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TARGETING ID PROTEINS IN NEURAL DIFFERENTIATION AND CANCER

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Alla mia famiglia

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GLOSSARY

Axon-inhibiting molecules: they include factors within CNS myelin that inhibit neurite outgrowth and promote the collapse of growth cones [NOGO receptor, NOGO 66, MAG (myelin-associated glycoprotein) and OMPG (oligodendrocytemyelin glycoprotein)], molecules that mediate axon-repulsive signals [UNC5A (netrin receptor) and SEMA3F (semaphoring 3F)] and factors that participate in the regulation of myelination (NOTCH and Jagged).

bHLH transcription factors: the basic helix–loop–helix (bHLH) proteins contain the HLH dimerization domain and a basic region that mediates DNA binding. They have been implicated in the regulation of differentiation, cell growth and survival, and tissue-specific gene expression. Genes that encode bHLH proteins are found in all major subdivisions of eukaryotes (metazoans, fungi and plants) but not in prokaryotes. They are categorized into distinct classes on the basis of biochemical and functional criteria.

Ephrussi box (E-BOX)

The Ephrussi box (E-box), is a hexameric DNA consensus sequence that is characterized by the consensus CANNTG, recognized by the bHLH factors.

Embryonic Stem cells: cells that are able to self-renew to form daughter stem cells and differentiate to generate diverse mature progeny.

ETS: they are transcription factors related to the transforming avian erytroblastosis virus transduced by the E26 avian retrovirus.

Floxed: Refers to a DNA construct in which a gene or gene segment is flanked by lox-P sites in the same orientation; Cre recombinase excises the segment between the lox-P sites.

Neural Crest: The neural crest comprises a bilaterally paired strips of cells that arise in the ectoderm at the margins of the neural tube. These cells migrate to many locations and differentiate into many cell types within the embryo.

PAX: They are developmental regulatory transcription factors characterized by a DNA-binding paired domain that was originally described in the Drosophila segmentation gene paired.

The ubiquitin–proteasome system: Ubiquitin (UB) is a 8-kDa protein that is first transferred to the ubiquitin-activating enzyme E1 in an ATP-dependent manner, and then to the ubiquitin-conjugating enzyme E2. Finally, the ubiquitin is covalently attached to the target protein by E3 ubiquitin ligase, leading to the formation of a poly-ubiquitin chain. The poly-ubiquitylated protein is recognized by the 26S proteasome and is destroyed in an ATP-dependent manner.

SUMMARY

Id proteins play a crucial role in developmental processes. They belong to the Helix-Loop-Helix (HLH) family of transcription factors, but are devoid of the DNA binding region. Acting as dominant negative regulators of bHLH proteins, Ids are involved in cellular mechanisms controlled by their partners. In particular, Ids derive their name by the ability to ***I***nhibit ***D***NA binding and ***I***nhibit ***d***ifferentiation.

Id biological functions are required in proliferating precursor cells throughout the embryo. In these cells Ids preserve self renewal and prevent fate determination, while their expression is extinguished upon cell cycle exit and differentiation. However, recent findings indicate that Id2 continue to be expressed in particular cells of mammals adult brain, suggesting that it may have highly specialized and still unknown functions.

To investigate Id2 role in adult nervous system, in the first part of this work we verified Id2 protein expression pattern in different rodent brain areas and started to generate an inducible knockout mouse model by the Cre/lox-P system. This technique allows Id2 ablation in a spatio-temporally regulated manner and we are confident that the inducible knockout will be a great tool for better understanding neural developmental networks.

When aberrantly expressed by tumour cells and tumour endothelium, Id proteins direct key phenotypic traits of cancer, such as deregulated proliferation, anaplasia, metastasis and neo-angiogenesis. Ids are considered as attractive targets for molecular cancer therapies design; however, inhibition of nuclear transcription factors is notoriously difficult to obtain. Since Id protein mechanism of action is a well defined set of protein-protein interactions, we supposed that targeting Ids dimerization domain could be a powerful inhibitory approach. To address this point, we used an HLH mutant domain, the 13I; firstly, we demonstrated that it is a potent and specific dominant negative inhibitor of Id

proteins. Subsequently, we employed 13I domain to model Id inhibition in human cancer cell lines. We found that, upon introduction into neuroblastoma cells, this domain is able to trigger neuronal commitment and differentiation, as well as to inhibit proliferation, increase responsiveness to all-*trans* retinoic acid (RA), and inhibit expression of the pro-angiogenic protein VEGF.

The principle outcome of this research is that we demonstrate the feasibility to interfere with Id functions by targeting their dimerization properties and that such an approach may have a therapeutic value in cancer cells.

INTRODUCTION

A family portrait

The proper development of an organism with a complex tissutal structure is the result of a dynamic balance between proliferation, differentiation and apoptosis. It is well known that the number of differentiated cells in a specific tissue is fixed by a genetic program. The acquisition of cellular diversity in a developing organism is defined by a combinatorial code of spatially and temporally co-expressed genes. The transcription of such genes is controlled by interactions between transcription factors and specific DNA recognition sequences in target promoters. Nevertheless proliferating, self-renewal cells capable of replacing damaged cells are always necessary. How can a cell know the right time to proliferate or differentiate is an ancient question. It has been said that, if a researcher can imagine the existence of a particular mechanism, the cell is likely to be already using it. Probably one of the major proof of concept for such an assumption is the existence of dominant negative molecules.

To date, many family of transcription factors involved in cell fate determination have been characterized; among them, the helix-loop-helix (HLH) domain proteins play a key role as differentiation regulators (Massari ME and Murre C, 2000). Members of this large family were identified in all eukaryotic subgroups, ranging from the yeast *Saccaromyces cerevisiae* to humans, suggesting an early evolutive origin. The HLH domain mediates homo- and hetero-dimerization. Its structure comprises two amphipathic α-helices, each 15-20 residues long, separated by a shorter intervening loop more variable in length and sequence. The HLH domain is usually adjacent to a short region of basic residues that recognizes specific palindromic sequences on DNA called E-boxes (CANNTG); hence these factors are also known as bHLH proteins (Littlewood T and Evan G, 1994).

Since bHLH factors have been discovered and characterized in a variety of different species, they are often identified by different

names. However, it is widely accepted a general classification in which bHLHs are divided in two major groups:

- Class A factors, also called E proteins. They are ubiquitously expressed. In Mammals, E proteins are encoded by three genes: *E2A*, with the two splicing variants E12 and E47, *HEB* and *E2-2*.

- Class B factors. They are tissue-specific, their expression being restricted to cells of a particular lineage. Well known examples are the myogenic factors MyoD and myogenin and the pro-neural factor NeuroD and Mash1.

The common view of tissue-specific regulation by bHLH family members holds that the dimerization of a bHLH protein of tissue-restricted expression (Class B) with an ubiquitously expressed one (Class A) is required for DNA binding and the following gene transcription.

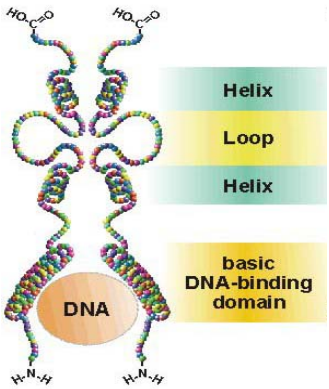


Figure 1. bHLH factors mechanism of action
Schematic representation of the dimer, formed by an E protein with a tissue-specific bHLH factor, complexed with DNA.
Once established, the dimer binds to E-box consensus sequences and activates transcription.

The biochemical properties of HLH-mediated dimerization were clarified by the resolution of crystallographic structures of bHLH homodimers complexed to DNA (Ellenberger T et al., 1994). The important notion emerged is that the presence of both basic regions is necessary for a stable DNA binding of bHLH complexes, since each monomer contacts a half of an E-box sequence.

However, it is known that there is no general rule without exceptions. Among the HLH factors, the exception is represented by Id proteins.

The family black sheeps: the Ids

In 1932 Muller postulated the existence of dominant negative mutations, to which he referred as *antimorphs* or *antagonistic mutant genes, having an effect contrary to that of the gene from which they were derived* (Muller HG, 1932). Id proteins are naturally occurring dominant negative regulators of bHLH transcription factors. We don't know whether Ids effectively originated as mutant version of bHLHs factors, but it is a very appealing possibility: it means that cells were able to convert a probably deleterious mutation into a useful mechanism.

Discovered in 1990, mammalian Ids are a family of four related proteins: Id1, Id2, Id3 and Id4. Their mechanism of action was almost immediately apparent from their amino acid sequence: Ids contain the HLH dimerization motif but lack the basic DNA binding domain. They form complexes with bHLHs transcriptional regulators; such complexes are unable to bind DNA and result to be transcriptionally inactive (Benezra R et al., 1990). Since most bHLH proteins turn on the expression of sets of genes involved in cell fate determination and differentiation, the term ***Id*** alludes to the ability of these proteins to **i**nhibit both **D**NA binding and **d**ifferentiation.

Noteworthy, the binding affinity of Id proteins for the ubiquitously expressed E proteins is higher than for the tissue-

specific bHLHs. Since tissue-specific bHLHs need E proteins as obligate partners to turn on transcription, by sequestering E proteins Ids may control the transcriptional repertoire of a variety of different cell types through the same biochemical mechanism.

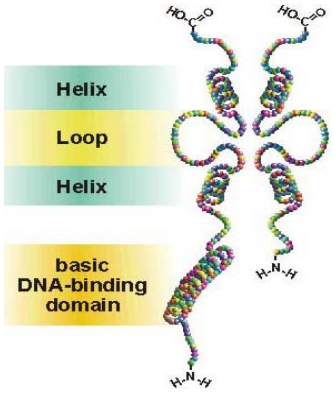


Figure 2. Id proteins mechanism of action
Schematic representation of the dimer, formed by an Id protein and a bHLH factor. Unable to bind to DNA, such dimer results trascriptionally inactive.

Although Id genes were isolated in several eukaryotic species, the most studied and better characterized are those of mammals; in humans, there are four genes located on different chromosomes: Id1 locus is at 20q11, Id2 at 2p25, Id3 at 1p36.1 and Id4 at 6p21-22. All four human IDs (reviewed in Ruzinova M and Benezra R, 2003) are small proteins, with sizes comprised between 13 and 20 kDa. Each of them possesses at least two splicing variants that seem have no evident biological relevance. They share high sequence homology within the HLH dimerization domain, but outside they display extensive sequence divergence. The HLH domains of IDs are more closely related to one another than to other bHLH proteins (Deed RW et al., 1994). Several studies revealed that, in addition to the bHLHs, Ids can bind to other classes of transcription factors, such as ETS and PAX transcriptional regulators (Norton JD, 2000). Moreover, Id2 interacts with the retinoblastoma tumour suppressor protein, pRB,

and the other pocket proteins, p107 and p130 (Iavarone A et al., 1994). The original picture in which Ids were represented solely as bHLH inhibitors became much more complex.

Terminal differentiation of a specific cell type is coupled with cell cycle withdrawal and arrest. Therefore it is not surprising that Ids not only act as negative regulators of cell differentiation programs, but as positive regulators of cell growth and proliferation as well (Zebedee Z et al., 2001).

How are Ids regulated?

There is an increasing interest on the mechanisms underlying regulation of Id protein activity. Cells can modulate Id proteins at different levels.

In many cell types, Id proteins expression is directly induced by bone morphogenetic proteins (BMPs), members of Transforming Growth Factor-β (TGF-β) super-family. TGF-β related proteins are pleiotropic signalling molecules that signal via phosphorylated Smad transcription factors and control several intracellular pathways. Ids exhibit wide variety of biological effects in many tissues; indeed, recent findings suggest that Id proteins act as conductors for controlling multiple cellular responses induced by BMPs and TGF-β (Miyazono K and Miyazawa K, 2002).

In addition to transcriptional control, other mechanisms affect Ids activity at post-translational level. An important one is the control of protein stability. Id proteins are very short-lived molecules, with an half-life comprised between 20-60 minutes. They are substrates of the ubiquitin (UB)-26S proteasome system, the major proteolytic machinery of eukaryotic cells (Bounpheng MA et al., 1999). Recently, a study revealed that the anaphase promoting complex/cyclosome (APC/C) is the ubiquitin ligase that primes Id proteins for proteasomal-mediated degradation.

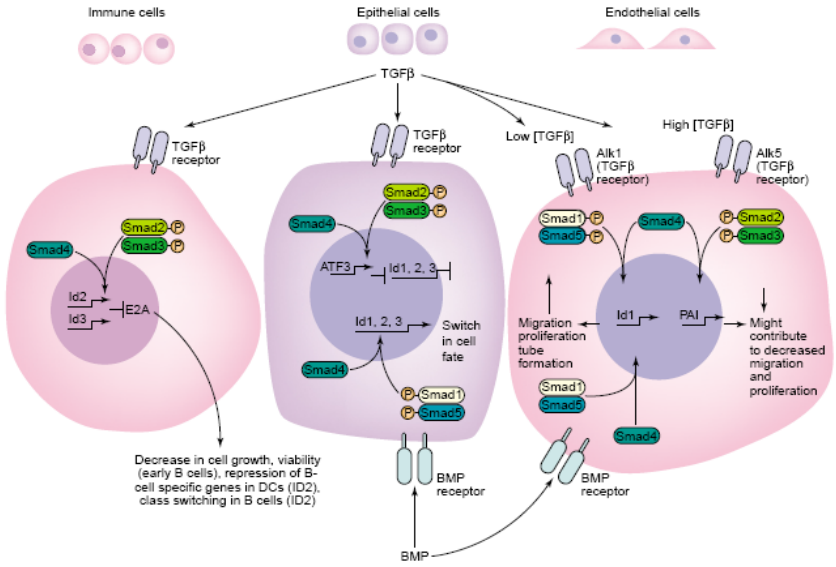


Figure 3. Regulation of Id genes expression by members of TGF-β family. *Schematic representation for the complex relationship between BMP and TGF-β signals on Id gene expression in different cell types. (From: Ruzinova M and Benezra R, 2003)*

APC/C and its co-activator CDH1 recognize all Ids, except Id3, through a conserved deconstruction-box (D box) motif, located at the HLH domain C-terminus. Although devoid of a D box Id3 is a substrate of the ubiquitin-proteasome system as well, being probably primed to degradation by other unknown ubiquitin ligases (Lasorella A et al., 2006). Dimerization with bHLH factors extends the half-life of Id3, and probably of the other Ids, suggesting that it protects Ids from degradation. This is likely to be due to the capability of bHLHs to act as molecular *chaperones* for Ids, which lack a nuclear localisation signal (NLS). bHLHs would sequester Ids within the nucleus, away from the degradation machinery (Deed RW et al., 1996).

However, this is a simplified view: several evidences point to Id proteins sub-cellular compartmentalization as a mechanism for

controlling their activity. Small proteins under 40kDa such as Ids, can cross the nuclear membrane. Therefore, they do not need an active transport machinery to shuttle between nucleus and cytoplasm. Since Ids exert their activity binding to and inhibiting mainly transcription factors, once localised in the cytoplasm, they would be considered inactive. Proteins capable to bind Id2 and sequester it within the cytoplasm, thus preventing its access to nuclear partners have been identified in different cell types. Two of them, enigma homolog (ENH) in the neuroblasts (Lasorella A and Iavarone A, 2006) and polycystin-2 (PC2) in kidney cells (Li X et al., 2005), are associated with the actin cytoscheleton. Another antagonist affecting cytoplasmic location of Ids in skeletal and cardiac muscle cells is the interferon-inducible protein p204 (Liu CJ et al., 2002). Association with ENH, PC2 and p204 impairs Id2 functional activity. It was suggested that the nuclear exclusion of Id proteins is necessary, even if not sufficient, to the differentiation program of specific cell types.

How to proliferate…via Id proteins

Id proteins appear to be critical players in cell growth (Yokota Y and Mori S, 2002). It is now clear that their activity is strongly integrated with mitogenic signalling cascades and cell cycle regulation. The first evidence was the observation that in human diploid fibroblasts ID1 and ID2 show a biphasic expression pattern after serum deprivation and re-addiction: peaks of expression are found at 2-3 hours and then at 12 hours following growth stimulation (Hara E at al., 1994). These two peaks correspond to the early G1 phase and the G1-S transition of the cell cycle.

This and other findings obtained in a variety of cell types *in vivo* and *in vitro*, indicate that Ids can be considered as essential positive regulators of proliferation (Lasorella A et al., 2001). Two mechanisms, illustrated in Fig. 4, were advanced to explain Ids capability to sustain cell cycle progression. The first one involves

the negative regulation of cyclin-dependent kinases inhibitors (CDKIs) expression, which Ids achieve by sequestering E proteins, Elk and ETS factors, thus blocking their recognition of E-box sites in CDKIs promoters. This mode of action was validated for Id1 inhibition of p21 and p16 transcription mediated by E2A and ETS1 (Prabhu et al., 1997; Ohtani et al., 2001). More recently, Id3 was shown to repress p27 transcription by sequestering Elk1 (Chassot et al., 2007). Because this ability relies on common biological activities, shared by all Ids, it seems plausible that the other family members would also function in a similar manner (Norton JD, 2000). CDKIs act by suppressing the activity of complexes between cyclins and cyclin-dependent kinases (CDKs), and their expression is associated with terminal differentiation and cell cycle exit. By inhibiting the transactivation of CDKIs genes, Ids allow CDK activation and stimulation of the G1/S transition.

The other mechanism involves the Id2 ability of to directly interact with pRB. During the G1 phase of the cell cycle, the un-phosphorylated form of RB associates with and inactivates E2F factors, which regulate a number of genes required for S phase progression. Sequential phosphorylation of RB by cyclinD/CDK4 and cyclinE/CDK2 complexes causes a conformational change in RB, which leads to E2F release and cell cycle progression. The ID HLH domain preferentially binds to the pocket domain of under-phosphorylated RB (Iavarone A et al., 1994). Among Ids, only Id2 and Id4 are able to bind to RB and the other pocket proteins, despite the fact of that all members of Id family share an highly homologous HLH domain and similar biological activity. As reported for viral oncoproteins such as the adenovirus E1A protein and the SV40 large T antigen, Id2 binding to under-phosphorylated RB, causes E2F release (Yokota Y and Mori S, 2002).

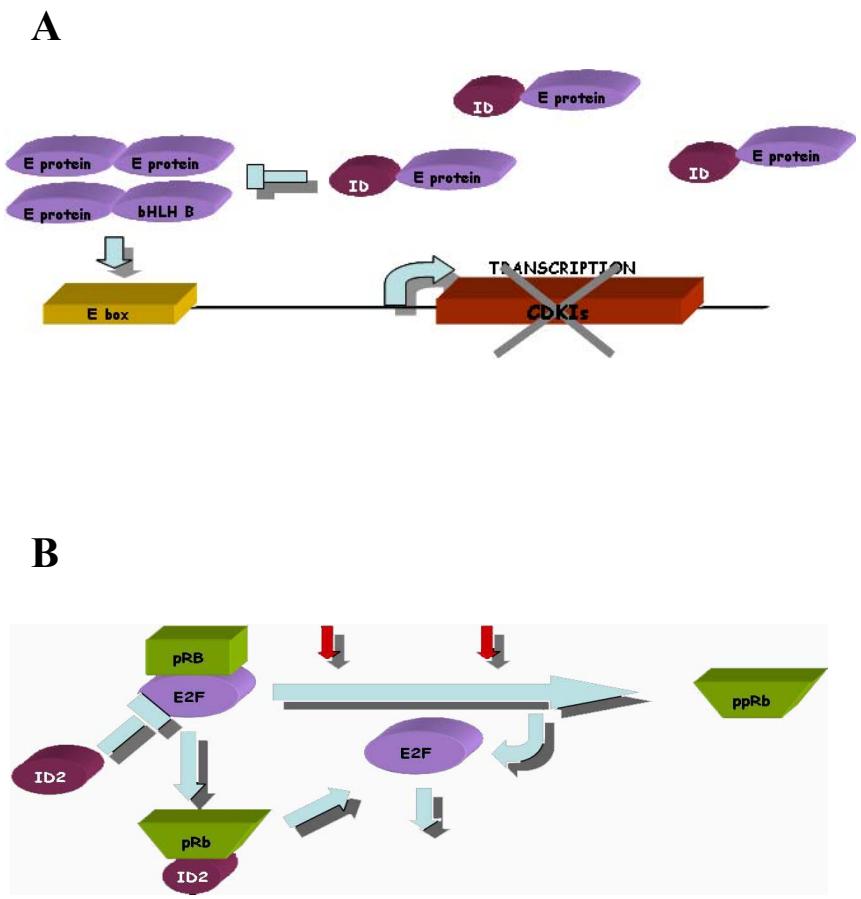


Figure 4. Schematic representation showing how Id proteins can promote cellular proliferation. *A) The bHLH factor-dependent mechanism: expression of CDKIs genes are enhanced by bHLH proteins, resulting in cell cycle arrest in the G0/G1 phase. Preventing the formation of complexes between bHLHs, Id proteins inhibit the transcription of CDKIs genes. B) The RB-dependent mechanism: Id2 competes with E2F for the binding to the hypo-phosphorylated form of pRB; this lead to the release of E2F from RB and the following activation of genes involved in cell cycle progression.*

All Id proteins except Id1 are themselves substrate for CDK2-mediated phosphorylation during early-S phase. This modification seems to be important for cell cycle progression: indeed, cells expressing mutant forms of Id2 and Id3 lacking these phosphorylation sites display S phase arrest and cell death (Deed R et al., 1997).

Id proteins and development: when a cell realizes that it's time to grow up

The developmental maturation of a precursor cell can be ideally divided in two distinct stages. Firstly, a proliferating cell commits to a specific lineage. This commitment is then followed by cell cycle arrest and terminal differentiation. Not surprisingly, Id proteins appear as crucial regulators of both steps in multiple cell lineages, such as myoblasts, B and T cells, dendritic cells, mammary epithelial cells and oligodendrocytes (Ruzinova M and Benezra R, 2003).

During early development, all Id family members are widely expressed, showing overlapping but distinct expression patterns. Such differences mainly depend on the cell developmental stage (temporal specificity) and its anatomical position (spatial specificity). Data obtained by *in situ* hybridization studies demonstrate a general feature of Id gene expression during development: while the distributions of Id1, Id2 and Id3 are overlapped and widespread throughout the organism from early gestation through birth, Id4 shows a unique expression pattern during embryogenesis (Yokota Y, 2001). It is generally hold that Ids are abundant in proliferating, undifferentiated cells, and that their levels become very low or undetectable in post-mitotic, terminally differentiated cells. In the adult organism, Id proteins are involved in maintaining self-renewal, pluripotency and undifferentiated state of progenitor cells (Jankovic et al., 2007; Ying et al., 2003). This means that Id expression is required for

survival of adult stem cells pools and for preventing premature differentiation and exhaustion.

Several murine models were established in order to better assess protein function *in vivo*. In particular, knockout mice clarified that Id function is essential for many aspects of mammals development and provided new insight into their role in specific cell lineages. Because of the functional redundancy among the four Id proteins, only crosses between mice that lack different Id genes are embryonic lethal. As reported in Table 1, Id1 and Id3 single knockouts are viable and fertile, while Id1/Id3 double null mice die around E13.5, displaying small brains accompanied by premature neuronal differentiation and defective brain angiogenesis. Id3 ablation causes defects in humoral immunity: Id3 null mice present low levels of IgG1 and IgG2a as well as a block in thymocyte maturation. The most severe phenotype for a single Id gene knockout is observed for Id2, the deletion of which results in ~25% perinatal lethality. The surviving mice are devoid of lymph nodes and Peyer's patches and exhibit a strongly reduced population of natural killer cells, Langerhans cells and splenic dendritic cells, although the whole splenic architecture appears to be normal.

Table 1. Id knockout mice phenotypes

Gene deleted	Phenotype	Reference
Id1–/–	No major abnormalities, elevated TSP-1 expression	(Volpert et al., 2002; Yan et al., 1997)
Id2–/–	Lack lymph nodes, NK cells and Peyer's patches; defects in spermatogenesis; loss of Langerhans cells	(Hacker et al., 2003; Yokota et al., 1999)
Id3–/–	Abnormalities in humoral immunity and T cell development	(Kee et al., 2001; Pan et al., 1999; Rivera et al., 2000)
Id1 –/–, Id3 –/–	Lethal E13.5, vascular defects in forebrain, premature maturation of neurons	(Lyden et al., 1999)
Id1/Id3 loss of single or multiple alleles	Defects in vascularization and growth of tumor xenografts	(Lyden et al., 1999)
Id2–/–, Rb–/–	Compensatory resolution of Rb–/– defects in neurogenesis and hematopoiesis. Rescue of Rb–/– embryonic lethality but severe muscle loss leads to postnatal demise	(Lasorella et al., 2000)

From: Sikder HA et al., Cancer Cell, 2003

Regarding to reproductive system, mammary glands develop normally in $Id2^{-/-}$ female mice, but, during pregnancy, immature

cells fail to give rise to mature lactating cells; on the other hand, males share general defects in spermatogenesis. By contrast, reflecting Id4 peculiar distribution, the Id4 null phenotype is restricted to the central nervous system. Adult knockout mice exhibit drastic alterations in brain morphology and size, mainly due to the presence of few neurons and glial cells.

The strange case of the nervous system…

The complex architecture of the nervous system requires that cell number is under a tight control during development, since the balance between divisions and cell cycle withdrawal of single cell type is vital for the entire organism. Developmental studies in different organisms revealed a novel picture of Ids roles in the nervous system.

Id genes are expressed in very complex and dynamic patterns during neural development (Yokota Y, 2001). During embryogenesis, Id1 and Id3 levels are high in proliferating, undifferentiated neural precursors, while their expression is extinguished upon cell cycle exit and differentiation (Riechmann V et al., 1995; Jen Y et al., 1997). As already mentioned, Id1: Id3 double null mice die at mid-gestation with clear evidence of premature differentiation, demonstrating the importance of Id proteins in setting the timing of neuronal differentiation. A similar function is ascribed to Id4 in the oligodendroglial lineage (Kondo T and Raff M, 2000). One remarkable feature is that Id2, continue to be expressed in post-mitotic, terminally differentiated neuronal and glial cell types. Surprisingly, a player involved in negative regulation of differentiation and positive expansion of immature cell populations, seems to exploit other functions in highly specialized cells. Experiments conducted by transiently over-expressing Id2 in cultured neural cells were not useful to clarify this apparent paradox: forced Id2 expression induces neither cell differentiation in cortical progenitors or cell survival in post-

mitotic cortical neurons, while inhibiting the induction of neuron-specific genes in undifferentiated cells (Toma JG et al., 2000).

In situ hybridization studies showed that Id2 mRNA is widely distributed throughout the brain (Kitajima K et al., 2006). It is present in all layers of cerebral cortex except layer 4; on cerebellum, Id2 is found in Purkinje cells, but not in the external granule, molecular or external granule cell layers; in the hippocampus only in interneurons and glial cells; finally, it is detected in cells from the olfactory bulb, the suprachiasmatic nucleus and the amygdaloid complex. One explanation of such a peculiar distribution might be the necessity of maintaining a silent ability to proliferate. This could be obtained by storing molecules which are active promoters of proliferation, such as Id2, under tight negative control of factors like pRB (Lasorella A et al.,2000). When required, certain stimuli might unleash Id2 and allow cells to quickly turn on proliferation.

Recently, new functions of Id2 were revealed in immature, post-mitotic neurons. In these cells, Id2 can stimulate axonal growth, by inhibiting bHLH-mediated transcription of axon inhibitory genes such as NOGO receptor, SEMA3F, NOTCH and Jagged 2 (Lasorella A et al., 2006). These findings might open new perspectives for therapeutic use of Id proteins in neurological defects; the idea being that Id2 activity may be beneficial to damaged neurons of patients presenting axonal alterations due, for example, to spinal cord injuries or Parkinson disease.

Moreover, a somehow unexpected connection between axonal growth and cancer progression is beginning to emerge. Numerous similarities exist between molecules implicated in axonal elongation and those that initiate tumour invasion and metastatic progression (Kerjan G et al., 2005). Id proteins could link some fundamental characters of cancer with key aspect of neurobiology (Iavarone A and Lasorella A, 2006). These and other ongoing works confirm Id proteins as exceptionally versatile molecules. Investigating their complex biology is expected to provide

important tools for better understanding how cells choose their own fate.

When cell loose control: Id proteins and cancerogenesis

Cancers arise by an evolutionary process in which somatic cells mutate and escape the restraints normally imposed to their expansion. Tumour cells use various strategies to disrupt pathways leading to terminal differentiation, a process known as *anaplasia*. This feature is associated with the ability to activate signalling mechanisms that sustain proliferation and cellular growth. But when can a cell be considered as "transformed"? In general terms, it is widely accepted that, beyond anaplasia and uncontrolled proliferation rate, one neoplastic cell should possess at least other two features: the first one, known as invasiveness, is the ability to produce and secrete proteases, thus disrupting the external matrix and invade neighboring tissues. Finally, neoplastic cells acquire the ability to promote and sustain growth of newly formed blood vessels. This property, called tumour angiogenesis, is essential for survival of such cells, which continuously need oxygen and nutrients supply for the expanding tumour mass (Hanahan and Weinberg, 2000).

It has long been envisaged that Id proteins could have a key role in cancer processes. A number of evidences (reviewed in Perk J et al., 2005) based on the analysis of tumour cell cultures, human cancer biopsies and animal tumour models, clearly indicate Ids as crucial components of the cell cycle machinery, the deregulated expression of which may confer a proliferative advantage to tumour cells.

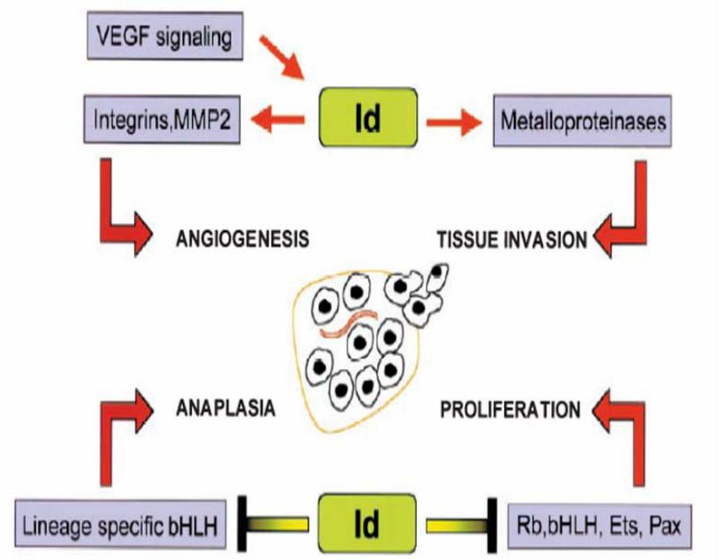


Figure 5. Scheme illustrating how Id signalling may control multiple essential traits of cancer. *Id proteins inactivating bHLH factors inhibit differentiation and promote proliferation. Furthermore, they can induce angiogenesis and tissue invasion.*

While no mutations in Id genes are found in many human tumour types, deregulated expression of Id mRNAs and proteins is found in a large variety of neoplasms, including also carcinomas of prostate, liver, endometrium cervix and thyroid, melanomas, Ewing's sarcomas, as well as in neural tumours and gastric adenocarcinomas (Fong S et al., 2004), as reported in Table 2.

The features of a malignant cell often recapitulate those of normal embryonal cells; it is particularly true for tumours arising in infancy, such as neuroblastomas, rhabdomyosarcomas and medulloblastomas. This suggests that the aberrant expression of Id genes might be recruited as an epigenetic mechanism for malignant transformation of embryonic tissues.

Table 2. Levels of Id mRNA or proteins in human primary tumours

Tumor type	Id	Detection
SCC, head and neck	Id1, Id2, Id3	IHC
SCC, esophageal	Id1	IHC
SCC, oral cavity	Id1	IHC
Melanoma	Id1	ISH
Hepatocellular carcinoma	Id1	IHC, qrt-PCR
Colorectal adenocarcinoma	Id1, Id2, Id3	IHC
Pancreatic cancer	Id1, Id2	Northern, IHC
Medullary thyroid cancer	Id1	IHC
Papillary thyroid cancer		Northern, IHC
Astrocytic tumor	Id1, Id2, Id3	IHC
Neuroblastoma	Id2	IHC, qrt-PCR
Ewing's sarcoma	Id2	Northern, IHC
Ovarian tumor	Id1	IHC
Cervical cancer	Id1	IHC
Endometrial carcinoma	Id1	IHC
Breast cancer	Id1	IHC
		ISH
	Id2	IHC
Prostate cancer	Id1	IHC, ISH
	Id2	IHC
Malignant seminoma	Id1 , Id2, Id3, Id4	IHC

Adapted from: Fong S et al., TRENDS in Molecular Medicine, 2004

As a counterpart, impaired Id activity would generate an unfavourable environment for cancer initiation and progression, possibly by limiting the pool of stem cells susceptible to malignant transformation (Iavarone A and Lasorella A, 2006).

It has been proposed that Id proteins could be defined as "*oncogenes*", mainly for their known ability to induce immortalization when ectopically expressed in several cell types, as primary rodent fibroblasts and primary human keratinocytes (Norton and Atherton, 1998; Alani et al., 1999). However, lack of direct genetic alterations suggests that aberrant Id genes expression is a consequence of the deregulation of upstream oncogenic signals (Perk J et al., 2005). Interestingly, genetic analysis indicates that

RB can restrain ID2 activity: studies based on knockout mouse models revealed that loss of RB function is partially compensated by loss of ID2 (Lasorella A et al., 2000). Such observations suggested the following mechanism. During normal differentiation, RB may prevent ID2 signalling, while the large accumulation of ID2 protein in tumour cells may override the tumour suppressor functions of RB (Lasorella A et al., 2001). Moreover, Id protein upregulation can be mediated by oncoproteins such as RAS, MYC and the chimeric protein Ewing's sarcoma (EWS)-ETS. In particular, it has been shown that Id1 and Id3 are critical downstream components of RAS signalling; RAS activates transcription of the Early Growth Response (EGR) factor which, in turn, induces Id1 and Id3 expression (Bain G et al., 2001). The connection between MYC and Id expression is clear: Id1, Id2 and Id3 possess in their promoter regions several MYC recognition sites (E-boxes). In particular, ID1 induction of by MYC appears to be required for breast cancer cells to enter S phase in culture. Furthermore, during neural crest development in *Xenopus laevis*, Myc maintains neural-crest progenitors in an immature state and Id3 overexpression can rescue the phenotype of c-Myc depleted embryos (Swarbrick A et al., 2005; Light W et al., 2005).

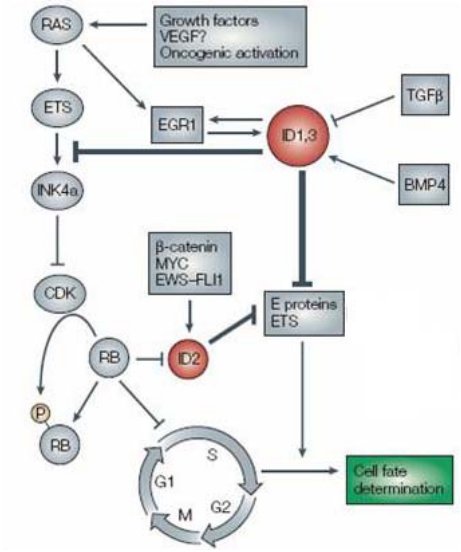


Figure 6. How Id proteins may control proliferation and differentiation of cancer cells. Id proteins functions in cancer cells can be regulated by RAS and MYC oncogenic pathways. ID2 is usually under the control of the tumour suppressor RB. Activation of oncogenic signallings or loss of tumour suppressors pathways may lead to IDs deregulated expression and following alteration in cell proliferation and differentiation programs (*From Perk J et al., 2005*).

However, it is still debated whether ID2 is the major effector of N-Myc in neuroblastomas. Several data suggest that ID2 is a primary transcriptional target of N-Myc, the amplification of which is the hallmark of unfavourable neuroblastomas. While it was reported that the aggressiveness of this type of tumour may depend on activation of N-Myc-ID2 pathway (Lasorella A et al., 2000 and 2002), other findings suggested that ID2 is an independent prognostic variable for neuroblastoma patients (Vandesompele J et al., 2003).

Probably the most exciting activities recently attributed to Id proteins are their roles in tumour angiogenesis and invasiveness. The first clue came from studies on embryonic lethality of Id1/Id3 double knockout mice, which invariably displayed haemorrhagic phenomena in the forebrain before death (Lyden D et al., 1999). Both Id1 and Id3 are expressed within the blood vessels of high grade tumours but not in those of normal adult tissues, suggesting that their expression could be required for tumour vascularization. Id proteins are up-regulated in response to Vascular Endothelial Growth Factor (VEGF) in the bone marrow and appear to be essential for the mobilization and proliferation of endothelial progenitor cells (EPCs), which enter into the bloodstream and migrate to the tumour site (Perk J et al., 2005).

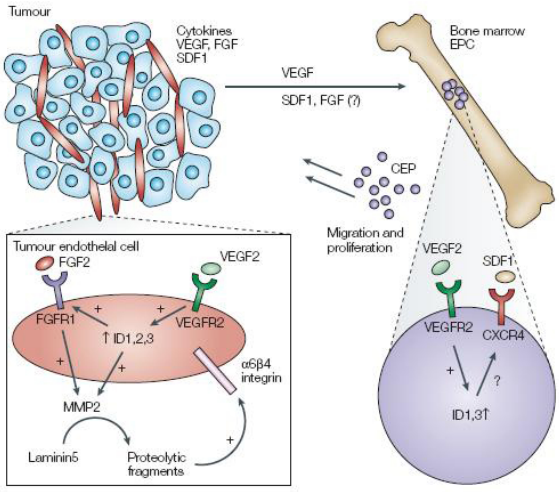


Figure 7. Id proteins functions in angiogenesis. Ids are required in peripheral endothelial cells, where they maintain expression of fibroblast growth factor receptor (FGFR1), matrix metalloprotese 2 (MMP2), laminin5 and α6β4 integrin, as well as in those endothelial ells derived from the bone marrow, where they are up-regulated in response to VEGF (*From Perk J et al., 2005)*.

It was reported that Id2 loss leads to down-regulation of VEGF in pituitary tumours, suggesting the existence of a feedback loop between Ids and VEGF (Lasorella A et al., 2005).

The invasiveness of a tumour reflects the ability of a restricted number of cancer cells to leave the tumour site, migrate and colonize distant organs. Establishment of metastasis is a multi-step process. Firstly, cancer cells enter the bloodstream, migrate to distant sites and exit from blood vessels by passing through the vascular wall into tissue parenchyma. Once in the new tissue, they may have the capacity to reinitiate tumour growth. Several evidences suggest that Id proteins participate in the metastatic process. It was shown that Id1 conferred to mammary epithelial cells the ability to migrate and invade the basement membrane of the surrounding normal cells, probably by inducing expression of matrix metalloproteases (Lin CQ et al., 2000). A recent study demonstrated that Id1 and Id3 are required for tumour initiation in two distinct mouse models of breast cancer with lung metastasis. Starting from the identification of ID1 as a Lung Metastasis Signature (LMS) gene in human breast cancer specimens, this work proved that Id1 and Id3 are necessary both in the context of primary tumour formation and during metastatic colonization of the lung microenvironment (Gupta GP et al., 2007).

AIMS

In this work we planned to interfere with Ids functions either in physiological and pathological conditions.

During normal development, Id proteins work maintaining self-renewal, pluripotency and undifferentiated state of progenitor cells, while their expression is extinguished upon cell cycle exit and differentiation. In the adult organism, the family member Id2 is expressed in certain terminally differentiated cell types of the nervous system, such as Purkinje cells in the cerebellum; however is still unclear why these cells retain its expression once they differentiated. On the other hand, several evidences indicate that Id proteins, when aberrantly expressed, have a role in cancer; at least four essential hallmarks of tumour cells are controlled by Id proteins: anaplasia, proliferation, invasiveness and angiogenesis.

In the first part, to get a better insight into Id2 neural functions in the adult brain, we decided to generate an inducible Id2 knock-out mouse model, by the loxP-Cre recombinase strategy (Sauer B, 1998). In knockout strategies, the endogenous locus in the mouse genome is targeted for lox-P modification by homologous recombination in ES cells, using a selectable marker. Once established the Id2 "floxed" mouse, the possibility to cross this animal with specific transgenic-Cre mice, will allow us to obtain a conditional spatio-temporal deletion of our interest gene in particular areas of the adult brain. This model will be an interesting tool to elucidate the mechanisms which regulate cell specification in adult nervous system.

The second part of this project is instead focused on modelling Id proteins inhibition in human cell lines derived from a tumour of the nervous system, the neuroblastoma, which arise from neural crest precursor cells. To this purpose, we have employed an HLH mutant domain, called 13I, previously isolated in our laboratory starting from the backbone of Heb HLH region (Ciarapica R et al., 2003); harbouring several aminoacidic substitutions in residues

critical for dimeriziation specificity, it was tested for binding affinity with different HLH proteins and choose for its ability to bind Id2 with high affinity.

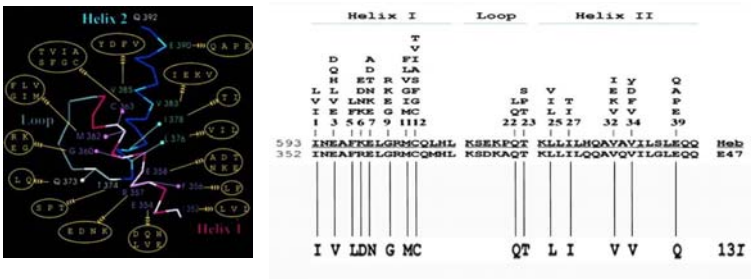


Figure 8. Sequence alignment of the HLH domains of Heb, E47 and the mutant 13I illustrating the residues introduced. *From Ciarapica R et al., JBC, 2003.*

Notably, the validity of this kind of approach was already demonstrated for an other domain, called Omomyc, also established in our laboratory, which acts as a potent inhibitor of c-Myc either *in vitro* and *in vivo* (Soucek L et al., 1998; 2002; 2004; 2008). In order to verify if our domain 13I can act as an effective dominant negative regulator of Ids in a cellular system, we planned to introduce it, by retrovirus infection, in two different neuroblastoma cell lines. Moreover, since neuroblastoma cells are usually used as models to recapitulate neural differentiation, we argued that targeting Id functions by impairing their protein-protein interactions may help us to clarify the role of their network in sustaining and maintaining the neoplastic features of neural tumours and if targeting Id proteins in this type of cancer could have a therapeutic value.

TARGETING ID PROTEINS IN NEURAL DIFFERENTIATION

RESULTS

Id2 protein expression levels in adult mouse brain

All four members of the Id gene family exhibit closely related but distinct expression patterns during mammalian embryonal development. Usually, their expression is high in neural progenitors and declines during differentiation. However, Id2 mRNA was reported to be present in some neuronal populations of adult rodent brain (Kitajima K et al., 2006). We investigated, in collaboration with Georgia Mandolesi at IRCCS S. Lucia in Rome, Id2 protein expression and localization in rodent adult brain.

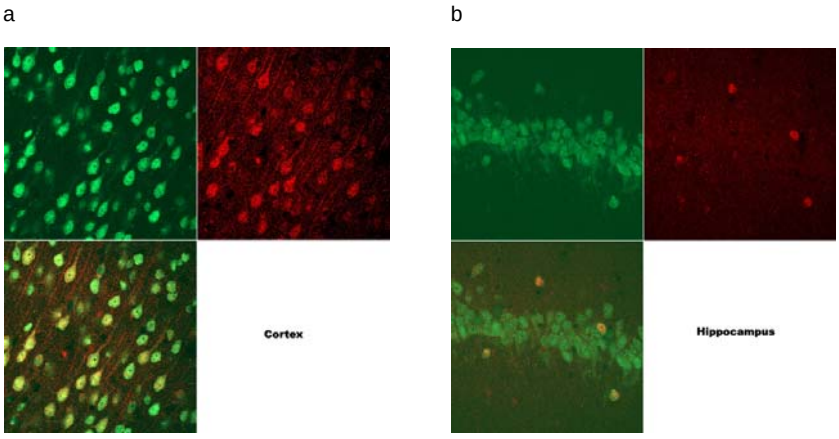


Figure 9. Expression of Id2 protein in rat adult brain. Immunohistochemical staining of sections from cortex, layer 1 (a), and hippocampus (b). Green: NeuN; Red: Id2; Yellow: Merge.

Sections from different brain areas were obtained by adult Wistar rats. Sagittal sections (30μm-tick) cut on a cryostat were mounted on glass slides and labelled with antibodies against Id2 and NeuN, a marker of post-mitotic neurons. As shown in Figure 10, confocal imaging revealed that Id2 is expressed in NeuN-positive neurons of all cortex layers, with exception of layer 4; cells expressing Id2

are dispersed throughout the hippocampus; it seems that these cells are interneurons and/or glial populations. Noteworthy, Id2 showed a peculiar distribution in the cerebellum (Fig.10), in which the only Id2 expressing cells are Purkinje cells, which do not express NeuN. Interestingly, Id2 is expressed either in the cell body and in the axonal processes and its localization appears mainly cytoplasmatic.

To get an insight into Id2 peculiar role of in these highly specialized cells, we set out to construct an inducible Id2 knockout mouse.

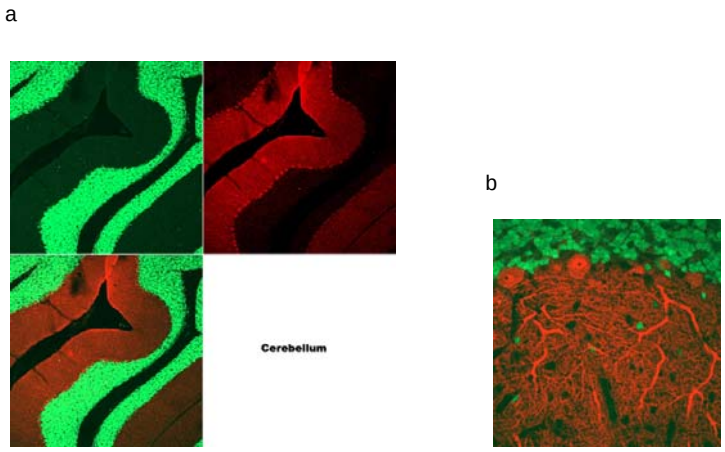


Figure 10. Id2 protein expression in adult brain cerebellum. a) Cerebellar section stained with NeuN (in green) and Id2 (in red) antibodies. Id2 is expressed specifically in Purkinje cells. b) Magnification of the merged stain.

Generation of an inducible Id2 knockout mouse by the Cre/loxP system

Generation of a targeting Id2 construct

This approach uses two different genetically engineered mouse strains to achieve a tissue-specific gene deletion. The first contains

the target endogenous genomic sequence modified, by homologous targeting in embryonic stem (ES) cells, so that the locus encoding for the interest gene is flanked by two lox-P sites, which are specific 34bp nucleotide sequences. Once flanked by two lox-P sites, sequences are said to be "floxed". The next step need the bacteriophage P1 recombinase known as *Cre*: this enzyme functions by recognizing two lox-P sites and mediating site specific recombination among them, leading to the excision of the region between them as a covalently closed circle and to target gene inactivation.

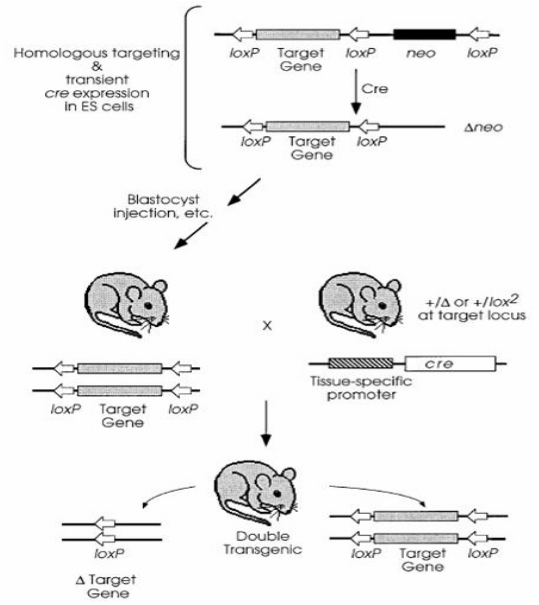


Figure 11. Generation of a conditional knockout mouse by the Cre-loxP strategy. Once obtained the targeted construct in which the interesting genomic sequence is flanked by two lox-P sites, this is electroporated into an embryonic stem cell line. After screening for integration of the construct, positive cells are injected into blastocysts and implanted into a pseudopregnant female. The mouse strain harbouring the floxed target gene can be crossed with different Cre-lines in order to achieve its spatio-temporal conditional deletion.

A fosmid clone harbouring the C57Bl/6 mouse Id2 genomic locus (identified as G135P61920E1 and annotated in Ensembl Mouse, http://www.ensembl.org) was obtained from C.H.O.R.I. BACPAC Resourches, Oakland CA, USA. The experimental plan was designed and carried out in collaboration with Dr. Liliana

Minichiello at EMBL Mouse Biology Unit in Monterotondo. The first step of the targeting strategy has been the construction of a plasmid harbouring the Id2 locus surrounded by two lox-P sites and a neomycin expression cassette flanked bytwo FRT sites.

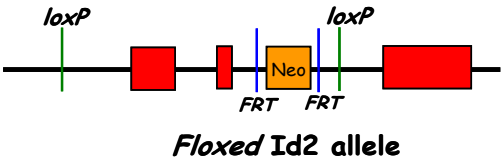


Figure 12. Scheme of the strategy chosen to target the Id2 allele.

The FRT sites are substrate of the FLP recombinase, which promotes the excision of the sequence present between them. Once screened the ES clones and isolated the recombinant ones, the Neo cassette is excised used FLP enzyme. The Neo selection marker is necessary for isolating the effectively targeted ES cells. However its presence might interfere with gene expression of neighboring genes. The presence of the two FRT sites, which are targets of the FLP recombinases, allows its removal after isolating the desired, Id2 targeted ES cells.

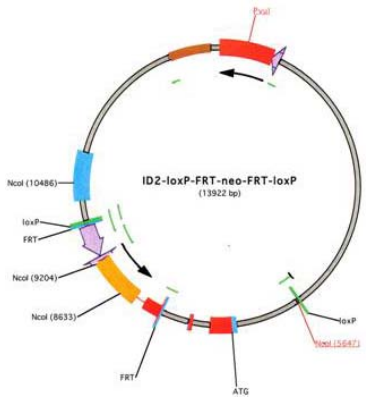


Figure 13. Map of the final targeted construct, about 14Kb, harbouring the floxed Id2 allele.

The construct with the targeted Id2 allele was analyzed by sequencing, in order to verify its integrity. The vector contains an unique *PvuI* recognition site, so that the digestion with this enzyme results in linearization of the construct.

Introduction of Id2 targeted vector into ES cells

The targeted construct was then introduced via electroporation into ES cells derived from the inner cell mass (ICM) of mouse blastocysts (129/Sv strain), which have the capacity to contribute to all embryonic tissue in chimeric mice. The electroporation was performed by application of a high voltage electrical pulse to a suspension of cells and DNA.
Stable transduction efficiency in ES cells using this technique is low, ranging from 10^{-3} to 10^{-4}. To identify cells that integrated the targeted construct in the genome, following electroporation, ES cells were plated onto gelatinized plates and selected for resistance to G418. Selected clones of neomycin-resistant ES cells were picked and plated in 96-well tissue culture plates in duplicated.

Screening of single ES clones for homologous targeting events

A critical step for identifying ES cell colonies harbouring a homologous recombination between the endogenous Id2 locus and the Id2 transgene, is the design of a Southern Blot strategy. The first step is to design a probe hybridizing partially to the genomic region just outside the target region, in order to discriminate between random and homologous integration, and to check for occurrence of deletions during the recombination process. This requires chose a strategy similar to the one used for the generation of the first Id2 knockout model (Yokota Y et al., 1999).

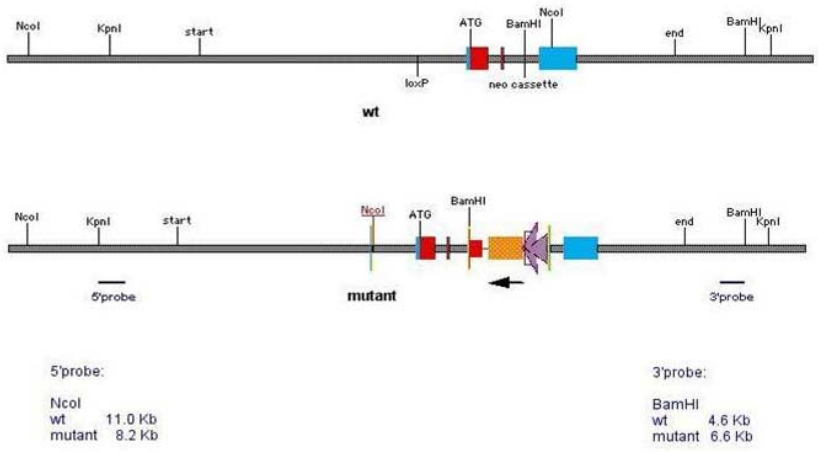


Figure 14. Scheme illustrating the Southern Blot strategy.

Two, a 5' specific and a 3' specific one, probes were obtained by PCR amplification from a DNA preparation of the fosmid clone, and checked by sequencing.
For screening with the 5' probe, genomic DNA from ES neomycin resistant clones was digested with the restriction enzyme NcoI, which is present within targeting vector near the first gene exon (Fig. 14). This means that in case of homologous recombination, the NcoI digestion pattern will change, showing a smaller fragment than the wild type locus. The same will occur for the 3'probe: in this case the genomic DNA has to be digested with BamHI.

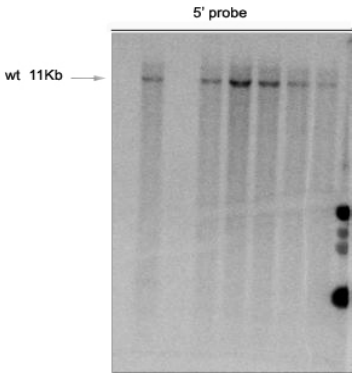


Figure 15. Southern Blot. *NcoI digestion of genomic DNA from ES clones hybridized with 5' radiolabelled probe. The fragment of 11Kb size represents the wild type allele.*

Figure 15 shows a representative Southern Blot experiment performed with a radiolabelled 5' probe on NcoI digested genomic DNA from neomycin resistant ES clones. Unfortunately, hybridization of about 200 ES clones tested, no one showed the occurrence of a homologous recombination event. Further studies will have to be performed.

TARGETING ID PROTEINS IN NEURAL CANCER

RESULTS

13I HLH domain is a dominant interfering inhibitor of Id proteins

The precise function of Id proteins in tumour cell biology is still debated. This issue is currently addressed by targeting Ids in mouse tumour models. The data suggest that hampering Id function has an impact on growth rate and invasiveness of tumour cells. However, the effect of Id inhibition in human cancer cell lines remains to be fully assessed. An Id interfering molecule, 13I, was isolated (Ciarapica R et al., 2003) from a library of mutant domains for its ability to strongly bind human ID2 *in vitro*. Notably, its binding affinity is higher for Id2 than for other bHLH proteins. This finding suggested us that 13I could act as an interfering dominant negative of Id proteins and we decided to investigate its effects in human cancer cell lines.

We initially tested whether the 13I domain is able to form complexes with ID2 in a cellular context, a necessary requirement for its use as an inhibitory molecule. To answer this question we performed an immunoprecipitation assay. To this aim, the 13I domain was cloned into the cβsFLAG expression vector, thus obtaining a FLAG-13I chimeric protein. HEK 293T cells were then transfected with a plasmid encoding ID2 and increasing amounts of the FLAG13I fusion vector. After lysis, cell extracts were precipitated with FLAG antibodies, and, shown in figure 16a, Western Blot analysis of precipitated proteins revealed that 13I binds ID2 in a dose-dependent manner.

These finding, suggesting that the 13I may act as a dominant negative for IDs, led us to test the ability of the 13I domain to interfere with ID2 protein biochemical properties. ID2 is particularly interesting in this regard due to its unique capability to bind the active, hypo-phosphorylated, form of the retinoblastoma tumour suppressor protein RB. During normal differentiation, RB inhibits ID2 activity. A large accumulation of ID2 proteins, instead

sequesters RB, impairing its control on cell cycle progression (Lasorella A et al., 2000).

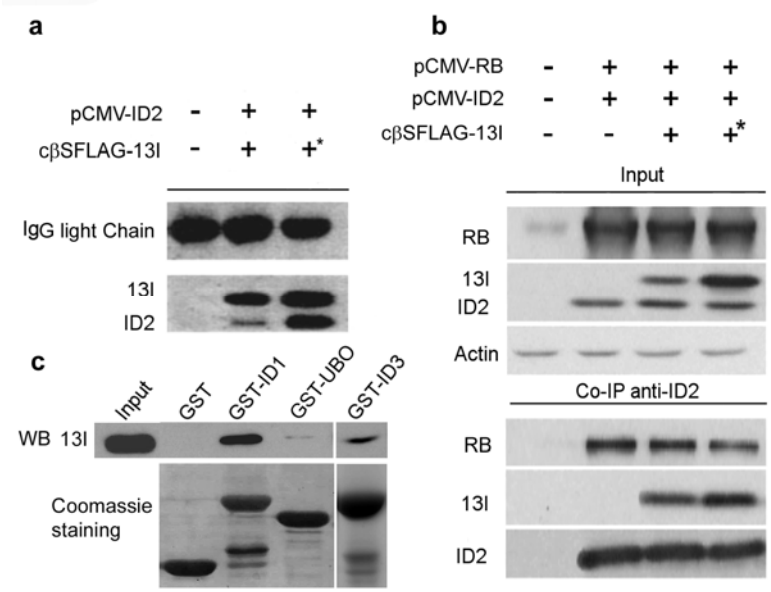


Figure 16. 13I domain interaction with ID proteins. a) *13I binds to ID2 intracellularly. 293T were transfected with pCMV-ID2 and, cell lysates were precipitated with FLAG antibodies and analysed by Western blotting; *Indicates that ID2 and FLAG-13I plasmids were employed in 1:3 relative amounts.* **b)** *ID2:RB complex formation is hampered by 13I domain. pCMV-ID2, pCMV-RB and cβS-FLAG13I transfected 293T cell extracts were immunoprecipitated with ID2 antibodies, and analysed by WB *Indicates that ID2, RB, and 13I expression plasmids were used in 1:1:3 relative amounts. (c) 13I selectively with Ids in vitro. A cβS-FLAG13I transfected 293T cell extract was utilised for pull down assays with GST, GST-ID1, GST-ID3, and GST-HEB dimerization domain (Coomassie staining). The amount of 13I bound to GST control and fusion proteins was detected by Western blotting with FLAG antibodies (WB).*

Interfering with ID2/RB complex formation may thus have a potential therapeutic interest, by freeing functionally active RB and restoring cell proliferation control in those pathological conditions characterised by a deregulated ID2 expression. To test the possibility that 13I may negatively interfere with protein-protein interactions mediated by the ID2 HLH domain, we analysed its ability to inhibit the ID2/RB association carrying out co-immunoprecipitation experiments (Fig. 16b). 293T cells were co-transfected with ID2 and RB and then with ID2, RB and increasing amounts of 13I domain. As shown in figure 16b, the ID2 antibody co-precipitates RB, as expected. RB co-precipitated by ID2 decreases in parallel with the 13I intracellular amount increase. This assay clearly demonstrates that 13I domain over-expression may be capable of inhibiting crucial ID2 functions.

ID family members are strictly related and share highly homologous HLH regions. This prompted us to investigate whether 13I binds to the other IDs as well. Experiments reported in Fig. 13c prove the interaction of 13I with ID1 and ID3. An other critical aspect of 13I biological mechanism of action needed to be assessed. Ids primary targets are the bHLH transcription factors, manly the E proteins, such as HEB and E47. To function as an ID specific inhibitor, 13I should not bind the E proteins as well. We investigated this issue by performing pull-down assays with GST-linked proteins, on extracts of 293T cells transfected with the FLAG-13I vector (Fig. 16c). We observed that 13I interacts with both ID1 and ID3, as expected, although with a stronger affinity for ID1. On the other hand, 13I binding to the HEB bHLH domain was barely detectable, indicating its ability to discriminate between ID and E proteins.

The next step was to ascertain its ability to affect ID function on gene expression which involves repression of E protein mediated transcription. potential as Id inhibition. This was addressed by transfecting 293T cells with a multimerized E-box luciferase reporter and different combinations of E47, ID2 and FLAG-13I expression vectors. Result are shown in figure 17: 13I effectively

relieves ID2 inhibition of E47 mediated transcription of the E-box luciferase reporter in a dose-dependent manner.

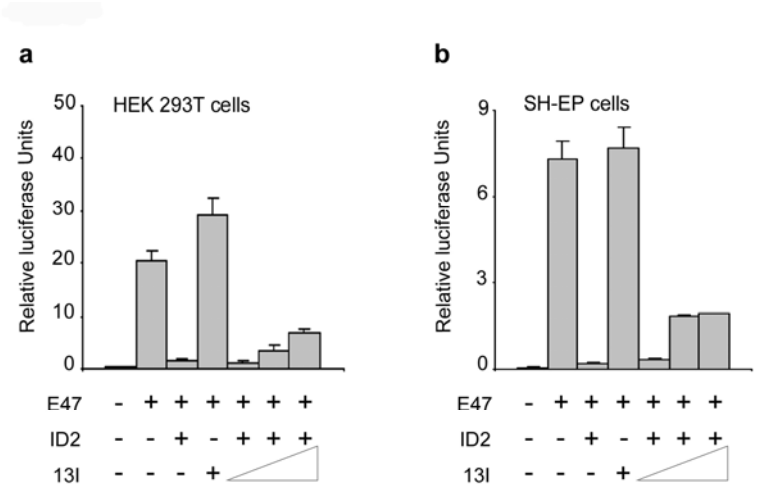


Figure 17. 13I relieves ID2-mediated repression of E protein dependent transcription. *HEK 293T (a) and SH-EP (b) cells were co-transfected with the 4RE-luciferase plasmid, with E47 and ID2 vectors, and increasing amounts (up to threefold) of 13I expression plasmid. Luciferase activities are displayed as means of triplicate assays, normalized for transfection efficiency by co-transfection of pGL3-Renilla.*

Notably, 13I does not impairs E47 activity *per se*. The slight increase in E47 dependent transcription observed in presence of 13I domain can be easily explained by postulating an inhibition of endogenous Ids. We obtained similar results in reporter assays performed on the neuroblastoma cell line SH-EP (Fi. 17b).

13I promotes neuroblastoma cell neuronal differentiation

ID proteins may contribute to the neoplastic cell phenotype by restraining differentiation, and promoting proliferation and invasiveness (Perk J et al., 2005). As the 13I domain is able to directly interact with ID proteins, it might be employed as a dominant negative, for interfering with ID function in cancer cells. Neuroblastoma (NB) cells were chosen as model for investigating this point. These cells are frequently used as *in vitro* models to recapitulate neural differentiation (Nakagawara A and Ohira M, 2004).

NB originate from neural crest precursors. Two cell types, neuroblastic (N) and substrate adherent (S), are found in human neuroblastoma cell lines (Ciccarone *et al.*, 1989). N type cells resemble proliferating neuroblasts, with small, loosely adherent cell bodies and several neurite like processes, and produce many neuron specific proteins. These features are enhanced upon RA treatment (Voigt *et al.*, 2000). They frequently over-express N-MYC. S type cells do not over-express N-MYC and may represent an embryonic, multipotent neural crest precursor (Ciccarone *et al.*, 1989). They do not display neuronal features, but have properties of melanocyte and glial neural crest cell lineages. They are epithelial in appearance, large, flattened and strongly adherent, with no neuritic processes, and do not differentiate in response to Retinoic Acid (RA) (Wainwright *et al.*, 2001). To test the impact of 13I expression, we examined two widely studied cell lines, LA-N-5 and SH-EP, which are N and S type, respectively (Wainwright *et al.*, 2001). N-MYC is amplified and over-expressed in LA-N-5 (Cohen *et al.*, 1988), while it is barely detectable in SH-EP cells (Vandesompele *et al.*, 2003).

A pBabePuro (pBP) vector expressing the 13I domain was introduced into SH-EP and LA-N-5 cells by retroviral infection. Cell populations stably infected with 13I and control retroviruses were isolated by puromycin selection.

Right panels in figure 18 show expression of 13I and ID1-ID3 in control and 13I infected cells, analysed by Western blotting. We observed that SH-EP and LA-N-5 controls produced all three IDs, although ID3 is barely detectable. However, 13I transformed cells produced higher amounts of ID1 and ID2 than the respective controls, implying that 13I may cause an ID up-regulation. This may either reflect changes in ID protein stability or suggest the existence of a negative feedback of IDs on their own production, as it was reported to happen for other HLH proteins, such as HES1 and MYC (Hirata et al., 2002; Penn et al., 1990).

This result suggested to us that 13I, by affecting Id functions, would hamper such feedback control, thus leading to higher ID1 and ID2 production in neuroblastoma cells. Anyhow, although the Western Blot revealed that control and 13I expressing LA-N-5 cells displayed two to threefold higher amounts than their SH-EP counterparts, the amounts of 13I domain produced were still in excess to the endogenous ID1 and ID2 in both cell lines.

Since we demonstrated that 13I domain is able to inhibit ID functions in a dose-dependent manner, we argued that its amount in the stably infected cell lines might be sufficient for interfering with ID activity. Cells stably producing 13I were therefore examined for a series of differentiation related characters, such as morphology and neuronal marker expression.

Morphology

About 20 days after infection with the pBP13I retrovirus, morphological changes became evident in both SH-EP and LA-N-5 cells. However, we found that 13I had quite a different impact on the two cell types.

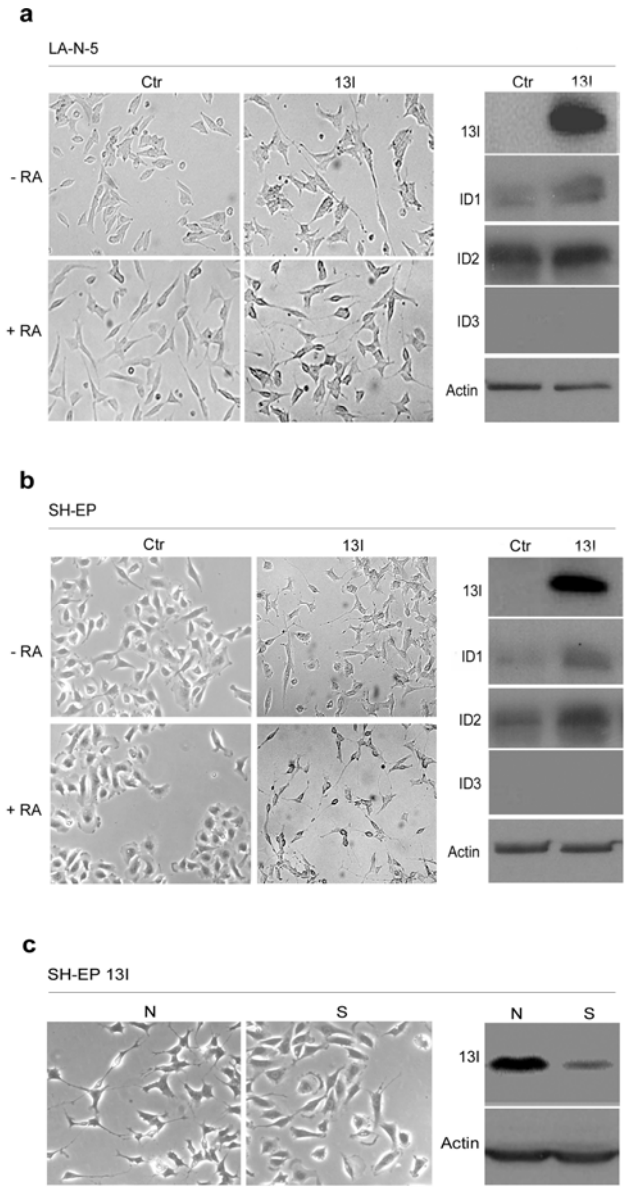


Figure 18. 13I promotes neuronal like morphological changes. LA-N-5 (a) and SH-EP (b) cells infected with empty (Ctr) and 13I expressing (13I) pBP retroviruses. Left panels show cell shape. (c) Two cell types, resembling S and N type neuroblastoma cells, are present in the SH-EP 13I cell population. Right panels display 13I and Id expression, detected by Western blotting.

LA-N-5 13I cells displayed more marked neuritic processes than control cells (Figure 18a, left panel). A four days RA treatment enhanced the neuronal features of LA-N-5 13I cells more significantly than control cells, as shown by their lower nucleus/cytoplasm ratio and increased neuritic extensions. While the neuronal characters of LA-N-5 cells were moderately reinforced by 13I, SH-EP 13I cells were strikingly different from control SHEP cells (Figure 18b, left panel). Remarkably, over 50% of SH-EP cells producing the 13I domain had lost the typical morphology of S-type cells, and acquired a tipical neuronal-like aspect, characterized by a small cell body, large nucleus/cytoplasm ratio, decreased adherence and multiple neurite-like processes. However, the most exciting feature acquired by 13I expressing SH-EP cells is that they became responsive to RA treatment: the neuronal characters were much more evident upon RA treatment, with round cell bodies and extended neuritic processes. SH-EP controls, instead, did not present a neuronal morphology and were unaffected by RA.

Nonetheless, a portion of SH-EP 13I cells still presented the typical flat shape and substrate-adherence of S type cells (Figure 18c, left panel), and we observed that their percentage increased upon serial passages (not shown). This suggested that the flat cells had a growth advantage. To evaluate whether this correlated to different 13I amounts, substrate adherent (S) were sorted from neuronal like (N) cells according to greater adhesion to plastic and larger size, and 13I expression analysed by Western blotting (Figure 18c, right panel). As we expected, the flat cells produced much less 13I.

Differentiation markers

Such a correlation between lower 13I amount, absence of neuronal characters, and better growth in culture strongly suggested to us that 13I may jointly promote differentiation and

restrain cell growth. To support this view, we analysed by immunofluorescence (Figure 19) the expression of two neuron specific proteins, neural cell adhesion molecule (N-CAM) and neurofilament 160 kD (NF-160), which are known to be absent in S type, and present in N type cell lines (Ciccarone *et al.*, 1989; Voigt *et al.*, 2000). The results we obtained clearly demonstrate that 13I is able to induce the expression of neuronal differentiation markers in both stably infected neuroblastoma cell lines. As expected, NF-160 and N-CAM signals were visible in LA-N-5 and undetectable in SH-EP control cells (Figure 19a and b, respectively). In LA-N-5 cells, the presence of 13I was associated to an increase in NF-160 expression, while N-CAM was unaffected (Figure 19a). RA treatment determined a significant enhancement of the NF-160 signal in both control and 13I expressing LA-N-5 cells, while the N-CAM signal was only strengthened in the 13I expressing cells, although modestly (Figure 19a). The presence of 13I in SH-EP cells was associated to significant NF-160 and N-CAM signals, undetectable in control cells (Figure 19b).

Moreover, 10 μM RA treatment caused a strong increase in NF-160 and N-CAM signals, as compared to their levels in absence of RA, in 13I expressing cells, while it was ineffective in control cells (Figure 19b).

13I domain affects neuroblastoma cell proliferation

Since SH-EP cells appeared to be more significantly affected by the 13I domain, we focused on this cell line to further evaluate its action on cell proliferation. It is well known that neuroblastoma cell differentiation is accompanied by changes in the cell cycle machinery leading to proliferative arrest (Wainwright *et al.*, 2001).

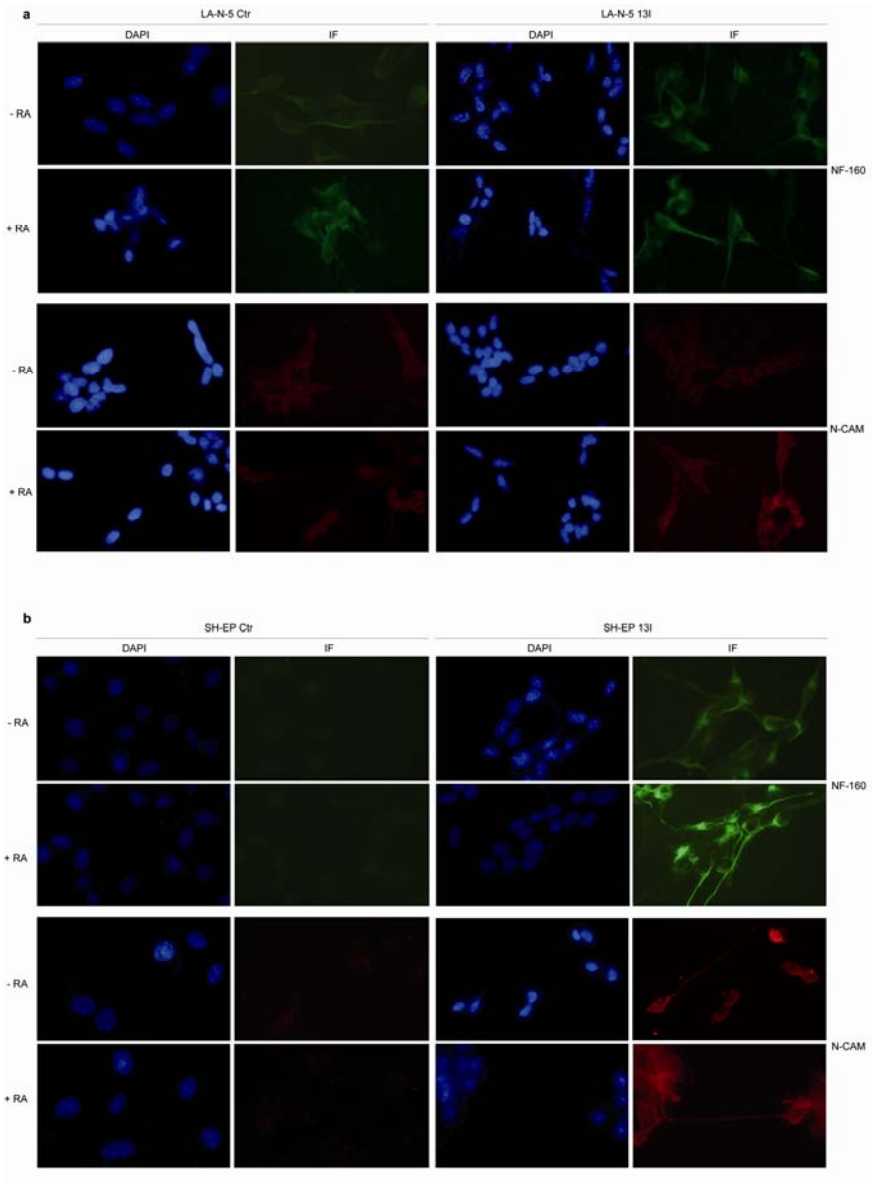


Figure 19. 13I stimulates neuron specific protein expression. *Control and 13I expressing LA-N-5 (a) and SH-EP (b) cells were stained with antibodies directed against the two neuronal markers NF-160, in green, and N-CAM, in red, in presence or absence of RA. In blue is shown the DAPI staining of the same cells displayed in the immunofluorescence (IF) pictures.*

Therefore, we expected that induction of SH-EP cell differentiation by 13I might be associated to a slower growth. To evaluate this aspect, control and 13I expressing cells were plated in equal amounts, grown in presence or absence of RA and counted for up to four days (Figure 20).

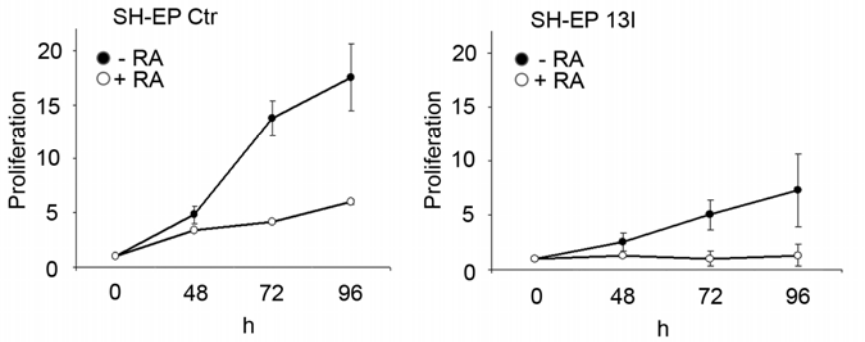


Figure 20. 13I impairs SH-EP cell proliferation. *Proliferation was assayed by cell count in control (left) and 13I producing (right) SH-EP cells, grown for up to four days in presence (○) and absence (●) of RA. Data are normalized to the cell number at time zero.*

SH-EP 13I cells grew more slowly than control cells in both conditions. After four days, the 13I cell number was reduced to 42% of control cells, in the absence of retinoic acid. RA treatment affected their proliferation even more significantly: while in its presence, proliferation of control cells was slowed down, SH-EP 13I cells were totally unable to proliferate.

To determine whether the impaired proliferation in SH-EP 13I could be ascribed to alterations in cell cycle regulators, we analysed the expression of cyclin D1, the cyclin dependent kinase (CDK) inhibitors $p21^{Cip1}$ and $p27^{Kip1}$, and the RB tumour suppressor by Western blotting. It is known that $p21^{Cip1}$, $p27^{Kip1}$ and cyclin D1 are modulated by Ids, and are targets of bHLH mediated control of

cell cycle and differentiation (Iavarone A and Lasorella A, 2006). Their expression was reported to change during neuroblastoma differentiation, and to vary among flat and neuronal like cell lines (De los Santos *et al.*, 2007; Wainwright *et al.*, 2001).

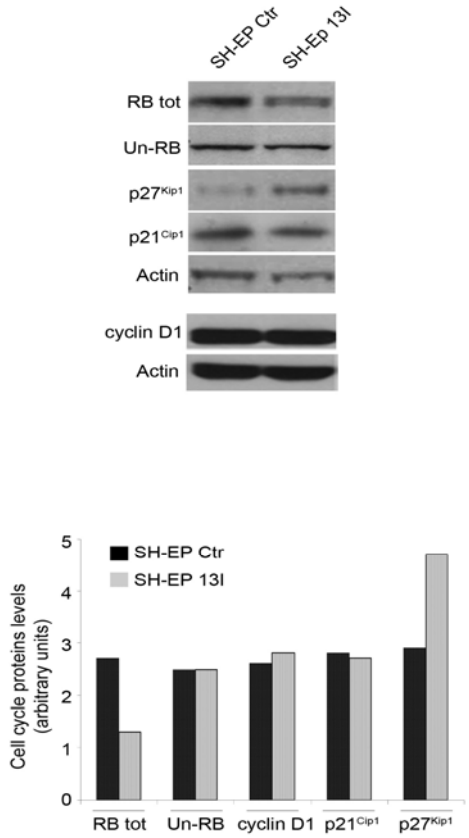


Figure 21. Figure 6. 13I affects the expression of cell cycle related proteins. *(a) Underphosphorylated RB and p27Kip1 are augmented in 13I expressing cells. Top: Western blot. Bottom: densitometric analysis of the same gel, normalised to the the Actin signal intensities.*

The Western blotting results are displayed in the upper panel in Fig. 21, while the lower panel shows the densitometric analysis. It can be observed that Cyclin D1 and $p21^{Cip1}$ are present in equal

amounts in 13I and control cells, and their levels are unaffected by RA treatment (not shown).
The CDKI $p27^{Kip1}$, on the contrary, was found in significant amounts in SH-EP 13I cells, while it was almost undetectable in control cells. We also measured $p27^{Kip1}$ mRNA levels by Real Time PCR experiments and found that it was increased in the 13I transformed cells (Fig. 22). This suggested to us that $p27^{Kip1}$ might be more relevant than $p21^{Cip1}$ and Ciclin D1 for explaining the properties of SH-EP 13I cells.

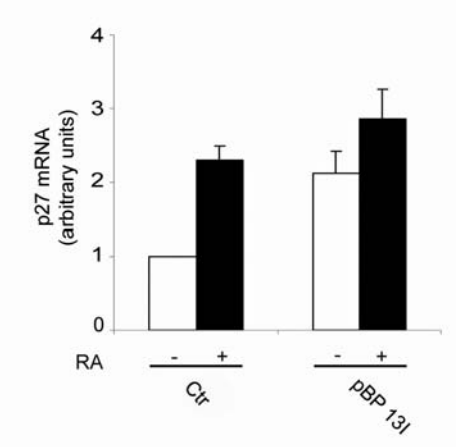


Figure 22. $p27^{Kip1}$ mRNA levels. *Real Time assays revealed that $p27^{Kip1}$ mRNA is increased in SH-EP 13I expressing cells respect to control, even in presence of RA treatment.*

Figure 21 (upper panel) also illustrates that the fraction of under-phosphorylated RB in SH-EP 13I cells is increased with respect to the global RB amount. This means that the balance between different, phosphorylated, RB species is shifted towards the ones that inhibit cell cycle progression and promote differentiation, in accordance with the reduced proliferation and enhanced differentiation of SH-EP 13I cells. These findings suggested that the accumulation of under-phosphorylated RB species in SH-EP 13I cells might be, at least in part, due to the

direct inhibition of Cyclin:CDK complexes mediated by the increased $p27^{Kip1}$ amount.

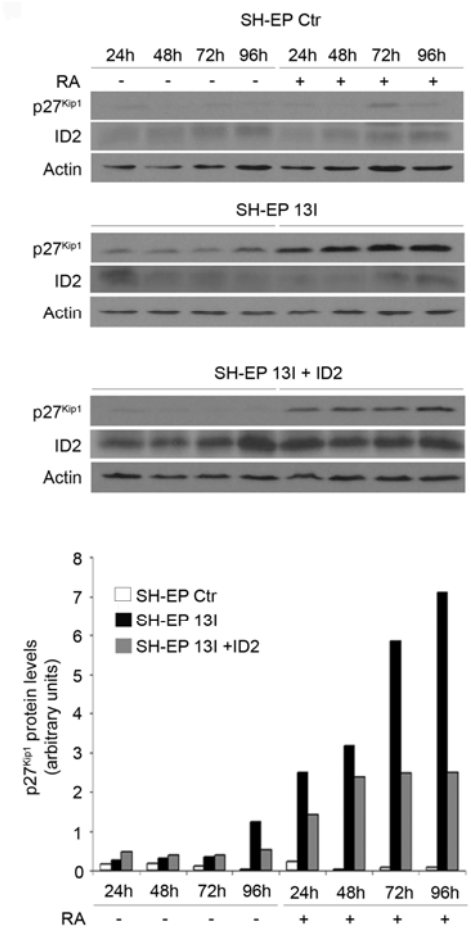


Figure 23. RA treatment upregulates the cell cycle inhibitor p27Kip1 in 13I expressing cells. *The upper panel shows Western blotting images of $p27^{Kip1}$ expression and the lower panel the intensity analysis, normalised to the the Actin signal intensities. $p27^{Kip1}$ amounts are higher in 13I producing cells and are strongly enhanced upon RA treatment, unlike control cells. Transfection of the ID2 expression vector (SH-EP 13I + ID2 cells) partly reverses p27Kip1 upregulation.*

Considering the crucial role that $p27^{Kip1}$ appears to have in SH-EP 13Icell properties, we decided to better investigate the relationship between $p27^{Kip1}$ expression and RA treatment in a time course experiment, the results of which are displayed in Fig. 23.

As expected, while $p27^{Kip1}$ protein level in control cells was not significantly affected by RA, this agent caused a progressive increase in 13I cells, amounting to about 3 fold after a 4 day treatment.

To verify such effects were due to 13I ability of impairing ID functions, we transfected SH-EP 13I cells with an ID2expression vector (Fig.23). We observed that ID2 expression entailed a marked reduction of $p27^{Kip1}$ (Figure 5b), suggesting that its up-regulation by 13I was a consequence of Id inhibition, at least in part.

13I domain down-regulates VEGF

Beyond the role of positive regulators of cancer cell proliferation, Id proteins are considered important players of tumour angiogenesis, since their expression promotes activation of pro-angiogenic genes in tumour endothelial cells (Ruzinova M and Benezra R, 2003). In neuroblastoma cells in particular, ID2 is able to up-regulate VEGF at mRNA and protein level as well (Lasorella A et al., 2005).

Since the data reported so far indicate that 13I is an effective ID inhibitor, we asked whether 13I influenced VEGF expression as well. To answer to this question, the amount of VEGF mRNA in 13I expressing and control SH-EP cells was measured by real time PCR (Figure 24). We observed that the VEGF mRNA amount in SH-EP 13I cells was only 20% of control cells.

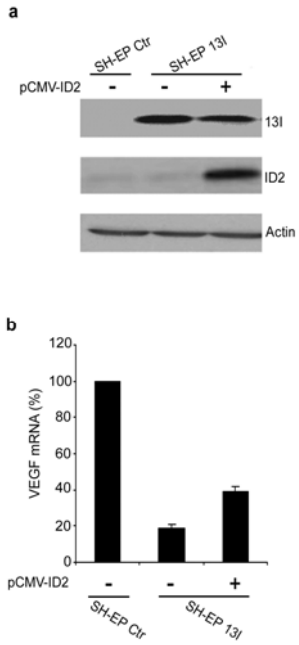


Figure 24. 13I inhibits VEGF mRNA expression in SH-EP cells. *(a) 13I and ID2 expression, detected by Western blotting in control and in 13I expressing cells, and in SH-EP 13I cells transfected with the ID2 expression vector pCMV-ID2. (b) VEGF expression, measured by real time RT-PCR. The VEGF inhibition by 13I is partially reversed upon ID2 over-expression.*

Notably, transfection of the ID2 expression vector in SH-EP 13I cells caused a twofold increase of VEGF mRNA after 48h, indicating that the decreased VEGF expression in 13I cells was caused, in large part, by the ability of the domain to impair ID2 functions.

Taken together, these results suggest that 13I domain may represent a tool to globally interfering with Id functions in neuroblastoma cells.

DISCUSSION

ID proteins: the crossroad of neural development and cancer

Id proteins (Id1-Id4) fascinate a number of researchers, mainly for their versatility.

They play crucial roles in development, when their function is required to preserve staminal characters and undifferentiated state of progenitors cells. However, by promoting and sustaining several key features of cancer cells, Ids also contribute to multiple steps of the tumourigenic process. An appealing point of view suggests that, through oncogenic signals leading to their aberrant activation in tumours, Id proteins are able to transfer the phenotypic traits of embryonic stem cells to cancer cells (Iavarone and Lasorella, 2006).

The aim of my PhD thesis has been to answer two major questions, the first one being: given the well established role of Ids as proliferation promoters and inhibitors of differentiation, why is Id2 expressed in terminally differentiated cells of the adult brain?

The second question concerns the possibility to consider Id proteins as targets for cancer therapy. Is it feasible to inhibit Id functions in human cancer cell lines by targeting their HLH dimerization domain and may such a strategy have an interest for cancer therapy?

An inducible Id2 knockout mouse model: if not there were Id2…

To address the first question on Id2 functions in adult brain, we decided to generate, by the Cre/loxP system, a conditional knockout mouse in which Id2 is deleted only in particular cells of the adult nervous system. Id2 role in adult brain was not addressed by studies on Id2 null mice, which showed retarded growth and neonatal lethality. The establishment of novel molecular

techniques for mouse genome manipulation allows to ablate a single gene, in a spatio-temporally controlled way.

The first step is to establish a "floxed" Id2 mouse, in which the Id2 genomic region is modified so that it is flanked by two loxP sites, according to standard technologies. A powerful feature of this approach is that the same loxP tagged animal can be used for gene ablation independently in a large number of different tissues or at different developmental times, by simply mating it with a corresponding *Cre* transgenic that displays the desired tissue or temporal specificity of expression.

To date, several transgenic lines are available, expressing *Cre* recombinase under the control of specific promoters in the nervous system (Table 3).

Table 3. Transgenic Lines Expressing the Cre Recombinase in the CNS

Gene	Expression Profile
Emx1-Cre	Cerebral cortex and hippocampus
D6-Cre	Neocortex and the hippocampus
Foxg1-Cre	Telencephalon, discrete head structures
Krox-20-Cre	Developing hindbrain and PNS
NSE-Cre	Wide recombination in brain
NSE-Cre	Pan-neuronal
mNF-H-Cre	Neurons of the CNS
Synapsin-Cre	Most neurons
KA1-Cre	Most neuronal cells of CNS
KA1-Cre	CA3 hippocampus
nestin-Cre	Neuronal and glial cells
GnRH-iCre	GnRH neurons
CamKIIα-Cre	Several lines with various patterns in neurons of the forebrain
CamKIIα-iCre	Forebrain
L7/pcp-2	Cerebellar Purkinje cells
GFAP-Cre	Astrocytes, some neural precursors
GFAP-Cre	Astrocytes, most neurons
GABA(A) Rα6-Cre	Granule cells of the cerebellum
D1-Cre	D1-expressing neurons
CamKIIα-CrePR	Neurons of the forebrain
ThyI-CrePR	Neurons of the CNS
GluRε3-CrePR	Granule cells of the cerebellum
GluRδ2-CrePR	Cerebellar Purkinje cells
PrP-CreERT	Various patterns in the CNS

Adapted from: Morozov A et al., Biol Psychiatry , 2003

In the nervous system, conditional genetic modifications are used to determine how individual molecules contribute to the function of defined neuronal circuits. In addition to solving the problem of embryonic or perinatal lethality, conditional gene inactivation provides opportunities for selective manipulation of specific neuronal circuits. We firstly verified the Id2 protein expression profile in rodent adult brain. The pattern we observed is suggestive of an important neural function in specific cell types, particularly in Purkinje cells.

Experiments were conducted in collaboration with Dr. Liliana Minichiello at EMBL in Monterotondo.

Starting from a fosmid clone of the Id2 genomic region, we have generated an optimized targeting vector for insertion into ES cells, and devised a Southern strategy. Following ES cell transformation, we are now screening cell clones in order to isolate the recombinant ones by Southern blot.

We are confident that this model will be a great tool to dissect and better understand Id2 functions in neuronal differentiation, and to elucidate its complex relationship with cancer. In this regard, recent evidences highlighted the numerous similarities existing between molecules responsible for axonal elongation, such as Semaphorins, and those involved in cancer metastatic progression. In immature post-mitotic neurons it was reported for Id2 a role as promoter of axonal growth (Lasorella A et al., 2006). In this view, a conditional Id2 knockout would also be used to assess eventual links between Id2 and the axon guidance molecules involved in cancer development.

Targeting ID HLH domain: looking for the Achille's heel of the tumour…

Undoubtedly, one of the major challenges of biomedical research is the treatment of cancer. Despite the progresses obtained in this field since 1940, when the chemotherapy era started with the use of nitrogen mustards and antifolate compounds as drugs, the scientific community is still looking forward to an effective cancer therapy. The growing understanding of the molecular pathogenesis of human malignancies started what is now known as the *molecular targeted-therapy revolution*. The term refers to a new generation of drugs designed to interfere with a specific molecular target, which is believed to have a critical role in tumour initiation or progression (Sawyers C, 2004). The advance of new technologies revealed the molecular bases underlying tumourigenic processes, mainly uncovering signalling networks that are dramatic altered in cancer cells. Moreover, identification of molecular signatures for many human tumours strongly suggests new attractive targets for drug design, and for more selective and personalised therapies.

Targeting transcription factors is a potentially powerful approach for cancer therapy. However, development of effective strategies is limited by the difficulty to inhibit them by conventional means, mainly due to their nuclear localization,. Transcription factors, with the possible exception of cytoplasmic steroid hormone receptors (SHRs), are widely considered *undruggable*. Nevertheless, somewhat is changing and a lot of efforts are spent in different approaches, such as antisense oligonucleotides, siRNAs, anti-gene, ribozyme gene transfer or small peptides.

Because of their peculiar biological functions, Id proteins are considered as excellent targets for anti-cancer drug design (Benezra R, 2001; Fong S et al., 2004; Iavarone A and Lasorella A, 2006). Multiple essential traits of cancer, such as anaplasia, sustained proliferation and invasiveness could be linked to

deregulated Ids expression. They are abundantly expressed in a variety of tumours, while they are almost undetectable in normal adult tissues, raising the hope that an anti-Id drug would be selective for cancerous cells, thus displaying a low toxicity. Finally, inhibiting Id proteins may allow to inhibition of cancerous mass and its vasculature simultaneously.

Recent studies demonstrated that two key hallmarks of tumour aggressiveness, invasion and metastasis, are impaired through forced reduction of Id mRNA levels in cancer cells (Tsuchiya T et al., 2005). Moreover, targeting Id1 with an antisense strategy in endothelial cells of two different tumour models led to enhanced haemorrhage, hypoxia and inhibition of primary tumour growth and metastasis (Henke E et al., 2008). These results encouraged us to accept the challenge and try a way to inhibit Id proteins in cancer cells.

To target Id functions in human neuroblastoma cell lines, we chose a different strategy. We decided to interfere with Id dimerization by means of 13I, a mutant HLH domain that was previously engineered in our laboratory (Ciarapica R et al., 2003). Id proteins carry out their roles in developmental and tumourigenic processes by forming complexes with a number of transcriptional regulators, and inhibiting them. Since formation of all these complexes is mediated by the Id dimerization domain, we argued that inhibiting protein-protein interactions involving the HLH region may effectively inhibit Id function.

Although employing an inhibitor affecting protein interactions is far less popular than down-regulating mRNAs by RNA interference, it may have some advantages. Disease relevant biological effects depend on complexes to which many proteins participate. Often, a single proteins is involved in a variety of protein-protein interactions, mediated by distinct functional domains, which can have quite different biological consequences. Therefore, it might be therapeutically more useful to inhibit only selected ones. This can best be obtained by molecules acting at the protein level. RNA interference is unable of discriminating among

functional domains of proteins, and may not have the required finesse in therapeutic use. Another reason is related to the possibility that a cancer cell may circumvent the inhibition of a single gene of a family, such as the Myc (c-, N, L-MYC) or the Id (ID1-4) family, by activating another family member. A reagent targeting protein-protein interactions has a good chance of inhibiting all family members, since their protein-protein interaction modules usually are almost identical. Relevant and encouraging results were obtained by targeting MYC with Myc dimerization mutant Omomyc (Soucek et al., 2004 and 2008), and RAS with a single antibody domain (Tanaka et al., 2007).

Making use of the 13I domain as an Id inhibitor, we demonstrated the feasibility of our approach and verified the potential therapeutic value of targeting Id proteins in neural cancer cells.

Firstly, we verified that 13I is a dominant negative inhibitor able to bind all Ids expressed in neuroblastoma cell lines. This indicates that its employment could prevent a cell from compensating the effects of inhibiting just one Id protein through the activities of the others. Furthermore, we verified that 13I binding is selective for Ids versus E proteins and, as expected, its expression counteracts ID2 inhibition of E47-dependent gene transcription. Finally, we demonstrated with a competition assay that 13I can interfere with ID2:RB complex formation in a dose dependent manner.

Taken together, these results prompted us to assess the impact of 13I expression in neuroblastoma cell lines, a tumour studied for the involvement of deregulated Id proteins in maintaining of a transformed phenotype (Iavarone A and Lasorella A, 2004).

Following retroviral-mediated infection of two different neuroblastoma cell lines, 13I domain was able to trigger neuronal commitment and differentiation of the S type SH-EP cells, while only modestly affecting the N type cells, LA-N-5. 13I stably expressing SH-EP cells acquired a markedly neuronal-like morphology, displaying neuritic extension and expression of

neuron specific markers. Moreover, while parental SH-EP cells do not differentiate in response to RA, these cells stopped proliferating and enhanced their neuronal features upon RA treatment in presence of 13I domain. It is interesting that the two cell types, S and N, usually co-exist within neuroblastoma tumours. S-type cells appear to be more resistant to chemotherapeutic drugs and are unable to differentiate in response to RA, used as treatment for neuroblastoma patients (Reynolds et al., 2003). Human malignancies are a very diverse group of diseases, even within histological classifications, and quickly display their diversity when exposed to chemotherapy. 13I additive effect on RA administration may have a great potential, since targeted therapies combined with conventional cytotoxic chemotherapeutics are now considered more effective than single agent approaches (Chabner BA and Roberts TG, 2005).

Notably, the decreased proliferation and enhanced differentiation of SH-EP 13I cells occurred concomitantly with the accumulation of $p27^{Kip1}$ and underphosphorylated RB. $p27^{Kip1}$ mediates differentiation and proliferative arrest induced by neurogenic bHLH transcription factors (Farah et al., 2000). Its expression is down-regulated by ID2 and ID3, in the latter case through the inhibition of the ETS transcription factors ELK1 (Chassot *et al.*, 2007). The $p27^{Kip1}$ accumulation appears to be due to an increased transcription, as the mRNA amount was augmented in the 13I transformed cells, suggesting that 13I may also affect the ID/ELK1 interaction. An increased $p27^{Kip1}$ expression may contribute to the accumulation of the under-phosphorylated, active form of RB, by inhibiting the CDKs involved in RB phosphorylation.

Furthermore, 13I down-regulated VEGF mRNA. Ids and VEGF are connected through a positive feedback loop, with VEGF up-regulating Ids (Sakurai *et al.*, 2004), and Ids, in turn, upregulating VEGF (Lasorella *et al.*, 2005). VEGF levels are considered as a tumour aggressiveness marker. Since Ids are expressed in tumour

endothelial cells, these findings suggest that 13I can affect metastatic progression.

To verify this assumption, we are planning to test its potential *in vivo*. Preliminary indications can come from experiments such as the tube test formation in Matrigel and clonogenic assays in soft agar. However, the delivery efficiency of 13I needs to be improved, since with retroviral infection we can not achieve a high 13I level expression. The weaker sensitivity of LA-N-5 to 13I may be explained considering that these cells produce more ID1 and ID2 than SH-EP cells, and the 13I amount might be insufficient for a full Id inactivation. A way to bypass, at least in part, this limitation, would be to clone our domain into a lentiviral vector. This tool will permit us to introduce the 13I even in cells weakly transducible, such as cancerous primary cultures.

In conclusion, this work demonstrate the feasibility of an approach based on Id proteins inhibition by targeting their HLH domain and suggest that it could be a therapeutic potential.

MATHERIALS AND METHODS

Antibodies and chemicals

ID1, ID2, ID3, HEB, cyclin D1, $p21_{Cip1}$ and actin rabbit antibodies (products sc-488, 489, 490, 357, 717, 397, 10731) were from Santa Cruz; N-CAM and NeuN antibodies (#AB5032 and #AB377, respectively) from Chemicon. RB, underphosphorylated RB, and $p27_{Kip1}$ mouse monoclonal antibodies were from BD (clones G3-245, G99-549, G173-524), FLAG antibodies (clone M2) affinity gel from Sigma, and anti NF-160 (MAB-10416) from Immunological Sciences. Horseradish peroxidase-conjugated donkey antirabbit and sheep anti-mouse antibodies, and rhodamine or fluorescein linked secondary antibodies were from Chemicon. All chemical were from Sigma, except when otherwise indicated.

Immunohistochemical assay

Adult rats (250-300gr, Wistar) were deeply anesthetized with intraperitoneal avertin injection and perfused through the aorta with ice-cold 4% parafolmaldehyde. The brain was removed from the skull, postfixed in 4% parafolmaldehyde by immersion for 2h, and then incubated into a 30% sucrose-PB for two nights. The brain was frozen on dry ice and 30µm- thick sagittal sections were cut on a sliding microtome to perform immunofluorescence experiments and confocal imaging. The sections were first incubated with 10% NDS (Normal Donkey Serum) in PBS (0, 02M) and Triton-X100 allo 0,25% (TPBS), primary antibodies in TPBS: anti-NeuN 1: 100 (mouse, Chemicon) to label all neurons (in the cerebellum Neu-N labelling is absent in the Purkinje cells) and anti- ID2 1:1000 (Santa Cruz, IC-20 rabbit). All incubations were performed at 4°C, one over night. After 3 washing steps in PBS 1X, the sections were incubated with Cy-2 and Cy-3 (Jackson Immuno Research) conjugated secondary antibodies (1:200), 2h at RT, mounted on poly-L-lysine coated slides, dried on air, coverslipped. The images were acquired by means of a confocal

laser microscope (Zeiss, LSM 510) with an oil objective 63x and 20x lens.

In plate DNA preparation

Confluent ES cells were lysed in 10 mMTris [pH 7.5], 10 mM EDTA [pH 8], 10 mM Na Cl, 0,5% sarcosyl and 100 µg/ml proteinase K (Boehringer); After overnight incubation at 55°C, 100 µl EtOH/NaCl (10 ml cold 96% EtOH + 150 µl 5M NaCl) were added and the plates were incubated 4 hours at room temperature. After precipitation and washes in 70% EtOH, plates were inverted and DNA was dried for 2-3 hours. About 30U of the appropriate restriction enzyme were added to each well and plates were incubated overnight at 37°C. Such DNA preparation was loaded on Southern Blot gels.

Southern blotting

Digested DNA was run on agarose gel (0,8%). Before blotting, gel was washed 10 minutes in 0,2M HCl, incubated 45 minutes in denaturing solution, washed in water and finally incubated in neutralization solution. The gel was then transferred to Hybond N+ membrane (GE) and blotted overnight. Next day, membrane was washed with 1,5M NaCl+1M Tris [pH7.5] and dried at 37°C; DNA was fixed by UV Xlink (UV Strata linker 1800). Probes were radioactively labeled with ^{32}P by Random Primers reaction (Roche). Blot were pre-hybridized in 0,5M Phosphate Buffer, 1mM EDTA, 3% BSAand 5% SDS for 1 hour at 65°C before adding the marked probe. Blot was hybridized overnight at 65°C. Next day the membrane was washed and signals were visualized by PhosphoImager.

Expression plasmids

13I was PCR amplified from the original phage clone (Ciarapica *et al.*, 2003) and fused to amino acids 634-682 of the HEB C-terminal region, known to stabilize the HLH domain (Goldfarb *et al.*, 1998). The resulting construct was inserted into the cβS-FLAG expression plasmid, from Dr M Cole (Dartmouth, USA), and sequenced for confirmation. The FLAG13I fragment was excised and cloned into the pBabePuro vector, thus obtaining pBP13I. pCMV-ID2 was from Dr L Vitelli (ISS, Rome), pCMV-ID1, pCMV-ID3, pGEX2T-ID1 and pGEX2T-ID3 from Dr A Iavarone (Columbia, USA), pCMV-RB from Dr M. Caruso (INMM, Rome), and pcDNA4/HisMaxA-E47 from Dr A Palamarchuk (CCC, Columbus, Ohio, USA).

Cells

SH-EP and LA-N-5 human neuroblastoma cells, provided by Dr C Dominici (Sapienza, Rome), were cultured in RPMI-1640, and 293T and Phoenix cells in DMEM, supplemented with 10% Foetal Calf Serum (EuroClone), at 37° C in a 5% CO_2 humidified incubator. 20 μg pBP and pBPFLAG13I DNA were introduced into $5\times10_6$ Phoenix cells in a 10 cm dish, through calcium phosphate transfection. Retrovirus containing supernatants were collected after 48 hours, passed through 0.45 μm Millipore filters, and used to infect sub-confluent cell cultures for 16 h, with 4 μg/ml polybrene. Cells were grown 16 more hours before addition of 1 μg/ml puromycin, which was kept for 4 weeks. Cells were treated with 10 μM RA for up to 4 days, by replacing the medium after 48 h. SH-EP and 293T cells were transfected by Lipofectamine 2000 (Invitrogen).

Western blotting

Samples were run on 10%, 12.5% and 15% SDS-PAGE gels and transferred to Hybond ECL membranes (GE). Membranes, preincubated in TBS solution containing 10% dry milk, were treated with appropriate primary and secondary antibodies. Actin antibodies were used as control. Staining was performed through the SuperSignal West Pico Chemiluminescent Substrate (Pierce). Densitometric analysis was performed by ImageJ software.

Co-immunoprecipitation

cβS-FLAG13I, pCMV-ID2 and pCMV-RB plasmids were transfected into 293T cells in a 10 cm dish. Two days after, cells were lysed in 10 mM NaCl, 10 mM Tris-HCl [pH 8], 0.3 M sucrose, 3mM $MgCl_2$, 0.5% NP-40, containing protease and phosphatase inhibitors. 1.5 mg protein samples were incubated at 4° C overnight with 1 μg ID2 antibody, conjugated to protein A/G Sepharose beads (Pierce), or anti-FLAG affinity gel, and briefly centrifuged. Pellets were washed 5X in PBS, suspended in SDS-polyacrylamide gel electrophoresis buffer and analysed by Western blotting.

Pull-down assay

E. coli BL21 cells harbouring plasmids expressing GST-linked ID1, ID3 and HEB dimerization region (aa 580-682) were grown to an OD_{600} of 0.6, and treated with 0.1 mM IPTG, 3-5 hours at 30°C. GST-fusion proteins were extracted from bacterial pellets and conjugated to Glutathione-Sepharose 4B beads (Amersham). 293T cells were transfected with cβS-FLAG13I DNA, and lysed in ice cold RIPA buffer containing protease and phosphatase inhibitors. Lysates were cleared by centrifugation, mixed with 10

μg GST control and fusion proteins coupled to Glutathione Sepharose beads, incubated 2 h at 4° C, and centrifuged. Pellets were washed 5X in PBS, run on polyacrylamide gels, and visualised by Coomassie staining and Western blotting.

Luciferase assay

Cells, seeded in 6-well culture plates, were cotransfected with the E-box dependent Firefly luciferase reporter 4RE-luc (courtesy of PL Puri, EBRI, Rome) and the Renilla luciferase reporter pGL3-*Renilla* for normalization. Different combinations of pcDNA4/HisMaxA-E47, pCMV-ID2, and increasing concentrations (up to threefold) of cβS-FLAG13I were added. Cells were lysed 48 h later, and Firefly and *Renilla* luciferase activities measured with the dual-luciferase reporter assay system (Promega).

Morphology and immunocytochemistry

Cells grown for 2 days on 35 mm dishes were visualised with an Eclipse TE200 microscope (Nikon), and images acquired through Metamorph software version 7.1 (Crisel). Cells grown on glass chamber slides (BD, #354118), were washed 3X in PBS, fixed 10' in 70% ethanol and treated 5' with 0.2% Triton X-100 in PBS. After 30' in PBS containing 3% bovine serum albumin, slides were incubated overnight at 4° C with NF-160 or N-CAM antibodies, washed, incubated 1 h at room temperature with rhodamine or fluorescein linked secondary antibodies, washed again, stained 2' with 200 ng/ml DAPI, and mounted in GelMount (Biomeda). Images were acquired by an Eclipse E600 fluorescence microscope (Nikon), through LUCIA software version 4.81.

Proliferation

Cells plated in triplicate onto 12 mm diameter multi-well plates ($2x10^4$ cells/well) were allowed to adhere overnight, harvested at different times, and counted with a hemocytometer in presence of 0.4% Trypan blue. Data reported are the mean of triplicate counts of at least three independent experiments.

RNA analysis

Cells were treated with Trizol (Invitrogen) and RNase-free DNase I (USB), 30' at 37° C. RNA was purified by phenol extraction and analysed by agarose gel electrophoresis. VEGF and $p27^{Kip1}$ mRNAs were measured by Real Time PCR with TaqMan Gene Expression Assays (Applied Biosystems) through an ABI PRISM 7700: 2' at 50° C, 10' at 95° C, 40 cycles of 15 sec at 95°C followed by 1' at 60° C. At least two independent amplifications were performed for each probe, with triplicate samples. Cycle threshold values were determined by automated analysis.

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