PTPIP51 – ein multifunktionales Protein im Gehirn

INAUGURAL-DISSERTATION
zur Erlangung des Grades eines Doktors der Medizin
des Fachbereichs Medizin
der Justus-Liebig-Universität Gießen

vorgelegt von
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Gießen 2011

Aus dem Institut für Anatomie und Zellbiologie

des Fachbereichs Medizin der Justus- Liebig- Universität Gießen

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Tag der Disputation: 14.03.2012

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1. Liste der Publikationen der kumulativen Doktorarbeit

(1) Koch P.*, Viard M.*, Stenzinger A., Brobeil A., Tag C., Steger K., Wimmer M. (2009). Expression profile of PTPIP51 in mouse brain. J Comp Neurol. 517(6):892-905. (*The first two authors contributed equally to this work, M. Viard= co-first author)

(2) Viard M., Kamm M., Bobrich M., Brobeil A., Petri P. Wimmer M. (2011). PTPIP51 – a multifunctional protein in brain tissue (eingereicht in Journal of Comparative Neurology)

(3) Petri MK., Koch P., Stenzinger A, Kuchelmeister K., Nestler U., Paradowska A., Steger K, .Brobeil A., Viard M., Wimmer M. (2011). PTPIP51, a positive modulator of the MAPK/Erk pathway, is upregulated in glioblastoma and interacts with 14-3-3β and PTP1B *in situ*. Histol Histopathol. (in Druck)

2. Einleitung

2.1 Protein-Tyrosin- Phosphatase interagierendes Protein 51

Das Protein-Tyrosin-Phosphatase interagierende Protein 51 (PTPIP51) wurde vor ca. 10 Jahren als Interaktionspartner der Protein-Tyrosin-Phosphatase-1B (PTP1B) identifiziert (Porsche, 2001; Stenziger et al., 2009).

Beim Menschen ist das Gen des PTPIP51 auf Chromosom 15 (15q15.1.) lokalisiert. Das Gen umfasst 13 Exone, von denen nur die Exone 2-12 kodierend sind. Es hat inklusive der nicht kodierenden Teile eine Länge von 19.373 Basenpaaren. Das entsprechende Protein umfasst bei vollständiger Expression 470 Aminosäuren. Aufgrund seiner Gensequenz ist PTPIP51 auch unter dem Synonym FAM82C (= family with sequence similarity 82) beziehungsweise FAM82A2 bekannt (Brobeil et al. 2011a). Die Proteinsequenz ist evolutionär nur gering verändert (evolutionär konserviertes Protein). Das Protein der Maus weist eine 84% Übereinstimmung zur Proteinsequenz der humanen Form auf (Stenzinger et al., 2009).

N-terminal verfügt das vollständige Protein über eine „mitochondriale Target-Sequenz“, die eine Assoziation an Mitochondrien ermöglicht. Neben dieser verfügt das Protein über weitere spezifische Domänen, wie die „conserved region1 (aa43-48)“, die „conserved region2 (aa146-154)“, die Tetratricopeptidregion (aa303 - aa447) und Tyrosine in den Positionen 53, 158, 176 und 300, sowie Serine an den Stellen 44, 46, 50, 212, 225 (Brobeil et al., 2011a). Bobrich et al. (2011, eingereicht) zeigten, dass eine Phosphorylierung des Tyrosin 176 die Interaktion von PTPIP51 und 14-3-3beta mit Raf-1 und somit die Aktivität des MAPK-Signalweges erniedrigt, wogegen eine Interaktion mit PKA zur Phosphorylierung an Serin46 führt und damit die Aktivität des MAPK-Signalweges erhöht.

Interessanterweise zeigte das PTPIP51 ein sehr spezifisches Expressionsmuster in zahlreichen Geweben. So wurde nachgewiesen, dass es in der embryonalen Augenentwicklung (Märker et al., 2008), in den verschiedenen Entwicklungsstadien der Plazenta, hier in diversen Zelltypen (Stenzinger et al., 2009), im Skelettmuskel, hier assoziiert mit dem Fasertyp IIa (Barop et al., 2009), in der Epidermis (Pfeiffer,

2006), in Fettgewebe (Bobrich et al., 2011) und in verschiedenen Zelltypen des Blutes (Brobeil et al., 2010,2011b) exprimiert wird. Zudem konnten Koch und Mitarbeiter (2008) PTPIP51-Protein und –mRNA Expression in verschiedenen Hauttumoren nachweisen, welche das PTPIP51 auch im klinischen Sinne höchst interessant erscheinen lassen.

PTPIP51 wird in den Perikaryen und Axonen von Neuronen im Nervengewebe exprimiert (Stenzinger et al., 2005). Das zentrale Nervensystem (ZNS), als zentrales Integrations-, Koordinations- und Regulationsorgan des Organismus besteht aus mehreren spezialisierten Regionen. Da das PTPIP51 in verschiedenen Organen sehr spezifische Expressionsmuster aufweist, stellte sich die Frage, ob die Expression des Proteins im ZNS möglicherweise lokal auf spezifische Regionen begrenzt vorkommt. Zur Klärung dieser Frage erschien eine systematische und detaillierte Analyse der Expression von PTPIP51 im zentralen Nervensystem sinnvoll. Um eine genauere Vorstellung der PTPIP51 Expression im Gehirn zu gewinnen verwendeten wir als Modell Gehirne von Mäusen.

2.2 Expressionsprofil von PTPIP51 im Mäusegehirn

In Wirbeltieren besteht das ZNS aus Gehirn und Rückenmark. Das Gehirn kontrolliert jedes Organ, entweder über Innervierung via Axonen, Sekretion von Neurotransmittern oder neuroendokrinen Hormonen. Die Zentralisierung erlaubt eine schnelle und koordinierte Anpassung an Änderungen in der Umgebung.
Das Gehirn ist anatomisch aufgeteilt in 6 Hauptregionen: das Telencephalon, das Diencephalon (Thalamus, Hypothalamus, Epiphyse und Hypophyse) das Mesencephalon (Mittelgehirn), das Kleinhirn (Cerebellum), die Pons (Metencephalon) und die Medulla oblongata.
Jede Region hat spezifische Funktionen und enthält spezialisierte Zellen wie die Purkinje Zellen (Cerebellum), Pyramidalzellen (Hippocampus, Kortex, Amygdala), magno- und parvocelluläre neurosekretorische Zellen (Nucleus paraventrikularis und supraopticus).
Das Nervengewebe besteht prinzipiell aus zwei Zelltypen: Neuronen und Gliazellen. Gliazellen sind nicht-neuronale Zellen, die die Homöostase aufrechterhalten, Myelin bilden, und den Support, sowie den Schutz der Neuronen darstellen. PTPIP51 wird im gesunden Nervengewebe nur in Neuronen exprimiert, was über die Co-Expression mit PGP9.5 Protein gezeigt wurde. In den Gliazellen fand sich keine Expression.
Die genaue Analyse der Expression des PTPIP51 im Gehirn erfolgte an Serienschnitten von Gehirnen weiblicher Mäuse. Diese Studie zeigte folgende spezifische Hirnareale mit einer vermehrten PTPIP51 Expression:

- Den piriformen Kortex, ein Hirnareal, das zur sekundären olfaktorischen Struktur gehört, welche in Zusammenhang mit Emotionen steht und mit Hyposmie bei Morbus Parkinson in Verbindung gebracht wird (Soudry et al,. 2011; Baba et al., 2011).
- Den Nucleus accumbens, ein Hirnareal, welches zum mesolimbischen System gezählt wird. Er spielt eine wesentliche Rolle bei Lernprozessen, der Impulsivität (Basar K. et al., 2010) und ist klinisch von Bedeutung beim Krankheitsbild der Schizophrenie und der Sucht (John und Manchanda, 2011).

- Die Colliculi superiores, bei Tieren auch als Colliculi rostrales bekannt, einem Kernbereich des Gehirns, welcher für die Verschaltung von optischen Reflexen und für die Entstehung von Sakkaden zuständig ist (Isa und Yoshida, 2009).
- Die Pedunculi cerebelli inferiores (untere Kleinhirnstiele). Sie stellen die Faserverbindung zwischen Kleinhirn und Medulla oblongata dar. Durch sie laufen als afferente Fasern der Tractus vestibulocerebellaris, der Tractus spinocerebellaris posterior und der Tractus olivocerebellaris, sowie als einzige efferente Fasern der Tractus cerebellovestibularis. Sie sind wichtig für die Erhaltung des Gleichgewichtes und der posturalen Stabilität.
- Das Genu nervi facialis, auch als inneres Fazialisknie bekannt, ein Hirnareal im Bereich der Medulla oblongata, in dem der Nervus facialis in seinem Verlauf durch den Abducenskern beeinflusst wird.
- Den spinalen trigeminalen Trakt, ein kompakter Faserverbund zusammengesetzt aus primär sensorischen Fasern der portio major des Nervus trigeminus.
- Den Nucleus paraventricularis, ein Kerngebiet im Hypothalamus, das lateral vom dritten Ventrikel liegt. Die magnocellulären und parvocellulären neurosekretorischen Zellen dieses Kerngebiets produzieren das Hormon Oxytocin und in geringen Mengen Vasopressin (auch antidiruetisches Hormon (ADH) genannt) (Russell und Leng, 2000). Oxytocin ist ein Hormon, welches über die Kontraktion der myoepithelialen Zellen der Mamma eine Milchausstoßung der Mamillen bewirkt. Vasopressin wiederum ist bekannt für seine vasokonstriktorische und damit blutdrucksteigernde Wirkung (Aisenbrey et al., 1981), sowie für die Reabsorption von Wasser aus den Sammelrohren der Niere. Beim Ausfall des Hormons kommt es zum Diabetes insipidus. Darüber hinaus enthält dieses Kerngebiet auch kleine Kerne, die das Corticotropin-releasing Hormon (CRH) sezernieren. Dieses wird primär über die Eminentia mediana an den primären hypophysären Pfortaderkreislauf abgegeben und sorgt auf diese Weise für die Ausschüttung von dem adrenokorticotropem Hormon und die Stimulierung des Sympathikus. Die Axone des Nucleus paraventricularis bilden zusammen mit denen des Nucleus supraopticus den Tractus hypothalamohypophysialis. Auch die

Nervenzellen des Nucleus supraopticus sind neurosekretorisch tätig und produzieren wie der Nucleus paraventrikularis Vasopressin und Oxytocin. Beide Kerngebiete sowie ihre axonalen Verbindungen zur Hypophyse weisen eine starke PTPIP51 Expression auf. Diese Hormone werden an ihre Transportproteine Neurophysin I und II gebunden (Fotheringham, 1991; Trembleau, 1994) und als Prohormone in Vesikel eingeschlossen um dann über die axonalen Nervenzellfortsätze zur Neurohypophyse (Hypophysenhinterlappen) transportiert zu werden (Brownstein et al., 1980; Dreifuss, 1975). In unserer Arbeit konnten wir eine Kolokalisation von PTPIP51 und Neurophysin zeigen und damit eine wahrscheinliche Beteiligung des PTPIP51 am axonalen Vesikeltransport nachweisen. Innerhalb der Granula wird das Hormon während des Transports vom Prohormon-Anteil abgespalten (Brownstein et al. 1980). Außer der sofortigen neurohypophysären Ausschüttung der Hormone werden auch einige Granula in den Nervenendigungen gelagert. Ihr Inhalt wird dann bei Depolarisation der Nervenendigungen durch Exozytose freigesetzt, wo sie als Neurotransmitter oder Neuromodulatoren fungieren (Buijs et al. 1982).

- Die Neurohypophyse (Hypophysenhinterlappen), sie ist ein Teil der Hypophyse, welche die von den Hypothalamuskernen produzierten Hormone Vasopressin und Oxytocin über den Tractus hypothalamohypophysialis erhält, speichert und bei Bedarf an die Blutbahn abgibt.
- Den Hippocampus, der im Temporallappen liegt und eine zentrale Schaltstation des limbischen Systems ist. Zum Hippocampus gehören mehrere Strukturen, unter anderem der Gyrus dentatus, das Ammonshorn und das Subiculum. Der eigentliche Hippocampus ist, als archicorticale Struktur, histologisch in drei Schichten aufgebaut. Die Nervenzellkörper liegen in der Pyramidenzellschicht (Stratum pyramidale). Die Hauptzellen sind hier glutamaterge Pyramidenzellen, die Dendriten radial sowohl nach innen als auch nach außen schicken. Als Eingangsschichten lagern sich nach außen an die Pyramidenzellschicht das breite Stratum radiatum und das schmalere Stratum lacunosum-moleculare an, nach innen das Stratum oriens, welches die Zellkörper der hemmenden Korbzellen enthält. Eine starke PTPIP51 Expression zeigten die Pyramidalzellen. In seiner

tangentialen Richtung wird der Hippocampus unterteilt in die CA1 bis CA4-Regionen. Im Hippocampus fließen Informationen verschiedener sensorischer Systeme zusammen, die verarbeitet und von dort zum Cortex zurückgesandt werden. Damit ist er enorm wichtig für die Gedächtniskonsolidierung, also die Überführung von Gedächtnisinhalten aus dem Kurzzeit- in das Langzeitgedächtnis. Im adulten Gehirn kann der Hippocampus neue Verbindungen zwischen bestehenden Nervenzellen bilden. Diese Neubildungen sind assoziiert an den Erwerb neuer Gedächtnisinhalte (synaptische Plastizität). Schädigung oder Abbauprozesse im Bereich des Hippocampus werden mit der Entstehung der Demenzerkrankung in Zusammenhang gebracht (Varela-Nallar L et al., 2010; Dhikav V et al., 2011). Darüber hinaus ist der Hippocampus in die Entstehung von Epilepsieerkrankungen (Oliveira et al., 2011), Stress, Emotionen (Loureiro M et al., 2011) und Depression (den Heijer T et al. 2011), sowie bei räumlicher Orientierung (Pereira AG et al, 2011) involviert.

- Das Kleinhirn, es hat zwei Hauptfunktionen für den menschlichen Organismus: einerseits die Koordination willkürlicher Bewegungen, andererseits die Kontrolle des Gleichgewichtes (Mauk et al 2000). Eine Beteiligung bei kognitiven und emotionalen Vorgängen wird ebenfalls diskutiert (Schmahmann und Sherman, 1998). Der zerebelläre Kortex besteht aus drei Schichten, der äußeren Molekularschicht, der Purkinjezell-Schicht und der Granula-Schicht. Die zerebellären Purkinjezellen spielen eine grundlegende Rolle in der motorischen Koordination und bei motorischen Lernvorgängen. Um diese Funktionen ausüben zu können gibt es zwei Arten von axonalen Fasern die zum Kleinhirnkortex projizieren. Zum einen die Kletterfasern, welche vom unteren Olivenkern ausstrahlen und Fehlersignale zur Feineinstellung (Präzision) des Bewegungsprogrammes übermitteln und zum anderen die Moosfasern. Die T-förmigen Axone der Moosfasern, auch Parallelfasern genannt, übertragen sensorische und motorische Informationen, die aus dem „pontocerebellar and spinocerebellar mossy fiber pathway“ (Watanabe, 2008) kommen.

Die stärkste PTPIP51-Immunoreaktion wurde in Purkinjezellen und ihren Dendriten beobachtet, wobei die Purkinjezellen durch ihre Calbindin-Expression identifiziert wurden. Yang und Goldstein (1998) beschrieben, dass Purkinjezellen eine starke Expression der „motor kinesein family member 3C (KIF3C)“ zeigen, insbesondere in Zellkörpern und Dendriten. KIF3C ist auch in retinalen Ganglienzellen exprimiert, welche ebenfalls PTPIP51-positiv sind (Märker et al., 2008).

Betrachtet man die oben genannten PTPIP51-positiven Regionen, so zeigt sich ein heterogenes Verteilungsmuster im ZNS. Die PTPIP51-positiven Hirnareale weisen unterschiedliche Funktionen auf, was einer einheitlichen Aufgabe/Wirkung des Proteins widerspricht.
Weitere funktionelle Studien zeigten eine partielle Kolokalisation von PTP1B und PTPIP51 in Neuronen. PTP1B wiederum ist an der Regulierung von axonalem Wachstum beteiligt (Pathre et al., 2001). Dies könnte ebenfalls für das PTPIP51 als bekanntem Interaktionspartner der PTP1B gelten (Porsche, 2001; Stenzinger et al., 2005). Ein weiterer Hinweis auf die mögliche Beteiligung von PTPIP51 an der Regulation des axonalen Wachstums hängt mit der Wirkung des „Ciliary Neurotrophic Factor” (CNTF) zusammen. Roger et al. (2007) zeigten eine CNTF-regulierte biphasische Expression von PTPIP51 in der Entwicklungsphase von Retinazellen. Die Neuronen in den PTPIP51-positiven Arealen wie zum Beispiel der piriforme Kortex, der Hippocampus, der Nucleus paraventrikularis und supraopticus, sowie das Kleinhirn exprimieren CNTF-Rezeptoren (Lee et al., 1997). CNTF spielt eine wichtige Rolle in der Proliferation, der Differenzierung und dem Überleben von Neuronen, sowie für das axonale Wachstum (Fuhrmann et al., 2003).
Das Immunoblotting von Kortex, Hippocampus und Kleinhirn mit der PTPIP51 zeigte drei verschiedene Isoformen mit Molekulargewichten von 30kDa, 34kDa und 52kDa.

Die vorhergehende Arbeit „PTPIP51 in brain tissue“ zeigte ein spezifisches Verteilungsmuster der PTPIP51-Expression im Mausgehirn. Basierend auf den oben genannten Hirnregionen und den möglichen bekannten Funktionen des PTPIP51 wie Proliferation, Differenzierung, Migration und Vesikeltransport (Koch et. al., 2009) stellt sich die Frage: Wie kann ein Protein an so vielen grundlegend unterschiedlichen Prozessen teilhaben? Eine mögliche Antwort auf diese Frage ist die Expression von Isoformen.

2.3 PTPIP51 im Gehirn – ein multifunktionelles Protein?

Im Immunoblot von Hirngewebe der Maus wurden im Cerebellum, Kortex und Hippocampus PTPIP51-Formen mit Molekulargewichten von 30, 34 und 52 kDa identifiziert. Dies impliziert die Existenz von Isoformen.

Die Initialisierung der Translation von mRNA benötigt ein AUG-Triplet mit spezifischer Umgebungssequenz (GCCRCCCaugG-R steht für Purinbasen) (Kozak 2005). Untersucht man nun den „coding open reading frame (ORF)“ von PTPIP51 nach intern liegenden Startcodons, so zeigen sich 6 AUG-Triplets (Brobeil et al., 2011a).

Weiterhin lassen sich mögliche Isoformen durch alternatives Spleißen erklären. Hierbei werden die Introns von den Spleißosomen aus der Prä-mRNA herausgeschnitten und die Exons zur reifen mRNA zusammengefügt. Diese dient dann als Matrize der Synthese des Proteins. Beim Zusammenfügen der Exone kann es zu Umorientierungen, Umlagerungen oder auch zum Auslassen von Exonen kommen, so dass unterschiedliche mRNAs entstehen und sich die Zahl der möglichen Proteine erhöht. Alternatives Spleißen führt dazu, dass viele Proteine in zahllosen Varianten vorkommen (Nilsen und Graveley, 2010). Ein Beispiel für die Entstehung von Isoformen durch alternatives Spleißen ist die für die Kontakte von Nervenzellen wichtige Proteinfamilie der Neurexine (Wei et al., 2010).

Die multiplen Isoformen in den verschiedenen Hirnregionen können mögliche unterschiedliche Funktionen in diesen Bereichen ausüben.

Zur Erfassung dieser Problematik erfolgten detaillierte Untersuchungen an Hirnregionen mit hoher PTPIP51-Expression, dem Kleinhirn und dem Hippocampus. Hierzu wurden peptidspezifische Antikörper gegen die N-terminale Region und gegen die C-terminale Region des PTPIP51 eingesetzt (Brobeil et al., 2011a).

Aminosäuresequenz des PTPIP51

MSRLGALGGARAGLGLLLGTAAGLGFLCLLYSQRWKRTQRHGR
SQSLPNSLDYTQTSDPGRHVMLLRAVPGGAGDASVLPSLPREG
QEKVLDRLDFVLTSLVALRREVEELRSSLRGLAGEIVGEVRCHM
EENQRVARRRRFPFVRERSDSTGSSSVYFTASSGATFTDAESE
GGYTTANAESDNERDSDKESEDGEDEVSCETVKMGRKDSLDL
EEEAASGASSALEAGGSSGLEDVLPLLQQADELHRGDEQGKR
EGFQLLLNNKLVYGSRQDFLWRLARAYSDMCELTEEVSEKKSY
ALDGKEEAEAALEKGDESADCHLWYAVLCGQLAEHESIQRRIQS
GFSFKEHVDKAIALQPENPMAHFLLGRWCYQVSHLSWLEKKTA
TALLESPLSATVEDALQSFLKAEELQPGFSKAGRVYISKCYRELG
KNSEARWWMKLALELPDVTKEDLA IQKDLEELEVILRD

Abbildung 1 Aminosäuresequenz des PTPIP51 und Antigensequenzen gegen den die Antikörper gerichtet sind.

Schema der Aminosäuresequenz von PTPIP51.
Antigensequenz gegen den der polyklonale PTPIP51(131-470)-Antikörper gerichtet ist (**rot**).
Antigensequenz gegen den der PTPIP51(2-14)-Antikörper gerichtet ist (N-Terminus, **grün**).
Antigensequenz gegen den der PTPIP51(446-470)-Antikörper gerichtet ist (C- Terminus, **blau** hinterlegt).

Bereits in der Westernblotuntersuchung des Kleinhirns, des Hippocampus und des Kortex zeigten sich mittels der peptidspezifischen Antikörper unterschiedliche Molekulargewichte. Korrespondierend zu diesen Ergebnissen konnten bereits einige andere Studien variierende Isoformmuster nachweisen wie zum Beispiel in der Muskulatur (52Da; Barop et al., 2009), in humanen HL-60 Zelllinien, für die akute promyeloische Leukämie (52, 45 and 38 kDa, Brobeil et al., 2011b) und in embryonalem Gewebe (30 kDa; Brobeil et. al., 2011a).

Zur genauen Analyse der morphologischen Verteilung und Funktion dieser möglichen PTPIP51-Varianten im ZNS, führten wir weitere Untersuchungen auf zellulärer und subzellulärer Ebene durch. Auch hier zeigte sich eine heterogene Verteilung der PTPIP51-Formen in den unterschiedlichen Hirnarealen.

In Purkinjezellen des Kleinhirns wie auch in den Pyramidalzellen des Hippocampus war eine positive Reaktion von PTPIP51 (aa131-470), des N-terminal spezifischen

Antikörpers im Soma wie auch im Dendrit zu sehen. Der C-terminal spezifische Antikörper hingegen wies nur eine Lokalisation im Soma nach.

Subzellulär waren beide PTPIP51-Formen mit dem Golgiapparat assoziiert. Der Golgiapparat ist an der Morphogenese der Dendriten und Axone beteiligt (Rosso et al., 2004; Tanabe et al., 2010). Zusätzlich zeigte die C-terminale Form eine Assoziation mit dem endoplasmatischen Retikulum. Die PTP1B, ein Interaktionspartner des PTPIP51, ist ebenfalls an das endoplasmtische Retikulum (ER) gebunden (Pathre et al., 2001). Mittels „Duolink proximity ligation assay" konnten wir die direkte Interaktion beider Proteine (PTPIP51 und PTP1B) in Purkinjezellen nachweisen. Bereits Pathre et al. (2001) sowie Fuentes und Arregui (2009) wiesen eine Beteiligung von PTP1B an Wachstum und Elongation der Axone nach. Außerdem spielt PTP1B eine wichtige Rolle im „Nerve Growth factor (NGF)" Signalweg (Shibata et al., 2008). Dieser Signalweg reguliert wiederum den Neuritenauswuchs, bewahrt sie vor Apoptose und nimmt somit eine funktionelle Rolle bei Gedächtnisprozessen ein (Chao et al., 2006; Shimoke et al., 2011). Analog zur intrazellulären Lokalisation der C- terminalen Form des PTPIP51 findet sich NGF ebenfalls am ER und Golgiapparat (Blöchl et al., 1996). Die Bindung von NGF an den Neutrophin-Rezeptor p75 (NTR) erhöht die enzymatische Aktivität von PTP1B. Neben den bereits erwähnten Funktionen ist PTP1B wichtig für das Überleben der Neurone. Es schützt Neurone vor dem Angriff von Amyloid (Chacon et al., 2011). Dieser Mechanismus ist noch nicht aufgeklärt (Chacon et al., 2011), könnte aber durch PTPIP51 vermittelt werden.

PTPIP51 interagiert mit 14-3-3beta in den Purkinjezellen des Kleinhirns. Die 14-3-3-Protein-Superfamilie umfasst 7 Isoformen, welche vermutlich im Kleinhirn an der neuronalen Proliferation, Migration und Differenzierung beteiligt sind (Umahara et al., 2009). Wie bereits von Yu et al. (2008) berichtet, interagiert PTPIP51 über 14-3-3beta mit Raf-1 und moduliert darüber die MAPK-Kaskade. Bemerkenswerterweise ist der NGF- Signalweg mit dem MAPK-Signalweg eng verlinkt (Xing et al., 1998). NGFs sind wichtig für Gedächtnisprozesse, welche insbesondere bei der Alzheimererkrankung negativ beeinflusst sind (Chao et al., 2006; Aggleton et al., 2010). PTPIP51 wird besonderes stark in der Hippocampusregion exprimiert. Diese Region ist ebenfalls an der Gedächtnisbildung beteiligt. Dies legt eine mögliche

Beteiligung des PTPIP51 über die Beeinflussung des NGF–Signalweges bei der Gedächtnisbildung nahe.

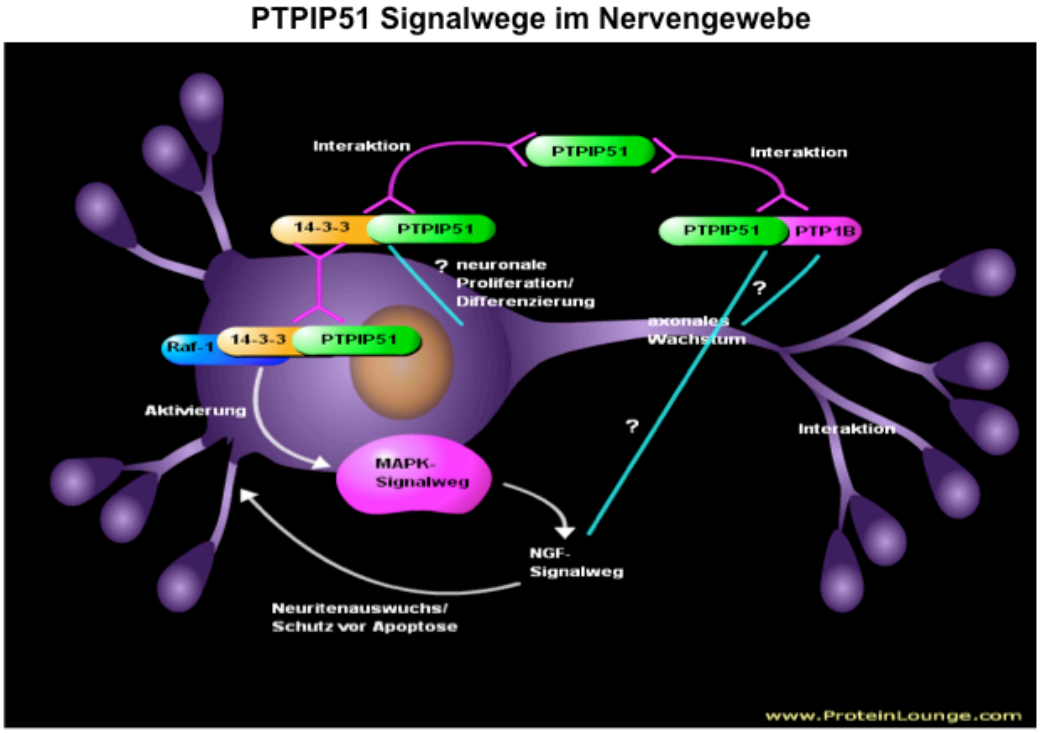

Abbildung 2 PTPIP51 Signalwege im Nervengewebe

Schema eines Neurons mit den entsprechenden Signalwegen.
Interaktion= lila Pfeil. Hypothese= blaue Linie. Aktivierung= weißer Pfeil.
(Abbildung erstellt mit dem Pathwaybuilder online tool. (www.proteinlounge.com)).

Die bereits in der vorhergehenden Studie aufgestellte Hypothese einer möglichen Beteiligung von PTPIP51 am axonalen Transport von Hormonen wurde über den Nachweis einer Interaktion von PTPIP51 und Neurphysin II, einem Transportprotein in den Neuronen des Nucleus paraventrikularis, verifiziert.

PTPIP51- Beteiligung am axonalen Transport

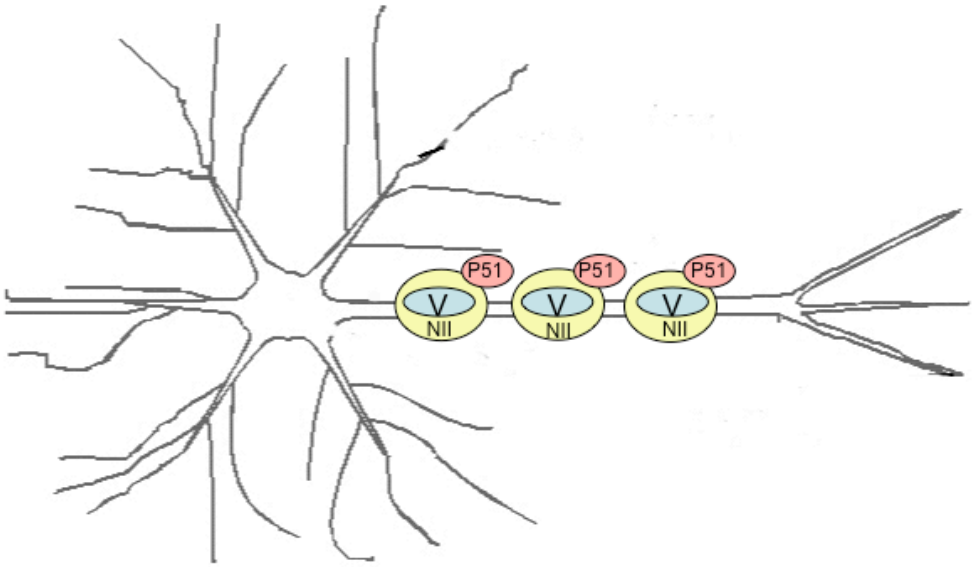

Abbildung 3 PTPIP51 und axonaler Transport von Vasopressin.

Schema eines Neurons mit Axon aus dem Bereich des Nucleus paraventrikularis/supraoptikus. V= Vasopressin. NII= Neurophysin II. P51= PTPIP51.

PTPIP51 als mitochondrial assoziiertes Protein induziert bei Überexpression Apoptose in HEK239T and HeLa Zelllinien (Lv et al., 2006). Dies ließ sich im Gehirn nicht bestätigen. Im Gehirn waren weder PTPIP51 (aa131-470), noch die peptidspezifischen PTPIP51-Antikörper an den Mitochondrien assoziiert.

Im Gehirn interagiert das PTPIP51 mit 14-3-3beta. Die 14-3-3 Proteine stehen im Verdacht über die Hemmung der Apoptose in Zusammenhang mit der Pathogenese von Karzinomen zu stehen. Dies basiert außerdem auch auf der Beobachtung einer Hochregulation der 14-3-3-Expression in einer Vielzahl von primären, humanen Nerventumoren wie zum Beispiel Astrozytomen, anaplastischen Astrozytomen, Glioblastomen. Die ausgeprägteste 14-3-3-Expression fand sich in Glioblastomen (Cao et. al., 2006). Dies und die von Koch et al. (2008, 2009a) gezeigte Beteiligung von PTPIP51 an der Pathogenese von Karzinomen bildete die Basis für eine Untersuchung des PTPIP51 im Glioblastom.

2.4 PTPIP51 in Glioblastomen

Das Glioblastoma multiforme (GBM) ist der häufigste und bösartigste primäre Gehirntumor in der westlichen Welt (Wang et al., 2010; Parsons et al., 2008). Im Verlauf der letzten Jahrzehnte wurde ein starker Anstieg in der Inzidenz verzeichnet. 18.820 neu aufgetretene Fälle von primären Gehirntumoren werden jährlich in den USA diagnostiziert, davon entfallen 60% auf Gliome, wobei es sich bei 30-40% um GBMs handelt (Khan et al., 2009). Bei GBM-Patienten beträgt die durchschnittliche Überlebenszeit trotz signifikanter technischer und medikamentöser Fortschritte im therapeutischen Bereich nach wie vor etwa nur ein Jahr (McLendon et al., 2007).

In humanen GBMs korrelieren die beiden Signalmoleküle 14-3-3beta und gamma mit dem Grad der Malignität (Yang et al., 2009). Aufgrund ihrer spezifischen Phosphoserin/Phosphothreonin Bindungsstellen besitzen die 14-3-3-Proteine die Fähigkeit mit vielen verschiedenen Proteinen zu interagieren. Hierzu gehört auch das PTPIP51. Im Mäusegehirn wurde diese Interaktion in situ nachgewiesen. Zwei weitere unabhängige Studien (Jin et al., 2004; Ewing et al. 2007) beschrieben PTPIP51 als Partner des 14-3-3beta, wobei dadurch die Interaktion mit dem Raf-1 vermittelt wird (Yu et al., 2008).

Die Expression von 14-3-3beta wird als Gradmesser für die Malignität von Glioblastomen gewertet. Deshalb ist es von höchstem Interesse zu überprüfen, ob eine erhöhte 14-3-3-Expression gleichzeitig mit einer erhöhten PTPIP51 Expression in Glioblastomen einhergeht. Zur Klärung dieser Frage wurden Proben von 20 Glioblastomen sowohl auf transkriptioneller, als auch auf translationeller Ebene mit Hilfe von Immunhistochemie, in situ Hybridisierung und RT-PCR untersucht.

Dabei wurden Grad 2-Astrozytome und GBM mittels RT-PCR analysiert, um eine potentielle Korrelation zwischen PTPIP51-Expression und Malignitätsgrad zu überprüfen.

Humane Glioblastomzellen weisen eine Interaktion zwischen PTPIP51 und 14-3-3 beta, sowie PTP1B auf. Beim Vergleich von GBM (WHO Grad IV-glioma) und niedriggradigen Astrozytomen (Grad II Glioma) zeigte sich eine zum Tumorgrad korrelierte Zunahme der Expression von PTPIP51 und 14-3-3beta in den höhergradigen Hirntumoren. 14-3-3beta vermittelt die Interaktion von PTPIP51 und

Raf-1, wodurch die Aktivität des MAPK-Signalweges moduliert wird (Lv et al., 2006; Stenzinger et al., 2009; Yu et al., 2008). Diese Hochregulation von 14-3-3-Proteinen ist mit einer reduzierten Apoptose-Kapazität assoziiert, da die Antagonisierung oder Ausschaltung der 14-3-3-Expression verstärkte Apoptose in kultivierten Gliomzellen bewirkt (Cao et al., 2010). Da der MAPK-Signalweg unter anderem Zellmigration kontrolliert, könnte eine Aktivitätserhöhung für Rezidive und die schlechte Prognose von GBM mitverantwortlich sein. Diffus auswandernde Tumorzellen sind in der Lage gesundes Hirngewebe zu infiltrieren, entgehen so der chirurgischen Extirpation und bilden neue Tumorherde. In Anbetracht dieser Fakten könnte die Interaktion von PTPIP51 und 14-3-3beta eine Rolle in der Migration und Proliferation von GBM-Tumorzellen spielen.

In den Endothelzellen von GBM typischen „glomerulumartigen Gefäßen“ ist eine deutliche Kolokalisation und Interaktion von PTPIP51 und 14-3-3beta zu beobachten. Diese abnorme Vaskularisierung und endotheliale Hyperplasie ist eines der Charakteristika und einer der Mechanismen für die Tumor-Angiogenese der GBMs (Wang et al., 2010). Die Endothelzellen von Tumoren exprimieren „Epidermal Growth Factor Receptoren (EGFR)“ (Dhara et al., 2006). Die untersuchten Proben wiesen eine partielle Kolokalisation von PTPIP51 und dem EGFR auf. EGFR kommt vermutlich eine zentrale Rolle in der Migration und der lokalen Infiltration von „brain tumor-initiating cells (BTICs)“ und somit in der Entstehung, der Therapieresistenz und Entstehung von Krankheitsrezidiven bei Hirntumoren zu (Mimeault M und Batra SK, 2011). Ausgehend von unseren Ergebnissen könnte PTPIP51 mittels 14-3-3beta vermittelter Interaktion mit Raf-1 und dem EGFR die Ras/Raf/MEK/ERK-Signalkaskade aktivieren. Diese Signalkaskade führt schlussendlich auf zellulärer Ebene zur einer Dysfunktion des Zellzyklus und einer erhöhten proliferativen Aktivität in GBM (Halatsch et al., 2004). Im Vergleich zum sekundären GBM, welches sich durch Tumorprogression aus niedriggradigeren Gliomen entwickelt, ist die EGFR-Genexpression in primären Gliomen etwa fünffach höher, was zu einer Überexpression in 40% der GBMs führt (Karpel-Massler et al., 2009). Neben dieser EGFR-Überexpression exprimieren 20% der Gliompatienten die Mutante EGFRvIII (Jutten et al., 2009). Diese Mutante, die auch in einigen anderen Epitheltumoren nachgewiesen wurde, ist permanent aktiviert (Yoshimoto et al., 2008; Hama et al., 2009). Epitheliale Tumoren exprimieren ebenfalls hohe Konzentration von PTPIP51 (Koch et al., 2008; 2009a).

In Gliomzellen konnte eine direkte Interaktion von PTPIP51 und PTP1B gezeigt werden. Die PTP1B ist ebenfalls in der Lage die MAPK-Kaskade über c-Src und Ras zu aktivieren (Stenzinger et al., 2009; Dubé et al., 2004; Dubé und Tremblay, 2004; Tonks und Muthuswamy, 2007; Zhao et al., 2008). Reichardt et al. (2003) konnten keine DNA-Amplifikation von PTP1B in humanem GBM nachweisen. Im Gegensatz dazu zeigt unsere Studie eine Erhöhung der PTP1B Expression. Dies wird auch bestätigt von Akasaki et al. (2006), die eine Überexpression von PTP1B in Gliomen zeigten. PTP1B partizipiert in der generellen Onkogenese durch Tyrosin-Dephosphorylierung von zentralen Signalmolekülen oder durch Hochregulation von zwei wachstumsfördernden Signalwegen (Arias-Romero et al., 2009). Im menschlichen Brustdrüsengewebe verknüpft PTP1B die insbesondere für die Onkogenese wichtige Rezeptor-Tyrosin-Kinase ErbB2 mit zellulären Signalwegen, welche wiederum atypische Zellteilung und Zellüberleben über Aktivierung von c-Src und den Übergang in einen Src-abhängigen Phänotyp fördern. Zusätzlich deaktiviert PTP1B den Ras/MAPK Signalweg Inhibitor (Tonks und Muthuswamy, 2007).

Die hier untersuchten Proben wiesen eine Kolokalisation von PTPIP51 und c-Src im Glioblastom auf. c-Src wiederum vermittelt die Phosphorylierung von EGFR und fördert dadurch ein fortschreitendes Tumorwachstum (Tice et al., 1999).

2.5 Zusammenfassung

Zusammenfassend lässt sich festhalten, dass PTPIP51 in mehreren spezifischen Gehirnarealen wie dem Kleinhirn, dem Hippocampus, dem Nucleus paraventrikularis, dem Nucleus supraopticus, dem Nucleus accumbens, dem piriformen Kortex, den Colliculi superiores, den Pedunculli cerebelli inferiores, dem Genu nervi facialis, dem spinalen Trigeminaltrakt und in der Neurohypophyse exprimiert wird. Diese lokal differente Expression ermöglicht die Beteiligung an einer Vielzahl von funktionellen Prozessen wie zum Beispiel optischen Reflexen, Lernprozessen, Gedächtnisbildung, Emotionen, Gleichgewicht, Orientierung, Hyposmie bei Morbus Parkinson. Schizophrenie und Depression.

Auf zellulärer und subzellulärer Ebene zeigt sich ebenfalls ein sehr heterogenes Expressionsmuster des PTPIP51. Das PTPIP51 interagiert mit der PTP1B, dem 14-3-3beta und dem Neurophysin II. Durch diese Interaktionen greift PTPIP51 in zahlreiche essentielle Signalwege wie den MAPK-, den CNTF- und den NGF-Signalweg ein. Deshalb ist eine der Hauptfunktionen des PTPIP51 im Nervengewebe wohl die Regulation von Proliferation, Differenzierung, Migration und des Vesikeltransports. Neben diesen Funktionen kann dem PTPIP51 bedingt durch seine Involvierung in diesen Signalwegen eine wichtige Rolle in der Pathogenese von Erkrankungen wie zum Beispiel dem Glioblastom und anderen Hirntumoren, der Alzheimer-Demenz und dem Morbus Parkinson zukommen. Dies macht das PTPIP51 nicht nur für die Grundlagenforschung, sondern auch für die Klinik interessant.

Die heterogene PTPIP51-Expression und dessen Funktionen lassen sich mit der Existenz möglicher Isoformen des PTPIP51 erklären und wird durch die unterschiedliche Verteilung mit peptidspezifischen Antikörpern nachgewiesener Formen gezeigt.

Auf Grund seiner weit gefächerten Funktionen im ZNS kann man das PTPIP51 auch als ein „multifunktionales Protein“ des Nervengewebes bezeichnen.

Diese Arbeiten stellen die Basis für weitere interessante Studien des PTPIP51 im ZNS dar.

So laufen bereits weitere Versuche mit kultivierten Glioblastomzellen, die die Wirkung von Chemotherapeutika (PD98059, Gefitinib, Cetuximab) auf die PTPIP51-Expression analysieren. Außerdem wird untersucht, ob die Expressionsrate des PTPIP51 in Ependymomen als möglicher Marker für verschiedene Stadien etabliert werden kann. Die Rolle von PTPIP51 bei der erhöhten Ausschüttung von Oxytocin in weiblichen Ammenratten soll weiteren Aufschluss über die Funktion im axonalen Transport geben.

Interessanterweise wurde PTP1B auch als Regulator des VCAM-1-Signalweges identifiziert. Es ist bekannt, dass die Interaktion von *α*-4 Integrin und VCAM-1 zur Ansammlung von T-Zellen an der Bluthirnschranke und damit zur perivaskulären Infiltration von Lymphozyten führt. Dadurch bedingt kommt es zum Beginn von Erkrankungen des ZNS (Vajkoczy und Menger, 2004). Antikörper gegen VCAM-1 und *α*-4 Integrin werden bereits in der medikamentösen Therapie von multipler Sklerose und anderen inflammatorischen Erkrankungen genutzt (Deem et al., 2007). Colucci et al. (2004) wiesen eine Korrelation zwischen dem klinischen Outcome von Patienten mit mulitpler Sklerose und der Konzentration von 14-3-3 im Liquor nach, so dass aktuell eine Studie begonnen wurde, in der die Expression von PTPIP51 und dessen Interaktionspartner 14-3-3 im Maus EAE-Modell in Abhängigkeit des MS-Stadiums untersucht wird.

Zusammenfassend kann man sagen, dass diese Arbeit eine wichtige Grundlage für viele weitere sehr interessante Projekte bezüglich des PTPIP51 im ZNS darstellt. Die kommenden Projekte werden nun das PTPIP51 in Relation zu Erkrankungen und mögliche Nutzung für Diagnose und Therapie stellen.

2.6 Summary

To sum up PTPIP51 is expressed in several specific brain areas: the cerebellum, the hippocampus, the paraventricular nucleus, the supraoptic nucleus, the nucleus accumbens, the piriformal cortex, the superior colliculus, the inferior cerebellar peduncle, the genu of the facial nerve, the spinal trigeminal tract, the neurohypophysis. This locally restricted different expression allows PTPIP51 to take part in a multitude of functions e.g. optical reflexes, learning, forming of memories, emotions, equilibrium, orientation, hyposmy in morbus Parkinson, schizophrenia and depression.

On cellular and sub- cellular level PTPIP51 displayed a heterogeneous expression. In brain PTPIP51 interacts with PTP1B,14-3-3 beta and neurophysin II. By these interactions PTPIP51 is involved in many essential pathways such as MAPK, CNTF and NGF. Therefore one of the main functions of PTPIP51 in nervous tissue seems to be the regulation of proliferation, differentiation, migration and vesicle transport. Besides these functions PTPIP51 plays a central role in the genesis of glioblastoma, Alzheimer disease and morbus Parkinson. This stresses the importance of basic and clinical research on PTPIP51.

The heterogeneous expression and function of PTPIP51 can be explained by possible isoforms. This subject was investigated by peptide specific antibodies.

Based on the broad spectrum of functions in the CNS PTPIP51 is a real “multifunctional protein”.

The presented manuscript is the basis for many interesting studies of PTPIP51 in CNS.

A current study with cultured glioblastoma cells investigates the effects of chemotherapeutics (PD98059, Gefitinib, Cetuximab) on the expression of PTPIP51. Another study tests whether the grade of PTPIP51- expression in ependymoma is a possible marker for its staging. The role of PTPIP51 in axonal transport is further investigated in nurse rats using their increased productions of oxytocin as a trigger.

Interestingly, PTP1B is known as a regulator of the VCAM-1-pathway. The interaction of *α*-4 integrin and VCAM-1 results in the accumulation of T-cells at the blood-brain-

barrier and in a perivascular infiltration of lymphocytes. This induces diseases of the CNS (Vjakoczy and Menger, 2004). Therefore, in medical therapy of multiple sclerosis and other inflammatory diseases antibodies against *α*-4 integrin and VCAM-1 are applied (Deem et al., 2007). According to Colucci and coworkers (2004) the clinical outcome of these patients is related to the concentration of 14-3-3 in the spinal fluid. Just now a study is done investigating the expression of PTPIP51 and its interaction partner 14-3-3 in relation to the stage of MS in the EAE mouse model.

In summary this PHD thesis is an important basis for many consecutive studies of PTPIP51 in CNS in relation to diseases and possibly for the use in diagnosis and therapy.

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4. Weitere Publikationen und Poster

A) weitere Publikationen

Koch P., Stenzinger A., Viard M., Märker D., Mayser P., Nilles M., Schreiner D., Steger K., Wimmer M. (2008). The novel protein PTPIP51 is expressed in human keratinocyte carcinomas and their surrounding stroma. J Cell Mol Med.12 (5B):2083-95.

Bobrich M., Schwabe S., Viard M., Kamm M., Brobeil A., Mooren FC., Krüger K., Tag C., Wimmer M. (2011). PTPIP51 – Connecting lipolysis and lipogenesis in adipose tissue (eingereicht).

B) Poster

Viard M. Localization of protein tyrosine phosphatase interacting protein 51 (PTPIP51) in mouse brain.(49th Symposium of the Society for Histochemistry Freiburg im Breisgau, Germany, 2007)

Koch P., Stenzinger A., Viard M., Mayser P., Wimmer M. Expression of the novel protein PTPIP51 in human keratinocyte carcinomas and their surrounding stroma. (102nd Annual Meeting of the Anatomische Gesellschaft, Giessen 2007)

Koch P., Stenzinger A., Viard M., Mayser P., Wimmer M. Expression of the novel protein PTPIP51 in human keratinocyte carcinomas and their surrounding stroma. (32nd FEBS Congress, Molecular Machines, Vienna 2007)

5. Danksagung

An erster Stelle möchte ich mich bei Frau Prof. Dr. Wimmer für die Überlassung des Themas, die Möglichkeit zu Publizieren und die herzliche Aufnahme in ihr Team sowie für die immerwährende fachliche und freundliche Betreuung meiner Arbeit bedanken.

Bei Frau Claudia Tag möchte ich mich für die Einarbeitung in die verschiedenen Methoden und ihre Hilfe in allen Belangen bedanken.

Auch den anderen Mitarbeitern des Instituts für Anatomie und Zellbiologie danke ich für ihre vielen guten Ratschläge und die sehr nette, freundschaftliche Arbeitsatmosphäre.

Zudem möchte ich mich bei Herrn Prof. Dr. Kaps und den Kollegen der Neurologie der Universitätsklinik Gießen bedanken, die mich in den letzten Monaten bei der Fertigstellung meiner Doktorarbeit unterstützt haben.

Mein weiterer Dank gilt Manuel Bobrich, Alexander Brobeil und Max Kamm für ihre fachliche Unterstützung, die mir in häufigen Diskussionen und Gesprächen zu Gute gekommen ist.

Julian Fugmann danke ich für seine Unterstützung bei computerfachlichen Fragen.

Meinen Geschwistern möchte ich für die aufbauenden Gespräche nach so manchen ernüchternden Tagen im Labor danken.

Ganz besonders bedanken möchte ich mich bei meinen Eltern, die mir mein Studium erst ermöglicht haben und ohne deren Unterstützung diese Arbeit nie zustande gekommen wäre.

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

7. Publikationen der kumulativen Doktorarbeit

7.1. Expression profile of PTPIP51 in mouse brain.

7.2. PTPIP51 – a multifunctional protein in brain tissue

7.3. PTPIP51, a positive modulator of the MAPK/Erk pathway, is upregulated in glioblastoma and interacts with 14-3-3β and PTP1B in situ

The Journal of Comparative Neurology 517:892–905 (2009)
Research in Systems Neuroscience

Expression Profile of PTPIP51 in Mouse Brain

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ABSTRACT

This study demonstrates the expression of the novel protein protein tyrosine phophatase-interacting protein 51 (PTPIP51) in mammalian brain tissue. Serial sections of the whole adult mouse brain were analyzed for PTPIP51 protein and mRNA by immunohistochemistry, immunoblotting, RT-PCR, and in situ hybridization. Recent investigations by Yu et al. (2008) describe PTPIP51 as being capable of activating Raf-1, thereby modulating the MAPK pathway. The role of Raf-1, as well as of 14-3-3, in neurological disorders is well established. PTPIP51 expression was confined to neurons in the following structures: the piriform cortex and their connections to the anterior commissure, nucleus accumbens, paraventricular and supraoptical nuclei, neurohypophysis, superior colliculus, genu of facialis nerve, spinal trigeminal tract, inferior cerebellar peduncle, and cerebellum. In the cerebellum, a subpopulation of Purkinje cells and their dendrites was strongly PTPIP51 positive. Moreover, PTPIP51 was found to be colocalized with vasopressin and its transport protein neurophysin II in the neuroendocrine nuclei and their connections to the neurohypophysis. The data presented here suggest a role of PTPIP51 in neuronal homeostasis, axonal growth, and transport. J. Comp. Neurol. 517:892–905, 2009. © 2009 Wiley-Liss, Inc.

Indexing terms: PTPIP51; PTP1B; CNTF; 14-3-3; Raf-1; neurophysin II; vasopressin; mouse brain; hypothalamus; hypophysis; nucleus accumbens; cerebellum

PTPIP51 is an evolutionarily conserved protein, which was shown to interact in vitro with two nontransmembrane protein tyrosine phosphatases, protein tyrosine phosphatase 1B (PTP1B) and T-cell protein tyrosine phosphatase (TcPTP; Porsche, 2001; Stenzinger et al., 2005). The interaction takes place in the region between amino acids 78 and 214. The protein is phosphorylated in vitro and in situ at Tyr176 by Src kinase and dephosphorylated by PTP1B and TcPTP (Stenzinger et al., 2009). In mammals, its expression is associated with specific tissues such as epithelia, testis, skeletal muscle, and nervous tissue (Stenzinger et al., 2005). PTPIP51 protein also plays a role during mammalian development (Märker et al., 2008), and both mRNA and protein could be traced during placental villi formation (Stenzinger et al., 2008) and in various carcinomas (Lv et al., 2006; Koch et al., 2008). Further experiments demonstrated a vitamin- and cytokine-mediated PTPIP51 expression in cultured keratinocytes (Stenzinger et al., 2006). Given these findings, we hypothesized PTPIP51 to be involved in cellular differentiation, motility, cytoskeleton formation, and possibly apoptosis.

Experiments by Lv and colleagues (2006) added evidence to this assumption by demonstrating that overexpressed PTPIP51 enhances apoptosis in HEK293 cells. Moreover, two independent studies by Jin et al. (2004) and Ewing et al. (2007) demonstrated an interaction between the two isoforms 14-3-3-β and 14-3-3-y and PTPIP51. Recent experiments by Yu et al. (2008) confirmed these findings by pull-down experiments and describe PTPIP51 as interacting with Raf-1 through 14-3-3, thereby modulating cellular motility and morphology via the mitogen-activated protein kinase (MAPK) cascade. Both the Ras/Raf/MEK/ERK pathway and the mammalian 14-3-3 superfamily play pivotal roles in neuronal development and maintenance as well as in many neurological disorders, including Alzheimer's and Parkinson's disease (Dougherty and Morrison, 2004; Mei et al., 2006; McCubrey et al., 2007; Samuels et al., 2008). As reported by several research groups, Ras signaling in particular influences neuronal plasticity, synaptic transmission, and short- and long-term memory of adult mice (Brambilla et al., 1997; Atkins et al., 1998; Giese et al., 2001; Dhaka et al., 2003).

In rat retina, transcription of *Ptpip51* is governed by ciliary neurotrophic factor (CNTF; Roger et al., 2007). CNTF, a neuropoietic cytokine of the interleukin-6 family, is widely expressed throughout the entire central nervous system (CNS; Sleeman et al., 2000). The cell type, however, that increases

Additional Supporting Information may be found in the online version of this article.

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Received 23 October 2008; Revised 2 February 2009; Accepted 10 August 2009

DOI 10.1002/cne.22201

Published online August 13, 2009 in Wiley InterScience (www.interscience.wiley.com).

TABLE 1. List of the Antibodies Used in This Study

	Immunogen	Antibody source	Clone	Dilution	Manufacturer
PTPIP51	Human recombinant PTPIP51 protein encoding amino acids (aa) 131-470	Rabbit polyclonal		1:400	Prof. H.W. Hofer, Biochemical Department, University of Konstanz
Vasopressin	Synthetic arginine-vasopressin	Rabbit polyclonal		1:1,000	Prof. Nürnberger, University of Frankfurt
PTP1B	Human recombinant protein tyrosine phosphatase 1B (aa 1-321)	Mouse monoclonal	107AT531	1:100	Abgent catalog No. AM8411
Neurophysin II	Raised against a peptide mapping near the C-terminus of neurophysin II of mouse origin (aa 78-128)	Goat polyclonal		1:1,000	Santa Cruz Biotechnology catalog No. sc-27093
Glial fibrillary acidic protein	Purified porcine glial filament from spinal cord	Mouse monoclonal	GA5	1:200	Chemicon catalog No. MAB3402
PGP9.5	Human recombinant protein, full-length PGP9.5	Mouse monoclonal	10A1	1:100	Neuromics catalog No. MO20002
Calbindin D	Bovine kidney calbindin-D	Mouse monoclonal	CB-955	1:2,000	Sigma catalog No. 015K4826
Antidigoxigenin-fluorescein Fab fragments	Immunization with digoxigenin	Sheep		1:200	Roche catalog No. 1207741
Alexa fluor 555 coupled to anti-rabbit antibody	IgG heavy chains from rabbit	Goat		1:800	Invitrogen catalog No. A21428
Alexa fluor 488 coupled to anti-mouse antibody	IgG heavy chains from mouse	Goat		1:800	Invitrogen catalog No. A11029
FITC anti-rabbit antibody	IgG from rabbit	Goat		1:400	Cappel catalog No. 55651
Cy3 donkey anti-goat antibody	IgG from goat	Donkey		1:400	Chemicon catalog No. AP180C

PTPIP51 expression in response to CNTF has not been determined yet. Interestingly, it mediates its action by the differential activation of the JAK-STAT and MAPK signaling pathway (Boulton et al., 1994; Bhattacharya et al., 2008).

Although the neuronal and ganglionic expression of PTPIP51 in rat peripheral nervous system as well as its localization in the hippocampal region of the CNS was already described in an organ distribution screening of PTPIP51 (Stenzinger et al., 2005), a detailed analysis of PTPIP51 in mammalian CNS is lacking. Therefore, we studied the cell- and tissue-specific expression of PTPIP51 mRNA and protein in adult mouse brain. Coimmunostainings with neurophysin II and vasopressin were performed to elucidate functional properties of PTPIP51 in specific regions of mouse brain.

MATERIALS AND METHODS

Tissue and section preparations

The study was performed on paraffin-embedded and cryosamples of mouse brain (n = 6; sex: female, age: 14 weeks), fixed in either Bouin fixative or paraformaldehyde. For both immunohistochemistry and in situ hybridization, the whole paraffin-embedded brain of each mouse was serially cut into 6-μm thin sections. Every tenth section was dried, deparaffinized in xylene, and rehydrated in graded alcohol prior to immunostaining and in situ hybridization, respectively. H&E-stained sections were used for orientation. PTPIP51-positive regions were identified by comparison with mouse brain maps: www.mbl.org/mbl_main/atlas.html; www.hms.harvard.edu/research/brain/atlas.html; www.brain-map.org/mouse/atlas.html.

PTPIP51 antibody production

The cDNA sequence encoding aa 131–470 was inserted into the BamHI and HindIII sites of the plasmid pQE30 and expressed as His6-tagged protein in the protease-deficient *Escherichia coli* expression strain AD202 [araD139DE(argF-lac)169 ompT1000:kan flhD5301 fruA25 relA1 rps150(strR) rbsR22 deoC1]. The protein was purified to electrophoretic homogeneity by chromatography on an Ni-agarose column (Porsche, 2001). Immunization of rabbits was performed with 0.5 mg of the purified protein in 0.5 ml RIBI adjuvant, followed by booster injections with 0.5 and 0.3 mg on days 14 and 21, respectively. The antiserum was collected on day 28. Monospecific antibodies were prepared following the method described by Olmsted (1981). Briefly, 2 mg of purified antigen was blotted on nitrocellulose after SDS electrophoresis. The protein band was marked with Ponceau solution and cut out. After blocking of the membrane strip with 1% low-fat milk powder in phosphate-buffered saline, the membrane was incubated with the antiserum for 1 hour, followed by extensive washing with Tris-EDTA-buffered saline. The antibodies were eluted with 0.2 M glycine (pH 2.0) for 2 minutes, followed by immediate neutralization with 1 M triethanolamine.

Immunohistochemistry

Prior to immunostaining, nonspecific binding sites were blocked with 0.1 M phosphate-buffered saline (PBS; pH 7.4) containing 5% bovine serum albumin and 5% normal goat serum. Indirect immunofluorescence was performed by overnight incubation with primary antibodies (see Table 1) diluted in PBS at room temperature, followed by washing in PBS and subsequent incubation for 1 hour at room temperature with the appropriate secondary antibodies (see Table 1). Then, the slides were washed in PBS, coverslipped in carbonate buffered glycerol at pH 8.6, and evaluated either by epifluorescence microscopy or by sequential confocal laser scanning microscopy.

The primary polyclonal antibody to PTPIP51 was visualized either by Alexa Fluor 555 secondary antibody or FITC anti-rabbit. Anti-mouse antibodies used for double staining were visualized by using Alexa Fluor 488 secondary antibody. The

primary monoclonal anti-goat antibody neurophysin II, used for identification of axonal transport, Cy3 donkey anti-goat was used as secondary antibody in combination with FITC anti-rabbit as secondary antibody for PTPIP51 visualization. Hypothalamic nuclei were identified by polyclonal anti-rabbit antibody to vasopressin. Nuclei were displayed through DAPI.

Antibody characterization

See Table 1 for a list of all antibodies used.

1) The specificity of the PTPIP51 antibody was tested by ELISA and by immunoblotting of the isolated purified recombinant protein staining bands with 52 kDa, 34 kDa, and 30 kDa. Immunoblotting of homogenates from porcine spleen tissue revealed bands of 48 kDa, 40 kDa, and 29 kDa (Hofer, Buerklen, and Welte, unpublished observations). The antibody binds to the EGFP fusion PTPIP51 protein expressed in HEK293 (Hofer and Schreiner, unpublished observations). Preabsorbing the PTPIP51 antibody against its antigen completely abolished the immune reaction in all tested samples (Stenzinger et al., 2005; Barop et al., 2009).

2) The calbindin antibody was derived from CB-955 hybridoma produced by the fusion of mouse myeloma cells and splenocytes from BALB/c mice immunized with purified bovine kidney calbindin-D-28K. The calbindin D antibody recognized on Western blot of rat brain extract a 28-kDa band at the expected molecular weight for calbindin-D. Recent publications by Kuwajima and coworkers (2006), Levin and coworkers (2006), and Soderling and colleagues (2003) demonstrated a staining pattern of cerebellar Purkinje cells in immunohistrochemistry of mouse brain and mouse embryo brain sections, comparable to our results. The antibody does not react with other members of the EF-hand family, such as calbindin-D9K, calretinin, parvalbumin, S-100a, S-100b, S100A2, and S100A6. Preabsorbation of this antibody with calbinidin-D28 kDa purified from chick and rat brains or from rat kidney completely abolished calbindin immunostaining in rat brain (manufacturer's data sheet; Pasteels et al., 1987).

3) The glial fibrillary acidic protein (GFAP) antibody recognized GFAP on Western blot of mouse brain tissue (50 kDa) and of astrocyte cultures (manufacturer's data sheet). As reported by Debus et al. (1983), the antibody was tested in Western blot against neurofilament polypeptides purified from porcine spinal cord. The antibody recognized a single band of porcine GFAP with an apparent molecular weight of 51 kDa in Western blot. In tissue sections of human brain and optic nerve, rat brain and spinal cord, and chicken brain, the antibody stained GFAP as well as tumor cells in human astrocytoma.

4) The neurophysin II antibody was raised in goat against the following peptide: RCQEENYLPSPCQSGQKPCGSGGRCAAVGICCSDESCVAEPECHDGFFRLT. It recognized on Western blot of mouse brain and rat pituitary tissue a single band of 23 kDa at the expected molecular weight (manufacturer's data sheet). Indirect ELISA testing was done with the immunogen neurophysin peptide (aa 78–128; manufacturer's information). This antibody was used for immunohistochemical detection of vassopressinergic neurons in the hypothalamus of mice (Russell et al., 2003) and for labelling arginine-vasopressin in isolated neurohypophyseal nerve terminals of the rat (Custer et al., 2007).

As described in Results, we performed preabsorption experiments with 2 μg of neurophysin II antigen (Sc-27093P) per 100 μl incubation mixture with diluted neurophysin II antibody (1:1,000). In the hypothalamic nuclei of mouse brain, neurophysin II antibody staining was completely knocked out by preabsorption to neurophysin II antigen.

5) The PGP9.5 antibody (clone 10A1) recognized single bands of 27 kDa on Western blot of four different human neural cell lines corresponding to the molecular weight of protein gene product 9.5 (Satoh and Kuroda, 2001). In immunohistochemistry, the antibody recognizes neurons in mouse embryos (Engleka et al., 2005).

6) The PTP1B antibody recognized a 50-kDa band on Western blot of mouse lung tissue corresponding to the expected molecular weight of PTP1B. Furthermore, the antibody recognized a single band on Western blot of isolated recombinant PTP1B protein (aa 1–321; manufacturer's data sheet). The specificity was tested by ELISA against the recombinant protein (manufacturer's information) and by preabsorbing the antibody against the blocking peptide (synthetic protein from the C-term region of human PTP1B). Preabsorbing the antibody resulted in a complete lack of staining of mouse brain sections as well as of the positive control tissue.

7) The vasopressin antibody was tested by RIA and immunocytochemistry. The antibody expressed only very weak cross-reactivities for oxytocin and vasotocin. For specificity tests, preabsorption of the antisera with its antigen was done (Schindler and Nürnberger, 1990). In addition, the specificity was confirmed by immunoblotting and by cross-absorption tests against oxytocin and mesotocin (Sephadex beads; Nürnberger, unpublished observations).

Preabsorption experiments for immunostaining

Specificity of the PTPIP51 immunoreactivity was controlled by preabsorbing the PTPIP51 antibody with the corresponding purified antigen at a concentration of 20 μg/ml for 18 hours at 4°C. To exclude cross-reactivity of the PTPIP51 antibody with the antigen neurophysin, the PTPIP51 antibody (1:400) was preabsorbed with a mixture of neurophysin I and neurophysin II blocking peptide (Santa Cruz Biotechnology, Santa Cruz, CA; catalog Nos. sc-7810 and sc-27093) in a concentration of each 2 μg/100 μl incubation mixture. As positive control, a normal incubation mixture including the same concentration of PTPIP51 antibody was used. To control the preabsorption process, the same procedure was performed with the antibody to neurophysin II.

Epifluorescence microscopy

A Axioplan 2 fluorescence microscope equipped with Plan-Apochromat objectives (Carl Zeiss, Jena, Germany) was used for photodocumentation. For visualization of the secondary antibody Alexa Fluor 555, an excitation filter with a spectrum of 530–560 nm and an emission filter with a spectrum 572.5–647 nm were used. Alexa Fluor 488 was visualized by an excitation filter with a range of 460–500 nm and an emission filter with a range of 512–542 nm.

Immunoblotting

Samples of brain tissue derived from medial neocortex, hippocampus, and cerebellum were separated on a 10% SDS-PAGE gel. Transfer on an Immobilon P membrane (Millipore, Bedford, CA) was performed according to Towbin et al. (1979). The membrane was blocked with 10% fat-free milk powder in PBS and subsequently incubated for 1 hour with antibodies

diluted in 0.5% fat-free milk powder in PBS. Incubation with polyclonal anti-PTPIP51 was done overnight at 4°C. Alkaline phosphatase-conjugated anti-rabbit immunoglobulins were applied for 1 hour at room temperature, and the reaction was visualized with the SigmaFast BCIP/NBT substrate. A prestained molecular weight marker (Fermentas, San Fransisco, CA; catalog No. SM0431) was used for calibration.

In situ hybridization

In situ hybridization was performed as described previously (Steger et al., 1998, 2000) using a stringency to achieve hybridization only with a homology greater than 80%. Briefly, 5-μm sections were partially digested with proteinase K, postfixed in 4% paraformaldehyde, and exposed to 20% acetic acid. After prehybridization in 20% glycerol, sections were covered with the DIG-labeled sense or antisense cRNA probes. Production of DIG-labelled cRNA probes was performed as described previously (Steger et al., 1998). PCR conditions were 1× 94°C for 3 minutes; 35× 94°C for 30 seconds, 66°C for 30 seconds, 72°C for 60 seconds; and 1× 72°C for 8 minutes with 5′-GTCTCTCACCTGAGCTGGCTAGA-3′ as forward primer and 5′-GGATAGCCAAATCCTCCTTCGTGA-3′ as reverse primer. The 247-nucleotide ER-product (nt positions 1126–1372, corresponding to aa 376–457) of the human PTPIP51 gene was subcloned in pGEM-T (Promega, Heidelberg, Germany). Plasmids were transformed in the XL1-Blue *E. coli* strain (Stratagene, Heidelberg, Germany) and extracted by column purification (Qiagen, Hilden, Germany). In vitro transcription of DIG-labelled PTPIP51-cRNA was performed with a 10× RNA-DIG Labelling Mix (Boehringer Mannheim, Mannheim, Germany) and RNA polymerases T3 and SP6. Vectors containing the PTPIP51 inserts were digested with NcoI and NotI (New England Biolabs, Frankfurt, Germany) for the production of sense cRNA and antisense cRNA, respectively. Both cRNAs were used at a dilution of 1:100 (the optimal concentration was determined by a dot-blot test) in hybridization buffer containing 50% deionized formamide, 10% dextran sulfate, 2× saline sodium citrate (SSC), 1× Denhardt's solution, 10 μg/ml salmon sperm DNA, and 10 μg/ml yeast t-RNA. Hybridization was performed overnight at 37°C in a humidified chamber containing 50% formamide in 2× SSC. Posthybridization washes were performed according to Lewis and Wells (1992). After blocking with 3% bovine serum albumin, sections were incubated (overnight at 4°C) with the anti-DIG Fab antibody conjugated to alkaline phosphate (Boehringer Mannheim). Staining was visualized by developing sections with nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate in a humidified chamber protected from light. Alternatively, the sections were incubated with anti-DIG-fluorescein, Fab fragments (catalog No. 11 207 741 910; Roche, Indianapolis, IN). For each test, negative controls were performed with DIG-labeled cRNA sense probes.

RNA extraction

RNA extraction from cryomaterial was performed with the RNA extraction kit RNeasy MINI (Qiagen).

First-strand synthesis

First-strand synthesis was performed by using Omniscript according to the manufacturer's protocol (Qiagen).

RT-PCR

RT-PCR was performed on an iCycler with Sybr green Supermix (Bio-Rad, Munich, Germany) to visualize the amplicons (Brehm et al., 2006). Per sample, 2 μl cDNA was used for amplification of PTPIP51. Cycling conditions were 94°C for 2 minutes, followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes. The following primers were employed: forward primer 5′-AGGGCATCTCGAAACGCATCCA-3′ and reverse primer 5′-CTTCTAGTTCTTCCAGGTCTTTTTG-3′, resulting in a 389-bp amplification product. PCR products were visualized by agarose gel electrophoresis. Amplification of a 90-bp β-actin product served as positive control, and negative controls included samples lacking reverse transcriptase.

Production of DIG-labelled cRNA probes

Production of DIG-labelled cRNA probes was performed as described previously (Steger et al., 1998). PCR conditions were 1× 94°C for 3 minutes; 35× 94°C for 30 seconds, 66°C for 30 seconds, 72°C for 60 seconds; and 1× 72°C for 8 minutes with 5′-GTCTCTCACCTGAGCTGGCTAGA-3′ as forward primer and 5′-GGATAGCCAAATCCTCCTTCGTGA-3′ as reverse primer. The 247-nucleotide ER product (nt position 1126–1372, corresponding to aa 376–457) of the human PTPIP51 gene was subcloned in pGEM-T (Promega). Plasmids were transformed in the XL1-blue *E. coli* strain (Stratagene) and extracted by column purification (Qiagen). In vitro transcription of DIG-labelled PTPIP51-cRNA was performed with the 10× RNA-DIG Labelling Mix (Boehringer Mannheim) and RNA polymerases T3 and SP6. Vectors containing the PTPIP51 inserts were digested with NcoI and NotI (New England Biolabs) for the production of sense cRNA and antisense cRNA, respectively.

RESULTS

This study demonstrates the expression pattern of PTPIP51 mRNA and protein in neurons making up specific regions of the adult mouse brain.

PTPIP51 mRNA expression pattern in mouse brain

In situ hybridization displayed the expression of PTPIP51 mRNA in distinct areas of mouse brain (Figs. 1, 5). A strong hybridization signal was detected in the paraventricular nucleus (Fig. 1A), piriform area (Fig. 1C), nucleus accumbens (Fig. 1E), cerebellum (Fig. 1G), and hippocampus (Fig. 5B). RT-PCR experiments (Fig. 2) corroborated the in situ hybridization results by demonstrating the transcription of PTPIP51 mRNA in the cerebellum and the pituitary gland. Brain tissue derived from the frontal lobe did not show a positive immunostaining for the PTPIP51 antigen and was devoid of the encoding mRNA (Fig. 2, lane Fb).

Immunohistochemical detection of PTPIP51 in serial coronal sections of mouse brain

The expression profile of PTPIP51 protein was analyzed by the use of serial sections of whole mouse brains. Every tenth section of the series was immunostained for the PTPIP51 antigen. Preabsorption experiments corroborated the specificity of the polyclonal PTPIP51 antibody. Preabsorbing the

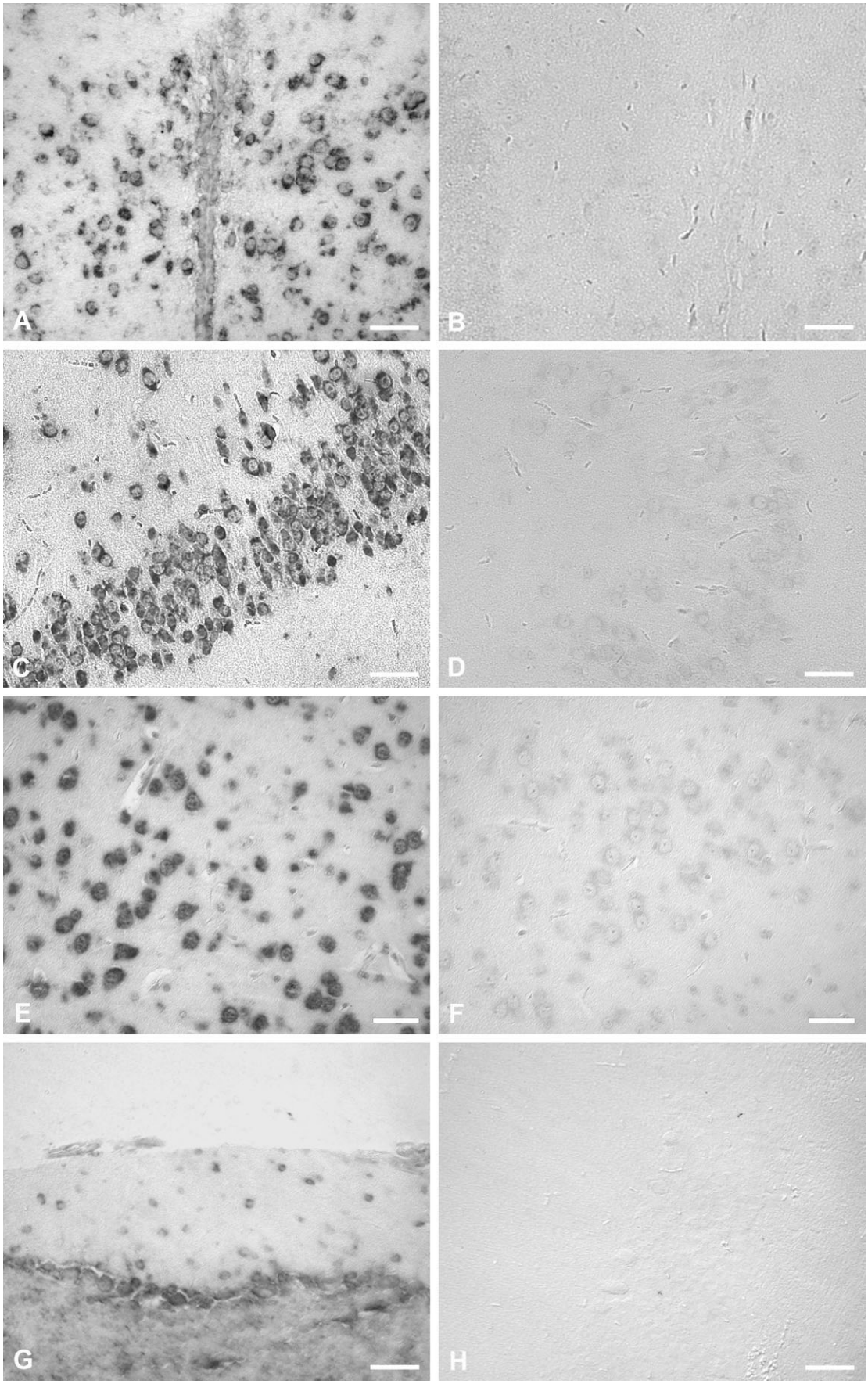

Figure 1.
PTPIP51 in situ hybridization of different mouse brain regions. **A,B:** Paraventricular nuclei. **C,D:** Piriform cortex. **E,F:** Nucleus accumbens. **G,H:** Cerebellum. A,C,E,G: Antisense probe. B,D,F,H: Sense probe. Scale bars = 50 μm.

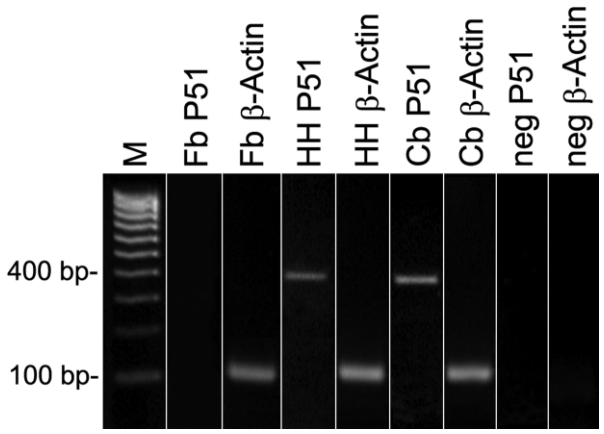

Figure 2.
Expression of PTPIP51 in mouse brain, frontal lobe, pituitary gland, hypothalamus, and cerebellum as detected by RT-PCR. RT-PCR was performed with primers specific to PTPIP51 as given in Materials and Methods. β-Actin was amplified as an internal positive control, and probes lacking reverse transcriptase served as negative controls (neg). Marker (M), samples of frontal lobe (Fb), hypophysis and hypothalamus (HH), and cerebellum (Cb). The white bar between the marker and the lanes indicates that the lanes were depicted from different gels.

polyclonal antibody with the corresponding antigen resulted in the elimination of the PTPIP51 immunoreactivity (Fig. 3). Combining fluorescence in situ hybridization and immunohistochemistry on distinct sections of mouse brain tissue further evaluated the antibody specificity. As exemplified in Figure 4, the neurons of the the piriform cortex express both PTPIP51 mRNA and its encoded protein within the same cell.

As shown in Figures 5 and 6, PTPIP51 expression was restricted to the following areas: the hippocampus (Fig. 5), specific regions within the piriform cortex (Fig. 4) including their connections to the anterior commissure (Fig. 6A), the nucleus accumbens (Fig. 6B), the paraventricular (Fig. 6C) and supraoptical (Fig. 6D) nuclei, the neurohypophysis (Fig. 6E), the zonal layer and superficial gray layer of superior colliculus, the genu of facial nerve, the hypoglossal nuclei, the spinal trigeminal tract, the inferior cerebellar peduncle, and the cerebellum (Fig. 6F). In the cerebellum, the strongest PTPIP51 signal was seen in Purkinje cells identified by double immunostaining with calbindin antibody (Fig. 7). All other brain areas displayed no detectable amount of the PTPIP51 antigen.

PTPIP51-positive cells were identified as neurons by coimmunostaining experiments with an antibody directed against the neuron-specific protein gene product 9.5 (PGP9.5) and the PTPIP51 antibody (Fig. 8). Accordingly, cells stained for glial cell labeling (glial fibrillary acidic protein; GFAP) showed no expression of PTPIP51 protein.

Colocalization of PTPIP51 with vasopressin and neurophysin II

The aim to study possible functional implications of PTPIP51 was met by coimmunolabeling of axonally transported peptide hormones, vasopressin and PTPIP51. PTPIP51 colocalizes with vasopressin and neurophysin II in the paraventricular and supraoptical region.

Vasopressin and PTPIP51. Staining of the paraventricular and supraoptical nuclei with the antibody to vasopressin showed a strict colocalization with PTPIP51 in cells and their arising fibers (Fig. 9C,D,G,H). This colocalization was also observed in the neurohypophysis (Fig. 10C,D).

Neurophysin II and PTPIP51. In the paraventricular and supraoptical nuclei, neurophysin II showed a strict colocalization with PTPIP51 protein within neurons and their arising fibers (Fig. 9A,B,E,F). This colocalization of neurophysin II and PTPIP51 also was observed in the neurohypophysis (Fig. 10A,B).

To exclude nonspecific binding of the PTPIP51 antibody to neurophysins, the specificity of the PTPIP51 reaction was controlled by preabsorbing the PTPIP51 antibody to neurophysin I and II antigen. As seen for the hypothalamic nuclei (Fig. 11), the PTPIP51 immunoreaction (Fig. 11A) is not blocked by preabsorption to neurophysin (Fig. 11B), whereas the neurophysin II antibody was completely knocked out by preabsorption to neurophysin II antigen (Fig. 11C,D).

PTP1B and PTPIP51. A minority of the neurons coexpressed PTPIP51 and PTP1B independent of the brain area investigated.

Immunoblotting of specific mouse brain regions

Immunoblotting experiments with samples from various regions of adult mouse brain (medial neocortex, hippocampus, cerebellum) revealed 30 kDa, 34 kDa, and 52 kDa bands of the PTPIP51 protein in all three regions (Fig. 12).

DISCUSSION

This study demonstrates for the first time the expression profile of PTPIP51 mRNA and its encoded protein in mouse brain. Positive immunoreactivity and mRNA expression could be traced in specific brain regions and were confined to neurons and their neurite extensions. Immunoblotting of different PTPIP51-expressing regions of mouse brain revealed three different molecular weight forms of PTPIP51 with a molecular mass of 30 kDa, 34 kDa, and 52 kDa, respectively. The 52-kDa band corresponds to the full-length form of PTPIP51 consisting of 470 amino acids and is located exclusively to mitochondria by a mitochondrial target sequence (Lv et al., 2006). The other isoforms are likely to be splice variants encoded by the same gene, which consists of 12 exons and is located on chromosome 15 (15q15.1). AUG triplets (devoid of Kozak sequences) are located at the beginnings of exons 1, 2, and 3 and could serve as alternative initiation sites. Exon 4 may also be an initiation site for protein translation, insofar as it contains an AUG triplet surrounded by a Kozak sequence. These initiation sites would lead to molecular protein masses of 52, 45, 38, and 30 kDa, which are close to the apparent masses of the PTPIP51 proteins as determined by SDS-PAGE. The band with ~34-kDa apparent molecular mass may represent the 38-kDa splice variant with a theoretical pI of 4.69 and potentially accelerated migration on the electrophoretic gel. This band was also observed in other tissues. These putative isoforms of PTPIP51 lack the mitochondrial target sequence and probably show a distinct subcellular localization. These theoretical considerations are in accordance with the observation that endogenous PTPIP51 is not exclusively located to mitochondria in mouse neurons. Subcellular localization of endogenous PTPIP51 may also be altered by the formation of pro-

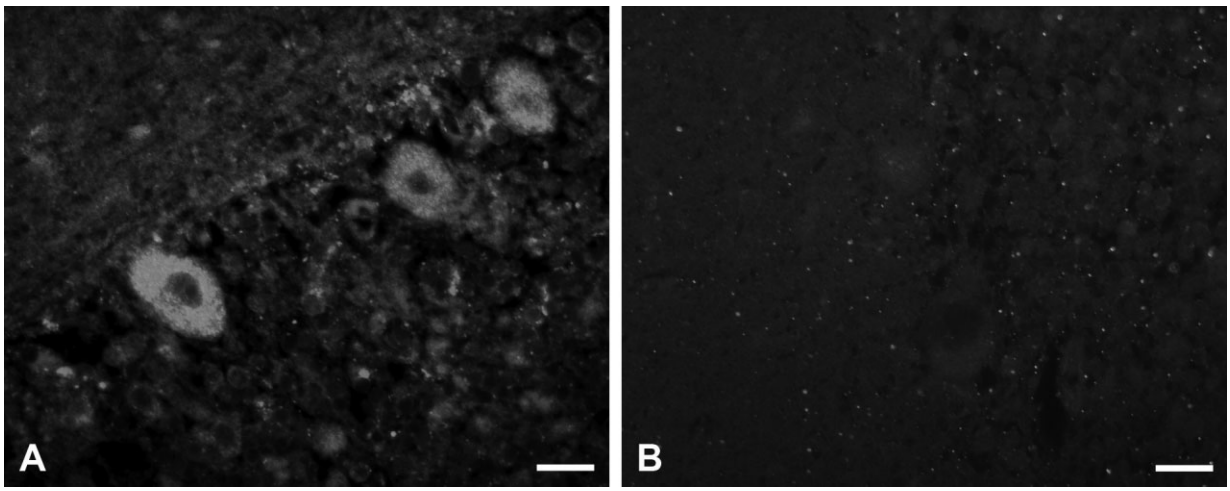

Figure 3.
Preabsorption experiments. **A:** Normal PTPIP51 immunoreactivity of neurons in mouse cerebellum. **B:** Preabsorption of the PTPIP51 antibody with the purified antigen. Scale bars = 20 μm.

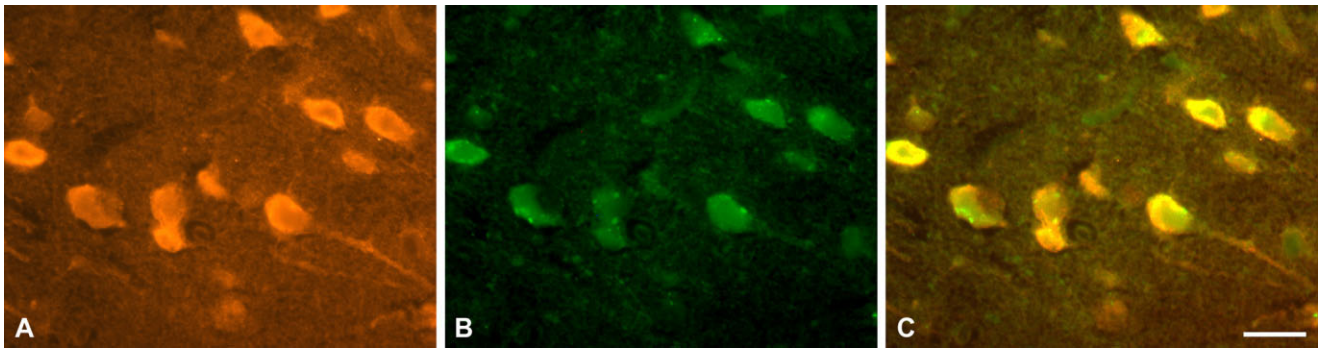

Figure 4.
PTPIP51 mRNA and protein in the piriform cortex of mouse brain. **A:** PTPIP51 immunostaining of neurons. **B:** Fluorescence in situ hybridization antisense probe of the same section. **C:** Merge of A and B. A magenta-green version of this figure is provided as Supporting Information for the assistance of color-blind readers. Scale bar = 20 μm.

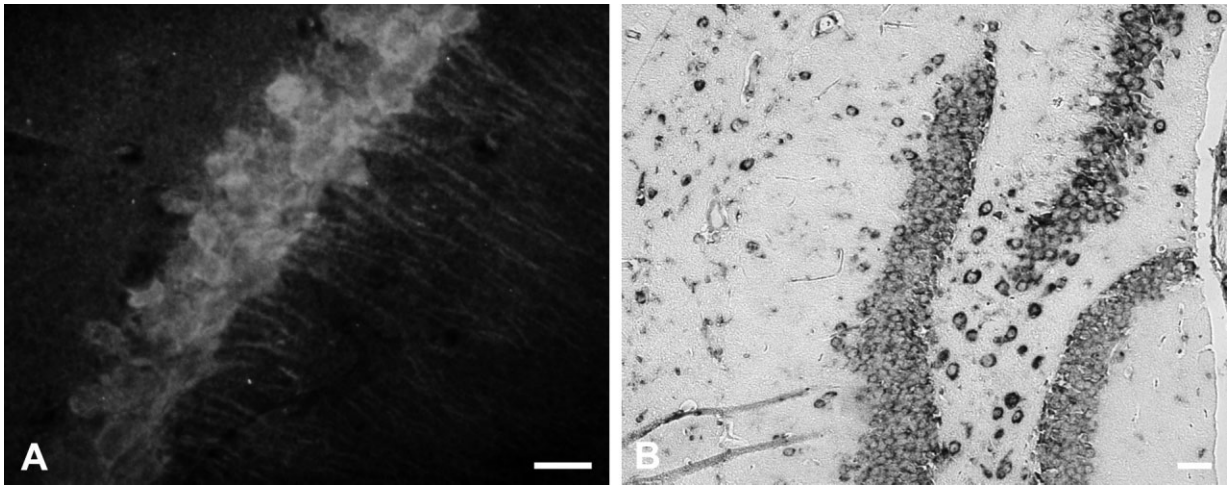

Figure 5.
PTPIP51mRNA expression and immunostaining in the hippocampus. **A:** Immunostaining. **B:** In situ hybridization. Scale bars = 20 μm in A; 50 μm in B.

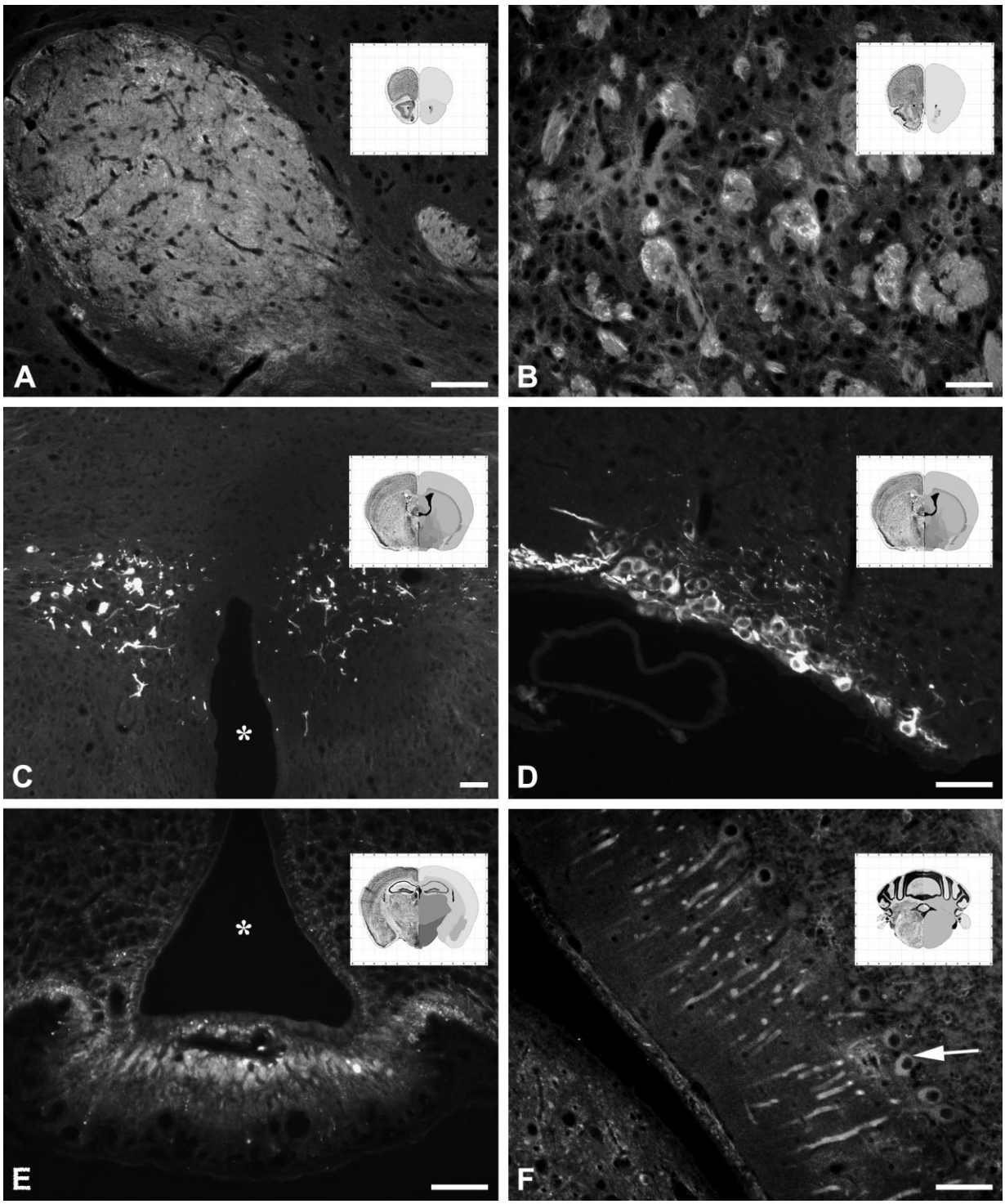

Figure 6.
PTPIP51 immunostaining of brain tissues. **A:** PTPIP51-positive fibers in anterior commissure. **B:** PTPIP51-positive cells in the area of nucleus accumbens. **C:** PTPIP51-positive cells and fibers arising from the paraventricular nucleus. **D:** PTPIP51-positive cells and fibers arising from the supraoptical nucleus. **E:** PTPIP51-positive fibers in neurohypophysis. **F:** PTPIP51-positive Purkinje cells and their fibers of cerebellum. Section was taken from nodulus. **Insets:** Modified diagrams of coronal sections from the Coronal Allen Brain Atlas, displaying the region shown in the figure. Asterisk, third ventricle; arrow, Purkinje cell. Scale bars = 50 μm in A,C–F; 20 μm in B.

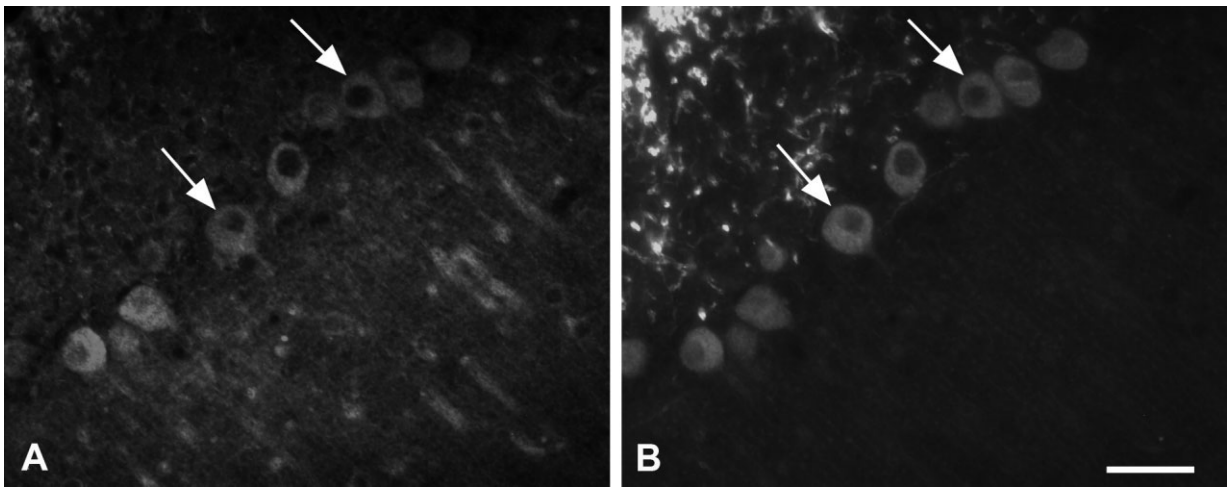

Figure 7.
Coimmunostaining of PTPIP51 with calbindin of cerebellar Purkinje cells. **A:** PTPIP51. **B:** Calbindin. Arrow, PTPIP51-positive Purkinje cells identified by calbindin staining. Scale bar = 20 μm.

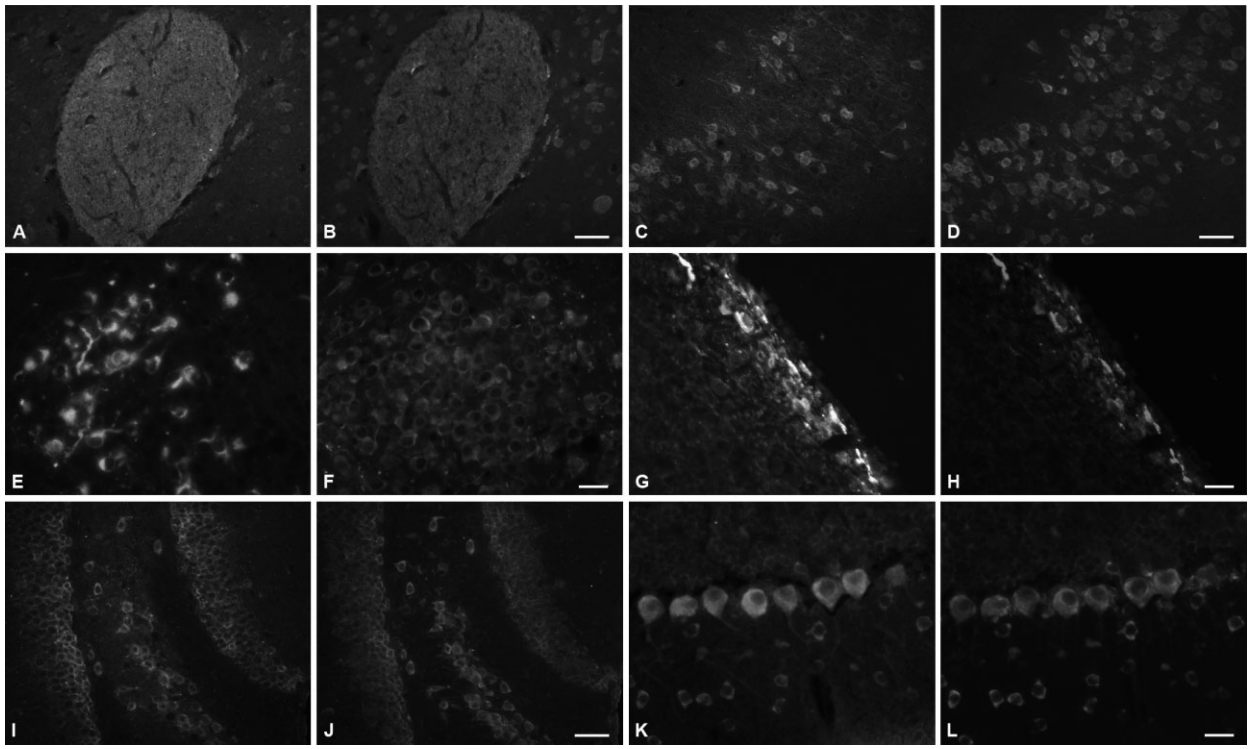

Figure 8.
Coimmunostaining of PTPIP51 with PGP9.5 in different brain regions. **A:** PTPIP51 staining of the anterior commissure. **B:** PGP9.5 staining of the anterior commissure. **C:** PTPIP51 staining of the piriform cortex. **D:** PGP9.5 staining of the piriform cortex. **E:** PTPIP51 staining of the paraventricular nuclei. **F:** PGP9.5 staining of the paraventricular nuclei. **G:** PTPIP51 staining of the supraoptical nuclei. **H:** PGP9.5 staining of the supraoptical nuclei. **I:** PTPIP51 staining of the hippocampus. **J:** PGP9.5 staining of the hippocampus. **K:** PTPIP51 staining of the cerebellum. **L:** PGP9.5 staining of the cerebellum. Scale bars = 50 μm in B (applies to A,B); 50 μm in D (applies to C,D); 20 μm in F (applies to E,F); 20 μm in H (applies to G,H); 50 μm in J (applies to I,J); 20 μm in L (applies to K,L).

tein complexes or by interaction with other proteins, thereby recruiting PTPIP51 to other cellular compartments.

First experiments found PTPIP51 as an interacting partner of PTP1B (Porsche, 2001; Stenzinger et al., 2005). In the mammalian brain, PTP1B is known as a regulator of axonal growth, which is mediated by cell–cell and cell–matrix adhesion molecules (Pathre et al., 2001). In agreement with previous observations (Stenzinger et al., 2005), the present study found

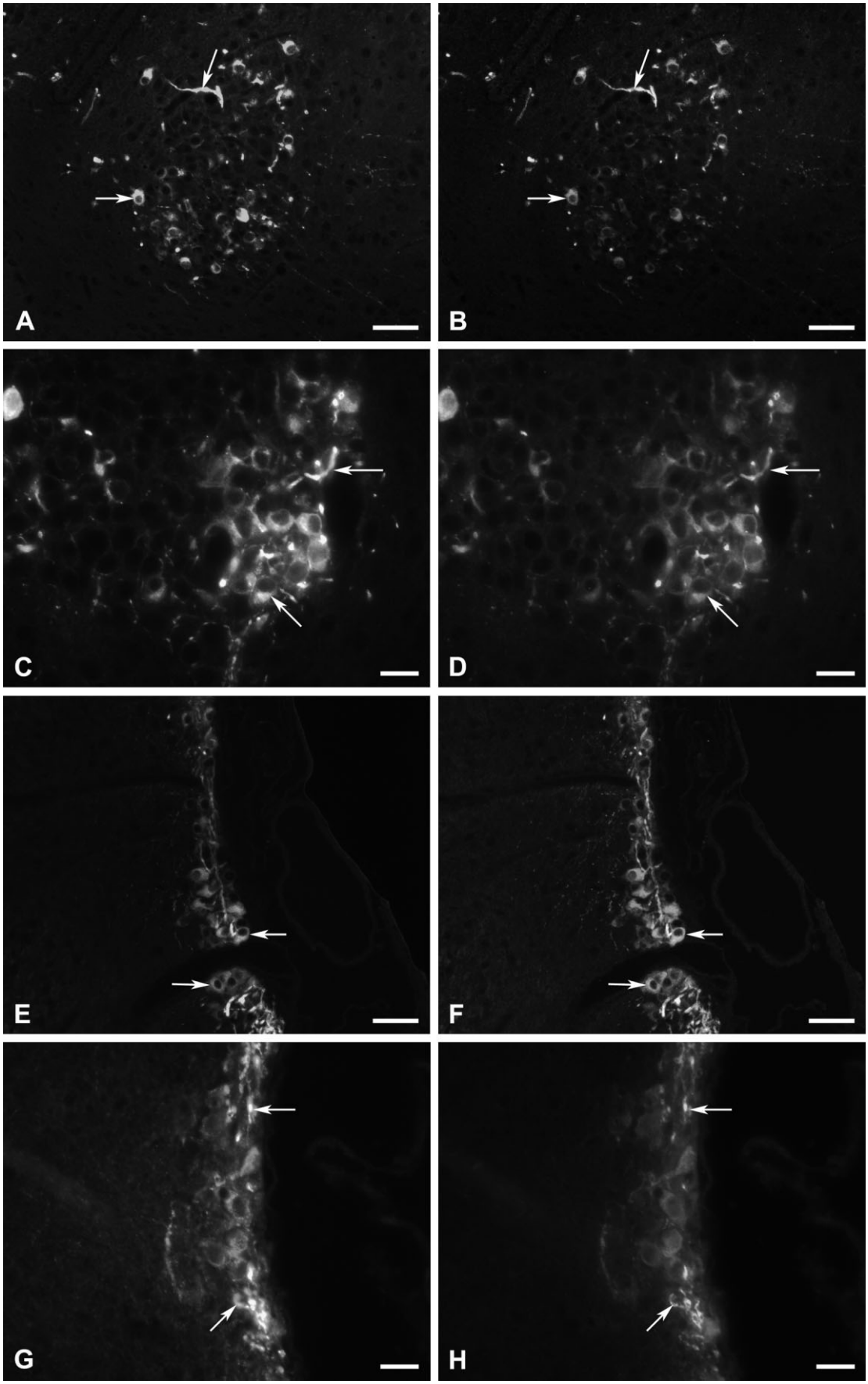

Figure 9.
Communostaining of PTPIP51 with neurophysin II and vasopressin of paraventricular (A–D) and supraoptical (E–H) nuclei. **A:** PTPIP51. **B:** Neurophysin II. **C:** PTPIP51. **D:** Vasopressin. **E:** PTPIP51. **F:** Neurophysin II. **G:** PTPIP51. **H:** Vasopressin. Arrow indicates double-labeled cells. Scale bars = 50 μm in A,B,E,F; 20 μm in C,D,G,H.

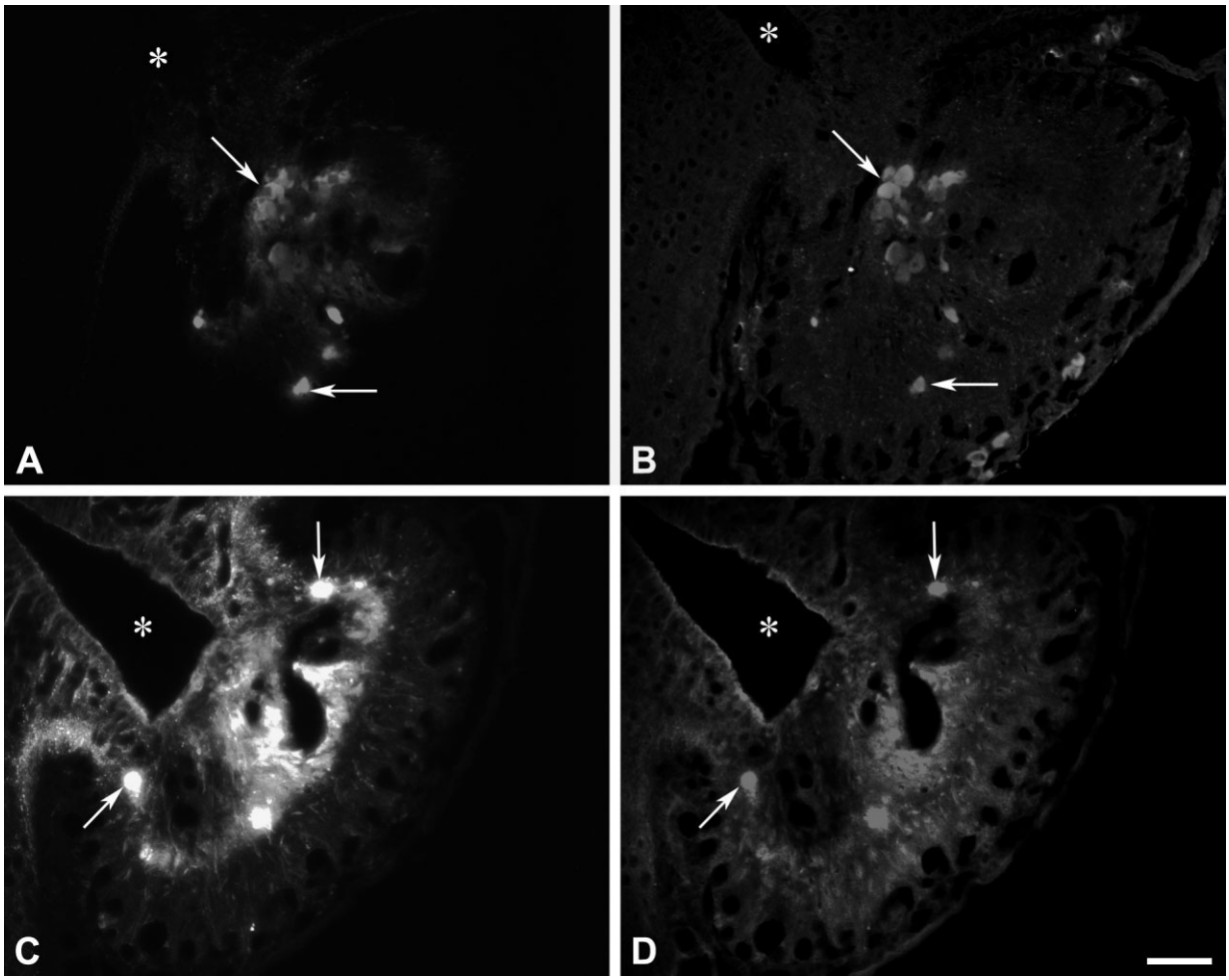

Figure 10.
Coimmunostaining of PTPIP51 with neurophysin II and vasopressin of neurohypophysis. **A:** PTPIP51. **B:** Neurophysin II. **C:** PTPIP51. **D:** Vasopressin. Asterisk, third ventricle; arrow, double-labeled cells. Scale bar = 50 μm.

PTPIP51-positive neurites throughout the nervous system, suggesting a role for PTPIP51 in this process. A coexpression of both proteins in mouse brain, however, was found in some but not all neurons, suggesting that PTPIP51 does not require continuous PTP1B interaction or can act independently of PTP1B signaling.

Vasopressin and oxytocin derived from magnocellular neurons (Russell and Leng, 2000) are transported together with their neurophysin carriers, neurophysin I and II (Fotheringham et al., 1991; Trembleau et al., 1994), in neurosecretory vesicles along the axon toward the neurohypophysis for secretion (Dreifuss, 1975; Brownstein et al., 1980). Axonal transport involves the formation of neurophysin–vasopressin complexes attached to the cytoskeleton. Insofar as PTPIP51-positive neurons and fibers displayed an identical localization of neurophysin II, PTPIP51 may act as a signaling partner mediating processes required for the axonal transportation of neuropeptides. The protein RMD-1 associates to microtubules by its TPR region and was recently shown to function in chromosome segregation of *C. elegans* (Oishi et al., 2007). Interestingly, RMD-1 belongs to the same protein family (FAM82) as PTPIP51 and exhibits some sequence homology with human PTPIP51 in the TPR region. It is therefore tempting to speculate that PTPIP51 may act as anchoring protein for microtubular transport processes. By its mitochondrial target sequence, PTPIP51 may also translocate mitochondria along the microtubular system. Motor proteins required for this endeavour (Gainer and Chin, 1998; Senda and Yu, 1999) are also involved in the motility of cilia (Mitchell, 2007; Scholey, 2008), which previous work has identified as PTPIP51-positive structures (Stenzinger et al., 2005). Pertubation and defects in the axonal transport in general can lead to diverse diseases, including amyotrophic lateral sclerosis and Alzheimer's and Huntington's diseases (Goldstein, 2001; Gerdes and Katsanis, 2005).

PTPIP51 expression was also traced in the cerebellum, with the most intense immunosignal in Purkinje cells and their dendrites. Interestingly, these cells have a strong signal for the motor protein kinesin family member 3C (KIF3C), especially in cell bodies and dendrites (Yang and Goldstein, 1998). KIF3C is also expressed in ganglion cells of the retina, which are PTPIP51 positive (Märker et al., 2008).

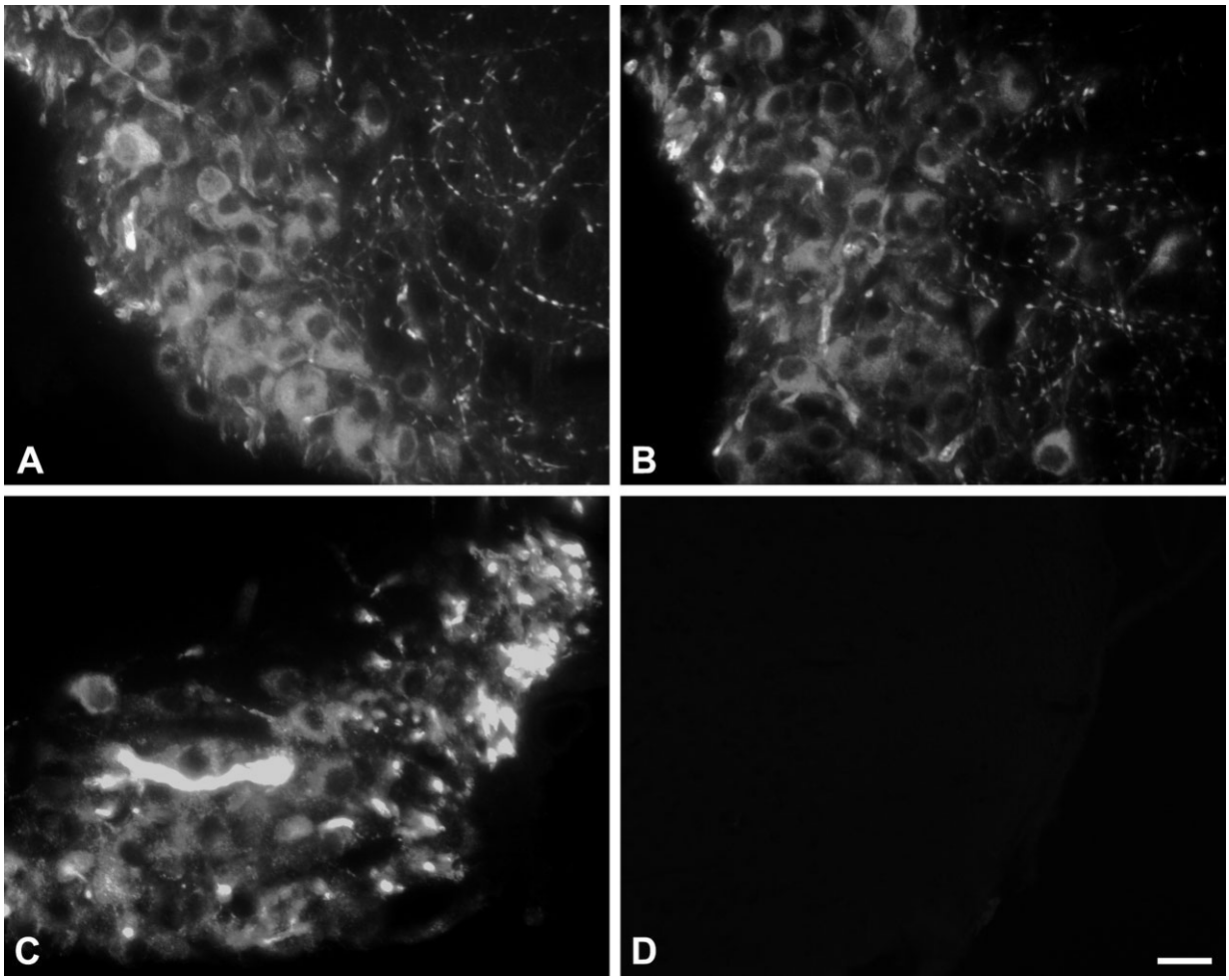

Figure 11.
Preabsorption of PTPIP51 antibody with the blocking peptides neurophysin I and neurophysin II in sections of the supraoptical nucleus. **A:** PTPIP51. **B:** Preabsorbtion of the antibody to PTPIP51 with blocking peptide neurophysin I and neurophysin II. **C:** Neurophysin II. **D:** Preabsorbtion of the antibody to neurophysin II with the blocking peptide neurophysin II. Scale bar = 50 μm.

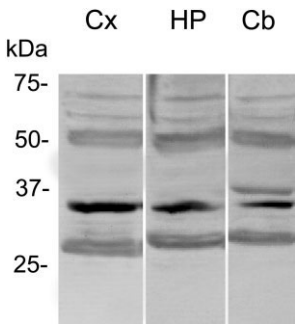

Figure 12.
Immunoblotting of PTPIP51 antigen in different regions of mouse brain. Cx, medial neocortex; HP, hippocampus; Cb, cerebellum.

As shown by Roger and colleagues (2007), PTPIP51 expression is regulated by CNTF in retinal cells; however, it is not known which retinal cell types increase PTPIP51 expression in response to CNTF. CNTF promotes neuronal survival, proliferation, and differentiation and is, amongst other trophic factors, an essential cytokine for the development and maintenance of the nervous system as well as for axonal growth (Weisenhorn et al., 1999; Sleeman et al., 2000; Markus et al., 2002a; Fuhrmann et al., 2003). The presence of CNTF receptor-α was described for rat brain regions such as the piriform cortex, hippocampal area, supraoptical and paraventricular nuclei, and Purkinje cells of the cerebellum (Lee et al., 1997), all of which were identified as PTPIP51-positive in our study. Additionally, CNTF receptors were traced in dorsal root ganglion neurons and in axons of rat peripheral nerves (MacLennan et al., 1996). As demonstrated by Stenzinger et al. (2005), dorsal root ganglion, trigeminal ganglion, motoneurons, and sensory neurons of sciatic nerve express PTPIP51 protein and mRNA. Lee et al. (1997) found CNTF receptors in retinal ganglion cells. Interestingly, M¨arker and colleagues (2008) could show the expression of PTPIP51 protein in retinal ganglion cells of mouse origin. In merging our data and the literature reviewed here, a functional interrelation of both proteins in mouse brain is not unlikely.

The data presented here suggest a function of PTPIP51 in mouse brain tissue distinct from the observations made by Lv and colleagues (2006), who reported that overexpression of PTPIP51 enhances apoptosis in HEK 293 cells. The findings rather point to a role for PTPIP51 as a modulator of the Raf-ERK pathway and interacting partner of PTP1B as well as 14-3-3-y and 14-3-3-β (Jin et al., 2004; Ewing et al., 2007; Yu et al., 2008), thereby influencing signal transduction, transcription, and protein trafficking for the processes discussed above, i.e., neuronal maintenance, neurite outgrowth, and axonal transport (Markus et al., 2002b; Dougherty and Morrison, 2004; Planchamp et al., 2008). Alterations in these pathways lead to multiple neurological disorders, including Alzheimer's and Parkinson's diseases, as well as malignant transformation (Seger and Krebs, 1995; McCubrey et al., 2007; Lyustikman et al., 2008). Further studies are aimed toward investigating the physiological function of PTPIP51 in neurons as well as its putative contribution to the neurological diseases mentioned above.

ACKNOWLEDGMENTS

We thank Hans-Werner Hofer (Department of Biology, Konstanz) for providing us with the antibody PTPIP51, Frank Nürnberger (Institute of Anatomy, Frankfurt) for the kindly gift of the vasopressin antibody and for providing the necessary information for this antibody, Phillip Grant (Institute of Anatomy and Cell Biology, Giessen) for critical reading of the manuscript, and Karola Michael (Institute of Anatomy and Cell Biology, Giessen) for help with the design of the figures.

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PTPIP51 – a multifunctional protein in brain tissue

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Keywords: Hippocampus, Cerebellum, 14-3-3, PTP1B, Neurophysin

Abstract:

Converging lines of evidence indicate Protein tyrosine phosphatase interacting protein 51 (PTPIP51) to be a multifunctional protein. We investigated the interaction of PTPIP51 with Protein tyrosine phosphatase 1B (PTP1B) and 14-3-3 beta in defined brain areas of adult female mice. In Purkinje-cells of the cerebellum PTPIP51 interacts with both proteins, indicating an involvement in nerve growth factor (NGF) signaling which plays an important role in memory processes. PTP1B activity is also known for its influence on neurons to resist the attack of amyloid.

Furthermore we can report an interaction of PTPIP51 with Neurophysin-2 in the paraventricular nucleus, implying its role in vesicle trafficking.

By the additional use of peptide specific antibodies, targeting C-terminus and N-terminus of PTPIP51, we were able to demonstrate different expression profiles, indicating the existence of PTPIP51 isoforms in mouse brain. The expression of those isoforms varied, depending on the region of the brain and the localization within the neuron, supporting the theory of an isoform based broad range of function.

Our results underline the hypothesis of PTPIP51 being a multifunctional protein with special emphasis on nervous tissue, therefore, being potentially involved in different essential cell functions such as axonal growth and axonal transport.

Introduction

Protein tyrosine phosphatase interacting protein 51 (PTPIP51), originally detected by a yeast two hybrid screen in order to identify potential substrates of Protein Tyrosine Phosphatase 1B (PTP1B) and T- cell Protein Tyrosine Phosphatase (TcPTP), has been proven to be expressed in various tissues (Stenzinger et al., 2009). However, up to now little is known about the definite function of PTPIP51 in brain tissue.

Previous studies by Koch et al. 2009 revealed a distinct expression of PTPIP51 in defined brain areas such as cerebellum, hippocampus and the nuclei of hypothalamus. On cellular level strongest PTPIP51 signals were seen in Purkinje cells of the cerebellum, neurons of the hippocampus, paraventricular and supraoptic

nuclei and their axons. Basic studies in brain tissue concerning the functions of PTPIP51 hinted at an involvement in essential cellular processes such as proliferation, differentiation, motility and axonal transport. Immunoblotting of medial neocortex, hippocampus and cerebellum of adult mouse brain revealed putative PTPIP51 isoforms with 30, 34 and 52 kDa (Koch et al., 2009). The existence of PTPIP51 isoforms may provide an explanation for the hypothesized broad spectrum of functions within these different neurons.

Previous in situ studies revealed an interaction of PTPIP51 with PTP1B in different tissues (Brobeil et al., 2010; Bobrich et al., 2011). In gliomas, PTP1B is expressed and interacts with PTPIP51 (Petri et al., in press) and it regulates axonal growth mediated by cell–cell and cell–matrix adhesion molecules (Pathre et al., 2001). Besides PTP1B, PTPIP51 is also known to interact with 14-3-3 beta and 14-3-3 gamma as demonstrated by two independent studies (Jin et al., 2004; Ewing et al., 2007). Furthermore, the work by Petri et al. (in press) could relate the extent of PTPIP51-14-3-3 beta interaction to the grade of glioma malignancy. It is well known that Purkinje cells express several 14-3-3 isoform transcripts during development primarily 14-3-3 beta (Watanabe et al., 1991, 1993a,b, 1994; Umahara et al., 2009).

The suggested role within axonal transport by the observed association of neurophysin and PTPIP51 is supported by the fact that PTPIP51 is alternatively known as regulator of microtubule dynamics protein 3 (RMD-3). RMD-3 belongs to the same protein family as regulator of microtubule dynamics protein 1 (RMD-1). RMD-1 was identified as a member of a new microtubule associated protein (MAP) family. Vesicle trafficking is functionally associated with coiled-coil proteins and PTPIP51 exhibits the same coiled-coil domains as RMD-1 (Gillingham and Munro 2003; Oishi et al., 2007). These facts provide evidence that PTPIP51 is involved in regulatory processes of vesicle trafficking through interactions with the microtubule cytoskeleton, as described before by Brobeil et al. (2010) and Koch et al. (2009).

Based on the above named findings, this study aimed to investigate the expression pattern of potential PTPIP51 isoforms in the brain tissues and the direct interaction of PTPIP51 with 14-3-3 beta, PTP1B and neurophysin II to clarify the function of PTPIP51 in brain tissue.

Materials and Methods

Animals were raised and housed under standard laboratory conditions. All animal experiments were performed in compliance with German law on the use of laboratory animals.

Tissue and section preparations.

Tissue and section preparation was performed as described by Koch et al. (2009). The study was performed with paraffin-embedded samples of mouse brain (n = 9; sex: female, genus C57Bl6J, age: 8-12 weeks), either fixed in Bouin fixative or 4% paraformaldehyde (PFA). For immunohistochemistry the paraffin-embedded brain of each mouse was cut into 6 µm thin sections, dried, deparaffinized in xylene and rehydrated in graded alcohol prior to immunostaining. PTPIP51 positive regions were identified by comparison with mouse brain maps: www.mbl.org/mblmain/atlas.html; www.hms.harvard.edu/research/brain/atlas.html;www.brainmap.org/mouse/atlas.html . Frontal and sagittal vibratome sections of cerebellum and hippocampus (thickness 50 µm) of adult female mice (6-8 weeks) were also performed. For this purpose animals were sacrificed after anaesthesia with pentobarbital and subsequently transcardial perfused and fixed overnight in 4% PFA.

PTPIP51 (aa 131-470) and peptide specific PTPIP51 antibodies

PTPIP51 (aa 131-470) antibody was produced as described previously (Koch et al., 2009). To identify different isoforms of the protein, antibodies against defined peptide sequences of PTPIP51 were used (BioLux, Stuttgart, Germany). The following sequence was used for the identification of the N-terminus: SRLGALGGARAGLGC (exon 2) and the following sequence was used for the identification of the C-terminus: CIQKDLEELEVILRD (exon 13). Identity and purity of each synthetized peptide were approved by ESI-MS and UV-analysis. Rabbits were immunized with the KLH-conjugated peptides. The specificity of each antibody was tested by ELISA and

Western blotTo verify the use of these peptide specific antibodies for immunostaining preabsorption experiments were performed (Brobeil et al., 2010). see Figure 1.

Immunoblotting

Brain tissue samples, derived from medial neocortex, hippocampus, and cerebellum were lysed and separated on 10% SDS-PAGE. According to Towbin et al. (1979) the transfer on an Immobilon P membrane (Millipore, Bedford, CA) was performed. For membrane blocking 10% fat-free milk powder in PBS was used and subsequently incubated for 1 hour. Incubation with either the PTPIP51 (aa 131-470) or the peptide specific PTPIP51 antibodies was done overnight at 4°C and washed three times with TBST for 5 min. The secondary antibody alkaline phosphatase-conjugated anti-rabbit were applied for 1 hour at room temperature, and the reaction was visualized with the SigmaFast BCIP/NBT substrate. For calibration, a prestained molecular weight marker (Biorad, Cat# 161-0374) was used (Stenzinger et al., 2005; Brobeil et al., 2010). Immunoblotting was performed four times (n=4).

Immunohistochemistry

Immunohistochemistry was performed as previously described by Koch et al. (2009). Prior to immunostaining nonspecific binding sites were blocked with 0.1 M phosphate buffered saline (PBS, pH 7.4) containing 5% bovine serum albumin and 5% normal goat serum. Samples were incubated overnight at room temperature with primary antibodies (see table 1) diluted in PBS, followed by washing in PBS (three time for 10 min) and subsequent incubation for 1 h at room temperature with the respective secondary antibodies (see table 1). The slides were washed in PBS, coverslipped in carbonate buffered glycerol at pH 8.6 and evaluated either by epifluorescence microscopy or by sequential confocal laser scanning microscopy.

PTPIP51 (aa131-470) and peptide specific PTPIP51 antibodies were visualized either by Alexa Fluor 555 secondary antibody or FITC anti-rabbit. Anti-mouse antibodies used for double staining were visualized by using Alexa Fluor 488 secondary antibody. The primary monoclonal anti-goat antibody neurophysin II used

for identification of axonal transport, was visualized using Cy3 donkey anti-goat as secondary antibody in combination with FITC anti-rabbit as secondary antibody for PTPIP51 visualization. Nuclei were displayed through Dapi. Every immunostaining was performed at least 15 (n=15) times to verify the results.

Duolink proximity ligation assay (DPLA)

Interaction of PTPIP51 with PTP1B, 14-3-3 beta and Neurophysin II was verified by proximity ligation assay kit Duolink II (PLA probe anti-rabbit minus, Cat.# 90602; PLA probe anti-mouse plus, Cat.# 90701; Dection Kit 563, Cat.# 90134). The DuoLink proximity ligation assay is based on the principle that proteins, localized within a certain on-site proximity to each other, precisely 40nm or less (Fredriksson S., 2009), perform protein-protein-interactions. To start the procedure, specimens are incubated with primary, antibodies from different species against the proteins that are investigated based on previously assumed interaction. Primary antibodies are recognized by species-specific PLA probes. PLA probes consist of antibodies, coupled with a DNA strand in either plus or minus orientation. Given that the distance between the antibodies is equal to or less than 40nm, the DNA strands can be hybridized using connector oligonucleotides. The connector oligonucleotides are subsequently connected by enzymatic ligation to form a circular template, which, after a multitude of amplifications, can be detected by a fluorophore labelled complementary oligonucleotide.

In this study we used PLA probe anti-rabbit minus, binding the primary PTPIP51(aa131-470) antibody, PLA probe anti-mouse plus, binding the primary PTP1B and 14-3-3 antibody and PLA probe anti-goat minus, binding the primary neurophysin II antibody. Methanol-fixed air-dried samples were pre-incubated with blocking agent for 1h. After washing in PBS for 10 min, primary antibodies to PTPIP51 (1:1000) and PTP1B (1:500) or 14-3-3 beta (1:100) and Neurophysin II (1:1000) were applied to the samples. Incubation was done overnight in a pre-heated humidity chamber at 37°C. Slides were washed three times in PBS for 10 min. Duolink PLA probes detecting rabbit or mouse antibodies were diluted in the blocking agent in a concentration of 1:5 and applied to the slides followed by incubation for 2h in a pre-heated humidity chamber at 37°C. Unbound PLA probes were removed by

washing three times in PBS for 10 min. For hybridization of the two Duolink PLA probes Duolink Hybridization stock was diluted 1:5 in high purity water and slides were incubated in a pre-heated humidity chamber for 15 min at 37°C. The slides were washed in TBS-T for 1 min under gentle agitation. The samples were then incubated in the ligation solution consisting of Duolink Ligation stock (1:5) and Duolink Ligase (1:40), diluted in high purity water, for 90 min at 37°C. Detection of the amplified probe was done with the Duolink Detection kit. Duolink Detection stock was diluted 1:5 in high purity water and applied for 1 h at 37°C. Final washing steps were done by SCC buffer and 70% ethanol. Every duolink proximity ligation assay was performed 5 times (n=5) to verify an interaction.

Epifluorescence microscopy

The Axioplan 2 fluorescence microscope equipped with Plan-Apochromat objectives (Carl Zeiss Jena, Germany) was used for photo documentation. For visualization of the secondary antibody Alexa Fluor 555 an excitation filter with a spectrum of 530-560nm and an emission filter with a spectrum 572-647nm were used. Alexa Fluor 488 was visualized by an excitation filter with a range of 460-500nm and an emission filter with a range of 512-542 nm.

Confocal laser scanning microscopy and 3D reconstruction.

Confocal microscopy was performed with a Leica TCS SP2 AOBS laser scanning microscope equipped with a Leica HCX PL APO CD 63x oil immersion lens (NA 1.4). Detector gain and amplifier offset were initially set to obtain pixel densities within a linear range. The confocal orthographic projections were done using Imaris software (Zürich, Switzerland). Confocal images were exported from the Leica software and stored as TIFF files. Figures were prepared in Photoshop CS2 (Adobe, San Jose, CA). Image brightness and contrast were adjusted

Results

This study demonstrates the expression pattern of PTPIP51 and supposed PTPIP51-isoforms, identified by peptide specific antibodies, in neurons from the hippocampus, cerebellum and paraventricular nucleus as well as the subcellular localization in the adult mouse brain. Furthermore, the direct *in situ* interaction of PTPIP51 with 14-3-3 beta, PTP1B and neurophysin II in brain was substantiated.

Immunoblotting of specific mouse brain regions

Immunoblotting experiments with samples from various regions of adult mouse brain (hippocampus, medial neocortex, cerebellum) revealed different molecular weights of 30, 34 and 52 kDa in all three regions, identified by the PTPIP51 (aa 131-470) antibody (Figure 2A). Using a C-terminus specific antibody, immunoblotting revealed different expression patterns in the three brain regions, with molecular weight of 70 kDa in hippocampus (Figure 2B1), 38 and 70 kDa in medial cortex (Figure 2B2) and 38, 65 and 70 kDa in cerebellum (Figure 2B3). As shown in Figure 2C, no region specific differences were recognized by the N-terminus specific antibody. All three investigated regions showed 3 bands with molecular weights of 25, 30 and 34 kDa, except the hippocampal tissue, which in addition displayed a 45 kDa band (Figure 2C1).

Immunostaining of PTPIP51 by peptide specific antibodies in mouse brain areas

To detect the region specific expression profile of PTPIP51, immunostainings, using the PTPIP51 (aa 131-470) and the peptide specific antibodies, were employed.

Hippocampus

The soma and the dendrites of pyramidal cells in hippocampus displayed positive reaction to the PTPIP51 (aa 131 – 470) antibody (Figure 3A-B), whereas staining against the C-terminus was restricted to soma (Figure 3C-D) and staining against the N-terminus was intense in the soma and dendrites (Figure 3E-F).

Cerebellum.

The soma and the dendrites of Purkinje cells in cerebellum displayed positive reaction to the PTPIP51 (aa 131 – 470) antibody (Figure 4A-B), whereas staining against the C-terminus was restricted to the perinuclear area (Figure 4C-D) and staining against the N-terminus was intense in the soma and dendrites (Figure 4E-F).

Subcellular localization of PTPIP51 (aa 131-470) in neurons of the cerebellum by confocal 3D reconstruction

Co-immunostaining of PTPIP51 (aa131-470) and antibodies identifying either Golgi apparatus, mitochondria or endoplasmic reticulum (ER) showed that PTPIP51 was neither associated with Golgi apparatus nor with mitochondria (Figure 5A,B). In addition, a partial colocalization of PTPIP51 (aa131-470) with the endoplasmic reticulum was observed (Figure 5C).

Subcellular localization of specific PTPIP51 peptides in neurons of hippocampus by confocal 3D reconstruction

Co-immunostaining of peptide specific PTPIP51 and antibodies identifying either Golgi apparatus, mitochondria or ER showed that the N-terminus specific peptide was not associated to the ER (Figure 6F), whereas a clear, yet only punctual, colocalization of the C- terminus specific peptide and the endoplasmic reticulum was found (Fig 5 C). In addition, a partial colocalization of PTPIP51 C- and N-terminus specific peptides with the Golgi apparatus was found (Figure 6A,D), interestingly not in mitochondria (Figure 6B,E).

Co- localization of PTPIP51 (aa 131- 470) and interacting partners in Purkinje cells of the cerebellum by confocal 3D reconstruction

Co-immunostudies of PTPIP51 (aa131-470) and either PTP1B and 14-3-3 beta showed a co- localization of PTPIP51 (aa131-470) with PTP1B and 14-3-3 beta (Figure 7) in Purkinje cells of cerebellum.

Interaction of PTPIP51 (aa 131- 470) with PTP1B and 14-3-3 beta in Purkinje cells of the cerebellum and neurophysin II in neurons of the paraventricular nucleus by Duolink proximity ligation assay (DPLA)

To confirm an interaction of PTPIP51 (aa131-470) with PTP1B and 14-3-3 beta a Duolink proximity ligation assay was performed with cerebellum of adult mouse. In Purkinje cells of all investigated samples PTPIP51 (aa131-470) was interacting with PTP1B as well as with 14-3-3 beta, as evidenced by a positive DPLA signal (fluorescence dots). Every dot corresponds to an interaction between PTPIP51 and PTP1B (Figure 8A), or as seen in Figure 8B an interaction between PTPIP51 and 14-3-3 beta.

To confirm an interaction of PTPIP51 and neurophysin II a Duolink proximity ligation assay was performed with the paraventricular nucleus of adult mouse. Every dot corresponds to an interaction between PTPIP51 and neurophysin II (Figure 8C)

Discussion:

PTPIP51 was described as a multifunctional protein (Brobeil et al., 2011a). Preceding studies of PTPIP51 expression in various tissues hinted to an involvement in essential cellular processes such as proliferation, differentiation, migration and apoptosis (Koch et al., 2009).

Here we present data that strongly suggests the expression of different PTPIP51 isoforms on cellular and subcellular levels in the central nervous system. In addition the interaction of PTPIP51 with 14-3-3 beta and PTP1B were analyzed to clarify the involvement of PTPIP51 in signaling processes within the nervous system.

Preliminary studies (Koch et al., 2009) reported the expression of PTPIP51 proteins with molecular weights of 30, 34, and 52 kDa in the medial neocortex, hippocampus and cerebellum indicating the prevalence of various PTPIP51 isoforms. Initiation of mRNA translation requires an AUG triplet with a specific surrounding sequence (GCCRCCCaugG - R stands for purine bases) as already described by Kozak (2005). Scanning the coding open reading frame (ORF) of PTPIP51 for internal start

codons displayed six additional AUG triplets (Brobeil et al., 2011a). Using alternative initiation codons calculated molecular weights of 45, 38 and 30 kDa were found (ProteinCaclculator v3, 3 http:// www.scripps.edu/-edputnam/protcalc.html; Stenzinger et al.2005).

Another mechanism that is suitable to explain the existence of additional PTIPIP51 isoforms is alternative splicing, a process, which is well known to create protein diversity (Nilsen and Gravely, 2010).

Katz et al. (2010) claim that multiple isoforms can often differ in their function. The differential expression of PTPIP51 isoforms could be the basis for the proposed multiple functions associated with their localization in the brain.

Immunoblotting of the cerebellum, hippocampus and cortex with the peptide specific antibodies confirmed these results.

The identified different molecular forms corresponded to calculated isoforms based on the leaky scanning mechanism, representing molecular weights of 52, 45, 38 and 30 kDa. Corresponding weights were seen in muscle tissue (52 kDa; Barop et al., 2009), in human HL-60 cell line representing acute promyelocytic leukemia (52, 45 and 38 kDa; Brobeil et al., 2011b) and in embryonic tissue (30 kDa; Brobeil et al., 2011a).

On subcellular level PTPIP51 antibodies revealed a heterogenous expression pattern.

The soma of the Purkinje cells and Pyramidal cells reacted positive for PTPIP51 (aa131-470) as well as for the C- and N- terminal specific antibodies. No C-terminal comprising isoform was detected in the dendrites. This suggests to a different function of PTPIP51 within the soma compared to the dendrite.

The C- and N- terminal specific peptides were associated with the Golgi- apparatus, which is involved in dendritic and axonal morphogenesis (Rosso et al., 2004; Tanabe et al., 2010). Additionally, the C- terminal specific peptide showed a colocalization with the ER.

PTP1B, an ER-bound protein tyrosine phosphatase, is known as a regulator of axonal growth, which is mediated by cell– cell and cell–matrix adhesion molecules

(Pathre et al., 2001). In Purkinje cells PTP1B was co-localized with PTPIP51, indicating an interaction of both proteins and it was corroborated by the duolink proximity ligation assay. Fuentes and Arregui (2009) showed an involvement of PTP1B in controlling growth cone dynamics and axon elongation in neurons. Furthermore, PTP1B plays an important role in nerve growth factor (NGF) signaling pathway, which has been reported to have the potential to elongate neurites, to prevent apoptosis and on functional level to influence memory processes (Chao et al., 2006; Shimoke et al., 2011). Intracellular, NGF is associated to ER and Golgi-apparatus (Blöchl et al., 1996). This resembles the expression pattern of PTPIP51 C-terminus. The constitutive secretion of NGF is confined to the neural soma and very proximal parts of the dendrites (Blöchl et al., 1996), underlying the colocalization with the C- terminus of PTPIP51. Dendrite patterning in hippocampal neurons is controlled by NGF, which activates the Src kinase, needed for NF-k-B activation and Hes1 expression (Chacón et al., 2010). In the context of Src-kinase-signaling, PTPIP51 has been shown to be an in vitro substrate of c-Src (Stenzinger et al., 2009).

Additional to its role in neurite outgrowth, PTP1B activity is also needed for neuron survival, assisting hippocampal neurons resist the attack of amyloid (Chacón et al., 2011). Binding of NGF to neutrophin receptor p75 (NTR) increases the enzymatic activity of PTP1B. Yet, the mechanism of this activation is still unknown (Chacón et al., 2011) and may involve PTPIP51.

The 14-3-3-protein superfamily comprises seven isoforms, which have been suggested to play a role in neuronal proliferation, migration and differentiation of the rat cerebellum (Umahara et al., 2009).

PTPIP51 interacted with 14-3-3 beta in the soma of Purkinje cells. As reported by Yu et al. (2008), PTPIP51 also interacts with Raf-1, an interaction that is mediated by 14-3-3 beta, thereby modulating the activity of the MAPK- cascade. Interestingly, NGF signaling is as well linked to the MAPK pathway (Xing et al., 1998). NGFs are important for memory processes, which are specifically affected in Alzheimer's disease (Chao et al., 2006; Aggleton et al., 2010). Since PTPIP51 is strongly expressed in the hippocampus area (Koch et al., 2009) and thus could be involved in memory process by affecting NGF signaling.

Previous studies revealed a colocalization of PTPIP51 with vasopressin and its carrier neurophysin II, suggesting a participation in axonal transport processes (Koch et al., 2009). By proving a direct interaction of PTPIP51 with the carrier protein neurophysin II in neurons of the paraventricular nucleus, we were able to verify this theory.

Lv et al. (2006) described PTPIP51 as a mitochondrial associated protein and inducer of apoptosis in HEK293T and HeLa cell lines. Since no colocalization of either PTPIP51 peptides with mitochondria was observed, a role in apoptosis does not appear to be the standout-function of PTPIP51 in brain tissue.

In summary, PTPIP51 is implied in the progression of dendritic and axonal growth, as well as axonal transport. PTPIP51 exerts these functions through interaction with key molecules PTP1B, 14-3-3 beta and Neurophysin ll. An additional function of PTPIP51 could be the modulation of NGF-signaling, therefore participating in memory processes and possibly being involved in the genesis of Alzheimer's disease. This functional diversity is probably based on the expression of multiple PTPIP51 isoforms and is strongly supported by the data presented here. In addition the multitude of such different functions can be explained by the expression of various isoforms and their different subcellular localization.

Following this, further experiments have to be performed to investigate the function of PTPIP51 in brain and thereby its potential involvement in Morbus Alzheimer or other pathological entities of the brain.

Acknowledgements

We are grateful to Mrs. C.Tag (Institute of Anatomy and Cell Biology, Giessen), for her excellent technical assistance.

Dedication

This publication is dedicated to Mr. Hans Werner Hofer who promoted the research on PTPIP51 with all his energy. He died much too early († 18.05.2011).

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Legends

Figure 1 Aminoacid sequence and antibody description

PTPIP51(aa1-470): Aminoacid sequence based on the coding exon sequence 2- 13. PTPIP51(131-470): antibody target sequence highlighted in black. PTPIP51(2-14): antibody target sequence highlighted in black. PTPIP51(446-470): antibody target sequence highlighted in black.

Figure 2 Immunoblotting of PTPIP51 (aa 131-470) and peptide specific antibodies in different regions of mouse brain.

1: hippocampus, 2: medial neocortex, 3: cerebellum.

A: Immunoblot probed with the PTPIP51 (aa 131-470) antibody. B: Immunoblot probed with the C-terminus specific antibody. C: Immunoblot probed with the N-terminus specific antibody.

Figure 3 Immunostaining of PTPIP51 (aa 131-470) and peptide specific antibodies in hippocampus

A,B) PTPIP51 (aa 131-470) staining in hippocampus. C,D) C-terminus specific antibody staining in hippocampus. E,F) N-terminus specific antibody staining in hippocampus. Bar: 20µm. *arrow:* dendrites.

Figure 4 Immunostaining of PTPIP51 (aa 131-470) and peptide specific antibodies in cerebellum

A,B) PTPIP51 (aa 131-470) staining in cerebellum. C,D) C-terminus specific antibody staining in cerebellum. E,F) N-terminus specific antibody staining in cerebellum. Bar: 20µm. *arrow:* dendrites.

Figure 5 Subcellular localization of PTPIP51 (aa 131-470) in neurons of the cerebellum analyzed by CLSM

A) Overlay of golgin, identifying Golgi apparatus and PTPIP51 (aa131-470). B) Overlay of SDH identifying mitochondria and PTPIP51 (aa131-470). C) Overlay PDI identifying endoplasmic reticulum and PTPIP51 (aa131-470). C1) Overlay, plane YZ. C2) Overlay, plane XZ. Bar: 3 µm.

Figure 6 Subcellular localization of PTPIP51 with peptide specific antibodies in neurons of the hippocampus analyzed by CLSM

A) Overlay of Golgin identifying Golgi apparatus and C-terminus specific antibody. A1) Overlay, plane YZ. A2) Overlay, plane XY. B) Overlay of SDH identifying mitochondria and C-terminus specific antibody. C) Overlay of PDI identifying endoplasmic reticulum and C-terminus specific antibody. C1) Overlay, plane YZ. C2) Overlay, plane XY. D) Overlay of Golgin and N-terminus specific antibody. D1) Overlay, plane YZ. D2) Overlay, plane XY. E) Overlay of SDH and PTPIP51 N-terminus specific antibody. F) Overlay of PDI and N-terminus specific antibody. Bar: 5 µm. arrows: co- localization points

Figure 7 Co-immunostaining of PTPIP51 (aa131-470) with PTP1B and 14-3-3 beta, in Purkinje cells of the cerebellum

A) PTP1B immunostaining. B) PTPIP51 (aa131-470) immunostaining. C) Overlay of PTPIP51 (aa131-470) and PTP1B. D) 14-3-3 beta immunostaining E) PTPIP51 (aa131-470) immunstaining. F) Overlay of PTPIP51 (aa131-470) and 14-3-3 beta. A-C Bar: 5 µm. D-F Bar: 10µm.

Figure 8 Duolink proximity ligation assay for PTPIP51 (aa 131-470) with PTP1B and 14-3-3 beta in Purkinje cells of the cerebellum and with Neurophysin II in neurons of the paraventricular nucleus (Interaction of both proteins is seen as dots).

A) PTPIP51 (aa 131-470) and 14-3-3 beta. B) PTPIP51 (aa 131-470) and PTP1B C) PTPIP51 (aa 131-470) and Neurophysin II.

Table 1: List of antibodies used in this study.

	Immunogen	Antibody Source	Clone	Dilution	Manufacturer
PTPIP51	Human recombinant PTPIP51 encoding amino acids (aa) 131-470	Rabbit polyclonal		1:400	Prof. HW Hofer, Biochemical Department, University Konstanz, Germany
PTPIP51 C-Terminus	Purified total IgG fraction CIQKDLEELEVILRD	Rabbit polyclonal		1:1000	BioLux, Stuttgart, Germany
PTPIP51 N-Terminus	Purified total IgG fraction SRLGALGGARAGLGC	Rabbit polyclonal		1:1000	
Anti PTP1B	Human recombinant protein tyrosine phosphatise 1B (PTP1B)	Mouse monoclonal	AE4-2J	1:40	CALBIOCHEM Germany Cat# PHO2
14-3-3 beta	Epitope mapping the C-terminus of human origin	Mouse monoclonal	A-6	1:100	Santa Cruz Cat# sc-25276
Neurophysin II	raised against a peptide mapping near the C-terminus of Neurophysin II of mous origin	Goat polyclonal	V-15	1:1000	SantaCruz USA Cat# sc-27093
anti-PDI	Recombinant full length protein (Rat)	Mouse monoclonal	RL77	1:50	Abcam Cat# ab5484
anti-Golgin-97	recognize the 97 kDa form of the golgin protein	Mouse monoclonal	CDF4	1:250	MoBiTeC Cat# A-21270
SDHB	30 kDa subunit	Mouse monoclonal	21A11	1:100	Invitrogen Cat# 518471
Secondary antibodies					
Alexa fluor 555	IgG heavy chains from rabbit	Goat		1:800	Invitrogen Cat# A21428
Alexa fluor 488	IgG heavy chains from mouse	Goat		1:800	Invitrogen Cat# A11029
FITC anti rabbit		Goat		1:400	Cappel Cat# 55651
Cy3 donkey anti-goat	IgG from donkey	Donkey		1:400	Chemicon Cat# AP180C

Figure 1

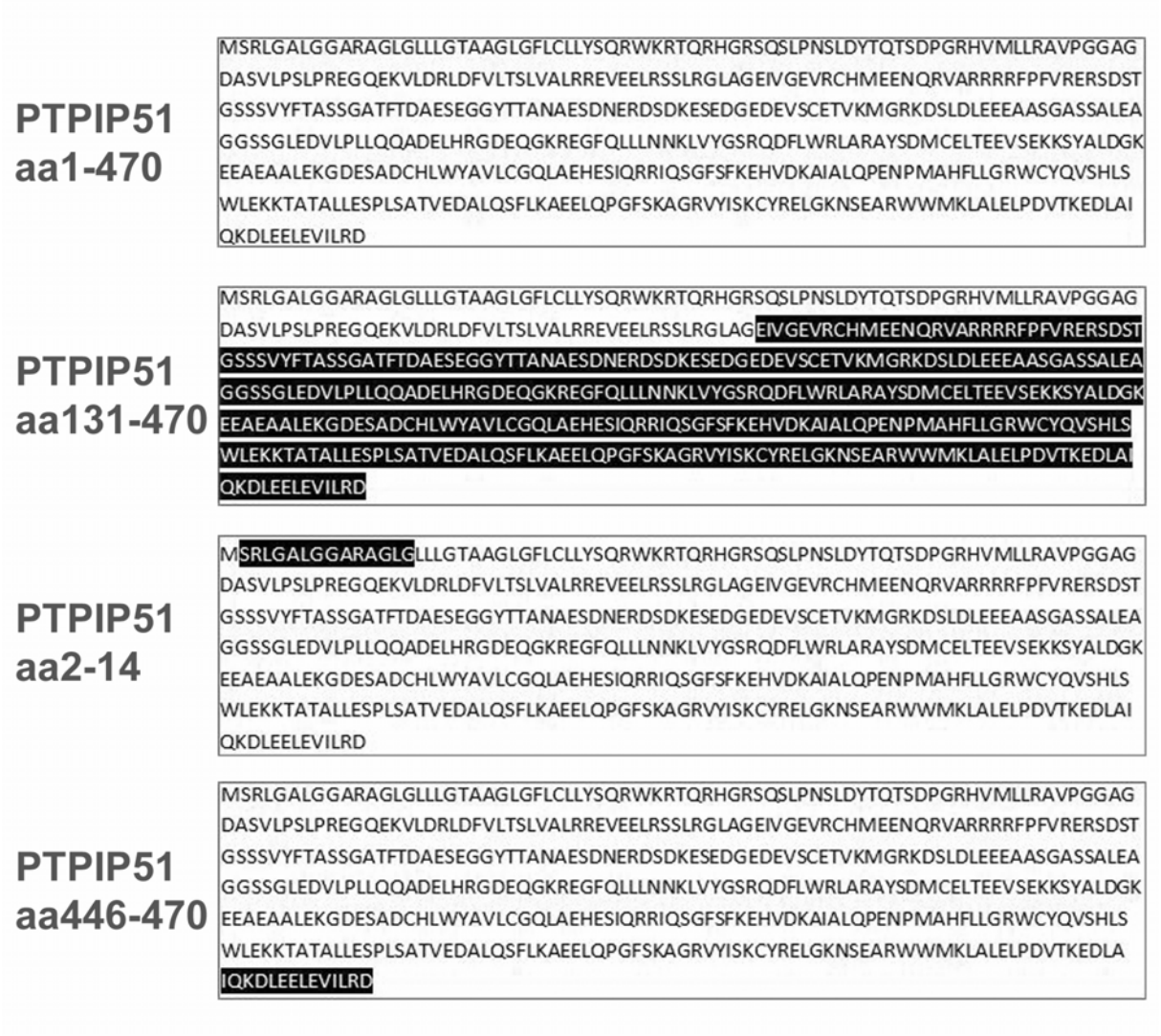

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Figure 2

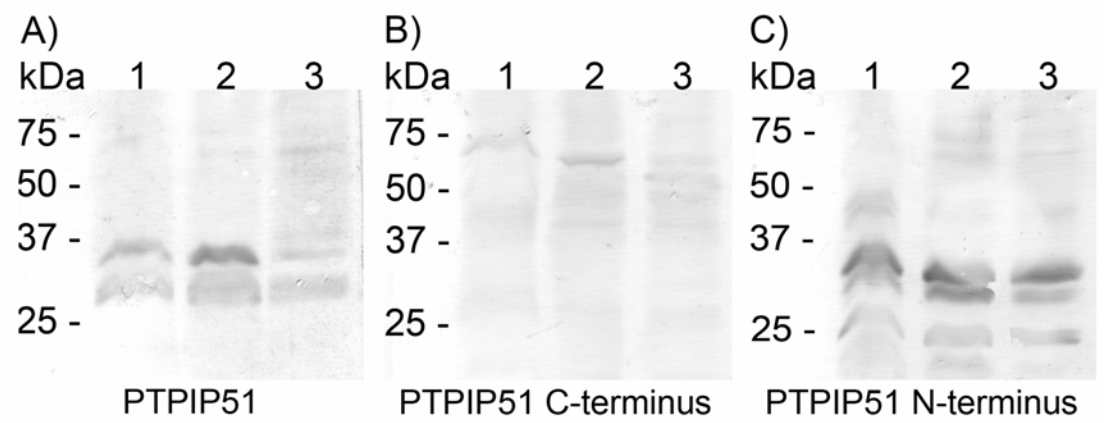

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Figure 3

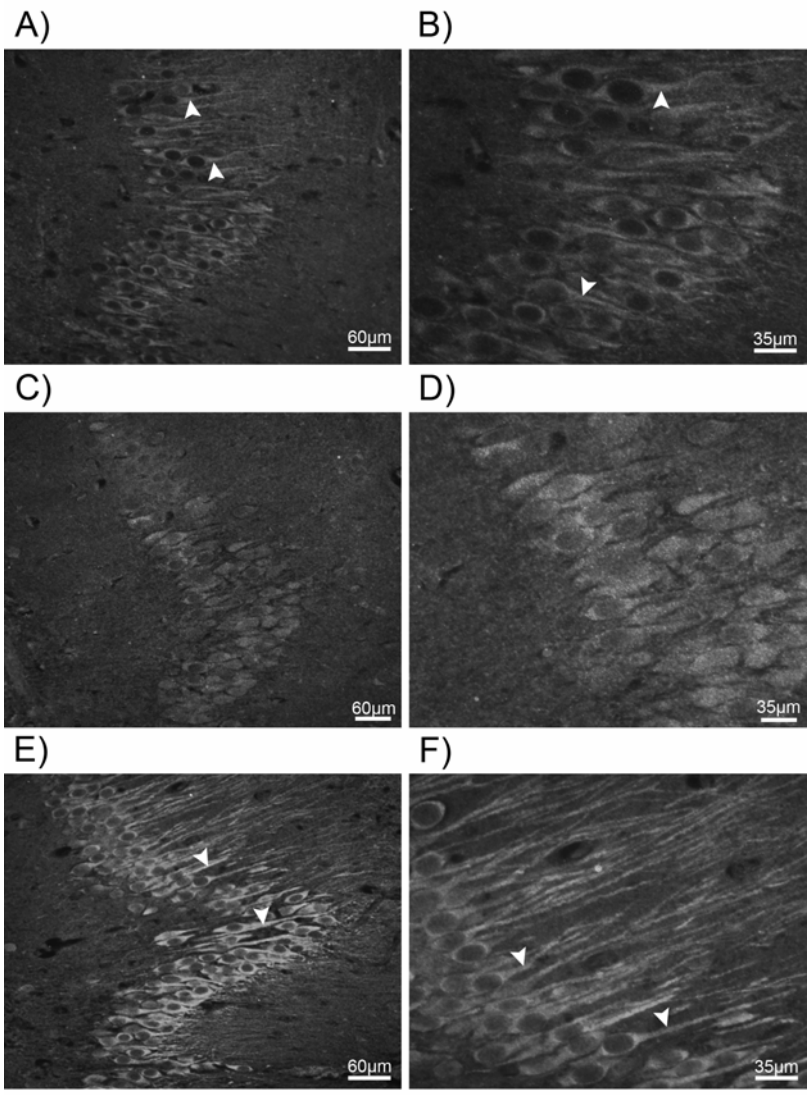

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Figure 4

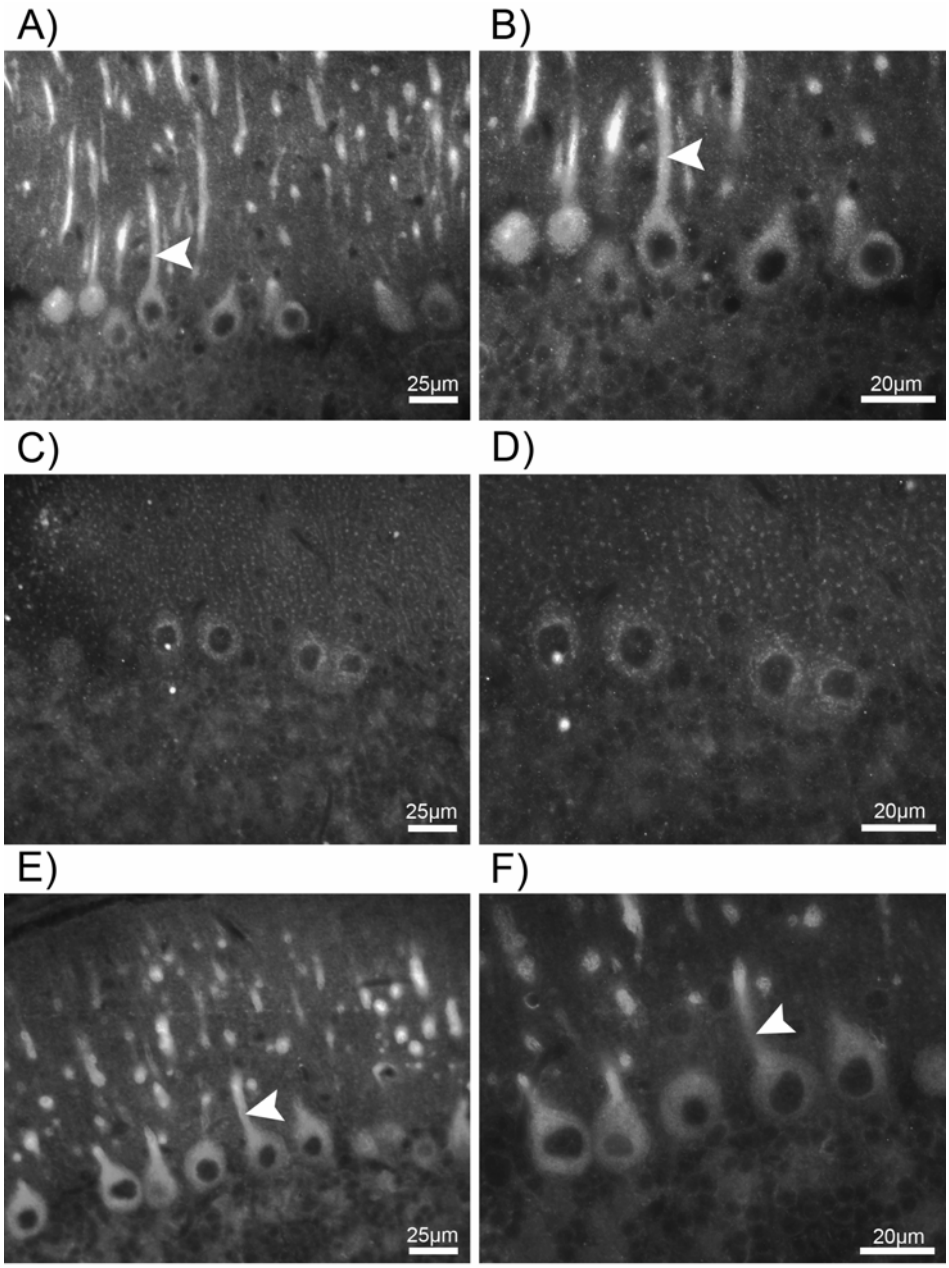

Figure 4 Immunostaining of PTPIP51 (aa 131-470) and peptide specific antibodies in cerebellum

A,B) PTPIP51 (aa 131-470) staining in cerebellum. C,D) C-terminus specific antibody staining in cerebellum. E,F) N-terminus specific antibody staining in cerebellum. Bar: 20µm. *arrow:* dendrites.

Figure 5

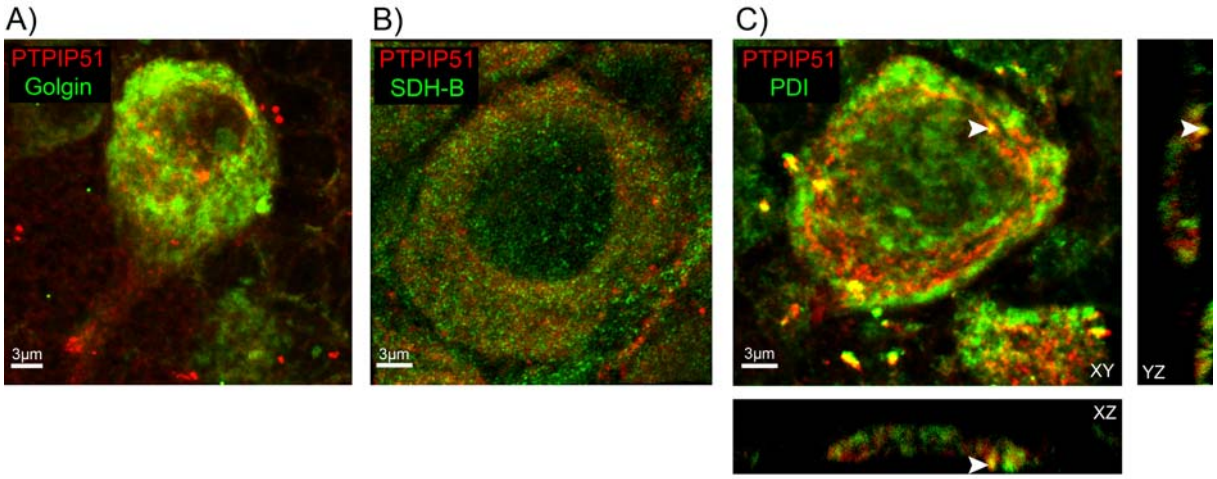

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Figure 6

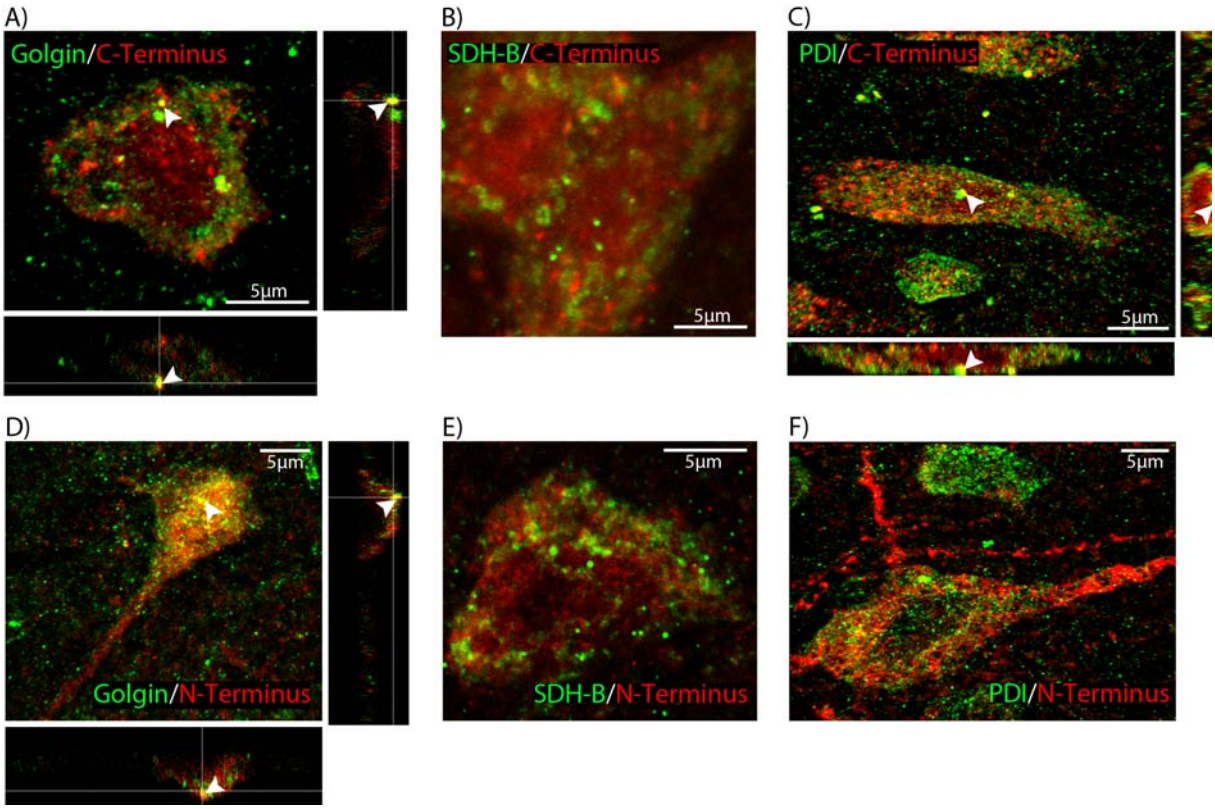

Figure 6 Subcellular localization of PTPIP51 with peptide specific antibodies in neurons of the hippocampus analyzed by CLSM

A) Overlay of Golgin identifying Golgi apparatus and C-terminus specific antibody. A1) Overlay, plane YZ. A2) Overlay, plane XY. B) Overlay of SDH identifying mitochondria and C-terminus specific antibody. C) Overlay of PDI identifying endoplasmic reticulum and C-terminus specific antibody. C1) Overlay, plane YZ. C2) Overlay, plane XY. D) Overlay of Golgin and N-terminus specific antibody. D1) Overlay, plane YZ. D2) Overlay, plane XY. E) Overlay of SDH and PTPIP51 N-terminus specific antibody. F) Overlay of PDI and N-terminus specific antibody. Bar: 5 µm. arrows: co- localization points

Figure 7

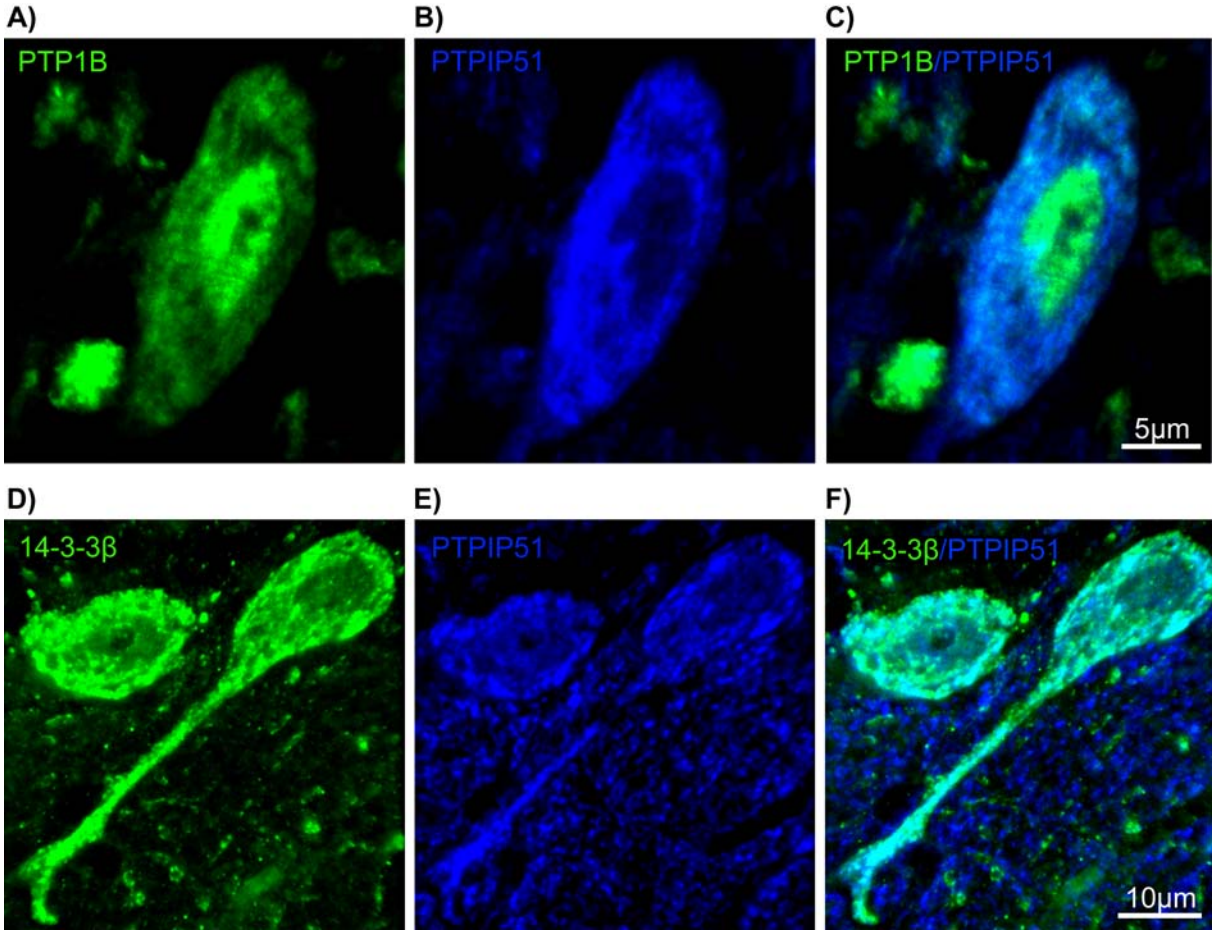

Figure 7 Co-immunostaining of PTPIP51 (aa131-470) with PTP1B and 14-3-3 beta, in Purkinje cells of the cerebellum

A) PTP1B immunostaining. B) PTPIP51 (aa131-470) immunostaining. C) Overlay of PTPIP51 (aa131-470) and PTP1B. D) 14-3-3 beta immunostaining E) PTPIP51 (aa131-470) immunstaining. F) Overlay of PTPIP51 (aa131-470) and 14-3-3 beta. A-C Bar: 5 µm. D-F Bar: 10µm.

Figure 8

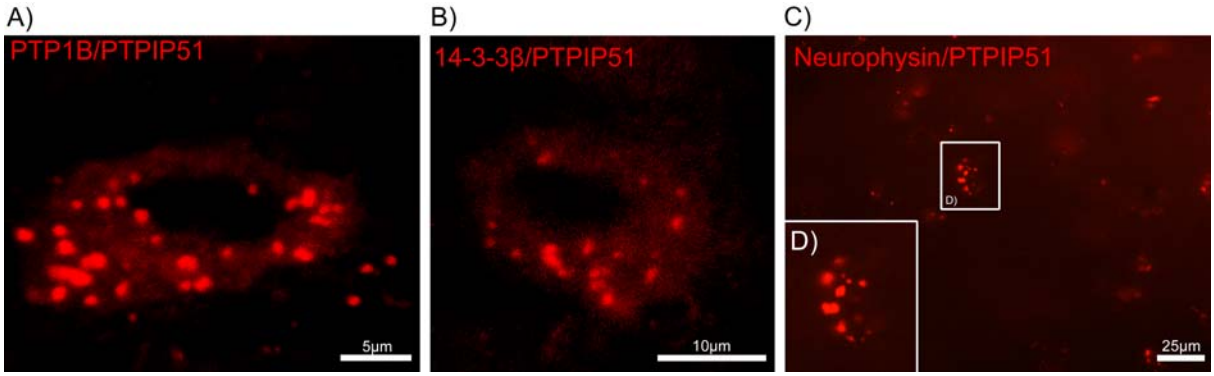

Figure 8 Duolink proximity ligation assay for PTPIP51 (aa 131-470) with PTP1B and 14-3-3 beta in Purkinje cells of the cerebellum and with Neurophysin II in neurons of the paraventricular nucleus (Interaction of both proteins is seen as dots).

PTPIP51, a positive modulator of the MAPK/Erk pathway, is upregulated in glioblastoma and interacts with 14-3-3β and PTP1B *in situ*.

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Running title: PTPIP51, 14-3-3β and PTP1B in glioblastoma

Keywords: glioblastoma, PTPIP51, 14-3-3β, EGFR, MAPK, Raf-1, glioma

Abstract

Glioblastoma multiforme (GBM) is the most common and most malignant primary brain tumour. Protein tyrosine phosphatase interacting protein 51 (PTPIP51) is an interaction partner of 14-3-3β, which correlates with the grade of malignancy in gliomas. In this study PTPIP51 and its interacting partners 14-3-3β, PTP1B, c-Src, Raf-1 as well as EGFR were investigated in human glioblastoma.

Twenty glioblastoma samples were analyzed on transcriptional and translational level by immunohistochemistry, *in situ* hybridization and RT-PCR. To compare PTPIP51 expression in gliomas of different malignancies, quantitative RT-PCR for grade II astrocytoma and GBM samples was employed. Additionally, we analyzed the correlation between PTPIP51 and 14-3-3β transcription, and checked for *in situ* interaction between PTPIP51 and 14-3-3β and PTP1B, respectively.

PTPIP51 and 14-3-3β mRNA showed a tumour grade dependent upregulation in gliomas. Glioblastoma cells displayed a strong immunoreaction of PTPIP51, which co-localized with 14-3-3β and PTP1B. The duolink proximity ligation assay corroborated a direct *in situ* interaction of PTPIP51 with both proteins, known to interact with PTPIP51 *in vitro*. The *in vitro* interacting partners Raf-1 and c-Src showed a partial co-localization. Besides, immune cells located in capillaries or infiltrating the tumour tissue and endothelial cells of pseudoglomerular vessels revealed a high PTPIP51 expression.

The upregulation of PTPIP51 and its connection with the EGFR/MAPK pathway by 14-3-3β via Raf-1 and by PTP1B via c-Src, argue for a functional role of PTPIP51 in the pathogenesis of human glioblastoma.

Introduction

The incidence of gliomas is increasing worldwide. 18,820 new cases of human primary central nervous system tumours are diagnosed annually in the United States of America, about 60% are gliomas and 30-40% of these account for glioblastoma (GBM) (Khan et al., 2009). GBM is the most frequent and most malignant form of

neuroepithelial tumour. The mean survival time of GBM patients is still around one year, despite significant advances in therapeutic options (McLendon et al., 2007).

PTPIP51 is a novel protein that has been shown to be expressed in many human cancers (Lv et al., 2006; Stenzinger et al., 2009). As demonstrated by Koch et al. (2008; 2009b) PTPIP51 is expressed in human keratinocyte carcinomas and prostate cancer. Comparing benign prostate hyperplasia with prostate carcinoma provided evidence that PTPIP51 expression is partially controlled by promoter methylation. Lv et al. (2006) demonstrated PTPIP51 mRNA-expression in various carcinomas.
PTPIP51 is evolutionary conserved and was shown to interact *in vitro* with the non-transmembrane protein-tyrosine phosphatase, Protein Tyrosine Phosphatase 1B (PTP1B) (Stenzinger et al., 2005; Stenzinger et al., 2009). The protein is phosphorylated *in vitro* and *in situ* at Tyr176 by Src kinase and dephosphorylated by PTP1B (Stenzinger et al., 2009). In HEK 293 and HeLa cells, PTPIP51 overexpression was shown to enhance apoptosis (Lv et al., 2006).

Recently, two independent studies by Jin et al. (2004) and Ewing et al. (2007) demonstrated the interaction between the two isoforms 14-3-3β, 14-3-3γ and PTPIP51. The study of Yang et al. (2009) demonstrated a correlation between the grade of malignancy and the expression of 14-3-3β and 14-3-3η in gliomas. In contrast, normal brain tissue was not found to express these two 14-3-3 isoforms. The isoforms β and η belong to a superfamily of 14-3-3 proteins, which are differentially expressed in many human tissues. 14-3-3 proteins have been implicated in numerous cellular processes, such as tumourigenesis, cell cycle control and apoptosis (Cao et al., 2008).

Yu and co-workers (2008) reported PTPIP51 to interact with Raf-1 through 14-3-3 protein, thereby modulating cellular motility and morphology by the mitogen activated protein kinase (MAPK) cascade. The MAPK/Erk pathway is involved in a variety of cellular functions such as growth, proliferation, differentiation, migration and apoptosis. This pathway has been extensively studied in glioblastoma cells (Lopez-Gines et al., 2008). The data available so far consider PTPIP51 to play a role in cellular differentiation, motility, cytoskeleton formation and apoptosis.
This study primarily aimed to investigate the PTPIP51-expression profile in GBM, applying RT-PCR, quantitative real time PCR, *in situ* hybridization and

immunohistochemistry. Moreover, cell specific co-expression of the proteins 14-3-3β, Raf-1, PTP1B, c-Src and EGFR were assessed at the mRNA and protein level. Furthermore, the direct interaction of PTPIP51 with 14-3-3β and PTP1B *in situ* was substantiated.

An earlier study by Koch et al. (2009a) displayed the expression of PTPIP51 to be restricted to neurons in specific areas of normal mouse brain and glial cells did not show an expression of PTPIP51.

Data obtained in the present study will set the base for further studies that aim to investigate the putative role of PTPIP51 in glioblastoma formation.

Material and Methods

Samples of twenty glioblastoma cases and samples of four astrocytomas were included in this study (Table 1).

Glioblastoma specimens were stained immunohistochemically for PTPIP51, 14-3-3β, c-Src, Raf-1, PTP1B, EGFR, apoptosis (TUNEL) and proliferation (Ki67). The cell specific expression of PTPIP51 was corroborated by means of *in situ* hybridization.

Four intraoperatively obtained tissue samples of primary glioblastoma and one of recurrent glioblastoma were examined for mRNA expression of PTPIP51, 14-3-3β, c-Src, Raf-1 and PTP1B. Moreover, to obtain quantitative data on the transcriptional activity of the 14-3-3 β and PTPIP51 gene in grade II astrocytoma (n=4 paraffin embedded samples) and glioblastoma (n= 5 paraffin embedded samples) kinetic PCR was employed.

Immunohistochemistry: The tumour tissue was fixed in neutral-buffered formalin for 48h, embedded in paraffin, sectioned and stained with H&E. The samples were categorized according to the WHO classification and diagnosed as GBM.
Paraffin samples were obtained from the Institute of Neuropathology, University Hospital Bonn. The patients had given informed consent that parts of the histological specimens can be used for research purposes. The sections were deparaffinized in

xylene and rehydrated in graded ethanol. Prior to the staining procedure, antigen retrieval using microwave-oven heating (2x5min, 800W) in 10mM standard sodium citrate buffer (pH 6.0) was carried out for all antibodies used in this study.
Nonspecific binding sites were blocked with phosphate-buffered saline (PBS) containing 5% bovine serum albumin and 5% normal goat serum. Immunoreaction with the primary antibodies (see Table 2) was performed overnight at room temperature, followed by incubation with the appropriate secondary antibodies (see Table 2) for 1 h at room temperature. Subsequently, the slides were coverslipped in carbonate buffered glycerol at pH 8.6.
The polyclonal antibody against PTPIP51 was raised as described and characterized in previous studies (Koch et al., 2009a).

Primary antibodies were visualized by Alexa fluor 488 and Alexa fluor 555 secondary antibodies. For each series of antibody staining sections were incubated with medium lacking PTPIP51 antibody, serving as an internal negative control. Nuclei were displayed by DAPI.

Apoptosis was detected using the *in situ* cell death detection kit ApopTag (Chemicon International, USA # S7110) which employs an indirect TUNEL method, utilizing an anti-digoxigenin antibody that is conjugated to a fluorescein reporter molecule. It provides indirect immunofluorescence staining. Results were analyzed by using fluorescence microscopy. The kit was used according to the instructions by the manufacturer.
Immunofluorescence analysis and photodocumentation were performed on an Axioplan 2 fluorescence microscope equipped with Plan-Apochromat objectives (Carl Zeiss Jena, Germany). Visualization of the secondary antibody Alexa fluor 555 was achieved with an excitation filter of 530-560nm and an emission filter with a range 572.5-647nm. Alexa Fluor 488 was visualized by an excitation filter with 460-500nm and an emission filter 512-542 nm.
Duolink proximity ligation assay (DPLA): Interaction of PTPIP51 with either PTP1B or 14-3-3β was detected by the proximity ligation assay kit DuoLink (Olink biosciences, Uppsala, Sweden, PLA probe anti-rabbit minus for the detection of the rabbit PTPIP51 antibody, Cat.# 90602; PLA probe anti-mouse plus for the detection of the mouse anti PTP1B or 14-3-3β antibody, Cat.# 90701; Detection Kit 563, Cat.# 90134). The DuoLink proximity ligation assay secondary antibodies only hybridise

when the two different PLA probes (probe anti-rabbit minus and probe anti-mouse plus) have bound to proteins in proximity closer than 40 nm. This proximity results in ligation forming a circular template and amplification step the fluorophore coupled testing probe binds the amplified oligonucleotide strands. Addition of the fluorescent labelled oligonucleotides that hybridize to the rolling circle amplification (RCA) product leads to a point-shaped signal that is visible in fluorescence microscopy.

Methanol-fixed air-dried samples were per-incubated with blocking agent for 1h. After washing in PBS for 10 min, primary antibodies for PTPIP51 and PTP1B, or PTPIP51 and 14-3-3β were applied to the samples. The antibodies were diluted in the blocking agent at a concentration of 1:500 and 1:100, respectively. Incubation was done overnight in a pre-heated humidity chamber. Slides were washed three times in PBS for 10 min. Duolink PLA probes detecting rabbit or mouse antibodies were diluted in the blocking agent at a concentration of 1:5 and applied to the slides following incubation for 2h in a pre-heated humidity chamber at 37°C. Washing three times in PBS for 10 min removed unbound PLA probes. For hybridization of the two Duolink PLA probes Duolink Hybridization stock was diluted 1:5 in high purity water and slides were incubated in a pre-heated humidity chamber for 15 min at 37°C. The slides were washed in TBS-T for 1 min under gentle agitation. The samples were incubated in the ligation solution consisting of Duolink Ligation stock (1:5) and Duolink Ligase (1:40) diluted in high purity water for 90 min at 37°C. Detection of the amplified probe was done with the Duolink Detection kit. Duolink Detection stock was diluted 1:5 in high purity water and applied for 1 h at 37°C. Final washing steps were done by SCC buffer and 70% ethanol.

H&E: The histomorphological characteristics were evaluated by hematoxylin and eosin staining.

In situ hybridization: In situ hybridization was performed as described previously (Koch et al., 2009a).

Reverse transcriptase-polymerase chain reaction (RT-PCR): For assessment of RT-PCR five glioblastoma tissue specimens were obtained during neurosurgical resection. The tissue samples were immediately transferred into RNA-later (Qiagen, Hilden, Germany) and stored deep frozen at – 20°C according to the manufacturer's instructions. All tissue samples used for RT-PCR were obtained from the Department of Neurosurgery, Justus-Liebig-University, Giessen. Prior to resection, patients had

given informed consent to using parts of the histological specimen for research purposes.

The RNA extraction was performed using the RNA extraction kit RNeasy MINI (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Qualitative reverse transcriptase PCR was performed on an iCycler using SYBR Green Supermix (BioRad, Munich, Germany) to visualize the respective amplicons. 2µl cDNA were used per sample. Cycling conditions were 94 °C for 2 min, followed by 40 cycles of 94 °C (PTPIP51); 58°C (PTP1B); 63°C (c-Src); 63,5°C (Raf-1) and 60°C (14-3-3β) for 30 sec, 55 °C for 30 sec and 72 °C for 2 min. The primers were employed for PTPIP51, PTP1B, c-Src, Raf-1 and 14-3-3β (see Table 3).

PCR products were visualized by agarose gel electrophoresis. While amplification of a 90 bp β-actin product served as positive control, negative controls included samples lacking reverse transcriptase.

Quantitative real time PCR*:* Five paraffin-embedded GBM tissues and four samples from patients with grade II astrocytoma were used for quantification of mRNA. The purification of RNA was done using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction.

The amplification of cDNA was carried out in 25 µl reaction volume on the iCycler iQ Real-Time PCR Detection System (Bio Rad, Munich, Germany). The final reaction tubes contained 100nM as PTPIP51, 14-3-3β and reference genes β-actin and GAPDH (see Table 3), 12.5µl iQ SYBR Green Supermix (Bio Rad) and 2µl of DNA template. The PCR conditions were 94°C for 3 min followed by 40 cycles for 30sec, 60°C for 30sec and 72°C for 1min. Melting curves were generated for both genes after amplification. Negative controls were included in each run. The selection of appropriate combination of reference genes for expression analysis of PTPIP51 in GBM and astrocytoma II tissue was carried out using NormFinder Program. PCR-products were additionally electrophoresed on a 3 % agarose gel and visualized by GelRed reagent.

Results

This study demonstrates for the first time the cell-specific expression of PTPIP51 mRNA and protein in human glioblastoma. Immunohistochemical experiments and in situ hybridization revealed a strong expression of PTPIP51 in GBM tumour cells and endothelial cells and immune cells. Additionally, the PTPIP51 expression profile was assayed in apoptosis by TUNEL and in proliferation with Ki67 by double immunostaining. Furthermore, we explored the interacting partners of PTPIP51, 14-3-3β, Raf-1, PTP1B, c-Src and EGFR. The transcription of the respective signalling partners was shown by qualitative reverse transcriptase PCR. The correlation of the tumour grade of gliomas with the quantitative expression of both, PTPIP51 and 14-3-3β, was examined by quantitative real time PCR comparing GBM and grade II astrocytoma.

In situ expression profile of PTPIP51 mRNA and protein:

Immunohistochemical staining of 15 GBM samples revealed PTPIP51 protein expression in tumour cells as identified by subsequent H&E staining of the sections (Fig.1 A-C). Probing glioblastoma samples with both PTPIP51 antibody and GFAP antibody confirmed the expression of PTPIP51 in malignant glial cells (Fig.1 D-F). Endothelial cells (Fig.2 J–L), smooth muscle cells (Fig.2 G–I) and immune cells also showed PTPIP51 expression. In endothelial cells of normal and of pseudoglomerular vessels PTPIP51 protein was restricted to the plasmamembrane and to the nucleus. Granulocytes located in capillary lumina or infiltrating the tumour, as well as the necrotic tissue, displayed a strong PTPIP51 immunoreaction (Fig.2 A-C). In contrast to granulocytes, B lymphocytes did not show any PTPIP51 expression (Fig.2 D-F).

Matching the protein expression profile, PTPIP51 mRNA was found in the cytoplasm of tumour cells and in the cytoplasm of endothelial cells as detected by in situ hybridization (Fig. 3).

In situ expression profile of PTPIP51 and its interacting partners:

PTPIP51 and 14-3-3β showed a strict co-localization. Both proteins were found in glioblastoma cells, endothelial cells and in immune cells. The glioblastoma cells displayed a strong cytoplasmic reaction (Fig. 4 A-C). In astrocytoma, a co-localization for PTPIP51 and 14-3-3β was seen, but to a much lower extent (Fig. 4 D-F).

To corroborate the interaction of PTPIP51 and 14-3-3β, a duolink proximity ligation assay was applied to GBM and astrocytoma sections. In all investigated samples, hybridized and amplified antibody-linked nucleotide strands were detected. Every dot corresponds to an interaction between PTPIP51 and 14-3-3β.

As seen in Figure 4 G and H, glioblastoma and astrocytoma cells displayed hybridized and amplified antibody-linked nucleotide strands, revealing the *in situ* interaction between PTPIP51 and 14-3-3β.

GBM tumour cells situated around pseudoglomerular vessels, as well as endothelial cells and immune cells, displayed a co-localization of PTPIP51 with PTP1B (Fig.5 A-C). To confirm an *in situ* interaction of PTPIP51 and PTP1B, a duolink proximity ligation assay was performed. As seen in Figure 5 D glioblastoma cells displayed hybridized and amplified antibody-linked nucleotid strands, revealing the interaction between PTPIP51 and PTP1B.

Raf-1 and PTPIP51 displayed a strong co-localization in the vessels surrounding tumour cells (Fig.6 A-C).

There was only a partial co-localization of PTPIP51 and c-Src (Fig.6 D-F). In contrast to high PTPIP51 expression in the cytoplasm of tumour and immune cells c-Src is only present in some of these cells.

A majority of tumour cells displayed a co-localization of PTPIP51 and EGFR (data not shown).

Stained by Ki-67, proliferating cells showed a strong expression of PTPIP51 in the cytoplasm with elevated concentration near the plasmamembrane (Fig.7 A-C).

TUNEL assay analysis of GBM sections did not show PTPIP51 positive cells executing apoptosis (Fig.7 D-F).

mRNA expression of PTPIP51 and its interaction partners:

Qualitative reverse transcriptase PCR analysis: The mRNA expression of PTPIP51 and its *in vitro* interaction partners was tested by reverse transcriptase PCR. As demonstrated in Figure 8, the samples 1, 2, 3 and 5 expressed a considerable amount of PTPIP51, PTP1B, 14-3-3β, Raf-1 and c-Src. A different expression pattern was observed in sample number 4, which had been obtained from a patient with a

recurrent GBM. The histopathological findings of this specimen showed healthy cerebral tissue with only singular tumour cells. In this case attenuated expression of PTPIP51 and PTP1B was found, c-Src was barely detectable, and Raf-1 was lacking (Fig.8).

Comparison of PTPIP51 expression levels in grade II astrocytoma and glioblastoma by quantitative PCR:

PTPIP51 expression levels of 5 GBM and 4 grade II astrocytoma samples were assessed by quantitative PCR. Candidate reference genes for normalization and the best combination of two genes were calculated according to their expression stability by the NormFinder program. The best fitting combination proved to be β-actin with GAPDH.

The results of expression analysis showed slightly elevated levels of PTPIP51 (mean value 5,23 +/- 1,01) in the group of GBM when compared to low grade astrocytoma (mean value 4,62 +/- 1,49). 14-3-3β expression in glioblastoma (mean value 3,42 +/- 1.10) was significantly higher than in grade II astrocytoma samples (mean value 0,88 +/- 2,74) (Fig. 9).

Discussion

Our results revealed elevated levels of PTPIP51 expression in GBM (grade IV glioma) samples when compared to low grade astrocytomas (grade II glioma), which correlated with the expression levels of 14-3-3β. This is consistent with recent studies displaying a tumour grade dependent expression of two isoforms of 14-3-3β and 14-3-3η in gliomas. Healthy cerebral tissue is completely lacking in both isoforms (Yang et al., 2009). The upregulation of 14-3-3 proteins seems to be associated with the reduced capacity of apoptosis, as antagonizing 14-3-3 or silencing its expression induces apoptosis in cultured glioma cells (Cao et al., 2010).

14-3-3β and 14-3-3γ mediate the interaction of PTPIP51 and Raf-1, thereby modulating the activity of the MAPK-cascade (Lv et al., 2006; Yu et al., 2008, Stenzinger et al., 2009;). The MAPK pathway plays an important role in cell migration

and seems to be one of the main reasons for recurrences and poor prognosis of glioblastoma. It is considered that migrating tumour cells infiltrate the healthy tissue surrounding the glioblastoma and in this way can escape surgical extirpation and give rise to regrowth. With regard to these findings the interaction of PTPIP51 with 14-3-3β, which was confirmed by the duolink proximity ligation assay, may mirror the role of PTPIP51 protein in migration and proliferation of GBM tumour cells.

A strong co-localization and interaction of PTPIP51 and 14-3-3β was also found in all endothelial cells of GBM-typical glomerulum-like vessels of glioblastoma.
This interaction may contribute to the well known high activation of the MAPK/Erk pathway via EGFR, resulting in dysfunction of cell cycle control and upregulation of proliferation in GBM.
The non-transmembrane Protein Tyrosine Phosphatase 1B (PTP1B), a known interacting partner of PTPIP51, is able to activate the MAPK cascade on c-Src and Ras level (Dubé et al., 2004; Dubé and Tremblay, 2004; Tonks and Muthuswamy, 2007; Zhao et al., 2008, Stenzinger et al., 2009). Reichardt and coworkers (2003) were unable to detect DNA amplification of PTP1B in human gliomas. However, in our study PTP1B expression was upregulated and highly co-localized with the PTPIP51 protein. The observed upregulation is consistent with the data reported by Akasaki and co-workers (2006), who also reported PTP1B to be overexpressed in gliomas. Furthermore, a direct *in situ* interaction of PTPIP51 and PTP1B in glioblastoma cells was corroborated by the duolink proximity ligation assay. These results underline the significance of our observations of upregulated PTPIP51 expression levels in glioblastomas.
PTP1B contributes to oncogenesis by the loss of tyrosine phosphorylation of key signalling proteins or by up-regulation of two growth-promoting pathways (Arias-Romero et al., 2009). In human mammary cells PTP1B links an important oncogenic receptor tyrosine kinase, namely ErbB2, to signalling pathways that promote aberrant cell division and survival by activation of Src and inducing a Src-dependent transformed phenotype. It deactivates the Ras/MAPK pathway inhibitor (Tonks and Muthuswamy, 2007).
C-Src mediates the phosphorylation of EGFR, thereby promoting tumour progression (Tice et al., 1999). The ultimate cellular response to the activation of EGFR signalling cascade via MAPK pathway is DNA synthesis and cell division (Halatsch et al.,

2004). In our samples PTPIP51 and EGFR were partly co-localized, suggesting a synergistic effect on cell proliferation, migration and oncogenic transformation. This may be exerted by sharing the same final signalling pathway, PTPIP51 via 14-3-3β interaction with Raf-1 and EGFR activating the Ras/Raf/MAPK/ERK pathway. Compared to secondary GBM, developed by progression from lower grade gliomas, EGFR gene amplification has been shown to be five times higher in primary glioblastoma, which leads to overexpression in 40 % of GBM (Karpel-Massler et al., 2009). Besides this EGFR overexpression, EGFR is expressed as the mutated EGFRvIII (epidermal growth factor receptor variant III) form of the receptor in 20 % of GBM cases (Jutten et al., 2009). This constitutively active mutant form of the EGFR, which is commonly expressed in glioblastoma, is also detected in a number of epithelial cancers (Yoshimoto et al., 2008; Hama et al., 2009) also known to express high concentrations of PTPIP51, e.g. non melanoma skin cancer, prostate cancer (Koch et al., 2008; 2009b) and breast cancer. EGFR signalling cascade via MAPK pathway is modulated by PTP1B through c-Src and by 14-3-3β through Raf-1 (Yu et al., 2008), both interaction partners of PTPIP51. This *in situ* interaction in GBM was substantiated in our study by duolink proximity ligation assay.
Glial malignant transformation might be correlated to the status of PTPIP51 gene promotor methylation, since high grade gliomas (GBM) also presented a higher mRNA expression of PTPIP51 in comparison to low grade gliomas (grade II astrocytoma).

To sum up, in neuroepithelial tumours, PTPIP51 expression increases with the grade of malignancy and PTPIP51 interacts *in situ* with 14-3-3β and PTP1B. The data presented in this study suggest an important role of PTPIP51 in glioblastoma formation.

Acknowledgments

We are grateful to Mrs. K. Michael (Institute of Anatomy and Cell Biology, Giessen) for help with the design of the figures and to Mrs. A. Peters (Department of Neurosurgery, Giessen) for help in providing the glioblastoma samples. The excellent

techncial assistance of Mrs. A. Erkel, Mrs. B. Fröhlich (Department of Urology and Pediatric Urology, Giessen), Mrs. A. zur Mühlen (Institute of Neuropathology, Bonn) and Mrs. C. Tag (Institute of Anatomy and Cell Biology, Giessen) is gratefully acknowledged.

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Legends

Fig. 1 Immunostaining of PTPIP51 in sections of human glioblastoma

A) PTPIP51 immunostaining B) H&E. staining of section A C) DAPI staining of section A D) PTPIP51 immunostaining E) GFAP staining of section D F) DAPI staining of section D Arrows: PTPIP51 positive glioblastoma cells, arrowheads: PTPIP51 positive endothelial cells, double arrowheads: PTPIP51 and GFAP positive reactive astrocyte, double arrow: PTPIP51 positive immune cell (neutrophil granulocyte). BAR = 50µm

Fig. 2 PTPIP51 expression in immune cells, smooth muscle cells and endothelial cells

A) PTPIP51 immunostaining B) granulocyte marker of section A C) Merge of A, B and DAPI D) PTPIP51 immunostaining E) B lymphocyte in section D detected by CD20 F) Merge of D, E and DAPI. BAR (A-F)= 10µm G) PTPIP51 immunostaining H) α-sma of section G I) Merge of G,H and DAPI J) PTPIP51 immunostaining K) endothelial cells detected by CD34 in section J L) Merge of J, K and DAPI. Arrowhead: neutrophil granulocytes; blank arrowhead: B lymphocytes; double arrow: smooth muscle cells; arrow: endothelial cells; white circle: lipofuscin granula . BAR (G-L) = 20µm

Fig. 3 In situ hybridization of PTPIP51 in sections of human glioblastoma

A) anti-sense probe B) sense probe of parallel section to A C) anti-sense probe D) sense probe in parallel section to C Arrows: PTPIP51 mRNA in glioblastoma cells, arrowhead: PTPIP51 mRNA in endothelial cells, double arrow: immune cells, asterisk: necrotic tissue. BAR = 50µm

Fig. 4 PTPIP51 and its interacting partner 14-3-3β - in situ co-localization analysis and Duolink proximity ligation assay in sections of human glioblastoma and astrocytoma

A) PTPIP51 immunostaining GBM B) 14-3-3β immunostaining of section A C) Merge of A and B D) PTPIP51 immunostaining astrocytoma E) 14-3-3β immunostaining of section D F) Merge of D and E. Arrows: PTPIP51 positive glioblastoma cells, arrowheads: PTPIP51 positive endothelial cells, double-arrows: immune cells. BAR = 50µm G) GBM duolink proximity ligation assay for PTPIP51 and 14-3-3β H) astrocytoma duolink proximity ligation assay for PTPIP51 and 14-3-3β. Interaction of both proteins is seen as orange dots (arrows) I) Negative control. White circles: lipofuscin granules. BAR = 10µm

Fig. 5 PTPIP51 and its interacting partner PTP1B in sections of human glioblastom

A) PTPIP51 immunostaining B) PTP1B immunostaining of section A C) Merge of section A and B D) Duolink proximity ligation assay for PTPIP51 and PTP1B. Interaction of both proteins is seen as orange dots (arrows). BAR (A,B, C) = 20µm, BAR (D) = 10µm

Fig. 6 PTPIP51 and its interacting partners Raf-1 and c-Src in sections of human glioblastoma

A) PTPIP51 immunostaining B) Raf-1 immunostaining of section A C) Merge of A and B D) PTPIP51 immunostaining E) c-Src immunostaining of section D F) Merge of D and E. Arrows: PTPIP51 positive glioblastoma cells, arrowheads: PTPIP51 positive endothelial cells, double-arrows: immune cells. BAR = 50µm

Fig. 7 PTPIP51 protein expression and investigation of proliferation and apoptosis by Ki67 antibody and TUNEL-assay

A) PTPIP51 immunostaining B) Ki67 immunostaining of section A C) Merge of A and B D) PTPIP51 immunostaining E) TUNEL-assay of section D, insert = H.E. staining of detail F) Merge of D and E. Arrows: PTPIP51 positive glioblastoma cells, arrowheads: PTPIP51 positive endothelial cells, blank arrowhead: apoptotic pericyte. BAR = 50µm

Fig. 8 Expression of PTPIP51 and its interaction partners as detected by reverse transcriptase-PCR in tissues of human glioblastoma

Qualitative reverse transcriptase-PCR was performed using primers specific to PTPIP51, PTP1B, Raf-1, c-Src and 14-3-3β as given in Materials and Methods section. β-actin was amplified as an internal positive control and probes lacking reverse transcriptase served as negative controls (N).

Fig. 9 Quantitative real time PCR analysis of PTPIP51 and 14-3-3β in glioblastoma and grade II astrocytoma

A: Diagram shows the results of quantitative expression analysis. Slightly elevated levels of PTPIP51 are seen in the group of glioblastoma (GBM) when compared to low grade astrocytoma. 14-3-3β expression in glioblastoma was significantly higher than in grade II astrocytoma samples.

Amplification of β-actin and GAPDH was used as a combination of reference genes.

B: Gel: bands exclusively detecting mRNA with the expected amplification size with the primers used in 7A. Negative control (N).

	Age (years)	Sex	Localization	Tumour specimens	WHO-Grade
1	65	M	central, left	GBM	IV
2	63	F	frontal, right	GBM	IV
3	78	F	frontal, right	GBM	IV
4	72	M	fronto-temporal, left	GBM	IV
5	76	M	temporal, right	GBM	IV
6	70	M	central, right	GBM	IV
7	60	M	temporal, right	GBM	IV
8	66	M	temporo-dorsal, left	GBM	IV
9	72	F	temporo-medial, right	GBM	IV
10	61	M	temporo-parietal, left	GBM	IV
11	42	M	central, left	GBM	IV
12	67	M	frontal, right	GBM	IV
13	78	M	central, right	GBM	IV
14	68	F	temporo-polar, left	GBM	IV
15	52	M	precentral/central, left	GBM	IV
16	33	M	temporo-medial, right	Astrocytoma	II
17	23	F	insula, left	Astrocytoma	II
18	44	F	corpus callosum, left	Astrocytoma	II
19	31	M	insula, right	Astrocytoma	II
20	55	M	occipital, left (hemianopsia)	GBM magnocellular	IV
21	55	F	parietal, right	GBM	IV
22	46	M	parietal/pre-central gyrus, left	GBM, relapse	IV
23	66	M	parietal, occipital right	GBM, relapse	IV
24	76	M	Broca region, left	GBM	IV

Table 1: Tumour specimens included in the study Tumours No. 1 – 19 were analyzed by immunocytochemistry and No. 11 – 19 by quantitative real time PCR. Tumours No. 20 – 24 were analyzed by reverse transcriptase PCR.

	Immunogen	Antibody Source	Clone	Dilution	Manufacturer
PTPIP51	Human recombinant PTPIP 51 protein encoding amino acids (aa) 131-470	Rabbit polyclonal		1:400	Prof. HW Hofer, Biochemical Department, University Konstanz, Germany
14-3-3 β	Epitope mapping the C-terminus of human origin	Mouse monoclonal	A-6	1:100	Santa Cruz Cat# sc-25276
PTP1B	Human recombinant protein tyrosine phosphatase 1B (aa 1 – 321)	Mouse monoclonal	107AT531	1:100	Abgent Cat#AM8411
Glial fibrillary acidic protein	Purified porcine glial filament from spinal cord	Mouse monoclonal	GA5	1:200	Chemicon Cat# MAB3402
Raf-1	Epitope mapping the C-terminus of human origin	Mouse monoclonal	E-10	1:50	Santa Cruz Cat# sc-7267
c-Src	Full-length human recombinant c-Src	Mouse monoclonal	17AT28	1:100	Santa Cruz Cat# sc-130124
EGFR	Plasma membranes of A431 cells	Mouse monoclonal	2E9	1:75	Santa Cruz Cat# sc-57091
Ki67	Human recombinant peptide corresponding to a 1002 bp Ki-67 cDNA fragment.	Mouse monoclonal	MIB-1	1:100	Dako Cytomation Cat# M 7240
CD20	CD20 protein	Mouse monoclonal	B9E9	1:100	Thermo Scientific Cat # MA1-7634
CD34	CD34 protein from human endothelial vesicles	Mouse monoclonal	QBEND-10	1:100	ThermoScientific Cat.# Ma35170
Granulocyte	nuclei from Pokeweed	Mouse			Santa Cruz Cat#

marker SPM250	nitrogen-stimulated human peripheral blood lymphocytes	monoclonal	his48	1:100	sc-65523
α-smooth muscle actin-FITC antibody	N-terminal synthetic decapeptide of α-smooth muscle actin	Mouse monoclonal	clone 1A4	1:100	SigmaAldrich Cat# F3777
Alexa fluor 555 Coupled to anti-rabbit antibody	IgG heavy chains from rabbit	Goat		1:800	Invitrogen Cat# A21428
Alexa fluor 488 Coupled to anti-mouse antibody	IgG heavy chains from mouse	Goat		1:800	Invitrogen Cat# A11029
ApopTag® Fluorescein In Situ Apoptosis Detection Kit					Chemicon International S7107

Table 2: List of the antibodies used in this study.

Primer RT-PCR (fresh tissue specimen)	size	template
PTPIP51 forward 5´-GCAGGTGGTGCTATCAGGTC-3´ reverse 5'-AGCTCCAGGGCCAACTTCATC-3'	232 BP	1294 –1525
PTP1B forward 5'-GGAGATGGAAAAGGAGTTC-3' reverse 5'-TGCTTTTCTGCTCCCACAC-3'	311 BP	177 - 487
14-3-3β forward 5'-ATTCGTCTTGGTCTGGCACT-3' reverse 5'-CAGGCTACAGGCCTTTTCAG-3'.	78 BP	689 – 766, 784 - 861
c-Src forward 5'-ATGGTGAACCGCGAGGTGCT-3' reverse 5'-GATCCAAGCCGAGAAGCCGGTCTG-3'	244 BP	1753 - 1996
Raf-1 forward 5'-CAGCCCTGTCCAGTAGC-3' reverse 5'-GCCTGACTTTACTGTTGC-3'	614 BP	1287 - 1900
β-actin forward 5'-TTCCTTCCTGGGCATGGAGT-3' reverse 5'-TACAGGTCTTTGCGGATGTC-3'	90 BP	2439 - 2528
Primer quantitative RT-PCR (paraffin embedded tissue)		
PTPIP51 forward 5'-TCAAGGAGCATGTGGACAAA-3' reverse 5'-ATAGCACCACCTGCCAAGAA-3'	80 BP	1228 - 1307
14-3-3β forward 5'-ATTCGTCTTGGTCTGGCACT-3' reverse 5'-CAGGCTACAGGCCTTTTCAG-3'.	78 BP	689 – 766, 784 - 861
β-actin forward 5'-TTCCTTCCTGGGCATGGAGT-3' reverse 5'-TACAGGTCTTTGCGGATGTC-3'	90 BP	2439 - 2528
GAPDH forward 5'-ATGCCAGTGAGCTTCCCGTTCA-3' reverse 5'-TGGTATCGTGGAAGGACTCATGA-3'	189 BP	628 - 794

Table 3: Primer used for RT-PCR and for quantitative real time PCR

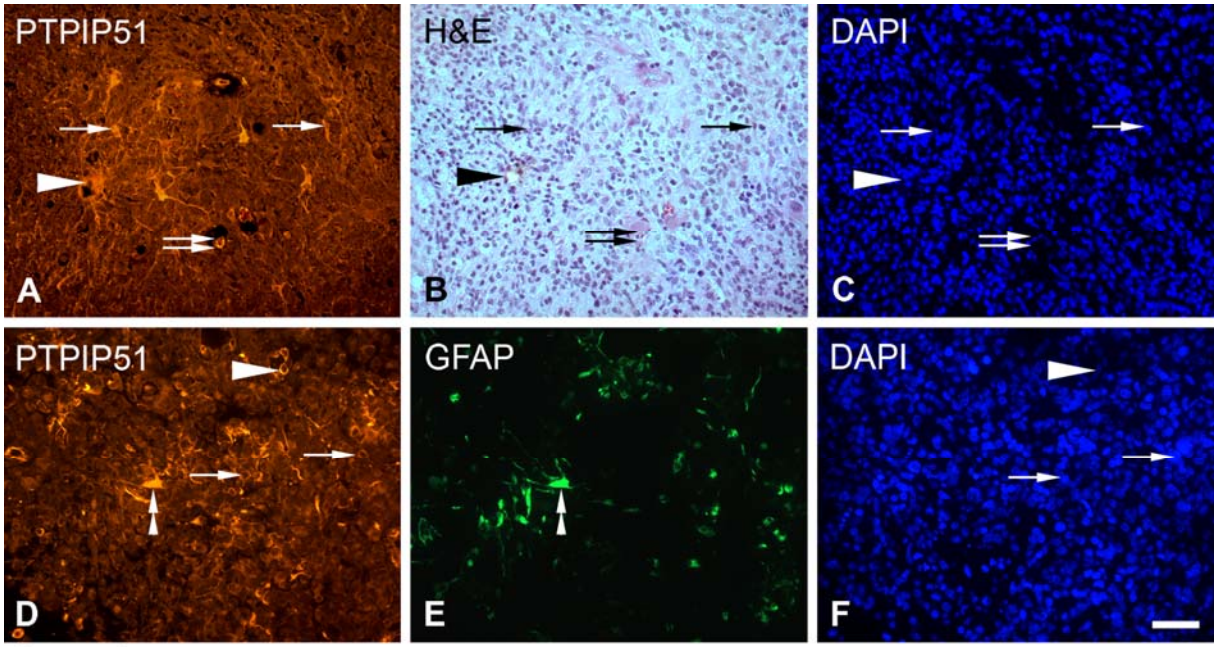

Figure 1

Fig. 1 Immunostaining of PTPIP51 in sections of human glioblastoma

A) PTPIP51 immunostaining B) H&E. staining of section A C) DAPI staining of section A D) PTPIP51 immunostaining E) GFAP staining of section D F) DAPI staining of section D Arrows: PTPIP51 positive glioblastoma cells, arrowheads: PTPIP51 positive endothelial cells, double arrowheads: PTPIP51 and GFAP positive reactive astrocyte, double arrow: PTPIP51 positive immune cell (neutrophil granulocyte). BAR = 50µm

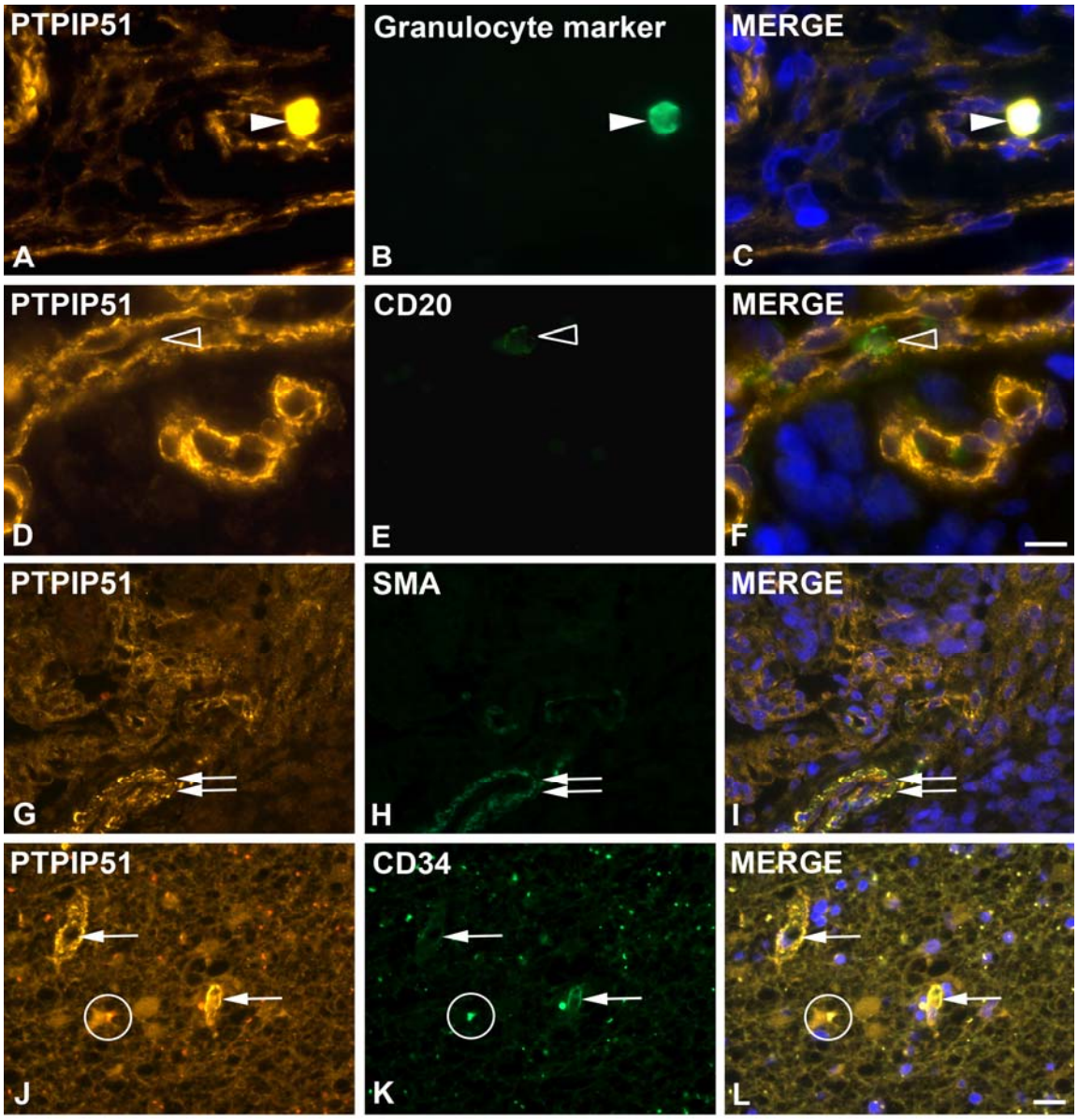

Figure 2

Fig. 2 PTPIP51 expression in immune cells, smooth muscle cells and endothelial cells

A) PTPIP51 immunostaining B) granulocyte marker of section A C) Merge of A, B and DAPI D) PTPIP51 immunostaining E) B lymphocyte in section D detected by CD20 F) Merge of D, E and DAPI. BAR (A-F)= 10µm G) PTPIP51 immunostaining H) α-sma of section G I) Merge of G,H and DAPI J) PTPIP51 immunostaining K) endothelial cells detected by CD34 in section J L) Merge of J, K and DAPI. Arrowhead: neutrophil granulocytes; blank arrowhead: B lymphocytes; double arrow: smooth muscle cells; arrow: endothelial cells; white circle: lipofuscin granula . BAR (G-L) = 20µm

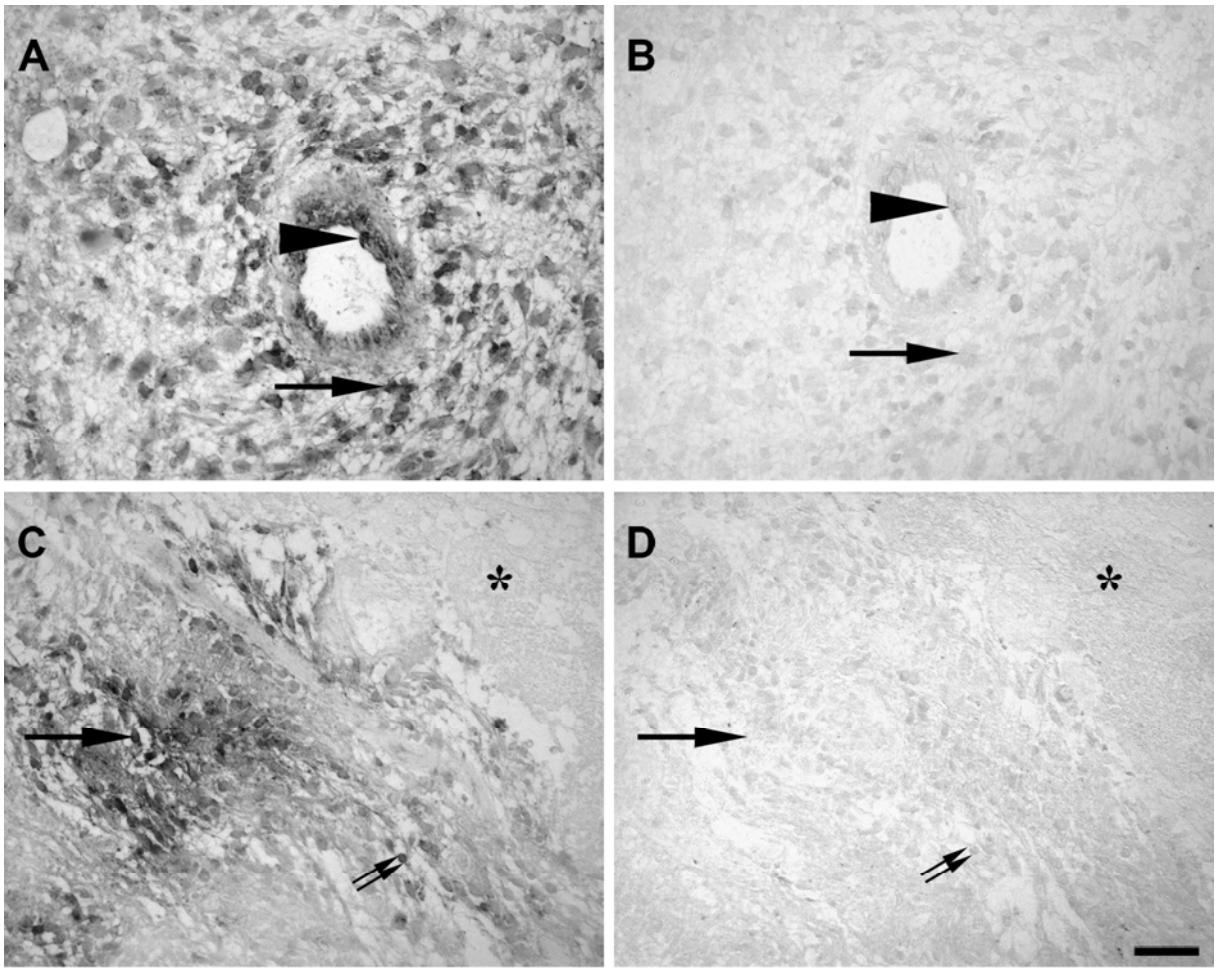

Figure 3

Fig. 3 In situ hybridization of PTPIP51 in sections of human glioblastoma

A) anti-sense probe B) sense probe of parallel section to A C) anti-sense probe D) sense probe in parallel section to C Arrows: PTPIP51 mRNA in glioblastoma cells, arrowhead: PTPIP51 mRNA in endothelial cells, double arrow: immune cells, asterisk: necrotic tissue. BAR = 50µm

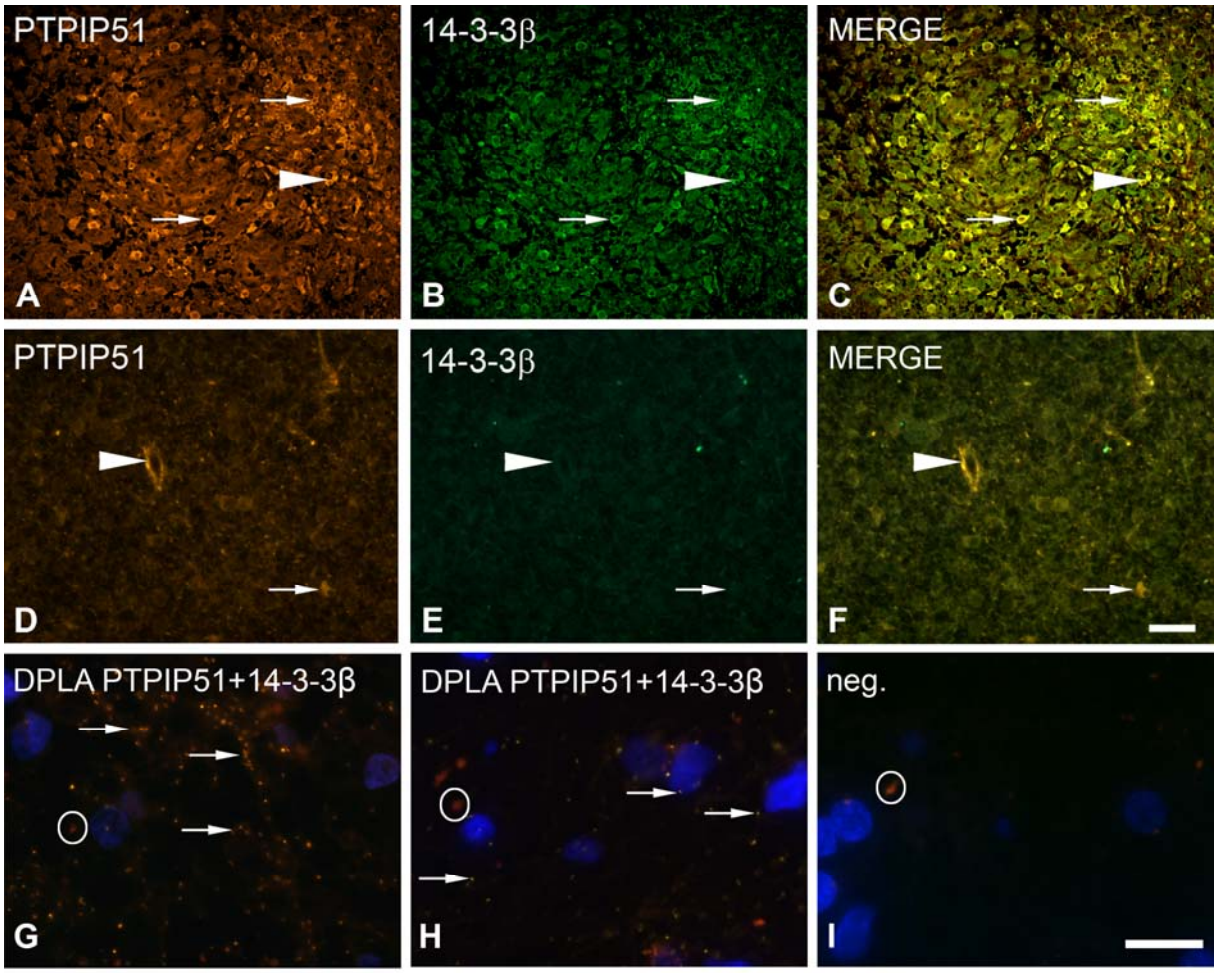

Figure 4

Fig. 4 PTPIP51 and its interacting partner 14-3-3β - in situ co-localization analysis and Duolink proximity ligation assay in sections of human glioblastoma and astrocytoma

A) PTPIP51 immunostaining GBM B) 14-3-3β immunostaining of section A C) Merge of A and B D) PTPIP51 immunostaining astrocytoma E) 14-3-3β immunostaining of section D F) Merge of D and E. Arrows: PTPIP51 positive glioblastoma cells, arrowheads: PTPIP51 positive endothelial cells, double-arrows: immune cells. BAR = 50μm G) GBM duolink proximity ligation assay for PTPIP51 and 14-3-3β H) astrocytoma duolink proximity ligation assay for PTPIP51 and 14-3-3β. Interaction of both proteins is seen as orange dots (arrows) I) Negative control. White circles: lipofuscin granules. BAR = 10μm

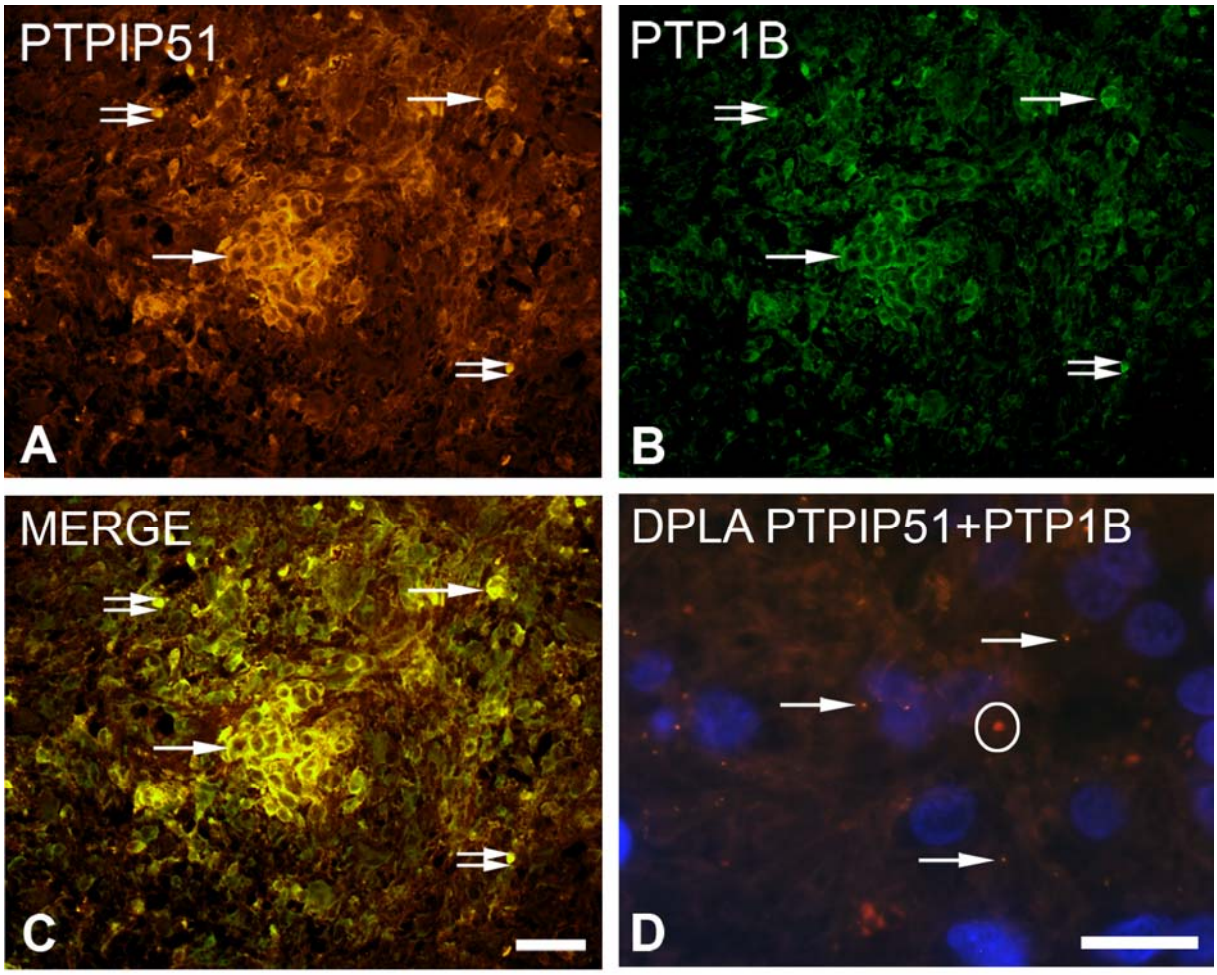

Figure 5

Fig. 5 PTPIP51 and its interacting partner PTP1B in sections of human glioblastom

A) PTPIP51 immunostaining B) PTP1B immunostaining of section A C) Merge of section A and B D) Duolink proximity ligation assay for PTPIP51 and PTP1B. Interaction of both proteins is seen as orange dots (arrows). BAR (A,B, C) = 20µm, BAR (D) = 10µm

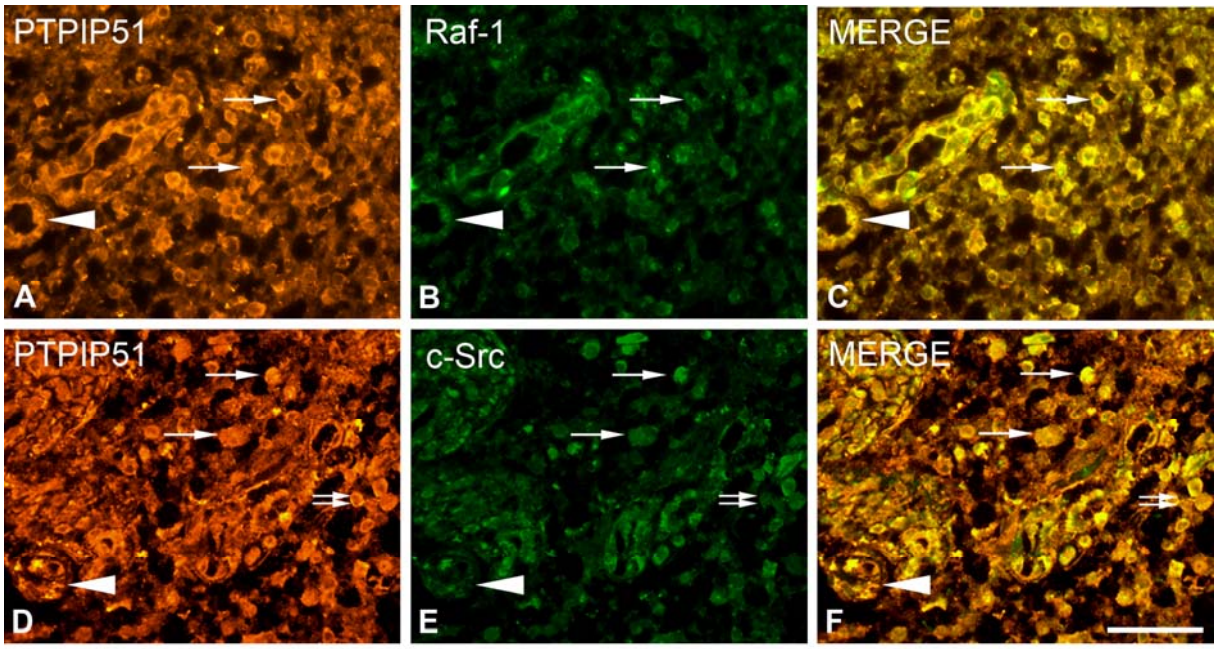

Figure 6

Fig. 6 PTPIP51 and its interacting partners Raf-1 and c-Src in sections of human glioblastoma

A) PTPIP51 immunostaining B) Raf-1 immunostaining of section A C) Merge of A and B D) PTPIP51 immunostaining E) c-Src immunostaining of section D F) Merge of D and E. Arrows: PTPIP51 positive glioblastoma cells, arrowheads: PTPIP51 positive endothelial cells, double-arrows: immune cells. BAR = 50µm

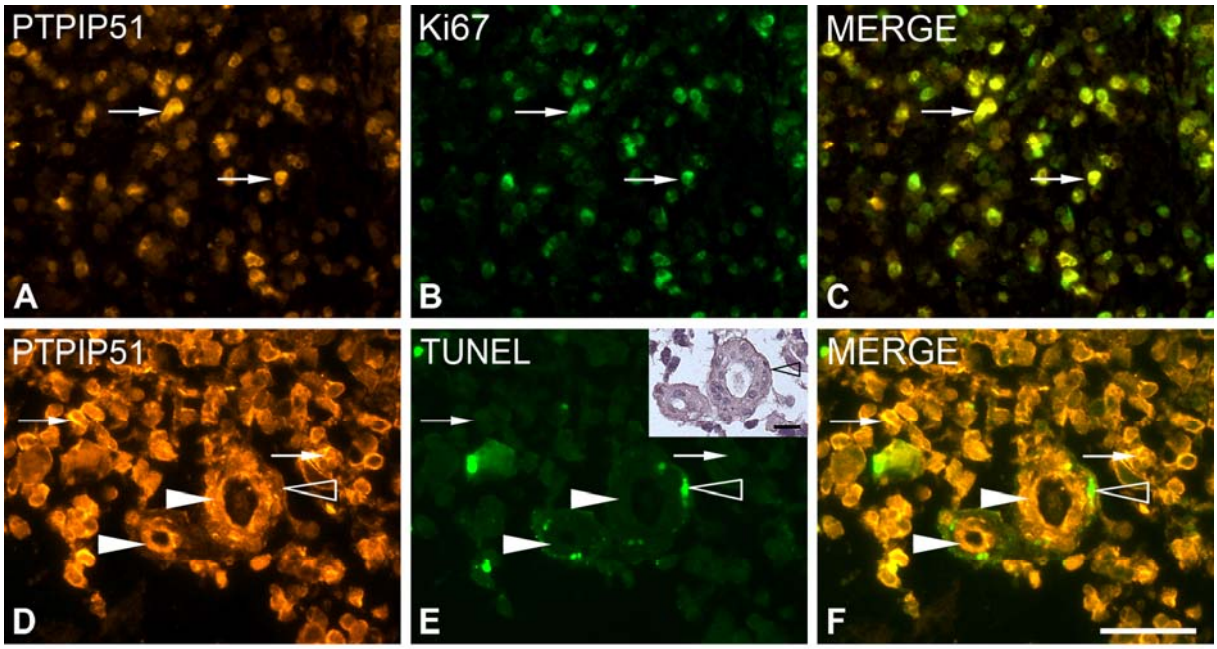

Figure 7

Fig. 7 PTPIP51 protein expression and investigation of proliferation and apoptosis by Ki67 antibody and TUNEL-assay

A) PTPIP51 immunostaining B) Ki67 immunostaining of section A C) Merge of A and B D) PTPIP51 immunostaining E) TUNEL-assay of section D, insert = H.E. staining of detail F) Merge of D and E. Arrows: PTPIP51 positive glioblastoma cells, arrowheads: PTPIP51 positive endothelial cells, blank arrowhead: apoptotic pericyte. BAR = 50µm

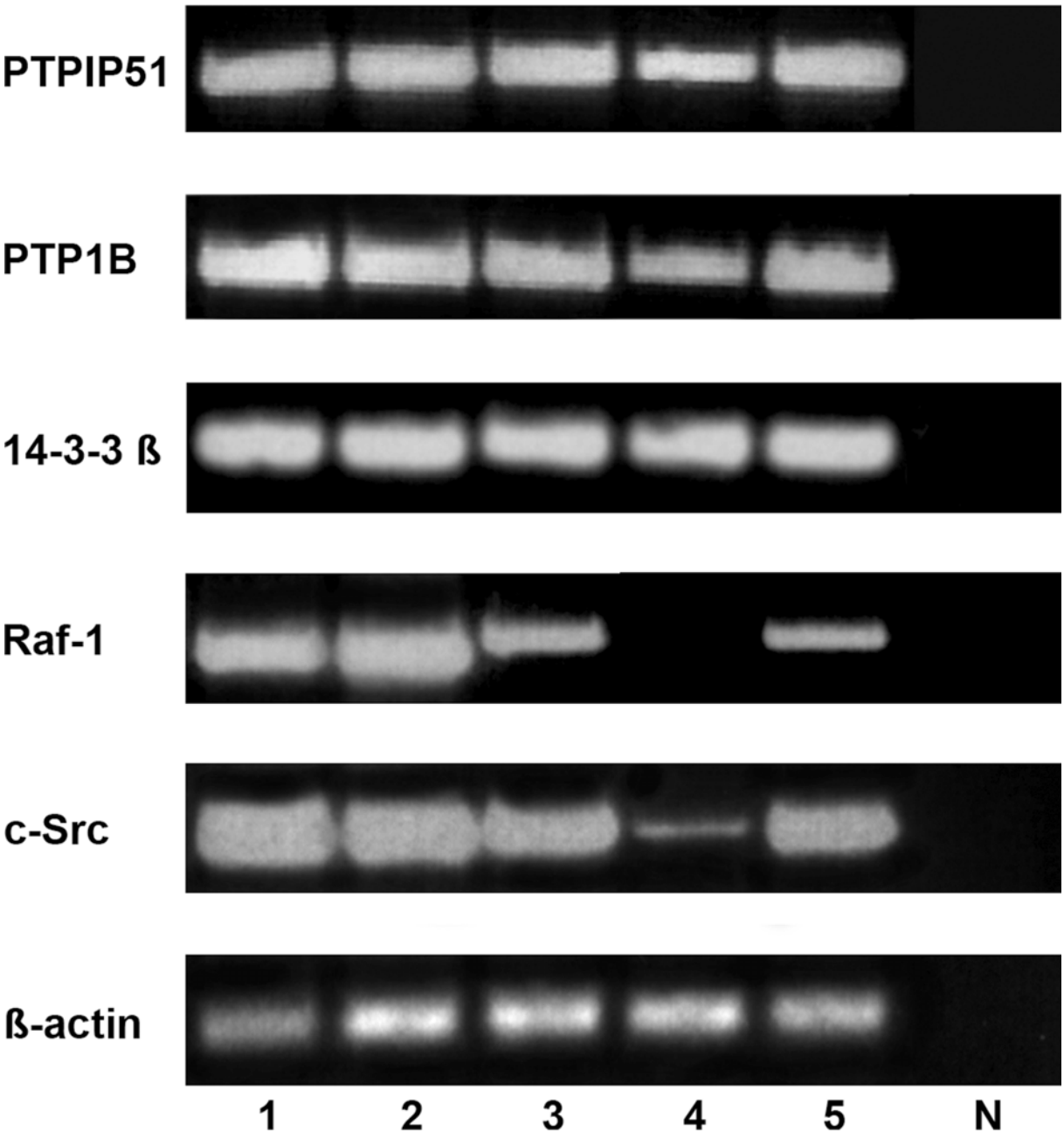

Figure 8

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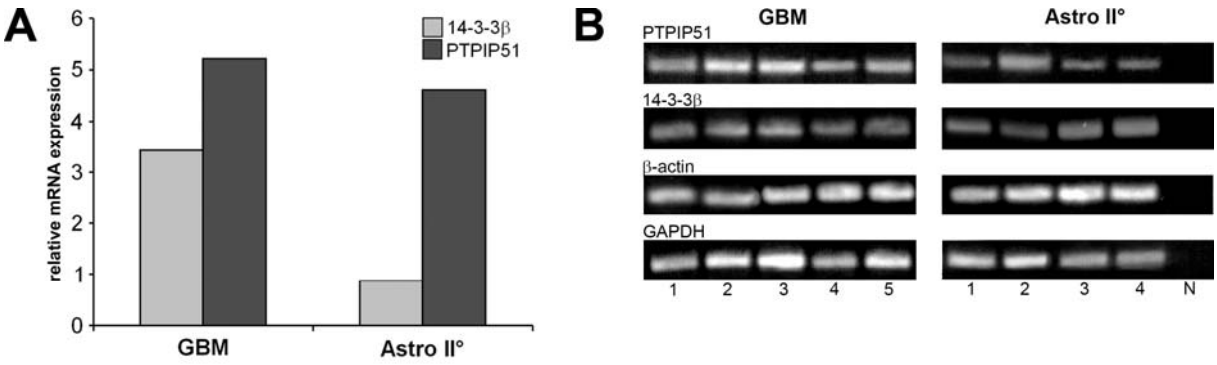

Figure 9

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