

UNIVERSITÀ DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN BIOCHIMICA E BIOLOGIA MOLECOLARE

CICLO XXV

**STRUCTURAL AND FUNCTIONAL TRAITS OF
NG2/CSPG4 PROTEOGLYCAN IN RELATION TO
ITS ROLE IN TUMORS**

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1. INTRODUCTION

Chondroitin Sulphate Proteoglycan-4 (CSPG4), also known as nerve/glial antigen 2 (NG2), High Molecular Weight-Melanoma Associated Antigen (HMW-MAA), or melanoma chondroitin sulfate proteoglycan (MCSP), is a cell surface proteoglycan with biological and clinical significance, which have been first characterized in a high-molecular-weight type 1 membrane proteoglycan in rat in 1981¹, and then identified with mouse monoclonal antibody (mAb) on human melanoma cells². It is a chondroitin sulfate proteoglycan that consists of an N-linked glycoprotein of 280 kDa and a proteoglycan component of about 450 kDa^{3,4,5} and plays an important role in cell proliferation, migration, and metastasis⁶. It is expressed on the surface of various types of immature progenitor cells and its expression decreases with terminal differentiation^{7,8}. NG2/CSPG4 has been supposed to be involved in platelet-derived growth factor (PDGF) signaling in oligodendrocyte precursor cells (OPCs)⁹, where its expression is widely used as a marker for these cells¹⁰. NG2/CSPG4 is mostly expressed by human malignant melanoma cells¹¹, tumors of neuroectodermal origin¹²⁻¹⁵, on tumour neovasculature by vascular pericytes in human malignant brain tumours¹⁶, on basal breast carcinoma¹⁷, squamous carcinoma of the head and neck (SCCHN)¹⁸, pancreatic carcinoma, some types of renal cell carcinoma, chordoma^{11,19}, chondrosarcoma cells¹⁹ and mesothelioma^{6,19}. Moreover, NG2/CSPG4 is highly expressed in uveal melanomas^{20,21} and soft tissue sarcomas²² where enhanced expression levels of NG2/CSPG4 proteoglycan provides a molecular factor that alone prospects a particularly unfavorable clinical^{22,23} and mediates a poor prognosis in childhood acute myeloid leukaemia (AML) patients²⁴.

Aberrant expression of NG2/CSPG4 in tumors and angiogenic vasculature was seen to be associated with an aggressive disease course in several malignancies, moreover NG2/CSPG4 is expressed on the surface of both tumor cells and pericytes, and its distribution in normal tissues can be considered relatively restricted, so to be proposed as an attractive candidate for simultaneously targeting the malignant and stromal cellular compartments within the tumour²⁵. Both the progenitor cells and the tumor cells are mitotic and in some cases highly motile. Many researches demonstrate the ability of NG2/CSPG4 to trigger rearrangements of the actin cytoskeleton²⁶⁻²⁸ and to interact with components of the extracellular matrix²⁹⁻³², suggesting that NG2/CSPG4 plays a role in both growth control and motility of cells³³. Because of its structure, distribution, and functions NG2/CSPG4 has been proposed to

promote tumor progression by multiple mechanisms and may represent a putative target for molecular therapy against cancer.

1.1 Gene-related traits

Human chondroitin sulphate proteoglycan 4 was first identified in 1981 on human melanoma cells³⁴, the complete cDNA and the primary amino acid sequences for rat ortholog of NG2/CSPG4 have been obtained in 1991³⁵, and in 1996 Pluschke and collaborators published the human sequence of NG2/CSPG4 expressed by human melanoma cells³⁶. The contiguous human cDNA spans 8,071 nucleotides and contains an open reading frame of 2,322 amino acids³⁵. The human predicted protein is an integral membrane molecule with a large N-terminal extracellular domain (2,221 amino acids), a single transmembrane domain (25 amino acids), and a short cytoplasmic tail (76 amino acids)³⁵, encoded by a single gene with 10 exons³⁷. To date no alternatively spliced variants have been described³⁷. The human NG2/CSPG4 gene is located on Chromosome 15q at position 75,966,663-76,005,189³⁸ (Fig. 1A). Sequence analysis shows that the proximal and distal regions of 15q share extensive ancient similarity³⁹. Many of the events by which the current duplication structure arose have been reconstructed, and from this kind of study researchers found that most of the intrachromosomal duplications seem to share a common ancestry. Moreover, it has been demonstrated that some remaining gaps in the genome sequence are probably due to structural polymorphisms between haplotypes⁴⁰. Data about Single Nucleotide Polymorphism (SNPs) inside NG2/CSPG4 gene have been extracted from the dbSNP database (NCBI, National Center for Biotechnology Information, Bethesda, <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp&cmd=search&term=>). The annotation of each SNP and its frequency in Caucasian populations were assessed in dbSNP NCBI and ENSEMBL (<http://www.ensembl.org/index.html>) databases. The outcome obtained from this research is reported in figure 1B. The system collected several polymorphisms in NG2/CSPG4 gene: they are above all synonymous or missense variants: the latter could yield codons coding for different amino acids compared to the template

NG2/CSPG4 sequence. Interestingly there are 5 stop gained SNPs in the region codifying the N-terminal portion of the protein, and likely these polymorphisms could cause the synthesis of a shorter protein possibly lacking the C-terminal portion compared to the full length one.

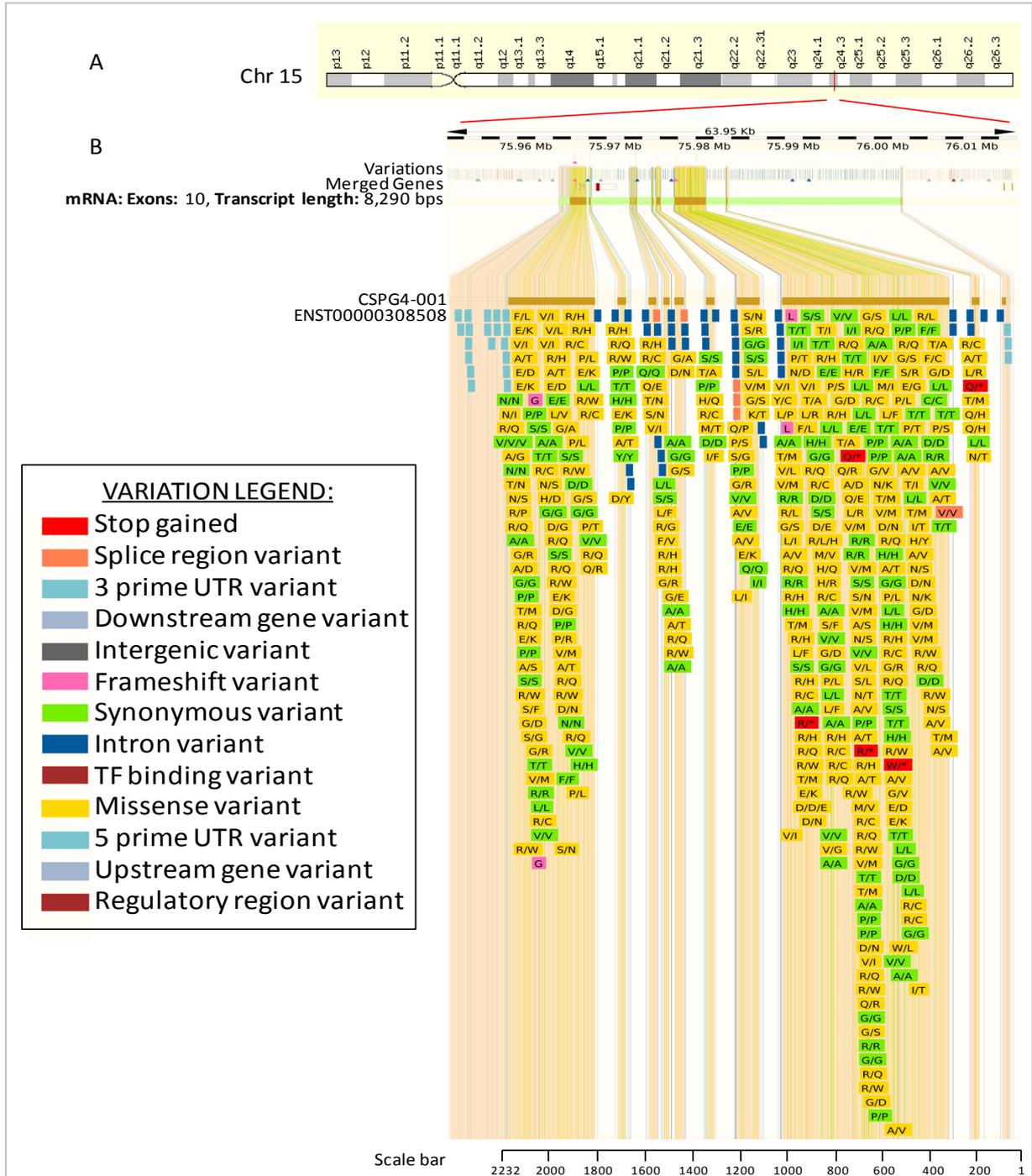


Figure 1: NG2/CSPG4 gene *in silico* study. A) Schematic representation of Chromosome 15. NG2/CSPG4 gene in genomic location, bands according to Ensembl, locations according to GeneLoc. Gene ID: ENSG00000173546 Location: Chromosome 15:75966663-76005189;-1. B) Genetic variation with polymorphisms within the gene for NG2/CSPG4. In the figure the gene is zoomed and shows the SNPs. Color legend of the modifications is on the left of the figure.

It is noteworthy that the NG2/CSPG4 has conserved its structural and functional properties through phylogenetic evolution, so similarity of NG2/CSPG4 with its counterparts in other animal species was considered and led to the observation that the NG2/CSPG4 homologue in rat and mouse shares over 80% amino acid sequence identity with the human sequence, and 90% amino acid identity with each other. Amino acid differences among the three are spread throughout the full-length coding sequence of each protein, suggesting that their primary structure is evolutionarily conserved^{11,41}. Here we show that the submission of the amino acid sequence of NG2/CSPG4 (Homo sapiens NP_001888.2 chondroitin sulfate proteoglycan 4 (NG2/CSPG4)) in MegaBlast (release 2.2.11) leads to the identification of 11 homologue sequences to the target. Multiple alignments of the elements were generated with ClustalW (v.1.83) and visualized with GeneDoc (Figure 2).

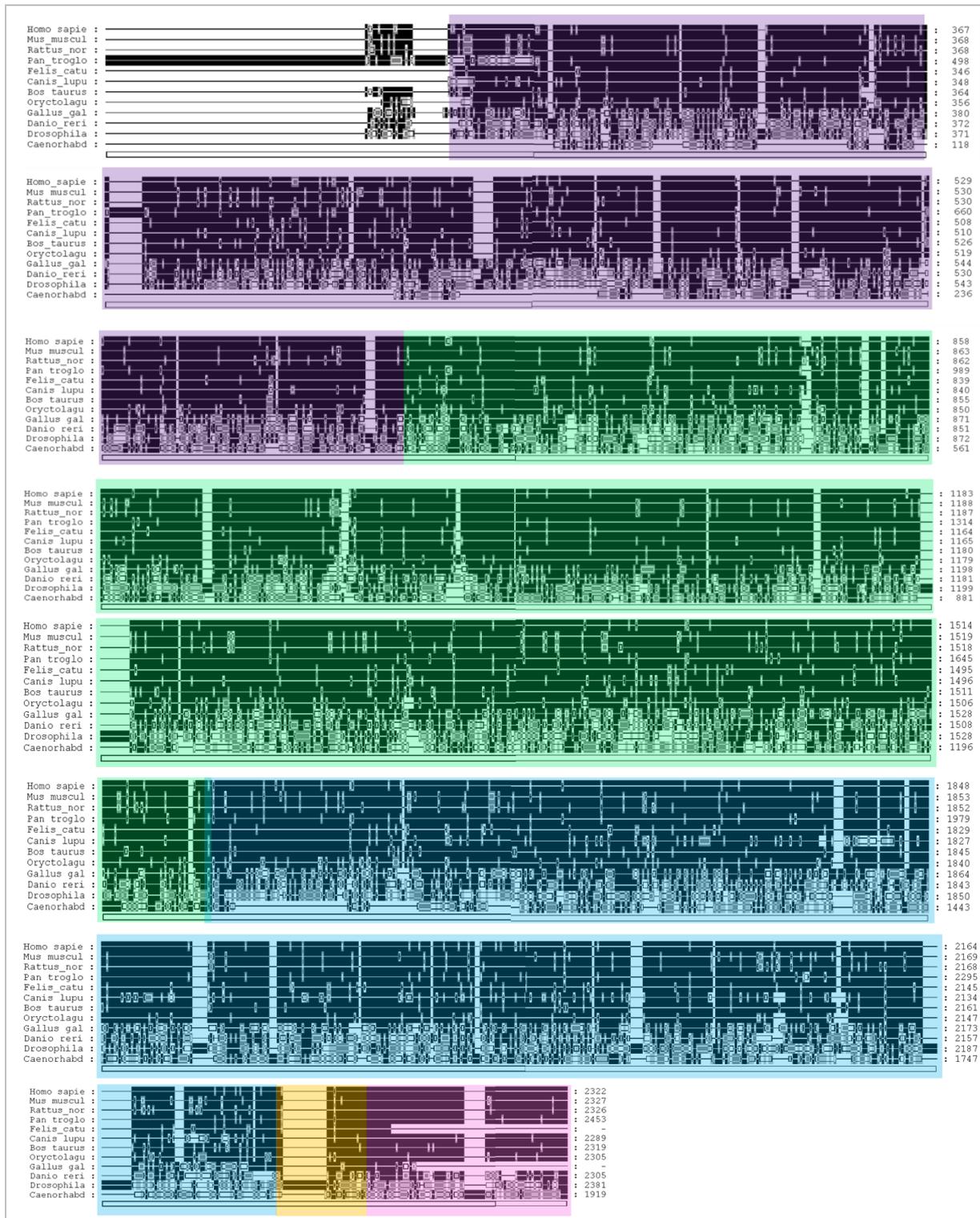


Figure 2: Alignment of amino acid sequences of NG2/CSPG4 homologues to the human NP_001888.2. Sequences were aligned by using CLUSTALW (www.ebi.ac.uk/clustalw) with default settings; the alignment is presented by GENEDOC (www.psc.edu/biomed/genedoc) and visualized in modality Summary view. Residues on black, dark gray, and light gray backgrounds indicate 100%, 80%, and 60% amino acid similarity, respectively. Color shapes of the regions respect the division of the sequence in domain as follows: D1=purple, D2=green, D3=light blue, Transmembrane domain=Yellow, Cytoplasmic tail=Pink.

Phylogenomics is an important tool for resolving the Tree of Life, and the transcriptome data set provides an opportunity to study the evolutionary history of the gene for NG2/CSPG4. Phylogenetic analysis was performed using traditional approach, that is a multiple sequence alignment (MSA) constructed using Clustal-X version 1.81, followed by tree reconstruction using Phylip Package. Our finding of human NG2/CSPG4 gene as sister to the clade containing dog, rat and mouse is consistent with recent studies¹¹, even if we could observe all the orthologues in the phylogenesis. In the tree of the orthologues shown in figure 3, we set *Ceanorabditis elegans* as outgroup since it is evolutionarily the fairest organism from *Homo sapiens*, moreover it respects the speciation and is coherent with the tree of life, since it localizes mammals in the same group in particular, as we expected, *Homo sapiens* and *Pan troglodytes* (Fig. 3).

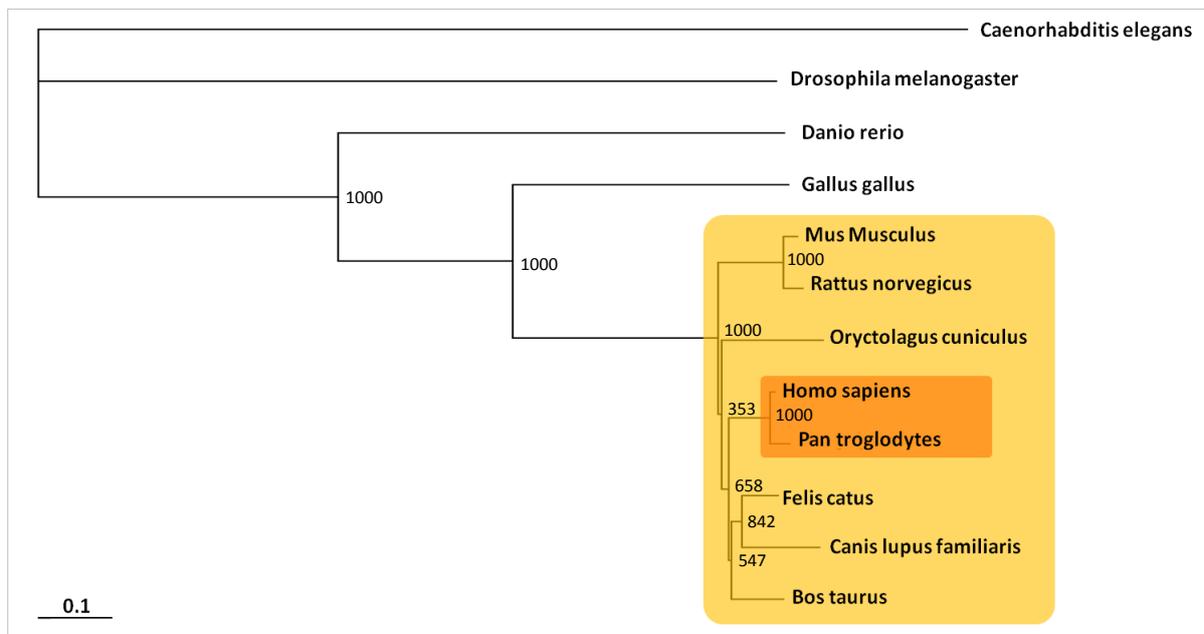


Figure 3: Phylogenetic Tree of the 11 sequences found to be homologous to the human protein sequence NP_001888.2 chondroitin sulfate proteoglycan 4 (NG2/CSPG4). The tree shows 12 orthologue genes where *Ceanorabditis elegans* has been defined as outgroup, since it is evolutionarily the fairest organism from *Homo sapiens*. As we expected, mammals are localized in the same group (bold in orange) above all *Homo sapiens* and *Pan Troglodytes* (bold in red). The tree respects the speciation and it is coherent with the tree of life. Scale bar= number of substitutions per site. Bootstrap index are reported above nodes.

1.2 Structural and functional characteristics

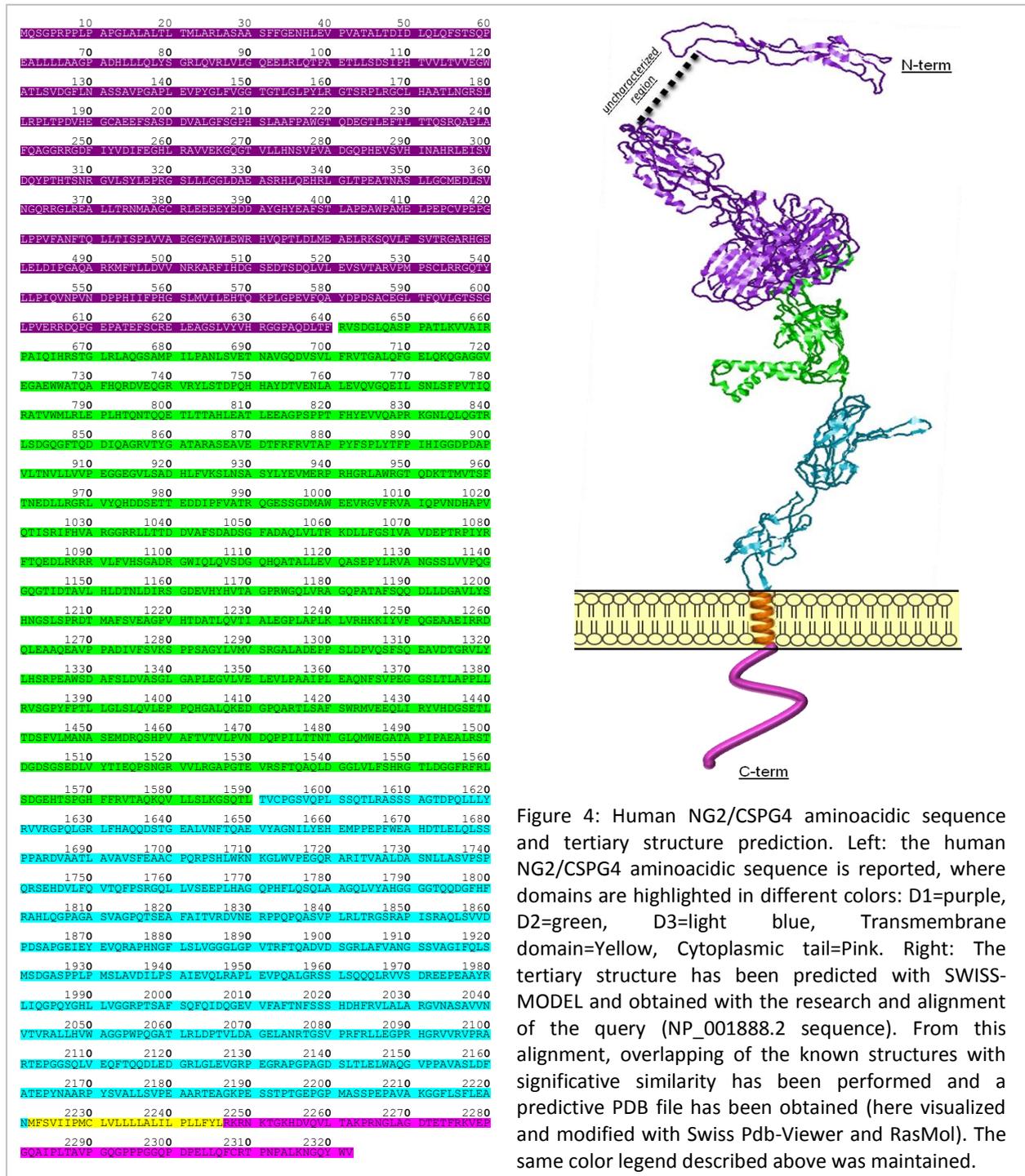
NG2/CSPG4 is a chondroitin sulfate proteoglycan with structural characteristics that make it unique among members of the proteoglycan family²⁸. Although many proteoglycans can be

grouped into families based on structural similarities (syndecans, aggrecans, glypicans, etc.), NG2/CSPG4 does not contain structural motifs common to any of these known groupings³⁵. NG2/CSPG4 could be expressed on the cell surface both with N-linked chondroitin sulphate chain and without any GAG chain, so to be considered a “part-time proteoglycan”³⁷. Figure 4 represents the aminoacidic sequence and the tertiary predicted structure of human NG2/CSPG4 (modified with Swiss Pdb-Viewer from the outcome obtained with SWISS-MODEL). As shown from this representation, NG2/CSPG4 is a type 1 transmembrane protein in which a 25-residue transmembrane domain (aminoacids 2222-2246, in yellow) separates a relatively short 76 amino acid C-terminal cytoplasmic domain (2247-2322, in pink) from an extensive 2225-residue N-terminal ectodomain that could be divided into three subdomains: an N-terminal globular domain (D1, aminoacids 1-640, in purple) stabilized by intramolecular disulfide bonding thanks to its 8 cysteins within the sequence³⁵, and containing two laminin G-type motifs that might be important for ligand binding⁴¹; a central extended domain(D2 aminoacids 641-1590, in green) containing the attachment site for the chondroitin sulfate chains⁴², and the binding sites of collagens V and VI^{30,43}; the globular juxtamembrane one-third (D3 aminoacids 1591-2221, in light blue)³⁵ containing N-linked oligosaccharides that bind galectin-3⁴⁴ and β 1 integrins⁴⁵, and the sites for proteolysis of NG2/CSPG4, leading to its release from the cell surface^{9,46}. This cleavage sites have been discovered in 1995 when Nishiyama and collaborators characterized three molecular forms of the NG2/CSPG4 core protein expressed by different cell lines⁴⁶: since then many researchers tried to give a significance to the cleavage of NG2 and its physiological and pathological implications³, as we delve further.

Glycosylation of NG2/CSPG4 with chondroitin sulphate (CS) chains occurs in the trans-Golgi compartment⁴⁷. Along the whole extracellular domain sequence there are 15 potential N-linked glycosylation sites (consensus sequence: NXS/T)⁴⁸.

The cytoplasmic domain of NG2/CSPG4 contains several structural features that should be critical for the function of the proteoglycan. Its carboxyl terminal tail contains the PDZ (postsynaptic density-95/discs large/zona occludens-1) binding motif QYWV, that can interact with PDZ domain-containing proteins⁴⁹ which usually play a scaffolding role. The NG2/CSPG4 cytoplasmic domain also contains multiple potential threonine

phosphoacceptor sites, including Thr₂₂₅₆/Thr₂₃₁₄ phosphorylated by PCK α and ERK 1/2, respectively, that seems to balance migration and proliferation of cells^{50,51}. Finally, there is also a proline-rich segment in the C-terminal half of the cytoplasmic domain, which may facilitate additional protein–protein interactions²⁶.



From the immunoprecipitations of NG2/CSPG4 from melanoma cells followed by immunoblotting analysis, it is easy to observe in literature the full length core protein (about 250kDa), the glycosylated form (about 450 kDa) and smaller molecules: 150 kDa proteins and smaller fragments that are proposed to be secreted or retained on the cell surface^{17,46,52}. Although little is known about the mechanisms of shedding or the functions of shed NG2/CSPG4³, it seems likely to play important roles in a number of physiological processes⁵³ and many other factors should be involved. Proteolytic shedding of NG2/CSPG4 results greatly enhanced in several types of injuries such as spinal cord injuries^{54,55}, multiple sclerosis⁵⁶ and tumors. Several studies regarding the expression of NG2/CSPG4 in the Central Nervous System (CNS) let suppose that the over-expression of chondroitin sulphate proteoglycans (CSPGs) is thought to create an environment non-conducive to axon regeneration in the injured CNS and a variety of CSPGs are known to be present at such sites, including NG2/CSPG4⁹. Asher and collaborators demonstrated the existence of large amounts of shed NG2/CSPG4 in the adult CNS, where is also used as a marker for OPCs⁹. They supposed it could have implications for in axon regeneration. There are evidence showing that in cells (juxtamembrane) D3 is to some extent inaccessible and imply that shed NG2/CSPG4, in which both inhibitory domains are accessible, would exert a greater axon growth-inhibitory effect than cell-associated NG2/CSPG4⁹. The researchers supposed that NG2/CSPG4 is involved in Platelet-Derived Growth Factor (PDGF) signaling in OPCs because the rate of NG2/CSPG4 shedding increased with cell density and NG2/CSPG4 expression was increased in the absence of PDGF⁹. Ectodomain shedding converts NG2/CSPG4 into a diffusible entity able to interact with the growth cone⁹. It has been demonstrated that soluble NG2/CSPG4 released from tumor cells or tumor-associated pericytes can stimulate endothelial cell migration in the tumor microenvironment by interacting with galectin-3 and $\alpha 3\beta 1$ -integrin on the endothelial surface^{44,57}. From a study of the effect of Transforming growth factor beta 1 (TGF β 1) on melanoma cells differentiation, it had been observed that TGF β 1 caused the increasing of synthesis and shedding of NG2-expressing human melanoma cells⁵⁸, even if the role of this events in tumors remains to be determined. To identify the conserved protein domains among the organisms, homologues were analysed by multiple sequence alignments using the programs ClustalW and GeneDoc. From

the alignments we could observe that the most conserved regions are: a) Interaction with COL6A2 region, (showed in figure 5 obtained with SeqLogos processing), b) Gly/Ser-rich (glycosaminoglycan attachment domain) region c) cystein-rich element in domain 1 and 3 of the ectodomain; d) phosphosytes in the cytoplasmic domain. Moreover it is evident that NG2/CSPG4 is conserved along the whole aminoacid sequence through the Evolution History, and this observation underlines that NG2/CSPG4 tertiary structure should be conserved too, as it resulted fundamental for its functions (Figure 2).

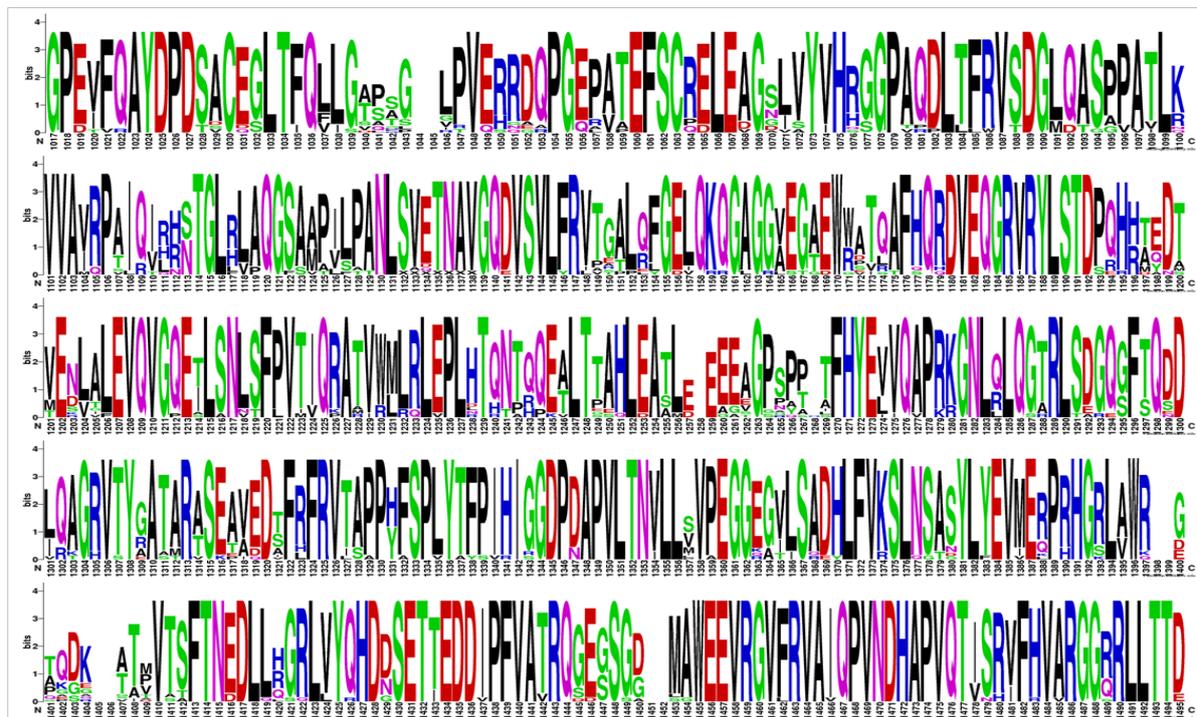


Figure 5: SeqLogos (<http://weblogo.berkeley.edu/logo.cgi>) of the Collagen VI binding region of NG2/CSPG4 (amino acid range: 574-1040) obtained from the multiple alignment in Figure 2. Legend: KRH=green, DE=blue, AVLPWF=red, STNQC=black. Most of the positions are conserved among all the organisms, underlining that the sequence and the folding of this region is particularly important for its functions

1.3 Putative molecular interactors

The singularity of NG2/CSPG4 structure suggests that it might also be unique in terms of its ability to participate in inter- and intra-molecular interactions. As a membrane-spanning molecule, NG2/CSPG4 has the potential to interact with its cytoplasmic domain, the large extracellular portion, and the glycosaminoglycan chain it carries on the core protein to

participate in signaling between the extracellular and intracellular compartments of the cell. NG2/CSPG4 guides the activation of important survival and growth pathways, in particular it is involved in integrin-regulated focal adhesion kinase (FAK), Extracellular signal-Regulated Kinases (ERK 1/2), and Phosphatidylinositol-3 kinases/Akt (PI3K/Akt) pathways, so that NG2/CSPG4 results implicated in regulating the growth, survival, proliferation, migration, as well as invasion of tumors cells⁴⁵ (Figure 6A and 6B). FAK serves to integrate signaling pathways between growth factor receptors and integrins⁵⁹ and is implicated in facilitating cell survival and regulating cell spreading, migration, and invasion⁶⁰. Integrins, a family of heterodimeric adhesion receptors that mediate both cell–ECM and cell–cell adhesion, initiate multiple cellular signals that profoundly influence shape, proliferation, differentiation, invasion, metastasis, apoptosis, and anoikis⁶¹ and it has been proved that NG2/CSPG4 acts as a coreceptor for $\alpha 4\beta 1$ -integrin to modulate cell adhesion and spreading, also to collagens⁴⁵, by mechanisms dependent on the small Rho family GTPase Cdc42 and the adaptor protein p130^{cas}. Both NG2/CSPG4 and $\alpha 4\beta 1$ -integrin are involved in modulating melanoma cell adhesion, migration, and invasion⁶². NG2/CSPG4 may stimulate a $\alpha 4\beta 1$ integrin-mediated adhesion and spreading by recruiting and activating a signaling cascade through CDC42, ACK1 and BCAR1⁶³. It has been supposed that NG2/CSPG4 could promote retraction fiber formation and cell polarization through Rho GTPase activation (RhoA, Rac, Cdc42, adptor protein p130cas) in the control of cell motility⁶³: the involvement of NG2/CSPG4 seems to lead to the signal transduction pathway activation thanks to the activation of this Rho GTPase family proteins. From these evidences it seems that NG2/CSPG4 enhances cell spreading and motility on one hand by PAK activated by Rac and Cdc42 and in parallel thanks to the tyrosine phosphorylation of p130^{cas}. These kinds of interactions have been supported also by to the employment of anti-NG2/CSPG4 monoclonal antibodies (mAbs), that lead to the hypothesis of the involvement of NG2/CSPG4 both in the phosphorylation of other molecules such as pPDK1, pAkt and pErk1, and in the activation of PKC α and β -catenin¹¹, in fact also thanks to the CSPG4-blocking mAb 225.28 it has been demonstrated the involvement of NG2/CSPG4 in *in vitro* and *in vivo* activation of FAK, ERK1/2, Akt, and the expression of PKC α in tumor cells¹⁷. The effectors of this signalling pathway are involved in cytoskeleton reorganization and in cell migration⁶².

Other recent studies have been performed to determine the link between NG2/CSPG4 and ERK expression and functions, with the relevance that NG2/CSPG4 expression in radial growth phase, vertical growth phase, or metastatic cell lines causes activation of ERK1/2, enhanced growth and motility which all require the cytoplasmic domain of the NG2/CSPG4 core protein. NG2/CSPG4 expression in a radial growth phase cell line also seemed to promote an epithelial-to-mesenchymal transition⁶⁴, a process which itself includes typical tumorigenic cell changes, such as enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components⁶⁵(Figure 6).

As we already mentioned, NG2/CSPG4 is able to interact both with components of the extracellular matrix and with the actin cytoskeleton. In 1996 Lin and collaborators demonstrated that NG2/CSPG4 is anchored to the actin cytoskeleton²⁷. A deeper examination of the distribution of NG2/CSPG4 during cell spreading suggests that NG2/CSPG4 can associate with two distinct types of actin-containing cytoskeletal structures: the first of these are actin- and myosin-containing stress fibers; the second are the radial processes extending from the cell periphery, and these projections contain actin, but unlike the cytoskeletal stress fibers, they do not contain myosin, and thus are a distinct domain of the actin cytoskeleton²⁸.

It has been investigated the expression of NG2/CSPG4 in microglial cells and its role on inflammatory reaction of microglia by analyzing the expression of the proinflammation cytokines (interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α)), chemokines (stromal cell-derived factor-1 α (SDF-1 α) and monocyte chemotactic protein-1 (MCP-1)) and inducible nitric oxide synthase (iNOS), finding out that NG2/CSPG4 mediates the induction of iNOS and inflammatory cytokine expression, but not the chemokine expression in activated microglia⁶⁶.

In a study dealing with tumor cell adhesion and proteolysis of the extracellular matrix proteins surrounding the cells in processes of tumor invasion, it has been determined, by antisense inhibition, that NG2/CSPG4 and membrane-type 3 matrix metalloproteinase (MT3-MMP) expressed by melanoma cells are required for invasion and degradation of type I collagen⁶⁷. MT3-MMP co-immunoprecipitated with NG2/CSPG4 in melanoma cells cultured on type I collagen or laminin. The association between MT3-MMP and NG2/CSPG4 was

largely disrupted by removing chondroitin sulfate glycosaminoglycan (CS) from the cell surface, suggesting that CSs mediate the association between the two cell surface core proteins. It has been shown that MT3-MMP possesses the potential to promote melanoma invasion and proteolysis and that the formation of a complex between MT3-MMP and NG2/CSPG4 may be a crucial step in activating these processes⁶⁷.

In human melanoma cells, NG2/CSPG4 interacts directly with different ECM components, such as collagens II²⁹, V⁴⁵ and VI²⁹, the heparin-binding domain of fibronectin (FN)^{31,32}, tenascin, and laminin via domains contained within the protein core of NG2/CSPG4²⁹, altering cellular morphology and proliferation. The best characterized among these ligands is type VI collagen^{29,68,69,70} which allows the proteoglycan to serve as an efficient cell surface receptor for ECM. Motility studies show that this interaction is functionally important and indicate that binding of type VI collagen to NG2/CSPG4 triggers signaling mechanisms that lead to enhanced cell migration²⁶. This interaction has been further characterized using a solid phase binding assay in which purified NG2/CSPG4 was shown to bind to pepsin-solubilized type VI collagen²⁹. Collectively, the available results support a role for NG2/CSPG4 as signal-transducing molecules that initiate or modify intracellular signal cascades important for cell adhesion, motility, and invasion (Figure 6).

From clinical evidence it has been highlighted that BRAF inhibitors induced the regression of metastatic lesions in about 70% of the patients affected from melanomas with the BRAFV600E mutation⁷¹. Ling and collaborators tried to develop a treatment strategy combining BRAF inhibitors and inhibitors targeting other signaling components/pathways determined to be abnormal in melanoma cells: with this aim they combined a BRAF inhibitor with a CSPG4-specific mAb demonstrating that *in vitro* response magnitude and duration of the BRAF inhibitor against BRAFV600E melanoma cells could be significantly enhanced by the employed anti-NG2/CSPG4 mAbs⁷¹.

There are evidence of the dependence of NG2/CSPG4 expression and those of Grb2⁷². This is a key molecule in intracellular signal transduction that has been found to link activated cell surface receptors to downstream targets by binding to specific phosphotyrosine-containing and proline-rich sequence motifs⁷³ like those contained in the cytoplasmic tail of NG2/CSPG4. Grb2 signaling is critical for cell cycle progression and actin-based cell motility,

and consequently, for more complex processes such as epithelial morphogenesis, angiogenesis and vasculogenesis⁷³, where also NG2/CSPG4 is involved.

The galectins are a family of mammalian lectins that contain conserved carbohydrate recognition domains (CRDs) responsible for oligosaccharide-dependent ligand binding⁷⁴. Researchers highlighted that the C-terminal carbohydrate recognition domain of galectin-3 is responsible for binding to the NG2/CSPG4 core protein. Within the NG2/CSPG4 extracellular domain, the membrane-proximal D3 segment of the proteoglycan contains the primary binding site for interaction with galectin-3. The interaction between galectin-3 and NG2/CSPG4 is a carbohydrate-dependent one, mediated by N-linked rather than O-linked oligosaccharides within the D3 domain of the NG2/CSPG4 core protein. NG2/CSPG4 stimulates endothelial cell motility and morphogenesis and this function of NG2/CSPG4 depends on formation of a complex with galectin-3 and $\alpha 3\beta 1$ -integrin to stimulate integrin-mediated transmembrane signaling⁴⁴. NG2/CSPG4, galectin-3, and $\alpha 3\beta 1$ -integrin form a complex on the endothelial cell surface, and the formation of this tri-molecular complex gives to NG2/CSPG4 the ability to activate endothelial cells in EC motility and morphogenesis processes⁵⁷. In conjunction with recent studies demonstrating the early involvement of pericytes in angiogenesis, these data suggest that pericyte-derived NG2/CSPG4 could be an important factor in promoting EC migration and morphogenesis during the early stages of neovascularization⁵⁷.

The intracellular domain of NG2/CSPG4 consists of the C-terminal 76 amino acids containing the PDZ binding motif QYWV, which binds to the PDZ domain of scaffold proteins such as syntenin^{75,76}, MUPP1⁷⁷, and GRIP1^{41,49,78} as summarized in figure 6:

- Syntenin: a PDZ domain protein that interacts with NG2/CSPG4 in oligodendrocytes, where syntenin-1 is necessary for normal rates of cells migration. The association of syntenin-1 with NG2/CSPG4, identified in a yeast two-hybrid screen, was confirmed by colocalization of both proteins within processes of oligodendroglial precursor cells and by co-immunoprecipitation from cell extracts. Syntenin-1 also co-localizes with NG2/CSPG4 in “co-capping” assays, demonstrating a lateral association of both proteins in live oligodendrocytes⁷⁵.

- MUPP1 (Multi-PDZ Domain Protein 1): a yeast two-hybrid screen was employed to identify ligands for the cytoplasmic domain of the NG2/CSPG4 and the existence of an NG2/CSPG4-MUPP1 interaction *in situ* is demonstrated by the ability of NG2/CSPG4 antibodies to co-immunoprecipitate both NG2/CSPG4 and MUPP1 from detergent extracts of cells expressing the two molecules. MUPP1 may serve as a multivalent scaffold that provides a means of linking NG2/CSPG4 with key structural and/or signaling components in the cytoplasm⁷⁷.
- GRIP1 (Glutamate receptor-interacting protein 1): it acts as a scaffolding molecule clustering NG2/CSPG4 and AMPA receptor in glial progenitor cells^{41,49}. NG2/CSPG4 may thus act to position AMPA receptors on glia towards sites of neuronal glutamate release⁷⁸.

Researchers showed that >90% of embryonic and adult NG2/CSPG4 cells in the adult central nervous system express Olig2, a basic helix–loop–helix transcription factor required for oligodendrocyte lineage specification. Studies of mice lacking Olig2 function showed a failure of NG2/CSPG4 cell development at embryonic and perinatal stages that were rescued by addition of a transgene containing the human OLIG2 locus. These findings demonstrated a general requirement for Olig2 function in NG2/CSPG4 cell development and highlight further roles for Olig2 transcription factors in neural progenitor cells⁷⁹. More recently it has been investigated the role of EGFR in oligodendrocyte regeneration and remyelination, characterizing endogenous neural progenitors that are capable of generating oligodendrocytes during normal development and after demyelination, identifying the molecular signals that enhance oligodendrogenesis from these progenitors⁸⁰. Through gain- and loss-of-function approaches, researchers explored the role of epidermal growth factor receptor (EGFR) signaling in adult myelin repair and in oligodendrogenesis in NG2/CSPG4 and OLIG2 signaling pathway. From the works performed, it comes out that EGFR signaling regulates oligodendrogenesis and remyelination by progenitors expressing both NG2/CSPG4 and Olig2⁸⁰.

There are many evidence of the effects that various growth factors have on NG2/CSPG4 activity in signal transduction⁸¹. Fibroblast growth factor (FGF)-2 differentially regulates

oligodendrocyte progenitor proliferation and differentiation in culture, and modulates gene expression of its own receptors, in a developmental and receptor type-specific manner. Three FGF receptors (types 1, 2, 3) are expressed in postmitotic, terminally differentiating oligodendrocytes⁸². Perivascular NG2/CSPG4 may serve as a dual modulator of the availability/accessibility of FGF at the cell membrane, as well as the resulting FGFR transducing activity as it has been demonstrated that in the absence of NG2/CSPG4, cells do not sequester FGF2 at the cell surface and therefore are unable to activate FGF receptors, leading to low proliferative responses⁸¹. The interaction between NG2/CSPG4 and the platelet-derived growth factor receptor- α (PDGFR α) on the surface of oligodendrocyte precursors and vascular smooth muscle cells had been previously proposed⁸³, and the disruption of this interaction with antibody against NG2/CSPG4 resulted in loss of signaling capability through the PDGFR α ^{84,85}. Paralysis can result from spinal cord injury and one of the causes is the local increase of glial cells leading to the formation of a glial scar, and the upregulation of CSPGs at the site of injury through which axons are unable to regenerate⁸⁶. NG2/CSPG4 was reported to physically interact with PDGFR α and its ligand PDGFAA, leading to enhanced PDGF-AA signaling activity^{87,88}. Moreover PDGF-BB overexpression is able to induce NG2/CSPG4 expression in neural precursors⁸⁹. The above data suggest a potential role for the proteoglycan NG2/CSPG4 in gliomagenesis. In addition, from more recent data, it has been demonstrated that NG2/CSPG4 is not required for PDGF-BB in the induction and progression of glial tumors⁹⁰.

Combined immunohistochemical and quantitative Western Blot analysis revealed major reductions in levels of core protein expression (>80% for 130-kDa neurocan, 145/80-kDa brevican, 300-kDa phosphacan) and immunoreactivity for NG2/CSPG4, neurocan, phosphacan and brevican within decorin-treated injuries compared with untreated controls⁹¹. Decorin pretreatment of meningeal fibroblasts *in vitro* also resulted in a three-fold increase in neurite outgrowth from co-cultured adult sensory neurons and suppression of NG2/CSPG4 immunoreactivity. It has been demonstrated the ability of decorin to promote axon growth across acute spinal cord injuries via a coordinated suppression of inflammation, NG2/CSPG4 expression and astroglial scar formation⁹¹. Inhibitory CSPGs and myelin-associated molecules are major impediments to axon regeneration within the adult

central nervous system⁹². The interaction of NG2/CSPG4 and decorin remains to be determined even if it seems that their expression patterns have a correlation.

Using *in vitro* and *in vivo* methods, Nolin et al. describe a therapeutic approach based on tissue plasminogen activator (tPA), an extracellular protease that converts plasminogen (plg) into the active protease plasmin. The researchers showed that tPA and plg both bind to NG2/CSPG4, which functions as a scaffold to accelerate the tPA-driven conversion of plg to plasmin. The binding occurs via the tPA and plg kringle domains to domain 2 of the NG2/CSPG4 core protein, and it is enhanced in some settings after chondroitinase-mediated removal of the NG2/CSPG4 proteoglycan side chains. Once generated, plasmin then degrades NG2/CSPG4, both in an *in vitro* setting using recombinant protein, and *in vivo* models of spinal cord injury⁹³. NG2/CSPG4 may modulate the plasminogen system by enhancing plasminogen activation and inhibiting angiostatin⁹⁴.

As described above, the complex mechanisms by which NG2/CSPG4 affects melanoma progression have started to be defined, in particular the association with other cell surface proteins and receptor tyrosine kinases (RTKs) and its central role in modulating the function of these proteins. NG2/CSPG4 is essential to the growth of melanoma tumors through its modulation of integrin function and enhanced growth factor receptor-regulated pathways including sustained activation of ERK 1/2. The activation of integrins, RTK, and ERK 1/2 function by NG2/CSPG4 modulates numerous aspects of tumor progression⁹⁵. One intriguing report demonstrated that NG2/CSPG4 expression in human melanoma cells is epigenetically regulated, involving changes in promoter methylation, although specific mechanisms involved in NG2/CSPG4 locus regulation remain to be identified⁹⁶. As transmembrane co-receptor on tumor cells the main mechanism that involves NG2/CSPG4 in a leading role is the cell migration that we consider more in deep in the next paragraph.

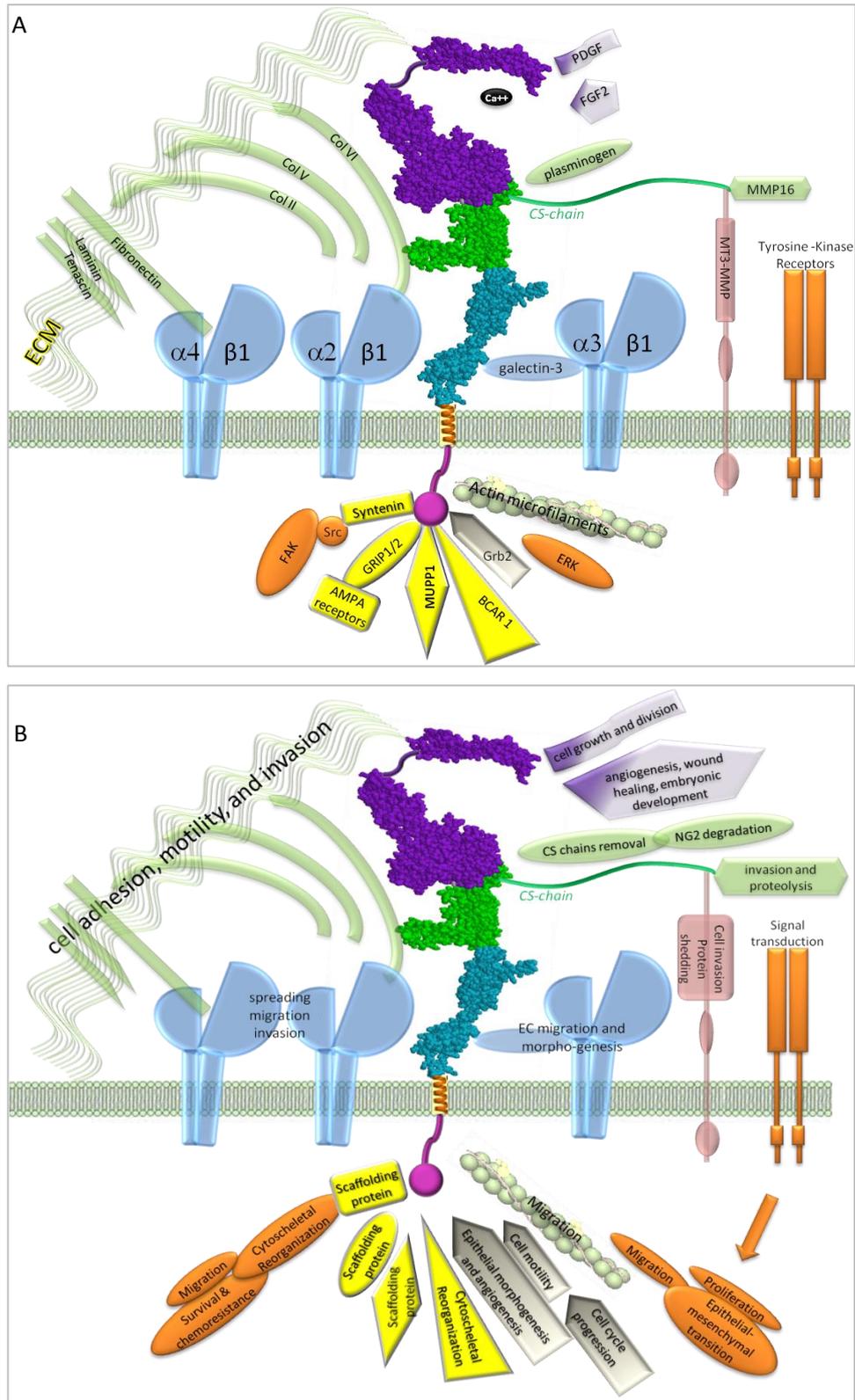


Figure 6: NG2/CSPG4 interactors molecules and functional significance. A) NG2/CSPG4 in the middle of the figure, shed with the color legend employed in figure2 and 4. Most of the interactors are reported with the same legend. ECM=Extracellular Matrix, ColII=Collagen II, ColV=Collagen V, ColVI=Collagen VI, $\alpha 2 \beta 1$, $\alpha 3 \beta 1$, $\alpha 4 \beta 1$ =integrins, MT3-MMP=Membrane-Type-3 Matrix Metalloproteinase, FAK=Focal Adhesion

Kinase, ERK=Extracellular signal-regulated Kinases, GRIP1/2= Glutamate receptor-interacting protein-1/2, AMPA=2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid, MUPP1= Multi-PDZ domain protein-1, FGF2= fibroblast growth factor-2, PDGFR α = Platelet-Derived Growth Factor Receptor- α , Grb2= Growth factor receptor-bound protein-2, BCAR1: breast cancer anti-estrogen resistance-1, CS-chain=Chondroitin sulphate chain. B) Here every NG2/CSPG4-interactor shown in A is replaced with the role that this interaction have in tumors cells. Most of the interaction reported lead to the regulation of cell migration, adhesion, spreading, proliferation.

1.4 Role of NG2/CSPG4 in cell proliferation and motility

Tumor cell invasion and metastasis is highly dependent on dynamic changes in the adhesion and migration of the malignant cells. Studies in this area have demonstrated that the recognition of extracellular matrix ligands, or adhesion promoting ligands expressed on neighboring cells (i.e. counter-receptors), involves complex molecular mechanisms. Some of these structures within extracellular matrix components act by binding integrins, whereas others bind additional receptors such as cell surface proteoglycans³². The vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these physiologic changes novel represents the successful breaching of an anticancer defense mechanism hardwired into cells and tissues⁹⁷. The first demonstration that NG2/CSPG4 can be important for cell motility came as a result of the finding that NG2/CSPG4 is a cell surface ligand for type VI collagen^{29,43,68,69,98} that binds the extended central D2 domain of NG2/CSPG4, as shown by studies in which recombinant deletion mutants of NG2/CSPG4 were expressed in rat B28 glioma cells⁴³. Since then several studies have been suggesting a role for NG2/CSPG4 in promoting the proliferation and motility that are characteristic of both normal progenitor cells and malignant tumor cells^{43,85,87,99}. NG2/CSPG4 seems to play a role in effective cell migration and many works on NG2/CSPG4 showed that melanoma cell attachment and spreading could be inhibited by NG2/CSPG4 antibodies^{4,100}. In addition NG2/CSPG4 was capable of triggering rearrangement of the actin cytoskeleton^{27,28,101}. Along with potentiating growth factor signaling and serving as a cell surface receptor for extracellular matrix components, NG2/CSPG4 seems to have the ability

to mediate activation of β 1-integrins. These molecular interactions would allow the proteoglycan to contribute to processes such as cell proliferation, cell motility and cell survival, typical of tumor cells⁵³. Post-translational modifications of NG2/CSPG4 provide an important means for regulating its interaction with extracellular and cytoplasmic binding partners. Phosphorylation and dephosphorylation have proved to be among the most versatile and functionally important types of posttranslational modifications^{102–104}. Two distinct Threonine phosphorylation events within the cytoplasmic domain of the NG2/CSPG4 proteoglycan seem to help to regulate the balance between cellular proliferation and motility. Evidences show that protein kinaseC- α could mediate the phosphorylation of NG2/CSPG4 at Thr₂₂₅₆, resulting in enhanced cell motility. Extracellular signal-regulated kinase seem to phosphorylate NG2/CSPG4 at Thr₂₃₁₄, stimulating cell proliferation. The effects of NG2/CSPG4 phosphorylation on proliferation and motility should be dependent on β 1-integrin activation⁵¹. Intriguingly, NG2/CSPG4 phosphorylated at Thr₂₂₅₆ was found to be co-localized with α 3 β 1-integrin in broad lamellipodia at the leading edges of motile cells⁵³. The phosphorylation at this site changes the distribution of NG2/CSPG4 on the cell surface. Whereas non-phosphorylated NG2/CSPG4 is localized to small membrane protrusions distributed over most of the cell surface, Thr₂₂₅₆-phosphorylated NG2/CSPG4 is largely associated with extensive lamellipodia at the cell periphery¹⁰⁴. This represents a translocation of NG2/CSPG4 from its co-localization with α 3 β 1 integrin on apical microprojections in non-motile cells, suggesting that NG2/CSPG4 phosphorylation at Thr₂₂₅₆ could be responsible for relocation of the NG2/CSPG4-integrin complex to lamellipodia, accompanied by increased cell motility⁵³ PKC- α -mediated. Thus NG2/CSPG4 phosphorylation at Thr₂₂₅₆ could be a key step for initiating cell polarization and motility⁵¹. Previous *in vitro* and *in vivo* studies demonstrated the involvement of NG2/CSPG4 in cell proliferation^{85,87,99}. A key finding with regard to the role of NG2/CSPG4 in cell proliferation was that the proteoglycan is capable of binding with high affinity to the growth factors FGF2 and PDGF-AA⁸⁸. Even if heparan sulfate proteoglycans act as co-receptor for members of the FGF family with the heparan sulfate chains¹⁰⁵, in the case of NG2/CSPG4, the core protein rather than the chondroitin sulfate chain is responsible for growth factor binding, with putative binding sites scattered throughout the D2 and D3 domains. As both

FGF2 and PDGF-AA are critical for expansion of the oligodendrocyte progenitor population, it was supposed that NG2/CSPG4 could be important for progenitor responsiveness to these two factors⁸⁴. This was also confirmed by the use of anti-NG2/CSPG4 antibodies that have effectively inhibited proliferation of oligodendrocyte progenitors⁸⁴. Other studies have shown that whereas the combined action of FGF2 and PDGF-AA was able to maintain wild type progenitors in their undifferentiated state, NG2/CSPG4 null progenitors began the process of differentiation even in the presence of the two growth factors. A similar set of findings using smooth muscle cell lines has been made by in our laboratory in the case of FGF2⁸¹. In the absence of NG2/CSPG4, cells do not sequester FGF2 at the cell surface and therefore are unable to activate FGF receptors⁸¹, leading to low proliferative responses. Phosphorylation of NG2/CSPG4 also plays a role in cell proliferation. ERK catalyzes phosphorylation of NG2/CSPG4 at Thr₂₃₁₄, stimulating cell proliferation⁵¹. The activation of $\alpha 3\beta 1$ -integrin is also required for this NG2/CSPG4-dependent increase in proliferation and NG2/CSPG4 phosphorylated at Thr₂₃₁₄ is co-localized with $\alpha 3\beta 1$ integrin on microprojections on the apical cell surface. It has been supposed that two distinct threonine phosphorylation events within the cytoplasmic domain of the NG2/CSPG4 proteoglycan seem to regulate the cellular balance between proliferation and motility. Evidences show that PKC- α could mediate the phosphorylation of NG2/CSPG4 at Thr₂₂₅₆, resulting in enhanced cell motility. Extracellular signal-regulated kinase seem to phosphorylate NG2/CSPG4 at Thr₂₃₁₄, stimulating cell proliferation. The effects of NG2/CSPG4 phosphorylation on proliferation and motility should be dependent on $\beta 1$ -integrin activation⁵¹, even if the details of these pathways remain to be determined.

1.5 Nature of NG2/CSPG4 isoforms

NG2/CSPG4 has been considered a putative target for immunotherapy against melanoma since the early 80's, and in more recent years several experiments have been performed to demonstrate that the inhibition of NG2/CSPG4 in several kinds of tumors leads to cell migration blocking^{6,71,100}, adhesion on fibronectin layer decreasing¹⁰⁶, reduction of tumors aggressiveness^{17,107-110}. Even if NG2/CSPG4 is a widely accepted immunotherapeutic target

for several cancer types, no anti-NG2/CSPG4 drug exists against tumors yet. From the employment of these mAbs in *in vivo* and *in vitro* experiments we could hypothesize the existence of different isoforms of the expressed protein among different cell lines. To this end table 1 summarize the antibodies employed in different experiments during the last 30 years. In many studies diverse mAbs have been employed with the aim to investigate the molecular and cellular heterogeneity of the NG2/CSPG4 synthesized by human cancer cells. The heterogeneity of the epitopes recognized by different mAbs was due, at least partly, to glycosylation of the antigen¹¹¹ but differences in core protein sequence cannot be excluded. In most of these works, where researcher tried to correlate the function of NG2/CSPG4 with its structural properties, they employed anti-NG2/CSPG4 monoclonal antibodies with the aim to block or, at least, interfere with the tumoral cells activities. In 1981 Bumol and Reisfeld performed a biosintetic study in M21 human melanoma cells employing the antibody 9.2.27 with high specificity for human melanoma cell surfaces. They could detect two different proteins: a 250kDa glycoprotein and a 400kDa high molecular weight proteoglycan. With these preliminary data they began to understand that the antigenic determinant recognized by monoclonal antibody 9.2.27 is located on a glycoprotein-proteoglycan complex which may have unique implications for the interaction of glycoconjugates at the human melanoma tumor cell surface¹¹². In 1987 Ferrone et al analyzed for the first time the effect of combinations of monoclonal antibodies to distinct determinants of the NG2/CSPG4 on the sensitivity of immunohistochemical assays to stain melanoma lesions. They employed mAbs 225.28, 657.9, and 902.5 recognizing distinct epitopes of the human high molecular weight melanoma associated antigen (HMW-MAA) to investigate the molecular and cellular heterogeneity of the NG2/CSPG4 synthesized by human melanoma cells. The mAbs 225.28, 657.9, and 902.5 immunoprecipitated the two characteristic components of the NG2/CSPG4 from an extract of human melanoma cells Colo38 ,while mAbs 657.9 and 902.5 precipitated Mr 250,000 and 90,000 proteins from the culture supernatant of Colo38 cells. MAb 225.28 precipitated a Mr 250,000 component but did not precipitate the Mr 90,000 component. Moreover thanks to a treatment of melanoma cells with glycosidases, they could observe that the heterogeneity of the epitopes recognized by the three monoclonal antibodies was, at least partly, due to

glycosylation of the antigen molecule, as they differentially bound the molecule. Additional experiments were then performed to determine the effect of the combination of the three anti-NG2/CSPG4 monoclonal antibodies on the staining of cultured melanoma cells and surgically removed melanoma lesions and showed heterogeneous distribution of the determinants recognized by mAbs 225.28, 657.9, and 902.5 on NG2/CSPG4 synthesized by a melanoma cell line¹¹¹. Three different anti-chondroitin sulphate monoclonal antibodies were used also in a study of Caterson and collaborators, each of which recognized a distinct epitope in native chondroitin sulphate¹¹³. Analyses indicated that these three antibodies reacted differently with chondroitin sulphates obtained from different sources, identifying three structurally distinct epitopes within chondroitin sulphates¹¹³. More significantly, the presence and, possibly, the location of these structures within the chondroitin sulphate chains varied with the source, developmental status and pathological status of cells that produce these molecules¹¹³. Antibodies against specific domains of the NG2/CSPG4 core protein have been employed to characterize three distinct molecular species of the proteoglycans expressed by different cell lines: in addition to the intact 300-kDa species, researcher identified a 290-kDa released form and a 275-kDa cell-associated form of the molecule, both of which lack the cytoplasmic domain⁴⁶. These works put the basis of a study where different anti-NG2/CSPG4 mAbs could be employed to identify different isoforms of the protein among tumors.

In 1981, Reisfeld obtained mAb 9.2.27 with high affinity for melanoma cells since it recognizes an epitope on the NG2/CSPG4¹¹⁴. This mAb has been widely employed from researchers to investigate distribution, structure and role of NG2/CSPG4 among tumors such as melanomas, breast cancers, glioblastoma (Tab. 1) and to investigate the putative effects that the mAb play in this cells. Recent findings show that this antibody seems to induces cell death in malignant melanoma cells through protein synthesis inhibition followed by some morphological and biochemical features of apoptosis¹¹⁵; it seems to have a specific cytotoxicity for NG2/CSPG4-positive cells¹¹⁶, and to suppress melanoma tumor growth in *in vivo* models^{107,108}.

Many anti-idiotypic mAbs have been product and employed for NG2/CSPG4 studies, such as TP41.2, TK7-371, MK2-23: they all showed specific blocking activity on different tumors and

resulted to be useful instruments for *in vivo* investigation of NG2/CSPG4 involvement in tumorigenesis, metastases and invasion processes (Tab. 1).

These and many other works listed in table 1 gave evidence of different NG2/CSPG4 forms expressed by different tumors: the 300 kDa fully processed form of the NG2/CSPG4 core protein, a 290 kDa piece that is secreted or shed into the medium and the 275 kDa piece that seems to be retained on the cell surface^{46,117}. It has been proposed that the cleaved 275 kDa form of NG2/CSPG4 remains associated with the cytoplasmic fragment via a non covalent interaction, allowing the larger extracellular fragment to remain at the cell surface¹¹⁷. The employment of antibody against NG2/CSPG4 in the last years led to several discoveries about the roles of this molecule in cancer development. There are evidence of a vaccine against NG2/CSPG4 that can trigger cell-mediated immune responses to this antigen, able to target not only tumor cells but also pericytes in the tumor vasculature¹¹⁸.

Even many bispecific antibody creation have been performed such as the atypical molecule rM28, a recombinant bispecific single-chain antibody directed to a melanoma-associated proteoglycan and to the costimulatory CD28 molecule on human T cells. The presentation of a CD28 antibody within a suitable recombinant, bispecific format could result in a “targeted supra-agonistic stimulation” of the CD28 molecule, which leads to effective tumor cell killing after induction of unspecifically lytic cells^{119,120}. More recently, several bispecific antibodies have been generated employing anti-NG2/CSPG4 monoclonal antibodies¹²¹ and among them a new bispecific T-cell engaging (BiTE) antibody resulted effective on tumor cell activities¹²². This antibody (MCSP-BiTE) binds to NG2/CSPG4 and human CD3, and when tested *in vitro* on human melanoma cell lines, it showed a specific cytotoxic activity against NG2/CSPG4-positive melanoma cell lines¹²². Finally for the first time a fully human scFv-Fc anti-NG2/CSPG4-specific antibody has been generated, by combining phage display scFv library technology and recombinant DNA cloning technology¹²³.

In summary, works employing anti-NG2/CSPG4 antibodies highlighted that: mAb 9.2.27 inhibits FAK activation⁴⁵, cell adhesion, and spreading¹²⁴, it induces clustering of NG2/CSPG4 and increases $\alpha 4\beta 1$ -integrin signaling; mAb 763.74 is an inhibitory antibodies, above all on 9.2.27 effects¹²⁵, and it is considered an anti-tumor antibody thanks to the evidence that it can induce a specific humoral response in patients with advanced melanoma and the

treatment significantly increased the survival of these patients^{126,127}; mAb 149.53 recognizes NG2/CSPG4 on cell membrane without any functional effect¹²⁸; mAb 225.28 has been tested above all on melanoma^{71,129} and triple-negative breast tumors¹⁷ where it has been seen to be able to block multiple signaling pathways important to cell growth, migration, and survival. NG2/CSPG4 has been shown to play an important role in growth, migration, and metastatic dissemination of tumor cells. It was used as a target for immunotherapy of melanoma because of its high expression in at least 80% of melanoma lesions with limited inter- and intra-lesional heterogeneity and its restricted distribution in normal tissues. Its clinical relevance is indicated by the statistically significant increase in survival of melanoma patients who developed NG2/CSPG4-specific antibodies following active-specific immunotherapy¹⁷.

We aimed to investigate whether NG2/CSPG4-specific mAbs can inhibit tumor recurrence and metastases. Our objectives included investigation of the existence of NG2/CSPG4 isoforms among tumors, as a useful instrument to study the effect of NG2/CSPG4-specific mAb on growth, adhesion, and migration of tumor cells, as well as activation of signaling pathways important for tumor cell growth, migration, and survival. To obtain new anti-NG2/CSPG4 mAbs we started a systematical production of mAbs with the Hybridoma technology, where immunization has been performed employing the extracellular portion of recombinant NG2/CSPG4 protein and from this immunization 63 clones have been selected and they have been characterized. We employed these mAbs to establish the expression patterns and subcellular distribution of NG2/CSPG4 in high aggressive tumors cells and tissues, and to investigate the existence of NG2/CSPG4 isoforms among different tumors.

The variable reactivity of mAbs against NG2/CSPG4 expressed from diverse cells or tissues, let us hypothesize that different isoforms of NG2/CSPG4 are expressed among the different tumors. Moreover Yang and collaborators found 18 punctiform differences between aminoacidic sequence of NG2/CSPG4 in A375 melanoma cell line and the sequence present in data bank GeneBank. One of the substitution found (C⁶³¹→R) may affect the folding of the NH2-terminal portion of the protein, which includes the laminin G domains⁴⁵. Taken together, these findings let us hypothesize the existence of isoforms among different cell lines.

Table 1. Summary of the anti-NG2/CSPG4 antibodies published.

ANTIBODY	ANTIBODY TYPE/ IMMUNOGEN	CELLS*	BANDING PATTERN	FUNCTIONAL EFFECTS
225.28	Anti-idiotypic antibody ¹¹¹	Colo38 ^{2,71,111,129-131} M21, SK-MEL-28, SK-MEL-37, 1520, MV3 ⁷¹ GP-1, GP-5, GP-6, and GP-8 ¹³² HS578T, MDA-MB-231, MDA-MB-435, and SUM149 ¹⁷	280 kDa, 240 kDa, 120 kDa, 80 kDa over 400 kDa, 300 - 700 kDa in range) ² , 280 kDa ¹³² , Precipitation of a 250 kDa component ¹¹¹	-Block of multiple signaling pathways important to cell growth, migration, and survival ⁷¹ . -Decrease of monocytes and granular eosinophils and increasing of circulating immune complexes ^{133,134} . -Antitumor effect on tumor metastases, spontaneous metastasis and tumor recurrence in breast cancer models in mice. Antitumor effect in the cell lines and in the mouse models ¹⁷ . -Inhibition of both tumor metastasis and tumor recurrence after surgical removal of orthotopic mammary primary tumors ¹⁷ . -Enhancement and prolongation the effects of the BRAF inhibitor in melanoma cells ⁷¹ .
657.9	Colo38 human melanoma cells ¹¹¹	Colo38 ¹¹¹	Precipitation of: 250 and 90 kDa component ¹¹¹	
902.5	Colo38 human melanoma cells ¹¹¹	Colo38 ^{111,129}	Precipitation of: 250 and 90 kDa component ¹¹¹	
9.2.27	M14 human melanoma cells ¹¹⁴	FEMX, SKMEL-28, LOX12, Melmet#1, MA-11, T47D, U87MG ¹³⁵ . OCM-1, OCM-3, OCM-8, Mel202, OMM-1 ¹¹⁶ . U87MG ¹³⁶ . A375 ^{23,25,44,62} M21 ^{4,107,108,112,137} . Chimpanzee melanoma cells ¹³⁸ . M2669-C1, M1477 ^{139,140} . FME ^{141,142} .	240, 400 kDa ¹¹² 97 kDa ^{143,144} 250 kDa ^{4,137-144}	-Activation of caspase-3 and PARP inactivation in FEMX and SKMEL-28 cells ¹³⁵ . -Induction of chromatin condensation in FEMX cells ¹³⁵ . -Specific cytotoxicity for NG2/CSPG4 -positive cells ¹¹⁶ . -Tumor growth suppression <i>in vitro</i> ²³ and <i>in vivo</i> ¹⁰⁷⁻¹⁰⁹ . -Three of nine malignant melanoma patients receiving the 9.2.27 monoclonal antibody showed an increase in antiglobulin titers. In patients developing antiglobulin responses, the response was rapid, typically being detectable within 2 weeks ¹¹⁰ .
TP41.2	Anti-idiotypic antibody ¹⁴⁵	Con, Gard, Gor, PPM-Mill, Phi, Rob, Hmeso, Ren ⁶ Colo 38 ^{145,146}		-Inhibition of cell adhesion resulting in decreased phosphorylation of FAK and Akt, reduced expression of cyclin D1 and apoptosis. -Reduced cell motility, migration, and invasiveness, and inhibited growth in soft agar. - <i>In vivo</i> treatment prevented or inhibited the growth of xenografts in SCID mice, with a significant increase in animal survival ⁶ .
7.1	Readus Cells (human marrow stroma cell line) ²⁴	COS, Pinkney, HeLa cells ²⁴ AML patient samples ^{24,147-150}	220- to 240-kDa ²⁴	

763.74	Anti-idiotypic antibody ¹²⁹	Colo 38 ^{100,129,131,145,151} , MV3 ¹⁰⁰ , MeWo ¹³¹ , MDA-MB-435 ¹⁷ , S5 and SK-MEL-28 ¹⁵²	250kDa ^{145,152} , 280 kDa, <440 kDa ¹⁷ , 450 kDa ¹⁵²	-Mediated cell-dependent cytotoxicity of melanoma cells ¹⁰⁰ . -Inhibition of spreading, migration and matrigel invasion of cells ¹⁰⁰ . -Ability to induce NG2/CSPG4-specific immune responses in BALB/c mice, in mice immunized with 763.74 bearing melanoma cells ¹⁵¹ . -Low concentration of mAb which reaches tumor lesions <i>in vivo</i> ¹³¹ .
TK7-371	Anti-idiotypic antibody ¹⁵³	Colo 38 in BALB/c mice and in rabbits ¹⁵³		-Elicitation of a delayed-type hypersensitivity reaction to NG2/CSPG4-bearing cells in syngeneic hosts and anti-HMW-MAA antibodies in BALB/c mice and in rabbits. -Induction of humoral immunity to self NG2/CSPG4 in patients with melanoma ¹⁵³ .
MK2-23	Anti-idiotypic antibody ¹⁵³	Colo38 ^{127,145,154-156}		-Induction of a delayed-type hypersensitivity reaction to NG2/CSPG4-bearing melanoma cells ^{154,155} . -Immune destruction of melanoma cells and interference with the metastatic potential of cells ¹²⁷ . -Reduction in the size of metastatic lesions in patients with malignant melanoma ^{157,158} .
155.8	plasma membrane-enriched fraction from human malignant melanoma cells M14 ¹⁵⁹	M14, L14, Melur cells ¹⁵⁹	250 kDa and a 400 kDa ¹⁵⁹	
149.53	Anti-idiotypic antibody ^{128,129}	Colo38 ¹²⁹⁻¹³¹ , MeWo ¹³¹ , GP-1, GP-5, GP-6, GP-8 ¹³²	280 kDa ¹³²	
MCSP-BiTE	bispecific T-cell engaging (BiTE) antibody that binds to NG2/CSPG4 and human CD3 ¹²²	Melanoma cell lines established from biopsied/resected metastatic lesions ¹²²		Cytotoxic activity in human melanoma cells ¹²² .
rM28	Recombinant bispecific single-chain antibody directed to NG2/CSPG4 and to the costimulatory CD28 molecule on human T ¹¹⁹	M21 (HLA A11/24, B15/35, DRB 04051/1301) SKMel63 (HLA A02/23, B49/50, DRB 04011/0701), Jurkat cells, OvGG ¹¹⁹ .		Tumor cell growth prevention ¹¹⁹ .
scFv-FcC21	scFv-Fc antibody ¹²³	Colo38, FO-1, M14, M21, Melur, MV3, and SK-MEL-28; LN443; PCI30; MDA-MB-231 and T47D; PPM-Mill; T24; PC3; MG-63; JY, LG-2, LKT13, Raji; ML-2 ¹²³		- <i>In vitro</i> inhibition growth and migration of tumor cells and <i>in vivo</i> growth of human tumor xenografts. These effects were mediated by inhibition of the activation of extracellular signal-regulated kinase and FAK signaling pathways that are critical for tumor cell growth and migration, respectively ¹²³ .

*Tumor cell lines in table 1 are as follows:

Human melanoma cell lines: Colo38, M14, M21, S5, SK-MEL-28, SK-MEL-37, 1520, MV3, A375, M21, FEMX, SKMEL-28, LOX12, Melmet#1, M2669-C1, M1477, FME, MeWo, SKMel63, Melur, FO-1

Human uveal melanoma cell lines: OCM-1, OCM-3, OCM-8, Mel202;

Cell line derived from a human uveal melanoma skin metastasis: OMM-1

Human breast cancer cell line: MA-1

Human triple-negative breast cancer cell lines : HS578T, MDA-MB-231, MDA-MB-435, and SUM149

Human ductal breast epithelial tumor cell line: T47D

Human glioblastoma multiforme cell line: U87MG

Human malignant mesothelioma cell lines: Con, Gard, Gor, PPM-Mill, Phi, Rob,Hmeso, Ren

Human marrow stroma cell line. Pinkney cell line

Human cervical cancer cell line: HeLa cells

Human Autologous lymphoblastoid cell line: L14

Human Ovarian carcinoma cell line: OvGG

Human glioma cell line: LN443

Human head and neck squamous cell carcinoma (SCCHN) cell line: PCI30

Human breast carcinoma cell lines: MDA-MB-231 and T47D

Human mesothelioma cell line: PPM-Mill

Human bladder carcinoma cell line: T24

Human prostate carcinoma cell line: PC3

Human osteosarcoma cell line: MG-63

Human B-lymphoid cell lines: JY, LG-2, LKT13, Raji

Human myeloid leukemia cell line: ML-2

Fibroblast-like cell line derived from monkey kidney tissue: COS

Guinea Pig Melanoma Cells: GP-1, GP-5, GP-6, GP-8

2. MATERIALS AND METHODS

2.1 *In silico* studies

- Genomic location of CSPG4 Gene and SNPs identification: research in Ensemble (<http://www.ensembl.org/index.html>) of the Gene ID: ENSG00000173546.

- CSPG4 Homologues research: submission of the aminoacid sequence of CSPG4 (Homo sapiens NP_001888.2 chondroitin sulfate proteoglycan 4 (CSPG4)) in MegaBlast (release 2.2.11) leads to the identification of 11 homologue sequences to the target.

Target sequence: Homo sapiens NP_001888.2 chondroitin sulfate proteoglycan 4 (CSPG4).

Omologue sequences:

- Pan troglodytes XP_001144835.3 chondroitin sulfate proteoglycan 4 (CSPG4)
- Bos taurus NP_001179711.1 chondroitin sulfate proteoglycan 4 (CSPG4)
- Felis catus XP_003986971.1 chondroitin sulfate proteoglycan 4 (CSPG4)
- Mus musculus NP_620570.2 chondroitin sulfate proteoglycan 4 (CSPG4)
- Rattus norvegicus NP_112284.1 chondroitin sulfate proteoglycan 4 (CSPG4)
- Oryctolagus cuniculus XP_002722087.1 chondroitin sulfate proteoglycan 4
- Canis lupus familiaris XP_544783.3 chondroitin sulfate proteoglycan 4 (CSPG4)
- Gallus gallus XP_423277.3 chondroitin sulfate proteoglycan 4
- Danio rerio XP_001923457.2 chondroitin sulfate proteoglycan 4
- Drosophila melanogaster AAF53672.3
- Caenorhabditis elegans C48E7.6

- Alignments: Alignment of aminoacidic sequences of CSPG4 homologues to the human NP_001888.2 using CLUSTALW (www.ebi.ac.uk/clustalw) with default settings; the alignment is presented by GENEDOC (www.psc.edu/biomed/genedoc) and visualized in modality Summary view (Fig. 2).

SeqLogos (<http://weblogo.berkeley.edu/logo.cgi>) of the Collagen VI binding region of CSPG4 (aminoacid range: 574-1040) obtained from the multiple alignment has been performed.

- Phylogenesis: first the distances (expressed as percent divergence) between all pairs of sequence have been calculated from a multiple alignment using CLUSTALW; then the NJ method has been applied to the distance matrix. BOOTSTRAP N-J TREE method has been

employed. Phylogenetic Tree of the 11 sequences found to be homologue to the human protein sequence NP_001888.2 chondroitin sulfate proteoglycan 4 (CSPG4) has been output in phylip format and visualized with TeeView (<http://www.treeview.net/>). The tree was rooted setting *Ceanorabditis elegans* as outgroup, since it is evolutionarily the fairest organism from *Homo sapiens*.

- NG2/CSPG4 structure prediction: a template selection search was performed using BLAST-P¹⁶⁰ against PDB¹⁶¹ database from NCBI interface simultaneously “Template Identification Tool” at SWISS-MODEL interface¹⁶² provided by Swiss Institute of Bioinformatics was utilized for template selection. In results, 21 significant hits with E-value zero were observed. SWISS-MODEL server was used to generate models and Swiss Pdb-Viewer (<http://spdbv.vital-it.ch/>) was employed to create a PDB file of the predicted tertiary structure of NG2/CSPG4. Structure has been visualized and modified with RasMol (<http://rasmol.org/>).

2.2 Tissue specimens

Paraffin-embedded of 22 weeks-old human fetal brain tissues and from glioblastoma multiforme lesions have been employed. For each kind of tissue, replicates of sixteen fresh tissue microtome sections, 10 mm-thick, 80 mm² wide, were scraped on ice using a wet scraper and immersed into the extraction buffer (20 mM Tris-HCl pH 8.8, 2% SDS, 200 mM DTT, 7M Urea, 1 tablet of “complete mini” Roche, 0.8 mM Pefabloc® SC). Lysates were collected in Eppendorf safe-lock tubes in a final volume of 800µl of extraction buffer and sonicated at the following conditions: 5% output, 5 sec pulse and 30 sec break cooling on ice, repeating 24 times to total sonication time of 2 min per sample. The material was then heated up to 100°C for 20 min and the extracts clarified for 15 min at 16,000xg at 4°C and quantified by the Bradford method¹⁶³.

2.3 Cell lines

Cell lines classification:

- A375, M2, COLO38: human malignant melanoma cell lines
- SK-UT-1: human uterus leiomyosarcoma cell line
- SK-LMS-1: human vulvar leiomyosarcoma cell line
- 143B: human osteosarcoma sarcoma cell line
- HT1080: human fibrosarcoma cell line

Cells listed above were provided by ATCC, and cultured in DMEM (Dulbecco's modified Eagle's medium, Lonza BioWhittaker®), low Glucose 1.0 g/L, supplemented with Penicillin 100 U/ml and Streptomycin 100 U/ml (Pen/Strep, Lonza BioWhittaker®), L-Glutamine 2 mM (Lonza BioWhittaker®), 10% (v/v) FBS (Fetal Bovine Serum, Gibco®).

Producing anti-NG2/CSPG4 mAbs hybridoma cells were cultured in DMEM (Dulbecco's modified Eagle's medium, Lonza BioWhittaker®), Glucose 4.5 g/L, with Penicillin 100 U/ml and Streptomycin 100 U/ml (Pen/Strep, Lonza BioWhittaker®), L-Glutamine 2 mM (Lonza BioWhittaker®) added with 20% FBS tested hybridoma serum and 0.01% oxaloacetic acid (that favors the Krebs cycle, increasing cellular respiration, thus, encouraging cell proliferation and antibody production) at first. By the time the nutrients are diminished to 10% FBS without oxaloacetic acid.

2.4 Various reagents

Commercial hybridoma cells producing the anti-NG2/CSPG4 mAb B5/M28 were obtained from ATCC. The antiserum against β -actin was purchased from Sigma-Aldrich. Anti-NG2/CSPG4 mAb 9.2.27 MAB2029, Millipore. Anti-FAK and anti-Paxillin, anti-Cofilin, anti-Fascin, have been purchased by abcam and employed following datasheets.

Secondary antibodies for immunocytochemistry staining, Alexa 488, Alexa 594 (mouse/rabbit) have been purchased from LifeTechnologies and employed as advised from datasheets. Antibodies employed in Flow Cytometry: Mouse IgG-PE Isotype control (cat 731624 Beckman Coulter), anti-NG2/CSPG4-PE (PN IM3454U-Clone 7,1 BD Biosciences).

Mouse anti-human chondroitin sulfate proteoglycan monoclonal antibody (9.2.27 MAB2029, Millipore). Secondary antibody AlexaFluor488. Chicken anti mouse IgG (H+L) from Invitrogen.

Reagents for apoptosis detection in microplate laser cytometer acumen®eX3: 0,7µg/ml PI and 1 µg/ml Hoechst have been employed for nucleous staining of respectively late apoptotic and living cells. Early apoptosis was detected employing reagents from Life technologies following manufacture specifications: Mitochondrial Membrane Potential Apoptosis Kit with Mitotracker™ Red & Annexin V Alexa Fluor®488; PARP FITC Apoptosis Kit - FITC Conjugated; CellEvent™ Caspase-3/7 Green Detection Reagent.

For the immunoprecipitation irrelevant mouse IgG antibody from Sigma has been employed.

2.5 Generation of full-length NG2/CSPG4 construct and recombinant protein production

Gateway® pDEST™ 26 Vector N-term-X6-His tag has been employed for the gene expression. Sequence from 1 to 6552 nucleotides of cDNA has been cloned, as it codes for the NG2/CSPG4 ectodomain from aminoacids 1 to 2184. The transfection has been performed in Human Embryonic Kidney 293 cells (HEK293) and Chinese hamster ovary cells (CHO) with Metafectene, for the production of the protein in an eukaryotic system. Recombinant protein has been purified with HisTrap HP columns prepacked with Ni Sepharose (GE Life Sciences).

2.6 Antibody production, purification and characterization

Female Balb/c mice were immunized with the recombinant extracellular portion of human NG2/CSPG4 (amino acids 1-2184 of the protein inserted into a pEF6V5his vector) produced in HEK293 cells by repeated intraperitoneal injections of the immunogen solubilized in complete Freud's adjuvant (Sigma-Aldrich, St Louis, MI, USA). From ELISA screenings of hybridoma clones generated by fusion of NS1 murine myeloma cells with spleen cells from mice that developed an anti-NG2/CSPG4 immune-response, we selected 63 clones for

further characterization. Reactivity traits of the clones were established by immunoblotting, using the immunogen, human melanoma cell lysates from A375 and M2 cell lines, and human sarcoma SK-UT-1, SK-LMS1, and HT1080^{NG2+} cell lines (see below). Moreover reactivity traits of clones was assayed by cell-E.L.I.S.A., Flow Cytometry, and immunocytochemistry. Clones were isotypized using the Pierce® Rapid ELISA Mouse mAb Isotyping Kit. Several of the clones were also used for the production of ascites fluids in Balb/c or nude mice. mAbs were purified with affi gel protein A (Bio-rad), and finally quantified.

2.7 Immunocytochemistry

Cells were seeded on coverslips and fixed with PFA 4% for 20 minutes. In the case of scratch assay coupled to immunocytochemistry staining, seeded cells were starved in absence of FBS overnight, then a scratch was done on the cells monolayer and after 24 hours of migration they were fixed in PFA 4%. Fixed cells were incubated with blocking buffer (5% Normal Goat Serum and 10% BSA in PBS) for 30 minutes at room temperature. In experiments with labeling inside the cell membrane, cells were permeabilized with an incubation of 5 seconds in Triton™ X-100 0,1%. Then primary antibodies, fluorescent secondary antibodies and Hoechst were applied as described above.

2.8 Immunoblotting

- Proteins from cells: cells were solubilized at 4°C with RIPA lysis buffer (50mM Tris, pH 7.4; 150mM NaCl; 0,5% Na-deoxycholate, 0,1% SDS, 1% Nonidet P-40, 2mM EDTA, 1 tablet of “complete mini” Roche, Pefabloc® SC 0,8mM) for 15 minutes. Lysates were clarified and the supernatant containing proteins was transferred to a fresh tube on ice, and quantified.
- Proteins from tissues: samples were prepared from paraffin-embedded 22 weeks-old human fetal brain tissues and from glioblastoma multiforme lesions. For each kind of tissue, replicates of sixteen fresh tissue microtome sections, 10 mm-thick, 80 mm² wide, were scraped on ice using a wet scraper and immersed into the extraction buffer (20 mM Tris-HCl

pH 8.8, 2% SDS, 200 mM DTT, 7M Urea, 1 tablet of “complete mini” Roche, 0.8 mM Pefabloc® SC). Lysates were collected in Eppendorf safe-lock tubes in a final volume of 800µl of extraction buffer and sonicated at the following conditions: 5% output, 5 sec pulse and 30 sec break cooling on ice, repeating 24 times to total sonication time of 2 min per sample. The material was then heated up to 100°C for 20 min and the extracts clarified for 15 min at 16,000xg at 4°C and quantified by the Bradford method.

- SDS-PAGE: samples were solubilized in 5X SDS-PAGE loading buffer (250mM Tris-HCl, pH 6.8, 2.5% SDS; 35% Glycerol, 0.025% (w/v) Bromophenol blue, 125 mM DTT) and resolved on Tris-HCl 5% or pre-cast 4-15% linear gradient gels (Bio-Rad), or native PAGE gels (Life Technologies, Inc.). Precision Plus Protein™ Dual Xtra standards (2–250 kDa), Unstained HiMark Standards (Life Technology Inc.) and Precision Plus Protein DualXtra standards (Bio-rad) were employed as molecular markers.

- Blotting: resolved proteins were transferred overnight onto nitrocellulose membranes and the membrane were saturated with blocking buffer (5% dry milk in TBS containing 0.1% Tween-20). Blocked membranes were incubated with the anti-NG2/CSPG4 mAbs (1:2 – 1:10 dilution, supernatants, or 1:150 dilution; ascites fluids) in blocking buffer. Membranes were incubated for 1 hour at room temperature with secondary antibodies conjugated to HRP (Sigma-Aldrich). Immunolabelled bands were visualized with the ECL Plus Chemiluminescence detection kit (Bio-rad). A polyclonal antiserum against β-actin was employed as a calibrator (diluted 1:400 in TBS-Tween 0,1%), following revealing with the secondary anti-rabbit antibodies conjugated to HRP (Sigma-Aldrich).

Experiments of Western Blotting/Scratch Assay-coupled were performed to study NG2/CSPG4 phosphorylation in immunosorted HT1080^{NG2+} sarcoma cells. HT1080^{NG2+} were seeded in three different plate dishes and treated with different growth conditions, with the aim to obtain three different protein expression patterns: 1) A plate dish was seeded with medium added with 10% FBS, and once it reached a 80% confluence the extraction of proteins was performed according to the protocol mentioned above with RIPA buffer in this section. Protein extract revealed the proliferating expression pattern (P). 2) The second dish was seeded with the same number of cells with complete growth medium containing 10% FBS, but in such a way that they were already at the 95% confluence, when the cells were

starved for 24 hours in serum-free medium, to block proliferation. Then a number of scratches were made with a small tip. After 48 hours of migration in serum-free medium, protein extraction was performed as usual. The protein expression pattern of migrating cells was obtained (M). 3) A third dish was seeded as control, following the same procedures described in point (2) but the scratch, so we could compare the protein expression pattern during the starvation without any scratch (S). Extracted proteins were quantified and 30µg for each of three different protein lysates were analyzed in SDS-PAGE using polyacrylamide gels (5%), as described above and the Western blotting was performed according to the protocol already described. To block the aspecific link of antibodies to the nitrocellulose membranes, they were incubated for 30 minutes at room temperature on a shaker using a blocking solution containing 2% of BSA. The hybridization with primary antibodies was performed over night at 4 °C on a shaker. Antibodies have been used in a 1:1000 dilution in 5% BSA in TBSTween 0,1%. The two primary antibodies employed were: the antibody anti NG2/CSPG4 sc-33038 (Santa Cruz Biotechnology, inc.) that reacts with all phosphorylated Threonines of NG2/CSPG4 of human origin; the Phospho-Threonine-X-Arginine Antibody #2351 (Cell Signaling Technology®), that detects endogenous levels of proteins containing the motive of NG2/CSPG4 belonging the Thr₂₂₅₆ we suppose is phosphorylated during cell migration. Then the membranes were incubated with secondary antibody anti-rabbit-HRP and the immunocomplexes highlighted by ECL Plus chemiluminescent detection system, as already described.

2.9 Cell-E.L.I.S.A.

Fixed cell-E.L.I.S.A.:

Cells were seeded in 96multiwell plates in complete growth medium (10000 cells/well). After the fixation with PFA2% for 30 minutes, the block of endogenous peroxidase have been performed with 3% H₂O₂ for 15 minutes at room temperature. Blocking aspecific sites has been performed dosing blocking buffer (PBS con2% di BSA; 10% sucrose; 0,1% NaN₃) to the cells for 30 minutes at room temperature. Cells were incubated over night at 4°C with the anti-NG2/CSPG4 mAbs, diluted as follows: surnatants 1:1 in blocking buffer, ascites 1:50

in blocking buffer. Streptavidin/Biotin system (Thermo Scientific TS-125-HR and TM-125-BN) and TMB (T4444 Sigma) revelation have been employed for the detection of the binding between mAbs and their antigen. They have been employed as recommended by the datasheet manufactured. All tests were done in triplicate.

Live cell-E.L.I.S.A.:

Cells were seeded in 96 multiwell plates in complete growth medium (melanoma cells cells=25000 cells/well; sarcoma=20000 cells/well). Blocking aspecific sites has been performed dosing sterile blocking buffer (PBS con 2% di BSA; 10% sucrose) to the cells for 30 minutes within the cell culture incubator. Then cells were incubated for 2 hours at 37°C with the anti-NG2/CSPG4 mAbs, diluted as follows: surnatants 1:1 in blocking buffer, ascites 1:50 in blocking buffer. After the fixation with PFA 2% for 30 minutes, the block of endogenous peroxidase have been performed with 3% H₂O₂ for 15 minutes at room temperature. Streptavidin/Biotin system (Thermo Scientific TS-125-HR and TM-125-BN) and TMB (T4444 Sigma) revelation have been employed for the detection of the binding between mAbs and their antigen. They have been employed as recommended by the datasheet manufactured. All tests were done in triplicate.

2.10 Flow Cytometry and cell sorting

All flow cytometry measurements were performed on a FACSCalibur (Becton Dickinson) and 10000 gated events were collected for each sample. When cells were used, a gate was set during acquisition on the forward scatter (FSC) vs. side scatter (SSC) plot to exclude fragment of dead cells.

FACS analysis of sorted cells

The positivity for CSPG4 was conferred analyzing F2 channel at value of 543 for HT1080 cells. Cells were detached from flask using EDTA 5mM and washed twicely with PBS and than resuspended in PBS at a concentration of 5×10^6 cells/ml.

50µl of cells was incubated for 15min at RT with 1µl of mouse IgG-PE or 20µl of antibody clone 7.1 + 30µl PBS. Than cells was washed with 2 ml PBS and resuspended in 300µl PBS and analyzed.

Cell Sorting

Cells HT1080 were detached from flask using EDTA 5mM and washed twicely with sorting Buffer (EDTA 2mM and BSA 0,5% in PBS without calcium and magnesium). Cells were counted (10×10^6) and resuspended in 240µl of buffer and incubated with 60 µl of Ab anti-NG2/CSPG4 7.1 for 30 minutes at 4°C, than cells were washed in 10 ml of buffer and resuspended in 3 ml of buffer in a sterile vial. Cells were analyzed with FACSAria cell sorter II (Becton Dickinson) and data were anlyzed with FACSDiva version 6.1.3. Several controls were performed to determine appropriated gates, voltages, flux and compensation. For the sorting we collected cells in two tubes: A (NG2/CSPG4 + 355173 cells), B (NG2/CSPG4 - 4214322 cells). We plated cell and made growth them for some days. Than we analyzed again cells with FACS Calibur. Immunsorted cell lines were obtained separating cells belonging to the same population of HT1080 according to the expression of NG2/CSPG4 using the patented MACS[®] columns and beads conjugated (Myltenyi Biotec GmbH, Germany). First, the cells were labeled with the primary anti-NG2/CSPG4 mAb 9.2.27. Subsequently, the cells were magnetically labeled with anti-mouse IgG MicroBeads. Then the cell suspension was loaded onto MACS[®] column, which was placed in a magnetic field of a MACS separator. The magnetically labeled cells were retained within the column, while the unlabeled cells were discarded. After removing the column from the magnetic field, the magnetically retained cells could be eluted as the positively selected cell fraction.

2.11 Immunoprecipitation and mass spectrometry

- Protein cross-linking: this procedure has been performed on the cells surface maintaining pH=8, employing Thermo Scientific Pierce BS3 (Sulfo-DSS) is bis(sulfosuccinimidyl)suberate 2mM for 30minutes at room temperature. The reaction was quenched with Tris-HCl 20mM pH=7.4 for 15 minutes at romm temperature.

- Protein extraction from cells: cells were solubilized at 4°C with Lysis Buffer (50mM Tris-HCl, pH 7.4; 150mM NaCl; 0,5% Na-deoxycholate, 1% Nonidet P-40, 1 tablet of “complete mini” Roche, Pefabloc® SC 0,8mM) for 20 minutes. Lysates were clarified and each supernatant containing proteins was transferred to a fresh tube on ice, and finally quantified by the Bradford method.
- Immunoprecipitation: pre-clearing has been performed adding 100µl protein A/G and 5µg of irrelevant mouse IgG antibody for each mg of sample protein and incubating for 30 minutes in agitation on ice. Samples were then centrifuged at 1000 g for 5 minutes at 4°C. Pellets were discarded and supernatant were saved for the immunoprecipitation. To each mg of proteins 120µl protein A/G were added, then samples were incubated overnight in agitation on ice with anti-NG2/CSPG4 purified mAbs at the final concentration of 2,5µg/ml. Immunoprecipitated proteins were centrifuged at 1000 g for 5 minutes at 4°C, and washed with the lysis buffer twice, always discarding the supernatant, as immunoprecipitated proteins remains in the pellet in a mix with protein A/G and anti-NG2/CSPG4 mAb. Finally proteins were resuspended in ultrapure MilliQ water and quantified. Then we used to reduce the pH of samples to permit the separation of NG2/CSPG4 from the protein A/G and the anti-NG2/CSPG4 mAb employed for the immunoprecipitation (buffer: 40%Acetonitril, 0,2%TFA, 4%TCA). Afterwards samples were purified with a microcon (cut-off 50 kDa), to obtain the protein in its elution buffer and water in a final volume of about 15-20 µL (final sample buffer: 12%ACN; 0,06%TFA; 1,2%TCA).
- Protein reduction: incubation of samples with 5mM dithiothreitol (DTT) for 1 hour at 60°C.
- Protein alkylation: incubation of samples with 18mM iodoacetamide (IAA) for 30 minutes at 25°C.
- Trypsin digestion: The complete digestion has been obtained in two step, first adding 16 µg/ml of trypsin to samples and incubating overnight at 37°C, then adding a second rate of enzyme at the same conditions for 8 hours. Reaction was stopped adding 13µl of 1% formic acid to each reaction tube.
- MALDI-TOF analysis: mass spectra were acquired using a 4800 Plus MALDI TOF/TOF™ mass spectrometer. Protein samples were prepared for MALDI-TOF mass spectrometry by mixing the sample solution with alpha-cyano-4-hydroxycinnamic acid (HCCA) solution. 1 µl

of this solution was dried on a metal target, forming cocrystals of matrix and protein. Details in sample preparation are given above. Spectra of proteins were calibrated externally using a mixture containing bradykinin fragment 1–7 (m/z 757.3997), angiotensin II (human) (m/z 1046.5423), P14R (m/z 1533.8582) and ACTH fragment 18–39 (human) (m/z 2465.1989). The mass spectra were acquired in positive mode in the range 700–3500 m/z. Data have been analyzed with the Open Source Mass Spectrometry Tool mMass^{164–166} that permitted also the research in MASCOT, a search engine that uses mass spectrometry data to identify proteins from primary sequence databases.

-Search Parameters. Type of search: Peptide Mass Fingerprint; Enzyme: Trypsin; Fixed modifications: Carboxymethyl (C); Variable modifications: Phospho (ST); Mass values: Monoisotopic; Protein Mass: Unrestricted; Peptide Mass Tolerance: ± 100 ppm; Peptide Charge State: 1+; Max Missed Cleavages: 1; Taxonomy: Human (Homo sapiens); Database: SwissProt.

The work flow followed for the analysis is outlined in figure 7.

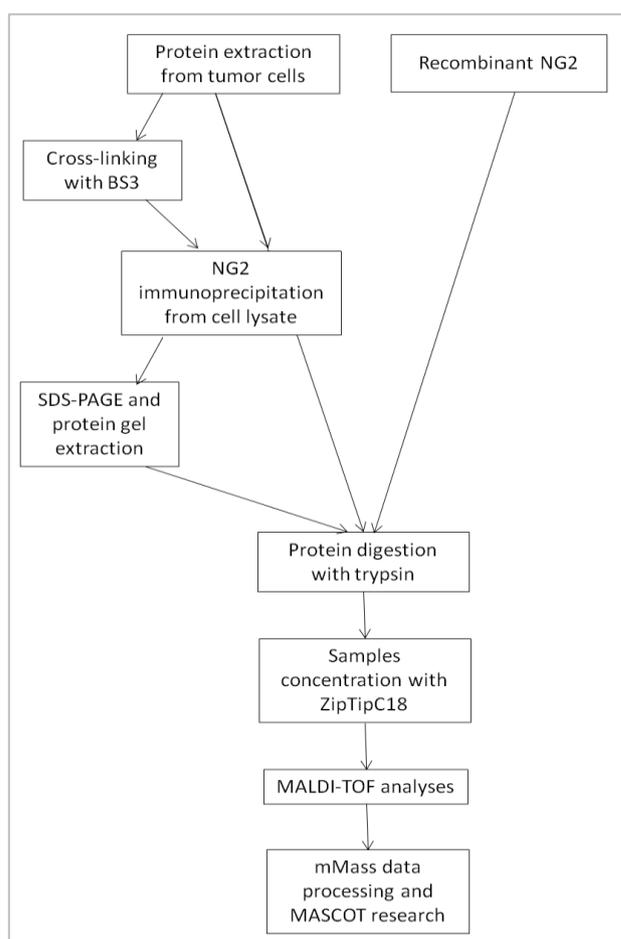


Figure 7: Work flow followed for the proteomic analysis. Three kinds of samples were prepared: proteins extracted from tumor cells and immunoprecipitated with anti-NG2/CSPG4 mAbs or the same sample run in a SDS-PAGE and extracted from gel. Protein extracted from tumor cells, crosslinked with BS3, and then immunoprecipitated with anti-NG2/CSPG4 mAbs, or the same sample run into a SDS-page and extracted from the gel. Recombinant NG2/CSPG4 ectodomain. Samples were digested with trypsin, purified and concentrated, and finally analyzed with MALDI-TOF.

2.12. Functional Studies

2.12.1 Apoptosis Assays

Cells were seeded on 96 multiwell plate and in their growth medium (1000 cells/well) and starved overnight in DMEM serum-free. The anti-NG2/CSPG4 purified mAbs were dosed on cells 18 µg/ml and incubated for 24 hours. Apoptosis markers were employed as described above, and apoptosis has been detected employing the MicroPlate Laser Cytometer acumen®eX3.

2.12.2 Cell Adhesion assay

Cells were seeded on Roche E-Plates microelectrode array in their growth medium (25000 cells/well). Then cells were starved in DMEM serum free for 24 hours. MABs administrating to starved cells: pure surnatants and diluted 1:100 in hybridoma growth medium ascites were used. Real-time monitoring of mAbs effect on cellular adhesion with the xCELLigence System (Roche).

2.12.3 Cell Migration assay

Cells were seeded (75000 cells/well) at 90% confluence in 48 multiwell plates in their growth medium. After the cells were adherent, a 24 hours starvation in serum free medium was performed to block cells proliferation. Then a surface incision was practiced at the diameter of the well in the longitudinal direction, with a small tip. One wash was performed with PBS 1X to remove cell debris produced by the operation^{167,168}. Every well was treated with different medium as follows: a) antibodies in surnatant were added in their own growth medium; b) antibodies in ascites were added 1:200 in the same growth medium used in point a.; c) growth medium with irrilevant IgG1 was used as negative control. The multiwell plate was placed under the microscope and fotograms of scratches were taken in regular intervals of 5 minutes over an extended period of 24 hours. The positions of individual cells were then marked in consecutive images, thus tracking positional changes of the cells over time. This tracking procedure was performed manually through “point and click” systems¹⁶⁹.

3. RESULTS

3.1 Selection of anti-NG2/CSPG4 clones and their characteristics

By conventional immunization of mice and hybridoma production, 63 clones and their subclones have been obtained and subclones were isotypized (Tab. 2).

Table 2. Anti-NG2/CSPG4 mAbs list. Some clones have been subcloned and isotypized.

CLONE	Subclone	Ig isotype		CLONE	Subclone	Ig isotype	
		H chain	L chain			H chain	L chain
NG 2161A4	-			NG 2164G7	-		
	B11	IgG1	k		A2	IgGM	k
	C12	IgG1	k		G4	IgG1	k
	H12	IgG1	k		H9	IgG1	k
NG 2161A8				NG 2164H5			
NG 2161B1					A8	IgG3	k
NG 2161C1					A11	IgG1	k
NG 2161D2					B9	IgG1	k
NG 2161D3				NG 2164H9			
	A7	IgG1	k	NG 2166G4			
	E10	IgG1	k		A5	IgG1	k
	G4	IgG1	k		B9	IgG1	k
	H9	IgG1	k		E5	IgG1	k
NG 2161D4					G5	IgG3	k
NG 2161D7	-			NG 2166G2			
	C10	IgG1	k	NG 2172A2			
	D3	IgG1	k	NG 2172B5			
	G1	IgG1	k	NG 2172B12			
	G10	IgG1	k		C10	IgG1	k
NG 2161E1					F10	IgG1	k
NG 2161F8					F12	IgG2b	k
NG 2161F9					G10	IgG2a	k
	C2	IgG1	k	NG 2172C2			
	D2	IgG1	k	NG 2172C3			
	D5	IgG1	k	NG 2172C10			
	E6	IgG1	k	NG 2172C12			
NG 2161G7				NG 2172D6			
NG 2161G8					B1	IgG1	k
NG 2161G11					B2	IgG1	k
NG 2161H2				NG 2172D11	G12	IgG1	k
NG 2161H3				NG 2172D12	-		
NG 2161H8					G10	IgG2a	k
NG 2163A1					G11	IgG1	k
NG 2163B1				NG 2172E8			
NG 2163C3				NG 2172E9			
NG 2163D7				NG 2172F4			
NG 2163E9				NG 2172F11			
NG 2163F4					B9	IgG2a	k
NG 2163H4					F3	IgM	λ
NG 2164A2					F9	IgG3	k
NG 2164A7					G2	IgG3	k
NG 2164B6					G3	IgG3	k
	B1	IgG1	k	NG 2172G3			
	F3	IgG1	k	NG 2172G4			
	D2	IgG1	k	NG 2172G6			
NG 2164C3				NG 2172H6			
	G8	IgM	k	NG 2172H12			
	H10	IgM	k	B5M28		IgG2b	k
	H12	IgG1	K				

The panel of anti-NG2/CSPG4 mAbs obtained have been employed for preliminary screenings of expressing-NG2 cells in cell-E.L.I.S.A. experiments on living and fixed cells (SK-LMS1, SK-UT-1, HT1080^{NG2+}, 143B, A375 and M2 cell lines). The results of these screenings are resumed in table 3. Each mAb has different abilities to recognize NG2/CSPG4 on tumor cell membrane. Antibodies have different patterns when tested on living cells and on 2% PFA-fixed cells: some antibodies, such as mAb 2161D7, are more reactive on their antigen when expressed on living cells, while others are more reactive when their antigen is expressed on fixed cells. Example of this behavior is mAb 2161A4 that links NG2/CSPG4 on SK-UT-1 cells only when they are fixed, while mAb 2161B1 behaves on contrary on A375 cells. We could observe that no one of the mAbs tested is able to recognize NG2/CSPG4 on M2 cells, even if this melanoma cell line expresses very high levels of NG2/CSPG4 (Tab. 3). Distribution of NG2/CSPG4 has been detected testing different mAbs on HT1080^{NG2+} and A375 cells (Fig. 8). In immunocytochemistry staining, as expected, we observed the distribution of NG2/CSPG4 on the cell membrane of both kinds of tumor cells and a different intensity of staining could be due to the efficiency that each antibody has in the binding of its antigen.

As we expected, NG2/CSPG4 is distributed above all on cell membrane, as we can observe when immunolocalized by all the positive anti-NG2/CSPG4 mAbs tested, apart from the negative results obtained with mAb 2161D2 on both cell lines. In some cases NG2/CSPG4 seems to immuno-localize in cytoplasmic regions (2161D3, 2164B6, 2172D6, 2166G4, 2161D7 on both cell lines) and in some other cases in perinuclear districts (2161D3, 2172D6, 2161D7 on both cell lines tested) (Fig. 8). No one among the tested anti-NG2/CSPG4 mAbs could bind to NG2/CSPG4 on M2 cell membrane (data not shown), and this confirms data previously obtained from the other screenings.

Flow Cytometry analysis, performed on several cell lines, confirmed what observed before on M2 cells, as no one among the tested antibodies could recognize NG2/CSPG4 expressed from this cell line (Tab. 3). Moreover, these screenings gave evidence of a great variability of results among the different cell lines studied, with the confirmation of our hypothesis concerning the putative existence of many forms cell-line specific of NG2/CSPG4 (Tab.3 and Fig. 9).

Table 3. NG2/CSPG4 relative expression on different tumors cell membrane. Data are expressed in percentage (%) compared to a negative control. n.d.: not determined data.

CLONE	Flow Cytometry							cell-ELISA (fixed cells)				cell-ELISA (alive cells)			
	SKLMS-1	SKUT-1	COLO38	HT1080 ^{NG2+}	A375	M2	143B	A375	SKUT1	143B	M2	A375	SKUT-1	SK-LMS-1	M2
NG 2161A4	0	0	9,18	3,5	0	0	0	24,6	54,04	37	20,2	38,2	0	23,5	0
NG 2161B1	59,4	78,8	51,06	89,2	96,8	0	86,1	41,1	37,8	39	0	0	22,325	28,971	0
NG 2161C1	20,07	8,05	12,15	n.d.	n.d.	n.d.	n.d.	0	0	5,3	0	7,36	4,46	0	0
NG 2161D2	3,11	11,65	18,8	0	n.d.	n.d.	n.d.	10,6	1,25	8,55	0	41,6	15	24,7	0
NG 2161D3	53,6	38,8	11,3	40,4	84	0	31,9	51,9	24,2	47,6	0	66,4	26,4	52,8	0
NG 2161D4	12,93	0,86	26,83	n.d.	n.d.	n.d.	n.d.	35,9	0	12,9	0	0	2,1	0	0
NG 2161D7	76,8	46,6	18,5	92,8	92,5	0	58,3	53,8	30,1	38	0	72,9	51,1	48,9	0
NG 2161E1	59,4	62,2	93,17	77,3	93,6	0	62,5	39,2	22,877	21,0	0	13,7	0	0	0
NG 2161F8	5,06	0,14	44,13	n.d.	n.d.	n.d.	n.d.	0	0	1,02	0	0	8,97	0	0
NG 2161F9	49,2	33	88,44	86,9	91	0	28,9	30,5	27,7	40,5	0	60,9	45,4	38,2	0
NG 2161G7	72,74	3,06	98,84	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3,81	0	0	0
NG 2161G8	23,01	3,36	47,46	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	0	0
NG 2161G11	10,47	9,44	12,74	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	5,65	0	0
NG 2161H2	4,3	6,6	97,8	40,4	46,8	0	5,5	39,8	25,15	36	0	4,06	0	0	0
NG 2161H3	26,99	6,34	7,67	n.d.	n.d.	n.d.	n.d.	54,1	58,01	35	0	4,95	0	6,36	0
NG 2161H8	82,6	52,2	21,34	95,2	93,6	0	63,8	42,0	49,87	34,4	0	5,12	0	4,69	0
NG 2163A1	67,49	6,7	39,67	n.d.	n.d.	n.d.	n.d.	49,8	14,79	12,5	19,2	0	0	2,93	0
NG 2163C3	33,3	18,8	98,29	30,9	80,8	0	13,8	51,2	31,1	24,2	0	6,46	0	6,17	0
NG 2163D7	42	55,5	42,26	65,4	82,9	0	18	46,1	40,8	39,5	0	62,3	35,3	50,5	0
NG 2163F4	1,4	5,5	26,15	23,8	7,4	0	0	49,7	32,5	11,4	0	4,35	0	3,53	0
NG 2163H4	100	65,5	81,38	107,1	87,2	0	76,3	58,4	41,7	49,8	0	14,6	31,59	23,06	0
NG 2164A2	100	67,7	80,44	98,8	97,8	0	83,3	55,6	45,7	46,2	6,57	6,12	25,62	7,4	0
NG 2164A7	8,13	16,56	15,26	n.d.	n.d.	n.d.	n.d.	44,9	22,92	32,4	0	13,7	20,99	21,87	0
NG 2164B6	84	56,6	70,44	92,8	94,6	0	62,5	51,2	25,6	11,4	0	38,3	50,4	42	0
NG 2164C3	92,7	43,3	81,14	89,2	96,8	0	83,3	42,9	17,2	10,4	0	93,8	33,6	20,1	0
NG 2164G7	76,8	52,2	88,17	92,8	91,4	0	48,6	42,3	26,91	49,8	0	9,14	12,73	13,4	0
NG 2164H5	9,36	1,03	10,51	91,5	n.d.	n.d.	n.d.	0	61,41	20,9	0	5,24	20,18	0	0
NG 2164H9	10,51	9,1	17,21	n.d.	n.d.	n.d.	n.d.	14,1	22,72	8,88	0	8,77	18,71	15,18	0
NG 2166G4	3,3	3,8	48,16	25,9	71,2	0	0	40,0	37,01	73,3	0	35,4	16,6	38,28	0
NG 2166G2	11,16	0,06	8,03	n.d.	n.d.	n.d.	n.d.		11,76	12,5	0	20,0	24,87	30,69	0
NG 2172A2	47,8	45,5	14,39	75	82,9	0	48,6	3,1	23	31,7	0	40,8	13,46	41,12	0
NG 2172B5	3,11	13,86	9,98	n.d.	n.d.	n.d.	n.d.	58,9	21	20,9	0	3,05	5,127	30,16	0
NG 2172B12	63,7	47,7	15,88	94	91,4	0	31,9	3,8	15,34	11,1	0	9,2	30,807	51,57	0
NG 2172C2	78,2	85,5	33,17	90,4	98,9	0	83,3	6,1	34,1	26,6	0	35,5	21,17	43,79	0
NG 2172C3	15,9	31,1	41,03	42,8	25,5	0	20,8	0	0,84	12,1	0	28,0	11,65	10,2	0
NG 2172C10	55	24,4	81,8	64,2	82,9	0	36,1	0	0	31,2	0	2,43	21,61	58,62	0
NG 2172C12	15,16	13,35	23,73	n.d.	n.d.	0	n.d.	46,3	30,7	60,3	0	30,0	18,23	29,93	0
NG 2172D6	46,3	43,3	53,14	85,7	95,7	0	43	44,3	17,2	20,1	0	33,5	23,01	21,23	0
NG 2172D12	0	0	12,5	0	0	0	0	0	0	20,8	0	12,5	24,21	0	0
NG 2172E8	23,11	1,38	45,78	n.d.	n.d.	n.d.	n.d.	38,7	11,6	24,2	0	7,45	17,39	23,47	0
NG 2172E9	1,56	0,06	17,18	n.d.	n.d.	n.d.	n.d.	46,8	13	22,3	4,54	5,03	10,5	0	0
NG 2172F4	3,14	4,08	21,64	n.d.	n.d.	n.d.	n.d.	33,3	0	18,1	0	6,73	8,86	0	0
NG 2172F11	0	0	74,88	0	0	0	0	3,2	14,51	10,3	0	0	23,33	18,42	0
NG 2172G3	78,2	38,8	90,15	89,2	91,4	0	47,2	0	1,78	0,88	0	23,6	31,52	19,05	0
NG 2172G4	73,9	33,3	5,16	89,2	93,6	0	38,8	0	0	11,1	0	31,4	50,33	22,34	0
NG 2172G6	33,3	70	9,84	83,3	79,7	0	31,9	0	0	5,45	0	27,9	40,05	22,34	0
NG 2172H6	75,3	85,5	87,14	97,6	101	0	69,4	6,3	20,6	7,1	0	18,0	11,91	28,21	0
NG2172H12	2,14	1,3	15,48	n.d.	n.d.	n.d.	n.d.	42,3	7,64	52,4	0	9,83	13,06	6,16	0

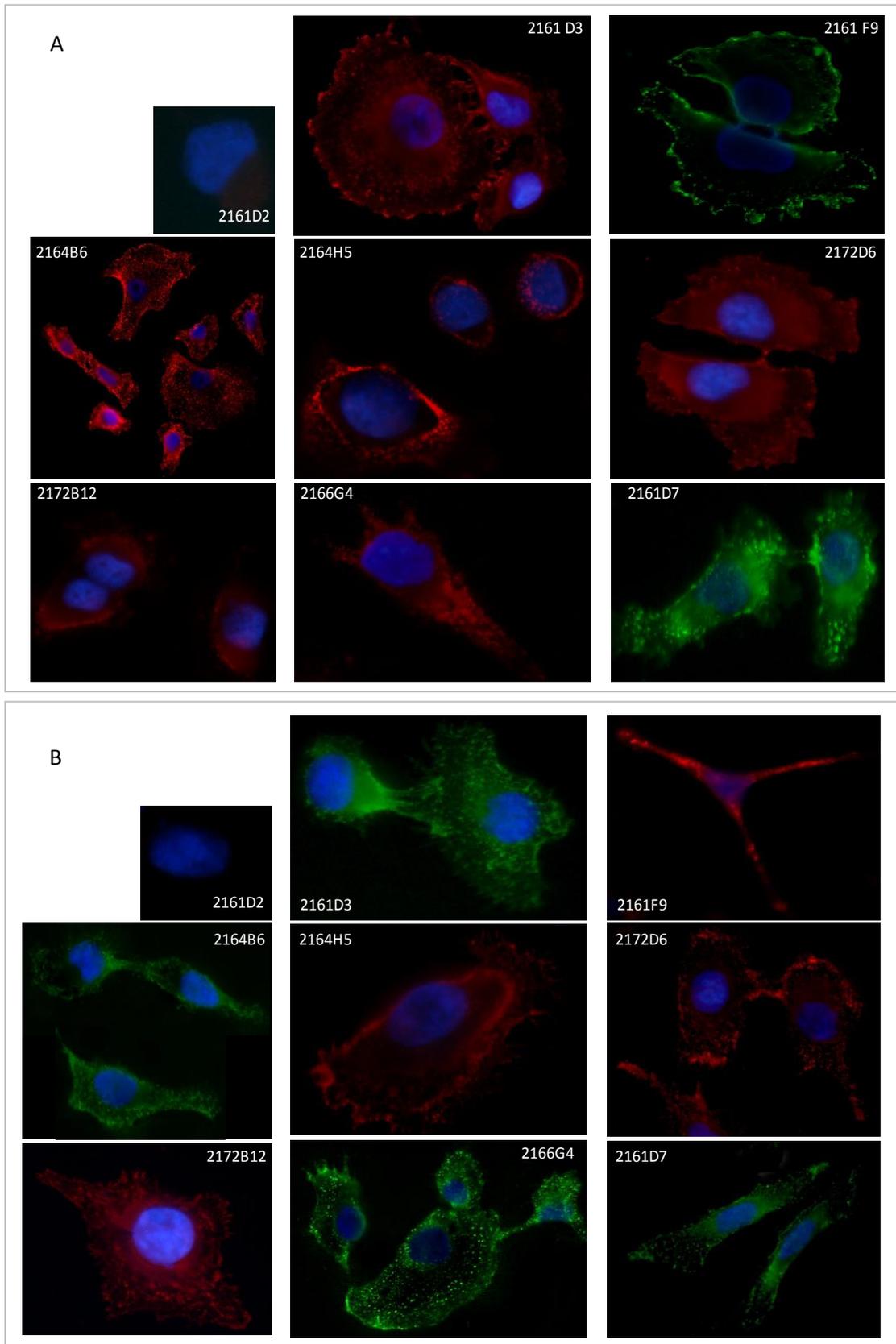


Figure 8: Anti-NG2/CSPG4 mAbs immunostaining. Distribution of NG2/CSPG4 on A375 (A) and HT1080^{NG2+} (B) 4%PFA-fixed cells, shown by immunofluorescence. Zoom 60X. Green= AlexaFluor-488, red= AlexaFluor-594, both directed to anti-NG2/CSPG4 primary mAbs. Blue= Hoechst staining of nucleus.

From immunochemistry assays we investigated the different isoforms that we hypothesized to be expressed by the different cell lines. As many evidence in the past showed that several forms of the protein are expressed in tumors (Tab. 1), we compared the reactivity of mAbs in different cell lines by Flow Cytometry assays as previously resumed in Table 3. Most relevant results have been plot in figure 9 where it is possible to observe that anti-NG2/CSPG4 mAbs employed seem to be able to recognize NG2/CSPG4 with various efficiency: for example, analyzing A375 cells, 2172B12 recognizes NG2/CSPG4 better than all other mAbs. On the other hand, comparing the level of NG2/CSPG4 recognized by a particular mAb on different cell lines we suppose that isoforms of NG2/CSPG4 could exist. An antibody could recognize or not a NG2/CSPG4 isoform depending on its specificity. For example the mAb 2166G4 recognizes NG2/CSPG4 on A375 cells but not on SK-UT-1 cells: perhaps these two cells posses different isoforms of NG2/CSPG4 (Fig. 9).

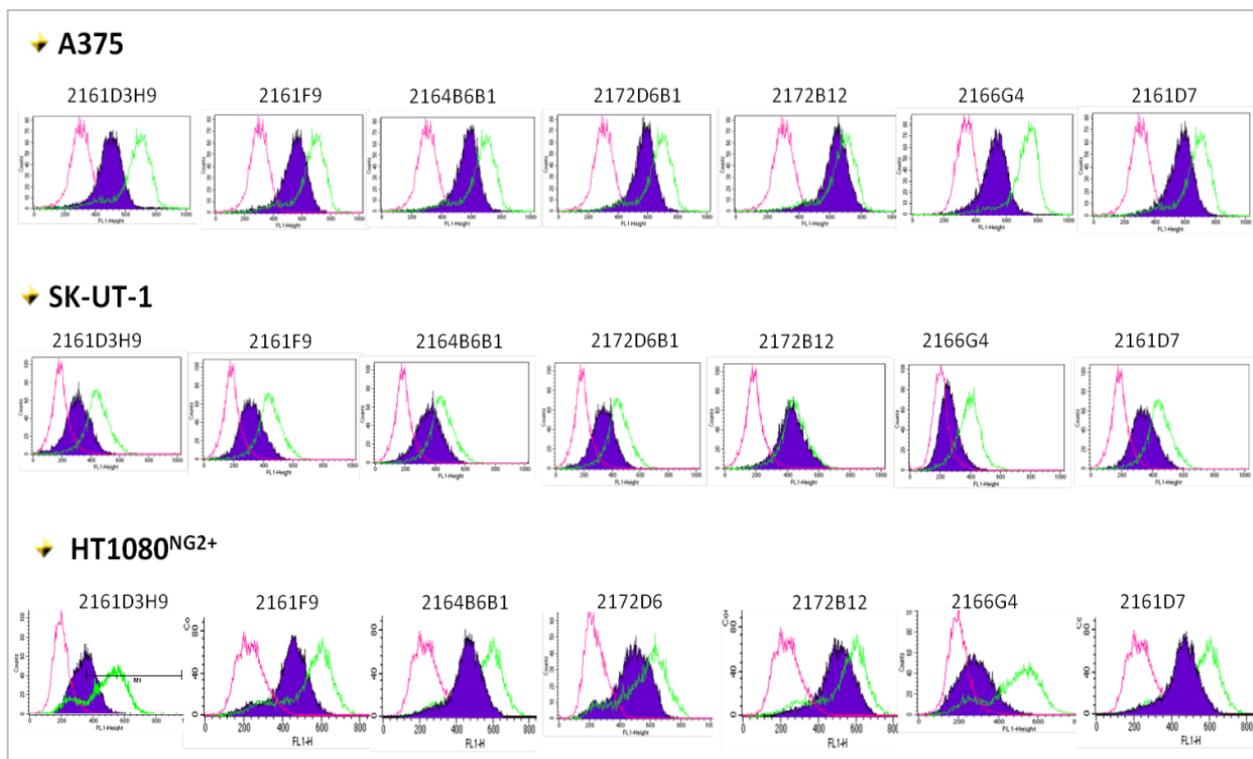


Figure 9: Flow Cytometry analysis. Pink: AlexaFluor488, Purple: anti-NG2/CSPG4 mAb + AlexaFluor-488, Green: Ab 9.2.27 (Millipore + AlexaFluor-488).

Screenings performed by western blotting confirmed what observed on different cell lines about different kinds of mAbs recognition of their antigens (Fig. 10). From A375 cell line banding pattern we can observe that it expresses mainly two NG2/CSPG4 isoforms: one heavier than 250 kDa and the other lighter than 250kDa. The following mAbs recognize both the isoforms: 2161F9, 2172D6, 2166G4, 2164H5, 2164B6, 2161D3, 2172A2, 2172E8, 2161D7. mAbs 2172B12 and 2161D2 recognize the lighter isoform on A375 cells, while mAb 2161G11 recognize only the heavier isoform. HT1080^{NG2+} cells do not show the lighter isoforms, and some antibodies link to the heavier isoforms: 2161F9, 2172B12, 2172D6, 2164H5, 2164B6, 2161D3, 2162D7. MAb 2161D2 recognizes only a degraded form of the protein (100kDa), while 2161F9 and 2172B12 bind to many isoforms of different weights ranging from 250 to 75 kDa. We aimed to test anti-NG2/CSPG4 mAbs on protein lysate obtained from tissue specimens of fetal brain and glioblastoma multiforme patients. From Western Blot we can observe that 2161F9 binds to the high molecular weight molecule on glioblastoma multiforme, while it binds to the lighter molecule on fetal brain. MAbs 2164B6, 2161D3, 2161D7 and 2166G6 have similar banding pattern on glioblastoma multiforme extracts, while 2164C3 mAb binds to lighter forms of the protein, ranging from 100 to 150 kDa. Results on fetal brain are moderate, and it seems that 2161D3 can not recognize NG2/CSPG4 on these tissues, while 2164B6, 2161D7, 2166G4 and 2164C3 have similar banding pattern ranging from 250kda to lower molecular weight peptides (Fig. 10).

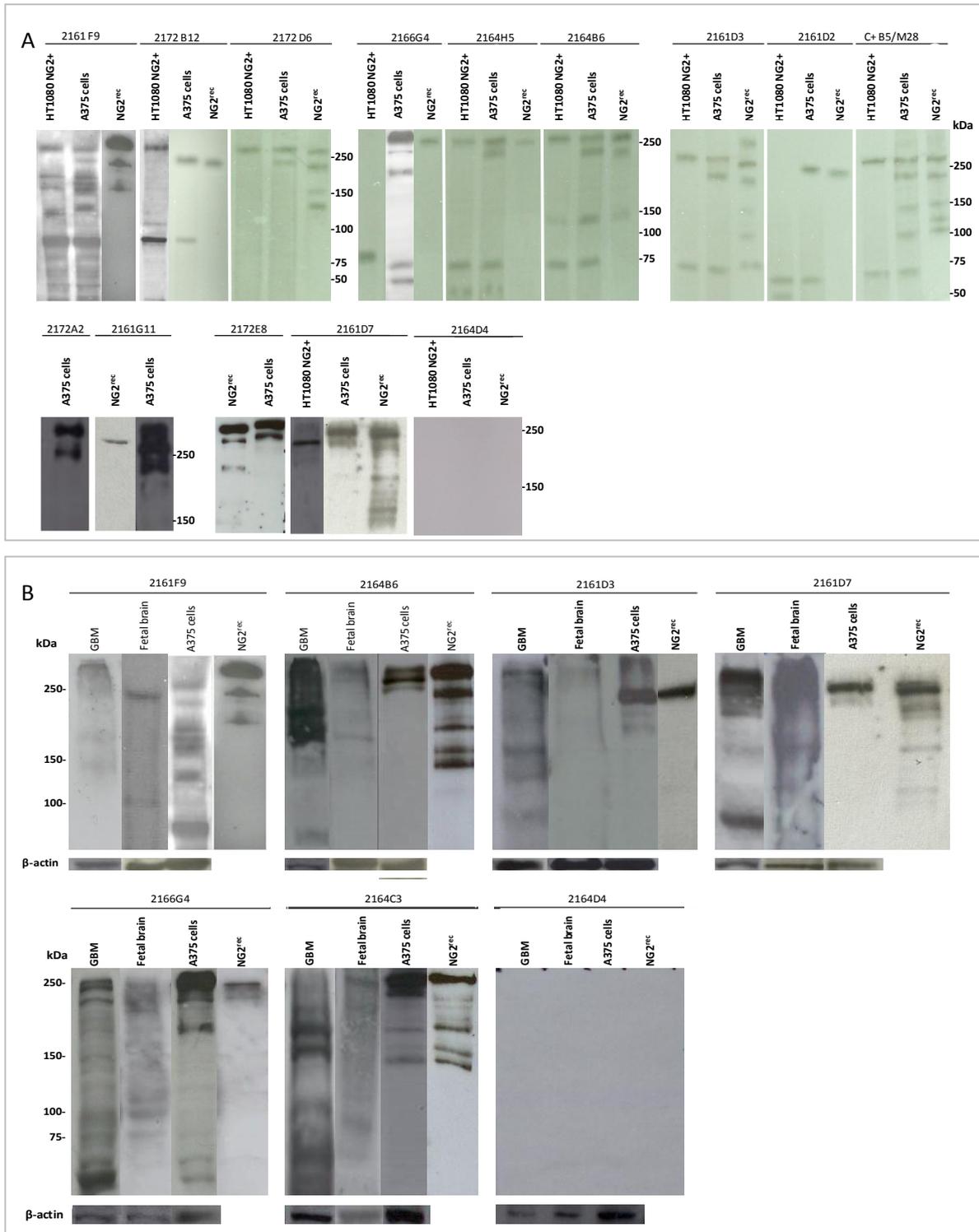


Figure 10: Identification of NG2/CSPG4 isoforms and their proteolytic fragments in cells and tissues. A) Identification of NG2/CSPG4 isoforms and their proteolytic fragments in tumors cell lysates (A375, HT1080^{NG2+}) and in recombinant NG2/CSPG4 (NG2^{rec}) used for producing the mAbs. Proteins were resolved by SDS-PAGE under reducing conditions on 5% or 4-15 gradient gels, followed by transferring onto nitrocellulose membranes and immunoblotting with the indicated anti-NG2/CSPG4 mAbs. Relative position of molecular weight markers is indicated to the side of each panel. B) Identification of NG2/CSPG4 isoforms and their proteolytic fragments in pericyte sprouts of fetal brain neovessels and in glioblastoma multiforme lesions (GMB). Sections from brain areas taken adjacently to sections with abundant angiogenesis and enrichment of

NG2/CSPG4 -expressing pericytes, and lysates of GMB were solubilized and the material treated as in A. A375 melanoma cells, and the NG2^{rec} were blotted in parallel for reference. Immunoblotting of β -actin with a commercial polyclonal ab was used for normalization of gel lane loading. Relative position of molecular weight markers is indicated to the side of each panel.

3.2 Characterization of isoforms and their interacting molecular partners

As a transmembrane molecule with co-receptor functions, NG2/CSPG4 has the potential to interact with both extracellular and cytoplasmic components and to participate in signaling between the extracellular and intracellular compartments of the cell, therefore it is considered a central factor in controlling the consequences of microenvironment on tumor progression. With the aim to find new molecular NG2/CSPG4-interactors, a proteomic analysis of A375 melanoma cells has been performed. Three kinds of samples were prepared: 1) proteins extracted from tumor cells and immunoprecipitated with anti-NG2/CSPG4 mAbs or the same sample run in a SDS-PAGE and extracted from gel. 2) Protein extracted from tumor cells, crosslinked with BS3, and then immunoprecipitated with anti-NG2/CSPG4 mAbs, or the same sample run into a SDS-PAGE and extracted from the gel. 3) Recombinant NG2/CSPG4 ectodomain (NG2^{rec}).



Figure 11: A) Preliminary western blotting to test the efficiency of immunoprecipitation (IP NG2/CSPG4) and of the crosslinking with BS3 (IP NG2/CSPG4 +BS3) employing NG2^{rec} as reference. Proteins were resolved by SDS-PAGE under reducing conditions on 5% gels, transferred onto nitrocellulose membranes and incubated with anti-NG2/CSPG4 mAb 2161D3. B) Coomassie-stained SDS-PAGE gel employed for protein extraction from gel and employment of samples for MALDI-TOF analysis. Proteins were resolved by SDS-PAGE under reducing conditions on 5% gels.

Samples were digested with trypsin, purified and concentrated, and finally analyzed with MALDI-TOF Mass Spectrometry. Figure 12A shows a pilot experiment performed as control. It shows the MALDI-TOF mass spectrum of peptides obtained from trypsin digestion of NG2^{rec} with a mass of 236936,5 \pm 1100 Da. The research in MASCOT confirmed the obtaining

of NG2/CSPG4 from this analysis. Figure 12B shows the spectrum of the peptide mass fingerprint of NG2/CSPG4 immunoprecipitated from A375 cells with anti-NG2/CSPG4 mAb 2161D3. The analysis with mMass and the research in MASCOT of the peaks led to the CSPG4_HUMAN achievement among the results (shown in green dots on the B spectrum, Fig. 12B). Figure 12C shows the spectrum of the peptide mass fingerprint of NG2/CSPG4 crosslinked with BS3 before the immunoprecipitation procedure from A375 total cell lysate. The analysis with mMass and the research in MASCOT of the peaks values let us obtain the CSPG4_HUMAN (shown in green dots on the spectrum) and a list of many other molecules with high score (over 50) that could be considered as putative interactors of NG2/CSPG4:

- **Integrin beta-5 (ITB5)**: it is a receptor for fibronectin. Values matched: 28. Protein sequence coverage: 28%.
- **Microtubule-associated protein 2 (MAP2)**: the exact function of MAP2 is unknown but MAPs may stabilize the microtubules against depolymerization. They also seem to have a stiffening effect on microtubules¹⁷⁰. Values matched: 33. Protein sequence coverage: 24%.
- **Alpha actin 3 (ACTN3)**: cross-linking protein which helps to anchor the myofibrillar actin filaments to a variety of intracellular structures¹⁷¹. Mass values matched: 29. Protein sequence coverage: 34%.
- **Actin related protein (ARP3B)**: it plays a role in the organization of the actin cytoskeleton. It may function as ATP-binding component of the Arp2/3 complex which is involved in regulation of actin polymerization and together with an activating nucleation-promoting factor (NPF) mediates the formation of branched actin networks. ARP3B seems to decrease the metastatic potential of tumors¹⁷². Values matched: 13. Protein sequence coverage: 31%.
- **Myosin-14 (MYH14)**: a cellular myosin that appears to play a role in cytokinesis, cell shape, and specialized functions such as secretion and capping¹⁷³. Mass values matched: 53. Protein sequence coverage: 28%.
- **Myomesin-2 (MYOM2)**: it is a 150KDa protein with undefined function. It contains 5 fibronectin type-III domains. Values matched: 52. Protein sequence coverage: 36%.

- **von Willebrand factor A domain-containing protein 5A (VMA5A)**: it may play a role as a tumor suppressor. Altered expression of this protein and disruption of the molecular pathway it is involved in, may contribute directly to modify tumorigenesis¹⁷⁴. Mass values matched: 23. Protein sequence coverage: 36%.
- **Spectrin alpha chain, non-erythrocytic 1 Protein (SPTN1)**: it seems to be involved in secretion, interacts with calmodulin in a calcium-dependent manner and is thus candidate for the calcium-dependent movement of the cytoskeleton at the membrane¹⁷⁵. Mass values matched: 49. Protein sequence coverage: 22%.
- **Plexin-A4 (PLXA4)**: a coreceptor for SEMA3A, necessary for signaling by class 3 semaphorins and subsequent remodeling of the cytoskeleton. It plays a role in axon guidance in the developing nervous system. Class 3 semaphorins bind to a complex composed of a neuropilin and a plexin. This plexin modulates the affinity of the complex for specific semaphorins, and its cytoplasmic domain is required for the activation of down-stream signaling events in the cytoplasm. It is a single-pass type I transmembrane protein¹⁷⁶. Recent studies show that Plexin-A4 promotes tumor progression and tumor angiogenesis by enhancement of VEGF and bFGF signaling¹⁷⁷. Mass values matched: 37. Protein sequence coverage: 23%.
- **Plasminogen-like protein A (PLGA)**: a secreted protein that may bind non-covalently to lysine binding sites present in the kringle structures of plasminogen. This may interfere with the binding of fibrin or alpha-2-antiplasmin to plasminogen and may result in the localization of activity at sites necessary for extracellular matrix destruction^{178,179}. Mass values matched: 9. Protein sequence coverage: 54%.
- **Synemin (SYNEM)**: it is a Type-VI intermediate filament (IF) which plays an important cytoskeletal role forming heteropolymeric IFs with desmin and/or vimentin. It interacts with cytoskeletal proteins including: alpha-dystrobrevin, dystrophin, talin-1, utrophin and vinculin. SYNEM is able to link these heteropolymeric IFs to adherens-type junctions¹⁸⁰⁻¹⁸². Mass values matched: 27. Protein sequence coverage: 20%.
- **Periostin (POSTN)**: it binds to heparin. Induces cell attachment and spreading and plays a role in cell adhesion. May govern extracellular matrix mineralization. It has been found to

be secreted in the extracellular space reaching the extracellular matrix^{183–185}. Mass values matched: 21. Protein sequence coverage: 32%.

- **Heparan sulfate glucosamine 3-O-sulfotransferase 3A1 (HS3SA)**: a single-pass type II membrane protein that catalyzes the transfer of a sulfo group to an N-unsubstituted glucosamine linked to a 2-O-sulfo iduronic acid unit on heparan sulfate¹⁸⁶. Mass values matched: 22. Protein sequence coverage: 53%.
- **ERC protein 2 (ERC2)**: it interacts through its C-terminus with the PDZ domain PDZ-containing proteins. It is a cytoplasmic protein distributed in cell junction and associated to cytoskeleton¹⁸⁷. Values matched: 38. Protein sequence coverage: 38%.
- **Zinc Finger (ZF) proteins** : ZNF510, ZNF624, ZNF658B, ZNF880, ZNF510, ZNF2, ZNF221, ZNF639, ZNF736, ZNF559, ZNF10, ZNF217, ZNFBTB, ZNF672, ZNF441, ZNF595, ZNF16, ZNF71, PR domain ZNF protein.

The two spectra in B and C are very similar, a part from some peaks that resulted to be peculiar of the sample run in 12C. For a deeper analysis, we followed the Workflow of cross-link search with the ion-tag model¹⁸⁸: from the peaks values list of IP-NG2/CSPG4 +BS3 (NG2/CSPG4 treated with a crosslinking agent), the common values obtained from IP-G2/CSPG4 (NG2/CSPG4 alone without any crosslinkation) have been subtracted and a new research in MASCOT has been performed with the pick values of the sample that was subjected to crosslinking. The results obtained are listed below and show important putative interactors of NG2/CSPG4 such as:

- **Fascin (FSCN1)**: a 55 kDa actin-bundling protein, that plays a role in the organization of actin filament bundles and the formation of microspikes, membrane ruffles, and stress fibers. It has been demonstrated to play a fundamental role in the formation of a diverse set of cell protrusions, such as filopodia, involved in cell motility and migration^{189–192}. Mass values matched: 27. Protein sequence coverage: 46%.
- **LIM domain and actin-binding protein (LIMA1)**: it binds to monomers and filaments of actin. LIMA1 increases the number and size of actin stress fibers and inhibits membrane ruffling. Inhibits actin filament depolymerization. Bundles actin filaments, delays filament

nucleation and reduces formation of branched filaments¹⁹³. This cytoskeletal protein co-localizes with actin stress fibers and focal adhesion plaques^{193,194}. Mass values matched: 40. Protein sequence coverage: 46%.

- **Metalloreductase (STEAP3)**: a six-pass membrane protein that localizes to vesicular-like structures at the plasma membrane and around the nucleus. Mass values matched: 6. Protein sequence coverage: 13%.
- **Erlin-1 (ERLN1)**: a component of the ERLIN1/ERLIN2 complex which mediates the endoplasmic reticulum-associated degradation (ERAD) of inositol 1,4,5-trisphosphate receptors (IP3Rs). ERLN1 is a endoplasmic reticulum membrane, a single-pass type II membrane protein that has been found in associated with lipid raft-like domains of the endoplasmic reticulum membrane and of cell membran¹⁹⁵⁻¹⁹⁷. Mass values matched: 7. Protein sequence coverage: 24%.
- **Galectins (Leg9b or leg9c)**: this is a family of proteins known to carry out intra- and extracellular functions, including inhibition of chronic inflammations, GVHD, and allergic reactions, through glycoconjugate-mediated recognition. From the cytosol they may be secreted by non-classical pathways, but they may also be targeted to the nucleus or specific sub-cytosolic sites. Mass values matched: 4. Protein sequence coverage: 14%.
- **Coiled-coil domain-containing proteins**: CC121, CCD18, TMC04, C144B, CCDC8.

These molecules seem to interact with NG2/CSPG4 weakly respect to those listed before, as their identification was obtained thanks to the employment of a crosslinking agent.

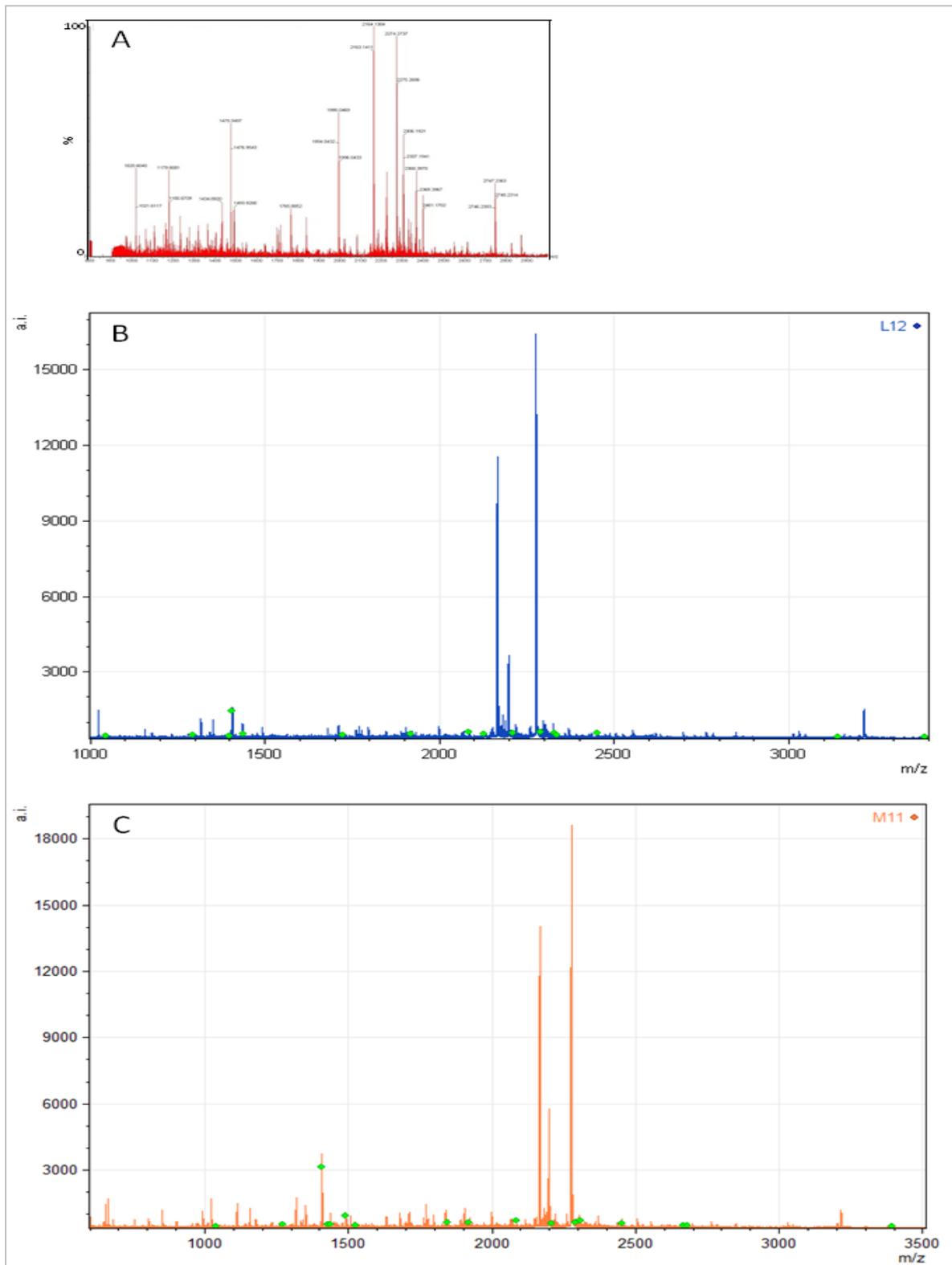


Table 4: MASCOT results. Sequences of the outputs with their match values and protein sequence coverage. Matched peptides shown in bold.

ITB5 Value matched=22 Protein sequence coverage=24%

1	MPRAPAPLYA	CLGLCALLP	RLAGLNICTS	GSATSCCEEL	LHPKCAWCS
51	KEDFGSPRSI	TSRCDLRANL	VKNGCGGEIE	SPASSFHLVR	SPLSSKSGS
101	SAGWDVQIMT	PQEIAVNLRP	GDKTTFQQLV	RQVEDYVDL	YYLMDLSIM
151	KDDLNIIRSL	GTKLAEMRK	LTSNFRLFGF	SFVDKDISPF	SYTAPRYQTN
201	PGIGYKLPFN	CVPSFGFRHL	LPLTRVDSF	NEEVRKORVS	RNRDAPEGGF
251	DAVLQAAVCK	EKIGWRKDAL	HLVFTTDDV	PHIALDGKLG	GLVQPHDQGC
301	HLNEANEYTA	SNQMDYPSLA	LLGKLEANN	INUFVAVTK	HYMLYKNFTA
351	IPGTTVEIL	DGDSKNIIQL	IINAYNSIRS	KVELSVWDQP	EDUNLFFAT
401	QDQGVSYVQ	RKCEGLKGD	TASFEVLEA	RSCPRTHEF	VFALRVPYGR
451	SDVLEGVYTN	RVCSGVGLE	PNSARCNCSG	TYVCGLCECS	PVGLTRCEC
501	QDGENQSVYQ	NLCREAEKGP	LCSGRGDSCS	NGCSCFESEF	GKIYGFPECC
551	DNFSCARNKG	VLCSGHGHECH	CCECKCHAGY	IQDCNCSSTD	ISTCRGRDQG
601	KSERGHCLC	GOCQCTEPGA	FCEMCEKPT	CPDASTCRD	CVCLLHSG
651	KPDNQCHSL	CRDEVTWVD	TIVKDDQEA	LCFYKAKDC	VMMFTYVELP
701	SGKSLNLTVR	EPECNGTANA	MTILLAVVGS	ILLVGLALLA	IWKLLVTHID
751	RREFAKQSE	RSRARVEMAS	NPLYRKPIST	HTVDFTFNFK	NKSYNGTVD

MYH14 Value matched=53 Protein sequence coverage=28%

1	MAAVTMSVPG	RKAPPRPGPV	PEAAQPFLLT	PRGPSAGGGP	SGSTSPQVEW
51	TARRLVVWPS	ELHGFEEAAL	RDEGEAEAV	ELAESGRRLR	LPRDQIQRMN
101	PPFKSKAEDM	AELTCLNEAS	VHLNLRERY	SGLYVTSGL	FCVIVINPYG
151	LPIYTEAIVE	MYRGGKRHEV	PHVYAVTEG	AYRSMQLDRE	DQSILCTGES
201	GAGKTENTKK	VIQYLAHVAS	SPKGRKEPVG	PGLERQLLQ	ANPILEAFGN
251	AKTVKNDNS	RFKFRIRINF	DVAGYVGAN	IETYLEKSR	AIRQAKDECS
301	HFHYQLLGG	AGEQLKADLL	LEPCSHYRFL	TNGPSSSPGQ	ERPFQTELE
351	SLRVLGFSE	EIISMLRMS	AVLQFGNIAL	AVLQFGNIAL	MREDAQAQKL
401	CRLLGLGVD	FSRALLPRI	KVGRDYVQKA	QTKEQADFL	EALAKATYER
451	LEFRLVLRNL	RALDRSPROG	KVALGILDIA	GFEIFQLNSF	EQLQINRYNE
501	KLQQLNHTM	FVLEQEYER	EGIDWFLDF	GLDLOPCIDL	IERPANPPGL
551	LALLDEECWF	PKATDKSFVE	KVAQEQQGHP	KFQPRHLRD	QADFSVLHYA
601	SKVDYKANEW	LMKNMPLND	NVAALLHQT	DRLTAEIWDK	VEGIVGLEQV
651	SSLGDGPPGG	RPRRGMFRTV	GQYKESLSR	LMATLSNTNP	SVFRCVPHN
701	EKRAGKLEPR	LVLDQLRCNG	VLEGRICRQ	GFNPRIHQE	FRQRYEILTP
751	NAIPKGFMDG	KQACEKMIQA	LELDPNLYRV	GSKRIFFRAG	VLAQLEERD
801	LKVTDIVSF	QAAARGLYLR	RAFQKRQQQ	SALSVMQRNC	AAYLKHLHWQ
851	WVWRLTKVCP	LLQVTRQDEV	LQARAQELQK	VQELQQQSAR	EVGELQGSRA
901	QLEERARA	EQAERAEALC	AEAEETGR	AARKQELV	VSELAARVGE
951	EEECRQMQT	EKKRQQHIQ	ELEAHLEAE	GARQLQLEK	VTEAKMKKF
1001	EEDLLEDQ	NSKLSKERK	LEDLRAEFS	QAEEEEKYK	SUNKRUKYE
1051	ATIADMEDRL	RKEEGRQEL	EKLKRLDGE	SSELOEQMVE	QQQRAEELRA
1101	QLGRKEELQ	AALARAEDG	GARAQLQSL	QAQALALEA	QDELESERVA
1151	RTKAEQRDR	LGEELEALRG	VLEDTLSTN	REQLRQRR	QEVTEKLTCL
1201	EEETRIHEAA	VQELRQRHGQ	ALGELAEQE	QARRGGKAW	KTRLALEAEV
1251	SELRAESSL	QTAHQEGEQ	RRRLQLQLE	VQGRAGDGER	ARAEAAEKLQ
1301	RAQAELENS	GALNEAEST	IRLSKELST	EQQLHDAQEL	LOETRAKLA
1351	LGSVRAMEA	EAAGLERQLE	EESAAERAG	RELQTAQQL	SEWRRQEE
1401	AGALEGEEA	RRRAAREEA	LQRLAEKTE	TVDRLEGR	RLQQLDATT
1451	MDLEQQRVL	STLEKQRKF	DQLAAEKAA	VLLAVEERER	AEAEGRRETA
1501	RLLQTRALE	EQEAARELE	RQNRALRAE	ELRASKDDV	GKSVHELERA
1551	CRVAEQAAND	LRAQVTELE	ELTAAEDAKL	RLVTVQALK	TOHERDLOGR
1601	DEAGERRRQ	LAKQLRDAE	ERDEERKRT	LVAARKKLE	GELEELKAQM
1651	ASAGQKEEA	VQKLRKMQAQ	MKELVREVE	TRTSREIF	QNRSEKRYE
1701	GLEAEVLRQ	EELAASDRAR	RQAQQDRDEM	ADEVANGNLS	KAAILEEKRQ
1751	LEGRGLQEE	ELEEQNSSE	LNDRYRKL	LQVSLTLE	SAERSFSAKA
1801	ESGRQQLERQ	IQELRGLGE	DAGARARHK	MTIAALESKL	AOAEEQLQEE
1851	TRERILSGK	VRRAEKRLKE	VVLQVEERR	VADQLRQLE	KGNLRVKQLK
1901	ROLEAEAAA	SRAQGRRL	QRELEVDTE	AESMNRVTT	LRNLRGRPL
1951	TFTRTRVRQ	FRLEGVASD	EEAEEAQGS	GPSPEPEGQ	PAHPQ

MAP2 Value matched=33 Protein sequence coverage=24%

1	MADERDEAK	APHWTSALPT	EASAHSHPEE	IKDQGGAGEG	LVRSANGFY
51	REDEGAFGE	HGSGQTYNT	KENGINGELT	SADRETAEEV	SARIVQVTA
101	EAVAVLKEQ	EKEAQHKDQ	AALPLAAET	ANLPPSPPPS	PASEQTVTVE
151	EDLTLAKME	FHDQQLTSP	TAEPDSQKKE	ESEKQSPKE	DLKHAALVSG
201	PETTKYTPDK	KDMQGETEEK	APLALFGHTL	VASLEDMKQK	TEPSLVVPGI
251	DLPKEPPTPK	EQKDWFIEMP	TEAKDEWGL	VAPISPGPLT	PMREKDVFD
301	PKWEGKQFD	SPMPSFQGG	SFTLPLDVMK	NEIVTETSPF	APAFLPDVK
351	KSLLQTSQPA	TADSKFKIE	PHEAKPKDMA	EAPPEAMTL	PKDAHIPVE
401	EHVMGKVEE	EKEAINQETV	QQRDTFSPG	QEPILTEKET	EKLKEEKTII
451	SDKEAVPKES	KPKPADEEI	GIQTSTEHT	FSEQKQDEPT	TMDLQKDSFP
501	VSLQAVTDS	AMTSKTLEKA	MTPEALIEK	SSIQELFEMR	VDDKDKIEG
551	GAATSAELOM	PFYEDKSGMS	KYFETSALKE	EATKSIPEGS	EKLKEEKTII
601	SVHESIDTMS	PMHKNQKDEF	QTGKESQSP	PAQEAQVSTL	AQSPYSDLPE
651	EPSSQERMF	TIPDKVYGEK	RDLSHKNKDD	LTLRSRLG	GRSIAEQSRM
701	SINLPMSCLD	SIALGFMNFR	GHDLSPLASD	ILTNSTGSDM	EGDVLVPGI
751	PALEKAPCFP	VESKEEQIE	KVKATGEEST	QAEISCESPF	LAKDFYKNGT
801	VMAPDLPEML	DIAGTRSLRA	SVSADAEVAR	RKSVPESTV	EDSTRYGLPV
851	IDENHIVIKT	VSQLEDLGYC	VFNKYTVPLP	SPVQDSENLS	GESGTFVEPT
901	DDKVRRLD	DLSLIEVLA	AAGRKVEDEF	VDKEASAHIS	GDKSGLSEK
951	QDEKANDRL	DTVLEKSEH	ADSKHAKKT	EEAGDEIETF	GLGVTYEQAL
1001	AKDLSIPTDA	SSEKAEKGLS	SVPEIAEVEP	SKKVEQGLDF	AVQGDQVVI
1051	SDFGQMASGL	NIDRRATEL	KLEATQDMTP	SSKAQOEADA	FMGVESGHMK
1101	EGTKVSETEV	KEKVAKPDV	HQEAQVKEES	YESSGEHESL	TMESLKADEG
1151	KKETSPESSL	IQDEIAVKLS	VEIPCPPAVS	EADLATDERA	DVQMEFIQGG
1201	KEESKETPDI	LHETIVSEPA	EIQSEEEIE	2A0EYDVKLLF	1201
1251	RSDTLQTDL	GVSGAREEFV	ETCPSEHKG	IESVVTIEDD	FITVVQTTTD
1301	EGESGSHSVR	FAALEQPEVE	RRSPHDEE	FEVEEAEEAQ	AEPKDGSPA
1351	PASPEREEVA	LEYKETENDY	DYKDETTDD	SIMDADSLWV	TDQDDRSIM
1401	TEQLETIPKE	EKAKEARRS	SLEKHKRKP	FKTGRGRIST	PERKVAKKEP
1451	STVSDEVR	KAKVYKAAEL	AKKTEVQASH	PSRKFILKPA	IKYTRPHTLS
1501	CRKRKTTAAG	GESALASVF	KQAKDKVSDG	VTSKPEKRS	LPRSPKPEK
1551	RRGVSGRDE	NSFSLNSSIS	SSARRTRSE	PIRRAGKSGT	STPTTGSTA
1601	TPGTPPSYS	SRTPTGTPG	SYRPTPTG	TPKSAILVPS	EKKVAIRPT
1651	KPSPATPKQL	LRNQPLD	KNVSKISGT	DNIKYQPKG	QSDVTKKID
1701	SIHVTSKGGS	RUNIRHRPGG	GRVKIESVKL	DFKEKAQAKV	GLQDNAHHVP
1751	SGGNKIDSQ	KLNFRHAKA	RVDHGAEIT	QSPGRSSVAS	PRRLSNVSS
1801	GSINLESQ	LATLAEDVTA	ALAKQGL		

MYOM2 Value matched=52 Protein sequence coverage=36%

1	MSLTVPFYQ	KRRHFDQSY	RNIQTRYLLD	EYASKRAST	QASSQKLSQ
51	RSSQVASSQ	TSLGGTICRV	CAKRVSTQED	EEQNSRYQ	SLVAAYGEAK
101	RQRFLELAH	LEEDVHLARS	QARDKLDKYA	IQQMMEDKLA	WERHTFEERI
151	SRAPIELVRL	RSHTVWERMS	VKLCFTVQGF	PTPVVQWYKD	GSLICQAAEP
201	KGAPPELVS	VHTLEINRAD	FDDATYASV	ATNAHQVST	NAAVVRRFRF
251	GDEEPRFVSG	LPIGLPSSM	IPYTHFDVQF	LEKGVTFRR	EGETVTLCT
301	MLVTPDLKRV	QPRAEWYRDD	VRLKSKWTK	MFGEGQASL	SFSLHKKDRE
351	GLTYLRISV	GGVSDHSFAL	FVLDADPLVT	GAFGAPMDLQ	GHANDRDYVI
401	VTWKPPNTTT	ESPVMGYFVD	RCEVGTNNVV	QCNDAPVKIC	KYPVTGLFEG
451	RSYFVRVAV	NSAGISRPSR	VSDAVALDP	LDLRRQAVH	LEGEKIAIV
501	QDLEDGDAQ	PGPPTGVHAS	EISRNVVLS	VEPPTPRGKD	PLMYFIEKSV
551	VGGSSQWRVN	AQTAVRSRPR	AVFDLMEGKS	WYFRVLSANR	HGLSEPSIT
601	SPIQAQDVT	VPSAPGRVLA	SRNKTSTVVV	QWDRPKHEED	LLGYVDCV
651	AGTNLWPCN	HKPIGYNRFV	VHGLTTEQY	IFRVKAVNAV	GMSENSQSD
701	VIKVQAALT	NCDGHSMTLG	WKVPKFSGGS	PILGYLLDR	AGIGEPSDPS
751	EVHHKNWHEV	NSSPSKPTL	TDVGLTEGL	YEFKIAAVNL	SSPVGKPVVSG
801	EHFKEAWTMM	PEPGAYDLT	FCEVRDLSL	KTWKAPVYSG	NANGVKPDS
851	FREEDAGEWI	TVNQTTANR	YLKVSLLQGG	KTVFVRVAV	NANGVKPDS
901	TSEEDYVLEAR	PGRKEISAGV	DEQGNILGF	DCQEMDASQ	FTWKSYSIE
951	SDDERFKIET	VGDSKLYLK	NPDKEDLGT	SVSVDTDGV	SSSFLDPEE
1001	LERLMAISNE	IKNPTIPLKS	ELAYEIFDKG	RFVRVQLQEH	LPDASRYFI
1051	INDREWSDE	IHIRIKCDKAT	GIAEMVMDRF	SIENEGTYT	QHDGKSSQD
1101	SSLVUGDAF	KTVLEAEFQ	RKEFLRQKGP	HFAEYHWDV	TEEVEVRLC
1151	KVANTKETE	FKWLKDDVLY	ETETPLNER	GICELLPKL	SKKDHGEYKA
1201	TLKDRDQGDV	SILEIAGKYV	DDMILAMSRV	CGKASAPLKV	LCTPEGRILQ
1251	CFMKYFTDEM	KVNWCHDKAK	ISSSEHMRI	GSEEMAWLQ	CEPTEKDKG
1301	YTFEFDGK	NHQPSSLDSG	QAFDEAFEF	QYFKAFAAE	KNRGRUGGL
1351	PDVVTIMEGK	TLNLTCTVFG	NPOPEWIFK	NDQDQLSEH	FSHVLIQAK
1401	VSMTIKGVTS	EDSGKYSINI	KNKYGGKID	VTVSVKHGE	KIPDMAQQQ
1451	AKPKLIPASA	SAAGQ			

ACTN3 Value matched=29 Protein sequence coverage=34%

1	MMVMVQPEGL	GAGEGRFAGG	GGGGEYMEQE	EDWDRLLD	PAWEKQQRK
51	FTAWCNSHLR	KAGQTIENIE	EDFRNGKLKM	LLEVISGER	LPDRDGMKMR
101	FKHIANVKA	LDFIASKGVK	LVSIGAEIV	DGNLKMTLGM	IWTILRFAI
151	QDISVEETSA	KEGLLWCQR	KTAPYRNWV	QNPHTSWKDG	LALCALUHRH
201	RPDDIYAKL	RKDDPIGNLN	TAFEVAEKYL	DIPKMLDAED	IVNTPKPEK
251	AIMTVVSCFY	HAFAGAEQAE	TAANRICKVL	AVNQENKUM	EYEKLADEL
301	LEWIRTVPW	LENRVGEPMS	SAMQRKLEDF	RDYRLLHKPP	RIQEKQLEI
351	NFNTLQTKLR	LSHRPAMFMS	EGLKVSJIAN	AWRGLQVEK	GYEDWLSIE
401	RRLQRLQHLA	EKRQKASLH	EAWTRGKEEM	LSQRDYDSAL	LQEVRAALLR
451	HEAFESDLAA	HQDRVIEHAA	LAQELNELDY	HEAASVNSRC	QAICDQWVNL
501	GLTLQQRDA	LERMEKLET	IDRLQEFAR	RAAFPNNWLD	GAVEDLQVNL
551	LHVSVEETQS	LLTAHQDFKA	TLPEADRERG	AIMGIQGEIQ	KICQTYGLRP
601	CSTNPYITLS	PDQINTKWD	VRKLVPSCDQ	TLQELARQQ	VNERLRQFA
651	AQANAIGPWI	QAKVEEVGRL	AAGLAGSLEE	QMAGLRQEQE	NIINYNKIID
701	REGDHLQLQ	ELNLFDSNHT	YYSMEHIRVG	VEQLTISAR	TINEVENQVL
751	TRDAKGLSQE	QISVFANKT	HYSDKQNGMM	EPDDFRACLI	SMGVDLGEVE
801	FARIMTMVDP	NAAGVVTFOA	FIDFMTRETA	ETDTEQVVA	SFKLAGDKN
851	YITPEELRE	LPAKQAEYCI	RRMVPYKGS	APAGALDYVA	FSSALYGESD
901					

VMA5A Value matched=23 Protein sequence coverage=36%

1	MYHFCGLLT	HREPVPLSKI	SVSNINIEFV	AGVSATLNEY	NEEKVPLEAF
51	FVFPMDDESA	VYSFEALVDG	KKVAELQDK	MKARTNEYKA	ISQGHQAFLL
101	EGDSSSRDVF	SCNVGNLQPG	SKAAVTLKY	QELPLEADGA	LEKRVPLAVLN
151	PRYQFSGSSK	DCLNWKPTI	VPVEDLPTYL	SMVATIDSQH	GIEKVSQNSCP
201	LSPTYLEGED	KTSAQVSLAA	GHKFDRDVEL	LYYNEVHTP	SVVLEMGMPN
251	MKPGHMLGDP	SAMVSYFNP	PEDQPSNTCG	EFFLMDRSG	SMQSPMSQSD
301	TSRLQIAAK	ETULLKSL	PIGCVFNIG	SGFSYEAQFP	ESVKYQTTM
351	EEALGRV KLM	QADLGGTEIL	APLQNIYRGP	SIPGHLQFL	VFTDGEVTD
401	FSVKEVRIN	RQKRCFSFG	IGEGTSTLS	KGIARSGGT	SEFTGKDRM
451	QSKALRTLK	SLQPVVEDVS	LSWHLPPLS	AKMLSPEQTV	IFRQJRSY
501	AQLTGRMPAA	ETTGVEQLKY	TLQKTFEDK	VTFPLQPKP	VNLTHRLAA
551	SKLQTKDMG	LRETASDCK	DANLNLSESG	VISFTAFIA	INKEINPKPV
601	GPLHRDVPDR	PILLGASAPL	KIKQCSGFRK	ALHSDRPPSA	SQPRGELMDC
651	KAKTFQMDY	SLCGSHKD	QSPGFGENH	LVQLYHQNA	NGSVDLNEQY
701	AKILGMSLEE	IMAAQPAELV	DSSGWATILA	VIVLHSHGKD	LCKEWELLER
751	KAVAWMRAHA	GSTMPVSKA	AITFLKSSVD	PAIFAF	

ARP3B Value matched=13 Protein sequence coverage=31%

1	MAGSLPCVV	DCGTGYTKLG	YAGNTEPOFI	IPSCIAIRES	AKVVDQAQR
51	VLRGVDDLDF	FIDGEAIDNP	TYATKWPIRH	GIEDWDLMLE	RFMEQVAVLA
101	LRAEPEDPHY	LMTEPELNP	ENREYLAIEI	FESFNVPDGY	IADVQVQVLA
151	ASWTSRQVGE	RTLGTVIDS	GDGVTYHIVP	AGEVYIGSI	KHPIAGRDI
201	TYIFQQLRE	REVGIPEQOS	LETAKAIEK		

SPTN1 Value matched=49 Protein sequence coverage=22%

1	MDP5GVKYLE	TAEDIQERRQ	QVLDYRHFKE	ELSTLRQKL	EDSVRFOFFQ
51	RDAEELEKWI	QEKLOQASDE	NYKDPTNLQG	KLQKHQAFEA	EYQANSQAINV
101	KLDGTGNLMI	SEGHFASETI	RTRLMELHRQ	WELLEKMRRE	KGKILQAOAK
151	LVQYLRCECD	VMDVWINDKEA	IVTSEELGQD	LEHVLEVQQK	FEFEQTDMAA
201	HEERVNEVNO	FAAKLQEQH	PEEELIKTKQ	DEVNAAQWL	KGIALQRQKG
251	LFGAAEVQRF	NRDVEDTISW	IKKEQQLMAS	DDFGDRLASV	QALLRKHGEL
301	ERDLAALDEK	VKALCAEADR	LQSQHPLSAT	QIQVKRELL	TNWEQIRTLA
351	AEHRARLND	YLRQRLADF	RDLSWVWTEM	KALINADELA	SDVAGAEALL
401	DRHQEHKGEI	DAHEDSFKSA	DESGQALLAA	GHYASDEVRE	KLVLTEESRA
451	ALLELWELRR	QQEQCQMDLQ	LFYRDTEQVD	NWMSKQAEFL	LNEDLGDSDI
501	SVEALLKHE	DPEKLSAQE	EKITALDEFA	TKLIQNNHYA	MEDVATRDA
551	LSRRNALHE	RAMRRRAQLA	DSFHLOQFRR	DSDELKSWVN	EKMKTATDEA
601	YKDPNSLQGG	VQKHQAFAE	LSANGSRIDA	LEKAGQKLD	VNHYAKDEVA
651	ARMNEVISLW	KKLEATELK	KQLREANQD	QQFNRNVEDI	ELWLYVEVGH
701	LASDDYQKDL	TNVQNLQKHK	ALLEADVAAH	QDRIDGITQJ	ARQFDQAGDL
751	DAENIKKQE	ALVARYEALK	EPMVARKQKL	ADSLRLOQLF	RDVDEETWI
801	REKPIAASD	NRGKDUVGQ	NLLKXHQALQ	AEIAGHEPRI	KAVTKQGNAM
851	VEEGHFAST	VKAKLHELNG	KWEALAKAKS	QRQDLEDSL	QAQGFADAN
901	EAE5WNRKE	PIVGTSDYK	DEDSAEALLK	KHEALMSDLS	AYGSSIQALR
951	FQAQSCRQV	APTDDETGKE	LVLALVDYQE	KSPREVTMKK	GDILLNST
1001	KDQWVWKEVN	DRQGFVPAAY	VKKLDPAQSA	SRENLEEQG	SIALRQEQID
1051	NQTRITKEAG	SVSLRMKQVE	ELYHSLLEIG	EKRKGMLKES	CKKFMLFREA
1101	NELQHWINEK	EAALTSVEEVG	ADLEQVEVLQ	KKFFDFQKDL	KAFNSRQKID
1151	NKVAEDLESE	GLMAEEVQAV	QQQVEYGMMP	RDETSKTAS	PWK5ARLVMH
1201	TVATFNSIKE	LYNERWSLQQ	LAERESQLLQ	SAHEVQRFRH	DADETEWIE
1251	EKNQALNTDN	QKHLKHEGFER	LQRKHGEGFER	DLAALGDKVN	DLAETKARI
1301	QSHPEAEADL	QKSTELNQA	VSSGLKGRADQ	RKAKLGDSDH	LQRFSLDFRD
1351	LSMSWINGIRG	LVSDLELAKD	W7SGAEALLER	HOEHRTEIDA	RAGTFQAFDQ
1401	FGQQLAHGH	YASPEIKQKL	DILDQERADL	EKAWVQRMM	LDQCLELQFL
1451	HROCEQENW	MAAREAFINT	EDKGDSDLSV	EALIKKHEDF	K2KAINVQEE
1501	IAAQAQFADQ	UAAAGHYAAG	DISSRNEVL	DRWRRLKAQM	IKRSALRQEL
1551	DTLQDQSRDV	DEIEAWISEK	LQTSADSEYK	DPTNIQSKHQ	KHQAFEAELH
1601	NADIRIRGVI	DMGNSLIERG	ACAGSEDAVK	ARLAAALADQW	QKLVQKSAEK
1651	SQKLKEANKQ	QNFNTGKIDF	DFWLSEVEAL	LASEDYGKDL	ASVNNLLKXK
1701	DLLEADISAH	EDRLKDLNSQ	ADSLMTSSAF	DT5VQKDKRD	TINGRQKIK
1751	SMAASRAKAL	NESHRLHQFF	RDMDDEESWI	KEKLLVGSSE	DYGRDLTGTV
1801	NLRKHKRLE	AELAAHEPAI	QGVLDTGKLL	SDNTIGKEE	IQQLAQQFVE
1851	HWKELQKLA	ARGORLESL	EYQQFVANVE	EEEAWINEKM	TLVASEDYGD
1901	FLAIIQGLK	KHEAFEDFT	VHKDRVNDVC	TNGQDLKKN	NHHEEISSK
1951	KMLGNKGVSD	LEKAAQORKA	KLDENSAFLQ	FNWKAADVES	WIGEKENSJK
2001	TDYGRDLSS	VQTLTKQET	FDAGLQAFQQ	EGIANITALK	DQLLAAKHVQ
2051	SKAIEARHAS	LMKRW5QLLA	NSAARKKLL	EAQSHFRKVE	DILFTAKKA
2101	SANF5WFENA	EEDLTPVRC	NSLEEIKALR	EAHDAFRSSL	SSAQADFNQL
2151	RELDRQKSF	RVASNPYWF	TMEALLETWR	NLQIKIIEER	LQKAEQRNQ
2201	EENDKLRQEF	AQHANAFFHW	IQETRYTLD	GSCMVESSTG	LSLEQLEATR
2251	KHQEIRAMRS	QKQIEDLGA	AMEEAALDNL	KYTHESTVGL	AOQWLDQDLQ
2301	5MRMQHNLEQ	QIQARNTGV	TEEALEKFSM	MFKHFDKDKS	GRLNHQEFKS
2351	CLRSLYGLDP	MVEEGEPPDE	FPAILDVYDP	NRDGHVSLQE	YMAFMISRET
2401	ENVKS5EEIE	SARFALS5EG	KPYVTKEELY	QNLTRQADY	CV5HMKPYVD
2451	5KGRPLTAF	DYVEFTRSLF	VN		

PLGA Value matched=9 Protein sequence coverage=54%

1	MEHKEVILL	LLFKSGGGE	PLDDYVNAQG	ASLFSVTKKQ	LGAGSRECA
51	AKCEEDKEFT	CRAFQVHSKE	QQCVIMAENK	KSSIIIRMRD	VVLFKEK

SYNEMIN Value matched=27 Protein sequence coverage=20%

1	MISWRLOTGP	EKAELQELNA	RLYDVVCRVR	ELERENLLE	EELRGRGRE
51	GLWAEQOARC	AEEARSLRQQ	LDLSWATAL	AEGERDALRR	ELELRLQDA
101	EERAAARGLD	AELGAQQREL	QELAGARAA	EALLGLQAE	RAGLDAHER
151	DVRELRARA	SLTMHFRARA	TGPAAPPRL	REVHDSYALL	VAESWRETVQ
201	LYEDEVRELE	EALRQGESR	LQAEETRLC	AQEAELRRE	ALGLEQLRAR
251	LEDALLMRRE	EYGIQAEERQ	RAIDCDEK	ATLTLAMADW	LRDYDQLLV
301	KTGLSEVAT	YRALLEGESN	PEIVWAHEV	ENMPSEFRNK	SYHYD5LLQ
351	RENERLFSR	QKAPLASFNH	SSALYSNL5G	HRGSQTGTSI	GGDARRGFLG
401	5GYSSATTQ	QENSYGKAVS	SQTNVRTFSP	TYGLRNTFA	QKTFPDRPK
451	AGDTREVPVY	IGEDSTIARE	SYRDRRDKVA	AGASESTRSN	ERTVLGKTK
501	EYKATREQER	NRPETIRTKP	EKMFDSKEK	ASEERNLRWE	LDLTKDEAR
551	QRESQOMKEK	AKEKDSPEK	SVREVEVPIS	LEVSQDRRAE	VSPKGLQTPV
601	KDAGGGTGRE	AEARELFRLL	GTSDATGSLQ	GD5M5TVAE	NIVT5LKQF
651	TQ5PETEASA	DSFPDTKVTY	VDRPELPER	KTKTEIVVES	KLTEDVD5SD
701	EAGDYL5LK	DIKEVGLK6K	SAEQMIGDII	NLGLKGREGR	AKVNVNVEI
751	EPV5V5GKE	PEEFSVPFKV	EEVEDV5PQY	WGLVKEE5G	G5D5V5FSV
801	QHRRTKQPE	NTHHVEETE	AGDSEGE5SG	FV5TPDEHPG	GHDRDQ55V
851	Q5IH5EEST	IRY5WQDEIV	QGTRRRTKQD	GAVGEV5VKP	LVD5P55LEG
901	DL5G5THWKEQ	AR5GEFHAEP	TVIEK5IPI	HEF55MKGVI	SSK5PRQ5LV
951	EVIGQLEETL	PERMRELSA	LTR5GQ5GPG	5V5VDV5KQV	GAG555VTLV
1001	AEV5N5QTV	ADRLDEELS	KDEASEMKA	VE5V5RESL5	QR55PAP55P
1051	DEG5EAP5A	AGIRFRWAT	LYP5G5ES	EVAG55SH5S	GQRTDQ55PV5
1101	ATVE555PTG	FQA5Q5LEDV	SQAARHKL6G	P5EV5WR5TMR	5YEG5TAEV5
1151	EL5SAG5D5LQ	AASPTGASRS	VRH5VTL5GPG	5PL5R5ELF6G	GPAP5CE5AW
1201	55PE5GPAES	SADMDG5GRH	STFG5RQ5FA	EKEIF5GPI	5AAG5KV5D5F
1251	ATEE55GTQT	SVRQ5LQ5PK	EGF5G5Q5FT	APL5DK5VELG	VIGD5VH5MEG
1301	LP5G555IRH	ISIG5RQ5HT	TQ5V5VH5L	PQ5G55G5DE	5TVH5G55AD
1351	VH5Q5H55HT5	GQRTV5M5TEK5	TFQ5V555P5	QD5SAGD5TG	AE5M555V5RS
1401	FR5H5R5G5P5E	TR555H5IAR	GPV5R5TFV5LA	GSAD555L6K	LD5R555TR5H
1451	APG5K55TF	TFQMDV5NVE	AIR5R5Q5EAG	ALG5V5DR55W	RD5ADR5NDQA
1501	V5G55FKASAG	EGDQAH5REQ	KEQAM5DKK5	QLQRM5DQ5RS	VISDEK5VAL
1551	LV5NEE5EEN	DGHWF			

POSTN Value matched=21 Protein sequence coverage=32%

1	MIFPLM5FL	LLLVNPIN	ANNHYDKILA	HSRIRGRDQ5	PNV5CALQ5IL
51	GT5KK5Y5F5C	KNW5YK5ICG	QKTTVLYECC	PGYMRMEG5K	GCPAV5L5PIDH
101	VY5TGL5V5GA	TTTQ5Y5DAS	KLRE5IE5GK	SFTY5FAPSNE	AWD5NLD5DIR
151	RG5L55N5V5E	LLNALH5SHMI	NKRMLTKD5K	NGM5I5P5MYN	NLGLF5IN5HYP
201	NGV5TV5NCAR	IHHNQIATN	LVFHV5IDRVL	TQIGT5IQD5F	IEA5EDL555F
251	RAA5555D5IL	EALGRDGHFT	GAFTPN5AFE	KLPR5V5LERI	MGDKV5ASEAL
301	MY5H5LNTLQ	CS5E5M5GAV	FET5L5G5TIE	IGD5G5D55TV	NGK5M5V5NKD
351	Y5R5N5V5IHL	IDQ5L5P5SA	KQV5IEL5GKQ	QITFT5DLV5AQ	LGL5AL5P5ND
401	GEY5TL5PVN	NAF5D5D5L5M	DQR5L5L5LQ	NH5LK5V5V5GL	NEL5NG5Q5LE
451	TIG5G5QL5RVF	VYRTVAG5EN	SCM5E5K55G5Q	RNGA5H5IFRE	IKP5AK55HL
501	KRAA55DK5RF5	TF5L5LEA5AD	LK5ELL5TOP5D	W5T5V5PT5NDA	F5KGM5555E5KE
551	I5UR5D5NALQ	N5IHL5L5TPG	V5F5IG5K5F5EP	V5T5MLK5T5GQ	55K5FL5K5V5ND
601	TLV5L5NEL5SK	ESD5IM5T5NGV	IHV5VD5K5L5YP	ADT5PV5G5NDL	5E5L5NK5I5K
651	Q5K5F5V5RG5T	F5KE5I5P5V5TYT	TK5I5TK5V5VEP	K5I5K5V5IE5G5LQ	PIK5TE5G5PTL
701	TK5V5K5IE5G5PE	FR5K5UE5G5T	TE5V5IH5G5PII	KY5TK5ID5G5V	P5VE5I5E5K5ETR
751	E5ER5IT5G5PEI	KY5TR5I55T5G5G	E5TE5E5L5K5L	Q5E5V5TK5V5TKF	I5EGD5GH5LFE
801	DE5EK5R5L5Q	D5TP5VR5K5LOAN	K5V5Q5G55R5R5L	REG5RSQ	

PLEXIN A4 Value matched=37 Protein sequence coverage=23%

1	MKAMPWNWTC	LLSHLMVGM	G5S5TLTR5QP	APL5SQK5RF	V5TR5G5E5PAE5G
51	FNHLV5DERT	GPHYLGAVNR	IKL55SD5LV	LVTHET5GDIE	NM5K5CY5P5PRI
101	VQTCNEPLTT	TNNVNM5ML	DYKENR5LIAC	GSLYQ5G5CKL	L5RED5L5K5LG
151	EPYH5K5E5HYL	SGV5NE5G5SVF	G5V5I55Y55N5LD	DKLFIAT5VD	GK5PE5Y5FT5S
201	5RKLTK5NSEA	DGM5F5V5F5HD	EFV5AS5MIK5IP	SDTFT5I5P5DF	D5I5Y5Y5G5F55
251	5NSV5Y5FL5TQ	PEM5V5P5G5ST	TK5Q5Y5T5SL	V5LCK5EDT5AF	NSV5Y5E5PI5G
301	ER5SG5VE5YRLL	QAAYL5SK5GA	V5LGR5LV5HP	DDDL5L5T5V5F5	G5Q5K5R5K5M5K5L
351	DESALCFIL	KQINDRIKER	LQ5CYR5GEGT	LDLAWL5K5VD	IPC5S5ALL5T
401	DDNF5CG5LDMN	APL5G5V5DM5VR	G5I5V5FT5DRD	RMT55V5I5V5Y	KNH5SL5F5M5T
451	5K5G5L5K5IRV	DG5PR5NALQY	ETP5Q5V5D5P5G	V5LRD5M5AF55D	H5E5Q5I5V55G5R
501	Q5L5TR5V5P5ESC	G5Q5Y5G5E5CL	G5S5D5PH5G5WC	V5LHNT5CTRKE	R5E5K5E55P5R5E
551	5ASE5MK5Q5CVR	LTV5HPN55IS	SQ5NV5LV5LE	TYN5V5P5EL5AG	VNCT5F5ED5SE
601	MDGL5V5GNQI	QC5Y5P5AA5KEV	PRIT5ENG5DH	H5V5QL5Q5L5K5	ETG5MT5F5AST5S
651	5FV5Y5NC55VHN	5CL5CV5E5SPY	R5HW5CK5Y5HV	CTH5DP5K5T5CF	Q5E5GR5V5K5P5ED
701	CPQ5L5RV5DKI	LV5P5E5V5IKPI	TLK5AKN5LPQ	Q5G5OR5G5Y5E	LNQ5G5E5Q5RV
751	PAL5RF5N555SV	QC5QNT55Y5SE	G5ME5NN5LP5VE	LTV5V5V5N5GH5FN	IDN5PA5Q5NK5VH
801	LY5K5GAM5RES	CG5LCK5AD5PD	FAC5G5WC5Q5G5P	Q5CTLR5Q5H5CA	Q5E5SQ5W5L5E5G
851	AK5K5CTN5PRI	TEI5P5V5T5GPR	EG5G5TK5V5IRG	ENL5G5L5FR5DI	ASH5VK5VA5G5VE
901	55P5LV5DG5YIP	AE5Q5V5CE5M5GE	AK5PS5QA5GFV	EIC5VA5CR5PE	F5M5AR555Q5LY
951	FM5TL5L55D5K	PSR5GPM5SGGT	QV5TIT5GT5NLN	AG5N5V5V5M5FG	KQ5CL5F5H5R5RS
1001	55V5IV5C5NT5S	S5DE5V5L5MK5V5	VQ5DRA5K5IHQ	D5LV5Q5Y5VEDP	T5IV5IE5P5EWS
1051	IV5SGNT5PIAV	WG5THL5DJQ	PQ5IRAK5HG5K	E5HIN5CE5VLN	ATE5MT5CQ5APA
1101	LAL5G5PDH5Q5SD	L5TER5PE5E5FG	IDL5NV5Q5SL	NK5T5N5FT5YP	NP5V5FE5AG5FS
1151	5L5I5E5L5K5P5GT	IL5K5GN5IUP	P5VAG5NV5KLN	Y5TV5LV5G5E5K5P	T5TV55D55Q5LL
1201	CE5PN5AR5GRH	KVM5AR5V5G5M5E	Y5SP5GM5V5IAP	DS5PL5P5AV	5I5AA5V5AG5L
1251	FIV5AV5UAY	KR5K5RES5DLT	LK5RLQ5M5Q5MDN	LES5RV5ALE5CK	E5AF5AE5LQ5TDI
1301	HEL5T5DL5D5GA	G5I5P5DF5YRT	TM5RV5L5F5PGI	DHP5V5LR5DEL5V	PQ5YR5Q5E5K5EY
1351	5K5L5FA5Q5IN	NK5V5L55F5IR	TV5L5Q5S5F5M	R5DR5GN5VASU	MTV5L5Q55L5K5
1401	AT5DV5L5Q5LLA	D5UD5KN5E5K	NHP5K5LL5RRT	E5V5AE5K5ML5TN	W5FT5FL5Y5K5F
1451	5K5E5G5E5P5L5F5	L5F5CA5K5Q5E5M5E	KG5PID5AIT5GE	AR5Y5L55E5DK	IRQ5DY5K5TL
1501	V5L5CV55P5DN5A	NS5PE5V5P5K5IL	NC5DT5IT5Q5V5KE	K5ILD5A5IF5KNV	PC5SHR5PK5AAD
1551	MD5LE5WR5Q5G5S	ARM5LQ5D5E5D	TK5K5I5ND5WKR	LNT5L5AH5Y5QV	D5G55V5L5V5K5
1601	Q5TAY5NAV5NN	5T5V5RS5AS5K	Y5EM5IR5Y5T5GS	P5D5L5RS5TPM	IT5P5D5L55G5K
1651	M5W5HL5KN5HEH	G5DQ5K5E5G5DR5G5	KM5V5E5I5L5TR	L5L5AT5K5L5G5LQ	F5V5D5D5L5ET5IF
1701	5TA5HR5G5ALP	L5A5I5Y5M5F5DL	DE5QAD5KH5GH	D5PH5V5R5HT5W5K5	N5CL5P5RF5V5VN
1751	M5IK5NP5Q5F5VD	HL5K5S5IT5DAC	LS5VA5Q5TF5MD	SC5T5SE5TR5K5S	CK5D55PN5K5L5Y
1801	AK5DIP5Y5KNW	VER5Y55D5IGK	MPA5IS5DQ5DMN	AY5LAE5Q5SR5MH	M5NE5F5T5M55AL
1851	5E5F55V5Y5G5KY	5E5E5L5G5PL5DH	DD5CQ5G5K5QLA	Y5K5E5Q5V5IT5LM	S5L5D5

H535A Value matched=22 Protein sequence coverage=53%

1	MAPP5G5P5ASAL	5T5SAE5PL5RS	IFR5K5FL5MLC	5L5L55LV5F5Y	CL5AER5CQ5TL5
51	GPV5V5L55G5GGG	EE5AG5P5G5G5V	L5AG5P5REL5AV	WP5AAA5QR5K5RL	LQ5LQ5P5W5R5R5R
101	PPAP5RD5D5GEE	AA5WEE5E5P5GL	5G5G5P5G55G5AG	5TV5AE5AP5GT	L5ALL5DE5G5K
151	QPQ5AI5I5VG	K5G5G5TR5ALLE	FLR5V5HP5D5VA	V5G5AE5P5H5F5DR	5YD5G5L5AW5WR
201	DM5L5P5RT5LD5Q	IT5ME5K5T55F5Y	V5T5RE5AP5L5IS	AM5SK5DT5KV	V5R5DP5V5TRAI
251	5D5Y5T5Q5L5SKR	PD5I5P5T5F5ESL	FK5NR5TAG5ID	TSW5A5IQ5IGI	Y5KH5L5E5H5W5P
301	HP5IR5Q5ML5FV	55GER5L55D5PA	GEL5GR5V5Q5DFL	KL5RII5TD5KH	F5Y5FN5K5T5G5L5R
351	CL5K5AE5G55SR	PH5L5G5K5T5GR	TH5PE5ID5REV5V	R5RL5RE5Y5R5F	NL5KF5Q5M5T5GH
401	DF5G5W5DG				

ERC2 Value matched=38 Protein sequence coverage=38%

1	MYG5ART5ITN	LE5G5PS5R5SPR	LP5RS5P5RL5GHR	RT55G5G5G5G5T	G5K5L5M5ENI5Q
51	5L5NA5AY5T55G	PM5Y5L5SD5HEG5V	AST5TY5PK5G5TM	TL5GR5AT5N5RAV	Y5G5R5V5T5AM5G5S
101	5PN5IAS5AG5L5	HT5DV5L5Y5TDQ	H5G5L5T5G55SH5	HHH5Q5V5P5M5LR	Q5VR5D5T5M5LDL
151	Q5AQ5K5EL5Q5RE	N5DL5LR5K5ELDI	K5D5K5L5G555M5N	5I5K5T5F5W55P5EL	K5K5ER5L5R5KE
201	AAR5M5V5L5K5EQ	M5RV5HEE5N5QH	LQ5L5T5Q5ALQD	E5LRT5Q5DL5NH	LQ5Q5E5G5N5RG
251	A5E5H5T5IELTE	EN5FR5L5Q5AEH	DRQ5AK5E5L5FL	R5K5T5E5EL5MR	I5E5TQ5Q5TL5NA
301	RD5E55IK5LLE	M5LQ55K5G5L5PSK	5L5E5DD5N5ER5TR	R5MA5E5E55Q5V5S	H5L5E5V5LD5Q5NE
351	5K5ENI5H5REEL	H5R5R5Q5LQ5PEP	AK5TK5ALQ5TVI	EM5K5DT5K5IASL	ER5N5IR5D5LEDE
401	IQ5ML5K5ANGVL	NT5EDR5E5E5IK	Q5IEV5Y5K55H5K	FM5K5T5K5ID5Q5K	Q5E5L5K5E55E5L
451	LAQ5TK5L5E5L	5N5Q5N5D5CK5QH	IEV5L5K5E5L5TA	MTV5L5K5AILQ5T	E5D5A5LR5L5RE
501	E5K5E5F5N5K5TK	K5Q5LQ5D5L5TEEK	5G5TL5AGE5R5DM	K5D5M5L5E5K5ERK	IN5V5LQ5K5IEN
551	LQ5E5Q5R5DKD5K	Q5L5TN5K5DR5VK	5LQ5D555N5TD	T5AL5T5L5E5AL	5E5K5I5E5R5L

FSCN1 Value matched=27 Protein sequence coverage=46%

1	MTANGTAEAV	QIQGLINCG	NKYLTAFAFG	FKVNASASSL	KKQIWTLEQ
51	PPDEAGSAAV	CLRSHLGRYL	AADKDGNVTC	EREVPGPCR	FLVAHDDGR
101	WSLQSEAHRR	YFGTEDRLS	CFAQTVSPA	KWSVHIAMHP	QVNIYSVTRK
151	RYAHLARPAA	DEIAVDRDVP	WGVDSLITLA	FQQQRYSVQT	ADHRLRHLDG
201	RLVARPEPAT	GYTLEFRSGK	VAFRDCEGRY	LAPSGPSGTL	KAGKATKVGK
251	DELFALEQSC	AQVVLQAANE	RNVSTRQGMID	LSANQDEETD	QETFQLEIDR
301	DTKKCAFTRH	TGKYWTLTAT	GGVQSTASSK	NASCYFDIEW	RDRRITLRAS
351	NGKFVTSKKN	GQLAASVETA	GDELFLMML	INRPIVFRG	EHFGIGCRKV
401	TGTLNANRSS	YDVFLQEFND	GAYNIKDSTG	KYWTVGSDSA	VTSSGDTVPD
451	FFFEFCYNNK	VAIKVGGRYL	KGDHAGVLKA	SAETVDPASL	WEY

LIMA1 Value matched=40 Protein sequence coverage=46%

1	MESSPFNRRQ	WTSLSLRVTA	KELSLVKNK	SSAIVEFSK	YQKAAEETNM
51	EKKRSNTENL	SQHFRKGTLT	VLKKNWENPG	LGAESHDTSL	RNSSTEIRHR
101	ADHPPAEVTS	HAASGAKADQ	EEQHPRSRL	RSPPEALVQG	RYPHKDGED
151	LKDSTESKK	MENCLGESRH	EVEKSEISEN	TDASGKIEKY	NVPLNRLKMM
201	FEKGEPTQTK	ILRAQSRAS	GRKISENSYS	LDDLEIGPGQ	LSSTFDSEK
251	NESRRNLELP	RLSETSICKR	MAKYQAAVSK	QSSSTNYTNE	LKASGGEIKI
301	HKMEQKENVP	PGEVCITHQ	EGEKISANEN	SLAVRSTPAE	DDSRDSQVKS
351	EVQQPVHPKP	LSPDSRASSL	SESSPPKAMK	KFOAPARETC	VECOQTVYPM
401	ERLLANQVVF	HISCFRCSYC	NNKLSLGYA	SLHGRIYCKP	HFNLQFKSKG
451	NYDEFGHPR	HKDLWASKNE	NEELERPAQ	LANARETPHS	PGVEDAPIAK
501	VGLAASMEA	KASSQEKED	KPAETKKLRI	AWPPPTLGS	SGSALEEGIK
551	MSKPKWPPED	EISKPEVPED	VLDLKKLRR	SSSLKERSRP	FTVAASFQST
601	SVISPKTVSP	PIRKGWSMSE	QSEESVGGRV	AERKQVENAK	ASKKNGNVGK
651	TTWQNKESKG	ETGKRSKEGH	SLEMENENLV	ENGADSDDED	NSFLKQSQSP
701	EPKSLNWSF	VDNTFAEEFT	TQNKSQDVE	LWEGEVVKEL	SVEEQKRRN
751	YDEDEDEE				

STAP3 Value matched=6 Protein sequence coverage=13%

1	MPEEMDKPI	SLHLVDSDS	LAKVPEAPK	VGILSGDFA	RSLATRLVGS
51	GFKVVGSRN	PKRTARLPS	AAQVTFQEEA	VSSPEVIFA	VFREHYSSLC
101	LSLDQLAGKI	LVDVSNPTEQ	EHLQHRNSNA	EYLASLPTC	TVVKAFNVIS
151	AWTLQAGPRD	GNRQVPICGD	QPEAKRAVSE	MALAMGFMPV	DMGSLASAW
201	VEAMPRLLP	AWKVPVTLAL	GLFVCFYAYN	FVRVDLQPYV	QESQNKFFLK
251	PVSVVNTTLP	CVAYVLLSLV	YLPGLAAL	QLRRGTYKQR	FPDWLDHWLQ
301	HRQIGLLSF	FCAALHALYS	FCLPLRAHR	YDLVNLAVKQ	VLANKSHLWV
351	EEVWRMEIY	LSLGLALGT	LSLLAVTSLP	SIANSLNWRE	FSFVQSSLGF
401	VALVLSLHT	LTYGWTRAFE	ESRYKFLPP	TFTLTLVPC	VVILAKALFL
451	PCISRLRAR	IRRGWEREST	IKFTLPTDHA	LAECTSHV	

FRLN1 Value matched=7 Protein sequence coverage=24%

1	MTQARVLVAA	VVGLVAVLLY	ASIHKIEEGH	LAVYYRGGAL	LTSPPGPGYH
51	MLPFITFR	SVQTLTQDDE	VKNVPCGTSG	GVMYIDRIE	VVNMLAPYAV
101	FDIVRNVTAD	YDKTLFNKI	HEHLENQFCSA	HTLQEVYIEL	FDQIDENLQK
151	ALQKDNLMA	PGLTIOAVRV	TKPIPEAIR	RNFELMEAEK	TKLLAAQKQ
201	KVVEKAEETE	RKKAIVIEAEK	IAQVAKIRFQ	QKVMEKETEK	RISEIEDAAF
251	LAREKAKADA	EYYAAHKYAT	SNKHKLTPEY	LELKYQAIA	SNSKIYFGSN
301	PNMFVDSSC	ALKYSDIRTG	RESSLPSKEA	LEPSGENVIO	NKESTG

LEG9C Value matched=4 Protein sequence coverage=14%

1	MAFSGQAPY	LSPAVPFSGT	IQGGLQDGFQ	ITVNGAVLSC	SGTRFAVDFQ
51	TGFSGNDIAF	HFNPRFEDGG	YVVCNTRQKG	TWGPPEERKM	MPFQKGMPPD
101	LCFLVQSSDF	KV/MVNGSLFV	QYFHRV/PFHR	VDTISVNGSV	QLSYISFQNP
151	RAVPVQPAFS	TVPFSQPVCF	PPRPRGRROK	PPSVRPNANPA	PITQTVIHTV
201	QSASGQMFQ	TPAIPMMYP	HPAYPMPFIT	TIPGGLYPSK	SIILSGTVLP
251	SAQRFHINLC	SGSHIAFHMN	PRFDENAVVR	NTQINNSWGS	EERSLPRKMP
301	FVRGQSFVW	ILCEAHCLKV	AVDQGHVFEY	YHRLRNLP	NKLEVGGDIQ
351	LTHVQT				

LEG9b Value matched=4 Protein sequence coverage=14%

1	MAFSGQAPY	LSPAVPFSGT	IQGGLQDGFQ	ITVNGAVLSS	SGTRFAVDFQ
51	TGFSGNDIAF	HFNPRFEDGG	YVVCNTRQKG	RWGPEERKM	MPFQKGMPPD
101	LCFLVQSSDF	KV/MVNGSLFV	QYFHRV/PFHR	VDTISVNGSV	QLSYISFQNP
151	RTVPVQPAFS	TVPFSQPVCF	PPRPRGRROK	PPSVRPNANPA	PITQTVIHTV
201	QSASGQMFQ	TPAIPMMYP	HPAYPMPFIT	TIPGGLYPSK	SIILSGTVLP
251	SAQRFHINLC	SGSHIAFHMN	PRFDENAVVR	NTQINNSWGS	EERSLPRKMP
301	FVRGQSFVW	ILCEAHCLKV	AVDQGHVFEY	YHRLRNLP	NKLEVGGDIQ
351	LTHVQT				

3.3 NG2/CSPG4 and cell migration

3.3.1 NG2/CSPG4-Threonine phosphorylation pattern

Post-translational modifications of NG2/CSPG4 provide an important means for regulating its interaction with extracellular and cytoplasmic binding partners. Phosphorylation and dephosphorylation have proved to be among the most versatile and functionally important types of post-translational modifications¹⁰². Two distinct threonine phosphorylation events

within the cytoplasmic domain of the NG2/CSPG4 proteoglycan seem to regulate the cellular balance between proliferation and motility. Evidences show that protein kinaseC- α could mediate the phosphorylation of NG2/CSPG4 at Thr₂₂₅₆, resulting in enhanced cell motility. Extracellular signal-regulated kinase seems to phosphorylate NG2/CSPG4 at Thr₂₃₁₄, stimulating cell proliferation. The effects of NG2/CSPG4 phosphorylation on proliferation and motility should be dependent on β 1-integrin activation⁵¹, but they need to be deeper investigated. To study NG2/CSPG4 phosphorilation sites on sarcoma cells during cell migration, Western Blotting have been performed on NG2/CSPG4 expressed from immunosorted HT1080^{NG2+} cells. Three different protein expression patterns were obtained using different growth conditions, and referring to section 2.8 we could obtain the following protein cell lysate from HT1080^{NG2+} showed in figure 13:

- Protein extract reflecting the proliferating expression pattern (P).
- Protein expression pattern of cells during the migration (M).
- Protein expression pattern during the starvation without any scratch (S).

Two primary antibodies have been used: the antibody anti NG2/CSPG4 sc-33038 (santa cruz biotechnology, inc.) that reacts with all phosphorylated Threonines of NG2/CSPG4 of human origin; the Phospho-Threonine-X-Arginine Antibody #2351 (Cell Signaling Technology®), which detects endogenous levels of proteins containing the motive of NG2/CSPG4 belonging the Thr₂₂₅₆ we suppose is phosphorylated during cell migration. In all three the expression patterns investigated, NG2/CSPG4 was phosphorylated at different sites, while after 72 hours of starvation and 48 hours of migration of HT1080^{NG2+} the protein resulted phosphorylated only at the site Thr₂₂₅₆ (Fig. 13, asterisk).

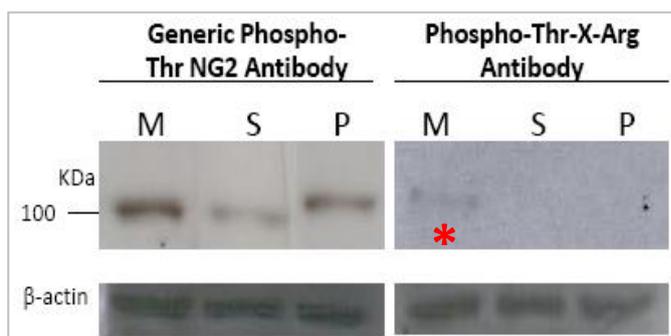


Figure 13: Immunoblotting showing HT1080^{NG2+} cells NG2/CSPG4 phosphorylation expression pattern. Left: the generic antibody anti-phosphorylated-NG2/CSPG4 has been employed, as control. It can be observed that in all the tested situation NG2/CSPG4 is phosphorylated. Right: the specific anti-phosphorylated-Thr₂₂₅₆-NG2/CSPG4 has been employed. It reacts only with protein cell lysate obtained from HT1080^{NG2+} after 72 hours of starvation and 48 hours of migration.

Immunoblotting of β -actin with a polyclonal antiserum was used for normalization. Relative position of molecular weight marker is indicated to left.

3.3.2 NG2/CSPG4 distribution during cell migration

To verify the distribution of NG2/CSPG4 on cells during HT1080^{NG2+} and A375 cells migration, staining of NG2/CSPG4 with mAbs have been performed on migrating cells in wound healing assays (Figure 14). We observed that, in cells engaged in migration, NG2/CSPG4 expression advices how the protein localized on the migration front (red arrows, Fig. 14).

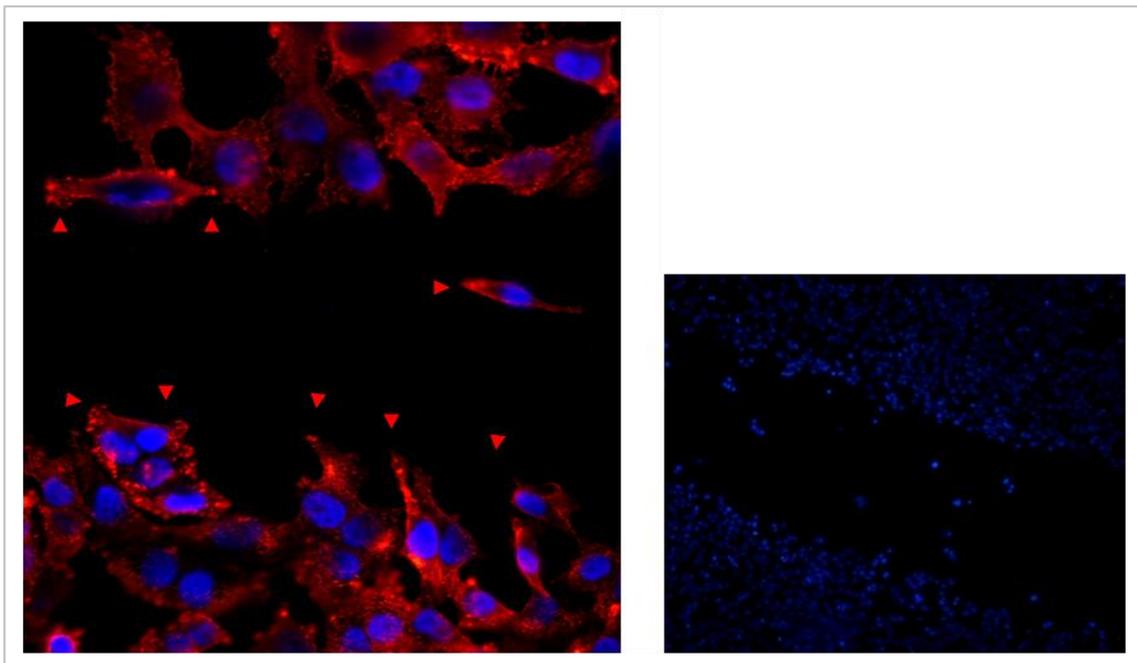


Figure 14: NG2/CSPG4 distribution during HT1080^{NG2+} cells migration in wound healing assay, shown by immunofluorescence. Cells have been seeded on glass coverlips in their complete growth medium. After the adhesion to the coverslips they were starved in serum-free medium for 24 hours, then a scratch has been done on the cell layer and cells were let migrate for 24 hours. Then paraformaldehyde-fixed cells were incubated with anti-NG2/CSPG4 mAb 2161D7 and stained with AlexaFluo-594 (red) to detect NG2/CSPG4. Nuclei were counterstained with Hoechst (Blue). Arrows indicate the distribution of NG2/CSPG4.

Slithering cells generate two major types of actin-based protrusive organelles, lamellipodia and filopodia, which have strikingly different actin polymerization machinery and are regulated by different signaling pathways^{198,199}. It has been supposed that cofilin and fascin could be essential elements in the dynamic reorganization of the actin cytoskeleton^{28,200}. As the transmembrane proteoglycan NG2/CSPG4 is able to interact with components of the extracellular matrix and with the actin cytoskeleton²⁸, we investigated its localization during

cell migrations compared with those of cofilin and fascin. Image 15 shows that NG2/CSPG4 immune-colocalize with both these molecules, above all in cellular protrusions.

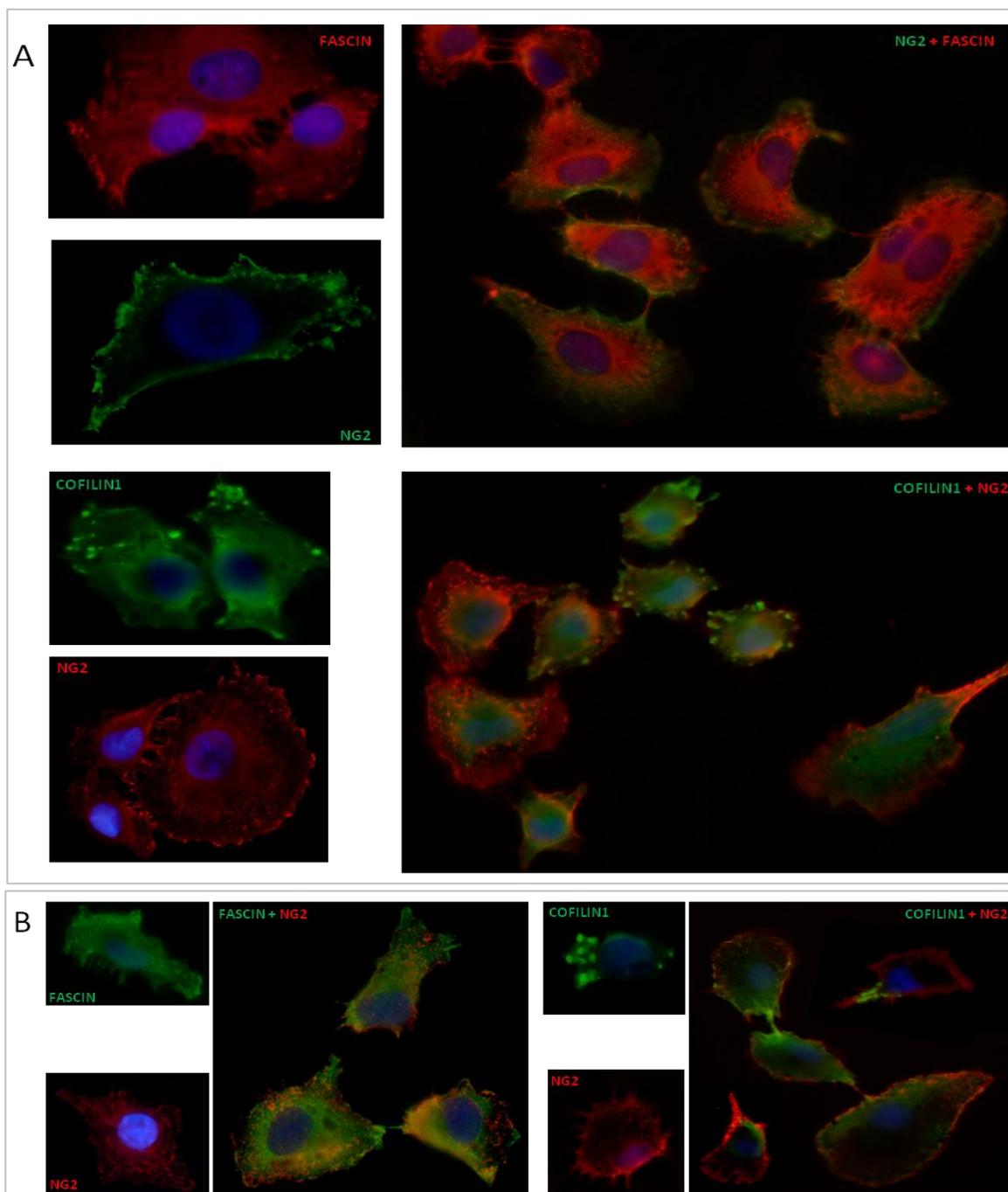


Figure 15: Immunofluorescent co-localization of NG2/CSPG4 with fascin and cofilin. Distribution of NG2/CSPG4 in A375 (A) and HT1080^{NG2+} (B) filopodia and lamellopodia is shown: starved and migrating cells have been fixed in 4% paraformaldehyde solution and then incubated with anti-fascin, anti-cofilin and/or anti-NG2/CSPG4 antibodies as reported in the panels. Immunoreactions have been revealed as follows: A) Red AlexaFluor-594 (Fascin and NG2/CSPG4 staining as indicated), Green AlexaFluor-488 (NG2/CSPG4 staining and cofilin staining as indicated), Hoechst (nuclei staining). B) Red AlexaFluor-594 (NG2/CSPG4 staining), Green AlexaFluor-488 (Fascin and cofilin staining), Hoechst (nuclei counterstaining).

3.4 Functional diversity of NG2/CSPG4 variants

3.4.1 Selection of anti-NG2/CSPG4 mAbs to induce cell apoptosis

As reported in Table 1, many anti-NG2/CSPG4 antibody have been developed and tested on melanoma and mesothelioma, in which it has been seen to be able to block multiple signaling pathways important to cell growth, migration, and survival. As nothing was investigated in sarcoma tumors in this contest, we performed a preliminary experiment to compare the effect that antibodies produced in our laboratory have on melanoma and sarcoma cells. With this aim, two mAbs and a negative control were tested on the following cells monolayer: A375 (Fig. 16A, left panel) and HT1080^{NG2+} (Fig. 16A, right panel) expressing NG2/CSPG4, and HT1080^{NG2-} (Fig 16A, central panel) unexpressing NG2/CSPG4 . Figure 16A shows that mAbs 2172D6 and 2161F9 had no effect on HT1080^{NG2-}, while seemed to detach A375 cells after 24 hours of treatment with the mAbs. It is clear from figure 16A, panel on the right, that mAbs 2172D6 and 2161F9 caused the detachment of HT1080^{NG2+} cells. To better understand the nature of this detachment we first investigated if detached cells went through apoptosis when treated with anti-NG2/CSPG4 mAbs (Figure 16B). Late apoptotic melanoma and sarcoma cells have been detected with PI. Data are reported in histogram in figure 16B on the left: 2161D2, 2164H5 tripled apoptotic cells number, compared to the control, when dosed on A375 and HT1080^{NG2+} cells; 2166G4 had no effect on HT1080^{NG2+} while redoubled A375 apoptotic cells number. All the other tested mAbs, namely 2161F9, 2161D3, 2172D6, 2172B12, 2164B6, caused late apoptosis only on HT1080^{NG2+} cells. Early apoptosis was detected in multiparametric assays, that allowed to detect both the phosphatidylserine translocation that links to the annexin V, and the Mitochondrial Membrane Potential changes (Fig 16B, right). We can observe that 2161F9 had no effect on A375 while 2161D2 had a weak effect on A375. The clones 2161D3, 2172D6, 2172B12 had a moderate effect on A375 apoptosis, and 2164B6, 2164H5, 2166G4 wew responsible of the doubling of early apoptotic A375 cells. All mAbs were effective on HT1080^{NG2+}, although mAb 2172B12 resulted to be the weakest among them.

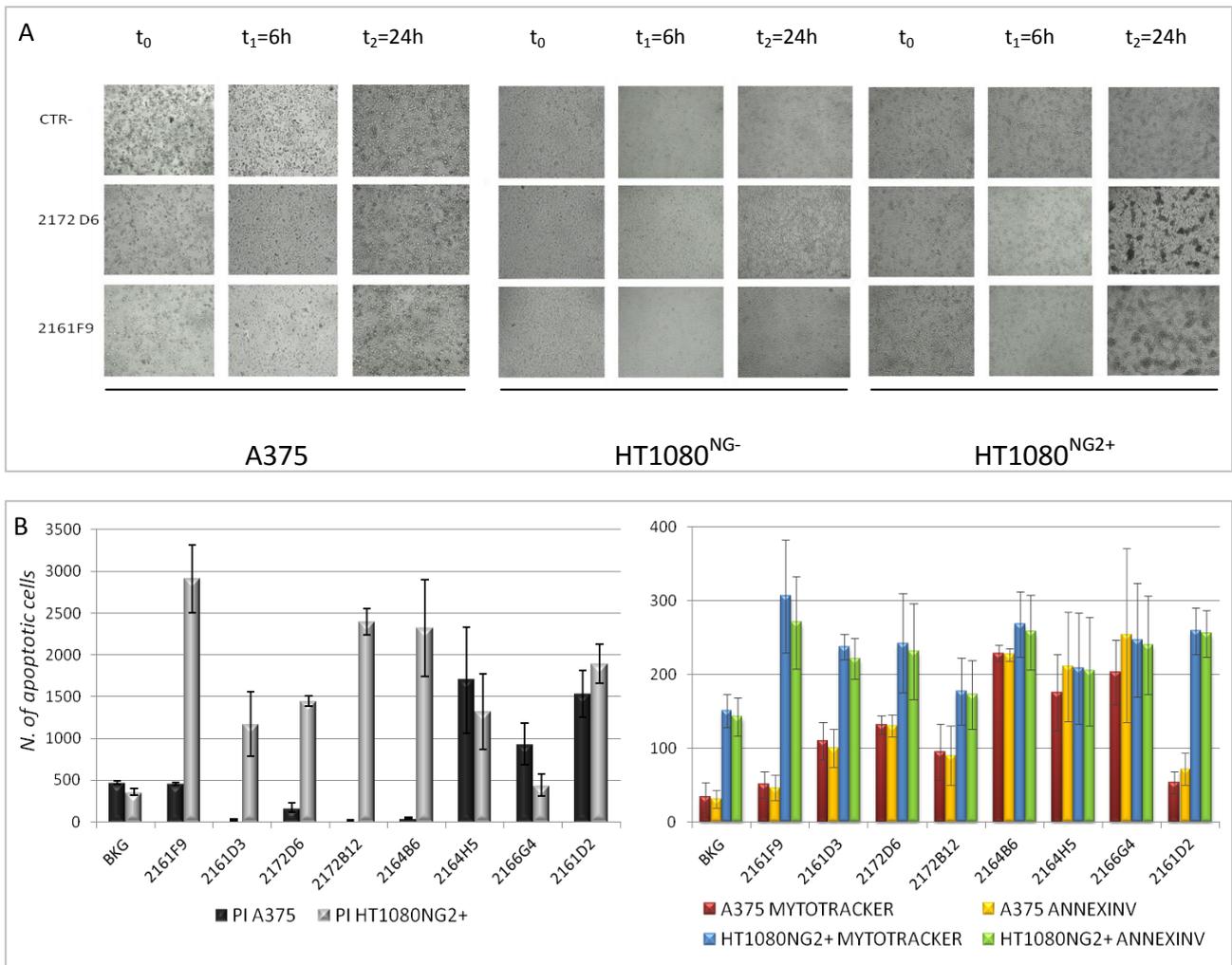


Figure 16: Effect of anti-NG2/CSPG4 mAbs on cultured tumor cells. A) Cells were seeded and treated with anti-NG2/CSPG4 mAbs 2171D6 and 2161F9 and a negative control. Pictures of the cell monolayer were taken at different times after the dosage (t_0 , $t_1=6h$, $t_2=24h$). Three cell lines have been tested: A375 (left) and HT1080^{NG2+} (right) cells highly expressing NG2/CSPG4, and HT1080^{NG2-} (center) that do not express NG2/CSPG4. The two mAbs caused the detachment of cells after 24 hours of dosage on cells expressing NG2/CSPG4, above all on HT1080^{NG2+}. B) Apoptosis assays. On cells treated with anti-NG2/CSPG4 mAbs, late (left) and early (right) apoptosis have been detected with Acumen technology. For Late apoptosis propidium iodide (PI) has been used to stain cells. For early apoptosis, phosphatidylserine translocation is detected with AlexaFluor-488 Annexin V, while the Mitochondrial Membrane Potential changes are detected with MitoTracker Red.

3.4.2 Effect of anti-NG2/CSPG4 antibodies on cell adhesion

As with normal cells, the adhesion of tumor cells influences their cytoskeletal organization, activation of signal transduction pathways within the cell and nuclear events leading to changes in mRNA transcription and protein synthesis³². NG2/CSPG4 is linked to cell adhesion and, although its cytoplasmic domains has no intrinsic kinase activity, it may serve as binding sites for kinases and cytoskeletal proteins. It seems to influence cell adhesion, and frequently influence integrin receptor functions and signaling²⁰¹. We investigated the effect that anti-NG2/CSPG4 mAbs have on HT1080^{NG2+} sarcoma cell adhesion with the aim to select those able to determine the detachment of tumor cells from their substrate. This study was performed using the xCELLigence System (Roche) and data analysis were performed using the RTCA Software. The output of the system permits to display and record in real time the dynamic monitoring of cell attachment and spreading observed after mAbs dosage. Results are shown in plots (CI versus timing of the experiment), and histograms (displaying the CI slope): the first gives evidence of the attachment/spreading of cells, the latter refers to the slope of the curves reported in the plots. In these experiments each antibody was tested in quadruplicated, and two independent experiments were performed. From the results, we can observe that some tested antibodies had an effect on HT1080^{NG2+} vital functions, in fact their dosage on cells avoided their proliferation. This behavior of cells was not observed when positive control mediums were dosed on HT1080^{NG2+}. The following mAbs had the described blocking activity: 2164H5, 2164B6, 2172B12, 2161D3. The most interesting effect was observed at the hands of mAb 2172D6, that determined the detachment of HT1080^{NG2+} cells as shown in figure 17.

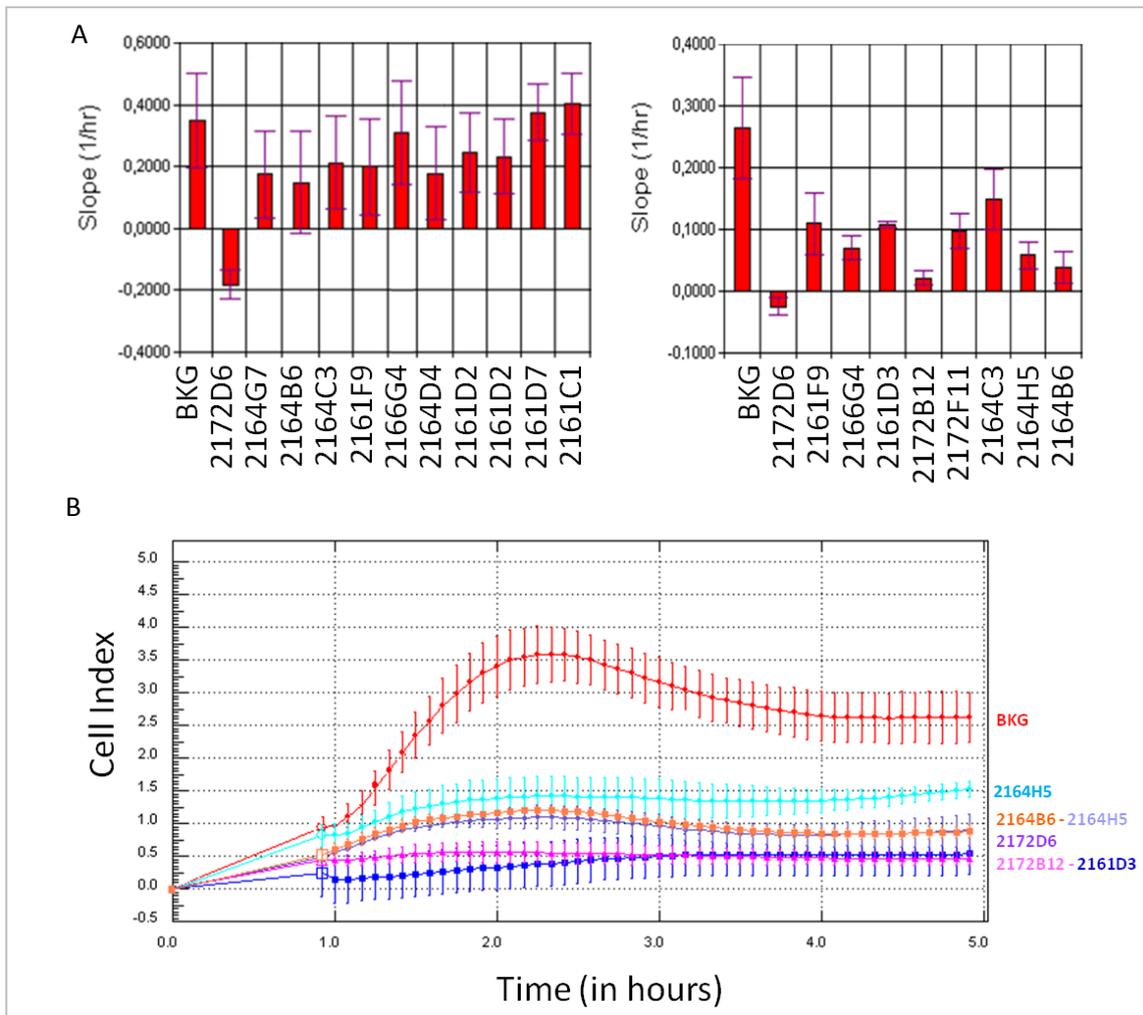


Figure 17: Anti-NG2/CSPG4 mAbs effect on sarcoma cells adhesion. Starved, adherent HT0180^{NG2+} cells were treated with mAbs and their adhesion was measured and monitored within 5 hours of treatment with the Xcelligence System (Roche). A) Histograms show the effect of all mAbs on cell index (CI) variation within 5 hours of dosage compared with the background (BKG= complete hybridoma growth medium containing an irrelevant IgG). B) Plot of the experiment shows the cell adhesion in real time within the 5 hours of mAbs dosage.

3.4.3 Effect of anti-NG2/CSPG4 antibodies on cell migration

Cell migration is a fundamental cellular process essential for tumor development and recurrence. Several studies suggest that NG2/CSPG4 plays a role in effective cell migration. We wanted to investigate if immunosorted cells from the same cell line differ in cell motility. From the wound healing assays performed on HT1080^{NG2+} and HT1080^{NG2-} sarcoma cells, treated in the same conditions, we could observe that cells expressing NG2/CSPG4 migrate

faster closing the scratch, compared to the ones that do not express NG2/CSPG4. Analysis refers to 24 hours of monitoring (Fig. 18).

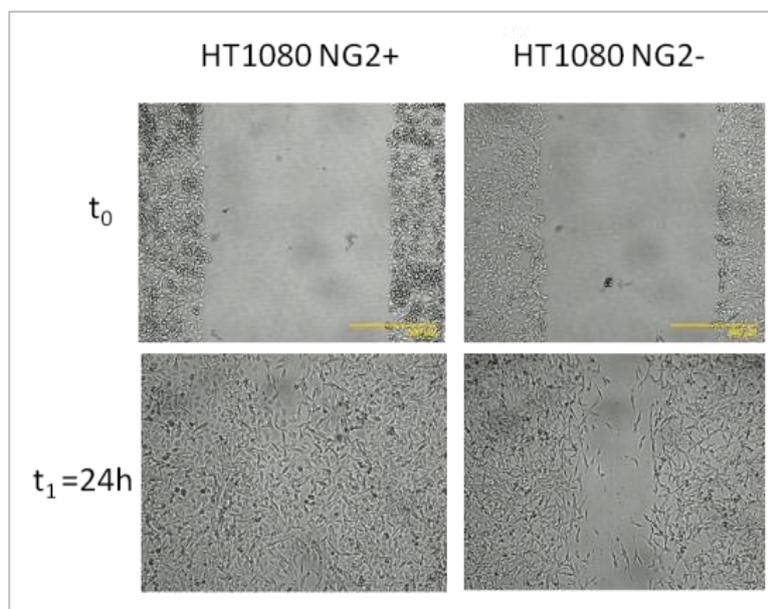


Figure 18: Wound healing assay. Sarcoma cells immunosorted and selected to express NG2/CSPG4 (HT1080^{NG2+}, on the left) and to do not express NG2/CSPG4 (HT1080^{NG2-} on the right) are compared in a wound healing assay. Cells were seeded at 90% of confluence and starved in serum-free medium for 24 hours. Their migration was measured and pictures were taken within 24 hours of migration. HT1080^{NG2+} cells covered the scratch while HT1080^{NG2-} cells did not.

For the anti-NG2/CSPG4 mAbs screenings, HT1080^{NG2+} sarcoma cells have been employed and, on their cell monolayer, mAbs were dosed with the aim to select those able to block cell migration. Time lapse microscopy has been employed for the monitoring and measure of cells migration in an experimental time of 24 hours. The migration rate of cells is measured via the mean displacement (MD, i.e. the mean distance (μm) traveled per minute) of the cell centroid. The migratory potential of cell populations can then be expressed as the average mean displacement (AMD) of all cells in the analysis. In order to determine how the manual selection of the centroid positions of cells influences the calculated migration rates of individual cells and cell populations, we manually marked cells using a point-and-click system¹⁶⁹. In this manual cell tracking method, we selected a subset of cells from each time-lapse video for analysis, which is then assumed to represent the whole cell population. Green lines in figure 19A represent the tracking of cells when incubated with different mAbs. We can observe that the tracking was highly influenced by the kind of dosed mAb, and from the tracking analysis it is possible to identify which mAbs resulted more effective (Figure 19). In the first histogram (Fig. 19B) cells speed for each different well is plotted; in

the second histogram (Fig. 19C) the covered distances is plotted for each mAb dosed on HT1080^{NG2+} cells. We can identify some anti-NG2/CSPG4 mAbs able to interfere with normal vital cell functions when incubated with HT1080^{NG2+}. In particular, the clones 2161F9, 2164C3, 2164H5, 2172B12 slowed down cell migration in wound healing assay. It is interesting to observe that 2161D3 and 2172D6 determined a complete block of migration and the detachment of cells after few hours of dosage.

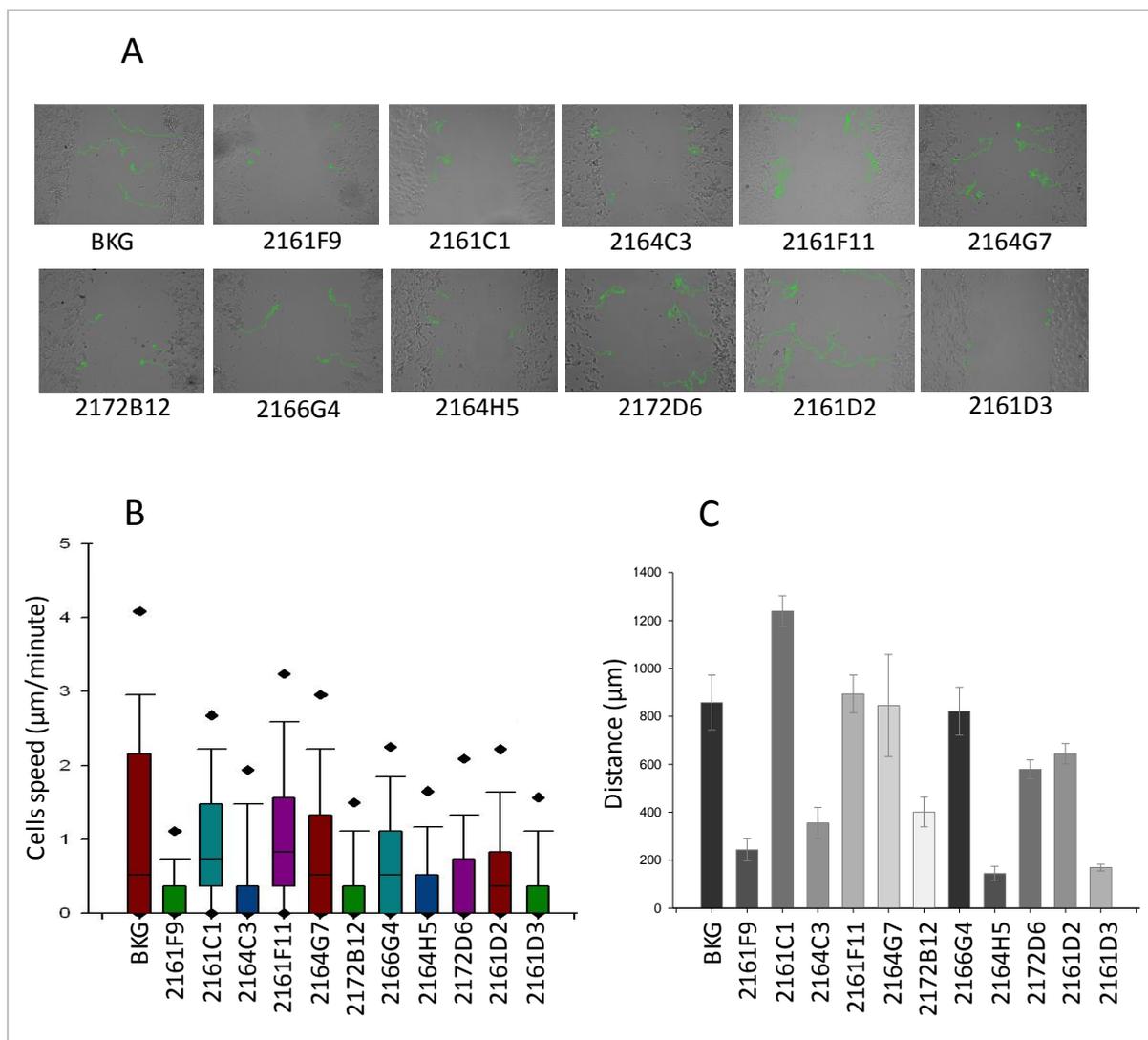


Figure 19: Effect of anti-NG2/CSPG4 mAbs on cell migration in wound healing assays. Starved HT1080^{NG2+} cells were scratch in the middle of their monolayer and their migration was monitored by a time lapse microscope in 24 hours. A) tracking of cells (highlighted in green) when incubated with different mAbs. B) Histogram of cell speed obtained from the tracking performed on migrating cells. C) Distance run from cells when incubated with the anti-NG2/CSPG4 mAbs.

4. DISCUSSION AND CONCLUSIONS

From the first studies on NG2/CSPG4 expression and function on melanoma cells^{1,35}, till the last works on other kinds of tumors such as mesothelioma^{6,123} and breast carcinoma¹⁷, a large panel of anti-NG2/CSPG4 antibodies has been developed (Table 1). This large collection of anti-NG2/CSPG4 antibodies has facilitated the characterization of the antigenic profile of the molecule and has provided a useful probe to dissect the antigenic heterogeneity of tumor cells and tumor lesions, as well as to define the functional role of distinct domains of NG2/CSPG4. To approach the molecular traits of NG2/CSPG4 isoforms expressed by different cell lines we made use of a subset of our anti-NG2/CSPG4 mAbs found to recognize NG2/CSPG4 variants expressed in sarcoma (143B, HT1080^{NG+}, SK-LMS-1, SK-UT-1) and melanoma (A375, M2) cell lines, as well as proteolytic fragments of its ectodomain generated under both physiological and pathological conditions. Comparative immunoblotting experiments substantiated the presence of isoforms of the PG and a certain fragmentation. Data were confirmed when immunoblotting experiments were performed involving tissue extracts derived from regions of the fetal brain containing accumulations of NG2/CSPG4-expressing neovascular pericytes and the above reference tumour tissues/cells, lesions of glioblastoma multiform and by A375 melanoma cells. In these cases degree of proteolytic processing of the PG seemed significantly lower in fetal brain neovessels when compared to tumour tissues and isolated tumour cell lines. This findings may be unexpected considering that although tumors produce excessive amounts of metalloproteinases and other proteases capable of degrading NG2/CSPG4, such enzymes also accumulate in sprouting angiogenic vessels. Notably, angiogenic NG2/CSPG4 isoforms of fetal brain appeared to be overall more glycosylated than their counterpart cancer-associated ones. The nature of this glycosylation and its biological significance remains to be defined. It remains similarly to be determined the precise molecular identity of the NG2/CSPG4 fragments generated by sprouting vessels and whether release of these fragments contributes to sequestering of extracellular signaling molecules (primarily growth factors), as reported for cell surface shedded syndecan fragments^{201,202}.

To investigate eventual molecular interactors of NG2/CSPG4 expressed in melanoma cells, we performed a broad-spectrum analysis of A375 cells protein expression. MALDI-TOF analyses lead to the identification of two types of interactors: one type seems to interact with NG2/CSPG4 strongly, since we could detect these molecules without the employment of cross-linking agent, the second panel of putative interactors seem to be linked to NG2/CSPG4 weakly, as their detection was possible only after a cross-linking treatment of cells before the protein pattern study. We do not know the nature of the interactions, neither if they are direct linked to NG2/CSPG4 or through other molecules, but this screening will give us a useful instrument for further more focused studies.

Independently from the kind of links between NG2/CSPG4 and its putative interactors, we can do some speculation about them. First we are glad to confirm some of the molecules found to be NG2/CSPG4-interactors in previous studies and reported in figure 6 such as: galectins, plasminogen-like protein (whose sequence in MASCOT data base could mimic those of plasminogen), and actins. Among the obtained results most of the proteins are involved in cell migration regulation and in the organization of cytoskeleton: Actin related protein, Microtubule-associated protein 2, Myosin-14, Myomesin-2, LIM domain and actin-binding protein, Plexin-A4, spectrin alpha chain, non-erythrocytic 1 Protein. Among these molecules, we are particularly interested in a in-deep analysis of the interaction between NG2/CSPG4 and Plexin A4, as it is a single-pass type I transmembrane protein¹⁷⁶ found to promote tumors progression and tumors angiogenesis by enhancement of VEGF and bFGF signaling¹⁷⁷. Moreover NG2/CSPG4 and plexin have been seen to be both involved together in injured central nervous systems²⁰³ and from our experiments a similar cooperation could be hypothesized in melanoma cells.

One of the most interesting results of this research has been the detection of fascin among the outcomes of the proteomic screening: in literature it has been observed that fascin- and NG2/CSPG4-positive structures are often localized to opposite poles of spreading cells, suggesting a possible role for the two classes of cellular extensions in the establishment of cell polarity during morphogenesis or migration²⁸. Moreover from our immunocytochemical studies we could detect a co-localization of NG2 and fascin both in

sarcoma (H1080^{NG2+}) and in melanoma (A375) cells as reported in section 3.3.2. Our observations are coherent with mass spectrometric results and could put the basis for a deeper investigation about NG2/CSPG4 and fascin relationship and their eventual interaction.

Another interesting finding is represented by the putative association of NG2/CSPG4 with the β 5-integrin: in literature many studies revealed a relationship between NG2/CSPG4 and other kinds of integrins (α 2-, α 3- and α 4- β 1 integrins as reported in figure 6) but nothing has been proposed about the relation between this specific β 5-integrin and NG2/CSPG4. Nevertheless in a study performed on the role of β 5-integrin, researchers injected A375 melanoma cell lines in mice lacking β 5-integrins for the study of tumorigenesis and metastases processes, reporting that the absence of this integrin did not block vessel development, which was in fact enhanced, even if the angiogenesis decreased²⁰⁴. As β 5-integrins usually block vessel formation, our findings of a putative interaction between NG2/CSPG4 and β 5-integrins could explain its inhibition, possibly due to the interaction with NG2/CSPG4 .

Concerning the detection, among the interactors, of erlin-1 (a single-pass type II membrane protein that has been found in associated with lipid raft-like domains of the cell membran¹⁹⁵⁻¹⁹⁷), periostin (that binds to heparin, induces cell attachment and spreading, plays a role in cell adhesion and may play a role in extracellular matrix mineralization found to be secreted in the extracellular space reaching the extracellular matrix¹⁸³⁻¹⁸⁵) and heparan sulfate glucosamine 3-O-sulfotransferase 3A1, we could hypothesize that NG2/CSPG4 could be involved in a pathway of endocytosis, maybe thanks to the involvement of TGF- β receptor through a clathrin-independent/lipid raft pathway, as it happens for many other proteoglycans²⁰⁵⁻²⁰⁹ .

We would be interested in the putative interaction between NG2/CSPG4 and the von Willebrand factor A domain-containing protein 5A (VMA5A), as this molecule has been found to play a role in tumorigenesis as a tumor suppressor, moreover researchers supposed that altered expression of this protein and disruption of the molecular pathway it is involved in, could contribute directly to modify tumorigenesis¹⁷⁴ .

PDZ domains are protein interaction modules that form sub-membranous complexes and regulate targeting and trafficking of cell surface molecules²⁰¹. It has been demonstrated that the intracellular C-terminal domain of NG2/CSPG4 contains the binding motif QYWV, which can interact with PDZ domain-containing proteins⁴⁹. This is compatible with one of the highest score putative interactors inferred with MALDI-TOF analysis, ERC protein 2, which interacts through its C-terminus with the PDZ domain PDZ-containing proteins and it is found to be above all distributed in cell junction and associated to the cytoskeleton¹⁸⁷. Like other PDZ-domain proteins found to interact with NG2/CSPG4, also ERC protein 2 could play a scaffolding role in this interaction.

It remains to be determined how NG2/CSPG4 would interact with the metalloredutase STEAP3, a six-pass membrane protein that localizes to vesicular-like structures at the plasma membrane.

Finally, among the molecules discovered from the analysis, we noticed that, from all samples analyzed, 19 zinc-finger proteins and 5 coiled-coil domain-containing proteins have been identified: this let us think that NG2/CSPG4 could have high affinity for these kinds of domains probably along its cytoplasmic tail and even if the functional role of these interactions is unknown, we could speculate that they could have a structural significance.

As outlined in Figure 6, NG2/CSPG4 has been demonstrated or hypothesized to interact with many extracellular and cytoplasmic molecules, with its cytoplasmic C-terminal tail, the N-terminal extracellular domain, and the chondroitin sulphate chain it carries on the core protein. These interactions have been proposed to activate many signalling pathways such as the integrin/focal adhesion kinase signalling and MAPK pathway signaling, leading to tumor progression through a variety of cellular functions: cytoskeletal reorganization, migration, proliferation, invasion, survival and chemoresistance, epithelial–mesenchymal transition^{45,64,85,95,101} (Fig. 6B). Our findings confirm the involvement of NG2/CSPG4 in these molecular signaling pathways, and open the way for further investigations regarding the new putative molecular interactors found and their role in cancer.

The panel of anti-NG2/CSPG4 mAbs produced in our laboratory, when screened, showed reactivity towards NG2/CSPG4 expressed both on melanoma and sarcoma cells, opening the way to investigation also in sarcoma tumors. As in the literature there is no information in the context of anti-NG2/CSPG4 target therapy on sarcoma tumors aimed at interfering with cell growth, migration, or survival, we screened *in vitro* our anti-NG2/CSPG4 mAbs on sarcoma cells. Since from the first evidences, mAbs 2161F9 and 2172D6 seemed to determine a detachment of cells when dosed on expressing-NG2/CSPG4 sarcoma cells, and from further investigations, we could distinguish mAbs able to interfere with cell adhesion from those able to cause sarcoma cells apoptosis. These data confirm the involvement of NG2/CSPG4 in sarcoma and melanoma cell adhesion and survival, suggesting that some of the antibodies could be employed in *in vivo* tests to select those that could be employed in immunotherapy strategies, above all against sarcoma. In this contest we can group antibodies depending on their blocking activities: 1) pro-apoptotic mAbs on sarcoma cells (2161F9, 2164H5, 2161D2); 2) apoptosis inducing and adhesion blocking mAbs on sarcoma cells (2161D3, 2172D6, 2172B12, 2164B6). We did not detect mAbs able to act only on the adhesion of cells without interfere with their survival. This effect could be due to the fact that the detached cells, revealed from the adhesion assays, could be all apoptotic cells, and this suggests that selected mAbs are so aggressive against sarcoma to induce cell death in 24 hours of dosage, while within 6 hours of administration in adhesion assays it is possible to detect also the effect on this function, even if the detachment is probably due to the pro-apoptotic action of mAbs on sarcoma cells.

Cell adhesion is a complex mechanism involved in a variety of processes including cell migration and invasion³², and previously works showed that cells expressing the NG2/CSPG4 proteoglycan can attach, spread, and migrate on surfaces coated with anti-NG2/CSPG4 mAbs, suggesting that engagement of NG2/CSPG4 can trigger the cytoskeletal rearrangements necessary for changes in cell morphology and motility²⁶. For this reason we first aimed to confirmed the involvement of NG2/CSPG4 in cell migration with a wound healing experiment on HT1080^{NG2+} cells compared to HT1080^{NG2-}. As we

expected, cells expressing NG2/CSPG4 migrated faster than their NG2/CSPG4-negative counterpart.

Numerous structures are involved in regulating protrusion of membrane at the leading edge of motile cells¹⁹⁹, and there are evidences in literature that lamellipodia localize on the leading edges of migrating cells, while numerous filopodia are present on trailing edges²⁸. To determine NG2/CSPG4 distribution in lamellopodia and filopodia, we coupled wound healing assays with immunocytochemical staining of NG2/CSPG4 mAbs together with fascin¹⁹² and cofilin²¹⁰, since these molecules are typically expressed in membrane protrusions during cell migration^{28,199}. Data show a co-immuno-localization of NG2/CSPG4 with both these molecules, suggesting a clear involvement of NG2/CSPG4 in migration of tumor cells and a probable function in cell membrane protrusion organization during cell migration. In the light of these results on migrating cells, screening of our anti-NG2/CSPG4 mAbs in wound healing assays have been performed to test their ability to interfere with sarcoma cells migration. These functional assays gave evident results of a blocking action, leading to the selection of a list of anti-NG2/CSPG4 mAbs able to slow down sarcoma cells migration: 2161F9, 2164C3, 2172B12, 2164H5, 2161D3, 2172D6 (weak effect).

Comparing these results with the groups previously identified as functional blocking anti-NG2/CSPG4 mAbs, we can observe that 2161D3 and 2172B12 are the most aggressive antibodies since they interfere with all the cellular functions investigated.

Our findings suggest NG2/CSPG4 as a promising new target to implement mAb-based immunotherapy of sarcoma, and we selected a panel of more aggressive antibodies able to inhibit tumor growth, adhesion and migration (mAbs 2161D3 and 2172B12), the interesting mAb 2164C3 which play a targeted action on block of sarcoma cell migration, and a panel of mAbs able to induce detachment of cells and finally their apoptosis: 2161F9, 2164H5, 2161D2, 2161D3, 2172D6, 2172B12, 2164B6. These groups of mAbs need to be tested *in vivo*, with the possibility to set the experimentation of an anti-NG2/CSPG4 drug against sarcoma, and a further molecular investigation to understand the nature of their different ability to block NG2/CSPG4 functions, that could be linked to

the different isoforms observed in our preliminary screening discussed above. Our findings of isoforms among the different cell lines would be in accordance with the *in silico* studies of the polymorphisms within the CSPG4 gene (Fig. 1) that show several missense SNPs coupled with 5 stop codon gains, which could lead to the expression of a NG2/CSPG4 molecule characterized by aminoacidic substitution, or even shorter forms expression of the protein. These observations and our findings let us hypothesize that NG2/CSPG4 isoforms we identified could be due to a different glycosylation pattern of the PG, to modification within the core-protein, and to shorter molecules expressed from different tumors in different contexts.

Our studies open the way for further studies aimed to the characterize these isoforms in the context of the *in vivo* validation of anti-NG2/CSPG4 target therapy against different kinds of tumors.

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Acknowledgements

I am grateful to Prof. Roberto Perris, as he gave me the possibility to work in his team, following me with confidence and patience.

I want to thank Dr. Domenica Mangieri, for her precious personal and scientific support.

Thanks to Dr. Daniela Zanocco for Flow Cytometry analysis.

Thanks to Dr. Davide Imperiale, for his important advices about Mass Spectrometry analysis.

Thanks to my colleagues Alessandra, Carlotta, Nicoletta, Silvia.

Thanks to the students who participated with passion to this project.

Thanks to my friends, the same of ever.

Thanks to my family, my pilasters.