it allows us to examine their structures to infer mechanistical information about NR and Mediator functioning.

Our work on RXR suggests that even at the base of *metazoan* evolution and in absence of a TR, NRs can regulate gene expression negatively. This underlines the importance of my second goal: to better characterise the cryptic negative regulation by NRs on the example of TRs.

Analysis of the TRβ LBD structure in patients with the mutation T273R showed that impairment of AF2 function and coactivator binding by TRβ results in deficient positive *and* negative regulation upon T_3 binding. At the same time, corepressor binding remained unchanged, possibly explaining the milder phenotype and absence of hyperthyroidism in these patients, compared to that in patients with mutations impairing T_3 binding.

This supports the proposed model of negative regulation, where gene expression is activated by corepressors and supressed by coactivators. As the principal coactivator, Mediator is thus likely to exert this repression on negatively regulated genes. Only two Mediator subunits are known to supress gene expression: MED3 and MED28. Having shown the functional conservation of *C. elegans* MDT-28, it seems that all animals regulating transcription

by NRs also have conserved this subunit, suggesting its importance.

What also remains unclear is how do NRs determine which genes are to be regulated negatively. This information can only be encoded in the DNA sequence, suggesting existence of distinct negative thyroid hormone response elements (nTREs). This is partially accepted although their sequences are unclear [12], [13]. Seeing as the specificity of NR binding to DNA is encoded in the position of the half-sites, the nTREs are read by RXR/TR dimers, too. Our study of RXR indicates that it might cooperate with its binding partners, in this case with TRβ, to reverse the action of coregulators. Structural analysis of TaRXR and human RXRs reveals that in contrast to other NRs with dedicated corepressor binding subdomains, all RXRs likely bind both coactivators and corepressors by AF2. This provides a filter for exclusivity of coactivator or corepressor binding by the dimer and has interesting mechanistic implications. RXR AF2 can bind both coactivator motif (LxxLL) and with lower affinity the corepressor motif (RID).

All these indirect indications allow to hypothesise one possible mechanistic model for the differential regulation on positively and negatively regulated genes (Figure 2). Importantly, the negative gene expression regulation by MED28 has been connected with dedifferentiation and carcinogenesis[14], [15]. Therefore, if this model is indeed valid, it would be of great interest in molecular and clinical oncology. More generally, all of the studied genes possess oncogenic potential and will certainly be the object of further studies.

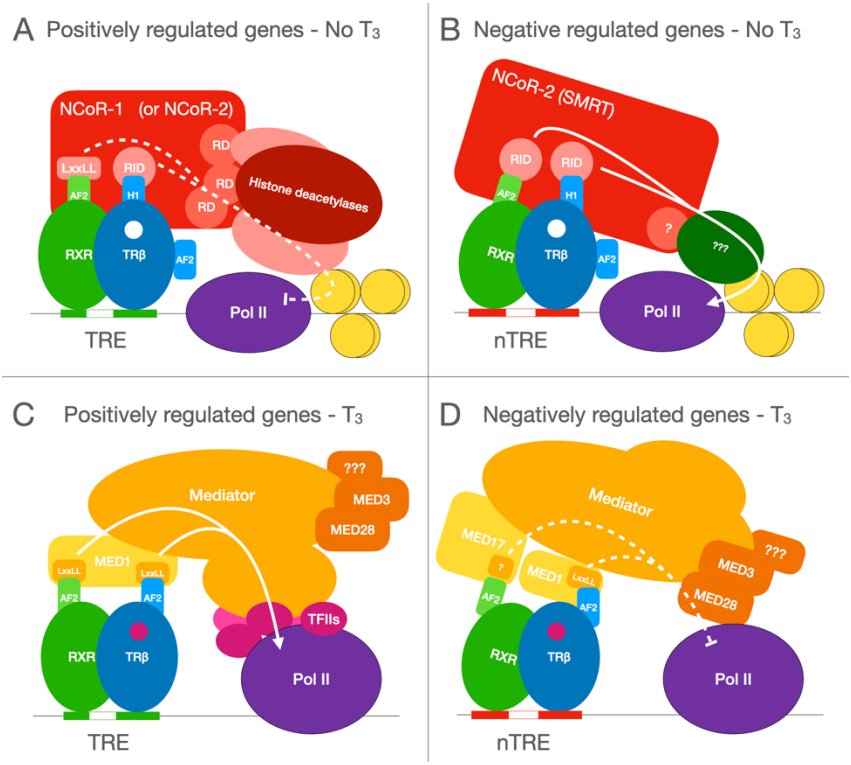

Figure 2. *A hypothetic model of transcription regulation on positive and negative TREs. Differential nTRE organisation causes alteration in RXR/TRβ heterodimer conformation. In absence of the ligand, NCoRs are bound to RXR/TRβ dimers, regardless of the signalling direction, but differently.* (A) *Positive TRE-bound RXR/TRβ can interact with NCoR1 via one RID and one LxxLL motif that would be preferentially bound by RXRs.* (B) *On negatively regulated genes, NCoR2 (SMRT) might be bound differently, e.g. via two RIDs, changing the conformation of NCoR2 and reverting the effect of NCoR2 on chromatin modifying complexes.* (C) *When T3 is bound to TRβ, NCoRs dissociate from its helix 1 (H1) and the AF2 subdomain changes conformation, actively binding the LxxLL motif on coactivators. It binds MED1, activating Mediator and therefore gene expression.* (D) *On negative TREs, the different conformation of the RXR/TRβ dimer might cause differential binding of the Mediator complex, e.g. by the MED17 subunit, which in turn activates MED28 and/or MED3 which are known to mediate transcriptional silencing.*

6. Conclusions

Regulation of gene expression in response to intercellular signals is indispensable for multicellular life. Regulation of transcription by NRs and their coregulators, especially the Mediator, is one of the most important and best evolutionary conserved regulatory pathways in *metazoa*. In this dissertation thesis, I describe the efforts of my colleagues and myself in elucidating certain aspects of this regulation.

I set out to assess and ascertain the evolutionary conservation of these mechanisms, to provide new evidence about the cryptic negative regulation by NRs and to establish possibilities for future research in this area.

In the process, we described a functional orthologue of RXR in the simplest known animal, *Trichoplax adhaerens*. We corrected a miss-annotation of a MED28 orthologue in *C. elegans*, while discovering the correct functionally conserved orthologue of MED28, as well as of Perilipin. And finally, we described a new mutation in the *THRB* gene in patients with RTHβ.

These findings allowed us to conclude that the principles of NR/Mediator-based gene expression regulation are homologous throughout *metazoa*, to elucidate the mechanisms of negative gene expression regulation and to hypothesise a working mechanistic model of both positive and negative regulations.

We introduced new model organisms to the field of NR research and we provided seminal concepts, which can further advance studies in molecular oncology.

7. List of my publications

In relation to this dissertation:

[A] **Kaššák F**, Chughtai AA, Kaššák S, Kostrouchová M. *Caenorhabditis elegans* perilipin is implicated in cold-induced lipolysis and inhibits autophagy in early embryos**.** *Folia Biologica.* 2020;66(5–6):179–85.; IF 0.709 (2021).

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Without relation to this dissertation:

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