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The Role of PP2A Phosphatase Activator (PTPA) in the biogenesis of PP2A in mammalian cells

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Abstract

The role of PTPA in the biogenesis of PP2A in mammalian cells

Protein phosphatase 2A (PP2A) is an essential intracellular serine/threonine phosphatase and a prime example of phosphatase multisubunit architecture. The PP2A holoenzyme is a heterotrimer built up by a well-conserved catalytic subunit, a scaffolding subunit and one member out of a family of regulatory subunits, which determines the substrate specificity, (sub) cellular localization and catalytic activity of the PP2A.

An activated monomer of the catalytic subunit would be unspecific and thus constitute a risk for the cell. In a previous study in yeast, it was shown that the catalytic subunit of PP2A is produced as a low active precursor that is activated by the protein Rrd (Rapamycin Resistant Deletion). Loss of RRD in yeast produced an unstable and metal-dependent form of PP2A with decreased enzymatic activity.

Activation of PP2A by RRD is tightly linked to PP2A holoenzyme formation, thus providing a mechanism to prevent free and active catalytic subunit from harming the cell (Fellner et al., 2003). This mechanism has only been shown in yeast and direct evidence for the same mechanism in mammalian cells is still missing.

However, complementation and RNA-interference experiments showed that PTPA (PP2A Phosphatase Activator), the mammalian homologue of Rrd fulfils an essential function in the regulation of PP2A, which is conserved from yeast to man.

The subject of this work was therefore to elucidate the role of PTPA in the biogenesis of PP2A in a mammalian cell culture system by inducible RNAi-mediated suppression of PTPA expression. Since it was hypothesized that decreased PP2A activity plays a role in the pathogenesis of Alzheimer disease, we chose neuroblastoma cell lines as experimental model system to establish a tetracycline-inducible knock-down of PTPA.

Summarizing my results, I designed and cloned two novel shRNA sequences targeting the 3´UTR of PTPA and was able to show potent knock-down of PTPA using these sequences. Additionally, corresponding mismatch sequences were designed and cloned to be used as control shRNA sequences. Also, cDNA of PTPA insensitive to shRNA was cloned to be used for complementation experiments.

Over time three PTPA knock-down clones lost their ability to suppress PTPA expression, we analyzed the possible reason for this, in order to avoid recurrence of this phenomenon:

I could demonstrate that the parental cell line -used in this study showed low inducibility and high leaky expression in comparison to other tetracycline repressor-expressing cell lines. After establishing a method for the detection for tetracycline in FCS (Fetal Calf Serum), I

could show that the FCS, used for the generation of the PTPA-knock-down clones, contained 10^{-10}g/ml tetracycline.

Taken together, promoter leakiness, tetracycline contaminated FCS, possible apoptotic effects of PTPA suppression and hence counter-selection against it could contributed to the loss of inducible PTPA suppression over time and explain the difficulties we faced in the establishment of stable PTPA knock-down cell lines.

However, after using approved tetracycline-free FCS, I was able to establish and characterize several PTPA knock-down cell lines in terms of cell proliferation, PP2A complex formation and activity of PP2A heterotrimers. Proliferation of these cell lines was only slightly affected by induction of PTPA knock-down. Moreover I observed that PP2A complex formation was not affected and enzymatic activity of PP2A towards serine-/threonine phosphorylated substrates was not reduced. However, this could be due to the fact that the PTPA protein levels were only reduced to about 50%.

In conclusion, further investigation beyond the limited timeframe of a diploma thesis, using other model systems and/or a potent and stable repression of PTPA are surely justified, considering the overwhelming importance of fully functional and tightly regulated PP2A for eukaryotic cells.

The role of PP2A regulatory subunit Bα in differentiation of neuronal cells

PP2A is built up by three different subunits, a catalytic, a structural and one out of many regulatory subunits. The regulatory B-type subunit is chosen out of a family consisting of four different members: Bα, Bβ, Bγ and Bδ. Unlike the Bβ and Bγ subunits, which exhibit a highly regulated spatiotemporal expression pattern, Bα is ubiquitously expressed during development and in the adult. Bα-containing PP2A heterotrimers are therefore considered to possess mainly "housekeeping" functions.

Interestingly, it was found that down regulation of Bα-containing PP2A heterotrimers is associated with Alzheimer´s Disease (AD)-affected brain regions, supporting the idea of a specific involvement of Bα-subunit in this process (Sontag et al., 1995, 1996, 1999, 2004; Merrick et al., 1997; Hiraga and Tamura 2000).

PP2A is considered to be the main phosphatase of tau, a major microtubules (MT) stabilizing protein. As tau-dephosphorylation is important for the stabilization of MTs, PP2A is thereby a critical factor in the maintainance of MT integrity. On the other hand, hyperphosphorylation of tau leads to the formation of neurofibrillary tangles, one of the major hallmarks in AD.

Additional evidence for the involvement of Bα-containing PP2A in AD came from Bα-silencing experiments in N2a and NIH3T3 cells that resulted in significant breakdown of acetylated and detyrosinated MTs, which is a typical hallmark of AD (Nunbhaki-Craig et al., 2007).

Thus, we wanted to analyze, if the ubiquitous Bα subunit plays a role in the process of functional differentiation, which is dependent on microtubules-stabilization and goes beyond its housekeeping functions.

Indeed, we were able to show that differentiation induced by serum-starvation or retinoic acid administration was significantly reduced upon RNAi-mediated knock-down of Bα subunit in N2a cells.

These results provide evidence for an involvement of PP2A subunit Bα in neuronal differentiation. Our results also back the hypothesis that reduced amounts of neuronal Bα-containing PP2A heterotrimers contributes to MT destabilization and therefore to formation of neurofibrillary tangles in Alzheimer´s disease.

Abstract in German

Die Funktion von PTPA in der Biogenese von PP2A in Säugetierzellen

Proteinphosphatase 2A (PP2A) ist eine essentielle Protein-Serin/Threonin Phosphatase und ein Paradebeispiel für die molekulare Architektur von Enzymen, die aus mehreren Untereinheiten aufgebaut sind.

PP2A setzt sich aus folgenden Untereinheiten zusammen: Aus einer katalytischen Untereinheit, einer strukturgebenden Untereinheit und einer von vielen regulatorischen Untereinheiten, die für Substratspezifität, subzelluläre Lokalisation und katalytische Aktivität verantwortlich sind.

Einer ungebundenen katalytischen Untereinheit fehlt die Substratspezifität, die sie erst durch Bindung an eine der vielen regulatorischen Untereinheiten erlangt. Freie und aktive katalytische Untereinheiten würden deshalb ein Risiko für Zellen darstellen.

In einer vorhergehenden Studie im Labor von E. Ogris wurde im Modellorganismus Hefe nachgewiesen, dass die katalytische Untereinheit in inaktiver Form und mit veränderter Konformation vorliegen kann (Fellner et al., 2003). Die Aktivierung der katalytischen Untereinheit durch den Aktivator RRD (Rapamycin Resistant Deletion) ist dabei eng an den Aufbau des Holoenzyms gekoppelt. So wird sichergestellt, dass die katalytische Untereinheit erst nach Bindung an die regulatorische Untereinheit aktiv wird. Die experimentelle Evidenz für dieses Modell wurde bisher nur in Hefe erbracht.

Erste Komplementierungsexperimente mit dem RRD-Säugetierhomolog PTPA („PP2A Phosphatase Aktivator“) in Hefe zeigten die konservierte Funktion von PTPA. RNA-interference Experimente bewiesen darüber hinaus die essentielle Funktion in Säugetierzellen. Dennoch, der direkte Beweis, dass die katalytische Untereinheit auch in Säugetierzellen in einer inaktiven Form vorliegt, die durch PTPA aktiviert wird, fehlt.

Das Thema dieser Arbeit war es daher, die Rolle von PTPA in der Biogenese von PP2A in einem Säugetier-Zellkultursystem zu studieren, indem die Expression von PTPA durch induzierbare RNAi gezielt supprimiert werden sollte.

Darüber hinaus wurde in von Alzheimer betroffenen Hirnregionen eine verminderte PP2A Aktivität festgestellt. Dies führte zu unserer Arbeitshypothese, dass PTPA als möglicher Aktivator von PP2A bei Fehlfunktion eine wichtige Rolle bei der Alzheimer´schen Krankheit spielen könnte. Um diesen Zusammenhang in einem Neuronen-ähnlichen System studieren zu können, wählten wir Neuroblastoma-Zelllinien als Modellsystem.

Zusätzlich zu der bereits von Fellner etablierten shRNA, die an den ORF von PTPA bindet, wurden zwei neue shRNA Sequenzen, die an die 3´UTR von PTPA binden, als Kontrolle

entworfen. Ich konnte zeigen, dass Expression beider shRNA Sequenzen signifikanten Knock-down von PTPA bewirkten.

Zusätzlich wurden ebenfalls als Kontrolle zu allen drei shRNA Sequenzen dazugehörige "mismatch" shRNA Sequenzen kloniert. Außerdem wurde eine RNAi-insensitive PTPA c-DNA hergestellt, durch die Komplementationstests durchgeführt werden können.

Die ersten PTPA knock-down Klone verloren innerhalb von vier Wochen ihre Fähigkeit die Expression von PTPA zu reduzieren. Um dieses Phänomen beim Etablieren der nächsten Klone zu verhindern, wurden die verantwortlichen Gründe untersucht:

Ich konnte zeigen, dass die verwendete Mutterzelllinie N2aTRex eine niedrige Induzierbarkeit und eine hohe Expression von Reportergenen in Abwesenheit des Inducers Doxycyclin aufweist.

Nach Etablierung einer Methode zur Detektion und Quantifizierung von Tetracyclin, konnte ich nachweisen, dass das von mir zur Herstellung der Klone verwendete FCS 10^{-10}g/ml Tetracyclin enthielt.

Sowohl die niedrige Induzierbarkeit und die Kontamination von FCS mit Tetracyclin, als auch die Tatsache, dass Suppression der PTPA Expression in HeLa Zellen Apoptose induziert könnten zu dem Verlust der Fähigkeit die Expression von PTPA zu supprimieren und erschwerter Etablierung von PTPA knock-down Klonen führen.

Dennoch konnte ich, unter Verwendung von garantiert Tetracyclin-freiem FCS, mehrere PTPA knock-down Klone etablieren und auf ihre Proliferationsrate, PP2A Komplexbildung und PP2A Aktivität untersuchen.

In meinen Experimenten konnte ich nach Erniedrigung des PTPA Expressionsniveaus auf 50% keine Änderung in Zellproliferation, PP2A Heterotrimer Komplexbildung und keine verminderte Aktivität von PP2A feststellen.

Weitere Untersuchungen in anderen Zelllinien und Modellsystemen sind daher nötig, um Funktion von PTPA und damit die regulierte Biogenese von PP2A in Säugerzellen aufzuklären.

Die Funktion der regulatorischen Bα Untereinheit von PP2A in neuronaler Differenzierung

PP2A setzt sich aus einer katalytischen (C), einer strukturgebenden Untereinheit (A) und einer von vielen regulatorischen Untereinheiten (B), die für Substratspezifität, subzelluläre Lokalisation und katalytische Aktivität verantwortlich sind, zusammen. Die Expression dieser verschiedenen regulatorischen Untereinheiten ist dabei einer genauen Kontrolle unterworfen.

Die B-Typ Familie setzte sich aus vier verschiedenen Varianten zusammen: Bα, Bβ, Bγ und Bδ. Im Gegensatz zu den regulatorischen Bβ und Bγ Untereinheiten von PP2A, die ein raum-zeitlich hochreguliertes Expressionsmuster aufweisen, wird die Bα Untereinheit sowohl im ganzen Körper als auch in der gesamten Entwicklung ubiquitär exprimiert.

Deshalb wurde lange angenommen, dass PP2A, das sich aus der A, C und Bα-Untereinheit zusammensetzt (Bα-PP2A) vor allen Dingen „Housekeeping"-Funktionen ausübt.

Interessanterweise wurde gezeigt, dass eine niedrige Expression von Bα-PP2A in Gehirnen mit den typischen pathologischen Kennzeichen der Alzheimerschen Krankheit (AD) assoziiert ist (Sontag et al., 1995, 1996, 1999, 2004; Merrick et al., 1997; Hiraga and Tamura 2000).

Es wird angenommen, dass PP2A die wichtigste Tau-Phosphatase darstellt, einem elementaren Mikrotubuli-stabilisierenden Protein. Da die Dephosphorylierung von Tau einen stabilisierenden Effekt auf Mikrotubuli besitzt, spielt PP2A eine wichtige Rolle in der Aufrechterhaltung der MT. Umgekehrt führt die Hyperphosphorylierung von Tau zur Bildung von Neurofibrillen, einem wichtigen pathologischen Zeichen von AD.

Zusätzliche Hinweise auf eine Beteiligung von Bα-PP2A in AD kamen von RNAi-Experimenten, in denen Suppression von Bα in N2a und NIH3T3 Zellen zu einem Zusammenbruch von azetylierten und detyrosinierten Mikrotubuli (MT) führte (Nunbhaki-Craig et al., 2007).

Um nachzuweisen, dass ubiquitäres Bα-PP2A nicht nur „Housekeeping" Funktionen ausübt, sondern auch in der Differenzierung von Neuronen, die von der Stabilisierung neu gebildeter MT abhängig ist, eine wichtige Rolle spielt, wurde die Expression von Bα in N2a Zellen durch RNAi gezielt supprimiert und das Differenzierungsverhalten analysiert.

Tatsächlich konnten wir in N2a Zellen eine signifikant verminderte Differenzierung nach Bα Suppression nachweisen.

Dieses Ergebnis zeigt, dass Bα-PP2A eine kritische Rolle bei der neuronalen Differenzierung spielt. Außerdem unterstützt dieses Resultat die Hypothese dass Bα-PP2A über die Regulation von MT eine wichtige Funktion in der Entwicklung von AD hat.

1. INTRODUCTION

Protein phosphorylation – which occurs on serines, threonines or tyrosines - is a reversible posttranslational modification that is used in cells for the regulation of multiple processes (e.g. cell cycle regulation or signal transduction). Phosphorylation can alter many different protein-specific properties, like catalytic activity, subcellular localization or binding activity of key regulatory proteins. The process of phosphorylation, which is catalyzed by protein kinases, is counteracted by protein-phosphatases, which bring about dephosphorylation.

Protein kinases have been studied extensively in the past. However in the last few years, it became clear that phosphatases play an equally important role in the regulation of all cellular processes (for reviews see Virshup, 2000; Millward et al., 1999 and Janssens and Goris, 2001).

Studies in yeast revealed the large number of 124 genes coding for protein serine/threonine kinases (PSTKs). PSTKs are built up by one amino acid chain, with a conserved catalytic and a regulatory domain. They largely obtain substrate specificity by changes in the regulatory domain.

Contrary to this, only 37 genes coding for protein serine/threonine phosphatases (PSTPs) have been found in yeast. PSTPs are typically multisubunit enzymes, in which the catalytic and regulatory subunits are encoded by different genes. A wide range of different substrate specificities in this case is achieved by the combinatorial assembly of a small number of catalytic subunits with a larger pool of regulatory subunits to form a big collection of hetero-multimeric holoenzymes.

According to their specificity, protein phosphatases are grouped into protein-serine/threonine phosphatases (PSTPases), protein-tyrosine phosphatases (PTPases) and dual specificity phosphatases (DSPases) (Barford et. al. 1998). The serine/threonine phosphatases are divided into the PPP and the PPM family. Protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and protein phosphatase 2B (PP2B), which differ in their sensitivity for inhibitors, requirement for cations and *in vitro* substrate specificity, belong to the PPP family of PSTPases. Protein phosphatase C (PP2C) and mitochondrial pyruvate dehydrogenase are members of the PPM family (for a review see Wera and Hemmings, 1995).

1.1. Structure and subunits of protein phosphatase 2A (PP2A)

Most PSTP´s are multisubunit enzymes in which the catalytic and the regulatory subunits are encoded by different genes. PP2A is a prime example of the PSTP multisubunit architecture.

Hence the term PP2A does not describe a single enzyme but rather a large family of distinct heterodi- and -trimeric complexes (Fellner et al., 2003).

The core enzyme is a heterodimer, consisting of a 36 kDa catalytic subunit ($PP2A_C$) and a 65 kDa regulatory A subunit ($PP2A_A$), which forms the scaffold to connect the catalytic and the different regulatory B subunits ($PP2A_B$). Association of one of several regulatory B subunits determines substrate specificity and subcellular localization of the PP2A holoenzymes (see Fig. A, adapted from Jahnsens and Goris, 2001) (Cegielska et al., 1994; Kamibayashi et al., 1994; Mayer Jaekel et al., 1994; Turowski et al., 1997).

Structural analysis of a heterotrimeric AB´C complex revealed a horseshoe-shaped A subunit holding the C and B subunit together at the upper surface of the molecule. The regulatory B´ subunit binds to the C subunit near the active site, thereby defining substrate specificity. (Cho et al., 2007).

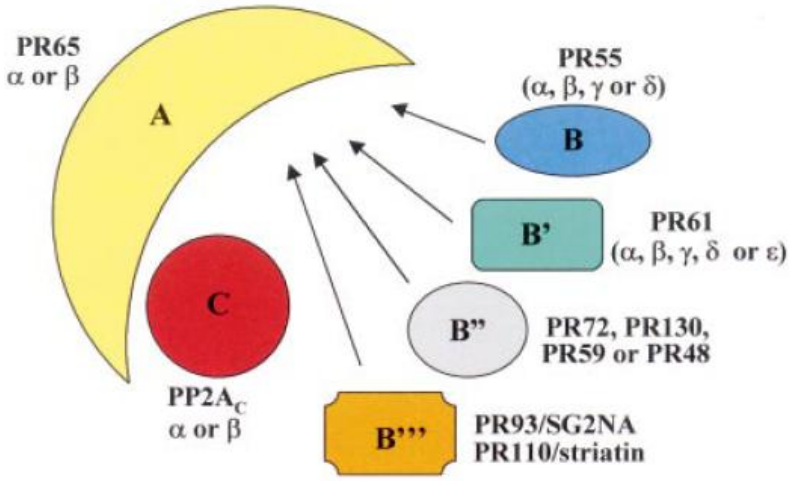


Fig. A Structure of PP2A

PP2A is built up by catalytic subunit C, structural subunit A and one member of the B/B´/B´´/B´´´ -type subunit families. In mammalia, A and C are encoded by two genes (α and β); the B subunits are encoded by four related genes (α, β, γ and δ); the B´ family are encoded by five related genes (α, β, γ, δ and ε); the B´´ and B´´´ subunit family contain three genes and two genes, respectively. (Adapted from Janssens and Goris, 2001)

1.1.1. Mammalian catalytic subunit

In mammals, there are two C subunit isoforms, α and β, which share 97% identity in their primary sequence. Both isoforms are ubiquitously expressed, with the α isoform more abundant that the β isoform (Arino et al., 1988; Khew-Goodall and Hemmings, 1988). It has been shown, that the structure of $PP2A_C$ is highly conserved from yeast to man, and maybe represents the most conserved of all known enzymes (MacKintosh et al., 1990; Orgad et al., 1990; Brewis et al., 1990; Sneddon et al., 1990: Cormier et al., 1991; Arino et al., 1993; Van Hoof et al., 1995; Ingels et al., 1995; Kinoshita et al., 1996). Deletion of the gene, encoding

PP2A catalytic subunit in mouse (Gotz et al.,1998) and yeast (Kinoshita et. al, 1996) is lethal, demonstrating that PP2A is essential.

Overexpression of $PP2A_C$ in mammalian cells has long been unsuccessful, due to an autoregulatory feedback cycle, ensuring relatively constant levels of PP2A (Baharians et al., 1998).

The overall structure of the catalytic subunit of PP2A resembles the one of PP1, although there are local structural differences (Xing et al., 2006). For example, the C terminus is unique and has a critical role in regulation of PP2A - its methylation facilitates B subunit binding by neutralizing charge repulsion.

The key structure of the catalytic centre of PP2A is a β-α-β-α fold. In the cleft, formed by the ß-sheets, three histidines, two aspartic acids and one asparagine are responsible for coordination of the binding of two divalent metal ions (Mn^{2+}). They are essential for catalytic activity and maintenance of the structural integrity of the enzyme (Cho et al., 2007).

Interestingly, addition of metal ions (like Mn^{2+} or Co^{2+}) (Cai, Chu et al. 1995; Endo, Connor et al. 1997) is only necessary for catalytic activity of PP2A when treated with reversible inhibitors like ATP, NaF or PPi or after long-term storage (Burchell and Cohen 1978; Hsiao, Sandberg et al. 1978; Brautigan, Ballou et al. 1982; Cai, Chu et al. 1995). This is probably due to a conformational change of the catalytic centre, which decreases the affinity of a metal ion to the active site and causes subsequent loss of the ions (Fellner et al., 2003).

1.1.2. Mammalian A subunit (PR65)

The A subunit is the structural subunit that links $PP2A_C$ subunit with one of the regulatory subunits. As is the case for the catalytic subunit, two distinct PR65 isoforms are present in mammalia, α and β, which share 86% sequence identity (Hemmings et al., 1990). Both are ubiquitously expressed. Binding of the different B subunits to the A subunit is mutually exclusive, as they compete for overlapping binding sites on the A subunit (Ruediger et al., 1994).

The protein is built up by 15 tandem repeats of a 39 amino acid sequence termed HEAT (huntingtin/elongation/A subunit/TOR). Its horseshoe-shaped architecture is due to stacking of the repeats.

1.1.3. Mammalian regulatory subunits

The most outstanding attribute about regulatory B subunits in mammalian cells is their variety, derived from the evolution of different subunit families.

At present, on the basis of sequence homology, four different families of B subunits have been identified, termed the B (B55/PR55), B' (B56/PR61), B" (PR48, PR59, PR72/130) and B'" PR93/PR110/striatin/SG2NA) families. These families lack sequence similarity, with the exception of two conserved A subunit binding domains (Li and Virshup, 2002).
Mammalian B subunits are expressed in a cell type/tissue-specific and developmental-dependent manner, thereby determining the activity and substrate specificity of PP2A in a spatial and temporal manner (Zolnierowicz and Hemmings, 1994; Strack et al., 1998).

The B or PR55 family

The B family is encoded by four different genes (Bα, Bβ, Bγ and Bδ). It was shown that Bα, and Bδ are ubiquitously expressed throughout the body, whereas Bβ and Bγ are predominantly expressed in the brain. Additionally Bβ and Bγ seem to have different functions in brain development, as the expression level of Bβ decreases and that of Bγ increases after birth (Strack et al., 1998).
Furthermore it is interesting, that the different isoforms also have different subcellular distributions. In contrast to Bα and Bβ, which are localized mainly in the cytosol, Bγ is highly enriched in the cytoskeleton.

The B´ or PR61 family

The B´ family is encoded by five distinct genes, their products named α, β, γ, δ and ε. This variety is further increased by the fact, that there are two isoforms of B´β (Bβ´1 and Bβ´2) and at least 3 splice variants of B´γ.
All B´ family members are built up by a highly conserved central region, which may be the interaction site for the A and also likely the C subunit. On the other hand the highly variable N- and C-terminal regions probably regulate subcellular targeting and substrate specificity. B´ family members α, β and ε are concentrated in the cytosol, while the γ isoforms are found in the nucleus and B´δ is localized both in the cytoplasm and in the nucleus.
The B´family members are also expressed in tissue specific manner: B´α and γ are highly enriched in heart and skeletal muscle. In contrast to that, B´β and δ are found mainly in the brain and seem to be developmentally regulated as indicated by differentiation experiments with N2a cells (McCright and Virshup, 1995; Csortos et al., 1996; Tehrani et al., 1996; McCright et al., 1996).

The B´´ or PR72 family

Five different B" isoforms are known in mammals: human PR72 and PR130, mouse PR59, human PR48, and the recently identified human G5PR (Hendrix et al., 1993; Kono et al., 2002)

Two members, PR72 and PR130, are splice variants from the same gene and differ only in their N-terminus. Both variants seem to be important in muscle, as indicated by the exclusive localization of PR72 and predominant expression of PR130 in heart and skeletal muscle (Hendrix, Mayer Jackel et al., 1993).

PR48 containing PP2A heterotrimers are localized to the nucleus and are selective for Cdc6, a component of the cell-cycle control system. As Cdc6 needs to be dephosphorylated to bind to the origin recognition complex and thus to initiate DNA replication, it is believed that PR48-containing PP2A is required for cell cycle progression (Yan et al., 2000).

PR59 was identified as an interaction partner of the retinoblastoma-like p107 protein. Overexpression of PR59 causes a G_1/S arrest and corresponds with an increased proportion of hyperphosphorylated and thus active p107 (Voorhoeve et al., 1999).

To sum up, many of the functions of B´´ indicate that they are predominantly related to the regulation of the G_1/S transition of cell cycle.

The B´´´ or PR93/PR110 family

Two other proteins, namely striatin (PR110) and S/G2 nuclear autoantigen (SG2NA or PR93) were identified and comprised as the new B subunit family B´´´ (Moreno et al., 2000). Both proteins are highly enriched in the brain and seem to have an important role in the modulation of calcium-dependent neuronal signaling and possibly remodeling of the cellular cytoskeleton (Castets et al., 1996; Moreno et al., 2000).

To summarize, each B subunit family consists of several highly related isoforms, therefore the total number of B subunits is further increased to a total of 18 (Fig. A, Janssens and Goris, 2001).

Consequently, more than 72 heterotrimeric holoenzymes, with different substrate specificities and subcellular localizations can be assembled.

This explains how the small pool of protein phosphatases can, by combinatorial assembly of holoenzymes with different substrate specificities, specifically counteract the activity of the large number of kinases in a cell.

1.2. PP2A subunits in yeast

In contrast to the diversity of PP2A subunits in mammalian cells, there are only few subunits in budding yeast Saccharomyces cerevisiae.

Yeast C subunit is expressed in two isoforms, Pph21 and Pph22. A third gene *PPH3* with high homology to *PPH21* and *PPH22* was found. Deletion of one of the two isoforms does not cause any major defects, but double deletion of both isoforms causes a severe proliferation defect. Additional deletion of *PPH3* results in cell death (Ronne H. et. al, 1991)

In yeast there is only one A subunit, named *TPD3* (tRNA processing deficient). Many cells in *tpd3Δ* strain become multi-budded and multi-nucleated, which points towards a defect in cytokinesis (van Zyl, Wills et. al, 1989).

The yeast homologues of the B and B´ subunit are encoded by *CDC55* and *RTS1* respectively. Deletion strains of either of the two subunits are viable but show defects in mitosis and exhibit high temperature sensitivity (Healy, Zolnierowicz et. al, 1991; Shu, Yang et. al, 1997; Eckert-Boulet, et. al, 2006; Wang et, al, 1997).

1.3. Regulation of PP2A biogenesis

PP2A is involved in many cellular processes; therefore a tight control is necessary.

Mechanisms to regulate PP2A comprise changes of its heterotrimer composition, phosphorylating the catalytic subunit (Chen et al., 1992; Guo and Damuni, 1993), phosphorylating the B subunits (McCright et al., 1996; Usui et al., 1998) and reversibly methylating/demethylating the C-terminus of the catalytic subunit (Favre et al., 1994) by methyltransferase LCMT (de Baere et al., 1999; Leulliot et al., 2004) and methylesterase PME-1, respectively (Ogris et al., 1999).

One of the most basic control mechanisms is an autoregulatory translational feedback loop of the catalytic subunit, which ensures relatively constant expression levels (Baharians and Schönthal, 1998).

Another important determinant is, that the stochiometric levels of the A, B and C subunits seem to be balanced, as was shown by RNAi experiments *in vivo* suppressing the A subunit, which lead to the degradation of the untargeted C and B subunits. This suggests that the PP2A subunits are stabilized in the holoenzyme, but are unstable as monomeric subunits (Li et al., 2002; Silverstein et al., 2002). This is supposed to be one of the control mechanisms, preventing free and unregulated C subunits, which would constitute a risk for a cell.

Yet, until holoenzyme assembly has taken place, there needs to be an additional surveillance system for free C subunit either newly translated or transiently free (if recycling occurs) (Fellner et al., 2003).

1.3.1. Posttranslational modifications

Modulation of PP2A by covalent modifications like phosphorylation or carboxymethylation takes place on its conserved C-terminal part. The advantage of regulating PP2A activity by posttranslational modifications is its high flexibility and responsiveness to other stimuli.

Phosphorylation

The $PP2A_C$ can be reversibly phosphorylated on Tyr^{307} located at its carboxy terminus, resulting in transient inactivation (Chen et al., 1992; Chen et al., 1994). External stimuli, like epidermal growth factors, insulin, interleukin-1 or tumor-necrosis factor α cause increased phosphorylation of PP2A C subunit (Guy et al., 1995). So, the transient inactivation of PP2A could be one way to speed up cellular communication through kinase cascades.
The regulatory subunits, particularly the B´ type subunit family, are also phosphorylated. This induces changes ranging from altering substrate specificity to increasing the activity of the PP2A holoenzyme (Usui et al., 1998; Xu et al., 2000).

Methylation

PP2A can be methylated on the carboxy group of Leu^{309}, located on the C-terminal residue. Methylation of PP2A is regulated by the counteractive activities of the PP2A methylesterase-1 (PME-1) and leucine carboxyl methyltransferase-I (LCMT-I) (Lee et al., 1993; Xie et al., 1994; Favre et al., 1994; Ogris et al., 1999; DeBaere et al., 1999).

Methylation of PP2A C subunit has been shown to be necessary for the formation of stable PP2A heterotrimers (Wei et al., 2001; Tolstykh et al., 2000; Wu et al., 2000; Yu et al., 2001). Deletion of *PPM-1* in yeast leads to a $PP2A_C$ subunit with decreased affinity to the A subunit and completely prevents binding of a B subunit to the AC dimer (Wu et al., 2000; Wei et al., 2001) and also leads to decreased catalytic activity in yeast (Fellner et al., 2003). In mammalian cells, methylation is required for the binding of B/PR55 and B''/PR72 whereas it seems to be dispensable for members of the B'''/striatin/SG2NA family (De Baere et al., 1999; Moreno et al., 2000).

So, the absence of methylation of free monomeric C subunit, isolated from *PPM-1* deletion strains, is accompanied by abolished heterotrimer formation and reduced catalytic activity. Contradictory to that, free monomeric C subunit, isolated from wild-type cells and therefore from pre-existing holoenzymes shows an increased catalytic activity. (Tung et al., 1985; Mumby et al., 1985)
Therefore it seems that activation of $PP2A_C$ and holoenzyme formation is dependent on PPM1 function.

1.3.2. PTPA, the PP2A phosphatase activator

In a study, published in 2003 by Fellner et al., it was shown, that yeast $PP2A_C$ requires a protein named RRD (rapamycin resistant deletion), to assemble into an active and specific PP2A complex. There are two genes in yeast, called *RRD1* and *RRD2*, comprising this function. Loss of PTPA function in yeast produces an unstable and metal-dependent form of PP2A with a decreased capacity to dephosphorylate serine/threonine phosphorylated substrates (Fellner et al., 2003).
Moreover, loss of the PTPA homologue in yeast leads to a $PP2A_C$ with an eightfold increase of activity towards tyrosine-phosphorylated substrates, compared to the wild-type protein. As phospho-tyrosines (P-Tyr) are more bulky than phosphoserines/–threonines (P-Ser/Thr), it has been suggested that PTPA induces a conformational change in PP2A.
The mammalian homologue PTPA (PP2A phosphatase activator) is able to partially rescue the phenotype of an *rrd1Δ/rrd2Δ* deletion strain. These results were summarized in a model, where PTPA/RRD induces a conformational change in the catalytic site of $PP2A_C$. This change narrows the catalytic site, so that only the more compact P-Ser/Thr substrates can enter. Additionally, this produces a high affinity conformation for metal ions, rendering the produced PP2A metal-independent (see Figure B) (Fellner et al., 2003).

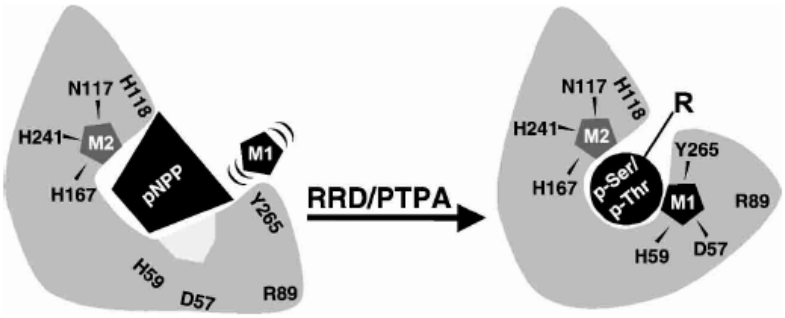


Figure B: Model of RRD/PTPA function

Para-nitrophenyl phosphate (pNPP) represents a bulky P-Tyr like substrate; P-Ser/P-Thr: phospho-serine / phospho-threonine substrate
M1 and M2 indicate metal ion 1 and metal ion 2, respectively, located at the catalytic core of $PP2A_C$. The indicated amino acid residues represent amino acids of PP2AC that are required for metal ion coordination and catalysis. (D) Asp; (H) His; (N) Asn; (R) Arg; (Y) Tyr. (Adapted from Fellner et al., 2003)

Recently, PTPA was reported to be a peptidyl prolyl cis/trans-isomerase, targeting Pro^{190} near the active site of the C subunit. This enzyme catalyzes the transformation between the cis- and trans-conformation of proline and thus can also cause a conformational change in the whole protein (Jordens et al., 2005). PPIases minimize the energetic cost of the rotation around the C-N bond by binding and stabilizing the transition state (Harrison and Stein, 1990). The molecular basis of PTPA function was defined further through crystal structures:

PTPA is a bilobal molecule, built up entirely by α-helices. Most of the conserved residues are found in a groove between the lobes. Within this groove the absolutely conserved Trp^{202} seems to have an important function, as it directly stacks with the $proline^{190}$ of the $PP2A_C$ subunit and its mutation causes significant reduction in PTPA´s capacity to activate PP2A and reduction in its *in-vitro* PPIase activity. However, this mutation does not disrupt the *in-vitro* PPIase activity, using another substrate, which could reflect a limitation of this assay for mutations or it could be argued, that in addition to the PPIase activity, another function is necessary to activate PP2A (Leulliot et al., 2006).

The activation of PP2A by PTPA provides a surveillance mechanism, keeping free C subunit in check, until holoenzyme assembly has occurred.

1.3.3. Activation and Holoenzyme formation of PP2A in yeast

Composition of the heterotrimer is mostly determined by availability of the different B subunits, thus determining localization and substrate specificity of PP2A. As mentioned above, the different B subunits exhibit specific temporal and spatial expression patterns. This regulates the assembly of particular PP2A complexes in an on-demand–fashioned manner.

Deletion of *RRD1* and *RRD2* in yeast results in a catalytic subunit with a conformation different from the wild-type C subunit and a decreased catalytic activity towards P-Ser/Thr.

Furthermore, knock-out of the gene, coding for A subunit in yeast, *tpd3* leads to reduced catalytic activity and to abolished holoenzyme assembly. This demonstrates its important function as a scaffolding protein but also indicates the importance of holoenzyme assembly for activation of PP2A (Hombauer et al., 2007).

Interestingly, deletion of *PPM-1* leads to disruption of holoenzyme assembly and to decreased catalytic activity of PP2A (Hombauer et al., 2007).

In contrast to that, deletion of PPE-1 in *Δtpd3* strains, leads, in absence of the holoenzyme, to a restoration of catalytic activity. On the other hand, deletion of PPE-1 in *rrd1/rrd2* knock-out strains leads to the production of inactive PP2A heterotrimers.

Moreover, a high level overexpression of PPE-1 in wild-type cells correlated with a significant increase in PPE-1:PPH21 complexes and a profound reduction of TPD3 and RRD2 in complex with PPH21 accompanied with a markedly reduced PPH21 phosphatase activity. This suggests that PPE-1 also competes for PPH21-binding with RRD and TPD3.

Putting these results together, Hombauer et al. proposed a model, where the activation of PP2A C subunit is tightly coupled to holoenzyme assembly and where both processes are regulated by its methylation status:

First the scaffolding subunit TPD3 builds a complex with RRD2 and PPH21 subunit, thereby repelling PPE-1. Next C subunit can be methylated by PPM1 and RRD2 can exert its function. Then, CDC55 or RTS1 binds to the complex, finishing the formation of an active heterotrimer. Thus the risk of an active and monomeric, thus unspecific C subunit is avoided by producing an inactive precursor, that must not be activated by RRD before holoenzyme assembly has occurred. Consequently, binding of free C subunit to PPE-1, keeping it demethylated prevents its untimely activation (Hombauer et al., 2007) (see Figure C).

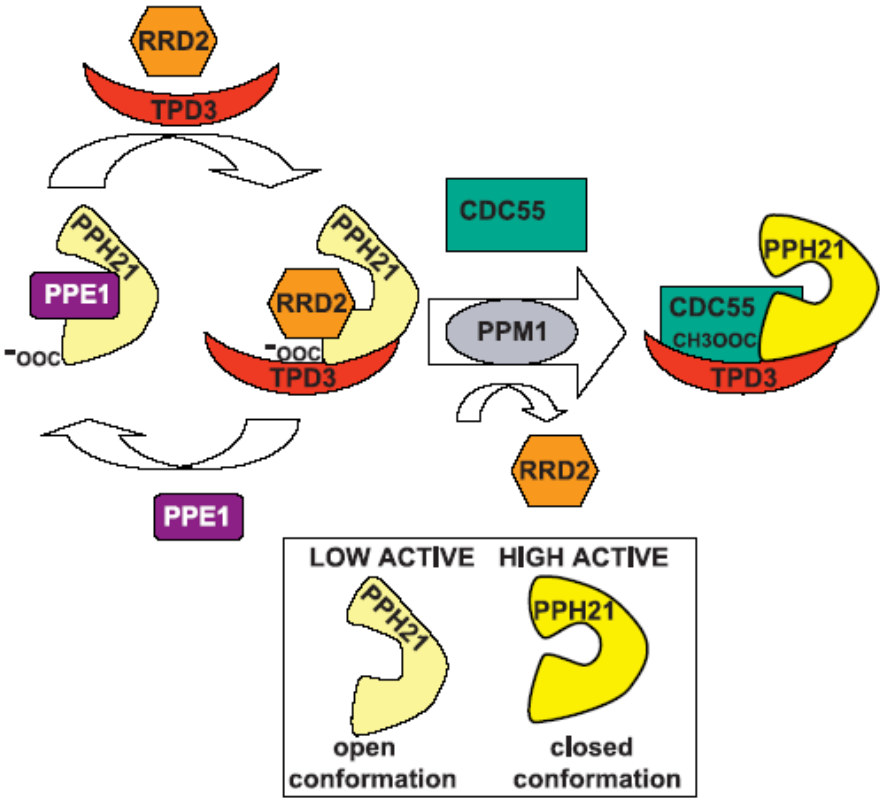


Figure C: RRD/PTPA-dependent generation of active PP2A C subunit is coupled to holoenzyme assembly and regulated by methyl-esterase/-transferase enzymes (Hombauer et al., 2007).

However, this model raises the question, what keeps the activated and unspecific AC dimer in check, until the regulatory B subunit assembles. A possible explanation could be, that phosphorylated B subunits are a substrate for the RRD2:TPD3:PPH21 complex, providing an

affinity that is high enough, to minimize the temporal gap to a neglectable period. By activating B-type subunit-specific kinases, the cell could therefore regulate the assembly of specific complexes.

1.3.4. Activation and Holoenzyme formation of PP2A in mammalian cells

The activation mechanism for PP2A in mammalian cells is still unknown and is subject to this study.

However, it was shown, that PTPA is able to partially complement the function of RRD1/2 in *rrd1/rrd2* knock-out in yeast. Moreover, RNA-interference experiments revealed, that PTPA is essential for mammalian cell survival in HeLa cells (Fig. D) (Fellner et al., 2003). However, direct evidence that PTPA fulfils the same function in mammalian cells, like RRD1/2 in yeast is still missing.

Therefore the major goal of this project was, to study the role of PTPA in the biogenesis of PP2A in mammalian cells, by suppressing its expression using RNAi.

As the suppression of PTPA by RNAi was shown to trigger apoptotic death in HeLa cells, we chose to use an inducible RNAi system in order to be able to analyze the PP2A biogenesis biochemically upon gradual suppression of PTPA.

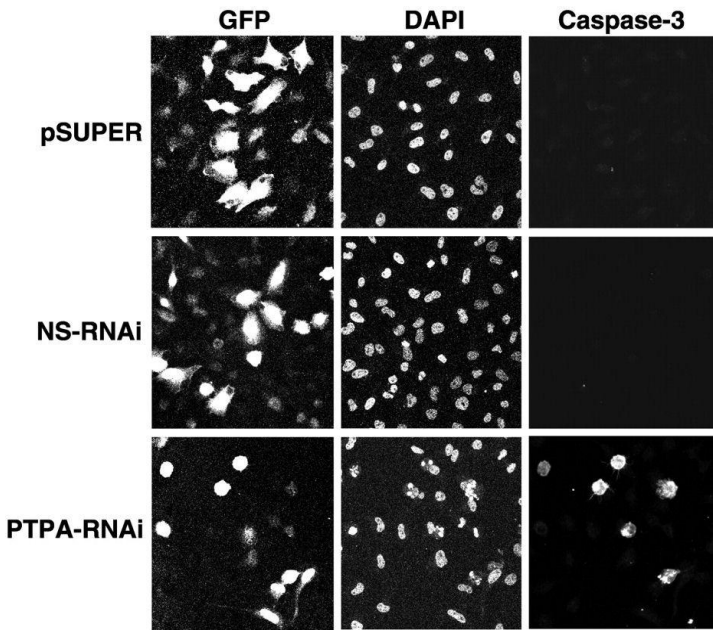


Fig. D Suppression of PTPA expression by RNAi triggers apoptotic cell death in mammalian cells.

HeLa cells were co-transfected with the pRETRO$_{Katze}$ vector containing the coding sequence of the puromycin resistance marker and of the green fluorescence protein (GFP) and either empty pSUPER vector (pSUPER), pSUPER-NS-PTPA containing a "nonsense"-targeting sequence (NS-RNAi) or pSUPER-PTPA containing a PTPA-targeting sequence (PTPA-RNAi). Cells were stained with antibody specific for active caspase-3 and counterstained with DAPI. GFP-staining indicates co-transfected cells. Confocal microscopy images of representative cells are shown. (Adapted from Fellner et.al, 2003)

1.4. RNA interference

RNA interference (RNAi) is an evolutionarily conserved mechanism used by the cell that permits a sequence-specific post-transcriptional down-regulation of target genes.

The discovery of RNAi was first noted in plants, where it was termed co-suppression (Napoli, 1990; Van Blokland, 1994) and later in the nematode *Caenorhabditis elegans* (Fire et al, 1998). An RNAi pathway has since been shown to be present in most eukaryotes.

1.4.1. Mechanism of RNAi

Briefly, the presence of double-stranded RNA (dsRNA) in cells results in the degradation of homologous mRNA und thus to silencing of target genes.

Described in more detailed, dsRNA is processed into 21-23bp short interfering RNA (siRNA) by a ribonuclease called Dicer (Myers et al., 2003). These siRNAs are subsequently incorporated into an effector complex called the RNA-induced silencing complex (RISC). The 3´ region of siRNA, referred to as the "seed region" first binds to the homologous mRNA, guiding the RISC complex to the appropriate target. The complex selectively degrades the bound mRNA strand, thereby inducing gene silencing (Fig. E) (Martinez, et al., 2002; Khvorova, et al., 2003; Schwarz, et al., 2002; for reviews see Hannon, 2002; Tijsterman, et al., 2002; Dykxhoorn, et al., 2003).

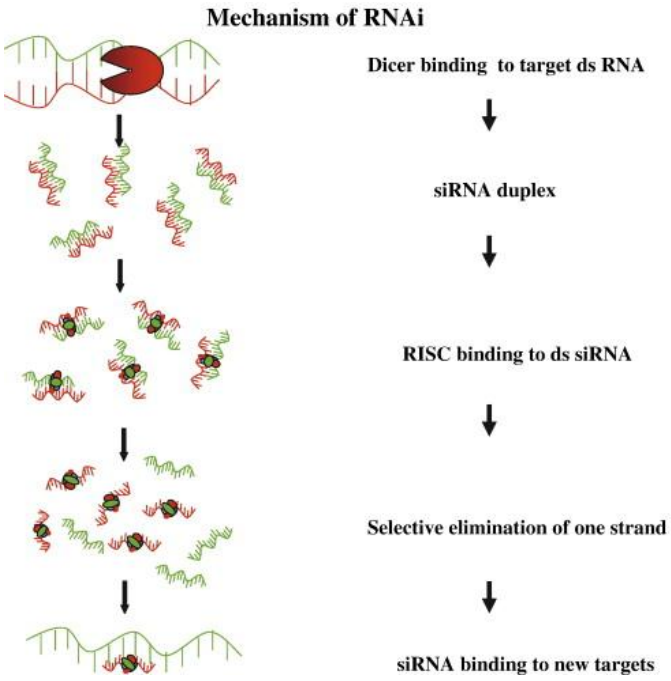


Fig. E: Schematic diagram of the mechanism of RNA interference.

Dicer binds to dsRNA and digests it into 21 to 23 nucleotide duplexes. These in turn are incorporated into the RISC, which has been suggested to eliminate one of the strands and so initiate a cyclical process as the siRNA associates with new target molecules (adapted from Kumar et al., 2007).

Three types of small RNA can be used to perform RNAi in mammalian cells: chemically synthesized siRNA or short hairpin RNA (shRNA) and micro-RNA adapted short-hairpin (shRNAmir) both cloned into plasmid DNA vectors.

The effects of synthetic siRNA are transient, lasting only for 3-5 days in cell culture and are therefore not sufficient for studies of proteins with long half-lives (Holen, et al., 2002). Another limitation inherent in transient transfection of siRNA is the variability of transfection efficiencies. Moreover, siRNA-concentrations, necessary for efficient knock-down are subject to concentration-dependent off-target effects (Jackson et al., 2003; Haley and Zamore, 2004; Persengiev et al., 2004).

The solution to all these limitations is the use of plasmids or viral vectors, coding for shRNA: First, vector-based RNAi is not transient and allows the co-expression of reporter genes, such as GFP or resistance genes, facilitating tracking and/or selection of transfected cells. And second, shRNA based systems are less susceptible to concentration-dependent off-target effects, as the processing of shRNA by the cellular machinery is a rate-limiting step (Jackson et al., 2003).

Most frequently, RNA-polymerase III promoters (U6, H1 or 7SK) mediated transcription of short hairpin structures with a stem of 19-29bp joined by a short loop of 4-10nt are used (Brummelkamp et al., 2002; Harborth et al., 2003; Paddison, et al., 2002).

The hairpin is formed by complementary regions, folding back on themselves, analogous to the natural micro-RNA. Recognition and processing of shRNA by the cellular RNAi machinery produces siRNA (Brummelkamp, et al., 2002; Miyagishi, et al., 2004).

1.4.2. Inducible RNAi systems

A doxycycline inducible form of the RNA polymerase III H1 promoter has been developed by replacement of a 19bp sequence between the TATA box and the transcription start site with a binding site (tetO) for the tetracycline repressor.

Binding of the repressor to the tetO site blocks transcription, whereas addition of the inducer tetracycline, or its derivative, doxycycline, leads to dissociation of the repressor, allowing expression of siRNA and in turn the down regulation of the target gene (Amar, et al., 2006).

As the tet-responsive polymerase III promoters display some level of leakiness, a vector, named pNTO with a more tightly regulated H1 promoter was recently described. This expression system contains two optimally placed tet operators and displays low basal transcriptional activity and effective silencing in the induced state (Figure G, adapted from Strack et al., 2004) (Strack et al., 2004). That system was used for the following study.

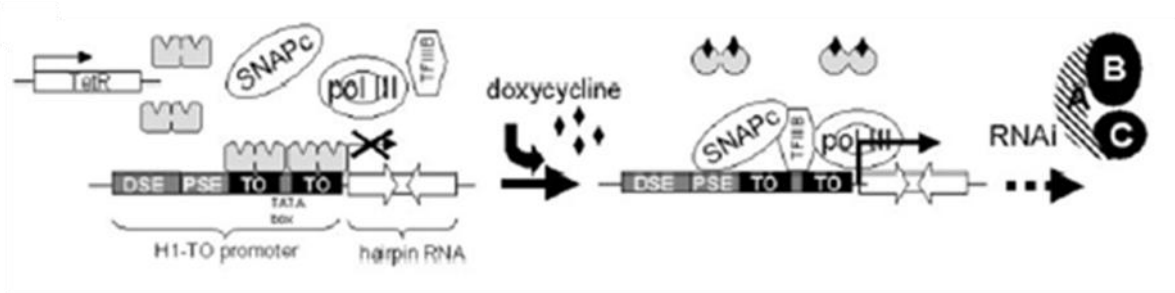


Fig. G. Schematic of inducible RNAi expressing vector pNTO

In the absence of inducer, tetracycline repressor (TetR) binds to the TO sites of the H1-TO promoter, blocking transcription of shRNA. Addition of doxycycline/tetracycline dissociates TetR from the promoter, allowing transcription of the shRNA to occur. (Adapted from Strack et. al, 2004)

As the pNTO system requires the function of the native TetR, a stable TetR-expressing cell line was generated by Petra Weidinger. N2a were transfected with the vector pcDNA^{TM}6/TR (Invitrogen) that contained the coding sequence of the native tet-repressor and a blasticidin resistance marker. After selection with blasticidin, single clones were isolated and tested for expression of tet-repressor and for their ability to block expression. Clone #14 (N2aTRex14) was chosen as the parental cell line to be used in the generation of PTPA knock-down cell lines.

1.4.3. Designing efficient siRNAs

Only 50 percent of siRNAs or shRNAs with exact complementarity to a target mRNA will result in effective silencing of the target mRNA (Elbashir, et al., 2002). Recently, various design rules have been identified to improve the frequency of designing functional siRNA:

Standard siRNAs are designed as 19-23bp oligonucleotides with 2nt 3′ overhangs and a preference for uridine residues in the 3′ overhang (Elbashir, et al., 2001). A GC content of about 50% should be ensured and strings of three or more G or C residues should be as well avoided as mRNA areas with complex secondary structures (Far and Sczakiel, 2003; Bohula, et al., 2003).

Moreover, different algorithms have been developed to further facilitating the process of designing efficient siRNA sequences (Reynolds, et al., 2004; Ui-Tei, et al., 2004; Boese, et al., 2005;Levenkova, et al., 2004; Chalk, et al., 2004; Naito, et al., 2004; Saetrom and Snove Jr., 2004; Yiu, et al., 2005; Santoyo, et al., 2005; Teramoto, et al., 2005).

Nevertheless, testing of siRNAs in an experiment is still the only effective validation for functional siRNAs.

1.4.4. Target validation

A major difficulty in using siRNA is that siRNAs and shRNAs can induce non-specific alterations in gene expression (Jackson et al., 2003; Sledz, et al., 2003; Bridge et al., 2003).
For this reason, all siRNA experiments should be controlled such, that an observed phenotype can be confidently linked to silencing of the targeted gene and not be due to an off-target effect of the siRNA.
As a first step, any siRNA being considered should be filtered through a sequence alignment algorithm (e.g., BLAST). Only siRNAs with as little identity with other transcripts as possible should be selected. A minimum of two mismatches (one centrally placed mismatched mutation and a second mismatch either downstream or upstream of the first mismatch) is usually sufficient to eliminate RISC-mediated cleavage (Rodriguez-Lebron E. and Paulson H. L., 2006)
As a negative control, so-called "mismatch-RNAi", where 3 critical positions of the siRNA 19-mer are changed, should be used. Studies in drosophila have shown that not all positions of a siRNA molecule are equally important for target recognition. Of particular importance is the centre of the siRNA (Elbashir, 2001). Additionally, it was shown, that specific residues at every third position (4, 7, 10, 13, 16, 19) of siRNAs greatly influence its RNAi activity (Katoh et al., 2007). Therefore, Positions 4, 10 and 16 were changed in the shRNA molecules used, to produce the respective "mismatch-shRNA".
Two ways to validate an RNAi induced phenotype are reproduction and complementation. The most commonly used control reproduces the phenotype with an additional, independent siRNA, targeting unrelated sequences of the target mRNA (Jackson et al., 2003). On the other hand, complementation or rescue experiments use the overexpression of siRNA-insensitive gene copies of the target gene, to eliminate the siRNA-mediated phenotype. This is mostly done by expressing a cDNA encoding the target gene, containing translationally silent mutations. When siRNAs are designed to target the untranslated region of an mRNA, wild-type cDNA can be used to rescue the phenotype (Lassus, et al., 2002).
Therefore, additionally to the RNAi sequence used by Fellner et al., targeting the C-terminal part of PTPA, two additional RNAi sequences were designed, targeting the 3´UTR of PTPA. This was done, to be able to reproduce the possible phenotype of PTPA knock-down with unrelated sequences and to be able to complement the possible phenotype by the overexpression of a wild-type cDNA copy of PTPA.
Moreover, mismatch RNAi sequences with residues 4, 10 and 16 exchanged were designed, cloned and used as a control for every RNAi sequence used in this study.

Additionally, in order to be able to complement the possible effects of the knock-down induced by RNAi targeting the C-terminal part of PTPA, we designed and cloned an RNAi-insensitive PTPA version.

1.5. PP2A in Alzheimer Disease (AD)

Alzheimer disease is characterized by progressive dementia that will end ultimately in the disability of the patient to master the own life and the need for long-time care. Dementia in AD is due to massive death of hippocampal and cortical neurons resulting in brain atrophy. The major histopathological hallmarks of AD neurons are amyloid plaques and neurofibrillary tangles.

Amyloid plaques are deposits of aggregated ß-amyloid peptide, a 42-amino-acid toxic peptide that descends from an abnormally processed ß-amyloid precursor protein.

Neurofibrillary tangles consist of an abnormally hyperphosphorylated intracellular protein named tau, tightly wound into paired helical filaments. Tau is a neuronal-specific microtubule (MT)-associated protein, which binds to microtubules and thereby increases their stability (for a review, see Tian et al., 2002).

Phosphorylation of tau leads to dissociation from the microtubule and therefore to reduced stability (Illenberger, et al, 1998; Biernat, et al., 1993; Leger, et al, 1997; Xie, et al., 1998). In healthy adults tau is phosphorylated on only a few sites. However, in AD affected brains, tau is hyperphosphorylated (Morishima-Kawashima, et al., 1995; Matsuo, et al., 1994), which is thought to impact microtubule assembly and protein trafficking, resulting in apoptosis (Mandelkow et al., 1995).

The hyperphosphorylation of tau is due in part to decreased tau phosphatase activity (Matsuo, et al., 1994). Several indications suggest an involvement of PP2A in this process:

PP2A was first shown to be capable to dephosphorylate tau, in a biochemical experiment, where AD brain derived hyperphosphorylated tau was dephosphorylated by PP2A (Gong et al., 1994).

Experimental indications that PP2A is involved in tau pathology *in vivo* came from analysis of AD affected brain regions, where decreased PP2A catalytic activity (Gong et al., 1993), PP2A mRNA expression (Vogelsberg-Ragaglia et al., 2001) and a decreased level of the main brain PP2A holoenzyme ABαC (Sontag et al., 2004) were demonstrated. Also PP2A activity negatively correlated to the level of tau phosphorylation in human brains (Sontag et al., 2004). As PP2A activity is accountable for 71% of total tau phosphatase activity in the human brain it was considered to be the major tau phosphatase (Liu et al., 2005).

Additionally, it was shown that $PP2A_C$ directly binds to tau and microtubules and that suppression of PP2A activity in a cell culture system by ocadaic acid (OA) or overexpression of SV40 small t, induces AD-like hyperphosphorylated tau, microtubule destabilization, modification of synapse structure, and neurodegeneration (Arias et al., 1993; Sontag et al., 1995; Sontag et al., 1996; Merrick et al., 1996; Merrick et al., 1997; Malchiodi-Albedi et al., 1997; Sontag et al., 1999).

Injection of OA into the brain causes tau hyperphosphorylation, neurodegeneration and memory impairment in rats (Arendt et al., 1995). Also, the expression of a dominant negative PP2A mutant in mice led to pathological tau hyperphosphorylation *in vivo* (Kins et al., 2001).

PP2A-dependent PI 3-kinase signaling also plays a crucial role in neuronal survival. It was hypothesized by E. Ogris and E. Sontag that disruption of normal brain PP2A functions by malfunction of PTPA could lead to the pathogenesis of AD and other neurodegenerative diseases.

Therefore, another goal of this study was the analysis of the tau phosphorylation status in cells with reduced PTPA levels. This would answer the question, whether the deregulation of PP2A is a cause or a consequence of the tau pathology.

To be able to study tau-phosphorylation in a cell culture system as similar as possible to the cellular situation in the brain, neuroblastoma (N2a) cells, having neuronal characteristics, were chosen as the parental cell line for all experiments.

1.6. The Role of PP2A B-type subunit Bα in differentiation of neuronal cells

Protein serine-/threonine phosphatase 2A (PP2A) is a multifunctional regulator of cellular signaling. Variable regulatory subunits associate with a core dimer of scaffolding and catalytic subunits and are postulated to dictate substrate specificity and subcellular location of the heterotrimeric PP2A holoenzyme.

In contrast to others, PP2A containing the regulatory subunit Bα, is expressed ubiquitously throughout the body, which indicates this enzyme to be a “housekeeping” phosphatase. Nevertheless, ABαC was identified as the major phosphatase for tau and therefore an important regulator of microtubule dynamics, implicating a possible role in neuronal differentiation.

Moreover, Schild et al. (Schild et al, 2006) showed that Bα protein levels were unaltered, while Bβ and Bγ protein levels are reduced after differentiation. On the other hand, overexpression of the PP2A regulatory subunit Bγ promotes neuronal differentiation by

activating the MAP kinase cascade (Strack et al. 2002), indicating partly redundant roles of the different B subunits.

To clarify the role of PP2A regulatory subunit Bα in neuronal differentiation, differentiation of the N2aTRex14 subline of N2a cells was analyzed upon RNAi-induced reduction of Bα.

2. MATERIALS AND METHODS

2.1. Tissue culture

2.1.1. Solutions, media and cell lines

10xPBS: Dissolve in ddH_2O: 80g NaCl, 2g KCl, 2g KH_2PO_4, 14,4g Na_2HPO_4, adjust to a final volume of 1l, adjust to pH 7.4, autoclave and store at RT.

DMEM: add 50ml FCS and 5mml antibiotic mix to 450ml of DMEM medium under sterile conditions (Sigma #D-5523).

Antibiotic mix: Dissolve 0,6g Penicillin-G and 1g Streptomycin-sulfate in 10ml 10xPBS, filter sterilize through a membrane filter (0.2µm) and store aliquots of 5ml at –20°C.

Trypsin: Dissolve 250mg trypsin in 25ml 10xPBS and add water to a total of 245ml. Stir for 2 hours. Add 5ml 1% Na-EDTA pH 7.4, mix and filter sterilize through a membrane filter (0.2µm). Store aliquots of 10ml at –20°C (Serva#37290).

Blasticidin: Dissolve 2,5mg/ml in H2O, filter sterilize through a membrane filter (0.2µm), use to an end concentration of 5µg/ml (Invitrogen #R210-01)).

Puromycin: Dissolve 10mg/ml in H2O, filter sterilize through a membrane filter (0.2µm), use to an end concentration of 5µg/ml for N2a cells and 0,8µg/ml for HeLa cells (concentrations derived from Fellner et al, 2003) (Sigma #P-7255).

Hygromycin B: Dissolve 50mg/ml in H2O, filter sterilize through a membrane filter (0.2µm), use to an end concentration of 200µg/ml for Hek293TRex cells (concentration according to recommendation of Invitrogen) (Calbiochem #400049).

Doxycycline: Dissolve 1mg/ml in H2O, filter sterilize through a membrane filter (0.2µm), use to an end concentration of 1µg/ml (Sigma #D-9891).

Geneticin: Dissolve 10mg/ml in DMEM +Pen/Strep, filter sterilize through a membrane filter (0.2µm), use to an end concentration of 600µg/ml for all cell lines (concentration according to recommendation of Invitrogen) (Gibco #11811-098).

All-trans retinoic acid: 1mM in DMSO, store light protected at -20°C, use to an end concentration of 1% (Sigma #95152-1G).

Cell lines:

HeLa:	human cervix carcinoma cell line
HeLaTRex:	human cervix carcinoma cell line stably expressing the Tet-Repressor (kindly provided by Michael Glotzer)

N2a:	human neuroblastoma cell line
N2aTRex14:	human neuroblastoma cell line, stably expressing the Tet-Repressor (established by Petra Weidinger)
HEK293TRex:	human embryonic kidney cell line, stably expressing the Tet-Repressor (kindly provided by Stefan Strack; Strack et al., 2004)
Pc12TRex:	rat chromaffin cell line, stably expressing the Tet-Repressor (kindly provided by Stefan Strack; Strack et al., 2004)

All cells are grown in DMEM+10% fetal calf serum. All media are supplemented with penicillin (100units/ml) and streptomycin (100µg/ml). All cells expressing the tet-repressor are grown in medium containing blasticidin (5µg/ml).

2.1.2. Propagation of cell lines

Cells are cultivated in an incubator at 37°C in an atmosphere with 7,5%CO_2.
It is important to split the cells every two or three days according to their doubling time so that the cells never become confluent.
Splitting of cells in Petri dish with 100mm diameter:
The medium is removed and cells are washed with 5ml PBS. Then 0,5ml Trypsin/EDTA is added. The cells are incubated at RT and detached by gentle knocking against the wall of the dish. When the cells are detached, the cells are rinsed down the plate with 10ml medium. After determination of the cell number, the cells are diluted as required and plated on new dishes.

2.1.3. Quantification of cell number

For the determination of cell number 20µl of the cell suspension is counted in a Thoma-chamber. Alternatively 50µl cell suspension is diluted in 5ml CASYton solution and the cells are counted by CASY cell counter.

2.1.4. Freezing/Thawing of cells

The cells are trypsinised and pelleted by centrifugation. The pellet is resuspended in 90%FCS/10%DMSO and incubated on ice for 20 minutes. Afterwards the cells are stored for at least one day at –80°C and are then transferred to liquid nitrogen. To thaw cells, the frozen tube is incubated in a 37°C water bath, until the cell suspension is liquid. Then it is diluted in

5ml medium and centrifuged. Finally, the cell pellet is resuspended in the appropriate volume of the according medium.

2.1.5. Transfection of cells

Cells are transfected using Invitrogen lipofectamine 2000 (Invitrogen #11668-027), using the following protocol for transfection in a 24-well format:

Cells are seeded at high density ($0,5\text{-}2x10^5$ cells on a 24-well) on the previous day, so that cells will be 90-95% confluent at the time of transfection.

For each transfection sample, 0,8µg DNA is diluted in 50µl DMEM without FCS/antibiotics and 2µl lipofectamine are diluted in 50µl DMEM without FCS/antibiotics. After 5 minutes incubation at room temperature, the diluted DNA is combined with diluted lipofectamine, mixed gently and incubated for 20 minutes at room temperature.

The lipofectamine/DNA mix is added to the cell directly and transfection is allowed to proceed for 4 hours, after which the medium is exchanged. Testing or antibiotic selection starts 24 hours post transfection.

To transfect in different tissue culture formats, use the volumes and amounts as shown below (Lipofectamine 2000, manual; Invitrogen):

Culture vessel	Volume of dilution medium (DMEM)	DNA	Lipofectamine 2000
24-well	2x 50µl	0,8µg	2µl
12-well	2x 100µl	1,6µg	4µl
6-well	2x 250µl	4µg	10µl
60-mm	2x 500µl	8µg	20µl
10-cm	2x 1,5ml	24µg	60µl

2.1.7. Differentiation of N2aTRex14 cells

Poly-L-lysine coating of plates and coverslips

Add 0,001% Poly-L-lysine solution (Stock: 0,01%; Sigma #P8920) to sterilized cover slips in multiwell or to a dish:

Culture vessel	Volume of Poly-L-lysine
24-well	300µl/well
6-well	500µl/well
60-mm	1ml
10-cm	2,5ml

Incubate dish with poly-L-lysine for 1 hour, remove and let dish dry for another hour under UV-light. Wash dish 4 times with ddH_2O and let again dry over night under UV-light.

Differentiation of Bα-knockdown cells

Cells are seeded on poly-L-lysine coated glass coverslips. After 24 hours, differentiation of N2aTRex14 cells is induced by starvation in medium supplemented with 1%FCS or with 1%FCS and 1% retinoic acid (RA).

Lightfield microscope pictures of cells are taken 24 and 48hours after starting differentiation (complete experimental outline: see Fig. H).

For estimates of differentiation state of, 4 random visual fields (on average 35 cells/picture) (20x objective) for each condition were viewed. The total number of cells visualized under phase contrast microscopy and the number of cells with neurites longer than the cell axis (differentiated) (see Fig. H) were counted in each visual field.

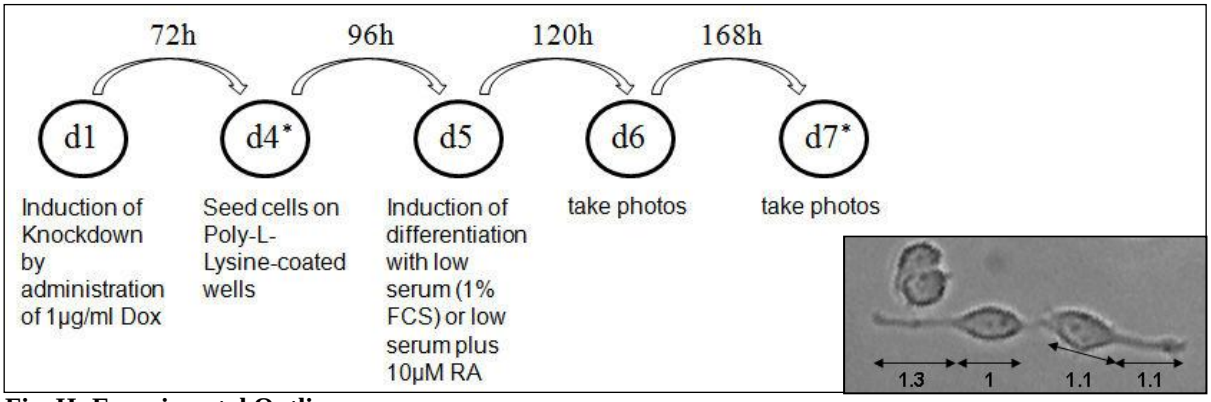


Fig. H: Experimental Outline

Knockdown of PP2A-Bα was induced on day1 by administration of 1µg/ml Dox. After 72 hours cells were seeded on Poly-L-Lysine-coated wells and induced to differentiate 24h later with low serum (1% FCS) or low serum plus 10µM RA. Doxycycline was added every day for 6 days to maintain knockdown.

Differentiation was scored on day 6 and 7 by taking photos and counting a minimum of 100 cells/experimental condition. Cells with neurite outgrowth longer than the cell body were considered differentiated (see Photo).

Lysates were prepared on day 4 and 7 (*) to prove that the differentiation starts with reduced levels of PP2A-Bα and that Bα-knockdown is stable over the experimental period.

2.2. Working with Bacteria

2.2.1. Solutions, media and bacterial strains

Luria Bertani (LB) media: 10g Bacto tryptone, 5g yeast extract, 5g NaCl in 1l H2O, autoclave and store at room temperature.

LB-plates: 10g Bacto tryptone, 5g yeast extract, 5g NaCl, 15g Bacto agar in 1l H2O, autoclave, cool to 50ºC, add antibiotics and pour plates. Store the plates at 4ºC.

Ampicillin-stock (100X): prepare a solution of 10 mg/ml in H2O, dissolve, filter sterilize through a membrane filter (0.2µm) and store aliquots of 5 ml at -20ºC. (Gerbu #1046)

Kanamycin-stock (200x): Prepare 10mg/ml in H2O, filter sterilize through a membrane filter (0.2µm) and store aliquots of 5ml at –20°C. (Sigma K-4000)

Tetracycline-stock (100x): Prepare 5mg/ml in 96% ethanol and store aliquots of 5ml at –20°C. (Sigma #T-3383)

Bacterial strains:

- XL-1 Blue: *end*A1, *gyr*A96, *hsd*R17(rK-mK+), *lac*, *rec*A1, *rel*A1, *sup*E44, *thi*-1, F´[*pro*AB, *lac*I^qZΔM15, Tn10 (Tet^r)] (www.neb.com)

-HB-101: *ara*14, *gal*K2, *hsd*S20(rB- mB–), *lac*Y1, Δ(*mcr*C- *mrr*), *mtl*-1, *rec*A13, *pro*A2, *rps*L20, *sup*E44, *xyl*-5, F– (www.neb.com)

2.2.2. Growth of bacteria

Bacteria are grown in liquid culture or on agar plates containing the appropriate selection antibiotics (Amp [100µg/ml]; Kan [50µg/ml]; Tet [25µg/ml] for plates and Amp [100µg/ml]; Kan [30µg/ml]; Tet [15µg/ml] for liquid culture) at 37°C.

2.2.3. Freezing of bacteria

To store transformed bacteria, 100µl glycerol and 900µl liquid culture are mixed and frozen at –80°C.

2.2.4. Transformation of heat shock competent bacteria

Thaw an aliquot of competent E. coli and incubate them immediately with 1-100ng plasmid DNA on ice for 30 minutes. Heat-shock the bacterial suspension on 42ºC for 90 seconds, then incubate the bacterial suspension 5 minute on ice. Add 1 ml LB media without antibiotics and incubate the bacteria suspension for 1 hour at 37ºC under shaking. Spread bacterial

suspension onto a LB plate containing the right selection antibiotic and incubate overnight at 37ºC.

2.3. Working with DNA

2.3.1. Buffer and Solutions

EDTA 0.5 M, pH 8.0: Dissolve 186.1g disodium ethylendiamine-tetra-acetate·$2H_2O$ in 800ml ddH_2O and adjust pH to 8.0 with NaOH (titrate with a diluted NaOH solution after addition of 18-19 NaOH pellets). Autoclave and store at 4ºC.

50X TAE: Dissolve in ddH_2O: 484g Tris, 114ml acetic acid, 200ml 0.5M EDTA pH 8.0, adjust to a final volume of 2l, store at RT.

PCI (Phenol:Chloroform:Isoamylalcohol) (25:24:1): Mix equal parts of equilibrated phenol and chloroform:isoamyl alcohol (24:1) and store under 0,1M Tris pH 8.0 at 4°C in the dark.

Miniprep solutions:

Solution I: Dissolve 0,9g Glucose in ddH2O, mix with 2,5ml 1M Tris-Cl (pH 8.0) and 2ml 0,5M EDTA (pH 8.0), fill up to 100ml with ddH_2O. Autoclave and store at 4°C.

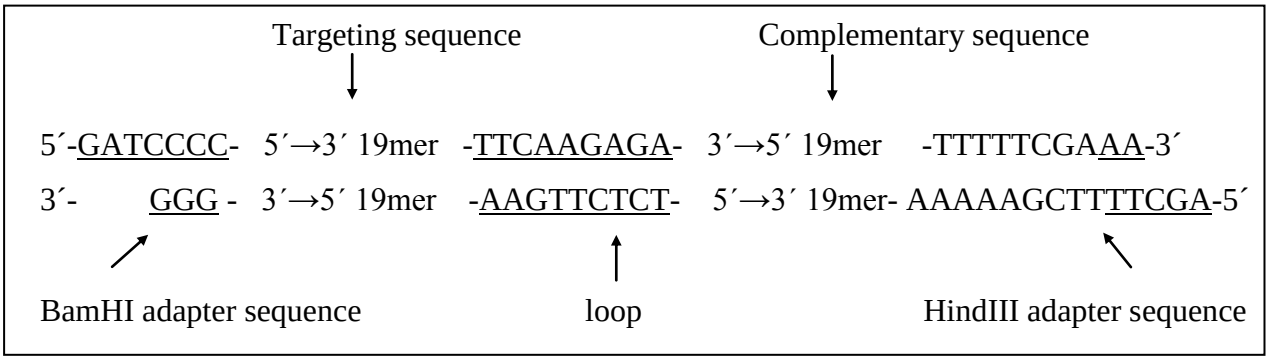
Solution II: Mix 20ml 1M NaOH with 5ml 20%SDS, fill up to 100ml with ddH_2O. Autoclave and store at 4°C.

Solution III: Mix 60ml 5M Potassium acetate with 11,5ml Glacial acetic acid and fill up to 100ml with ddH_2O. Store at 4°C.

2.3.2. Primers

sh-DNA

All shDNAs were designed to the following scheme:



shPTPA-1

Position of targeted sequence in ORF: 903-921

Sequence derived from and already confirmed in Fellner et al., 2003

shPTPA-1forward (PTPA-CtfwPetra/#630):	5´-GATCCCC-GTTCCCTGTGATCCAGCAC-TTCAAGAGA-GTGCTGGATCACAGGGAAC-TTTTTGGAAA-3´
shPTPA-1reverse (PTPA-CTrevPetra/#631):	5´AGCTTTTCCAAAAA-GTTCCCTGTGATCCAGCAC-TCTCTTGAA-GTGCTGGATCACAGGGAAC-GGG-3`
shControl-1forward (PTPA-Ctmisfw/#632):	5´-GATCCCC-GTTTCCTGTAATCCAACAC-TTCAAGAGA-GTGTTGGATTACAGGAAAC-TTTTTGGAAA-3´
shControl-1reverse (PTPA-Ctmisrev/#633):	5´AGCTTTTCCAAAAA-GTTTCCTGTAATCCAACAC-TCTCTTGAA-GTGTTGGATTACAGGAAAC -GGG-3`

shPTPA-2

Position of targeted sequence from start codon ATG in ORF: 1254-1272

Entry in database, from which name was derived: AY035997

shPTPA-2forward (mmPTPA1360fw/#644):	5´-GATCCCC-GGACACCCTGCTCTTGTCA-TTCAAGAGA-TGACAAGAGCAGGGTGTCC- TTTTTGGAAA-3´
shPTPA-2reverse (mmPTPA1360rev/#645):	5´AGCTTTTCCAAAAA-GGACACCCTGCTCTTGTCA-TCTCTTGAA-TGACAAGAGCAGGGTGTCC-GGG-3`
shControl-2forward (mmPTPA1360misfw/#646):	5´-GATCCCC- GGAGACCCTACTCTTATCA–TTCAAGAGA-TGATAAGAGTAGGGTCTCC- TTTTTGGAAA-3´
shControl-2reverse (mmPTPA1360misrev/#647):	5´AGCTTTTCCAAAAA-GGAGACCCTACTCTTATCA-TCTCTTGAA-TGATAAGAGTAGGGTCTCC-GGG-3´

shPTPA-3

Position of targeted sequence from start codon ATG in ORF: 1692-1710

Entry in database, from which name was derived: AY035997

shPTPA-3forward (mmPTPA1798fw/#648)	5´-GATCCCC-GAGACTCCGGGAGGCAGTA-TCAAGAGA-TACTGCCTCCCGGAGTCTC- TTTTTGGAAA-3´
shPTPA-3reverse (mmPTPA1798rev/#649):	5´AGCTTTTCCAAAAA-GAGACTCCGGGAGGCAGTA-TCTCTTGAA-TACTGCCTCCCGGAGTCTC-GGG-3`
shControl-3forward (mmPTPA1798misfw/#650):	5´-GATCCCC-GAGTCTCCGAGAGGCGGTA-TTCAAGAGA-TACCGCCTCTCGGAGACTC - TTTTTGGAAA-3´
shControl-3reverse (mmPTPA1798misrev/#651):	5´AGCTTTTCCAAAAA-GAGTCTCCGAGAGGCGGTA-TCTCTTGAA- TACCGCCTCTCGGAGACTC-GGG-3`

sh-Bα mismatch:

Position of targeted sequence from start codon ATG in ORF: 1199-1217

sh-BαControlforward (PP2A-Balphamis_for/#664):	5´-GATCCCC-GTGACAAGCTAAAGATAGA-TTCAAGAGA-TCTATCTTTAGCTTGTCAC-TTTTGGAAA-3´
sh-BαControlreverse (PP2A-Balphamis_rev/#665):	5´AGCTTTTCCAAAAA-GTGACAAGCTAAAGATAGA-TCTCTTGAA-TCTATCTTTAGCTTGTCAC-GGG-3`

Nucleotides changed in mismatch-oligonucleotides are underlined.

New PTPA targeting sequences PTPA2 and 3 were designed using the following algorithms:

- Ambion
- Jack
- OptiRNAi
- Emboss
- MPI
- GenScriptDharmaconQiagen
- SiDirect

Sense and antisense fragments were hybridized and cloned into the BamHI-HindII sites of a linearized H1TO promoter-driven RNAi-ready pNTO vector (Strack). Vector inserts were verified by sequencing. Sequence sh-PTPA1, targeting the C-terminal region of PTPA was already confirmed to induce knock-down of PTPA transiently in HeLa cells by Fellner et al. (Fellner et al., 2003). Sequences sh-PTPA2 and 3 target the 3´untranslated region (3´UTR) of PTPA.

Sequencing

H1 forward (#385): 5´-GAA TCG CGG GCC CAG TGT CA-3`

T7 forward: 5´-GTA ATA CGA CTC ACT ATA GGG C-3`

Sp6 reverse: 5´-GAT TTA GGT GAC ACT ATA G -3`

Site-directed mutagenesis

PTPAmutPCR_BstXIfw (#783): 5´-CTTGATCAGGAAGCAGAA-3´

Sp6 reverse: 5´-GAT TTA GGT GAC ACT ATA G -3`

mmPTPAmut1fw (#759):

Nucleotides changed from C G G

5´-GAGTGCCTGGAGAAGTTTCCTGTAATCCAACACTTCAAGTTCGGGAGC -3´

mmPTPAmut1rev (#760):

5´-GCTCCCGAACTTGAAGTGTTGGATTACAGGAAACTTCTCCAGGCACTC -3´

Nucleotides changed from wild-type PTPA are underlined.

2.3.2. PCR (Polymerase Chain Reaction)

PCRs were performed using: 1xTaq Polymerase buffer w/o $MgCl_2$ containing $(NH_4)_2SO_4$ (Fermentas), 0.25mM dNTPs (of each), 5pmol/µl primer (forward and reverse), 2mM $MgCl_2$, 5 units Taq polymerase (Fermentas) and 0.5-1µg DNA as template.

The PCR program: 95ºC 2min, followed by 32-35 cycles of subsequent steps of denaturing at 95ºC 30 sec, annealing at X(*)ºC for 1min and extension at 72ºC for 2 min. The cycling is followed by one single extension step at 72ºC for 10 minutes, pause at 4ºC.

Site-directed mutagenesis by PCR

As a template for site-directed mutagenesis the vector pcDNA3, coding for the expression of mammalian PTPA (pcDNA3_mPTPA#1; mouse PTPA) is used.

To mutate PTPA on position 906, 912 and 918, three PCR reaction steps are performed. The first reaction (PCR1) is carried out using a forward primer upstream of the site to be mutated (BstXIfw) and a reverse primer, targeting the site to be mutated, with three exchanged bases (mmPTPAmut1rev). Correspondingly the equivalent PCR (PCR2) is done with a reverse primer downstream of the site to be mutated (Sp6) and a forward primer, again targeting the site to be mutated, with three exchanged bases (mmPTPAmut1fw). Primers targeting the site to be mutated are designed to produce a shPTPA1-insensitive form of PTPA, but to produce silent mutations. Products of PCR reaction 1 and 2 are purified, using Promega PCR Clean-up system.

The third reaction is performed using 25ng of each PCR product and primers PTPAmutPCR_BstXIfw and Sp6.

After the third PCR, vector pcDNA3_mPTPA#1and PCR product are digested with BstXI and XhoI and ligated. For a full graphic representation see Figure I. The generated vector is sequenced using primer Sp6 (see attachment for map).

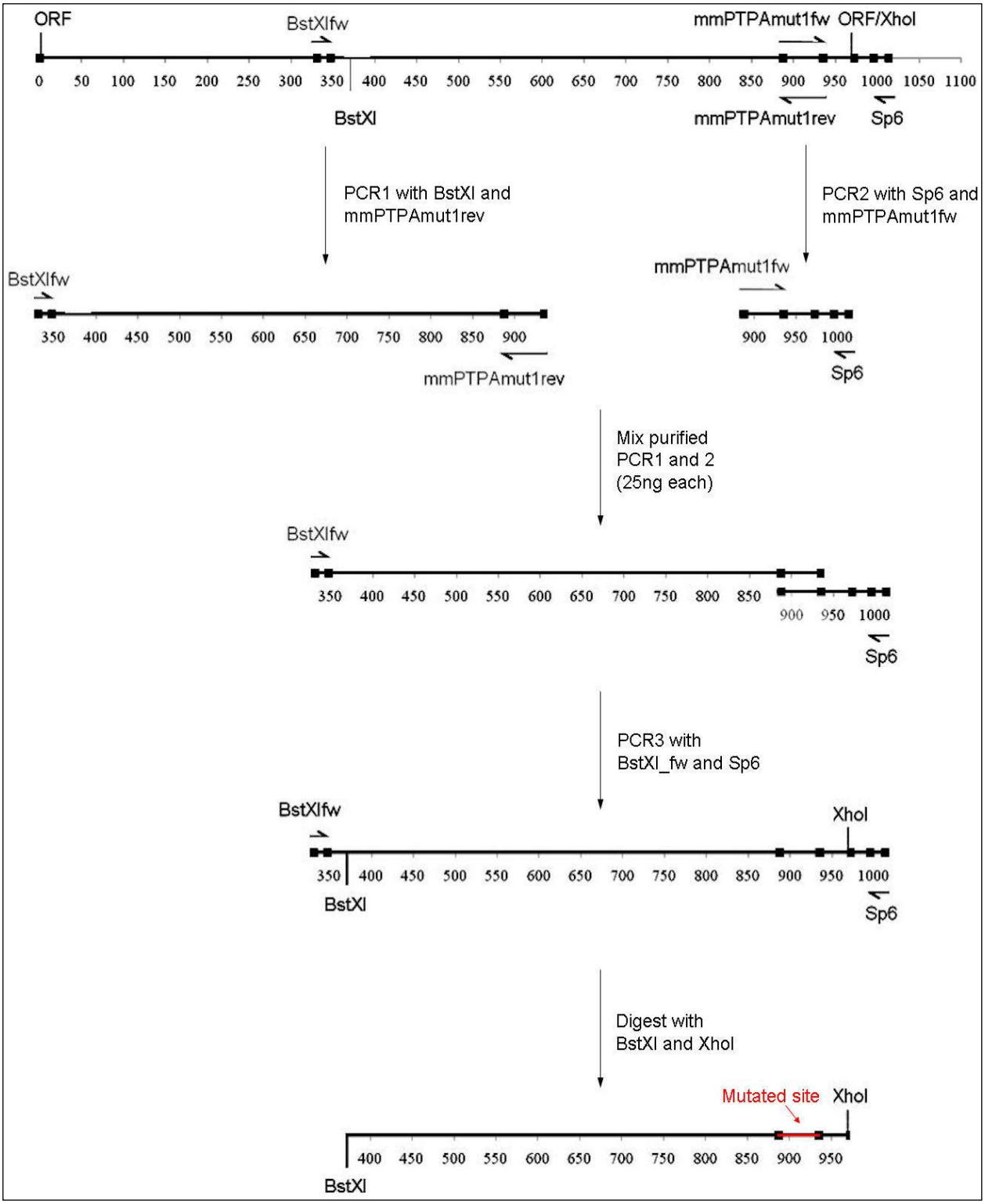


Fig. I: Scheme of site-directed PTPA mutagenesis using the vector pcDNA3_mPTPA#1

2.3.3. Sequencing

For sequencing 0,5µg template DNA was mixed with 2µl Big dye Terminator Mix, 4µl (1pmol/µl) primer, 4µl 5x sequencing buffer and filled up to total volume of 20µl with ddH_2O. For sequencing sh-RNA expressing vectors, 0,5µg template DNA was mixed with 2µl Big dye Terminator Mix, 0,4µl (10pmol/µl) primer, 4µl 5x sequencing buffer, 1µl DMSO, 12,5µl 2M betain and filled up to total volume of 20µl with ddH_2O.

The sequencing program: 95ºC 2min, followed by 30 cycles: 95ºC 30 sec, 50ºC 30 sec, 60ºC 1-2 min, 60ºC for 4 minutes, pause at 4ºC.

After this amplification using the Big dye system, the sequencing reaction is sent to VBC-genomics where sequencing is performed.

2.3.4. Methods of cloning and manipulation of DNA

Restriction digests

For digesting 1 µg plasmid DNA use 1-5 U of the requested restriction enzyme and prepare the reaction mix as suggested by supplier. The final volume of the reaction is usually 100 µl for preparative purposes or 30 µl for control digests. Incubate for 1-2 hours at the recommended temperature. Usually 1 unit of enzyme digests 1 µg of plasmid DNA in 1 hour when incubated at the temperature optimum. For digestion of miniprep DNA, Rnase is used at an end concentration of 0,5mg/ml. Stop the reaction by adding 1/10 volume of loading dye.

Analyze the restriction digests on an agarose gel (0.8-3% in TAE buffer) depending on the expected fragment sizes.

DNA precipitation

Add 1/10 volume of Na-acetate pH 5.2 to the DNA-solution and mix. Then add 2 volumes of cold 95% ethanol, mix and keep the mixture for at least 20 minutes at -20ºC. Centrifuge at 14.000 rpm at 4ºC for 10 minutes. Discard the ethanol and dry the DNA pellet on air. Resuspend the DNA in a suitable amount of buffer or ddH_2O.

Elution of DNA fragments using QIAquick gel extraction kit

Use the protocol described by manufactures QIAGEN, QIAquick gel extraction kit #28706.

Ligation of DNA fragments with T4 DNA ligase

Mix plasmid DNA and insert at ratio 1:3-1:5 in a final volume of 20 µl containing 1 µl (400 U) T4 DNA ligase and 1X T4 ligase buffer. Incubate at 16ºC overnight. Use half of the mixture for transformation of competent *E. coli.*

Annealing of Oligonucleotides

The ordered oligonucleotides are diluted to 100µM with ddH2O according to the datasheet. Then 2µl (200pmol) of the corresponding forward and reverse oligonucleotides are mixed with 8µl 5x annealing buffer and filled up with ddH_2O to a total volume of 40µl (end concentration of [$5*10^{-3}$fM]. The oligonucleotides are annealed by boiling the mixture in a water bath at 96°C for 5min and then cooled down to room temperature under constant stirring.

2.3.5. Plasmid Isolation from bacteria

DNA plasmid isolation using alkaline lysis

For small scale DNA plasmid preparation the self made MINI preparation solutions and following protocol were used.

6ml overnight culture of bacteria is centrifuged 5min with 5000rpm. The pellet is resuspended in 100µl buffer 1 and transferred to an eppendorf tube. 200µl of buffer 2 is added, the tube is inverted and incubated for a maximum of 5 minutes at room temperature. After adding 150µl buffer 3 and vortexing, the solution is centrifuged at 14000rpm for 10 minutes. The supernatant is mixed with 300µl isopropanol by vortexing. After centrifugation at 14000rpm for 15 minutes the pellet is washed with 70% ethanol. After centrifugation for 3 minutes at 14000rpm, the pellet is dried resuspended in 50µl ddH_2O.

DNA plasmid isolation using the MIDI preparation kit QIAGEN

For large scale DNA plasmid preparation the MIDI preparation kit (QIAGEN #12145) was used. Follow the instructions as described in the manual.

2.4. Working with proteins

2.4.1. Solutions

30% Acrylamide: Dissolve 292g acrylamide and 8g bisacrylamide in 1l ddH_2O. Add ion-exchange resin (BioRad AG 501-X6) to the final solution and store at 4ºC in the dark.

1M Tris pH 8.8: Dissolve 242.3g Tris in ddH_2O, adjust to pH 8.8 with HCl and fill up to 2l with ddH_2O. Autoclave and store at 4ºC.

1M Tris pH 6.8: Dissolve 60.5g Tris in ddH_2O, adjust to pH 6.8 with HCl and fill up to 500ml with ddH_2O. Autoclave and store at 4ºC.

20% SDS: Dissolve 40g SDS in ddH_2O. Stir and heat slightly, if necessary. Add ddH_2O to a final volume of 200ml and store at RT.

10% APS: Dissolve 1g ammonium persulfate in 10ml ddH_2O and store at 4ºC.

10X running buffer: 250mM Tris, 2M glycine, 35mM SDS in ddH_2O.

Transfer buffer: 25mM Tris, 190mM glycine, 20% methanol in ddH_2O.

GSD 3X stock (protein sample buffer): 4.5M glycerol, 10ml 20% SDS, 0.33M dithiotreitol (DTT) and 10ml ddH_2O. Add a bit of bromophenol blue and neutralize with a few drops of 1M Tris pH 6.8 (the solution should appear blue). Dilute 1:3 in ddH_2O before use or dilute with the sample.

3% NFDM blocking solution: Dissolve 30g non fat dry milk (NFDM) in 1l PBS containing 0.05% Tween 20 (PBS-T), add 50µl 20% sodium azide, mix and store at 4ºC.

0.5% NFDM antibody incubation solution: Dissolve 5g non fat dry milk (NFDM) in 1l PBS-T, add 1ml 1% thimerosal, mix and store at 4ºC.

IP-wash buffer: 20mM Tris-HCl pH 8.0, 135mM NaCl, 10% glycerol in ddH_2O. Filter-sterilize and store at 4°C.

IP-lysis buffer: 20mM Tris-HCl pH 8.0, 135mM NaCl, 10% glycerol and 1% (w/v) NP-40 in ddH_2O. Filter-sterilize and store at 4°C.

PMSF stock (100X): Dissolve 0.697g of phenylmethylsulfonylfluoride (PMSF) in 20 ml isopropanol (2-propanol), make aliquots and store at RT in dark (Roche #10837091001).

Aprotinin-stock (100X): 10ml Aprotinin (10 TIU (trypsin inhibitor units)/ml; Sigma), aliquot and store at 4ºC (Sigma A-6279).

Complete™ stock (25X): Dissolve one Complete™ Protease inhibitor cocktail tablet (Roche #11836145001) in 2ml IP-buffer and store at 4ºC. Prepare fresh every two weeks.

Basic buffer used for ß-Gal assay: 25mM tricine, 0,5mM EDTA, 0,54mM Na-Tripolyphosphate, 16,3 mM $MgSO_4*7H_2O$, 0,1% triton X-100 in ddH_2O, adjust to pH 7.8 with NaOH. Autoclave and store at 4ºC.

0,1M Na-Phosphate buffer pH 7.5: Dissolve 1,78g $Na_2HPO_4*2H_2O$ in 50ml ddH_2O (0,2M) and 0,31 g $NaH_2PO_4*H_2O$ in 10ml ddH_2O (0,2M). Mix 41ml of 0,2M $Na_2HPO_4*2H_2O$ and 9ml of 0,2M $NaH_2PO_4*H_2O$. Adjust to pH 7.5. Add 50ml ddH_2O. Autoclave and store at 4°C.

100x Mg^{2+} solution: 0,1M $MgCl_2$, 4.5M ß-mercaptoethanol in ddH_2O. Store at 4°C.

1xOPNG (o-nitrophenyl-ß-D-galactopyranoside) solution: Dissolve 4mg/ml o-nitrophenyl-β-D-galactopyranoside in 0,1M Na-phosphate buffer pH 7.5. Prepare fresh, store at 4°C (Sigma N1127-25G).

ß-galactosidase assay buffer: Mix 3µl 100xMg solution, 66µl 1xOPNG solution and 201µl 0,1M Na-phosphate buffer pH 7.5 per sample [OPNG: 3,24mM].

1M Na_2CO_3 solution: Dissolve 52g Na_2CO_3 in 500ml ddH_2O. Autoclave and store at 4°C.

2.4.2. Preparation of protein lysates of mammalian cells

Cells are washed with 5ml PBS and 5ml IP-wash. Add IP-lyse, containing the following protease inhibitors: For 1ml IP-lyse add 10µl PMSF- (final concentration: 2mM), 5µl aprotinin-stock (final concentration: 0.05TIU/ml) and 40µl complete-stock. The dish is incubated on the shaker for 10minutes at 4°C. The cells are scraped off with a rubber scraper and the cell debris is transferred to a 1,5ml tube and again incubated on the shaker for 10minutes at 4°C. After 10 minutes centrifugation with maximum speed at 4°C, the supernatant is transferred to a new tube and proteins concentration can be determined.

2.4.3. Determination of protein concentration (Bradford)

Mix 1µl of cell lysate with 1ml of Bradford reagent solution (dilution 1:4 in ddH_2O; Bio-Rad Protein Assay Dye Reagent #500-0006). Mix and incubate for 10 minutes at RT. Measure the optical density at 595nm. For each lysate at least three samples are measured.

2.4.4. SDS-PAGE (SDS-Polyacrylamide gel electrophoresis)

Clean the glass plates with ddH_2O and ethanol and assemble the gel unit. Mix the components for a separating gel except APS and TEMED. Degas the mixture (5 minutes vacuum) and add APS and TEMED. Mix and carefully apply the mixture between the glass plates, leaving 2.5 cm space for the stacking gel. Overlay the separating gel with 1ml ddH_2O to ensure an even surface. After polymerization of the separating gel, remove the water and fill in the stacking

gel. Insert the comb and avoid air bubbles. After polymerization carefully remove the comb and wash the slots with ddH_2O in order to remove unpolymerized acrylamide. Insert the gel unit into the running unit and fill the chambers with 1x running buffer.
Samples are prepared by mixing the volume of whole cell lysate equivalent to 60µg protein with 3xGSD. Each sample is filled up to the same volume with IP lyse solution.
Load the samples and run the gel overnight at 6-9 mA per gel and stop when the front is 15cm past the stacking gel.

Separation gel:	15%	10%	7.5%
Acrylamide/Bis (30%)	20ml	13.4ml	10.1ml
1M Tris pH 8.8	15ml	15ml	15ml
ddH2O	5ml	11.7ml	15ml
20% SDS	200µl	200µl	200µl
10% APS	134µl	134µl	134µl
TEMED	26µl	26µl	26µl

Stacking gel:	
Acrylamide/Bis (30%)	1.7ml
1M Tris pH 6.8	1.25ml
ddH2O	7.1ml
20% SDS	50µl
10% APS	50µl
TEMED	10µl

2.4.5. Western blot analysis

Western transfer

Carefully remove the glass plates and take out the gel. Cut off the gel below the stacking gel and 15 cm from there. Assemble the western sandwich in the following order:
Support pad, 2 sheets of 3mm Whatman chromatography paper, gel, nitrocellulose membrane (Schleicher&Schuell 13.5x15 cm), again 2 sheets of 3mm paper and the other support pad (all components soaked with transfer buffer). Avoid air bubbles during the assembly.
Insert the western sandwich into the transfer unit with the membrane at the plus pole. Transfer the proteins at a constant current of 500mA at 4°C for 3.5 hours.

Antibody incubation

Wash the membrane after the transfer shortly with PBS-T (PBS containing 0.05% Tween 20) and incubate with 3% milk (blocking solution) for approximately 1 hour, rocking, at RT. After blocking, wash the blot once with PBS-T and incubate it either overnight at 4°C or for 1 hour at room temperature with the appropriate primary antibody, diluted as listed below, in 0.5% milk. After incubation, remove the primary antibody and wash the blot three times for 5

min with PBS-T. Incubate the blot then with secondary antibody, diluted as indicated below in 0.5% milk for 1 hour at room temperature under low shaking.

antibody	Directed against	dilution	Type of antibody	kDa
2G9	Bα, δ-subunit (human)	1:5000	mouse, monoclonal	50
ß-Actin	ß-Actin (human)	1:10.000	mouse, monoclonal (Sigma; A1978)	35
PTPA	PTPA (mouse)	1:10.000	rabbit, polyclonal	40,6
SAT20	C-subunit	1:10.000	rabbit, polyclonal, purified	36
4C10	A-subunit	1:100	mouse, monoclonal	65
B´-subunit (p56)	B´-subunit	1:1000	rabbit, polyclonal, purified	56
B´´-subunit (p59)	B´´-subunit	1:1000	rabbit, polyclonal, purified	59
10E2	HDAC1	1:100	mouse, monoclonal	62
PME-1	PME-1	1:10.000	rabbit, polyclonal	44
Hsp70	Hsp70	1:2000	mouse, monoclonal Upstate, P34932	70
16B12	HA-tag	1:10.000	mouse (monoclonal) (Abcam, ab2477)	
Secondary antibody		dilution	Type of antibody	
HRP anti-mouse	IgG, Fcγ fragment-specific	1:5000	Goat; Jackson #115-035-008	
HRP anti-rabbit	IgG, Fcγ fragment-specific	1:5000	Goat; Jackson #111-035-008	
ALEXA anti-mouse		1:20 000	Goat; Invitrogen A-21057	
ALEXA anti-rabbit		1:20 000	Goat; Invitrogen A-21076	

Immunodetection by enhanced chemoluminescence (ECL):

After incubation with one of the secondary antibodies coupled to horse radish peroxidase, wash the blot three times for 10 minutes with PBS-T and dry the blot between two 3MM Whatman-sheets. Mix equal amounts of the two ECL solutions (oxidizing reagent and enhanced luminol reagent) and incubate the blot for 1 minute with this mixture. Take out the membrane, wrap it in saran wrap and expose the blot to X-ray films.

Wash the membrane once in PBS-T and incubate for 30min with blocking solution at RT. Wash again with PBS-T and incubate with a new primary antibody or dry the blot between two 3mm Whatman sheets.

To quantify expression levels of proteins, the X-ray films are scanned with the Canon CanoScan 4200F. The bands are then quantified by using Image Quant 5.0 software.

Immunodetection using the LiCor infrared imaging system

After incubation with the secondary antibody (ALEXA), wash the blot three times for 10 minutes with PBS-Tween and scan the blot with medium resolution on LiCor Odyssey Infrared Imaging System. Evaluate your data using the supplied application software.

Incubate the membrane for 30 min with blocking solution, wash with PBS-T and incubate with a new primary antibody or dry the blot between two 3mm Whatman-sheets.

2.4.6. Immunoprecipitation with crosslinked beads

In order to use equal amounts of whole cell protein for the immunoprecipitation, equilibrate the native yeast lysates to equal concentrations with IP-buffer plus protease inhibitors and add 500µl of the dilution to 40 µl of anti-HA (12CA5) antibody cross-linked to BSA-coated protein A sepharose beads (1:1 suspension in PBS) and incubate for 1 hour, rocking, at 4°C.

Then centrifuge the mixture for 1 minute at 1000 rpm (Eppendorf centrifuge 5417C). Carefully remove the supernatant. Wash the beads once with IP-buffer supplemented with protease inhibitors, three times with cold TBS. After the final centrifugation, carefully remove the supernatant. Use the immunoprecipitates for protein phosphatase assays or boil the beads directly in 60µl minimum 1X GSD for 5 min at 95°C, if they are used for SDS-PAGE. Before loading the sample on a SDS-polyacrylamide gel, boil the samples again for 5 minutes and centrifuge for a few seconds at maximum speed.

2.4.7. Protein phosphatase assays

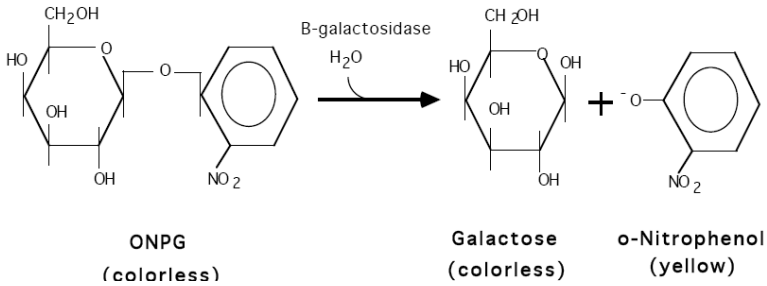
To determine catalytic activity of PP2A complexes from mammalian cells, immunoprecipitate similar amounts of HA-tagged C subunit and resuspend the immunoprecipitate in 400µl cold TBS.

To test the catalytic activity of PP2A complexes towards phosphorylase a transfer 100µl of the 400µl immunoprecipitate suspension to a new 1.5ml safelock tube containing 15µl 1:1 protein A sepharose beads (in PBS). Centrifuge at 1000 rpm for 1 minute and remove the supernatant. Resuspend the beads in 40µl phosphorylase a buffer. To start the protein phosphatase reaction, add 20µl of the ^{32}P-labeled phosphorylase a (approx. 40µM) to the tube. Vortex immediately and incubate the reaction in a heating block for 15 min at 30°C, shaking. Stop the reaction by adding 180µl cold 20% TCA. Vortex the tube and place on ice for at

least 10 min. Centrifuge the tube at 14000 rpm for 10 min at 4°C. Transfer 200µl of the clear supernatant to a scintillation vial containing 5ml Ecosint H (National Diagnostics) and determine the amount of radioactivity released in the assay as $^{32}P_i$ in a scintillation counter.
Spin the remaining part of the immunoprecipitate at 1000 rpm for 1 minute and remove the supernatant. Boil the beads in at least 60µl 1X GSD for 5 minutes at 95°C. Analyze the sample by SDS-PAGE and immunoblotting to determine the amount of catalytic subunit used in the phosphatase assay.

2.4.8. ß-galactosidase-assay

The amount of ß-galactosidase expressed, can be assayed by measuring hydrolysis of the chromogenic substrate, o-nitrophenyl-ß-D-galactoside (ONPG) as shown below (Miller, J. 1972. Experiments in Molecular Genetics, p. 352-355. Cold Spring Harbor Laboratory, NY).



Cell lysis

Cells are washed with basic buffer. 100µl basic buffer/6-well are added, cells are scraped off with a rubber scraper and transferred to a 1,5ml tube. Cells are lysed by two consecutive freeze/thaw cycles in liquid nitrogen, with thawing them the first time at room temperature and the second time on ice. Then the lysate is centrifuged for 10minutes at 14000rpm at 4°C. The supernatant is transferred to a 1ml tube. Protein concentration is measured according to the protocol above.

ß-galactosidase assay

270µl ß-galactosidase assay buffer are mixed with 30µl protein lysate. According to the expected signal, different amounts of protein are used for the assay. Different liquid levels are filled up to 30µl with basic buffer.
In order to avoid different starting time points, the lysates are pipetted onto the cap of the tube and then centrifuged down.
The reaction mix is incubated for 1hour at 37°C at 400rpm. Stop the reaction by adding 500µl 1M Na_2CO_3. Absorbance is measured at OD_{420}. To compare signals, OD_{420} per µg whole cell lysate used, is calculated.

3. RESULTS

3.1. Inducible RNAi

A shRNA sequence (shPTPA1) targeting PTPA was established by Fellner et al. to transiently knock-down PTPA in HeLa cells (Fellner et al., 2003). As this sequence targets a sequence in the ORF of PTPA that is 100% identical between mouse and human, it was considered to be also functional in mouse cells. To determine the functionality of this sequence in mouse N2a cells, cells were transfected with either the constitutive pSUPER vector expressing a hairpin with a 19bp targeting sequence for the PTPA mRNA or the empty vector (pSUPER; Brummelkamp et al., 2002). HeLa cells were used as a positive control. As transient transfection of cell never reaches 100%, we selected transfected cells in order not to underestimate the effects of the siRNA. Geneticin selection takes about 7-10 days to kill all non-transfected cells. In contrast to that, selection with puromycin kills 99% of all non-transfected cells within 2 days. Therefore we co-transfected a vector coding for the expression of the puromycin resistance gene in a ratio of 1:5, to be able to analyze the effect of the shRNA in a short timeframe. 24 hours after transfection, puromycin was added to the cells. Less than 5% of wild-type PTPA levels could be detected in lysates of both cell lines 4d post transfection of the shPTPA construct (Fig.1), indicating functionality of the knock-down construct in N2a cells.

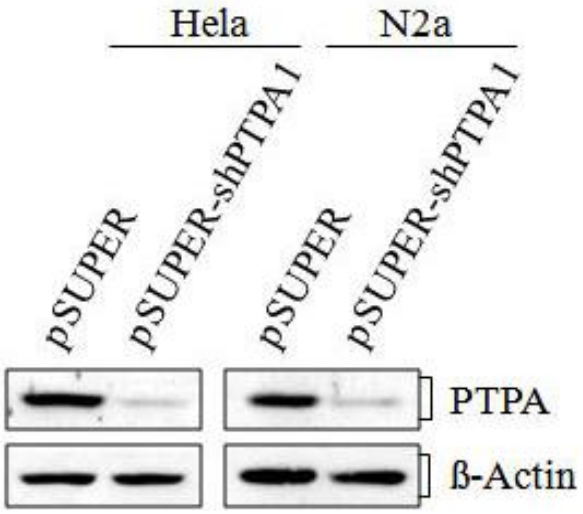


Fig.1: Downregulation of PTPA in N2a and HeLa cells, transfected with pSUPER-shPTPA1

N2a and HeLa cells were co-transfected with the pNTO^{Puro} vector (see attachment for map) containing the coding sequence of the puromycin resistance marker and either empty pSUPER vector (pSUPER) or pSUPER-shPTPA1, containing PTPA-targeting sequence# 1 (shPTPA1). After transfection cells were grown in medium supplemented with puromycin for 48h. Whole cell lysates were analyzed by SDS-PAGE and immunoblotting with anti-PTPA and ß-Actin (loading control) antibody. (n=1)

Studying the phenotypic appearance of the cells after 48h of puromycin selection we could observe rounded cells, apoptotic bodies and surface blebbing with some cells. However these cells were easily detached by rinsing the plate and therefore were considered to be apoptotic, because of a lack of puromycin-resistance. As there were viable cells visible, it was concluded, that there was no apoptotic effect of PTPA knock-down in these cells.

Attempts to isolate transfected cells by co-transfection with a vector coding for the expression of green fluorescence protein (GFP) and sorting by fluorescence activated cell sorting (FACS) failed, because of low sorting efficiency due to a damaged laser.

Next, the inducible vector pNTO, containing the sequence sh-PTPA1 was analyzed for its ability to knock down PTPA in N2a cells following the same protocol as above. Again HeLa were used as a positive control. N2a and HeLa cells were transfected with the pNTO vector, containing sh-PTPA1 (pNTO-shPTPA1) or a mismatch sequence, with changes from C to T at position 4 and from G to A at position 10 and 16 of the 19bp-sequence (pNTO-shControl-1). Since there was no empty pNTO^{Neo} vector at hand, to be used as a negative control, cells were transfected with the empty vector pCI^{Neo}. Again for selection of transfected cells, pNTO^{Puro} was co-transfected in a ratio of 1:5. 24 hours after transfection, puromycin was added to the cells. As the inducible vector was transfected into cell lines, that do not express the tet-repressor, no induction with Dox was needed for shRNA-expression.

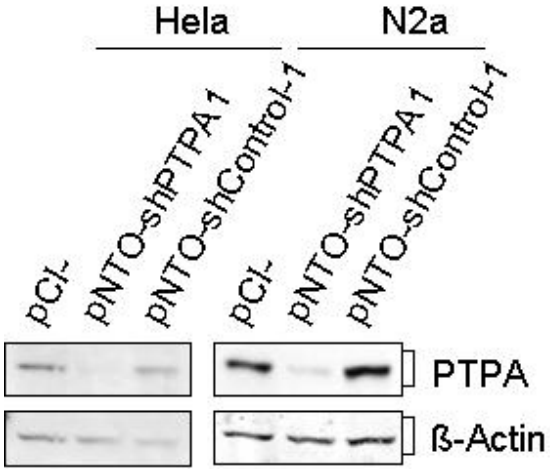


Fig.2: Downregulation of PTPA in N2a and HeLa cells, transfected with pNTO-shRNA1

N2a and HeLa cells were transfected with empty pCI vector (pCI), pNTO-shPTPA1, containing PTPA-targeting sequence#1(sh-PTPA1) or pNTO-shControl1 containing the corresponding mismatch sequence (see attachment for maps).

Again some of the cells displayed a phenotype similar to apoptotic cells, yet again these cells could easily be detached from the plate and therefore was probably due to the puromycin selection.

In contrast to the cells transfected with the mismatch control pNTO-shControl1, pNTO-shPTPA1 transfected cells showed significant knock-down of PTPA, indicating that the vector system and sequence can be used for the establishment of stable knock-down cell lines (Fig.2).

3.1.1. Testing of sequences targeting the 3`UTR of PTPA

In addition to sequence shPTPA1, two new PTPA targeting sequences from the entire PTPA including the 5' and 3' untranslated regions were designed and tested for their ability to induce knock-down of PTPA. The 5' and 3' untranslated regions were chosen as possible phonotypical effects of PTPA knock-down could easily be controlled by a rescue experiment with a wild-type cDNA copy of PTPA.

To evaluate the capacity of the newly designed shRNAs targeting the 3´UTR of PTPA to knock-down PTPA, N2a cells were transfected with the pNTO vector, containing sh-PTPA2 or 3 (pNTO-shPTPA2/3) or a corresponding mismatch sequence (pNTO-shControl-2/-3) or the empty vector pCI^{Neo}. For selection of transfected cells, $pNTO^{Puro}$ was co-transfected in a ratio of 1:5. 24 hours after transfection, puromycin was added to the cells.

Sequence sh-PTPA1 (pNTO-shPTPA1) and corresponding mismatch control were used as a positive and a negative control.

A 60 and 67% knock-down could be detected in lysates 4d post transfection of the shPTPA2 and shPTPA3 construct, respectively. While levels were unchanged in shControl-3 transfected cells, levels of shControl-2 transfected cells were reduced to less than 70% (Fig.3). Consequently, significant knock-down was achieved in shPTPA1, shPTPA2, shPTPA3 transfected cells, when compared to the respective shControl and to wt-PTPA levels. Nevertheless, shControl-2 also induces a significant knock-down, therefore shPTPA1 and 3 were used to produce stable PTPA knock-down cell lines.

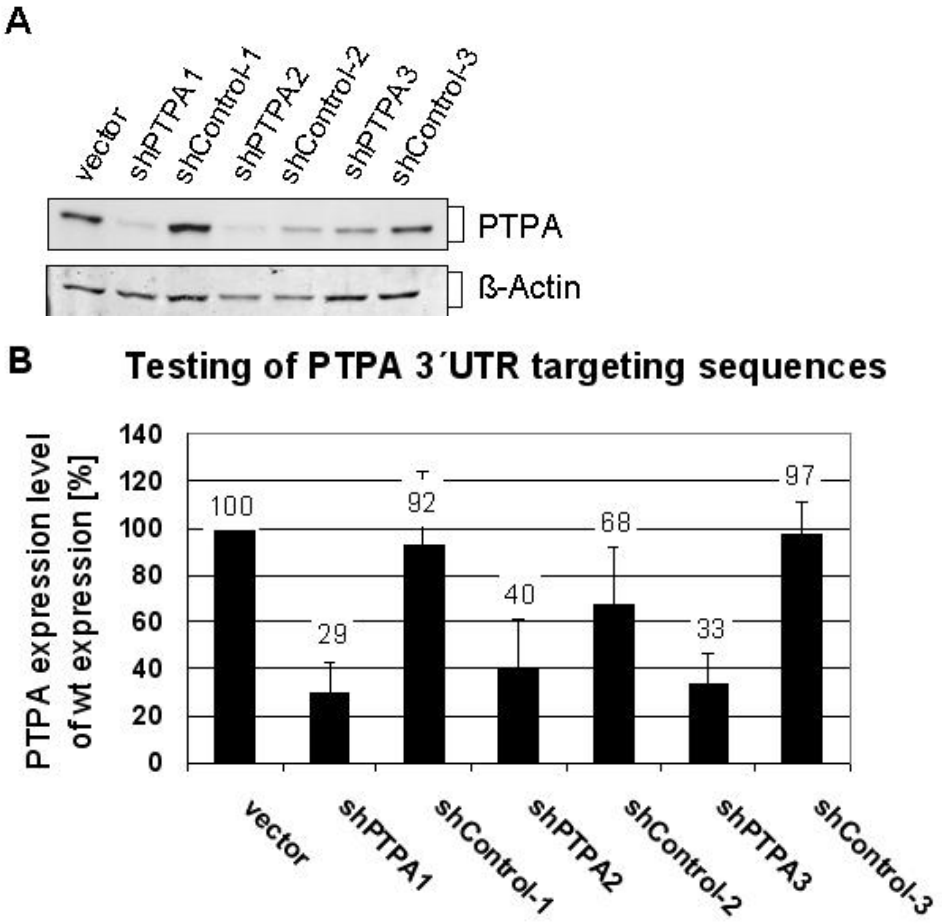


Fig.3. Testing of different shPTPA constructs
N2a cells were transfected using the same protocol as above, with either empty pCI vector (vector), pNTO-shPTPA1-3 (sh-PTPA1-3) or pNTO-shControl1-3 (sh-Control 1-3).
(A) Whole cell lysates were analyzed by SDS-PAGE and immunoblotting with anti-PTPA and ß-Actin (loading control) antibody. (B) Densiometric quantification of PTPA knock-down; the values are presented as a percentage of the PTPA expression level in control vector transfected N2a, which was set 100%. (n=5; mean ±S.D.) Data were analyzed using the Student t-test. Differences with p-values less than 0.05 were considered statistically significant:
(shPTPA1 p=3*10-4; shControl-1 p=0,6; shPTPA2 p=3*10-4; shControl-2 p=0,03; shPTPA3 p=3*10-4; shControl-3 p=0,7)

3.1.2. Cloning of RNAi-insensitive PTPA for rescue experiments

It is important to determine whether the phenotype of knocking-down PTPA in cell lines is due only to loss of PTPA function and not to possible RNAi-off-target effects. Therefore, rescue experiments with RNAi-insensitive PTPA have to be performed. For sequence shPTPA3, the ORF can be used directly for the rescue expression whereas with shPTPA1 targeting the coding region this sequence has to be replaced in the ORF with a wobbled sequence to be refractory to the shRNA. Therefore PTPA was mutated by site-directed PCR-mutagenesis (see materials and methods).

Mutated PTPA was cloned into the vector pcDNA3_mmPTPA and sequenced. The sequence proofed to be correct.

3.2. Generation of N2aTRex cell lines with inducible PTPA knock-down

N2aTRex cells were transfected with the pNTO vector, containing shPTPA1, 3 or the corresponding mismatch control sequences shControl1/3

For selection of transfected cells, cells were grown in medium supplemented with 600µg/ml geneticin for two weeks. Next, cells were seeded at clonal density and at least 10 single clones/shRNA were isolated.

RNAi expression was induced with 1µg/ml doxycycline for 4 days. Whole-cell lysates were analyzed by SDS-PAGE and immunoblotting for PTPA knock-down.

For shPTPA3 out of 10 clones, none showed considerable knock-down of PTPA. For shPTPA1, three out of 14 clones showed significant knock-down of PTPA upon induction with doxycycline. This knock-down was demonstrated in at least three independent experiments.

However, in all three clones, continuously proliferating in culture or stored in liquid nitrogen and recultivated, the ability to inducibly knock-down PTPA got lost over a period of 4 weeks (Fig.4), probably due to counterselection or epigenetic silencing of the knock-down construct.

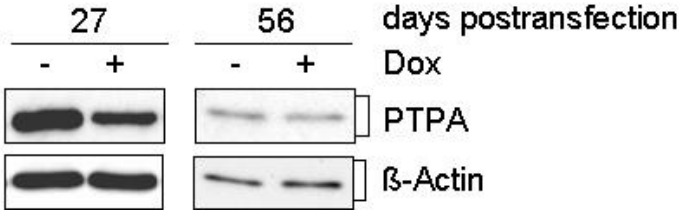


Fig.4: Loss of PTPA knockdown in N2aTRex cells stably transfected with pNTO-shPTPA1

N2aTRex14 cells were transfected with the pNTO vector containing shPTPA1. After single clone isolation, this clone showed significant PTPA knockdown after induction with doxycycline (Dox). 4 weeks later, no knock-down could be induced.

An explanation for this could be that the system is leaky, which means, that PTPA-targeting RNAi is constitutively expressed at low levels. As knock-down of PTPA causes apoptosis in HeLa cells (Fellner et al., 2003), this could lead to a clonal evolution towards a cell with decreased ability of PTPA knock-down, e.g. by silencing of the coding sequence for the shRNA.

In order to avoid repeated loss of knock-down, the reason for loss of PTPA knock-down was analyzed:

RNAi expression in the absence of doxycycline can be induced by two factors. First, leakiness of the H1TO promoter in N2aTRex14 cells can be caused by either low expression level of tetracycline repressor (tet-repressor) or by low affinity of the repressor to the operator.

Although a wt-repressor was initially introduced into N2a cells to produce the TetR expressing N2Trex cell lines, a mutation could have happened during the selection process and accidentally picked by choosing N2aTRex14. Second, the FCS used could contain tetracycline, thereby activating the expression of shRNA and thus the knock-down of PTPA which is harmful to the cells.

3.2.1. Inducible Promoters in N2aTRex14 cells

To analyze, if the TetR would be able to block expression of shRNA in the absence of the inducer, N2aTRex14 cells, were compared with three other cell lines expressing the tet-repressor: HeLaTRex, HEK293TRex and Pc12TRex (kindly provided by S. Strack).

To test for leakiness, activity of a tetracycline-inducible ß-galactosidase (ß-Gal), was compared in Dox-treated and untreated populations of these four cell lines.

The cell lines were transfected with 0,5µg-8µg of the vector pcDNA5/TO-LacZ. 24 hours after transfection, cells were split and one half induced with doxycycline. After 24 hours of induction, whole cell lysates were analyzed for expression of ß-Gal. As a control for transfection efficiency and as a negative control cells were co-transfected with pCRUZ-GFP, coding for the expression of GFP.

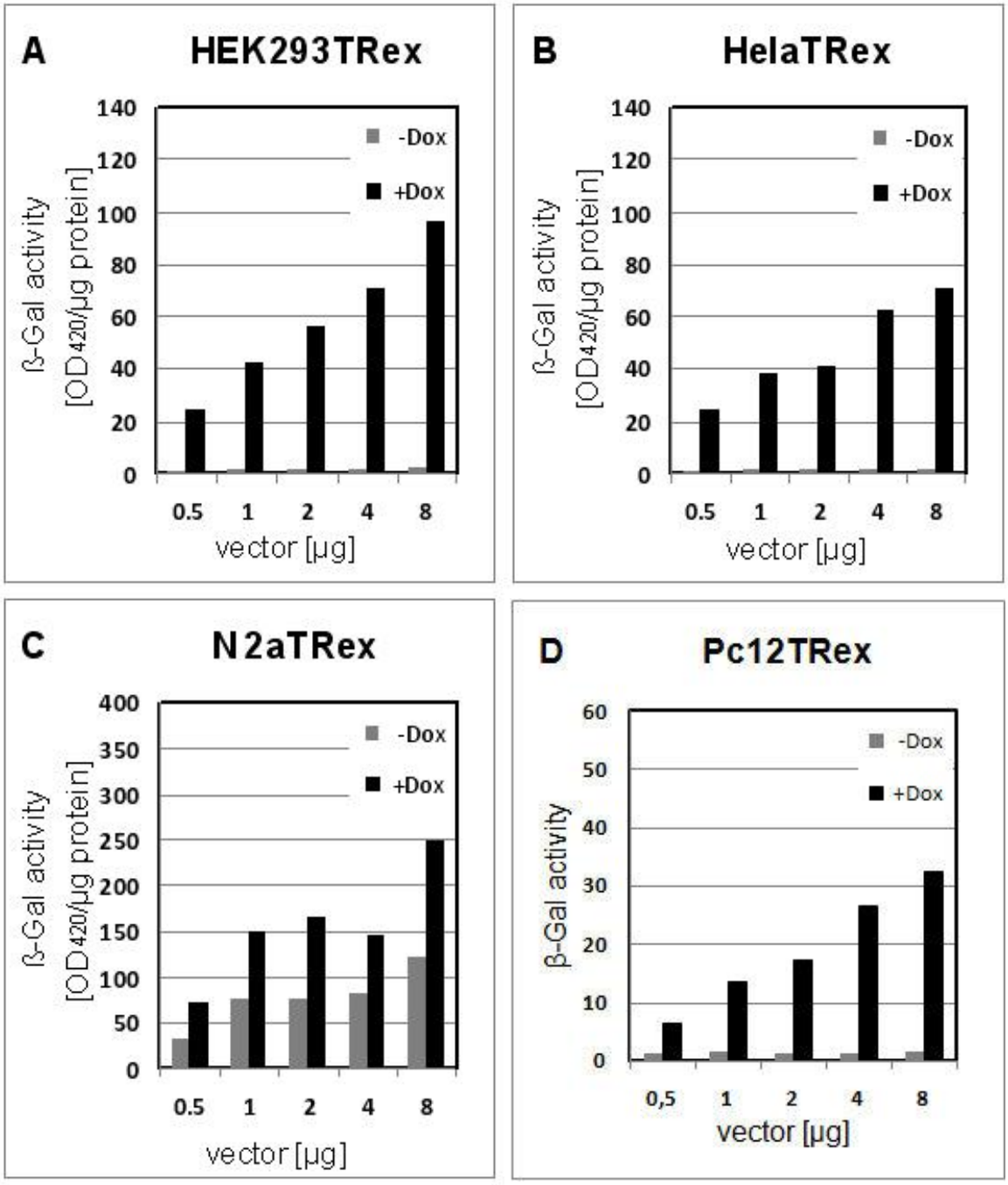


Fig.5 A-D. Leaky expression of ß-Gal in different cell lines

HEK293TRex, HeLaTRex, N2aTRex and Pc12TRex14 were transfected with 0,5-8µg of the vector pcDNA5/TO-LacZ (see attachment for map), where the reporter construct ß-Gal is under the control of a tet-inducible promoter. 24 hours post transfection, cells were split and half of them induced with doxycycline for 24 hours.
After 24 hours of induction, whole cell lysates were analyzed for the expression of ß-Gal. Data are presented as a function of ß-Gal activity (OD_{420}*10.000/µg protein) versus µg vector transfected.
(n=3)

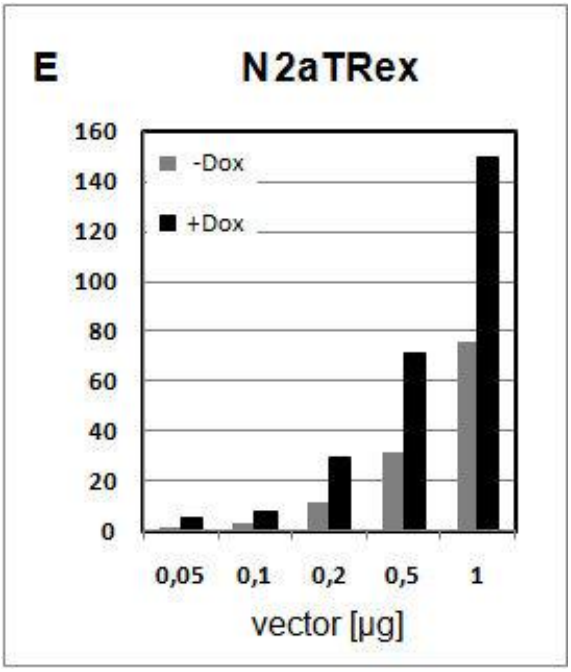


Fig.5E: Leaky expression of ß-Gal in N2aTRex cells

N2aTRex14 were transfected with 0,05-1µg of the vector pcDNA5/TO-LacZ, to show that difference in leaky expression is not due to out titration of the TetR due to higher transfection efficiency. Data are presented as a function of ß-Gal activity (OD_{420}*10.000/µg protein) versus µg vector transfected (n=3)

Cell lines HEK293TRex, HeLaTRex and Pc12TRex showed no or very low leaky expression of ß-Gal even with high amounts of vector transfected (Fig.5A, B and D). The ß-Gal activity of induced cells in HEK293TRex cells increased about of 86 times, in HeLa cells about 51 times and in Pc12TRex cells about 17 times, when compared to uninduced cells.

Interestingly, the cell line N2aTRex14, exhibited high leaky expression, increasing with the amount of vector transfected (Fig. 5C). Inducibility (signal of induced cells/signal of uninduced cells) of the ß-Gal expression increased only 2-fold independent of the amount of DNA transfected.

Yet, the overall ß-Gal activity/µg DNA transfected is higher in the cell line N2aTrex14 compared to the other two, which was most probably due to higher transfection efficiency in N2aTRex14. Considering the transfection efficiencies, which were 81% in N2aTRex14, 26% in HeLaTRex and 46% in HEK293TRex on average, these differences adjust to the same level of ß-Gal expression in the three cell lines.

As higher amounts of the vector could possibly outtitrate the tetracycline-repressor, and therefore be the cause for low inducibility of ß-Gal expression in N2aTRex14 cells, the experiment was repeated with lower amounts of DNA, from 0,05µg to 1µg DNA. Even then, the inducibility of expression stayed low at about 2-fold (Fig.5E).

This becomes even more obvious when comparing absolute ß-Gal activity levels:

Both Hek293TRex and HeLaTRex cells show very low leaky ß-gal activity of about 1,2 when their induced ß-Gal activity was at about 40. In contrast to that, the N2aTRex cell line shows leaky ß-Gal activity of 12 when exhibiting an even lower induced ß-Gal signal of 30.
This suggested that leakiness of this system is not due to a low expression of the tet-repressor, but rather to a less tight tet-promoter in N2aTRex14 cells, compared to others. This was confirmed by the fact, that N2aTRex14 cells expressed even higher amounts of tet-repressor than the cell line HeLaTRex (Fig.6).

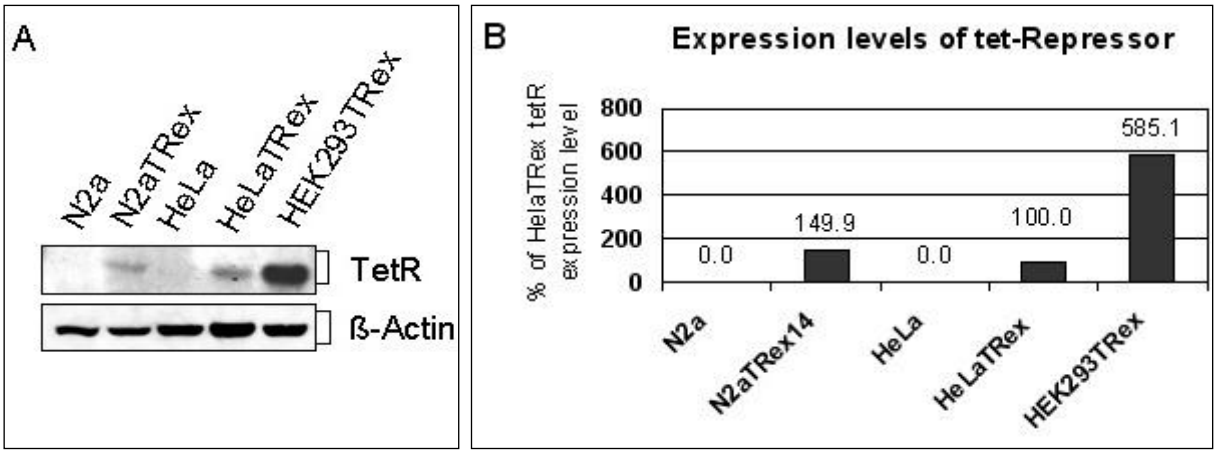


Fig.6: Comparing tetracycline-repressor expression of different cell lines

(A) Expression levels of tetracycline-repressor in N2aTRex, HeLaTRex and HEK293TRex were analyzed by SDS-PAGE and immunoblotting with anti-tetracycline-repressor (TetR) and ß-Actin (loading control) antibody. N2a and HeLa cells were used as negative controls. (B) Densiometric quantification of tetracycline expression in N2aTRex, HeLaTRex and HEK293TRex. The values are presented as a percentage of the tetracycline-repressor expression level in HeLaTRex cells, which was set 100%. (n=1)

To confirm the leakiness using another system, different cell lines stably expressing the tet-repressor were transfected with a vector (pcDNA5/TO/HA-$C_{SU,}$ see attachment for cloning strategy), coding for a Dox-inducible HA-tagged C subunit of PP2A. 24 hours after transfection, cells were split and one half was induced with doxycycline for another 24 hours. Immunoblotting after 24 hours of Dox-stimulation indicates again that N2aTRex14 possesses a high expression level, even in the absence of doxycycline, whereas the other cell lines robustly repress transgene expression in the absence of doxycycline (Fig.7).

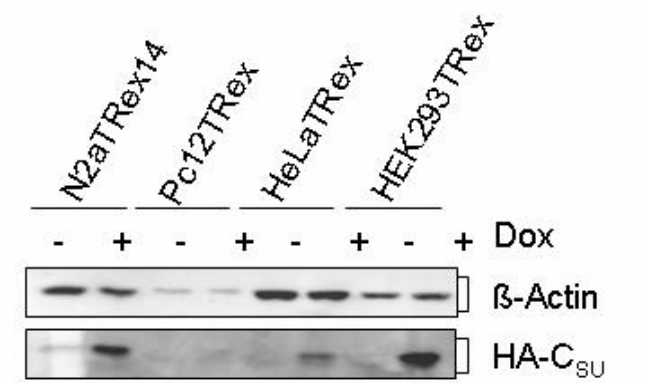


Fig.7: Leaky expression of different tet-repressor expressing cell lines in a pol III promoter based system

N2aTRex, HeLaTRex, HEK293TRex and Pc12TRex were transfected with the vector pcDNA3/TO/HA-CSU (see attachment for map). Whole cell lysates after 24 h of doxycycline treatment were analyzed for expression of HA-tagged C subunit by SDS-PAGE and immunoblotting with anti-HA and anti-ß-Actin antibody (loading control).

To summarize, the cell line N2aTrex14 showed high leaky expression, when compared to other tetracycline-repressor expressing cell lines. Of all cell lines, HEK293TRex exhibited least leaky expression and highest level of inducibility (81-fold).

Thus, HEK293TRex were used as parental cell line for the generation of PTPA knock-down cell lines. The cell line HEK293TRex was transfected with the vector pNTO-PTPA1 or the corresponding mismatch control pNTO-Control-1. Cells were grown as single cells progeny in medium supplemented with geneticin for 2 weeks. RNAi expression was induced with 1µg/ml doxycycline for 4 days and whole cell lysates were analyzed by SDS-PAGE and immunoblotting for PTPA knock-down.

Out of 11 clones tested, 7 showed significant knock-down of PTPA to fewer than 50% upon induction with doxycycline: HEK293TRex14+pNTO-shPTPA1 clone #1,8,9,12,26,28,30 and 29. Three of them with highest PTPA repression upon RNAi induction with doxycycline (HEK293-PTPA#1,8 and 9) are shown in Figure8.

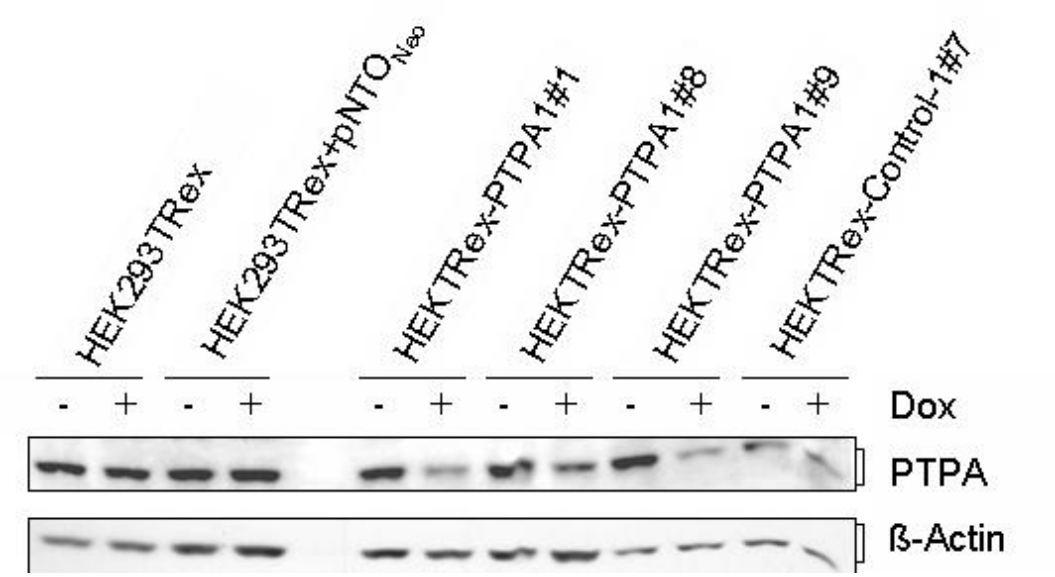


Fig.8 Stable and inducible PTPA knock-down cell lines based on HEK293TRex cells

HEK293TRex cells were transfected with the vector pNTO, containing the PTPA-targeting sequence#1 (shPTPA1) and the corresponding mismatch sequence (shControl-1). 24hours post transfection cells were grown as single cells in medium supplemented with geneticin for 2 weeks. Single clones were induced for 4 days with doxycycline and whole cell lysates were analyzed by SDS-PAGE and immunoblotting with anti-PTPA and ß-Actin (loading control) antibody (immunoblot done by Michaela Kugler).

In addition, N2aTRex14 were also used for a new round of transfection and single-clone isolation with tetracycline-free FCS (see 3.2.2), as they are of neuronal origin and can be used in future to address the question of whether PTPA deregulation is involved in tau hyperphosphorylation and thus AD pathology.

3.2.2. Analysis of non-approved-tetracycline-free FCS for presence of tetracycline

One explanation for the observed loss of inducible PTPA knock-down in the previous functional PTPA-loss-of-function (PTPA-LOF) cells is, that RNAi expression in the absence of doxycycline was induced by tetracycline present in the not tetracycline-free approved FCS (non-approved FCS) used and because of this PTPA was suppressed even in the absence of Dox. The pro-apoptotic effect of PTPA suppression could act selectively on those cells, which able to knock-down PTPA and could thus drive a clonal evolution towards cells that lost their ability to knock-down PTPA.

Differential proliferation of tetracycline-sensitive and /-resistant bacterial strains with increasing FCS-concentrations

To address the question, whether there was tetracycline present in non-approved FCS, the proliferation rate of the tetracycline-sensitive bacterial strain HB-101 upon addition of FCS or

approved tetracycline-free FCS was analyzed. The strain Xl-1, which differs from HB-101 among other things, in its tetracycline-resistance, was used as a control.

For Xl-1 no difference in proliferation rate was detectable when adding 0-20% of either sera to the medium. Addition of 50% non-approved FCS and tetracycline-free FCS resulted in a decrease of proliferation rate to about 56% and 86%, respectively (Fig.9A). As there was no change in proliferation rate upon addition of tetracycline from 0-15µg/ml (Fig.9C), this change was most probably not due to tetracycline, but rather to an additional, unknown factor. This factor was capable to influence also the proliferation of Xl-1 negatively.

Proliferation rate of HB-101 increased or stayed at 100% with rising concentrations up to 25% of either sera, but then dropped down to 10% and 90% upon addition of 50% of non-approved FCS and approved tetracycline-free FCS, respectively (Fig.9A).

To determine the possible tetracycline-dependent effect on proliferation of HB-101, the effect of tet-free FCS was subtracted from the effect of non-approved FCS on proliferation. The resulting function is linear, as indicated by the applied trend line (Fig.9B).

In contrast to that, addition of tetracycline to HB-101 led to an exponential decrease in proliferation rate (Fig.9C).

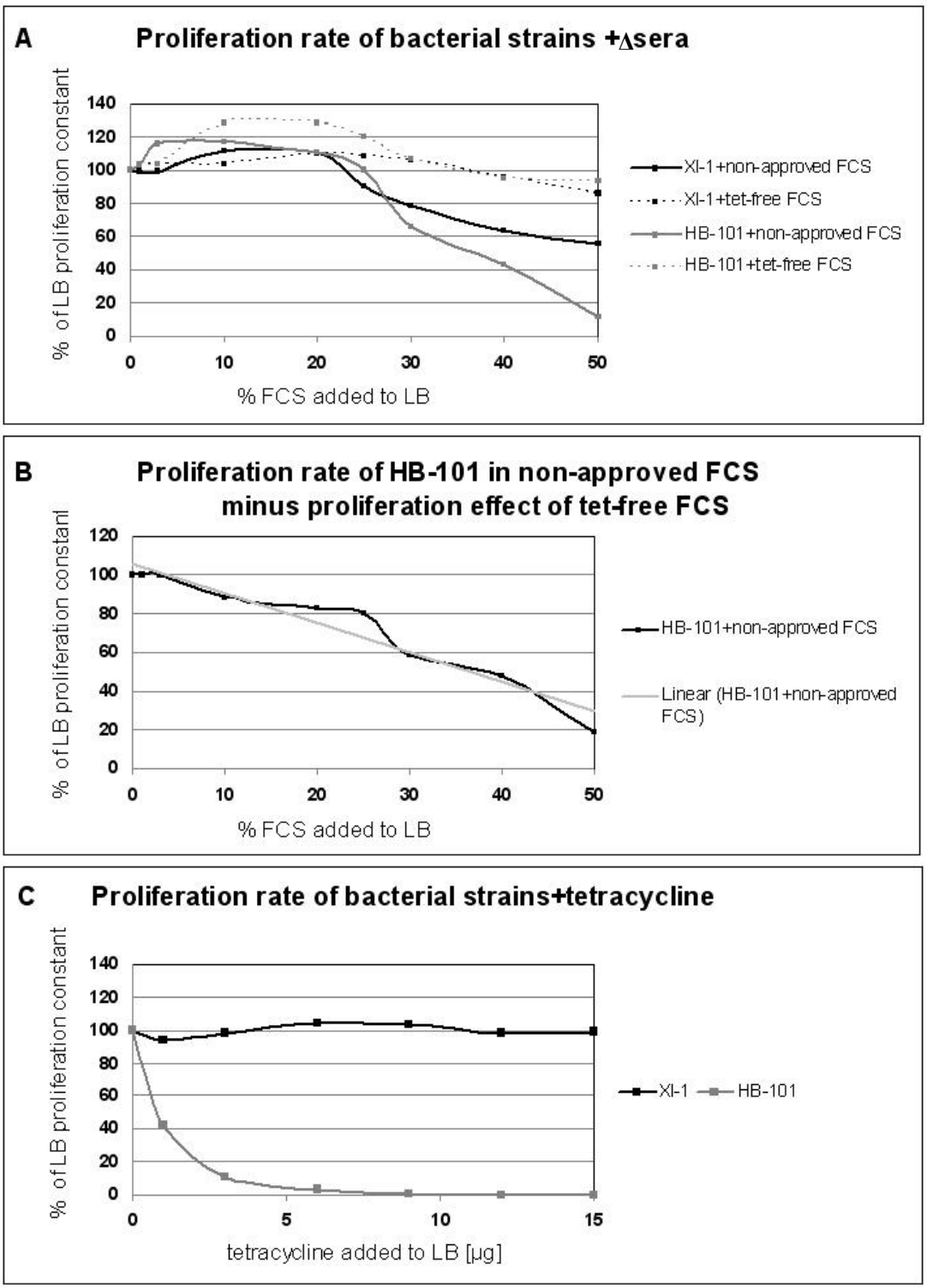


Fig.9 Proliferation rate of Xl-1 and HB-101 changed differentially upon incubation with increasing amounts of non-approved, tet-free FCS or tetracycline

Both strains were grown in LB medium at 37°C for at least 7 hours. Proliferation of bacterial culture was quantified by measuring cell density at OD_{550} every hour. Proliferation constants were determined by calculating the slope in the linear range of a plot of time versus OD_{550} (proliferation curve). The values are presented as a percentage of the proliferation constant of bacteria, grown in 100% LB media, which was set 100%.(n=3; mean)
(A) Both strains were grown in LB medium, supplemented with 0-50% non-approved or tet-free FCS.
(B) Growth constants of HB-101 in non-approved FCS were adjusted, by subtracting the effect of tet-free FCS on the strain. The applied trend line shows, that the line has a linear form.
(C) Both strains were grown in LB medium, supplemented with 0-15µg/ml tetracycline.

To sum up, treatment with approved tetracycline-free FCS had, besides small differences, the same effect on both strains. Contrary to that, normal FCS had a different effect: Addition of 50% FCS to the medium led to a 44% decrease in Xl-1 and a 90% decrease of proliferation in HB-101. So, there was a proliferation rate decrease, which seemed to be both tetracycline-dependent and -independent.

Proliferation rate of HB-101 dropped down to 10%, with 3µg/ml tetracycline added. Therefore, it could be argued, that in the medium, containing 50% non-approved serum, the same amount of tetracycline was probably present. However, this is in contrast to the fact that addition of 25% FCS to HB-101 did not lead to a decrease of proliferation to 25%, which could be expected if there were 1,5µg/ml tetracycline in the serum, but proliferation dropped down to only 80% (Fig.9B). Therefore it was concluded, that the observed decrease in proliferation of HB-101 with 50% non-approved FCS added was not only due to tetracycline, but rather to an additional factor present only in non-approved FCS. This is further supported by the fact that addition of increasing concentrations of non-approved FCS did not lead to an exponential, but to a linear decrease in proliferation rates.

It was concluded, that this method was not appropriate to determine the levels or show existence of tetracycline in non-approved FCS.

Differential induction of ß-galactosidase in tetracycline-inducible HEK293TRex cells upon addition of non-approved and approved tetracycline-free FCS

In order to analyze the non-approved FCS for the presence of tetracycline, HEK293TRex cells were stably transfected with the reporter construct pcDNA6/TO-LacZ described above, to establish a cell line, which would allow the detection of tetracycline. 24hours post transfection cells were grown medium supplemented with geneticin for 2 weeks. Afterwards single clones were picked.

As the level of tetracycline in the non-approved FCS is probably very low, we looked for a HEK293TRex/TO-LacZ clone with high expression levels of ß-galactosidase upon addition of doxycycline and very low expression in the absence of doxycycline/tetracycline, i.e. high inducibility.

To do so, ß-Gal activity was analyzed in whole cell lysates of eight single clones, either induced with doxycycline or left uninduced (Fig.10A).

Out of eight clones picked, clones #6 and #7 were chosen because of low background and high signal strength (Fig.10B).

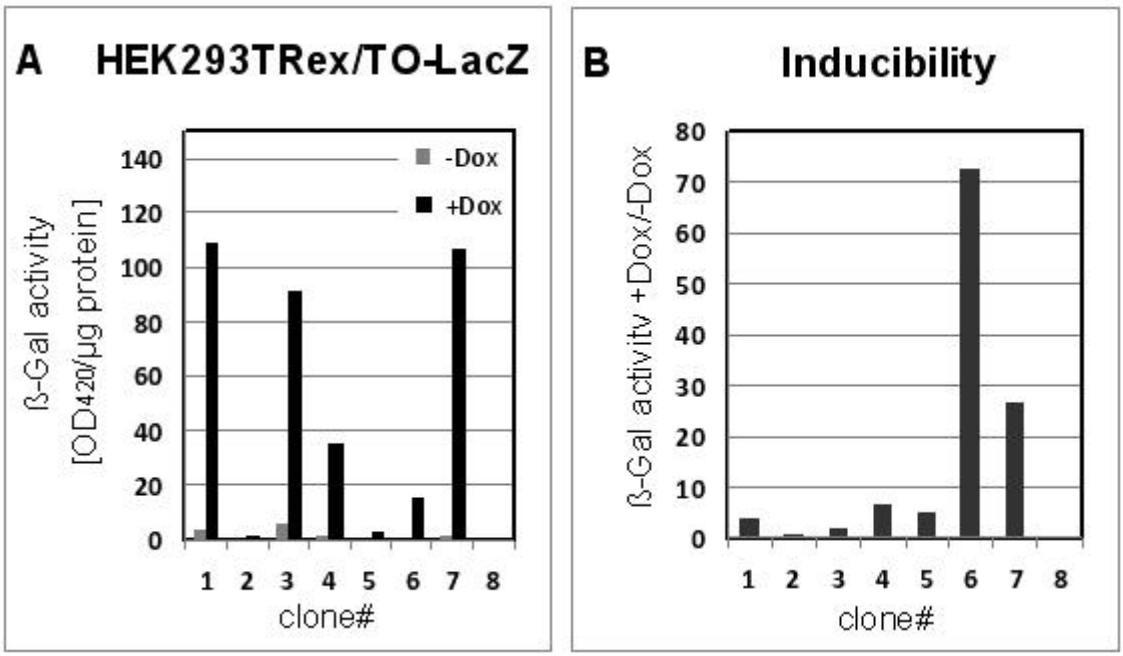


Fig.10 Generation of a tetracycline-detection tool

HEK293TRex cells were transfected with the vector pcDNA3/TO-LacZ, coding for the reporter protein ß-Gal under the control of a tet-inducible promoter. After transfection, cells were grown in medium supplemented with 600µg/ml geneticin for 2 weeks and single clones were picked. After induction of ß-Gal expression with 10^{-6} g/ml doxycycline in clones 1-8 for 24hours, whole cell lysates were analyzed for ß-Gal expression by a ß-Gal assay.
(A) ß-Gal expression (arbitrary units) in single clones HEK293TRex-TO-LacZ#1-8 in the uninduced state (-Dox) and induced state (+Dox). Clone#1, 3 and 7 showed highest ß-Gal expression level upon treatment with doxycycline.
(B) Inducibility of HekTRex/TO-LacZ clones #1-8 was derived by calculating signal ratio of induced cells/uninduced cells. Clones #6 and 7 showed highest inducibility

Next, HEK293TRex/TO-LacZ clone #6 and #7 (HekTRex/TO-LacZ#6/7) were further characterized by inducing the cells with 10^{-6}-10^{-15} g/ml doxycycline and measuring ß-Gal activity in whole cell lysates after 24 hours of induction.

To ensure an excess of substrate, compared to amount of enzyme, 2µg whole cell lysate were used from cells induced with doxycycline-concentrations from 10^{-6}-10^{-9} g/ml. To be able to detect subtle differences in signal strength in cells induced with 10^{-9}-10^{-15} g/ml doxycycline, 500µg whole cell lysate were used.

The ß-Gal activity decreased with decreasing doxycycline concentrations from 10^{-8} to 10^{-12} g/ml. From a doxycycline concentration of 10^{-12}µg/ml only background signal was detectable (Fig.11 and 12).

However, as can be seen in the flattening of the curve in Fig. 11, ß-Gal activity was underestimated in lysates of HEK293TRex/TO-LacZ#7 cells induced with 10^{-6} and 10^{-7} g/ml doxycycline by adding too less substrate to the reaction. The same is true for HEK293TRex/TO-LacZ#6 cells induced with 10^{-6} to 10^{-8} g/ml doxycycline.

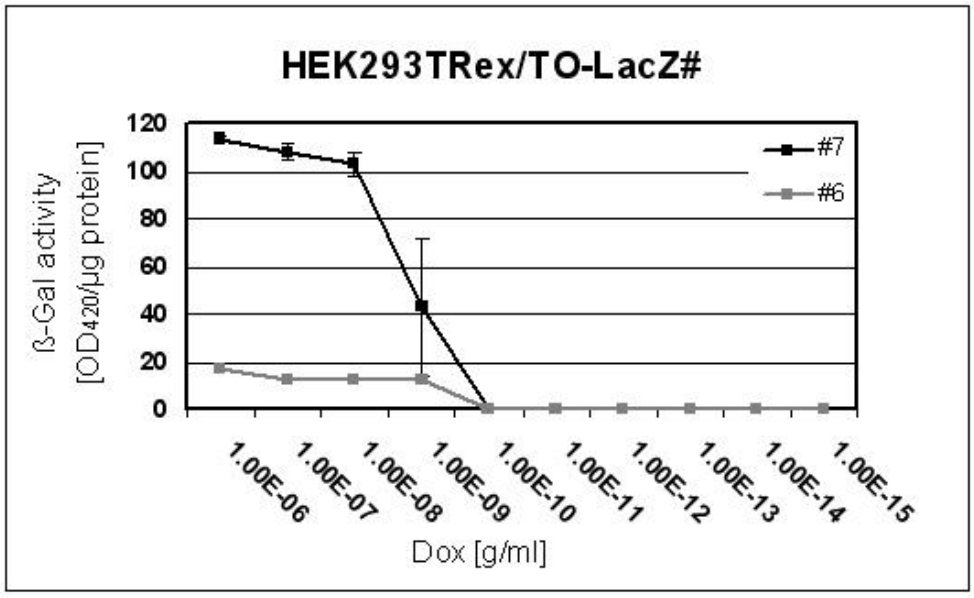


Fig.11 Standard curve for ß-Gal assay in HEK293TRex/TO-LacZ#6 and #7

Cells were grown in medium supplemented with 10% approved tetracycline-free FCS and incubated with doxycycline concentrations from 10^{-6}-10^{-15}g/ml. After 24 hours of doxycycline induction, whole cell lysates were analyzed for ß-Gal activity. The x-axis shows ß-Gal activity (arbitrary units), the y-axis shows the doxycycline concentration, cells were incubated with. (n=2; mean ±S.D.)

To determine the presence and concentration of tetracycline in non-approved FCS, cells were grown in medium supplemented with non-approved FCS or approved tetracycline-free FCS as a negative control. After 24 hours of proliferation in the two different sera, cells were lysed and analyzed for ß-Gal assay activity. 500µg of whole cell lysate were used for one assay.

There was a significant difference in ß-Gal activity between whole cell lysates of cells grown in approved tetracycline-free FCS or in non-approved FCS. While ß-Gal activity in cells grown in approved tet-free FCS was in the background range, ß-Gal activity of cells grown in non-approved FCS was in the range of 10^{-10}µg/ml doxycycline (Fig.12).

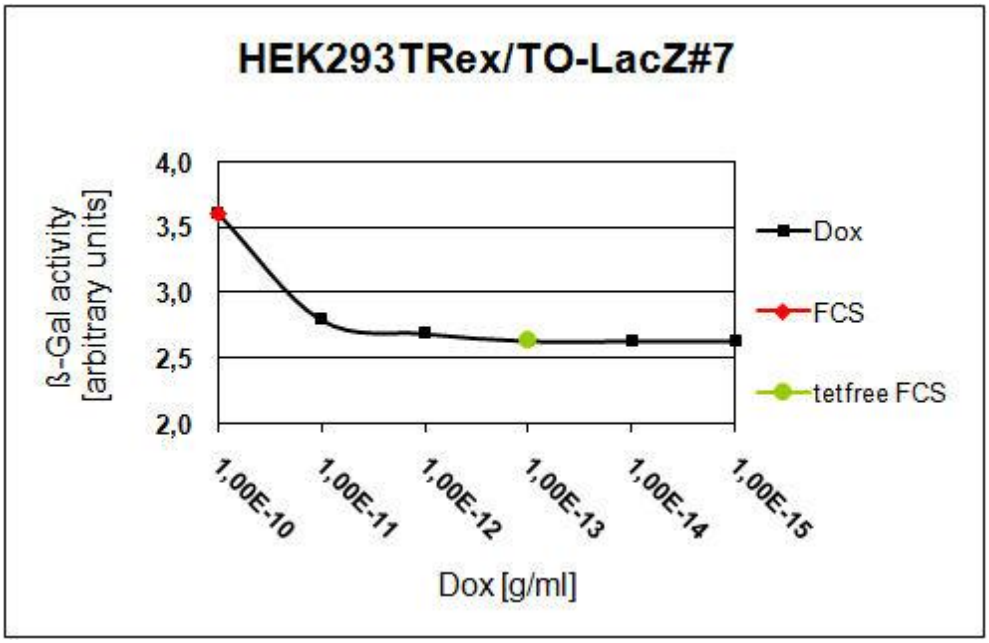


Fig.:12 Non-approved FCS contained a tetracycline concentration equivalent to 10^{-10}g/ml doxycycline

ß-Gal signals of whole cell lysates of HEK293TRex/TO-LacZ#7 cells, induced with Dox concentrations from 10^{-9}-10^{-15}g/ml were used to establish a standard curve. HEK293TRex/TO-LacZ#7 cells were grown in serum supplemented with either 10% approved tet-free FCS (tet-free FCS) or in non-approved FCS (FCS) for 24 hours. ß-Gal activity in whole cell lysates was compared to signals of the standard curve.
Lysates of cells grown in approved tetracycline-free FCS showed no ß-Gal activity. But lysates of cells grown in non-approved FCS showed ß-Gal activity that resembled induction with $1x10^{-10}$ µg/ml doxycycline.

This result was further confirmed by using the cell line HekTRex-LacZ#6. Consequently, for the next round of N2aTRex14 transfection with shPTPA and single cell isolation, approved tetracycline-free FCS was used.

3.2.3. Generation of stable PTPA knock-down cell lines

To generate stable PTPA knock-down clones, N2aTRex14 cells were co-transfected with the pCRUZ^{Neo}-GFP vector containing the coding sequence of GFP and either the vector pNTO, containing the PTPA-targeting sequence#1 (shPTPA1) or the corresponding mismatch sequence (shControl-1). 24hours post transfection cells were GFP-sorted (FACS facility at the IMP) and grown as single cells progeny in medium supplemented with geneticin for 2 weeks. RNAi expression was induced with 1µg/ml doxycycline for 4 days and whole cell lysates were analyzed by SDS-PAGE and immunoblotting for PTPA knock-down.
Three out of 90 clones tested, showed significant knock-down of PTPA upon induction with doxycycline (Fig.13):
N2aTRex14+pNTO-shPTPA1 clone #18 and #30 (shPTPA#18/30) showed a 2,5x reduction of PTPA levels when induced with doxycycline. PTPA levels of N2aTRex14+pNTO-

shPTPA1 clone #89 (shPTPA#89) showed a 3,8x reduction upon induction of shRNA expression with doxycycline (Fig.13).

The ability to knock-down PTPA upon induction with doxycycline and the expression of GFP remained stable over at least three months.

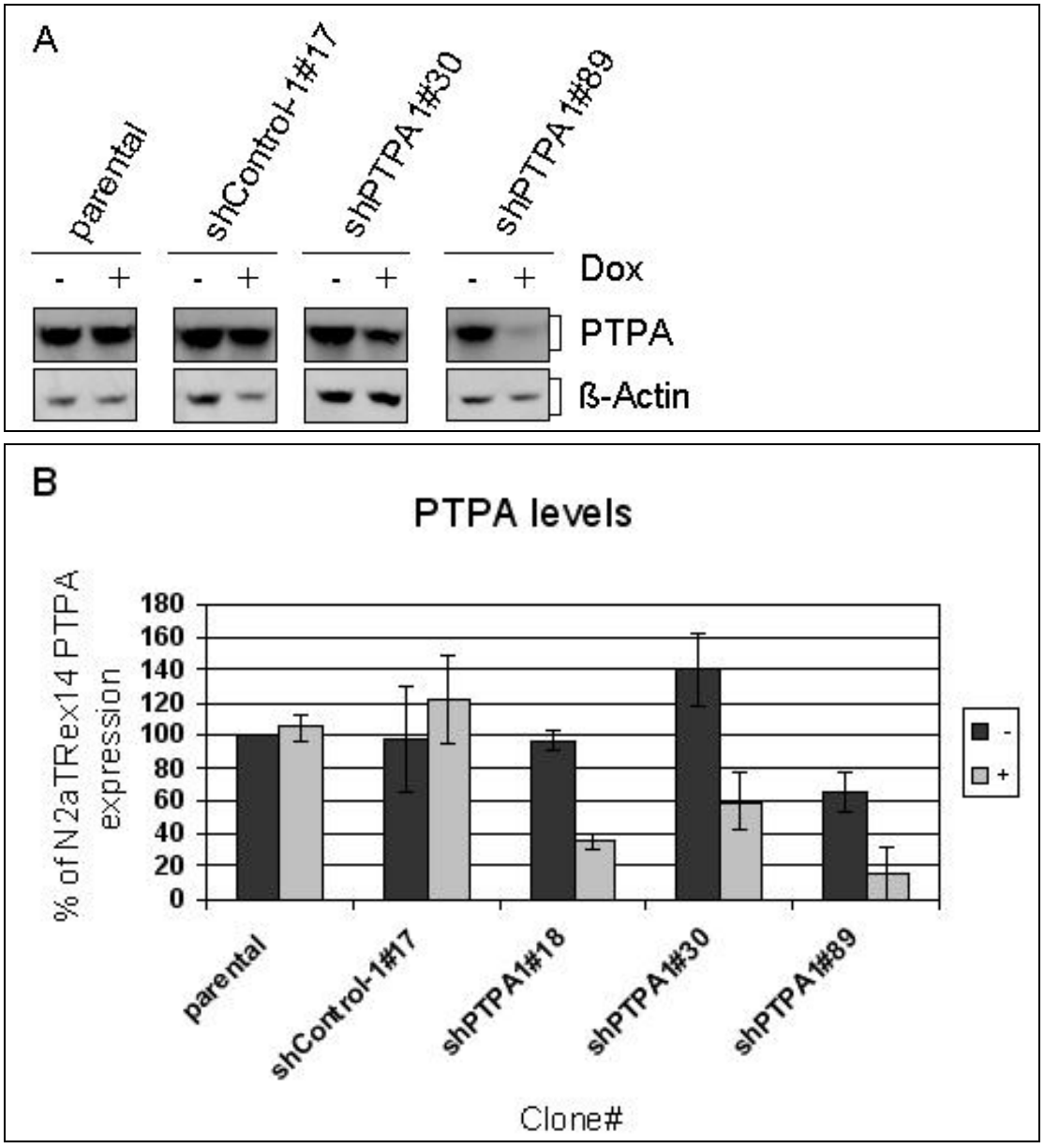


Fig.13 Stable and inducible PTPA knock-down cell lines

N2aTRex14 cells were co-transfected with the pCRUZ vector containing the coding sequence of GFP and the vector pNTO, containing the PTPA-targeting sequence#1 (shPTPA1) or the corresponding mismatch sequence#1 (shControl-1). 24hours post transfection cells were GFP-sorted and grown as single cells in medium supplemented with geneticin for 2 weeks. (A) Single clones were induced for 4 days with doxycycline and whole cell lysates were analyzed by SDS-PAGE and immunoblotting with anti-PTPA and ß-Actin (loading control) antibody. (B) Quantification of PTPA expression of the five cell lines. The values are presented as a percentage of the PTPA expression level in uninduced N2aTRex14, which was set 100%. (n=3; mean ±S.D.)

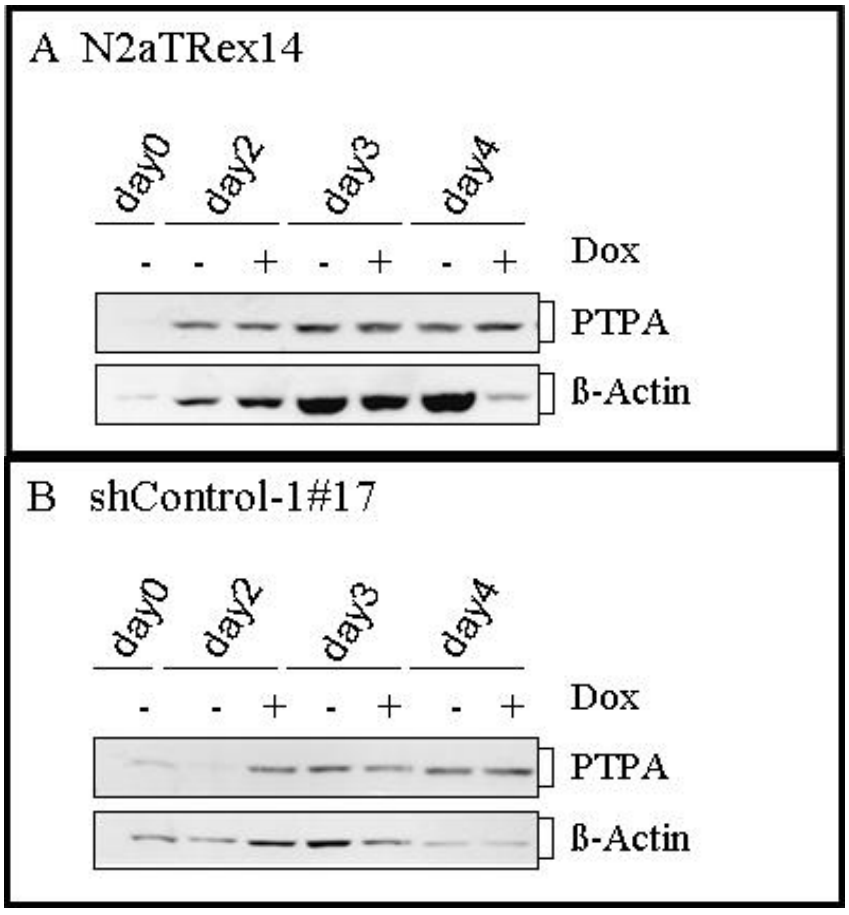
However, the pCRUZ-GFP vector, the cells were co-transfected with, contained the coding sequence for neomycin-resistance. The same resistance gene was also encoded on the shRNA-expressing vector.

This experimental error could have contributed to the low efficiency in the establishment of stable PTPA knock-down clones, as cells that have lost the vector pNTO-shPTPA could still be neomycin-resistant through retaining pCRUZ^{Neo}-GFP. Additionally, GFP is under the control of the strong CMV promoter and high expression levels of GFP are known to have a toxic effect on the cells. Due to time constraints I was unable to repeat the generation of PTPA knock-down clones without the use of pCRUZ^{Neo}-GFP vector.

3.3. Effect of PTPA suppression on PP2A biogenesis in mammalian cells

3.3.1. Analysis of the effect of PTPA knock-down on proliferation

To examine the effect of PTPA loss on PP2A in more detail, we relied on these 3 clones. Cell lines N2aTRex14 (parental) and shControl1 (mismatch control) were used as controls.



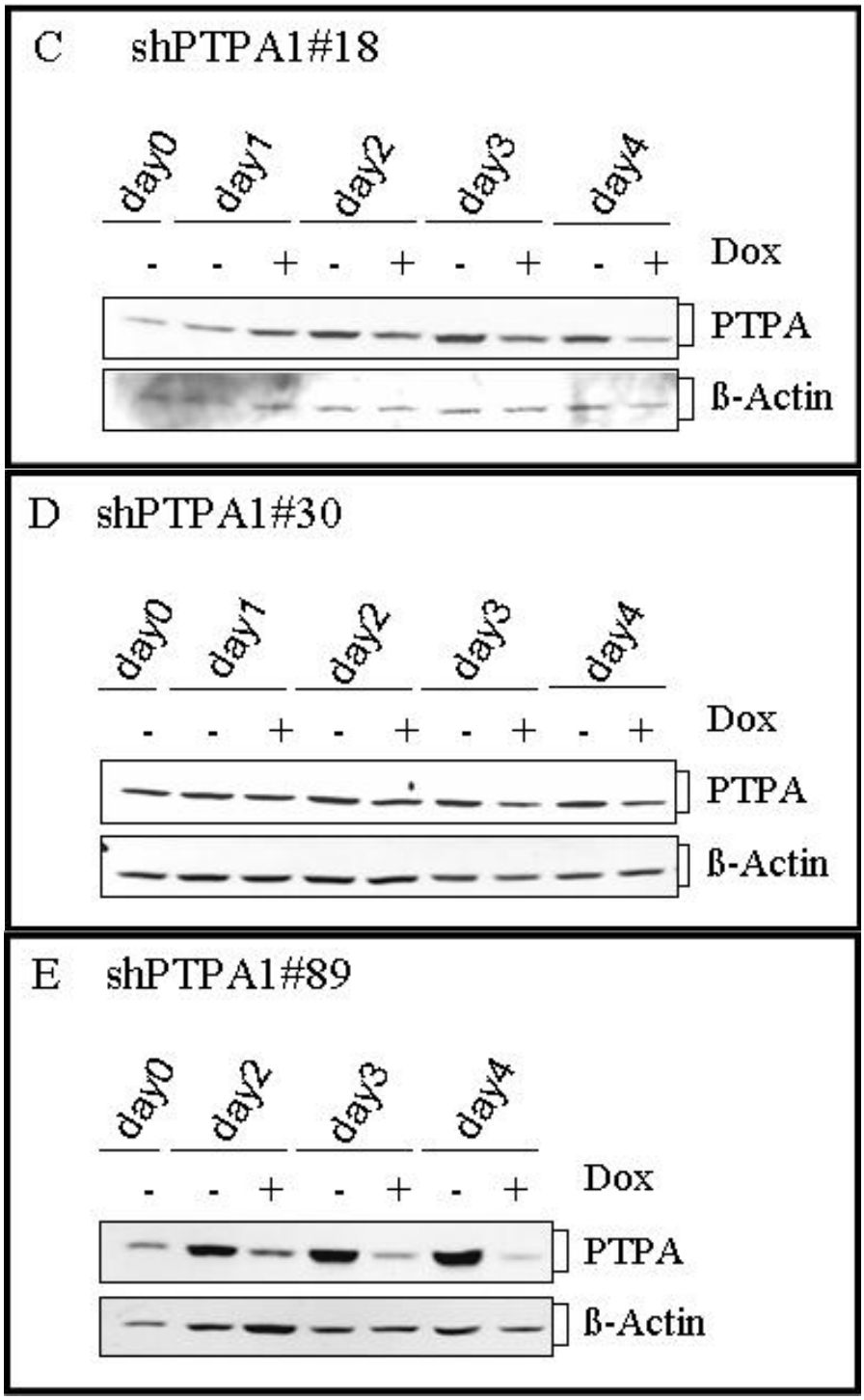
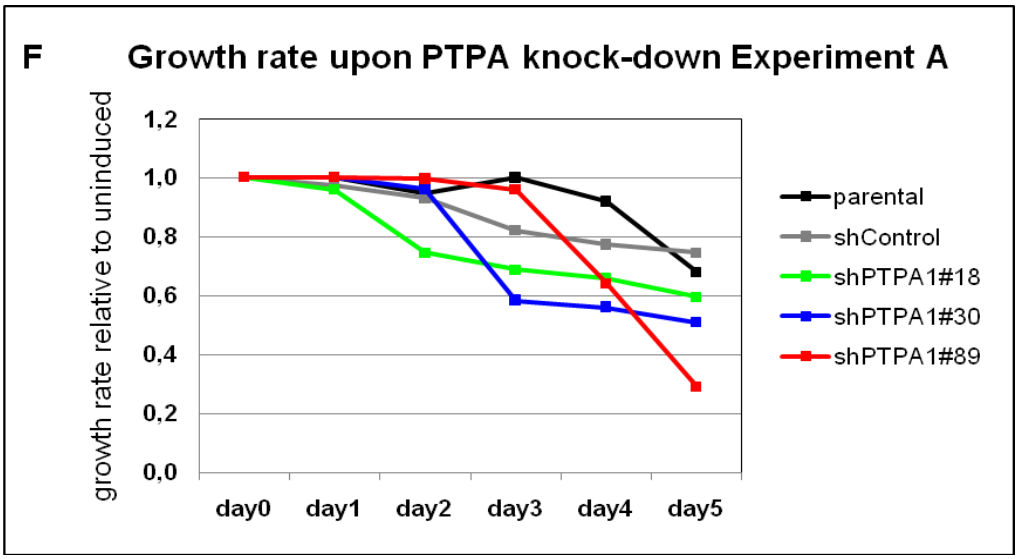


Fig.14 Timecourse of PTPA knock-down and proliferation in PTPA knock-down and control cell lines

$1*10^5$ cells/plate were seeded and induced next day with doxycycline (+Dox). Un-/induced cells were counted (D), lysed on day0-4 and analyzed for knock-down of PTPA by SDS-PAGE and immunoblotting, using anti-PTPA and anti-ß-Actin (loading control) antibody (A-E).



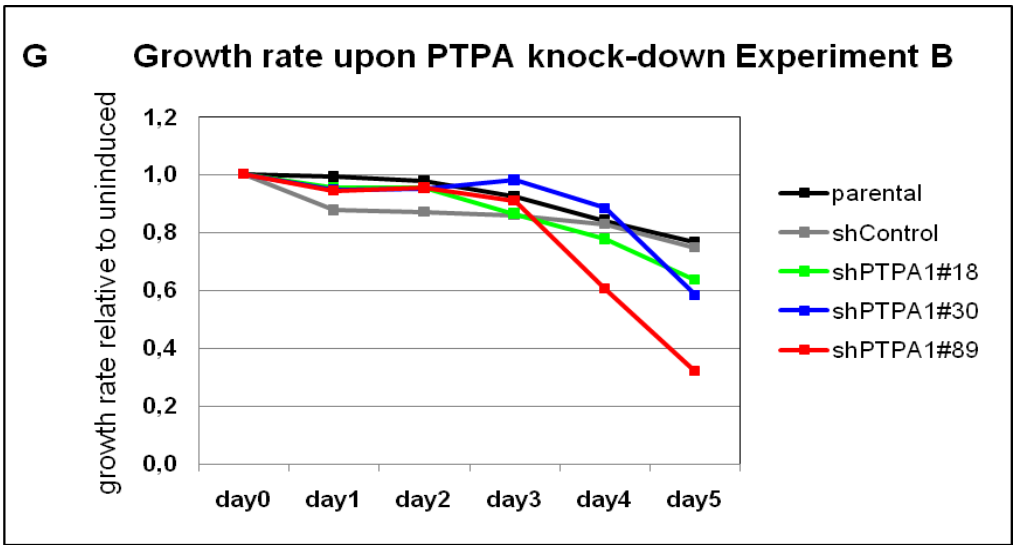


Fig.14 F-G: Timecourse of PTPA knock-down and proliferation in PTPA knock-down and control cell lines

Proliferation rate of cell lines upon doxycycline treatment, relative to uninduced cells

Analysis of differences in cell proliferation upon induction of PTPA knock-down was performed two times with different results.

In the first experiment cell proliferation upon induction of PTPA knock-down showed a reduction of proliferation rate to about 80% of the uninduced cells on day 4 and 5 upon doxycycline treatment for the two control cell lines, probably due to its cytotoxic effect (Fig.14F) (Ermak et al., 2003).

On day4 of shRNA-induction, proliferation of shPTPA1#18 and #30 decreased to 65% and 68% when PTPA- expression decreased to 87% and 70% respectively (Fig.14C, D and F). However, the conclusion that PTPA knock-down results in decreased proliferation was conflicted by the fact, there was only a slight decrease in proliferation to 66% in clone

shPTPA1#89, which shows the strongest decrease of PTPA levels – down to 24% compared to PTPA levels in uninduced cells.

Nevertheless, cell proliferation upon PTPA knock-down in clone shPTPA #89 was reduced on day 5 to 29%, although PTPA levels did not change markedly from day4 to 5. Maybe the effect of PTPA suppression on cellular processes is delayed in time (Fig.13F).

To summarize this experiment, a negative effect of PTPA suppression on cell proliferation could be observed for all shPTPA clones from day3 to day5.

However, upon repetition of the experiment, such an effect on all cell lines could only be observed on day5 (Fig. 14G).

Considering these slightly conflicting data and the fact, that this experiment was only repeated twice, we can only suggest, that it is probable that PTPA suppression has a negative effect on cell proliferation of these clones. In order to completely demonstrate this, this experiment needs to be repeated.

3.3.3. Complex assembly in PTPA knock-down cells

Although deletion of *RRD1* and *RRD2* in yeast results in a catalytic subunit with a conformation a decreased catalytic activity towards P-Ser/Thr, complex assembly is largely unaffected in these cells. To confirm these data in mammalian cells, first PP2A complex formation was assessed.

Antibodies directed against the C subunit interfere with PP2A complex formation and thus cannot be used for the analysis. Therefore hemagglutinin (HA)-tagged C subunit was expressed in the PTPA knock-down clones. HA tagged C subunit cannot be expressed at unphysiologically high levels because a translational control mechanism adjusts the total C subunit amount to constant levels (Baharians and Schönthal, 1998).

First, it was tested, how fast the expression level of HA-tagged C-subunit decreases after transient transfection and if thus would be affected by the suppressed PTPA levels.

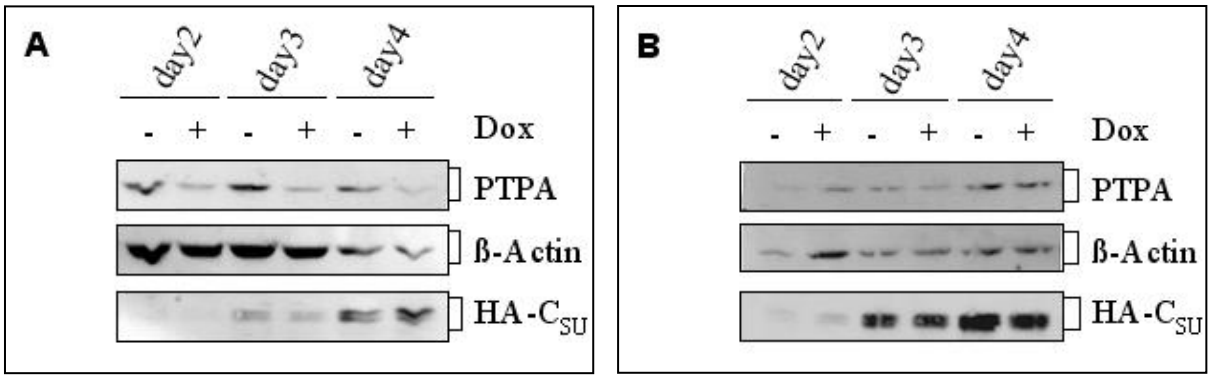


Fig.15 HA-tagged C subunit expression decreased with time

Analysis of the effect of PTPA knock-down on the rate of reduction of PP2A C subunit after transient transfection:
shPTPA1#30 cells (A) and Control-1 cells (B) were seeded in a 6-well each and the cells of three wells of each 6-well plate were induced. On three consecutive days after induction (day2-4), one well with uninduced cells and and one well with induced cells were transfected with the vector pcDNA3/HA-C_{SU} (see attachment for map), coding for the expression of mammalian HA-tagged C subunit. On day5, whole cell lysates were tested for the expression level of HA-tagged C subunit by SDS-PAGE and immunoblotting

As there was no difference in HA-C_{SU} expression between uninduced and induced cells, it was concluded that induction of PTPA-knock-down has no effect on the C subunit expression of HA-tagged C subunit (Fig. 15A).

shPTPA1#30 cells, transfected on day2 showed a low level of HA-C_{SU} expression on day5, whereas cells transfected on day4, exhibited a high level of HA-C_{SU} expression on day 5 (Fig. A,B). As the same effect was observed in a shControl-1 cell line, this effect was considered to be independent of the PTPA knock-down. This means, that the expression level of transiently transfected HA-C_{SU} decreases with every day passing after transfection.

So, two strategies were followed: PTPA knock-down clones were induced for three days and then transiently transfected or were stably transfected with the vector pcDNA3/HA-C_{SU}.

Using the first strategy, parental cell line N2aTRex14, mismatch control cell line shControl1 and PTPA knock-down cell line shPTPA1#30 were stably transfected with empty vector pcDNA3 (pcDNA3) or vector pcDNA3/HA-C_{SU} (HA-C_{SU}). Cells were induced with doxycycline for four days. To determine whether loss of PTPA affects PP2A complex assembly, HA-tagged C subunit of PP2A was immunoprecipitated from lysates of untreated and doxycycline-treated cells. The immunoprecipitates were analyzed by immunoblotting for the presence of the A and various B-type subunits.

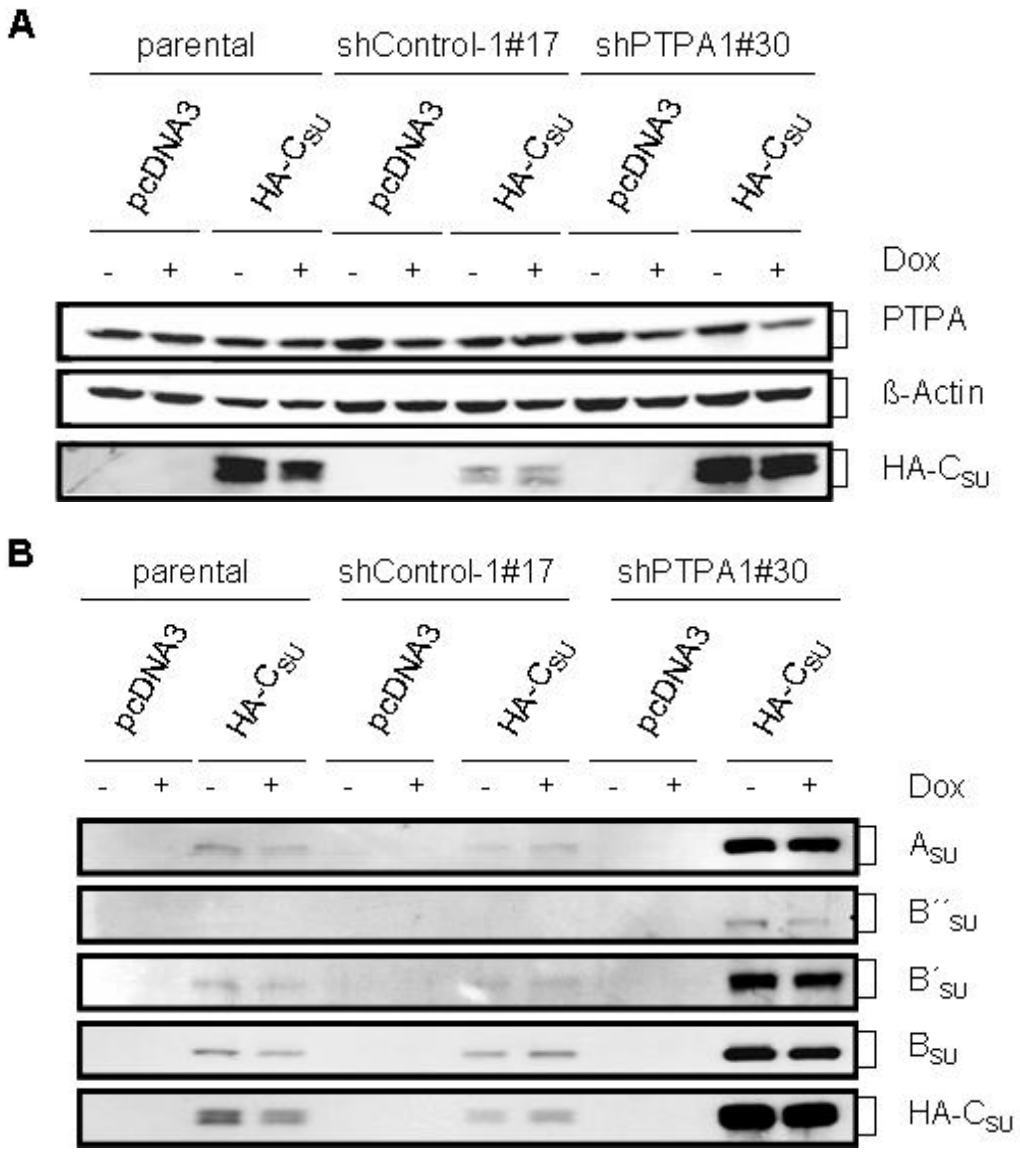


Fig.16 The heterotrimeric PP2A complex assembles correctly in PTPA knock-down cells

(A) Parental cell line N2aTRex14, mismatch control cell line shControl1 and PTPA knock-down cell line shPTPA1#30 transfected with empty vector pcDNA3 (pcDNA3) or vector pcDNA3, coding for HA-tagged C subunit (HA-C_{SU}). Cells were treated with doxycycline for four days. Whole cell lysates were analyzed for knock-down of PTPA by SDS-PAGE and immunoblotting. Blots were sequentially incubated with anti-HA, anti ß-Actin (loading control) and anti-PTPA antibody.
(B) Anti-HA tag immunoprecipitates from lysates of parental cell line N2aTRex14, shControl1 and shPTPA1#30 transfected with empty vector pcDNA3 (pcDNA3) or vector pcDNA3, coding for HA-tagged C subunit (HA-C_{SU}) were analyzed by 10%SDS-PAGE and immunoblotting. Blots were sequentially incubated with anti-HA, anti-A subunit, anti-B, B´ and B´´ antibody.

PP2A complex assembly was not impaired in the parental and the mismatch control strain, but also not in the PTPA knock-down strain shPTPA1#30, which exhibited a PTPA knock-down to 50% of the PTPA expression level of the uninduced state of the cell line (Figure16).

3.3.4. Catalytic activity of mammalian C subunit in PTPA knock-down cells

The immunoprecipitates from cell lines either stably or transiently transfected with pcDNA3/HA-C_{SU}, were further analyzed for catalytic activity towards the serine-14-phosphorylated substrate phosphorylase a, a substrate that is dephosphorylated by all PP2A holoenzymes. This experiment was repeated only twice, one with transiently and once with stably pcDNA3/HA-C_{SU}-transfected PP2A knock-down clones.

Using cell lines stably transfected with the vector pcDNA3/HA-C_{SU}, with a PTPA reduction to 50%, a reduction in catalytic activity of PP2A to 60% and 30% was shown in shPTPA1#30 cells, compared to the parental and mismatch control cell line, respectively. However, catalytic activity of PP2A complexes isolated from induced PTPA knock-down strain shPTPA#30 was not different from the uninduced state (Fig.17A).

However, different results were obtained for the cell line shPTPA1#89, transiently transfected with the vector pcDNA3/HA-C_{SU}, when PTPA knockdown was already induced for three days to of 17% of the PTPA level in the parental cell line.

A difference of 41% between the uninduced and the induced state of PTPA knock-down cell line shPTPA1#89 was shown. Nevertheless, an increase catalytic activity of PP2A to 166% and 125% was shown in shPTPA1#89 cells, compared to the parental cell line, probably due to experimental mistakes (Fig.17B).

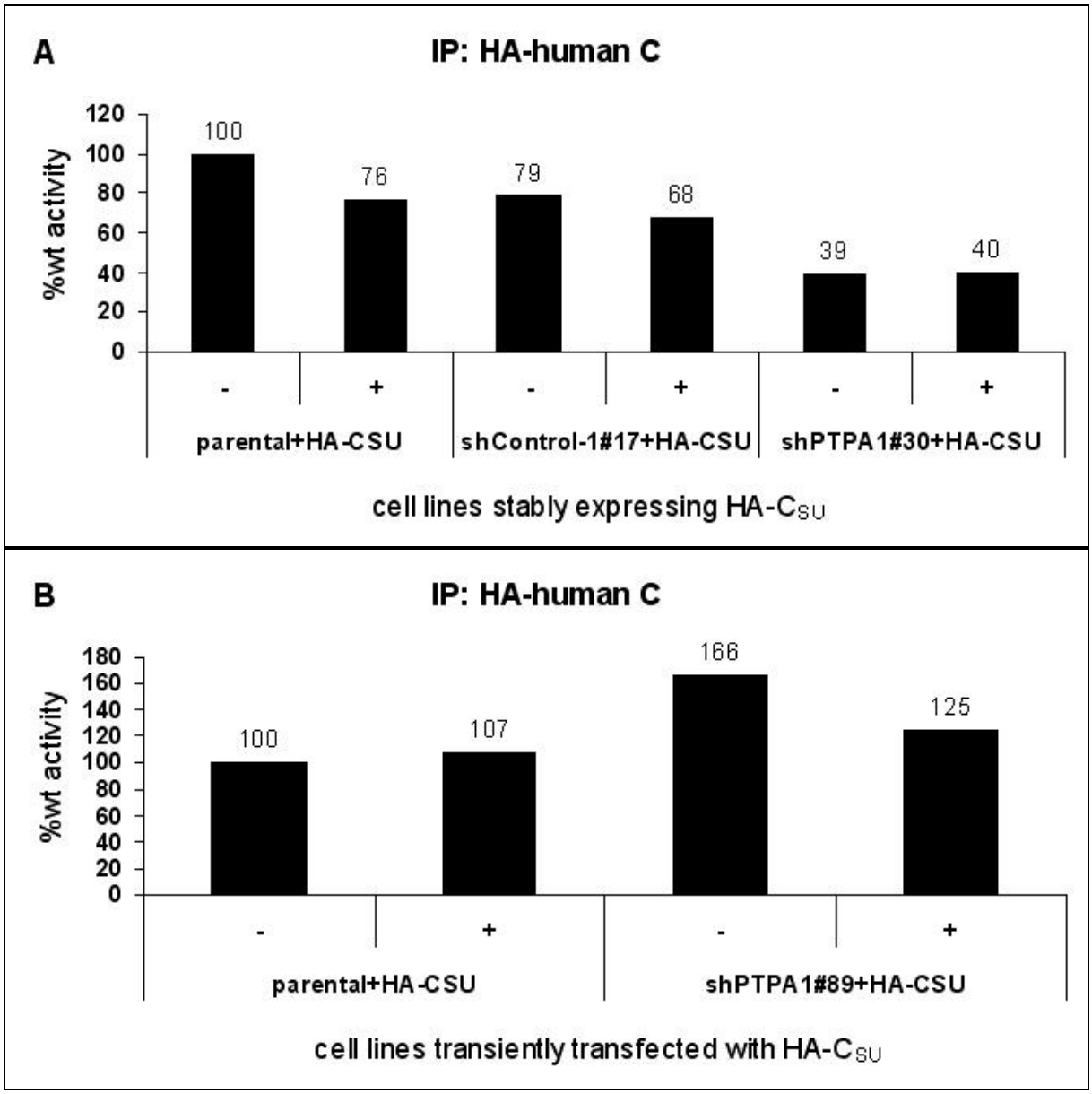


Fig.17 Catalytic activity of the heterotrimeric PP2A complex is not altered in PTPA knock-down cells

(A) Anti-HA tag immunoprecipitates from lysates of parental, mismatch control (shControl1) and PTPA knock-down (shPTPA1#30) strains, stably transfected with pcDNA3/HA-C_{SU}, either uninduced (-) or induced with doxycycline for 4 days (+) were analyzed by phosphatase assays toward the substrate phosphorylase a

(B) Anti-HA tag immunoprecipitates from lysates of parental and PTPA knock-down strain #89 (shPTPA1#89), transiently transfected with pcDNA3/HA-C_{SU} either uninduced (-) or induced with doxycycline for 4 days (+) were analyzed by phosphatase assays toward the substrate phosphorylase a

The assay values were normalized to the amount of the respective immunoprecipitated protein as determined by immunoblot analysis and are presented as a percentage of the activity for PP2A complexes purified from the wild-type strain, which was set 100%.

However, this experiment was done only twice (once for each PTPA knock-down cell line) and needs to be repeated.

3.4. Effect of Bα-knock-down on differentiation of N2aTRex14

N2aTRex were transfected with the vector pNTO^{Puro}, containing shRNA targeting the PP2A subunit Bα (Bα-targeting sequence from Strack et al., 2004). Control cells were transfected with the corresponding mismatch sequence, followed by selection with 5µg/ml puromycin and single cell expansion.

Out of 12 clones analyzed, 7 showed significant downregulation of the Bα subunit upon induction with doxycycline for three days.

To analyze the effect of Bα-Knockdown on differentiation, knock-down was induced for 3 days (day1-4) by supplementing 1µg/ml doxycycline.

Bα -Knockdown was shown in whole-cell lysates prepared before and after differentiation (day 4 and day7), by SDS-PAGE and immunoblotting with anti-Bα and ß-Actin (loading control) antibody (Fig.18).

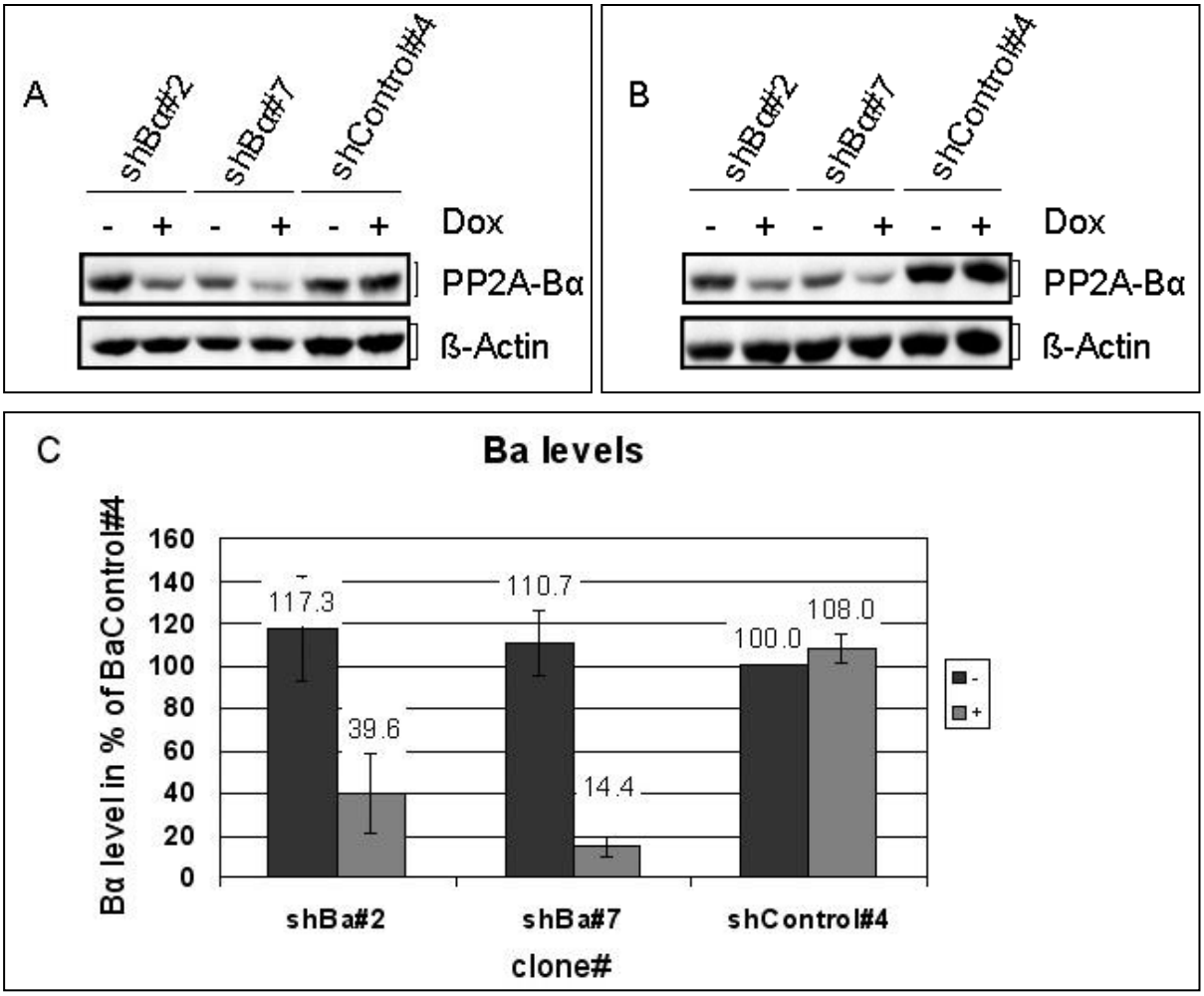


Fig.18: Knockdown of PP2A-Bα by doxycycline-inducible shRNA

(A) Protein levels of PP2A-Bα and Bδ in undifferentiated N2aTRex cells after 3d of doxycycline administration. Shown are 2 representative clones and a mismatch control. (B) Protein levels of PP2A-Bα in differentiated N2aTRex cells after 7d of doxycycline administration. (C) Quantification of Bα expression of the three presented cell lines; The values are presented as a percentage of the Bα expression level in uninduced shControl#4 cells, which was set 100%. (n=3; mean ±S.D.)

On day4, cells were seeded on poly-L-lysine. On day5 differentiation was induced by growing cells under starvation with low serum (1%FCS) or low serum plus 10µM retinoic acid (RA) for 48 hours. Pictures of cells are taken 24 and 48 hours after starting differentiation (day6 and 7, respectively). Differentiation state of cells is determined by comparing the number of cells with neurites longer than the cell axis (differentiated) to the total number of counted cells (differentiated and undifferentiated) (Fig.19A-F, Fig. 20A-F and Fig.21A-B) (for a complete experimental outline, see Fig. D in materials and methods).

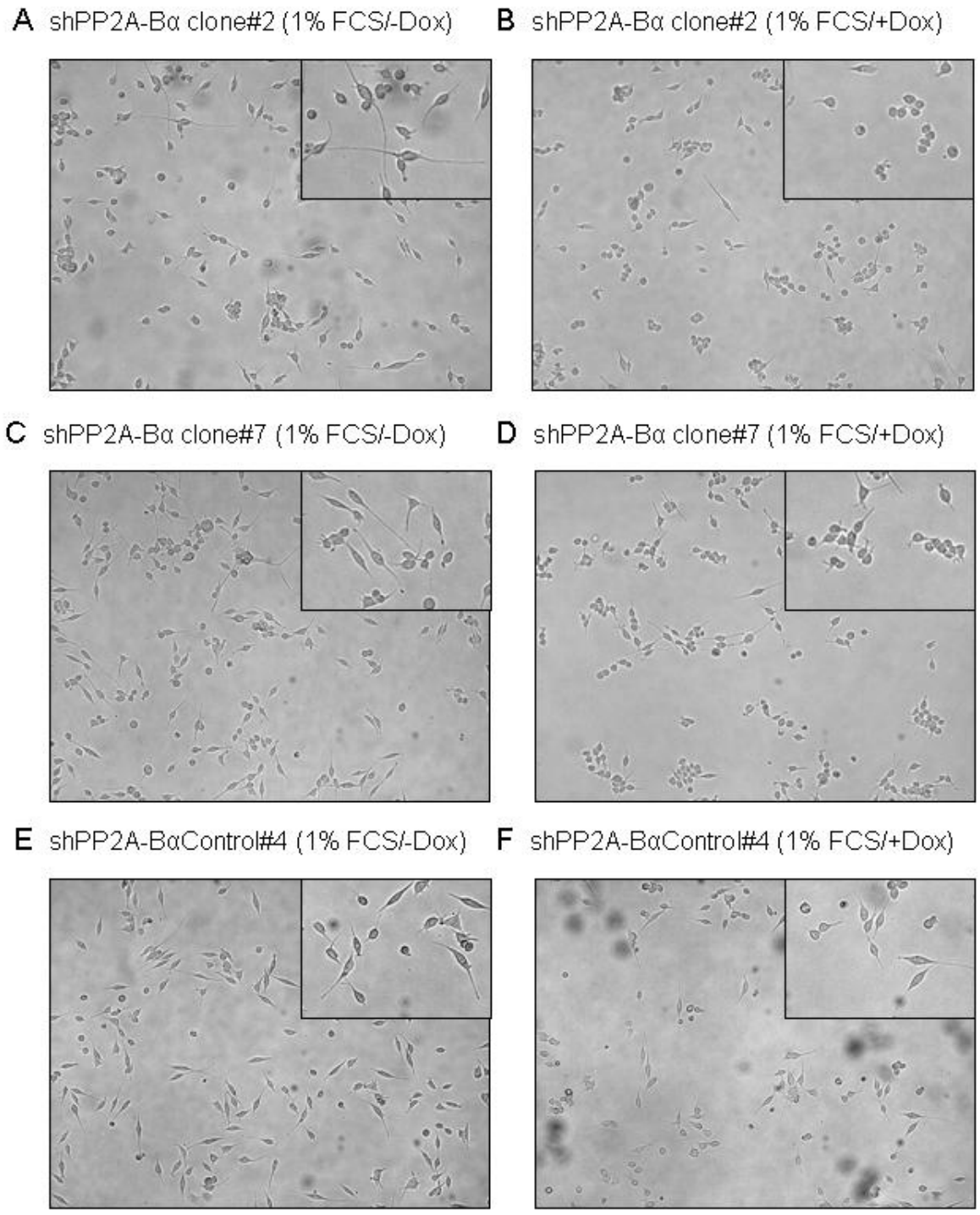


Fig.19: Representative lightfield microscope pictures of neuronal differentiation of Bα-knockdown clones Bα-knockdown clones

Expression of shRNA was induced for 3 days with 1 ug/ml Doxycycline (+Dox) (B, D, F) or cells were left untreated (A, C, E). Afterwards, differentiation was induced with low (1%FCS) serum for 48 hours.

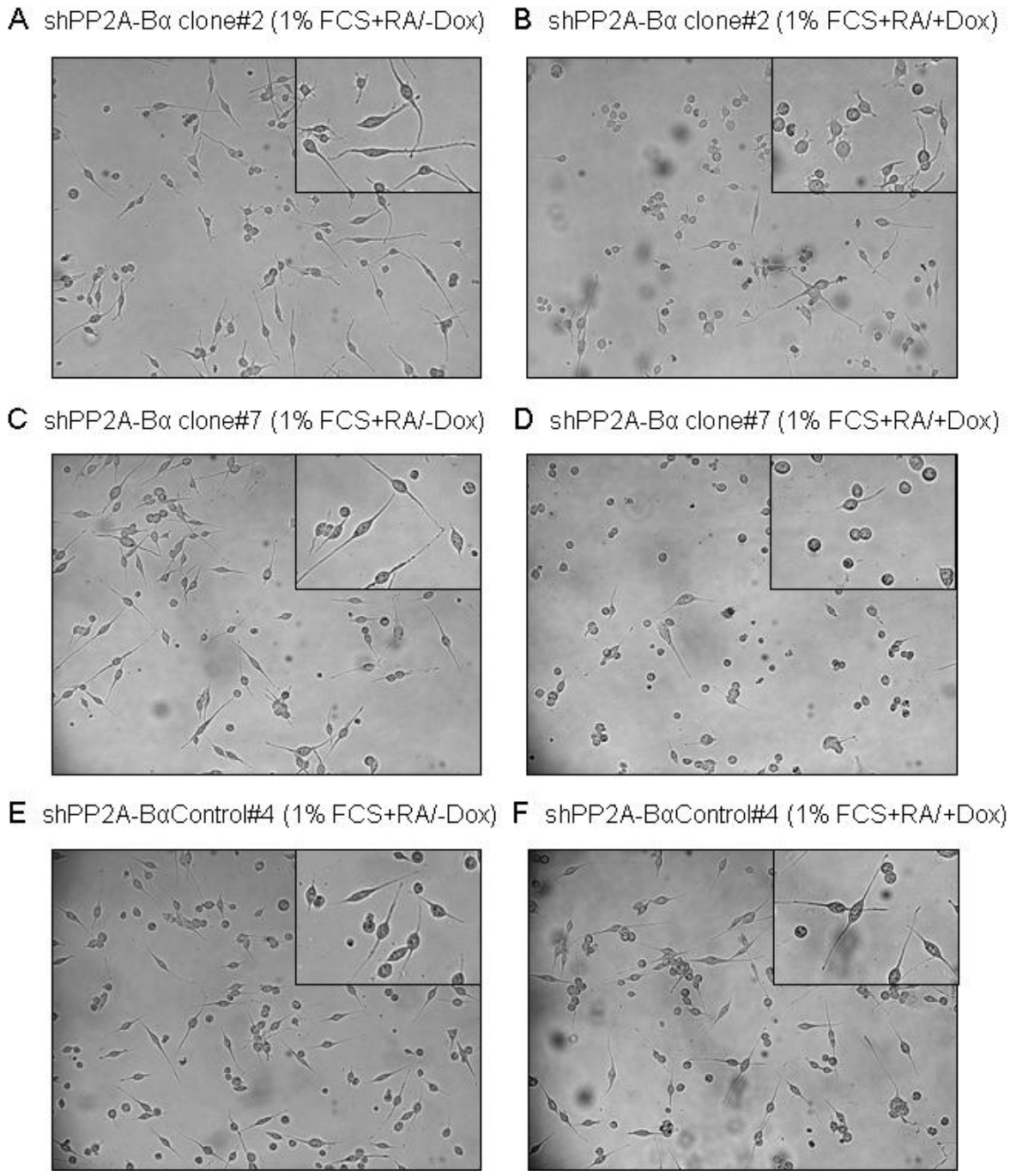


Fig.20: Representative lightfield microscope pictures of neuronal differentiation of Bα-knockdown clones Bα-knockdown clones

Expression of shRNA was induced for 3 days with 1 ug/ml Doxycycline (+Dox) (B, D, F) or cells were left untreated (A, C, E). Afterwards, differentiation was induced with low (1%FCS) serum and retinoic acid (RA) for 48 hours.

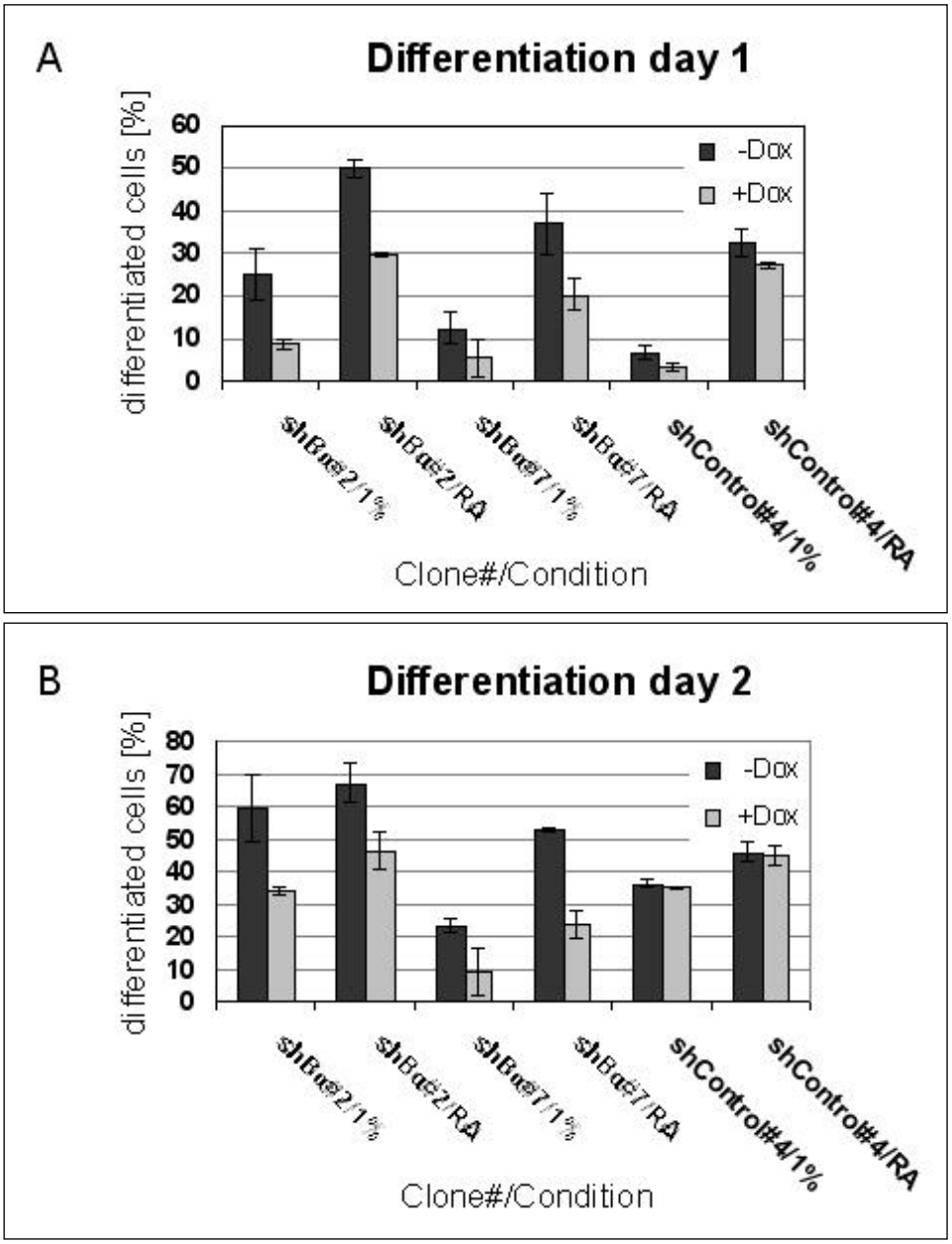


Fig.20: Uninduced (-Dox) Bα-knockdown clones shBα#2, shBα#7 exhibit faster differentiation, compared to differentiation upon Bα-knock-down induced by doxycycline (+Dox).

No significant difference was detected in mismatch control strain shControl#4. Differentiation was induced by low serum (1%) or low serum +10µM retinoic acid (RA) for 24 hours (A) and 48 hours (B)
Shown are means ± S.D. from four independent experiments. Data were analyzed using the Student t-test. Differences with p-values less than 0.05 were considered statistically significant:
(A) P-values, 24h differentiation: shBα#2/1% p=0,01; shBα#2/RA p=0,0001; shBα#7/1% p=0,098; shBα#7/RA p=0,025; shControl#4/1% p=0,82; shControl#4/RA p=0,19;
(B) P-values, 48h differentiation: shBα#2/1% p=0,013; shBα#2/RA p=0,012; shBα#7/1% p=0,027; shBα#7/RA p=0,0003; shControl#4/1% p=0,24; shControl#4/RA p=0,76;

Differentiation of uninduced Bα-knockdown-clones shBα#2 and #7 was faster, compared to clones with Bα-knockdown induced with doxycycline. Mismatch control cell line shControl#4 exhibited no significant difference of differentiation upon treatment with doxycycline.

4. DISCUSSION

The role of PTPA in the biogenesis of PP2A in mammalian cells

In most cell types, PP2A is responsible for the majority of dephosphorylations. Due to its overwhelming importance for normal cell physiology, it is not surprising that malfunction or dysregulation of PP2A has been associated with a plethora of pathologies, ranging from neuronal diseases, like Alzheimer and Parkinson`s disease to cancer. Moreover, several viruses use PP2A to deregulate cellular pathways in the host, underscoring the general importance of PP2A in signal transduction (for a review see Jahnssens and Goris, 2001; Arroyo and Hahn, 2005). Thus, deepened understanding about the biology of PP2A is of high importance to define novel therapeutic targets.

PP2A is a prime example for a multisubunit enzyme that obtains its substrate specificity by combining one out of only two different catalytic C subunits with a vast number of different regulatory subunits. This explains how one catalytic subunit can process the broad range of different PP2A-substrates with a high degree of specificity. Free non-complexed catalytic C subunit is known to harm cells, probably due to its unrestricted and unspecific activity (Ronne, Carlberg et al. 1991). Egon Ogris and colleagues demonstrated the synthesis of the catalytic subunit as a low-activity form in yeast that is fully activated only through interaction with Rrd (Fellner et al., 2003).

Although it was shown that PTPA - the mammalian homologue of Rrd – can partially compensate for Rrd1/2 loss in yeast, direct evidence that PTPA fulfils the same function in mammals is still missing. Therefore the major goal of this work was to study the role of PTPA in the biogenesis of PP2A in mammalian cells.

As suppression of PTPA via RNAi leads to apoptosis in HeLa cells (Fellner et al, 2003), we took advantage of a doxycycline-inducible knockdown construct to overcome this limitation and to be able to perform biochemical studies.

Moreover, PP2A is the major phosphatase involved in modulating the phosphorylation status of tau, a microtubule-associated protein. Emerging evidence suggests that hypoactivity of PP2A results in hyperphosphorylated tau, which then accumulates to form neurofibrillary tangles, a histopathological hallmark of AD neurons (for a review, see Tian et al., 2002). As the upstream function of Rrd is crucial for the biogenesis of active and fully functional PP2A in yeast, E. Ogris and E. Sontag postulated that malfunction of PTPA could be causative for the observed PP2A deregulation in brains affected by AD.

To establish a possible functional link between PTPA activity and regulation of tau phosphorylation via PP2A, we selected the neuronal N2a cell line for PTPA loss-of-function approaches.

Successful knockdown of PTPA was demonstrated elsewhere (Fellner et al, 2003). We cloned this targeting sequence into an inducible expression vector and were able to reproduce PTPA repression. In addition two other newly designed targeting sequences also displayed significant levels of protein knockdown if transiently transfected.

To be able to perform complementation experiments we also established an RNAi-resistant form of PTPA, where single nucleotides of the coding sequence were substituted without changing the protein sequence.

The neuronal N2a cells bearing the tetracycline-sensitive repressor were firstly chosen as the parental cell line for knockdown experiments, but showed very high leaky expression and low inducibility (factor 2) in my hands. Thus, I used HEK293TRex as an additional model. Although this cell line is not of neuronal origin, it is nevertheless very instrumental due to observed high inducibility (factor 86) and absence of leakiness.

In a first attempt, I established 3 inducible knock-down N2a clones, able to suppress PTPA upon doxycycline treatment. This ability was lost over a period of 4 weeks, probably due to the presence of tetracycline in the FCS the medium was supplemented with. Tetracycline could activate the doxycycline-dependent expression of shRNA and thus the knock-down of PTPA constitutively, driving a selection for cells with decreased PTPA knock-down, e.g. by epigenetic silencing of the shRNA construct.

Indeed, we could show the presence of 10^{-10}µg/ml tetracycline in the FCS used. Using approved tetracycline-free FCS, we were able to generate three inducible PTPA knock-down clones that were stable over a period of at least three months.

Interestingly, 2 of these 3 clones exhibited a 2 to 3-fold increase of proliferation rate in the uninduced state, when compared to the parental cell line (data not shown), probably due to an additional genetic event favoring proliferation/survival. This could involve either random or insertional mutagenesis due to incorporation of the shRNA transgene. Although no such increase of proliferation was shown in our third clone, the possibility of an additional genetic event seems likely, given that in total only three out of 90 clones tested were positive for inducible PTPA knock-down. Maybe an additional genetic insult is needed for N2aTRex to tolerate PTPA knock-down.

Consistent with these observations, the yield of knockdown-proficient clones was much higher in HEK293TRex, which are devoid of leaky expression.

However, it is also likely, that the low percentage of PTPA knock-down clones in all stable neomycin-resistant clones is due to an experimental mistake , namely the co-transfection of these cells with the pCRUZ^{Neo}-GFP vector, initially used to sort transfected cells, because both vectors coded for a neomycin-resistance gene and therefore the shPTPA-expressing vector could be lost without losing the resistance and high ectopic GFP-expression levels have been shown to have a toxic effect on cells.

Therefore, the higher rate of HEK293TRex clones able to knock-down PTPA could be due to the fact, that these cells were not co-transfected with pCRUZ^{Neo}-GFP.

All three shPTPA clones based on the N2aTRex cell line were used to analyze proliferation rate, PP2A complex assembly and PP2A activity upon PTPA suppression.

I could not detect a significant reduction of proliferation upon PTPA knock-down, also PP2A complex assembly seemed not to be compromised. Moreover, knockdown of PTPA to half the protein amount did not result in altered PP2A activity, as determined by phosphatase assays. With a reduction of PTPA to 17% only a small decrease in phosphatase activity could be detected.

Taken together, this suggests that even low protein levels suffice to fulfill required functions.

Additional studies in other cell systems are warranted to further elucidate these questions. Based on this study, the cell line HEK293TRex seems to represent a very promising model system to address the regulation of PP2A by PTPA.

The role of PP2A regulatory subunit Bα in differentiation of neuronal cells

The Bα-containing heterotrimer ABαC is the major phosphatase for tau and therefore involved in maintaining microtubule integrity. In this manner, Bα could be important in neuronal differentiation in addition to its general “housekeeping” functions, which are evident from its ubiquitous spatiotemporal expression

In contrast to the A, C and Bα subunits, which show ubiquitous spatiotemporal expression patterns, the Bβ and Bγ subunits are differentially regulated. The latter two are detectable mainly in the brain and are subject to developmental regulation, with Bβ levels decreasing and Bγ increasing sharply after birth (Strack et al, 1998). This regulation suggests distinct functions of PP2A holoenzymes in brain development and functional maturation, depending on the included B subunit.

I was interested whether the ubiquitous Bα subunit plays a role in the process of functional differentiation, which goes beyond its “housekeeping” phosphatase functions

Indeed, I could demonstrate a role for the Bα subunit of PP2A in this process, as differentiation induced by serum-starvation or retinoic acid was markedly reduced after RNAi-mediated Bα-knockdown in N2a cells.

However, differentiation was reduced, but not abolished upon knockdown of Bα. This could be explained by functional compensation through Bβ and/or Bγ. In this respect, it is important to note, that Bγ was shown to co-localize with cytoskeletal substrates that are important in the establishment and maintenance of neuronal connections (Strack et al, 1998).

Although – as mentioned above - some regulatory B subunits are expressed in a tissue- and developmental stage-specific pattern, any given mammalian cell is believed to express several dozen distinct PP2A heterotrimers formed by the combination of the 20 PP2A subunit genes. This complexity presents a considerable challenge, particularly because functional redundancy can complicate the interpretation of genetic ablation experiments.

Further experiments using single and combined loss of different B subunit(s) are needed to conclude which one(s) are predominantly involved in neuronal differentiation during brain development.

Furthermore microtubule (MT) destabilization is linked with a substantial number of diseases, including Alzheimer´s disease (reviewed in Mandelkow et al., 2003). An involvement of PP2A in this process is indicated by the fact that PP2A directly binds to and regulates MT dynamics and that down regulation of PP2A enzymes containing Bα subunit occurs in Alzheimer´s disease (Sontag et al., 1995, 1996, 1999, 2004; Merrick et al., 1997; Hiraga and Tamura 2000).

Nunbhakdi-Craig et al. have found that silencing of the PP2A Bα subunit in N2a and NIH3T3 cells is sufficient to induce breakdown of acetylated and detyrosinated MTs (Nunbhaki-Craig et al., 2007). Our results provide evidence for the importance of Bα for differentiation of N2a cells. As axon growth critically depends on stable microtubule assembly, they also support the hypothesis that reduced amounts of neuronal Bα-containing PP2A heterotrimers could contribute to MT destabilization in Alzheimer´s disease.

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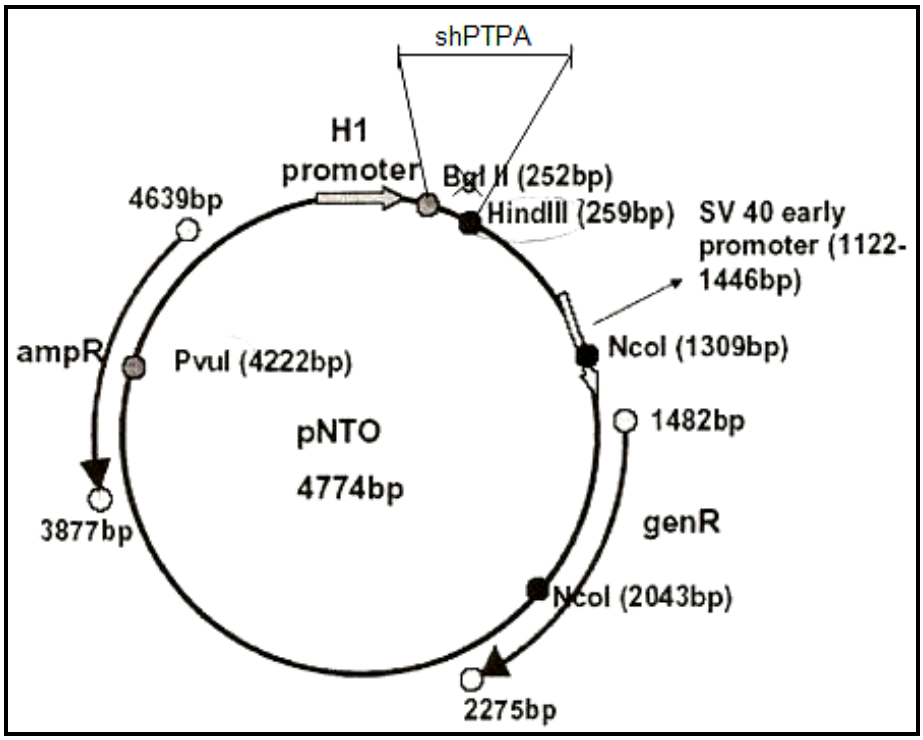
6. APPENDIX

pNTO^{Neo}-CT/CTMIS/1360/1360Mis/1798/1798MIS:



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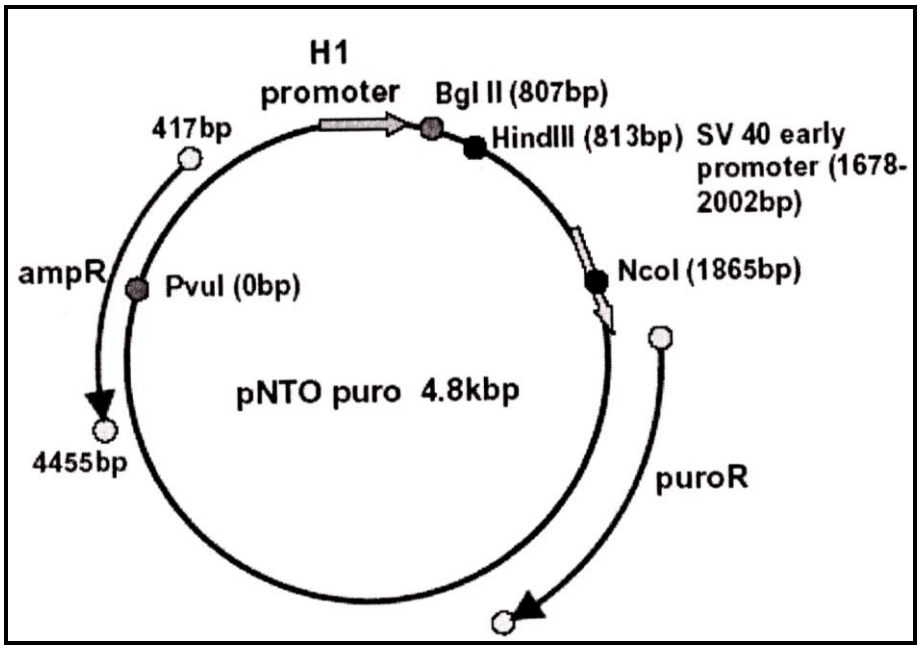

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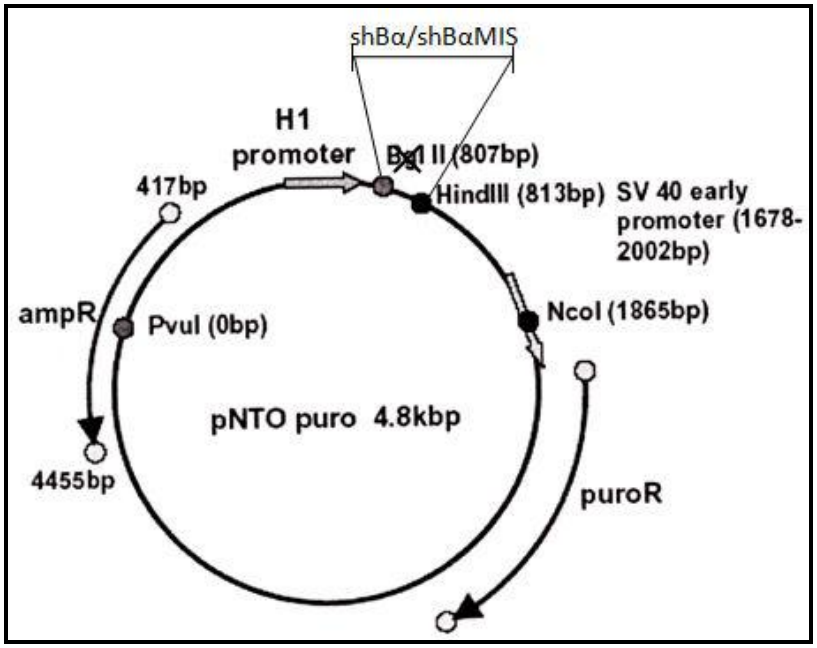
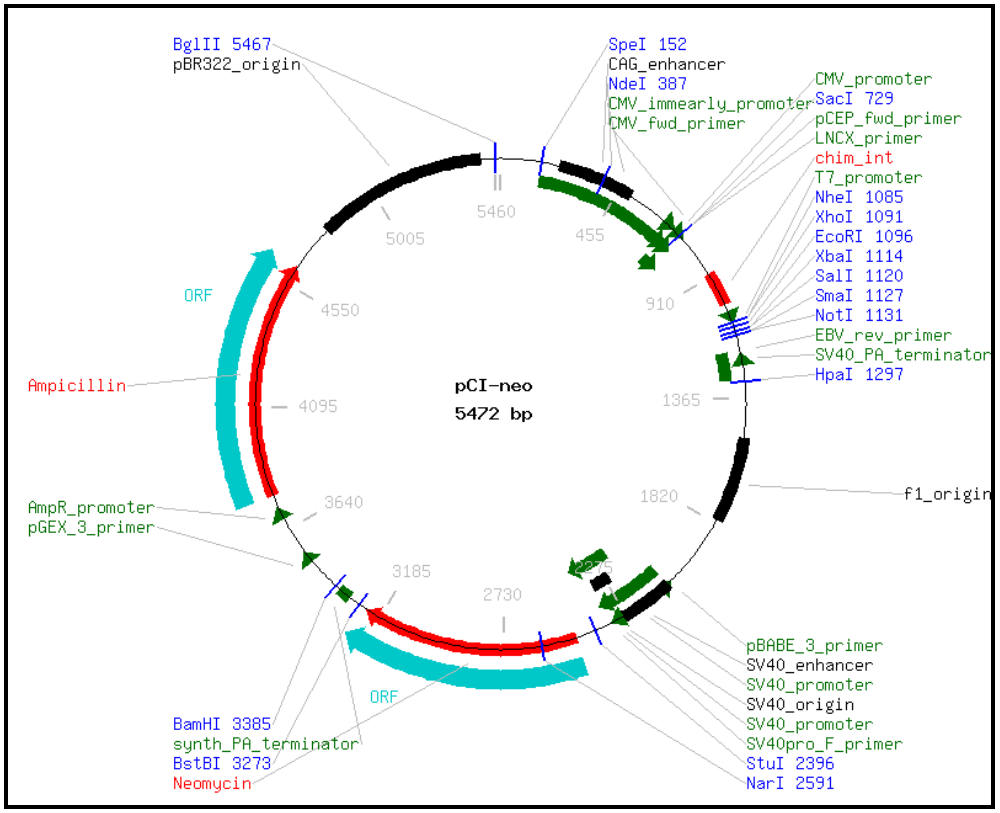
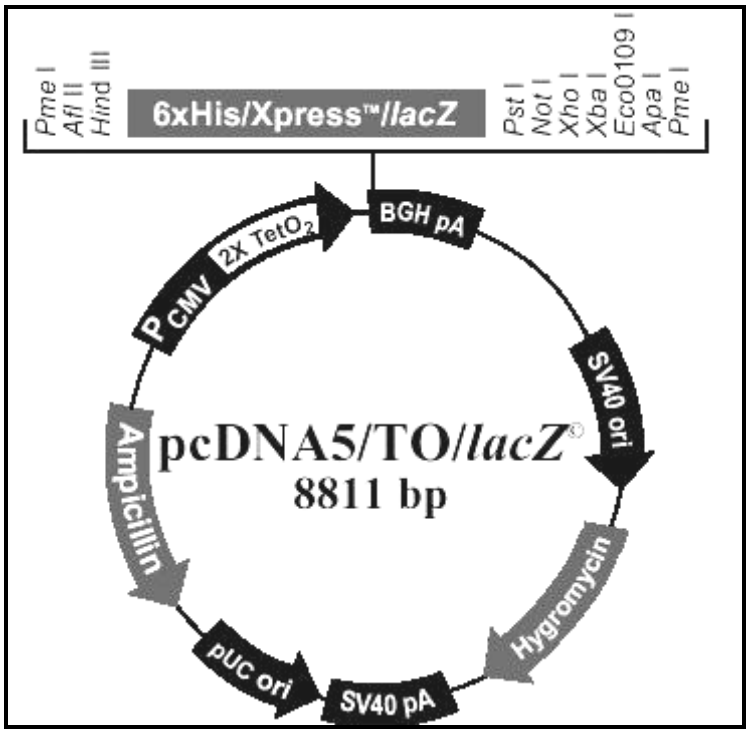


pCI^{Neo}:



pcDNA5/TO-LacZ: Invitrogen



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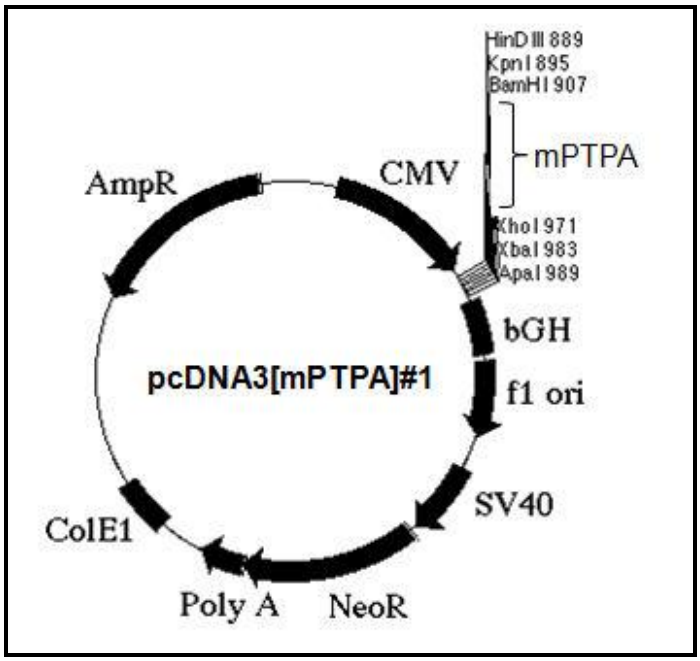
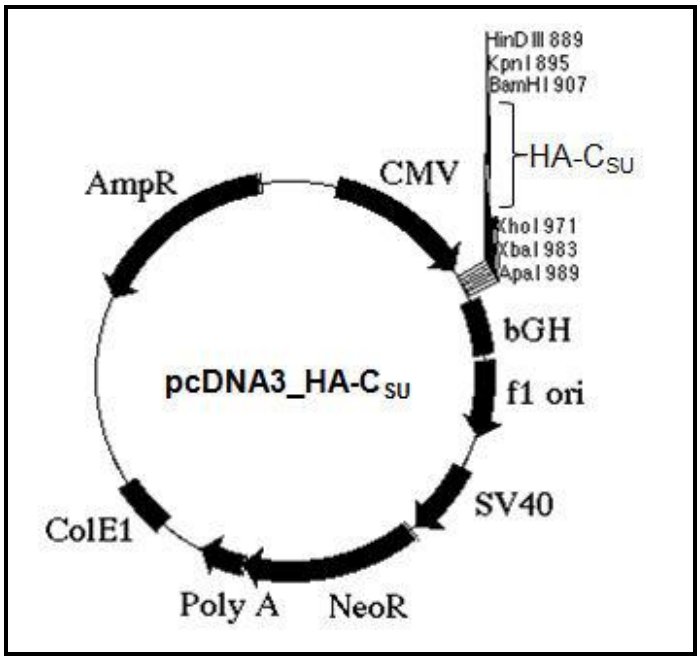


pcDNA3/HA-C_{SU}:



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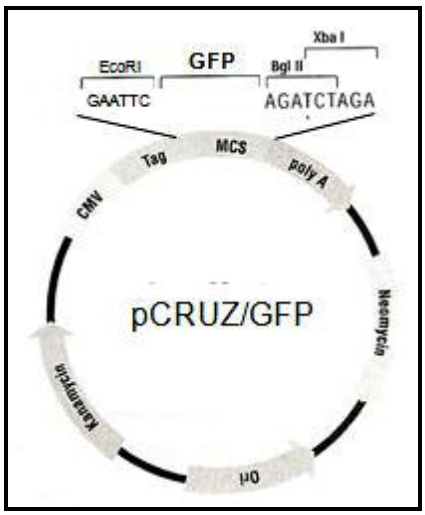

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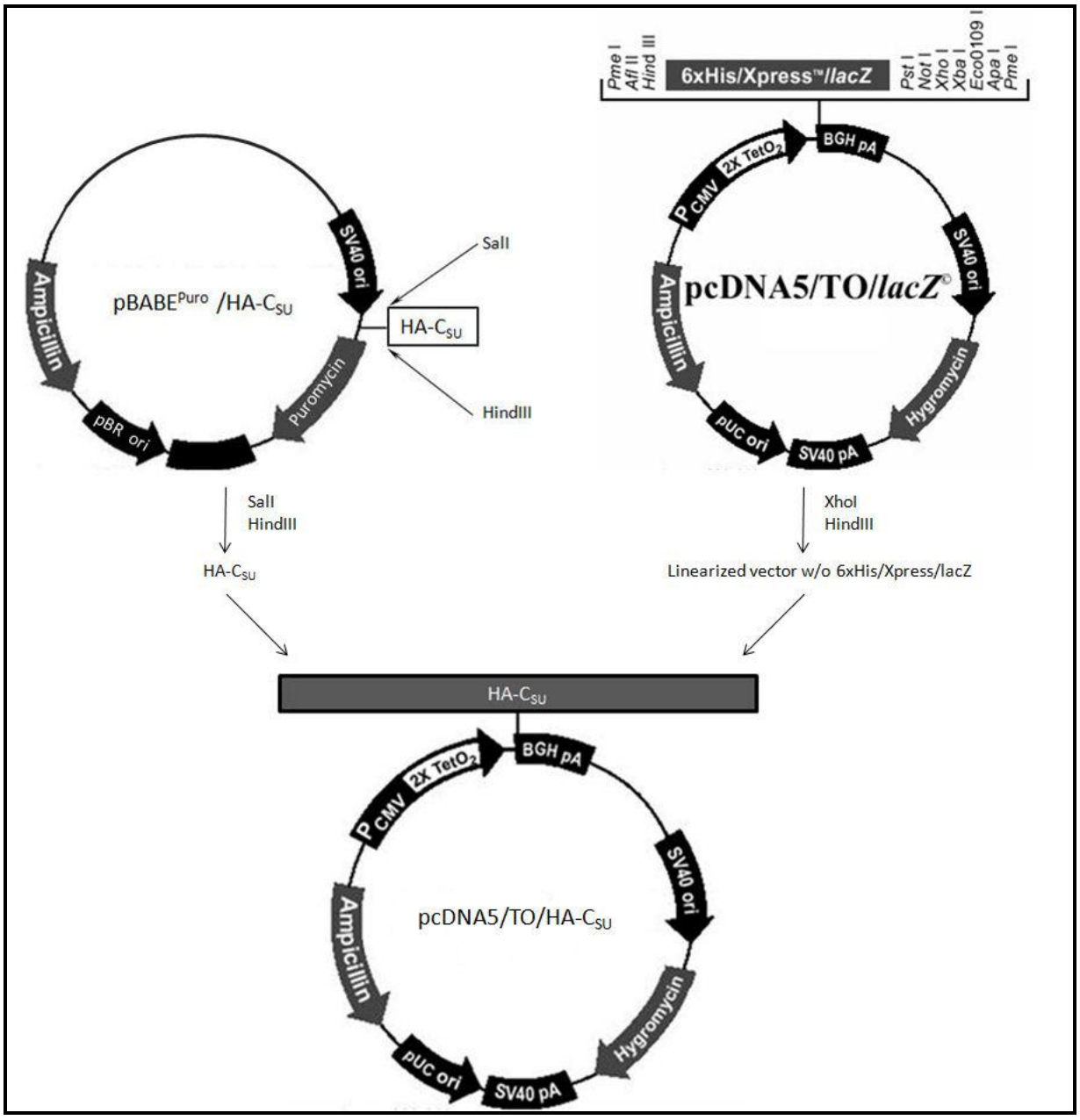

7. CURRICULUM VITAE

Personal Details

Name	Mitterhuber	
First name	Martina	
Sex	Female	
Nationality	Austria	
Date and place of birth	Sep 4, 1982	Eberstalzell, Austria
Address	Elinstrasse 4 A-4614 Marchtrenk	
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Education

2002-2008		Study of molecular biology at the University of Vienna, Austria
	Main subjects	cell biology, immunology
	Minor subjects	neurobiology
Diploma thesis		Lab Egon Ogris, Max F. Perutz Laboratories (MFPL), Vienna, Austria. "The Role of PTPA in the biogenesis of PP2A in mammalian cells"
1997 - 2002		Secondary College for Food technology and cereal industry

Professional training / Holiday courses

2007/03 -2008/04		Diploma thesis with Egon Ogris, Max. F Perutz Libraries (MFPL), Vienna, Austria
	Department	Cell signalling
	Supervisors	Prof. Egon Ogris, Dr. Stefan Schüchner
	Project	The Role of PTPA in the biogenesis of PP2A in mammalian cells
2007/01-2007/02		Internship at the University of Vienna
	Department	Immunology
	Supervisor	Prof. Egon Ogris
	Project	Generation of monoclonal antibodies specific for disease-causing mutant proteins

2006/07- 2006/09		Internship at the University of Vienna in the group of Prof. G. Wiche
	Department	Cell biology
	Supervisors	Dr. Konieczny Patryk
	Projects	Changes in protein expression underlying functional defects in desmin knockout mice
2006/01- 2006/02		Lab rotation in the Institute for Brain Research in Vienna
	Department	Neurobiology
	Supervisors	Dr. Johannes Berger
	Projects	General techniques in neuroscience
2005/07-2005/08		Internship at the General Hospital in Linz (AKH)
	Department	Molecular biology
	Supervisor	Univ-Doz. Dr. Jörg Berg
	Projects	Establishing a qPCR based method for quantifying the effect of chemotherapy on non-hodgkin lymphoma patients
2004/07-2004/08		Internship at the Wagner-Jauregg Hospital in Linz
	Department	Pathology
	Supervisor	Dr. Christine Webersinke
	Project	Generation of internal controls for the detection and identification of bacterial pathogens in human samples

relevant hands-on training in either molecular biology, cell biology, genetics, biochemistry, analytical and organic chemistry is furthermore contained in the syllabus of the study

Languages

German	Mother tongue
English	Fluent in spoken and written language
Spanish	Basic skills

Private interests

Travel, dance, music, literature