Charles University in Prague
First Faculty of Medicine

Thyroid hormone receptors and selected interacting proteins in glial tumors: The analysis of the expression and regulatory potential

PhD thesis

Petr Libý, M.D.

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CONTENTS

Abbreviations

aa – amino acids; bp – base pair; CDK – cyclin dependent kinases; cDNA – complementary deoxyribonucleic acid; CT – treshold cycle; EGFR – epidermal growth factor receptor; CGH - comparative genomic hybridization; G – gliosis;
G I, GII,GIII or GIV – astrocytoma grade I, II, III or IV; GBM – glioblastoma multiforme; GTF – general transcription factors; HAT – histone acetyltransferase; HGA – high grade astrocytoma; HGG – high grade glioma; HDAC3 – histone deacetylase 3; kD – kilo Daltons; IAP – The Inhibitors of Apoptosis Protein Family; LBD – Ligand binding domain; LGA – low grade astrocytoma; LOH – loss of heterozygosity, mb – multiple bands, NHR – nuclear hormone receptor, NR – nuclear receptor; NCoR – nuclear receptor corepressor; PCR – polymerase chain reaction; PDGFR - Platelet-derived growth factor receptor; PML – promyelocytic leukemia; Pol II – RNA polymerase II; Q-PCR – quantitative polymerase chain reaction; RNAi – RNA interference; RXR – retinoid X receptor; RT – reverse transcription; RTK – receptor thyrosine kinases; SKIP – Ski-interacting protein; SMRT – silencing mediator of retinoid and thyroid hormone receptor; TF – transcription factor; TFIIB - transcription factor IIB; TR – thyroid hormone receptor; TRE – thyroid responsive element; TSA – trichostatin A;WB – Western blotting; WHO – The World Health Organization

1 SYNOPSIS

Thyroid hormone plays a crucial role in brain development. Contrary to that, a significantly milder phenotype is caused by elimination of both thyroid receptor genes, the TRα and TRβ, in knock-out animals, indicating that unliganded receptors severely affect the brain development.

The work described in this thesis was part of a scientific endeavour aimed at the elucidation of the hypothetical possibility that the pathologic involvement of thyroid receptors may be an important part of tumorigenesis of human astrocytic tumors. We investigated the expression of thyroid hormone receptors and their natural dimerization partners, the RXRs, in a collection of bioptic samples. We assayed the level of receptor expression by quantitative PCR, Western blots and by immunocytochemistry in non-malignant gliosis samples, in low grade and high grade gliomas. Special care was given to conduct all examinations in a way that complies with very stringent ethical rules and in all instances, supported and enlarged,

and not restricted, the diagnostic process. Samples used for the molecular biology methods were obtained from frozen sections prepared by cryocut apparatus and allowed histopathological control any time during the procedure. The samples were also, because of this, very limited and this did not allow in some cases, to complete all the sets of experiments.

Thyroid hormone receptors` cDNAs of α1, α2, β1 and β2 of expected sizes were efficiently amplified from all examined cases. Quantitative PCR showed a big diversity in the expression of thyroid receptors in all groups of examined samples. Contrary to control experiments performed on total RNA extracted from cell lines, including the glioblastoma cell line U373, which allowed clear quantification, many tumor samples showed disturbed melting curves indicating that no single fragment of mRNA was reverse transcribed and amplified. Such results were not included in the comparison of the expression of studied transcripts. Analysis of quantifiable experiments indicated that TRα1, TR α2, TR β1 and TR β2 were expressed in the studied samples. A trend, although not statistically significant assuming the null hypothesis, of increased expression of TRα2 and decreased expression of TR β1 was observed. On the protein level, expression of thyroid receptors was studied using commercial antibodies as well as by antibodies obtained from cooperating laboratories. Western blot analysis identified proteins of expected size in case of TRβ1 and TRα2 (TRβ2 was not assayed). The trend of protein expression was similar as that found by Q-PCR, the hallmark was big diversity of the expression levels, the increase of TRα2 and decrease of TRβ1. Antibodies directed against TRα1 detected multiple proteins. On the cellular level, a strong and characteristic expression pattern was obtained in case of TRα2. It was found predominantly in the cytoplasm, although nuclear presence was also detected. The pattern did not differ between non-malignant samples and tumors, but was dramatically stronger in tumors. Strong intranuclear staining was obtained for TRα1 in tumors. The results were compatible with the possibility of formation of multiple protein isoforms of TRα1, but can not be taken as a support of such situation since unspecific interactions could not be excluded. We started experiments aimed at direct mass spectroscopic characterization of proteins recognized by antibodies.

RXRα, RXRβ and RXRγ, dimerizing partners of thyroid receptors, were found present and rather increased in tumors compared to non-tumorous tissues on mRNA level. The increase of RXRγ in tumors was statistically significant ($P<0.05$).

A similar expression pattern was found in the U373 glioblastoma cell line. Levels of detected TRα1, TRα2, TRβ1, TRβ2, RXRα, RXRβ and RXRγ were similar as the highest

values observed in malignant astrocytoma. Transfection experiments showed that thyroid receptors in U373 cells are capable of activation of transcription from thyroid responsive promoters but exogenous receptors are necessary for efficient activation of exogenous thyroid responsive promoters. Contrary to that, RXRs seem to be present in sufficient numbers of copies for efficient transcription activation from endogenous responsive promoters.

Next we searched for the expression pattern of cofactors that have hypothetical potential to modulate the function of thyroid receptors. We focused on SKIP, an evolutionarily conserved cofactor shown to interact with several NHRs, Survivin, that was shown to be functionally linked with SKIP and thyroid receptor mediated gene expression and HDAC3, which is implicated in TR dependent transcription silencing. Interestingly, SKIP was found to be elevated in glial tumors and to follow the elevated expression of Survivin, similarly as it is in *C. elegans*, where, the homologoue of Survivin, *bir-1* is organized with *SKIP* in an operon.

The analysis of the expression of HDAC3 showed markedly elevated and deregulated expression in glial tumors on the level of mRNA, protein and on cellular level. While the distribution of HDAC3 was both nuclear as well as cytoplasmic and moderate in intensity in non-malignant tissues and low grade gliomas, high grade tumors expressed HDAC3 in a focally deregulated pattern that included strongly pronounced cytoplasmic localization. Confocal microscopy and additional colocalization analysis detected nuclear HDAC3 in all tumors examined. We conclude that HDAC3 expression is elevated in human astrocytic tumors and its expression pattern is deregulated at the cellular level in high grade gliomas.

In an attempt to visualize the transcription regulation function of Survivin, which is critically influencing the cell cycle, that in turn projects to transcription regulation, we turned to the heterologous system, *C. elegans*. Here we searched for genes whose expression is affected by BIR-1 loss of function using whole genome microarray experiments in synchronized L1/L2 larvae. *C. elegans* larvae have at the end of the L1 stage most cells postmitotic. The only somatic cell type that is dividing during larval transitions are the seam cells, specialized epidermal cells that keep blast cell (stem cell) character. First we worked out conditions that did not affect cell divisions of seam cells and we used these conditions on large scale larval cultures inhibited for bir-1 by RNA interference. Extracted total RNA was used for whole genome microarrays (Affymetrix). The microarray experiment was done at Microarray facility, NIDDK, NIH. Microarrays identified several collagen and ribosomal genes as candidate targets of *bir-1* inhibition in L1/L2 larval stage. The decreased expression of selected collagen genes in *bir-1* inhibited larvae was confirmed by quantitative RT-PCR.

Next, we generated transgenic lines expressing *bir-1* mRNA under a heat shock regulated promoter and searched if *bir-1* overexpression has potential to augment the expression of genes that showed decreased expression in worms treated by *bir-1* RNAi. Overexpression of *bir-1* resulted in a pronounced increase (2 to 5 times) of the expression of these genes. Our findings support the concept that BIR-1, a protein generally regarded as a mitotic factor, is involved in the regulation of transcription during normal development of *C. elegans* and has a strong potency to affect transcription of developmentally active genes if overexpressed.

In conclusion, these analyses indicate that thyroid hormone receptors are likely to be important active players in the regulation of transcription in astrocytic tumors. Our findings argue for often elevated expression of all forms of thyroid receptors. Although TRβ1 shows tendency to be downregulated in a majority of astrocytomas, in some cases it is strongly upregulated. We hypothetisize that such deregulated expression of thyroid receptors may be explained by incorporation of thyroid receptors in multiple pathways and mechanisms that may be further critically regulated by interaction of thyroid receptors with critical interacting proteins. Indeed, our search for the expression of selected proteins that may critically diverge the function of thyroid receptors found elevated and deregulated HDAC3, SKIP and Survivin.

2 INTRODUCTION

2.1 Glial tumors

Glial tumors, gliomas, are heterogenic group of brain tumors of presumed astrocytic, oligodenroglial and ependymal cell origin. Among these, devastating, diffusely infiltrating astrocytomas, are the most frequent intracranial neoplasms. They account for more than 60% of all primary brain tumors. The ability of astrocytoma cells to diffusely migrate throughout the brain is their hallmark and a rubust scientific and theraupeutic challenge. The incidence of glial tumors varies geographically but is usually among 5 to 7 new cases per 100,000 a year.

The World Health Organization (WHO) divides astrocytomas according to their histopathological features into four groups and variants (Kleihues and Cavenee, 2000) (table 1). The benign non-tumorous grouping or proliferation of glial cells is termed gliosis.

Pilocytic astrocytoma, grade I, seen in paediatric patients and young adults, is circumscribed, slowly growing and is classified as benign. Although if reccurencently present in hypothalamus it could be lethal.

Diffuse astrocytoma, glioma grade II, also termed low grade astrocytoma, is diagnosed when one criterion, ususally, nuclear atypia is present. This tumor has the potential to transform to anaplastic astrocytoma and glioblastoma. The usual range of survival is more than 5 years.

Anaplastic astrocytoma, grade III, usualy shows two criteria, most often nuclear atypia and mitotic activity. Survival is ranged from 2 to 5 years.

For Glioblastoma multiforme (GBM), grade IV, three criteria: nuclear atypia, numerous mitoses and endothelial proliferation and/or necrosis are typical. This tumor can develop from lower grade astrocytomas (secondary glioblastoma) in younger patients or directly after a short period of clinical history (primary glioblastoma) in older patients. Mutations of several genes participating on different genetic pathways were linked to specific features of both, primary and secondary GBM (tab.2).

The ablility of glioma cells to migrate through normal brain tissue, without disrupting brain cytoarchitecure, is a key attribute of low and high grade astrocytomas (Schrerer, 1940) and is not known in any solid tumor that metastasize to the brain. Glioma cell invasion bears conspicuous resemblance to the massive migration of glial and neuronal cells during embryogenesis (Hatten, 1999), thus implicating a reactivation of the mechanisms regulating early glial migration.

Angiogenic burst in GBM si likely conencted with the vascular endothelial growth factor (VEGF) and its receptors, VEGFR1 and VEGFR2, and angiopoietins, Ang1 and Ang2, and their receptor system. VEGF has been shown to be critical for the earliest stages of vasculogenesis, promoting endothelial cell proliferation, differentiation, migration, and tubular formation (Maher et al., 2001). Angiopoietin-1 (Ang1) promotes remodeling and stabilization of VEGF-induced vessels (Suri et al., 1996). On the contrary, angiopoietin (Ang2) destabilizes mature vessels. In the presence of VEGF activity, the Ang2 effect is followed by sprouting and ingrowth of new vessels (i.e., neovascularization) (Maher et al., 2001). This is tested as a new potential therapeutical approach. It is speculated that acquisition of EGFR activation may constrain, through a functional link to VEGF, the induction of angiogenesis in GBM.

WHO grade	**WHO designation**	**Histological criteria**
I	Pilocytic astrocytoma	
II	Diffuse astrocytoma	One criterion present, usually nuclear atypia
III	Anaplastic astrocytoma	Two criteria, usually nuclear atypia and mitotic activity
IV	Glioblastoma multiforme	Three criteria: nuclear atypia, mitoses, endothelial proliferation and/or necrosis

Tab. 1. The World Health Organization (WHO) grading system of astrocytomas (Kleihues and Cavenee, 2000).

Diffuse astrocytoma (WHO grade II)	**Anaplastic astrocytoma (WHO grade III)**	**Secondary GBM (WHO grade IV)**	**Primary GBM (WHO grade IV)**
p53 mutation amplification of **PDGFR-α** **gain of chromosome 7q** **amplification of chromosome 8q**	***p53*** mutations ***p16*** deletion ***RB*** alterations ***p^{14}ARF*** deletion ***CDK4*** amplification ***PTEN/MMAC1*** mutations **LOH on chromosome 10q and 22q** ***EGFR*** amplification **Deletion on chromosome 6**	***p53*** mutation Overexpression of ***PDGF-A***, ***and PDGFR-α*** **LOH 19q** ***RB*** alteration **LOH 10q** ***PTEN*** mutation ***DCC*** loss of expression ***PDGFR-a*** amplification	amplification, overexpression of ***EGFR*** amplification, overexpression of ***MDM2*** ***p16*** deletion **LOH 10p and 10q** ***PTEN*** mutation ***RB*** alteration

Tab. 2. Molecular patology of brain tumors. These basic molecular deviations were described in diffusely inflitrating astrocytomas. Mutations of several genes participate in the evolution of primary and secondary glioblastoma (modified from (Kleihues and Cavenee, 2000))

2.2 Transcription in glial tumors

Although histological appearance of these tumors might be in certain aspects similar, the intrinsic biology, mercilessly, dictates the course of the disease, the prognosis of patient. It is generally disccussed among cooperating specialists from different scientifical and clinical fields that molecular biology research in diffusely infiltrating astrocytomas is of immense importance, because neurosurgery, radiotherapy and chemoterapy, although, nowadays, tremendously advanced, haven´t changed the length of survival of malignant astrocytoma patients much for decades, since pioneering neurological surgeons`s Hurvey Cushing`s and Percival Bailey`s histological classification in 1926 (Bailey and Cushing, 1928).

Throughout the years, the monumental work on brain tumors shed more light on what, actually, happens on cellular and on subcellular levels. The components of cell regulatory and apoptotic pathways were found to be demaged in diffusely infiltrating astrocytomas. Growth factor pathways of PDGF and EGF are implicated in gliomagenesis and progression. EGFR is amplified in approx. 60% of glioblastomas and some anaplastic astrocytomas (Wong et al., 1987). Normally, the highest EGFR expression occurs during the embryonic and early perinatal period (Burrows et al., 1997). About 40% of GBM express truncated forms of EGFR, called EGFRvIII, deltaEGFR, or del2-7EGFR.

The p53 pathway is implicated in the development of glial tumors. Allelic loss of chromosome 17p and mutations of p53, primarily missense, are detected with the same frequency in diffuse, anaplastic astrocytomas and secondary GBM (Louis et al., 1993), suggesting that inactivation of p53 is an early event in gliomagenesis.

MDM2, a 54 kD protein, directly inhibits p53 transcriptional activity. p53 induces transcription of MDM2 (Barak et al., 1994; Zauberman et al., 1995). This negative feedback loop regulates the activity of the p53 protein and expression of MDM2.

The Retinoblastoma gene, *RB1*, located at 13q encodes the 107-kD retinoblastoma protein, pRB, a major regulator of the cell-cycle progression. It is mutated in approx. 25% of HGA (Henson et al., 1994).

p27 regulates progression from the G1 to the S phase by directly inhibiting cyclin E-dependent kinases and indirectly inhibiting cyclin D-dependent kinases (Sherr, 2000).

G1 cyclin-dependent kinase family members, CDK4 and 6 are targeted in gliomas. The CDK4 gene is amplified 10- to 100-fold in about 15% of HGG (Nishikawa et al., 1995; Reifenberger et al., 1994). In some tumor cases without amplification of CDK4 or loss of RB, CDK6 amplification has been observed, thus suggesting that CDK4 and CDK6 are functionally substitutable in gliomagenesis (Costello et al., 1997).

Other genes inplicated in gliomagenesis are p15 and p16, cell cycle regulatory proteins, coded by genes *CDKN2A* and *CDKN2B*, respectively, residing on chromosome 9p21, a site that is significantly associated with interstitial and homozygous deletions in HGA (James et al., 1999). The hypermethylation of the CpG islands in 5` region of the *CDKN2A* and *CDKN2B* genes is another mechanism which inhibits CDKN2A and CDKN2B and resulting transcription silencing (Costello et al., 1997; Herman et al., 1996).

$p14^{ARF}$ is a tumor suppressor, that directly binds to MDM2 and blocks the degradation of p53, leading to its stabilization. $p14^{ARF}$ enhances apoptosis in a p53-dependent manner (Kamijo et al., 1997; Pomerantz et al., 1998; Zhang et al., 1998) induces G1 and G2 phase

cell-cycle arrest (Kamijo et al., 1997; Quelle et al., 1995) and blocks oncogenic transformation (Pomerantz et al., 1998).

Loss of heterozygosity (LOH) of 10q is found in 75% - 90% in HGG (Fults and Pedone, 1993; Hata et al., 2006)

Mutations of PTEN, the tumor supressor are found in anaplastic astrocytomas and both primary and secondary glioblastomas (Fan et al., 2002a; Ohgaki and Kleihues, 2007).

Mxi1 is a candidate tumor supressor gene located on 10q. It is a member of the Mad (Mxi1) family of proteins, which are strong antagonists of Myc dependent oncoproteins in vivo(Schreiber-Agus and DePinho, 1998; Schreiber-Agus et al., 1998).

DMBT1 (deleted in malignant brain tumors) is another putative tumor suppressor gene located on 10q and has been found deleted in 50% - 80% of anaplastic astrocytomas and GBMs (Brat et al., 2004; Brat et al., 2007; Fan et al., 2002b).

19q chromosome is demaged by allelic losses in 40% of HGA tumors (Henson et al., 1994). The putative tumor supressor gene has not been identified yet, although three interesting genes ANOVA (Ueki et al., 1997), a protein serine threonine phosphatase gene (Yong et al., 1995), and the EHD gene (Pohl et al., 2000) are residing there. Although mutational analyses of these genes have not discovered deletions or mutatations so far.

LOH of chromosome 22q occurs in 20%-30% of gliomas of all grades (Fults et al., 1990; James et al., 1988), proposing the presence of a tumor suppressor gene involved in the early stages of gliomagenesis.

Recently Maléndez and coworkers used comparative genomic hybridization (CGH) on cDNA microarrays and detected overexpression of novel amplified genes SLA/LP, a gene described as Soluble liver antigen/liver pancreas antigen and STIM2, Stromal interaction molecule 2, located on 4p15, and TNSF13B, a gene for Tumor necrosis factor superfamily member, 13b, and COL4A2, a gene coding for collagen type IV, alpha 2 (13q32-34). It was speculated that deregulation of these genes could be important in the development and progression of GBM (Ruano et al., 2006).

2.3 Regulation of transcription by NHRs

Regulation of gene transcription is critical for proper development, growth, tissue maintenance and metabolism. Two major regulatory levels of transcription are the level of modification of chromatin proteins and the level of assambly of the Pol II transcription complex. Another regulatory level is based on chromatin organization.

NHRs are ligand-inducible transcription factors. They can directly interact as monomers, homodimers or heterodimers with the retinoic X receptor (RXR) with DNA response elements (RE) of target genes or by interacting with different signaling pathways.

The large nuclear hormone receptor (NHR) superfamily is subdivided according to the sequence alignment and phylogenetic tree into 6 distinct subfamilies (Nuclear Receptor Nomenclature Committee, 1999; (Escriva et al., 2000; Germain et al., 2006). In humans, 48 NRs were identified along with the sequencing of the human genome, and 24 have been found to be liganded receptors (Germain et al., 2006).

NHR`s typical modular structure consists of 5 to 6 domains termed A to F, from the N- to the C-terminal end. These domains have high homology among different NR with regions of conserved sequence and function. The DNA-binding domain (DBD, region C) and the ligand-binding domain (LBD, region E), containing the transcription activation function 2 (activation function 2, AF-2), are the most important and most highly conserved domains. The variable N-terminal A/B domain, containing a transcriptional function 1 (activation function 1, AF-1) and the D region are less conserved. The C-terminal F region, adjacent to the E domain, is not part of all receptors and its role is insufficiently uderstood (Germain et al., 2006).

The NHR superfamily includes the thyroid hormone and steroid receptors, retinoic acid, vitamin D receptors and members which may have no ligand or their ligands are not known yet. These receptors are classified as „orphan“ receptors (Aranda and Pascual, 2001; Beato et al., 1995). Recently, ligands for some of these receptors were detected („adopted orphans“), demonstrating that products of lipid metabolism like fatty acids, leukotrienes, prostaglandin and cholesterol derivatives, bile acids, pregnanes or even benzoate derivatives may bind to nuclear receptors and thus regulate gene transcription. Some of these chemical substances are products of intracellular metabolism. This partially explains why these ligands had remained elusive longer then classic hormones and also it suggests that some ligands still may not have been discovered. Other receptors` ligands are still not known or may not exit („true orphans“). Also they may act in a constitutive mode or could be activated by different ways, for example

by phosphorylation mediated by hormones and growth factors that stimulate various signal transduction pathways (through receptor thyrosine kinases, RTK) (Aranda and Pascual, 2001).

NHRs are ligand-inducible transcription factors. They can directly interact as monomers, homodimers or heterodimers with the retinoic X receptor (RXR) with DNA response elements (RE) of target genes or by interacting with different signaling pathways.

The transcriptional regulation by NHRs is mediated through interactions with coregulator molecules, coactivators or corepressors (surveyed in section 1.5). Corepressors are co-players in multisubunit regulatory complexes. Some corepressors mediate histone deacetylase activity (Lazar, 2003). Deacetylation results in chromatin condensation and is connected to transcriptional repression. When the ligand is bound to the LBD of the receptor, the receptor`s conformation is changed enabling the recruitment of coactivator complexes. Some of these are chromatin remodeling factors or present histone acetylase activity while others may interact straightly with the basic transcriptional machinery. Upon recruitment of coactivator complexes to the target promoter the chromatin unwinds, allowing transcriptional activation.

The orphan receptors along with research of new agonistic ligands for classical ligand-inducible transcription factors, compose important targets for drug discovery. NHRs` success as a drug target is stressed by the widespread use of retinoic acid for RARα, targeted in acute promyelocytic leukemia, the synthetic antagonist tamoxifen for estrogen receptor alpha (ERα), targeted in breast cancer, dexamethasone for glucocorticoid receptor (GR) targeted in inflammatory diseases, or thiazolidinediones for peroxisome proliferator-activated receptor gamma (PPARγ) targeted in type II diabetes (Bernal, 1999).

2.4 TRs and RXRs

Thyroid hormone receptors (TRs, class I, NR1A) and Retinoid X Receptors (RXRs, class II, NR2B) are members of the nuclear hormone receptor superfamily (NHR). The ligand for thyroid hormone receptors is the thyroid hormone (TH, 3,5,3`-triiodothyronine) and for RXRs it is the 9-cis-retinoic acid.

Thyroid hormone has a cardinal function in differentiation, growth, and metabolism. TH has principal impact on the developing brain in utero and during the neonatal period (Bernal, 1999). Thyroid hormone receptors are the cellular homologs of the viral oncogene product *v*-

erbA. In the family of NHRs, Thyroid receptors are not only the closest to retinoid receptors, but also use the Retinoid X receptor as a dimerization partner. Retinoid receptors regulate fundamental developmental events such as the body plan (lateral symmetry) and limb development (Lohnes et al., 1995; Mendelsohn et al., 1992). Thyroid receptors, as most Nuclear Hormone Receptors are potent activators of transcription, but may also, in absence of the ligand or in a mutated form, as it is in the case of v-erbA, be critical transcription repressors (Alenghat et al., 2006).

Thyroid receptors are coded by two separate genes. The *THRA* gene located on the 17th chromosome encodes for TRα and *THRB* on the 3rd chromosome encodes TR. Two major TRα isoforms, TRα1 and TRα2, are formed by alternative splicing of the initial TRα RNA transcript. TRα1 bind T3 and mediate TH-regulated gene expression. TRα2, also c-erbAα-2, does not bind a TH because a 122- aa carboxy terminus replaces a region in TRα-1 that is critical for TH binding. TRα2 also binds TREs weakly but is not able to transactivate TH-responsive gene expression and may act as an inhibitor of TH action possibly by competing for binding to TREs. There are also two TRβ isoforms, TRβ1 and TRβ2, originating by the use of the alternate promoter in the *THRB* gene. These isoforms vary in N-terminal region, otherwise they are identical. Both bind TREs and TH with high affinity and specificity and are able to mediate TH-dependent transcription (Velasco et al., 2007; Yen, 2001).

TRs heterodimerize with Retinoid X Receptors (RXRs). RXRs are members of the NHR superfamily bearing high homology to RARs (Kliewer et al., 1992). 9-cis-retinoic acid, the ligand of RXRs, is bound with high affinity to the receptors (Levin et al., 1992). There are three RXRs genes: RXRα, RXRβ and RXRγ (Chambon, 1996) . TR/RXR hterodimerizing broadens the spectrum of genes that can be regulated by T3 (Diallo et al., 2007).

The T3-mediated transcritptional activation of target genes can be enhanced by increasing the phosphorylation level of cells (Lin et al., 1992). Yen, speculates in his review, that the mechanism for this enhanced transcritpional activation could involve phosphorylation of TR, RXR, or coactivators (Yen, 2001). It has been shown that TR can be phosphorylated in vitro and in vivo (Glineur et al., 1989; Glineur et al., 1990). The human TRβ1 can be phosphorylated in vivo and in vitro but the phosphorylation site was not determined (Yen, 2001). Phosphorylation by protein kinase A can decrease v-erbA and chick TRα-1 monomer binding to TREs. TRβ1 can interact with MAPK protein and the liganded receptor can enhance phosphorylation of itself by MAPK (Davis et al., 2000; Lin et al., 2003; Shih et al., 2001) . It was shown that phosphorylation modulates rapidly DNA binding by TRα2. The

increased dominant negative action of TRα2 is observed in non-phosphorylated status, so that phosphorylation may serve as a powerful switch modulating the expression of T3-responsive genes (Katz et al., 1995; Xu and Koenig, 2005). Ligand-independent gene transcription was seen in neuroblastoma (Pastor et al., 1994). TRα2, similarly as TRα1, is an RNA binding protein using the same 41 amino acid segment distal to the second zinc finger as its RNA binding domain. The unique C-terminal end of TRα2 contains multiple (potentially 9) sites for phoshorylation by protein kinase CK2. CK2 dependent phosphorylation of TRα2 abolishes its RNA binding. Unphosphorylated TRα2 is localized predominantly in the nucleus and phosphorylated TRα has cytoplasmic localization (Xu and Koenig, 2005).

2.5 Cofactors and transcription machinery

The cofactors (also termed coregulators), coactivators or corepressors, play a crucial role in the regulation of transcription by nuclear receptor/accessory protein complex. The ways how they do it remain in many aspects unclear.
Unliganded TRs can repress basal transcription by interacting directly with TFIIB, a key component of the basal transcription machinery (Baniahmad et al., 1993; Petty, 1995; Tong et al., 1995)

Corepressors

The first identified transcriptional corepressors were a 270-kDa protein called nuclear receptor corepressor (NCoR), identified by Rosenfeld nad collegues (Horlein et al., 1995) and Silencing Mediator of Retinoid and Thyroid Receptors (SMRT), first identified by Evans and collegues (Chen and Evans, 1995). NCoR and SMRT bear structural resemblance, are large molecules that bind to NRs in absence of the ligand and contain autonomous, transferable repression domain (Lazar, 2003). NCoR/SMRT, can interact with other repressors, such as Sin3, TBL1, GPS2, HDAC1, 2, 3, 4, 5 and HDAC7. Further proteins with corepressor function for NHRs are Alien (Dressel et al., 1999), Hairless (Potter et al., 2001), LCoR (Fernandes et al., 2003), and SUN-CoR (Zamir et al., 1997) and RIP140 (Steel et al., 2005).

Coactivators

The steroid receptor coactivators, SRC family members, are coactivators connected with regulation of transcription by NHRs. SRC1 also known as NCoA-1 is also, among others, interacting with RXRs. Another TR coregulator is SRC2, known as GRIP-1 in mouse and

TIF-2/NCoA-2. Although SRC1 and SRC2 bear about 50% amino acid homology in carboxyl terminus and their functions are partially overlaping, they also regulate different pathways. The third member of the SRC family is SRC3 expressed higly also in hipocampus and olphactory bulbs. The mouse homolog is named p/CIP and human isoforms are designated RAC3, ACTR, AIB-1, and TRAM. SRC3 is also interacting with RXRs (Li and Shang, 2007; Moore and Guy, 2005).

Thyroid Hormone Receptor-associated Protein (TRAP) complex is a large multisubunit complex that is a general coactivator for different transcription factors (Ito and Roeder, 2001). TRAP220 is an important member of this complex.

Peroxisome Proliferator-activated Receptor-g Coactivator-1 (PGC-1) has tissue-specific expression and has been linked with adaptive thermogenesis and hepatic gluconeogenesis and other important physiological pathways (Herzig et al., 2001; Yoon et al., 2001).

Thyroid Hormone Receptor-binding Protein (TRBP), alternatively PRIP, is mentioned in literature mentioned as a general coactivator interacting with different transcription factors beside NR.

p300/CBP are able to link NR and transcriptional machinery and possess histone acetyltransferase activity therby may modify chromatin for gene transcription. Other coactivators of TR are the Androgen Receptor Activator 70 (ARA70), Trip1/Sug1, RAP46/BAG-1, E6-AP and TLS (Moore and Guy, 2005).

2.6 The regulatory network of Thyroid receptors

Regulation of expression by nuclear hormone receptors is involved in major developmental pathways of Metazoan species (McKenna et al., 1999). Retinoid receptors are part of regulatory cascades in limb, heart, liver as well as brain development. Thyroid hormone is indispensable for mammalian brain development. v-erb-A, the viral form of thyroid hormone receptor is a powerful oncogene contributing to tumor transformation in avian erythroblastosis (Yen, 2001).

The role of thyroid receptors in brain development and function suggests its possible involvement in biology of brain tumors. Members of nuclear hormone receptor family were detected in cell lines originating from brain cells (Magrassi et al., 1993). T4-5 deiodinase was also detected in human brain tumors (Mori et al., 1993). Depletion of thyroid hormone inhibits proliferation of astrocytoma cells in vitro via an induction of p21 (WAF1/CP1) (Toms

et al., 1998). On the other hand, thyroid hormone induces expression of mitogenic growth factors, acidic and basic fibroblast growth factor (aFGF and bFGF), TNF-beta and TGF-beta (Trentin et al., 2001). Alien is a correpressor that is under the regulation of thyroid hormone in rat brain (Tenbaum et al., 2003). Thyroid hormone upregulates protein synthesis in astrocytes (Trentin and Alvarez-Silva, 1998) and ecto-5'-nucleotidase/CD73 (Wink et al., 2003) in C6 glioma cells which can serve a proliferation signal. Suppression of thyroid function reduces IGF-1 levels in glioma patients and enhances sensitivity to tamoxifen as is linked to increased survival of glioma patients (Hercbergs et al., 2003). The levels of T3 and T4 and activities of iodothyroinine deiodases were found inhibited in human glial tumors (Nauman et al., 2004).

In pituitary, thyroid hormones regulate negatively expression of beta subunit of TSH (Shupnik et al., 1986) (Shupnik and Ridgway, 1987). This regulation is executed on both transcriptional and posttranscriptional levels (Shupnik and Ridgway, 1987; Staton and Leedman, 1998) and such negative regulation may be the basis for TR regulation also in brain tumors. T3 regulates negatively the expression of TRH in the hypothalamus (Yamada et al., 1989). Several promoters including TRH were shown to contain negative response elements (nTRE) (Iwasaki et al., 1996). These negative response elements include sites that bind most likely TRs as monomers (Darling et al., 1989) suggesting that imbalance in the expression of TRs and their heterodimerization partners may contribute to negative regulation of gene expression. T3 also affects negatively polyadenylation of TSHβ mRNA and its stability (Leedman et al., 1995).

Expression of specific cofactors, T3-dependent correpressors may be another possibility for TR mediated transcription repression in brain tumors, that, however, remains undocumented. In this part of study we attempted to characterize expression of major TRs` and RXRs` isoforms and selected putative interacting proteins, SKIP, Survivin and HDAC3 in human diffusely infiltrating astrocytomas. HDAC3 gave straightforward data that were submitted for publishing and are separately included in section 5.2. Basic survey on TR´s and RXR's is presented in section 1.4.

The *THRA* gene (NT_010755.15) is localized on chromosome 17, spans 31858 bp and contains 10 exons. 16 possible transcripts are deposited in public databases. Two major isoforms originate from this gene by alternative splicing of last two exons. A 5-kb *THRA1* mRNA encodes a 410-amino acid protein and a 2.7-kb *THRA2* mRNA encodes a 490-amino acid protein.

The *THRB* gene (NT_022517.17) is localized on chromosome 3, spanning 377409 bp and contains 18 exons. 20 possible transcripts are deposited in public databases. Two known isoforms of *THRB* gene formate by use of alternate promoter in *THRB* gene. TRβ1 protein contains 461 aminoacids and its molecular weight is 52788 Da. The TRβ2 protein consists of 476 amino acids and has molecular weight of 54356 Da (ExPASy database, 2007).

The *RXRA* gene (NT_019501.13) resides at 9q34.3, spans 114822 bp and contains 12 exons. 18 possible transcripts are deposited in public datatbase. Only one isoform is known so far.

The *RXRB* gene (NT_007592.14) is located at 6p21.3, spanning 7868 bp and contains 9 exons. 9 transcripts are listed in NCBI database. SwissProt lists 2 isoforms, long and short, emerging by alternative splicing, further isoforms seem to exist.

The *RXRG* gene (NT_004487.18)is located at 1q22-q23, spans 44881 bp and contains 12 exons. NCBI database lists 7 transcripts and SwissProt retreive only one isoform.

Survivin is a member of the inhibitors of apoptosis protein family (IAP) that regulates cell division and inhibit apoptosis in mammals. Survivin (also BIRC5; NT_010641.15) gene is located on cromosome17q, spanning 12240 bp containing 5 exons. About 35 possible transcripts are listed in databases.

SKIP, SKI-interacting protein, is splicing cofactor and transcriptional coactivator on several cellular and viral promoters. Its gene, (NT_026437.11), highly conserved among species, is located on 14q and codes for 536 aminoacid protein of molecular weight of 62 kD found in many tissues. SKIP was originally identified using 2-hybrid screen as a protein interacting with SKI oncoproteins what is considered as important fact for SKI`s transforming activity. This interaction interface, found centrally within locus of aminoacids 171-353 , is called SNW domain. SKIP interacts with pRb and by cooperation with SKI is able to overcome pRb induced transcriptional repression. SKIP plays an distinct role in transformation activity of v-Ski and EBNA2 (Epstein-Barr virus encoded latency protein). Combination of SKIP and SKI can successfully overcome G1 arrest. It is necessary for EBNA and NotchIC activation of CBF-1 repressed promoter. Ski is a protooncogene found in genome of an avian acutely transforming retrovirus. Cellular Ski binds DNA in a mammalian nuclear extract, in associacion with other proteins and regulates transcription.

2.7 Enzymes as effectors in transcription regulation

Cancer growth and behavior, in addition to genetic changes that induce loss or gain of gene function, depends on a cancer specific transcription profile. The behavior of cancer cells is fundamentally affected by a wide range of repressed genes and other sets of upregulated genes that form together a cancer-specific transcriptom (Kioussis and Festenstein, 1997; Wolffe and Matzke, 1999). Gene transcription is regulated at several levels. Genomic regions may be excluded from transcription by organizing chromatin in such a way to make it inaccessible to transcription factors and co-factors. The cell lineage depends in part on this transcriptionally inaccessible chromatin (Higgs et al., 2006). Gene expression further depends on general and cell- and tissue-specific transcription factors and co-factors.

In addition to the formation of sterically active or repressive transcription complexes, the transcription complexes bind enzymes that modify chromatin proteins. Molecular regions of histones H2A, H2B, H3 and H4 are enzymatically modified. These posttranslational modifications include acetylation, phosphorylation, methylation, ubiquitination, biotinylation and other modifications (Nightingale et al., 2006). Non-histone proteins (including constituents of transcription complexes) are also often enzymatically modified (Fu et al., 2004). Enzymatic modifications may dramatically alter protein-protein interactions, the stability of the DNA-nucleosome structure and the accessibility of transcription regulatory proteins to DNA. Acetylation of lysines predominantly localized at the N-termini of nucleosomal histones H3 and H4 is generally associated with transcriptionally active chromatin (Jenuwein and Allis, 2001). Transcription factors, such as nuclear hormone receptors associated with their agonistic ligands, bind co-activators displaying histone acetylation activity or binding histone acetyl transferases (HATs) that directly acetylate lysines of nucleosomal histones. Acetylation can be enzymatically reversed by histone deacetylases (HDACs), which are often constituents of repressive transcription complexes. Transcription-repressive complexes that include nuclear hormone receptors in their unliganded status bind nuclear hormone receptor co-repressors SMRT and NCoR; these co-repressors bring histone deacetylases to the promoters of regulated genes and function as repressors (Chen et al., 2005; Heinzel et al., 1997; Zamir et al., 1996)

To date, two major protein families possessing histone deacetylation activity were identified. The first family includes HDAC class I and II (de Ruijter et al., 2003) and the second HDAC class III, a Sir2 family of NAD^+-dependent protein deacetylases, called sirtuins (Grubisha et al., 2005). HDAC class I consists of HDAC1, HDAC2, HDAC3 and

HDAC8, which are most closely related to *Saccharomyces cerevisiae* transcription co-factor RPD3 and are involved in the regulation of transcription in the majority of vertebrate tissues. HDAC class II consists of HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10 and is closely related to *Saccharomyces cerevisiae* histone deacetylase HDAC1. Members of HDAC class II are more tissue-restricted, compared to members of class (de Ruijter et al., 2003). Sirtuins differ from HDACs class I and class II in catalysis of the reaction in which NAD^+ and an acetylated substrate are converted into a deacetylated product, nicotinamide, and O-acetyl ADP-ribose. This highly conserved mechanism is used in the regulation of chromatin organization, gene expression, cell cycle regulation, apoptosis and ageing (Grubisha et al., 2005). Members of all classes of HDACs were shown to participate in both targeted and non-targeted genome-wide deacetylation of histones.

Several lines of evidence indicate that enzymes involved in histone acetylation and deacetylation (HATs and HDACs) participate in the establishment of cancer specific transcription regulation. Chromosomal translocations and re-arrangements lead to the formation of genes coding for fusion proteins that interact with HATs and mistarget them to promoters of proliferative genes (Iyer et al., 2004). Nonfunctional HATs are created in Rubinstein-Taybi syndrome, which is linked to various malignances (Eckner, 1996; McManus and Hendzel, 2001; Petrij et al., 1995). Translocations 15/17 results in fusion proteins with PML, where RARα functions as a repressor (Lin et al., 2001). The acetylation status of chromatin can be increased by inhibition of HDACs by small molecules, such as trichostatin A, valproic acid, suberoylanilide hydroxamic acid (SAHA), and butyrate, which interfere with HDACs' enzymatic activity. Inhibition of histone deacetylases by HDAC inhibitors was shown to reverse several characteristics connected with the malignant phenotypes of cancers. TSA increases the radiosensitivity of glioblastoma cell lines (Kim et al., 2004). Inhibition of histone deacetylase activity by the HDAC inhibitor FK228 induces apoptosis and suppresses cell proliferation of human glioblastoma cells *in vitro* and *in vivo*. Sodium butyrate and TSA inhibit vascular endothelial growth factor (VEGF) secretion from human glioblastoma cells in cell cultures (Sawa et al., 2004; Sawa et al., 2002). Another inhibitor of HDAC activity, 4-phenylbutyrate, modulates expression of glial fibrillary acidic protein and connexin 43 and enhances gap-junction communication in human glioblastoma cells (Asklund et al., 2004).

Searches in the ESTs have revealed an approximately a 3-fold increase in incidence of most HDACs in brain tumors. HDAC3 has been found 3.1 times more often in brain tumors compared to non-tumorous brain tissue (de Ruijter et al., 2003). HDAC3 mediates the

repressive function of unliganded thyroid hormone receptors (Ishizuka and Lazar, 2003; Lazar, 2003) and can be bound to the thyroid hormone receptor in a ligand-independent manner, through the interaction with cyclin D1 (Lin et al., 2002). HDAC3 is also co-immunoprecipitated with HDAC4, 5 and 7 and forms a complex together with SMRT and NCoR (Guenther et al., 2001) .

In this work, we studied the expression of HDAC3 in glial tumors and benign glial tissues on the mRNA, protein and cell levels. We show that the expression of HDAC3 is elevated in glial tumors. High-grade gliomas (grade III and IV) also expressed more isoforms of HDAC3 compared to non-malignant tissues. While HDAC3 was found in normal glial cells and non-malignant gliosis exclusively as relatively weak and uniform nuclear/cytoplasmic staining, high grade astrocytomas showed focally deregulated expression, consisting of strong cytoplasmic HDAC3 expression. Confocal microscopy showed co-localization of HDAC3 in nuclei visualized by DAPI in all examined tumors. Thus, elevated expression of HDAC3 may be indispensable for the growth of malignant human glial tumors.

2.8 Multipartite molecular interactions regulate transcription and development

Regulation of gene transcription is critical for proper development, growth, tissue maintenance and metabolism. The molecular execution of this process depends on a coordinated assembly of the polymerase II (Pol II) basic transcription machinery consisting of general transcription factors (GTFs, TFII A, B, D, E, F and H) and a multi-subunit Mediator complex. This highly dynamic complex comprising up to 30 proteins mediates connection of the basic Pol II transcription complex with transcription factors that recognize specific regulatory sequences in gene promoters - response elements (Malik et al., 2005; Roeder, 2005) and transcription cofactors, a diverse group of proteins that interact with transcription factors. Tissue and metabolic state-specific transcription depends not only on the actual presence of transcription factors (TFs), but also on stimuli coming from the cell membrane and the organism. Posttranslational modifications and spatially restricted availability of transcription factors and cofactors translate to activated or repressed transcription. The ability of proteins to form complexes that activate or repress gene-specific transcription is complemented by modifications that lead to the removal of interacting proteins and the disintegration of protein complexes.

Transcription cofactors form a complex protein network that enhances or represses transcription and are designated depending on the identified role in transcription as coactivators or corepressors. Transcription cofactors may function as activators or repressors depending on the cellular and molecular context and on the structure of the gene promoter.

SKIP, the SKI- interacting protein, is an evolutionarily highly conserved transcription and splicing cofactor affecting transcription regulation by Notch, TGFβ and nuclear receptors in metazoan species. It was previously shown that *C. elegans* SKIP (CeSKIP, *skp-1*) is indispensable for normal embryonic and larval development and has an overlapping developmental phenotype with CHR3 (NHR-23) a nuclear receptor that regulates embryonic and larval development (molting and larval transitions) (Kostrouchova et al., 2002). Surprisingly, *skp-1* is organized in an operon together with *bir-1*, a homologue of the human Survivin (Fraser et al., 1999; Speliotes et al., 2000). Inhibitor of apoptosis proteins (IAPs) proteins were originally identified in studies of baculoviruses as proteins able to inhibit apoptosis (Birnbaum et al., 1994; Crook et al., 1993). Contrary to other IAPs, BIR-1 and Survivin are small proteins consisting of two main domains, a BIR domain and a small helical region (Chantalat et al., 2000; Muchmore et al., 2000; Verdecia et al., 2000). Both regions are likely to mediate interactions with other proteins. Survivin was first shown, in accordance with the function of IAPs, to prevent apoptosis through its ability to bind and inhibit caspases (Ambrosini et al., 1997). Surprisingly, the most prominent function of both Survivin and BIR-1 is the regulation of mitotic events, spindle formation and chromosome segregation (Fraser et al., 1999; Li et al., 1998; Speliotes et al., 2000). Survivin has been found to regulate the stress response and to interact with the Hsp90 protein (Fortugno et al., 2002)

Since *bir-1* is expressed together with SKIP (*skp-1*) in non-dividing cells we search for functional connections between these two proteins. Approximately one third of *C. elegans* genes are organized in operons that often bring functionally linked genes under the regulation of a common promoter (Blumenthal et al., 2002). *bir-1* inhibition induces phenotypes that are part of loss of function phenotypes of CeSKIP (*skp-1*) and CHR3 (*nhr-23*) (Kostrouchova et al., 2003b). It was also shown that *bir-1* inhibition is connected to a decrease in the expression of several transgenes as well as endogenous genes and a decrease of the phosphoacetylated histone H3. In a heterologous transfection system, *bir-1* expression increases the level of phosphoacetylated histone H3 and activates transcription from thyroid hormone- regulated promoters. Its effect is potentiated by the co-expression of human SKIP.

In this study, we searched for genes affected by *bir-1* inhibition during the L1 larval stage using whole genome microarrays. We identified several developmentally important

collagen genes as *bir-1* transcriptional targets. Genes whose expression is repressed in *bir-1* inhibited cultures are strongly activated by the forced expression of *bir-1* in transgenic lines expressing *bir-1* from heat shock-regulated promoters. These data support the involvement of BIR-1 in transcription regulation during normal development of *C. elegans* and indicate that non-physiological levels of *bir-1* are able to dramatically affect the transcription profile.

3 HYPOTHESIS AND AIMS

In addition to specific mutations which cause loss of function of tumor suppressor genes and gain of function of cancer promoting genes, cancer biology depends on cancer supporting transcription profile. Thyroid and retinoid receptors are major differentiation regulatory transcription factors involved in regulation of differentiation. Since thyroid hormone is a critical factor for brain development, we decided to study the expression and function of thyroid receptors in glial tumors. The aim of this project was to characterize expression pattern of thyroid hormone receptors and selected putative cofactors in glial tumors and characterize their function.

4 EXPERIMENTAL DESIGN AND DEVELOPMENT OF THE PROJECT

Focused attempt to characterize the expression of TRs and RXRs in human glial tumors was carried out.

I developed cooperation with the Department of Neurosurgery of the 1st Faculty of Medicine, Charles University and Central Military Hospital in Prague and established a routine for collection of samples obtained during intracranial operations. I was personally present at most operations, participated on collecting samples in a way that was ensuring that only material which would not be used for any other examination was donnated for this study. The informed consent of the use of the bioptic material for scientific purposes was obtained from each patient prior to the study in accordance and with the approval of the Ethics Committee of the Central Military Hospital, Strešovice, Prague and the Ethics Committee of the 1st Faculty of Medicine, Charles University and Faculty Hospital.

We identified the presence of TRs/RXRs in diffusely infiltrating astrocytomas and benign gliosis. We observed that the expression pattern was strikingly deregulated, with varying levels of expression and in some tumors considerably elevated. There was indication

of a tendency of elevated levels of TRα2, TRα1, RXRα and RXRγ while TRβ1 was slightly decreased.

This led us to conclussion that thyroid receptors and RXRs, are likely to be part of cancer biology, as modified proteins or based on interaction with proteins which have potential to diverge their function.

In search for such proteins, we have focused on SKIP, the evolutionarily conserved cofactor of nuclear receptors, Survivin, which is functionally linked to SKIP in *C. elegans* (Kostrouchova et al., 2002; Kostrouchova et al., 2003a) and HDAC3, which is known to be able to bind to thyroid receptors in absence of the ligand through the interaction with Cyclin D1 and inhibit thyroid hormone dependent transcription (Lin et al., 2002). HDAC3 expression was significantly elevated in malignant tumors and we decided to study its expression in detail. The study confirmed that HDAC3 is elevated and deregulated on cellular level in malignant astrocytomas.

Survivin and SKIP were found deregulated and elevated as well. We decided to prolongate a study earlier done in our laboratory that showed that BIR-1, the *C. elegans* orthologue of human Survivin, regulates transcription and development. In order to visualize the BIR-1 transcriptional function in non-dividing cells, we performed a genome wide analysis of *bir-1* loss of function and identified numerous collagen and ribo somal genes as its targets. Subsequently, we showed that genes that were repressed by *bir-1* inhibition are dramatically induced by its overexpression in transgenic animals.

I conducted or participated on most experiments described in this study with the exception of microarray experiments. The project described in this thesis was part of the greater project of the whole research group.

5 MATERIALS AND METHODS USED IN THIS THESIS

5.1 Methods used in work addressing the expression of TRs, RXRs and SKIP, Survivin in glial tumors

Biopsy samples

For this study, biopsy samples from 32 patients who had undergone therapeutic surgery for epileptic refractory lesions or brain tumors at the Department of Neurosurgery of the 1st Faculty of Medicine, Charles University and Central Military Hospital in Prague were used. This collection included 5 cases of benign gliosis obtained from patients surgically treated for refractory epilepsy, 9 cases of astrocytma grade II and 18 high grade astrocytomas (17 of grade IV and one of grade III). Where possible, part of the material was frozen immediately after surgical removal and stored in liquid nitrogen. An informed consent of the use of the bioptic material was obtained from each patient prior to the study in accordance and with the approval of the Ethics Committee of the Central Military Hospital, Strešovice, Prague, and the Ethics Committee of the 1st Faculty of Medicine, Charles University and Faculty Hospital. Parallel formaldehyde-fixed paraffin-embedded (FFPE) biopsy material was processed for histopathologic examination and the diagnosis was established at the Department of Pathology, Central Military Hospital, Strešovice, Prague. Histopathologic diagnoses were established in accordance with WHO classification (Kleihues and Cavenee, 2000). All glial tumors included in this study were gliomas of astrocytic origin.

The frozen material was used for the preparation of frozen non-fixed sections in a Leica CM 1850 cryostat machine and haematoxylin-eosin-stained sections and biochemical methods.

Extraction of nucleic acids and preparation of protein lysates

Sections of the frozen material were cut and collected in at least two pre-chilled eppendorf tubes (20 to 40 sections/tube). One tube was used for extraction of total RNA using RNA STAT 60 (Tel-Tex, Houston, Tx) and the second tube was adjusted with 2x Laemmli buffer and a cocktail of protease inhibitors (Boehringer Mannheim, Germany) as recommended by the manufacturer. The quality of total RNA stained by ethidium bromide was controlled using agarose gel electrophoresis.

Reverse transcription

Five μg of total RNA was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Ca) primed by random hexamers as recommended by the producer.

Real-Time PCR

Quantitative PCR was performed similarly as described by Sun et al. (Sun et al., 2004) with modifications. The amplified fragments were eluted from agarose gels using electrophoresis and a semi-permeable membrane. The amount of DNA was determined spectrophotometrically. The purified DNA was used to determine the standard curves for each amplified region. Real-time PCR was preformed in PTC0200 DNA EngineR Thermal Cycler equipped with ALS0296 96+Well Sample Block and the DyNAmoTM HS SYBR Green qPCR Kit, which contains a hot start version of a modified Thermus brockians DNA polymerase to prevent extension of non-specifically bound primers during the reaction setup. The characterization of amplification included calculating the number of copies in samples and melting point temperatures, using the computer program Opticon MonitorTM Version 3.0. Each sample was analysed at least two times. The number of copies was expressed in relation to the total RNA used for reverse transcription. The expression of beta tubulin was determined in each sample with these primers: TUBB: gatccgggaagag and ccgtgtctgacaccttgggt. The primers were taken from the RTPrimerDB: Real Time PCR Primer and Probe Database (http:// medgen.ugent.be/rtprimerdb/index.php). The amplified fragments were sequenced directly using ABI Prism automated sequencer. (kindly done by Ms. Helena Myskova in the sequencing facility of the Institute of Inherited Metabolic Disorders).
Coding sequences of TRs and RXRs were retrieved from Genbank. Sequences designated by accession numbers were: 1/ TRα1: X55005; 2/TRα2: NM_003250; 3/TRα common: X55005 common region for TRα1 and TRα2; 4/TRβ1: BC106930; 5/TRβ2: X74497; 6/TRβ common: BC106930 common region for TRβ1 and TRβ2; 7/RXRα: NM_002957; 8/RXRβ: NM_021976; 9/RXRγ: NM_006917

The primers for the amplification of specific regions for convetional and real-time PCR were designed using Primer premier software. The primers were (listed our laboratory code: 5` to 3` sense primer; and lab code: 5` to 3` antisense primer):
1/ TRα1: 05/073: tcaaccaccgcaaacacaa; 05/074: gtggggcactcgactttc
2/ TRα2: 05/119: cgtcaaccaccgcaaacac; 05/120: gagacttcccgcttcacca

3/ TRα common: 05/038: tccgacgccatctttgaa; and 05/039: tgtttgcggtggttgacg

4/ TRβ1: 05/032: gcccagaccttccaaa; 05/068: ttcctggcactgatttcgc

5/ TRβ2: 05/123: cggttgtgatgctcaggct; 05/124: ccttttttttgagaatacgatggc

6/ TRβ common: 05/046: aaaatgggggtcttgggg; 05/047: caggctcctatcatccgca

7/ RXRα: 04/055: gcttccttcaccaagcaca; 04/056: ctggtcgactccacctcatt

8/ RXRβ: 04/057: ctgaagatgtgaagccac; 04/058: ctgatagcggcagtactgacag

9/ RXRγ: 04/059: agtgtcagcagttcagaggaca; 05/007: catgacaaggcacttctgata

10/ Surivivin: 06/015: accaggtgagaagtgaggga; 06/016: aacagtagaggagccaggga

11/ SKIP: 05/063: tcacagaaggtcgccgc; 05/064: ctccttgctgagatggtgtg

Western blot analysis

Western blots were performed using a standard protocol: samples were mixed with 2xLaemmli sample buffer (Laemmli, 1970) and 10x protease cocktail to reach 1x Laemmli sample buffer concentration and boiled for 7 min. Protein concentration was estimated using BCA Protein analysis kit (Pierce, Rockford, IL) as recommended. Thirty to 120 ug of protein were loaded for each sample, separated by electrophoresis and blotted onto a nitrocellulose membrane using a Mini Protean II apparatus (Bio-Rad, Hercules, CA). Remaining gels were stained by Coomassie blue. The membranes were then incubated in 1% Tween 20 in phosphate-buffered saline pH 7.4 (T-PBS) containing 5% (w/v) dried non-fat milk overnight at 4^{o}C.

The primary antibodies used in this study were:

anti TRα1 mouse monoclonal IgM (04/027, a kind gift from Onno Baker);

anti TRα1 mouse monoclonal IgG (cat.no. sc-740, Santa Cruz Biotechnology);

anti TRα1 rabbit polyclonal IgG (cat.no. sc-772, Santa Cruz Biotechnology);

anti TRα2 mouse monoclonal IgM (04/028, a kind gift from Onno Baker);

anti TRα2 goat polyclonal IgG (cat.no. sc-10821, Santa Cruz Biotechnology);

anti TRβ1 mouse monoclonal IgM (45/03, a kind gift from Onno Baker);

anti TRβ1 mouse monoclonal IgG (J52, cat.no.sc-738, Santa Cruz Biotechnology);

The primary antibody was diluted 1:100 to 1:5 000 in T-PBS containing 5% milk (w/v) and the membranes were incubated for 1 h with this primary antibody at room temperature, washed six times in T-PBS for a total duration of 1 h and incubated with secondary antibody (against mouse IgM, mouse IgG, rabbit IgG and goat IgG) labelled by horseradish peroxidase

(Sigma) diluted 1: 6 000 to 1: 20 000 in T-PBS for 1 h at room temperature and washed six times for a total of 1 h. After wash in T-PBS, the secondary antibody was visualized using ECL-Plus chemiluminescent system (Amersham, Pharmacia, Uppsala, Sweden) or Supersignal purchased from Pierce.

Immunohistochemistry

Standard FFPE tissue sections (7 μm) were dewaxed and hydrated according to the standard protocols. The mouse monoclonal IgM anti TRα1 (in our lab marked 04/027) and mouse monoclonal IgM anti TRα2 (marked 04/028) antibodies used for detection of TRα1 and TRα2 immunoreactive proteins were diluted 1:200 in AntibodyDiluent (DAKO, Glostrup, Denmark). Secondary detection was performed using the horseradish peroxidase-labeled anti-rabbit IgG antibody, diluted 1:500, and visualized by DAKO DAB system . Nuclei were stained by Weigert's haematoxyllin.

Transfections

Cell cultures. U373 cells (obtained from ATCC) were cultured in Dulbeco Minimal Essential Medium supplemented with 10 % fetal calf serum and Gentamycine 10 μg/ml. For experiments testing hormone induction, the fetal calf serum (FCS) was deprived of thyroid hormones as described (Kostrouchova et al., 2003b).

Transfection experiments were done in 24 well plates. Cells were plated at 4.10^3 per well and transfected 16-18 hr later using FuGENE transfection reagent (Roche, Indianapolis, IN). Plasmids, ME-TRE – Luciferase (used as a reporter) and mRXRα and rat TRα1 were kind gift from Dr. K. Ozato and Dr. P. Yen. The transfection assay was performed using the Dual Luciferase kit (Promega, Madison, WI) with CMV-Renilla luciferase as an internal control. The FB 12 (Berthold, Bundoora, VIC, Australia) was used for quantification. Triiodothyronine was desolved in DMSO/ethanol and used at concentration 10^{-6} mol/l. The solvent was used in control experiments.

5.2 Methods used in work addressing the expression of HDAC3 in glial tumors

Biopsy samples

For this study, biopsy samples from 45 patients who had undergone therapeutic surgery for epileptic refractory lesions or brain tumors at the Department of Neurosurgery of the 1st Faculty of Medicine, Charles University and Central Military Hospital in Prague were used for this study. This collection included 35 glial tumors, four non-malignant gliosis removed for therapeutic purposes, one case of suspected glioma tissue (histologically classified as normal tissue but later a glioma was found in the vicinity of the previously removed tissue), two meningeomas, two oligodendrogliomas and one lymphoma.

Extraction of nucleic acids and preparation of protein lysates

Procedure fully described above in section 4.1

Reverse transcription

Procedure fully described above in section 4.1

Amplification and characterization of HDAC3

Sequences of HDAC3 isoforms were retrieved form Genbank. Four transcripts were chosen from transcripts available in Genbank (April 10, 2006), three identical in CDS to isoforms originally reported by Yang et al. (1997) and an N-terminally deleted isoforms. Sequnces are represented by accession numbers:

1/ AF039703.1 (CDS identical to U75697, originally denominated main isoform of HDAC3);

2/ U75696.1 originally denominated HDAC3 isoform A (HDAC3A);

3/ AF005482.1 originally denominated HDAC3 isoform C (HDAC3C);

4/ AF130111.1 (a short isoform potentially coding for an N-terminally deleted protein) that we name isoform D (HDAC3D).

Since there are other nomenclatures of HDAC3 isoforms that recognize up to 13 transcripts (ACEview) and this study was not aimed at full characterization of expression of human HDAC3, we used the nomenclature of HDAC3 isoforms in keeping with the original

description by Yang and coworkers (Yang et al., 1997) and added a new name for the N-terminally abrogated isoform as isoform HDAC3D. The primers for the amplification of specific regions of HDAC3 isoforms were (listed
5' ->3` as the sense and the antisense primers):
1/ gttcatcttgtgtctccatcccga (HDAC3 sense)
2/ catgtgccgcttccactccgagg (HDAC3, HDAC3A, HDAC3C sense)
3/ ccaggatgccaatcacaatgtcgt (HDAC3, HDAC3A, HDAC3C antisense)
4/ ttgctccttgcagagatgcgcct (HDAC3, HDAC3A, HDAC3C antisense)
5/ gcattgacccatagcctggtcct (HDAC3, HDAC3A sense)
6/ gcctcagttacacatccaaactctaca (HDAC3C sense)
7/ gaagtccactacctggttgataac (HDAC3, HDAC3A, HDAC3C, HDAC3D antisense)
8/ aaactcgagccactcttaaatctccacatcg (HDAC3, HDAC3A, HDAC3C, HDAC3D antisense)

The primers for quantitative PCR were: 1/ HDAC3 (all four isoforms): ccgaaatgttgcccgctgctg and aggtgcatggttcagcatctt; 2/ HDAC3 + HDAC3A: acccatagcctggtcctgc and aggatgccaatcacaatg; 3/ HDAC3 + HDAC3A + HDAC3C: gtgccgcttccactccga and aggatgccaatcacaatg; 4/ HDAC3D: ccgcctcagttacacatcca and tcatcaatgccatcccgc

The expression of glycerol-phosphate dehydrogenase, beta tubulin and beta actin was determined in each sample with these primers:
1/ GAPDH: ccgtctagaaaaacctgcc and gccaaattcgttgtcatacc
2/ TUBB: gatccgggaagag and ccgtgtctgacaccttgggt
3/ ACTB: ccatcatgaagtgtgacgtgg and gtccgcctagaagcatttgcg
The primers were taken from the RTPrimerDB: Real Time PCR Primer and Probe Database (http:// medgen.ugent.be/rtprimerdb/index.php).
The amplified fragments were sequenced directly using ABI Prism automated sequencer

Western blot analysis

The primary anti-HDAC3 antibody H3034 (Sigma, St. Louis, MO) was diluted 1:1000 in T-PBS containing 5% (w/v) milk and the membranes were incubated for 1 hr with this primary antibody at room temperature, washed six times in T-PBS for a total duration of 1 hr and incubated with the goat anti-rabbit antibody labeled by horseradish peroxidase (Sigma) diluted 1:2000 in T-PBS for 1 h at room temperature and washed six times for total of 1 h.

After the wash in T-PBS, the secondary antibody was visualized using ECL-Plus chemiluminescent system (Amersham, Pharmacia, Uppsala, Sweden) or Supersignal purchased from Pierce.

Films were exposed from 10s to 1 hr and developed using an automated developer and analyzed using NIH Image (Image J) program available on http://rsb.info.nih.gov/ij/.

Histological analysis

Standard FFPE tissue sections (7 μm) were dewaxed and hydrated according to the standard protocols. U373 glioblastoma cells (a kind gift from prof. Alexi Šedo) were grown in DMEM containing 10 % FCS and gentamycine (100 μg/ml) in 5 % CO_2 humidified atmosphere. For histology, the cells were cultured in glass chambers as described (Mandys and Elleder, 1980) and fixed in 4 % paraformaldehyde for 5 min, cold acetone for 2 minutes, and stored dry.

Immunohistochemistry

The rabbit polyclonal anti-histone deacetylase 3 H3034 antibody (Sigma) used for detection of HDAC3 immunoreactive proteins was diluted 1:2000 in AntibodyDiluent (DAKO, Denmark). Secondary detection was performed using horseradish peroxidase labeled anti-rabbit IgG antibody, diluted 1:500, and visualized by DAKO DAB system Nuclei were stained by Weigert's haematoxyllin.

Immunofluorescence

HDAC3 primary antibody was diluted 1:1000, secondary detection used the goat anti-rabbit IgG antibody labelled by Alexa Fluor 488 (Invitrogen-Molecular Probes, Carlsbad, CA.) diluted 1:500 in TBS pH 7.6. For fluorescent detection, the nuclei were stained by DAPI (4'6-diamidino-2-phenylindole) diluted 1:1000 and added at the end of the procedure before mounting for 2 minutes. The staining protocol was the same for tissue sections and cultured cells.

Bright field, epifluorescence and laser scanning confocal 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. Complete available area of each sample was assessed. Three areas were selected for subjective evaluation of HDAC3 positivity. One area was selected for the highest staining pattern, one for the lowest staining pattern and one selected randomly. Each area was observed using a 40x objective, and all cells in the field scored for subjective positivity of HDAC3 staining in the bright field and epifluorescence illumination. Standard barrier filter sets were used for DAPI and Alexa Fluor 488 visualization. Nikon Eclipse E400 microscope with Cool Snap (RS Photometrics, Ontarion, NY) camera was also used for subjective evaluation of a complete set of samples by a second histologist.

A Nikon Eclipse TE2000-E microscope with C1*si* confocal head, Apo TIRF 60x (N.A. 1.49) objective and appropriate 450+/-15 and 515+/-15 band pass filter sets was used for confocal image acquisition. The image sampling density (*xyz*) was corrected to conform to the Nyquist criterion. The acquisition settings were kept constant for all image acquisitions except for a limited number of sections that had too weak or too intensive DAPI staining. The setting for HDAC3 recording was kept constant throughout the experiment. The overall evaluated volume from each sample was: four areas of 50 μm^2 (*xy*) were analyzed in six consequitive 150 nm *z* steps with the maximum intensity plane in the middle. Sixteen cases that were available in paraffin- embedded sections and were also present in the collection examined biochemically were evaluated in three separate locations as complete *z* stacks consisting of 40 stacks averaged 10 times for both channels. The efficiency of used filter sets to prevent spectral cross-talk of DAPI and Alexa Fluor 488 was checked using the spectral mode and the linear unmixing algorithm of C1*si*.

Co-localization map construction

Co-localization maps using single pixel overlap coefficient values (in the range 0-1) (Manders et al., 1993) were calculated using Huygens Proffesional Software (SVI, Hilversum, The Netherlands) from representative maximum intensity *z* level *xy* sampled dual channel images. The overlap coefficient values were scaled with a lookup table (LUT) as demonstrated in Figs. 9 and 10. Prior to this analysis, non-specific background was subtracted from the DAPI channel of the images, and the images were again checked for potential

spectral cross-talk. The background was also determined from the DAPI-stained sections that were processed without the primary antibody.

5.3 Methods used in the study addressing the transcription role of BIR-1 in *C.elegans*

Strains

The *C. elegans* Bristol N2 strain was used whenever not specifically stated and were maintained as described (Brenner, 1974). The strains SU93- *jcls 1*[*ajm-1: :gfp; unc-29(+)*; *rol-6(su1006*)] expressing AJM: :GFP transgene (Mohler et al., 1998) and JR667- *unc-119 (e2498: :Tcl); wls51* [*unc-119(+); scm: :gfp*] expressing the transgene in the nuclei of seam cells were kindly supplied by the *Caenorhabditis* Genetics Center.

The transgenic strain carrying the transgene *pnhr-23: : gfp* that is expressed in epithelial cells including seam cells was prepared as described (Kostrouchova et al., 1998). The lines expressing *bir-1* mRNA from heat shock-regulated promoters were prepared by amplifying *bir-1* cDNA from wild-type N2 worms,which was after subcloning and verification by sequencing re-cloned into the heat-shock promoter vector pPD49.83. Two independent transgenic lines were generated by microinjecting 100 ng/ul of plasmid DNA and a marker plasmid pRF4, *rol-6* (*su1006*) using an Olympus IX70 inverted microscope equipped with a PC-10 Narishige Microinjection system (Narishige, Tokyo, Japan, and Olympus, Prague, Czech Republic).

RNA-mediated interference (RNAi)

The constructs for *bir-1* RNAi were prepared as described (Kostrouchova et al., 2003b). The coding region lacking the ATG sequence was amplified, cloned in pCR4-TOPO (Invitrogen, Carlsbad, CA), confirmed by sequencing and re-cloned in the L4440 vector (a kind gift from Dr. A. Fire, Stanford University). The ssRNA was prepared from linearized DNA by *in vitro* transcription reactions using T3 DNA-dependent RNA polymerase (DNA digested by *Sma*I) and T7 DNA dependent RNAs were mixed polymerase (DNA digested by *Xba*I) (Promega, Madison, WI). Both sense and antisense RNAs were mixed together at 68°C for 10 min and 37°C for 30 min. dsRNAi was purified by phenol-chloroform extraction, precipitated with ethanol, and the pellet was diluted in water to an approximate concentration of 2 μg/μl. The dsRNAs for RNAi were injected into the gonad of adult hermaphrodites as

recommended (Mello et al., 1995) and embryos of microinjected animals were scored for *bir-1* loss-of-function mitotic phenotypes.

Large scale RNA interference

For large-scale culture *bir-1* inhibition, the method of inhibition by feeding of bacteria producing dsRNA to the synchronized cultures was used. *bir-1* cloned in L4440 vector was transformed to HT1115 *Escherichia coli* and induced by isopropyl-D galactoside as described (Fire et al., 1998). The control cultures were transformed with L4440 vector carrying the 100bp long non-specific sequence and a linker sequence that were induced equally as plates used for RNA interference. Agarose plates were used instead of standard worm plates. The synchronized L1 larval cultures were obtained by harvesting embryos from wild-type hermaphrodites and synchronization by incubation without food overnight in 1xphosphate-buffered saline (PBS). The resulting L1 larvae were collected by centrifugation, the density estimated by counting the number of larvae in a defined volume under the microscope, and equal numbers of larvae were used for paired control-RNA inhibition experiments. The cultures were incubated at 22°C overnight and the developmental stages were determined. No delay in development was observed in any culture. The initial experiments were used to determine the amount of bacterial food that was set to last equally in experimental and control cultures just before the cultures were harvested. Remaining bacterial food was removed by washing the worms three times in water and cultures were left to digest the remaining bacteria during a 30-min incubation time. The resulting cultures were pelleted by short centrifugation at 4^{o} C and frozen. Aliquots of cultures were kept on new plates with induced bacteria for control of known *bir-1* loss-of-function phenotype that could be detected in later stages.

Total RNA isolation

Synchronized larval cultures of *C.elegans* were grown on 2% agarose-capped plates as described; at appropriate culture times, the plates were washed with water, larvae pelleted by short centrifugation at 4°C and frozen at -80°C. The frozen pellet was resuspended in 0,5 ml of resuspension buffer (0,5% SDS; 5% 2-mercaptoethanol; 10 mM EDTA; 10 mM Tris/HCl (ph 7,5) with 12,5 υl of proteinase K (20 mg/ml)). The mixtures were mixed by vortexing for 60 s and incubated for 60 min at 55°C. Proteins were separated by phenol-chloroform extraction and nucleic acids precipitated in ethanol in a final volume of 1350 υl. The 350 υl

of water soluble phase was mixed successively with 1 υl of glycogen (20 mg/ml), 35 ul of 10% NaOAc and 950 υl of cold ethanol. The samples were incubated for 30 min on dry ice and centrifuged for 20 min at 4°C at 10,000 *g*. The air-dried pellet was dissolved in water and treated with 1 unit of DNase (Promega) per 1 ug of total RNA for 30 min at 37°C. The DNase was inactivated and extracted by a second round of chloroform extraction and ethanol precipitation and the total RNA was resuspended in DEPC (diethyl pyrocarbonate)-treated autoclaved water. The quality of total RNA stained by ethidium bromide was controlled using agarose gel electrophoresis.

Reverse transcription

Five υg of total RNA were reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen) primed by random hexamers as recommended by the producer.

Microarrays experiments

The microarrays experiments were kindly done at The Miroarray Core Facility, NIDDK, by Dr. M.C. Cam and G. Poy. The standard protocol was used (http://microarray.niddk.nih.gov). The c.elegans whole genome array representing 22,500 transcripts according to GenBank Release 121 were purchased as re-annotated arrays from Affymetrix (Cat.No. 900383 *C.elegans* Genome Array)(www.affymetrix.com). The data from three independent experiments paired with controls were processed. One experimental set differed from all other data sets and was not used for analysis of data. The data were analyzed using the Statistical analysis program (ANOVA) and the Gene Spring computer program.

The data analyzed by the computer program were labelled as Present/Absent (P,A), Increased/Decreased (I, D), Not Changed (NC), Moderate Increase (MI) and Moderate Decrease (MD) Data were also printed as raw values recorded by the scanner, the P value of data and the P value of change and fold of the change were calculated. The data of selected genes were also evaluated manually by exporting the values to the Excel computer program (Microsoft) and analysed.

Quantitative PCR

The amplicons of two control transcripts, the large subunit of RNA Pol II, *ama-1*, and actin *act-1* and selected transcripts identified in the microarrays experiments were designed according to WormBase (Table 4). The selected regions were amplified using PCR, eluted from agarose gel using electrophoresis and the semi-permeable membrane, and the amount of DNA was determined spectrophotometrically. The purified DNA was used to determine a standard curve for each amplified region. Real-time PCR was preformed in PTC200 DNA EngineR thermal cycler equipped with an ALS0296 96-well sample block or PTC 200 Chrome 4 (Bio-Rad, Hercules, CA) and the DyNAmoTM HS SYBR Green qPCR Kit. The modified *Thermus brockians* DNA polymerase was used to prevent extension of non-specifically bound primers during reaction setup. The amplification, including the number of copies in the samples, was characterized using the computer program Opticon MonitorTM Version 3.0. Each sample was analyzed by at least three independent analyses. The number of detected copies was normalized according to *ama-1* and *act-1* expression

The amplification reaction was done in a final volume of 20 μl, containing 500 fmol primers and 250 ng of total RNA. The cycling consisted of an initial 15 min denaturation at 95°C, followed by 10 s denaturation at 94 °C, annealing at 59 °C for 30 s, extension for 30 s at 72 °C and consisted of 45 cycles. The melting curves were established by the incubation at 72°C for 10 min and successive denaturation by increasing the temperature from 72°C to 95°C by steps of 1°C for 3 s and final incubation at 72°C for 10 min.

Tab. 4. Primers used for quantitative PCR

gene	sense	5´→ 3´	antisense	5´→ 3´
dpy-2	05-069	gaaatcgcaaacgagtggg	05-070	tgcggatgcctgacaaaa
dpy-4	05-071	ccgccgtctgcttctca	05-072	gttgtaggtagaacgggcgg
dpy-7	05-074	ttggacgaggctcacgaa	05-075	attgttggttgtcggattgag
dpy-8	05-077	atgcgggatttctacgacg	05-079	cggaactgcgtcaccct
dpy-13	05-108	cgtcttctccgttatcgcc	05-109	gcagcatccctcgcatc
col-94	05-093	tcaggcttaccgcttcgt	05-094	tggtggtggggtgattggct
col-125	05-096	cctaccgcttcgttgcct	05-097	ggcagcaagcatcacatcc
col-144	05-099	tctcgttaccgcctctgct	05-100	cggaagatagagttgaatgggtt
col-166	05-102	ccagtctcgcttcttcctcttt	05-103	gcccacctgctttctaccct
col-167	05-106	cgccccaatgctctacaactac	05-107	gcgagaacttgagtgcgggtaa
ama-1	4684	ttccaagcgccgctgcgcattgtctc	4685	cagaatttccagcactcgaggagcgga
act-1	5293	atgtgtgacgacgaggttgccgc	5294	gctcattgtagaaggtgtgatgcc

6 RESULTS

6.1 The Expression of TRs, RXRs and Selected Putative Interacting Proteins, SKIP and Survivin, in Human Astrocytic Glial Tumors

6.1.1 The expression of TRs, RXRs, SKIP and Survivin at the mRNA level

In this study 32 biopsies of limited volume were analysed. Five samples were glial non-malignant tissues removed during therapeutic surgery, nine were low-grade astrocytomas and eighteen high-grade astrocytomas (grades III and IV). Part of the tissue was used for extraction of total RNA and reverse transcribed. The Real-time PCR method was optimalized firstly on U373 glioblastoma cell line. Secondly, the bioptical tissue had to be used for more accurate optimalization. Only the samples with melting profile characteristic for the specific amplification products were ranked. Values were normalized according to the expression of β-tubulin. Selected amplified sequences were directly sequenced and the specificity of the amplification confirmed.

Thyroid hormone receptors` cDNAs of α1, α2, β1 and β2 and cDNA`s of SKIP and Survivin were efficiently amplified from all examined cases. We observed a large diversity in the expression of selected genes in all groups of examined samples.

A trend, although not statistically significant assuming the null hypothesis, of increased TRα1 expression, especially in low grade astrocytomas, was observed. The TRα2 isoforms were found elevated in LGA. Obtained copy numbers suggests that TRα2 isoform is also the most transcribed isoform in astrocytomas. The region of TRα, common to TRα1 and TRα2, was found slightly eleveted in LGA and decreased in HGA.

Interestingly, a trend of TRβ1 expression was decreased with the severity of the disease (statistically significant, with 96% probability in student *t*-test, if controls compared against all tumors). A trend of TRβ2 expression was found, statistically not significant, elevated in astrocytomas. The region of TRβ common to TRβ1 and TRβ2 was decreased statistically significantly (95% of probability) in HGA although elevated in LGA.

RXRα, β and γ were found present in controls and tumors, with increased expression in tumors. RXRβ was the most expressed isotype. The increase of RXRγ was statistically significant ($P<0.05$) in high-grade astrocytomas.

The expression of SKIP and Survivin was found elevated in tumors. The connection of Survivin with cancer is widely documented and accepted. Also our results showed a trend of elevation in tumors (although statistically not significant in student t-test), with variable expression among studied cases.

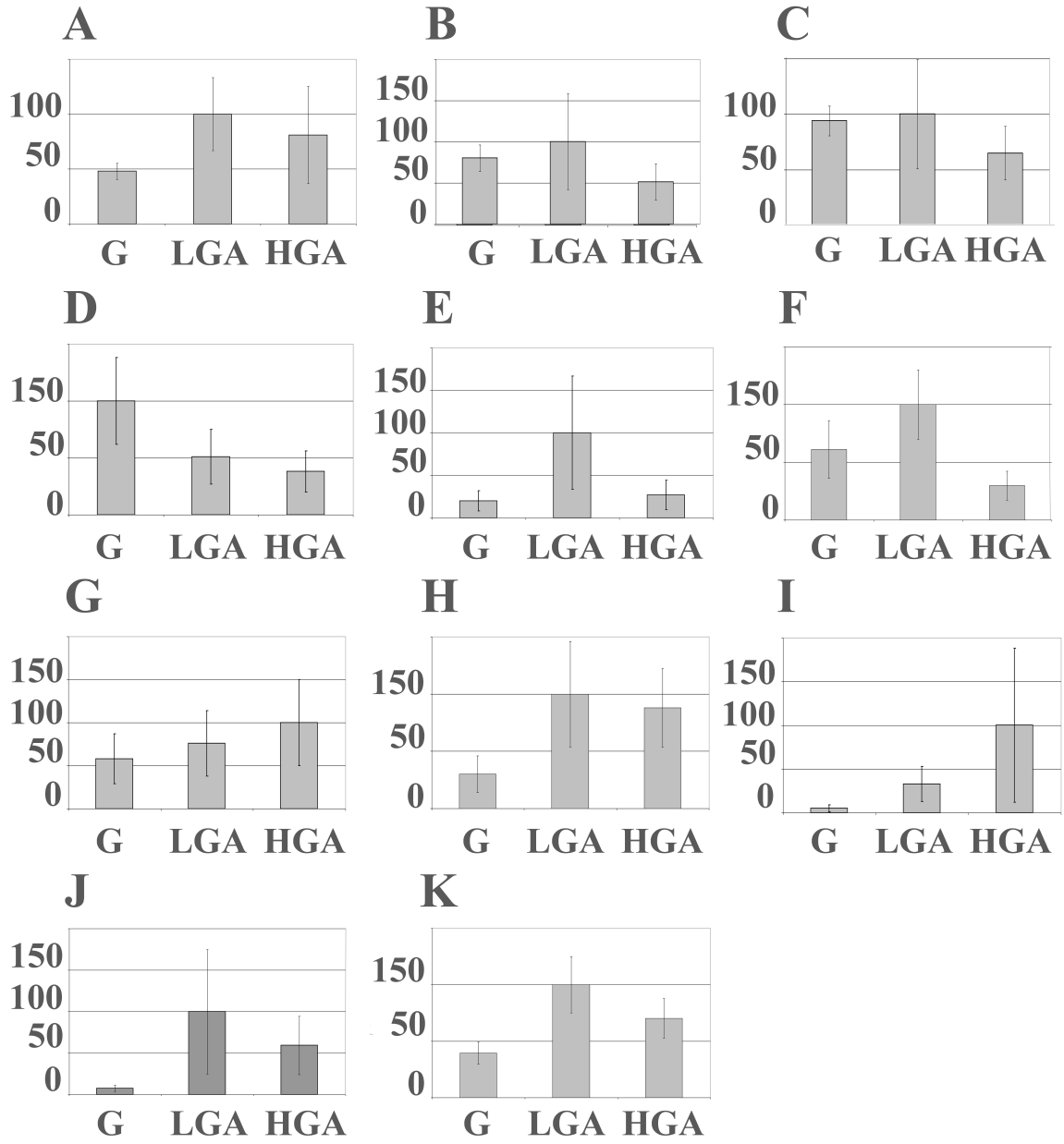

Fig.1. Quantitative PCR of TRs, RXRs, SKIP and Survivin in human astrocytic tumors. Average values given as percent of highest values found and standard deviations are shown for the group of glioses (G), low-grade astrocytomas (LGA) and high-grade astrocytomas (HGA). Values were normalized according to the expression of β-tubulin. Panel A is showing expression of TRα1; Panel B-TRα2; Panel C-TRα (the region common for TRα1 and TRα2);

Panel D-TRβ1; Panel E-TRβ2; Panel F-TRβ (the region common to TRβ1 and TRβ2); Panel G-RXRα; Panel H-RXRβ; Panle I-RXRγ; Panel J-SKIP, Panel K-Survivin;

6.1.2 The expression of TRα1, TRα2 and TRβ1 at the protein level

We prepared a collection of one non-malignant gliosis, three diffuse astrocytomas (WHO grade II), one anaplastic astrocytoma (WHO grade III) and three glioblastomas (WHO grade IV).

Different antibodies, commercial as well as antibodies obtained based on cooperation, were used. Interestingly, anti TRα1 monoclonal antibody (04/027, a kind gift from Onno Baker), detected multiple proteins with lower and higher molecular mass than predicted size of TRα1 (47,7 kD) (Tab. 3, Fig. 2A and B). Two low-grade samples, anaplastic astrocytoma and one glioblastoma showed a faint band between 42 to 60 kDa (Fig.2 Panel A). These and additional bands became strongly detectable in all cases after prolongated exposure period (Fig. 2B). Multiple immunoreactive bands were detected as well in U373 glioblastoma cell line (not shown). The result was compatible with the possibility of formation of multiple protein isoforms of TRα1, but can not be taken as support of such situation as unspecific interactions could not be excluded. We started experiments for direct mass spectroscopic characterization of proteins recognized by antibody.

Immunoreactive bands of expected size of TRα2 (50,7 kDa) were detected in all samples except one diffuse astrocytoma (case 9) and control tissue (case 19) (Fig. 2C).

A single band of expected size of TRβ1 (55 kD) was detected in all samples. This protein was expressed strongly in one anaplastic astrocytoma (case 11) in concordance with high TRβ1 expression at mRNA level in this case (Fig. 2D).

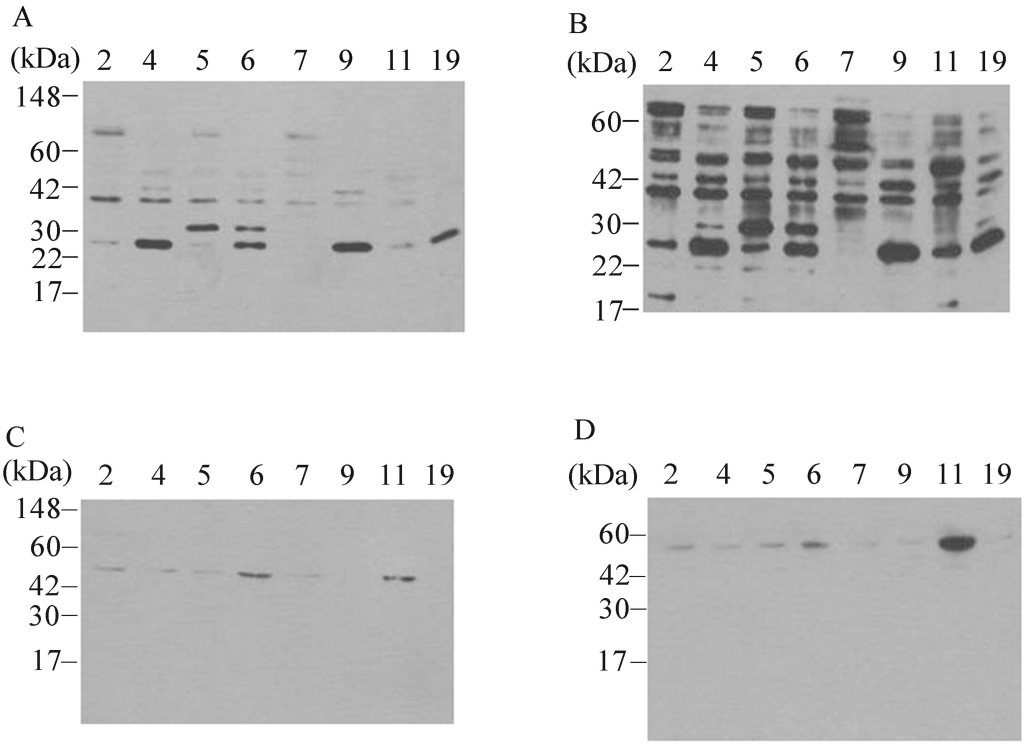

Fig. 2. Expression of TRα1, TRα2 and TRβ1 detected by Western blot analysis. Cases 2, 4 and 5 are GBMs. Cases 6,7, 9 are LGAs and case 11 is astrocytoma GIII. Case 19 is non-tumorous gliosis. Panel A shows multiple immunoreactive bands detected by antibody against TRα1. Cases 4, 6, 7 and 11 showed faint immunoreactive band between 42 to 60 kDa (Predected size of TRα1 is 47,4 kDa). Additional proteins smaller than expected size were seen; B – The same antibody against TRα1 after longer exposure time. C – The antibody against TRα2 revealed immunoreactive bands of expected size in all samples except cases 9 (LGA) and 19 (gliosis). D – All analysed cases showed band corresponding to expected TRβ1. The highest positivity was detected in case 3 (anaplastic astrocytma, GIII).

case	**6**	**7**	**9**	**11**	**2**	**4**	**5**	**19**	**U373**
histology	GII	GII	GII	GIII	GIV	GIV	GIV	gliosis	cell line
TRα1 47,7 kD	+,mb	+,mb	+,mb	+,mb	+,mb	+,mb	+,mb	+,mb	+,mb
TRα2 50,7 kD	+	+	-	+	+	+	+	-	not done
TRβ1 55 kD	++	+	+	++++	++	+	+	+	not done

Tab.3. The detection of thyroid receptors in human astrocytic tumors by Western blot analysis. TRα1, TRα2 and TRβ1 in gliosis, LGAs, HGAs and U373-MG
mb – multiple bands, ++++ - strong expression was observed in case 11, anaplastic astrocytoma.

6.1.3 The expression of TRα1 and TRα2 at the cellular level

Eight samples (the same as in WB) were examined by histochemistry using the peroxidase method. Monoclonal anti TRα1 (04/027) and anti TRα2 (04/028) antibodies were used (the same as for WB). One control material (case 3) was evaluated as benign gliosis under the reparative process, glioma was excluded by pathologist.

Interestingly, a strong and characteristic pattern was obtained in case of TRα2. It was found predominantly in the cytoplasm, although nuclear presence was also detected (Fig. 3B, D, F). The pattern did not differ between control tissue and tumors, but was considerably stronger in tumors (Fig. 3D and F).

The TRα1 immunoreactive proteins were detected abundantly in both, nuclei and cytoplasm (Fig. 3A, C and E), in all samples, but dramatically strongly in glioblastomas (Fig. 3A).

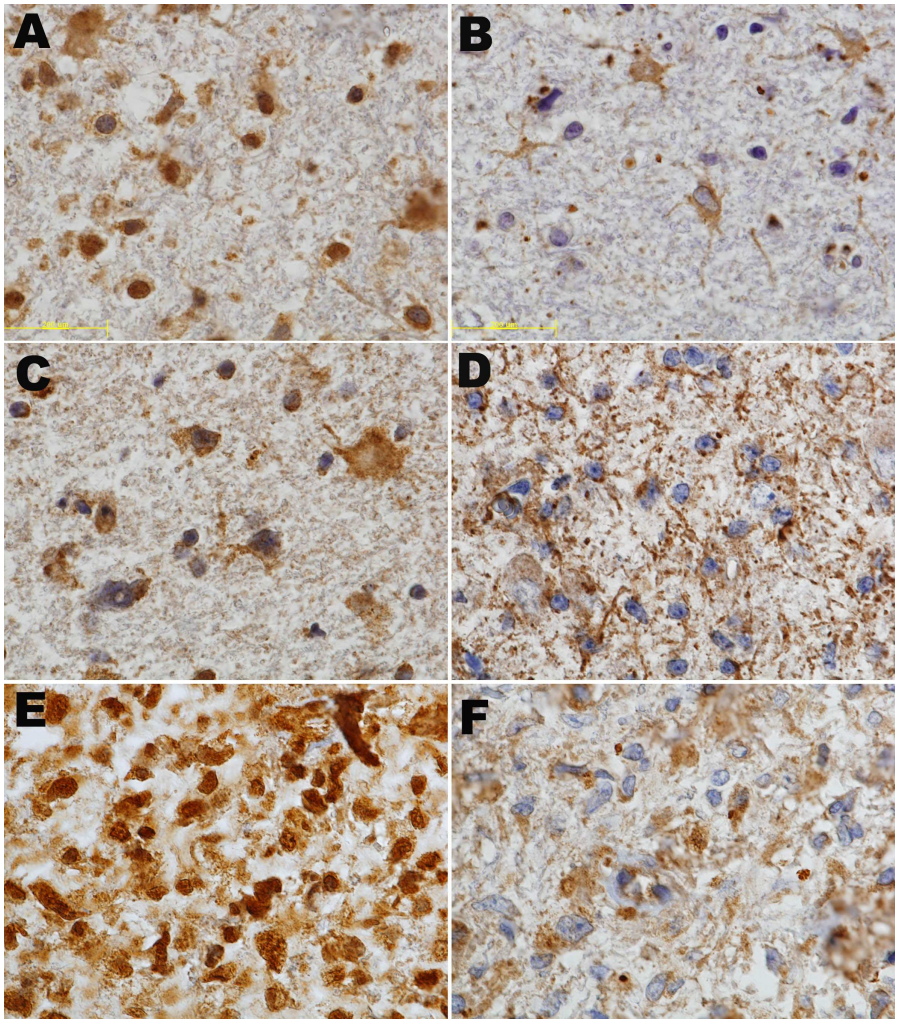

Fig.3. Immunohistochemistry. Panel A – TRα1 immunoreactivity was observed abundantly in cytoplasm and nuclei in bening gliosis. B – Interestingly, TRα2 immunoreactivity was observed predominantly in cytoplasm and was also seen in nuclei in benign gliosis. C - TRα1 immunoreactivity in low-grade astrocytomas is observed abundantly in cytoplasm and nuclei. D – strong TRα2 immunoreactivity in cytoplasm and vestigially in nuclei in the same tumor as in panel C (LGA). Panel E shows strong TRα1 positivity in cytoplasm and nuclei in GBM and panel F- TRα2 cytoplasmic immunoreactivity in the same GBM case.

6.1.4 Functional analysis of TRs in U373 glioblastoma cell line

We searched if thyroid hormone receptors found in the U373 cell line are functional in regulation of thyroid hormone responsive genes. We trasfected U373 cells with exogenous thyroid responsive promoter, vectors without an insert or vectors coding for thryroid receptor apha 1 and RXRα and assayed the effect of T3 on expression of the reporter that codes for fire fly luciferase. For the normalization of results, a control vector coding for Renilla luciferase was used. We did two independent 48 hours experiments. Treatment with thyroid hormone increased the transcription from thyroid dependent promoter approximately by 50%. Additional cotransfection with RXRα led to further increase of the expression to approximately 80% increase. In many cell lines, such expression of transcription from exogenous throid responsive poromoters is not visible. Our results indicate that endogenous thyroid receptors are functional in U373 cells. In some studies, it was proposed that estrogen receptors may also be influenced by thyroid hormone but these would certainly not be cooperative with additional RXRs as was observed in our experiments.

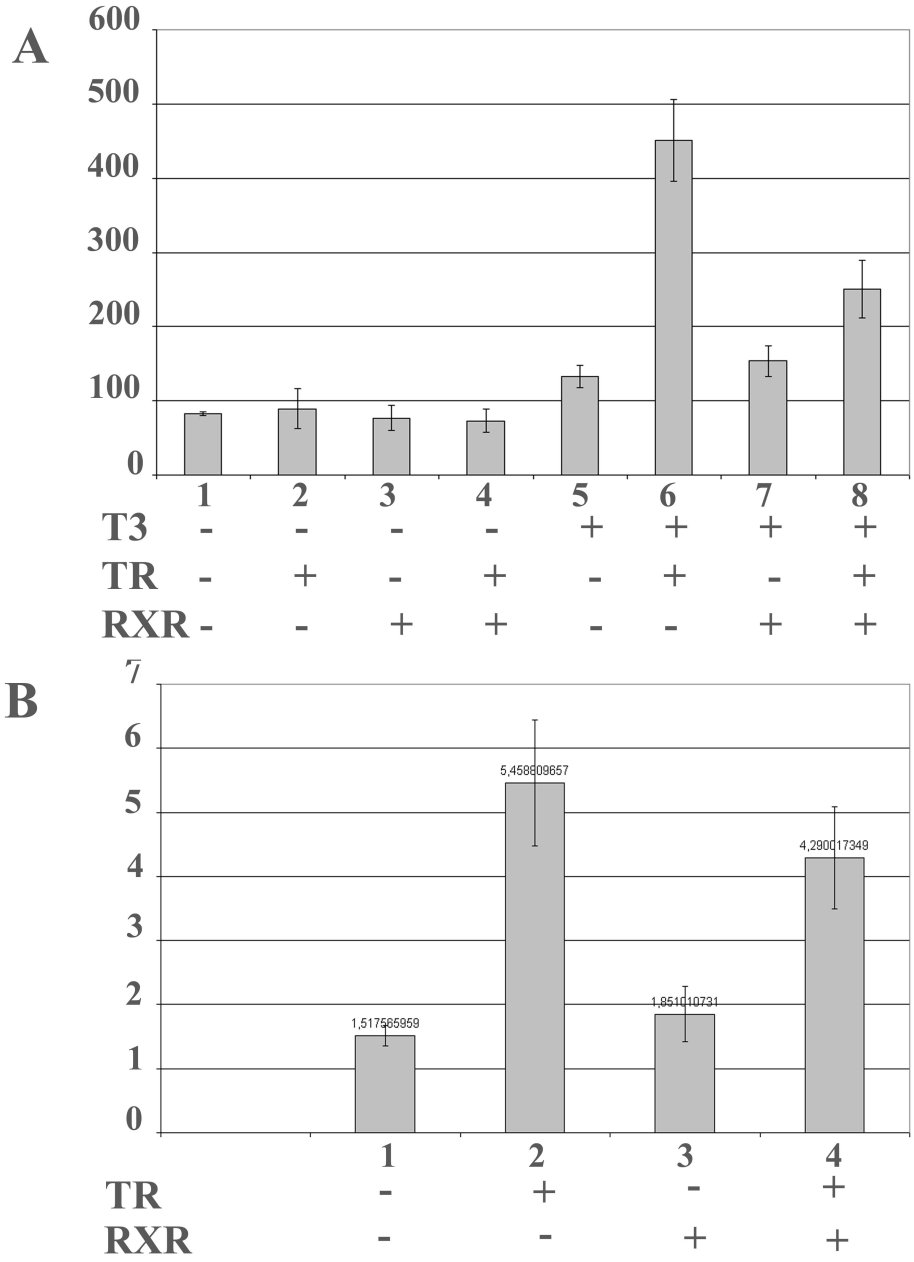

Fig.4. Functional analysis of thyroid hormone receptors in U373 glioblastoma multifome cell line. Panel A – Analysis of the expression of the exogenous reporter consisting of fire fly luciferase regulated by a thyroid hormone responsive promoter. Treatment with thyroid hormone (T3) and cotransfection with exogenous thyrodid hormone receptor alpha one or RXRα are indicated below the graph. Panel B - analysis of the inducibility of transcription from endogenous and exogenous thyroid receptors in U373 cells. Results are shown in fold expression – 1 represents 100% of the expression observed without T3.

6.2 Elevated and Deregulated Expression of HDAC3 in Human Astrocytic Glial Tumors

6.2.1 Characterization of HDAC3 expression in non-malignant glial tissues and glial astrocytic tumors at the mRNA level

The *HDAC3* gene (NT_029289.10), which is localized on chromosome 5, spans 16770 by and contains at least 15 exons. More than 380 partial sequences and 17 possible transcripts are deposited in public databases. There is evidence of existence of at least four types of HDAC3 transcripts that contain open reading frames (Fig. 5A). The HDAC3 isoforms deposited in databases can be represented by four sequences: the basic isoform, known as HDAC3 (Yang et al., 1997), which is represented in this work by AF039703.1 yielding a 1920 by long open reading frame coding for a 428 amino acid protein with a calculated molecular weight of 48.8 kDa; HDAC3 isoform A (U75696.1) that has a 1941 by long CDS, coding for a protein with 429 amino acids and a calculated molecular weight of 49.1 kDa; HDAC3 isoform C (AF005482.1) with 1981 by long CDS, coding for a protein with 371 amino acids and a predicted molecular weight 42.3 kDa; and a short isoform lacking the N-terminal half of the CDS, represented by AF 130111.1. For the purpose of this study, we named the shortest isoform HDAC3 isoform D.

The coding region of isoform D starts in the 7^{th} exon and uses the methionine that is present in all isoforms. The protein sequence derived from the open reading frame of isoform D is composed of 223 amino acids (calculated molecular weight 25.6 kDa) that form the C-terminal part of all remaining isoforms. Isoform D lacks the N-terminal sequence that was shown to be indispensable for histone-deacetylating function of HDACs (Zhang et al., 2005) (Fig. 5B).

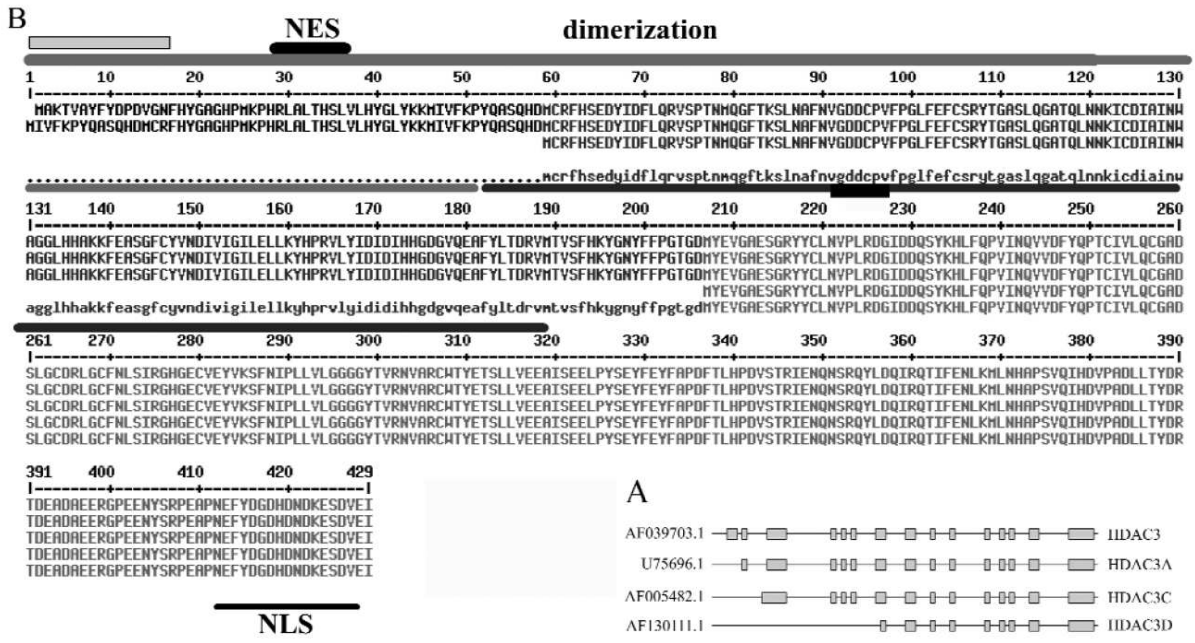

Fig. 5. A – Schematic representation of four isoforms of HDAC3 mRNA. Accession numbers and denomination used by Yang et al. (1997). B - Multiple alignments of proteins derived from mRNA shown in A. The sequences represent HDAC3, HDAC3A, HDAC3C and HDAC3D (from the top to the bottom). The bottom line shows the consensus calculated by the MultAline computer program. The sequence given in capital letters in the consensus line indicates the C-terminal part shared by all four isoforms. Note that HDAC3 and HDAC3A differ only in 15 or 16 N-terminal amino acids, respectively. HDAC3C starts at position 59 of HDAC3A and may differ in function since the N-terminal nuclear export sequence (NES) is missing in HDAC3C, but contains an additional nuclear export sequence (indicated by the dark bar in position 220-230) that is localized in the centre of the molecule. HDAC3, HDAC3A and HDAC3C may be expected to be functional deacetylases since both regions necessary for HDAC activity are present (Zhang et al., 2005; Zou et al., 2006). Contrary to that, HDAC3D lacks the N-terminal half of the molecule and is most likely a non-functional deacetylase. The C-terminus common to all four isoforms contains the nuclear localization signal (NLS).

We prepared a collection of four glial non-malignant tissues removed during therapeutic surgery, six low-grade gliomas and eleven high-grade gliomas (grades III and IV). Frozen sections were cut using a Leica Cryocut II apparatus and stored in eppendorf tubes. Total RNA was prepared and cDNA was made using Superscript II reverse transcriptase and random hexamers as primers.

Primers were designed to amplify HDAC3 and HDAC3A (from the start of a shared region to the stop codon), HDAC3C and HDAC3D. Similarly, primers for the amplification of the region common to all four isoforms were designed (Fig. 6A). Selected amplified sequences were characterized by direct sequencing and specificity of the amplification confirmed for all studied isoforms. Since the two longest isoforms are likely to be functionally similar if not identical, we searched for the expression of both HDAC3 and HDAC3A in all examined samples. PCR targeted to region B amplified the expected fragments from all examined samples and the specificity was confirmed by sequencing (Fig. 6).

Quantitative PCR directed at the region common to all four isoforms also amplified the expected fragments from all samples and the specificity of the amplification was confirmed by direct sequencing. For quantitative PCR, the standard curve for increasing number of copies per reaction was determined together with the assayed samples and the threshold set at the start of the efficient amplification. With the exception of one case that was histologically classified as grade II glioma, but was recurrent, all non-malignant glial tissues and grade II gliomas showed less than 10,000 copies per 150 ng of total RNA (Fig. 7A). Contrary to that, seven out of 11 gliomas of grade III and grade IV yielded more than 24,000 copies per 150 ng of total RNA (Fig. 7D). Interestingly, four of six examined gliomas of grade II had levels lower than those found in non-malignant tissue in 150 ng of total RNA.

Analysis of HDAC3 expression compared to the histological type of the examined tissues supported the elevated HDAC3 expression in high-grade gliomas (fig. 7B). Expression of HDAC3 followed the expression of two housekeeping genes, glycerol 3-phosphate dehydrogenase, and β-actin, in keeping with the possibility that HDAC3 elevated expression is part of an overall disturbed expression profile in glial tumors. Normalization against ß-tubulin alone followed the pattern of elevated HDAC3 expression in total RNA (Fig. 7C). The relative level of HDAC3 expression compared to β-tubulin also indicated elevated levels of HDAC3 in high-grade gliomas (Fig. 7D). A similar trend was observed for quantitative analysis of the expression of all three long isoforms or two longest isoforms (HDAC3 and HDAC3A), indicating expression of multiple isoforms of HDAC3 in high-grade gliomas.

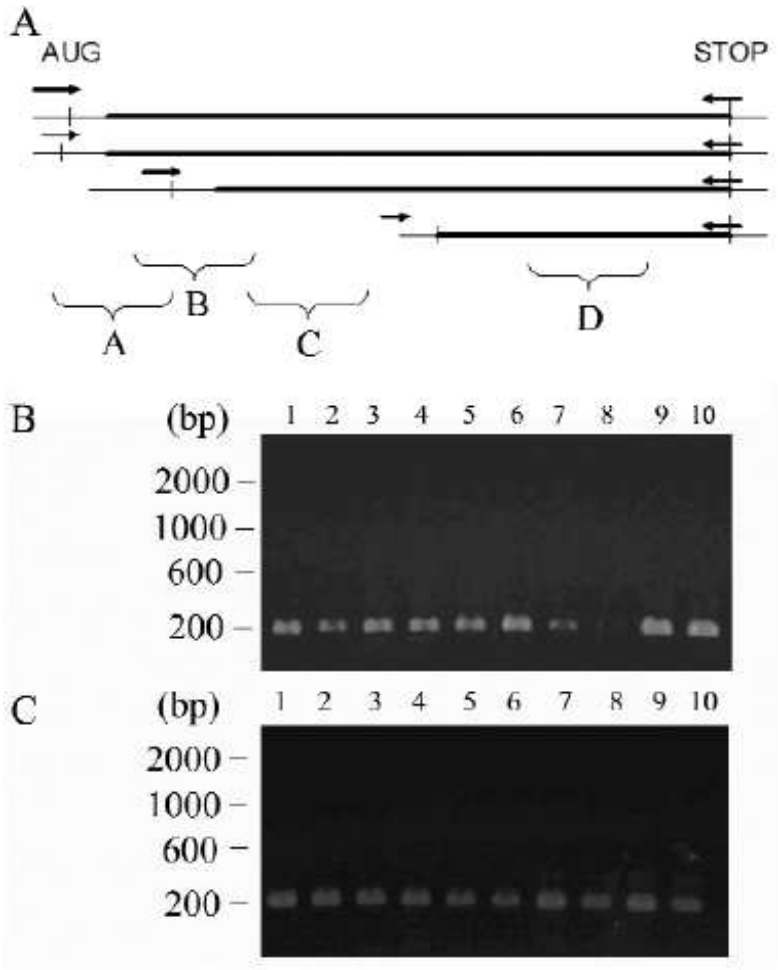

Fig. 6 A. - Schematic representation of regions selected for quantitative PCR (regions A to D) and cloning of complete coding sequences of all four isofonns (arrows). B and C - Amplification of the region common to all three long isoforms (region C in panel A), showing the presence of these isoforms in all samples. Samples 1 to 10 correspond to cases coded as NCH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 (containing gliosis tissue - NCH3, as well as grade II - NCH 7, 8, 9 and grade IV gliomas - NCH 2, 4, 5, 10, 11) in panel B and NCH 17, 18, 19, 20, 21, 22, 24, 25, 26 and 27 (containing gliosis tissue - NCH 19, 20, 27, and low- -NCH 21, 22 - and high-grade gliomas - NCH 17, 18, 24, 25, 26) in panel C. The results indicate efficient amplification of region C indicated on panel A from all examined samples. The specificity of amplification was confirmed for selected cases by direct sequencing.

Analysis of HDAC3 expression compared to the histological type of the examined tissues supported the elevated HDAC3 expression in high-grade gliomas (fig. 7B). Expression of HDAC3 followed the expression of two housekeeping genes, glycerol 3-phosphate dehydro-genase, and β-actin, in keeping with the possibility that HDAC3 elevated expression is part of an overall disturbed expression profile in glial tumors. Normalization against β-tubulin alone followed the pattern of elevated HDAC3 expression in total RNA (Fig. 7C). The relative level of

HDAC3 expression compared to ß-tubulin also indicated elevated levels of HDAC3 in high-grade gliomas (Fig. 7D). A similar trend was observed for quantitative analysis of the expression of all three long isoforms or two longest isoforms (HDAC3 and HDAC3A), indicating expression of multiple isoforms of HDAC3 in high-grade gliomas.

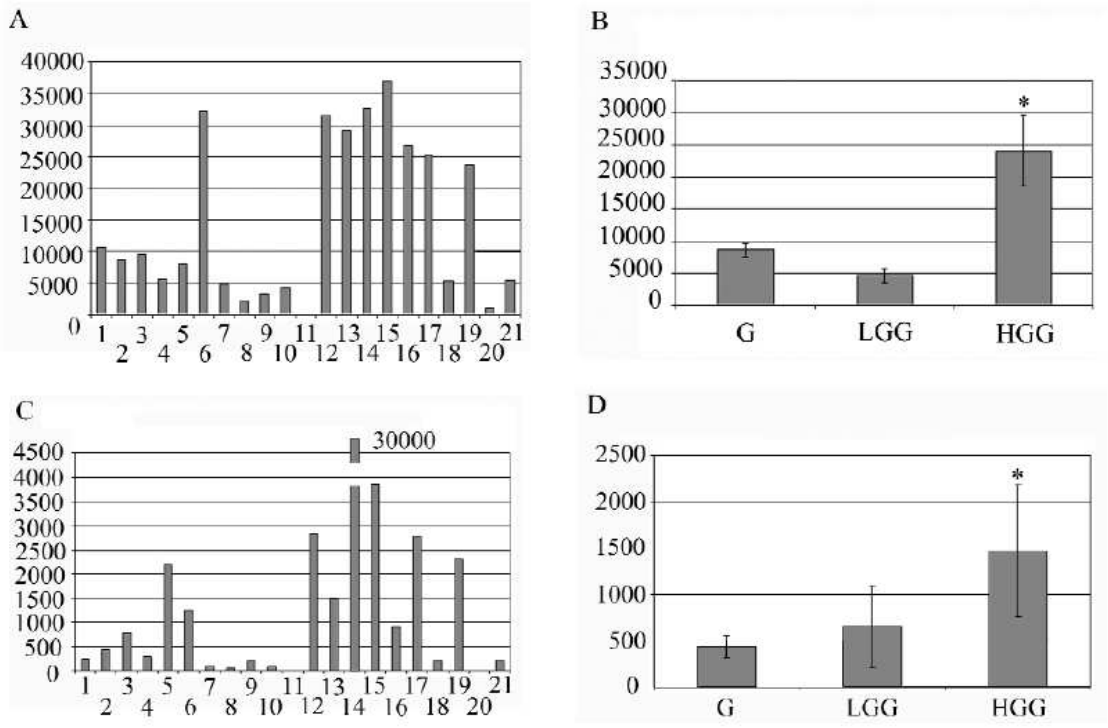

Fig. 7. **Quantitative PCR for region D shown in panel Fig. 6A.** Panels A and C show quantification of region D in cases numbered from 1 to 4 as follows: NC1I 3, 19, 20, 27 (non-malignant gliosis), 5 to 10 low-grade gliomas NCH 6, 7, 8, 9, 21, 22, and 11 to 21 high-grade gliomas NCH 1, 2, 4, 5, 10, l l, 17, 18, 24, 25, 26. Bar 6 indicates a case with histological features of low-grade glioma, which was however recurrent. All values in this panel are expressed as calculated numbers of copies per 150 ng of total RNA. B - Quautification of HDAC3 mRNA expressed as average values in glioses (G), low-grade gliomas (LGG) and high-grade gliomas (HGG). Standard deviation is indicated. HGG show elevated values of HDAC3 mRNA compared to glioses and LGG at 95 % probability in Student's t-test. C - The same experiment as shown in panel A normalized for values found for ß-tubulin mRNA. A similar trend as in readings normalized for total RNA is found. D - Average values and standard deviations calculated for the group of glioses, LGG and HGG from values normalized for ß-tubulin (shown in C). Asterisk indicates values significantly different from values found in glioses and LGG at 95 % probability in the Student's t-test. The highest value obtained for case NCH 5 was not included in this calculation

6.2.2 Detection of HDAC3 expression at the protein level by Western blot analysis

Western blot analysis with a rabbit polyclonal antibody revealed a faint band corresponding to a protein with an approximate size of 50 kDa in non-malignant glioses but prominent expression in tumour samples (Fig. 8A and B). Two non-malignant samples had a prominent band at 25 kDa (NCH19, 20) that presumably corresponds to a protein of N-terminally abrogated isoform D. On longer exposure films, a band with a size of approximately 58 kDa was also observed. Contrary to that, three out of five gliomas of grade II displayed 2-fold increased expression of the 50 kDa protein compared to the mean values seen in non-malignant samples (Fig. 9A) and two expressed a 25 kDa HDAC3 immunoreactive protein (Fig. 8A). All nine examined gliomas of grade III and IV expressed the 50 kDa HDAC3 and five of the nine gliomas had 2-fold the levels found in non-malignant tissue (Fig. 9A). Two samples contained a strongly expressed 48 kDa protein and a 42 kDa protein (Fig. 8B). Four out of nine cases had elevated levels of a 25 kDa protein. Densitometric analysis from two analyses prepared from the same material for each case revealed elevated expression of 38-50 kDa proteins (Fig. 9A) in high-grade tumours. Analysis of HDAC3/HDAC3A and HDAC3C expression related to the histological type of assayed tissue showed an increasing trend of HDAC3 expression in high-grade gliomas (Fig. 9B), indicating significantly elevated expression of the three longest isoforms in tumours and a trend for higher expression of HDAC3 in high-grade tumours. The result was significant in the Student's t-test with 95 % probability**.**

In order to know whether HDAC3 is inherent to glial cells and not to other cell types that may contaminate the tumour samples, we studied HDAC3 expression in the cultured U373 human glioblastoma cell line. Quantitative PCR yielded values that corresponded to the highest values found in tumours. Similarly, HDAC3 at the protein level was found to be expressed in U373 cells as a prominent 50 kDa protein. Contrary to that, the 25 kDa protein was not observed in U373 cells despite the fact that the short isofotm HDAC3D was amplified from U373 cells and confirmed by sequencing.

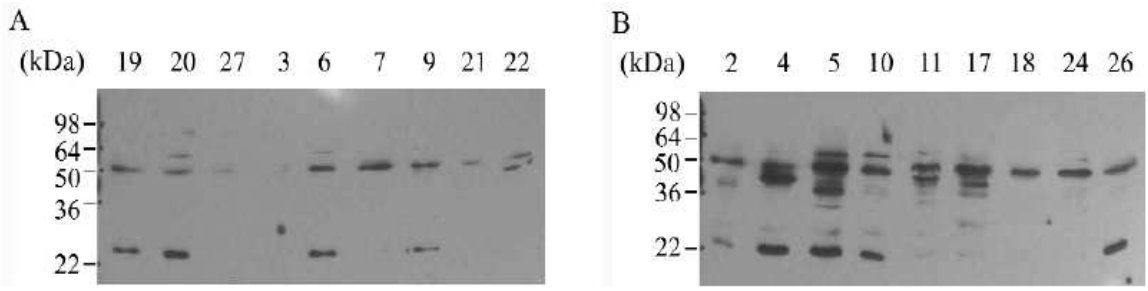

Fig. 8. Expression of HDAC3 detected by Western blot analysis. HDAC3 was detected by a rabbit polyclonal antibody specific for the C-terminus of the HDAC3 molecule. Panel A shows results obtained from non-malignant gliosis (cases NCH 19, 20, 27 and 3) and low-grade gliomas (cases 6, 7, 9, 21, 22) and panel B from high-grade gliomas (cases NH 2, 4, 5 10, 11, 17, 18, 24 and 26). Tumours show higher expression of HDAC3 species in the range 48 to 54 kDa HDAC3 expression is well visible in tumours and is strongly pronounced in the majority of high-grade gliomas.

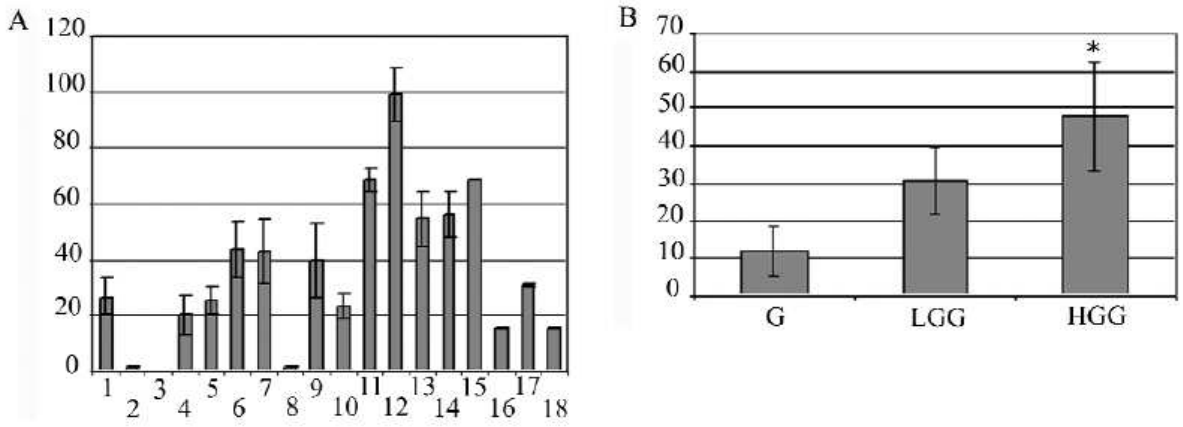

Fig. 9. A - Densitometric analysis of 48 to 54 kD HDAC3 proteins in cases shown in Fig.8. Values show mean and standard deviations of two independent Western blots. Bars numbered as 1 to 18 correspond to cases NCH 19, 20, 27, 3 (G), 6, 7, 9, 21, 22 (LGA), 2, 4, 5 10, 11, 17, 18, 24 and 26 (HGA) (as in Fig. 8). B – Analysis of the average expression of HDAC3 in non-malignant gliosis (G), low grade gliomas (LGG) and high grade gliomas (HGG). The values found in high-grade gliomas are significant in the Student t-test with 95% probability.

6.2.3 Analysis of HDAC3 expression at the cellular level

Thirty-five tumours were examined by histochemistry using the peroxidase method and by immunofluorescence. Immunohìstochemistry detected HDAC3 weakly in the cytoplasm and nuclei in normal brain glial cells as well as in gliosis and low- and high-grade gliomas. For an orientation analysis, all samples were evaluated as described in Methods. Cases where the observer classified 50 % of cells as cells containing cytoplasmic or nuclear staining that differed from control sections were classified as potentially HDAC3-positive. All evaluated tumours fulfilled this criterion.

For immunofluorescence, the samples were processed and evaluated as described in Methods. Nonmalignant glial cells were weakly stained predominantly in the nuclei. Non-malignant gliosis showed stronger labelling for HDAC3 both in the nuclei and the cyto-plasm. High-grade gliomas were strongly stained in the cytoplasm and had a pronounced focal character, containing areas with prominent cytoplasmic staining and areas with stronger nuclear HDAC3 localization. Although many fields examined were clearly characterized by observers as nuclear HDAC3 fluorescence,
a partial cross-talk from DAPI channel could not be excluded using the epifluorescence band pass filter sets. Analysis using the criterion of 50 % subjectively positive cells indicated that all cases of glial tumours were positive for HDAC3 staining.

Next, we analysed whether U373 cells showed staining for HDAC3 similar to the cells in the examined tumours. Epifluorescence detected HDAC3 in the cytoplasm of all cells. Nuclei were also stained but in some nuclear staining seemed to be compared to the cytoplasm (not shown).

Confocal microscopy

We employed confocal microscopy in order to evaluate the co-localization of HDAC3 staining with the nuclear compartment strongly labelled by DAPI. Analysis of U373 cells showed that HDAC3 staining was clearly detectable throughout the cytoplasm and was accumulated around the nucleus in many cells (Fig. 10A1 and B1). Nuclear staining was also clearly detectable (Fig. 10 A1 and B1). Co-localization maps (Fig. 10 A4 and B4) using single pixel overlap coefficient values were calculated as described in Material and Methods.Co-localization maps revealed nuclear HDAC3 staining in all examined nuclei (Fig. 10 A4).

Detection of HDAC3 expression in human filial tumors by confocal microscopy

A collection of samples that included all cases studied biochemically (except for three non-tumorous glioses that were not available as paraffin-embedded material) was analysed as described in Methods. Analysis confirmed the HDAC3 expression pattern detected by immunohistochemistry and epifluorescence in the cytoplasm.

A low and relatively uniform pattern was observed in normal glial tissue and in non-malignant gliosis, more intensive labelling in low-grade gliomas and strong focally accented labelling in high-grade gliomas. The nuclear labelling was also confirmed in all areas studied (Fig. 11 panels A1 to F1). Co-localization maps clearly showed nuclear localization of HDAC3 in all examined tumours (Fig. 11 panels A4 to F4).

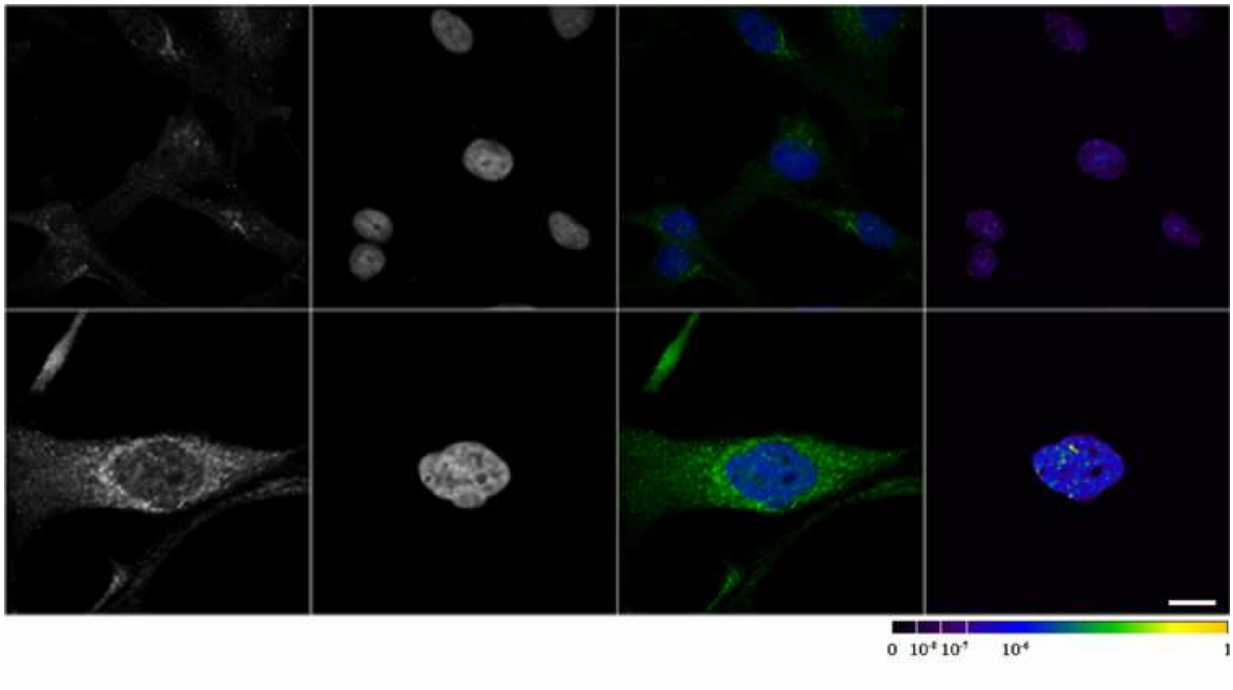

Fig. 10. Analysis of HDAC3 expression in U373 cells by immunofluorescence (confocal microscopy)
Panels A1 and B 1 show representative planes of HDAC3 data acquisition as described in Methods, panels A2 and B2 show DAPI recordings, panels A3 and B3 show a simple merge view and panels A4 and B4 show the co-localization maps constructed as described in Methods. The lookup table (under the figure) represents values of a single pixel overlap coefficient, e.g. the contribution of each single pixel to the overall value of the overlap coefficient. Note that the overall image overlap coefficient value ranges from 0-1. The co-localization map shows every single pixel contribution to the overall overlap coefficient value scaled with an appropriate lookup table (refer to its scale beneath the Figure). This representation of the channel overlap is superior to the simple RGB merge for a number of reasons (e.g. single channel pixel intensity value inequity, etc.). Scale bar corresponds to 10 µm.

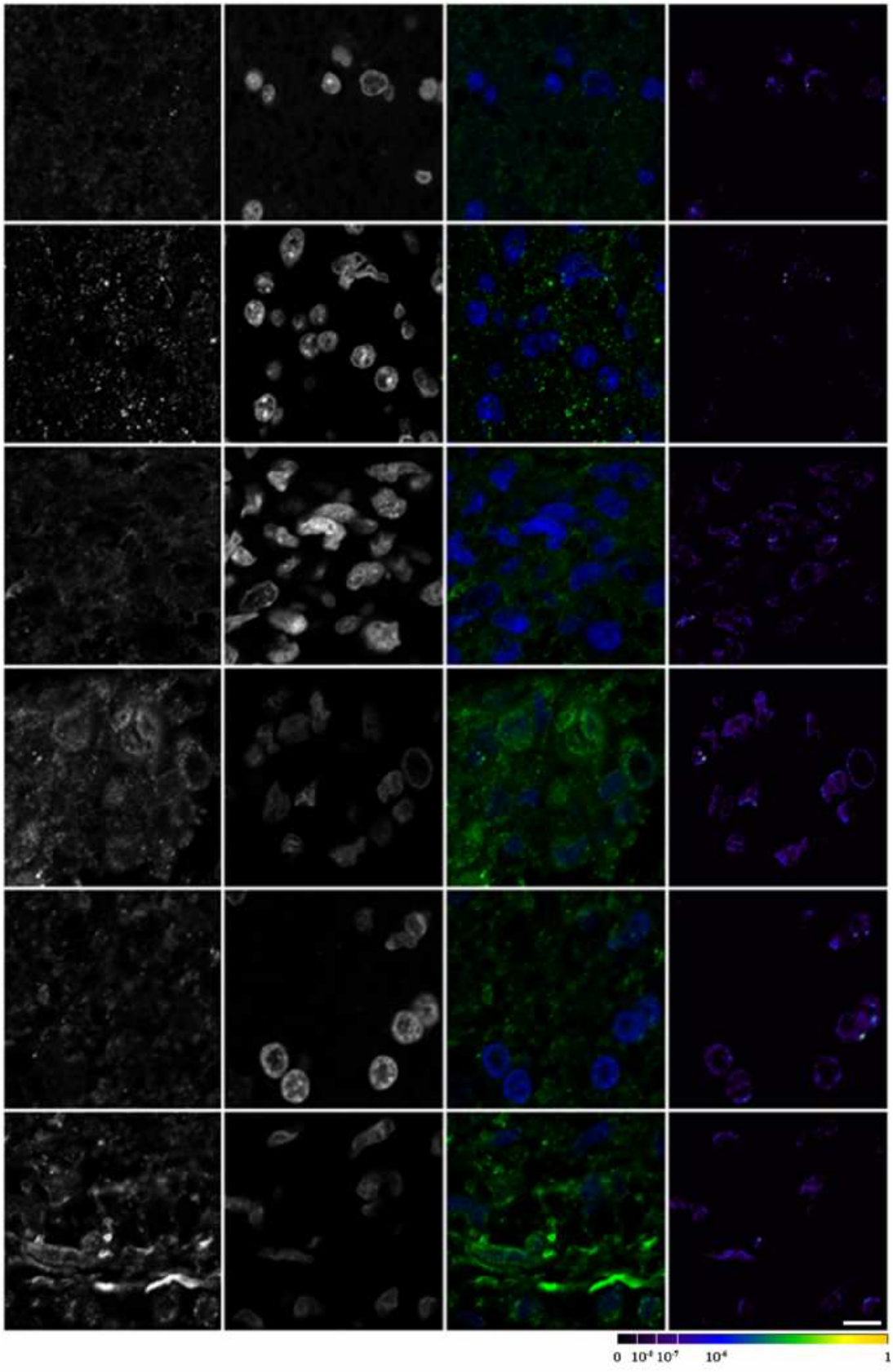

Fig. 11. Expression of HDAC3 in normal human glial tissue, non-tumorous gliosis and human glial tumours at the cellular level by confocal microscopy. Panels Al to A4 show recordings from a standardized field in a sample from normal brain tissue, panels B1 to B4 show

recordings from nou-tumorous gliosis, panels C1 to C4 show recordings from low-grade glioma, panels D, E and F show recordings from three representative fields taken from the analysis of high-grade gliomas. Left panels (A1 to F1) show HDAC3 staining, panels A2 to F2 show DAPI detection and panels A3 to F3 show composite pictures. Panels A4 to F4 show co-localization maps calculated as described in Material and Methods and in legend to Fig. 10. While in normal glial tissue and gliosis the staining for HDAC3 is weak in intensity and uniform, the glioma shows strong cytoplasmic and nuclear staining, and the co-localization map clearly shows HDAC3 in the nucleus. Focal character of HDAC3 detection in high-grade gliomas is seen in panels D to F. Panels D1 to F1 show strong cytoplasmic detection of HDAC3. Co-localization maps show nuclear HDAC3 in all examined areas of high-grade gliomas (panels D4 to F4). Scale bar corresponds to 10 µm.

6.3 Analysis of The Regulatory Potential of Bir-1 (The Orthologue of Human Survivin) in Regulation of Transcription in *C.elegans*

6.3.1 Inhibition of the bir-1 causes decrease in expression of several proliferative genes in L1 larvae

The effect of bir-1 inhibition on L1 larvae

Embryos of wild-type hermaphrodites were prepared using a standard protocol and synchronized L1 larvae were prepared. Synchronized cultures were incubated on a bacterial lawn of control E. *coli* transformed with vector L4440 carrying the cloning site and a non-specific sequence flanked by bacterial promoters on both sides. For *bir-1* inhibition, a 350 by sequence with the majority of *bir-1* cDNA was used. The production of dsRNA was induced by IPTG in both control and *bir-1*-inhibited cultures. Worms treated with *bir-I* or control RNAi did not differ visibly in the time of L1lL2 molt. Only a slight dpy phenotype was observed in a proportion of bir-l-inhibited worms (Fig. l2A). The development of seam cells that divide at the end of L1 stage was monitored in JR667 and SU93 transgenic larvae L1 prepared similarly as wild-type larvae (Fig. 1 B, D). These experiments showed that inhibition of *bir-I* by the feeding method does not affect cell divisions of seam cells.

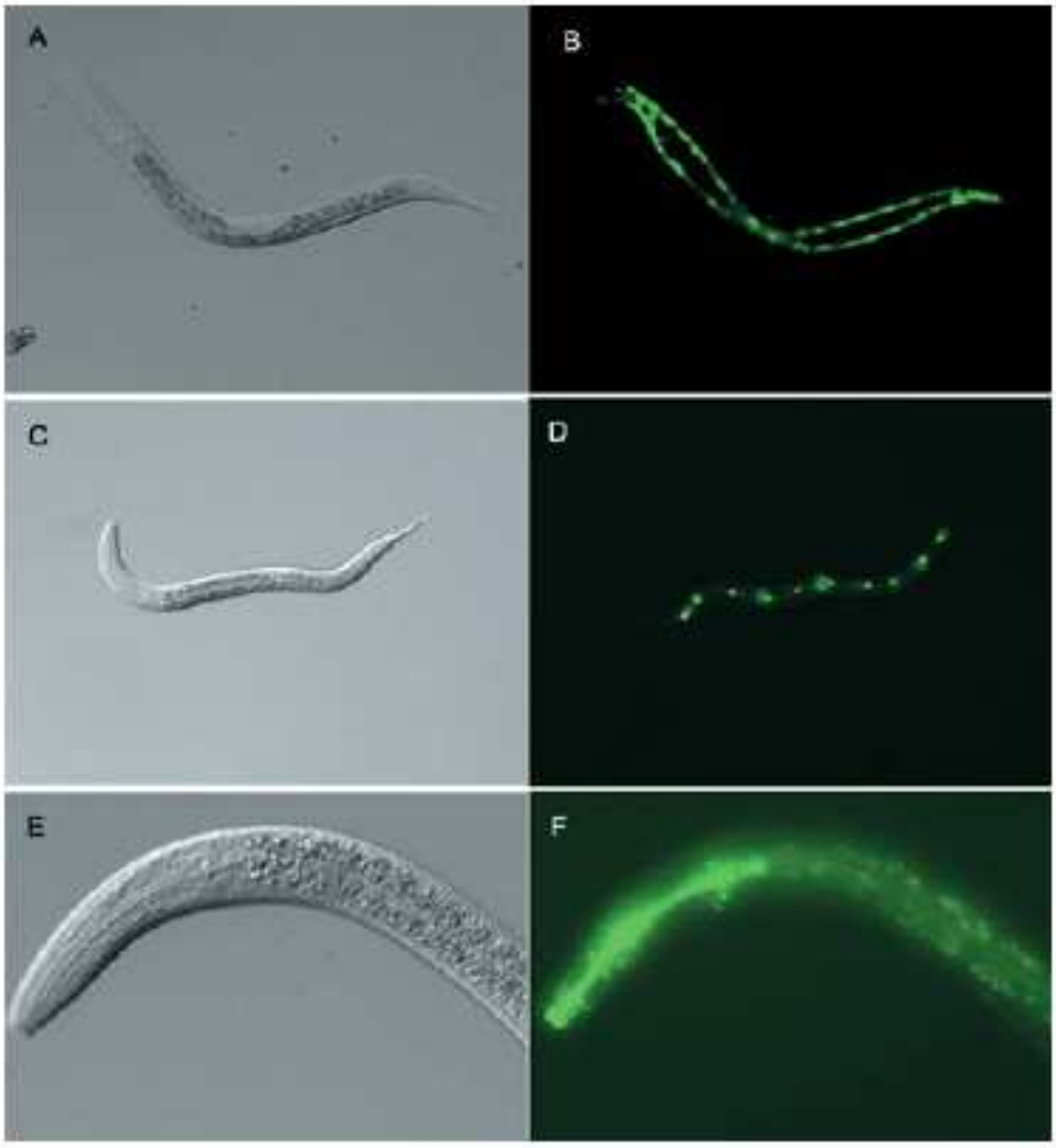

Fig. 12. **Morphological analysis of *bir-1*-inhibited larvae.** Panel A - L2 larva of transgenic line expressing *pnhr-23::*gfp (Kostrouchova et al., 1998) treated with *bir-1* RNAi for 24 h shown in Nomarski contrast. A weak dpy phenotype is visible with no other apparent morphological change. Panel B - the same larva that is shown in panel A
in fluorescence. Seam cells are visualized by the expression of the transgene. Note the properly formed seam cells in both lateral sides of the larva. Panel C - L2 larva of JR667 line shown in Nomarski contrast. A weak dpy phenotype is visible in the proximal part of the animal. Panel D - the same larva shown in panel C in fluorescence microscopy. The transgene JR667 marks 10 seam cells during the middle part of L2 larval development, indicating that the dpy phenotype induced by *bir-I* inhibition is independent of seam cell development. Panel E - the head of an SU93 larva L2 in Nomarski contrast showing a weak dpy phenotype. Panel F - the head of the larva shown in panel E in fluorescent microscopy. The transgene marking the cell membrane of epithelial cells indicates a proper development of seam cells at conditions used in this study.

bir-1 inhibition by RNA interference induces whole-genome transcription changes

To determine the transcription profile by microarrays, three independent experiments were done and evaluated as three control versus *bir-1* RNAi pairs. The statistical analysis labelled the results as Present/Absent (P, A), Increased/Decreased (I, D), No Change (NC), Moderate Increase (MI) and Moderate Decrease (MD). The detection and change P values of the absolute majority of data indicated marginal differences in the analysis of the data. One set of experimental data showed a major problem in evaluation or preparation of the experiment and was eliminated from the analysis. The computer program recognized 67 annotated spots as decreased in both evaluated *bir-1* RNAi experiments and 226 annotated probes as increased in *bir-1* RNAi experiments.

Interestingly, genes recognized as decreased were mostly found in groups of genes that showed highest readings on Affymetrix microarrays. The analysis of values obtained by Affymetrix microarrays showed that the group of 30 gene probes with highest values showed decreased readings in *bir-1* RNAi (Fig. 13A) while the genes with average readings showed both increased and decreased or unchanged values in *bir-1*RNAi-treated cultures (Fig. 13B).

The tendency of decreased readings in RNAi-treated cultures for genes with highest values on Affymetrix microarrays were not observed in our unrelated microarray experiments (not shown).

Genes that were identified as genes inhibited in *bir-1* RNAi included several collagen genes and genes with a known dpy phenotype: *dpy-2, dpy-3, dpy-4, dpy-7, dpy-8, dpy-9, dpy-10, dpy-13, dpy-15, dpy-17, col-1, col-3, col-10, cot-117, col-92, col-93, col-94, cot-125, col-144, co1-154, col-160, col-166, col-167, col-169,* genes for several ribosomal proteins: *rps-1, rps-4, rpl-2, rpl-21* and enzymes (e.g. phosphoheptose isomerase).

Next, we amplified coding regions of selected collagen genes that were identified by microarray analyses and selected genes that were suitable for quantitative PCR according to the amplified fragment (that was confirmed by direct sequencing) and the character of the melting curves. Ten collagen genes fulfilled these criteria and were used for further analysis: *dpy-2, dpy-4, dpy-7, dpy-8, dpy-13, col-94, col-125, col-144, col-166,* and *col-167.* Manual evaluation of raw data obtained using Affymetrix microarrays indicated a 20 to

40 % decrease in the expression in *bir-l*-inhibited worms (Fig. 13C).

Quantitative RT-PCR confirmed the decreased expression of all evaluated collagen genes (Fig.14). However, the expression of several collagen genes that were identified by microarrays as genes inhibited in *bir-1* RNAi-treated larvae could not be quantified because unspecific sequences were amplified as indicated by multiple bands detected in the final PCR products and by the character of the melting curves.

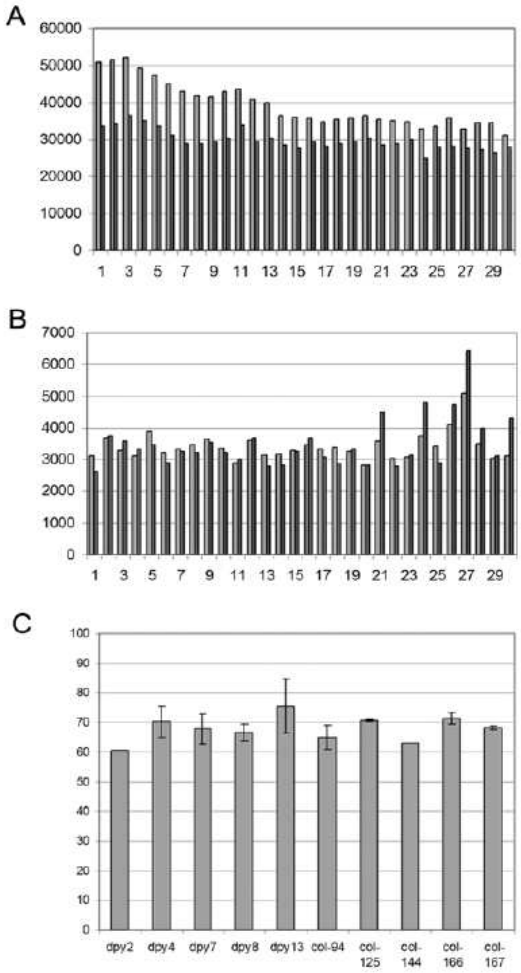

Fig. 13. Whole genome expression analysis by Affymetrix microarrays.
Panel A - an average of three evaluated control values of the gene probes with highest readìng values (light bars) and an average of values obtained for corresponding gene probes of two *bir-l*-inhibited cultures (dark b a r s). Note that 30 gene probes with highest reading values show decreased expression in *bir-l*-inhibited cultures. Panel B - an example of values of 30 gene probes that show average reading values (around 3,000 units in Affymetrix arrays). The values obtained for control experiments are similar to the values of cultures that were treated with *bir-1* RNAi (dark bars). Several gene probes show an increase in *bir-1* RNAi-treated cultures. Panel C - raw data obtained by Affyrnetrix microarrays for selected collagen genes that were evaluated manually in each paired experiment and expressed as percent of control values in the particular experiment and SD estimated. The values show 20 to 40 % decrease in *bir-I*-inhibited cultures.

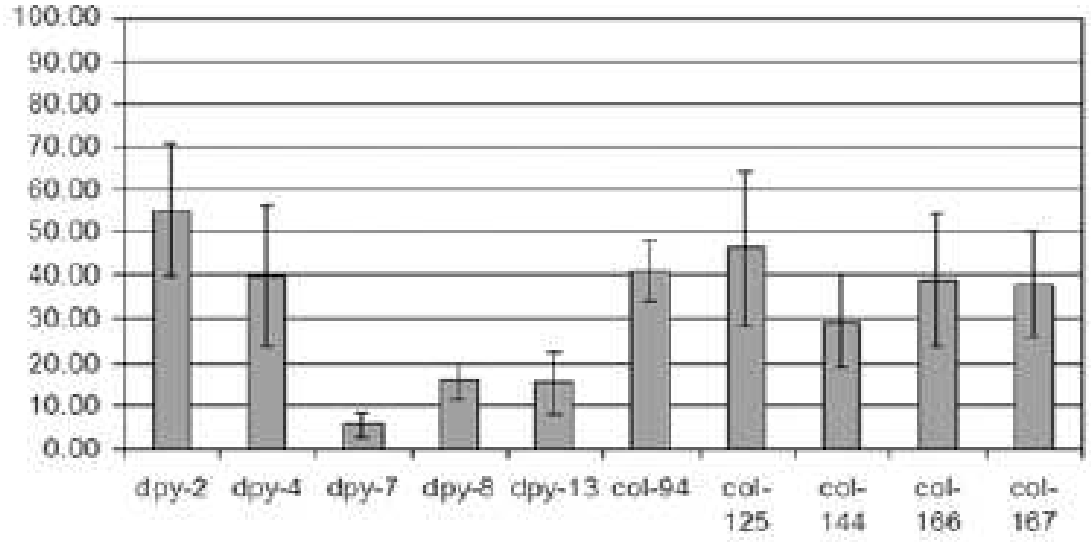

Fig. 14 Validation of data obtained by microarray analysis using quantitative PCR. Average values and SD from a minimum of three independent experiments are shown. Ten selected collagen genes were evaluated and the results are expressed as percent of values found in control experiments. There is a 60 to 90% decrease of the expression of evaluated genes in *bir-1* RNAi-treated cultures.

6.3.2 Overexpression of BIR-1 has the potential to dramatically upregulate developmentally active collagen genes

bir-1 overexpression induces a dramatic increase in the expression of collagen genes whose expression is sensitive to *bir-1* RNAi during normal development

In order to see whether genes that showed decreased expression in *bir-l*-inhibited larvae are also sensitive to elevated levels of BIR-1, we generated transgenic lines expressing *bir-1* mRNA from a transgene regulated by a heat-shock inducible promoter and confirmed the presence of the transgene by direct sequencing. A short heat-shock induction (30 min at 34°C) strongly induced the expression of all ten examined collagen genes normalized to the expression of actin *(act-1)* (Fig. 14). This induction and an almost identical expression pattern were observed in experiments normalized according to the expression of a large subunit of Pol *II, ama-1*. Interestingly, the expression of transcripts used for the normalization of results *(act-1* and *ama-1)* was elevated in experiments arranged to determine the number of amplified copies in equal amounts of starting material (based on total RNA), indicating that the observed difference in the expression of collagen genes may actually be larger.

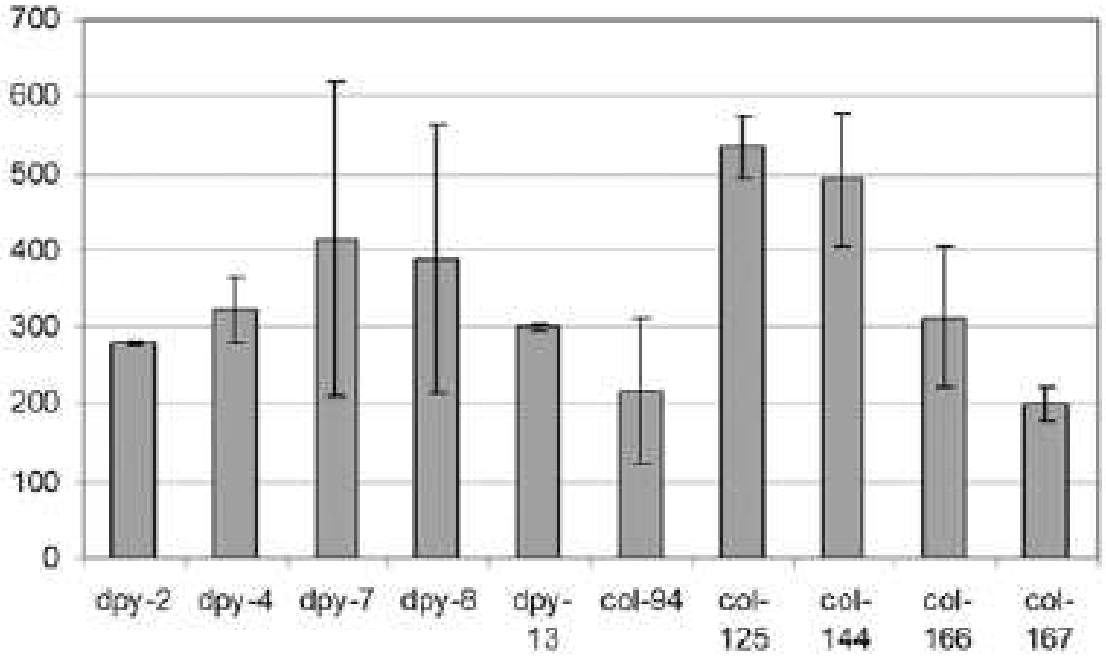

Fig. 15 The expression of selected collagen genes in larvae overexpressing *bir-1* shown as percent of control values. Average values from a minimum of three independent experiments and SD are shown. The expression of all evaluated genes show a pronounced increase in animals with *bir-1* overexpression compared to control wild-type larvae.

7 DISCUSSION

In this study we characterized the expression pattern of the TRs/RXRs genes and selected putative interacting proteins in astrocytic brain tumors.

We found that four major TR isoforms are expressed in diffusely infiltrating astrocytomas and benign gliosis. Three RXRs genes are expressed in benign gliosis as well as in tumors.

Our data show that the expression pattern of these NRs is dramatically deregulated, with variable levels of expression in tumors of the same grade. Clearly, in some tumors TRs and RXRs are strongly elevated.

Thyroid receptors are suspected to be important factors in development of many malignancies since their discovery which showed that thyroid receptors are cellular holmologues of v-erb A (Sap et al., 1986; Weinberger et al., 1986). Numerous studies attempted to find mutated forms of TRs in cancers. It is concluded that mutations and deletions are not a frequent event in cancers (Gonzalez-Sancho et al., 2003). Some studies indicated, however, that decreased expression of throid receptors, namely TRβ1 may be caused by methylation of the TRβ1 gene in breast cancers (Li et al., 2002). Further, in head and neck tumors, uveal melanoma, breast, small cell lung, renal cell, uterine cervical, ovarian and testicular cancers were found somatic deletions in chromosome 3p, where *THRB* resides. On the basis of theses results it was speculated that *THRB* is a tumor suppressor in human cancers(Gonzalez-Sancho et al., 2003). TRβ RNAs loss or substantial reduction was detected in eight samples of colon carcinoma compared to control tissue (Markowitz et al., 1989) . Loss of heterozygosity of *THRA* locus was found in sporadic breast cancer (Futreal et al., 1994; Futreal et al., 1992). In acute promyelocytic leukemia and acute poorly differentiated leukemia was found a translocation affecting THRA in 17q (Dayton et al., 1984).

Elevated expression of THRA compared to normal hepatic tissue was observed in study including six hepatocellular carcinomas (Arbuthnot et al., 1989). In the same type of cancer was found increased TRβ1 expression (Lin et al., 1999). In normal and hyperplastic thyroid tissue were detected significantly higher transcription levels of TRβ RNAs compared to thyroid tumors (Bronnegard et al., 1994). The mean expression values of TRβ and TRα RNAs were found significantly lower, however the expression of TRβ1 and TRα1 proteins was higher in thyroid tumors compared to normal thyroid tissue (Puzianowska-Kuznicka et al., 2002). In one study, focused on expression of isoforms in two samples of TSH-secreting pituitary tumors and six controls, authors found no differences in RNA levels in controls and

tumors, contrary to protein expression, which was detected only in controls. This suggests a post-transcriptional error in TR RNA processing in TSH-secreting adenomas (Gittoes et al., 1998). In 20 cases of human renal clear cell carcinoma TRα RNA was decreased and TRα1 protein`s expression elevated in tumors. TRβ1 protein was hardly detectable in all samples including 30% cancers with TRβ overexpressed on mRNA level (Kamiya et al., 2002). TRα2 was found overexpressed in nasopharyngeal carcinoma cell lines. TRα2 RNA was detected in 4.2% normal nasopharynx epithelium biopsie, in 18,5% primary and in 62% recurrent tumors (Lee et al., 2000). Forced expression of TRα1 in nasopharyngeal carcinoma cells reduced proliferation, suggesting that TRa1`s role may be tumor-suppressing in nasopharyngeal carcinogenesis (Lee et al., 2002)

Furthermore, the suspicion that TRs could be implicated in carcinogenesisis, may be reflected by observations made on transgenic animals defective for specific isoforms of thyroid receptors. While mice expressing the v-erb A develop hepatocellular tumors (Barlow et al., 1994, no higher incidence of malignancies is reported for TR Knock-Out animals [Forrest, 2000 #882).

Initial analysis of expression of steroid and thyroid receptors in several brain tumors and glial cell lines indicated that these genes are commonly coexpressed in brain tumors (Magrassi et al., 1993), but detailed study of the expression of thyroid and retinoid receptors in glial tumors is not available. The findings presented in this study suggest, that thyroid receptors are deregulated in many astrocytic tumors and are likely to be part of the oncogenesis of glial tumors. We hypothesized that this may reflect a possibility of abnormal use of thyroid receptors in the regulatory network. Abnormal use of thyroid receptors may be caused by interaction with transcription cofactors. We turned our attention to histone deacetylases especially HDAC3.

Our results show that HDAC3 is expressed at high levels in malignant human astrocytic glial tumors. HDAC3 was found to be expressed in multiple isoforms on both mRNA and protein levels. Four different transcripts are listed in the expert-curated NCBI database and more than 17 transcripts were found in more than 130 types of HDAC3 ESTs.
The originally reported HDAC3, HDAC3A and HDAC3C were amplified using specific primers and reverse transcription-PCR and their sequences were confirmed by direct sequencing. Primers designed to amplify the N-terminally deleted HDAC3D mRNA led to the amplification of the expected fragment in one case but not in the remaining cases.

HDAC3D was also found to be expressed in the U373 glioblastoma cell line on the mRNA level but surprisingly not on the protein level.

In keeping with the amplified cDNAs for the three long isoforms of HDAC3, Western blots detected proteins with sizes corresponding to the expected proteins derived from HDAC3, HDAC3A and HDAC3C. In some cases, a protein of approximately 25 kD was detected by HDAC3 C-terminus specific antibody and can be expected to correspond to HDAC3D. In most cases that expressed the 25 kD protein, primers designed to amplify a 183 bp long fragment amplified longer fragments, ranging from 400 to 600 bp. Sequencing showed that at least in some cases, these fragments contained the coding sequence of HDAC3D.

Since HDAC3 and HDAC3A differ only in 15 or 16 amino acids, respectively, it is likely that their functions and cellular regulations are very similar if not identical. HDAC3C differs from the two longer isoforms by a deletion of 58 (57 compared to HDAC3A) N-terminal amino acids, but can be expected to be a functional HDAC based on a comparison to domains shown to be irreplaceable for the histone deacetylase function (Fu et al., 2004; Zou et al., 2006). The shortest isoform, denominated HDAC3D, may correspond to the 25 kDa protein that is clearly recognized by Western blot in approximately one half of the cases in this study. It is unlikely, that HDAC3D is a functional histone deacetylase, since it is missing domains necessary for histone deacetylase activity and can be expected to function as a negative regulator of some HDAC3 isoforms or even multiple histone deacetylases. Since our analyses failed to amplify the predicted cDNA of HDAC3D in several cases that expressed the 25 kDa protein recognized by HDAC3 antibody, its existence needs to be confirmed by direct proteomic methods.

The findings of this study indicate that the mRNA of HDAC3 is elevated in tissues of high grade gliomas compared to non-malignant gliosis and low grade gliomas. This was observed using quantitative PCR directed to amplify the region that is common to all four isoforms, three long isoforms and two longest isoforms from equal amount of total RNA extracted from tissues. We interpret these findings as increased presence of HDAC3 in high grade glioma tissues but not necessarily per cell or nucleus. The high expression of HDAC3 in malignant tumors was supported by the expression of HDAC3 on protein level detected by Western blots.

Detection of HDAC3 by commercial antibodies and fluorescent microscopy (epifluorescence as well as confocal microscopy and colocalization analyses) show rather low nuclear expression of HDAC3 in histologically normal glial tissue. Contrary to that, gliomas,

especially high grade gliomas showed strong cytoplasmic HDAC3 expression. Confocal microscopy and colocalization maps detected HDAC3 in nuclei of an absolute majority of cells of glial tumors examined. Our data are in keeping with the possibility that HDAC3 may be indispensable for proliferation of glial cells and may play important roles in the malignant transformation of glial cells and growth of astrocytic tumors. This is supported by findings that inhibition of histone deacetylation by histone deacetylase inhibitor 4-phenylbutyrate, exerts anti-proliferative and differentiation inducing effects on a glioblastoma cell line that is connected with the induction of connexin 43 expression and inter-cellular communication via gap-junctions. Also, the non-phosporylated forms of connexin 43 and glial acidic fibrillary protein were induced by 4-phenylbutyrate (Asklund et al., 2004). HDAC3 is an effector in the transcription repression by thyroid receptors not occupied by ligand (Ishizuka and Lazar, 2003). Cheng and coworkers have shown that cyclin D1 can bring HDAC3 into the complex of thyroid receptors in a ligand independent manner thus overruling the transcription activation function of liganded thyroid receptors. Thus, the nuclear HDAC3 localization may be expected to have a potency to specifically inhibit a number of differentiation pathways.

An interesting feature observed in our experiments was the focal character of elevated cytoplasmic HDAC3 expression.

Histone deacethylases were shown to function not only in the nucleus, but also in the cytoplasm. HDAC inhibitors disrupt the complex between HDAC/PP1 and in consequence dephosphorylation of Akt I U87MG gliolbastoma and PC-3 prostate cancer cells. The HDAC inhibitors that were assayed differed in potency to activate the Akt dephosphorylation. TSA showed the highest effect compared to HDAC42 and SAHA (Chen et al., 2005). The proposed mechanism includes interaction of HDAC with protein phosphatase 1 (PP1) in an active, phosphorylated form and excludes HDAC from interaction with other proteins, including Akt. Akt was shown to interact with HDAC1 and HDAC6 (Chen et al., 2005). HDAC3 activity, phosphorylation status and cellular localization were shown to depend on the interaction with PP4 (Gao et al., 2005). The activity is dependent not only on the phosporylation status, which may regulate the interaction of class II HDACs with 14-3-3 proteins (Bertos et al., 2001; Gagnon et al., 2003; Grozinger and Schreiber, 2000), but also on the activity of corepressors of transcription factors (Guenther et al., 2001; Ishizuka and Lazar, 2003). It was recently shown that HDAC3 is also localized at the plasma membrane and may be phosphorylated by Src (Longworth and Laimins, 2006). HDAC3 expression, activity and cytoplasmic localization is connected with TNF signaling. HDAC3 inhibits the activation of MAPK11-mediated activating transcription factor-2 and expression of TNF

(Mahlknecht et al., 2004). In the opposite way, regulation of nuclear translocation of HDAC3 by IκBα is required for the inhibition of PPARγ function by TNF (Gao et al., 2005).

In this study, we found HDAC3 in both nuclear and cytoplasmic localizations in all examined cases of high grade gliomas. It can be speculated that the dual deregulated expression of HDAC3 may be important for biological properties of malignant gliomas.

Experiments included in this thesis show that BIR-1, a homologue of the human cancer related protein Survivin, regulates expression of several developmentally active collagen genes during the normal development of C. elegans and has a strong potential to affect gene expression when expressed at non-physiological levels in transgenic animals.

C. elegans offers a versatile model for studies of transcription regulation in both dividing and non-dividing cells. The development of C. elegans proceeds through an embryonic stage and four larval stages. Adult C. elegans worms have a constant number of somatic cells, 956 in hemaphrodites and 1031 in males. The cell lineages follow a developmental scheme that result in the formation of specialized tissues and cells in a tightly regulated pattern. At the end of the embryonic stage, the newly hatched L1 larva has approximately 550 cells, and the subsequent cell divisions in the L1 stage form an animal that has most cells developed. Only a limited number of cells continue to divide during a narrowly framed time window. Seam cells, specialized hypodermal cells that form two lines on the side of the larvae asymmetrically divide once in each larval stage and form a new seem cell that keeps the blast cell character and a hypodermal cell that fuses with the lateral hypodermal syncytium. Proper development of seam cells is critical for normal body shape and motility.

Most cells can be regarded as postmitotic in the larval stages of C. elegans and the separation of dividing and non-dividing cells offers a system for studies of protein function related to cell division and postmitotic functions.

BIR-1 and Survivin execute critical functions during cell division: the regulation of the spindle formation and the proper separation of chromosomes. These functions are dramatic and since the cell cycle projects to complex cellular events including transcription regulation may obscure non-mitotic events. Inhibition of BIR-1 and Survivn function leads to mitotic arrests, defects in karyokinesis and cytokinesis including the formation of aneuploid cells and nuclei-free cell bodies. The other functions of Survivin include potent inhibition of apoptosis and a newly discovered role in the regulation of stress response.

Our previous work identified a new function of BIR-1, that is not related to mitotic functions: the regulation of transcription and development in non-dividing cells (Kostrouchova et al., 2003). Structural data that are available for Survivin indicate its wide

ability to interact with other proteins. Survivin is small 123 aa protein that forms a zinc finger BIR domain and a laterally postioned helix. Both domains are likely to effectively bind other proteins including Bir1 itself that was in crystalographic studies found unexpectadley dimerizing through the region that forms a tip of triangularly shapd molecule. Survivin and BIR-1 were shown to form complexes with more than one protein. BIR-1 interacts with a small protein CSC-1 (Romano et al., 2003) and INCENP (Romano et al., 2003) and together with both proteins is involved in docking the Aurora B kinase Air-2 to chromosomes (Speliotes et al., 2000).

The binding function of Survivin includes binding of caspases and interaction with mitochondrial proteins. It seems likely that the interaction has inactivating consequences. The antiapoptotic functions seem to have a wide spread role in most human cancers. The high expression of Survivin is generally linked to a higher malignancy and progression in most cancer types. Both Survivin and BIR-1 were shown to reside both in the cytoplasm and in the nucleus. While the cytoplasmic expression of Survivin is reported as an unfavorable criterion in most studied cases, the nuclear expression was found to be related to a better prognosis.

Despite that Survivin is generally found to be overexpresssed in most cancers, its elevated expression in transgenic mice does not induce cancer formation itself but elevates the sensitivity to cancer promoting events and is connected with changes of gene expression in microarrays.

The structure and potentially multiple protein interaction potential of BIR-1/Survivin suggests that its elevated expression and spacially restricted presence may induce fundamental changes in the availability of regulatory proteins and may change the capacity of interacting proteins to participate in protein complexes. Since BIR-1 is able to bind Air-2 and phosporylate histones H3 on P10, we hypothesized that kinase docking may be a part of BIR-1's transcriptional role in non-dividing cells. Histone H3 phosphorylation is the critical event for chromatin compaction during mitosis which is generally connected to the silencing of transcription but may also be linked to transcription activation. Phospohoacetylated histones H3 in the form of K9-Ac S10-P and S10-P K14-Ac were found to be part of transcription activation and it was suggested that phosphorylation of histone H3 may even precede the acetylation and may actually be the starting event in transcription activation (Hauser et al., 2002). Alternativelly, both phosphorylation and acetylation events may be functionally separated and project synergistically but independently to the promoters of genes (Thomson et al., 2001). The phosphorylation of histone H3 may be connected to regulatory events that exceed modification of chromatin events similarly as acetylation of histones that represent a

modification generally related to transcription activation, is part of posttranslational modifications of both chromatin proteins and transcription factors as shown for nuclear hormone receptors. Phosphoacetylation of histones in nucleosomes regulated by c-fos and c-jun proteins was shown to be part of transcription activation (Clayton et al., 2000).

The role of Survivin in the regulation of gene expression was also observed in mammalian systems. Survivin enhances the level of p21ras mRNA as well as its activated form on the protein level (Temme et al., 2005). Survivin regulates the expression of p53 and its protein family members both on mRNA and protein levels (Fu et al., 2004). The involvement of Survivin in the regulation of gene expression via the up-regulation of SP-1 mediated gene transcription was shown on colon cancer cells (Asanuma et al., 2004).

The results reported here suggest that BIR-1 may be involved in contacts with multiple transcriptionally active proteins. As reported here, *bir-1* inhibition repressed transcription of most but not all genes that showed the highest transcriptional activity in microarrays and decreased the values recorded for 67 genes. A larger group of genes comprising 227 members was identified as genes potentially transcriptionally inhibited by BIR-1. Together with the findings that most transgenes and transfected reporters are more transcriptionally active in presence of BIR-1, this suggests that BIR-1 is likely to activate the ongoing active transcription. Multiple contacts of BIR-1 with transcriptionally active proteins may project to elevated expression of specific genes in *bir-1* RNAi.

In conclusion, the data reported here support the active role of BIR-1 in transcription regulation during *C. elegans* development and indicate that BIR-1 interferes with transcription regulation when expressed at high levels. BIR-1 and Survivin can substitute for each other in several systems (Speliotes et al., 2000). It is therefore likely that Survivin, strongly overexpressed in tumors, has profound effect of cancer specific transcriptome.

8 CONCLUSION

Our results document that TRs/RXRs are expressed and deregulated in human astrocytic tumors.

Cofactors that have potential to modulate the regulatory functions of thyroid receptors, namely SKIP, Survivin and HDAC3 are elevated and deregulated in human astrocytic tumors.

The involvement of BIR-1 in regulation of transcription of developmentally active genes confirmed.

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10 List of author`s publications and presentations

Publications

Libý P., Kostrouchová M., Pohludka M., Yilma P., Hrabal P., Sikora J., Brožová E., Kostrouchová M., Rall J.E., Kostrouch Z. (2006) Elevated and Deregualted Expression of HDAC3 in Human Astrocytic Glial Tumours . Folia Biol 52, 21-33

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