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(54) **Title:** IL-34 RECEPTOR ASSAYS AND USES THEREOF

(57) **Abstract:** Methods are disclosed for identifying activators and inhibitors of actions of interleukin-34 (IL-34) that are independ-
ent of the colony stimulating factor- 1 (CSF-1) receptor (CSF-1R) and play a role in development, homeostasis and disease.



IL-34 RECEPTOR ASSAYS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 61/826,356, filed May 22, 2013, the content of which is herein incorporated by reference in its entirety.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under grant numbers CA32551, 5P30-CA13330 and SIG 1S10RR019352 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The invention relates to assays for activators and inhibitors of actions of interleukin-34 (IL-34) that are independent of the colony stimulating factor-1 (CSF-1) receptor (CSF-1R) and play a role in development, homeostasis and disease.

BACKGROUND OF THE INVENTION

[0004] Throughout this application various publications are referred to in parentheses. Full citations for these references may be found at the end of the specification before the claims. The disclosures of these publications are hereby incorporated by reference in their entireties into the subject application to more fully describe the art to which the subject application pertains.

[0005] The CSF-1R kinase (1,2) plays a critical role in the regulation of macrophage and osteoclast production and function (3-6) as well as the development and regulation of other cell types (7-11). The existence of an additional CSF-1R ligand was proposed based on the greater severity of phenotype of homozygous null CSF-1R mice, compared to the phenotype of homozygous CSF-1-null mutant mice (12). A second ligand for the CSF-1R, interleukin-34 (IL-34), with no apparent sequence similarity to any other growth factor, was subsequently identified (13). While IL-34 and CSF-1 compete for binding to the CSF-1R and have similar CSF-1R-mediated effects, they exhibit significant tissue specific and developmental differences in their expression patterns (14). In addition, whereas CSF-1-

deficient mice exhibit partial loss of microglia, CSF-1R-deficient mice have no microglia (15). This observation, together with the high expression of IL-34 in brain suggested an important role of IL-34 in microglial development. In agreement with this, IL-34-deficient (*IL-34*^{-/-}) mice were shown to exhibit severe deficits in microglia (16,76). Despite the similarity of IL-34 and CSF-1 in their CSF-1R-mediated effects (14,17), *IL34* mRNA is expressed at a significantly higher level than either *Csf1* or *Csf1r* mRNA in several regions of the early postnatal and adult brain (14) and IL-34 protein is often expressed in regions where there is minimal expression of the CSF-1R or CSF-1-reporter proteins and IL-34 is significantly more active in suppressing neural progenitor cell proliferation and neuronal differentiation than CSF-1 (9).

[0006] Protein tyrosine phosphatase receptor type zeta (PTP- ζ) (18,19), a cell-surface receptor and a chondroitin sulfate (CS) proteoglycan (CSPG), is highly abundant in the brain (20), primarily expressed on neural progenitors and glial cells (21-23) and binds to and signals through the action of multiple ligands (24) including the growth factor, pleiotrophin (PTN) (25,26), the cell-surface protein, contactin (CNTN) (27) and the extracellular matrix (ECM) protein, tenascin-R (TN-R) (28). The binding of some of these ligands involves the CS glycosaminoglycan (GAG)-moiety of PTP- ζ (25,29). Ligand binding to PTP- ζ leads to increased tyrosine phosphorylation of down-stream targets, including β -catenin, β -adducin, Src-family kinases (SFK), focal adhesion kinase (FAK), paxillin and extracellular signal-regulated kinase-1/2 (Erk-1/2) (30-37). PTP- ζ is up-regulated in many human cancers, including glioblastomas, and regulates their proliferation and migration (38-40).

[0007] The present invention addresses the need for activators and inhibitors of actions of IL-34 that are independent of the CSF-1R receptor and play a role in development, homeostasis and disease.

SUMMARY OF THE INVENTION

[0008] The invention provides methods for determining whether or not an agent is a candidate agent for inhibiting interaction between interleukin-34 (IL-34) and protein tyrosine phosphatase receptor type zeta (PTP- ζ) comprising: contacting cells that express PTP- ζ on their surface and that do not express colony stimulating factor-1 receptor (CSF-1R) with IL-34 in the presence of the agent and in the absence of the agent, and measuring a cellular response induced by IL-34, wherein an agent that reduces a cellular response induced by IL-34 is a candidate agent for inhibiting interaction between IL-34 and PTP- ζ , and wherein an

agent that does not reduce a cellular response induced by IL-34 is not a candidate agent for inhibiting interaction between IL-34 and PTP- ζ .

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] Fig. 1A-1C. Interaction of IL-34 with PTP- ζ in solubilized membrane fractions of mouse brain. (A) N-octyl- β -D-glucoside (OG)-solubilized membrane fractions of mouse brain were incubated (4⁰C, 16h) with either immobilized polyclonal rabbit anti-mIL-34 antibody beads that had been preincubated with mIL-34 (lanes 1 & 2), or with biotinylated mIL-34 (lanes 3, 4). The SDS eluates of the IL-34 immunoprecipitates or biotinylated IL-34 complexes (recovered with neutravidin beads) were analyzed by SDS-PAGE with silver staining. The IL-34-associated proteins identified with 99% certainty by mass spectrometry were PTP- ζ and TN-R (Fig. 7). Western blots (WB) indicate the PTP- ζ (L, long isoform, P, phosphacan/soluble isoform and the ~225-kDa short isoform) and TN-R staining bands. Multimeric (52) (slower migrating bands, ~400 kDa) and alternatively spliced (faster migrating bands, 160/180 kDa) variants of TN-R were co-immunoprecipitated, or pulled down. (B) Scheme depicting various PTP- ζ isoforms. *Upper panel*, the long isoform (active) containing a carbonic anhydrase domain (CA) and a fibronectin type III repeat (F), a transmembrane domain (TM), protein tyrosine phosphatase domains (PTP1 and PTP2) and three glycosaminoglycan (GAG) addition sites. *Middle*, phosphacan or the secreted isoform lacking the TM and PTP domains. *Lower*, the short isoform (active) missing 860 amino acids of the long isoform. 3F8 and C-209 antibodies recognize the extracellular and intracellular regions of PTP- ζ , respectively and the 473-HD antibody all three isoforms. 3F8 is directed against rat phosphacan and is not as effective in detecting mouse PTP- ζ . C-209 antibody recognizes the short isoform from the mouse brain membrane lysates infrequently. (C) Co-immunoprecipitation of PTP- ζ with IL-34. The OG-solubilized mouse brain membrane fraction was incubated with mIL-34 (4⁰C, 16h), immunoprecipitated with immobilized anti-PTP- ζ (3F8), or isotype control (mIgG1) antibodies and the immunoprecipitates analyzed by gradient SDS-PAGE and western blotting. L, long isoform; P, phosphacan and S, short isoform.

[0010] Fig. 2A-2C. IL-34 binds cell-surface PTP- ζ in U251 human glioblastoma cells. (A) Interaction of IL-34 with PTP- ζ in OG-solubilized U251 cell membrane fractions. Membranes lysates were incubated with biotinylated hIL-34 (4⁰C, 16h), the complexes captured with neutravidin beads (4⁰C, 6h), eluted with SDS and analyzed by SDS-PAGE and

silver staining, or by western blotting (WB) with antibodies to PTP- ζ . Arrowhead, non-specific band; asterisk, PTP- ζ proteolytic product (51); L, long isoform; NG, non-glycosaminoglycan form; S, short isoform. (B) Reduced PTP- ζ expression in PTP- ζ KD U251 clones. Left panel; PTP- ζ and control (EF1 α) western blots of OG-solubilized whole cell lysate from cells expressing scrambled, or PTP- ζ (KD1 and KD2) shRNAs. Right panel, quantitation of the combined intensities of the three bands (L, NG, S) from two independent experiments (average $\pm$ range). (C) Flow cytometric analysis of hIL-34 binding to PTP- ζ KD U251 lines. Serum-starved control (scrambled) and KD (1 & 2) cells were either untreated, incubated with 5 μ g/ml biotinylated hIL-34, then subsequently incubated with streptavidin-conjugated APC-Cy7 prior to flow cytometric analysis, gating on viable cells. G.M., geometric means of signal intensities of duplicate experiments (average $\pm$ range).

[0011] Fig. 3A-3B. IL-34 inhibits growth and clonogenicity of U251 glioblastoma cells in a PTP- ζ -dependent manner. (A) Cell proliferation assay. Control (left panel) or PTP- ζ KD (right panel) cells were incubated with the indicated factors for the indicated times and the viable cell numbers assessed by trypan blue exclusion staining. (B) Clonogenic assay. Left panel, micrographs of colony forming assays of U251 cells incubated with vehicle or IL-34. Right panel, histograms showing average colony counts from triplicate experiments. (A) and (B), Means $\pm$ SD; n=3; *, significantly different from cells incubated with vehicle alone, $p < 0.05$.

[0012] Fig. 4A-4D. IL-34 inhibits PTP- ζ -dependent U251 motility. (A) Inhibition of U251 monolayer wound healing by IL-34. Left panel, numbers above the horizontal lines indicate the fraction of the initial wound width at the time of scratching. Scale bar, 100 μ M. Right panel, quantitation (means $\pm$ SD; n=3). (B) Quantitation of wound healing by control and PTP- ζ KD U251 cells at 9h, in the presence of PTN or IL-34 (means $\pm$ SD; n=3). (C) Inhibition of haptotactic migration by IL-34. Migration is expressed as a percent of cells migrated in the BSA control (means $\pm$ SD; n=3, ≥ 15 fields per condition). (D) Inhibition of random migration by IL-34. Migration is expressed as a percent of cells migrated in no GF (vehicle) control (means $\pm$ SD; n=3, ≥ 10 fields per condition). *, $p < 0.05$ and **, $p < 0.01$.

[0013] Fig. 5A-5C. IL-34 binding to PTP- ζ enhances tyrosine phosphorylation of FAK and Paxillin, in U251 cells. (A) Kinetics of tyrosine phosphorylation in response to IL-34. SDS-PAGE and western blot analysis of NP-40 lysates from U251 cells treated with IL-34 or PTN harvested at the indicated times and stained as shown. FAK, focal adhesion

kinase; Pax, paxillin. (B) Immunoprecipitates of FAK (left panel) and paxillin (right panel) from NP-40 lysates of serum-starved U251 cells incubated with hPTN or hIL-34 (0.1 nM) western blotted with antibodies to phosphotyrosine (pY), FAK or Pax. (C) Abrogation of the IL-34 and PTN-induced phosphorylation of FAK and paxillin in U251 cells expressing PTP- ζ shRNA. Normalized to the total FAK and Pax expression and expressed as fold stimulation of levels in control (vehicle-treated) cells (dotted line).

[0014] Fig. 6A-6C. IL-34 binds to PTP- ζ in a CS-dependent manner. (A) Effect of Chondroitinase ABC on specific PTP- ζ isoforms. Anti-PTP- ζ western blot of U251 membrane lysates incubated with and without chondroitinase ABC (0.3 U/ml, 37°C, 1h). L, long isoform; NG, non-glycosaminoglycan isoform; S, short isoform. (B) CS is required for IL-34 binding to PTP- ζ . Serum-starved U251 cells were either incubated with, or without ChABC (4.2 U/ml) and subsequently with 2 μ g/ml biotinylated hIL-34, prior to further incubation with streptavidin-conjugated APC-Cy7 and flow cytometric analysis, gating on viable cells. The vertical line defines the level of non-specific binding of biotinylated IL-34. (C) Competition with CS and the failure to compete with CSF-1 and PTN. 2×10^5 serum-starved U251 cells were pre-incubated with a 16 molar excess of IL-34 in the presence of increasing concentrations of CS, or with a 16 molar excess of CSF-1 or PTN, prior to washing and binding of biotinylated-IL-34 (2 μ g/ml). Geometric means (G.M.) of signal intensities of duplicate experiments (average $\pm$ range) were used to calculate percentage inhibition. *, $p < 0.05$.

[0015] Fig. 7A-7B. LC-MS/MS peptide hits for TN-R and PTP- ζ . (A) TN-R protein sequence from mouse (SEQ ID NO:10). (B) PTP- β/ζ protein sequence from mouse (SEQ ID NO:11). The carbonic anhydrase homology domain (CA), the fibronectin type III repeat (F) and the two phosphatase domains (PTP1 and PTP2), are boxed. The consensus glycosaminoglycan (GAG)-addition sites are *underlined* and the transmembrane domain is *italicized*. The several N-linked glycosylation sites are not indicated. The peptide stretch missing in the short isoform is bolded.

[0016] Fig. 8A-8C. U251 cells lack the CSF-1R. (A) Failure of hIL-34 to bind to CSF-1R on U251 cells. N-octyl- β -D-glucoside (OG)-solubilized membrane fractions of BAC1.2F5 (lanes 1 & 2) and U251 (lanes 3 & 4) cells were incubated overnight at 4°C with biotinylated mIL-34 (lanes 1 & 2) or biotinylated hIL-34 (lanes 3 & 4). The biotinylated IL-34 complexes were recovered by incubation with neutravidin and SDS eluates containing the complexes analyzed by SDS-PAGE and western blotting (WB) with an antibody that

equivalently recognizes both mCSF-1R and hCSF-1R. (B) Absence of the CSF-1R in U251 cells. N-octyl- β -D-glucoside (OG)-solubilized membrane fractions of U251 human glioblastoma (lane 1) and NIH-3T3-hCSF-1R (lanes 2-4) cells were incubated overnight at 4°C with anti-hCSF-1R antibody (lanes 1, 2) or biotinylated hIL-34 (lanes 3, 4). The biotinylated IL-34 complexes were recovered by incubation with neutravidin and SDS eluates of IL-34 pull-down and CSF-1R immunoprecipitates analyzed by SDS-PAGE and western blotting (WB) with antibodies to hCSF-1R. (C) Further verification of the lack of CSF-1R expression on U251 cells. Flow cytometric analyses of 2×10^5 viable 3T3-hCSF-1R and U251 cells were incubated with 5 μ g/ml of rat monoclonal anti-hCSF-1R antibody, or rat IgG1 (isotype control) for 30 min at 4°C, washed with PBS and further incubated with 5 μ g/ml of anti-rat IgG1-conjugated FITC for 30 min at 4°C.

DETAILED DESCRIPTION OF THE INVENTION

[0017] The invention provides a method for determining whether or not an agent is a candidate agent for inhibiting interaction between interleukin-34 (IL-34) and protein tyrosine phosphatase receptor type zeta (PTP- ζ) comprising:

contacting cells that express PTP- ζ on their surface and that do not express colony stimulating factor-1 receptor (CSF-1R) with IL-34 in the presence of the agent and in the absence of the agent, and

measuring a cellular response induced by IL-34,

wherein an agent that reduces a cellular response induced by IL-34 is a candidate agent for inhibiting interaction between IL-34 and PTP- ζ , and

wherein an agent that does not reduce a cellular response induced by IL-34 is not a candidate agent for inhibiting interaction between IL-34 and PTP- ζ .

[0018] The terms PTP- ζ , and PTP- β/ζ are used interchangeably in this application.

[0019] The cells can be, for example, glioblastoma cells, such as, e.g., U251 human glioblastoma cells.

[0020] The cells can have been transfected with nucleic acid encoding human PTP- ζ . Human PTP- ζ has the amino acid sequence (ACCESSION P23471, VERSION P23471.4 GI:229485537, SEQ ID NO:12):

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1  mrlkrflac iqllecvrld wangyyrqr klveeigwsy tgalnqknwg kkyptcns pk
61  qspinidedl tqvvnlnkkl kfqgwdktsl entfihntgk tveinlndy rvsggvse mv
121 fkaskitfhw gkenmssdgs ehslegqkfp lemqiycfda drfssfeeav kgkgklrals
181 ilfevgteen ldfkaiidgv esvsrfgkqa aldpfillnl lpnstdkyyi yngsltsp pc
241 tdtvdwivfk dtvsisesql avfcevltmq qsgyvmlmdy lqnnfreqqy kfsrqvfssy
301 tgkeeiheav cssepenvqa dpenytsllv twerprvvyd tmiekfavly qqldgedqtk

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361 hefltdgyqd lgailnnllp nmsyvlqiva ictnglygky sdqlivdmpt dnpeldlfpe
 421 ligteeiike eeegkdieeg aivnpgrdsa tnqirkkep q istthynri gkyneaktn
 481 rsptgrsefs gkgdvpntsl ntsqpvtkl atekdislts qvtelppht vegtasln d
 541 gsktvlrsp mnlsgtaesl ntvsiteyee eslltsfkld tgaedssgss patsaifis
 601 enisqgyifs senpetityd vlipesarna sedstssgse eslkdp smeg nvwfpsstdi
 661 taqpdvgsgr esllqntyte irvdesekt ksfsgpvm qgpsvtdlem phystfayfp
 721 tevtphafp ssrqdvlst vnvvsyqtq pyvnetplq psyssevfpl vtplldnqi
 781 lntpaass dsalhatpvf psdvsvfesi lssydgapll pfssasfsse lfrhlhtvsq
 841 ilpqysate sdkvplhasl pvaggdlle psaqysdvl sthaasetl efgsesgvly
 901 ktlmfsqvep psdammhar ssgpepsyal sdnegsqhif tsvysaipv hdsvgvtyqg
 961 slfsgpship ipksslitpt asllqpthal sgdgewsgas sdseflpdt dgltniss
 1021 pvsvaeftyt tsfvgddnka lskseiign etelqipsfn emvypsestv mpnmydnvkn
 1081 lnasqetsv sisstkgmfp gslahtttkv fdheisqvpe mnfsvqptht vsqasgdtl
 1141 kpvlsansep assdpassem lspstqllfy etsasfstev llqpsfqasd vdtllktvlp
 1201 avpsdpilve tpkvdkisst mlhlivnsa ssenmlhsts vpvfdvspts hmhsaslqgl
 1261 tisyasekye pvllksesh qvpslysnd elfqtanlei nqahppkgrh vfatpvlid
 1321 eplntlinkl ihsdeiltst kssvtgkvfa giptvasdtf vstdhsvpig nghvaitav
 1381 phrdgsvtst klfpksats elshsaksda glvggedgd tddgdgddd drgsdglsh
 1441 kemscssyre sqekvmndsd thenslmdqn npisyslsen seednrvtv ssdsqtgmdr
 1501 spgkpsang lsqkhdgke endiqtsal lplspeskaw avltsdeesg sgqgtsdln
 1561 enetstdfs adnekdadg ilaagdseit pgfpqspts vtsensevf vseaasns
 1621 hesriglaeg lesekkavip lvivsalfti clvvlvgili ywrkcfqah fyledstsp
 1681 vistptpif pisddvgaip ikhfpkhvad lhassgftee fetlkefyqe vqscvdlgi
 1741 tadssnhpdn khknryiniv aydhsrvkla qlaekdgklt dyinanyvdg ynrpkayiaa
 1801 qgplkstaed fwrmiwehmv evivmitnlv ekgrkcdqy wpadgsee y nflvtqksvq
 1861 vlayytrnf tlrtkikkq sqkgrpsgrv vtqyhtqwp dmvgpeyslp vltfvrkaay
 1921 akrhavgpvv vhsagvgrt gtyivldsm lqqiqhegtvn ifglkhirs qmnylvqtee
 1981 qyvfhdtlv eailsketev ldshihayvn allipgpakg tklekqfql sqsniqqsd y
 2041 saalkqcnre knrtssiipv ersrvgissl sgegtidyina symgyyqsn efuitqphll
 2101 htikdfwrm wdhnaglvvm ipdgqmaed efvywpnkde pinesfktv lmaehkcls
 2161 neekliiqdf ileatqddyv levrhfqcpk wpnpdspisk tfelisvike eanrdgpmi
 2221 vdehggvta gtfcalttm hqlekensvd vyqvakminl mrpgvfadie qyqflykvil
 2281 slvstrqeen pstslsnga alpdgniaes leslv

[0021] Cellular response induced by IL-34 can include any of the following. IL-34 can inhibit, for example, one or more of cell proliferation, clonogenicity and/or motility. IL-34 can induce tyrosine phosphorylation of a protein, such as, for example, focal adhesion kinase (FAK) and/or paxillin. IL-34 can also, for example, stimulate differentiation of neural progenitor cells.

[0022] The agent can be, for example, an antibody, a monoclonal antibody, a polyclonal antibody, an antibody fragment, a F(ab')₂ fragment, a Fab' fragment, a peptide, a peptide nucleic acid, or small chemical compound (e.g., have a molecular weight of 5,000 daltons or less).

[0023] The agent can bind to a chondroitin sulfate glycosaminoglycan (GAG) moiety on PTP- ζ or to a GAG mimic.

[0024] The agent can be useful, for example, for treatment of a disease or disorder or a defect in neural or microglial development or a defect in homeostasis. The disease or disorder can be, for example, cancer, inflammation, a central nervous system disease, brain

injury, neuro-degeneration, a memory deficit, glioblastoma, multiple sclerosis, schizophrenia, or autoimmune disease.

[0025] This invention will be better understood from the Experimental Details that follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims that follow thereafter.

EXPERIMENTAL DETAILS

Introduction

[0026] An unbiased proteomic approach identified PTP- ζ as an IL-34-interacting membrane protein in mouse brain. Using shRNA-mediated suppression of PTP- ζ expression in a CSF-1R-less U251 human glioblastoma cell line, IL-34 was demonstrated to bind specifically to cell-surface PTP- ζ to initiate downstream signaling that leads to the inhibition of cell proliferation, clonogenicity and motility. IL-34-binding to PTP- ζ is dependent on the presence of the CS GAG moiety on PTP- ζ . The demonstration of the existence of a novel IL-34 receptor increases the scope of biological effects of IL-34 in development, homeostasis and disease.

Materials and Methods

[0027] *Reagents* – Purified mouse IL-34 (mIL-34), human IL-34 (hIL-34) and purified polyclonal rabbit anti-mIL-34 antibodies were from FivePrime Therapeutics, Inc., CA, USA) and human PTN (hPTN) was from R&D (Minneapolis, MN). Growth factors were suspended in phosphate buffered saline (PBS) as vehicle. mIL-34 and hIL-34 were biotinylated using a 10 molar excess of EZ-Link Sulfo-NHS-LC-LC-Biotin (Thermo Scientific, MA, USA) (15', 20°C) following manufacturer's instructions. The rabbit anti-C-terminal CSF-1R peptide antibody (C-15) to the mouse CSF-1R (mCSF-1R) and human CSF-1R (hCSF-1R), used for western blotting and immunoprecipitation, has been previously reported (41). Other antibodies used for western blotting were directed against phosphotyrosine (pY-100) and β -catenin (Cell Signaling), pY118paxillin and pY397FAK (Life Technologies, NY, USA); hCSF1R (2-4A5), β -adducin, FAK and TN-R (Santa Cruz Biotechnology Inc., CA, USA); paxillin and PTP- ζ (C-209) (BD Biosciences), PTP- ζ (3F8) (DSHB, the University of Iowa); PTP- ζ (473-HD) (Santa Cruz Biotechnology Inc., CA, USA)(42); EF1 α (43). Bovine serum albumin (BSA), was from Gemini (CA, USA), Puromycin dihydrochloride, trypan blue, crystal violet, DAPI, shark cartilage CS salts,

Proteus vulgaris chondroitinase ABC (chABC) and phalloidin were from SIGMA (MO, USA). Polybrene was from Santa Cruz Biotechnology, Inc. (CA, USA). Neutravidin-Ultralink beads were from Thermo Scientific (MA, USA). Streptavidin-conjugated APC-Cy7 was from Biolegend (CA, USA). LIVE/DEAD[®] Fixable dead cell stain kits were from Molecular Probes (NY, USA). HTS FluoroBlok[™] inserts and 24-well and 6-well tissue culture dishes were from BD Biosciences, Franklin Lakes, NJ. Accutase was from Stem Cell Technologies (Vancouver, BC, Canada). Human PTP- ζ and CSF-1R extracellular domains (ECD) fused immunoglobulin Fc domains (hPTP- ζ -ECD-Fc and hCSF-1R-ECD-Fc) were prepared as described previously for the hCSF-1R-ECD-Fc (13). Human recombinant CSF-1 (hCSF-1) was a gift from Chiron Corp. (Emeryville, CA, USA). EDC/NHS, HBSP and HBSP+ buffers were from GE Healthcare Biosciences, Pittsburgh, PA, USA.

[0028] *Sample preparation for LC-MS/MS-identification of the receptor* - Sub-cellular fractionation was carried out to isolate the membrane fraction from a pool of 2 postnatal day 7 and 2 postnatal day 60 mouse brains. Briefly, mouse brain tissue was homogenized in homogenization buffer (65 mM Tris, 150 mM sodium chloride, 1 mM EDTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 1 mM benzamidine, pH 7.4) and the homogenate centrifuged (1000xg, 3 min, 4⁰C). The supernatant was further centrifuged (100,000xg, 30 min, 4⁰C) and the pellet dissolved in 2% N-octyl- β -D-glucoside (OG), prior to centrifugation (100,000xg, 30 min, 4⁰C). The supernatant, containing 26 mg of the OG-solubilized membrane lysate was first pre-cleared by incubation with 60 μ g of anti-CSF-1R peptide antibody (C-15) (4⁰C, 16h) and then incubated with 24 μ g of mIL-34 non-covalently bound to 40 μ g of immobilized polyclonal rabbit anti-mIL-34 antibody (4⁰C, 16h). mIL-34-anti-mIL-34 antibody complex was serially washed using 0.1 M glycine-HCl, pH 2.2 and 8 M urea and subsequently eluted with 1% SDS. The glycine-HCl and urea washes did not result in dissociation of proteins from mIL-34-anti-mIL-34 antibody complex, as determined by SDS-PAGE and LC-MS/MS. The denatured, reduced and alkylated SDS eluate was further concentrated by ultracentrifugation using 100 kDa cut-off filters and subjected to SDS removal, concentration, trypsinization and detergent extraction with ethyl acetate, as described elsewhere (44,45) followed by LC-MS/MS.

[0029] *Nanoelectrospray LC-MS/MS analyses and protein identification*- Tryptic digests were loaded and separated using the UltiMate, FAMOS, Switchos nano-HPLC system (LC Packings, Dionex; Sunnyvale, CA), connected on-line to a LTQ Linear Ion Trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) and equipped with a nanospray

source. The mobile phases consisted of 5% acetonitrile/water, 0.1% formic acid (A) and 80% acetonitrile/water, 0.1% formic acid (B). After injection (15 µl of sample) and loading onto a C18 trap column, 0.3 mm I.D. X 5 mm, the tryptic peptides were separated on a C18 analytical HPLC column (75 µm I.D. X 15 cm; Pepmap, 3 µm, 100 Å; LC Packings, Dionex; Sunnyvale, CA). The flow rate for loading and desalting was 15 µl/min for 30 minutes while the analytical separation was performed at 250 nl/min. The gradient used was: 2% to 55% B in 65 min; held at 55% B for 10 minutes; increase to 95% B in 5 min and then held at 95% B for 5 minutes. The HPLC eluent was electrosprayed into the LTQ using the nanospray source. After an initial MS-survey scan, m/z 300-1800, MS/MS scans were obtained from three most intense ions using a normalized collision-energy of 35%. DTA files were generated from the raw data files, merged and searched against all species of the NCBI database (July 2, 2010) using Mascot (version 2.3). The search parameters were: fixed modification - carboxymethyl Cys; variable modifications – N/Q deamidation, oxidized Met, pyro-glu from Q and pyro-glu from E; 2 missed cleavages; peptide mass tolerance of ± 3.5 Da and ± 0.6 Da for the product ions. Scaffold (version 3, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95% probability and protein identifications were accepted at greater than 99% probability and with unique significant peptide hits ($p < 0.05$). Utilizing these criteria 9 proteins were identified excluding trypsin and keratin (6 of them were membrane proteins). The two proteins identified with the highest protein score were TN-R and PTP- ζ . The Mascot protein score for TN-R (3 matching protein accession numbers: gi|148707401, gi|226958549 or gi|61216646; 139 kDa) was 932 with protein sequence coverage of 15% protein. The protein score for PTP- ζ (gi|124486807) was 555 with 5% coverage.

[0030] *Cell lines, cell culture conditions and cell treatments* - The U251, SNB19 and U87MG human glioblastoma cell lines were a gift of Dr. J. Segall, Albert Einstein College of Medicine, NY, USA). NIH-3T3-hCSF-1R cells (46) were a gift from Dr. Martine Roussel, St. Jude Children's Research Hospital, Memphis, TN. Mouse BAC1.2F5 macrophages (47) were cultured in 36 ng/ml hCSF-1 as described (48). U251 cells were cultured in DMEM-high glucose (GIBCO, Grand Island, NY, USA) supplemented with 10% FCS and passaged when confluent. Prior to stimulation with hIL-34 or hPTN, cells were depleted of growth factors by incubation in DMEM-high glucose supplemented with 0.2% BSA for 16h, except where otherwise indicated. Following stimulation, cells were washed in ice-cold PBS and recovered

by scraping and centrifugation, except where otherwise indicated. For chABC-treatment, serum-starved U251 cells were incubated with 4.2 U/ml chABC (37°C, 1h 30') and washed extensively, before processing for flow cytometry as described below. For treating membrane lysates, 0.3 U/ml chABC was used.

[0031] *Generation of U251 PTP- ζ knock-down (KD) cells* - Lentiviral particles (5×10^4 IFU) carrying a pool of three different PTP- ζ shRNA or scrambled shRNA plasmids (Santa Cruz Biotechnology, CA, USA) were used to infect 3×10^4 U251 cells (50% confluent) in the presence of 5 μ g/ml polybrene in 6-well dishes (37°C, 16h). Vector-containing cells were selected using 5 μ g/ml of puromycin dihydrochloride and the resistant colonies were further sub-cloned by serial dilution method in 96-well plates. The efficiency of knock-down was estimated by western blotting whole cell lysates from the puromycin resistant clones.

[0032] *Immunoprecipitation and Western blot analysis* - Membrane fractions of mouse brain and of BAC1.2F5 macrophages, NIH-3T3-hCSF-1R cells or U251 cells were solubilized in homogenization buffer (65 mM Tris, 150 mM sodium chloride, 1 mM EDTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 1 mM benzamidine, pH 7.4) containing the appropriate concentration of OG (brain membrane 2%; cell membrane 1%) and incubated (4°C, 16h) with either immobilized purified polyclonal rabbit anti-mIL-34 antibody beads (preincubated with mIL-34), biotinylated mIL-34, biotinylated hIL-34 (a gift from FivePrime Therapeutics, Inc., CA, USA), or anti-hCSF-1R antibodies. The biotinylated IL-34 complexes were recovered by incubation with neutravidin-agarose and SDS eluates of IL-34 pull-down and immunoprecipitates analyzed by SDS-PAGE and western blotting (WB). For co-immunoprecipitation experiments, mouse brain membrane lysates were pre-incubated with mIL-34 (4°C, 16h), prior to incubation with anti-PTP- ζ (3F8) antibodies. For stimulation and/or immunoprecipitation experiments, serum-starved U251 cells were incubated with hPTN or hIL-34 (120 ng/ml) at 37°C and NP-40 cell lysates (using 1% NP-40, 10 mM Tris-HCl, 50 mM NaCl, 30 mM Na₄P₂O₇, 50 mM NaF, 100 μ M Na₃VO₄, 5 μ M ZnCl₂, 1 mM benzamidine, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin, pH 7.2) were subjected to immunoprecipitation using antibodies to FAK and paxillin.

[0033] *Flow cytometry* - For cell-surface IL-34 binding, serum-starved U251 cells were gently harvested with 2 mM EDTA in PBS, pH 7.4, washed and 1×10^6 cells were pre-incubated with a 10 molar excess of hIL-34 in Hank's Balanced Salt Solution (HBSS) (Life Technologies) in the presence of 1% BSA (4°C, 1 h). After extensive washing, specific IL-34 binding was detected by incubating the cells with 2 μ g/ml biotinylated hIL-34 (4°C, 1 h), and,

subsequently, with 5 µg/ml streptavidin-conjugated APC-Cy7. Flow cytometry was performed using FACS Canto II (BD Biosciences, NC, USA) (gating on viable cells). The FlowJo software (Treestar, USA) was used for data analysis. For detection of hCSF-1R expression, serum-starved 2×10^5 U251 cells were incubated with 5 µg/ml rat anti-hCSF-1R monoclonal antibody (2-4A5) or control rat IgG₁ (e-Biosciences, CA, USA) (4°C, 45'), then subsequently incubated with 5 µg/ml FITC-conjugated F(ab')₂-anti-Rabbit IgG (e-Biosciences, CA, USA) prior to flow cytometric analysis as previously described. For the analysis of FLAG-tagged IL-34 and CSF-1 binding to U251 cells by flow cytometry, the expression, purification and quantitation of the concentrations of IL-34-FLAG and CSF-1-FLAG proteins in the medium of the transfected 293T cells, as well as the detection of cell binding with biotin-labeled anti-FLAG M2 antibody, were carried out as described (17).

[0034] *Cell proliferation and clonogenic assays* - U251 cells were seeded at 25% confluency in DMEM-high glucose supplemented with 10% FCS in 24-well tissue culture dishes. 24h later, cells were washed twice with PBS and medium was replaced with DMEM-high Glucose supplemented with 1% FCS and vehicle (PBS) or hIL-34 (20 ng/ml) or hPTN (20 ng/ml). Cell proliferation was assessed by counting viable (Trypan-Blue excluding) cells harvested at the indicated times. For the clonogenic assays, semi-confluent U251 cells were exposed to a 16h pulse of hPTN (20 ng/ml), hIL-34 (20 ng/ml) or vehicle (PBS). After that, cells were harvested by Accutase digestion, filtered through a 40 µm mesh to ensure single cellularity and subsequently seeded at 1000 cells/well into 6-well dishes in the presence of 25% conditioned medium (from the 16h pulse). The number of colonies composed of >50 cells was scored by crystal violet staining 8 days later.

[0035] *Cell migration assays* - For wound healing assays, serum-starved (16 h) monolayer cultures of U251 cells were scratched and the wound allowed to heal in the continued absence of serum and in the presence of either hPTN or hIL-34 (200 ng/ml). For haptotactic migration assays, 10^5 serum-starved U251 cells were assayed (37°C, 4h) in a 24-well transwell chamber. Inserts were pre-coated with BSA (20 µg/ml, no growth factor) or hPTN (5 µg/ml) or hIL-34 (10 µg/ml) for 2 h at room temperature prior to the assay. For the random migration studies (37°C, 4h), hPTN or hIL-34 (1 µg/ml) was added to both sides of the transwell chamber. Cells were scraped from the upper side of the chamber and the lower side was stained with DAPI and phalloidin. Phalloidin-stained cells were counted using a fluorescence microscope.

[0036] *Nanoelectrospray LC-MS/MS analyses and protein identification-* Tryptic digests were loaded and separated using the UltiMate, FAMOS, Switchos nano-HPLC system (LC Packings, Dionex; Sunnyvale, CA), connected on-line to a LTQ Linear Ion Trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) and equipped with a nanospray source. The mobile phases consisted of 5% acetonitrile/water, 0.1% formic acid (A) and 80% acetonitrile/water, 0.1% formic acid (B). After injection (15 μ L of sample) and loading onto a C18 trap column, 0.3 mm I.D. X 5 mm, the tryptic peptides were separated on a C18 analytical HPLC column (75 μ m I.D. X 15 cm; Pepmap, 3 μ m, 100 Å; LC Packings, Dionex; Sunnyvale, CA). The flowrate for loading and desalting was 15 μ L/min for 30 minutes while the analytical separation was performed at 250 nL/min. The gradient used was: 2% to 55% B in 65 min; hold at 55% B for 10 minutes; increase to 95% B in 5 min and hold at 95% B for 5 minutes. The HPLC eluent was electrosprayed into the LTQ using the nanospray source. After an initial MS-survey scan, m/z 300-1800, MS/MS scans were obtained from three most intense ions using a normalized collision-energy of 35%. DTA files were generated from the raw data files, merged and searched against all species of the NCBI database (July 2, 2010) with Mascot (version 2.3). The search parameters used were: fixed modification - carboxymethyl Cys; variable modifications – N/Q deamidation, oxidized Met, pyro-glu from Q and pyro-glu from E; 2 missed cleavages; peptide mass tolerance of $\pm$ 3.5 Da and $\pm$ 0.6 Da for the product ions. Scaffold (version 3, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability and protein identifications were accepted at greater than 99.0% probability and unique significant peptide hits ($p < 0.05$). With these criteria 9 proteins were identified excluding trypsin and keratin (6 of them were membrane proteins). The 2 proteins identified with the highest protein score were TN-R and PTP- ζ . The Mascot protein score for TN-R (3 matching protein accession numbers: gi|148707401, gi|226958549 or gi|61216646; 139 kDa) is 932 with protein sequence coverage of 15% protein. The protein score for PTP- ζ (gi|124486807) is 555 with 5% coverage.

[0037] *PTP- ζ shRNA sequences are as follows-*

Hairpin sequence (SEQ ID NO:1):

5'-GATCCCCAGATTTCTACCACAACATTCAAGAGATGTTGTGGTAGAAATCTGGTTTTT-3'

Corresponding siRNA sequences:

- Sense: 5'-CCAGAUUUCUACCACAACATT-3' (SEQ ID NO:2)

- Antisense: 5'-UGUUGUGGUAGAAAUCUGGTT-3' (SEQ ID NO:3)

Hairpin sequence (SEQ ID NO:4):

5'-GATCCCCACAGAGATGGTTCTGTATTCAAGAGATACAGAACCATCTCTGTGGTTTTT-3'

Corresponding siRNA sequences:

- Sense: 5'-CCACAGAGAUGGUUCUGUA-3' (SEQ ID NO:5)
- Antisense: 5'-UACAGAACCAUCUCUGUGG-3' (SEQ ID NO:6)

Hairpin sequence (SEQ ID NO:7):

5'-GATCCCGAAGGAAGTGTCAACATATTCAAGAGATATGTTGACAGTTCCTTCGTTTTT-3'

Corresponding siRNA sequences:

- Sense: 5'-CGAAGGAACUGUCAACAUA-3' (SEQ ID NO:8)
- Antisense: 5'-UAUGUUGACAGUCCUUCG-3' (SEQ ID NO:9).

[0038] *Surface plasmon resonance (SPR) binding analyses* – SPR binding analyses were performed at 25°C on Biacore instruments. For binding of hIL-34 and hCSF-1 to immobilized hPTP- ζ - and hCSF-1R- ECD-Fcs (Biacore T100), all flow cells of a CM4 sensor chip were activated with EDC/NHS (7 min, 10 μ l/min) and recombinant Protein A (Pierce, 21184, 50 μ g/ml in 10 mM Na acetate, pH 4.5) was applied (7 min, 10 μ l/min). Following immobilization ($\geq$ 2500RU of Protein A per flow cell), all flow cells were blocked with 1M ethanolamine-HCl, pH 8.5. hPTP- ζ -ECD-Fc (25 μ g/ml) and hCSF-1R-ECD-Fc (7 μ g/ml) in HBSP were captured ($\sim$ 300 RU and $\sim$ 97RU respectively) on individual flow cells and binding analyses performed with different concentrations of recombinant hCSF-1 or hIL-34 in HBSP+ or HBSP+ with 3 μ g/ml CS (shark cartilage, Sigma, C4384). The protein A surface was regenerated with 10 mM Glycine-HCl, pH 1.5. For binding of hIL-34 to brevican, different concentrations of hIL-34 in HBSP+ were passed over brevican immobilized on the chip. Binding of hTN-R to IL-34 and to hPTP- ζ ECD-Fc (Biacore 3000) was carried out at 25°C by immobilizing IL-34 (pH 7.0) and hPTP- ζ ECD-Fc (pH 4.05) directly onto flow cells of a CM5 sensor chip ($\geq$ 2800RU per flow cell) and passing over different concentrations of hTN-R. Kds were calculated according to the steady state model using BIAevaluation Software (GE Lifesciences). All sensorgrams were double-referenced.

[0039] *Immunofluorescence microscopy* – Anesthetized mice were transcardially perfused with ice-cold 0.25 mg/ml heparin in PBS (10 ml) followed by 4% paraformaldehyde (50 ml), their brains harvested, post-fixed overnight in 4% PFA, equilibrated in 20% sucrose, flash frozen in a cryomatrix resin and stored at -80°C prior to use. For immunofluorescence

microscopy, specimens were cryosectioned (30 μ m sections), immunostained with a mature neuronal marker (NeuN, mIgG1, 1:100, Millipore); an adult neural stem cell marker (GFAP, mouse IgG2b, 1:300, Millipore); IL-34 (polyclonal rabbit IgG, 1:500, Five Prime Therapeutics, CA, USA); PTP- ζ (3F8, mouse IgG1, 1:1250, Developmental Studies Hybridoma Bank, IA); TN-R (polyclonal rabbit IgG, 1:50, Santa Cruz Biotechnology, CA); photographed either with an Olympus BX51 microscope (Tokyo Japan) coupled with a Sensicam digital camera (Cooke Corporation, Michigan, USA), or with a Zeiss Duo V2 Laser Confocal Microscope. Images were subsequently processed with Image J and Photoshop CS5 software programs.

[0040] *Statistics* - Student's *t* test was used to assess the statistical significance of the data sets.

Results

[0041] *Mouse IL-34 (mIL-34) associates with mouse brain PTP- ζ and TN-R* - Compared with the expression patterns of CSF-1 and the CSF-1R, IL-34 expression in postnatal mouse brain, is spatially, temporally and quantitatively distinct (9,14). This prompted a search for additional IL-34 receptor(s) in detergent-solubilized postnatal mouse brain membranes. To identify novel IL-34-interacting proteins, mouse brain membrane lysate, depleted of the known IL-34 receptor, CSF-1R, was subjected to affinity chromatography with mIL-34 non-covalently bound to an immobilized polyclonal rabbit anti-mIL-34 antibody. Bound proteins were eluted with SDS and processed for mass spectrometry (MS). The two eluted proteins identified with highest certainty were PTP- ζ , a cell-surface RPTP and its ECM ligand, TN-R (Fig. 7). SDS-PAGE and silver staining of the SDS eluates of the mIL-34-anti-mIL-34 antibody affinity purification, or of IL-34-associated proteins prepared by the alternative approach of binding to biotinylated-mIL-34 and capturing the complexes with immobilized neutravidin contained a diffuse band of ~400 kDa, another broad band at ~225 kDa, as well as ~160/180 kDa species (Fig. 1A, lanes 1-4, upper panel).

[0042] Due to alternative splicing, PTP- ζ exists in three isoforms, one soluble and two membrane-spanning molecules (Fig. 1B). The ~400-kDa band was confirmed by western blotting to cover stainable bands corresponding to both the long (49) and phosphacan isoforms of PTP- ζ (50,51) as well as multimeric TN-R (52). The ~225-kDa band co-migrated with a band that stained with the 473-HD antibody (42), that sensitively stains the short PTP- ζ isoform and which also stained in the region of the ~400 kDa band corresponding to the long receptor and the phosphacan isoforms. The 160/180-kDa proteins, with the apparent M_r

of the monomeric TN-R isoforms (52), co-migrated with the faster bands western blotted with the anti-TN-R antibody (Fig. 1A, lanes 1-4, lower panels), corresponding to the monomeric TN-R isoforms. To confirm the PTP- ζ binding results, mouse brain membrane lysate was incubated with mIL-34 and a reciprocal co-immunoprecipitation experiment was performed, immunoprecipitating with 3F8, an antibody that recognizes both the soluble and the long membrane-spanning isoforms of rat PTP- ζ (53) (Fig. 1B) and western blotting with anti-IL-34 and anti-TN-R antibodies (Fig. 1C). These results show that mIL-34 forms a complex with the larger membrane-spanning isoform of PTP- ζ and with TN-R. However, consistent with earlier reports that the TN-R binding to PTP- ζ is ligand-independent (28,54), TN-R binding to PTP- ζ was IL-34-independent (Fig. 1C).

[0043] SPR analysis of hIL-34 binding to the full-length hPTP- ζ ECD-Fc ($K_d \sim 10^{-7}M$) and the hCSF-1R-ECD-Fc ($K_d \sim 10^{-12}M$) revealed dose-dependent binding to both, whereas hCSF-1 only bound to the hCSF-1R-ECD-Fc ($K_d \sim 10^{-11}M$). Similar analyses of the binding of IL-34 to two other proteoglycans identified by MS, hTN-R ($K_d \sim 10^{-6}M$) and human brevican ($K_d = 3 \times 10^{-6}M$), revealed lower affinity binding, whereas the interaction of TN-R with the hPTP- ζ ECD-Fc was of higher affinity ($K_d \sim 10^{-8}M$), but lower than previously reported (28,54).

[0044] *Human IL-34 (hIL-34) binds to cell-surface PTP- ζ on U251 glioblastoma cells*
 - Since membrane-spanning PTP- ζ , rather than TN-R, is a known signal-transducing receptor for several ligands (24-28), it was determined whether PTP- ζ also functions as a receptor for IL-34. As PTP- ζ is upregulated in human glioblastomas (38-40), glioblastoma cell lines U251, SNB19 and U87MG were tested for PTP- ζ expression. All the tested cell lines expressed high levels of PTP- ζ . U251 was selected for human IL-34 (hIL-34)-binding studies since it does not express the CSF-1R (Fig. 8). Supporting the observations in mouse brain membrane (Fig. 1A), biotinylated-hIL-34 primarily formed complexes with the long, membrane-spanning, ~ 400 kDa PTP- ζ (50) and to a lesser extent with the non-glycosaminoglycanylated 300 kDa (55) and the short 220 kDa (49) isoforms (Fig. 2A) in U251 membrane lysates. However, TN-R was not observed in the biotinylated-hIL-34 pull-down fractions of U251 membranes, suggesting that IL-34 probably binds to PTP- ζ in a TN-R-independent manner. Clones stably-expressing PTP- ζ shRNA (KD cells) expressed lower levels of PTP- ζ protein than clones expressing scrambled shRNA (scrambled cells) (Fig. 2B). Scrambled cells expressed higher levels of total soluble PTP- ζ than uninfected cells,

indicating that lentiviral infection *per se* causes cellular PTP- ζ upregulation. Consistent with the dependence of IL-34 binding on PTP- ζ expression, flow cytometric studies demonstrated that the ability of biotinylated hIL-34 to bind to the cell-surface of intact U251 cells was reduced in PTP- ζ KD cells (Fig. 2C), particularly in KD2 cells. Thus these results show that hIL-34 binds to PTP- ζ at the surface of intact U251 cells. The specificity and binding of IL-34 to U251 cells was also investigated in binding experiments with FLAG-tagged human IL-34 and FLAG-tagged human CSF-1 (17). At equivalent concentrations, IL-34-FLAG showed robust binding, whereas CSF-1-FLAG failed to bind and IL-34-FLAG exhibited dose-dependent binding, covering a wide concentration range (0.1 pM-1 nM).

[0045] *hIL-34 inhibits U251 proliferation, clonogenicity and motility in a PTP- ζ -dependent manner* - PTP- ζ -signaling is involved in neuronal migration (56) and neuritogenesis (57) in mouse and in the *in vitro* and *in vivo* growth of human glioblastomas (39,58,59). As previous studies have shown that PTN inhibits the growth of glioblastomas, to determine the functional relevance of PTP- ζ receptor engagement by IL-34, the effects of IL-34 and PTN were tested on the U251 glioblastoma cells expressing either PTP- ζ or scrambled (control) shRNAs. Either IL-34 or PTN treatment slightly, but significantly, reduced the growth of scrambled U251 cells over a 96 h time period (~20% reduction in IL-34 vs vehicle-treated control cells, Fig. 3A left panel), while not affecting the growth of the PTP- ζ KD U251 cell lines (Fig. 3A right panel). The effects of IL-34 and PTN on the colony-forming-ability of infected U251 cells were also examined. After a 16 h pulse with IL-34 or PTN, cells were seeded at clonal density and the colonies formed at 8 days were stained and counted. IL-34 or PTN treatment strongly decreased the clonogenicity of scrambled U251 cells (reductions of 68% for IL-34 and 53% for PTN, vs vehicle control cells), without significantly affecting the clonogenicity of PTP- ζ KD cells (Fig. 3B, left and right panels).

[0046] As PTN was also previously shown to affect glioblastoma cell migration (38-40), to investigate the role of IL-34 in PTP- ζ -mediated glioblastoma cell migration, the effects of IL-34 and PTN were first compared on the wound-healing rates of scrambled and PTP- ζ KD cells (Fig. 4A). In the absence of added ligand(s), KD clones exhibited a slower rate of wound healing, indicating that the constitutively active PTP- ζ receptor facilitates U251 migration (e.g. time taken for 50% wound closure [t_{50}] for KD2 cells was >20 h, compared to 5.5 h for scrambled cells). Consistent with ligand-induced inactivation of the receptor (30,32,60), PTN (t_{50} = 10 h), or IL-34 (t_{50} = 11.5 h) significantly inhibited wound

healing in uninfected cells (vehicle $t_{50} = 7.5$ h). Furthermore, neither IL-34, nor PTN could suppress PTP- ζ KD cell wound healing (Fig. 4B), thereby indicating that suppression of healing by either ligand is mediated through PTP- ζ . To determine whether IL-34 and PTN suppress directed migration a haptotaxis assay was used (Fig. 4C), in which PTP- ζ ligands were shown to be more effective in regulating migration than in a conventional chemotaxis assay (61,62). Both IL-34 as well as PTN, when coated on the bottom of the membrane, suppressed migration of U251 cells (Fig. 4C). To determine whether IL-34 and PTN also inhibit random migration, migration of the cells was examined through membranes containing these growth factors on both sides. Both IL-34 as well as PTN inhibited the random migration of the cells (Fig. 4D). Together, these results demonstrate that IL-34 suppresses proliferation, clonogenicity and motility of U251 cells *in vitro*, in a PTP- ζ -dependent manner.

[0047] *hIL-34 enhances PTP- ζ -mediated tyrosine phosphorylation of FAK and paxillin in U251 cells* - To function as a receptor for IL-34, IL-34 binding to cell surface PTP- ζ should trigger intracellular signaling. Consistent with the reduction of PTP- ζ phosphatase activity by ligand binding (30,33,60), PTP- ζ ligand binding has previously been shown to trigger intracellular protein tyrosine phosphorylation (31,32,34,35). Following incubation of U251 cells with PTN or IL-34 for various times at 37°C, a similar ligand-induced tyrosine phosphorylation of proteins was observed, including those with apparent M_r s of ~190, ~125, ~120, ~70 and ~42 kDa, that peaked within the first 5 minutes of stimulation (Fig. 5A). PTP- ζ ligands have been shown to increase the tyrosine phosphorylation of FAK, in lung and prostate carcinomas and endothelial cells (31,37,40) and of paxillin, in osteoblastic cells (63). Either PTN or IL-34 also increased the tyrosine phosphorylation of FAK (~125 kDa) and paxillin (~70 kDa) in U251 cells (Figs. 5 A, B). In contrast, there was no detectable increase in the tyrosine phosphorylation of the putative PTP- ζ substrates, β -catenin or β -adducin (32,34), in response to either ligand. IL-34-mediated activations of FAK and paxillin were abolished in the PTP- ζ -KD2 cell lines (Fig. 5C), demonstrating that tyrosine phosphorylation of these proteins, induced by IL-34, is mediated by PTP- ζ .

[0048] *hIL-34 binds to PTP- ζ in a chondroitin sulfate-dependent manner* - PTP- ζ is a proteoglycan receptor for several ligands (24-28) Furthermore, the PTP- ζ CS chains are known to affect binding to some of these ligands (25,29). The possible requirement of CS for

IL-34 binding was therefore tested. Consistent with the previously reported presence of CS on PTP- ζ (19,64), treatment of solubilized U251 membranes with chondroitinase ABC increased the mobility of a significant fraction of the large PTP- ζ isoform (Fig. 6A). To determine the requirement of CS for cell surface binding, intact U251 cells were incubated with enzyme buffer alone, or with chondroitinase ABC, to remove cell surface CS. Treatment with chondroitinase ABC reduced binding of biotinylated IL-34 to the level seen in IL-34 competed cells (background levels) (Fig. 6B). Pre-incubation of U251 cells with a 16 molar excess of IL-34 blocked the subsequent binding of biotinylated IL-34, whereas pre-incubations with a 16 molar excess of CSF-1, or of PTN, were without effect (Fig. 6C). Consistent with the removal of binding sites by preincubation with chondroitinase ABC, pre-incubation of IL-34 with 3 μ g/ml of shark cartilage CS blocked IL-34 inhibition of biotinylated IL-34 binding (Fig. 6C). Thus the CS GAG moiety of PTP- ζ is involved in IL-34 binding. SPR analysis further confirmed the inhibition by CS.

[0049] *Comparative expression profiles of PTP- ζ , TN-R and IL-34 in adult brain -*

Previous studies have shown that PTP- ζ is primarily expressed in neural progenitors and glial cells (21-23) as well as in a subset of cortical neurons (22,25). The expression of TN-R overlaps with PTP- ζ expression in rostral brain regions (77-79). IL-34 expression is primarily observed on mature neurons (9,16,76), including regions of the brain where PTP- ζ is expressed (23). IL-34 expression profiles are distinct from those of its cognate receptor, CSF-1R and also of CSF-1, and that it is preferentially increased in specific areas of early postnatal and adult forebrain, thereby suggesting the presence of an alternative signaling receptor (9). As PTP- ζ functions as a cell-surface receptor for IL-34 and also interacts with TN-R, the expression profiles of IL-34, PTP- ζ and TN-R were analyzed in 8-10 week-old mouse brains. PTP- ζ and TN-R were co-localized in OB, cerebral cortex, RMS and the CA3 region of the hippocampus that have previously been shown to express IL-34 (9). In addition, PTP- ζ expression remained prominent in distinct subcortical structures (thalamic and subthalamic nuclei), midbrain and brain stem nuclei (inferior colliculus, pontine nuclei, locus coeruleus and vestibular nuclei), as well as the cerebellum, and displayed distinct co-localization patterns with both IL-34 and TN-R. Mature post-mitotic neurons of the cerebrum were labeled by all three of these markers. These observations are consistent with a previous study that localized IL-34 expression preferentially to mature neurons of the adult cerebral cortex, extending from layers 2-5. In addition, IL-34 and PTP- ζ co-localization was

particularly prominent in layer 5 of the cortex. Consistent with the existence of the secreted PTP- ζ isoform, PTP- ζ expression was also observed in the ECM of the cerebral cortex in concert with IL-34. IL-34 and PTP- ζ appeared to be uniformly distributed in the ECM of layer 5 and at the periphery of mature neuronal cell bodies. In contrast, in cortical layer 6, PTP- ζ expression was reduced in the ECM and neuronal soma, whereas IL-34 expression was virtually absent. Expression of TN-R was evident in cerebral cortical layers 2-5, most prominently in layer 4, where it was co-localized with IL-34 in both the ECM and at the periphery of mature neuronal somas. Its cellular and extra-cellular expression decreased in cortical layer 5. Finally, in contrast to the expression profiles of IL-34 and TN-R, PTP- ζ staining was also seen in GFAP+ adult neural stem cells present in the anterior SVZ, as well as in those neural species migrating through the RMS to the OB (21,23), but not in GFAP+ astrocytes in the CC.

Discussion

[0050] The differential and higher expression of IL-34, compared to CSF-1 and CSF-1R expression in brain coupled with the more pronounced effects of IL-34 compared with CSF-1 on neural progenitor cell self-renewal and differentiation (9,14), raised the possibility that IL-34 might signal via an alternate receptor. The present studies identified PTP- ζ , a cell-surface CSPG, as a second functional receptor for IL-34. IL-34 selectively interacts with PTP- ζ in membrane lysates from both mouse brain as well as U251 human glioblastoma cells. It binds to intact U251 cells, stimulates their phosphotyrosine signaling and suppresses their tumorigenic properties in a PTP- ζ -dependent manner. Furthermore, IL-34, but not CSF-1, binds PTP- ζ *in vitro* ($K_d \sim 10^{-7}M$) and at the cell surface and whereas IL-34 and CSF-1 compete for binding to the CSF-1R (14,65,66), pre-incubation with a 16-molar excess of CSF-1 failed to compete for IL-34 binding to U251 cells. Thus PTP- ζ fulfills the criteria required for it to function as the postulated IL-34 receptor.

[0051] PTP- ζ is primarily expressed on neural progenitor and glial cells (21-23), as well as on a subset of cortical neurons (22,25). In contrast, IL-34 is expressed primarily on neurons (9,16) and also in the regions of the brain where PTP- ζ is expressed (23). Interestingly, IL-34 signaling via PTP- ζ in U251 glioblastoma cells causes a suppression of clonogenicity, similar to the effect of CSF-1 or IL-34 on isolated CSF-1R-expressing neural progenitors (9). This suppression of clonogenicity is correlated with stimulation of cellular tyrosine phosphorylation in either setting. Indeed, IL-34 was shown to be significantly more

active in suppressing neural progenitor cell proliferation than CSF-1 (9), consistent with an additional action of IL-34 via the PTP- ζ receptor on the neural progenitor cells.

[0052] PTP- ζ -signaling plays a contrasting role in hematopoietic stem cells, leading to their expansion (67). A positive regulatory role of PTP- ζ in proliferation and migration was also reported in some glioblastomas (31,40), which could reflect the role of other known PTP- ζ co-receptors, such as the integrins $\beta 1$ (63) and $\beta 3$ (38), in governing these biological responses. Addition of IL-34 to U251 cells led to an increase in the tyrosine phosphorylation of FAK and paxillin and a suppression of cell motility. Although an increase in tyrosine phosphorylation of FAK is associated with an increase in proliferation and motility in other cells, it has been shown to inhibit these responses in glioblastomas (68). Also, similar to the present observations in U251 cells, increased tyrosine phosphorylation of paxillin is correlated with inhibition of motility in macrophages (69) and FAK (Y397) dephosphorylation promotes tumor metastasis (68). Thus, signaling via the PTP- ζ receptor can have contrasting biological effects in specific cellular contexts, possibly dependent on the differential expression of PTP- ζ co-receptors and the activation of specific signaling pathways.

[0053] In view of the striking dependence of the IL-34-PTP- ζ interaction on the CS GAG chain, it was of interest that IL-34 also bound to other CS and HS proteoglycans with low affinity. The CS inhibition of PTP- ζ binding and the finding that binding to brevican and glypican was effectively blocked by heparin, suggest that the low affinity binding of IL-34 to proteoglycans involves the electrostatic interactions between IL-34 and the proteoglycan GAG chains and that the nature of the GAG chain is the likely determinant of this interaction. This electrostatic interaction may also be an important part, but not the sole component, of the high affinity interaction of IL-34 with PTP- ζ and the reason for the CS inhibition that was observed.

[0054] Like IL-34, PTN also exhibits GAG-dependent binding to PTP- ζ . However, in contrast to IL-34, CS-C, but not shark cartilage CS, inhibits this binding (25). Furthermore, competition experiments showed that PTN fails to compete for the binding of IL-34 (Fig. 6C), suggesting that IL-34 binding could involve a different PTP- ζ CS GAG-moiety, not recognized by PTN. Interestingly, the CS-A replaces CS-C on the PTP- ζ receptor during development from embryonic to post-natal brain and this is correlated with a decrease in the

expression of PTN (64,70). In contrast, IL-34 expression increases progressively during brain development (9).

[0055] The identification of PTP- ζ as a novel receptor for IL-34 necessitates a reevaluation of the possible role of IL-34/ PTP- ζ signaling in tissues in which both ligand and receptor are expressed. Obviously the CNS is an important organ system because of the significant expression of both IL-34 and PTP- ζ in brain and because PTP- ζ has been implicated in several disease settings in the CNS. For example, it is expressed in remyelinating oligodendrocytes (OL) and *PTP- ζ* -deficient mice display a delayed recovery from demyelinating lesions in a model of experimental autoimmune encephalomyelitis (71). Furthermore, the soluble PTP- ζ isoform has been shown to be necessary for maturation for OL progenitors to differentiated myelin-secreting oligodendrocytes *in vitro* (72) and *PTP- ζ* -deficient mice exhibit increased myelin breakdown (73). In addition, *PTPRZ1* gene in humans is a schizophrenia-susceptibility gene (74) and PTP- ζ regulates tyrosine phosphorylation of voltage-gated sodium channels in neurons (75). Given the present demonstration that IL-34 modulates tumorigenic properties of glioblastoma cell line U251, the fact that PTP- ζ is expressed in neuroblastomas (18) and other tumors (37,40), such as prostate and lung cancer, is highly relevant. IL-34 is also important for Langerhans cell development (16, 76), probably via RPTP-zeta.

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What is claimed is:

1. A method for determining whether or not an agent is a candidate agent for inhibiting interaction between interleukin-34 (IL-34) and protein tyrosine phosphatase receptor type zeta (PTP- ζ) comprising:

contacting cells that express PTP- ζ on their surface and that do not express colony stimulating factor-1 receptor (CSF-1R) with IL-34 in the presence of the agent and in the absence of the agent, and

measuring a cellular response induced by IL-34,

wherein an agent that reduces a cellular response induced by IL-34 is a candidate agent for inhibiting interaction between IL-34 and PTP- ζ , and

wherein an agent that does not reduce a cellular response induced by IL-34 is not a candidate agent for inhibiting interaction between IL-34 and PTP- ζ .

2. The method of claim 1, wherein the cells are glioblastoma cells.

3. The method of claim 1, wherein the cells are U251 human glioblastoma cells.

4. The method of claim 1, wherein the cells have been transfected with nucleic acid encoding human PTP- ζ .

5. The method of any of claims 1-4, wherein IL-34 inhibits one or more of cell proliferation, clonogenicity and/or motility.

6. The method of any of claims 1-4, wherein IL-34 induces tyrosine phosphorylation of a protein.

7. The method of claim 6, wherein IL-34 induces tyrosine phosphorylation of focal adhesion kinase (FAK) and/or paxillin.

8. The method of claim 1, wherein IL-34 stimulates differentiation of neural progenitor cells.

9. The method of any of claims 1-8, wherein the agent binds to a chondroitin sulfate glycosaminoglycan (GAG) moiety on PTP- ζ .
10. The method of any of claims 1-9, wherein the agent is useful for treatment of a disease or disorder or a defect in neural or microglial development or a defect in homeostasis.
11. The method of claim 10, wherein the disease or disorder is cancer, inflammation, a central nervous system disease, brain injury, neuro-degeneration, a memory deficit, glioblastoma, multiple sclerosis, schizophrenia, or autoimmune disease.

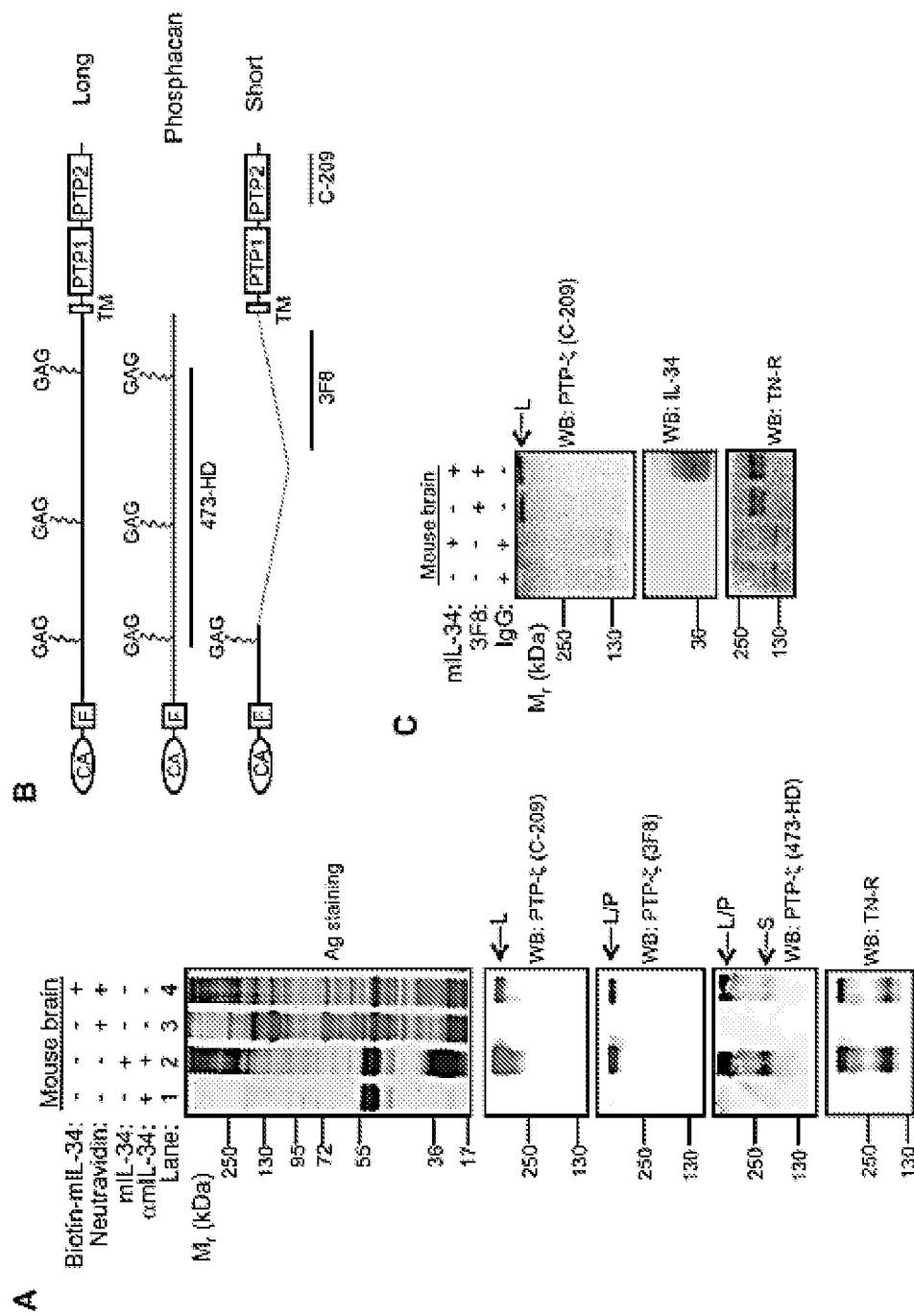


FIGURE 1A-1C

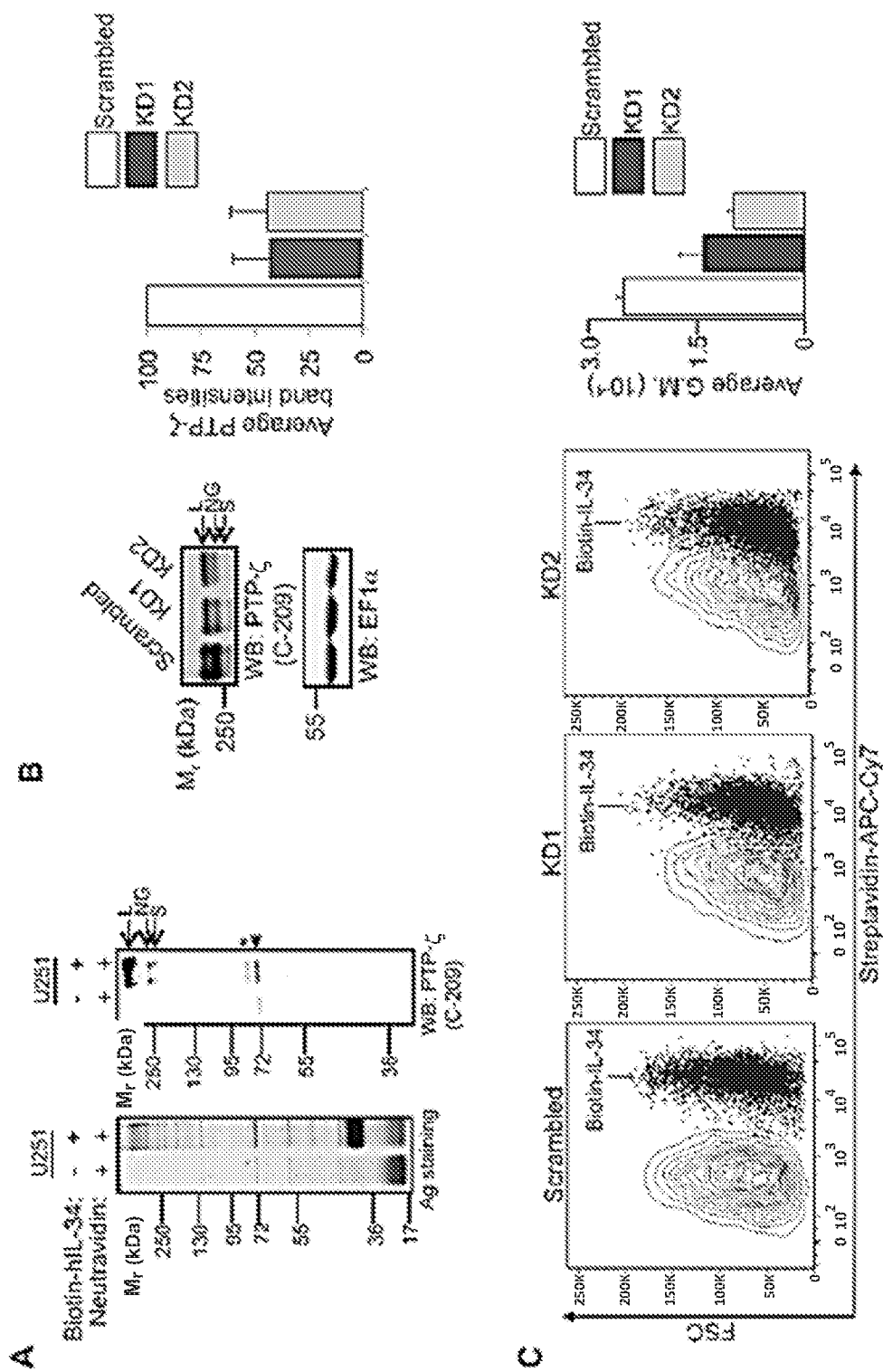


FIGURE 2A-2C

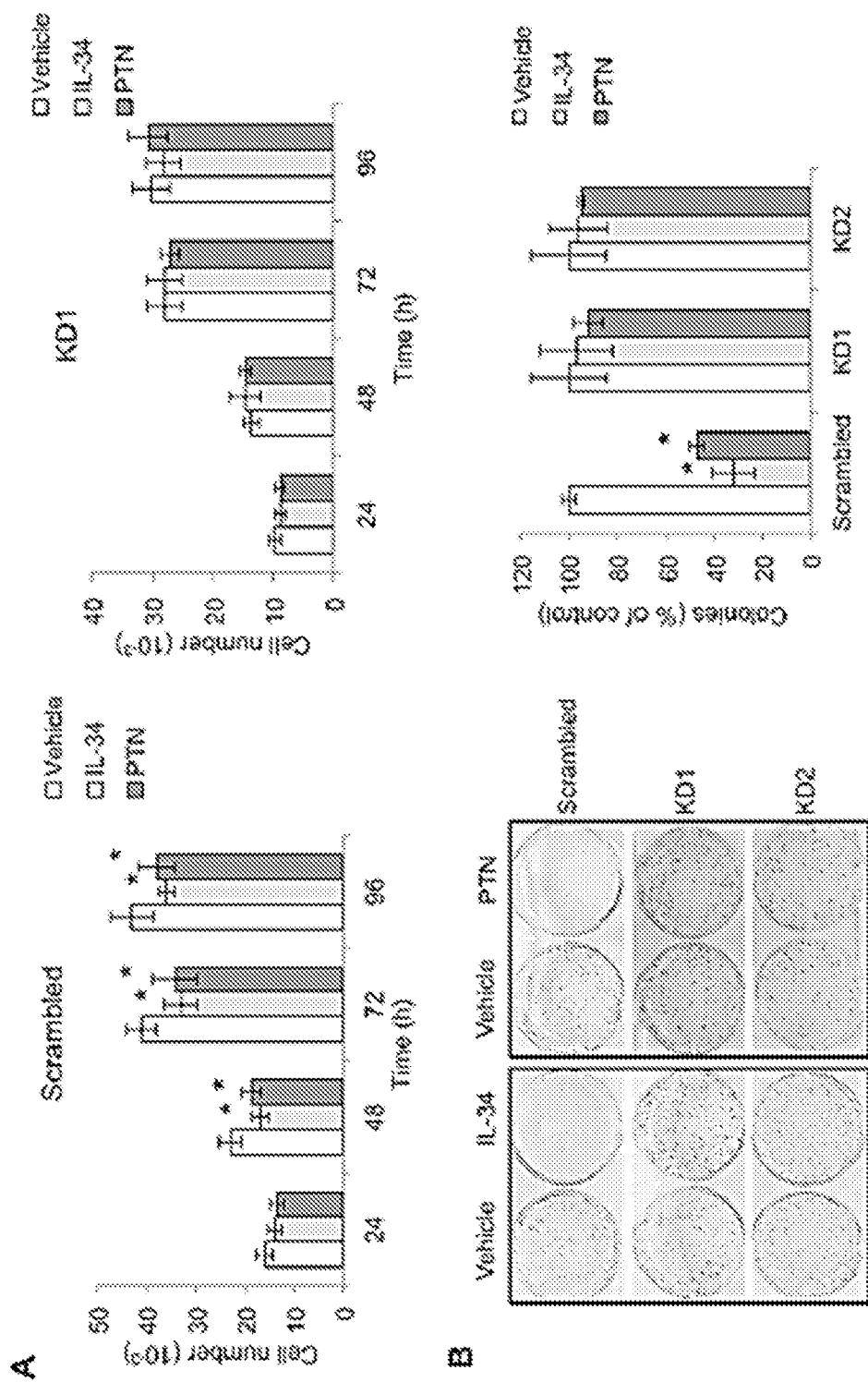


FIGURE 3A-3B

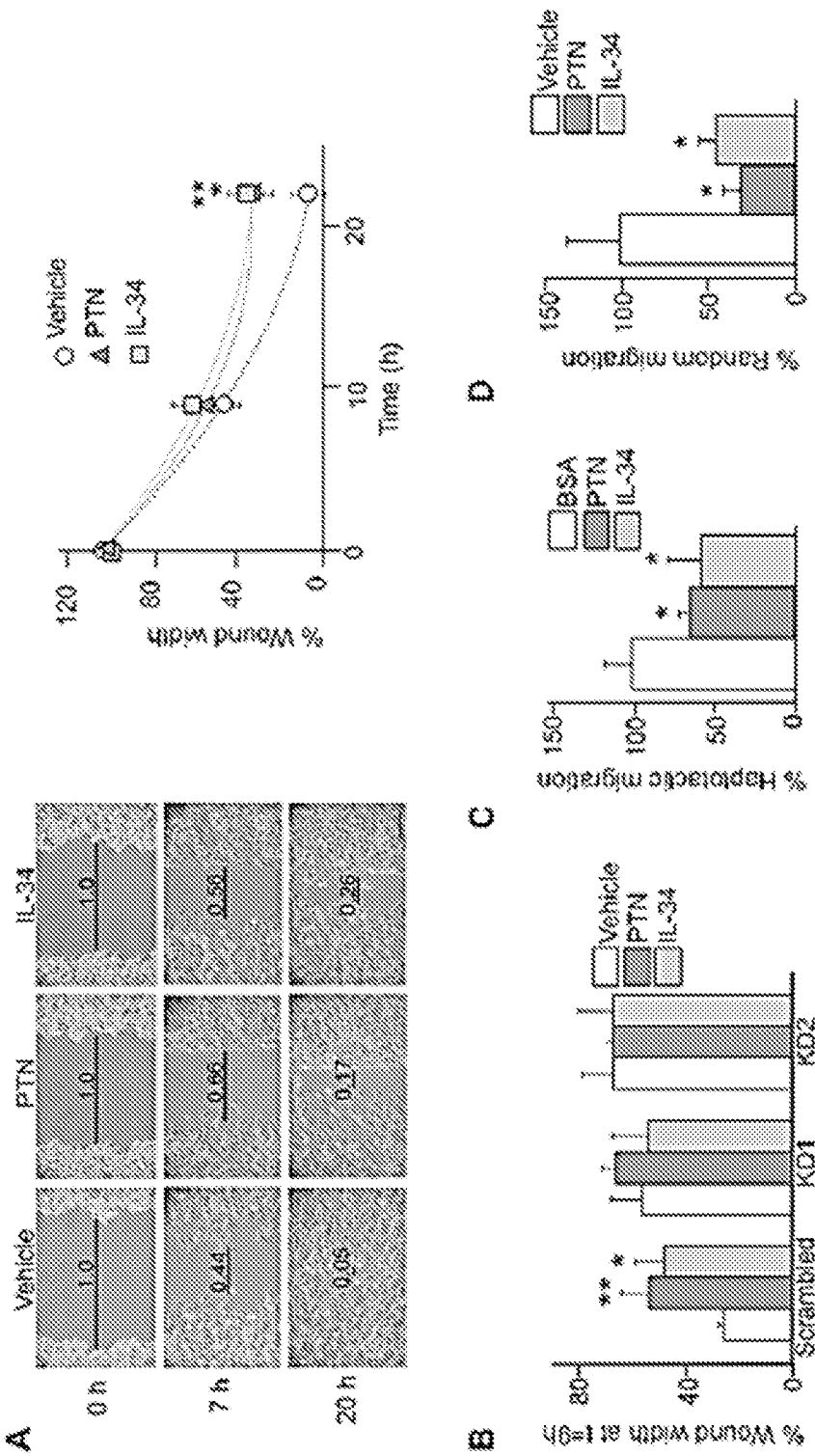


FIGURE 4A-4D

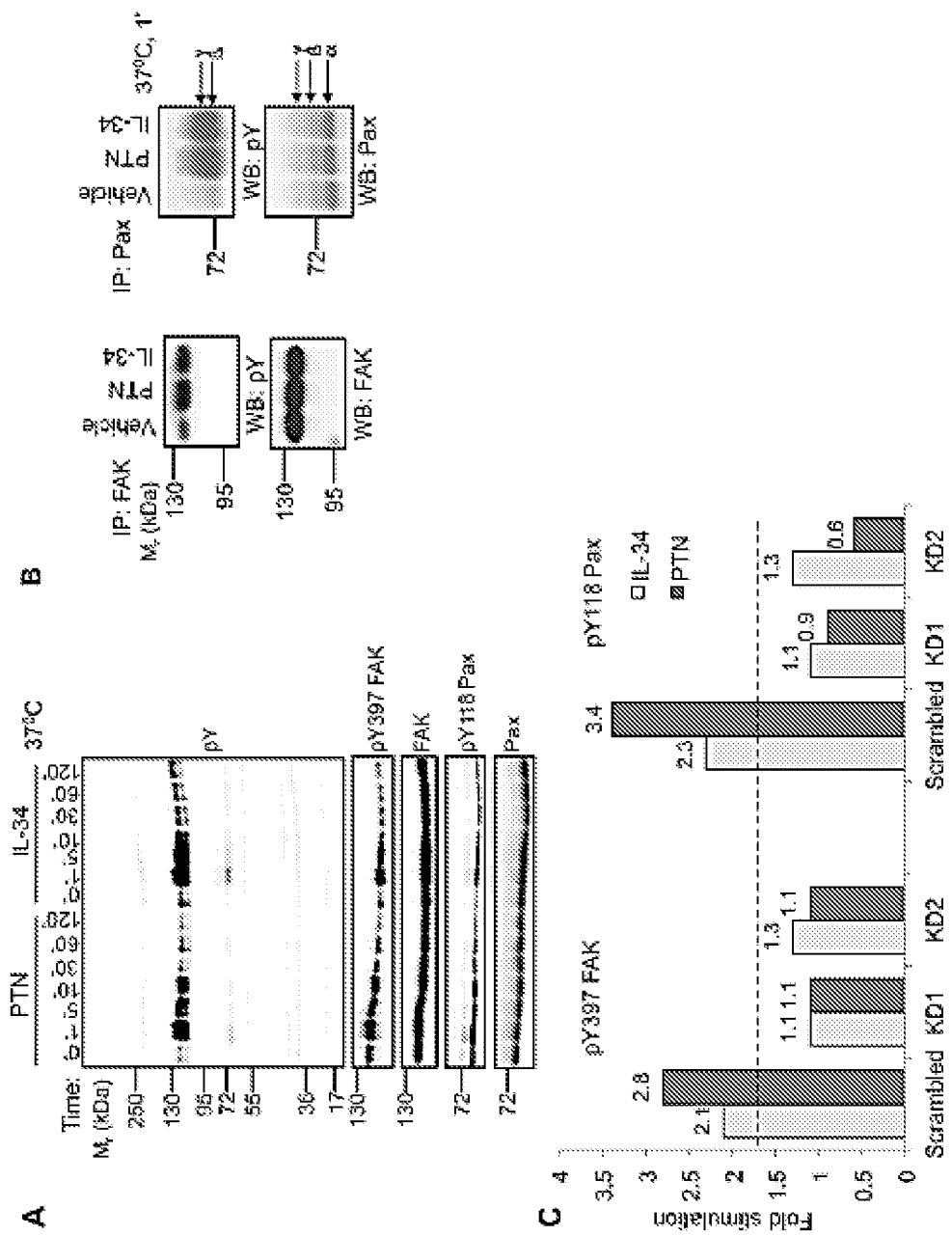


FIGURE 5A-5C

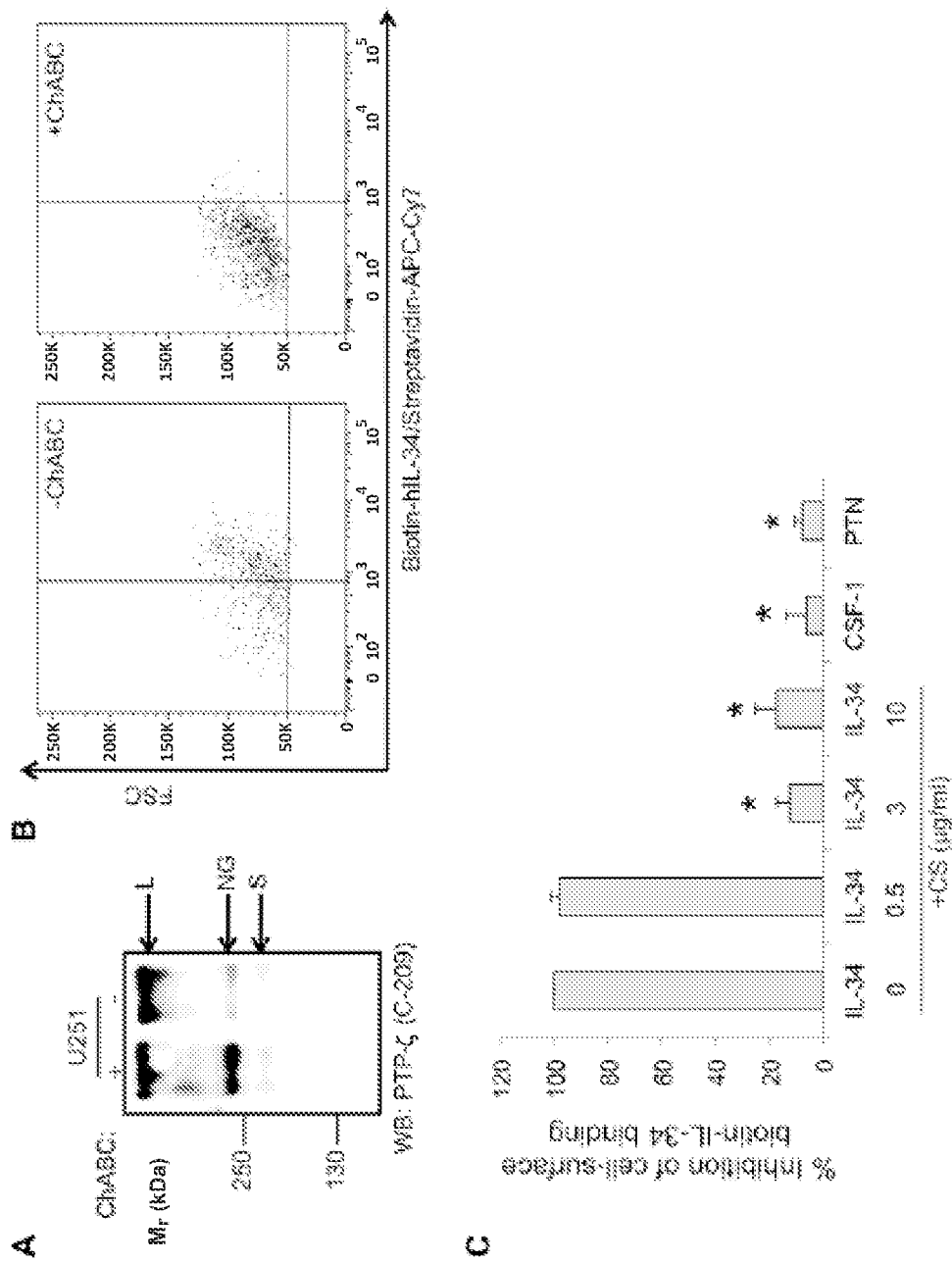


FIGURE 6A-6C

A	1	B	I	CA	F	PTP1	PTP2
51	51	51	51	51	51	51	51
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201	201	201	201	201	201	201	201
251	251	251	251	251	251	251	251
301	301	301	301	301	301	301	301
351	351	351	351	351	351	351	351
401	401	401	401	401	401	401	401
451	451	451	451	451	451	451	451
501	501	501	501	501	501	501	501
551	551	551	551	551	551	551	551
601	601	601	601	601	601	601	601
651	651	651	651	651	651	651	651
701	701	701	701	701	701	701	701
751	751	751	751	751	751	751	751
801	801	801	801	801	801	801	801
851	851	851	851	851	851	851	851
901	901	901	901	901	901	901	901
951	951	951	951	951	951	951	951
1001	1001	1001	1001	1001	1001	1001	1001
1051	1051	1051	1051	1051	1051	1051	1051
1101	1101	1101	1101	1101	1101	1101	1101
1151	1151	1151	1151	1151	1151	1151	1151
1201	1201	1201	1201	1201	1201	1201	1201
1251	1251	1251	1251	1251	1251	1251	1251
1301	1301	1301	1301	1301	1301	1301	1301
1351	1351	1351	1351	1351	1351	1351	1351
1401	1401	1401	1401	1401	1401	1401	1401
1451	1451	1451	1451	1451	1451	1451	1451
1501	1501	1501	1501	1501	1501	1501	1501
1551	1551	1551	1551	1551	1551	1551	1551
1601	1601	1601	1601	1601	1601	1601	1601
1651	1651	1651	1651	1651	1651	1651	1651
1701	1701	1701	1701	1701	1701	1701	1701
1751	1751	1751	1751	1751	1751	1751	1751
1801	1801	1801	1801	1801	1801	1801	1801
1851	1851	1851	1851	1851	1851	1851	1851
1901	1901	1901	1901	1901	1901	1901	1901
1951	1951	1951	1951	1951	1951	1951	1951
2001	2001	2001	2001	2001	2001	2001	2001
2051	2051	2051	2051	2051	2051	2051	2051
2101	2101	2101	2101	2101	2101	2101	2101
2151	2151	2151	2151	2151	2151	2151	2151
2201	2201	2201	2201	2201	2201	2201	2201
2251	2251	2251	2251	2251	2251	2251	2251
2301	2301	2301	2301	2301	2301	2301	2301

FIGURE 7A-7B

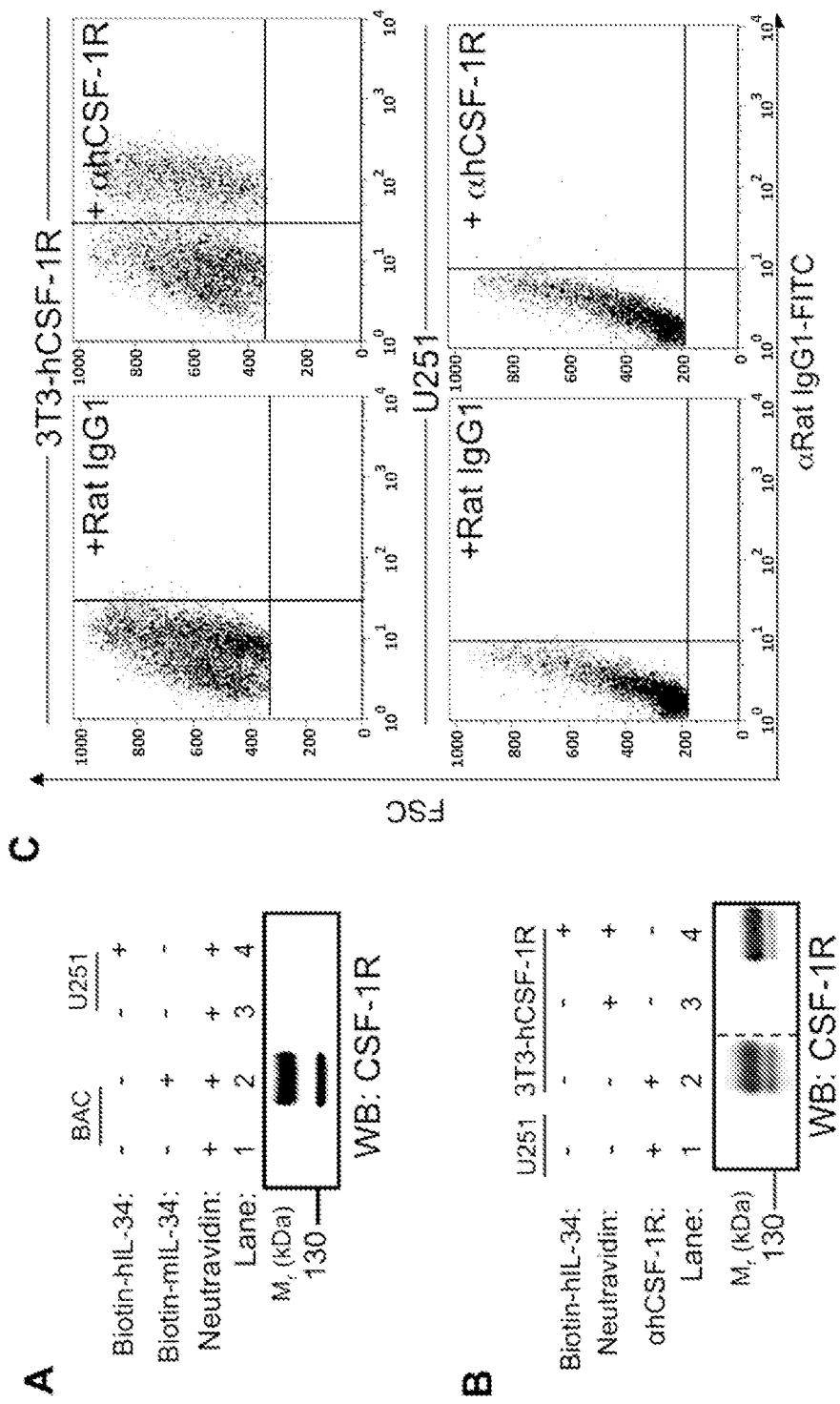


FIGURE 8A-8C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 14/38590

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 33/567, C12N 5/07, A61K 39/395 (2014.01)

CPC - A61K 38/00, A61K 2039/505

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - G01N 33/567, C12N 5/07, A61K 39/395 (2014.01)

CPC - A61K 38/00, A61K 2039/505

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/7.21, 435/335, 424/143.1, 424/155.1, 424/158.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST; PatBase; Google Scholar

search terms - tyrosine phosphatase, receptor, z, zeta, receptor-type protein-tyrosine phosphatase ?, PTP-?, PTPZ, PTPRZ, PTP, PTPR, HPTP, RPTPB, RPTPbeta, phosphacan, HTPZP\$, PTPRZ\$, HPTPzeta, PTPzeta, PTPRzeta, HPTPz, PTP18, Protein tyrosine...

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2013/011021 A1 (STEIDL et al.) 24 January 2013 (24.01.2013) abstract; p. 5, para 2 and 5, p. 11, last para, p. 12, para 1 and 3.	1-8
A	Nakamichi et al., 'Spleen serves as a reservoir of osteoclast precursors through vitamin D-induced IL-34 expression in osteopetrotic op/op mice' PNAS vol. 109 no. 25 10006-10011 (June 19, 2012) p. 10006, col 2, para 1; p. 10008, para 2.	1-8
A	Ulrike 'RNA interference targeting protein tyrosine phosphatase zeta/receptor-type protein tyrosine phosphatase beta suppresses glioblastoma growth in vitro and in vivo' Journal of Neurochemistry, 2006, 98, 1497-1506. abstract.	1-8
A	Maeda et al., 'A Receptor-like Protein-tyrosine Phosphatase PTP zeta/RPTPbeta Binds a Heparin-binding Growth Factor Midkine: INVOLVEMENT OF ARGININE 78 OF MIDKINE IN THE HIGH AFFINITY BINDING TO PTPz' J. Biol. Chem. 1999, 274:12474-12479. p. 12474, col 2, para 1.	1-8
A	US 2010/0130527 A1 (LEHRER et al.) 27 May 2010 (27.05.2010) para [0215], Table 4.	1-8
P,X	Nandi et al., 'Receptor-type Protein-tyrosine Phosphatase zeta Is a Functional Receptor for Interleukin-34' J. Biol. Chem. 2013, 288:21972-21986. originally published online June 6, 2013. abstract.	1-8

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

25 August 2014 (25.08.2014)

Date of mailing of the international search report

01 OCT 2014

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 14/38590

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

a. (means)

☐

on paper

☒

in electronic form

b. (time)

☐

in the international application as filed

☐

together with the international application in electronic form

☒

subsequently to this Authority for the purposes of search

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 14/38590

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 9-11
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.