

The Stem Cell Marker CD133 (Prominin-1) is Phosphorylated on Cytoplasmic Tyrosine-828 and Tyrosine-852 by Src and Fyn Tyrosine Kinases[†]

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ABSTRACT: CD133 (prominin-1) is a transmembrane glycoprotein expressed at the surface of normal and cancer stem cells, progenitor cells, rod photoreceptor cells, and a variety of epithelial cells. Although CD133 is widely used as a marker of various somatic and putative cancer stem cells, its contribution to fundamental properties of stem cells such as self-renewal and differentiation remains unknown. CD133 contains a short C-terminal cytoplasmic domain with five tyrosine residues, including a consensus tyrosine phosphorylation site that has not yet been investigated. In this study, we show that CD133 is phosphorylated in human medulloblastoma D283 and Daoy cells, in a Src family kinase-dependent manner. The cytoplasmic domain of CD133 is tyrosine phosphorylated in Daoy cells overexpressing Src and Fyn tyrosine kinases, as well as in vitro using recombinant proteins. Deletion of the C-terminal cytoplasmic domain of CD133 considerably reduced its phosphorylation by Src. To identify the tyrosine phosphorylation sites in CD133, we used matrix-assisted laser desorption/ionization quadrupole time-of-flight (MALDI Q-TOF) and liquid chromatography tandem mass spectrometry (LC-MS/MS). Analysis of tyrosine-phosphorylated CD133 by mass spectrometry and site-directed mutagenesis identified tyrosine-828 and the nonconsensus tyrosine-852 as the major tyrosine phosphorylation sites both in vitro and in intact cells. Identification of CD133 as a substrate for Src-family tyrosine kinases suggests that the cytoplasmic domain of CD133 might play an important role in the regulation of its functions.

CD133 (prominin-1), a 115–120 kDa five-transmembrane domain glycoprotein encoded by the *PROM1* gene, was identified independently by two research laboratories in 1997 (reviewed in refs (1–4)). Prominin-1 was isolated from mouse neuroepithelial cells in an effort to identify new proteins specifically localized in microvilli of the apical surface of epithelial cells (5). CD133 (originally named AC133) was identified as a novel cell surface marker for human hematopoietic stem and progenitor cells (6,7). However, CD133 expression is not limited to neuroepithelial and hematopoietic stem/progenitor cells, as originally observed, but is also expressed in adult epithelial cells (5,8–10), nonepithelial cells such as rod photoreceptor cells (11,12), and endothelial precursor cells

(13,14). Recently, several laboratories have successfully used various cell surface markers (e.g., CD133, CD34, CD44, CD24) to isolate putative cancer stem cells (CSCs) from a wide variety of cancer types (15,16). Among these, CD133 has emerged as the most important marker, being expressed in a number of CSCs identified to date in brain (17,18), pancreas (19), prostate (20,21), lung (22), liver (23,24), and colon tumors (25,26). In brain, CD133 is highly expressed in CSCs from medulloblastomas (27) and glioblastomas (28,29), and has been used to isolate and characterize the highly tumorigenic potential of these CSCs. However, it is not clear at this point how CD133 contributes to CSCs characteristics such as resistance to therapy (29–31), self-renewal and differentiation (32).

Although the expression of CD133 at the surface of several cancer stem cells suggests a role of the protein in the biological properties of these cells, the function of CD133/prominin-1 remains unknown. CD133 does not have any known enzymatic activity, extracellular

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ligands, or intracellular binding proteins. However, the preferential localization of CD133 in plasma membrane protrusions (5,33), and its ability to specifically interact with plasma membrane cholesterol in cholesterol-based lipid rafts (34), suggest an involvement of CD133 as a regulator of plasma membrane topology (2). By establishing and maintaining membrane protrusions, CD133 may thus be involved in cell polarity and migration (35) via cell–cell and cell–extracellular matrix interactions.

CD133 is expressed in adult retinal photoreceptor cells, and a rare single-nucleotide deletion identified in an Indian pedigree was found to cause retinal degeneration due to impaired photoreceptor disk morphogenesis (11). More recently, a R373C missense mutation in the prominin-1 gene was identified in patients with familial macular degeneration (36). Interestingly, in transgenic mice expressing the mutant prominin-1 human gene, the outer segment disk membranes were overgrown and mis-oriented indicating that CD133/prominin-1 plays a critical role in the organization of retinal rod photoreceptor disks (36).

Murine prominin-1 and human CD133 are highly similar in term of topologies, although their amino acid sequence showed only 60% identity. CD133 and prominin-1 cDNAs both encode for five-transmembrane domain proteins with a 85 amino acids extracellular N-terminus, a 52 amino acids cytoplasmic C-terminus, and two large extracellular loops containing eight consensus sites for *N*-linked glycosylation (5,7). Other features revealed by Procite analysis include a putative signal peptide at the N-terminus and, interestingly, five tyrosine residues in the cytoplasmic C-terminus domain including a tyrosine phosphorylation consensus site [R/K]xxx[D/E]xxY (37–39). In this study, we report that CD133 is phosphorylated within its C-terminal cytoplasmic tail, in a Src family kinase-dependent manner. The phosphorylation of CD133 tyrosine-828 may regulate its interaction with SH2-domain containing proteins, which may be involved in intracellular signaling events.

MATERIALS AND METHODS

Antibodies and Reagents. Rabbit monoclonal anti-CD133 antibody (C24B9) was purchased from Cell Signaling Technology (Danvers, MA); mouse monoclonal antiphosphotyrosine antibody (PY99) was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Src mouse monoclonal antibody (GD11) and recombinant human Src expressed in Sf21 cells were from Upstate Biotechnology (Lake Placid, NY). Antimouse and anti-rabbit horseradish peroxidase-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and enhanced chemiluminescence reagents were from Perkin-Elmer Life Sciences (Boston, MA). PP2, PD98059, SB203580, and LY294002 were from Calbiochem (La Jolla, CA). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). All other reagents were from Sigma-Aldrich (Oakville, ON, Canada).

Cell Culture and Transfection. Medulloblastoma cell lines D283 (HTB-185) and Daoy (HTB-186) were

purchased from the American type Culture Collection and cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum and 1 mM pyruvate. Transfections were performed using FuGENE HD reagent (Roche Applied Science) following the manufacturer's instructions. Stably transfected Daoy cells expressing high levels of CD133 were selected and maintained in 1 mg/mL G418.

Immunoprecipitation and Western Blotting. Cells were lysed on ice in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, and 1 mM NaF) and the lysates were clarified by centrifugation at 16000g for 20 min. For immunoprecipitation, equal amount of proteins were incubated in lysis buffer overnight at 4 °C in the presence of 1–2 µg/mL of specific antibodies, and the immune complexes were collected by incubating the mixture with protein A- or G-coupled Sepharose beads. Bound proteins were solubilized in Laemmli sample buffer, boiled for 3 min, and separated by SDS–PAGE. The proteins were transferred onto polyvinylidene difluoride membranes, blocked overnight at 4 °C in Tris-buffered saline-Tween 20 (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 3% (w/v) bovine serum albumin, and incubated for 2 h at room temperature with the specified primary antibodies. Immunoreactive bands were revealed after a 1 h incubation with horseradish peroxidase-conjugated antimouse or antirabbit secondary antibodies, and the signals were visualized by enhanced chemiluminescence. Densitometric analysis was performed using the IPLab software.

Cloning of CD133 C-Terminus Deletion Mutants. To construct CD133 cDNAs in which either half (26 amino acid residues) or the entire C-terminal cytoplasmic domain (52 amino acid residues) is deleted, a stop codon was introduced by PCR after Val-813 or Met-839. PCR products were amplified by PfuUltra polymerase (Stratagene) using pCR3.1-Uni-AC133 (a generous gift of Dr. Denis Corbeil, Dresden, Germany) as the template using the following primers: 5'-CTTTCTGTTGGGTG CAGCAGGAAG-3' (forward) and 5'-GACTCGAGT CACATATTTTTCATGGGTATAGTTTC-3' (reverse) to generate a 630 bp product (CA26); and 5'-CTTTCTG TTGGGTGCAGCAGGAAG-3' (forward) and 5'-GACTCGAGTCATACCGCAAAAATTAGAGC-3' (reverse) to generate a 708 bp product (CA52). The PCR products were digested, gel purified, and then inserted in *BsgI* and *XhoI* sites of pCR3.1-Uni-AC133 to generate pCR3.1-Uni-CD133CA26 and pCR3.1-Uni-CD133CA52.

Cloning of NH₂-Terminal Flag Tagged-CD133. To construct the Flag-CD133 cDNA, a Flag peptide (DYKDDDDK) was added between the signal peptide and the extracellular N-terminal domain of CD133. First, a 114-bp nucleotide sequence corresponding to the CD133 signal peptide (Met₁ to Ser₁₉) was fused to a Flag-tag at the C-terminus was amplified using hCD133 forward primer A (5'-CGTTTAAACTTAAGCTTGAG-3') and hCD133 reverse primer B (5'-CTTATCGTCGT CATCCTTGTAATCTGAAAAGGAGTTCCCGCAC AG-3') with full-length CD133 cDNA as a template.

Next, a 660-bp nucleotide sequence corresponding to Flag-tag followed by CD133 sequence from Gly-20 to Asn-231 was amplified using the hCD133 forward primer C (5'-GATTACAAGGATGACGACGAT AAGGGAGGGCAGCCTTCATCCACAG-3') and hCD133 reverse primer D (5'-GTTCAGATCTGT GAACGCCTTGTCCTTGG-3'). The above two PCR products were purified from agarose gels, and used as templates to amplify a 750-bp nucleotides sequence, with hCD133 forward primer A and hCD133 reverse primer D. The PCR product was purified (Qiagen) and ligated in *Hind*III and *Bgl*II sites of the original template pCR-Uni-AC133. Flag-CD133CA26 and Flag-CD133CA52 were constructed by subcloning a *Hind*III-*Bgl*II fragment containing the N-terminus of CD 133 and the Flag-tag, from pCR3.1-Uni-FlagCD 133 into pCR3.1-Uni-CD133CA26 and pCR3.1-Uni-CD133CA52, respectively.

Mutagenesis. Mutations of CD133 cDNA were introduced using the QuikChange Lightning site-directed mutagenesis kit (Stratagene). Mutations were introduced into a set of primers that were used for mutant strand synthesis using the template plasmid pCR3.1-Uni-AC133 and *Pfu*Ultra HF DNA polymerase (Stratagene). After mutant strand synthesis, the template plasmid was removed by *Dpn*I digestion. The mutated plasmids were transformed into XL10-Gold. Mutations were confirmed by sequencing (Bio S&T, Montreal, QC, Canada).

Expression of GST and GST-CD133CD Proteins in Bacteria. The plasmid used to express a GST¹ fusion protein containing CD133 C-terminal cytoplasmic domain (CD133CD; amino acids 814 to 865) was constructed using PCR amplification by *Pfu*Ultra polymerase (Stratagene). A 172-bp PCR product was amplified using pCR3.1-Uni-AC133 as the template and with the following primers: 5'-CGGGATCCAACT GGCTAAGTACTATCGTTCG-3' (forward) and 5'-GG AATTC AATGTTGTGATGGGCTTGT-3' (reverse). The PCR product was digested, gel purified, and then inserted in *Bam*HI and *Eco*RI sites of pGEX4T-3 to generate pGEX-CD133CD. pGEX4T-3 and pGEX-CD133CD were transformed into *Escherichia coli* BL21 RP (Stratagene). The production of GST or GST fusion protein was induced by adding 1 mM isopropyl β -D-thiogalactoside (IPTG) to the culture medium, followed by an additional incubation at 37 °C for 2 h. Bacteria were pelleted by centrifugation at 6000g, resuspended in cold PBS, and sonicated. 1% Triton X-100 was added to the lysate and the insoluble material was removed by centrifugation at 12000g for 15 min. GST and GST-CD133CD proteins were purified on a glutathione-Sepharose column followed by dialysis in PBS. An equal volume of glycerol was added to the purified proteins before storage at -20 °C.

In Vitro Phosphorylation. Phosphorylation of recombinant GST-CD133CD was performed by incubation of 10 μ g of GST-CD133CD with 0.25 μ g of recombinant active Src in 50 μ L of kinase buffer (20 mM Hepes-

NaOH, pH 7.5, 5 mM MgCl₂, 1 mM DTT, 0.1 mM ATP, and 0.1 mM Na₃VO₄) for 1 h at 30 °C. The CD133 cytoplasmic domain was released from the GST-CD133CD fusion protein by incubation with thrombin protease for 1 h at 37 °C. The mixtures were subjected to SDS-PAGE on 15% polyacrylamide gels, and the bands corresponding to unphosphorylated and phosphorylated CD133CD were excised with a razor blade.

In-Gel Digestion of Proteins. Bands were cut into 1 mm cubes and washed twice with water for 20 min. Gel pieces were destained and then dehydrated with 75 μ L of acetonitrile. Samples were reduced with 50 μ L of 10 mM DTT for 30 min followed by alkylation with 50 μ L of 55 mM iodoacetamide for 20 min. After being washed and dehydrated in ammonium bicarbonate and acetonitrile, respectively, gel pieces were digested for 4.5 h with 6 ng/ μ L of trypsin (Promega) in 100 mM ammonium bicarbonate. Peptides were extracted with 30 μ L of formic acid (FA) solution (1% FA in 2% acetonitrile) for 30 min. Peptides were extracted twice more with 12 μ L of formic acid solution and 12 μ L of acetonitrile for 30 min for a final volume of 60 μ L.

Chromatography. Twenty microliters of digest solution was loaded on to Zorbax 300SB-C18 5 \times 0.3 mm desalting column and washed for 5 min at 15 μ L/min with 3% ACN:0.1% FA. Peptide separation was done on a New Objective Biobasic C18 10 \times 0.075 mm Integrafit analytical column. Gradient elution was from 10% ACN:0.1% FA to 95% ACN:0.1% FA in 30 min using an Agilent 1100 Nanoflow system.

Mass Spectrometry. The instrument used for LC-MS/MS was a QTRAP 4000 from Sciex-Applied Biosystems (Concord, ON). Information-dependent ms/ms analysis was done for three of the most intense ions selected from each full scan MS with dynamic exclusion for 90 s. The survey scan used was the enhanced MS scan from 375 to 1600 m/z at 4000 amu/s using Dynamic Fill time. Collision energy was determined using a rolling collision energy equation. MS/MS data was acquired for three scans from 70 to 1700 m/z with 20 ms trap fill time and Q0 trapping activated. The instrument used for peptide mass fingerprint analysis was a MALDI Q-TOF Ultima (Waters, Milford, MA) equipped with a 337 nm Nitrogen laser working at 10 Hz. The samples were spotted in a 1:1 ratio of sample to matrix solution of 10 mg/mL alpha-cyano-4-hydroxycinnamic acid in 50% ACN:0.1% trifluoroacetic acid).

Database Searching. Peak lists generated with Mascot distiller 2.1 were searched against the NCBI nr database of human taxonomy protein sequences (192056 sequences) from July 2007. Mascot 2.1 search parameters used were Trypsin with a single miscleavage, carboxyamidomethylation of cysteines as fixed modification, oxidation of methionines and phosphorylation of tyrosine as variable modifications, 1.5 Da precursor and 0.8 MS/MS fragment tolerances.

RESULTS

CD133 Is Phosphorylated by Src Family Tyrosine Kinases in Medulloblastoma Cells. Because CD133 C-terminal cytoplasmic domain contains five tyrosine residues,

¹GST, glutathione S-transferase; MS, mass spectrometry; MS/MS, tandem mass spectrometry; LC, liquid chromatography.

including one consensus site for tyrosine kinase ([R/K]xxx[D/E]xxY), we sought to examine the possibility that CD133 is actually phosphorylated in medulloblastoma cells treated with the tyrosine phosphatase inhibitor pervanadate. CD133 was immunoprecipitated from D283, a medulloblastoma cell line that express a high level of endogenous CD133, and from Daoy medulloblastoma cell line transiently transfected with human CD133 cDNA. CD133 was immunoprecipitated using an anti-CD133 rabbit monoclonal antibody, and the extent of tyrosine phosphorylation was determined by immunoblotting using an antiphosphotyrosine antibody (PY99). As shown in Figure 1A, pervanadate treatment caused a marked increase of tyrosine phosphorylation of both endogenous CD133 in D283 cells and exogenous CD133 in Daoy cells. To identify kinases directly or indirectly involved in the phosphorylation of CD133, cells were pretreated with the Src family tyrosine kinase (SFK) inhibitor PP2, the Erk inhibitor PD98059, the MAPK inhibitor SB203580, or the PI3K pathway inhibitor LY294002, and then treated with pervanadate. As shown in Figure 1B, tyrosine phosphorylation was markedly reduced in cells preincubated with the SFK inhibitor PP2 but not significantly in cells preincubated with other inhibitors. To provide further evidence for the involvement of SFK in CD133 phosphorylation, Daoy cells were transiently transfected with CD133 along with Src, Fyn, or kinase-inactive dominant-negative forms of Src and Fyn. As shown in Figure 2, CD133 immunoprecipitated from cells cotransfected with wild-type Src and Fyn was tyrosine-phosphorylated, whereas coexpression of CD133 with inactive forms of Src and Fyn failed to induce the tyrosine phosphorylation of this protein. In Daoy cells, transfected CD133 appears as two bands of 116 kDa and 105 kDa after immunoblotting with CD133-specific mAb C24B9 (Figure 2). Deglycosylation of CD133 with peptide-N-glycosidase F yield a single 94-kDa band, indicating that the difference between the two forms was due to differential N-glycosylation (data not shown). Similar results have been previously reported for mouse prominin transfected in CHO cells (5). Interestingly, Src overexpression induced the phosphorylation of the two glycosylated forms of CD133, while Fyn expression only induced the phosphorylation of the higher molecular weight, that is, more glycosylated form.

To ascertain whether the phosphorylation sites are located in the cytoplasmic tail of CD133, Src and wild-type CD133 or CD133 mutants lacking half (CA26) or the entire C-terminal cytoplasmic domain (CA52) were cotransfected in Daoy cells. CD133 was isolated by anti-CD133 immunoprecipitation and probed for tyrosine phosphorylation with antiphosphotyrosine antibody. As shown in Figure 3, wild-type CD133 was heavily tyrosine-phosphorylated in cells overexpressing Src kinase, whereas deletion of the 26 or 52 amino acid residues of the cytoplasmic domain resulted in an almost complete loss of tyrosine phosphorylation. Similar results were obtained using Flag-tagged version of wild-type CD133 and deletions mutants CA26 or CA52 (data not shown).

Mapping of Phosphotyrosine Residues In Vitro. To map the tyrosine residues phosphorylated by Src, we

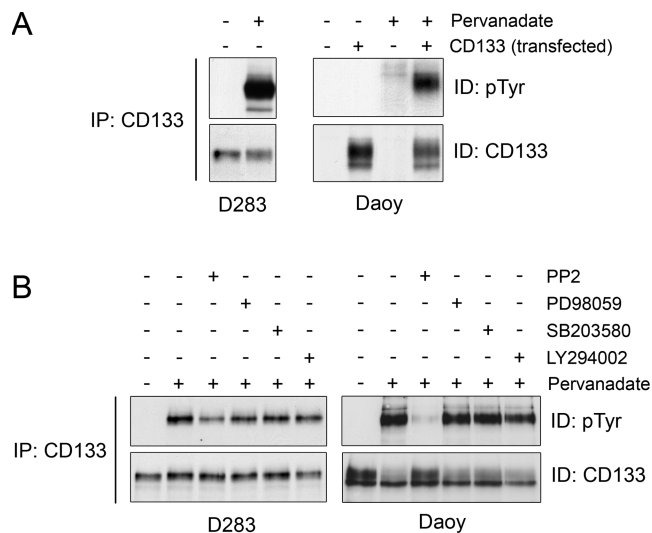


FIGURE 1: Tyrosine phosphorylation of endogenous CD133 in pervanadate-treated D283 and of exogenous CD133 in Daoy medulloblastoma cells. (A) Daoy medulloblastoma cells were transfected with a plasmid encoding for human CD133 or with empty vector, as indicated. Untransfected D283 or transfected Daoy medulloblastoma cells were treated with or without 0.2 mM pervanadate for 10 min, and cell lysates were immunoprecipitated (IP) with an anti-CD133 monoclonal antibody. The immunoprecipitates were immunoblotted with antiphosphotyrosine (pTyr) antibody, and the same membrane was reprobed with anti-CD133 antibody. (B) Untransfected D283 or Daoy medulloblastoma cells transfected with CD133 were pretreated with 10 μ M PP2, 50 μ M PD98059, 10 μ M SB203580, or 10 μ M LY294002 for 1 h prior to treatment with 0.2 mM pervanadate for 10 min. Anti-CD133 immunoprecipitates (IP) were immunoblotted with antiphosphotyrosine, and the same membrane was reprobed with anti-CD133 antibody. ID, immunodetection.

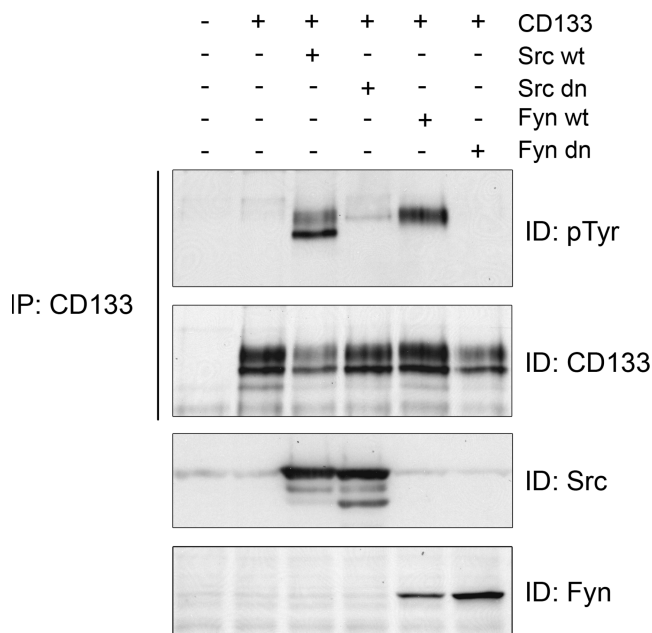


FIGURE 2: Tyrosine phosphorylation of CD133 by Src and Fyn. Daoy cells were transfected with either empty vector, wild-type CD133, alone or together with wild-type Src (Src wt), a dominant-negative form of Src (Src dn), wild-type Fyn (Fyn wt) or a dominant-negative form of Fyn (Fyn dn). Twenty-four hours post-transfection, the cells lysates were immunoprecipitated (IP) with anti-CD133 monoclonal antibody, the immunoprecipitates were immunoblotted with antiphosphotyrosine (pTyr) antibody, and the same membrane was reprobed with anti-CD133 antibody. Levels of transfected Src and Fyn were monitored in lysates by immunoblotting using specific monoclonal antibody. ID, immunodetection.

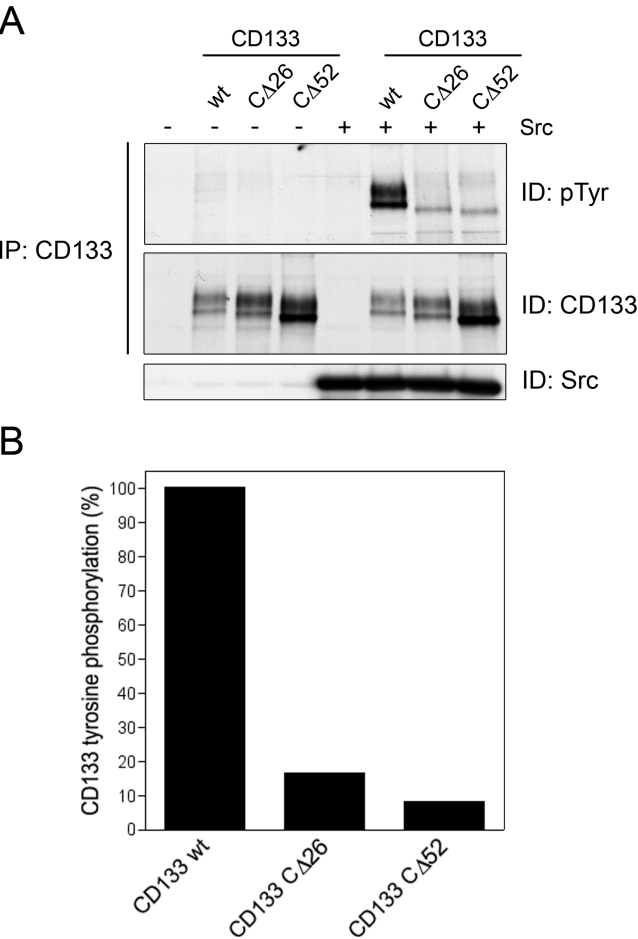


FIGURE 3: Tyrosine phosphorylation of CD133 C-terminal cytoplasmic domain by Src. (A) Daoy cells were transfected with either empty vector, wild-type CD133, CD133 mutants lacking half (CA26), or the entire C-terminal cytoplasmic domain (CA52), alone or together with Src. 48 h post-transfection, the cells lysates were immunoprecipitated (IP) with anti-CD133 monoclonal antibody, the immunoprecipitates were immunoblotted with antiphosphotyrosine (*pTyr*) antibody, and the same membrane was reprobed with anti-CD133 antibody. Levels of transfected Src were monitored in lysates by immunoblotting using an anti-Src monoclonal antibody. (B) Densitometry analysis of data shown in A. Values are normalized to wild-type CD133. ID, immunodetection.

expressed the C-terminal cytoplasmic domain of CD133 (amino acids 814–865) fused to GST and performed in vitro phosphorylation using purified recombinant Src kinase. The CD133 cytoplasmic domain (CD133CD) was cleaved from the GST fusion protein by thrombin. An aliquot of each protein mixture was separated by SDS–PAGE followed by immunoblotting using antiphosphotyrosine antibody and the remaining mixtures were separated on a second gel for preparation of peptides for mass spectrometry after staining with Coomassie Blue. As shown in Figure 4, the GST-CD133CD is heavily tyrosine phosphorylated by Src, resulting in a reduced electrophoretic mobility. Thrombin cleavage of unphosphorylated and phosphorylated GST-CD133CD generated peptides with an apparent molecular weight of 7.5 kDa and 14 kDa, respectively, but only the 14-kDa peptide contained phosphotyrosine residues. These non-phosphorylated and phosphorylated CD133CD bands were digested with trypsin and the resulting tryptic peptides were compared by MALDI Q-TOF. As shown in

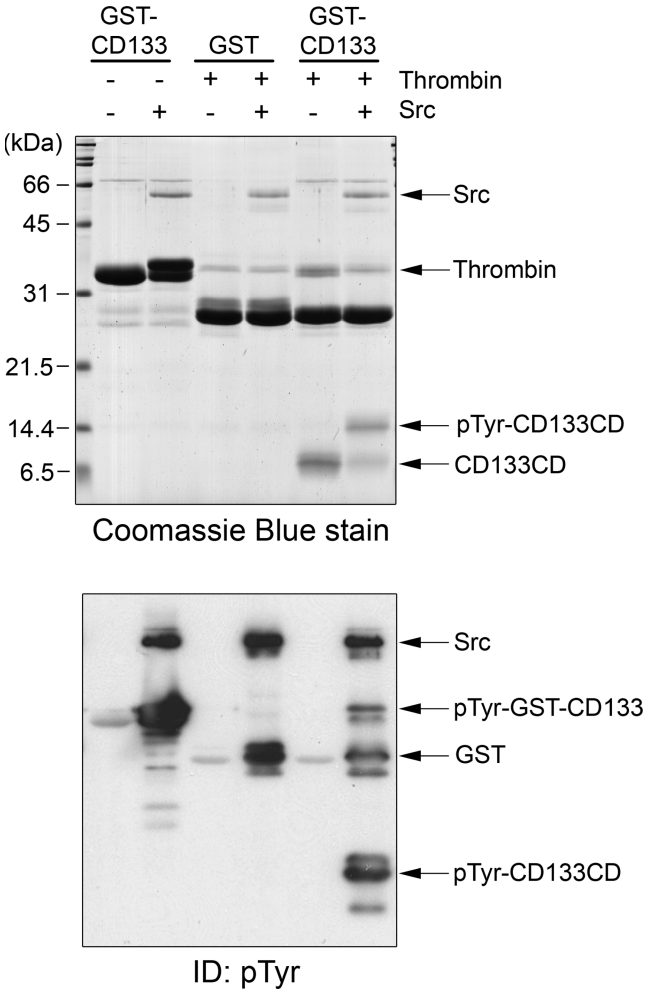


FIGURE 4: Tyrosine phosphorylation of CD133 by Src in vitro. The CD133 cytoplasmic domain (amino acids 814–865) fused to GST was incubated with or without purified recombinant Src and ATP in vitro, and the samples were subjected to SDS–PAGE and stained with Coomassie blue (upper panel) or immunoblotted with antiphosphotyrosine antibody (*pTyr*, lower panel). As indicated, GST-CD133CD and GST (as a control) were also incubated with thrombin to cleave the CD133 cytoplasmic domain from the GST moiety. ID, immunodetection.

Figure 5, three peptides had their peptide mass increased by 80 Da, the mass of one phosphate group, upon phosphorylation by Src. Peptides of 1935.89 Da (corresponding to amino acids 849–865), 2075.91 Da (corresponding to amino acids 822–837), and 3211.41 Da (corresponding to amino acids 838–865) were detected at a reduced relative intensity in phosphorylated CD133, while the corresponding phosphorylated peptides of 2015.86 Da, 2155.87 Da, and 3291.38 Da were not detected in the unphosphorylated sample. Although these results indicate phosphorylation sites at tyrosine 828 and tyrosine 852, we performed further analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) to confirm results obtained by MALDI-TOF. The sequences obtained from MS/MS were analyzed and peptides containing phosphotyrosine residues were identified. As shown in Figure 6, MS/MS analysis revealed a peptide MDSEDV-pY-DDVETIPMK (amino acid residues 823–837) phosphorylated at tyrosine-828, and a peptide DHV-pY-GIHNPMVTSPSQH (amino acid residues 849–865) phosphorylated at tyrosine-852,

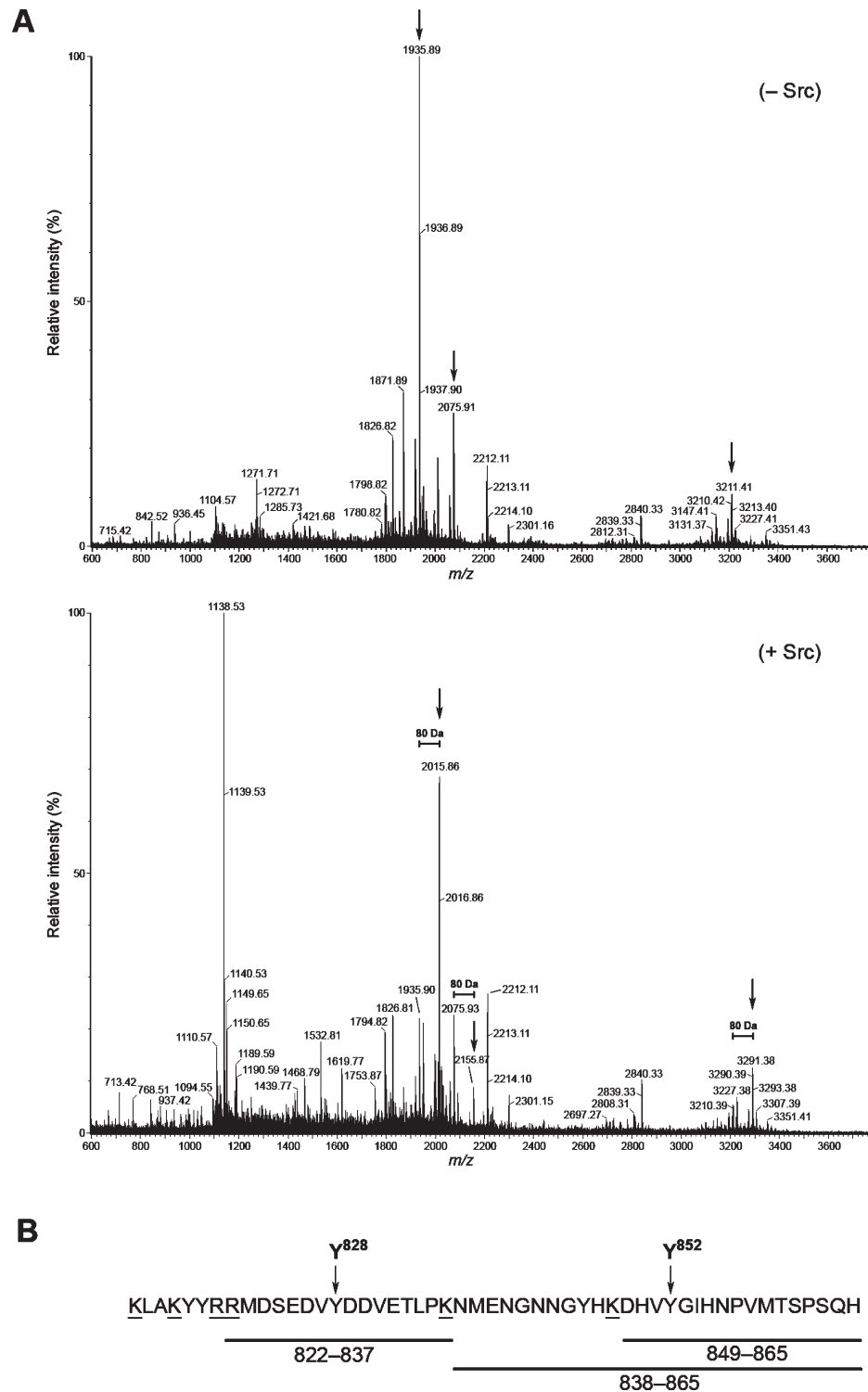


FIGURE 5: MALDI Q-TOF analysis of CD133 cytoplasmic domain phosphorylated by Src in vitro. (A) Unphosphorylated (-Src) and phosphorylated (+Src) CD133CD peptides described in Figure 4 were digested with trypsin and analyzed by a MALDI Q-TOF mass spectrometer. The peptides modified by phosphorylation (increased mass of 80 Da) are indicated by arrows. (B) The amino acid sequence of CD133 C-terminal cytoplasmic domain (amino acids 814–865) with lysine and arginine residues (trypsin cleavage sites) underlined. Peptides corresponding to 1935.89, 2075.91, and 3211.41 Da are indicated by bars labeled with the corresponding amino acids numbers. Deduced phosphorylation sites are indicated by arrows with amino acid number.

confirming unambiguously the results obtained by MALDI-TOF.

Tyrosine 828 and 852 Are Phosphorylated by Src Kinase In Vivo. To confirm that the mapping results obtained in vitro correspond to intracellular events, we generated five full-length human CD133 constructs in which the five tyrosine residues present in the cytoplasmic

tail were individually substituted to a phenylalanine residue, and expressed these proteins with Src in Daoy cells. All these CD133 mutants were expressed at similar levels at the surface of Daoy cells, as determined by FACS analysis (data not shown). As shown in Figure 7, tyrosine phosphorylation of CD133 Y828F and Y852F mutants was considerably reduced,

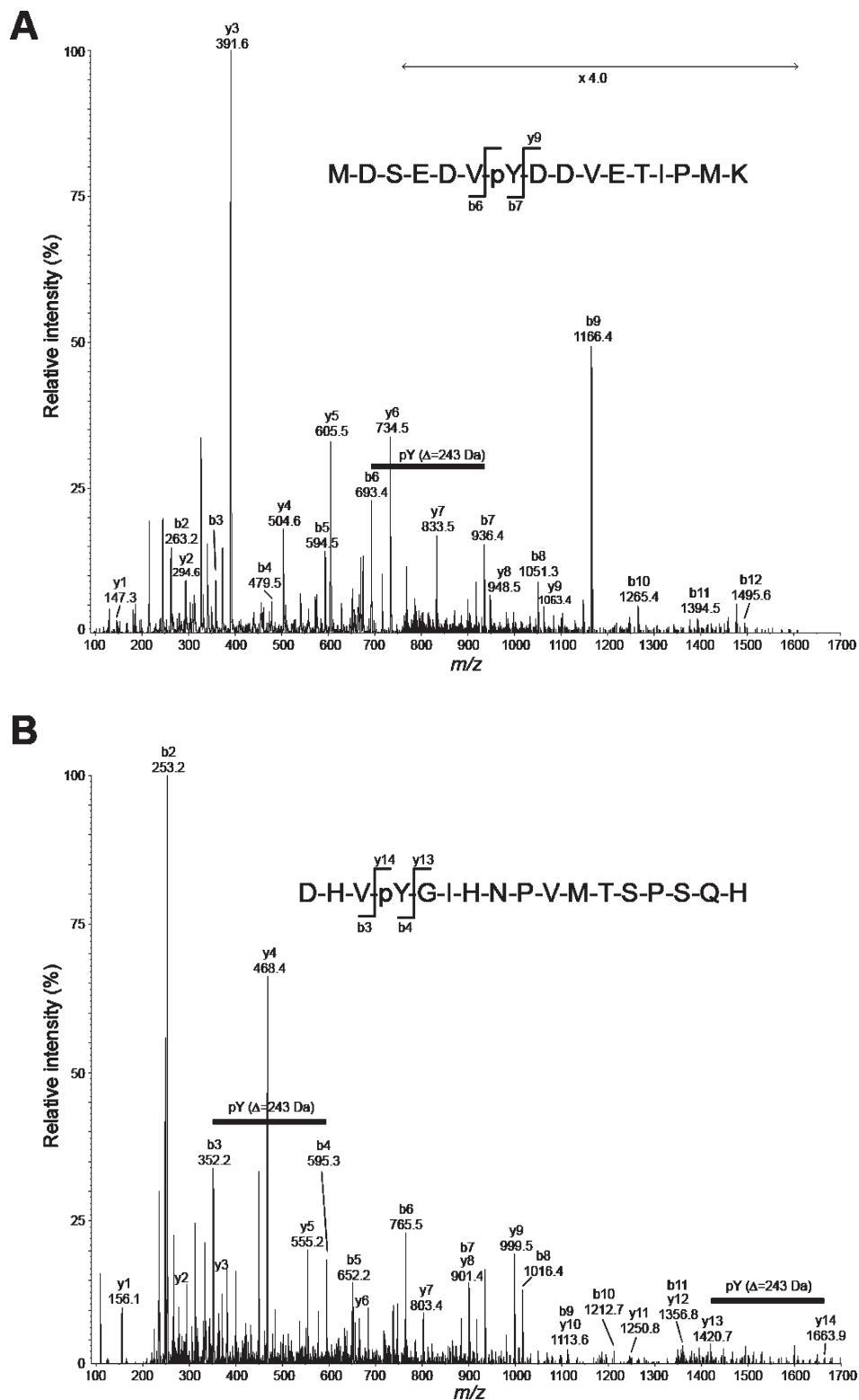


FIGURE 6: Identification of tyrosine residues of CD133 phosphorylated by Src by LC-MS/MS. The CD133 cytoplasmic domain phosphorylated by Src was digested with trypsin, and the tryptic peptides were analyzed by LC-MS/MS. (A) MS/MS spectrum of the human CD133 peptide MDSEdV-pY-DDVETIPMK phosphorylated at tyrosine residue 828. (B) MS/MS spectrum of the human CD133 peptide DHV-pY-GIHNPVMTSPSQH phosphorylated at tyrosine residue 852. In both figure panels, the phosphotyrosine mass difference of 243 Da between the appropriate b-ions or y-ions is indicated by a bar labeled pY.

indicating that Src and Fyn phosphorylates these tyrosine residues *in vivo*. Amino acid sequences around tyrosine-828 and tyrosine-852 are conserved in rat, mouse, chimpanzee, and human CD133 orthologs (40), suggesting that these residues are important for its function.

DISCUSSION

CD133 is a transmembrane protein used as a marker for the isolation and characterization of cancer stem cells, especially in brain tumors. The function of CD 133 in normal and cancer stem cells is still unknown

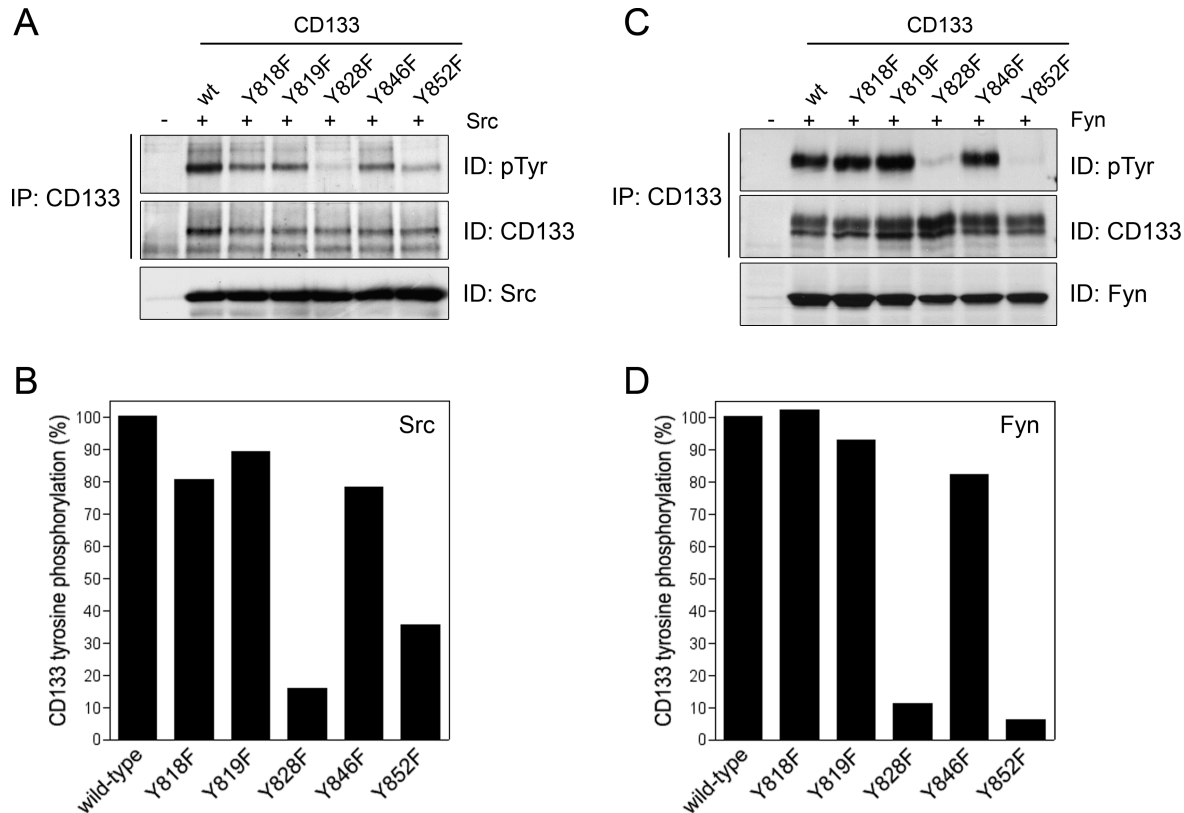


FIGURE 7: Identification of tyrosine residues of CD133 phosphorylated in intact cells. Wild-type (*wt*) or tyrosine to phenylalanine mutant CD133 plasmids were transfected into Daoy cells together with Src (A) or Fyn (C). Cell lysates were immunoprecipitated (IP) with anti-CD133 antibody and the immunoprecipitates were immunoblotted with antiphosphotyrosine (*pTyr*) antibody and subsequently reprobed with anti-CD133 antibody. Levels of transfected Src or Fyn were monitored in cell lysates by immunoblotting using specific antibodies. ID, immunodetection. (B and D) Densitometry analysis of data shown in A and C, respectively. Values are normalized to wild-type CD133.

but it likely involves interactions with the extracellular matrix or with neighboring cells. This is supported by the cellular localization of CD133 in protrusions of the plasma membrane and by the fact that most of CD133 is exposed to the extracellular environment. Little is known about the role of the C-terminal cytoplasmic domain of CD133 in the regulation of its function. In mouse MDCK epithelia cells, prominin-1 delivery to the apical plasma membrane and its retention in microvilli were unaffected by deletion of the C-terminal cytoplasmic domain (41). We hypothesized that CD133 C-terminal cytoplasmic domain might serve as a signaling unit upon phosphorylation of specific tyrosine residues and association with SH2 domain containing signaling proteins. In this paper we report that CD133 (prominin-1) endogenously or exogenously expressed in medulloblastoma cells is phosphorylated by Src and Fyn, two members of the Src-family tyrosine kinases.

Phosphorylation of the juxtamembrane tyrosine-828 within the CD133 cytoplasmic domain was expected since this region contains a [R/K]xxx[D/E]xxY motif recognized by tyrosine kinases. The tyrosine-828 is located within a YDDV motif, which upon phosphorylation can serve as a binding site for the SH2 domains of cytoplasmic targets such as Src, Fyn, and Nck. Phosphorylation of tyrosine-852 was unexpected since this region does not contain a known recognition motif for tyrosine kinases. However, phosphorylation of tyrosine-852 is unlikely to be artifactual since it is

detected *in vitro*, and since a point mutant substituting the tyrosine 852 to a phenylalanine considerably reduced the tyrosine phosphorylation of CD133 *in vitro*. The significance of tyrosine-852 phosphorylation is difficult to assess. The amino acid sequences surrounding tyrosine-852 (YGHI) do not conform to binding sites for SH2 domain-containing proteins. It is possible that phosphorylation at tyrosine-852 plays a different role from phosphorylation at tyrosine-828, a role that would not require binding to adaptor molecules.

The role of CD133 has been recently investigated in metastatic melanoma cells (42). Down-regulation of CD133 by short hairpin RNA in metastatic melanoma cells reduce cell growth, motility, metastatic capacity, and decrease the formation of spheroids under stem cell culture conditions. Down-regulation of CD133 in melanoma cells was shown to increase the gene expression of several Wnt inhibitors, suggesting a link between CD133 and Wnt pathways (42). Whether CD133 directly or indirectly interacts with Wnt pathways remains unknown, but it is tempting to speculate that tyrosine phosphorylation of CD133 in response to an extracellular ligand might activate intracellular signaling pathways, such as the Wnt pathway, that would influence cell growth, mobility, and metastatic potential. Further studies on the impact of tyrosine phosphorylation of CD133 on cellular functions should provide new insights on the role of this modification in the regulation of the function of the enzyme.

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