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Contents

Abbreviations

Abbreviation	Meaning
aa	amino acid
ARID1A	AT-rich interactive domain-containing protein 1A
ARs	androgen receptors
C. elegans	Caenorhabditis elegans
CAR	constitutive androstane receptor
CBP	cAMP response element binding protein
CNS	central nervous systém
COUP	chickem ovalbumin upstream promoter
CREB	cAMP response element binding
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DBD	DNA binding domain
ERs	estrogen receptors
FXR	farnesoid X receptor
GRIP1	glucocorticoid Receptor-interacting protein 1
GRs	glucocorticoid receptors
HAT	histone acetyl-transferase aktivity
HNF-4	hepatocyte nuclear factor 4
hTLX	human TLX protein is an orphan nuclear receptor that is expressed in vertebrate forebrains
LBD	ligand binding domain
LXR	liver X receptor
MCM2	minichromosome maintanace protein 2
MRs	mineralocorticoid receptors
NCOA	nuclear Receptor Co-activator
NCoR	nuclear receptor co-repressors
NHR	nuclear hormone receptor
NHRs	nuclear hormone receptors
NR	nuclear receptor
NRs	nuclear receptors
NSCs	neural stem cells
PBS	phosphate buffered saline
PCAF	p300/CBP-associated factor
PCR	polymerase chain reaction
PNR	photoreceptor-specific nuclear receptor
PPAR	peroxisome proliferator-activated receptor
PRs	progesteron receptors

Abbreviation	Meaning
PXR	pregnane X receptor
RAR	retinoic acid receptor
RAR	retinoic acid receptor
RORα	RAR-related ophan receptor-α
RORβ	RAR-related ophan receptor-β
RXR	retinoid X receptor
S. mediterranea	*Schmidtea mediterranea*
SF1	steroidogenic factor 1
SMARC	SWI/SNF-related, Matrix-associated, Actin-dependent Regulator Chromatin
Smed-tlx-1	*S. mediterranea* homologue of nematode, insect and vertebrate genes *NHR-67, tailess and Tlx*, respectively- gene and/or transcript
SMED-TLX-1	*S. mediterranea* homologue of nematode, insect and vertebrate genes *NHR-67, tailess and Tlx*, respectively - protein
SMRT	silencing mediator of retinoid and thyroid hormone receptor
SRC	steroid receptor co-activators
SWI/SNF	SWItch/Sucrose NonFermentable
T3	triiodothyronine
T4	thyroxine
TIF2	transcriptional mediator/intermediary factor 2
tll	tailess, insect homologue of the chordate *Tlx*
tlx	tailles homolog
TR	thyroid hormone receptor
TRAM-1	thyroid hormone receptor activator molecule 1
UTR	untranslated region
VDR	vitamin D receptor

Abstrakt

Jaderné receptory zahrnují rozsáhlou rodinu transkripčních faktorů, které jsou silnými regulátory tkáňového metabolismu, homeostázy a vývoje tkáně živočišných druhů včetně člověka. Jsou zvláště zajímavé pro svoji schopnost reagovat na vyvázání hormonů, metabolitů, xenobiotik či uměle vytvořených molekul a převést interakci s těmito malými lipofilními molekulami do specifického regulačního signálu.

Při hledání jaderných receptorů, jejichž úloha by mohla být kritická pro nervovou tkáň u bezobratlých a zachovalá během vývoje živočichů, jsme identifikovali blízký homolog TLX obratlovců u ploštěnky *Schmidtea mediterranea*. Ploštěnky představují velmi slibný biologický model pro studium tkáňové homeostázy a regenerace. Ploštěnky jsou schopny vstřebávat vlastní tkáně a použít je jako zdroj energie během hladovění, a pomocí neoblastů znovu vytvořit celé svoje tělo při dostatku potravy. Informatická analýza veřejně přístupných dat sekvenovacího projektu *Schmidtea mediterranea* ukázala, že genom planarií obsahuje minimálně jeden gen s vysokým stupněm podobnosti s genem *tlx* obratlovců. Klonovali jsme kompletní CDS (coding DNA sequence of cDNA) a charakterizovali jsme gen funkčně. Ukázali jsme, že TLX (NR2E1) vykazuje u ploštěnky a obratlovců vysokou podobnost v jejich celé délce kódující sekvenci a odvozeného proteinu. Zjistili jsme, že TLX u ploštěnky, který jsme nazvali *Smed-tlx-1*, je exprimován v hlavičce, jakož i v kaudální části těla. Exprese *Smed-tlx-1* v hlavičce je přitom alespoň 10x vyšší ve srovnání s kaudální částí. Exprese *Smed-tlx-1* se po podání potravy zvyšuje přibližně dvakrát v obou těchto částech těla ploštěnky. Experimenty s RNA interferencí dále ukázaly, že *Smed-tlx-1* je kritický pro zachování stavby těla během cyklů hladovění/krmení a pro integritu oblasti mozku a očí ploštěnky.

V druhé části práce jsme se u glioblastomových buněčných linií zabývali expresí a distribucí TLX v buňkách. Zjistili jsme, že TLX se v buňkách nachází v několika formách proteinu, což svědčí o možných posttranslačních modifikacích TLX. V imunofluorescenční a kolokalizační studii jsme ukázali, že TLX je lokalizován do jádra i cytoplasmy a že jeho intracelulární distribuce může být regulována.

Naše výsledky ukazují, že funkce NR2E1 v homeostáze a vývoji nervové tkáně je evolučně zachovalá a že funkční mechanismus by mohl zahrnovat regulaci jeho intracelulární distribuce.

Klíčová slova

Astrocytom, buněčná reprodukce, diferenciace, homeostáza tkáně, imunofluorescence, NR2E1, *Schmidtea mediterranea*, TLX.

Abstract

Nuclear receptors constitute a large family of transcription factors that are powerful regulators of animal tissue metabolism, homeostasis, tissue maintenance and development. They are particularly attractive for their ability to respond to the binding of hormones, metabolites, xenobiotics and artificially prepared molecules and transmit the interaction with these small lipophylic molecules to specific regulatory potential.

In search for nuclear receptors that are likely to be critical for neural tissues in invertebrates and conserved during the evolution of animals, we have identified a close homologue of vertebrate TLX in a planarian *Schmidtea mediterranea*. Planaria represent very promising biological model systems for studies on tissue maintenance and regeneration. Planaria are able to resorb their tissues and use them as sources of energy during fasting and they re-build their bodies from neoblasts when food is plentiful.

Our search in *Schmidtea mediterranea's* publicly accessible genome sequencing data indicated that planarian genome contains at least one gene with a high degree of similarity to vertebrate TLX. We cloned full length CDS (coding DNA sequence of cDNA) and characterized the gene functionally. This showed that the planarian and vertebrate NR2E1 are highly similar in their entire coding sequence and the derived protein molecule. We found that the planarian TLX, that we name *Smed-tlx-1* is expressed in heads, as well as in tails of animals. *Smed-tlx-1* expression is at least 10times bigger in heads than in tails of animals and in both body parts increases approximately twice during the feeding phases. Inhibition of Smed-tlx-1 by RNA interference revealed that *Smed-tlx-1* is critical for sustaining the body plan during fasting – feeding cycles and for integrity of brain areas and eyes.

In the second part of the study, we studied the expression and intracellular distribution of TLX in glioblastoma cell lines. We have found that TLX is detected in multiple protein forms suggesting that they may be posttranslationally modified. Using immunofluorescence and collocalization studies, we show that TLX is localized in the nuclei as well as in the cytoplasm and we have found indications that TLX intracellular distribution may be regulated.

The results indicate that NR2E1 function in regulation of maintenance and development of neural tissues is evolutionarily conserved and its mechanism of function may include its regulated intracellular distribution.

Key words

Astrocytoma, cell reproduction, differentiation, immunofluorescence, NR2E1, *Schmidtea mediterranea*, tissue maintenance, TLX.

Synopsis

The work that constitutes this thesis is a part of the effort to contribute to the elucidation of mechanisms by which NRs regulate metabolism, tissue homeostasis and development. The work presented in this thesis represents an attempt to identify conserved mechanisms that may be critical for regeneration and maintenance of neural tissues. These mechanisms are likely to be important not only at normal or optimal conditions of the organisms' development and function but also at pathological conditions involving wound healing, neurodegenerative diseases, and tumors.

Nuclear receptors constitute a large family of transcription factors including receptors that regulate their target genes in response to binding of specific ligands, hormones, metabolites and xenobiotics. They are expressed in a large variety of cell types at various levels and constitute a regulatory network including nuclear receptors, transcription cofactors, ligands and interact with other regulatory pathways including transduction signaling pathways and metabolic pathways.

In search for NRs that are likely to be critical for neural tissues, we have chosen planaria for our research. Planaria have been used as classical biological systems from the earliest years of systematic biological research. Contemporary development of genetic and genomic tools brings this model again into the focus of modern biology and makes it especially attractive for studies on regeneration and tissue maintenance. The genome sequencing project that is currently underway on *Schmidtea mediterranea* is providing enormous wealth of data that may speed up the research on this organism.

Our search for NRs in *Schmidtea mediterranea* indicated that their genome contains close homologues to most vertebrate NRs. However, many receptors seem to be divergent in their structure and function. With respect to neural tissue, the predicted closest homologue of vertebrate TLX seemed to be especially well conserved in a part of its predicted sequence but diverged at both N and C termini of the predicted derived proteins. This would suggest a diverged functionality of *S. mediterranea* TLX.

The structure of the DNA binding domain of NR2E subclass of NRs differs from all other NRs by including additional amino acids. This is not an exception in the evolution of Metazoan species. For example, *Platyhelminthes* have NRs similar to thyroid hormone receptors that have two DNA binding domains in their molecule, a situation that is not found in vertebrates. The sequence of NR2E NRs in *C. elegans* and in *Drosophila* seems to be divergent from the vertebrate counterparts and differs also in the overall size of the molecule. On the other hand, the DNA binding domain of NR2E NRs seems to be conserved between various Metazoan phyla indicating that this receptor evolved before these phyla separated during Evolution.

To learn more about the TLX in *S. mediterranea*, we decided to clone its mRNA and characterize it functionally. This led to finding that *S. mediterranea* has a surprisingly close homologue to vertebrate TLX. Expression analysis indicated that *Smed-tlx-1* is expressed at both ends of the planarian body, in heads and in tails, although about 10 times bigger expression was detected in heads of animals that contain primitive brains, compared to tails.

Planaria have specialized cells, called neoblasts that have the potential of stem cells for all planarian cell types. These cells are able to reproduce and rebuild the complete planarian body plan from small fragments. They are also able to serve as the pool of progenitors for growing the planarian tissues. *Planaria* are able to use their bodies as energy sources in a regulated way during fasting and they re-grow from neoblasts at favorable conditions.

Our expression analysis showed that the expression of *Smed-tlx-1* is augmented during feeding periods compared to fasting. This indicated that Smed-TLX-1 may be more important for growing periods then during the animal regression. Inhibition of *Smed-tlx-1* by RNAi revealed its function during the growing period in feeding-fasting cycles and a role for development of neural tissues and eyes.

This part of the study indicated that the developmental role of NR2E is likely to be evolutionarily conserved.

In the second part of the study, we attempted to expand the knowledge of the NR2E function in the model of human glioblastoma cells.

TLX was shown to be a strong repressor required for maintenance of neural stem cells. On the other hand, the role of vertebrate TLX for activation of transcription of specific genes was also shown. We have chosen the model of human glioblastoma/astrocytoma cells as several lines of evidence also indicate that vertebrate TLX promotes the tumorigenic potential of glial cells, and that TLX is expressed in astrocytic tumors and glioblastoma cell lines. Its expression was also linked to worse prognosis of patients with malignant astrocytic tumors.

Astrocytes are formed from the common neural progenitors – neural stem cells representing a cell type with features known for mesenchyme. They have potential of multiplication and remodeling of brain tissue and wound healing. This feature gives them in their malignant variant an extreme malignant potential and a tendency of fast spreading.

Using Western blot analysis and two antibodies designed to recognize different domains of the protein, we have found that TLX is expressed in human astrocytic cell lines at various levels and may be posttranslationally modified.

Immunofluorescence analysis detected human TLX in nuclei as well as in the cytoplasm. Immunofluorescence and correlation to the staining of DNA by DAPI or the expression of minichromosome maintenance protein 2 (MCM2) revealed that the ratio between nuclear and cytoplasmic TLX differs between individual cells and during particular phases of the cell cycle. The cells of individual cell lines also showed a different predominant pattern of TLX staining. This indicated that TLX moves between the nucleus and the cytoplasm and its distribution may be regulated. TLX signal greatly increased in the extrachromosomal compartment during mitosis suggesting a possibility that TLX is efficiently synthesized during G2 and/or TLX molecules bound to chromatin are released from chromatin during chromosome compaction. Our results suggest that TLX function is further regulated by intracellular distribution.

Based on the work presented in this thesis, we conclude that:

NR2E1 function in regulation of neural tissues is evolutionarily conserved from planaria to man.

Smed-TLX-1 regulatory function is revealed in proliferative phases of tissue growth.

hTLX shows signs of translocations between the nucleus and the cytoplasm suggesting regulated intracellular distribution and the regulation of TLX on the protein level.

Introduction

Regulation network that controls cell fate and tissue maintenance including neuronal tissue is based on interaction of cell survival, renewal and differentiation stimuli (Reed 1999; Watt and Hogan 2000; Gage 2000; Pellettieri and Sanchez Alvarado 2007; Klein and Simons 2011) . Each of these steps is controlled by positive and negative stimuli that enable the organism to protect cells necessary for generation of new cells capable of sustaining the tissue integrity and directional programming of specific subpopulations of cells important for tissue functionality. Although the regulatory pathways of this process are known to a great detail, new regulatory molecules are being discovered by large scale studies.

Nuclear receptors (NRs), also termed nuclear hormone receptors (NHRs), are powerful regulators of animal development and metabolism. They are involved in regulation of specific cell functions as well as integration of developmental and metabolic processes at the level of organism. Their structure contains highly conserved DNA binding domain (DBD) that is coordinated by two zinc ions in a form consisting of two “zinc fingers” and a carboxy terminal ligand binding domain (LBD) composed of 12 helices. Several members of nuclear receptor (NR) family are hormonal receptors, such as steroid hormone and thyroid hormone receptors, retinoid receptors and Vitamin D receptor. A growing number of NR family members are recognized as receptors capable to bind small molecules that, dependent on the receptor-ligand binding affinity modulate the receptor transcriptional functions (Antebi 2006; Kininis and Kraus 2008; McEwan 2009).

Ligands with very big affinity that fulfill the criteria of hormonally active compounds, usually derived from metabolites or molecules obtained with food, execute regulatory functions at the local or tissue restricted level as well as at the level of entire organism (Jacobs and Lewis 2002) . Specific ligand binding properties of many NRs evolved during evolution.

The superfamily of NRs includes members that are highly conserved in distant animal phyla as well as receptors that apparently diversified (Escriva et al. 2000; Robinson-Rechavi et al. 2003; Escriva et al. 2004). Some NRs, like RARs related HR3 NRs seem to have similar

functions in insects and nematodes while other including the multiplied HNF4 related NRs in *C. elegans* seem to have acquired new functions (Antebi 2006).

Another class of NRs, RXRs are conserved between fungi, *Cnidaria* and vertebrates, but their orthologue is missing in many nematode species (Antebi 2006). In case of RXR, the ligand binding specificity, its DNA binding specificity and dimerization capabilities are conserved between *Cnidaria* and vertebrates (Kostrouch et al. 1998).

It can be speculated that the regulatory network governing the reprogramming of cells in various tissues include growth factors, cytokines and timing, and that the specification directing network is based on NRs and their ligands. This network is likely to be complex and include several coherently functioning factors. For its analysis, systems that are efficient and inexpensive are necessary. Such systems are represented by invertebrate model organisms that may allow visualization of conserved regulatory mechanisms that may be tested later on vertebrate models.

Rationale behind the dissertation

Nuclear receptors are important transcription factors that show both enormous variability and conserved features. While the variable features are likely to be phyla of genus specific, the conserved are likely to reflect core mechanisms that govern the very basic regulatory pathways of Metazoan cells (Sluder and Maina 2001; Enmark and Gustafsson 2001).

Combination of classical biology, genetics and genomics can put a new light on data that seem to be petrified. This is especially true for the rapidly growing genome sequencing projects that are revealing data accessible to the computer meta-analysis.

One such model is the model of *Schmidtea mediterranea*. *S. mediterranea* is a flatworm, platyhelminth. Many platyhelmints are dangerous parasites, difficult to control and causing suffering of animals including modern as well as ancient man (Eckert et al. 2000; Olson et al. 2001). But non-pathogenic flat worms of genus *S. mediterranea* becomes a very

powerful model organism for its capabilities to regenerate tissues and especially to re-grow entire organism from small fragments of body (Newmark and Sanchez Alvarado 2002; Salo 2006; Baguna 2012). This requires complex rearrangement of tissues and restoration of new body plan from multipotent or totipotent cells, the neoblasts (Sanchez Alvarado et al. 2002; Cebria et al. 2007; Wenemoser and Reddien 2010; Wagner et al. 2011). This capability of regression and re-growing is also taking place in adaptation of the animals to critical food restriction (Nimeth et al. 2004; Pellettieri et al. 2010; Gonzalez-Estevez et al. 2012).

These animals are able to sustain their existence under starvation (during which the number of cells and the animal size are decreasing) by utilization their own tissues as the energy supply while protecting the pluripotent cells that are able to support the complete re-growing of animals when the food supply is restored (Baguna and Romero 1981; Romero and Baguna 1991; Oviedo et al. 2003; Nimeth et al. 2004; Pellettieri et al. 2010; Fraguas et al. 2011; Gonzalez-Estevez et al. 2012). This process requires a complex regulatory network that sacrifices some cells while protects other (for detailed reviews concerning *S. mediterranea* see Newmark and Sanchez Alvarado 2002; Salo 2006; Pellettieri and Sanchez Alvarado 2007; Sanchez Alvarado 2007; Baguna 2012).

As shown in the first part of this dissertation, we were able to show that the nuclear receptor Smed-TLX-1, a homolog of TLX in vertebrates, plays the important role in a regeneration of *S. mediterranea*. This finding immediately turned our attention to vertebrates including humans. Namely, recent studies have indicated that brain stem cells in vertebrates depend critically on a transcription factor TLX, that belongs to the superfamily of nuclear hormone receptors (Shi et al. 2004; Qu and Shi 2009; Chavali et al. 2010). In the second part of this dissertation, we have therefore chosen the model of human glioblastoma (astrocytoma) cells as several lines of evidence also indicated that vertebrate TLX promotes the tumorigenic potential of glial cells, and that TLX is expressed in astrocytic tumors and glioblastoma cell lines (Liu et al. 2010; Park et al. 2010). Its expression was also linked to worse prognosis of patients with malignant astrocytic tumors (Park et al. 2010).

Nuclear receptors

Nuclear receptors are intracellular proteins that are activated by ligands (different molecules that also include lipophilic hormones), interact with DNA and this interaction leads to the modulation (usually activation) of transcription of respective genes (Giguere et al. 1986; Green and Chambon 1987). Nuclear receptors may be therefore taken as ligand binding transcription factors. However, a ligand does not exist or is not known so far in the case of many nuclear receptors (Giguere et al. 1988; Mangelsdorf and Evans 1995; Horard and Vanacker 2003). These receptors are known as orphan nuclear receptors. If the endogenous ligand of these receptors is found then these receptors are known as adopted orphan receptors (Chawla et al. 2001).

From the year 1999 a Unified nomenclature system for the nuclear receptor superfamily is available (Auwerx et al. 1999) which divides nuclear receptors into 7 subfamilies and is “open”, i.e. it anticipates other nuclear receptors to be discovered in the future (mainly orphan nuclear receptors). The system is based on the structural similarity between individual receptors from an evolutionary point of view. Although this is the recommended nomenclature system, older classifications, or their newer modifications, are still also used (Mangelsdorf et al. 1995; Chawla et al. 2001; Mahajan and Samuels 2005; Wierman 2007). These classifications evolved gradually as individual receptors were isolated. According to these classifications nuclear receptors are classified by different parameters such as the type of bound ligand, the location where the ligand binds the receptor (i.e. in the cytoplasm or in the nucleus), the mechanism by which the receptors bind DNA (i.e. as homodimers, heterodimers or monomers) or by the sequence with which they bind DNA. Presently mainly due to practical and didactic reasons, “dimeric” and the “ligand derived” classifications are the ones we encounter most frequently.

The “dimeric” classification is based on different mechanisms of dimerization of nuclear receptors and divides receptors into 4 classes. The first class includes nuclear receptors that dimerize as homodimers and their response elements (i.e. binding sites of NRs - discussed in detail later in the text) are arranged as inverted repeats. The second class includes receptors that heterodimerize with RXR and their response elements are mostly

arranged as direct repeats. The third class again includes receptors that dimerize as homodimers but their response elements are arranged mainly as direct repeats. The fourth class includes nuclear receptors that bind as monomers (Mangelsdorf et al. 1995).

The two most common variants of the "ligand derived" classification we nowadays encounter are dividing NRs either into 4 or 3 groups. The 4 group variant divides receptors into steroid receptor group, non-steroid or TR/RAR/VDR receptor group, adopted orphan receptor group and orphan receptor group (Chawla et al. 2001; Germain et al. 2006). The 3 group variant divides receptors into steroid receptor group, non-steroid (also called class II) receptor group and orphan receptor group (Kliewer et al. 1998; Mahajan and Samuels 2005; Wierman 2007). Even though these ligand based classifications are not universal, interestingly many representatives of the same group resemble each other also in additional above mentioned parameters such as dimerization or receptor localization.

As it is apparent from the previous paragraphs, for the proper orientation in the NRs field, knowledge of the old classifications, which are unfortunately not universal (especially in not taking fully in account the continually expanding group of non-steroid and orphan receptors), but are otherwise well understandable and easy to remember. The nomenclature system pointing out the evolutionary aspect of the classification meets the criteria of universality, but is much more complex. This classification with many tens of identified genes/gene products is using code name.

These 3 types of classifications of nuclear receptors, i.e. "dimeric", "ligand derived" and "evolutionary", will be discussed in detail in the following text. Indeed, one encounters in the literature different classifications used by various laboratories. Such informations are frequently confusing for the reader.

The structure of nuclear receptors

As soon as the first nuclear receptors were sequenced (Hollenberg et al. 1985; Conneely et al. 1986; Sap et al. 1986; Weinberger et al. 1986; Green et al. 1986; Krust et al. 1986; Koenig et al. 1987; Petkovich et al. 1987; Giguere et al. 1987; Arriza et al. 1987; Lubahn et al. 1988; Giguere et al. 1988; Baker et al. 1988; Trapman et al. 1988), it was obvious that their sequences in certain domains strongly overlap. The first two described domains that showed significant conservation were designated as the DNA binding domain and the ligand binding domain, also termed hormone-binding domain or steroid-binding domain (Hollenberg et al. 1985; Krust et al. 1986; Danielsen et al. 1986; Kumar et al. 1986; Kumar et al. 1987; Green and Chambon 1987). Especially the DNA binding domains of different receptors were found to be conserved which can be explained through their similar DNA binding sites (for many receptors basically identical or very similar). The ligand binding domains of different receptors are conserved only to some extent. Accordingly, different chemical characteristics of the binding ligands are encountered (Krust et al. 1986; Danielsen et al. 1986; Kumar et al. 1986; Kumar et al. 1987; Green and Chambon 1987; Evans 1988; Germain et al. 2006).

With the advancement of time other domains were described. Currently for the description of nuclear receptors the classification into 5 main domains is used (Krust et al. 1986; Kumar et al. 1986; Kumar et al. 1987; Danielsen et al. 1987; Evans 1988; Ruff et al. 2000; Germain et al. 2006): N-terminal domain, DBD, hinge domain, LBD, C-terminal domain which are schematically depicted in Figure 1.

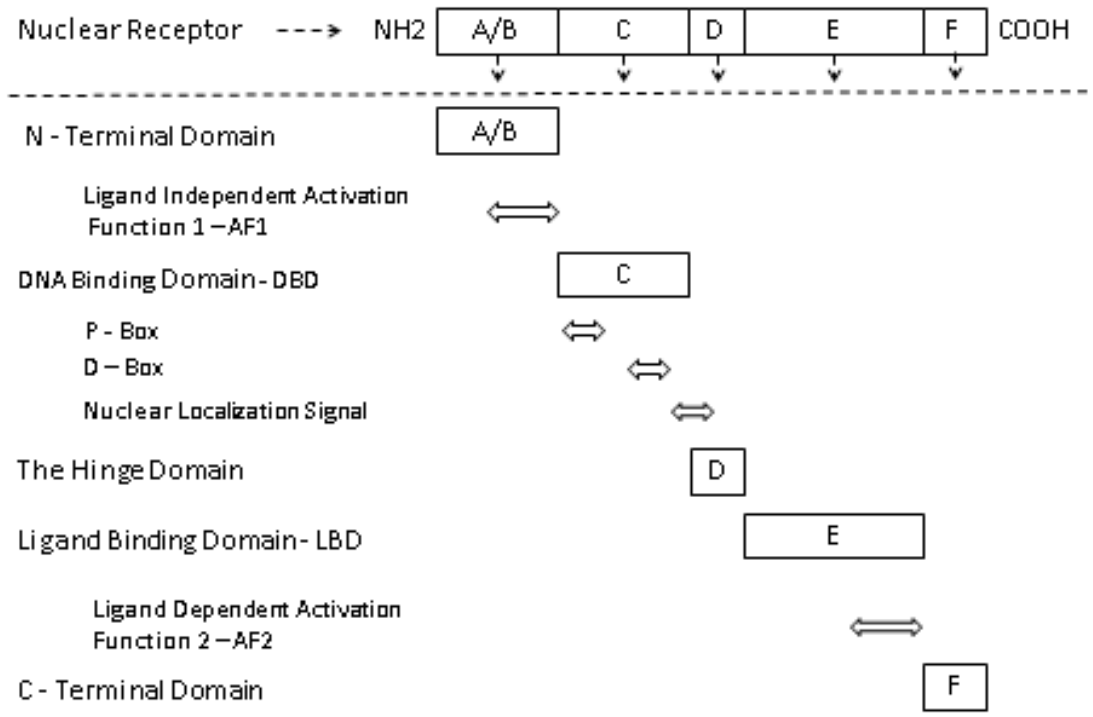


Figure 1. Scheme of the nuclear receptor domains.

N – terminal domain

The N- terminal domain, also called the A/B domain, is not well conserved (Evans 1988). The function of this domain is not entirely clear. In some receptors it appears to function as a hormone independent trans-activation domain, which is derived from the subregion of this domain, called activation function (AF1) (Ruff et al. 2000; Germain et al. 2006).

DNA binding domain

In contrast with the previous domain, this domain, also called C domain, is the one which is the most conserved. This domain includes sequence for 2 zinc fingers (Krust et al. 1986; Danielsen et al. 1986; Evans 1988; Green et al. 1988; Danielsen et al. 1989; Luisi et al. 1991). The main function of this domain is the recognition of specific DNA sites and binding to these sites and also the actual dimerization of the receptors.

The conservation of the DBD domain was the base for an accelerated search for new nuclear receptors and made possible the identification of receptors for which the ligand is so far not found, i.e. orphan receptors (Giguere et al. 1988; Evans 1988).

As it was said the structure of the DBD domain consists of 2 zinc fingers. This is very important, because these two structures are very well recognizable in the amino acid sequence, and thus also in its nucleotide sequence. Because we are looking for zinc fingers, the region has to be rich in cysteine molecules, which are ordered in a specific way, to allow four of them to bind to one Zinc cation (Hartshorne et al. 1986; Krust et al. 1986; Danielsen et al. 1986; Evans 1988; Luisi et al. 1991) as shown in the Figure 2.

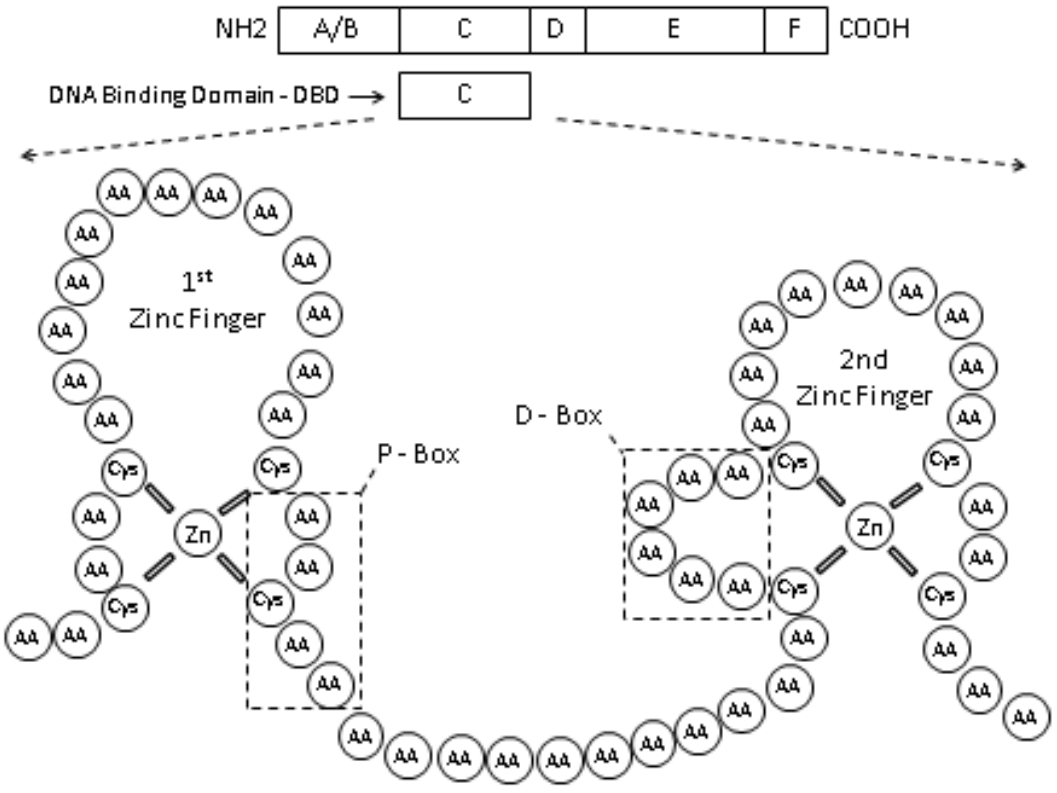


Figure 2. Schematic picture of the DBD region of a nuclear receptor.

Thanks to the experiments based on amino acid substitutions, 2 important subregions within both zinc fingers were recognized. The first subregion is called Proximal Box (P-Box) and comprises 5 amino acids which are located at the carboxy end of the first Zn

finger, the second subregion is called Distal Box (D – box) and is located between the first 2 cysteins of the second zinc finger. Both boxes are shown in Figure 2.

The main function of the P-Box is that its amino sequence determines the nucleotide sequence of the DNA that the receptor will bind (Umesono and Evans 1989; Mader et al. 1989; Ruff et al. 2000). For example a substitution of 2 amino acids, which are between the 2 cysteins at the carboxy end of the first zinc finger, changes the receptor from recognizing sequences of DNA typical for glucocorticoid receptor to sequences typical for estrogen receptor (Danielsen et al. 1989). Moreover, if we exchange glycine with glutamate at the first position of the D-box, the receptor will now recognize not only sequence of DNA typical for glucocorticoid receptor, but also for estrogen receptor (Umesono and Evans 1989).

The main function of the D-Box is in the dimerization of the nuclear receptors and also contributes to recognition of certain DNA sequences (Umesono and Evans 1989; Dahlman-Wright et al. 1993).

The hinge domain

This region, also called D domain (not to be confused with D-box), is a short not well conserved region between the DBD and LDB (Krust et al. 1986; Germain et al. 2006). Its name comes due to a fact that this region is very flexible and enables extensive bending of the protein in its 3D structure. In many receptors this region also includes a larger fraction of the nuclear localization signal (Zhou et al. 1994).

Ligand binding domain

LBD, also known as domain E, is the second most conserved domain within the nuclear receptors and typically consists of 12 α helices (H1 – H12) (Bourguet et al. 2000) (Renaud et al. 1995; Bourguet et al. 1995). However, it should be noted that some NRs in their LBD domains may miss several helices while the number of helices may be higher in other NRs (Giguere 1999; Ruff et al. 2000; Jin and Li 2010). This domain has many functions as ligand recognition, dimerization, ligand dependent activation function 2 (AF2), heat shock binding function and in some cases also repression function (Renaud et al. 1995; Wurtz et al. 1996).

In particular it is necessary to point out the importance of the helix H12 (Renaud et al. 1995; Brzozowski et al. 1997; Germain et al. 2006), which basically equals the AF2 domain (also termed AF2 helix), and has a unique feature. After the agonist is bound, this helix, which is originally flanking loosely on the surface of the nuclear receptor, is now, also thanks to the interplay of other helices, reverted inside forming together with other helices a special binding groove for the transcription co-activators (Brzozowski et al. 1997). This step is very crucial in the ligand dependent activation process and will be discussed in a bit more detail in the nuclear receptors co-activators section. On the contrary, when the antagonists bind to some of the nuclear receptors, helix H12 is also translocated, but so that it actually blocks the co-activator binding site through mimicking interaction with co-activator (Brzozowski et al. 1997; Shiau et al. 1998; Pike et al. 1999).

C – terminal domain

This domain, also called F domain, is a very short, not well conserved region, which is present only in some nuclear receptors. Although its function still remains unclear, some publications report its connection mainly with antagonist action (Nichols et al. 1998).

Binding sites of the nuclear receptors

Binding sites, also termed response elements, are target sequences in promoter region of different genes, which are searched and bound by the nuclear receptors. A typical response element consists of two six nucleotide half-sites which are separated usually by 1 to 5 nucleotides. The actual orientation, sequence and distance of the two half-sites are the 3 crucial features which define the type of the nuclear receptor that will bind (Koenig et al. 1987; Glass et al. 1988; Luisi et al. 1991; Mangelsdorf et al. 1991; Yu et al. 1991; Umesono et al. 1991; Mangelsdorf et al. 1995).

Orientation of the half-sites

In most cases the sequence of the first half-site repeats in the second half-site in three different ways, that is as direct repeat (the sequence is identical), as inverted repeat

(the sequence repeats as palindrome) and as everted repeat, i.e. the sequence repeats as inverted palindrome (Forman and Evans 1995). This corresponds to the fact that most of the nuclear receptors bind to response elements as dimers, i.e. each receptor is bound to one of the two half-sites and that their orientation depends on the mutual orientation of the repeats. The uncommon monomeric receptors bind only to single half-site, but to do so, typically there has to be a special short (2 to 3 nucleotides) upstream signal attached to the regular half-site sequence (Luisi et al. 1991; Mangelsdorf et al. 1991; Umesono et al. 1991; Mangelsdorf and Evans 1995; Mangelsdorf et al. 1995). Examples of different half-site orientation are shown in Figure 3.

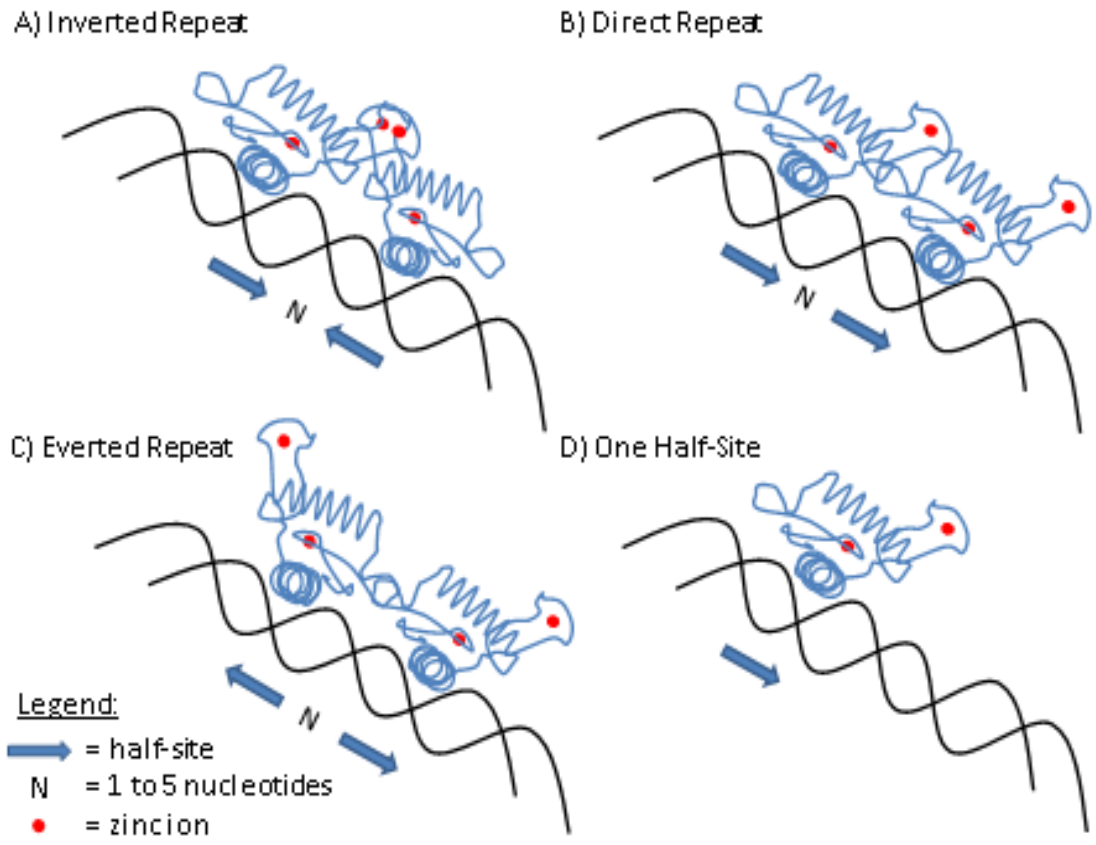


Figure 3. Different orientation of the half-sites of the DNA binding sites (only DBD domains are shown).

Sequence of the half-sites

There are basically two universal motifs of the half-sites, one is “AGAACA”, the other is “AGGTCA” (Evans 1988; Luisi et al. 1991; Ruff et al. 2000; Germain et al. 2006). While the

first motif is present exclusively almost only in response elements designed for steroid receptors (the exception are estrogen receptors), the other motif is present in response elements designed for all the other nuclear receptors and also estrogen receptors (Luisi et al. 1991; Mangelsdorf et al. 1991; Yu et al. 1991; Umesono et al. 1991; Mangelsdorf and Evans 1995; Mangelsdorf et al. 1995).

Distance of the two half-sites (1 to 5 rule)

Within the frame of the binding site, we still need to describe the "spacer code", which basically means the number of nucleotides present between the actual half-sites. This form of coding is particularly important in the non-steroid nuclear receptor group. Here the sequence and orientation of the half-sites are identical, so the only way how to differentiate between response elements of various nuclear receptors is the actual spacing between the half-sites. This code was originally deciphered by Evans laboratory (Umesono et al. 1991) and was termed 3-4-5 rule, as the spacing of 3 nucleotides coded vitamin D receptor response element, spacing of 4 nucleotides coded thyroid hormone response element and the spacing of 5 nucleotides coded retinoic acid receptor response element. As newer nuclear receptors were discovered, the rule expanded to 1-2-3-4-5 rule, or also 1 to 5 rule (Luisi et al. 1991; Zhao et al. 2000; Germain et al. 2006). Examples of the 1 to 5 rule for different spacers and corresponding NRs are shown in Figure 4.

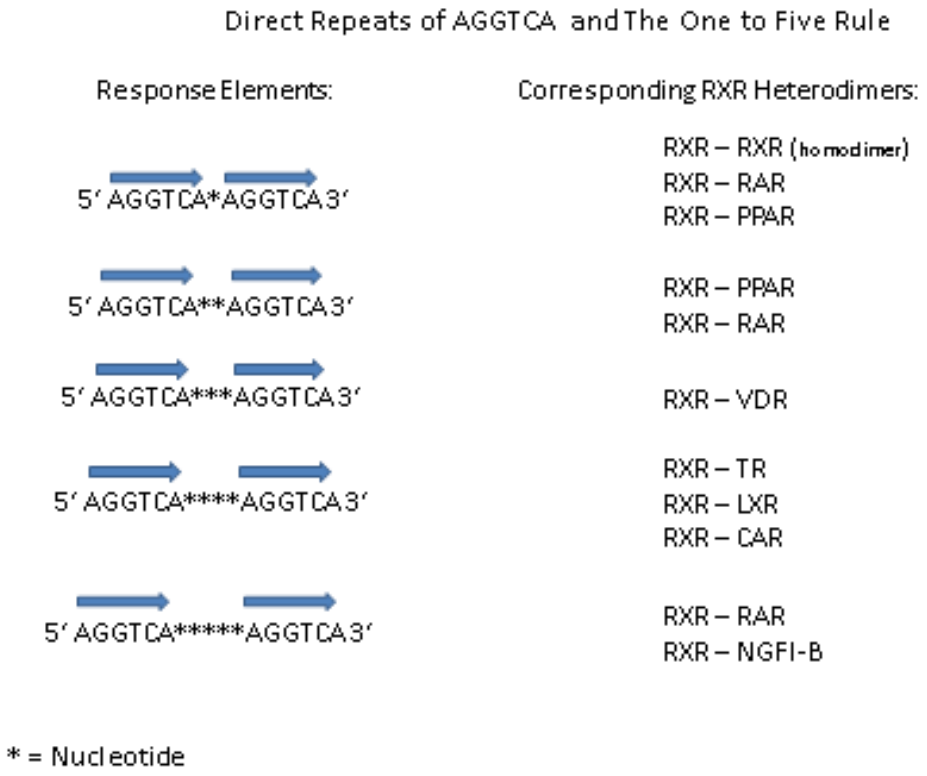


Figure 4. Examples of the 1 to 5 rule for different spacers and corresponding NRs.

"Dimeric" classification of the nuclear receptors

As mentioned earlier there are several ways how to divide the superfamily of Nuclear Receptors. First the "dimeric" classification is discussed. This classification, which was originally proposed by Mangelsdorf et al. 1995, divides receptors into 4 classes and is based on the pattern in which the receptors dimerize.

The downside of this original classification was that most of the receptors are able to exist in multiple dimerization states, i.e. as heterodimers, homodimers or even monomers, and thus can simultaneously fit into more than one of the discussed classes (Perlmann and Jansson 1995).

The original classification will be discussed first and then a more recent list of the nuclear receptors and their different dimerization capacities will be given in Table 1.

Class I nuclear receptors

This class, also called Steroid Receptor Class, encompasses the glucocorticoid receptors (GRs), estrogen receptors (ERs), progesterone receptors (PRs), androgen receptors (ARs) and mineralocorticoid receptors (MRs).

All these receptors appear to act as homodimers and are found in the cytosol in their ligand free state (Jensen et al. 1968; Jensen and Desombre 1972; Jensen and Desombre 1973; Funder 1993; Beato and Klug 2000; Germain et al. 2006). When the ligand is bound they undergo conformational change and are translocated into the nucleus and all bind to hexameric inverted repeats which are spaced with 3 nucleotides (Funder 1993; Ruff et al. 2000; Beato and Klug 2000; Germain et al. 2006).

Except ERs, all other steroid receptors recognize “AGAACA” hexameric half-site motif (Ruff et al. 2000; Germain et al. 2006). The ERs recognize “AGGTCA” half-site motif (Ruff et al. 2000; Germain et al. 2006). A schematic picture documenting class I nuclear receptors is given in Figure 5.

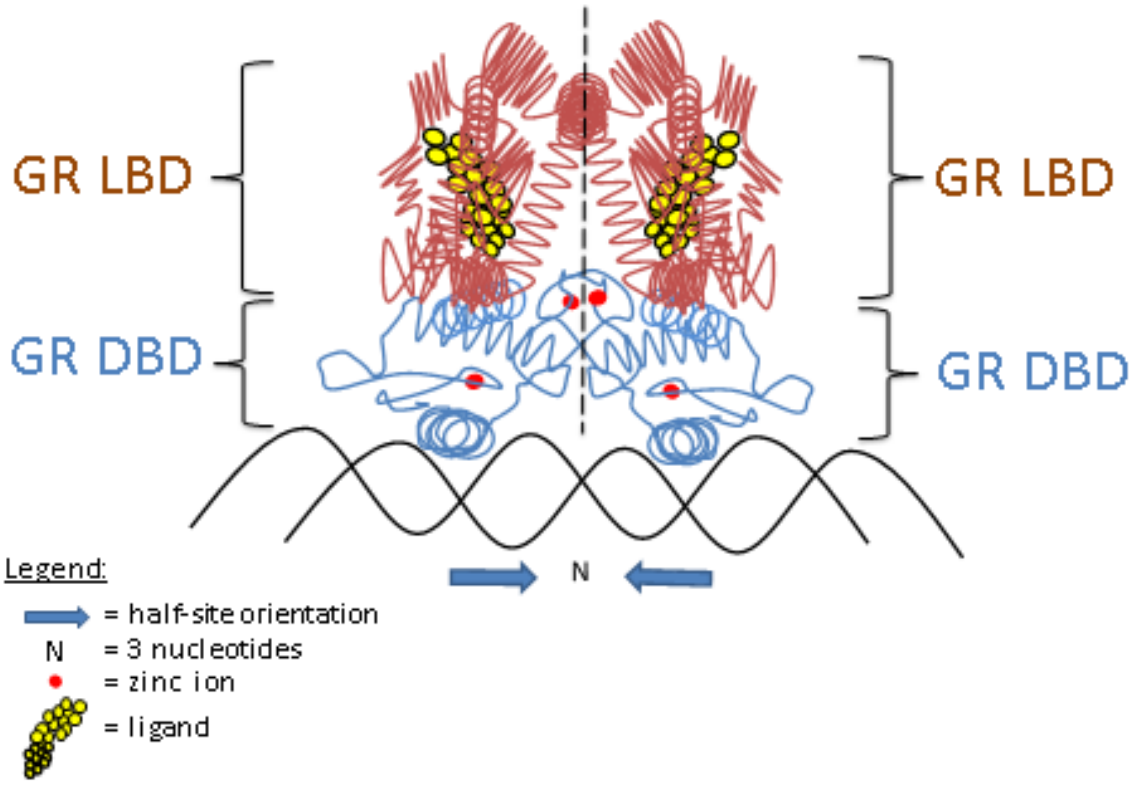


Figure 5. Class I nuclear receptors (homodimers and inverted repeats).

But how is the specificity of glucocorticoid, progesterone, androgen and mineralocorticoid hormone action solved if the response elements of these receptors are identical? Several levels of modulation come into consideration to possibly answer the fact that expression of some genes is under a strict control of one steroid hormone (Truss and Beato 1993). First level could be simply selective hormone degradation by tissue specific enzymes which is typically seen in mineralocorticoid targeted tissues, where this degradation selectively blocks the action of glucocorticoids in favor of mineralocorticoids (Funder et al. 1988). Other more complex levels can involve interplay of different co-activators and/or co-repressors, receptor phosphorylation, DNA methylation, histone modifications and all other possible epigenetic mechanisms (Truss and Beato 1993; Funder 1993; Xu et al. 1999).

Class II nuclear receptors

The main feature of the class II nuclear receptors is that they bind to DNA as heterodimers, in most cases retinoic X receptor (RXR) being the second dimerization partner (Kliewer et al. 1992; Mangelsdorf and Evans 1995). Therefore these receptors are also called as RXR Heterodimers. This class encompasses "classical" nuclear receptors as vitamin D receptor (VDR), thyroid hormone receptor (TR), retinoic acid receptor (RAR), "adopted orphan" receptors as constitutive androstane receptor (CAR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), farnesoid X receptor (FXR) and some still "orphan" receptors as nerve growth factor-induced clone B (NGFI-B) (Mangelsdorf and Evans 1995).

In contrast to the class I nuclear receptors, most of these receptors are located in the nucleus even in their free-ligand state commonly bound with co-repressors. Typically after the ligand binding, co-repressors are dissociated from the receptor while co-activators come into play (Kurokawa et al. 1995).

As discussed in more detail in the section about binding sites, the "AGGTCA" half-site motifs of these receptors are in most cases oriented as direct repeats (Umesono et al. 1991; Mangelsdorf and Evans 1995; Mangelsdorf et al. 1995). The binding site specificity of the receptors is then adjusted by the spacing of the half-sites, which follows the 1 to 5 rule.

Whether RXR occupies the 5' or 3' half-site in the heterodimer partnership depends on the other partner as well as on the spacer size, e. g. with VDR, TR and RAR (3, 4 and 5 spacer size, respectively) RXR occupies the 5' site (Kurokawa et al. 1993; Perlmann et al. 1993; Mangelsdorf and Evans 1995). Interestingly in case of RAR/RXR heterodimer with 1 nucleotide spacer, RXR occupies the 3' site (Kurokawa et al. 1994).

Note that the position and partner of RXR in the dimerization complex also determines the overall responsiveness of RXR to its ligand, i. e. whether to be responsive or silent (Kurokawa et al. 1994). Moreover RAR/RXR heterodimer with spacer 1 is unresponsive to ligands and acts as constitutive repressor (due to association with co-repressor) (Kurokawa et al. 1995).

A schematic picture documenting class II nuclear receptors is given in Figure 6.

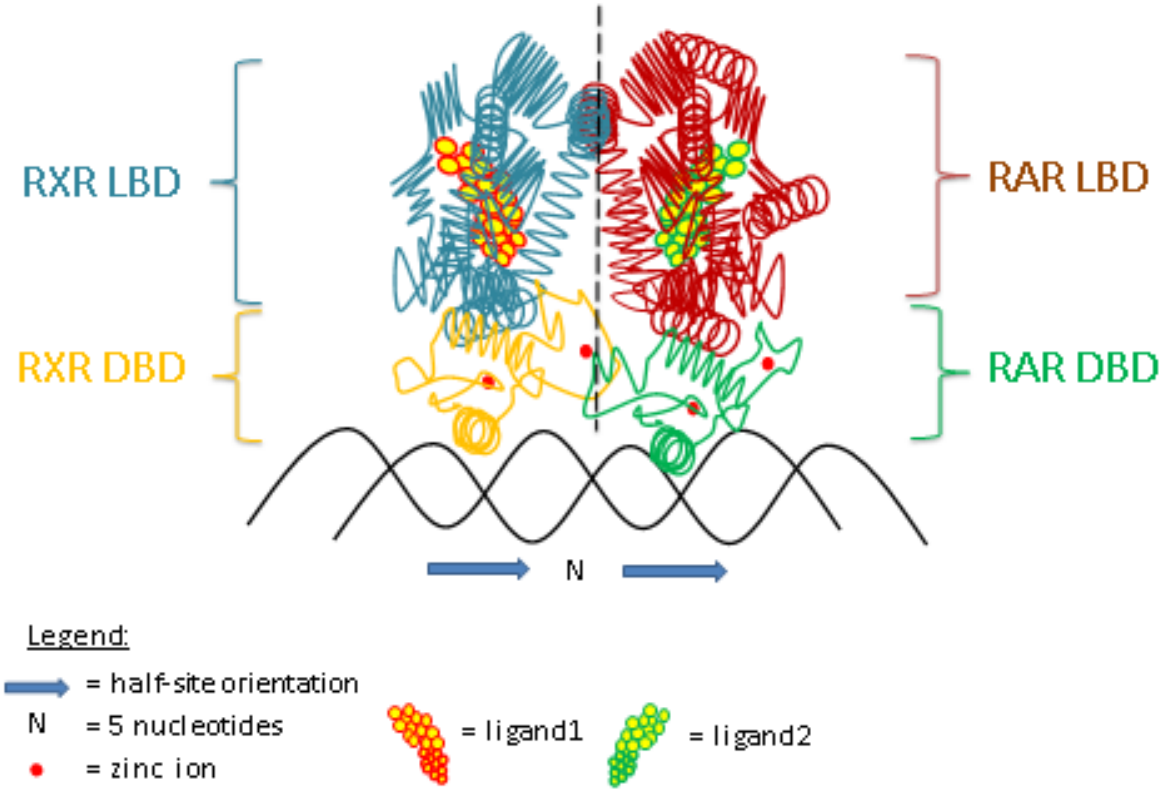


Figure 6. Schematic example of class II nuclear receptors.

Class III nuclear neceptors

This class, also called Homodimeric Orphan Receptor class, consists mainly of "adopted orphan" or still "orphan" receptors, which bind as homodimers to half-sites oriented most commonly as direct repeats (Mangelsdorf and Evans 1995). This class encompasses NRs such as RXR homodimers, chicken ovalbumin upstream promoter (COUP) homodimers, hepatocyte nuclear factor 4 (HNF4) homodimers, testicular receptors 2 (TR2) (Mangelsdorf and Evans 1995). A schematic picture documenting class III nuclear receptors is given in Figure 7.

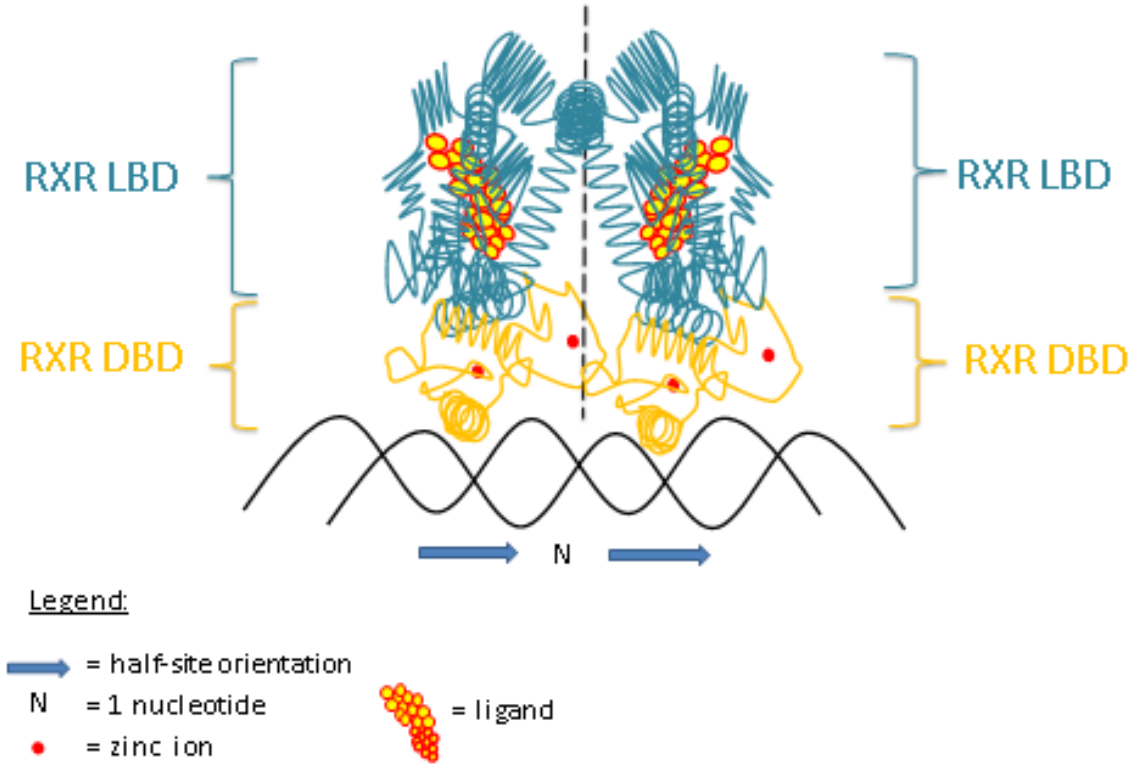


Figure 7. Schematic example of class III nuclear receptors.

Class IV nuclear receptors

This class, also called Monomeric Orphan Receptor Class, consists of "adopted orphan" or "orphan" receptors, that typically bind as monomers to single half-site which has to have a special short (2 to 3 nucleotides) upstream signal attached to the regular "AGGTCA" half-site motif (Wilson et al. 1993; Mangelsdorf and Evans 1995; Mangelsdorf et al. 1995). Thus this short nucleotide upstream signal specifies the nuclear receptors that will bind.

This class encompasses steroidogenic factor 1 (SF1), RAR-related orphan receptor-α (RORα), RAR-related orphan receptor-β (RORβ), homologue of the drosophila tailless gene (TLX) (Mangelsdorf and Evans 1995; Mangelsdorf et al. 1995). A schematic picture documenting class IV nuclear receptors is given in Figure 8.

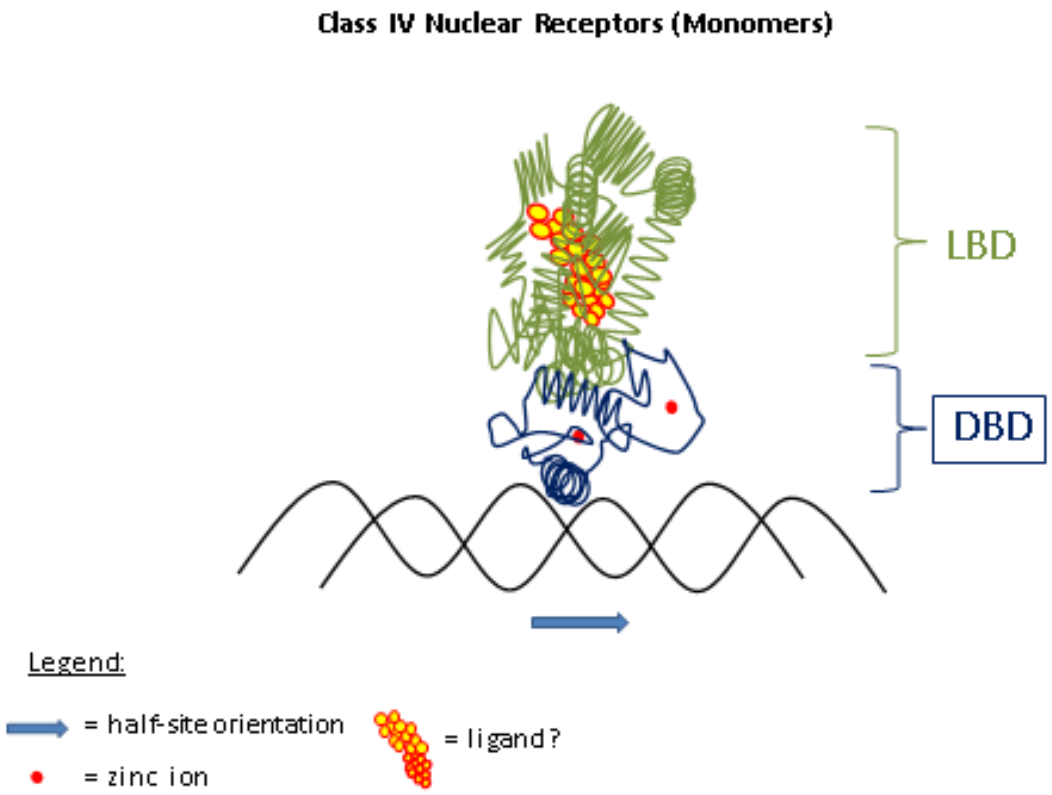


Figure 8. Schematic example of the Class IV nuclear receptors (monomers).

As already mentioned, many of the nuclear receptors can exist in multiple dimerization states and thus can be representatives of more than one of the dimerization classes. Table 1 shows a more recent list of the nuclear receptors together with their DNA binding abilities.

Table 1. List of the nuclear receptors together with their DNA binding abilities (Due to the size of this table, its continuation is shown in the following page).

Abbreviation	Name of the Nuclear Receptor	Monomeric	Homodimeric	Heterodimeric
AR	Androgen receptor	no	yes	no
CAR	Constitutive androstane receptor	yes	no	yes, with RXR
COUP-TF1	Chicken ovalbumin upstream promoter-transcription factor I	no	yes	yes, with COUP-TF2 and also with RXR
COUP-TF2	Chicken ovalbumin upstream promoter-transcription factor II	no	yes	yes, with COUP-TF1 and also with RXR
DAX1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1	no	yes	yes
EAR-2	V-erbA-related gene	no	yes	yes
ERRα	Estrogen-related receptor-α	yes	yes	no
ERRβ	Estrogen-related receptor-β	yes	yes	no
ERRγ	Estrogen-related receptor-γ	yes	yes	no
ERα	Estrogen receptor-α	no	yes	no
ERβ	Estrogen receptor-β	no	yes	no
FXR	Farnesoid X receptor	no	no	yes, with RXR
FXRβ	Farnesoid X receptor-β	no	no	yes, with RXR
GCNF	Germ cell nuclear factor	no	yes	no
GR	Glucocorticoid receptor	no	yes	yes, with RXR
HNF4α	Hepatocyte nuclear factor-4-α	no	yes	no
HNF4γ	Hepatocyte nuclear factor-4-γ	no	yes	no
LRH-1	Liver receptor homolog-1	yes	no	no
LXRα	Liver X receptor-α	no	no	yes, with RXR
LXRβ	Liver X receptor-β	no	no	yes, with RXR
MR	Mineralocorticoid receptor	no	yes	yes, with GR
NGFIB	Nerve Growth factor IB	yes	yes	yes, with RXR and also with NURR1 and NOR1
NOR1	Neuron-derived orphan receptor 1	yes	yes	yes, with RXR and also with NGFIB and NURR1
NURR1	Nuclear receptor related 1	yes	yes	yes, with RXR and also with NGFIB and NOR1
PNR	PNR - Photoreceptor-specific nuclear receptor	no	yes	no
PPARα	Peroxisome proliferator-activated receptor-α	no	no	yes, with RXR
PPAR-β/δ	Peroxisome proliferator-activated receptor-β/δ	no	no	yes, with RXR
PPARγ	Peroxisome proliferator-activated receptor-γ	no	no	yes, with RXR
PR	Progesterone receptor	no	yes	no
PXR	Pregnane X receptor	no	no	yes, with RXR

Abbreviation	Name of the Nuclear Receptor	Monomeric	Homodimeric	Heterodimeric
RARα	Retinoic acid receptor-α	no	no	yes, with RXR
RARβ	Retinoic acid receptor-β	no	no	yes, with RXR
RARγ	Retinoic acid receptor-γ	no	no	yes, with RXR
Rev-Erb-α	Rev-Erb-α	yes	yes	no
Rev-Erb-β	Rev-Erb-β	yes	yes	no
RORα	RAR-related orphan receptor-α	yes	yes	no
RORβ	RAR-related orphan receptor-β	yes	yes	no
RORγ	RAR-related orphan receptor-γ	no	yes	no
RXRα	Retinoid X receptor-α	no	yes	yes
RXRβ	Retinoid X receptor-β	no	yes	yes
RXRγ	Retinoid X receptor-γ	no	yes	yes
SF1	Steroidogenic factor 1	yes	no	no
SHP	Small heterodimer partner	no	no	no
TLX	TLX - tailless homologue	yes	yes	no
TR2	Testicular receptor 2	no	yes	yes, with TR4
TR4	Testicular receptor 4	yes	yes	yes, with TR2
TRα	Thyroid hormone receptor-α	yes	no	yes, with RXR
TRβ	Thyroid hormone receptor-β	no	yes	yes, with RXR
VDR	Vitamin D receptor	no	no	yes, with RXR

"Ligand derived" nuclear receptor classifications (2 variants)

Two commonly used examples of "ligand derived" classifications are given here. First classification divides nuclear receptors into 4 groups, second "simplified" classification divides receptors into 3 groups.

Four group "ligand derived" nuclear receptor classification

Although this classification has some minor flaws, it is used because of its simplicity and usefulness. This classification divides the nuclear receptor superfamily basically into 4 groups (there is also a simplified version of this classification, which will be discussed at the end of this subsection). The first group includes classical steroid hormone receptors, the second group includes classical non-steroid hormone receptors (also called TR/RAR/VDR group), the third group includes the "adopted orphan nuclear receptors" and the fourth one includes the "orphan receptors" (Chawla et al. 2001; Germain et al. 2006). The shortcoming of this classification is mainly due to the orphan receptors group, because as time goes by,

new ligands are found for the originally unliganded orphan receptors, and thus these receptors should be continuously reclassified into the “adopted orphan” group.

1. *Steroid hormone nuclear receptor group*

This group equals the class I receptors from the “dimeric” classification. It encompasses all the classical steroid hormone nuclear receptors, i.e. GRs, ERs, PRs, ARs and MRs. All the ligands of this group are accepted as high affinity hormonal ligands.

2. *Non-steroid hormone or TR/RAR/VDR nuclear receptor group*

This group encompasses classical non-steroid hormone nuclear receptors, which are: VDR, TRα, TRβ, RARα, RARβ and RARγ (Chawla et al. 2001; Germain et al. 2006) . Although especially in the case of VDR, the term non-steroid is questionable, because vitamin D is actually produced from cholesterol, but on the other hand its final active structure does not preserve the four ring steroid structure, as in the case of the steroid hormone nuclear receptor group (Norman 2008). All the ligands of this group are also accepted as high affinity hormonal ligands.

3. *Adopted orphan nuclear receptor group*

The representatives of this group are: RXRα, RXRβ, RXRγ, CAR, PXR, PPARα, PPARγ, PPAR-β/δ, LXRα, LXRβ and FXR. All these nuclear receptors were originally regarded as orphan, but later on for most of them endogenous ligands were found, revealing their crucial function especially in metabolic regulation (Chawla et al. 2001). The ligands of this group are accepted mostly as low-affinity dietary ligands and are categorized as xenobiotics (CAR, PXR), fatty acids (PPAR), oxysterols (LXR) and bile acids (FXR). Thus the CARs and PXRs are tough as xenobiotic regulators, PPARs as fatty acid regulators, LXRs as cholesterol regulators and FXRs as bile acid regulators (Chawla et al. 2001).

4. *Orphan nuclear receptor group*

This group encompasses nuclear receptors which were first discovered only on the basis of sequence similarity with the already known classical steroid hormone receptors but their proper ligand was still not found or there are some candidates for the ligand already in nomination but these possible ligands were not yet generally accepted and thus their corresponding receptor was not yet reclassified. On the contrary, there are some receptors which are, mostly on the basis of structural analysis, believed to be truly unliganded and thus called "true orphans" (Chawla et al. 2001; Germain et al. 2006).

The representatives of the orphan nuclear receptor group are: Rev-Erb-α, Rev-Erb-β, HNF4α, HNF4γ, TR2, TR4, TLX, PNR, COUP-TF1, COUP-TF2, EAR-2, ERRα, ERRβ, ERRγ, GFIB, NURR1, NOR1, SF1, LRH-1, GCNF, DAX1, SHP, RORα, RORβ and RORγ (Chawla et al. 2001).

Although RORs are still most commonly classified as orphans, there is increasing evidence that all three receptors have their natural ligands. The most discussed possible ligands for RORα are melatonin and cholesterol and its derivatives, for RORβ retinoids, and for RORγ retinoids and hydroxycholesterols (Wiesenberg et al. 1995; Stehlin-Gaon et al. 2003; Lau et al. 2004; Jin et al. 2010).

Intensive search for new ligands continues and only time will tell how many of these nuclear receptors will remain truly orphan.

Three group "ligand derived" nuclear receptors classification

This simplified but often used version of the previous classification divides the nuclear receptors into 3 groups. The only difference is that this classification fuses group 2 and group 3 into one group. Actually the adopted orphan nuclear receptor group is inserted into the non-steroid nuclear receptor group (Kliewer et al. 1998; Mahajan and Samuels 2005; Wierman 2007).

1. *Steroid nuclear receptor group*

This group equals the group 1 from the dimeric classification and contains all the known classical nuclear hormone receptors: GRs, ERs, PRs, ARs and MRs.

2. *Non-steroid or class II nuclear receptor group*

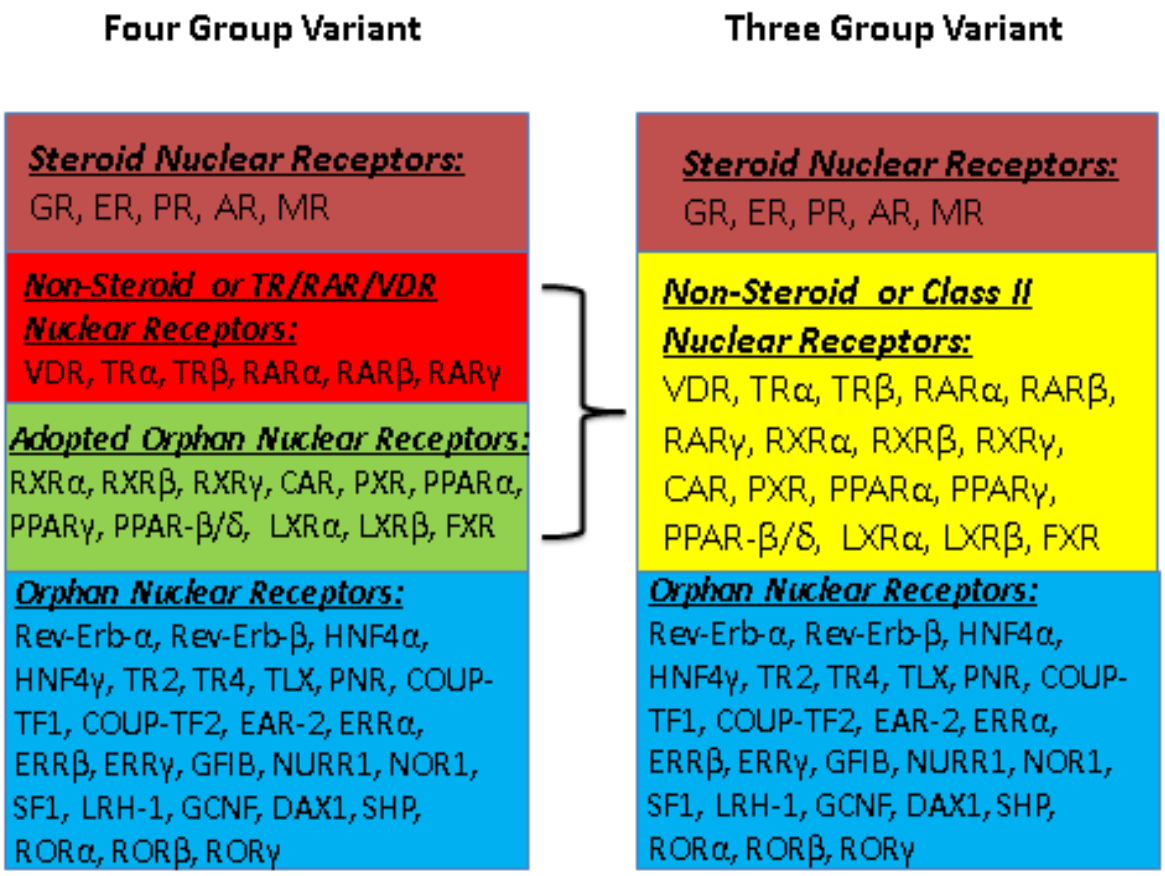
This is the fused group in comparison with the previous classification and contains all the classical non-steroid hormone receptors as well as all the adopted orphan nuclear receptors: VDR, TRα, TRβ, RARα, RARβ, RARγ, RXRα, RXRβ, RXRγ, CAR, PXR, PPARα, PPARγ, PPAR-β/δ, LXRα, LXRβ and FXR. As discussed earlier, the commonly used term "non-steroid" is in this case even more questionable, because now really some of the receptors, like LXRs and FXRs, have actually oxysterols and bile acids as their endogenous ligands.

3. *Orphan nuclear receptor group*

This group contains all the orphan receptors and also some NRs, as for example RORs, which have, with a serious portion of certainty, their ligands already identified but still there was not yet a general consensus to reclassify them.

This group equels the group 4 from the previous classification and contains these receptors: Rev-Erb-α, Rev-Erb-β, HNF4α, HNF4γ, TR2, TR4, TLX, PNR, COUP-TF1, COUP-TF2, EAR-2, ERRα, ERRβ, ERRγ, GFIB, NURR1, NOR1, SF1, LRH-1, GCNF, DAX1, SHP, RORα, RORβ and RORγ. Table 2 recapitulates the two, still often used "ligand derived" classifications.

Table 2. "Ligand Derived" Nuclear Receptor Classification, the two variants.



"Evolutionary" classification

A universal and flexible classification, which would easily categorize newly discovered nuclear receptors and which would not be dependent on the knowledge of the actual ligands nor DNA binding abilities of the nuclear receptors, was needed. Thus in 1999 a Unified Nomenclature System for the Nuclear Receptor Superfamily was proposed (Auwerx et al. 1999). This new nomenclature not only recategorizes the already known nuclear receptors but also proposes new, more universal coded names for them.

In contrast with the previous systems this system is based on the sequence homologies between different nuclear receptors in their two most conserved regions, i.e. the DBD and LBD regions, i.e. domains C and E (Auwerx et al. 1999). Thus any newly discovered nuclear receptor can be now immediately categorized and properly named, although its function and other characteristics may still be a mystery.

When the phylogenetic tree was constructed, in total 7 subfamilies emerged, although one of the subfamilies, the subfamily NR0, is artificial with no evolutionary sense and consists of incomplete receptors, i.e. those, which are missing either DBD or LBD regions. In contrast the nuclear receptors of the other 6 subfamilies, i. e. subfamily NR1 - NR6, have within each subfamily strong evolutionary relations (Auwerx et al. 1999).

Because the DBD region is the one most conserved, and this classification system is based firstly on the sequence similarities of this region, no wonder that certain associations between DNA binding properties and subfamily membership exist (Table 3). Needless to say that these subfamily associations are powerful tools used in the prediction approaches.

Table 3. New Classification proposed by The Nuclear Receptor Nomenclature Committee with the new official coded names. The older names and known ligands are also mentioned (due to the size of the table, it is shown in this page and next three pages).

Nuclear Receptor Subfamilies	Nuclear Receptor Groups	Present Name	Abbreviation of the Historic Name	Historic Name	Ligands
NR1. Thyroid hormone receptor-like	1A. Thyroid hormone receptors	NR1A1	TRα	Thyroid hormone receptor-α	T3, T4
		NR1A2	TRβ	Thyroid hormone receptor-β	T3, T4
	1B. Retinoic acid receptors	NR1B1	RARα	Retinoic acid receptor-α	all-trans-retinoic acid
		NR1B2	RARβ	Retinoic acid receptor-β	all-trans-retinoic acid
		NR1B3	RARγ	Retinoic acid receptor-γ	all-trans-retinoic acid
	1C. Peroxisome proliferator-activated receptor	NR1C1	PPARα	Peroxisome proliferator-activated receptor-α	fatty acids, eicosanoids as leukotriene B4
		NR1C2	PPAR-β/δ	Peroxisome proliferator-activated receptor-β/δ	fatty acids and eicosanoids

Nuclear Receptor Subfamilies	Nuclear Receptor Groups	Present Name	Abbreviation of the Historic Name	Historic Name	Ligands
		NR1C3	PPARγ	Peroxisome proliferator-activated receptor-γ	fatty acids, prostaglandin J2, eicosanoids, eicosapentaenoic acid
	1D. Rev-Erb receptors	NR1D1	Rev-Erb-α	Rev-Erb-α	orphan
		NR1D2	Rev-Erb-β	Rev-Erb-β	orphan
	1F. RAR-related orphan receptors	NR1F1	RORα	RAR-related orphan receptor-α	cholesterol,cholesterol sulphate, melatonin
		NR1F2	RORβ	RAR-related orphan receptor-β	all-trans-retinoic acid
		NR1F3	RORγ	RAR-related orphan receptor-γ	25-hydroxycholesterol
	1H. Liver X receptor-like receptors	NR1H2	LXRβ	Liver X receptor-β	oxysterols
		NR1H3	LXRα	Liver X receptor-α	oxysterols
		NR1H4	FXRα	Farnesoid X receptor-α	oxysterols
		NR1H5 (only pseudogene in humans)	FXRβ	Farnesoid X receptor-β	oxysterols, bile acids
	1I. Vitamin D receptor-like receptors	NR1I1	VDR	Vitamin D receptor	1,25-dihydroxyvitamin D3
		NR1I2	PXR	Pregnane X receptor	xenobiotics, steroids, benzoates
		NR1I3	CAR	Constitutive androstane receptor	xenobiotics,androstanes
NR2. Retinoid X receptor-like	2A. Hepatocyte nuclear factor-4 receptors	NR2A1	HNF4α	Hepatocyte nuclear factor-4-α	orphan
		NR2A2	HNF4γ	Hepatocyte nuclear factor-4-γ	orphan
	2B. Retinoid X receptors	NR2B1	RXRα	Retinoid X receptor-α	9-cis-retinoic acid

Nuclear Receptor Subfamilies	Nuclear Receptor Groups	Present Name	Abbreviation of the Historic Name	Historic Name	Ligands
		NR2B2	RXRβ	Retinoid X receptor-β	9-cis-retinoic acid
		NR2B3	RXRγ	Retinoid X receptor-γ	9-cis-retinoic acid
	2C. Testicular receptors	NR2C1	TR2	Testicular receptor 2	orphan
		NR2C2	TR4	Testicular receptor 4	orphan
	2E. Tailless-like receptors	NR2E1	TLX	TLX - tailless homologue	orphan
		NR2E3	PNR	PNR - Photoreceptor-specific nuclear receptor	orphan
	2F. COUP-TF-like receptors	NR2F1	COUP-TF1	Chicken ovalbumin upstream promoter-transcription factor I	orphan
		NR2F2	COUP-TF2	Chicken ovalbumin upstream promoter-transcription factor II	orphan
		NR2F6	EAR-2	V-erbA-related gene	orphan
NR3. Estrogen receptor-like	3A. Estrogen receptors	NR3A1	ERα	Estrogen receptor-α	17β-estradiol
		NR3A2	ERβ	Estrogen receptor-β	17β-estradiol
	3B. Estrogen-related receptors	NR3B1	ERRα	Estrogen-related receptor-α	orphan
		NR3B2	ERRβ	Estrogen-related receptor-β	orphan
		NR3B3	ERRγ	Estrogen-related receptor-γ	orphan
	3C. 3-Ketosteroid receptors	NR3C1	GR	Glucocorticoid receptor	aldosterone, corticosterone, cortisol, deoxycortisone

Nuclear Receptor Subfamilies	Nuclear Receptor Groups	Present Name	Abbreviation of the Historic Name	Historic Name	Ligands
		NR3C2	MR	Mineralocorticoid receptor	aldosterone, corticosterone, cortisol, progesterone
		NR3C3	PR	Progesterone receptor	progesterone
		NR3C4	AR	Androgen receptor	dihydrotestosterone, testosterone
NR4. Nerve growth factor IB-like	4A. Nerve growth factor IB-like receptors	NR4A1	NGFIB	Nerve Growth factor IB	orphan
		NR4A2	NURR1	Nuclear receptor related 1	orphan
		NR4A3	NOR1	Neuron-derived orphan receptor 1	orphan
NR5. Fushi tarazu F1-like	5A. Fushi tarazu F1-like receptors	NR5A1	SF1	Steroidogenic factor 1	orphan
		NR5A2	LRH-1	Liver receptor homolog-1	orphan
NR6. Germ cell nuclear factor	6A. Germ cell nuclear factor receptors	NR6A1	GCNF	Germ cell nuclear factor	orphan
NR0. DAX-like	0B. DAX-like receptors	NR0B1	DAX1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1	orphan
		NR0B2	SHP	Small heterodimer partner	orphan

Co-regulators of the nuclear receptors

Although finding response elements and binding on proper sites of DNA is a very important step for nuclear receptors in controlling transcription, as with other DNA binding transcription factors it is only the first step. The other steps which are inevitable for proper control of the transcription are dependent on the interaction between the AF-2 domain of the nuclear receptors and the constantly expanding group of transcription co-regulators,

consisting mostly of proteins and in some cases also RNAs (Xu et al. 2009) . These co-regulators are then divided into subgroups of co-activators and co-repressors. These co-activators or co-repressors then typically start enzymatic cascades which ultimately lead to chromatin modifications and general transcription machinery modulations which both are the very important steps of the target gene expression regulation (McKenna et al. 1999).

Note that despite the above mentioned co-regulator mediated nuclear receptor expression regulation there are some nuclear receptors, which can directly interfere with the general transcription factors (Schulman et al. 1995; McEwan and Gustafsson 1997).

Co-activators of the nuclear receptors

Out of the general transcription co-activator group, which is a huge and encompasses heterogeneous collection of proteins and RNAs, a considerable portion is also participating in the nuclear receptor regulated expression (Sterner and Berger 2000). The co-activators, which are recruited by the nuclear receptors, are commonly divided into 3 major categories.

First category consists of co-activators which are able to alter the transcription through modifying the chromatin structure with their ATPase and helicase activity (Ostlund Farrants et al. 1997; Tang et al. 2010). This category typically encompasses members of the SWItch/Sucrose NonFermentable (SWI/SNF) family, as AT-rich interactive domain-containing protein 1A (ARID1A) or „SWI/SNF-related, Matrix-associated, Actin-dependent Regulator Chromatin“(SMARC), subfamily A, number 1 (SMARCA1) also known as BRG1 (Collingwood et al. 1999).

Second category consists of co-activators which themselves or in association with other co-activators display histone acetyl-transferase aktivity (HAT) and thus through acetylation of lysine remnants on the histone N-terminal tails are able to regulate gene expression (Chan and La Thangue 2001). This class encompasses proteins as p300 (also known as EP300), cAMP response element binding (CREB) protein (CBP or CREBBP), P300/CBP-associated factor (PCAF) and a whole family of co-activators called p160/steroid receptor co-activators (SRC) family of proteins (also called p160 family), which includes enzymes as Nuclear Receptor Co-activator 1 (NCOA1), also known as SRC1 or F-SRC-1 (Onate

et al. 1995); as NCOA2 also known as Glucocorticoid Receptor-interacting protein 1 (GRIP1), or SRC2, or Transcriptional mediator/intermediary factor 2 (TIF2); and as NCOA3 also known as SRC3 or Thyroid Hormone Receptor Activator molecule 1 (TRAM-1) (Takeshita et al. 1997; Chen et al. 1999).

Third category represents a special co-activator unit, through which nuclear receptors, but also other numerous DNA binding transcription factors, are able to control transcription on the level of RNA polymerase II holoenzyme (Kim et al. 1994). This co-activator is called „Mediator complex", also known as thyroid hormone receptor-associated protein/vitamin D receptor-interaction protein (TRAP/DRIP) complex, and comprises of many various protein subunits, out of which some are being able to directly bind with RNA polymerase II. This is consistent with the fact, that Mediator complex is essential for successful transcription of nearly all class II genes (i. e. genes that code for proteins), because of its irreplaceable transcription associated function in recruiting general transcription factors and RNA polymerase II to the promoter region of the target gene, and also in stabilizing general transcription factors and RNA polymerase II during initiation and elongation processes (Kim et al. 1994). On the other hand, some subunits of the Mediator complex are able to bind with the activation domains (activation functions) of the DNA binding transcription factors (including nuclear receptors) and thus through these multiple interactions mediator complex serves as an important interface through which nuclear receptors can modulate different components of the transcription machinery (McKenna et al. 1999; Malik and Roeder 2000; Germain et al. 2006).

In summary, and in very simplistic manner, nuclear receptor co-activators can basically affect transcription by chromatin remodeling, histone modification and by interaction with the basal transcription machinery.

Co-activator interaction with nuclear receptors

As mentioned earlier, after the nuclear receptor is activated by ligand binding, AF2 helix (helix H12 of LBD domain) in association with other helices of the LBD domain is transposed from periphery more inwards where it also interacts with the bound ligand (McInerney et al. 1998; Nolte et al. 1998). With this conformational change, AF2 helix and

nearby helices (notably helix 3 and helix 4) prepare a special binding groove for the transcription co-activators. The interaction between activated nuclear receptor and co-activator is enabled due to interaction of this special co-activator binding groove with α helical amino acid LXXLL motif of the co-activator, where L stands for Leucine and X for any amino acid (Heery et al. 1997; McInerney et al. 1998; Nolte et al. 1998). This LXXLL motif, also called nuclear receptor box, is highly conserved and is characteristic for the nuclear receptor co-activator family. It is also not rare, that the amino acid sequence of many co-activators encompasses more copies of this motif; moreover search for this motif in amino acid sequences of different proteins discovered many new transcription co-activators (McInerney et al. 1998; Nolte et al. 1998).

Co-repressors of the nuclear receptors

Some nuclear receptors that are constitutively bound to DNA, as RAR and TR, are able - when unliganded - also repress the transcription (Kurokawa et al. 1995; McKenna et al. 1999). This repression is done through transcription co-repressors as nuclear receptor co-repressors (NCoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT). When RAR and TR receptors are unliganded, their LBD binds the above mentioned co-repressors which in turn typically bind transcription complexes that contain histone deacetylases (HDACs) and thus through histone deacetylation these co-repressors are able to counteract the effect of co-activators (Nagy et al. 1997; Xu et al. 1999).

Co-repressor interaction with nuclear receptor

Similarly as co-activators, co-repressors possess also an α helical LXXLL-like motif which is LXX H/I IXXX L/I (where L stands for Leucine, H stands for Histidine, I stands for Isoleucine and X stands for any amino acid) , also termed CoRNR box (Perissi et al. 1999; Cohen et al. 2001). Interestingly due to this motif, in some unliganded nuclear receptors, the repressors are able to bind to the same, but slightly transformed, groove of the LBD as co-activators, and thus start the transcription repression process. Then when the ligand is bind to the nuclear receptor, the conformational change of the groove releases co-repressor in favor of the co-activator (Perissi et al. 1999).

TLX (NR2E1) nuclear receptor

TLX belongs to the group of orphan nuclear receptors. It was first identified in the neuroepithelial embryonic tissue firstly in chicken, and immediately also in mice to confirm its universal presence in vertebrates (Yu et al. 1994). This protein showed strongest homology with the insect tailless (TLL) receptor in Drosophila melanogaster where the amino acid sequence comparison indicated 60% similarity in the DBD region and 41% similarity in LBD region. Comparison with other to that date known nuclear receptors, as EAR3/COUP, RXRalfa and RARalfa did not exceed 60% similarity for DBD and 41% similarity for LBD (Yu et al. 1994). Interestingly, *tlx* similarly as earlier cloned *tll* (Pignoni et al. 1990) differs from the rest of the nuclear receptors especially in the P and D boxes of the DBD domain. In both receptors, in P box normally present lysine is replaced by a different amino acid and in the D box, which in the rest of the nuclear receptor superfamily normally consists of 5 amino acids, are 2 amino acids extra, making a total of 7 amino acids per P box (Yu et al. 1994). Even more striking evidence of homology was the result of *in vivo* test, where ectopic expression of vertebrate TLX in fly embryos could perfectly mimic the function of Tll (Yu et al. 1994).

In developing mice the *tlx* gene is expressed in telencephalon and dorsal midbrain and also in the optic cups and nasal placodes (Monaghan et al. 1995). Even more convincing argument to think about TLX in connection with central nervous system (CNS) was, that although artificially *tlx* knock-out mice were able to develop and live, they displayed serious changes in behavior, i. e. increased aggressiveness for both genders although more serious for males (even killing their littermates), lack of maternal instincts in the female cases, and also seriously impaired spatial learning abilities (Monaghan et al. 1995; Roy et al. 2002). In correspondence with the behavior and memory deficits, anatomical studies of the mutant mouse reported decreased sizes of the rhinencephalic and limbic structures (Monaghan et al. 1997; Roy et al. 2002) and also reduction of neocortical thickness by 20% (Land and Monaghan 2003). Similarly same defects in mouse behavior and brain malformations were also observed in cases of spontaneous *tlx* mutations (Young et al. 2002). All these newer findings were more and more supporting the original prediction that TLX is very probably essential for proper proliferation or survival of certain subpopulations of neural progenitor cells in these affected regions of CNS (Monaghan et al. 1997).

But what were these subpopulations? The most important step to answer this question started by the identification of mammalian adult neural stem cells (NSCs) (Eriksson et al. 1998; Doetsch et al. 1999). This naturally led to a search for different regulation pathways which would keep these cells renew and keep their pluripotent potential. And finally laboratory of Shi pointed to TLX as to a very promising and crucial regulator of adult neural stem cells renewal and preservation (Shi et al. 2004). The reasons to point to TLX were several. First, thanks to β-galactosidase reporter artificially knocked into the *tlx* locus the expression of *tlx* could be visualized through LacZ staining. This pointed out locations in dentate gyrus and in subventricular zone which are also places where adult NSCs typically reside (Eriksson et al. 1998; Doetsch et al. 1999; Shi et al. 2004). Moreover colocalization of β-galactosidase and nestin, a marker of proliferation CNS progenitors indicated TLX expression in adult NSCs or progenitor cells (Shi et al. 2004). Next, TLX expressing cells isolated from the normal mice adult brains could proliferate, self-renew, have pluripotent neuronal potential and expressed nestin. In contrast *tlx* null cells from the mutant mice could not proliferate and expressed GFAP, an astrocyte marker (Shi et al. 2004). Moreover successful rescue of *tlx* null cells was done through infection with lentiviral vector expressing TLX. As the result, infected cells regained their ability to proliferate and express nestin again (Shi et al. 2004).

The laboratory of Shi convincingly proved by means of several different approaches that TLX directly represses expression of astrocyte specific genes and that possibly transcriptional repression is crucial in preserving the undifferentiated state of neural stem cells (Shi et al. 2004).

The role of TLX as primarily transcription repressor was in correspondence with older (Yu et al. 2000) and also subsequent studies which pointed out interaction of TLX with co-repressors such as atrophin1 (Atn1) (Zhang et al. 2006; Estruch et al. 2012), HDACs (Sun et al. 2007) and lysine-specific demethylase 1 (LSD1) (Yokoyama et al. 2008).

The original *tlx* expression studies (Yu et al. 1994; Monaghan et al. 1995) suggested that TLX has probably very important function also in the visual system. This was confirmed by Yu et al. (2000). The authors proved, while using *tlx* -/- mice, that TLX is a key component of retinal development and vision (Yu et al. 2000). TLX in mice is expressed in retinal

progenitor cells and is also present in two types of eye glial cells, Muller cells and astrocytes. TLX appeared to be critical for the maturation of these cells (Miyawaki et al. 2004). Moreover TLX has been shown to be required for proper coordination of retinal proliferation and differentiation to prevent retinal dystrophy (Zhang et al. 2006). Strikingly, experimental knock in of bacterial artificial chromosome carrying single copy of human *tlx* completely restored (evaluated by retinal histology and electroretinograms) the retinas of *tlx* null mice. Interestingly enough, no such effect was observed on other CNS deformities (Schmouth et al. 2012).

No wonder that this intimate relation between TLX and adult NSCs proliferation and renewal lead to speculations that TLX could play an important role in tumor development. And indeed, it was demonstrated that neural stem cell-specific overexpression of TLX in transgenic mice leads to neural stem cell expansion and glioma-like lesions in aged mouse brains (Liu et al. 2010). If this overexpression is also combined with p53 knock out, then these lesions typically progress to invasive gliomas. In addition, the expression study with human malignant astrocytomas was performed. Its results revealed that in 9 cases out of 41 primary glioblastomas the expression of TLX was increased (Liu et al. 2010). Another study reported increased mRNA TLX levels in 5 out of 7 human glioma cell lines and also increased TLX mRNA levels in 2 out of 6 glioma stem cells derived from patients with gliomas (Park et al. 2010). Moreover, the data analysis of 297 glioma patients taken from the Repository of Molecular Brain Neoplasia Data (REMBRANDT) database of the National Cancer Institute revealed correlation of increased TLX expression levels and poor survival prognosis suggesting TLX as a possible diagnostic marker (Park et al. 2010).

Specific Aims

Following the identification of partial sequence of NR2E1/TLX in *Schmidtea mediterranea*, we aimed at cloning full length cDNA and characterization of *S. mediterranea* NR2E1 functionally. Next we attempted to study NR2E1 in human astrocytic cell lines at the cell biology level to establish its expression pattern.

Specific hypotheses

NRs represent a very interesting group of transcription factors that can be influenced by small pharmacologically accessible compounds. NRs are typically expressed in large variety of cell types yet regulate very specific developmental and metabolic processes. Numerous examples document a potential of NRs to accept new roles during evolution of Metazoa. Some core mechanisms of function of NRs are conserved and can be identified in distant organisms. Conserved mechanisms are more likely to be functionally important than mechanisms that are genus, or phyla specific. Finding of pathways that are conserved for NRs may be of great importance.

NRs are transcription factors found only in Metazoa. They are not found in yeast. They are present in Cnidaria (jellyfish, corals and anemones), sponges and all animals higher in the evolutionary tree that were studied for the existence of NRs.

Since *S. mediterranea* genome is partially sequenced and accessible online, we decided to search for conserved NRs.

We hypothesized that some mechanisms of NRs may be conserved from Platyhelminthes to man, and accessible for a focused study.

In the following we provide the two studies entitled "Search for factors with regulatory potential on neuronal reprogramming and regeneration using an invertebrate model of *Schmidtea mediterranea*" and "Expression of TLX (NR2E1) in human astrocytoma cell lines." In order to simplify the matters, we shall use in the following text a shorter title for the first study, namely "Search for TLX (NR2E1) in *Schmidtea mediterranea*."

Materials and methods

Materials and methods of the part I: Search for TLX (NR2E1) in *Schmidtea mediterranea*

Animals and animal cultures

Asexual strain of *Schmidtea mediterranea* was kindly provided by Dr. Francesk Cebria. Animals were kept in tap water (supplied by Veolia in the district Prague 4 which source is the water supply facility Želivka). Tap water from this region proved to be more suitable for *S. mediterranea* than three bottled still water products including one declared as ideal for infants. Animals were fed in one week intervals with beef liver tissue that was stored in frozen aliquots, de-frozen and briefly washed of blood before use. Approximately 1 g of liver tissue was used per 100 animals in 200 ml to 500 ml Beaker flasks. The water was filtered before use (45 μm filters Corning, Corning, NY) and kept for up to 14 days in open glass bottles.

Beaker flasks were exchanged every two weeks. Animals were kept in partially shaded air-conditioned room at 20^{o} C and protected against direct light. For preparation of bigger quantities of animals, some animals were cut into several pieces and left to regenerate.

Total RNA preparation

Total RNA was prepared using two protocols. For cDNA preparation, the total RNA was prepared using proteinase K digestion, DNase treatment and RNA extraction as described (Brozova et al. 2006). Animals were anesthetized and killed by fast freezing on dry ice. For reverse transcription – quantitative PCR, the total RNA was prepared both using the proteinase K – DNAse- RNA extraction protocol as well as extraction using the Trizol (Invitrogen, Carlsbad, CA).

Reverse transcription

cDNA was prepared from 3 μg of total RNA using the SuperScriptII and/or SuperScriptIII kits from Invitrogen according to manufacturer's recommendations. For preparation of cDNA intended for cloning the complete coding sequence of *Smed-tlx-1*, both random hexamers as well as poly T primers were used. For quantitative RT-PCR only random hexamers were used.

Amplification

Amplification of complete coding sequence of *Smed-tlx-1* was done by nested PCR strategy. Design of primers is indicated in Fig. 1. Primers for amplification of predicted sequence available in SmedGD database (http://smedgd.neuro.utah.edu) (Sanchez Alvarado et al. 2002; Robb et al. 2008) were also used but did not yield an efficiently amplified fragment in nested PCR. We therefore employed the 3' RACE method from cDNA prepared using Transcriptor First Strand cDNA Synthesis Kit (Hoffman-La Roche, Basel, Switzerland). For amplification of 5' ends of cDNAs, we used splice leader sequences SL1 and SL2 (Zayas et al. 2005). SL1: CGGTCTTATCGAAATCTATATAAATCTTATATG and SL2: CGGTCTTATCGAAATCTATATAAAAATTATATG and primers designed according to a short segment that showed significant homology to most known TLX homologues.

The following primers were used for the cloning:

4-2010	ATGACAGTAACAAAGCAATCATTATTCAG
8-2010	GTACCTTGCAAGGTGTGTCAAGACCATTCA
9-2010	TTGCAAGGTGTGTCAAGACCATTCATCGG
13-2011	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
5 -2011	TGGATCATGTCAGCAGACTTTGTCAG
19-2011	CATCTCTCTCATTTTAGGCAATCA
20-2011	CATGATTGAATGATTTTGAGTACAG
21-2011	TCATTTTAGGCAATCATATCCGATA
22-2011	TTGAATGATTTTGAGTACAGGCCGA

Cloning and sequencing

Amplified fragments were visualized on agarose gels stained by ethidium bromide, isolated using electroelution and ligated using pCR2.1 kit (Invitrogen) and transformed to Top10 competent bacteria (Invitrogen). Miniprep DNA was isolated and plasmid DNA assayed for presence of inserts using DNA restriction and agarose gel electrophoresis. Candidate clones were sequenced in DNA sequencing facility of the Institute of Inherited Metabolic Disorders.

Quantitative PCR

Quantitative PCR was done using the Universal Probe Library technique (Hoffman-La Roche, Basel, Switzerland) and was performed on LightCycler 1.2 instrument equipped with the software LightCycler 4.1 (Hoffman-La Roche, Basel, Switzerland).

The conditions for amplifications were used as follows: Pre-denaturation cycle 95 ^{o}C for 10 min, 45 cycles consisting of steps of denaturation at 95 ^{o}C for 15s, annealing at 60^{o}C for 30s and elongation at 72^{o}C for 1s. Then the reactions were cooled at 37^{o}C for 15s. The fluorescence was detected at the end of each 60^{o}C step. All UPL probes are detected using 480-530 nm channel.

For Smed-tlx-1 primers # 7523 (tctgctcgatctctgtttaacact) and 7524 (agcaccgacacgatctcttt) and probe #47 were used. For normalization of results the Ura4 mRNA was used, which was amplified with primers #7561 (gcctgctcaaacgcagttat) and #7562 (atggtaaatgcggccttaaa) and probe #53 and #11.

Standard curves were prepared as described previously (Vohanka et al. 2010).

RNA interference

RNA interference was induced using the standard *C. elegans* protocol (Timmons et al. 2001; Liby et al. 2006). The sequence covering the complete DNA binding domain and 2/3 of the LBD (SacI – XhoI) was cloned into L4440 vector and transformed into HT115 bacteria, both kindly provided by Dr. Andrew Fire. Production of dsRNA was induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma, St. Luis, Mo) for 4 hr, bacteria were pelleted at 1500

xg at 4°C, kept refrigerated at 4°C and used for maximum of two weeks. For induction of RNA interference, bacteria were fed to animals in mixture containing one part of homogenized beef liver tissue prepared using glass homogenizer (10 ml). Liver homogenate was prepared using 10 ml glass homogenizer from 1 g of liver tissue supplemented with 500 µl deionized autoclaved water, homogenate was centrifuged in aliquots 1.5 ml in Eppendorf tubes at 10,000 xg for 7 minutes, clear supernatant discarded and upper portion of the pellet containing the small cellular fractions used for bacteria/DNA mixtures. Some cultures were fed using pelleted bacteria without the liver extract.

Control samples were prepared by similar way using the bacteria producing nonspecific dsRNA from empty L4440 vector.

In vivo imaging

Animals were viewed using Olympus SZX10 (Olympus Czech Group, Prague, Czech Republic) stereo microscope equipped with an Andor Clara CCD camera (Andor Technology, Belfast, Northern Ireland).

Modeling

Modeling together with informatics was performed using the following sites http://www.mat.univie.ac.at/~neum/protein.html, http://www.biochem.ucl.ac.uk/~shepherd/old-2001/bioinf-prot-pred.html, Clustalw website www.ebi.ac.uk/Tools/msa/**clustalw**2/. The three dimensional model was calculated using Modeller computer program http://salilab.org/modeller/ based on structures deposited to PubMed structural database http://www.ncbi.nlm.nih.gov/pubmed/.

Materials and methods of the part II: Expression of TLX (NR2E1) in human astrocytoma cell lines

Glioblastoma cell lines and cell cultures

The glioblastoma cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) with the Individual Material Transfer Agreement licensed to the Laboratory of Molecular Pathology, First Faculty of Medicine, Charles University in Prague. Cells were propagated as recommended by the supplier. Following initial propagation, cell were frozen in aliquots and secondary cultures used for experiments as cultures that did not overpass 20th passage.

The following cell lines were used for the study: A-172, LN-299, U-373, U-87 MG, U-138 MG.

The individual cell line characterization:

A-172: A cell line derived from 53 years old male patient diagnosed as glioblastoma. The cell line is characterized by an adherent type of cell culture extensively used for studies on apoptosis. This line has functional p53 pathway that can be restored by treatment with glycerol (Ohnishi et al. 2002). Additional information about this line can be found at http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=CRL-1620&Template=cellBiology.

LN-299: A cell line derived from a glioblastoma of 60 year old Caucasian female patient taken from the right frontal parieto-occipital cortex in 1979. The cells exhibit mutated p53 (TP53) and possible homozygous deletions in the p16 and p14ARF tumor suppressor genes. The oncogenic background of the cell line: p53 + (mutated, CCT (Pro) --> CTT (Leu) mutation at codon 98), PTEN + (wild type), p16 - (deleted), p14ARF - (deleted).

Additional information can be found at http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=CRL-2611&Template=cellBiology.

U-373: This line was obtained originally from ATCC (as HTB-17) and used in the laboratory for numerous studies. The authenticity of this cell line was questioned in the literature (Ishii et al. 1999) as similarities between U-373 MG and another glioblastoma, U-

251 were reported. The cell line U-373 MG, obtained from the original lab in Uppsala has differing genetic properties from the ATCC® HTB-17™ (denominated as U-373 MG). Following further investigations, ATCC stopped distribution of this cell line. Since U373 cell line was extensively used for numerous published studies, and the doubts concerning its origin, the line was used in the study and the denomination U373 is also used in this thesis. Additional information about this line can be found at http://www.hpacultures.org.uk/products/celllines/generalcell/detail.jsp?refId=08061901&collection=ecacc_gc.

U-87 MG: This cell line (also known as HTB-14) is derived from a glioblastoma of 44 years old Caucasian male patient. This is a hypodiploid cell line with the modal chromosome number of 44 occurring in 48% of cells. The rate of higher ploidy was 5.9%., Twelve markers were common to all cells, including der(1)t(1;3) (p22;q21), der(16)t(1;16) (p22;p12), del(9) (p13) and nine others. The marker der(1) had two copies in most cells. There was only one copy of normal X. N1, N6 and N9 were not found. Additional information about this line can be found at http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=HTB-14&Template=cellBiology.

U-138 MG: This cell line is derived from a glioblastoma of 47 years old Caucasian male patient. It is characterized as hyperdiploid to pentaploid with several markers; the stemline chromosome number is near triploid with the 2S component occurring at 9.8%., Five markers {t(11;5), t(8q;4), t(19;?18), M1 and M2} were common to most S metaphases. One chromosome 4 could be found in every S metaphase. Chromosome composition was very uniform among cells. This cell line is characteristic with very slow growth. Additional information about this line can be found at http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=HTB-16&Template=cellBiology.

The electronic sources linked to the cell line characteristics were accessed lastly on August 23, 2012.

Western blotting

Western blot analysis was performed as described previously (Pohludka et al. 2008). Cells were grown in 75 cm2 tissue culture flasks, washed by 3 x 10 ml PBS, scraped and harvested by centrifugation.

Immunofluorescence and image analysis

Cells were grown in 12-well tissue culture plates with sterilized round cover slips with a diameter 1 cm positioned at the bottom of culture wells prior the installation of cell suspension prepared from parent cultures similarly as for regular cell propagation.

Cells attached to the coverslips were washed three times by 1 ml of PBS, fixed in 4% fresh paraformaldehyde in PBS (pH 7.4) for 15 minutes at room temperature. Cultures selected for immunodetection of gamma tubulin were postfixed in cold methanol for 10 minutes at -20 C and acetone at -20 C for 1 minute. Cells were quickly transferred into cold PBS to avoid a complete drying. All cells were pretreated with 10 % fetal calf serum in PBS containing 0.1 % of Tween 20 (Sigma Aldrich, St. Louis, Mo, USA). In some experiments, cells were fixed in cold methanol (+ 4 C) for 5 min without preceding formaldehyde fixation.

Immunodection was performed on drops of 50 μl of diluted primary or secondary antibodies diluted in PBS (diluted 1:100 and 1:1000, respectively). The incubation was done in wet chambers on prafilm stripes at room temperature. Following the incubation, cells were washed 4 x 3 minutes on 500 μl drops of PBS. Slides were mounted on microscopic slides in fluorescent mounting medium (Dako, Glostrup, Danmark). Slides stained for DNA were mounted in a 25 μl drop of DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) diluted from the stock solution 1:1000 (at the estimated final concentration 0.5 μg/ml). The slide edges were covered with a thin layer of nail polish to prevent fast drying of specimens.

Antibodies used in the study: anti human TLX antibodies ab86276 and ab30942 were obtained from Abcam (Cambridge, UK) and used as recommended by the supplier. Antibody recognizing MCM2 (purified mouse anti-BM28 antibody) was obtained from BD Transduction Laboratories, BD Biosciences Pharmingen (San Diego, Ca, USA) and used as recommended by the supplier. Antibody specific for gamma tubulin TU-30 was a kind gift from Dr. Pavel Dráber (Institute of Molecular Genetics, Czech Academy of Sciences, Prague) and used as

published (Novakova et al. 1996). The antibody is also available from Exbio (Vestec, Czech Republic).

Secondary antibodies were as follows: Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L), Alexa Fluor 568 Goat Anti-Rabbit igG (H+L) were purchased from Life Technologies (Grand Island, NY, USA) and used as recommended by the supplier.

Donkey anti mouse Cy5 antibody was purchased from Merck (Billerica, MA, USA) and used as recommended by the supplier.

DAPI was purchased from Sigma Aldrich (St. Louis, Mo, USA) and used at final concentration (dilution 1:1000 from a stock solution containing 10 mg/ml).

Table 4. The cell lines were examined under the following scheme.

Glioblastoma cell lines used for immunodetection	Nomarski optics, antiTLX (ab86272), DAPI	Nomarski optics, antiTLX (ab30942), DAPI	Nomarski optics, antiTLX (ab30942), antiBM28, DAPI	Nomarski, antiTLX (ab30942), anti gama tubulin TU30, DAPI
A172	+	ND	ND	+
LN299	+	ND	ND	+
U373		+	+	+
U87MG	ND	+	+	+ (Note, the experiment with DAPI was not done)

In addition to the experiments listed in the Table 4,comp two glioblastoma cell lines were also analyzed for co-localization of TLX and MCM2 using antibody ab30942 and anti-BM28 antibody.

Concerning fluorescence microscopy, the slides were examined using an Olympus AX-70 microscope equipped with a DP-30 monochrome CCD camera and Analysis (Olympus, Tokyo, Japan) software for image acquisition. All immunodetection experiments were made in three parallel versions as 1/ a complete experiment with one or two different primary antibodies, the appropriate one two secondary antibodies, and with DAPI staining, 2/ as a second version without the DAPI staining and 3/ as a negative control in which only secondary antibodies were used. In the case of U-87 MG, the experiment of immunodetection of TLX and gamma tubulin was not done in the version with DAPI staining. For the image analysis, ImageJ computer program found at http://rsbweb.nih.gov/ij was used.

Results

Results of the part I: Search for TLX (NR2E1) in *Schmidtea mediterranea*

Cloning *S. mediterranea* Smed-tlx-1

In search for members of nuclear receptors in *S. mediterranea*, we identified partial DBD sequence of NR2 class of NRs in the chromosomal region v31.016829:1526..2067. The sequence was included in a predicted transcript classified as TLX homologue in the genome browser SmedGD. In accordance with the proposed *S. mediterranea* nomenclature, we named the gene *Smed-tlx-1*. However, the predicted sequence differed on both 5' and 3' ends substantially from TLX homologues known from many animal species sequenced so far. In order to amplify larger segments of cDNA, we designed primers from the predicted form of *Smed-tlx-1* cDNA and attempted to amplify larger segments by reverse-transcription PCR. Only primers designed according to the apparently conserved sequence of the DNA binding domain and first part of the hinge region lead to amplification of the predicted sequence. For full cDNA amplification, we used 3' RACE method (with the polyA derived anchor primer). We hypothesized that the predicted sequence containing codons of the first two cysteins of the DBD may be divided into two separate exons and used the sequence positioned more toward the 3' direction for amplification of the sequence in both directions. The strategy for amplification is shown in the Figure 9 (see kindly this and the following figures at the end of this part of results). For amplification of 5' end of the cDNA, we attempted to use splice leader sequences, known to exist in *S. mediterranea.* Attempts to amplify the 5' UTR of the cDNA using splice leader sequences failed for apparent efficient amplification of unrelated messages amplified by SL primers from both 5' and 3' directions. For amplification of 5' coding sequence, additional primers were designed based on homology search for conserved amino acids found in various TLX homologues and search in SmedGD genomic database. This strategy led to cloning of a sequence that shows signs of mature spliced message as well as high similarity to known TLX homologues. The sequence was deposited to GenBank and is available under the Accession Number that will be released upon the Online publication of the article.

The Clustal analysis shows a remarkable conservation of derived amino acid sequence between the SMED-TLX-1 and other members of the NR2E family (Figure 10).Both DNA binding domains and ligand binding domain show high degree of conservation. The DBD clearly indicates that the SMED-TLX-1 differs together with other NR2E class members from other NRs. The second zinc finger is broader, the D box contains more amino acids than most other NRs and the sequence corresponding to T box is also clustering NR2E proteins together. The molecular signature of SMED-TLX-1 thus has a form C-X2-C-X13-C-X2-C-X18-C-X13C-X9-C-X2-C-X4-C-X3-GM.

Comparison of SMED-TLX-1 sequence with other members of NR2 family members indicated that TLX in *S. mediterranea* is related to vertebrate orthologues as well as to the *C. elegans* orthologue, NHR-67 (Figure 11). Comparison of SMED-TLX-1 with representatives of TLX homologues, Photoreceptor nuclear receptor (PNR) and *C. elegans* genes NHR-67, FAX-1 and NHR-111 shows that SMED-TLX-1 clusters clearly with TLX not with PNR group of genes (Figure 12).

Contrary to most members of NR superfamily of proteins, SMED-TLX-1 contains 13 amino acids in the D box region. This region contains 7 amino acids in vertebrate Tlx and in *Drosophila Tll*. Analysis of the predicted molecular structure suggests, however that this inclusion should not interfere with DNA binding of the receptor (Figure 13).

The overall structure of LBD is keeping with 12 helices conserved molecular signature of NRs and remarkably conserved is also the carboxy-terminus of the sequence suggesting the conserved properties in binding of interacting proteins (Figure 10).

Smed-tlx-1 expression is regulated in response to fasting/feeding state

In order to assess the expression of *Smed-tlx-1*, we designed primers according to Roche Universal Library strategy. For analysis we used the region localized in LBD. In animals kept under once a week feeding cycle and expression analysis just before the new feeding, the *Smed-tlx-1* was found to be relatively abundantly expressed in both proximal part of the body as well as in the tail region suggesting expression pattern not restricted to the brain

region. Nevertheless, the expression in head was approximately 10 to 20 times bigger compared to the tail region (Figure 14A).

Next, we analyzed the expression of *Smed-tlx-1* during the fasting and fed states. *Smed-tlx-1* appeared to be expressed in both states with accented expression following feeding (Figure 14B).

SMED-TLX-1 is critical for tissue maintenance and integrity of organism in feeding/fasting cycles

For initial analyses of SMED-TLX-1 biological functions, we employed the RNAi method shown previously to be effective in induction of gene loss of function in *S. mediterranea*. We used the same setting as for *C. elegans* except that the bacteria producing dsRNA were in some experiments mixed with liver homogenate. Several feeding schemes were used.

Surprisingly, strong effect of *Smed-tlx-1* inhibition appeared during the fasting period following the single feeding with the dsRNA producing bacteria. The most apparent effect was shrinking the head area of inhibited worms (Figure 15 A, B, C, E). Animals also showed slow movement and changes of the body shape in form of strictures or even defects that were seen in both head and tail areas. Further culture of RNAi affected animals led to substantial recovery of affected worms but huge morphological defects were observed especially in the head area (Figure 15 G, H). They included smaller and malformed eyes and irregular shape of the head. Some animals lack one or both eyes and animals with three underdeveloped eyes were also seen (Figure 15 J, K). Repeated feeding with dsRNA producing bacteria led to gross defects and death of most animals. Some animals developed also abnormalities in the tail area. They included irregularities of the body shape and protrusions in a form of thin tail like projections on the dorsal side of the body. The critical function of SMED-TLX-1 in the regressive phase of the fasting-feeding cycles was more obvious when longer periods of starvation were applied following the RNAi. There was no effect of unspecific dsRNA produced by bacteria in control cultures (Figure 15 D, F, I).

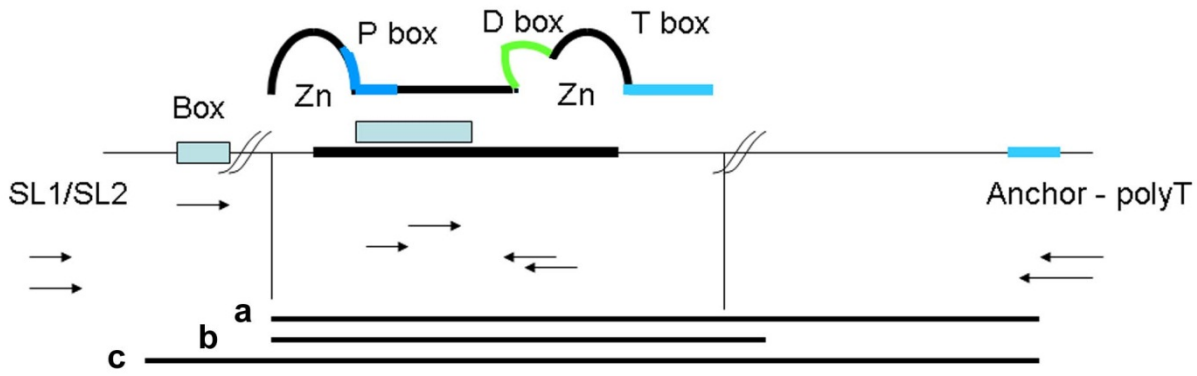


Figure 9. Schematic representation of cloning strategy. The two structural motifs coordinated by zinc ions ("zinc fingers") of the DNA binding domain are indicated by two arches, the P box by blue lines and D box by green line. T box – the region important for increased affinity of binding to response elements is indicated by blue line at the 3' position in the DNA binding domain. The region marked as thick line is the region identified as NR2E class of NHRs by computer searches with high degree of probability. Primers designed for amplification of cDNA in 3' and 5' direction were designed and used in amplification PCRs as indicated. The 3' region was amplified using poly-T (Anchor) primer and 5' terminal region was amplified using primers derived from conserved sequences detected by computer alignments. Dark lines labeled as a, b and c represent amplified cDNA regions covering the region including the complete DBD and LBD (a), a short transcript that contains a stop codon and may code for a protein lacking most LBD (b) and the complete coding region of a cDNA containing the conserved 5' motif, complete DBD and complete LBD (c).

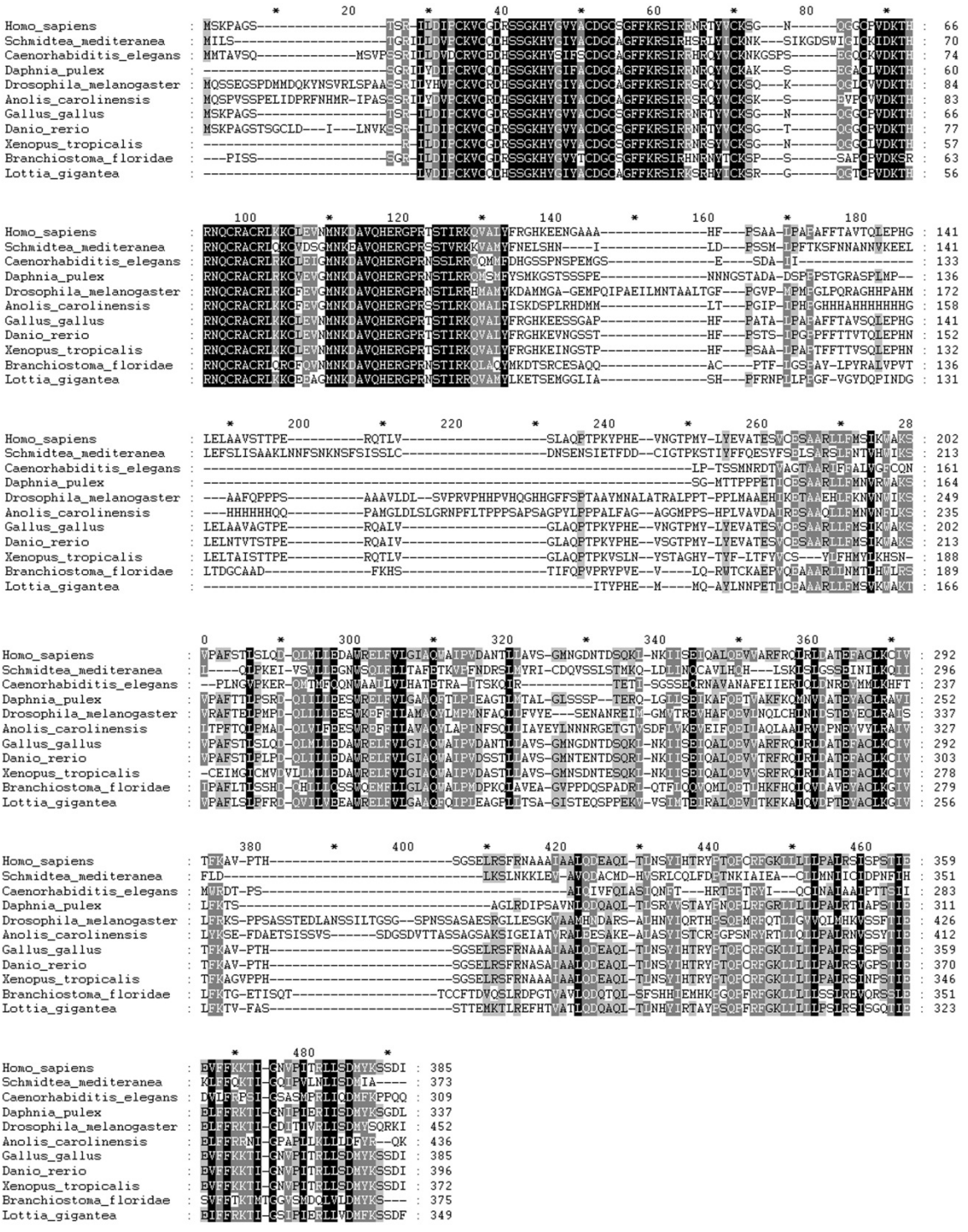


Figure 10. Clustal analysis of SMED-TLX-1 (see this Figure on the next page). Two conserved regions are apparent. DNA binding region (aminoacid - aa - in the position 35 – methionine in the position 112 in numbering unifying all aligned sequences) shows high degree of conservation as well as the C terminal region (aa 450 – 480) which is likely to be involved in protein infarction with transcriptional cofactors. Two regions adjacent to the DNA core region on both sides show also high degree of homology of SMED-TLX-1 with other members of NR2E subclass of NRs.

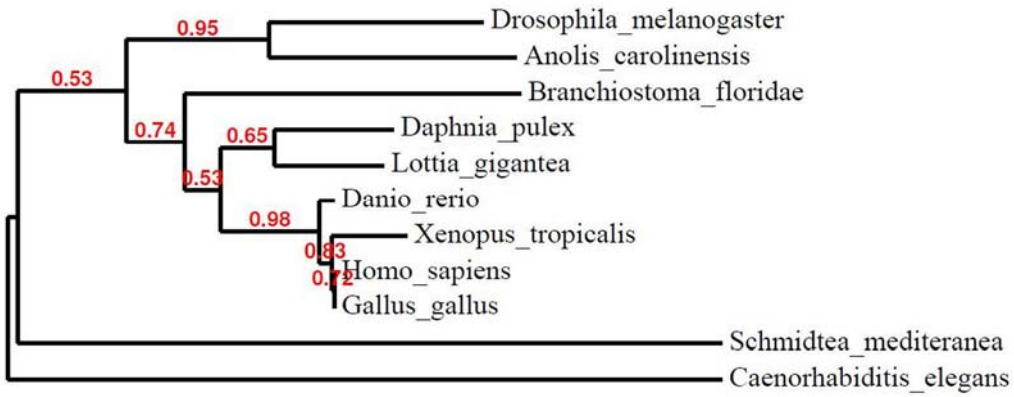


Figure 11. Phylogenetic tree derived from Clustal analysis shown in Fig. 18. Close relation of SMED-TLX-1 to from representative NR2E1 family members from various phyla are shown. *S. mediterranea* SME-TLX-1 is positioned next to *C. elegans* homologue NHR-67. All proteins aligned in this figure are homologues of TLX recognized by BLASTP program in Swiss Prot Database.

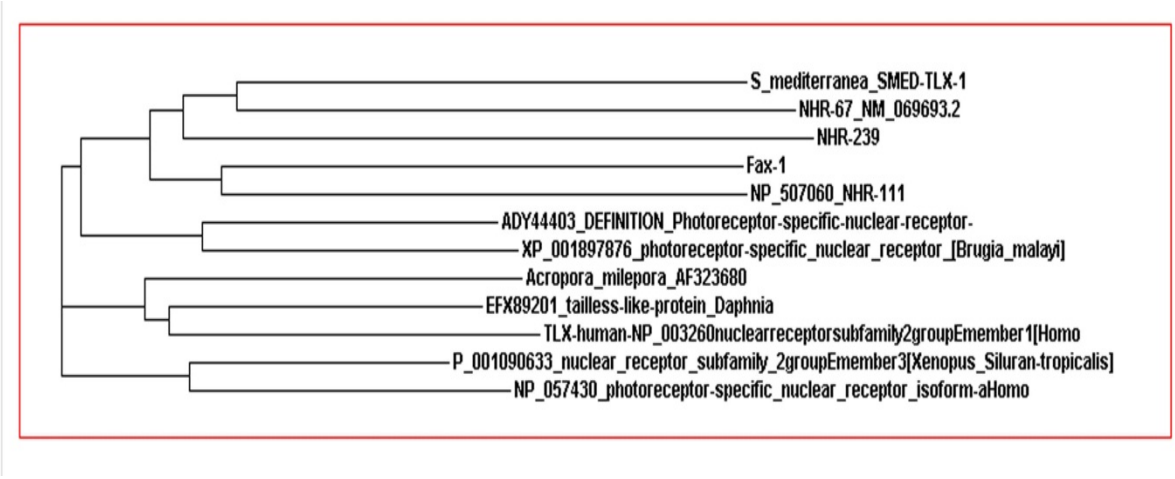


Figure 12. Phylogenetic tree of selected representatives of NR2E class members. The two main subgroups including NR2E1 members (TLX/*tailless*) and NR2E1 (photoreceptor nuclear receptors PNR) group differently. SMED-TLX-1 shows closer relation together with NHR-67 and to NR2E1 subclass. The two other *C. elegans* homologs, FAX-1 and NHR-111 group closer to PNR related proteins.

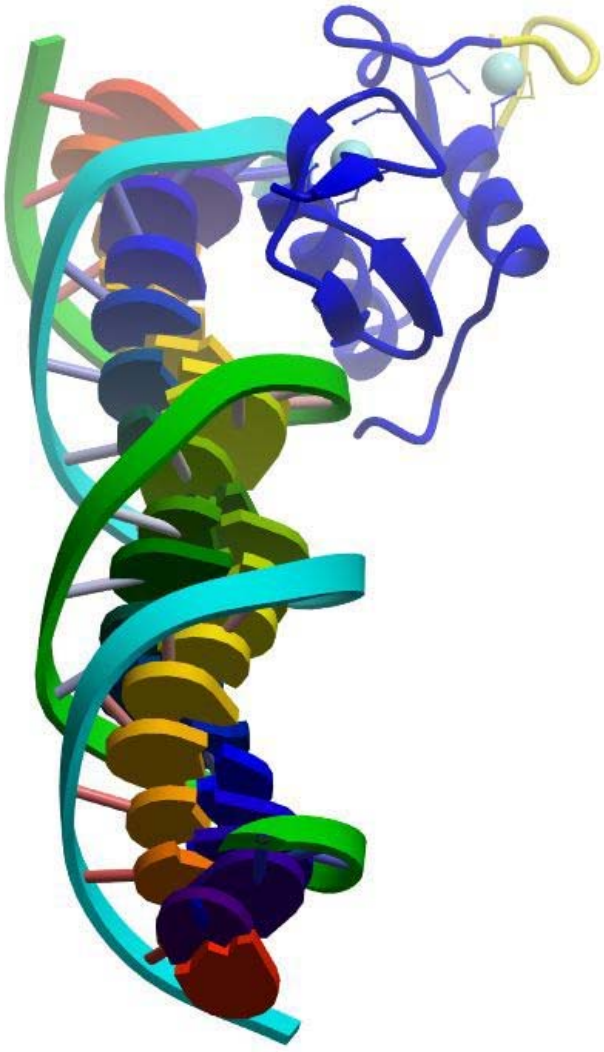


Figure 13. Model of DNA binding domain of RXR alpha exchanged for SMED-TLX-1. The loose loop marked by yellow represents the region composed of 13 aa in the predicted D box of SMED-TLX-1. The model shows that this protein motif is localized on the opposing side of DBD that is not involved in contact with DNA.

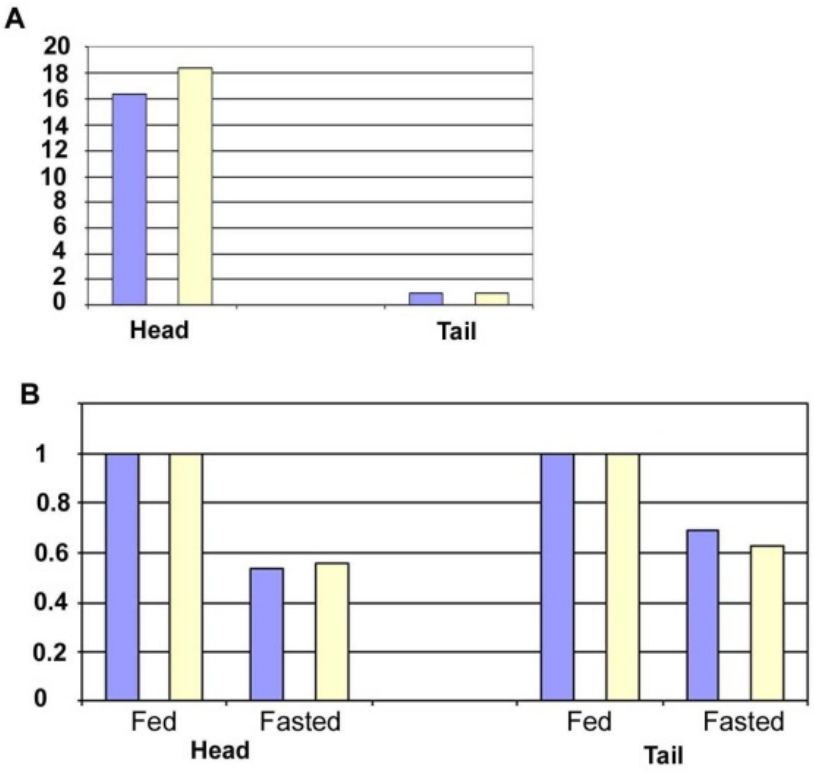


Figure 14. Expression analysis of *Smed-tlx-1* in RT-qPCR. A – comparison of *Smed-tlx-1* expression in most proximal part of head and most distal part of tail. Two different regions, primer pairs and UPL probes were used (dark and pale columns). The analysis shows high expression of *Smed-tlx-1* expression in the region containing brain and eyes and well detectable expression of *Smed-tlx-1* in tail region. B – analysis of *Smed-tlx-1* expression in fed (set as 1) and fasting animals. The two probe strategy shows expression of full length cDNA in both states and elevated expression during animal growing (fed) state in both head and tail regions.

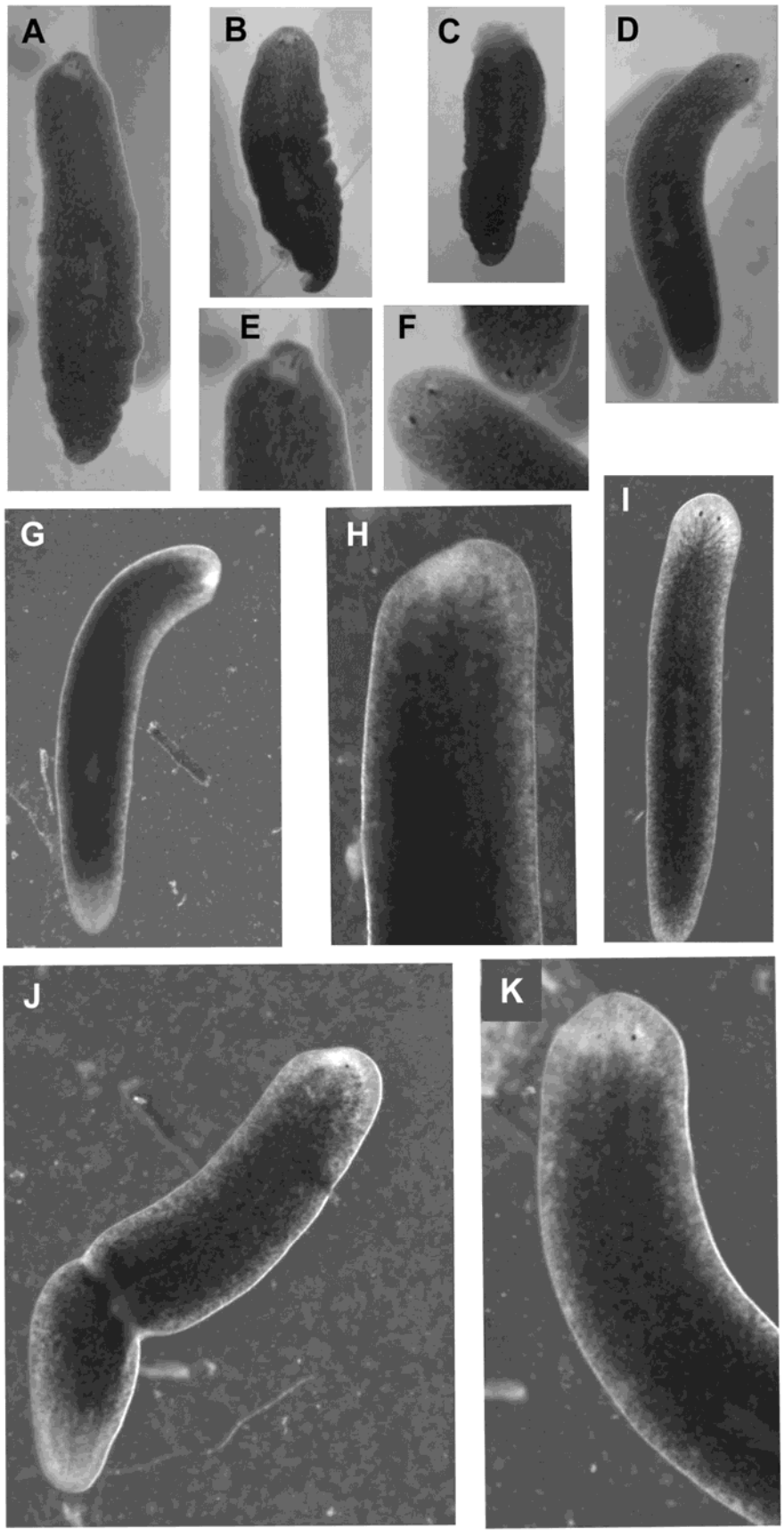

A
B
C
D
E
F
G
H
I
J
K

Figure 15. (see preceding page) *In vivo* imaging of *S. mediterraneae* inhibited for *Smed-tlx-1* function by RNAi (panels A, B, C, E, G, H, J and K) and control animals (panels D, F and I). During the starvation period following the RNAi induced by feeding, animals developed morphological defect in head areas (panel A and detail shown in panel E), defects in head as well as in tail areas (B), including a complete disintegration of head regions (C). After re-feeding animals developed irregularly, some with missing eyes, irregular shape of proximal body part (G) and irregular texture of internal structures in proximal body part (H). Frequently, irregular development of eyes was observed (J, K).

Results of the part II: Expression of TLX (NR2E1) in human astrocytoma cell lines

TLX is expressed in glioblastoma cell lines at various levels and shows signs of posttranslational modifications or multiple isoform expression

The cells cultured at conditions recommended by the supplier showed similarities as well as differences in growth. All cells grew as adherent cultures and the basic cell shape was similar: triangular or spindle shaped cells with predominantly two or three spicular projections. Some cells however had a round cell body usually projecting into flat irregular or almost rectangular pseudopodia like extension. Cell lines also showed great differences in growth rate. U-373 cells grew very fast, followed by U-87 MG and A-172, LN-299 grew as the slowest from the named cell lines and U-138 grew even less effectively. Differences were observed in the grouping of cells in the culture: U-373 grew continuously as a relatively uniform lawn of cells. A-172 were similar as U-373 but grew less effectively. Cells of the line U-87 MG grew as separated groups of cells and cells of the line LN-299 grew mostly as individually separated cells.

The cells were cultured at two different conditions, in log phase and at confluence. Cell cultures were harvested in the two growth phases and used for Western blot analysis.

Surprisingly, antibody 1 (ab86276) yielded almost no signal in Western blots made on 70 μg of loaded total protein (Figure 16). Only a weak signal at the expected size of 42 kDa was observed after overnight chemiluminescence exposures in U-373 cells. Antibody 2 (ab30942) detected TLX in all cell lines examined (Figure 16).

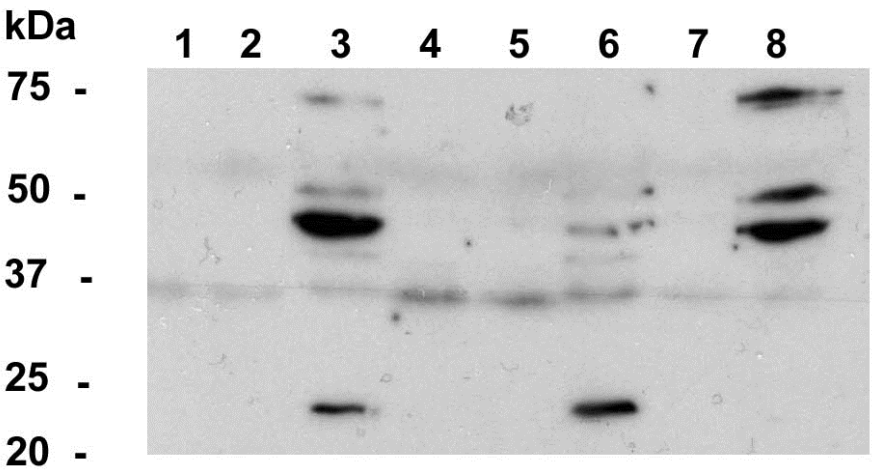


Figure 16. Immunoblot detection of TLX in protein lysates from U-373, U-87 MG and A-172 cells. 70 µg of total protein was loaded in each lane. Lanes 1, 2 and 3 are lysates from U-373 cells, lanes 4, 5, and 6 from U-87 GM cells and 7 and 8 from A-172 cells. Primary antibody was ab86276 in lanes 2, 5 and 7 and ab30942 in lanes 3, 6 and 8. Primary antibodies were omitted in lanes 1 and 4. Protein with a predicted size of 42 kDa is recognized by ab30942 in all cell lines examined. Additional bands with size 22 kDa are detected in U-373 cells and U-87 MG, and a band 50 kDa is detected in all three cell lines, although very weakly in U-87 MG cells. A band with an approximate size of 75 kDa is detected in U-373 cells and A-172 cells.

Western blots performed on protein lysates from cells grown to complete confluence detected additional bands in addition to the predicted form of TLX with the size of 42 kDa. The additional bands had approximate sizes of 50 kDa, 75 kDa and 24 kDa proteins. The pattern of detected proteins differed between the cell lines. The slowly growing U-87 MG had the weakest detection of all forms of immunodetected proteins and fast growing U-373 and A-172 had the strongest expression of proteins detected by the 30942 antibody but not the same pattern: both lines had strong expression of 42 kDa protein, A-172 lacked the small protein (24 kDa) but had very strong expression of 50 kDa protein (Figure 16). This raised a possibility of existence of multiple protein isoforms or posttranslationally modified forms of TLX in glioblastoma cell lines.

TLX is detected in the nucleus as well as in the cytoplasm and shows signs of nucleocytoplasmic relocation during the cell cycle phases

Indirect immunofluorescence with two different antibodies was used for analysis of TLX intracellular distribution. Keeping with the results obtained in Western blot analyses, the

antibody 1 (ab86276) gave a very weak signal (Figure 17, Figure **18**). Although this antibody was at the beginning of the study considered as unfit for the study, later evaluation indicated that the labeling pattern of cells keeps with the labeling pattern obtained with the second antibody (Figure 19,Figure 20), although the signal is very low.

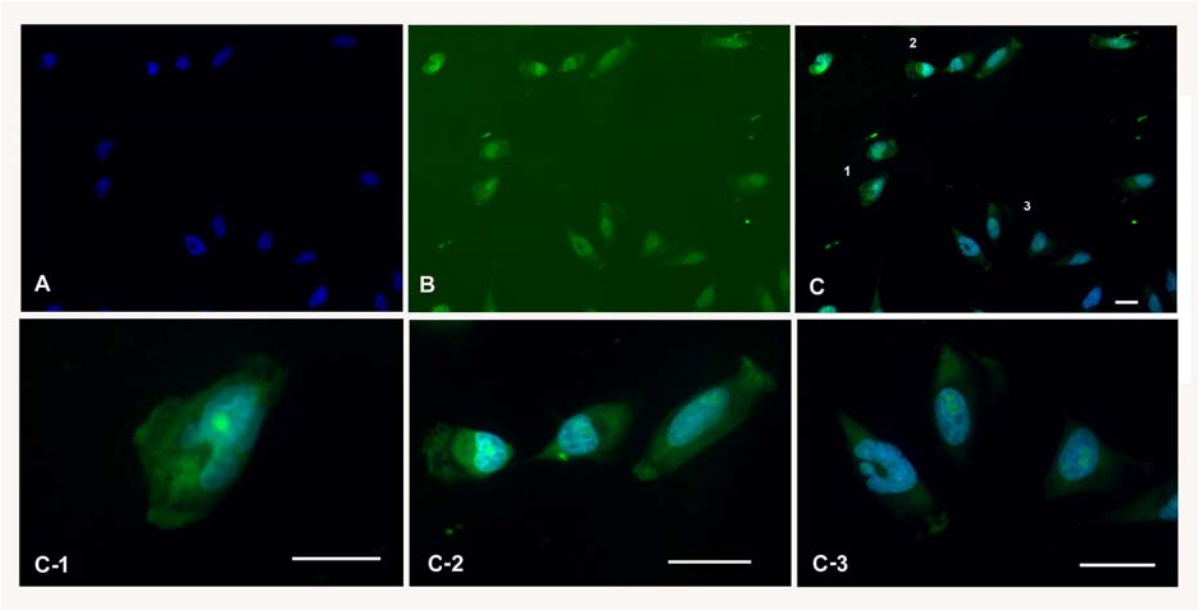


Figure 17. Immunodetection of TLX in LN-299 cells using the ab86276 antibody. A – DAPI staining. B – Detection of TLX. C – composite figure of panels A and B. Regions marked by numerals 1, 2 and 3 indicate areas magnified in C-1, C-2 and C-3. The scale bar in panel C3 represents 30 μm. The predominant nuclear staining of TLX in panel B is seen in all cells. Areas with distinct TLX staining are also clearly visible in the cytoplasm and at specific regions of cell membrane. Nuclear staining of TLX is partially overlapping with DAPI signal but with variable intensity and colocalization panels C-1, C-2 and C-3 show intranuclear domains with accumulated TLX signal and low DAPI staining.

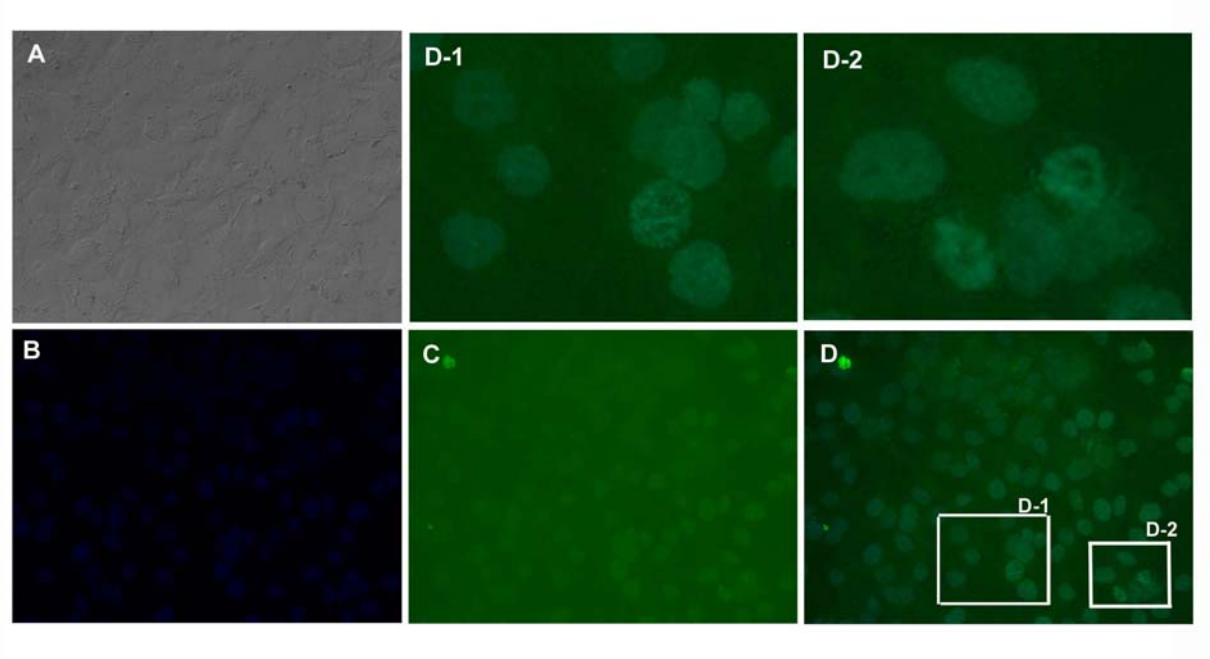


Figure 18. Immunodetection of TLX in A-172 cells using antibody ab86276.

A – Nomarski optics of the recorded area. B – DAPI staining. C – Immunodetection of TLX. D – Composite figure of panels B and C. Regions D1 and D2 are magnified and shown as panels D-1 and D-2 (The scale bars the bottom lines of the rectangles D-1 and D2 and represent 60 µm and 45 µm, respectively). Weak but easily distinguishable detection of TLX is seen in most cells in panel C and D. Increased TLX versus DAPI signal is visible in cells with compacted DAPI positive staining in panels D-1 and D-2.

Antibody 2 (ab30942) gave clear positive signal that was found in all cell lines in cell nuclei as well as in the cytoplasm (Figure 19,Figure 20).

For determination of intracellular distribution of TLX, the colocalization with DAPI or MCM2 was performed (Table 5). DAPI staining very effectively labels DNA rich in A-T. Although the labeling of DNA is not strictly quantitative, it gives good determination of the nuclear compartment and accumulated DNA in it. Although the immunodetected TLX overlap with DAPI staining in the first approximation, detailed view revealed uneven distribution of TLX and DAPI staining indicating that in certain areas of nuclei TLX is more abundant in nuclear compartments with low DAPI signal. This indicates that TLX stains more

Table 5. Intracellular localization of hTLX in glioblastoma cell lines detected by immunofluorescence. The detected pattern is marked in the following way: N – nuclear staining, C – cytoplasmic staining. G1, G2, S, and M (mitosis) indicate specific cell cycle phases for the detected TLX expression. EC indicate extrachromosomal staining during mitosis. ND - not done, + indicates that the corresponding immunodetection protocol was performed.

Glioblastoma cell lines used for immunodetection	Nomarski optics, antiTLX (ab86272), DAPI		Nomarski optics, antiTLX (ab30942), DAPI		Nomarski optics, antiTLX (ab30942), antiBM28, DAPI		Nomarski, antiTLX (ab30942), anti gama tubulin TU30, DAPI	
		Results		Results		Results		Results
A172	+	N, C	ND		ND		+	N, C, EC, N at G1, S and G2
LN299	+	N, C	ND		ND		+	N, C, EC, N at G1, S and G2
U373	ND		+	N, C, EC	+	N, C, EC	+	N, C, EC, N at G1, S and G2
U87MG	ND		+	N, C, EC	+	N, C, EC	+	N, C, EC, N at G1, S and G2

nuclear domains depleted of A-T rich DNA that is likely to be predominantly non-coding DNA.

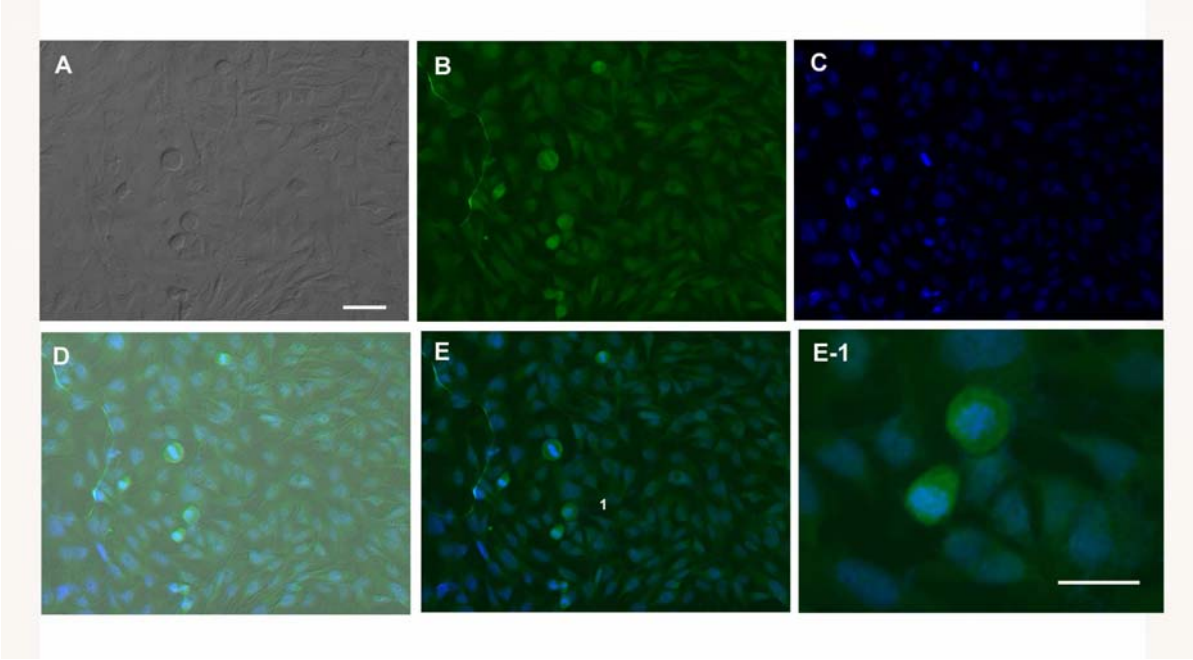


Figure 19. Immunodetection of TLX in U-373 cells. A – Nomarski optics of the recorded area. B – detection of TLX using ab30942 antibody. C – DAPI staining. C – composite figure of panels A, B and C. E – Composite figure of panels B and C. The numeral 1 indicates the region magnified in E-1. Scale bars represent 60 µm and 15 µm in panels A and E-1 respectively. Predominant nuclear staining for TLX is visible in most cells. Two, probably recently divided cells in the panel E-1 have strongly increased labeling pattern for TLX. TLX is also detected in the cytoplasm of most cells.

In order to relate the intracellular distribution of TLX during the G0/G1 cell cycle phase and S/G2 phase, we performed simultaneous immunodetection of TLX and MCM2 or gamma tubulin (Table 5). MCM2 is expressed more efficiently in G1 phase and as a component of the pre-replication complex shows higher granular staining of replicated DNA. During gradual progress of S phase, it is liberated from DNA and becomes gradually degraded (Masata et al. 2011). Gamma tubulin is greatly accumulated in centrosomes and allows the determination of cells in G0/G1, S and/or G2 phases, G2, and in mitosis based on

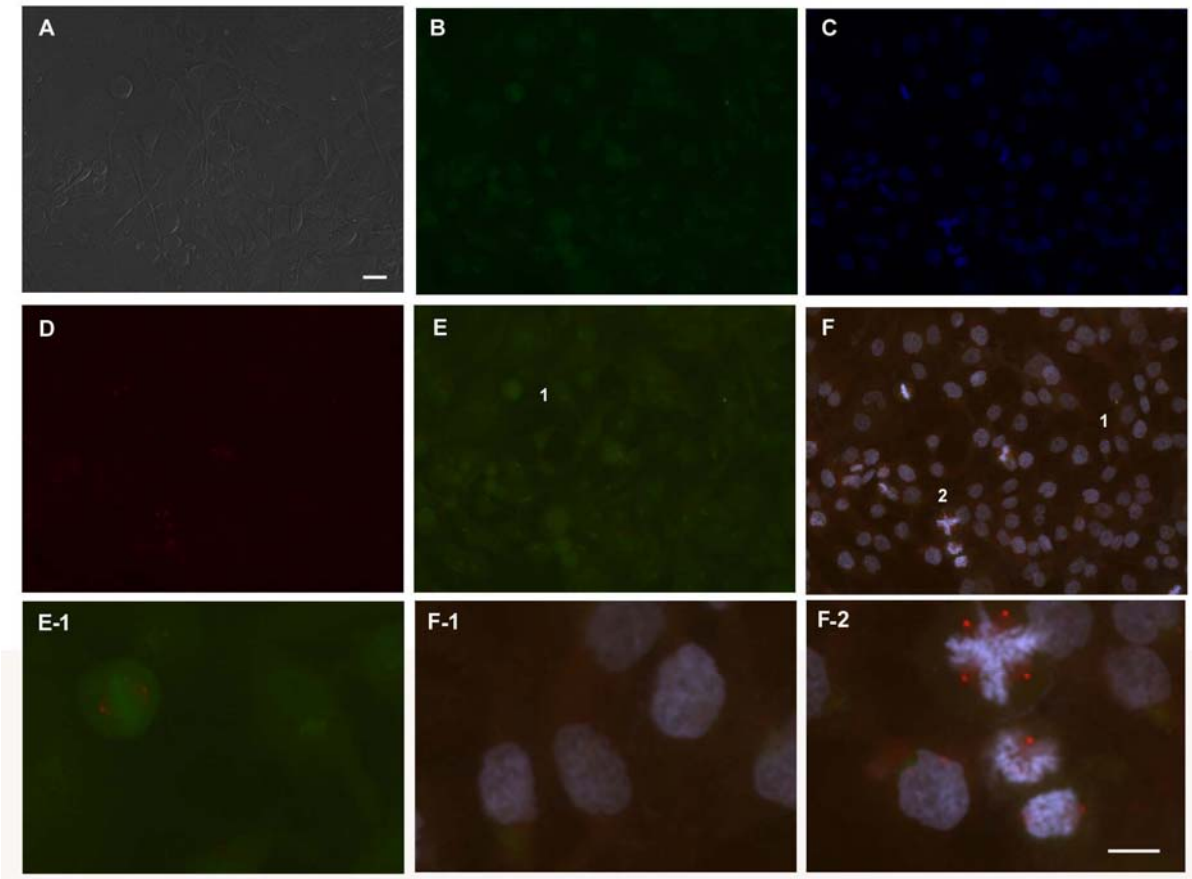


Figure 20. Immunodetection of TLX in A-172 cells using ab30942 antibody and simultaneous detection of gamma tubulin using the TU-30 antibody. A – recorded area in Nomarski optics, B – detection of TLX using the ab30942 antibody, C – DAPI staining, D – detection of gamma tubulin, E – composite figure of panels B and D showing the TLX detection in relation to centrosomes detected by anti-gamma tubulin antibody, F – composite figure of panels B, C and D showing TLX detection, centrosomes and DNA stained by DAPI. Panel E-1 shows the area 1 from panel E at higher magnification. F-1 and F-2 show corresponding areas from panel F. Scale bar represent 60 µm and 15 µm in panels A and F-2, respectively. Most cells show nuclear staining for TLX and weaker but clearly present cytoplasmic staining. The mitotic cell marked by the numeral 1 in the panel E and magnified in the panel E1 shows elevated extrachromosomal TLX staining. The region F1 with a weak nuclear staining mostly overlapping with DAPI staining. The region F2 shows a tetraploid cell undergoing mitosis with elevated cytoplasmic TLX detection, one cell with a duplicated centrosome and a cell with two centrosomes on opposite cell poles.

centrosome number (one per cell during the G0/G1 phase and two per cell during S phase and in G2), position (two centrosomes positioned next to each other in S and G2 phase) and

appearance (mature centrosomes give stronger signal for gamma tubulin) (Hinchcliffe and Sluder 2001; Azimzadeh and Bornens 2007).

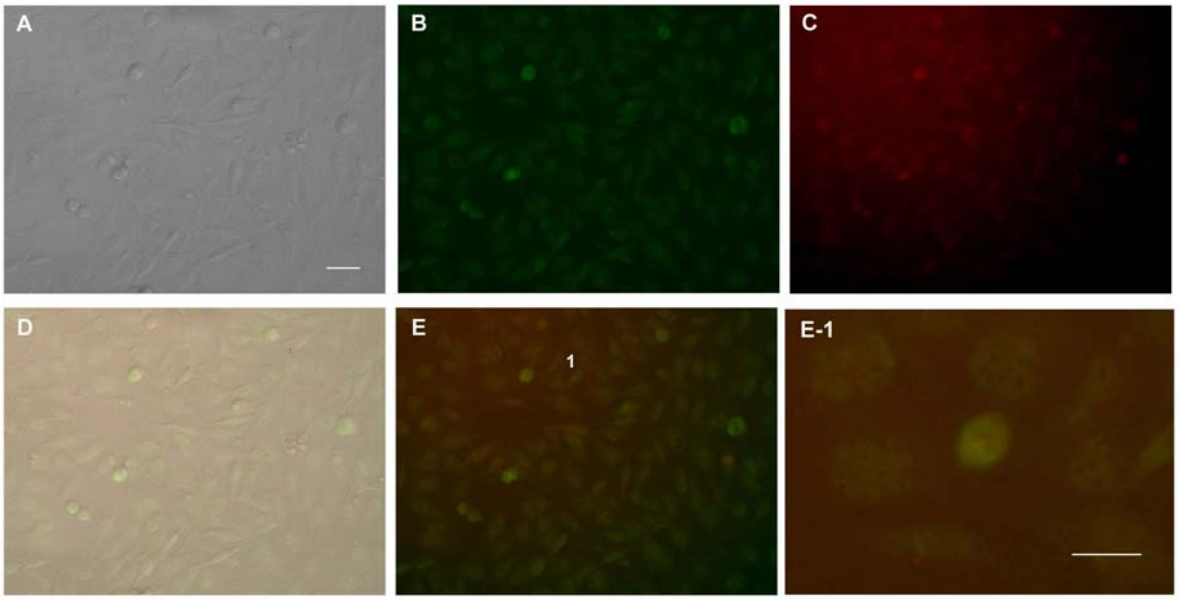


Figure 21. Immunodetection of TLX and MCM2 in U-373 cells. A – Recorded area in Nomarski optics. B – Detection of TLX using ab30942 antibody. C – detection of MCM2. D – Composite figure of panels A, B and C. E – Composite figure of panel B and C. Numeral 1 indicate area that is magnified in E-1. The scale bars represent 20 µm and 10 µm in panel A and E1, respectively. The detection of TLX greatly increases in cells undergoing mitosis. In comparison to MCM2 staining, that is diffuse in most nuclei; TLX staining is more restricted although the whole nuclear compartment labeled by MCM2 is also stained with TLX.

The colocalization of TLX with MCM2 keeps with the expression of TLX in G1 phase (Figure 21) and the labeling pattern clearly supported the nuclear localization of TLX, although cells with predominantly cytoplasmic expression were found in all cell lines. Interestingly, the ratio of TLX/MCM2 immunodetection revealed as color change in merged composite figures showed that some cells are more MCM2 positive then for TLX indicating that TLX is expressed also in G2 phase (Figure 21, Figure 22).

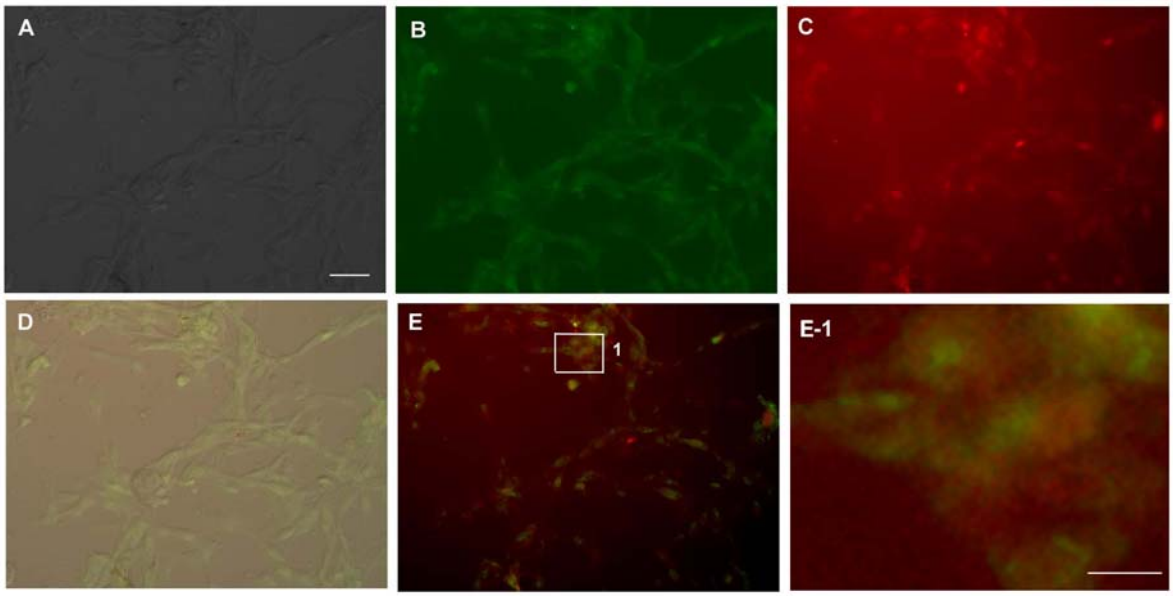


Figure 22. Immunodetection of TLX and MCM2 in U-87 MG cells. A – Recorded area in Nomarski optics. B – Detection of TLX using ab30942 antibody. C – detection of MCM2. D – Composite figure of panels A, B and C. E – Composite figure of panel B and C. Numeral 1 indicate area that is magnified in E-1. The scale bars represent 40 µm and 20 µm in panel A and E-1, respectively. The detection of TLX greatly increases in cells undergoing mitosis. In comparison to MCM2 staining, that is diffuse in most nuclei; TLX staining is more restricted although the whole nuclear compartment labeled by MCM2 is also stained with TLX. Cells in the panel E-1 show separation of TLX and MCM2 labeling.

Although the nuclear localization was predominant in most cells, in all cells examined, the cytoplasmic TLX was also observed. The cytoplasmic detection of TLX was in some cell cultures very prominent and the TLX signal was found in close relation to the cell membrane and specific peripheral cytoplasmic domains (Figure 17, panels C-2 and C-3). Analysis showed that the ratio between nuclear and cytoplasmic immunodetected TLX (measured as mean integrated optical density of the recorded immunofluorescence) was very variable and frequently, the signal was bigger in the cytoplasm compared to the nucleus.

Discussion

Discussion of the part I: Search for TLX (NR2E1) in *Schmidtea mediterranea*

Smed/TLX is important for keeping body plan and tissue integrity during the regression and growth phases of planarian life.

In this work, we cloned and partially characterized a member of NR2E class of NHRs in *Schmidtea mediterranea*. Analysis of its primary sequence as well as its three dimensional model indicate a very high degree of homology between its counterparts in various Metazoan species including coral, arthropods, nematodes and chordates. The DBD of SMED-TLX-1 is conserved to the extent that suggests a conserved binding to the response elements similarly as shown in case of nematode orthologue NHR-67 (DeMeo et al. 2008). Expression of Smed-tlx-1 is augmented in proximal part of body but it is not restricted to this region. Keeping with this, the developmental consequences of Smed-tlx-1 inhibition by RNAi leads to multiple phenotypic changes including shrinkage of the brain area, body shape defects and in following regeneration phase defects of head morphology, defective development of eyes, slow movement and defects of the body shape in the tail area.

S. mediterranea is able to cope with food restriction by resorbing its own tissues while the stem cells and tissues important for normal planarian life sustain functionality and allow the animal to re-grow when the food supply is restored (Pellettieri et al. 2010). This process includes apoptotic cell death and removal of dead cells by phagocytosis. The survival of cells that are supporting the organism functionality and next regeneration includes the anti-apoptotic protein BCL2. This basic mechanism is reminiscent of rearrangement of tissues during amphibian metamorphosis (Das et al. 2002; Nakajima et al. 2005; Rowe et al. 2005) as well as development of organs during chordate ontogenesis (Nagasawa et al. 1997).

The close relationship between SMED-TLX-1 and its nematode, insect and chordate homologues (NHR-67, tailless and TLX) indicate that other mechanistic relationships may be also conserved. The insect tailless and vertebrate TLX are predominantly transcriptional repressors and both regulate development by affecting additional nuclear receptors; tailless

is repressing the knirps (Moran and Jimenez 2006) and TLX retinoid receptor beta 2 (RAR beta2) (Kobayashi et al. 2000).

The search for homologues in C. elegans indicates that the functions supported by NR2E class members may diversified between several NHRs, NHR-67 is the most obvious orthologue of TLX/tailless in C. elegans. Other homologues found in C. elegans genome are FAX-1, NHR-111 and NHR-239 and possibly other NHRs.

NHR-67 regulates proper development of vulva cells in a regulatory circuit based in inhibitory function of NHR-67 and COG-1 (Fernandes and Sternberg 2007). The circuit includes another NR, the NHR-113. This NHR is highly conserved between nematode species but is only distantly related to ERR and RARs (Ririe et al. 2008).

NHR-67 deficiency affects L3 and L4 stage development probably by affecting the cell migration program, left-right specification and timing (Sarin et al. 2009).

NHR-67 controls specification of gustatory neurons and affects the left/right (L/R) asymmetric subtype diversification. NHR-67 regulates positively the expression of a sensory neuron-type-specific selector gene, che-1, which encodes a zinc-finger transcription factor. The neuronal function of NHR-67 is broader and covers many neuronal types (Kato and Sternberg 2009).

Although tailless/TLX seem to be primarily repressors, the existence of direct transcription activation function is well documented. TLX activates NAD+-dependent histone deacetylase directly by acting on sirt1 promoter (Iwahara et al. 2009).

In addition to this, TLX is recruited to the Oct-3/4 promoter in hypoxia and augments its expression. This induces proliferation of progenitor cells and preserves their pluripotency (Chavali et al. 2010).

The DBD of SMED-TLX-1 is almost identical to human TLX with the exception of the D box which contains 13 amino acids compared to vertebrate TLX which has only 7 aa in the corresponding region. The C. elegans homologue, NHR-67 has 10 amino acids in this region. The model based on known structure of related NHRs suggests that this inclusion is unlikely to affect the DNA binding although may have consequences for dimerization on DNA response elements as well as protein-protein interactions in this domain (Figure 13). The LBD

of SMED-TLX-1 is also highly conserved. The LBD has all 12 helices and the helix 12 involved in cofactor binding is conserved (Figure 10).

The remarkable conservation of SMED-TLX-1 suggests that additional mechanisms that are functioning in *S. mediterranea* tissue maintenance and renewal may also be conserved between Turbellaria and vertebrates.

Discussion of the part II: Expression of TLX (NR2E1) in human astrocytoma cell lines

hTLX is found in both the nucleus and the cytoplasm

Our experiments detected hTLX in the nucleus as well as in the cytoplasm. Although TLX seems to be a relatively uncomplicated NR with only one known expressed isoform, its regulated localization would be in line with several other NRs that are spatially regulated. Estrogen receptors, thyroid receptors and retinoic acid receptors were shown to be regulated not only by their ligands but also at the level of protein intracellular distribution (Maruvada et al. 2003). This is similarly true for a number of proteins that interact with NRs and modulate their function http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0022395.

Using specific antibodies and Western blot analyses, we identified several immunoreactive proteins that are likely to represent posttranslationally modified forms of hTLX. Existence of posttranslationally modified forms of TLX would support also its regulated intracellular localization. Nuclear receptors as well as other transcription factors are ubiquitinated and sumoylated by specific E3 ligases dependent on the type of modification preferentially retained in nuclei or transported into the cytoplasm. The regulation of AR activity by E3 ligase and sumoylation is well documented (Yang et al. 2011) as well as the connection of specific sumoylation and RAR alpha intracellular distribution and transcription reulation activity (Zhu et al. 2009).

Although it cannot be excluded that the additional proteins recognized by anti-TLX antibodies in Western blot analyses are TLX unrelated, it seems more likely that they represent specific protein forms of TLX. The differences in the protein patterns obtained with anti-TLX antibodies, their dependence on particular cell lines examined and culture conditions, are in line with this possibility. The existence of posttranslational regulation of TLX was already proposed (Obernier et al. 2011).

Immunolocalization of TLX in human astrocytic cell lines indicated that

TLX is mostly intranuclear, but is also detected in specific cases in the cytoplasm and at specific cell membrane domains.

TLX is expressed in both G1 and G2/S cell cycle phases.

Correlation of TLX expression with immunodetected centrosomes allowed to determine the G0/G1 and S/G2 cell cycle phase of the particular examined cell. Immunodetection of gamma tubulin which is accumulated in centrosomes allows to distinguish G0/G1 during which phases the cell has only one centrosome and S/G2 phases. Centrosome development and maturation can also be visualized using anti gamma tubulin antibodies (Azimzadeh and Bornens 2007).

Immunolocalization of TLX in human astrocytic cell lines indicated that TLX is mostly intranuclear, but is also detected in specific cases in the cytoplasm and at specific cell membrane domains. Comparison of the staining during the particular cell cycle phases was done in cells that showed clear cell cycle stage based on the finding of only one centrosome (G1/G0), or two centrosomes duplicating and maturating during the S and G2 phase at one cell pole. In prophase, centrosomes start to move to opposite poles.

The nuclear staining was found in both G0/G1 and S/G2 phases. Nevertheless, it was possible to detect many cells with TLX detected more strongly in the cytoplasm, compared to the nucleus. The immunodetection of TLX greatly increased during the mitosis in extrachromosomal cellular compartment as well as in the cytoplasm of newly divided cells. The lack of TLX was shown to be linked with elevated expression of factors that prevent cell cycle entry and downregulation. The homeobox transcription factor Dlx2, that is critical for neuronal cell differentiation was also downregulated suggesting a dual role of TLX in different populations of mouse brain cells (Obernier et al. 2011). This suggested a dual role of TLX, blocking the negative regulators of cell cycle entry and progression and differentiation in G2 phase. Both functions may be dependent on a transcription repression function of TLX. This is keeping with the concept that TLX promotes cell division and prevents differentiation of neural cell progenitors. Increased expression of TLX was found in glioblastoma cell lines and its elevated expression was also related to unfavorable disease course in human glioblastomas. It can be speculated that TLX may perform different roles in the formation of initial cancer progenitors, regulation of cell cycle progression in G0/G1, and differentiation in S/G2 (Liu et al. 2010; Park et al. 2010).

Cell lines derived from tumors are very valuable models of cell biology, although they are not normal cells. Many mechanisms that can be visualized in cell lines derived from tumors function in cultured cells and may be recognized and studied. Thus the cell specific intracellular distribution of TLX may reflect a situation in cell undergoing asymmetric mitosis. It can be expected that preferential relocation of TLX to one daughter cell would profoundly alter the cell fate of daughter cells. The immunodetection pattern observed in our astrocytic cell lines may be in line with this concept.

TLX detection increases during the mitosis

The sudden increase of the TLX immunodetection in extrachromosomal compartment during mitosis indicates that TLX expression is relatively high in G2 phase of the cell cycle but for some unknown reason is not detected by immunofluorescence. The epitope may be masked by protein complexes. The separation of TLX immunodetection signal and MCM2, that is known to be relocated from chromatin during S phase, supports this possibility. Recently, similar intracellular distribution was shown also for GR (Matthews et al. 2011).

The distribution of TLX in the nuclear compartment is likely to be linked to specific nuclear domains. Co-localization with both DAPI stained DNA rich compartments and whole nucleus stained with MCM2 (Masata et al. 2011) indicated that TLX is distributed unevenly in the nuclei and in certain cells is accumulated in domains with low DNA staining. The biological meaning of this finding has to be determined.

Conclusions

Conclusions of the part I: Search for TLX (NR2E1) in *Schmidtea mediterranea*

TLX is an important player in *S. mediterranea* maintenance of neuronal tissue and points at conserved mechanisms that are likely to be shared between planaria and vertebrates.

Modulation of reprogramming by TLX and its regulatory cascade could be a prime target for research in surgical reconstructions.

Conclusions of the part II: Expression of TLX (NR2E1) in human astrocytoma cell lines

hTLX is detected in both the nucleus and the cytoplasm.

The nucleo-cytoplasmic distribution varies between examined astrocytic cell lines and between cells in the same cell line what suggests that the particular intracellular localization of TLX may be connected to cell metabolic stage or cell cycle phase.

It seems likely that the observed pattern of TLX intracellular localization reflects its regulated intracellular transport.

Multiple protein forms of TLX were detected by Western blot.

The results suggest that the regulated intracellular distribution and the regulation of TLX on the protein level are likely to be connected to the mechanism by which TLX regulates tissue proliferation and maintenance.

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