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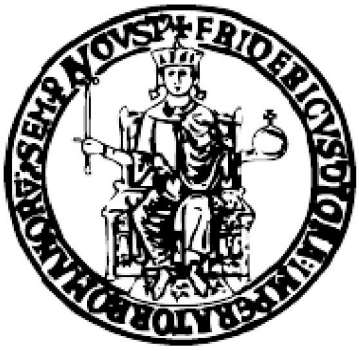

"Titolo della tesi"

Discovery of new therapeutic targets in Neuroblastoma by means of proteomic approach and functional studies

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List of abbreviations

NB: neuroblastoma

RA: retinoic acid

ATRA: all-trans-retinoic acid

2D-DIGE: two dimensional-differencial in gel electrophoresis

MS: mass spectrometry

VBP-1: Von Hipple-Lindau binding protein 1/prefoldin 3

TrkA: tyrosin kinase receptor A

TrkB: tyrosin kinase receptor B

BDNF: brain derived neurotrophin factor

NGF: nerve growth factor

Gal-1: galectin-1

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Abstract

Neuroblatoma (NB) is an embryonal tumor of the sympathetic nervous system which arises from the neural crest cells. This disease rappresents the most common extracranial tumor in infants, accounting for 8% to 10% of all childhood cancer and for approximately 15% of cancer deaths in children.

NB is a heterogeneous tumor for which biology dictates clinical behaviour.

It comprises cases with divergent outcome ranging from spontaneous differentiation to metastatic forms with poor prognosis. The deep knowledge of NB biology is imperative toward the development of novel therapy.

The most favourable subset of NB (stage 4S) can spontaneously differentiate in neurons or regress to a benign tumour phenotype. Retinoic Acid (RA) is a known neural differentiation-inducing agent actually used in NB therapy. In order to get new insights in the molecular mechanism driving neuronal differentiation in vitro, two-Dimensional Differential In-Gel Electrophoresis (2D-DIGE) analyse was performed on the cytosolic and nuclear protein expression patterns of NB cells following RA treatment. The combination of a proteomic approach and sub-cellular fractionation of the proteome provides the identification of 33 differentially expressed proteins during RA treatment in NB. The identified proteins have important

roles in a variety of pathways which may have role on NB development and in NB RA-induced differentiation. The results also strength the use of proteomics to discover new putative protein targets in cancer.

Expression of Trk receptors is an important prognostic factor in NB. TrkB and its ligand BDNF (brain derived neurotrophin factor) are preferentially expressed in NB with poor prognosis, conferring invasive and metastatic potential to the tumor cells as well as enhancing therapy resistance. TrkA in contrast is high expressed in tumor with good outcome. Galectin-1 (Gal-1), a very promising cancer target, is involved in modulating cell proliferation, cell death and cell migration and was found to be up-regulated in patients with aggressive, relapsing NB. Gal-1 is a down-stream mediator of TrkB signalling, since its espression is increased in human SY5Y NB cells upon activation of ectopically expressed TrkB (SY5Y-TrkB), but not TrkA (SY5Y-TrkA). Functional studies here presented underlined the Gal-1 role to mediate invasion and migration in TrkB over-expressing NB cells, thus being activated by BDNF. This establishes Gal-1 as a potential therapeutic marker in high-risk TrkB-expressing NB.

Introduction

1.Neuroblastoma

1.1 Neuroblastoma

Neuroblastoma (NB) is an embryonal tumor of neuroectodermal cells derived from the neural crest and destined for the adrenal medulla and sympathetic nervous system (Brodeur 2003; Maris et al., 1999).

This disease is the most common extracranial solid tumor in the pediatric population accounting for 8% to 10% of all childhood cancer and for approximately 15% of cancer deaths in children. The incidence per year is 10,5 per million children less than 15 years of age. 30% of all cases occur in the first year of life and nearly half of the newly diagnosed patients are between the age 1 and 4 years (Stiller et al., 1992; Park et al., 2008).

The etiology of NB remains obscure. Despite the fact that NB is sometimes diagnosed in the perinatal period, no environmental influences as prenatal exposures that impact on disease recurrence have been identified consistent (Connelly et al., 2007).

The peculiarity of this pediatric tumor is the heterogeneity: clinical variability and genetic abnormalities acquired by the tumor cells are at basis of the complex biology of NB. During the past two decades, the major biological and genetic features of the tumor have been

identified and therapeutic strategies have been proposed to treat NB. A deeply knowledge of the molecular mechanism of development are actually determinant to develop new therapy less toxic and more effective (Park et al., 2008; Maris et al., 2007; Brouder 2003).

1.2 Pathology: Cell of origin and stem cell hypothesis

NB are drived from precursor or immature cells of the sympathetic nervous system (SNS), and primary tumors can be found at any location of SNS structures as ganglia, paraganglia and adrenal medulla.

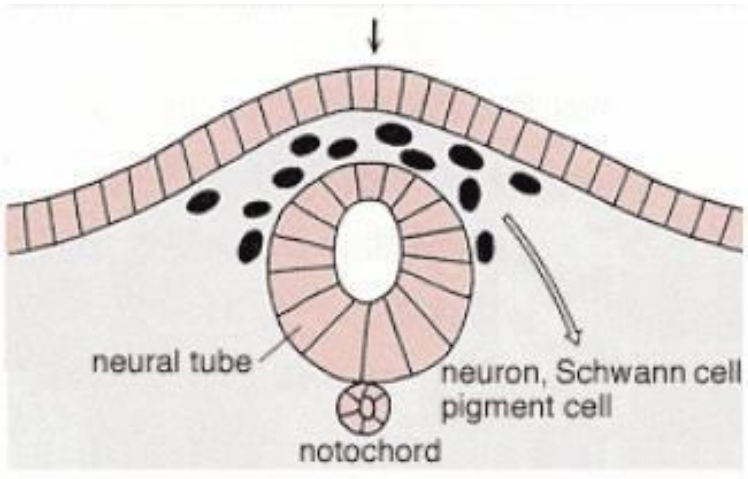


Figure 1 Neural crest cells formation during embryonic development

The sympathoadrenal lineage originates form the neural crest cells which appears at the dorsal edge of the neural fold at the third embryonic week (Figure 1). These cells migrate along define routes in the interstitial space of the embryo, and exposed to a variety of

environmental cues, they can develop into such diverse tissues or cell types as the peripheral nervous system, melanocytes, smooth muscle, cartilage, and skeletal structures of the head (Reviewed in Mora et al., 2004).

The SNS is composed of neurons, chromaffin cells, and small intensely fluorescent cells (SIF). The neurons (ganglio cells) make up the ganglia proper, which comprise the paravertebral sympathetic chain ganglia and the para-aortic trunkus and pelvic ganglia. The SIF cells are located in the sympathetic ganglia, either solitarily or in clusters, but their function has not yet been determineted. Sympathetic neuroblasts/ganglion cells are also present in the medulla of the developing human adrenal gland, but these cells apparently disappear during development. Chromaffin cells make up two structures: the paraganglia and the adrenal medulla. The paraganglia are the main source of catecholamines outside the central nervous system, and thereafter production is taken over by the adrenal gland (Reviewed in Mora et al., 2004).

Recently, the cancer stem cell hypothesis has provided an intruiging alternative explanation for NB heterogeneity. Although the presence of cancer stem cells with leukemia and some solid tumors has been estabilished, NB stem cells have not been clearly identified (Johnsen et al., 2009). Notch, Sonic hedgehog, and Wnt/b-catenin pathways are implicated in embryonal tumorigenesis; NB stem cells arising from normal neural crest stem cells, could partly preserving and

dysregulating these pathways (Allenspach et al., 2002; Blanc et al., 2005). The identification and characterization of cancer stem cells in NB could permit a targeted approach to more effective treatment.

2. Neuroblastoma Heterogeneity

2.1 Clinical presentation and staging

Due to their neural crest cell lineage, NBs may occur in the adrenal medulla (most common location) or anywhere along the sympathetic ganglia. Additional sites of origin include the neck, chest, and pelvis. Mostly infants present thoracic and cervical primary sites. One percent of patients have no detectable primary tumor. Early symptoms, when present, are typically nonspecific symptoms such as general malaise, weight loss, and unexplained fever. In general severe symptoms do not occur until the tumor has reached a critical size and/or developed metastases (Titilope et al., 2007).

NB diagnosis is confirmed by pathologic definition from tumor tissue or NB tumor cells in a bone marrow sample and by the setting of increased urine or serum catecholamines or catecholamine metabolites (dopamine, vanillylmandelic acid, and homovanillic acid) (Park et al., 2008).

The Shimada classification, modified as the International Neuroblastoma Pathology Classification (INPC), has been widely

used to describe and predict NB behaviour and prognosis (Shimada et al., 1999). This classification system takes in consideration histologic features such as the degree of cellular differentiation, schwannian stroma and the mitosis-karyorrhesis index (MKI), in addition to the age of the patient. According to INPC, NB is a tumor composed of neuroblastic cells forming groups of nests separated by stromal stepta with none to limited Schwannian proliferation. However, at the time of diagnosis and to establish appropriate treatment protocols, the generally accepted method is the International Neuroblastoma Staging System (INSS) (Brodeur et al., 1993).

The INSS definitions for NB stage are shown in Figure 2. Completely resected tumors are classified as Stage 1, and partially resected regional tumors with or without regional nodal involvement are classified as Stage 2. Stage 3 is rappresented by tumors that have spread across the middle of the body and present liver, bone and regional lymph node involvement. Stage 4 disease is defined as distant nodal or hematogenous spread of disease. Stage 4S is represented by localised primary tumor and an unique pattern of dissemination limited to liver, skin, and minimal bone marrow involvement. The tumors of Stage 4S have a potential for spontaneous regression and/or neuronal differentiation, even after no, or minimal therapy intervention, in marked contrast with the disseminated aggressive disease (Evans et al., 1980; Yamamoto et al., 1998).

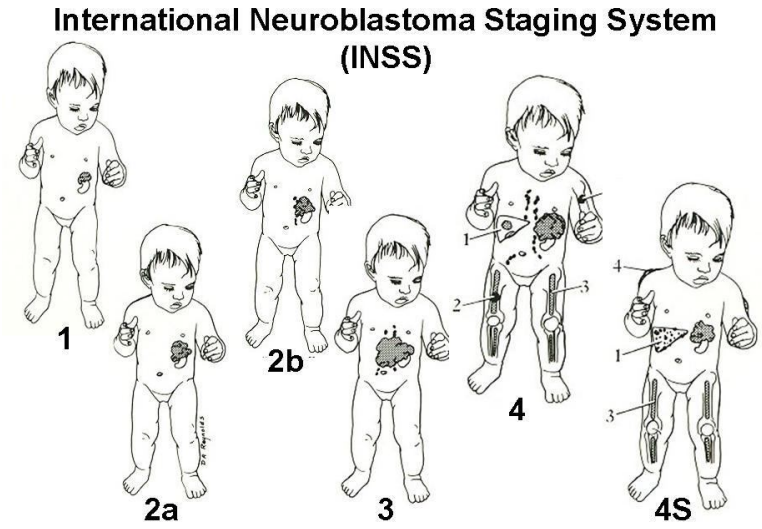


Figure 2 International Neuroblastoma Staging System (INSS)

2.2 Genetic Heterogeneity

NB is a very heterogeneous tumor which present different genetic alterations in the population. Despite extensive data correlating genomic alterations with disease outcome, no bona fide target genes have been identified for NB, with the exception of MYCN (subsequently described) (Figure 3).

Disorders resulted from abnormalities in the development of neural crest cells in embryo such as Hirscsprung disease, neurofibromatosis type 1, and congenital central hypoventilation syndrome have been reported in association with NB. Anyway, not specific abnormality has been identified with increased frequency, suggesting that NB has a different origin (Clausen et al., 1989; Rohrer et al., 2002; Trochet et al., 2005).

Familial forms of NB are rare, accounting for about 1% of all cases.

The median age at diagnosis of patient with familiar NB is 9 months, which contrasts with a median age of 18 months for NB in the general population. There are two reported pedigrees of familial NB which show a linkage of NB predisposition to chromosomal region 16p12-p13, found in North American families and linkage to chromosome 12p and 2p reported in studies of European pedigrees (Maris et al., 2002; Longo et al., 2007).

Recently, a whole-genome linkage scan in a panel of NB pedigrees identified a significant linkage signal at 2p23-24. In this region germline mutations were identified in ALK gene (Mosse et al., 2008). Subsequently, in about 8% of all sporadic NB tumors, somatic ALK mutations or amplification were identified (Caren et al., 2008; Chen et al., 2008; Janoueix-lenosey et al., 2008). ALK is a member of the tyrosine kinase family and mutations provide a therapeutic target in NB (George et al., 2008). Most NBs occur spontaneously. Somatic changes, such as gain of alleles and activation of oncogenes, loss of alleles or changes in tumour-cell ploidy have been shown to be important in the development of sporadic NB.

DNA content is most prognostic in NB for patients who are 1-2 years old. Near-triploid NB (55%) are characterized by whole chromosome gains and losses without structural genetic aberrations. These tumors are more often localized and show a favourable outcome. Near-diploid NB (45%) are characterized by the presence of genetic aberrations, such as MYCN amplification, 17q gain, and chromosomal losses and

show unfavourable outcome (Brodeur 2003; Bagatell et al., 2005; Look et al., 1991).

The MYCN oncogene, located on the distal short arm of chromosome 2, locus 2p24, is present in an increased copy number in 30% to 40% of stage 3 and 4 NB (Schwab et al., 1984; Weiss et al., 1997). MYCN oncoprotein is a transcription factor which forms heterodimers with MAX protein, leading to activation of gene targets ODC, MCM7 and MRP1 and to progression through the G1 phase of the cell cycle. Even if MYCN has a short half-life, the extremely high steady-state levels (100 times normal) in amplified tumor cells probably ensures that cells stay in cycle and do not enter G0. In absence of MYCN, MAX forms homodimer that is transcriptionally repressive (Wenzel et al., 1994).

Gain of the entire chromosome 17 or gain of parts of chromosome 17q occur in greater than 80% of NBs. The partial 17q gain most often results from unbalanced translocation of 17q21-25 to chromosome 1 (Meddeb et al., 1996; Van Roy et al., 1994) and identifies unfavourable NB (Vandesompele et al., 2005). Obvious candidate genes on 17q are the NM23 and the BIRC5 (survivin) gene (Adida et al., 1998; Godfriend et al., 2002).

Loss of tumor suppressor regions is reported in NBs for many chromosomal regions. The most frequently affected regions are chromosome 1p (30-40%), 4p (20%), and 14q (25%). Chromosome 1p loss occurs more frequently in older children who have stage 3 and

4 NB (Gehring et al., 1995; Caron et al., 1996). In almost all samples with MYCN amplification, concomitant 1p loss is demonstrated, but loss of chromosome 1p can also occur in MYCN-single copy cases. In the past, many attempts were made in order to delineate a shortest region of deletion (SRD) on 1p36 (Okawa et al., 2008). One of the most promising candidate NB suppressor genes in this region is CHD5 (Bagchi et al., 2007; Fujita et al., 2008). This gene is a member of the chromatin remodelling family and is expressed mostly in the nervous system. Whitin region, KIF1B gene, a member of the kinesin 3 family, was identified as a haploinsufficient candidate tumor suppressor gene (Munirajan et al., 2008).

Chromosome 11q loss is demonstrated in approximately 40% of patients. It is inversely correlated with MYCN amplification and therefore identifies an additional high-risk subset of patients characterized by advanced stage, older age, and unfavourable pathology (Attiyeh et al., 2005; McArdle et al., 2004). Recently, two common regions of deletion on the long arm of chromosome 11 were delineated and CADM1 was identified as a candidate tumor suppressor gene (Michels et al., 2008; Nowacki et al., 2008).

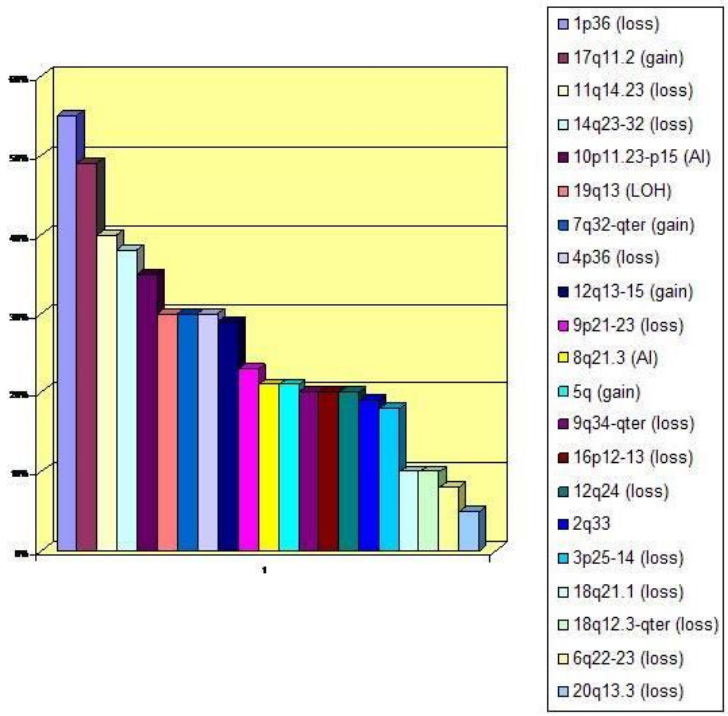


Figure 3 Mostly frequent genetic abnormalites in Neuroblastoma

Genome-wide association studies (GWASs) have shown a significant association between clinically aggressive NBs and common single nucleotide polymorphism (SNPs) or variants at chromosome region 6p22 (Maris et al., 2008). A second susceptibility locus for the development of aggressive NB tumors was identified, BARD1 locus at 2q35. This susceptibility is linked to six SNPs located in introns 1, 3 and 4 of BARD1 (Capasso et al., 2009). The BARD1 protein heterodimerizes with the familial breast cancer gene product BRC1 and is essential for the tumor suppressor activity of BRC1 (Wu et al., 1996).

2.3 Tumor Biology

A number of biological pathways regulating cancer seem to be distrupted or affected in NB, including tumor differentiation, apoptosis, drug resistence, angiogenesis, and metastasis. Insight into the molecular regulation of these biological pathways will lead to the identification of novel drug targets.

NB has the highest rate of spontaneous regression observed in human cancers, so delayed activation of normal apoptotic pathways might be important phenomenon involved in spontaneous regression and therapy resistance. Major elements of apoptotic signalling cascade with abnormal expression include BCL2 family, survivin and caspase 8. BCL2 is highly expressed in most NBs, and the level of expression is inversely related to the proportion of cells undergoing apoptosis and the degree of cellular differentiation. The BCL2 protein might also be important to acquire resistance to chemotherapy (Castle et al., 1993).

Caspase-8 is mainly affected by inactivation due to epigenetic silencing. CpG-island hypermethylation of gene promoters is a frequent mechanism for functional inactivation of genes. In NB, this mode of inactivation has been demonstrated not only for caspase 8 but also for the four TRAIL apoptosis receptors, the caspase-8 inhibitor FLIP, the RASSF1A tumor suppressor, p73, RB1, DAPK, CD44, p14ARF, and p16INK4a (Eggert et al., 2001; Hopkins-Donaldson et al., 2000; Teitz et al., 2000). Because many of these genes are

involved in apoptotic signalling and therapy responsiveness, gene hypermethylation might be a major event leading to resistence. Therefore, the antitumor effects of demethylating agents, including decitabine, are being investigated in preclinical studies.

Acquired resistence to chemotherapeutic agents may be conferred by enhanced drug efflux due to overexpression of classical multidrug resistence proteins, including multidrug resistence gene 1 (MDR1) and the gene for multidrug resistance-related protein (MRP1) whose potential clinical significance in NB has been addressed in several studies (Goldstein et al., 1990; Haber et al., 2006).

Several additional factors have been shown to contribute to the treatment resistance in NB, including the expression of oncogene such as MYCN, TrkB/BDNF signalling, or loss of p53 expression (Jaboin et al., 2002).

Neurotrophin signalling mediated by neurothrophin tyrosine kinase receptors TrkA, TrkB and TrkC and the ligands NGF (nerve growth factor), BDNF (brain derived nerve factor), NT3 (neurotrophon 3), respectively, has a central role in normal neuronal development (Nakagawara et al., 1994;1993).

Alterations of these pathways have been found to be responsible of regulating the differentiation or the malignant transformation of sympathetic neuroblasts to NB. Since the clinical and biological roles of Trk receptors and their ligands have been extensively investigated, they have been identified as important prognostic factor in NB. High

expression of TrkA was correlated with younger age, lower stage and absence of MYCN amplification. Furthermore, TrkA levels was correlated with favourable outcome and a combination of TrkA expression and MYCN amplification provided even greater prognostic power. By contrast, full-length TrkB expression was strongly associated with MYCN amplified tumors. Because these tumors also present high level of ligand BDNF, this might present an outcome or paracrine loop, thereby providing some survival and growth advantage (Nagakawara et al., 1993, 1994).

NB aggressive phenotype correlates to high expression of important players of tumor adhesion and migration such as CD44 and NM23-H1, and of regulators of local invasiveness and metastasis such as metalloproteinases (mainly MMP9) (Gross et al., 2000; Almgren et al., 2004; Chantrain et al., 2004). High expression of proangiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) which enhance tumor angiogenesis, are also correlated with more aggressive phenotype (Ribatti et al., 2004). These observations make angiogenesis and invasion inhibitors an attractive treatment option that is being evaluated in preclinical studies.

During the past decade, one set of small-non-coding RNAs (miRNA) has been studied heavily in the contest of cancer. Their expression correlates to prognosis, diagnosis and response to treatment and, like coding genes, they can function as oncogenes or tumor suppressor

genes. Overexpression of the miR-17-92 cluster has been reported in several cancer and is responsible of cycle progression and blocking of apoptotic signalling (Hayashita et al., 2005). In NB miR-17-92 cluster expression is directly activated by MYCN through a direct binding to conserved E-box elements in miR-17-92 promoter. Treatment with antagomir-17 abolished the growth of MYCN-amplified and therapy-resistant NB in vivo, indicating that targeting miR-17-92 is useful in the treatment of NB (Fontana et al., 2008).

3. NB therapy

Current treatment for high-risk NB consists of a coordinated sequence of chemotherapy, surgery, and radiation (Matthay et al., 1999).

Over half of children presenting NB have widespread metastatic disease. Although the majority of these children respond well to intensive chemotherapy, with complete remission of metastatic disease in many cases, most appear to harbour occult minimal residual disease which eventually results in disease relapse. Although outcome has improved with intensive multimodal treatment protocols, prognosis remains poor with long-term survival in only around 30-40% of children with high-risk disease. Several rationally chosen biologic agents are in ongoing clinical trials for recurrent NB, including histone deacetylase inhibitors, Trk tyrosine kinase

inhibitors, and anti-angiogenic agents, to optimize treatment of minimal residual disease.

Immunotherapy is an attractive therapeutic option for NB as it potentially offers a much more specific and less toxic treatment than conventional therapies. The genetic alterations in cancer cells that confer upon them their malignant properties also result in the expression of proteins, or tumor antigens, that differ from normal cells and have the potential to be identified by the immune system as foreign (Boon et al., 1997). GD2 glycosphingolipid is abundantly expressed on the cell membranes of virtually all human NB cells, providing a good target for mAb targeted therapies. To date, three monoclonal antibodies direct against GD2 have been used clinically in children with NB, showing most promise when used in children with minimal residual disease (Modak et al., 2007).

The efficacy of isotretinoin (cis-RA) in treating minimal residual NB has been demonstrated, establishing a standard use of non cytotoxic therapy. Alternative retinoid derivatives, including fenretinide, have been tested and show promising response rates in recurrent disease (Matthay et al., 1999).

3.1 Retinoic Acid

Retinoic Acid (RA) is a derivate of Vitamin A (retinol) belonged to a Retinoid family, which can exist in alternate stereoisomeric forms (all-trans, 9-cis or 13-cis). Retinol is usually ingested and up-taken by

a system involving retinol binding protein (RBP) and the transmembrane protein STRA6 (Kawaguchi et al., 2007). During animal development, RA is synthesized from retinol in two steps of NAD-dependent oxidation, each catalyzed by different families of enzymes. The first step is the reversible oxidation from retinol to retinal by the alcohol dehydrogenases (ADH) which can metabolize 9-cis and 13-cis retinol as well as all-trans retinol, and by the short-chain dehydrogenases/reductase (SDR) which can only catalyze the oxidation of all-trans retinol (Yaung et al., 1994). The second step is the irreversible oxidation of retinal to retinoic acid (RA) by the aldehyde dehydrogenase family (ALDH) (Duester et al., 1996). Other proteins, such as cellular retinoid binding proteins (CRBP) interact with retinoids regulating the availability of RA in vivo and mediating the transport of RA to the nucleus (Napoli et al., 1999). The availability of RA is also controlled through its degradation by proteins of the cytochrome P450 which catalize the oxidation of RA in a wide range of metabolites whose biological activity is controversial (White et al., 1997). RA functions are mediated by heterodimers of two members of the nuclear hormone receptors superfamily-RAR and RXR (reviewed in Gronemeyer et al., 2004) (Figure 4).

Co-activators and co-repressors also regulate the activity of RA. Many co-activators such as CBP/p300 and pCAF contain histone acetyltransferase activity, able to remodel chromatine to a more open conformation, allowing assembly of large protein complexes with the

basal transcription machinery (Kamei et al., 1996; Blanco et al., 1998). The heterodimers binds to specific DNA elements in the regulatory regions of target genes, called RA response elements (RAREs). In vitro, RAR can bind both all-trans and 9-cis RA, whereas RXR can only binds 9-cis RA. During embryonic development, RA activities is to regulate transcription of two classes of gene targets: genes coding for factors involved in the RA pathways (synthesis, metabolism and signalling) and transcription factors containing homeobox domains crucial in the developing embryo. RA has long been thought to act over distance in the form of gradient along the anteroposterior (AP) body axis in the vertebrate embryo: high concentrations in the posteriormost part of the embryo activate a specific group of genes whereas low concentrations in the anterior part active other genes (Chen et al., 1994). The responsiveness to RA depends on the sensitivity of given tissue which varies both temporally and spatially during development.

Alteration of RAR expression or function has been observed in a variety of cancers. In the case of acute promyelocytic leukemia, it has been shown that a major cause of this disease is a genetic translocation resulting in the production of a fusion protein containing RARalpha (Pandolfi et al., 2001). In some tumors, RA can induce the expression of RARbeta, and this is associated with growth suppression. However in many tumors RARbeta is silenced either

through deletion, mutation or methylation within the promoter sequence (Nkayama et al., 2001).

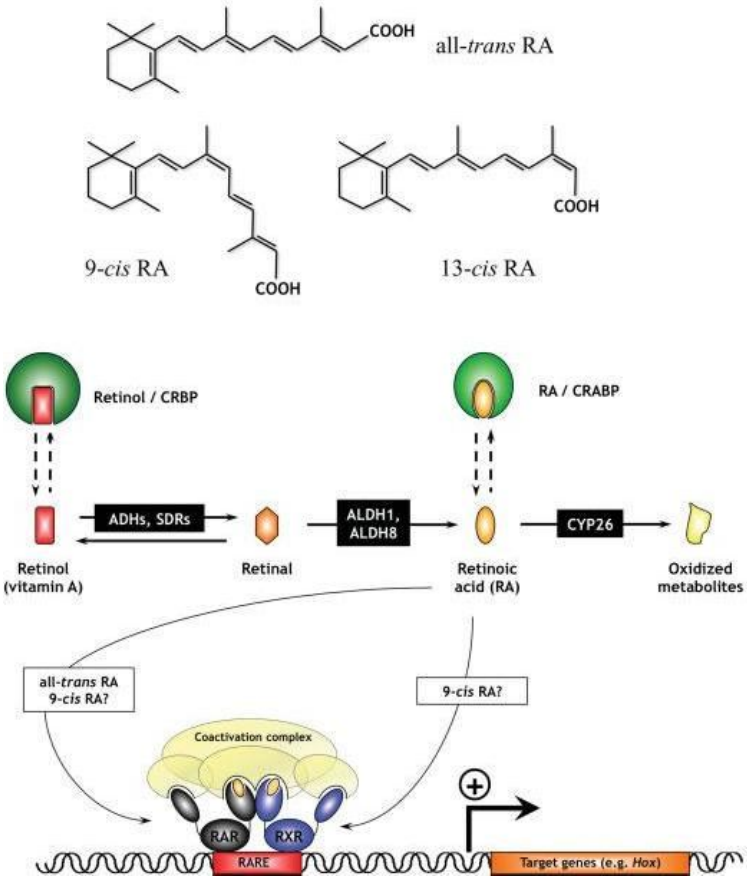


Figure 4. Retinoic Acid: Mechanism of Action

It has also been shown that retinoic acid can restore "normal" functions (differentiation) in certain tumors, such as NB (Sidell et al., 1982), melanoma (Ludwig et al., 1980) and acute promyelocytic leukemia (PML) (Breitman et al., 1980).

In NB cell lines RA induces differentiation up-regulating the transcription factor AP-1 and by activating JNK pathway via MAP

kinase which is required for neurite outgrowth (Huang et al., 2003; Yu et al., 2003). RA also activate Erk1/2 pathway in SHSY5Y NB cells during neuronal differentiation and alter the activity of the phosphoinositide-3 kinase/Akt pathway which is associated with enhanced cell survival (Singh et al., 2003; Lopez-Carballo et al., 2002). One of the major biological effects of RA is to inhibit cell proliferation, by inhibiting cyclin D and stimulating p27 expression which block progression through the G1 phase of the cell cycle (Zhou et al., 1997).

In clinical practice, all-*trans* retinoic acid (ATRA) is mainly used for patients with acute PML (Huang et al., 1989). In the treatment of NB patients, ATRA has been used as a chemotherapeutic agent with same success (Reynolds et al., 2003), but 13cis-RA is preferred due to its more favourable pharmacokinetics (Veal et al., 2002; Reynolds et al., 1994; Matthay et al., 1999) . Furthermore, studies comparing the activities of 13cis-RA and ATRA in NB cell lines have demonstrated similar potencies of these retinoids, in terms of cellular differentiation, growth arrest and regulation of tumour markers such as MYCN (Yuza et al., 2003).

4. Neurothrophin receptors

The family of neurotrophin tyrosine kinase (NTK) receptors consists of TrkA (NTRK1), TrkB (NTRK2), and TrkC (NTRK3). The primary

ligands for these receptors are nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and neurotrophin-3 (NT3), respectively; neurotrophin-4/5 (NT4) functions through TrkB (Kaplan et al., 1991a, 1991b; Klein et al., 1991a, 1991b) (Figure 5). Although TrkC is the primary receptor for NT3, it also binds and activates TrkA and TrkB. Another transmembrane receptor (P75) binds all the neurotrophins with low affinity. P75 can interact directly with TrkA, TrkB, and TrkC in both extracellular and intracellular domains, and this interaction may contribute to the formation of high-affinity receptors or otherwise alter the function of Trk receptors (Hantzopoulos et al., 1994).

Trk expression is crucial for the normal development of the peripheral nervous system. Knockout mice for TrkA, TrkB, or TrkC display unique but overlapping patterns of abnormalities involving the central and peripheral nervous system, but the TrkB knockout is the most severe (Klein et al., 1994, 1993; Smeyne et al., 1994). Nevertheless, TrkA expression is most important for the development of normal sympathetic neurons (Fagan et al., 1996; Schober et al., 1997). Indeed, NGF is necessary for the survival and differentiation of sympathetic neurons both in vitro and in vivo (Levi-Montalcini, 1987). For sympathetic neurons, TrkC (±TrkA) is expressed early, TrkB expression is rarely if ever seen (Dixon et al., 1994; Schober et al., 1997), and TrkA expression predominates in later stages.

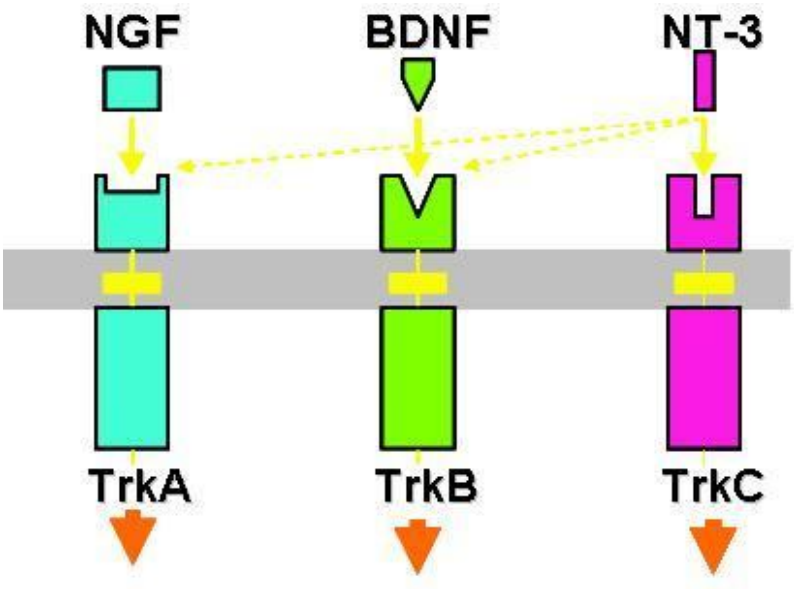


Figure 5 Trk receptors and ligands

TrkA, TrkB, and TrkC have distinct isoforms that affect the function of the receptors. The neuronal TrkA isoform (TrkA-I) lacks exon 9 (790 aa), whereas TrkA-II nonneuronal isoform is 796 aa, and has enhanced responsiveness to NT3 (Clary et al., 1994). There is also an early developmental form (TrkAIII) that splices out exons 6, 7, and 9 and is constitutively active independent of ligand. TrkB is expressed as both full-length (kinase-intact) and truncated (kinase-deleted) isoforms (Nagakawara et al., 1995; Allen et al., 1994; Middlemas et al., 1991). Both may act as dominant-negative inhibitors of full-length TrkB kinase activity, because expression of truncated TrkB receptors inhibits BDNF-induced neurite outgrowth (Fryer et al., 1997). TrkC also has full-length and truncated isoforms, similar to those found for TrkB (Menn et al., 1998).

Upon binding of their specific ligands, Trk receptors dimerize and autophosphorylate cytoplasmic tyrosines. Signal transduction is processed through a diverse array of signaling pathways, including the Ras/MAPK-, the inositol (1,4,5) triphosphate (IP3)-dependent Ca2C release and the PI3K/AKT pathway (Kaplan et al., 1997). Ras/MAPK are activated downstream of TrkA pathway. MAPK translocates to the nucleus to participate in the activation of transcription factors that regulate NGF-inducible genes, resulting in survival and neuronal differentiation. Activated TrkB receptor recruits PI3K via adaptor proteins such as Shc and Gab-1. Alternatively, PI3K can be activated downstream of Grb2-binding or of Ras G-proteins. PI3 kinase activity is critical for TrkB receptor-mediated survival signalling (Fryer et al., 1997) utilizing lipid second messengers that activate the serine threonine kinase Akt, also known as protein kinase B (PKB) (Figure 6).

Critical elements of the Trk signaling pathway may vary between different tissues leading to a cell-type-specific response. For example, stimulation of TrkA by its specific ligand, nerve growth factor (NGF), leads to differentiation of neurons and NB cells, whereas it induces proliferation of fibroblasts and apoptosis of medulloblastoma cells (Muragaki et al., 1997; Micera et al., 2001). In contrast to its role in NB, TrkB is associated with a favorable biology and good prognosis in medullary thyroid carcinoma (McGregor et al., 1999).

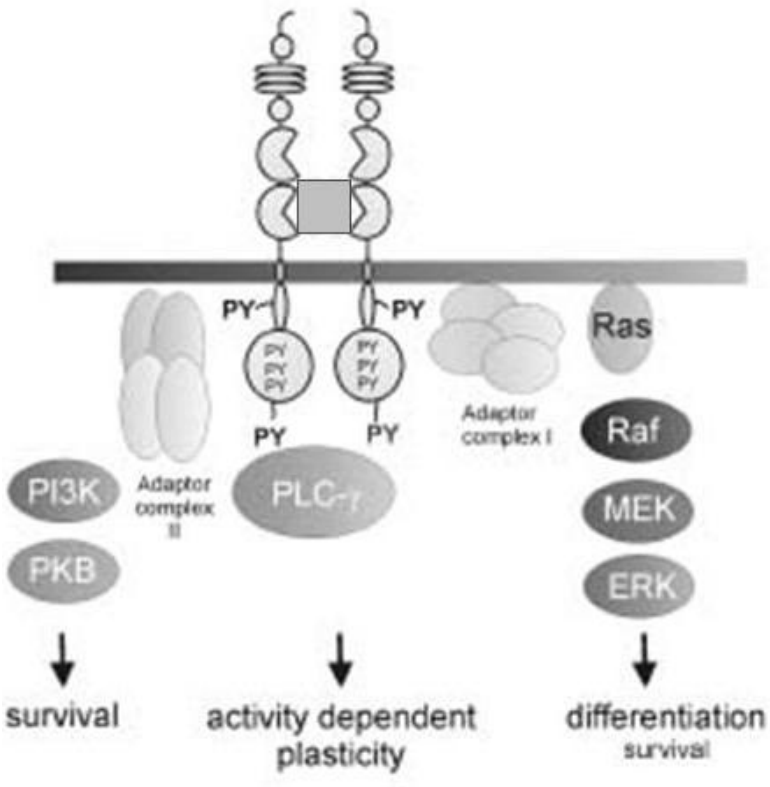


Figure 6 Transduction signals of Trk receptor

4.1 Biological role of Trk receptors in neuroblastoma

In order to understand complex regulation patterns via tyrosine receptor TrkA or TrkB signalling in NB, pathways and the resulting phenotypes have been well studied in stable Trk-expressing SY5Y cells (Eggert et al., 2000, 2002).

From the reported observations in these cellular models, activation of TrkA by NGF resulted in neuronal differentiation, growth inhibition, inhibition of angiogenesis and sensitization to drug-induced apoptosis (Eggert et al., 2000, 2002). Whereas, activation of TrkB by its ligand brain-derived neurotrophic factor (BDNF) mainly resulted in

enhanced proliferation and chemotherapy resistance of the cells (Ho et al., 2002). The biological phenotype of the transfectants corresponds well with the excellent prognosis of primary neuroblastomas expressing high levels of TrkA, and the poor prognosis of neuroblastomas expressing TrkB. These models represent the starting point to clarify the molecular basis of aggressiveness in NB.

5. Galectin-1

Galectins are conserved family of lectins, carbohydrate binding proteins with an affinity for beta-galactoside. Fifteen mammalian galectins have been defined to date which share consensus of amino-acid-sequences and are composed of one or two carbohydrate recognition domain (CRD) responsible for beta-galactoside binding. Regarding to biochemical structure, some galectins contain one CRD and exist as monomers (galectin-5, -7, -10) or as homodimers (galectin-1, -2, -11, -13, -14, -15) whereas other galectins contain two CRD connected by a short linker region (galectin-4, -6, -8, -9, -12). In contrast, galectin-3 uniquely occurs as chimeric protein with one CRD and a non-lectin domain involved in oligomerization of this protein. CRD of all galectins share an affinity for the N-acetyl-lactosamine, but different modifications to this minimum disaccharide ligand can also be recognised, showing the fine specificity of certain galectins for

tissue- or developmentally-specific ligands (Reviewed in Rapoport et al., 2008) (Figure 7).

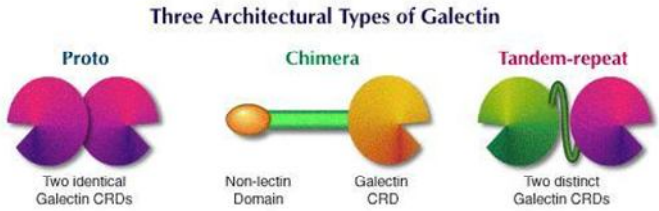


Figure 7 Galectins Family

Galectin-1 (Gal-1) is encoded by the LSGALS1 gene located on chromosome 22q12. The homodimeric Gal-1 protein can spontaneously dissociate at low concentrations into a monomeric form that is still able to bind to carbohydrates but with a lower level of affinity.

Gal-1 shows the characteristics of typical cytoplasmic proteins as well as an acetylated N-terminus and a lack of glycosylations. It has been described in cell nuclei and cytosols and it also translocates to the intracellular side of cell membranes. Gal-1 is secreted and can be found on the extracellular side of all cell membranes as well as in the extracellular matrices of various normal and neoplastic tissues. Gal-1 lectin activity, related to its carbohydrate-binding site, is observed when it is extracellular, whereas the protein-protein interactions of Gal-1 concern its intracellular functions (Reviewed in Rabinovich 2005).

Expression of Gal-1 has been well documented in many different tumor types including astrocytoma, melanoma and prostate, thyroid, colon, bladder and ovary carcinomas. Interestingly, in most cases such expression correlates with the aggressiveness of these tumors and the acquisition of metastatic phenotype (reviewed in Liu and Rabinovich 2005; Rabinovich 2005; Salatino et al. 2008) (Figure 8).

Intracellular Gal-1 may play a key role in the initiation of transformed phenotype of tumors. It has been found that Gal-1 interacts with oncogenic H-RAS and contribute to membrane anchorage of H-RAS. Furthermore, in tumor cells both the membrane association of H-RAS and cell transformation are increased by over-expression of Gal-1. Intracellular Gal-1 causes biphasic modulation of cell growth, depending on the dose involved. In NB and stromal bone marrow cells high doses of recombinant Gal-1 (1uM) inhibit cell proliferation independently of Gal-1 sugar binding activity; low doses (1nM) of Gal-1 are mitogenic and are susceptible to inhibition by lactose. The paradoxical positive and negative effects of Gal-1 on cell growth are highly dependent on cell type and cell activation status, and might also be influenced by the relative distribution of monomeric versus dimeric, or intracellular versus extracellular forms.

Extracellular Gal-1 has an important role to modify adhesion, motility, and invasion processes. Gal-1 increases the adhesion of various normal and cancer cells to the extracellular matrix via cross-linking of glycoproteins (integrins) exposed on the cell surface (Hughes, 2001).

Gal-1 causes the increased motility of glioma cells and the reorganization of the actin cytoskeleton associated with an increased expression of RhoA protein (Yamaoka et al., 2000). An important role of Gal-1 is the function as a homeostatic agent by modulating innate and adaptative immune responses (Juszynski et al, 2007; Rodig at al, 2008). The effects of Gal-1 on immune and inflammatory cells are likely to be due to the binding and cross-linking on the cell surface of T cells of the glycoproteins as CD2, CD3, CD7, CD43 and CD45, in a carbohydrate-dependent manner. This binding leads to apoptosis of activated T cells, thus contributing to tumor immuno-escape (He and Baun, 2004)..

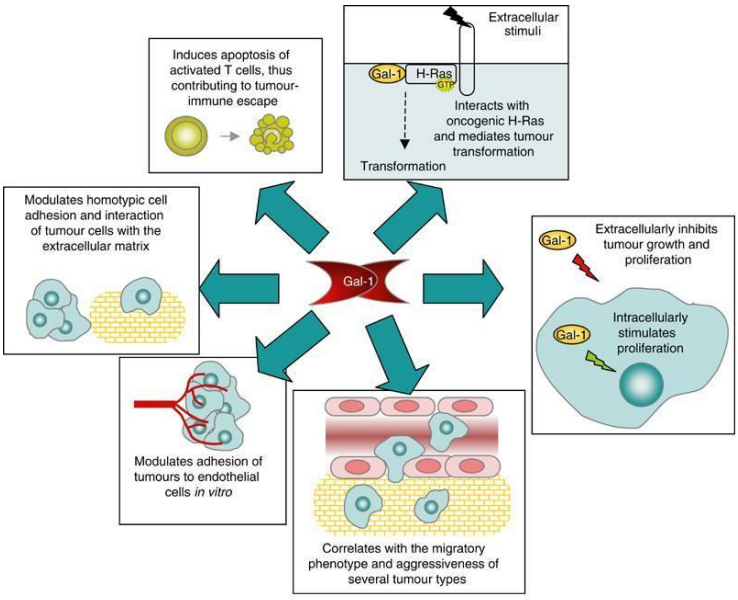


Figure 8 Galectin-1 and cancer progression

Taken in account the association of high expression of Gal-1 to tumor progression, it still remains to be elucidated how expression of Gal-1

in tumor tissue or tumour-associated stroma may actively influence disease outcome in terms of our understanding of its multifunctional modes of actions.

Drugs able to inhibit Gal-1 function have been defined. Actually present in clinical trials, anginex is a peptide that targets Gal-1 and shows an anti-angiogenic activity in vivo (Thijssen et al, 2006). Farnesylthiosalicylic acid (FTS) which disrupts Ras membrane anchorage imparing H-Ras(12V)-Gal-1 interactions (Paz et al, 2001) has proven anticancer activity in various model systems and is now proceeding to clinical development.

A current challenge is the design of more specific and potent Gal-1 inhibitors for therapeutic propose to be assessed in clinical practice (in association with cytotoxic agents) in the near future.

6. Discovery of new therapeutic targets in Neuroblastoma by means of proteomic approach and functional studies.

NB is a heterogeneous tumor for which biology dictases clinical behaviour, ranging from spontaneous regression/differentiation to metastatic forms with rapid progression and poor prognosis. Overall survival is excellent for patients who have low- and intermediate-risk NB with a general trend toward minimization therapy whereas less of

40% of high-risk NB patients survive because of the high risk of minimal residue disease. Actually, several rationally chosen biologic agents are in ongoing clinical trials for recurrent NB, including histone deacetylase inhibitors, Trk tyrosine kinase inhibitors, and anti-angiogenic agents.

The goal of this thesis has been to suggest new ways to improve the current therapeutic protocols as the use of low-toxic drugs (differentiation agents) and as impairing NB by means of targeting downstream mediators of the mainly involved pathways (Trk pathways).

6.1 First Aim

The good knowledge of the molecular mechanism of differentiation in NB is imperative to improve the efficacy of therapy whose targets could be the drivers of the differentiation program.

Retinoids are used to treat minimal residual NB and are estabilished as standard for the use of noncytotoxic therapy (Reynolds et al., 1991).

Advances in understanding the molecule biology of NB have been supported by the use of high-throughput, array-based methods not only to improve prognosis but also to identify key targets that can efficiently be exploited therapeutically. For example, gene expression profiling on NB specimens have been described as identifying the molecular signatures of high-risk and low-risk tumours and novel prognostic markers (Ohira et al., 2003, 2005; Hiyama et al., 2004;

Riley et al., 2004; Wei et al., 2004; Takita et al., 2004; Schramm et al., 2005b). Anyway, the search for markers at the transcriptional level is less reliable than at the protein level, as there is a "long" and unpredictable route from RNA to proteins, and very often protein expression does not correlate with mRNA expression. Indeed, several proteins can be encoded by the same gene, through splice variants and post-translational modifications that cannot be directly predicted from gene sequence. These observations have lead to search for markers at translation levels, by using a comparative proteomic approach. Actually, two-dimensional difference gel electrophoresis (2D-DIGE) coupled with mass spectrometry (MS) is the best proteomic approach, extensively used to identify potential biomarkers of various cancers (Kumar et al., 2009; Kohnke et al., 2009; Ploussard et al., 2010). 2D-DIGE advantages over traditional 2D-PAGE are: 1) multiple pre-labelling of samples; 2) introducing a pooled internal standard; 3) co-detection; and 4) a wider dynamic range. In spite of this, one of the limitations of DIGE-based proteomics is the low identification rate of low-abundance proteins (Hoorn et al., 2006).

In the first part of my thesis, my aim was to gain further insight into the molecular mechanisms of NB differentiation induced by ATRA, in order to underline which pathways and molecular markers are responsible of this process. I combined the advantage of 2D-DIGE proteomic approach to the advantages of sub-cellular fractionation. The sub-cellular fractionation of the proteoma in cytosol and nuclear

fractions gave the possibility to show a major number of proteins on the same gel and to enrich the low-abundant proteins in the extract.

6.2 Second Aim

Trk tyrosine kinase receptors represent the most important prognostic marker in NB. The expression of neurothrophin brain-derived neurotrophic factor (BDNF) and its tyrosine receptor TrkB are often detected in NB tumors derived from patients with an unfavourable prognosis. In the other hand, high expression of tyrosine receptor TrkA is correlated to favourable biological features and patient survival.

Although mediators of Trk pathways have been elucidated in the past years, there is still to be searched the potential markers leading to the aggressive behaviour of TrkB expressing NB despite the TrkA expressing NB (Schulte et al., 2005). To elucidate markers of NB aggressiveness is an important step to develop effective therapy reducing the risk of minimal residue. In previous studies Galectin-1 emerged as new interesting TrkB receptor target: Gal-1 mRNA was up-regulated in patients with aggressive, relapsing NB and protein was up-regulated in an in vitro model of aggressive TrkB expressing NB cells.

The aim of the second part was the functional study of Gal-1, down-stream mediator of TrkB signalling, in order to establish Gal-1 as a potential therapeutic marker in high-risk TrkB-expressing NB.

Material and Methods

1.Cell Culture

1.1 Cell Culture and characteristics of stable transfectants

The human LAN-5 NB cell line was grown in RPMI medium. The human SK-N-BE and SH-SY5Y NB cell lines were grown in Dulbecco's modified Eagle's medium. Full-length TrkB or TrkA cDNA was cloned into the retroviral expression vector, pLNCX, and transfected in human NB SH-SY5Y (SY5Y) cells by electroporation. Stably transfected cells were selected in RPMI medium supplemented with 500 mg/ml geneticin. As negative control, SY5Y cells were transfected with the empty vector (SY5Y-vec). Surviving, drug-resistant SY5Y cells were subcloned to obtain single-cell clonal lines (Schulte et al., 2005). Cells were maintained in an atmosphere containing 5% CO2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, antibiotics. The stable clones were also supplemeted with geneticin to maintain selection pressure.

The cells were passaged 1:4 twice weekly and routinely fed 24 h before each experiment.

1.2 Neuronal Differentiation

Neuronal differentiation was induced in cells at 80% confluence by 5 μM ATRA (Sigma) dissolved in dimethyl sulfoxide (DMSO). All experiments were performed under dim light, and the tubes containing retinoids were wrapped in aluminum foil. The cells were washed three times in 10 mL ice-cold phosphate-buffered saline (PBS) and harvested 0, 8, 24, and 48 h after RA treatment, using a cell scraper. This time course was chosen to reflect primary or early responses of NB cell lines to the RA treatment. Each experiment was performed in triplicate. Three cellular plates for each experimental point (0, 8, 24, and 48 h) were harvested, pooled, and centrifuged for 5 min at 2000*g* at 4 °C. The pellets were frozen at -80 °C until sample preparation.

2. Two Dimensional-differential in gel electrophoresis (2D-DIGE)

2. 1. Cytosolic Protein Fraction Preparation

The cell pellets were incubated on ice in ice-cold lysis buffer (250 mM sucrose, 3 mM imidazole, pH 7.4, and 1 Mm EDTA) in the presence of a protease inhibitor cocktail (Complete Mini EDTA-free, Roche). The cellular pellets were disrupted by 8-10 passes through a 22-guage needle, and then centrifuged at 16 000*g* for 20 min at 4 °C.

The pellets obtained were considered as the nuclear fraction, while the post-nuclear supernatant (PNS) contained the cytosolic fraction and cell membranes.

The PNS was ultracentrifuged at 100 000*g* for 45 min at 4°C. The supernatant obtained here was used as the cytosolic fraction. The cytosolic fraction was solubilized in 7 M urea, 2M tiourea, 40 mM Tris-HCl, and 4% CHAPS and incubated for 30 min under agitation at room temperature.

2.2 Nuclear Protein Fraction Preparation.

The nuclear pellet obtained above was resuspended in 7 M urea, 2 M tiourea, 40mM Tris-HCl, pH 8.5, 0.24% Triton X-100, and 4% CHAPS. After 30 min of incubation at room temperature, 10 mM spermine was added, and the solution was incubated for 1 h at room temperature. The precipitated material was removed by ultracentrifugation at 100 000*g* for 45 min at 4 °C. The solubilized cytosolic and nuclear fractions were reduced by 5mM tributylphosphine (TBP) for 90 min and were alkylated by 10 mM iodacetamide (IAA) for 90 min, in tubes wrapped in aluminum foil. After, the samples were precipitated for 90 min in a mix of acetone/methanol (8:1) at -20 °C and centrifuged at 13 400*g* for 30 min, at 4 °C. The pellets were air-dried and solubilized in 7 M urea, 2 M tiourea, 3% CHAPS, and 30 mM Tris-HCl. Protein concentrations were determined using the Bradford method (Bio-Rad).

2.3 Cy-Dye Labeling of Cytosolic and Nuclear Fraction Extracts

The pH of the samples was adjusted to pH 8.5 with 0.1M NaOH or HCl. Typically, 50 µg of extract was labelled with 400 pmol Cy2 (standard mixture of extracts), Cy3, and Cy5 (untreated and RA-treated cells). The labelling reaction was carried out on ice in the dark for 30 min, and was stopped with 1 mM lysine (final concentration). The samples were then mixed, and supplemented with 0.5% carrier ampholytes pH 3-10 (Bio-Rad) and 1% bromophenol blue. The final volume was adjusted to 380 μL with 7 M urea, 2 M tiourea, and 3% CHAPS.

2.4 2-D Gel Electrophoresis, Imaging, and DIGE Analysis.

The immobilized pH gradient (IPG) gel strips (length, 17 cm; thickness, 0.5 mm), nonlinear pH gradient range (NL) pH 3-10 (Bio-Rad) were passively rehydrated with 150 μg of tripartite-labelled sample (50 μg for each labelled sample and 50 μg internal standard) in the dark overnight. Isoelectric focusing (IEF) was carried out with a Protean IEF Cell (Bio-Rad), with a low initial linear voltage up to 1000 V in 5 h, and then by applying a voltage gradient up to 10 000 V with a limiting current of 50μA/strip. The total voltage applied was 76

000 V/h for each strip, and the temperature was set to 20 °C. The strips were equilibrated in 6 M urea, 2% SDS, 20% glycerol, and 0.375 M Tris-HCl (pH 8.8), for 30 min in the dark. Equilibrated IPG strips were transferred onto 18/20 cm 11% polyacrilamide gels, within low-fluorescence glass plates (ETTAN-DALT, GE Healthcare). The second-dimension SDSPAGE was performed using a Peltier-cooled DALT II electrophoresis unit (GE Healthcare) at 1 W/gel overnight. The gels were scanned with a Typhoon 9400 variable mode imager (GE Healthcare) using mutually exclusive excitation/emission wavelengths for Cy2 (488 nm/520 nm), Cy3 (532 nm/580 nm), and Cy5 (633 nm/670 nm). Images were normalized and analyzed for statistics, and differentially expressed spots were quantified using the DeCyder 5.0 software (GE Healthcare). A DeCyder differential in-gel-analysis (DIA) module was used for pairwise comparisons of each sample (Cy3 and Cy5) to the Cy2 mixed standard present in each gel. The DeCyder biological variation analysis (BVA) module was then used to simultaneously match all of the protein-spot maps from the gels, and to calculate average abundance ratios across triplicate samples. Proteins with molecular masses from approximately 20-150 kDa were resolved, and approximately 3000 spots/gel were detected using the DeCyder image analysis software. To avoid false positives, only spots with a fold change equal or above 1.3 and a p value e0.075 (Student's t test) were taken in account. A manual sorting based on the quality of the protein spots was also performed.

3 Protein Identification by MS.

For preparative protein separations, 1 mg of unlabeled sample was used to passively rehydrate the IPG strips. The first and second dimension runs were conducted as described above. After 2-D electrophoresis, the separated proteins were visualized using the universal staining methods of anionic dyes (Coomassie Colloidal Blue; Pierce). The resolved polypeptides were fixed in 10% acetic acid/40% methanol solution for 12-16 h before staining. The staining in Colloidal Coomassie was performed for 3 days, and then the gels were washed three times in deionized H2O. Selected protein spots were excised from the gels and washed in 50 mM ammonium bicarbonate, pH 8.0, in 50% acetonitrile until completely distained. The gel pieces were re-suspended in 50 mM ammonium bicarbonate, pH 8.0, containing 100 ng of trypsin, and incubated for 2 h at 4 °C and overnight at 37 °C. The supernatants containing the resulting peptide mixtures were removed, and the gel pieces were re-extracted with acetonitrile. The two fractions were then collected and freezed.

3.1. MALDI MS Analysis.

MALDI mass spectra were recorded on an Applied Biosystem Voyager DE-PRO mass spectrometer equipped with a reflectron analyzer and used in delayed extraction mode. One microliter of peptide sample was mixed with an equal volume of R-cyano-4-

hydroxycynnamic acid as matrix (10 mg/mL in 0.2% trifluoroacetic acid (TFA) in 70% acetonitrile), applied to the metallic sample plate, and air-dried. Mass calibration was performed using the standard mixture provided by the manufacturer. Mass signals were then used for database searching using the MASCOT peptide fingerprinting search program (Matrix Science, Boston, MA), available on the Internet.

3.2. LC-MS/MS Analysis.

The unknown protein spots from peptide mass fingerprinting were further analyzed by LC-MS/MS using a Q-TOF Ultima hybrid mass spectrometer (Micromass, Waters) equipped with a Z-spray source and coupled online with a capillary chromatography system (CapLC, Waters). The peptide mixture (10 μL) was first loaded onto a reverse phase trap-column (Waters) at 10 μL/min using 0.2% formic acid as eluent. The sample was then transferred to a C18 reverse-phase capillary column (75 μm, 20 mm) at a flow rate of 280 nL/min and fractionated using a linear gradient of running buffer B (0.2% formic acid in 95% acetonitrile) in running buffer A (0.2% formic acid in 5% acetonitrile) from 7% to 60% in 50 min. The mass spectrometer was set up in the data-dependent MS/MS mode to alternatively acquire a full scan (*m*/*z* acquisition range from 400 to 1600 Da/e) and a tandem mass spectrum (*m*/*z* acquisition range from 100 to 2000 Da/e). The three most intense peaks in any full scan were selected as precursor

ions and fragmented by collision energy. Raw MS and MS/MS spectra were elaborated by the ProteinLynx software, provided by the manufacturers that generated a peak list containing all of the fragmentation data that was used for database searching using the MASCOT MS/MS ion search software for protein identification.

3.3 Protein Identification by Bionformatic Tools

Raw data from nanoLC-ESI-MS/MS analyses were converted into a Mascot format text to identify proteins by means of a Mascot software version 2.1 in home, MatrixScience.30 The protein search both from nanoLC-ESI-MS/MS and MALDI MS analyses was governed by the following parameters: nonredundant protein sequence data base (NCBInr -20061017 database with 4 051 787 sequences and 1 396 484 404 residues downloaded; Sprot- 50.9 database with 235 673 sequences and 86 495 188 residues downloaded); specificity of the proteolytic enzyme used for hydrolysis (trypsin); taxonomic category of the sample (*Homo sapiens*); no protein molecular weight was considered; up to 1 missed cleavage; cysteines as *S*-carbamidomethylcysteines; unmodified N- and C-terminal ends; methionines both unmodified and oxidized; putative pyroGlu formation by Gln; precursor peptide maximum mass tolerance of 150 ppm and a maximum fragment mass tolerance of 100 ppm.

4. Data Mining.

Hierarchical cluster analysis was performed using a tool available on the Internet (http://gepas.bioinfo.cnio.es/). The distance between protein expression profiles was calculated using Correlation Coefficient (linear), and the UPGMA algorithm was used to construct dendrograms.35,36. The proteins were classified according to the DAVID 2.1 beta annotation system (http://david.niaid.nih.gov/david/ease.htm). This tool adopts the Fisher exact test to measure the protein enrichment in annotation terms. A Fisher exact test P=0 represents perfect enrichment. If the P-value is equal to or smaller than 0.05, a protein would be considered strongly enriched in the annotation categories.

5. Western Blotting.

Cells were washed three times with ice cold PBS and scraped into extraction buffer (50 mM Tris-HCl, pH 7.5, 150mMNaCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, and complete protease inhibitor cocktail (Roche)), transferred to 1.5 mL microcentrifuge tubes, vortexed for 15 min at 4 °C, and centrifuged at 16 100g for 30 min at 4 °C. Proteins extract concentrations were determined by the Bradford assay (Bio-Rad). Thirty micrograms of total protein lysates was diluted 1:1 with Laemmli SDS-PAGE sample buffer, loaded onto 12%

polyacrylamide gels, and blotted onto polyvinylidene difluoride membranes (PVDF; Bio-Rad). Membranes were blocked with 5% non-fat milk (Bio-Rad) in PBS, pH 7.6, and 0.2% Tween-20 (PBS-T), and then incubated with specific commercial goat anti-PRX II (peroxyredoxin-2), SgII (secretogranin II), NF-L (68 kDa neurofilament), EF-2 (elongation factor-2), prefoldin subunit 3 and Gβ2 (guanine-nucleotide-binding protein beta subunit 2) antibodies, a mouse anti-PCNA (proliferating cell nuclear antigen) antibody (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA), and a rabbit anti-EF1R (eukaryotic elongation factor) antibody (1:100) (Upstate) at 4 °C overnight. Antihuman Gal-1 goat polyclonal antibody (R&D Systems, Wiesbaden, Germany) was used 1:200. After a washing step in PBS-T, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated antigoat, anti-mouse, anti-rabbit antibody (1:10 000) (Santa Cruz Biotechnology), and the immunoblots were visualized using ECL detection kits, with enhanced chemiluminescence (Pierce). A mouse β-actin antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the control for equal loading. The protein bands images on X-ray films were acquired with the GelDoc 2000 system (Bio-Rad). The densitometric measurements were performed by Quantity One 4.5 tool (Bio-Rad).

6. siRNA transfection

Cell lines were transiently transfected with siRNAs directed against Gal-1 or with an unrelated control siRNA (NTC) using lipofectamine buffer according to the manufacturer's protocol (Qiagen, Hilden, Germany). An ALEXA-labeled scrambled siRNA (Qiagen) was used to control for transfection efficiency and as a control for Gal-1-mediated effects. Untreated cells and cells transfected with lipofectamine buffer only were included as negative controls. Cells were plated at $2x10^5$ per well onto six-well plates or at $10x10^5$ onto twenty-four-well plates (Corning, Lowell, MA, USA) on the day before transfection. The siRNA was transfected at day 1, and total RNA and protein were extracted at day 4. The knockdown efficacy was monitored by reverse transcriptase PCR and by western blotting.

7. **Cell proliferation analysis**

Ten thousand cells per well were seeded in 96 multiwell plates. After 24 h, the cells were treated with the respective neurotrophins (NGF 100 ng/ml, BDNF 50 ng/ml), or rGal-1 (2 mg/ml) (rGal-1 was a gift of Juergen Kuhlmann (Max-Planck- Institute, Dortmund, Germany)). Metabolic activity as a surrogate marker for cell proliferation of treated samples was assessed after 24 h using the MTT (3-(4,5-

dimethylthiazol-2-yl), 5-diphenyltetrazolium bromide) assay. Cell vitality of siRNA-transfected cells was assessed 72 h after transfection. Each value represents six replicates, and each experiment was repeated at least two times.

8. Reverse transcriptase PCR and microarray analyses

Microarray experiments and analyses were performed previously (Schulte et al., 2005). Total RNA from 102 primary, untreated NB tumor samples was prepared using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription, labelling of total RNA and subsequent hybridization to Affymetrix U133v2 chips according to manufactures.

Total RNA from NB cell lines was prepared using Qiagen RNaeasy Mini Kit (Qiagen). cDNA synthesis was performed by standard protocols. Relative quantification of Gal-1 mRNA expression was achieved using Taqman Assay Hs00355202_m1 (Applied Biosystems, Foster City, CA, USA) and the DCt method. All assays were carried out in triplicate. mRNA expression was normalized to the averaged GAPDH expression in each sample.

9. Cell invasion assay

Cell invasiveness was determined using Matrigel invasion chamber inserts (24-well format with 8 mm pores, BD Biosciences, Heidelberg, Germany). Eighty thousand cells were seeded onto the upper chamber in RPMI 1640 medium containing 5% fetal calf serum. siRNA-transfected cells were seeded at day 4 after transfection. The medium in the lower chamber contained15% fetal calf serum in RPMI1640 with or without the respective neurotrophins (NGF 100 ng/ml, BDNF 50 ng/ml). rGal-1 (2 mg/ml) or anti-Gal-1 neutralizing antibody (1:500) or isotype immunoglobulin-G control were added once at the beginning of the experiment to the upper compartment. Cells migrating to the lower membrane surface were stained with hematoxylin/eosin after 24 h. Cells were counted in three representative areas per membrane at 40x magnification using a light microscope. The experiments were carried out in duplicate and two independent sets of experiments for each type of invasion assay were carried out.

10. Cell migration assay

A total of 100 000 cells were plated in 24-well tissue plates and maintained in RPMI medium. At 80–90% confluence, the tip of a micropipette was used to create a linear scratch resulting in two cell fronts that were 2mm apart from each other. The cells were washed with phosphate-buffered saline to remove floating cellular debris and

fed for an additional 24 h with either RPMI medium only or RPMI medium supplemented with the appropriate neurotrophins (NGF 100 ng/ml, BDNF 50 ng/ml). Cell migration was judged by photographs taken immediately after scratching and at designated times after scratching using a digital camera.

11. Immunohistochemistry

After antigen retrieval, immunohistochemical stainings were carried out on 4 mm sections with a Techmate 500 immunostainer (DAKO, Glostrup, Denmark). Gal-1 antibody was used at a dilution of 1:500. The antigen–antibody binding was visualized by means of the avidin–biotin complex (ABC method) using AEC (3-amino-9-ethylcarbazol) as chromogen. Immunoreactions were visualized with the ABC complex diluted 1:50 in phosphate-buffered saline (Vectastain, Vector, Burlingame, CA, USA). Gal-1 immunostaining was evaluated using a semiquantitative scoring system. Briefly, the Gal-1 staining intensity of NB cells (in NB) and ganglionic cells (in ganglioneuromas) was assessed and scaled 0–3 (0=no expression, 1=weak expression, 2=moderate expression and 3=strong expression). All slides were reviewed independently by two examiners in a blinded manner.

Results

1. DIGE Analysis of Differentially Expressed Proteins following ATRA Treatment in the LAN-5 NB Cell Line

To determine the molecular mechanisms involved in ATRA-induced differentiation in NB, I examined changes in the proteome of LAN-5 NB cells.

1.1 Subcellular fractionation

I analyzed LAN-5 cells proteome in two different cellular fractions, enriched in either cytosolic or nuclear proteins. I checked the quality of these fractions by Western blotting using specific markers (Figure 9). The biochemical protein markers for nuclei included lamin β (LM β), which is a marker of structural components of the nuclear matrix, and the transcriptional factor SP1, which is a marker of the soluble nuclear fraction. LMβ and SP1 were mostly detected in the nuclear fraction; on the contrary, the cytosolic biochemical protein marker enolase (ENO) is mostly present in the cytosolic fraction, and only seen at low levels in the nuclear extract (Figure 9A).

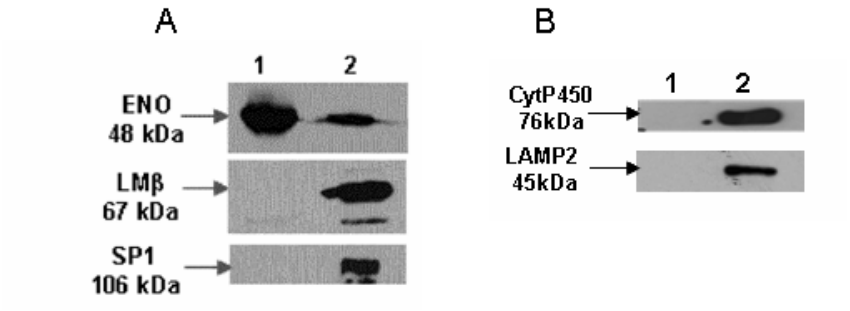


Figure 9. Checking the cytosol and nucleus protein fractions. *Western blotting was performed to test the cross-contamination of cytosolic (lane 1) and nuclear (lane 2) protein extracts from the LAN-5 NB cell line. The biochemical protein markers used for the nuclei were anti-LMβ and anti-SP1 antibodies, and an anti-ENO antibody was used for the cytosolic fraction (A). Anti-cytochrome P450 and anti-LAMP-2 proteins were used as marker of mitochondria and lysosomes, respectively (B).*

To checked the enrichment of some organelles, I used cytochrome P450 reductase as a marker of mitochondria and lysosome-associated membrane glycoprotein 2 (LAMP-2) as a marker of lysosomes. I observed that the nuclear fraction was also enriched in these organelles (Figure 9B).

1.2 Two-Dimensional Differential In-Gel Electrophoresis (2D-DIGE) analyse

The differential expression of the LAN-5 cytosolic and nuclear proteins as a function of ATRA treatment was analyzed at 8, 24, and 48 h of ATRA treatment using DIGE in a pH range of 3.0-10.0. The samples were labelled according to the scheme shown in Table 1. To increase biological and statistical significance of the results, I prepared the protein lysates from three independent treatments of LAN-5 NB cultures. Accordingly, each experiment required two sets of gels (see Table 1).

Table 1

	Gel n°	Cy2	Cy3	Cy5
1 Experiment	1	pooled standard	0 h	8 hs
	2	pooled standard	24 hs	48 hs
2 Experiment	3	pooled standard	0 h	8 hs
	4	pooled standard	24 hs	48 hs
3 Experiment	5	pooled standard	8 hs	0 h
	6	pooled standard	48 hs	24 hs

Table 1 Experimental Design of 2D-DIGE.

The protein extracts to compare were pre-labelled with either Cy3 or Cy5 fluorescent dyes. Each Cy3/Cy5-labeled sample pair was co-mixed with a Cy2-labeled pooled standard sample containing an equal amount of all 12 samples analyzed both for cytosolic and nuclear fractions. The Cy2/Cy3/Cy5 labelled samples run together on the same gel. Furthermore, I interchanged the labelling design in the third experiment to reduce the effects of preferential binding of dyes to proteins. For each gel, the Cy3, Cy5, and Cy2 images were imported into the DeCyder DIA (difference in-gel analysis) module to reveal the differentially expressed protein spots featured in each gel.

To identify the differentially expressed protein spots across the six gels, both for cytosolic and nuclear fractions, the results from the intra-gel comparison (six DIA files) were imported into the BVA module of DeCyder Software. For the cytosolic and nuclear fractions, one Cy2 image was selected as the master image, and the other five internal standard images were matched sequentially to it (Figure 10).

Approximately, 3000 protein spots were detected for both the cytosolic and the nuclear fractions. The protein spots were then filtered for the statistically relevant trend of regulation (*p*value 0.075; Student's paired *t* test) among the various ATRA treatment time points used. The analysis allowed us to find 126 statistically relevant and differentially expressed spots: 58 from the cytosolic fraction, and 68 from the nuclear fraction, with fold changes >1.3 or e1.3 (48 vs 0 h). In particular, 24 spots were more highly expressed and 34 spots were less expressed in the cytosolic fractions, with 43 spots and 25 in the nuclear fractions, respectively.

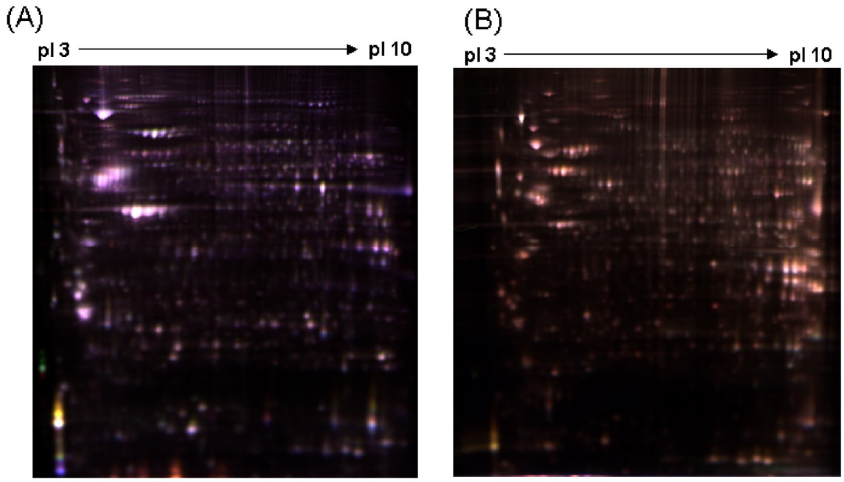


Figure 10. 2D-DIGE gel images. *Isoelectric focusing was performed on immobilized pH gradient IPG gel strip, NL pH 3-10, and the proteins were further separated by 11% SDS-PAGE in the second dimension. (A) Overlaid images of Cy3- and Cy5-labelled cytosolic protein extracts; and (B) as for A, for a nuclear protein extracts.*

1.3 Hierarchical Clustering Analysis

The RA-induced proteome changes were classified on the basis of the expression profiles of these 126 protein spots using hierarchical clustering. As shown in Figure 11, the DeCyder analysis data from

both DIGE experiments (cytosol and nucleus) divide along two main branches, with each dividing further into two branches, showing evidence of four major categories: two up-regulated categories in the upper dendrogram, and two down-regulated categories in the lower dendrogram. I observed that in the two cellular compartments there are two sub-clusters denoting proteins highly expressed at the 8 h and then lower expressed at 48 h, and proteins that are lower expressed at 8 h and then highly expressed at 48 h.

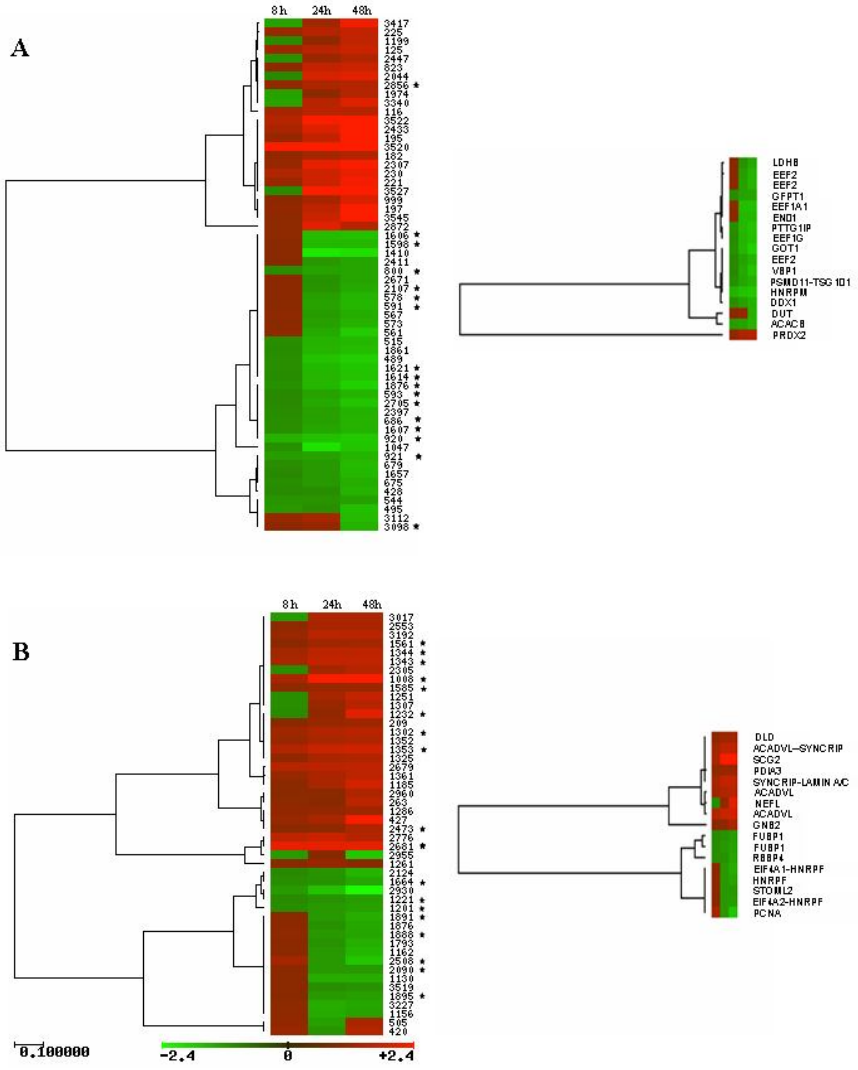


Figure 11. Hierarchical clustering analysis. *Hierarchical clustering analysis of protein-spots expression profiles in the cytosol (A) and nucleus (B) of the Lan5 cell line following 8, 24 and 48 hs of RA treatment (by column, as indicated). The up-regulated proteins are in red, the down-regulated proteins are in green. The expression profiles of the identified protein spots, indicated by asterisks, are shown on the right side of the Figure.*

2. Mass Spectrometry

To identify the differentially expressed proteins, 38 spots of interest were excised from the preparative gels, and in-gel trypsin digestion and mass spectrometry (MS/MS) analysis were performed for protein identification. Successful identification was achieved if at least five peptides of experimental MS/MS data matched the internal sequence of the theoretical candidate protein. Mass spectrometric analysis identified 33 proteins corresponding to 17 protein spots from the cytosolic fraction, and 16 from the nuclear fraction. The results of the protein identification are given in Tables 2 and 3, and the positions of the differentially expressed spots picked in the 2D gel are shown in Figure 12.

In this study, I was not able to determine the identities of the protein components of other differentially expressed spots using either the MALDI-TOF or LC-MS/MS mass spectrometric techniques. This may in part be due to insufficient amounts of protein in the spots, and also to the scarcity of tryptic digestion sites. In several cases, some well-separated spots of similar mass but different charge were identified as the same proteins. This may imply alternative post-

translational modifications, such as phosphorylation or multiple expression forms.

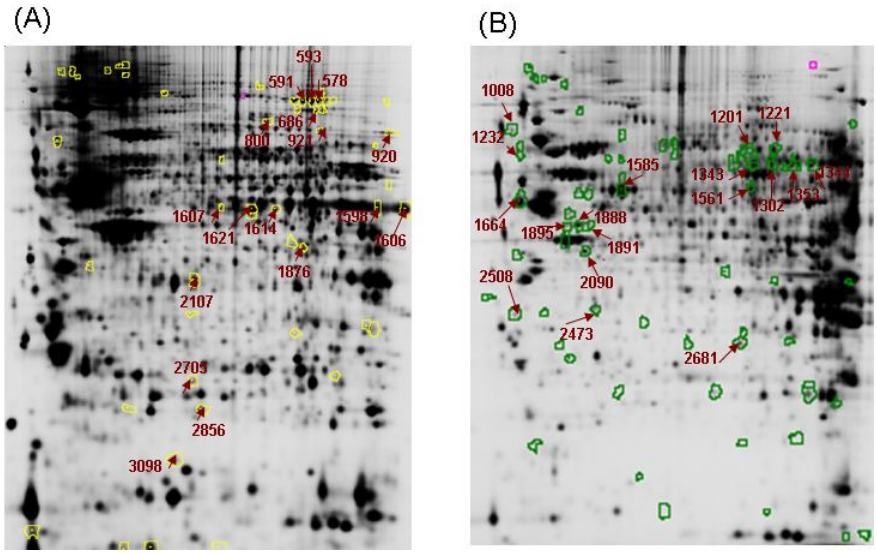


Figure 12. The differentially expressed protein spots upon ATRA treatment. *The differentially expressed protein spots upon ATRA treatment for cytosolic (A; 58) and nuclear fractions (B; 68). The arrows show the picked-up spots on the preparative gels and then identified: 17 spots for the cytosolic fraction and 16 spots for the nuclear fraction*.

These possibilities have not been studied further to date. Similarly, some different proteins co-migrated in the same spot under our experimental conditions, and the identified pituitary tumor-transforming gene protein-binding factor PTTG1IP was detected in a spot which differed from its theoretical molecular mass and p*I*. This could reflect proteolytic degradation of the protein or post-translational modifications, such as glycosylation.

Table 2. Proteins identified in the cytosolic fraction

Master Number	Protein Name	ID NCBI	ID SPROT	Theor. MW (kDa)	ExperimMW (kDa)§	Theor pI	Experim pI §	MS score	MS match	Fold 48H/0H	P value
	Catalytic activity (Transaminase activity)										
800*	Glucosamine--fructose-6-phosphate aminotransferase 1	gi/183082	Q06210	77,5	_	6,39	_	166	10	-1,34	0,00014
1876	Aspartate aminotransferase, cytoplasmic	gi/105387	P17174	46,35	_	6,81	_	188	14	-1,87	0,03
	Nucleic acid binding (DNA binding)										
1607*	Tumor susceptibility gene 101	gi/60655269	Q99816	44,1	_	6,06	_	276	12	-1,52	0,023
1598	Enolase 1 Variant	gi/62896593	P06733	47,5	_	7,01	_	125	12	-1,6	0,03
	Magnesium ion binding										
3098	dUTP pyrophosphatase	gi/181844	P33316	15,5	_	6,13	_	126	8	-1,52	0,036
	Nucleic acid binding (RNA binding)										
686	DEAD box protein 1, DDX1 protein	gi/33877837	Q92499	78,8	_	8,27	_	151	17	-1,52	0,046
920	Heterogeneous nuclear ribonucleoprotein M	gi/187281	P52272	77,9	_	8,99	_	115	16	-1,78	0,021
	Protein binding										
2705	Prefoldin subunit 3	gi/48429043	P61758	18,5	_	6,63	_	94	8	-1,73	0,0097
	Catalytic activity (Hydrolase activity)										
1607*	Proliferation-associated protein 2G4	gi/5453842	Q9UQ80	44,2	_	6,13	_	261	13	-1,52	0,023
	Catalytic activity (Oxidoreductase activity)										
2856	Peroxiredoxin-2	gi/1617118	P32119	18,5	_	5,19	_	109	8	1,51	0,078
2107	L-lactate dehydrogenase B chain	gi/49259212	P07195	36,8	_	5,86	_	184	14	-1,39	0,035
	Translation elongation factor activity										
591					88,4		7,37			-1,56	0,014
593					88,3		7,52			-1,53	0,0036
578	Human Elongation Factor 2	gi/31108	P13639	96,3	88,8	6,41	7,65	94	11	-1,55	0,00017
1621					50,3		6,65			-1,73	0,018
1614	Elongation factor 1-gamma	gi/15530265	P26641	50,5	50,4	6,25	6,95	202	12	-1,69	0,022
1606*	Elongation factor 1, alpha 1	gi/48734733	P68104	50,5	_	9,14	_	241	18	-1,69	0,031
921	acetyl-CoA carboxylase beta	gi/1399290	O00763	89,8	_	6,01	_	80	14	-1,55	0,037
	Catalytic activity (Ligase activity)										
1607*	26S proteasome non-ATPase regulatory subunit 11	gi/2150046	O00231	47,7	_	6,08	_	318	20	-1,52	0,023
	Molecular function unknown										
1621	Pituitary tumor-transforming gene protein-binding factor	gi/21411022	P53801	21,2	_	9,14	_	202	5	-1,73	0,018

Table 3. Proteins identified in the nuclear fraction

Master Number	Protein Name	ID NCBI	ID SPROT	Theor. MW (kDa)	Experim MW(kDa)§	Theor pI	Experim pI§	MS score	MS match	Fold 48H/0H	P value
	Nucleic acid binding (RNA binding)										
1895					45,7		5,04			-1,27	0,0037
1888					45,8		5,12			-1,35	0,014
1891	Heterogeneous nuclear ribonucleoprotein F	gi/76780063	P52597	46	45,7	5,38	5,17	103	9	-1,44	0,0000072
1891	Eukaryotic initiation factor 4A-I	gi/77735407	P60842	46	_	5,38	_	186	7	-1,44	0,0000072
1895	Eukaryotic initiation factor 4A-II	gi/16198386	Q14240	46,6	_	5,38	_	256	11	-1,27	0,0037
1343*					58,3		6,52			1,68	0,02
1302*					58,8		6,68			1,59	0,00029
1353*					58,5		6,87			1,87	0,0055
1344*	Heterogeneous nuclear ribonucleoprotein Q	gi/21619168	O60506	58,95	58,6	7,18	7,06	325	7	1,65	0,00035
	Structural constituent of of cytoskeleton										
1232	68 kDa neurofilament protein	gi/24658018	P07196	61,6	_	4,64	_	111	8	2,12	0,0018
	Nucleic acid binding (DNA binding)										
1201					62,1		6,43			-1,29	0,013
1221	FUSE-binding protein 1	gi/37078490	Q96AE4	67,65	62	7,18	6,7	178	13	-1,26	0,0099
2508	Proliferating cell nuclear antigen	gi/2914387	P12004	29,1	_	4,57	_	115	8	-1,75	0,003
	Structural molecule activity										
1343*	lamin A/C	gi/55957499	P02545	69,5	_	6,4	_	386	7	1,68	0,02
	Nucleotide binding										
1561	Dihydrolipoyl dehydrogenase, mitochondrial	gi/71042410	P09622	50,7	_	6,35	_	86	7	-1,38	0,037
	Protein binding										
1664	Histone-binding protein RBBP4	gi/30583457	Q09028	47,95	_	4,74	_	149	10	-1,38	0,0037
	Metal ion binding										
1008	SecretograninII, Chromogranin C	gi/134464	P13521	70,85	_	4,67	_	83	8	3,13	0,00032
	Signal transducer activity										
2473	Guanine nucleotide-binding protein beta subunit 2	gi/20357529	P62879	38,1	_	5,6	_	148	11	1,44	0,01
2090	Stomatin (EPB72)- like 2	gi/14603403	Q9UJZ1	38,65	_	6,88	_	125	11	-1,21	0,054
	Catalytic activity (Hydrolase activity)										
1585	Disulfide isomerase ER-60	gi/860986	P30101	57,1	_	6,1	_	118	13	1,23	0,0027
	Catalytic activity (Oxidoreductase)										
1353*					58,5		6,87			1,87	0,0055
1302*					58,8		6,68			1,59	0,00029
1344*	Very long chain acyl CoA dehydrogenase	gi/3273228	P49748	70,85	58,6	8,88	7,06	600	10	1,65	0,00035
2681	brain and muscle Ah receptor nuclear translocator-like protein	gi/7512308		31,6	_	9,42	_	94	7	2,14	0,041

The asterisk (*) indicates the spots that were identified by LC-MS/MS analysis.
(§) Experimental MW and pI, calculated by DeCyder 5.0 software are reported only for proteins identified in more than one spot, in order to assess post-translational modifications.

3. Gene Ontology

I performed functional classification of the proteins identified according to the DAVID 2.1 beta annotation system. Statistical analysis (Fisher exact test) of the 17 cytosolic proteins identified (Figure 13A) indicated that some functional categories are overrepresented in this list, such as translation elongation factor activity, catalytic activity, transaminase activity, transferase activity, and the transferring of nitrogenous groups. Moreover, the functional category RNA binding was overrepresented in the list of the 16 nuclear proteins identified (Figure 13B).

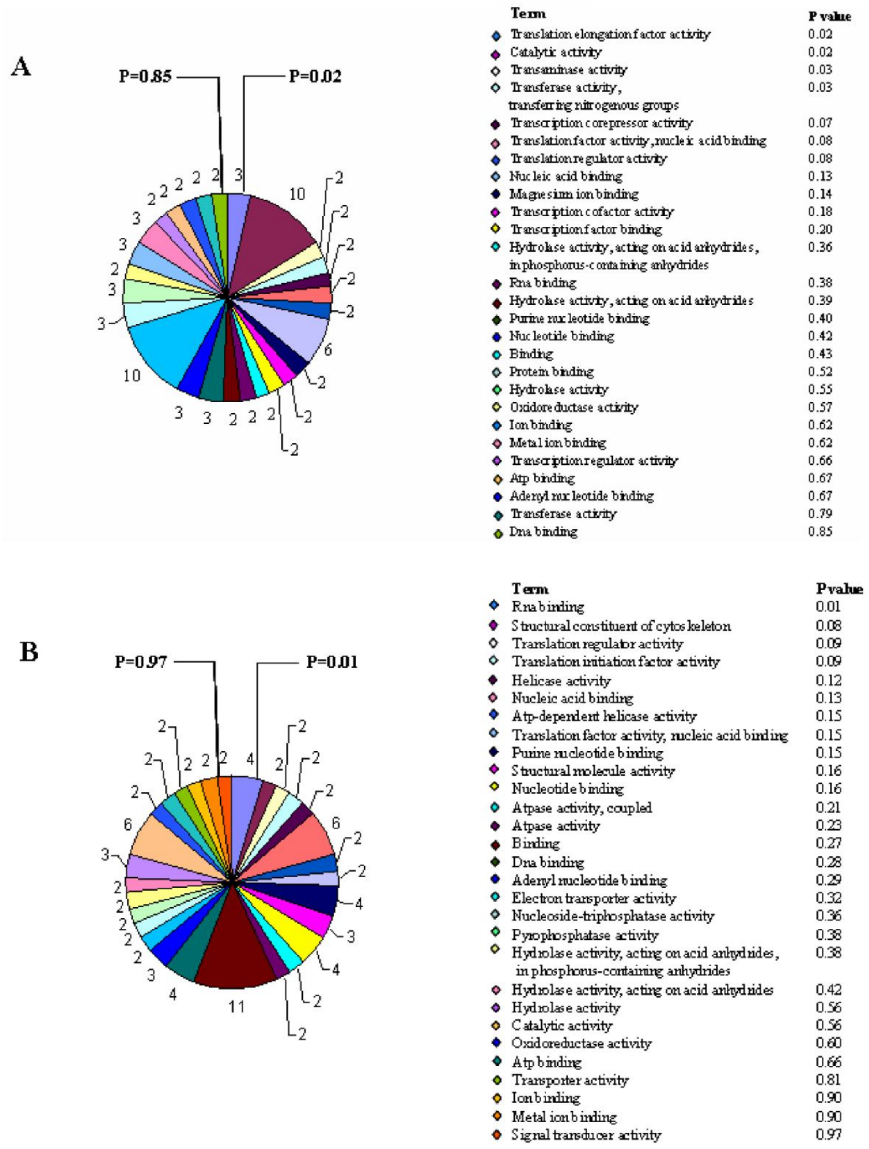


Figure 13. Gene ontology. *The proteins identified were classified using the DAVID 2.1 beta annotation system. The Fisher exact test was used to determine the protein-enrichment in annotation terms. The functional categories are sorted by P value. Statistical analyses (Fisher exact test) of the cytosol proteins (A) and the nuclear proteins (B) are shown. The graphic shows the number of proteins within each functional category.*

4. Gene Mapping

To examine the correlations between the proteins showing RA-induced differential expression and the chromosomal rearrangements or epigenetic regulatory loci in NB, I searched for the physical location of the genes coding for the differentially expressed proteins, using UNIGENE searching of the NCBI genome database. This revealed that the DLD (dihydrolipoamide dehydrogenase), ENO1 (enolase variant 1), DDX1 (ATPdependent RNA helicase), ACACB (acetyl-CoA carboxylase beta), PSMD11 (26S proteasome non-ATPase regulatory subunit 11) genes map to allelic imbalance chromosomal regions involved in NB (Table 4).

Table 4 Mapped Chromosomal regions of genes coding for the identified proteins.

Protein name	Gene Name	Mapped chromosomal regions
Glucosamine--fructose-6-phosphate aminotransferase 1	GFPT1	2p13
Aspartate aminotransferase, cytoplasmic	GOT1	10q24.1-q25.1
Tumor susceptibility gene 101	TSG101	11p15
Enolase 1 Variant	ENO1	1p36.3-p36.2 *
dUTP pyrophosphatase	DUT	15q15-q21.1
DEAD box protein 1, DDX1 protein	DDX1	2p24 *
Heterogeneous nuclear ribonucleoprotein M	HNRPM	19p13.3-p13.2
Prefoldin subunit 3	VBP1	Xq28
Proliferation-associated protein 2G4	PA2G4	12q13
Peroxiredoxin-2	PRDX2	19p13.2
L-lactate dehydrogenase B chain	LDHB	12p12.2-p12.1
Human Elongation Factor 2	EEF2	19pter-q12
Elongation Factor 1-gamma	EEF1G	11q12.3
Elongation Factor 1, alpha 1	EEF1A1	6q14.1
acetyl-CoA carboxylase beta	ACACB	12q24.11 *
26S proteasome non-ATPase regulatory subunit 11	PSMD11	17q11.2 *
Pituitary tumor-transforming gene protein-binding factor	PTTG1IP	21q22.3
Heterogeneous nuclear ribonucleoprotein F	HNRPF	10q11.21-q11.22
Eukaryotic initiation factor 4A-I	EIF4A1	17p13
Eukaryotic initiation factor 4A-II	EIF4A2	3q28
Heterogeneous nuclear ribonucleoprotein Q	SYNCRIP	6q14-q15
68 kDa neurofilament protein	NEFL	8p21
FUSE-binding protein 1	FUBP1	1p31.1
Proliferating cell nuclear antigen	PCNA	20pter-p12
lamin A/C	LMNA	1q21.2-q21.3
Dihydrolipoyl dehydrogenase, mitochondrial	DLD	7q31-q32 *
Histone-binding protein RBBP4	RBBP4	1p35.1
SecretograninII, Chromogranin C	SCG2	2q35-q36
Guanine nucleotide-binding protein beta subunit 2	GNB2	7q21.3-q22.1
Stomatin (EPB72)- like 2	STOML2	9p13,1
Disulfide isomerase ER-60	PDIA3	15q15
Very long chain acyl CoA dehydrogenase	ACADVL	17p13-p11
brain and muscle Ah receptor nuclear translocator-like protein	§	§

*Genes coding for the identified proteins that are mapped to regions known to be altered in NB.

5. Validation and Analysis in Different Human NB Cell Lines.

I performed validation of the data for some of the identified proteins based on the availability of good commercial antibody by Western blotting: PRX II (peroxyredoxin-2), SgII (secretogranin II), NEF-L

(68kDa neurofilament), eEF-1R (eukaryotic elongation factor), PCNA (proliferating cell nuclear antigen), EF-2 (elongation factor-2), prefoldin subunit 3 and Gβ2 (guanine nucleotide-binding protein beta subunit 2) (Figure 14A). I performed semi-quantitative analysis of the protein expression on total amount of proteins at the four time points of the differentiation: 0, 8, 24, and 48h. The bands were quantified by densitometry to obtain an integral optic density (IOD) value which then was normalized with respect to β-actin value (Figure 14B).

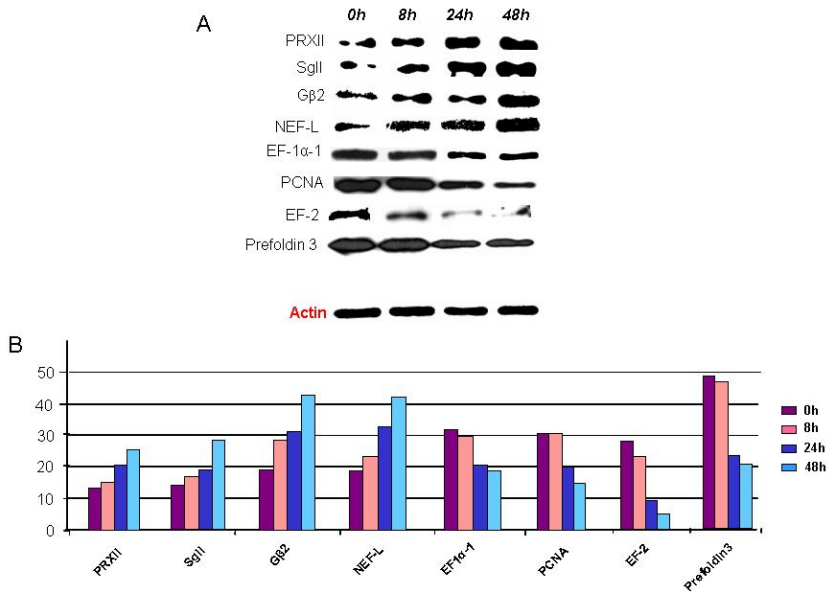


Figure 14. Western blot analysis of selected proteins in LAN-5 cells. *Western blot analysis of selected proteins was performed in LAN-5 cells, not treated or treated with 5 μM RA for 8, 24 and 48hs. The β-actin was used as the loading control (A). The bands were quantized by densitometry. The bar graph shows integral optic density (IOD) value for each band, normalized with respect to β-actin expression (B).*

All of these proteins showed differential expression patterns in the LAN-5 cell line, according to the DIGE results. I also analyzed the expression patterns of these selected proteins in two other NB cell lines, SH-SY5Y and SK-N-BE, not treated and treated with RA for

48h (Figure 15A). Here, the proteins TSA, SgII, NF-L, EF1R, PCNA, and EF-2 showed the same trends seen in the LAN-5 cells, while the prefoldin 3 and Gβ2 proteins were differentially expressed in the SH-SY5Y cells, and not in the SK-N-BE cells. Semi-quantitative analysis of the protein expression at the two extreme time points of differentiation: 0 and 48 h, in LAN-5, SH-SY-5Y, and SK-N-BE cells was shown in Figure 15B.

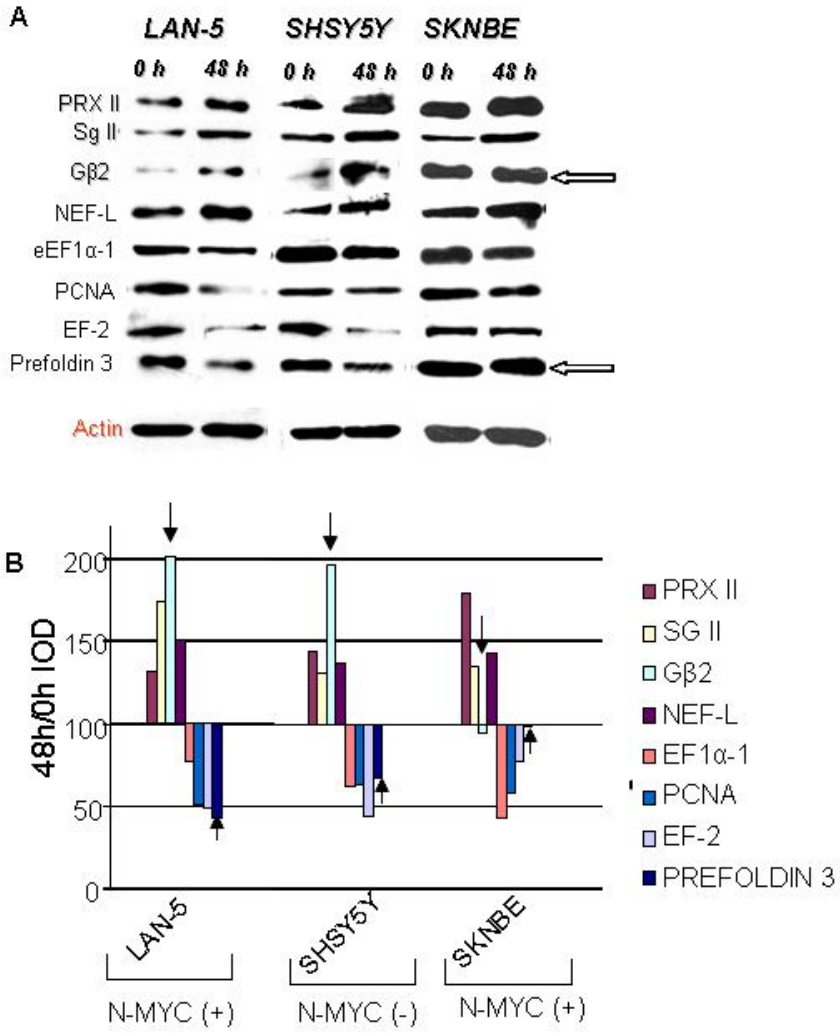


Figure 15. Western blot analysis of selected proteins was performed in LAN-5, SH-SY5Y and SK-N-BE cells. *Western blot analysis of selected proteins was performed in LAN-5, SH-SY5Y and SK-N-BE cells, with (+) and without (-) N-MYC amplification, either not treated or treated with 5 μM RA for 48hs. The β-actin was*

used as the loading control (A). The bar graph shows the IOD as fold of induction expressed at 48hs of RA differentiation. Proteins expression level at 0h was assigned equal to 100% (B). The arrows show two proteins which were found differentially expressed in LAN-5 and SH-SY5Y cells and not differentially expressed in SK-N-BE cells.

I also checked the variation of expression of prefoldin 3 and DDX1 proteins in the cytosol and nuclear fraction separately, upon ATRA treatment at 48h. As observed in Figure 16, prefoldin 3 expression decreases in cytosol and does not change in nucleus fraction, whereas DDX1 expression increases in nucleus and decreases in cytosol fraction.

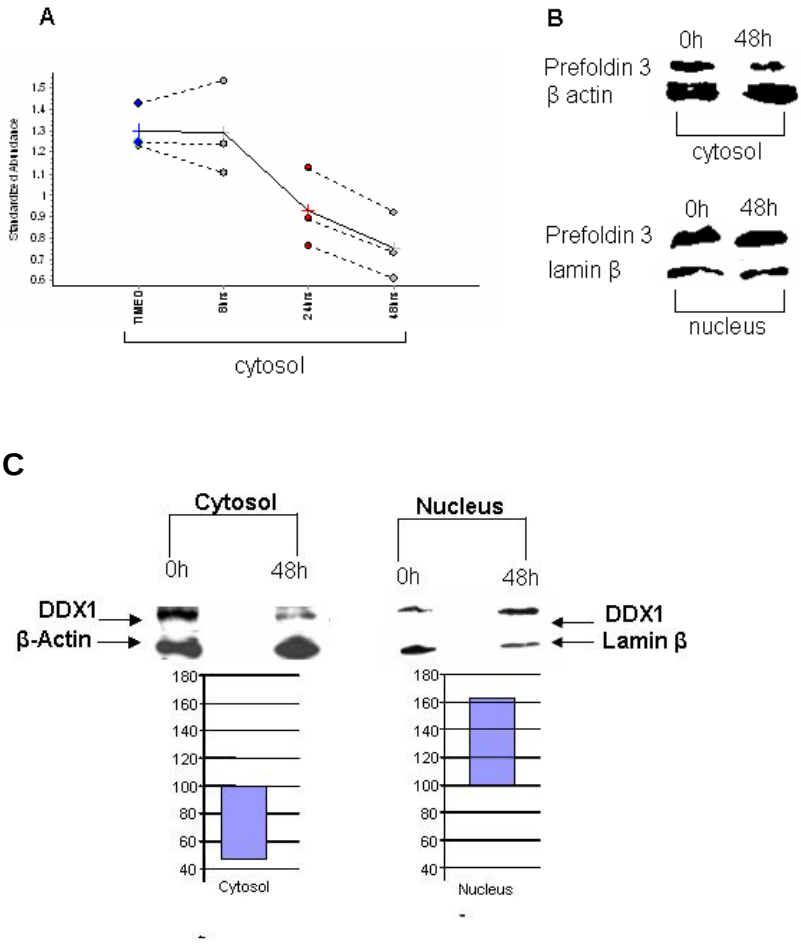


Figure 16. Prefoldin 3 and DDX1 ATRA differentially expression. *Prefoldin 3 DIGE differentially expression in cytosolic fraction was output by Biological*

Variation Analysis (BVA). Each point represents the standardized log abundance (sample/internal standard) of a protein spot obtained for each of the 12 gels analysed: 3 gels loaded proteins at 0h, 3 gels loaded proteins at 8hs RA, 3 gels loaded proteins at 24hs RA and 3 gels loaded proteins at 48hs RA. A paired Student's t-test was applied to all samples, yielding a p-value 0.0097 and fold 48h/0h -1.73 (A). Western blotting of prefoldin 3 (B) and DDX1 (C) was performed on LAN-5 nuclear and cytosol fractions, not treated or treated with 5 μM RA for 48hs. The β-actin and lamin β were used as the loading control. In (C) the bands were quantized by densitometry. IOD value was normalized respect to b-actin expression values.

6. VBP1 survival correlation in primary NB

Gene coding Prefoldin 3 is called VBP-1, as Von Hippel-Lindau binding protein 1. I analysed the correlation of VBP-1 mRNA expression to survival in a cohort of 102 primary NBs (Affymetrix U133v2 chips). The macroarray experiment was already performed (Schulte et al., 2005; Schramm et al., 2005b). I found a statistically significant correlation of VBP-1 high espression to bad survival. (p-value 0,00048) (Figure 17).

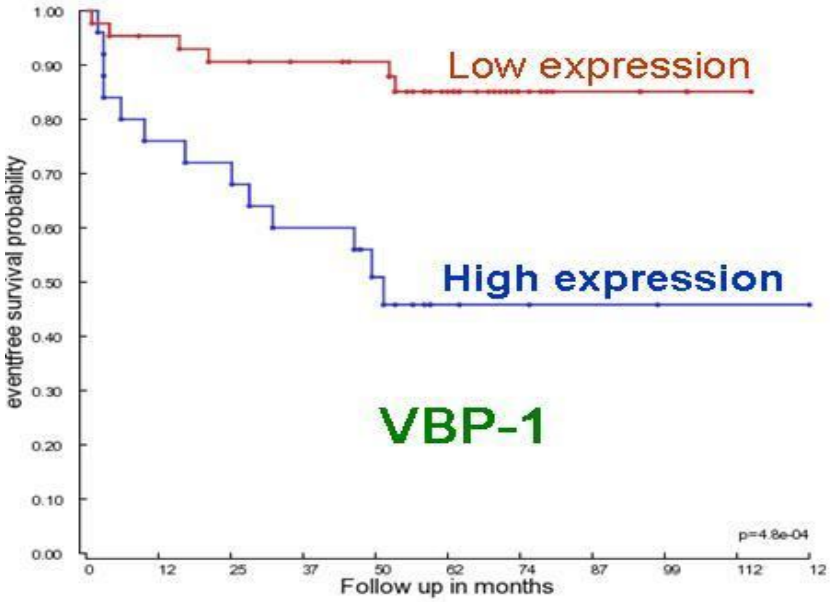


Figure 17 VBP1 (Von Hippel-Lindau binding protein 1) expression survival in NB. *The survival curve of 102 patients with NB shows a significant association of high expression of VBP-1 to patients that have less month of follow up. The value of expression of VBP-1 was analysed by a software.*

7. Gal-1 mRNA expression correlates with neurotrophin receptor expression in primary NB

I analysed the correlation between Gal- 1 and Trk receptors mRNA expression in a cohort of 102 primary NBs (Affymetrix U133v2 chips). Gal-1 expression correlated significantly with the levels of TrkB expression ($P=4x10^{-9}$) and was anticorrelated with TrkA expression ($P=6x10^{-3}$, Figure 18).

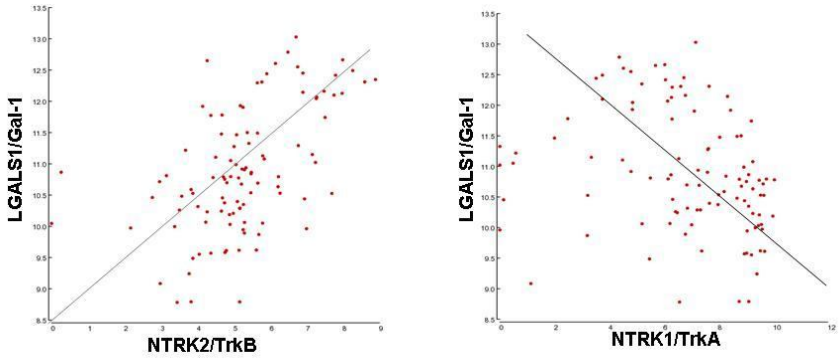
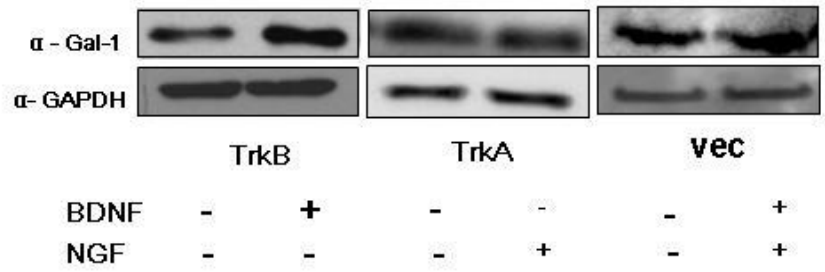


Figure 18 Correlation of Gal-1 and Trk expression in primary NB. *Gal-1/LGALLS1 expression was significantly correlated with TrkB/NTRK2 expression ($P=4x10^{-9}$) and significantly anticorrelated with TrkA/NTRK1 (P=0,006) in a cohort of 102 primary NB.*

In addition, in our model system SY5Y, Gal-1 protein and mRNA levels increased on activation of TrkB by BDNF in SY5Y-TrkB, but not on activation of TrkA by nerve growth factor (NGF) in SY5Y-TrkA or in the vector control SY5Y-vec (Figure 19a, 19b).

A



B

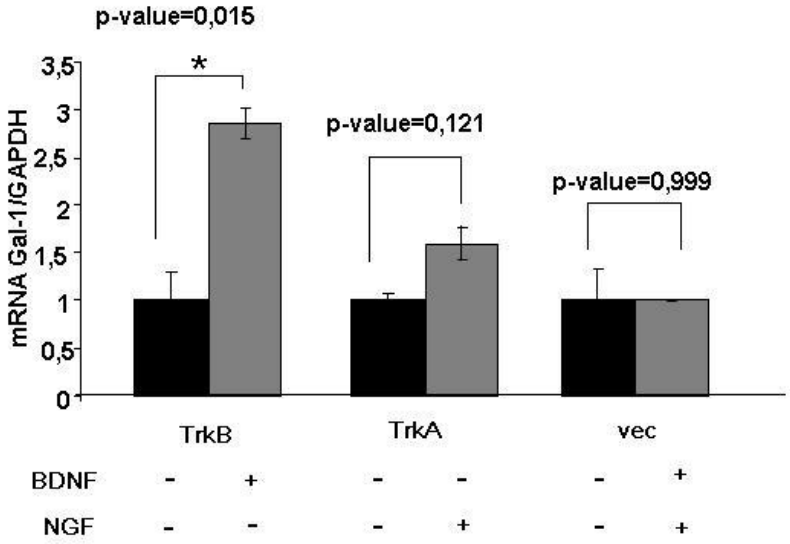


Figure 19. Gal-1 expression in SY5Y cells. *Gal-1 expression was evaluated by western blotting (A). and RT-PCR (B). Gal-1 levels significantly increased (P0 0,015, t-test) on stimulation of SY5Y-TrkB with BDNF. No significant changes in Gal-1 were observed in SY5Y-TrkA or in control-transfected cells (SY5Y-vec) following neurothrophin treatment.*

Furthermore, recombinant Gal-1 (rGal-1) induced Gal-1 mRNA expression only in SY5Y-TrkB, but not in SY5Y-TrkA cells, suggesting a feedback activation loop (Figure 20a). Interestingly, this effect could be reverted if the sugar-binding capacity of Gal-1 was blocked by the addition of lactose, whereas cell proliferation was not

affected by rGal-1 (Figure 20b). Taken together, Gal-1 expression was found to be tightly linked to TrkB expression in vivo and in vitro.

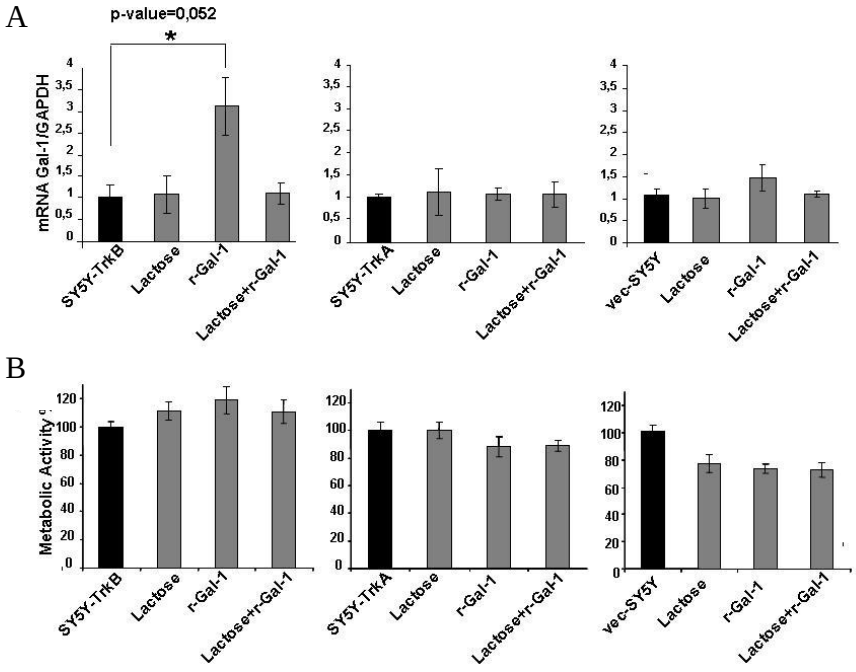


Figure 20. rGal-1 mediated effects. *Addition of r-Gal-1 to medium of TrkB-expressing cells significantly (P=0,05, t-test) increases expression of Gal-1, which can be inhibited by competition with lactose(A), although not significantly altering cell proliferation measured by MTT assay. rGal-1 does not affect Gal-1 expression in SY5Y-TrkA or control cells (SY5Y-vec) (B).*

8. Gal-1 protein is highly expressed in primary NB

To analyse Gal-1 expression in situ, I hybridized a tissue microarray containing 92 primary NBs with a Gal-1-specific antibody. There were distinguishable patterns of Gal-1-staining intensity in the tumor cells (Figure 21) with a trend toward elevated expression in higher tumor stages, but statistical analyses revealed no significant correlation between Gal-1 protein expression and patient survival or clinical parameters, including age at diagnosis, stage or MYCN

amplification (data in text only). Interestingly, Gal-1 expression was also high in stromal septae, in particular, in differentiating tumors.

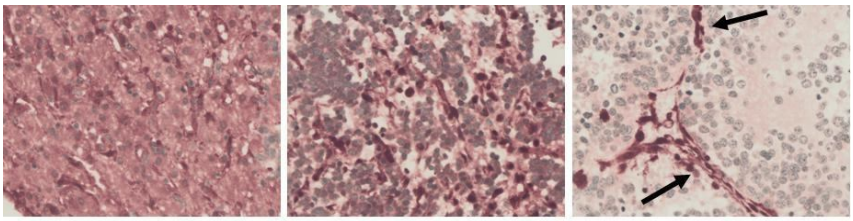


Figure 21. Immunohistochemical staining of Gal-1 in primary NB. *Three rappresentative examples of a total 92 NB tumors analysed are shown. The left and the middle pictures show sections from INSS stage 4 NB, poorly differentiated; left: MYCN-amplified tumor with 1p del; middle: MYCN single copy with normal 1p. the pictures on the right is derived from an INSS stage 1, differentiating NB with single copy MYCN. Note the extracellular and cytoplasmic staining of Gal-1, as well as the positively stained septae (marked by arrows in the right picture).*

9 Gal-1 contributes to BDNF-mediated invasion of SY5Y-TrkB cells

The invasive capacity of SY5Y-TrkA and SY5Y –TrkB cells was assessed using a Boyden chamber assay. Although neurotrophin receptor activation resulted in very few migrating cells in SY5Y-TrkA and empty vector control, migration of SY5Y-TrkB cells was strongly enhanced on TrkB activation by BDNF (Figure 22).

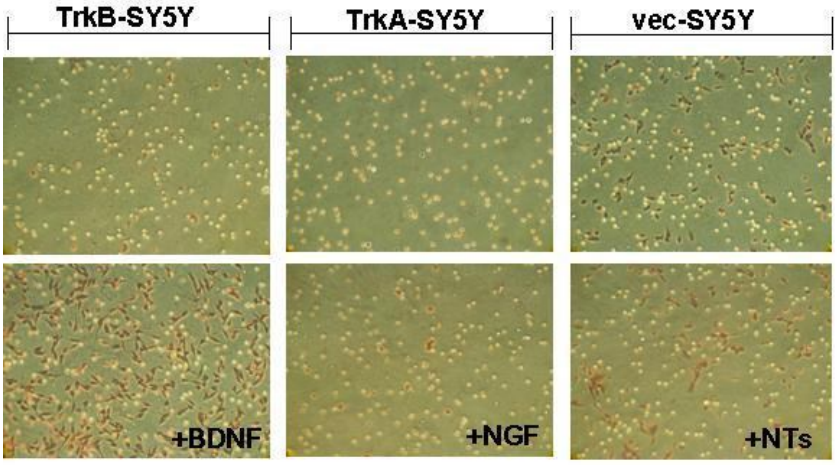


Figure 22.**Invasion mediated by TrkB stimulation**. *Stimulation of TrkB receptor by BDNF is required for the invasion of SY5Y-TrkB cells, whereas NGF treatment of SY5Y-TrkA or neurotrophin treatment of control cells (SY5Y-vec) has only a minor effects on the invasive capacity properties.*

The addition of rGal-1 alone modestly enhanced the migratory capacity of TrkB cells, whereas blocking Gal-1 function using a neutralizing anti-Gal-1 antibody strongly reduced BDNF-mediated migration of SY5Y-TrkB cells. (Figure 23). rGal-1 and BDNF cooperatively induced cell migration in SY5Y-TrkB (Figure 23b, lower right part). I also aimed to assess whether the intracellular or extracellular function of Gal-1 is required for invasion. For this purpose, I knocked down the intracellular pools of Gal-1 by siRNA and added rGal-1 simultaneously. The down regulation of Gal-1 by siRNA strongly reduced the invasiveness of BDNF-activated TrkB-expressing cells, which could be restored by the addition of rGal-1 (Figure 24). In summary, this suggests a strong impact of extracellular Gal-1 on the invasive capacity of TrkB-expressing NB cells.

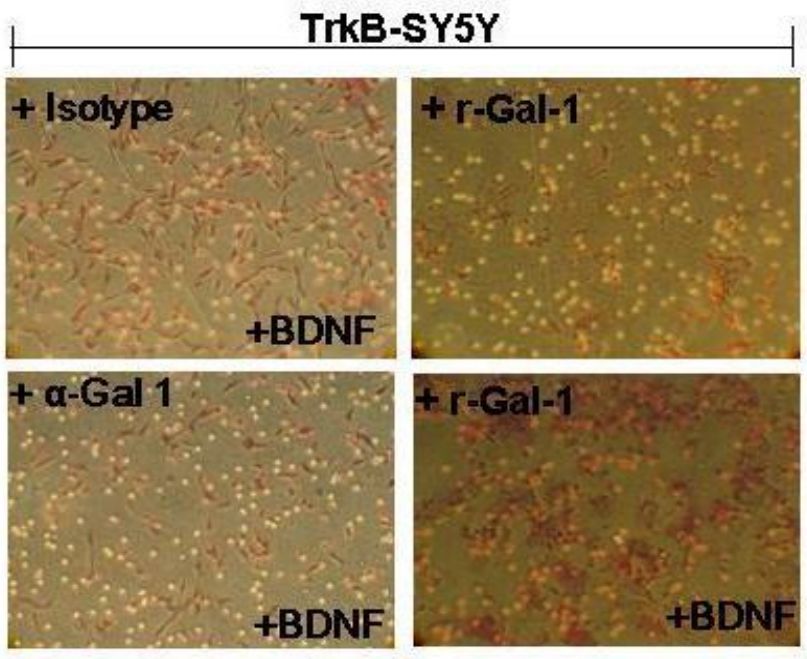


Figure 23 Gal-1 contributes to BDNF-mediated invasion.. *A neutralizing antibody directed against Gal-1 reduces the invasive capacity of BDNF-stimulated SY5Y-TrkB cells. Addition of rGal-1 to the medium of unstimulated SY5Y-TrkB cells increases the number of invading cells but it is not sufficient to restore fully the invasive capacity of SY5Y-TrkB cells in presence of BDNF.*

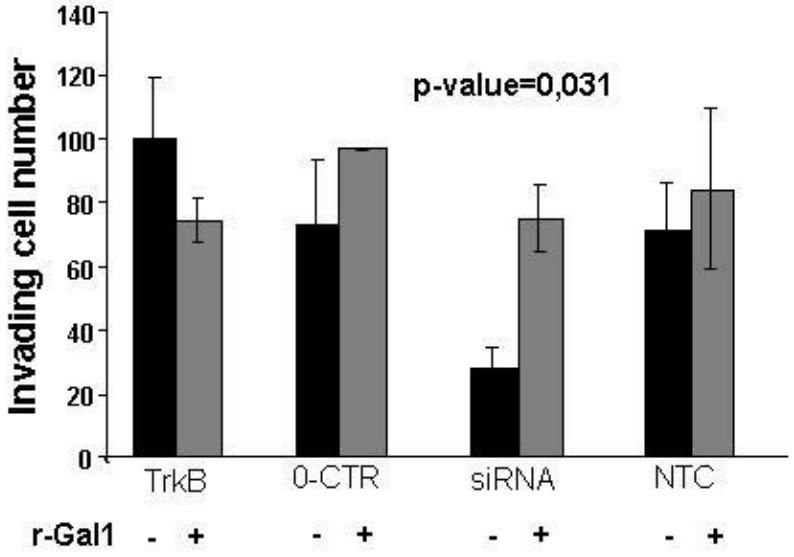


Figure 24. Extracellular Gal-1 effect on TrkB mediated.invasion. *Inhibition of BDNF-triggered invasion mediated by a siRNA directed against Gal-1 can be overcome by addition of recombinant Gal-1 (rGal-1; CTR= control using an unrelated siRNA sequence, NTC= control using transfection reagent only). Only when intracellular Gal-1 levels were depleted by a Gal-1 specific siRNA, addition of rGal-1 significantly increased the number of invading cells (p= 0.03, t-test).*

9.1 Gal-1 silencing by siRNA

I used three different siRNA directed against Gal-1 mRNA. The efficiency of silencing was checked by RT-PCR and Western Blotting as shown in Figure 25. The mostly effective siRNA were siRNA3 and siRNA7 which were used for the described experiments.

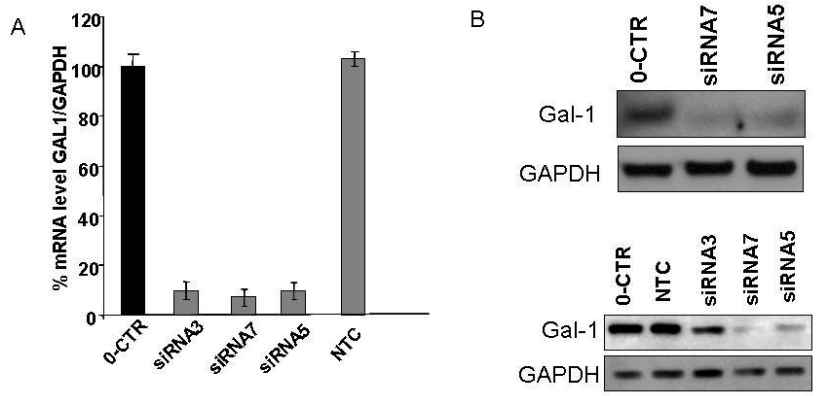


Figure 25. Gal-1 expression silencing. *Gal-1 expression was silenced by using separatly three different siRNA against three different regions on Gal-1 transcript. The knockdown efficacy was checked by RT-PCR (A) and by Western Blotting(B). A non-targeting siRNA- control (NTC) and buffer transfected control (0-CTR) were used to assure the target specificity.*

10. Downregulation of Gal-1 abrogates migration of SY5Y-TrkB cells

Next, I analysed the migration capacity of Trk-expressing SY5Y cells in a 'wound-healing' assay. After scratching, SY5Y-TrkA or SY5Y-TrkB was incubated in the presence or absence of neurotrophins. BDNF treatment of SY5Y-TrkB cells enhanced migration, which was impaired by transient siRNA-mediated silencing of Gal-1, but not by a

control siRNA (Figure 26a). SY5Y-TrkA cells as well as SY5Y empty vector cells did not migrate irrespective of neurotrophin treatment (Figure 26b). These experiments show that Gal-1 expression is an essential parameter for the migration capacity of SY5Y-TrkB cells. BDNF-mediated up-regulation of Gal-1 expression strictly depends on TrkB receptor activation.

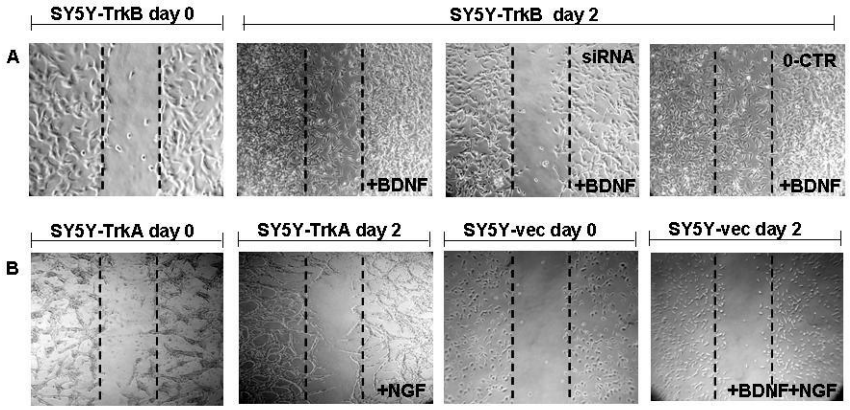


Figure 26. Gal-1 effect on TrkB mediated.migration *(A) BDNF treatment of SY5Y-TrkB cells enhances migration within 2 days following scratching. This is impared by si-RNA-mediated silencing of Gal-1 compared with untreated control or unrelated si-RNA. (B) NGF treatment of SY5Y-TrkA cells does not enhance migration and neither does NT treatment of control cells (SY5Y-vec).*

11. Gal-1 is strictly dependent on TrkB pathway

The contribution of an activated and functional TrkB signaling pathway to the regulation of Gal-1 expression was assessed using K252a, a general inhibitor of Trk signaling. The activation of TrkB by BDNF induced an increased phosphorylation of Akt (p-Akt) in SY5YTrkB cells that was inhibited by K252a (Figure 27). Akt activation could be restored only partially by the simultaneous addition of BDNF and K252a. I did not find Akt activation in control-

transfected cells (SY5Y-vec) on neurotrophin addition, but only an increase in p-Akt on treatment with K252a. BDNF mediated up-regulation of Gal-1 expression in SY5YTrkB was abrogated when the TrkB receptor was inhibited by K252a alone or by the simultaneous addition of BDNF and K252a (Figure 27). These findings strongly suggest that Gal-1 up-regulation is strictly dependent on a functional TrkB pathway.

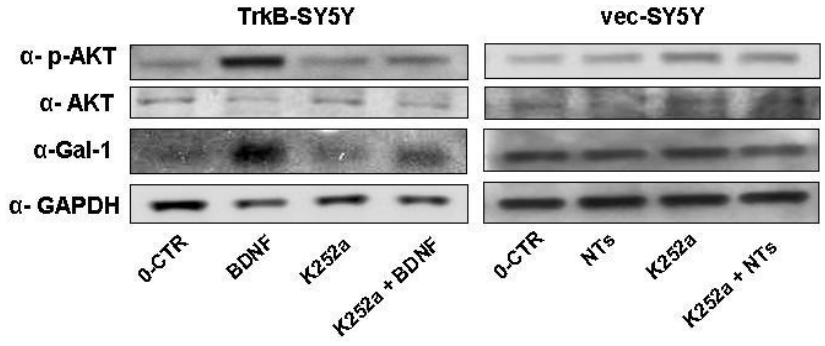


Figure 27. Gal-1 is strictly dependent on TrkB pathway activation *BDNF-mediated activation of TrkB induces an increased p-Akt in SY5Y-TrkB cells that is inhibited by TrkB inhibitor K252a. No activation of Akt can be seen in control cells (SY5Y-vec) without TrkB expression following neutothrophin (NTs= simultaneous addition of BDNF+NGF) treatment. Expression of Gal-1 is reduced when TrkB signalling is inhibited bt K252a or by simultaneous addition of BDNF and K252a in SY5Y-TrkB cells, but nit in the controls (SY5Y-vec).*

Discussion

NB is an heterogeneous tumor which shows a wide range of genetic alterations and biological phenotypes. Even if there are so many altered pathways and so many biological markers known to be involved in molecular mechanism of this tumor, to date there isn't been defined a common mechanism of insurgence and progression.

Standard therapy for patients who have low- and intermediate-risk NB involves tumor primary resection and moderate-dose chemotherapy including cisplatin, doxorubicin, etoposide, and cyclophosphamide.

Standard therapy for patients who have high-risk NB involves chemotherapy in escalated dose intensity (anthraciclines, alkylators, platinum compounds, and topoisomerase II inhibitors), surgical resection and radiotherapy to the primary tumor. Minimal residual NB are treated by isotretinoin (cis-RA) or monoclonal antibodies directed against gangliosidase GD2.

The knowledge of NB biology is imperative toward the use of novel therapies for high-risk NB. Several biologic agents are in ongoing clinical trials for recurrent NB, including histone deacetylase inhibitors, Trk tyrosine kinase inhibitors, and anti-angiogenic agents.

These agents will be moved into-front line therapy to optimize treatment of minimal residual disease.

The goal of this thesis has been the discovery of novel therapeutic targets in NB therapy, using the mounting knowledge of NB tumor biology as the differentiation/regression ability and the well-characterized TrkA and TrkB pathways whose mediators are responsible of two biological phenotypes.

In the first part, I applied a systematic approach as proteomics to get deeper in the molecular mechanism of ATRA-induced differentiation in NB cells in order to clarify pathways and molecular targets most drivers of NB differentiation. In the second part, I focused on the functional study of Gal-1 which is a down-stream mediator of TrkB pathway, whose expression is mostly associated with high-risk group patients. Although Trk inhibitors are actually used in clinical trials, specific targeting TrkB pathway by means of Gal-1 silencing should be a novel therapeutic strategy to be suggested in the current protocols.

1.First Aim:

Comparative proteomic expression profile in All-trans Retinoic Acid differentiated NB cell line.

NB shows the highest rate of spontaneous regression of any human tumour, mainly due to differentiation and maturation of these highly malignant cells in neurons. It is of interest to study the molecular pathways driving spontaneous regression in NB in order to unravel the molecular basis of NB development and the driver-pathways and markers to be suggested as targets in differentiation therapy.

ATRA, the most commonly used anti-neoplastic agent in NB therapy, can induce neural differentiation in NB cell lines in vitro (Sidell et al., 1982). Systematic approaches at the transcription and translational levels have been used to identify the target genes for NB progression. Moreover, studies of differentiation induced by ATRA have been performed in PML cells and in mouse stem cells using a proteomic approach.

The main aim of our study was to gather insights into the molecular mechanisms of NB differentiation using 2-D DIGE technology. I used the human LAN-5 NB cell line amplified for the oncogene N-MYC and responsive to ATRA treatment (Cesi et al., 2002; Hettmer et al., 2004), and monitored the cytosolic and nuclear protein expression in those cells at the early phase of ATRA differentiation. I used 5 μM ATRA, according to the known pharmacological doses used in phase I trials of RA administrated to NB patients (Villablanca et al., 1995).

I detected a total of 58 spots in the cytosolic fraction and 68 in the nuclear fraction that showed differences in their relative expression between the control and RA-treated LAN-5 cells; 33 of these proteins

were identified (17 in the cytosolic fraction and 16 in the nuclear fraction). In agreement with the neuronal-orientated differentiation induced by ATRA, I observed at early phase of differentiation a substantial up-regulation of the NEF-L and SgII proteins, which are known to be neuronal markers (Pagani et al., 1992; Giudici et al., 1992; Jang et al., 2004), and the down-regulation of the proliferating cell nuclear antigen PCNA, an auxiliary protein of DNA polymerase. The highest levels of PCNA were seen in advanced NB stage tumours with an amplified N-myc gene (Keim et al., 1993; Mejia et al., 2003). Our results suggest that the decreased levels of PCNA may reflect differences in proliferative activity and that the suppression of proliferation is an obligatory step in the differentiation of these cancer cells. Far upstream element Binding Protein 1 (FBP) stimulates the expression of c-myc, a transcription factor involved in cell growth, proliferation, differentiation and apoptosis (Ducan et al., 1994). FBP down-regulation upon ATRA differentiation was observed in our model systems as well has also been seen in differentiated haematopoietic stem cells (Tao et al., 2004).

Closed examination of the list of differentially expressed identified proteins shows that those of greatest relevance are annotated to the "translational elongation factor activity" and "RNA-binding" functional categories. Most of the proteins related to these categories were down-regulated during RA differentiation. Among these, the initiation factor IF-2, the elongation factors eEF-1gamma and eEF-

1alpha1, the heterogeneous nuclear ribonucleoprotein hnRNP F, and the eukaryotic initiation factors eIF4A-1 and eIF4A-2 are known to be down-regulated by ATRA in acute promyelocytic cells (Harris et al., 2004; Zheng et al., 2005; Guo et al., 2001). In particular, IF-2, eEF-1gamma and eEF-1alpha1 have roles in the elongation stages of the protein synthesis mechanism, and hnRNP F, eIF4A-1 and eIF4A-2 have roles in mRNA processing and transport. This may suggest that ATRA-induced differentiation of NB could share these six effectors with ATRA-induced differentiation in PML cells.

A group of enzymes involved in biochemical metabolism was found to be significantly down-regulated, implicating the suppression of related biochemical pathways in ATRA-treated cells (dihydrolipoyl dehydrogenase, glucosamine-fructose-6-phosphate aminotransferase 1, aspartate aminotransferase, enolase variant 1, L-lactate dehydrogenase B chain, acetyl CoA carboxylase beta). The down-regulation of lactate dehydrogenase (LDH) is also interesting, as it is a characteristic serum marker that is useful in facilitating diagnosis, prognosis and monitoring disease progression in children affected by NB.

Several genomic alterations in NB have been reported to correlate with prognosis, including amplification of MYCN oncogene, gain of chromosome 17q and loss of chromosome 1p36. Other recurrent changes have also been suggested to have relevance to the development and progression of these tumours (Bown et al., 2001;

Maris et al., 1999). Our results show that some of the genes coding for the identified proteins mapped to chromosomal regions that are known to be altered in NB. Among these, the acetyl CoA carboxylase beta ACACB gene which is mapped to allelic imbalance NB region (17p13-p11) and the ENO1 gene into a loss chromosomal NB region (1p36.3-1p36.2). Moreover, enolase variant 1, which was down-regulated in our system, has been seen to be up regulated in undifferentiated haematopoietic cells (Tao et al., 2004). The DLD, DDX1 and PSMD11 genes mapped to gain NB regions (Mosse et al., 2005; Chen et al., 2004; Hackett et al., 2003). The dihydrolipoyl dehydrogenase DLD protein level, which is down-regulated in our model system, has been seen to increase in the central nervous system of rats after oxidative stress (Poon et al., 2006). The DDX1 gene coding for the DEAD box protein 1 which is down-regulated in our model system, has been shown to be over-expressed in a subset of unfavourable NBs and in retinoblastoma cell lines (Godbout et al., 1998); it has been mapped to chromosome 2p24 and found often co-amplified with the proto-oncogene MYCN in patients with a worse prognosis (Pandita et al., 1997; Manohar et al., 1995) than in patients with only the MYCN gene amplified (Squire et al., 1995; George et al., 1996). The role of DDX1 in the tumorigenic process is not known though; it is a putative RNA helicase, predicted to be involved in RNA binding and in the export of mRNA from the nucleus to the cytoplasm (Scott et al., 2003; Bleoo et al., 2001). It is both

cytoplasmatic and nuclear in DDX1-MYCN amplified NB and RB cell lines. However, it is known to localize specifically to the nucleus in non MYCN amplified cell lines. In LAN-5 cells, I observed a correlation between MYCN amplification and DDX1 nuclear translocation upon ATRA treatment. These findings may support the hypothesis of its putative RNA shuttle function into the nucleus as a sign of good prognosis upon ATRA differentiation.

Other down-regulated proteins identified in our study have been associated to the aggressiveness of several tumours. This would suggest that the cells are regressing from their tumoral state, following RA treatment. Among these, there are pituitary tumour-transforming gene protein-binding factor PTTG1IP and ribonucleoprotein hnRNP F. PTTG1IP is a prognostic indicator in thyroid cancer, even if its precise contribution to tumorigenesis has not yet been explored (Stratford et al., 2005). Also, increased expression of hnRNP F has been seen in more aggressive colorectal tumours. This suggests hnRNP F as a potential marker for colorectal cancer progression (Balasubramani et al., 2006). Furthermore, the DDX1, eIF4A1, eIF4A2, eEF1-gamma and LDH genes are detected in several NB cDNA libraries. Moreover, eIF4A1 mRNA has been shown to be consistently over-expressed in human melanoma cells in vitro (Eberle et al., 1997), in hepatocellular carcinoma (Shuda et al., 2000; Yoon et al., 2006) and in early-stage non–small-cell lung cancer (Ji et al., 2003).

The SH-SY5Y and SK-N-BE human NB cell lines are able to undergo neuronal differentiation in presence of ATRA. The SH-SY5Y cell line is not amplified (Biedler et al., 1973) and the SK-N-BE cell line is amplified for the oncogene MYCN (Biedler et al., 1976) an important determinant of RA response in vitro and patient prognosis in vivo (Bordow et al., 1998). Either has often been used as one of the models for the analysis of neuronal function and differentiation. According to it, I saw that the neural markers SgII and NEF-L and the proliferating marker PCNA, were differentially regulated by RA in the SH-SY5Y and SK-N-BE cell lines as compared to the LAN-5 cell line. In the three cell lines I also observed the PRDX2 protein up-regulation which has been seen in the SH-SY5Y cell line upon treatment with the anti-neoplastic drug ectoposide (Urbani et al., 2005) and during differentiation of embryonic stem cells to neural cells by ATRA (Guo et al., 2001). Moreover I observed that the ATRA treatment down-regulates the EF1α and EF-2 proteins and this effect has just been reported in promyelocitic cells.

After ATRA treatment, Gβ2 and prefoldin subunit 3 were differentially expressed in the SH-SY5Y cells as compared to the LAN-5 cells, although they were not differentially expressed in the SK-N-BE cell line. Here I assay three independent cell lines: two N-MYC duplicated and one without amplification. Indeed the three cell lines have different tumoral origins and the un-regulation of these two proteins in SK-N-BE cells might suggest that NB ATRA-induced

differentiation may involve different pathways. Gβ2 is an important regulator of certain signal-transduction receptors and effectors and is ubiquitously expressed in human tissues. Nothing of this protein was known to be associated with the tumour process or to be regulated by RA. Prefoldin subunit 3/vbp1 protein is a chaperone that captures proteins in unfolded state and transfers them to cytosolic chaperonin for functional folding. To our knowledge, the role of prefoldin proteins is not known in differentiation per se or in neural differentiation. I saw that it decreased in cytosolic fraction upon RA treatment. Thought proteomic approach it was found that proteins containing prefoldin structures increased during neural differentiation (Oh et al., 2006). Additionally, it was shown that the chaperonin prefoldin 3 binds the Von Hippel-Lindau (VHL) tumor suppressor gene product (Woodward et al., 2000; Brinke et al., 1997). VHL is involved in the ubiquitination and subsequent proteasomal degradation via the VHL ubiquitination complex (Iwai et al., 1999; Lisztwan et al., 1999) and in the down-regulation of transcriptional elongation (Maxwell et al., 1999). Because prefoldin 3 functions as a chaperone protein, it may play a role in the transport of the Von Hippel-Lindau protein from the perinuclear granules into the cytoplasm for the ubiquitination of hypoxia-inducible factor, an important step in the development of angiogenic tumours. VHL alteration gene leads to VHL disease which is associated with various rare neoplasias, including haemangioblastoma of the central nervous

system, retinal angioma, clear cell renal carcinoma and pheochromocytoma (Gnarra et al., 1996) (OMIM 193300). VHL gene is mapped on chromosome 3p25 and the loss of this region is a non-random alteration associated to aggressive NBs (Spits et al., 2003). Furthermore, its mRNA expression level is a promising marker to predict patient survival in NB (Hoebeeck et al., 2006). It is known the inhibition of endogenous expression of VHL protein in SHSY5Y cells reduced neuronal properties. In conclusion VHL protein has a neuronal differentiating potential to transform NB cells into functional neuron-like cells (Murata et al., 2002; Kanno et al., 2000). To date, the role of prefoldin 3 in NB has not been investigated. It is known to have a cytoplasmatic location, but in presence of VHL protein has a nuclear location (Tsuchiya et al., 1996). As shown in Figure 16, prefoldin 3 has a preferential location in the nuclear compartment upon ATRA differentiation while in the cytoplasm is observed a decreased expression. This data underline the potential new role of prefoldin 3 in the nuclear compartment during differentiation process.

Gene coding prefoldin 3 is VBP-1 (Von Hippel-Lindau binding protein 1). I also checked the correlation of VBP-1 expression in a cohort of 102 primary NB to survival, founding that high expression of VBP-1 is significantly correlated to bad survival. This observation and the previously shown VBP-1 down regulation in NB cells following ATRA-induced differentiation, lead to suggest VBP-1 as an important marker in the treatment of minimal residual disease.

Moreover the speculation on the role of prefoldin 3 together with VHL protein in the progression of NB might be an important issue for the future studies on NB tumor.

2. Second Aim:

Galectin-1 is a major effector of aggressiveness in TrkB-expressing NB

Another aspect of NB is the treatment of more aggressive forms which are resistant to chemotherapy. To minimize the presence of minimal disease residues, it is necessary to develop a therapeutic strategy hitting the aggressive tumors in a multistep way.

Aggressiveness defined by tissue invasion and metastasis is a hallmark of cancer cells (Hanahan and Weinberg, 2000). In NB, several factors have been identified to be associated with aggressive tumor behaviour, including deletion or epigenetic silencing of caspase-8 (Eggert et al., 2001; Teitz et al., 2000), expression of the HGF/c-met axis (Hecht et al., 2004; Hecht et al., 2005) as well as expression of full-length TrkB and its cognate ligand BDNF (Matsumoto et al., 1995). The latter is also associated with amplification of the MYCN oncogene, which is the strongest independent marker of outcome in NB (Nakagawara et al., 1994). On the other hand, expression of the neurotrophin receptors TrkA

(Nakagawara et al., 1993) and TrkC (Yamashiro et al., 1997) are found in favourable NBs, with high expression of TrkA been accompanied by massive changes in the transcriptome of NB (Schramm et al., 2005b). This biological diversity between TrkA and TrkB expression could also by verified in vitro (Lucarelli et al., 1997; Schramm et al., 2005a). Taken together, identification of effector targets differentially regulated between TrkA and TrkB expressing NBs could serve as drug targets for NB treatment. A cohort of 102 primary NBs was screened for differentially expressed genes between TrkA and TrkB and compared the results to gene and protein lists obtained in a NB cell line model, SY5Y, with ectopic expression of either TrkA or TrkB (Schulte et al., 2005; Sitek et al., 2005). One of the most promising candidate genes identified in this analysis was Galectin-1 (GAL-1), which has been suggested as a cancer target before (Rabinovich, 2005; Salatino et al., 2008). I found a direct and significant correlation of mRNA expression of TrkB and Gal-1 in primary NB, while TrkA and Gal-1 expression were significantly anti-correlated (Figure 10). This clearly stresses the importance of the in vitro findings and suggested a functional link between Trk expression and Gal-1 expression also in the primary tumors. This is important, because inhibition of Gal-1 functions by siRNA has been shown to inhibit tumor angiogenesis, which can be accomplished by siRNA (Mathieu et al., 2007) or a Gal-1 specific peptide (Thijssen et al., 2006).

Gal-1 expression has also been correlated to tumor aggressiveness (Chiang et al., 2008; Jung et al., 2007). In invasive breast cancer, Gal-1 was found in cancer-associated stromal cells (Jung et al., 2007), which is in accordance with our findings in NB (Figure 13). However, in contrast to the situation in breast cancer, I could not establish a link of stromal Gal-1 expression to NB progression. Nevertheless, the results prompted us to investigate the consequences of modulating Gal-1 functions in our model system.

BDNF-mediated activation of TrkB increased Gal-1 expression on mRNA and protein levels in SY5Y-TrkB cells but not in neurotrophin-treated SY5Y-TrkA or control-transfected cells, in line with previous shown results in a proteomic study (Sitek et al., 2005). Therefore, I not only focused on the differences between TrkA- and TrkB-expressing cells but also analysed the consequences of neurotrophin treatment in our model systems. BDNF markedly increased the invasiveness of SY5Y-TrkB cells and this capacity was reduced by neutralising Gal-1 function (Figure 15). rGal-1 alone was not sufficient to induce the full invasive capacity of SY5Y-TrkB cells, suggesting that other BDNF-induced factors are involved in invasion as well. Previously, MCSP and c-Met have been suggested to be TrkB-regulated and to contribute to invasion in NB (Hecht et al., 2005; Schulte et al., 2005). These current findings identify Gal-1 as a third player contributing to TrkB-mediated invasiveness in NB.

Since Gal-1 can exert its functions extracellularly as well as intracellularly I conducted a rescue experiment, in which the intracellular pools of Gal-1 were depleted by siRNA, and rGal-1 was added simultaneously. While a siRNA directed against Gal-1 prevented invasion of SY5Y-TrkB cells, this effect could be completely rescued by addition of rGal-1 (Figure 16). This suggests a major importance of extracellular Gal-1 for invasion either in a autocrine or paracrine fashion. Similar results were obtained for the migratory capacity of Trk-expressing NB cells. BDNF-induced migration of SY5Y-TrkB cells was strongly reduced in the presence of a siRNA directed against Gal-1 in a scratch assay, whereas neurotrophin-treated SY5Y-TrkA cells or vector control cells were not able to migrate (Figure 18). Therefore, both the migratory and invasive capacity of SY5Y cells were highly dependent on the activation of TrkB by BDNF and these functions were strongly modulated by Gal-1.

Furthermore, I analysed the consequences of interfering with BDNF signalling using the Trk-specific small molecule inhibitor, K252a. BDNF-mediated phosphorylation of Akt was used as a marker for TrkB activation in SY5Y-TrkB cells, in which inhibition of Akt sensitizes to chemotherapy (Ho et al., 2002; Jaboin et al., 2002). It has been previously shown that the chosen concentration of K252a (100 nM) is sufficient to inhibit Trk phosphorylation in other model systems (Stephan et al., 2008). Following treatment with K252a,

phosphorylation of Akt was strongly reduced in BDNF-activated SY5Y-TrkB cells and this could not be seen in neurotrophin-treated SY5Y cells without TrkB expression (Figure 19). A minor increase in Akt phosphorylation following K252a treatment in the absence of Trk receptors might be attributed to its described effect on serine/threonine protein kinases (Tapley et al., 1992). As Gal-1 levels also decrease following K252a treatment, I conclude that Gal1- induction is directly dependent on activated TrkB in SY5Y-TrkB cells.

In summary, I have shown that Gal-1 is correlated to the Trk receptor status in primary NB and that Gal-1 is a major effector of TrkB-mediated aggressiveness. Blocking Gal-1 function clearly and strongly reduced the migratory and invasive capacity of TrkB-expressing NB cells. There are already several strategies available to interfere with Gal-1 functions in vivo including siRNAs and small molecule inhibitors. These should be analysed to evaluate Gal-1 as a target for the treatment of NB.

research articles Journal of proteome research

Comparative Proteomic Expression Profile in All-*trans* Retinoic Acid Differentiated Neuroblastoma Cell Line

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Neuroblastoma (NB) is an infant tumor which frequently differentiates into neurons. We used two-dimensional differential in-gel electrophoresis (2D-DIGE) to analyze the cytosolic and nuclear protein expression patterns of LAN-5 cells following neuronal differentiating agent all-*trans*-retinoic acid treatment. We identified several candidate proteins, from which Gβ2 and Prefoldin 3 may have a role on NB development. These results strength the use of proteomics to discover new putative protein targets in cancer.

Keywords: neuroblastoma • all-*trans*-retinoic acid • 2D-DIGE • LAN-5 human neuroblastoma cell line

Introduction

Neuroblastoma (NB) is manifested in childhood as an extracranial solid tumor of the sympathetic nervous system that can show extraordinary clinical and biological heterogeneity.[1] The majority of NBs are aggressive metastatic tumors with poor clinical outcome, despite intensive multimodal therapy. The most favorable subset of this embryonic tumor (stage 4S) can spontaneously differentiate or regress to a benign tumor phenotype, even after no, or minimal, therapy.[2−4] This has generated considerable interest in agents that are able to regulate these important biological processes.

Vitamin A and its analogues (the retinoids) have roles in cell proliferation, differentiation, and apoptosis in normal tissues during embryonic development.[4] It has also been shown that retinoic acid can restore "normal" functions (differentiation) in certain tumors, such as NB,[5] melanoma,[6] and acute promyelocytic leukemia (PML).[7] In clinical practice, all-*trans* retinoic acid (ATRA) is mainly used for patients with acute PML.[8] In the treatment of NB patients, ATRA has been used as a chemotherapeutic agent with some success,[9] but 13*cis*-RA is preferred due to its more favorable pharmacokinetics.[10−12] Furthermore, studies comparing the activities of 13*cis*-RA and ATRA in NB cell lines have demonstrated similar potencies of these retinoids, in terms of cellular differentiation, growth arrest, and regulation of tumor markers such as MYCN.[13]

RA effects appear to be mediated by two families of nuclear retinoic acid receptors (RARs and RXRs) that form a part of the steroid/thyroid/vitamin D superfamily.[14] These receptors function as homo/heterodimers and directly modulate transcriptional activity by binding to the RA response elements (RAREs). RA affects NB differentiation either through the transcriptional regulation of genes directly involved in the differentiation process or that control the differentiation process.[5,15] Over the past two decades, a large number of NB cell lines have been generated, which have diverse biological characteristics. These NB cells provide "good" model systems both for the unraveling of the molecular basis of NB development, and for the development of therapeutic protocols based on NB differentiation.

Recently, there has been significant progress in the development of systematic approaches to study NB development, at both the transcriptional and translational levels. Gene expression profiling on NB specimens have been described as identifying the molecular signatures of high-risk and low-risk tumors[16−18] and novel prognostic markers.[19−21] The search for markers at the transcriptional level is less reliable than at the protein level, as there is a "long" and unpredictable route from RNA to proteins, and very often protein expression does not correlate with mRNA expression. Indeed, several proteins can be encoded by the same gene, through splice variants and post-translational modifications that cannot be directly predicted from gene sequence. The most commonly used comparative proteomic approach is two-dimensional difference gel electrophoresis (2D-DIGE) coupled with mass spectrometry (MS), which provides a good proteomic tool for the investigation of novel proteins that might serve as candidates for tumor markers. Previously, 2D-PAGE analyses of protein changes were performed to study chemoresistance,[22] to search for markers for tumor diagnosis in NB cell lines,[23,24] and to study quantitatively and qualitatively differences in healthy and pathological NB mouse samples.[25,26] Moreover, proteomic characterization of differentiation induced by ATRA has been described in promyelocytic cells[7,27,28] and in mouse embryonic stem cells.[29] However, very little is known about the large-scale protein

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ORIGINAL ARTICLE

Galectin-1 is a major effector of TrkB-mediated neuroblastoma aggressiveness

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Expression of Trk receptors is an important prognostic factor in neuroblastoma (NB) and other cancers. TrkB and its ligand brain-derived neurotrophic factor (BDNF) are preferentially expressed in NB with poor prognosis, conferring invasive and metastatic potential to the tumor cells as well as enhancing therapy resistance. Galectin-1 (Gal-1) has emerged as an interesting cancer target, as it is involved in modulating cell proliferation, cell death and cell migration, all of which are linked to cancer initiation and progression. We previously identified Gal-1 mRNA to be upregulated in patients with aggressive, relapsing NB and found that Gal-1 protein was upregulated in human SY5Y NB cells on activation of ectopically expressed TrkB (SY5Y-TrkB), but not TrkA (SY5Y-TrkA). Here, we report that Gal-1 mRNA levels positively correlated with TrkB expression and anticorrelated with TrkA expression in a cohort of 102 primary NB. Immunohistochemical analyses of 92 primary NB specimens revealed high Gal-1 expression in stromal septae and in neuroblasts. BDNF-mediated activation of TrkB enhanced invasiveness and migration *in vitro*, which could be impaired by transient transfection using Gal-1-specific siRNA or a neutralizing antibody directed against Gal-1. The addition of recombinant Gal-1 (rGal-1) in the absence of BDNF partially restored migration and invasive capacity. Using the Trk inhibitor K252a, we could show that the upregulation of Gal-1 protein strictly depended on activated TrkB. Our data suggest that targeting Gal-1 might be a promising strategy for the treatment of aggressive NB.
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Introduction

Galectin-1 (Gal-1) is a multifunctional protein involved in various aspects of tumorigenesis and has been described as a promising cancer target (reviewed in Liu and Rabinovich (2005); Rabinovich (2005); Salatino *et al*. (2008)). It can act as an extracellular matrix protein to modulate cell adhesion (Hughes, 2001) as well as intracellularly by interaction with H-ras causing Ras membrane anchorage and cell transformation (Paz *et al*., 2001). Gal-1 function has been found to be intricately linked to essential biological processes in tumor cells. It regulates complex signaling pathways involved in tumor–host interaction (Juszczynski *et al*., 2007; Rodig *et al*., 2008) and angiogenesis (Thijssen *et al*., 2006). By forcing activated T cells to undergo apoptosis (He and Baum, 2004), Gal-1 expression has been hypothesized to contribute to the tumor-immune escape. Inhibition of Gal-1 function resulted in T-cell-mediated tumor rejection (Rubinstein *et al*., 2004). Gal-1 has been shown to contribute to tumor angiogenesis in a mouse melanoma model (Le Mercier *et al*., 2008), and selective peptide inhibitors to Gal-1 have been developed that show profound antiangiogenic activity *in vivo* (Thijssen *et al*., 2007). Intracellularly, Gal-1 can associate with H-ras and direct the transport of farnesylated H-ras to the Golgi apparatus in various systems (Belanis *et al*., 2008). Inhibition of H-ras/Gal interaction by small molecule inhibitors, such as farnesylthiosalicylate, (Paz *et al*., 2001) has proved anticancer activity in various model systems and is now proceeding to clinical development.

In neuroblastoma (NB), the most common tumor of childhood, neurotrophin receptors of the Trk family are key players determining biology as well as patient prognosis. Expression of TrkA/NTRK1 is associated with favorable biology and excellent patient outcome, whereas expression of TrkB/NTRK2 correlates with unfavorable tumor biology and poor patient outcome (Nakagawara *et al*., 1993, 1994). Therefore, proteins that are differentially regulated in TrkA- and TrkB-expressing NBs are excellent candidate targets for NB therapy.

We have developed model systems for differential Trk expression in the neurotrophin receptor null human

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