Interplay of PTEN subcellular localization and catalytic activities *in vivo*

Miriam Kristina Stumpf

"The beauty of a living thing is not the atoms that go into it,
but the way those atoms are put together."

- Carl Sagan, Cosmos -

For Enric

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Cover design by Miriam Stumpf: The "PTEN zipper" in angiogenesis: wild type catalytic activitiy and subcellular localization of PTEN is required for correct blood vessel formation in zebrafish. Functional hypo- or hyperactivity of PTEN induces hyperbranching or stalled vessel phenotypes, respectively.

Interplay of PTEN subcellular localization and catalytic activities *in vivo*

Samenspel van PTEN subcellulaire localisatie en katalytische activiteiten *in vivo*

(met een samenvatting in het Nederlands)

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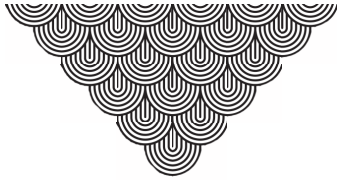

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Introduction to PTEN function in human health and disease

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This thesis focuses on PTEN (Phosphatase and Tensin homolog), an essential lipid- and protein dual-specificity phosphatase, the main antagonist of PI3K (Phosphoinositide3 -kinase) in higher eukaryotes. Phosphorylation, the covalent addition of a phosphoryl-group to a lipid or protein target molecule is catalyzed by kinases, while dephosphorylation, the hydrolysis of an added phosphate group, is catalyzed by phosphatases. Both reactions happen continuously in our body and in all other organisms, from prokaryotes to eukaryotes, in order to regulate the multitude of processes that are going on in (and in between) each living cell. In fact, transduction of external stimuli into an intracellular reaction via phosphorylation cascades is one of the oldest known concepts of molecular biology. It is most typically based on an extra- or intracellular ligand/receptor interaction, which activates a specific intracellular signaling pathway. Post-translational modification of proteins, such as phosphorylation, methylation, acetylation, oxidation, ubiquitination, SUMOylation, myristoylation or palmitoylation, plays an important role during signal transduction, since it is the perfect tool to reversibly change the conformation, subcellular localization, enzymatic activity and other characteristics of a protein and thereby convey a spatially and temporarily controlled message to the cell.

1. PTEN, the main suppressor of PI3K signaling

The PI3K/Akt(PKB) signaling pathway is stimulated by a diversity of growth factors and mitogens, such as insulin, IGF-1 (insulin like growth factor), EGF (epidermal growth factor), HGF (hepatocyte growth factor), PDGF (platelet derived growth factor), VEGF (vascular endothelial growth factor), GPCRs (G-protein coupled receptors) and the Raf/Mek/Map kinase pathway, all with the ultimate goal to enhance cell growth, survival and proliferation [1-9]. Upon activation of one of the before mentioned receptors or pathways, PI3K phosphorylates the phospholipid phosphatidylinositol(4,5)-bisphosphate (PIP2) to phosphatidylinositol(3,4,5)-trisphosphate (PIP3) [10]. The addition of a phosphoryl group at the D3 position of the inositol ring converts PIP2 into the second messenger molecule PIP3 [11], which recruits the downstream kinase Akt/Protein kinase B (PKB) to the cell membrane via its phospholipid binding pleckstrin homology (PH)-domain. Akt is subsequently phosphorylated on threonine 308 (T308) and serine 473 (S473) and thereby activated [2, 12-14]. Activated Akt, also termed p-Akt, phosphorylates a large number of downstream targets, with either inhibitory effects, for example on GSK3 (glycogensynthase-kinase3), FOXO (forkhead-box-protein O), BAD (Bcl-2-associated-death promotor), TSC2 (Tuberous Sclerosis Complex 2) [15-18] or in an activating manner, for example IKK-β (inhibitor of nuclear factor kappa-B kinase subunit beta) or SKP2 (S-phase kinase-associated protein 2) to stimulate cell proliferation and survival [19, 20].

Components of the PI3K/Akt(PKB)/PTEN signaling axis are frequently mutated in sporadic cancers, indicating the importance of a strict regulation of this signaling pathway in multicellular organisms [21]. The dual-specifity protein- and lipid- phosphatase PTEN is the major antagonist of PI3K/Akt(PKB) signaling, as it directly counteracts PI3K function at the cell membrane by dephosphorylating PIP3 back to PIP2 [22] (**Fig 1**).

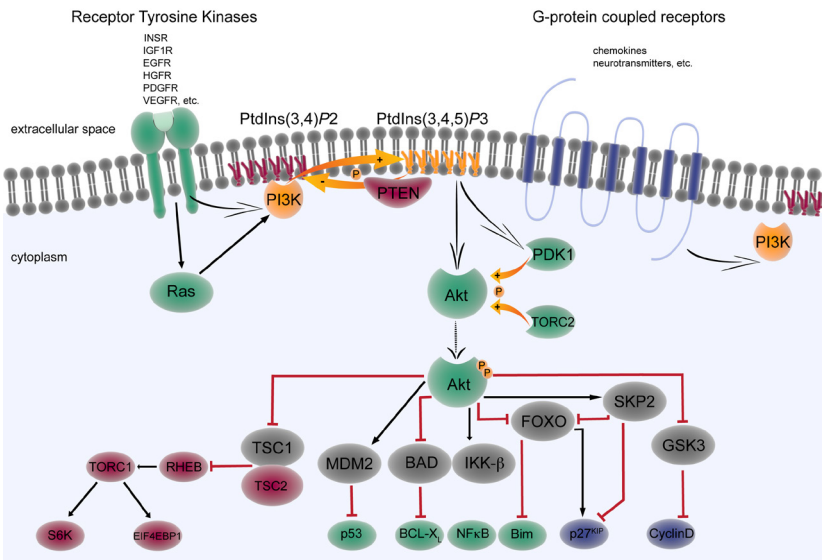

Fig 1. PTEN has a central role in negative regulation of PI3K signaling

PI3K is activated and recruited to the cell membrane upon activation of RTKs, the MAPK pathway and G-protein coupled receptors. Once activated, PI3K phosphorylates PIP2, converting it into the lipid second messenger PIP3. PTEN antagonizes PI3K signaling at this early step by dephosphorylating the newly generated PIP3 pool back to PIP2, thereby preventing all subsequent steps in the PI3K/Akt(PKB)-pathway. Akt binds to PIP3 via its PH – domain and the PIP3-enriched regions within the cell membrane further recruit PDK1, which phosphorylates Akt on Thr380 and partially activates Akt. Full activation of Akt occurs upon subsequent phosphorylation at Ser473 by TORC2. Activated Akt (p-Akt) phosphorylates a wide range of downstream targets (some examples are represented in this figure in grey). Akt activates MDM2, IKK-β and SKP2 while it suppresses TSC1, BAD, FOXO and GSK3. Taken together, Akt signaling affects many different cellular processes, such as cell survival, cell cycle, protein synthesis and others that are not represented in this figure, as for example cell metabolism, and thereby promotes cell survival and proliferation.

PTEN further exerts its role as a tumor suppressor by phosphatase-independent functions in the cytoplasm and the nucleus, by promoting DNA damage repair, genome stability, apoptosis, cell cycle progression or autophagy [23-29]. There exists a tight network of mechanisms that controls PTEN subcellular localization and activity according to the temporal and physiological context in which the cell or the whole organism is situated.

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2. PTEN protein structure and domains

The tertiary structure of PTEN is composed of various functional domains, beginning with the N-terminal lipid binding domain [30], which contains a PIP2-binding motif that partially overlaps with a putative noncanonical NLS (nuclear localization signal)- like sequence, followed by the phosphatase domain, which contains the PTP (protein tyrosine phosphatase) signature motif HCXXGXXR required for the protein's phosphatase activity, which is located at the bottom of the catalytic pocket. The protein's core region consists of a Ca^{2+}-independent C2 domain, which assists in positioning the PTP domain on the cell membrane by interaction with phospholipids within the lipid bilayer [31]. Finally, the C-terminal tail is subject to a number of post-translational modifications and contains two PEST homology domains and various caspase 3 cleavage sites, which makes the C-terminal tail a regulatory region of PTEN protein conformation status, protein stability and subcellular localization [32-37]. The C-terminal PDZ domain of PTEN faciliates protein-protein interactions with other PDZ domain containing proteins, for example with the scaffold protein MAGI-2 (membrane-associated guanylate kinase inverted 2), which enhances the PI3K antagonizing activity of PTEN [38-41], or with NHERF (Mammalian Na/H Exchange Regulatory Factor), which recruits PTEN to PDGFR (platelet derived growth factor receptor) to inhibit the activation of PI3K signaling [42]. A big portion of our knowledge on the tertiary structure of human PTEN today is based on the resolution of its 2.1 Å crystal structure bound to L(+)-tartrate in 1999 [31], which represented a hug leap in the emerging field of PTEN research. Not only did it reveal characterisitcs of the PTEN active site that distinguish it from the catalytic domain of all other DSP (dual specificity phosphatase) and PTP (protein tyrosine phosphatase) family members, it also led to the identification of the C2 domain and its role for PTEN membrane localization. Remarkably, some regions within the PTEN N- and C-terminus (amino acids 6-15 and 353-403) could not be resolved in the crystal structure because they are highly unstructured [31, 43-47] (**Fig 2**).

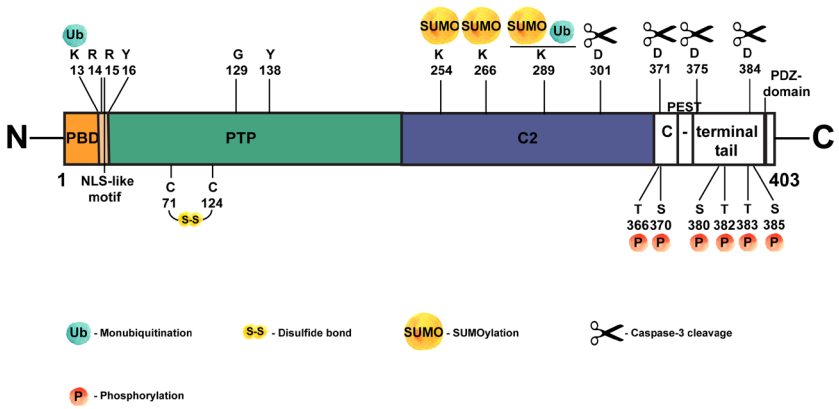

Fig 2. Post-translational modifications of PTEN
A schematic representation of the PTEN domain structure and the most relevant post-translational modifications for this thesis. The N-terminal PBD-domain harbors an NLS-like motif constituted of the key residues Lys13, Arg14, Arg15 and Tyr16, which is involved in the regulation of PTEN subcellular localization and catalytic activity. Monoubiquitination at Lys13 has been reported to play a special role in PTEN nuclear localization. The catalytic activity of PTEN crucially depends on the active site Cys124 in the PTP domain, which is regulated by reversible oxidation of PTEN. Upon oxidation (for example caused by reactive oxygen species (ROS)), a disulfide bond forms between Cys71 and Cys124 and leads to inactivation of PTEN phosphatase activity. The disulfide bound is reduced by thioredoxin (Trx) [111]. Cys124 and Gly129 are frequently found mutated in spontaneous cancer and PHTS patients. The mutations G129E and Y138L differentially affect lipid- and protein phosphatase activity of PTEN. While PTEN G129E only retains protein phosphatase activity, Y138L retains the capability to dephosphorylate phospholipids. The residues Lys254, Lys266 and Lys289 have been identified as SUMOylation sites that are involved in regulating PTEN subcellular localization. In addition, Lys289 has also been identified as a mono-ubiquitination site. Protein stability of PTEN is largely regulated by the C-terminal tail, which contains various PEST motifs and caspase-3 cleavage sites. Caspase-3 cleavage of PTEN at Asp301, Asp371, Asp375 and Asp384 occurs during apoptosis. Phosphorylation at the neighbouring CK2 phosphorylation sites Ser370 and Ser385 may be regulating this partial and putatively activating proteolysis of PTEN [33]. These and other C-terminal phosphorylation sites (Ser380, Thr382 and Thr383) are involved in regulating PTEN conformation and thereby its subcellular localization and catalytic activity. Thr366 and Ser370 further underlie regulation by autodephosphorylation and have recently been suggested to mediate an intermediate catalytic status of PTEN when dephosphorylated while the other four phosphorylation sites remain phosphorylated [47]. The C-terminal PDZ domain faciliates protein-protein interactions with other PDZ-domain containing proteins, such as MAGI-2.

3. Regulation of PTEN subcellular localization and phosphatase activity

In proliferating cells, PTEN localizes predominantly to the cytoplasm, where it can be found in its closed conformation, with the C-terminal tail folded back on the N-terminus and the core region [33, 47-50]. Nuclear localization of PTEN, in contrast, has been associated with differentiated, quiescent cells and, in line with these findings, absence of PTEN from the nucleus is frequently associated with advanced tumor stage in cancer patients [51-54]. It has been demonstrated that C-terminally dephosphorylated PTEN adopts an open conformation, which switches the protein's subcellular localization predominantly to the cell membrane and the nucleus [24, 37] (**Fig 3**). Whereas the mechanism of PTEN membrane localization [37] has been unraveled, it is still unknown how PTEN translocation to the nucleus occurs. Various models, ranging from passive diffusion [55], to import via MVP (major vault-protein) [56], and Ran-GTPase-dependent import [24] have been suggested. The mechanism(s) employed for PTEN translocation to the nucleus might be context-dependent and need to be further determined in the future. Apart from the known PIP3- and Akt- antagonizing role, PTEN has been shown to exert some of its cytoplasmic and nuclear functions independently from its lipid phosphatase activity, especially those functions involved in suppressing cell migration and invasion [57-61]. It has in fact been reported that the nuclear PIP3-pool is insensitive to dephosphorylation by PTEN [62], suggesting that PTEN in the nucleus exerts its functions mainly through protein-protein interactions, for example with p53 (cellular tumor antigen p53) [63] or CENP-C (Centromere protein C) [25], or possibly via its protein-phosphatase activity, as for example by direct dephosphorylation of the tran-

scription factor CREB (Cyclic AMP-responsive element-binding protein 1) [64]. Recent studies suggest, though, that PTEN could be its own main protein target but the identity of other suggested protein substrates of PTEN remains to be determined definitively [61, 65]. Autodephosphorylation of PTEN on threonine 366 (T366), however, seems to be yet another mechanism to enhance its catalytic activity towards phospholipids [61].

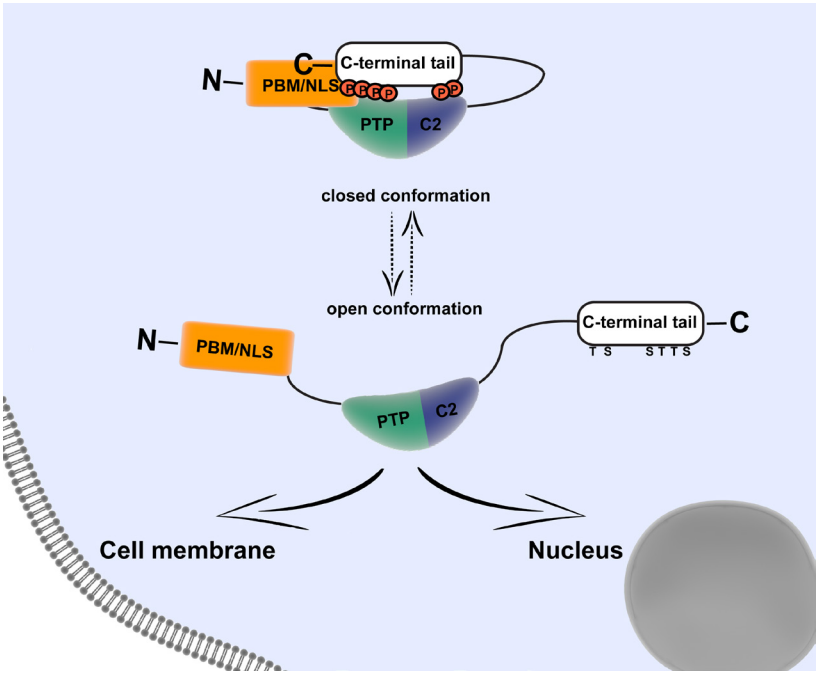

Fig 3. PTEN conformation determines its subcellular localization and catalytic activity

PTEN in the cytoplasm exists predominantly in its closed conformation, in which the six C-terminal phosphorylation sites are phosphorylated and mediate the interaction with basic amino acid stretches on the PTEN core region and the N-terminus. As a consequence, the C-terminal tail is folded back onto the PTP and C2 domains and the N-terminus and blocks any possible interaction of PTEN with the cell membrane or its substrates. PTEN in its closed conformation is completely inactive. Partial dephosphorylation, at Thr366 and S370, has been recently suggested to partially open the conformation of PTEN and thereby make the catalytic site accessible for soluble substrates [47]. Complete dephosphorylation of the C-terminal tail renders open conformation PTEN, which preferentially localizes to the cell membrane and the nucleus, where it excerts its full spectrum of catalytic activities.

Overall, the regulation of PTEN subcellular localization is clearly a key mechanism to control its variety of cellular functions. Among the post-translational modifications not only phosphorylation, but also monoubiquitination, SUMOylation and oxidation have been suggested to influence PTEN localization [35, 36, 66-68]. Monoubiquitination on two lysines, K13 and K289, has been reported to enhance nuclear localization of PTEN [66]. Mutational studies, however, could only confirm this observation for K13, not for K289 [50].

Polyubiquitination on the other hand, leads to degradation of PTEN in the proteasome [69, 70] or to drastically decreased PTEN phosphatase activity, in case of atypical poly-ubiquitination by RFP (E3 ubiquitin-protein ligase TRIM13) [71]. Nuclear accumulation of PTEN has been further associated with its SUMOylation status on Lys254, Lys266 and Lys289 [35, 36].

Another important modification involved in controlling PTEN transcriptional levels is methylation. Hypermethylation of CpG islands, and thereby inactivation of the PTEN promotor, is a frequently observed event in tumors with apparantly intact *pten* alleles [72-77]. Additionally, a vast network of microRNAs is involved in controlling PTEN protein translation[78].

4. PTEN in human development, germline mutations of *PTEN* and PHTS

The human *PTEN* gene, localized on chromosome 10q23.3, spans 9 exons that code for a 403 amino acid long gene product [79] which is frequently mutated or deleted in spontaneous cancers and in cancer susceptibility syndromes. Pten loss of hetero-zygosity (LOH) in mice results in enhanced tumor formation [80] and it has been further reported that already small alterations in gene dosage result in haploinsufficiency [81]. A complete lack of Pten, in fact, is incompatible with life in higher eukaryotes, such as mice [82-84], *C. elegans* [22, 85-87] or zebrafish [88] and the embryos or larvae, respectively, die at early developmental stages. Germline mutations of *PTEN* cause PTEN hamartoma tumor syndrome (PHTS), a disease spectrum that includes the autosomal dominant Cowden syndrome (CS), a syndrome with an estimated incidence of about 1:200.000, which is characterized by multiple hamartomas and a high risk of breast (25-50%), thyroid (10%) and endometrial (5-10%) cancers throughout the patients lifespan and Bannayan-Riley-Ruvalcaba syndrome (BRRS), an autosomal dominant syndrome which typically becomes manifest in macrocephaly, mental retardation, lipomatosis, haemangiomas and speckled penis. *PTEN* germline mutations also frequently underlie Proteus-like syndromes which are characterized by congenital malformations, hamartomatous overgrowth of multiple tissues, connective tissue and epidermal naevi, and hyperostoses. Hamartomas are benign tumors that are characterized by an imbalance in the proportion of individual cell types within a tissue [89]. Tissues that are especially affected by germline *PTEN* mutations are the central nervous system, the thyroid gland and the skin. Both benign hamartomas as well as an increased incidence of sporadic cancers have been reported for these tissues in PHTS patients. Arterious-venous malformations are commonly found in BRRS, especially often in Proteus-like syndrome and occasionally in CS patients. Kidney malformations, such as horseshoe kidney and multiple ureters have been detected in CS patients and the increased incidence of renal clear cell carcinoma

further confirms a certain role of PTEN during kidney development [90]. PHTS is not considererd a genetically homogeneous disease, on the contrary, a variety of different mutations within the gene locus but also in the promotor region of *PTEN* have been identified in patients with a similar clinical presentation [91, 92]. Interestingly, so far only CS patients were found to harbour germline *PTEN* promotor mutations, while large chromosomal deletions or rearrangements involving *PTEN* have only been found associated with BRRS [89]. While *PTEN* germline mutations are found in about 80% of patients diagnosed with CS, further (additional) gene mutations, for example in *SDHB* (Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial), *SDHD* (Succinate dehydrogenase [ubiquinone] cytochrome b small subunit, mitochondrial), *AKT1*, *PI3KCA* (PI3K-alpha) or *KLLN* (Killin) can underlie the PHTS spectrum disorders, especially in Cowden syndrome like patients [91]. *PTEN* germline mutations have further been associated with autism spectrum disorders, where PTEN dysfunction affects neuron soma size, length and density of dendritic spines and thereby brain plasticity [93-95].

5. Spontaneus mutations of *PTEN* and cancer

PTEN is one of the most frequently mutated tumor suppressor genes in human cancer and can be found partially or totally lost in a broad spectrum of tumors, including glioblastomas, endometrial and prostate cancer [96, 97]. In a few cancer types, however, mutation of *PTEN* is a rather uncommon event, for example in tumors of the upper gastrointestinal tract, lung or liver cancer [90]. Interestingly, there further exist tissues that are frequently affected by *PTEN* mutations in spontaneous cancer, for example the CNS (glioblastoma multiforme) or the blood (T cell acute leukemia), but that do not show a higher cancer susceptibility in PHTS patients. This phenomenon could be indicative of differential tissue-specific or developmentally regulated functions of PTEN. Based on studies in glioblastomas, it was initially suggested that *PTEN* mutation is predominantly associated with advanced tumor stages. Anyhow, it has been demonstrated in subsequent studies of endometrial and other types of cancer, that *PTEN* mutations can be detected as early as in pre-neoplastic lesions [98-100]. It is not surprising to find *PTEN* mutations across all the different tumor stages, taking into account the variety of functions that PTEN exerts to prevent tumor formation, growth and spreading, from inhibition of cell proliferation (for example by suppressing membrane PIP3 levels [101, 102] or by inducing G1 cell cycle arrest by decreasing CDK2 levels [103, 104]) to inhibition of angiogenesis (by decreasing stromal VEGFA levels [105, 106]). Homozygous loss of *PTEN*, however, is embronically lethal [82] and acute loss of PTEN function in otherwise healthy tissue induces cell senescence due to an innate failsafe mechanism via activation of the p53/p21^{CIP} pathway [107, 108].

Therefore, complete loss of PTEN is not initially selected for during tumorigenensis, unless there already exist mutations in other oncogenes or tumor suppressor genes that allow the cell to circumvent permanent cell cycle arrest. That is also why loss of heterozygosity (LOH) can be much more frequently found than loss of both copies of *PTEN* and why PTEN expression and function is usually only partially affected in tumor tissue [109]. In fact, heterozygous expression of a wild type Pten allele with a phosphatase inactive Pten allele (*Pten*$^{+/C124S}$ or *Pten*$^{+/G129E}$) in mice rendered similar but more severe tumor spectra in the affected animals in comparison to mice with only one intact Pten allele (*Pten*$^{+/-}$). The dominant-negative effect of the phosphatase inactive Pten mutants is likely exerted by Pten heterodimerization [110]. These findings illustrate the importance of understanding the molecular consequences of certain cancer-associated mutations *in vivo* and may help to find new personalized treatment and prevention strategies for patients affected by *PTEN* spontaneous or germline mutations.

6. Outline of this thesis

The work described in this thesis focuses on the mechanisms and the relevance of PTEN subcellular localization for the regulation of its lipid- and protein phosphatase activity during zebrafish embryonic development.

Chapter 1 provides a general introduction to signal transduction, the PI3K/Akt (PKB)/PTEN axis, PTEN phosphatase-dependent and –independent functions and regulation of those functions through changes in protein conformation and subcellular localization. Last, we give a short résumé on the role of PTEN in human health and disease.

In **Chapter 2** we describe the role of Pten in zebrafish cancer and development. To this end, we give an overview of developmental and pathological processes that have been successfully studied in zebrafish *pten* models. Further, we introduce techniques that we and others have developed for modulation of Pten expression *in vivo* and for the establishment of zebrafish cell lines from tumors. The chapter concludes with an outlook on what we can expect to learn about the function of Pten from using the zebrafish model system.

Chapter 3 comprises a comprehensive mutational and functional analysis of the PTEN N-terminus, including a panel of tumor-related mutations in this region, employing *S. cerevisiae* and mammalian cells as model systems. In this study we identify categories of PTEN N-terminal mutations with distinct PIP3 phosphatase and nuclear accumulation properties and provide the basis for a systematic analysis of tumor-related and experimentally engineered PTEN mutations.

In **Chapter 4** we describe the identification of importin alpha3 as a factor involved in PTEN nuclear translocation by immunofluorescence and confocal microscopy performed in mammalian cells. Systematic introduction of point mutations in the importin

alpha 3 substrate binding pockets and subsequent co-expression with PTEN allowed us to further narrow down the region of importin alpha 3 that is important for nuclear accumulation of PTEN to the minor binding pocket.

In **Chapter 5** we unveil a differential requirement of Pten lipid and protein phosphatase activity during zebrafish development. Using *pten* zebrafish lines, we performed rescue assays and screened the Pten lipid phosphatase inactive mutant Ptenb G129E and the protein phosphatase inactive mutant Ptenb Y138L via confocal microscopy and immunoblotting for their capacity to prevent the typical angiogenesis and pleiotropic phenotype of *pten* double homozygous embryos when microinjected at the one-cell stage. We propose that the role of Pten during angiogenesis mainly consists of suppressing PI3K signaling via its lipid phosphatase activity, whereas the complex process of embryonic development requires lipid and protein phosphatase activity of Pten.

In **Chapter 6** we characterize open conformation PTEN, its subcellular localization and function, *in vivo* during zebrafish development. We report that open conformation PTEN, PTEN QMA, shows enhanced localization to both the cell membrane and the nucleus and we present a new Pten-associated vascular phenotype, termed stalled vessel phenotype, that is likely caused by a gain of function of PTEN QMA, conferred by its enhanced recruitment to the cell membrane. The findings of this chapter demonstrate the requirement of tightly regulated and equilibrated Pten activity during zebrafish development and angiogenesis.

Finally, **Chapter 7** provides a summarizing discussion on the work presented here and its implications for future research.

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2

Pten function in zebrafish: Anything but a fish story

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Abstract

Zebrafish is an excellent model system for the analysis of gene function. We and others use zebrafish to investigate the function of the tumor suppressor, Pten, in tumorigenesis and embryonic development. Zebrafish have two *pten* genes, *ptena* and *ptenb*. The recently identified N-terminal extension of human PTEN that may facilitate cell membrane transfer, appears not to be conserved in zebrafish Ptena or Ptenb. Mutants that retain a single wild type *pten* allele develop tumors, predominantly hemangiosarcomas. Homozygous double mutants are embryonic lethal. Zebrafish embryos lacking functional Pten display enhanced proliferation of endothelial cells, resulting in hyperbranching of blood vessels. In addition, *ptena*–/– *ptenb*–/– mutant embryos display enhanced proliferation of hematopoietic stem and progenitor cells and concomitant arrest of differentiation, although Pten-deficient cells commit to all blood cell lineages. Zebrafish is an ideal model for intravital imaging and future work using *ptena*–/–*ptenb*–/– mutants will enhance our understanding of the function of Pten *in vivo*.

1. Introduction

PTEN is one of the most frequently mutated tumor suppressor genes in human cancer [1,2]. Nonsense and missense mutations in *PTEN* have been identified in many different types of cancers [3–5] and additionally, missense mutations have been identified in rare human autosomal dominant cancer syndromes known as Cowden's disease, Bannayan–Zonana and Lhermitte–Duclose disease [6–8]. Reduction of PTEN activity in mouse by as little as 20% already results in increased tumor susceptibility [9], indicating that PTEN function is crucial for homeostasis.

Whereas PTEN has many different functions in cell signaling at distinct subcellular locations – at the cell membrane or in the nucleus – and even outside the cell (this issue of Methods), PTEN is best known for its lipid phosphatase activity. PTEN dephosphorylates phosphatidylinositol(3,4,5)trisphosphate (PI(3,4,5)P3) and is selective for the 3-position [10,11]. Hence, PTEN counteracts catalytic activity of phosphatidylinositol-3-kinase (PI3K) and PTEN is an antagonist of PI3K signaling (**Fig 1**).

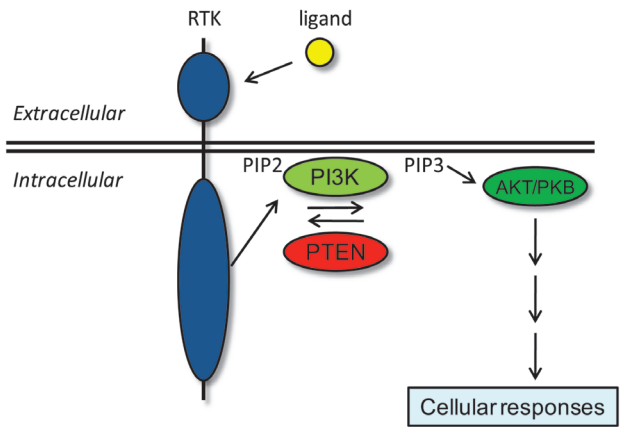

Fig 1. PTEN antagonizes PI3K/Akt (PKB) signaling.
Simplified scheme of PTEN signaling. Ligand binding to its receptor leads to receptor activation and subsequently to PI3K activation, resulting in ehanced levels of phosphatidylinositol(3,4,5)-trisphosphate (PIP3) and activation of downstream signaling via Akt.
PTEN dephosphorylates PIP3 and loss of PTEN in cancer results in hyperactivation of the signaling pathway.

Inactivation of PTEN by mutation results in activation of downstream signaling, which is commonly reflected by phosphorylation and thus activation of Akt, which is also known as PKB [12,13]. Further downstream signaling results in enhanced cell survival and proliferation, which is consistent with *PTEN* being a tumor suppressor gene.

PTEN is expressed ubiquitously and mouse knockouts lacking functional PTEN are embryonic lethal around day 8.5 of embryogenesis [14–16], indicating that PTEN is essential for normal development. Likewise, genetic mutants have been generated in other model organisms, including *Caenorhabditis elegans* and *Drosophila*, that have helped to delineate the function of Pten in development and disease [17,18].

We use zebrafish, *Danio rerio*, to investigate the function of Pten. Zebrafish is a model that facilitates experimental approaches to investigate gene function, because of intrinsic properties, including relatively short generation time, high fecundity, transparency of the embryos, rapid embryonic development in aqueous medium, easy delivery of chemical compounds and circumstantial advantages, including availability of the genome sequence and availability of transgenic indicator lines expressing fluorescent proteins under the control of tissue-specific promoters [19].
Here, we will review the role of Pten in zebrafish cancer and development and we will provide an outlook on what we can expect to learn about the function of Pten from the zebrafish model system.

2. *Pten* gene and Pten protein

The zebrafish genome encodes two *pten* genes, *ptena* and *ptenb*, that are both highly homologous to human *PTEN* [20,21]. The alignment of Ptena and Ptenb with human PTEN illustrates that the three protein sequences are highly homologous throughout [21]. The phosphatase domain and the tensin homology domain contain only a few amino acid substitutions. The tensin homology domain in Ptena contains an additional loop that is 21 and 20 residues longer than in human PTEN and Ptenb, respectively, in an unstructured region of the protein [22]. Moreover, splice variants of both genes have been reported, giving rise to an additional 23 residues in the tensin homology domain of Ptena and Ptenb, which appears not to interfere with Pten function [23]. Importantly, Ptena and Ptenb display catalytic activity with a preference for the 3-position of PI(3,4,5)P3, like human PTEN [21]. Recently, it was discovered that human PTEN dimerizes [24]. Which regions of PTEN and which amino acid residues are essential for dimerization remain to be determined. Given the high sequence conservation between Ptena and Ptenb, it is highly likely that Ptena and Ptenb homo- and heterodimerize. Future research will have to establish whether or not homo- or heterodimerization occurs and whether this affects Pten function in zebrafish.
A long form of human PTEN was identified with a 173 amino acid N-terminal extension, resulting from an alternate translation initiation site [25–27]. This long form of PTEN was recently dubbed PTEN-L to distinguish it from the PTEN protein that has already been studied for more than two decades [28]. The N-terminal extension of PTEN-L bestows it with the capacity to traverse the cell membrane [25] and/or to regulate mitochondrial function [27]. The N-terminal extension of PTEN-L is encoded by sequences immediately 5' to the start ATG in exon 1 of the *PTEN* transcript and makes use of an alternative CUG translation initiation site. This sequence is conserved in mammals [25]. Translation of genomic sequences to the 5'-side of the start ATG of *PTEN* of a

selection of species until the first in-frame stop codon confirmed that mammalian sequences are highly conserved. The first in-frame stop is localized at 196–237 amino acids from the start ATG and all mammalian species contain the alternative CUG translation initiation site in frame with the start ATG (**Fig 2**).

The 5' sequence of the lizard *Anolis carolinensis pten* encodes a stretch of 203 amino acids until the first stop codon, which is somewhat conserved, albeit sequenc conservation is not as high as between mammalian sequences. The N-terminal extension of *Xenopus laevis Pten* contains 73 amino acids in-frame upstream of the start ATG, which is poorly conserved. Whether reptiles and amphibians actually encode an N-terminal extension that functions like the extension in human PTEN-L remains to be determined. Sequences to the 5'-side of the start ATG of chicken (*Gallus gallus*) are missing in ENSEMBL (data not shown) and therefore it remains to be determined whether birds encode PTEN-L. Zebrafish *ptena* and *ptenb* encode short stretches of 5 and 35 amino acids, respectively, which are not conserved at all. Likewise, the *pten* genes of other fish species do not encode conserved sequences to the 5' side of the start ATG (**Fig 2**). Hence, we conclude that fish species do not express Pten-L. The lack of endogenous Pten-L may put the zebrafish model in a unique position to study the function of the N-terminal extension. For instance, comparison of ectopic expression of PTEN-L and PTEN-S in zebrafish may provide insight into functional differences that result from the presence or absence of the N-terminal extension.

3. Modulation of Pten expression

One of the great advantages of the zebrafish as a model system is the rapid modulation of target protein expression by morpholino-mediated knockdown [29]. Antisense morpholinos are designed, aimed at either the start ATG or splice sites. Morpholinos are extremely stable in cells due to their backbone, which contains a morpholine ring, making morpholinos resistant to cellular nucleases. Morpholinos are routinely microinjected into zebrafish embryos at the one-cell stage, resulting in protein knockdown for up to 4 days. Usually, protein knockdown is transient and incomplete, resulting in expression of some residual target protein. Moreover, morpholinos do not target existing protein, such as maternally deposited target protein.

Two non-overlapping morpholinos each have been designed to target the 5'UTR of Ptena and Ptenb [20]. Antisense-morpholino-mediated knockdown of Ptena or Ptenb led to enhanced Akt phosphorylation. Distinct developmental defects were observed in response to Ptena or Ptenb knockdown [20], which is surprising, given the largely overlapping expression pattern and the similarity in enzymatic activity of Ptena and Ptenb [21].

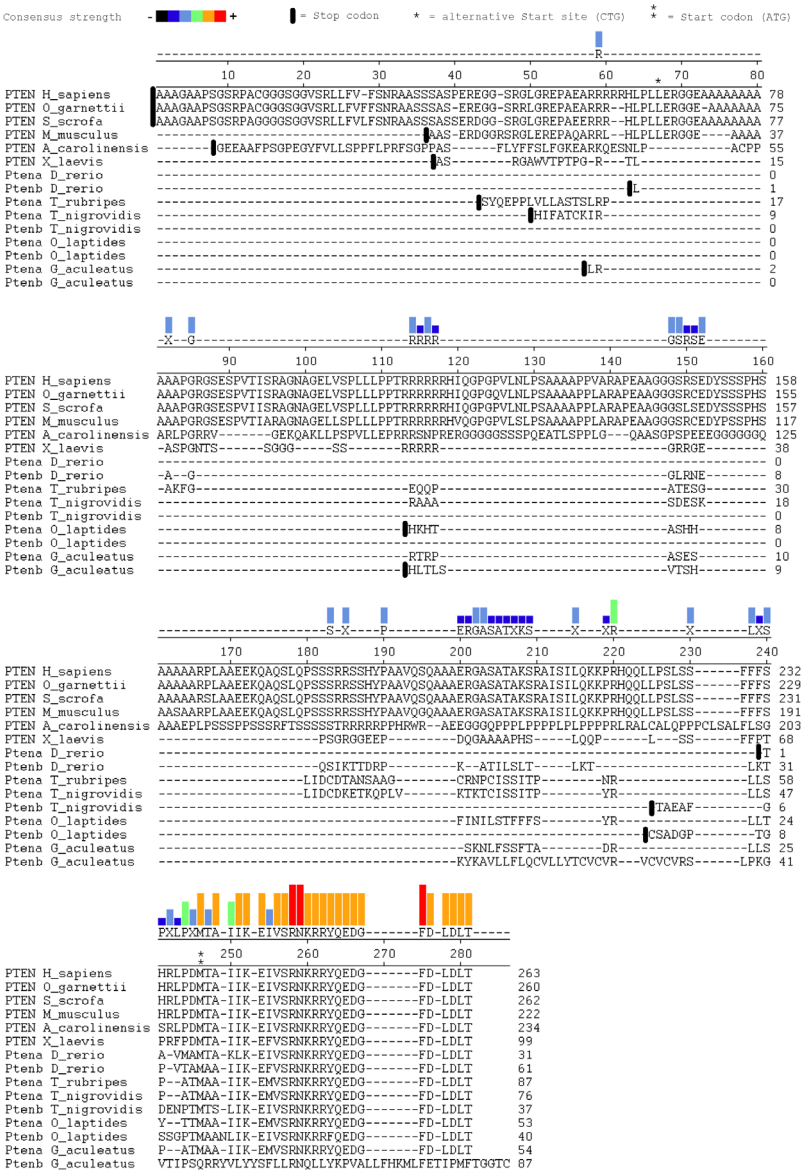

Fig 2. PTEN-Long is evolutionarily conserved among mammals but not teleosts.
Clustal-W alignment of the 5′ N-Terminal sequences until the first in-frame Stop codon (position in the sequence indicated by **I**) of Human PTEN (NM_000314.4) with genomic sequences from the Northern greater galago (*O. garnettii*), Pig (*S. scrofa*), Mouse (*M. musculus*), Lizard (*A. carolinensis*), Frog (*X. laevis*), Zebrafish (*D. rerio*), Fugu (*T. rubripes*), Green spotted puffer (*T. nigroviridis*), Mekada (*O. laptides*) and Stickleback (*G. aculeatus*). The consensus strength is represented by a color code as indicated in the figure (black for low homology, red for highest homology). All genomic sequences were obtained from the ENSEMBL database (www.ensembl.org). For translation into amino acid sequences, the Expasy Translate tool was used (http://web.expasy.org/translate)and the sequences were aligned with the MegAlign software from Lasergene. For Human PTEN, the mRNA sequence NM_000314.4 was used because the genomic sequence from the ENSEMBL database contains a frameshift at position 1592. The alternative CUG start codon is indicated with an asterisk (position 65 in human PTEN) and the ATG proper is indicated with two asterisks (position 238 in human PTEN).

We decided to isolate genetic mutants lacking functional Ptena and Ptenb. Target-selected gene inactivation [30] was used to identify *ptena* and *ptenb* nonsense mutations well upstream of the catalytic site cysteine. The mutants that were identified contain a mutation in exon 2 of *ptena* (Arg43 → stop) or in exon 3 of *ptenb* (Tyr65 → stop) [21]. Given their locations in the *pten* genes, these are considered to be null mutations. These mutants were bred to homozygosity. Homozygous *ptena*–/– mutants or *ptenb*–/– mutants are viable and fertile and the embryos do not display developmental defects, suggesting Ptena and Ptenb have redundant functions, which is in stark contrast to the morpholino data. More and more evidence is emerging that morpholinos may have off-target effects [31]. The standard in the field is that two non-overlapping morpholinos are used that induce similar phenotypes. The knockdown efficacy is monitored by assessment of splicing at the morpholino target site and/or target protein expression is assessed. By now, compelling evidence is available that despite all controls, the morpholino-induced developmental defects are not always reproduced in genetic mutants. Likewise, zebrafish lacking the function of a single *pten* gene, i.e. *ptena*–/– or *ptenb*–/– zebrafish are viable and fertile. We have maintained zebrafish lines with only a single wild type *pten* gene for at least ten generations without any noticeable developmental defects. Yet, morpholino-mediated knockdown of Ptena or Ptenb induced massive developmental defects [20]. Moreover, injection of the same Ptenb morpholino, targeting the 5'UTR, by itself induced gastrulation cell movement defects, which could be rescued by a PI3K inhibitor as well as by dominant negative Cdc42. Surprisingly, the Ptenb morpholino had the same effect in homozygous *ptenb*–/– embryos from an incross of homozygous *ptenb*–/– fish, lacking functional Ptenb [32]. The most logical explanation is that the Ptenb morpholinos target Ptenb as well as one or more unknown additional targets. Yet, formally it cannot be excluded that a truncated Ptenb protein is produced in *ptenb*–/– embryos that is knocked down by Ptenb morpholino, as concluded by *Yeh et al.* [32]. Ptenb knockdown using the same Ptenb morpholino was also reported to cause abnormal myeloid development [33]. Knockdown of Ptena, but not Ptenb, using distinct morpholinos targeting the start ATG resulted in enhanced thrombocyte count [34]. The reports about off-target effects of morpholinos and the lack of developmental defects in single *ptena*–/– or *ptenb*–/– mutants cast considerable doubt on the Pten morpholino results described above, and these knockdown results should be confirmed in *ptena*–/– or *ptenb*–/– mutants.

Whereas homozygous single *pten* mutants do not display developmental defects, homozygous *ptena*–/–*ptenb*–/– double mutants display pleiotropic defects that are associated with enhanced cell proliferation and survival. *Ptena*–/–*ptenb*–/– embryos die around 6 days post fertilization, indicating that Ptena and Ptenb indeed have redundant functions. In the single mutants, Ptena compensates for the loss of Ptenb

and vice versa, but embryos cannot survive without functional Pten [21]. Moreover, expression of Ptena or Ptenb by microinjection of synthetic mRNA encoding Ptena or Ptenb at the one-cell stage in *ptena*–/– *ptenb*–/– double mutants both rescue the developmental defects, indicating that Ptena and Ptenb have redundant functions ([35] and unpublished results). The observed developmental defects in *ptena*–/– *ptenb*–/– embryos are largely due to enhanced PI3K signaling, because inhibition of PI3K using LY294002 rescues the developmental defects to a large extent [21].

4. Tumorigenesis in Pten mutant zebrafish – hemangiosarcoma

Given the role of PTEN as a tumor suppressor in human cancer [1] and [2], and given that reduction of PTEN expression levels by as little as 20% enhances tumor susceptibility in mouse [9], it is not surprising that *pten* mutant zebrafish are more prone to cancer. However, enhanced tumor susceptibility only becomes evident upon loss of three of the four *pten* alleles, i.e. in *ptena*+/– *ptenb*–/– and *ptena*–/– *ptenb*+/– fish [36]. A cohort of 294 *ptena*+/– *ptenb*–/– fish were observed and 10% of these zebrafish with a single wild type *pten* allele developed a tumor within the first year of their life. These tumors occurred often, but not exclusively, close to the eye. The tumor-bearing fish were fixed, sectioned, mounted and stained using standard histopathological techniques. Pathological analysis and immunohistochemistry of sections using an endothelial marker (CD31) and a cell proliferation marker (PCNA) demonstrated that the tumors consist of an overgrowth of endothelial cells that form proper blood-filled vessels that are connected to blood circulation. Pathologically, these tumors were diagnosed as hemangiosarcomas, which is consistent with an enhanced incidence of hamartomas in human patients with germline *PTEN* mutations. Some tumors were excised and surprisingly, a single wild type *pten* allele was still detected in these tumors, indicating that tumor formation was not associated with loss-of-heterozygosity, but rather was due to haploinsufficiency. Not all *ptena*+/–*ptenb*–/– fish develop tumors. Apparently, additional factors are involved in tumor development. It is noteworthy that tumors often occur close to the eye, where the *rete mirabile* is localized, a meshwork of blood vessels. At this particular location, as a result of the abundance of blood vessels, it is not unlikely that substantial (vascular endothelial) growth factor signaling is occurring that may drive tumor formation in the event that three of the four *pten* alleles are lost.

Loss of tumor suppressor genes is often not sufficient to drive tumorigenesis. Additional events are required. Expression of oncogenic MYC in zebrafish induces T-cell acute lymphoblastic leukemia (T-ALL) [37]. Likewise, inducible expression of MYC induces T-ALL and removal of MYC leads to regression of tumors [38]. Inducible expression of MYC oncogene in *ptena*+/– *ptenb*+/– mutant zebrafish does not accelerate the onset of MYC-induced T-ALL. However, tumor regression upon removal of oncogenic MYC in

double heterozygous *ptena*+/– *ptenb*+/– fish is greatly reduced. Hence, mutation of *pten* promotes loss of MYC oncogene dependence in T-ALL.

5. Zebrafish cell lines

Zebrafish constitutes an excellent model system for *in vivo* analyses. Sometimes it is desirable to assess molecular biological or biochemical traits in an *in vitro* cell culture system, rather than in a whole organism *in vivo*. For this purpose, only a few stable zebrafish cell lines are available. Most human cell lines are derived from tumors. Tumorigenesis in haploinsufficient *pten* mutant zebrafish prompted us to develop a protocol for the establishment of zebrafish cell lines from tumors [39]. Crucial steps in the protocol are the isolation of the tumor, dissociation of the cells from the tumor, the composition of the medium and suppression of bacterial infection, particularly in the initial phase of establishing cell lines from zebrafish tumors. We have adapted the protocol to isolate stable cell lines from single zebrafish embryos, which will facilitate *in vitro* analysis of complex mutants (**Fig 3**). Using this protocol, we established several cell lines from tumors of *pten* haploinsufficient mutants. Interestingly, we confirmed the *ptena*+/– *ptenb*–/– genotype in the tumor-derived cell line, indicating that indeed tumor formation was not due to loss-of-heterozygosity, but rather was due to *pten* haploinsufficiency [36].

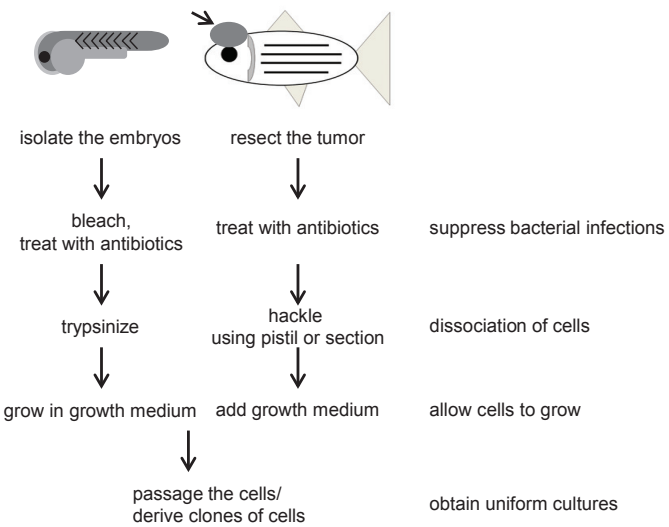

Fig 3. Outline of the protocol to derive cell lines from zebrafish tumors or single zebrafish embryos.
Crucial steps of the protocol are indicated with their purpose on the right. For details of the protocol, see [39].

6. Pten in angiogenesis

Zebrafish embryos lacking functional Pten display pleiotropic defects that are associated with enhanced proliferation and survival [21]. Since *pten* haploinsuffcient zebrafish develop hemangiosarcomas, consisting of blood-filled vessels, we investigated the vasculature in *ptena–/– ptenb–/–* embryos, because of its endothelial origin. Zebrafish are best used for their intravital imaging potential, as the embryos are transparent and develop rapidly. Transgenic lines are available that express fluorescent proteins in all endothelial cells. One of these lines, Tg(*kdrl:eGFP*) expresses Green Fluorescent Protein under the control of the *kdrl* promoter [40]. The entire vasculature is fluorescently labeled in these embryos and confocal time-lapse imaging allows assessment of vasculo- and angiogenesis in real-time. In *ptena–/–ptenb–/–* mutants, initial blood vessel formation occurs normally, but at 3 and 4dpf, excessive sprouting and filopodia formation are observed, resulting in hyperbranching. This is particularly obvious in the trunk region where intersegmental vessels display massive overgrowth (**Fig 4**) [35]. These defects are consistent with hemangiosarcomas in *pten* haploinsufficient adult zebrafish.
Interestingly, hyperbranching at 4dpf is rescued to a large extent by microinjection of synthetic mRNA encoding Ptena at the one-cell stage, by treatment of the embryos with PI3K inhibitor, LY294002, or with the VEGFR-selective inhibitor, Sunitinib. Surprisingly, *vegfaa* expression is highly upregulated in *ptena–/–ptenb–/–* embryos, which may explain why particularly endothelial cells are affected in these mutants. Moreover, *vegfaa* expression is upregulated in hemangiosarcomas in adult *ptena+/–ptenb–/–* haploinsufficient zebrafish, providing support for the hypothesis that Vegf signaling is enhanced in zebrafish with *pten* inactivating mutations, which may contribute to tumorigenesis in these fish.

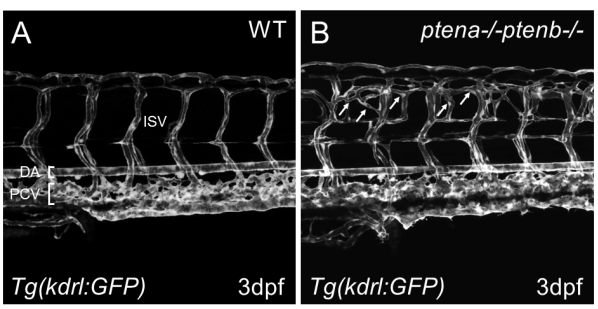

Fig 4. Hyperbranching of the vasculature in ptena–/– ptenb–/– zebrafish embryos. Tg(*kdrl:GFP*) embryos expressing GFP in all endothelial cells of the vasculature were imaged at 3dpf. An area of the trunk and tail region is shown here; anterior to the left, dorsal up. The dorsal aorta (DA), posterior cardinal vein (PCV) and one of the intersegmental vessels (ISV) are indicated. **(A)** Wild type and **(B)** ptena–/– ptenb–/–. Note the increase in blood vessels (hyperbranching) particularly in the dorsal side of *ptena–/–*ptenb–/– embryos (some of which are highlighted with arrows), compared to wild type, indicating hyperproliferation of endothelial cells.

7. Pten in hematopoiesis

PTEN is frequently found to be mutated in hematologic malignancies, including bone marrow failure and leukemia [41] and [42]. Zebrafish is an ideal system to investigate the early stages of hematopoiesis because zebrafish embryos can survive without circulating red blood cells until the larval stage (10–12 dpf). Therefore, we investigated hematopoiesis in zebrafish embryos lacking functional Pten, using molecular markers, (immuno)histological staining and transgenic lines. In zebrafish, Hematopoietic Stem and Progenitor Cells (HSPCs) emerge from the floor of the dorsal aorta and transiently colonize the caudal hematopoietic tissue (CHT) [43], [44] and [45]. These HSPCs expand and commit to most blood lineages, they differentiate and supply the developing embryos with mature blood cells. Subsequently, HSPCs migrate again to colonize the thymus and kidney marrow to produce blood cells for the duration of adult life. Hematopoiesis in mammals is similar in that HSPCs emerge from the aorta [46], transiently colonize the fetal liver and migrate to the thymus and bone marrow to produce definitive blood cells [47].

Using the *Tg(CD41-eGFP)* line, which expresses low levels of GFP in HSPCs and high levels of GFP in thrombocytes [48], we demonstrated that HSPCs colonize the CHT in *ptena–/– ptenb–/–* embryos [49]. Moreover, whole mount immunohistochemistry using phosphoHistone-3 specific antibodies indicates that HSPCs hyperproliferate in *ptena–/– ptenb–/–* embryos, which accounts for the enhanced number of HSPCs that is observed at 4dpf. A panel of markers indicated that the HSPCs engage in all blood lineages we tested, including thromboid, myeloid and lymphoid. However, *ptena–/– ptenb–/–* embryos do not contain fully mature blood cells, in that definitive blood cell markers are not expressed in these cells. Together, these results indicate that in embryos lacking Pten, HSPCs hyperproliferate and do not differentiate terminally, which is consistent with the loss of a tumor suppressor. Strikingly, inhibition of PI3K at late stages rescues the ability of *ptena–/– ptenb–/–* HSPCs to differentiate terminally.

Dong et al. [50] reported similar results, in that *ptena–/– ptenb–/–* embryos exhibit an increased number of myeloid cells, which however are immune deficient. Inhibition of PI3K corrects expansive myelopoiesis in the *ptena–/– ptenb–/–* embryos.

The function of Pten in zebrafish hematopoiesis is consistent with the role of PTEN in mammalian hematopoiesis. Conditional mouse knockouts have been generated in which functional PTEN is deleted in adult bone marrow cells or in fetal liver. In these mice, HSCs lacking PTEN are driven into the cell cycle, which results in depletion of HSCs. These conditional PTEN-deficient mice die of a myeloproliferative disorder that resembles acute myeloid/lymphoid leukemia. Hyperproliferation of HSPCs in the CHT of Pten mutant zebrafish is reminiscent of the expansion of bone marrow HSCs in conditional mouse models.

8. Outlook

Whereas we and others have addressed several important questions regarding Pten function using zebrafish embryos as a model, many more questions remain. Specific benefits of zebrafish as a model system should be exploited in full to obtain new insights into the function of Pten. We envisage particularly that intravital imaging of zebrafish (mutants) will lead to important new findings. Many transgenic lines have been derived with tissue- or cell type-specific expression of fluorescent proteins that will facilitate analysis of the function of Pten *in vivo*. Moreover, transgenic multi-color cell-tracing lines have been generated that will allow assessment of the clonality of Pten-deficient cells and tissues [51] and [52]. Another area that is relatively unexplored currently is the analysis of the effect of cancer drivers in Pten-deficient background. This will undoubtedly lead to the formation of tumors other than hemangiosarcomas and all the benefits of the zebrafish as a model will then facilitate the analysis of the underlying mechanism of tumorigenesis on the one hand and the cell biology of cancer, particularly with respect to stem cells, on the other.

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3

A functional dissection of PTEN N-Terminus: Implications in PTEN subcellular targeting and tumor suppressor activity

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Abstract

Spatial regulation of the tumor suppressor PTEN is exerted through alternative plasma membrane, cytoplasmic, and nuclear subcellular locations. The N-terminal region of PTEN is important for the control of PTEN subcellular localization and function. It contains both an active nuclear localization signal (NLS) and an overlapping PIP2-binding motif (PBM) involved in plasma membrane targeting. We report a comprehensive mutational and functional analysis of the PTEN N-terminus, including a panel of tumor-related mutations at this region. Nuclear/cytoplasmic partitioning in mammalian cells and PIP3 phosphatase assays in reconstituted *S. cerevisiae* defined categories of PTEN N-terminal mutations with distinct PIP3 phosphatase and nuclear accumulation properties. Noticeably, most tumor-related mutations that lost PIP3 phosphatase activity also displayed impaired nuclear localization. Cell proliferation and soft-agar colony formation analysis in mammalian cells of mutations with distinctive nuclear accumulation and catalytic activity patterns suggested a contribution of both properties to PTEN tumor suppressor activity. Our functional dissection of the PTEN N-terminus provides the basis for a systematic analysis of tumor-related and experimentally engineered PTEN mutations.

Introduction

The PI3K/PTEN/AKT pathway is involved in the etiology and progression of a wide variety of human tumors, on account of genetic and epigenetic alterations in the genes that govern the pathway, resulting in aberrant expression levels or activity of its components. In the case of PI3K and AKT, these include protein overexpression or hyperactivity, mainly due to gene amplification or gain-of-function mutations [1–3]. In the case of PTEN, about 30% of human tumors display protein downregulation or loss-of-activity, mainly due to total or partial gene loss or gene transcription, decreased mRNA or protein stability, and loss-of-function mutations [4–6]. Remarkably, the genes encoding p110α (*PIK3CA*, α catalytic subunit of PI3K), *AKT1*, and *PTEN*, are all targets for germline hereditary mutations in patients with PHTS (PTEN Hamartoma Tumor Syndrome) [7]. Thus, the PI3K/PTEN/AKT pathway has arisen as a major axis for therapeutic intervention in both sporadic and hereditary cancer [8]. The major role of PTEN as a potent tumor suppressor relies on its function as a lipid phosphatase, dephosphorylating the 3' position of phosphatidylinositol 3,4,5-trisphosphate (PIP3) to generate phosphatidylinositol 4,5-bisphosphate (PIP2), which directly antagonizes the oncogenic activity of PI3K [9,10]. The N-terminal portion of PTEN, together with the C-terminal phosphorylatable PTEN region, is crucial to exert its PIP3 phosphatase activity in cells, being involved in the dynamic regulation of the PTEN open/closed conformational status, which dictates PTEN subcellular localization, stability, and function [11–17]. C-terminal cleavage of PTEN by caspase-3 triggers PTEN nuclear accumulation in a manner dependent on Ran GTPase and a PTEN N-terminal nuclear localization signal (NLS; residues 8–32) [18,19]. PTEN N-terminus also harbours a PIP2-binding motif (PBM; residues 6–15) that mediates binding to membranes, as well as allosteric activation of the enzyme [20–23]. Interestingly, a cytoplasmic localization signal has also been described within this region (residues 19–25) [24]. Nuclear PTEN regulates genome stability, DNA repair, cell cycle, gene expression, and apoptosis [6,25–27], and the loss of PTEN nuclear localization associates in several cancers with tumour progression and poor clinical outcome [28,29]. However, most of PTEN nuclear functions rely on protein-protein interactions rather than on catalysis. Remarkably, monoubiquitylation of Lys13 at the NLS motif is required for PTEN nuclear entry [30], as well as for PTEN secretion via exosomes, a novel PTEN export pathway [31]. In addition, a novel PTEN isoform (PTEN-Long, PTENα; PTEN-L) has been discovered that displays an extended N-terminus. This longer PTEN form can be targeted to the mitochondria and can also be secreted and transferred, as a functional enzyme, from donor to acceptor cells, which is relevant for PTEN-mediated tumor suppression and potential PTEN-based antitumor therapies [32–34].

Here, we have performed a comprehensive functional analysis of the PTEN N-terminal

region (residues 2 to 43) using both a humanized *S. cerevisia*-based system and human cancer cell lines. We have uncovered the functional properties of tumor-associated mutations targeting the PTEN N-terminus, and functionally dissected this important region. Our results illustrate the multifaceted role of the PTEN N-terminus in the regulation of PTEN tumor suppression.

Results

Functional analysis of tumor-associated N-terminal PTEN mutations

In this study, we have monitored PTEN subcellular distribution and PIP3 phosphatase activity using in parallel mammalian and yeast cell systems (**Fig 1**). When overexpressed in COS-7 cells, wild type PTEN displays a cytosolic or a nuclear/cytosolic distribution; in contrast, the PTEN 1–375 truncation, which mimics a PTEN product of caspase-3 cleavage, accumulates in the nucleus in an N-terminus-NLS-dependent manner (**Fig 1A**) [18,19]. Heterologous galactose-inducible expression of p110α-CAAX (constitutively active, prenylatable form of the p110α catalytic subunit of PI3K) results in high toxicity in *S. cerevisiae* yeast cells as a result of the depletion of essential PIP2 pools. Co-expression of wild type PTEN restores PIP2 levels and suppresses PI3K toxicity. Conversion of PIP3 to PIP2 by PTEN can be monitored either by the suppression of PI3K-dependent yeast growth inhibition (**Fig 1B**) or by the relocation of a heterologous Akt reporter from the plasma membrane (**Fig 1C**). This allows for rapid and reliable assessment of PTEN activity *in vivo* [36,39,40].

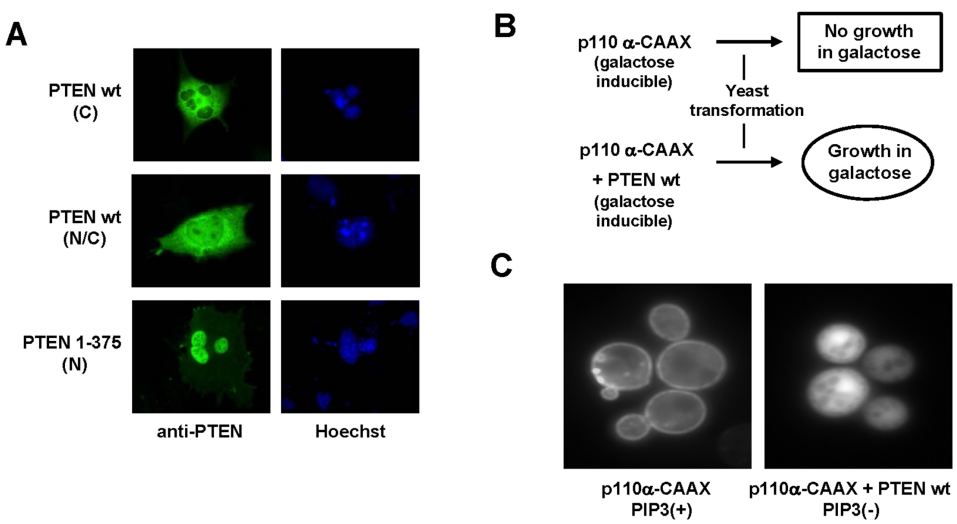

Fig 1. Depiction of the experimental systems used in this study
(A) Subcellular localization of PTEN in COS-7 cells. Ectopically expressed PTEN wild type (wt) mainly localizes in the cytoplasm (C) of COS-7 cells (60–70% of transfected cells; upper panels), and some cells show nuclear/cytoplasmic (N/C) localization (30–40% of transfected cells; middle panels). Cells were transfected with plasmids containing the indicated PTEN forms, and subjected to immunofluorescence using anti-PTEN antibodies (green) or Hoechst nuclear staining (blue). By contrast, PTEN 1–375 accumulates in the nucleus of COS-7 cells (80% of transfected cells; lower panels). This feature was used to test the influence of amino acid substitution mutations on PTEN nuclear accumulation. **(B)** Schematic showing the basis of the PTEN PIP3 functional assay in the yeast *S. cerevisiae*. Yeast cells expressing p110α-CAAX (a constitutive active form of the p110α catalytic subunit of mammalian PI3K under the control of GAL1 promoter) do not grow, and this can be reverted by expressing PTEN wild type. This feature was used to indirectly measure PTEN phosphatase activity, by reconstituting yeast viability. **(C)** Indirect measurement of PIP3 hydrolysis by PTEN in yeast is visualized by GFP-Akt1 reporter distribution. In the presence of p110α-CAAX alone (left panel), the GFP-Akt1 reported distributes in the plasma membrane as a result of PIP3 acccumulation [PIP3(+)], whereas in the presence of p110α–CAAX + PTEN wt, GFP-Akt1 displays a diffuse cytoplasmic distribution as a result of PIP3 hydrolysis [PIP3(-)].

The PTEN N-terminal region contains motifs important for PTEN subcellular localization and function (**Fig 2A**). To analyze the contribution of this region to PTEN tumor suppressor function *in vivo*, we performed a functional analysis of a panel of tumor-associated N-terminal PTEN mutations (COSMIC database; http://cancer.sanger.ac.uk/cosmic) (**Fig 2**; **Table 1**; **Table 2**). The nuclear/cytosolic localization of the mutations was tested in mammalian COS-7 cells. On a PTEN wild type background (1–403), which mostly displays cytoplasmic localization, the mutations L23F, M35R, and G36R showed increased nuclear localization (**Fig 2B**; **Table 1**).

Table 1. Subcellular localization of tumor-associated PTEN mutations motif on a PTEN 1–403 background.

	Subcellular localization (%)*		
Mutation	**N**	**C**	**N/C**
wt	0	67	33
S10N	0	72	28
K13E	0	70	30
R15I	0	68	32
R15S	0	92	8
Y16C	2	94	4
L23F	8	25	67
D24Y	0	61	39
I33S	3	70	27
A34D	0	83	17
A34V	0	74	26
M35R	34	2	64
G36R	47	7	46
L42P	0	88	12

*Percentage of COS-7 cells showing nuclear (N), cytoplasmic (C), or nuclear/cytoplasmic staining (N/C) is indicated. Note that, in the context of PTEN wild type (wt) 1–403, most of the mutations displayed predominant cytoplasmic localization. The mutations L23F, M35R, and G36R favored PTEN 1–403 nuclear accumulation. Mutations are from COSMIC database (http://cancer.sanger.ac.uk/cosmic)

In contrast, on a PTEN 1–375 background, a different pattern of PTEN nuclear/cytosolic partition in such mutations was observed (**Fig 2B** and **2C**; **Table 2**). The mutations K13E, A34D, and L42P, fully abrogated the nuclear accumulation of PTEN 1–375. A partial inhibition of nuclear accumulation was observed with the R15I, R15S, D24Y, I33S, M35R, and G36R mutations, whereas the nuclear/cytoplasmic distribution of S10N, Y16C, L23F, and A34V mutations was not significantly altered.

It is likely that changes in both nuclear active shuttling and binding to plasma mebrane account for the different subcellular distribution patterns of these PTEN mutations. Next, the PIP3 phosphatase activity of the tumor-associated N-terminal PTEN mutations was assessed using the *S. cerevisiae* heterologous reconstitution system. In these experiments, the mutations were tested in a PTEN 1–403 background, and the expression levels of all mutants were similar to the expression levels of wild type PTEN (**Fig 2E**), suggesting that the tested tumor-associated N-terminal PTEN mutations do not affect PTEN protein stability. As shown in **Fig 2D** and **2E**, and **Table 2**, the mutations K13E, R15I, R15S, D24Y, I33S, A34D, M35R, G36R, and L42P totally abrogated PTEN activity in the yeast model, whereas mutations S10N, Y16C, L23F, and A34V partially reduced PTEN activity. Interestingly, the latter tumor-associated mutations that did not strongly affect PTEN activity, did not significantly modify the PTEN 1–375 nuclear/cytosolic distribution either, pointing towards an overlapping tumor suppressor role of the PTEN N-terminus for its PIP3 catalytic activity and its nuclear localization *in vivo* (**Table 2**).

Table 2. PIP3 functional analysis and subcellular localization of tumor-associated PTEN mutations.

Mutation	PIP3 in vivo activity*	Subcellular localization†	Tumor type/disease#
wt 1–403	+	C	
wt 1–375	+	N	
S10N	+/-	N	LC,NHML,EC
K13E	-	C	*CS*,Glb,EC
R15I	-	N/C	Glb,EC
R15S	-	N/C	*CS*,*ASD*,Glb,CC
Y16C	+/-	N	Glb,NHML
L23F	+/-	N	Glb
D24Y	-	N/C or C	*BRR*,*CS*,EC,OC,BD
I33S	-	N/C	Glb,EC
A34D	-	C	*BRR*,EC
A34V	+/-	N	EC
M35R	-	N/C	*JPS*,Glb
G36R	-	N/C	*CS*,*LDD*,Glb,SS
L42P	-	C	Glb

* PIP3 *in vivo* activity in yeast was monitored by the reconstitution of the p110α-CAAX-induced lack-of-growth phenotype (+, reconstitution; +/-, partial reconstitution;-, no reconstitution). Data of mutations are on a PTEN 1–403 background, from Fig 2 and from [36].

† Major subcellular localization (N, nucleus; C, cytoplasm; N/C, nucleus/cytoplasm) was determined by immunofluorescence on mammalian COS-7 cells. Data of mutations are on a PTEN 1–375 background, from Fig 2.

From COSMIC (http://cancer.sanger.ac.uk/cosmic) and HGMD (http://www.hgmd.cf.ac.uk) databases. Abbreviations: BD, bladder cancer; BRR, Bannayan-Riley-Ruvalcaba syndrome; CC, colon carcinoma; CS, Cowden syndrome; EC, endometrial cancer; Glb, glioblastoma; JPS, juvenile polyposis syndrome; LC, lung carcinoma; LDD, Lhermitte-Duclos disease; NHML, non-Hodgkin's malignant lymphoma; OC, ovarian carcinoma; SC, stomach carcinoma; SS, synovial sarcoma. In italic, germ-line mutations.

Functional analysis of PTEN N-terminal region by comprehensive mutagenesis

To further dissect the involvement of PTEN N-terminal region in its function, we performed a full Ala-scanning mutagenesis of the PTEN region from residue 2 to residue 43 (Ala residues were mutated to Val). The mutated region spans the N-terminal PTEN tail and

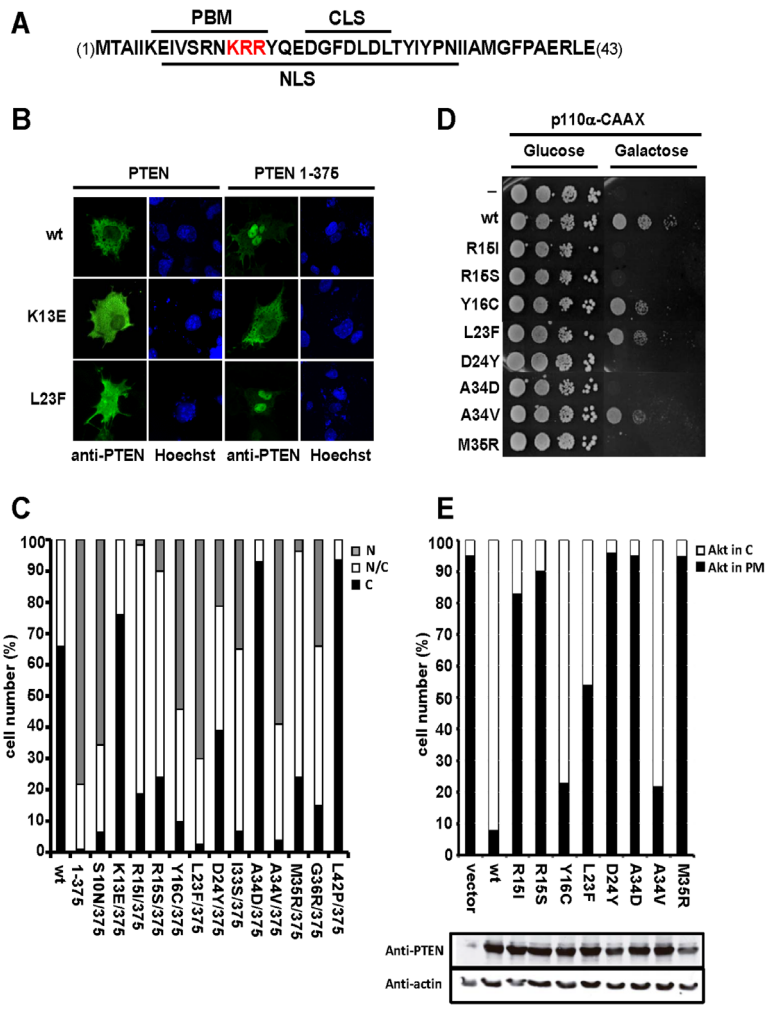

Fig 2. Subcellular localization and functional analysis of tumor-associated N-terminal PTEN mutations. (A) Amino acid sequence of PTEN N- terminus (residues 1–43). The nuclear localization signal (NLS), the PIP2-binding motif (PBM), and the cytoplasmic localization signal (CLS) are indicated. The KRR motif (residues 13–15) is in red. **(B, C)** The influence of tumor-associated PTEN mutations on PTEN subcellular localization was assessed by immunofluoresecence on mammalian COS-7 cells. In B, COS-7 cells transfected with wild type (wt; 1–403) or mutated PTEN (1–375, or mutations in a 1–375 background) were analysed by standard immunofluorescence microscopy using anti-PTEN 425A mAb (green). Nuclei were stained with Hoechst (blue). In C, the nuclear/cytoplasmic distribution of PTEN mutations was scored from COS-7 cells processed as in B; N, nuclear localization; C, cytoplasmic localization; N/C, nuclear/cytoplasmic localization. **(D, E)** The influence of tumor-associated PTEN mutations in the *in vivo* PTEN PIP3 phosphatase activity was assessed in yeast. In D, *S. cerevisiae* drop growth experiments were performed on co-transformants expressing p110α-CAAX and the indicated PTEN mutations, under repression (Glucose) or induction (Galactose) of the expression of the heterologous mammalian proteins. Expression of p110α-CAAX results in high toxicity in yeast cells as a result of the depletion of essential PIP2, which is converted to PIP3. Co-expression of wild type PTEN hydrolyses PIP3 and restores PIP2 levels and viability. In E, PIP3 hydrolysis by PTEN in yeast cells was monitored indirectly using a GFP-Akt1 reporter protein. Results are shown as percentage of yeast cells that display GFP-Akt1 at the plasma membrane (PM). The bottom panel shows the equivalent expression in the yeast of all PTEN mutations, as assessed by immunoblot using anti-PTEN and anti-actin (as a loading control) antibodies.

the three N-terminal β-strands at the PTP domain (**Fig 2A** and **Fig 3C**). Both subcellular localization and yeast reconstitution experiments were performed. Mutations at the basic residues of the N-terminus NLS region, including K13A, R14A, and R15A, as well as mutations E7A, Y16A, D24A, Y27A, I28A, P30A, N31A, I32A, I33A, and L42A, inhibited nuclear entry of PTEN 1–375, whereas mutations N12A, E18A, M35A, and A39V also inhibited nuclear accumulation but to a lesser extent (**Fig 3A**; and [18]). On the other hand, the cytoplasmic localization of PTEN 1–403 shifted partially to the nucleus in the mutations D19A, G20A, and F21A (**Table 3**), in consistence with a previously mapped cytoplasmic localization signal (CLS) at these residues [24]. Thus, several regions at the PTEN N-terminus are important for PTEN nuclear localization.

Regarding the PIP3 phosphatase activity of the PTEN mutations in the yeast *in vivo* assay, a set of mutations, including R15A, Y16A, D24A, I32A, M35A, P38A, and A39V, displayed complete loss-of-function, whereas the rest of the mutations partially compromised (A3V, I4A, I5A, K6A, S10A, Q17A, I33A, and L42A) or did not considerably affect PTEN activity on PIP3 in yeast (**Fig 3B**). The expression of all mutant versions was similar to the expression of wild type PTEN (**Fig 3B**). Therefore, many of the mutations differentially affected PTEN nuclear accumulation and PTEN phosphatase activity *in vivo*, suggesting a distinctive control of these two processes. Also, mutations R15A, Y16A, D24A, I32A, I33A, M35A, A39V, and L42A abrogated both nuclear accumulation and PIP3 phosphatase activity of PTEN, indicating that the relationship between these two properties extends beyond the NLS- and the PBM-residues (**Fig 3C**). Together, these results provide a map of the contribution of particular residues at distinct PTEN N-terminal regions to both subcellular localization and catalytic activity *in vivo*. PTEN N-terminal mutations can thus be categorized in three subgroups: a group affecting PIP3 phosphatase activity, a group involved in nuclear accumulation, and a third group involved in both phosphatase activity and nuclear localization.

Differential involvement of residues at the PTEN N-terminal KRR motif in PTEN phosphatase activity and subcellular localization

Since specific residues from the positively charged motif at the PTEN N-terminus NLS (Lys13Arg14Arg15; KRR motif) (**Fig 2A**) are important for nuclear localization or phosphatase activity *in vivo*, we performed additional amino acid substitutions in this motif aimed to change its charge and/or its bulk. As shown in **Fig 4A**, charge conservative replacement K13R and R14K mutations, as well as K13E mutation, affected the nuclear localization of PTEN 1–375. It should be mentioned that inhibition of nuclear accumulation on PTEN 1-375/K13R associated with increased plasma membrane localization, as evidenced by confocal microscopy (**Fig 4B**). On the other hand, mutation R15K did not inhibit PTEN 1–375 nuclear accumulation, and increased the nuclear

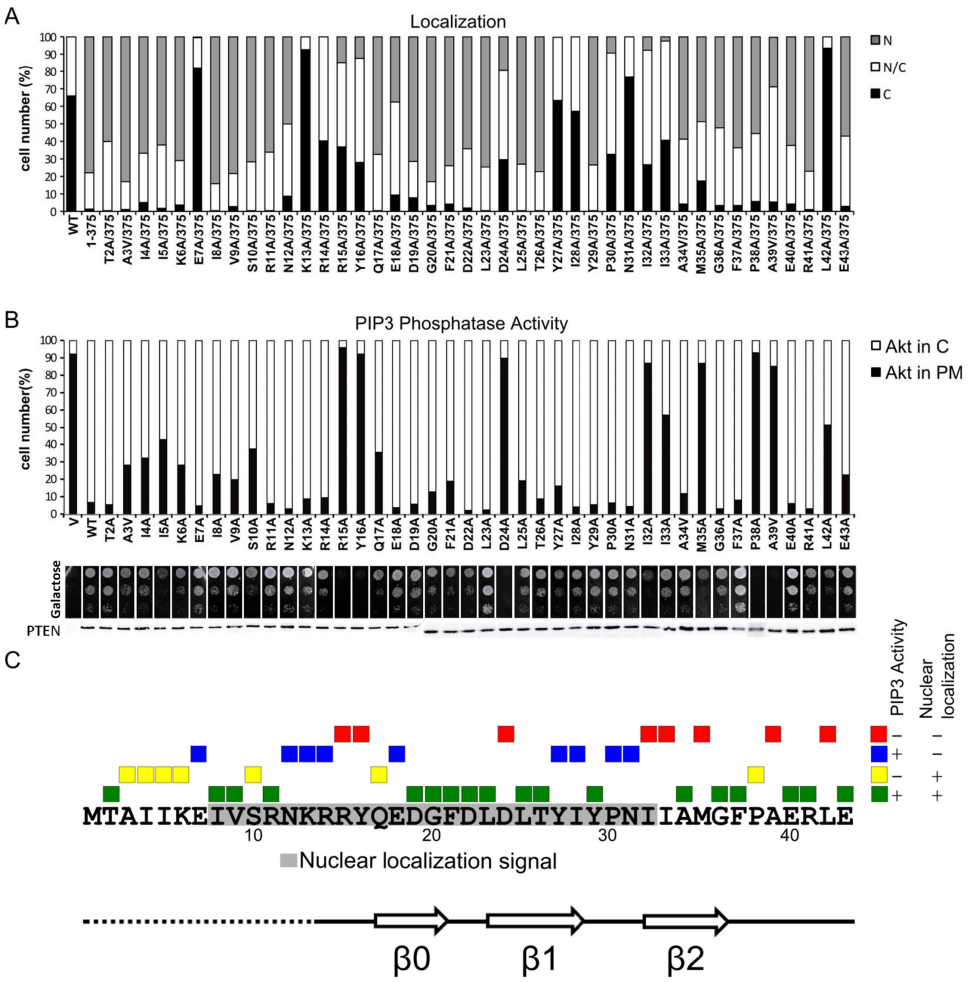

Fig 3. Subcellular localization and functional analysis by Ala-scanning of PTEN N-terminal region reveals distinct subgroups of PTEN mutations. (A) Nuclear/cytoplasmic distribution of PTEN N-terminal mutations, monitored as in Fig 2C. Nuclear/cytoplasmic distribution of mutations V9A to E18A is as in [18]. **(B)** Influence of PTEN N-terminal mutations in the *in vivo* PTEN PIP3 phosphatase activity, assessed in yeast. In the upper panel (bars graph), the PIP3 phosphatase activity of PTEN N-terminal mutations was monitored as in Fig 2E. In the middle panel (drop growth), growth was monitored as in Fig 2D. The activity of mutations K6A to E18A is as in [36]. In the bottom panel, the equivalent expression in the yeast of all PTEN mutations, as assessed by immunoblot using anti-PTEN antibodies, is shown. **(C)** Scheme summarizing the Ala-scanning functional results and the distinct subgroups of PTEN mutations. PTEN N-terminal amino acid sequence (residues 1–43) is shown, and the NLS (grey) motif is indicated. The functional consequences of each Ala- substitution are indicated with a colour code: green (+ +), no effect; yellow (- +), normal PIP3 phosphatase activity but impaired nuclear accumulation; blue (+ -), impaired phosphatase activity but normal nuclear accumulation; red (- -), impaired phosphatase activity and nuclear accumulation. For nuclear localization in the background of PTEN 1–375, we consider positive those mutants that showed over 50% of cells with nuclear localization. For *in vivo* activity on PIP3, we consider positive those mutants that showed under 30% of cells with Akt at the plasma membrane and significantly rescued p110α–induced growth inhibition. The first Met (M) was not mutated, and corresponds to PTEN wild type.

accumulation of PTEN 1–403 (**Fig 4A**, **Table 4**). Thus, Lys13 and Arg14, but not Arg15, are essential for PTEN nuclear accumulation. Combined mutations rendering either a KKK (R14K/R15K) or an AAA (K13A/R14A/R15A) motif were also excluded from the nucleus, whereas the mutation rendering a RAA (K13R/R14A/R15A) motif displayed a partial nuclear accumulation. In contrast to the results obtained for PTEN nuclear accumulation, the R15K mutation did not display phosphatase activity *in vivo*, whereas the activity of the K13R and R14K mutations was not diminished in copparison with wild type PTEN. In fact, the K13R mutation even displayed an increased ability to counteract PI3K activity in the yeast model. Finally, the activity of mutation K13E was totally absent (**Fig 4C**). The expression of all mutations at the KRR motif was similar to the expression of wild type PTEN (**Fig 4C**). These results confirm that Arg15, but not Lys13 or Arg14, is essential for the PIP3 phosphatase activity of PTEN *in vivo*. This is in agreement with the finding that Arg15 is critical for targeting PTEN to the plasma membrane [41]. Together, our results reveal a differential and complementary involvement of PTEN residues Lys13, Arg14, and Arg15 in PTEN nuclear localization and phosphatase activity in cells.

Functional analysis of PTEN N-terminal mutations in mammalian cells

To test the function on mammalian cells of PTEN N-terminal mutations displaying distinctive nuclear accumulation, stable human osteosarcoma U2OS Tet-Off cell lines expressing PTEN 1-375/L23F and 1-375/N31A mutations were generated, and cell proliferation and soft-agar focus formation assays were performed. As a comparison, cells expressing empty vector, PTEN wild type, or PTEN 1–375 were also analysed (**Fig 5A**). As shown above, the L23F mutation displays compromised/partial PIP3 catalytic activity in the yeast, without affecting the nuclear accumulation of PTEN 1–375 in mammalian cells. Conversely, the N31A mutation did not affect PIP3 catalytic activity in the yeast, but impaired the nuclear accumulation of PTEN 1–375 in mammalian cells (**Figs 2** and **3**). Cells expressing PTEN 1–375 grew slower on plastic and formed less colonies in soft agar than cells expressing PTEN wild type. Remarkably, cells expressing PTEN 1-375/L23F behaved in both assays as empty vector cells, indicating a complete loss-of-function of this mutation in mammalian cells in terms of cell growth inhibitory capacity. On the other hand, cells expressing PTEN 1-375/N31A behaved as PTEN wild type cells, formed less colonies in soft agar than cells expressing PTEN wild type, displaying better growing properties than PTEN 1/375 cells (**Fig 5B** and **5C**). These results support the notion that partial loss of PIP3 phosphatase activity of PTEN is enough to confer a loss-of-function phenotype in mammalian cells, and suggest that impairment of PTEN nuclear accumulation may affect the full capacity of PTEN to control cell growth.

Table 3. Subcellular localization of Ala-scanning PTEN mutations on a PTEN 1–403 background.

	Subcellular localization (%)*		
Mutation	**N**	**C**	**N/C**
T2A	0	65	35
A3V	0	60	40
I4A	9	71	21
I5A	0	71	30
K6A	0	68	32
E7A	1	82	18
I8A	0	72	28
V9A	0	65	35
S10A	0	69	31
R11A	0	54	46
N12A	0	71	29
K13A	0	90	10
R14A	0	81	19
R15A	0	74	26
Y16A	0	77	23
Q17A	0	80	20
E18A	0	75	25
D19A	17	12	71
G20A	47	3	50
F21A	61	2	37
D22A	9	66	25
L23A	0	61	39
D24A	6	75	19
L25A	2	64	34
T26A	0	77	23
Y27A	0	57	44
I28A	0	65	35
Y29A	0	64	36
P30A	0	68	33
N31A	0	50	50
I32A	0	63	37
I33A	3	70	27
A34V	0	74	26
M35A	0	70	30
G36A	0	64	36
F37A	0	69	31
P38A	1	70	29
A39V	0	55	45
E40A	0	65	35
R41A	0	70	30
L42A	0	78	22
E43A	0	71	29

*Percentage of COS-7 cells showing nuclear (N), cytoplasmic (C), or nuclear/cytoplasmic staining (N/C) is indicated. Note that, in the context of PTEN 1–403, most of the mutations did not affect the localization of PTEN wild type (wt), with the exception of some mutations in the cytoplasmic localization signal at residues 19–25, which favored PTEN 1–403 nuclear accumulation

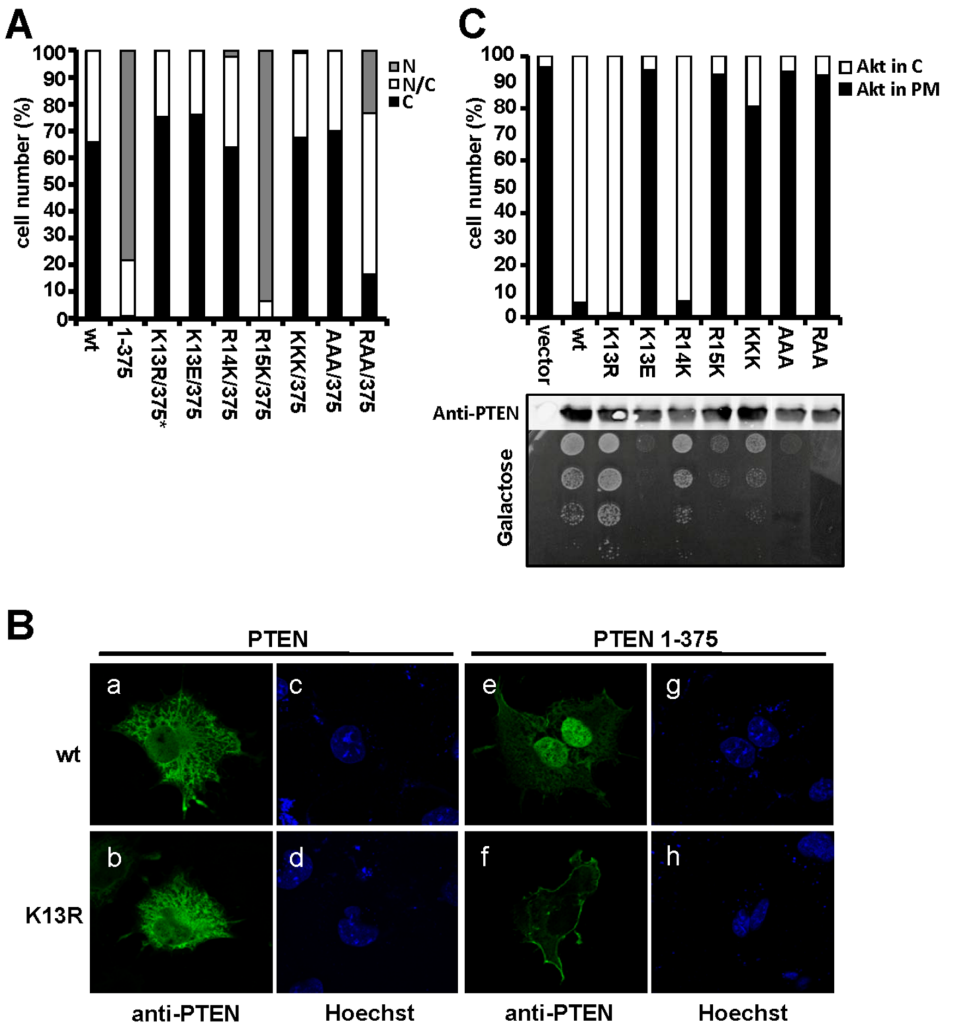

Fig 4. Subcellular localization and functional analysis of PTEN mutations at the N-terminal KRR motif.
(A) Arg13 and Lys14 are essential for nuclear localization. The nuclear/cytoplasmic distribution of PTEN mutations at the KRR motif was monitored by standard immunofluorescence microscopy, as in Fig 2C. *Note that standard microscopy does not provide information on plasma membrane localization for K13R (see B, panel f). KKK, R14K/R15K; AAA, K13A/R14A/R15A; RAA, K13R/R14A/R15A. **(B)** COS-7 cells transfected with PTEN wild type (wt) or mutations were analysed by confocal immunofluorescence microscopy using anti-PTEN 425A mAb (green). Nuclei were stained with Hoechst (blue). Note the plasma membrane staining on the PTEN 1–375/K13R mutation (panel f). **(C)** Arg15 is essential for PIP3 phosphatase activity. The influence of PTEN mutations at the KRR motif on the *in vivo* PTEN PIP3 phosphatase activity was assessed in yeast. In the upper panel (bar graph), the PIP3 phosphatase activity of PTEN mutations at the KRR motif was monitored as in Fig 2E. The middle panel shows the equivalent expression in the yeast of all PTEN mutations, as assessed by immunoblot using anti- PTEN antibodies. In the bottom panel (drop growth), growth was monitored as in Fig 2D.

Table 4. Subcellular localization of PTEN mutations at the N-terminal KRR motif on a PTEN 1–403 background.

	Subcellular localization (%)*		
Mutation	**N**	**C**	**N/C**
K13R	8	46	46
K13E	0	75	25
R14K	0	73	27
R15K	44	18	38
KKK	0	72	28
AAA	0	74	26
RAA	0	75	25

*Percentage of COS-7 cells showing nuclear (N), cytoplasmic (C), or nuclear/cytoplasmic staining (N/C) is indicated. KKK, R14K/R15K; AAA, K13A/R14A/R15A; RAA, K13R/R14A/R15A. Note that the mutation R15K favored PTEN 1–403 nuclear accumulation.

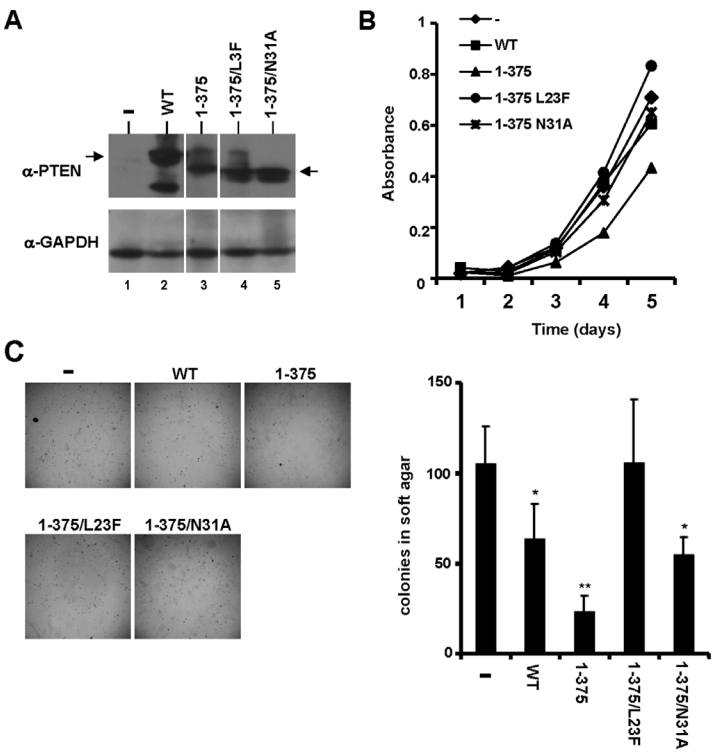

Fig 5. Functional analysis in mammalian cells of PTEN mutations displaying distinctive nuclear accumulation and PIP3 phosphatase activity. (A) The ectopic expression of PTEN wild type (WT) and mutations in U2OS clones was monitored by immunoblot with an anti-PTEN antibody (upper panel). The arrows indicate the migration of the full-length (residues 1–403) or the truncated (residues 1–375) recombinant PTEN. Expression of GAPDH is shown in the lower panel as a loading control. The figure shows non-adjacent bands from the same blot. **(B)** Proliferation of the distinct U2OS clones during 5 days of culture. Data are shown as the mean ± SD of the absorbance from three experiments, corrected for background.-, empty vector. **(C)** Growth in soft agar of the distinct U2OS clones. Cells were plated on soft agar, and pictures (X40 magnification) were made after 3–4 weeks of growth (upper part). The bottom graph shows the quantification of the number of colonies per plate for each clone. Data are shown as the mean ± SD from three independent experiments.** p<0.001, * p<0.05 with respect to PTEN wild type.-, empty vector.

Discussion

PTEN is the second most frequently mutated tumor suppressor gene in human cancers. Although tumor-associated gross mutations in *PTEN* abrogate all its tumor suppressor functions by means of complete loss of the PTEN protein, the phenotype caused by disease-linked *PTEN* point mutations is more variable. This could be relevant for the implementation of specific therapies and preventive measures to patients and affected families. For instance, most of the germline *PTEN* point mutations found in patients with PHTS abrogate PTEN PIP3-phosphatase activity. By contrast, some patients with autism spectrum disorders are also carriers of *PTEN* germline point mutations and such variants do not result, in general, in total loss of PTEN PIP3 phosphatase activity [42–44]. In addition to PTEN intrinsic catalysis, other biological properties of PTEN, including protein-protein interactions and subcellular localization, may be determinant for its lipid phosphatase activity and tumor suppression *in vivo*. This makes multifunctional analyses of the PTEN variants found in patients necessary.

The N-terminal region of PTEN contains overlapping NLS/PBM motifs that regulate PTEN subcellular localization and catalytic activity, and this region is targeted by tumor-associated mutations with high incidence (15% of *PTEN* total mutated samples target residues 1–40; about 40% of these being missense mutations; COSMIC database). Moreover, some *PTEN* tumor-associated mutations at PTEN N-terminus display a reduced ability to bind to the plasma membrane [45]. Our functional analysis of the PTEN N-terminus illustrates that tumor-associated mutations in this PTEN region are selected for loss-of-function, in terms of PIP3 phosphatase activity in a yeast-based *in vivo* setting and nuclear localization in mammalian cells, indicating the additive relevance of both properties in PTEN-mediated tumor suppression. This is in agreement with the distinct lipid phosphatase-independent tumor suppressor functions ascribed to nuclear PTEN [46–51]. By contrast, tumor-associated mutations targeting other PTEN regions, including the ATP-binding sites, show enhanced nuclear accumulation [52]. The lack of nuclear PTEN in many human cancers has been documented, and proposed as a factor of poor prognosis for some tumor types [27]. It would be interesting to contrast these findings on PTEN nuclear exclusion in human tumors with the analysis of the phosphatase activity of the non-nuclear remnant PTEN protein. The KRR residues 13–15 in the PTEN NLS/PBM, as well as the β2-sheet residues 31–36, are targeted in tumors for loss-of-function mutations (**Table 1**). The KRR residues are likely to mediate direct interactions with PIP2 or nuclear transporters, required for PTEN catalysis and nuclear accumulation [18,20,23,53]. On the other hand, the PTEN residues at the β2-sheet are highly hydrophobic and not solvent-exposed [54], suggesting that mutations at this region compromise functional PTEN local folding. In this regard, our Ala-scanning functional analysis suggests that the integrity of Lys13, Arg14, and residues 31–36, is

more relevant for nuclear accumulation than for PIP3 phosphatase activity *in vivo*. On the other hand, integrity of Lys15 and Tyr16 seems to be more important for PIP3 phosphatase activity than for nuclear accumulation. We have found several functional categories of PTEN mutations in our Ala-scanning analysis, including mutations that did neither affect nuclear accumulation nor PIP3 phosphatase activity (+ +), mutations that compromised both nuclear accumulation and PIP3 phosphatase activity (- -), and mutations that discriminated between these two functions (+ -) (- +) (**Fig 3C**). In conclusion, although PTEN-PIP2 binding and PTEN-nuclear transport mechanisms share recognition motifs, our results suggest the existence of specific molecular determinants for each of these two activities on the PTEN N-terminus. In line with this notion, the K13R mutation favored PTEN targeting to the plasma membrane in a nuclear-PTEN-mutation background (PTEN 1-375/ K13R), but not in the cytosolic wild type-PTEN background (PTEN K13R). A similar phenomenon has been found for other PTEN mutations, including mutations at the PTEN N-terminus, which increased their accumulation at the plasma membrane when combined with a PTEN C-terminal-phosphorylation defective mutation [41,42]. Interestingly, sandwiched between clusters of residues important for nuclear localization at the PTEN N-terminus, a cytoplasmic localization signal has been described at residues 19–25 [24]. Our finding that PTEN mutations in this region partially shift to the nucleus support the idea that it could behave similarly to the nuclear exclusion motifs defined in other PTEN regions [18]. This documents the complexity of PTEN N-terminus as a determinant to drive mechanisms of subcellular targeting.

We have tested in mammalian cells the tumor suppressor capacity of PTEN mutations displaying differential PIP3 phosphatase activity and nuclear accumulation properties. The caspase-3 mimicking PTEN C-terminal truncation, PTEN 1–375, which accumulates in the nucleus, displayed enhanced cell growth inhibition, when compared to PTEN wild type. Since the presence of an intact PTEN C-terminal tail keeps PTEN in a closed inactive conformation, which is neither competent to go to the nucleus nor to the plasma membrane [11,13,14,17], it is difficult to delimitate the contribution of the nuclear accumulation of PTEN 1–375 on its tumor suppressor activity. Our results using PTEN 1-375/L23F and 1-375/N31A mutations indicate that both the PTEN PIP3 phosphatase activity and the PTEN capacity to accumulate in the nucleus are important for PTEN tumor suppression.

Also of interest is our finding that Ala-substitution of residues at the non-crystalized disordered N-terminal PTEN region, including Ala3, Ile4, and Ile5, affects PTEN PIP3 phosphatase activity *in vivo*, suggesting the existence of additional PTEN regulatory elements in this region. This is sustained by the presence of PTEN mutations targeting these PTEN N-terminal residues in tumors (COSMIC database). Importantly, residues within the unique region of the PTEN longer isoform, contiguous to the region studied

here, are also targeted by mutations in tumors [32], although the intrinsic PIP3 catalytic activity of these mutations is not altered when assessed in the yeast [55]. Further molecular and *in vivo* analyses are necessary to fully understand the regulatory mechanisms imposed by the standard PTEN N-terminus, and by the adjacent residues from the PTEN longer isoform, to PTEN tumor suppressor function in mammalian cells. Our comprehensive functional analysis of PTEN N-terminus provides a foundation to understand and exploit the N-terminal properties of PTEN to intervene therapeutically in PTEN-mediated tumor suppression.

Materials and Methods

Cell culture, transfections and plasmids

Simian kidney COS-7 (ATCC CRL-1651) cells were grown in DMEM containing high glucose supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Human osteosarcoma U2OS-derived UTA6 Tet-Off cell line containing Tet-Off plasmid was provided by R. Farràs [35]. U2OS Tet-Off stable cells were grown in the same medium as COS-7 cells, supplemented with 10% FBS, 200 µg/ml geneticin (Invitrogen) and 100 µg/ml hygromycin (Sigma). Cells were grown at 37°C, 5% CO_2. COS-7 cells were transfected by the DEAE-dextran method and processed after 48h. To generate stable cell lines overexpressing ectopic PTEN wild type and mutations, U2OS Tet-Off cell line was transfected with pTRE2hyg plasmids using the calcium phosphate method, and pooled clones were selected in the presence of 100 µg/ml hygromycin. The *Saccharomyces cerevisiae* strain YPH499 (*MATa ade2-101 trp1-63 leu2-1 ura3-52 his3-Δ200 lys2-801*) was used for heterologous expression of mammalian proteins. YPH499 yeast cells were grown in synthetic complete (SC) medium, containing 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate supplemented with appropriate amino acids and nucleic acid bases, and added 2% glucose (SD), galactose (SG) or raffinose (SR), as required. Yeasts were transformed by standard procedures, and drop growth assays were performed as described [36]. pRK5 PTEN constructs have been previously described [18]. The PTEN N-terminal amino acid substitution mutations were made by PCR oligo-nucleotide site-directed mutagenesis, and mutations were confirmed by DNA sequencing. pTRE2hyg PTEN plasmids were made by subcloning into pTRE2hyg of PCR-obtained PTEN cDNAs from the corresponding pRK5 constructs. YCpLG myc-p110α-CAAX and pYES2 PTEN plasmids have been described [36]. Cloning of PTEN mutations into pYES2 was made from the corresponding pRK5 PTEN mammalian expression vectors.

Immunofluorescence and microscopy techniques

To monitor PTEN subcellular location in mammalian cells, immunofluorescence was performed as previously described, using mouse monoclonal anti-PTEN 425A and fluorescein-conjugated anti-mouse antibody [18,37]. For standard microscopy, a Zeiss fluorescence microscope (Thornwood, NY) was used. For confocal microscopy, a Leica confocal microscope (TCS-SP2-AOBS, Mannheim, Germany) was used. For quantitation of PTEN subcellular distribution, at least 100 positive cells were scored for each experiment. Cells were rated as showing nuclear staining (N), cytoplasmic staining (C), or staining within both the nucleus and the cytoplasm (N/C), as illustrated in Fig 1A. Nuclei were identified by Hoechst 33258 (Molecular Probes, Eugene, OR) staining. All pictures were taken under a 400X magnification. Measurement of green fluorescent protein (GFP)-Akt1 plasma membrane localization in yeast, as an indirect indicator of cellular PIP3 levels, was performed by fluorescence microscopy, as described [36]. 150 cells were examined and scored for each condition or experiment for either cytoplasmic or membrane-associated localization, as illustrated in Fig 1C. Cells were examined under an Eclipse TE2000U microscope (Nikon) and digital images were acquired with Orca C4742-95-12ER charge-coupled device camera and HCImage software (Hamamatsu).

Immunoblot

Whole cell protein extracts from U2OS cells overexpressing ectopic PTEN and mutations were prepared by cell lysis in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% IGEPAL CO-630 (Nonident P-40), 2 mM Na_3VO_4, 100 mM NaF, 1mM PMSF, 1 µg/ml of aprotinine, 20 mM $Na_4P_2O_7$), followed by centrifugation at 15200g for 10min and collection of the supernatant. Yeast cell extracts were obtained by standard procedures. Proteins (50–100µg in the case of mammalian cells; 20–50µg in the case of yeast) were resolved in 10% SDS-PAGE under reducing conditions and transferred to PVDF membranes. Immunoblot was performed using anti-phospho-Ser473-Akt + anti-phospho-Thr308-Akt and anti-Akt antibodies (Cell Signaling Technologies), anti-PTEN 425A mAb (Andrés-Pons et al., 2005) or rabbit polyclonal anti-PTEN antibodies (Upstate), anti-GAPDH (Santa Cruz Technology) or anti-actin C4 (MP Biomedicals, France) antibodies, followed by horseradish peroxidase (HRP)-conjugated anti- rabbit or anti-mouse (Calbiochem) antibodies. For determination of phospho-Akt content, bands were quantified using ImageQuantTL software (Amersham Biosciences).

Cell growth assays

Cell growth of U2OS cells was measured as described [38]. For soft-agar growth assays, U2OS Tet-Off cells were plated at a density of 15000 cells per well (12-well plates) in 2 ml of medium with 20% FBS and 0.7% cell culture-tested agar (Sigma), onto the solidified bottom layer of 1.4% agar. Colonies were stained after 3–4 weeks with 0.05% crystal violet. For cell proliferation assays, the 3-[4,5-Dimethylthiazol-2-yl]-2,5-dephenyltetrazolium bromide assay (MTT) was used according to the manufacturer's protocol (Roche Applied Science). Cells were plated at a density of 2500 cells per well (96-well plates) with complete medium, and cell proliferation was determined at 1–5 days. Yeast cell growth was monitored on solid media by serial dilution- drop growth assays, as described [36].

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Author contributions

Conceived and designed the experiments: AG IRE MS MM VC RP. Performed the experiments: AG IRE MS. Analyzed the data: AG IRE MS MM VC RP. Contributed reagents/materials/analysis tools: AG IRE MS MM VC RP. Wrote the paper: AG MM VC RP.

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4

PTEN nuclear localization is increased by Importin alpha3 via minor binding pocket

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Abstract

PTEN is an essential tumor suppressor that acts at the plasma membrane and is frequently mutated in human cancers. Recently, PTEN was found to localize to the nucleus and lack of nuclear PTEN is associated with more aggressive tumor formation. Multiple mechanisms have been reported that regulate nuclear localization of PTEN. Here, we investigated the role of the classical protein import pathway by immunofluorescence and confocal microscopy, and found that it is a major mechanism employed for PTEN nuclear accumulation in COS-7 cells. Importin α3 co-expression with PTEN in mammalian cells increased PTEN nuclear localization and the N-terminal region of PTEN was sufficient for this increased nuclear accumulation. Upon mutation of the importin α3 minor binding pocket, but not the major binding pocket, nuclear accumulation of PTEN decreased, indicating that the minor binding pocket of importin α3 is indispensable for the observed importin α3-dependent nuclear localization of PTEN. We conclude that PTEN is transported to the nucleus via a mechanism particularly involving the N-terminal region of PTEN and the classical protein import pathway and that the minor binding pocket of importin α3 mediates this PTEN nuclear import mechanism.

Introduction

The lipid- and dual-specificity protein phosphatase PTEN is an essential tumor suppressor [1, 2] that exerts its most prominent function, the dephosphorylation of the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3) [3], at the cell membrane, thereby antagonizing PI3K/PKB(Akt) signaling [4]. Nonetheless, during the past decade it has become more and more evident that localization of PTEN in the nucleus is required for cell function in specific contexts, such as for example ischemia, to promote cell survival [5], or in response to apoptotic stimuli, to promote apoptosis [6]. Absence of PTEN from the nucleus has further been associated with advanced disease stage and it has been rated as a poor prognosis factor in various cancer types [7-10]. Given the functional relevance of PTEN nuclear localization, we were interested in investigating the mechanism of PTEN nuclear translocation. Various models have been proposed for PTEN nuclear import [11-13], varying from simple passive diffusion [11] to a specific import mechanism implying the major vault protein (MVP) [12, 13]. Our previous work indicates that the small GTPase Ran is involved in PTEN nuclear import, possibly employing the classical nuclear import pathway [6].

In contrast to small proteins (<40 kDa), which can freely diffuse through the nuclear pore complexes (NPCs), bigger proteins and other proteins that require a regulated nuclear import, depend on specific nuclear transport factors, termed karyopherins, to translocate to the nucleus. Many karyopherins belong to the β-karyopherin superfamily that consists of 20 different β-karyopherins in mammals [14]. Among them, karyopherin subunit beta1 (importin β1) is the only one that uses karyopherin α (importin α) isoforms as adaptors [15], having the advantage to widen its range of substrates without losing substrate specificity [16, 17]. Of all of the different nuclear transport mechanisms, the classical import pathway is the best studied one, with many specific cargo proteins identified [18-30]. Its directionality is conferred by the Ran-GDP/Ran-GTP gradient between cytoplasm and nucleus [31], which is built up by the unequal distribution of the Ran guanine nucleotide exchange factor (Ran-GEF, nucleus) [32] and Ran GTPase activating protein (Ran-GAP, cytoplasm) [33] within the cell. For nuclear import, the cargo protein can bind directly to importin β [34-36] and translocate to the nucleus. Alternatively, the cargo protein binds to importin α and thus indirectly to importin β1, through the importin β binding site of importin α (αIBB), which also contains the protein's classical nuclear localization signal (NLS). In an unbound state of importin α, its NLS sequence is folded back on the αIBB domain in an auto-inhibitory manner, inhibiting the binding to importin β. To date, the exact chronological order of events during formation of the ternary complex remains elusive. However, in the ternary complex of cargo, importin α and importin β, importin β provides the sites to bind the FxFG repeats on the inner nuclear pore complex (NPC) [37-39], where it is recognized

and can enter the nucleus in an energy requiring process. Within the nucleus, Ran-GTP binds to the ternary complex and upon this interaction, the complex disassembles and the cargo protein is released into the nucleus [40]. Importin β can exit the nucleus autonomously, while importin α with the bound Ran-GTP requires its specific nuclear export factor CAS to get back to the cytoplasm, where a new cargo protein can be bound upon hydrolysis of Ran-GTP [41].

In previous experiments, we have shown that the small GTPase Ran is involved in nuclear import of PTEN [6]. C-terminally truncated PTEN (PTEN 1-375) and mutant PTEN with the 5 C-terminal CK2 phosphorylation sites mutated to alanines (S370A, S380A, T382A, T383A, S385A, PTEN QMA) both accumulate in the nucleus. Co-expression of these constructs with dominant-negative Ran Q69L [42] leads to a strong decrease in PTEN nuclear localization, indicating that Ran GTPase activity is required for PTEN nuclear import. To test whether the classical import pathway via importin α/β was involved, the cells were co-transfected with PTEN QMA or PTEN 1-375 and wild type importin α1 or importin α5 or with αIBB-domain mutants, Δ1-70 importin α1 or Δ1-70 importin α5, that are not able to interact with importin β. Whereas co-expression of PTEN QMA or PTEN 1-375 with wild type importin α1 or importin α5 decreases their nuclear accumulation, co-expression of the Δ1-70 importin α mutants does not affect nuclear localization of PTEN QMA and PTEN 1-375. Co-expression of PTEN wild type with wild type or mutant importin α1 or importin α5 does not affect PTEN WT subcellular localization at all. These results indicate that the classical importin α/β pathway may be involved in PTEN nuclear import but that the studied isoforms importin α1 and importin α5 are not the ones that transport PTEN [6].

Following up on these findings, we were intrigued to investigate which importin α isoforms are involved in nuclear translocation of PTEN and which regions of importin α and of PTEN are required for this interaction.

In our study, we used immunofluorescence and confocal microscopy to investigate the involvement of importins in PTEN nuclear transport. We found that importin α3, but not importin α6, increased nuclear localization of PTEN upon co-transfection in COS-7 cells. Using site-directed mutagenesis and chimeric fusion proteins, we mapped the involved regions to the minor binding pocket of importin α3 and the PTEN N-terminus. Based on our data, we conclude that importin α3 mediates PTEN nuclear localization via its minor binding pocket.

Results

Characterization of the subcellular localization of various PTEN constructs in COS-7 cells

We generated constructs that encode mutants of PTEN that allow us to study the involvement of the N- and C-terminus in nuclear import of PTEN and to more efficiently screen for the importin isoforms involved in this process. Wild type PTEN consists of two domains, the PTP-domain (7-185) and the C2-domain (186-352) and some unstructured areas (1-7) and (353-403) that could so far not be resolved in the crystal structure (**Fig 1**). The N-terminus, the PTP domain and the C-terminus contain motifs involved in regulating PTEN subcellular localization. Post-translational modifications may induce conformational changes that may regulate subcellular localization. For instance, phosphorylation of PTEN is the most extensively studied post-translational modification and the C-terminus contains various phosphorylation sites that are phosphorylated by CK2 [45]. We mutated five of those known PTEN phosphorylation sites to alanines (S370A, S380A, T382A, T383A and S385A), presumably leading to loosening of the previously postulated intramolecular interactions between the C-terminal tail, the C2 domain and the N-terminal tail and thereby to the exposition of certain regions on the protein that contain the NLS-like sequence and various NES-motifs. This 'open conformation' [46-51] mutant of PTEN was termed PTEN QMA [6, 45, 52] (**Fig 1**). To study the involvement of the N-terminus of PTEN in nuclear import, we fused the first 32 amino acids of PTEN to a GST-GFP fusion protein, resulting in PTEN 32-GST-GFP [6]. GST-GFP was deliberately chosen for its size (53kDa), that requires an active import mechanism, and for its cytoplasmic localization (**Fig 1**).

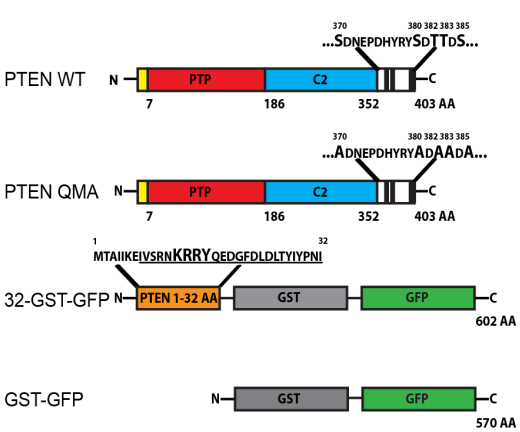
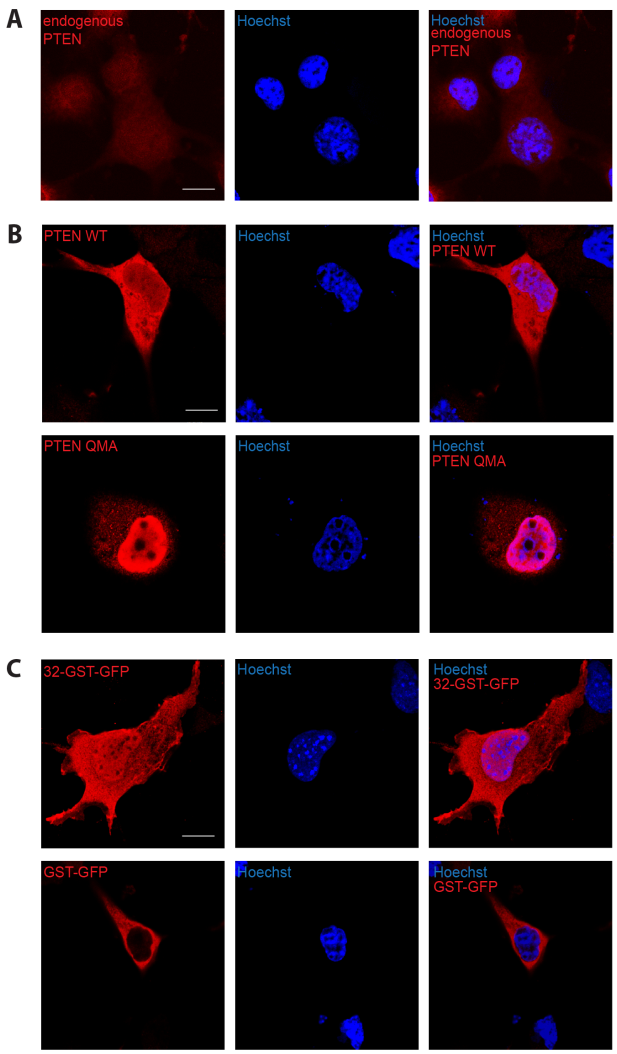


Fig 1. Schematic representation of PTEN WT, PTEN QMA and PTEN-GST-GFP fusion constructs.
WT PTEN consists of the PTP-domain (red) and the C2 domain (blue). The N-terminus (yellow) and the PTP-domain contain (partially overlapping) stretches of amino acid sequences required for nuclear localization, membrane localization, as well as the PIP2-binding motif. The C2-Domain (blue) contains various phospholipid binding motifs, e.g. for PIP3, and other motifs that regulate PTEN subcellular localization. The C-terminus (black and white) contains various sites that can be post-translationally modified, caspase 3 cleavage sites and a PDZ binding domain for interaction with PDZ domain containing proteins. The residues mutated in PTEN QMA (S370A, S380A, T382A, T383A and S385A) are the known phosphorylation sites for CK2 within its C-terminal tail. Joint mutation of these residues to alanines results in an 'open conformation' protein structure. The PTEN 32-GST-GFP construct consists of the first 32 amino acids of the N-terminus of PTEN (orange), fused to a GST-moiety (grey) and GFP (green). The GST-GFP fusion protein is a control without PTEN sequences.

In order to characterize subcellular localization of these constructs, we used the SV40 transformed african green monkey kidney cell line (COS-7), for its excellent suitability for microscopy. COS-7 cells were transfected and subcellular localization of the PTEN constructs was characterized by immunofluorescence using PTEN- or GST-specific antibodies and Hoechst counterstaining of the cell nuclei. Subsequently, subcellular localization was classified as nucleo-cytoplasmic (N/C), cytoplasmic (C) or nuclear (N) and quantified. In non-transfected control COS-7 cells, endogenous PTEN was weakly detected and showed a nucleo-cytoplasmic localization pattern (**Fig 2A**).



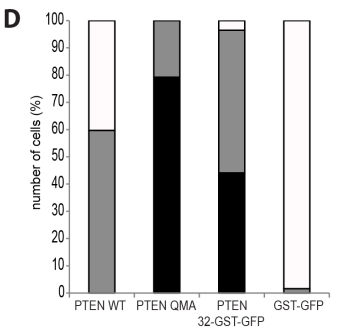


Fig 2. Involvement of the N- and C-terminus in nuclear localization of PTEN. Subcellular localization of the different PTEN constructs in COS-7 cells. Immunofluorescence microscopy, using anti-PTEN 2824 N-terminal polyclonal antibody (2824), was performed. **(A)** Non-transfected COS-7 cells, showing expression of endogenous PTEN; nuclei were stained with Hoechst (blue). **(B)** COS-7 cells transfected with PTEN WT (upper panels) or PTEN QMA (lower panels) and stained with PTEN-specific antibody (red) or Hoechst (blue); overlays in the panels to the right. **(C)** PTEN 32-GST-GFP (upper panels) or GST-GFP (lower panels) were transfected in COS-7 cells. Cells were stained using a GST-specific antibody (red) or Hoechst (blue); overlays are shown in the panels to the right. **(D)** The subcellular localization of PTEN or GST-GFP constructs was determined in 100 or more cells per condition. The graph represents the percentage of cells showing one of the three subcellular localization patterns, cytoplasmic (C), depicted in white; nucleo-cytoplasmic (N/C), depicted in grey or nuclear (N), depicted in black.

Transfected PTEN WT resulted in a great increase in fluorescence, which also localized mainly to nucleus and cytoplasm (N/C; 59.8%). PTEN QMA with an open conformation, due to mutation of its C-terminal phosphorylation sites, predominantly showed nuclear localization (N; 79.3%) (**Fig 2B**). Fusion of the N-terminal first 32 amino acids of PTEN to GST-GFP (32-GST-GFP) was sufficient to induce a nucleo-cytoplasmic distribution of this construct (N/C; 52.3%), which resembles that of PTEN WT. GST-GFP was mainly excluded from the nucleus and localized to the cytoplasm (C; 98.3%) (**Fig 2C**). These results, in accordance with previously published data [6], indicate that the 'open conformation' of PTEN facilitated its nuclear translocation and that the 32 N-terminal amino acids of PTEN may contain an NLS-like sequence, since fusion of PTEN 1-32 to GST-GFP was sufficient for the nuclear accumulation of an -otherwise cytoplasmic- protein (GST-GFP).

Importin α3 specifically increases nuclear PTEN levels

To investigate if importins are involved in the nuclear transport of PTEN, COS-7 cells were co-transfected with PTEN WT, PTEN QMA, PTEN 32-GST-GFP or GST-GFP and importin α3. Immunofluorescence experiments were performed to visualize and to quantifiy subcellular localization of the respective cargo proteins. For the quantification, the cells were classified into three categories of cargo subcellular localization: cytoplasmic (C), nucleo-cytoplasmic (N/C) and nuclear (N). Co-expression of importin α3 led to redistribution of PTEN WT from a nucleo-cytoplasmic (N/C; 59.8%) and cytoplasmic (C; 40.3%) expression pattern towards a more nucleo-cytoplasmic (N/C; 59.8%) and nuclear (N; 33.5%) pattern (**Fig 3A**). The localization of PTEN QMA remained predominantly nuclear (N; 79.3%) when co-expressed with importin α3 (N; 83.4%) (**Fig 3A**). PTEN 32-GST-GFP, which showed predominantly nucleo-cytoplasmic (N/C; 52.3%) localization in COS-7 cells, accumulated more strongly in the nucleus (N; 83.4%) upon co-transfection of importin α3 (**Fig 3B**), indicating that the N-terminus of PTEN is involved in its importin α3-dependent nuclear accumulation. In contrast, GST-GFP, which localized in the cytoplasm, remained cytoplasmic (C; 98.3%) despite co-expression of importin α3 (C; 99.2%) (**Fig 3B**).

Co-expression of importin α3 increased nuclear localization of PTEN. To investigate whether enhanced nuclear accumulation of PTEN is specific for importin α3, importin α6 was co-transfected with PTEN. Neither PTEN WT nor PTEN QMA (**Fig 4A, 4C**) showed increased nuclear localization upon co-transfection of importin α6, suggesting that importin α6 is not involved in the transport process. PTEN 32-GST-GFP subcellular localization was not significantly increased by co-expression of importin α6 either (**Fig 4B, 4C**). These results indicate that importin α3, but not importin α6, mediates nuclear accumulation of PTEN and implicate the N-terminal 32 residues of PTEN in the process.

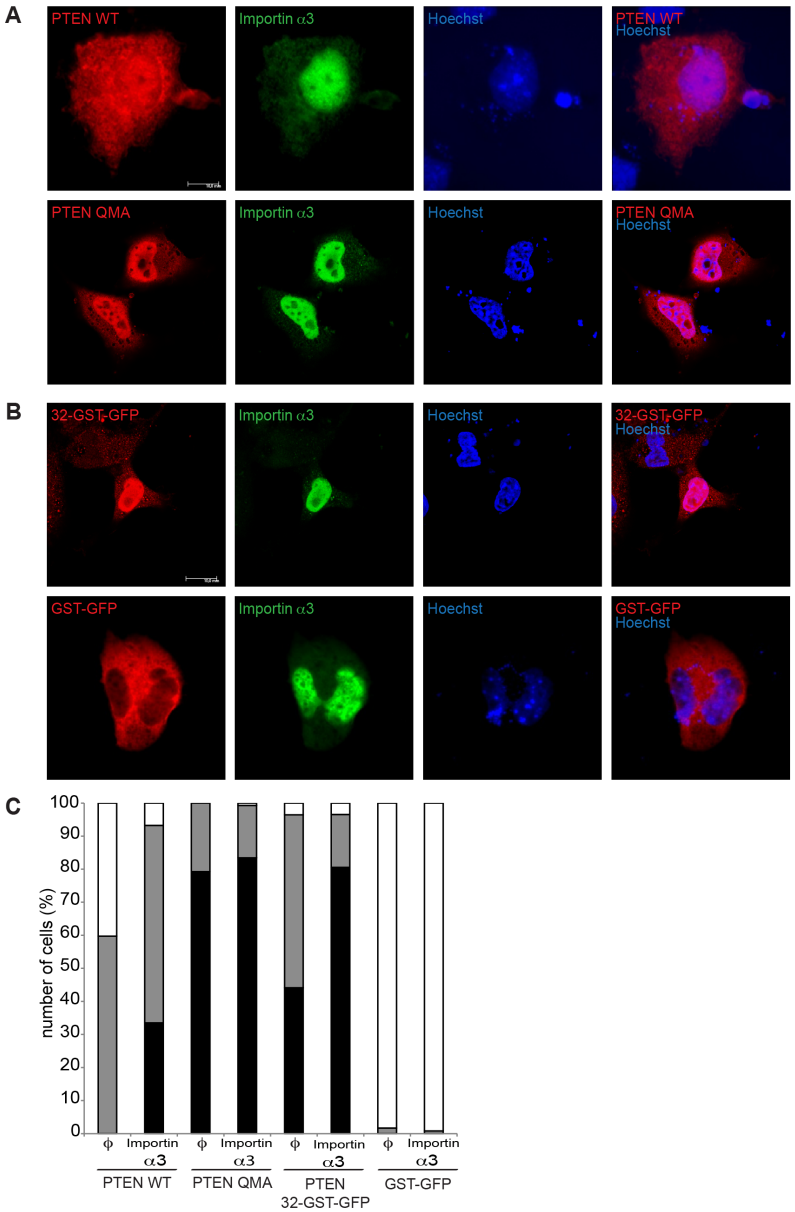


Fig 3. Importin alpha3 co-expression increases PTEN nuclear accumulation.
PTEN WT, PTEN QMA, 32-GST-GFP and GST-GFP were co-transfected with an expression vector for importin α3 and subcellular localization was determined by immunofluorescence as in Fig. 2. **(A, B)** Representative pictures of transfected cells: PTEN WT, PTEN QMA, 32-GST-GFP and GST-GFP, red; importin α3, green; Hoechst, blue and an overlay are shown. **(C)** Quantification of the subcellular localization of at least 100 cells per condition. Control (ϕ, empty vector) is compared to importin α3 co-transfected. The graph represents the percentage of cells showing one of the three subcellular localization patterns, cytoplasmic (C), depicted in white; nucleo-cytoplasmic (N/C), depicted in grey or nuclear (N), depicted in black.

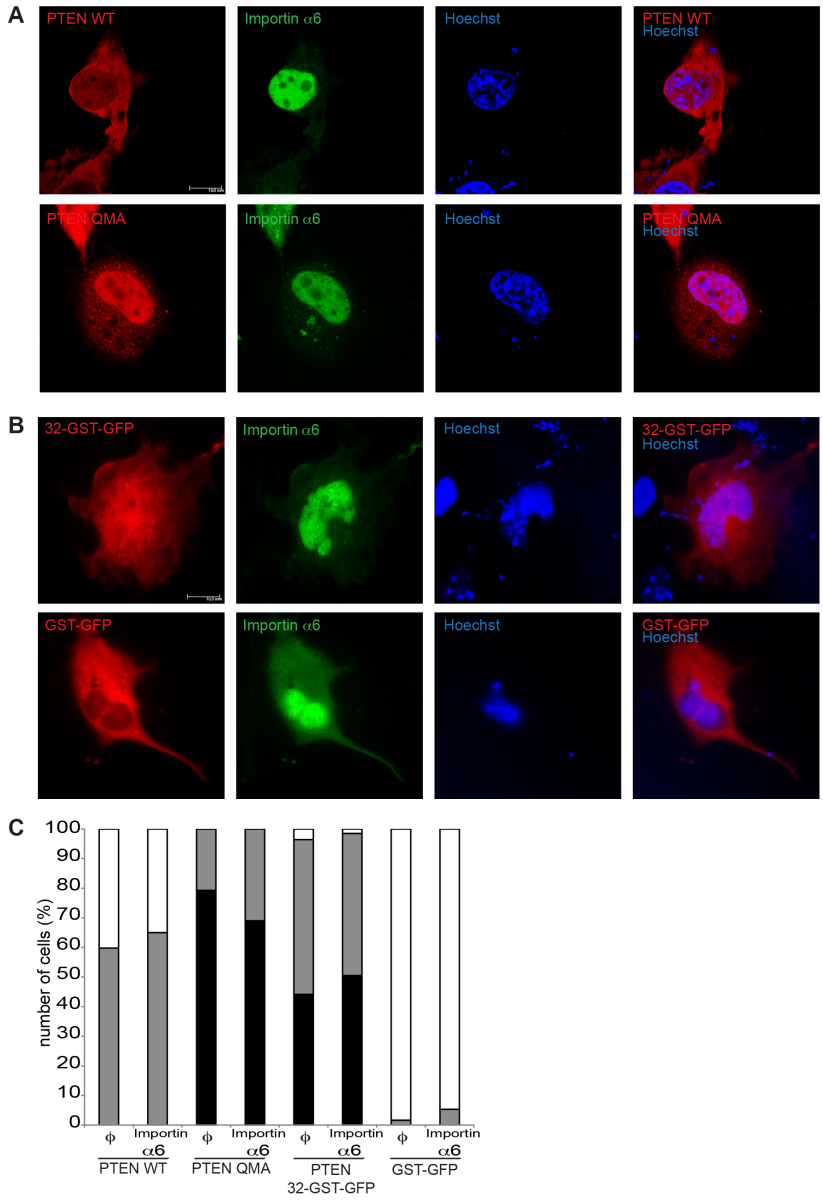


Fig 4. PTEN subcellular localization is not affected by importin alpha6 co-expression.
PTEN WT, PTEN QMA, 32-GST-GFP and GST-GFP were co-transfected with an expression vector for importin α6 and subcellular localization was determined by immunofluorescence as in Fig. 2. **(A, B)** Representative pictures of transfected cells: PTEN WT, PTEN QMA, 32-GST-GFP and GST-GFP, red; Importin α6, green; Hoechst, blue and an overlay are shown. **(C)** Quantification of the subcellular localization of at least 100 cells per condition. The graph represents the percentage of cells showing one of the three subcellular localization patterns, cytoplasmic (C), depicted in white; nucleo-cytoplasmic (N/C), depicted in grey or nuclear (N), depicted in black. Control (ϕ, empty vector) is compared to importin α6 co-transfected.

Point mutations in the importin α3 minor binding pocket abolish increased PTEN nuclear localization

To investigate which region of importin α3 is required for bringing forth the observed increase in nuclear localization of PTEN, we generated mutations in the known cargo binding pockets of the protein. The importin α protein family members share a highly conserved protein structure, consisting of the αIBB domain and ten tandem armadillo repeats (Arm) (**Fig 5A**). One armadillo repeat consists of three α-helices (H1, H2 and H3) that are encoded by a stretch of ~ 40 amino acids [53]. The H3 helices of the ten stacked armadillo repeats form the inner concave surface of the importin α3 tertiary structure where the cargo proteins are bound (**Fig 5B**). Some of the Armadillo repeats directly contribute to the interaction with cargo proteins via the major binding pocket (Arm 2-4) or the minor binding pocket (Arm 6-8). The major binding pocket preferentially interacts with a monopartite classical NLS, which typically consists of a stretch of basic amino acids (e.g. like that of the simian virus 40 large T antigen; PKKKRRV) [54, 55]. Both binding pockets are required to bind to a bipartite NLS, which differs from a monopartite NLS in the disruption of the motif by a stretch of random amino acids. We introduced point mutations in the well conserved WXXXN motif of the H3 helix [56-58] of Arm 3 or Arm 8, changing it to AXXXA, to disrupt cargo binding at either of the two binding pockets (**Fig 5A, 5C**).

We co-expressed the importin α3 binding binding pocket mutants importin α3 Arm3 (major binding pocket mutated), importin α3 Arm8 (minor binding pocket mutated) or importin α3 Arm3+8 (both binding pockets mutated) with either PTEN QMA or PTEN 32-GST-GFP in COS-7 cells and performed immunofluorescence experiments to detect PTEN and the importin α3 binding pocket mutants (**Figs 6A, 6B**). Co-transfection of the major binding pocket mutant importin α3 Arm3 induced increased nuclear localization of PTEN, like wild type importin α3, whereas co-transfection of the minor binding pocket mutant importin α3 Arm8 abolished nuclear localization of PTEN. Quantification of these results (**Fig. 6C**) indicated that PTEN QMA nuclear localization (N; 35.1%) was significantly decreased upon co-expression of the minor-binding pocket mutant importin α3 Arm8 (N; 2.5%), with concomitant predominantly cytoplasmic (C; 49.4%) localization of PTEN QMA, compared to co-expression of WT importin α3 (N; 49.3%) or importin α3 Arm3 (N; 51.9%). Combined mutation of both binding pockets (importin α3 Arm3+8) also abolished PTEN QMA nuclear localization (N; 0.2%), indicating that mutation of the importin α3 minor binding pocket is involved in PTEN nuclear accumulation. Further experiments, co-expressing the PTEN 32-GST-GFP chimeric protein with either WT importin α3 or the respective binding pocket mutants of importin α3 (importin α3 Arm3, importin α3 Arm8, importin α3 Arm3+8) confirmed the role of the importin α3 minor binding pocket and the autonomy of the PTEN N-terminus in driving

importin α3-dependent nuclear localization of PTEN. The increased nuclear accumulation of PTEN 32-GST-GFP upon co-expression of WT importin α3 (N; 22.9%) (17.3% increased compared to empty vector control) was completely abrogated upon co-expression of the minor binding pocket mutant (importin α3 Arm8) with PTEN 32-GST-GFP (N; 1.1%). Combined mutation of both binding pockets (importin α3 Arm3+8) did not significantly increase the negative effect on PTEN QMA nuclear localization (N; 0.2%). Again, this indicates that an intact importin α3 minor binding pocket is required for nuclear accumulation of PTEN and that the PTEN N-terminus is sufficient to drive importin α3-dependent nuclear localization of PTEN.

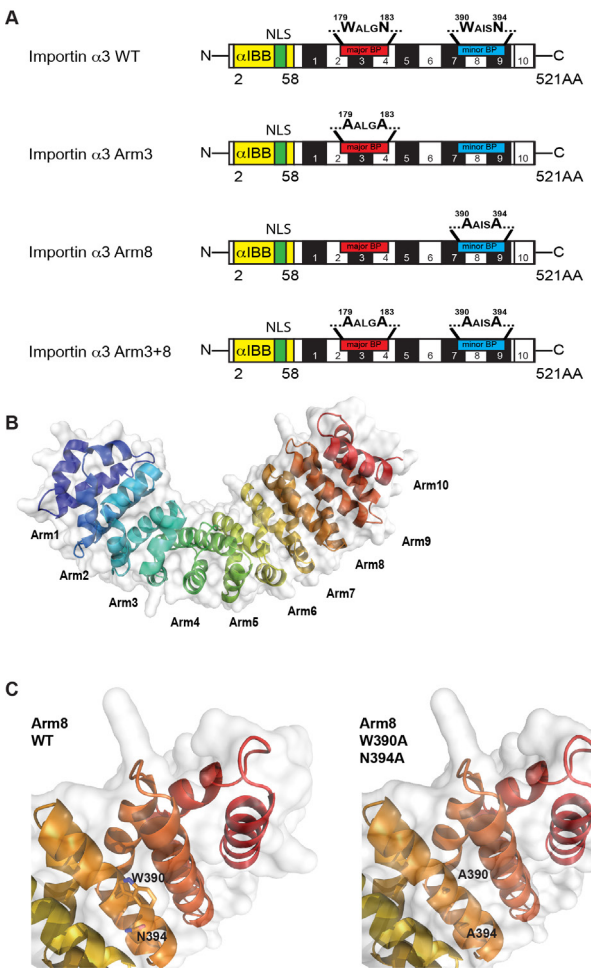


Fig 5. Protein structure of importin alpha3 and mutagenesis of its major and/or minor binding pocket.
(A) Schematic representation of importin α3. The αIBB domain, which is required for interaction with importin β1 and contains the NLS motif, and the 10 Armadillo repeats (Arm) are indicated. The major binding pocket, which preferentially binds cargo proteins that contain a monopartite NLS, is formed by Arm repeats 2-4. The minor binding pocket is formed by Arm repeats 7-9. We mutated the well conserved WXXXN motif to AXXXA in Arm repeat 3 and/or 8 to disrupt the major binding pocket and/or minor binding pocket, respectively.
(B) The tertiary structure of importin α3 is depicted using PyMOL. The αIBB-domain is deleted from this model for better visualization.
(C) Point mutations introduced in the conserved WXXXN cluster, exemplified for Arm 8 (minor binding pocket) of wild type importin α3 (left) and importin α3 W390A, N394A (right), are depicted using PyMOL. Note the loss of bulkiness in the tertiary structure at this position.

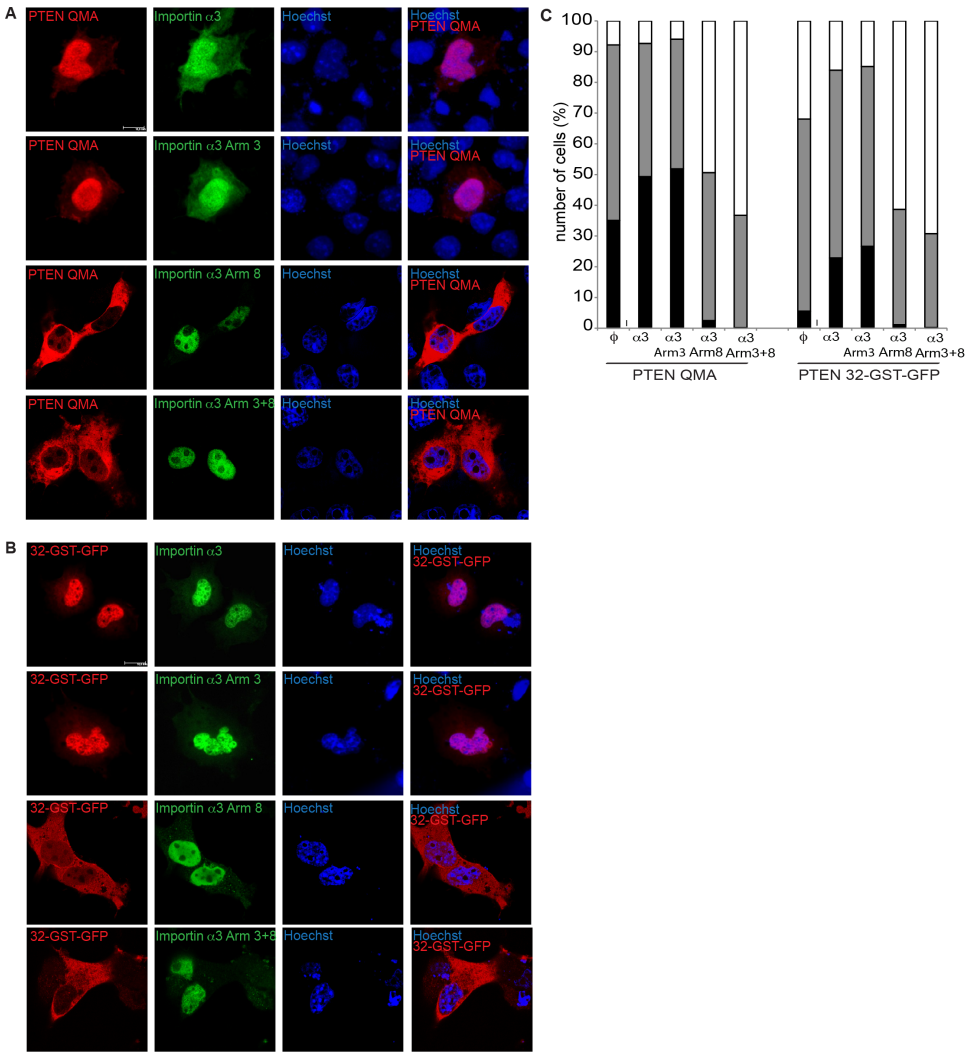


Fig 6. Mutation of the minor binding pocket of importin alpha 3 abolished the increase in PTEN nuclear localization.
PTEN QMA or 32-GST-GFP were co-transfected with importin α3 (mutants) and subcellular localization was assessed by immunofluorescence as in Fig. 2. Representative immunofluorescence pictures are shown in **(A)** and **(B)**. **(C)** Quantification of the subcellular localization of PTEN QMA and 32-GST-GFP upon co-transfection of empty vector (ϕ) and importin α3 (mutants): cytoplasmic (C), depicted in white; nucleo-cytoplasmic (N/C), depicted in grey or nuclear (N), depicted in black.

Discussion

In this paper, we used COS-7 cells to study the mechanism of PTEN subcellular localization via immunofluorescence and confocal microscopy. We found that importin α3 is involved in PTEN nuclear import and that not only full length PTEN nuclear accumulation increased upon co-expression of importin α3, but also a chimeric protein, consisting of the 32 N-terminal amino acids of PTEN (PTEN 32-GST-GFP), responded to importin α3 co-expression with increased nuclear accumulation. These results suggest that the PTEN N-terminus has an important role in the nuclear import process. We further identified the minor binding pocket of importin α3 as the essential site to promote nuclear accumulation of PTEN, since the introduction of point mutations in a well conserved amino-acid motif abrogated PTEN nuclear localization. Similar mutation of the major binding pocket did not have this effect.

Previous studies have pointed to the importin α/β classical import pathway as a possible mechanism for PTEN nuclear translocation in mammalian cells [6]. Here we identified importin α3 as an important player in nuclear transport of PTEN and hence as an important regulator of Pten's different subcellular functions. It is known that the importin α/β classical import pathway is employed for the regulation and subcellular localization of many other proteins that require regulated and rapid translocation from one subcellular compartment to the other in order to fulfill their specific function, as for example transcription factors (various members of the STAT family [19, 22, 34, 59], Zac1 [23], or NFκB [20]), other transcriptional regulators (e.g. RNA helicase A [21]), cell-cycle regulating proteins (e.g. cdc25 [60], p53 [26] and $p27^{KIP}$ [61]) or DNA damage repair pathway proteins (e.g. XPA, involved in nucleotide excision repair [28]). Further, also cell-type specific functions, such as the regulation of the circadian clock (by e.g. nuclear import of mCRY2) [62] or of stem cell identity [63-65] are regulated by the importin α/β pathway. Therefore it is not surprising that various viruses high-jack this transport machinery for their own replication, among them HIV, Rota- and Dengue virus [66-68]. The import process is regulated at many different levels, ranging from the highly auto-regulated formation of the ternary complex, to post-translational modifications as acetylation [69], ubiquitination [26] or phosphorylation [60, 61] of the cargo protein, or competitive inhibition of one importin by another (as in the case of nuclear import of Snail) [70]. Not only the import process itself, but also importin α gene- [25] and protein expression [71] and its subcellular localization within the cell [72, 73] are highly regulated. Import of PTEN via the importin α/β pathway provides the cell with the tools to regulate PTEN functions via changes in subcellular localization of the protein on a variety of levels, fine-tuning PTEN functions to the needs of the cell in a rapid and reversible manner. Importins of the α subfamily act as specific adaptors to transport classical nuclear localization signal (NLS)-containing proteins to the nucleus [16, 74-76]. The flexible N-terminal IBB domain of importin α

interacts either *in trans* with importin β or *in cis* with its classical NLS binding sites in an autoinhibitory manner and is an important player in controlling the assembly and disassembly of the ternary complex [74, 77]. The C-terminal domain in contrast is highly structured and consists of ten Arm repeats, some of them forming the minor and the major binding pocket that bind cargo proteins [39, 57, 76, 78]. Research to date has shown that there are various types of NLSs. A classical NLS usually consists of a cluster of basic amino acids, predominantly lysines (K) and arginines (R) that can either form one motif (monopartite NLS) or that form two motifs, interrupted by a stretch of 10-12 random amino acids (bipartite NLS) [79, 80]. An atypical NLS has less predictable motifs [81] and a PY-NLS is recognized by a completely different karyopherin (karyopherin β2) and pathway [14]. There are various online tools to predict a proteins' NLS and NES sequences. In the case of PTEN, so far no classical NLS has been identified. Prediction of the NLS, using NLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi; [82]), suggests that the amino acids 10-19 (SRNKRRYQED) form a monopartite NLS with a score of 6 on a scale of 1-10 (with higher scores representing more NLS activities). This score predicts a nucleo-cytoplasmic localization of PTEN, which coincides with our experimental data in COS-7 cells. Note that the online tool is based on yeast experimental data and that therefore it may not be very accurate for the mammalian importin α/β pathway, despite the high level of conservation of the importin α/β pathway in eukaryotes. Recent studies, employing an *in vitro* virus (IVV) mRNA display method, led to the identification of three novel classes of importin α-dependent NLS motifs. The PTEN KRRY motif highly resembles one of the new consensus sequences (class 4 monopartite atypical NLS, KRKY). Remarkably, this motif only binds to the importin α minor binding pocket [81], supporting our findings that the minor binding pocket of importin α3 is involved in nuclear import of PTEN. Unfortunately, we were not able to obtain unambiguous results concerning the direct interaction between importin α3 and PTEN due to technical difficulties. We cannot exclude the possibility that there is no direct interaction between importin α3 and PTEN but that instead another unknown protein is required to mediate this interaction within the ternary complex. However, our experimental data, paired with the previous publications and the online prediction, gives strong indications that transport of PTEN to the nucleus occurs via the PTEN N-terminus and the minor binding pocket of importin α3.

Materials and Methods

Constructs

N-terminally Flag-tagged importin constructs were derived by cloning of the Flag tag in frame with full length importin cDNA into the XbaI/SalI sites of pRK5 vector. The binding pocket mutations of importin α3 were obtained by mutating the highly conserved WXXXN motif of the Armadillo repeats 3 and/or 8 to AXXXA [α3 Arm3 (W179A, N183A), α3 Arm8 (W390A, N394A), α3 Arm3+8 (W179A, N183A, W390A, N394A)] by site-directed mutagenesis and verified by sequencing. PTEN QMA (S370A, S380A, T382A, T383A and S385A), PTEN 1-32-GST-GFP and GST-GFP have been previously described [6, 43, 44].

Immunofluorescence

COS-7 cells were grown on Poly-L-Lysine (Sigma-Aldrich) coated coverslips in Dulbecco's modified Eagle's medium supplemented with 5% FCS during 24h and then transfected with Lipofectamine 2000 transfection reagent (Invitrogen). After repeated washing with 1x PBS, the cells were fixed 24h after transfection by adding 4% PFA during 20 min at room temperature. The cells were blocked and washed with PBS/3% BSA. The primary antibodies used were anti-PTEN 2824, (polyclonal antibody, rabbit, 1:200 [6] or anti-GST (91G1, rabbit, Cell Signaling, 1:200) and anti-Flag (M2, mouse, Sigma, 1:200) and as secondary antibodies anti-rabbit-Alexa568 (1:200) and anti-mouse IgG-FITC (F0257, Sigma, 1:200) were used. The nuclei were counterstained with Hoechst 33342 (Invitrogen, 1:1000).

Confocal microscopy

A Leica TCS-SPE confocal microscope with a 63x oil objective was used for imaging and quantification of the immunofluorescence experiments. FITC and Alexa568 signal was detected using a 488 nm and 516 nm laser line, respectively, for excitation. The images were taken with a 2.5x zoom factor.

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5

Differential requirement for Pten lipid and protein phosphatase activity during zebrafish embryonic development

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Abstract

The lipid- and protein phosphatase *PTEN* is one of the most frequently mutated tumor suppressor genes in human cancers and many mutations found in tumor samples directly affect PTEN phosphatase activity. In order to understand the functional consequences of these mutations *in vivo*, the aim of our study was to dissect the role of Pten phosphatase activities during zebrafish embryonic development. As in other model organisms, zebrafish mutants lacking functional Pten are embryonically lethal. Zebrafish have two *pten* genes and *pten* double homozygous zebrafish embryos develop a severe pleiotropic phenotype around 4 days post fertilization, which can be largely rescued by re-introduction of *pten* mRNA at the one-cell stage. We used this assay to characterize the rescue-capacity of Pten and variants with mutations that disrupt lipid, protein or both phosphatase activities. The pleiotropic phenotype at 4dpf could only be rescued by wild type Pten, indicating that both phosphatase activities are required for normal zebrafish embryonic development. An earlier aspect of the phenotype, hyperbranching of intersegmental vessels, however, was rescued by Pten that retained lipid phosphatase activity, independent of protein phosphatase activity. Lipid phosphatase activity was also required for moderating pAkt levels at 4 dpf. We propose that the role of Pten during angiogenesis mainly consists of suppressing PI3K signaling via its lipid phosphatase activity, whereas the complex process of embryonic development requires lipid and protein phosphatase activities of Pten.

Introduction

PTEN (Phosphatase and tensin homolog) is one of the most frequently mutated tumor suppressor genes in spontaneous cancers [1, 2] and germline mutations of *PTEN* have been associated with PTEN hamartoma tumor syndromes (PHTS), such as Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome [3–5], and with autism-spectrum disorders [6, 7]. Moreover, homozygous loss of germline *PTEN* is incompatible with life in higher eukaryotes [8–12], illustrating its essential functions for multicellular organisms since early embryonic development.

A prominent function of the tumor suppressor PTEN is its lipid phosphatase activity towards phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3), which makes PTEN the main antagonist of the cell proliferation and cell survival promoting phosphatidylinositol-3-kinase (PI3K)/ Akt (also known as protein kinase B, PKB) signaling pathway [13]. Little-known, in contrast, is its dual-specificity protein phosphatase activity against phosphotyrosine (pTyr), phosphoserine (pSer) and phosphothreonine (pThr), which was discovered and characterized [14] shortly after the identification of *PTEN* as one of the most frequently mutated tumor suppressor genes in human cancers. Nonetheless, the main attention so far has been drawn to the lipid phosphatase activity of PTEN, partly due to the early identification of germline mutations in *PTEN* in Cowden syndrome patients, that are particularly affecting the protein's lipid phosphatase activity [3, 15]. As in other members of the PTP (protein tyrosine phosphatase) family, the catalytic core of the PTEN PTP domain contains a cysteine, in this case Cys124, which is essential for dephosphorylation of substrates [16, 17]. Mutation of PTEN Cys124 to serine, C124S, which has been associated with spontaneous cancer [18], completely abolishes PTEN phosphatase activity towards inositol phospholipids or phosphorylated proteins [19]. Another point mutation within the catalytic core, G129E, which has been first identified in Cowden syndrome patients, only affects lipid- but not protein phosphatase activity of PTEN [3, 20]. In tumor specimens, Gly129 has been found mutated to either glutamate, G129E; arginine, G129R [14] or valine, G129V (http://cancer.sanger.ac.uk/cosmic). Due to its specific loss of lipid phosphatase activity but not protein phosphatase activity, PTEN G129E has to date been a valuable tool to study the contribution of each of the two enzymatic activities of PTEN *in vitro* [14, 20–25] and *in vivo* [26]. Upon the discovery of the PTEN related phosphatase TPIP (Transmembrane phosphoinositide 3-phosphatase and tensin homolog) [27], which lacks protein phosphatase activity, and based on the homologous region shared with PTEN, *Leslie et al.* generated a PTEN mutant, Y138L, which conserves PTEN lipid phosphatase activity but lacks PTEN protein phosphatase activity [28]. Mutation of Tyr138 to cysteine, Y138C, has further been identified in a small cell lung carcinoma cell line, indicating that, though

apparently with less frequency, also lack of PTEN protein phosphatase activity is positively selected for in some types of cancer [29]. These three phosphatase mutants of PTEN, C124S, G129E and Y138L, have been used in different studies, *in vitro* and *in vivo*, to functionally dissect PTENs distinct phosphatase activities [26, 28, 30, 31]. However, rescue experiments in living organisms using all three phosphatase mutants of PTEN, have not been done systematically yet. In cell based assays, the requirement for lipid phosphatase activity of PTEN to suppress cell proliferation and colony formation was confirmed definitively [22, 32, 33], whereas the importance of the protein phosphatase activity in this process is debatable and depends on the kind of assay performed [28, 29, 34]. However, the PTEN protein phosphatase activity seems to be required for glial cell migration [35–37], for regulating differentiation of neuronal progenitor cells [31], for suppression of cell invasion *in vitro* (Matrigel assay) and for suppression of endothelial to mesenchymal transition (EMT) *in vivo* [28, 29, 38]. The identity of the protein substrates of PTEN remains to be determined definitively. Some of the protein phosphatase-dependent functions of PTEN have recently been attributed to PTEN autodephosphorylation rather than to dephosphorylation of other target proteins [29, 37, 39–41].

The role of PTEN lipid and protein phosphatase activity during embryonic development has not been studied in much detail yet, since it requires the availability of a suitable animal model. Analysis of mouse Pten knockouts and knockins during embryonic development is hampered by embryonic lethality of Pten knockouts at day E8.5 [8] and because development takes place *in utero*. We use zebrafish to study protein function during development. Zebrafish is a great model, because of extra-uterine development, the number of embryos generated (~200 per female, weekly), the transparency of the egg and the variety of techniques to (re-) introduce genetic information (synthetic RNA, DNA) at the one-cell stage.

Zebrafish have two *pten* genes, *ptena* and *ptenb*, that have redundant functions [9, 10]. We generated *ptena*–/– and *ptenb*–/– fish lines by target-selected gene inactivation (TSGI) and inbred them to obtain *ptena*+/–*ptenb*–/– and *ptena*–/–*ptenb*+/– fish lines that we use for our functional rescue experiments [9, 42, 43]. While their heterozygous and single homozygous siblings are viable and fertile, double homozygous embryos that lack all Pten activity develop a pleiotropic phenotype, characterized by massive heart edema, craniofacial defects, aberrant pigmentation and shorter body axis. Those embryos die around 5 days post fertilization (dpf). We previously characterized the pleiotropic phenotype of double homozygous *ptena*–/–*ptenb*–/– embryos at different developmental stages [9], unveiled the formation of hemangiosarcomas in adult *pten* haploinsufficient zebrafish [44] and further studied the role of Pten in zebrafish angiogenesis [43] and hematopoiesis [45].

Angiogenesis, the formation of new blood vessels from the existing vasculature, is a common hallmark of solid tumor progression and the underlying signaling pathways,

especially VEGFR-signaling, have been extensively studied as therapeutic targets. We recently described that *pten* double homozygous embryos have constitutively elevated pAkt and *vegfaa* levels [9, 43, 44], accompanied by enhanced angiogenesis that can be visualized from 3dpf onwards using confocal microscopy in *ptena–/–ptenb–/–* zebrafish embryos in the transgenic Tg(*kdrl: eGFP*) background [43]. This vasculature hyperbranching phenotype is rescued by treatment with the PI3K-specific inhibitor LY294002 but also by microinjection of synthetic *pten* mRNA [43].

In this study we investigated which Pten phosphatase activity, lipid or protein phosphatase, is required for normal zebrafish embryonic development and angiogenesis. We tested the capacity of phosphatase activity mutants of Pten to rescue the pleiotropic phenotype at 4dpf when injected as synthetic mRNA at the one-cell stage. Wild type Pten, but none of the phosphatase mutants, was able to rescue the pleiotropic phenotype at 4dpf. In contrast to the pleiotropic phenotype at 4dpf, the hyperbranching vessel phenotype at 3dpf was rescued by both lipid phosphatase active Pten constructs, Pten wild type and Pten Y138L. Further, lipid phosphatase active Pten decreased activated Akt (pAkt) levels in *pten* double homozygous zebrafish embryos at 4dpf. We conclude that Pten lipid phosphatase activity is required to regulate correct vessel formation and pAkt levels during zebrafish embryogenesis, whereas the Pten protein phosphatase activity seems dispensable for regulating angiogenensis. For correct zebrafish embryonic development, however, both lipid- and protein phosphatase activities appear to be required.

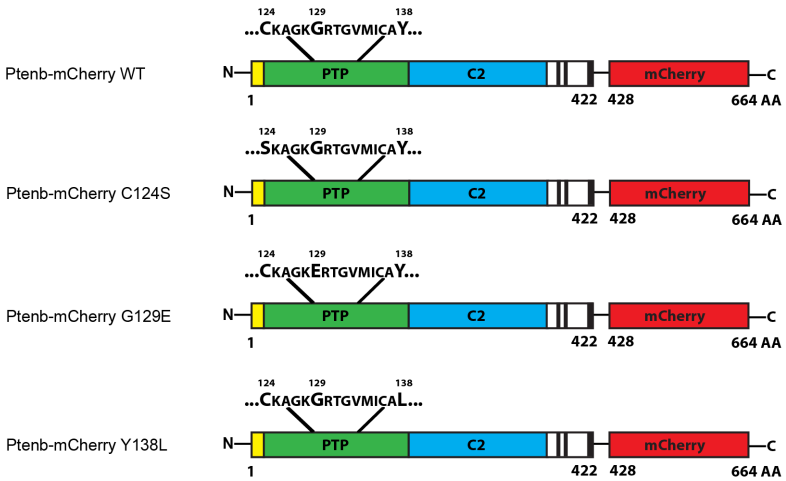
Results

Pten phosphatase mutants

In order to study the role of Pten phosphatase activities during zebrafish embryonic development, we introduced point mutations, that are known to disrupt -fully or partially- the enzymatic activity of human PTEN, in the PTP domain of the zebrafish homologue, Ptenb (**Fig 1A**). The sequence conservation of the catalytic domain between human PTEN and zebrafish Pten is extremely high. All residues that contact the substrate, including Asp92, His93, Lys125, Ala126, Gly127, Lys128, Gly129, Arg130, Thr167, Ile168 and Gln171, and particularly the catalytic site cysteine of PTEN, Cys124, [49] are conserved in Ptenb. Therefore, we believe that mutation of C124S, G129E or Y138L in Ptenb affects the catalytic activities of these mutants in a similar manner as in human PTEN. We previously reported that zebrafish, as many other teleosts, have two *pten* genes, *ptena* and *ptenb*, that have redundant functions during embryonic development [9] and that both *ptena* and *ptenb* largely rescued the morphological defects in *ptena–/–ptenb–/–* zebrafish [43]. For our approach, we used *ptenb* and mutants. We first generated

constructs encoding fluorescent protein-tagged Ptenb, using either eGFP or mCherry, which allowed us to check for correct microinjection and protein expression. Subsequently we created the phosphatase mutants of Ptenb-mCherry and Ptenb-eGFP (not depicted in the schematic) by site-directed mutagenesis (**Fig 1A**). Mutation of the catalytic cysteine, Cys124, to serine abolishes all phosphatase activity, whereas mutation of Gly129 to glutamate renders a lipid phosphatase inactive Ptenb that retains protein phosphatase activity. Mutation of Tyr138 to leucine on the contrary abolishes protein phosphatase activity, but not lipid phosphatase activity (**Fig 1B**).

A



B

	PTP-activity: lipid	PTP-activity: protein
Ptenb-mCherry WT	✓	✓
Ptenb-mCherry C124S	✗	✗
Ptenb-mCherry G129E	✗	✓
Ptenb-mCherry Y138L	✓	✗

Fig 1. Schematic representation of the Ptenb-mCherry WT, C124S, G129E and Y138L constructs and their enzymatic activities. (A) Wild type Ptenb (long splicing variant, 422 amino acids) consists of the N-Terminus (yellow), the PTP-domain (green), the C2 domain (blue) and the C-terminus (black and white). The red fluorescent protein mCherry (red) is C-terminally tagged in frame to all Ptenb constructs. The point mutations of interest were introduced into the PTP-domain by site-directed mutagenesis. **(B)** Phosphatase activities of the Ptenb mutants: Wild type Ptenb posseses phosphatase activity against phospholipids and phosphorylated peptide sequences, which can both be completely abrogated by mutation of the catalytic cysteine, Cys124 to serine (C124S). Mutation of Gly129 to glutamate (G129E) results in loss of Ptenb lipid phosphatase activity while protein phosphatase activity is retained. Mutation of Tyr138 to leucine (Y138L), renders a protein-phosphatase-dead Ptenb that retains lipid phosphatase activity.

Both Pten phosphatase activities are required to rescue the *ptena–/–ptenb–/–* pleiotropic phenotype

For our functional rescue assay, we microinjected embryos of a *ptena+/–ptenb–/–* zebrafish incross at the one-cell stage with 300pg of synthetic Ptenb-mCherry WT or phosphatase mutant mRNA. At 4dpf, we performed brightfield microscopy of the whole embryos to assess their overall phenotype. Double homozygous *ptena–/–ptenb–/–* embryos displayed severe developmental defects, including massive heart and abdominal edemas, craniofacial defects, aberrant pigmentation and reduced body axis extension (**Fig 2A**, non-injected control (NIC)). These defects became visible at 4dpf and were likely provoked by aberrant cell proliferation and enhanced cell survival [9]. Microinjection of 300 pg synthetic *ptenb* mRNA largely rescued the pleiotropic defects of *ptena–/–ptenb–/–* embryos, whereas injection of 300 pg *ptenb* mRNA did not induce any phenotypes in siblings (**Fig 2A**, WT). Although most features of the pleiotropic phenotype were rescued by re-introduction of wild type Ptenb, a complete rescue was not achieved. This is probably due to a decrease in mRNA levels over time and to a certain degree of mosaicism that may occur during the early cleavage stages. For quantification (**Fig 2B**), we therefore considered a highly improved phenotype, like the one of the Ptenb-mCherry WT-injected embryo depicted in Fig 2A, as "rescued". In this assay, we used the phosphatase mutants of Ptenb in parallel to WT Ptenb in order to assess their capacity to rescue the *ptena–/– ptenb–/–* phenotype. Both lipid-phosphatase inactive mutants, Ptenb-mCherry C124S and Ptenb-mCherry G129E, were not capable of rescuing the pleiotropic phenotype at all. The *pten* double homozygous embryos microinjected with these two mutants closely resembled the non-injected double homozygous siblings (**Fig 2A**). Microinjection of Ptenb-mCherry Y138L seemed to considerably alleviate the severity of the phenotype but did not fully rescue it. Yet, compared to the non-injected controls, Y138L injection is a great amelioration. Ptenb-eGFP G129E and Ptenb-mCherry Y138L RNA were co-injected each at half of the dose of the single injections to avoid inadvertant effects of microinjection of too much synthetic mRNA. However, these co-injections did not rescue the *pten* double homozygous phenotype either. In fact, the phenotype appeared much more severe than the one of the Ptenb-mCherry Y138L injected embryos but not as severe as the non-injected control.

According to Mendelian law, one quarter of the offspring of a *ptena+/–ptenb–/–* incross, 25%, is expected to be double homozygous. Counting the number of embryos displaying the *pten* double homozygous phenotype, compared to the total number of embryos in each experimental condition allowed us to assess the rescue capacity of each Ptenb-mCherry construct and to perform statistical analysis (**Fig 2B**). As expected, the occurrence of the *pten* double homozygous phenotype in the non-injected control was about

25%, which was significantly reduced, to about 10%, by microinjection of Ptenb-mCherry WT. Ptenb-C124S and Ptenb- G129E did not rescue the morphological defects in *ptena–/–ptenb–/–* embryos. Although there appeared to be a positive effect of Ptenb-mCherry Y138L expression on the number of phenotypes observed, this was not statistically significant and neither was co-expression of Ptenb-G129E and Ptenb-Y138L. We conclude that for normal embryonic development in zebrafish, both phosphatase activities of Pten are required, because none of the Ptenb phosphatase mutants rescued the morphological defects in *ptena–/–ptenb–/–* mutants to the same extent as wild type Ptenb.

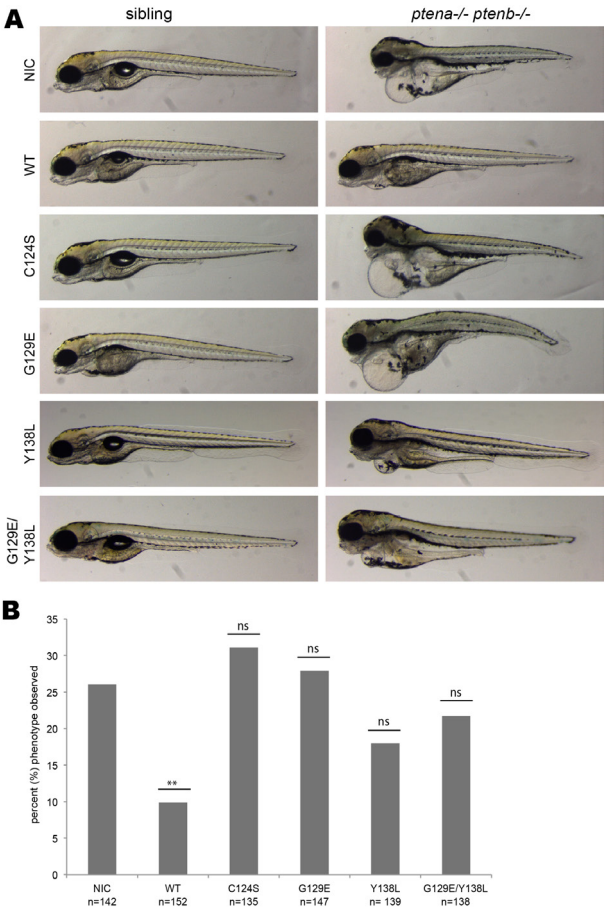


Fig 2. Both Pten phosphatase activities are required to rescue the ptena-/-ptenb-/- pleiotropic phenotype. Zebrafish embryos from a *ptena+/– ptenb–/–* incross were injected with either Ptenb-mCherry WT, Ptenb-mCherry C124S, Ptenb-mCherry G129E or Ptenb-mCherry Y138L encoding synthetic mRNA at the one-cell stage.
(A) At 4dpf the embryos were submitted to brightfield microscopy and analyzed for the pleiotropic phenotype. Pictures show representative, genotyped embryos. Non-injected control embryos (NIC) were included for reference.
(B) Quantification of the embryos showing the typical *ptena-/- ptenb-/-* pleiotropic phenotype at 4dpf. In the non-injected control (NIC), approximately 25% of the embryos showed the characteristic phenotype (Mendelian segregation). Only WT Ptenb-mCherry rescued the pleiotropic *ptena-/-ptenb-/-* phenotype significantly at 4dpf. The statistical significance of each of the conditions compared to the non-injected control was determined using two-tailed Fisher's exact test and is indicated in the bar graph (ns = not significant, * = p-value < 0,05, ** = p-value < 0,01, ***= p-value < 0,001).

Pten lipid phosphatase activity is sufficient to rescue the hyperbranching vasculature phenotype of *ptena–/– ptenb–/–* zebrafish embryos

Morphological defects in *ptena–/–ptenb–/–* mutants develop relatively late during embryonic development, at 4dpf. The mCherry signal is already hard to detect at this stage, indicating that a big proportion of the injected mRNA might already have been degraded. Therefore, we decided to focus on a feature of the phenotype that emerges earlier during development: the hyperbranching of the vasculature at 3dpf, which we imaged using a *kdrl:eGFP* transgenic zebrafish line [43]. The characteristic trait of this phenotype is the correct development of the intersegmental vessels (ISV), which subsequently produce excessive sprouting, starting at around 70–72 hpf, and quickly evolve into a branch-like meshwork that intrudes into the somite tissue (**Fig 3**; NIC). Like the pleiotropic phenotype at 4dpf, hyperbranching of the vasculature was largely rescued by re-introduction of Pten [43] (**Fig 3**). We microinjected Ptenb WT, Ptenb C124S, Ptenb G129E and Ptenb Y138L or a combination of Ptenb G129E and Y138L RNA in zebrafish eggs of a *ptena+/–ptenb–/–* incross with transgenic Tg(*kdrl:eGFP*) background, at the one-cell stage. At 3 dpf, the embryos were subjected to confocal *in vivo* imaging of the vasculature (**Figs 3** and **4**). The Z-projections of the stacks were analyzed for the occurrence of the hyperbranching phenotype and accordingly categorized into either "normal" or "hyperbranching" phenotype for quantification and statistical analysis (**Fig 5**). Representative images of embryos of this experiment at 3dpf are shown (**Figs 3** and **4**). Both enzymes retaining lipid phosphatase activity, Ptenb WT (**Fig 3**) and Ptenb Y138L (**Fig 4**), were able to significantly suppress the hyperbranching vasculature phenotype at 3dpf (**Figs 3–5**). Microinjection of the other Ptenb mutants (**Fig 4**) that lack Pten lipid phosphatase activity, Ptenb C124S and Ptenb G129E, did not suppress excessive sprouting of the intersegmental vessels. Also, co-injection of Ptenb G129E and Ptenb Y138L each at half the normal dose did not significantly rescue the hyperbranching phenotype. This might be due to the fact that only half the amount of lipid phosphatase active Ptenb Y138L was injected compared to single injection of Ptenb Y138L. Therefore, we co-injected Ptenb G129E and Ptenb Y138L each at the normal dose. This, however, did not rescue the hyperbranching phenotype (**S1 Fig**), suggesting that Ptenb G129E might have a dominant-negative effect over Ptenb Y138L or that both phosphatase activities have to be present in the same molecule (see discussion).

From the z-stack images, we assessed the number of embryos showing hyperbranching of the intersegmental vessels at 3dpf and calculated the percentage of the observed phenotype according to the total number of embryos in each experimental condition (**Fig 5**). Again, as expected, the occurrence of the hyperbranching phenotype in the non-injected control is about 25% and can be significantly reduced by microinjection of

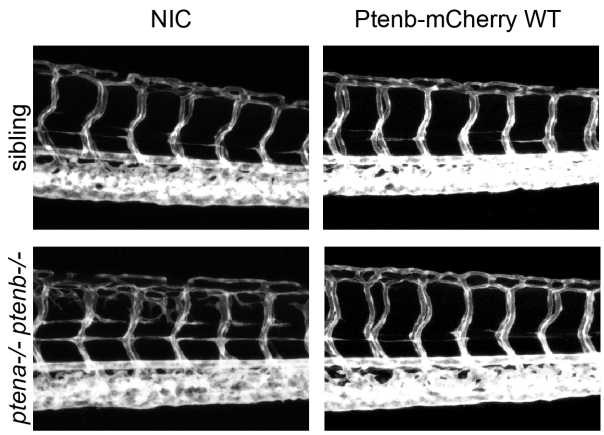


Fig 3. The hyperbranching vasculature phenotype, observed in *pten* double homozygous zebrafish embryos at 3dpf, can be rescued by wild type Pten. Zebrafish embryos from a Tg(*kdrl:eGFP*) *ptena+/–ptenb–/–* incross were microinjected at the one-cell stage with 300pg synthetic mRNA, encoding Ptenb-mCherry. At 3dpf the embryos were analyzed for the hyperbranching vessel phenotype by confocal live imaging on a Leica TCS-SPE microscope (anterior to the left, 20x objective, 2µm z-stacks). Subsequently, the embryos were genotyped. Pictures show the trunk region distal from the urogenital opening of representative embryos; non-injected control embryos (control) were included for reference.

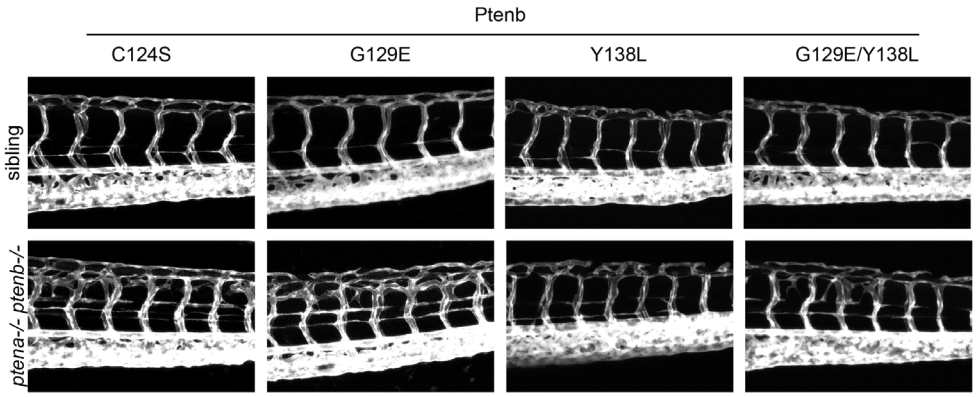


Fig 4. Pten lipid phosphatase activity is required to rescue the hyperbranching vasculature phenotype observed in *pten* double homozygous zebrafish embryos at 3dpf. Zebrafish embryos from a Tg(*kdrl:eGFP*) *ptena+/–ptenb–/–* incross were microinjected at the one-cell stage with 300 pg synthetic mRNA encoding Ptenb-mCherry WT (not shown here, see Fig 3) or with either Ptenb-mcherry C124S, Ptenb-mcherry Y138L, Ptenb-mCherry G129E or with 150 pg of each, Ptenb-eGFP G129E and Ptenb-mCherry Y138L. At 3dpf, the embryos were analyzed for the hyperbranching vessel phenotype by confocal live imaging on a Leica TCS-SPE microscope (anterior to the left, 20x objective, 2µm z-stacks). Pictures show the trunk region distal from the urogenital opening of representative genotyped embryos. Both Ptenb WT (shown in Fig 3) and Ptenb Y138L rescue the hyperbranching phenotype observed at 3dpf.

of Ptenb-mCherry WT, to about 5%. From the quantification of hyperbranching, it appears that there might be a slight difference between the rescue capacity of Ptenb WT (5% phenotype observed) and Ptenb Y138L (8% phenotype observed). However, comparing the two conditions directly to one another and performing two-tailed Fisher's exact test revealed that this difference is not significant. Neither are the differences between Ptenb G129E (20% phenotype observed) and Ptenb G129E/ Ptenb Y138L injection (27% phenotype observed), which further confirms the observation that co- microinjection of the two complementary phosphatase mutants did not restore a Ptenb wild-type-like rescue capacity.

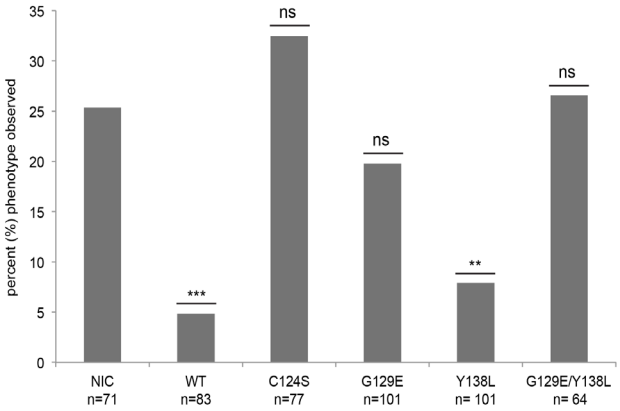


Fig 5. Only lipid phosphatase-active Pten rescues the hyperbranching vasculature phenotype at 3dpf. Quantification of the number of embryos showing the typical *ptena–/–ptenb–/–* hyperbranching intersegmental vessel phenotype at 3dpf. Both Ptenb-mCherry WT and Ptenb-mcherry Y138L rescue the characteristic *ptena–/–ptenb–/–* hyperbranching phenotype while the other phosphatase mutants do not. The statistical significance was determined using two-tailed Fisher's Exact test. (ns = not significant, * = p-value < 0,05, ** = p-value < 0,01, *** = p-value < 0,001).

Wild type Pten and Pten Y138L restore physiological pAkt levels in *ptena-/-ptenb-/-* double homozygous zebrafish embryos

To assess the ability of the different Ptenb phosphatase mutants to antagonize PI3K/Akt-signaling *in vivo*, the embryos were genotyped at 4dpf, and the phosphorylation of downstream Akt in each embryo was detected by immunoblotting (**Fig 6**). These data further confirmed that both lipid phosphatase active Ptenb, Ptenb WT and Ptenb Y138L, but none of the other phosphatase mutants were capable of decreasing pAkt levels *in vivo*. We conclude that there may be a causal link between the characteristic hyperbranching of intersegmental vessels at 3dpf in *ptena–/–ptenb–/–* embryos and elevated PIP3/pAkt signaling, which is consistent with the notion that Pten lipid phosphatase activity, but not protein phosphatase activity, is required to suppress the *ptena–/–ptenb–/–* hyperbranching phenotype.

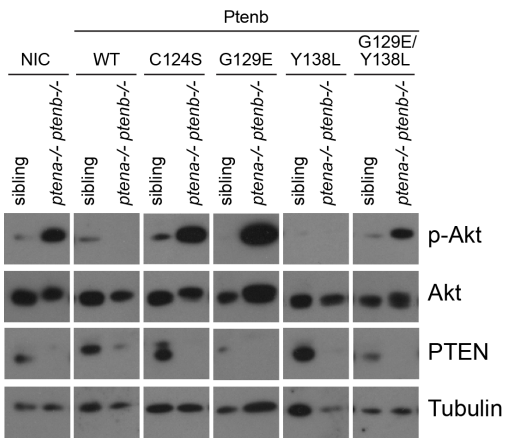


Fig 6. Only lipid phosphatase-active Pten restores normal pAkt levels at 4dpf. At 4dpf, single embryos from a *ptena+/–ptenb–/–* incross were cut in half. The trunk region was used for genotyping and the anterior half was lysed and processed for immunoblotting. Lysates from siblings and *ptena–/–ptenb–/–* embryos were run side by side on gels and blotted. The membranes were probed with phosphospecific anti-pAkt antibody (directed against pSer473), and subsequently sequentially stripped and probed with Akt-specific antibody, Tubulin-specific antibody as a loading control and PTEN-specific antibody, which only detects endogenous Ptena. The elevated pAkt levels in *pten* double homozygous embryos (see non-injected control) could be reduced to physiological levels by microinjection of either Ptenb WT or Ptenb Y138L RNA, indicating that both constructs have similar lipid phosphatase activities.

Discussion

So far, the contribution of the distinct phosphatase activities of PTEN to its cellular functions has been mainly studied *in vitro* [14, 20–25, 28, 29, 31] and the few *in vivo* approaches did not include the PTEN protein phosphatase deficient mutant PTEN Y138L [26, 30, 50]. In order to address whether Pten lipid and protein phosphatase activity are required for correct embryonic development, we investigated the capacity of Pten mutants to functionally rescue the pleiotropic phenotype of *ptena–/–ptenb–/–* embryos. We found that Pten lipid and protein phosphatase are both required for normal embryonic development at 4dpf and that lipid phosphatase activity is required for normal angiogenesis at 3dpf. Consistent with our results at 4dpf, *Tibarewal et al.* reported that in Matrigel *in vitro* assays with UM87G cells [29], only re-expression of wild type PTEN but of none of the PTEN phosphatase mutants, not even co-expression of PTEN G129E and PTEN Y138L, suppressed cell invasion in spite of the fact that PTEN Y138L restored normal pAkt levels. Based on these *in vitro* results and the finding that the main protein substrate of PTEN could be its own C-terminal tail, more specifically Thr366, *Tibarewal et al.* speculated that both phosphatase activities might have to be present on the same PTEN molecule in order to fulfill certain biological functions. In fact, they demonstrated that PTEN Y138L is hyperphosphorylated on Thr366, caused by its lack of protein phosphatase activity, and that mutation of this residue to alanine, T366A, restored the capacity of PTEN Y138L to suppress cell invasion [29]. Anyhow, we realized that in our experiments the mCherry signal was already quite difficult to detect at 4dpf, suggesting that at these relatively late developmental stages exogenous mRNA might have been degraded and hence the rescuing effect of Pten might be diminished. The limited lifespan of mRNA molecules *in vivo* is a common drawback of the microinjection technique and is the reason why achieving a long-lasting and complete rescue by microinjection of synthetic mRNA is virtually impossible.

To avoid the possible effects of RNA instability on the rescue capacity of Pten at later stages in development, we focused on an aspect of the *ptena–/–ptenb–/–* phenotype that emerges earlier during embryonic development: the characteristic hyperbranching of the intersegmental vessels at 3dpf [43]. Angiogenesis, the sprouting of new blood vessels from existing vasculature, is a highly regulated process during zebrafish embryonic development that requires a fine-tuned interplay between different signaling pathways, including Vegf signaling (vascular endothelial growth factor; artery and lymph vessel formation) and Bmp signaling (bone morphogenetic proteins; vein formation), as well as secondarily involved pathways, including for example Notch signaling, Semaphorin-Plexin signaling and the PI3K/Akt(PKB)/PTEN-axis. Upon binding of Vegfa to VEGFR2, the Vegfa receptor, PI3K signaling gets activated (as well as phospholipase Cγ1 and Src family kinases), promoting proliferation and differentiation of vascular

endothelial cells and thereby inducing angiogenesis [51–53]. The work of various groups previously unveiled that PTEN, the main antagonist of PI3K signaling, suppresses this pathway *in vitro* [54] and lack of PTEN has been associated with enhanced angiogenesis in gastric cancer patients [55]. We reported that *pten* double homozygous zebrafish develop an ectopic vascular hyperbranching phenotype [43] and that this phenotype is rescued either by re-introducing *pten* mRNA at the one-cell stage or by treating embryos with LY294002, a specific PI3K inhibitor, thereby proving that Pten suppresses angiogenesis *in vivo* mainly via decreasing phosphorylated Akt levels.

We were intrigued to find out whether the lipid phosphatase activity of Pten is sufficient to rescue the hyperbranching vessel phenotype or whether Pten requires both phosphatase activities in order to suppress angiogenensis. Our results revealed that wild type Ptenb and Ptenb Y138L, both possessing lipid phosphatase activity, were the only Ptenb constructs that significantly prevented the development of hyperbranching trunk vasculature in the double homozygous *pten* embryos at 3dpf. This indicates that Pten lipid phosphatase activity is required to suppress angiogenesis *in vivo*.

Detection of pAkt levels at 4dpf by immunoblotting further confirmed that both Ptenb WT and Ptenb Y138L, are capable of restoring physiological pAkt levels at this developmental stage. Immunoblotting suggested that Ptenb Y138L was more active than Ptenb WT, since it also suppressed pAkt levels in the microinjected siblings. It would be interesting to address this question in further studies. To our surprise, co-microinjection of Ptenb-eGFP G129E and Ptenb-mCherry Y138L did not significantly rescue the hyperbranching vessel phenotype at 3dpf, whereas microinjection of Ptenb-mCherry Y138L by itself did rescue. This may be due to a dominant negative effect of Ptenb G129E on Ptenb Y138L, for example by dimerization [26]. *In vivo* studies in mice revealed that both mutations of Pten that abolish lipid phosphatase activity, C124S and G129E, have a dominant negative effect over wild type Pten in a heterozygous setting (*Pten*$^{+/C124S}$ or *Pten*$^{+/G129E}$), rendering mice with an amplified tumor spectrum compared to mice affected by Pten loss of heterozygosity (Pten$^{+/-}$), an effect that can be explained by PTEN heterodimerization [26]. However, another explanation for the observed lack of a full rescue upon co-expression is that both phosphatase activities may need to be present in the same Pten molecule. Complete PTEN phosphatase functionality seems to be especially important to control complex biological processes such as invasion [29] and embryonic development.

We further believe that, as proposed by *Tibarewal et al.*, certain biological functions of PTEN could either depend on lipid phosphatase-independent mechanisms, exerted by protein-protein interactions, or on lipid phosphatase activity of PTEN towards a specific spatial PIP3 pool that does not correlate with pAkt levels, as for example membrane ruffling and cell polarity [29]. This hypothesis would also explain why the Y138C mutation of PTEN has been positively selected for in the metastatic small cell lung cancer cell line

NCI-H196 [29, 34, 41, 56, 57]. *Lyu et al.* recently reported that PTEN protein phosphatase activity but not lipid phosphatase activity is essential for the regulation of neuronal progenitor cell differentiation and that PTEN likely exerts its protein phosphatase-dependent function by dephosphorylating CREB [31]. Ultimately, cell motility, invasion, cell polarity and differentiation are all crucial processes that have to be highly regulated for correct embryonic development and therefore it is not surprising that some features of the *pten* double homozygous phenotype are controlled by PI3K/Akt-independent PTEN functions. Our results indicate that the role of Pten in antagonizing angiogenesis signaling is crucially dependent on its ability to dephosphorylate PIP3, whereas its role in zebrafish embryonic development likely depends on additional functions of Pten that are independent from suppressing the PI3K/Akt-signaling axis.

Materials and Methods

Ethics statement

All procedures involving experimental animals described in this manuscript were approved by the local animal experiments committee (Koninklijke Nederlandse Akademie van Wetenschappen-Dierexperimenten commissie protocol HL05.1501) and performed according to local guidelines and policies in compliance with national and European law.

Fish line

Zebrafish were maintained and the embryos were staged as previously described [46]. The lines Tg(*kdrl:eGFP*) and *ptena*–/–, as well as *ptenb* –/– were previously described [9, 47, 48].

Constructs, mRNA synthesis and Microinjections

The Ptenb-mCherry and Ptenb-eGFP construct were obtained by amplification of *ptenb* from the vectors described in [9]. The PCR-product and the pCS2+mCherry or pCS2+eGFP vectors were digested with BglII and *ptenb* was subsequently ligated into pCS2+mCherry and pCS2+eGFP. The point mutations in the PTP domain were introduced by site-directed mutagenesis. The constructs were linearized with NotI and to synthetize 5' capped sense RNA, the mMessage mMachine SP6 kit (Ambion) was used. mRNA injections were performed at the one-cell stage as described using a total of 300 pg of mRNA.

Lysis

Zebrafish embryos at 4dpf were anesthetized with 16mg/ml 3-amino benzoic acid ethylesther (MS-222) and cut in half (just below the yolk sack extension). The tail was lysed for genotyping and the head/trunk region was subjected to a protein lysis protocol for subsequent immunoblotting (see below).

Immunoblotting

Zebrafish embryos were lysed at 4dpf in 25mM HEPES (pH7,4), 125mM NaCl, 0,25% Deoxycholate, 10mM MgCl2, 1mM EDTA, 1% Triton X-100, 10% Glycerol buffer containing proteinase and phosphatase inhibitors. Samples were lysed for 30min and subsequently subjected to sonication with the Bioruptor (settings: high intensity, 15min, 1min on/off cycles). Samples were run on a 10% SDS-PAGE gel, transferred to a PVDF membrane and stained with Coomassie Blue to verify equal loading. The blots were probed with antibodies specific to pAkt (1:2000), Akt (1:1000) and PTEN (1:1000); (all Cell Signaling) and Tubulin (1:3000); (Calbiochem). For signal detection, enhanced chemiluminescence (Thermo Scientific kit) was diluted 1:2 in home- made ECL.

Confocal microscopy and Analysis

Zebrafish embryos were anesthetized at 3dpf and laterally mounted on glass bottom dishes (Greiner bio one) in 0,5% agarose (type V, Sigma Aldrich) in E3 embryo medium containing 16mg/ml 3-amino benzoic acid ethylesther (MS-222) to block contractile movements. Confocal microscopy was performed using a Leica TCS SPE, 20x objective. Z-stacks (step size 2µm) of the vasculature in the tail region, right below the yolk sack extension, were acquired for every embryo at 28.5°C. Image J software (http://rsb.info.nih.gov./ij/) was used to generate z- projection images of the zebrafish embryonic vasculature.

Statistics

Significance of the frequency of occurrence of the "hyperbranching" vasculature phenotype in the different experimental conditions compared to the non-injected control was assessed using two-tailed Fisher's exact test. Results were considered significant when $p<0.05$ (p values * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Acknowledgements

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Author Contributions

Conceived and designed the experiments: MS JdH. Performed the experiments: MS. Analyzed the data: MS JdH. Contributed reagents/materials/analysis tools: MS JdH. Wrote the paper: MS JdH.

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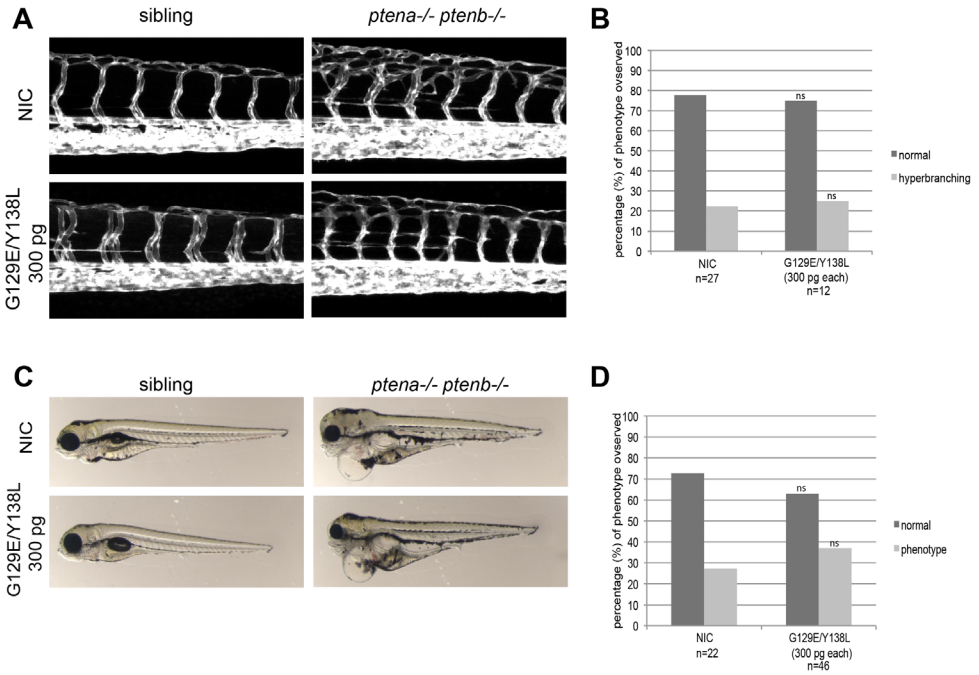
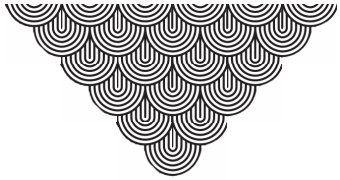
Supporting information



S1 Fig. Co-microinjection of Ptenb-mCherry G129E and Ptenb-mCherry Y138L did not rescue developmental defects in *ptena-/-ptenb-/-* zebrafish embryos.
(A) Zebrafish embryos from a Tg(*kdrl:eGFP*) *ptena*+/–*ptenb*–/– incross were microinjected at the one-cell stage with mRNA encoding Ptenb-mCherry G129E and Ptenb-mCherry Y138L (300 pg each). At 3dpf the embryos were analyzed for the hyperbranching vessel phenotype by confocal live imaging on a Leica TCS-SPE microscope (anterior to the left, 20x objective, 2µm z-stacks). Subsequently, the embryos were genotyped. Pictures show the trunk region distal from the urogenital opening of representative embryos; non-injected control embryos (NIC) were included for reference. **(B)** Quantification of the number of embryos showing the typical *ptena*–/–*ptenb*–/– hyperbranching intersegmental vessel phenotype at 3dpf. Two-tailed Fisher's Exact test indicated no statistically significant difference between NIC and G129E/Y138L-injected embryos. **(C)** The morphology of NIC embryos and G129E/Y138L-injected embryos was assessed at 4dpf by brightfield microscopy. Pictures show representative, genotyped embryos. Non-injected control embryos (NIC) were included for reference. **(D)** Quantification of the embryos showing the typical *ptena*–/–*ptenb*–/– pleiptropic phenotype at 4dpf. Two-tailed Fisher's Exact test indicated no statistically significant difference between NIC and G129E/Y138L-injected embryos.



6

Fine-tuning of Pten localization and phosphatase activity is essential for zebrafish angiogenesis

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Abstract

The lipid- and protein phosphatase PTEN is an essential tumor suppressor that is highly conserved among all higher eukaryotes. As an antagonist of the PI3K/Akt cell survival and proliferation pathway, it exerts its most prominent function at the cell membrane, but (PIP3-independent) functions of nuclear PTEN have been discovered as well. PTEN subcellular localization is tightly controlled by its protein conformation. In the closed conformation, PTEN localizes predominantly to the cytoplasm. Opening up of the conformation of PTEN exposes N-terminal and C-terminal regions of the protein that are required for both interaction with the cell membrane and translocation to the nucleus. Lack of Pten leads to hyperbranching of the intersegmental vessels during zebrafish embryogenesis, which is rescued by expression of exogenous Pten. Here, we observed that expression of mutant PTEN with an open conformation rescued the hyperbranching phenotype in *pten* double homozygous embryos and suppressed the increased p-Akt levels that are characteristic for embryos lacking Pten. In addition, in *pten* mutant and wild type embryos alike, open conformation PTEN induced stalled intersegmental vessels, which fail to connect with the dorsal longitudinal anastomotic vessel. Functional hyperactivity of open conformation PTEN in comparison to wild type PTEN seems to result predominantly from its enhanced recruitment to the cell membrane. Enhanced recruitment of phosphatase inactive mutants to the membrane did not induce the stalled vessel phenotype nor did it rescue the hyperbranching phenotype in *pten* double homozygous embryos, indicating that PTEN phosphatase activity is indispensable for its regulatory function during angiogenesis. Taken together, our data suggest that PTEN phosphatase activity needs to be carefully fine-tuned for normal embryogenesis and that the control of its subcellular localization is a key mechanism in this process.

Introduction

PTEN is a major tumor suppressor that antagonizes the PI3K/Akt (also known as protein kinase B, PKB) pro-survival and -proliferation signaling pathway by dephosphorylating the lipid second messenger phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3) to phosphatidylinositol (4,5)-bisphosphate (PIP2) [1]. In many cancer types, one or multiple components of this pathway can be found mutated. Upon loss of PTEN function, enhanced PIP3 signaling potentially drives cells into excessive proliferation and tumorigenesis by enhanced activation of the downstream effector, Akt [2,3]. Dephosphorylation of PIP3 is an event that occurs upon binding of PTEN to phospholipid-rich areas at the inner cell membrane [1,4]. There are other functions of PTEN, such as the regulation of DNA damage repair, genome stability, apoptosis, cell cycle progression or autophagy, that have been attributed to its nuclear localization [5-11].

During the past decade, it has become evident that controlling PTEN protein conformation and thereby its subcellular localization is a key regulatory mechanism to manage the variety of PTEN cellular functions. In its closed conformation, PTEN is predominantly cytoplasmic, while opening up its conformation results in re-location of PTEN to the cell membrane and the nucleus [8,12-15]. The conformational status of PTEN is predominantly regulated by post-translational modifications, especially by (de)-phosphorylation of the C-terminal tail, which alters the intramolecular interactions within PTEN [13,14,16,17]. PTEN is phosphorylated by multiple kinases, including GSK3, CK2 and ATM [11,18-20]. In the context of conformational changes, especially the phosphorylation by CK2 is an important event [19]. Phosphorylation on S370, S380, T382, T383 and S385 renders PTEN in its closed conformation, having the C-terminus folded back onto the N-terminus. Upon dephosphorylation of PTEN at these positions, the molecule adopts the open conformation, in which newly exposed side chains and amino acid motifs relocalize PTEN to either the cell membrane or the nucleus, depending on the cellular context [12,15,21]. Mutation of these five phosphorylation sites to alanines (S370A, S380A, T382A, T383A and S385A) mimics constitutive dephosphorylation at these sites and renders a PTEN mutant termed PTEN QMA or 'open conformation PTEN' [19,22,23]. One of the motifs that is potentially exposed upon opening of the PTEN protein conformation is the positively charged lysine-arginine-arginine (KRR) cluster within the N-terminus, which is involved in regulation of PTEN nuclear localization [24,25]. It has been shown that mono-ubiquitination of lysines within the N- and C- terminal tail, especially lysine 13, K13, regulates nuclear translocation of PTEN [26]. However, the regulation and mechanism of PTEN nuclear translocation as well as the identity of nuclear targets of PTEN remains to be determined definitively.

In this study, we investigated the functional *in vivo* consequences of expressing open conformation PTEN. We chose zebrafish as a model organism because of its high fecun-

dity and its fantastic suitability for live cell imaging. Further, we have generated *pten* knockout zebrafish lines that facilitate functional characterization of Pten mutants. Like many other genes, *pten* has been duplicated during teleost evolution, resulting in two *pten* genes with redundant functions in zebrafish, *ptena* and *ptenb* [27,28]. By target selected gene inactivation (TSGI), we identified nonsense mutations in both *pten* genes, each resulting in a premature stop codon. We generated *ptena-/-* and *ptenb-/-* fish lines [28] that we incrossed to obtain *ptena*+/–*ptenb*–/– and *ptena*–/–*ptenb*+/– fish lines for our functional rescue assay. While their heterozygous and single homozygous siblings are viable and fertile, double homozygous embryos that lack all Pten activity develop a pleiotropic phenotype, characterized by massive edema formation, craniofacial defects, aberrant pigmentation and shorter body axis. Double homozygous *pten* mutant embryos are embryonic lethal around 5 days post fertilization (dpf) [28]. We previously studied the role of Pten in zebrafish angiogenesis and found that Pten is required for cell proliferation and migration during the formation of new blood vessels from the existing vasculature [29]. Zebrafish embryos that lack functional Pten are characterized by constitutively elevated pAkt and *vegfaa* levels, accompanied by enhanced angiogenesis that can be visualized from 3dpf onwards using confocal microscopy in *ptena*–/–*ptenb* –/– zebrafish embryos in the transgenic Tg(*kdrl:eGFP*) background [29,30]. Treatment with the PI3K-specific inhibitor LY294002, as well as microinjection of synthetic *pten* mRNA rescued the vasculature hyperbranching phenotype [29]. Recently, we reported that Pten lipid phosphatase activity is required for normal angiogenesis, whereas the complex process of embryonic development requires both lipid and protein phosphatase activity of Pten [31]. Here, we decided to screen functional mutants of Pten for their ability to rescue angiogenesis defects in Tg(*kdrl:eGFP*) zebrafish embryos that lack functional Pten.

PTEN is an important antagonist of pro-angiogenic signaling and likely exerts its role both in a cell autonomous way, activated by upstream Dll4/Notch signaling in stalk cells at the sprouting front to control cell proliferation [32], and in a non-autonomous way by suppressing *vegfaa* levels [29] in the surrounding tissue. Angiogenesis during embryonic development underlies the same, evolutionary conserved signaling pathways, as angiogenesis that is induced in adult ischemic tissues or by emerging tumors [33-35]. This is why PTEN is essentially important to suppress the angiogenic switch, a requisite for solid tumor growth and why lack of PTEN is associated with increased microvascular density in glioblastoma models [36] or gastric cancer biopsies [37]. Thus, a detailed understanding of how and when PTEN activity is fine-tuned during embryonic angiogenesis might potentially give new insights into its deregulation and therapeutic potential during tumor angiogenesis.

In this study, we investigated the rescue capacity of open conformation PTEN and found that its expression in *pten* double homozygous embryos rescued the characteristic

hyperbranching vasculature phenotype and the increased levels of pAkt. Surprisingly, we also observed a significantly increased number of embryos exhibiting stalled intersegmental vessels that failed to connect with the dorsal longitudinal anastomotic vessel (DLAV). This phenotype was not observed upon microinjection of an equal dose of wild type PTEN mRNA. Further experiments suggest that specifically the enhanced membrane localization of PTEN induced the stalled vessels defect. We demonstrate that enhanced membrane localization of phosphatase active PTEN dramatically increased its biological function in suppression of angiogenic sprouting. Tight temporal and spatial regulation of PTEN activity during angiogenesis may therefore play an essential role.

Results

Open conformation of PTEN enhances both its membrane and nuclear localization *in vivo*

Previously, we have used the ability of Pten mutants to rescue the hyperbranching phenotype in zebrafish embryos lacking endogenous Pten as a read-out for Pten function [31]. Modulation of the conformation of PTEN is an established regulatory mechanism. Here, we investigated the consequences of expressing mutant PTEN with constitutively open conformation in live zebrafish embryos. Wild type zebrafish Ptenb-mCherry and phosphatase-inactive mutant Ptenb-mCherry C124S (**Fig 1A**) were used as positive and negative controls, respectively. Constructs encoding human PTEN-mCherry, which is highly homologous (81%) to zebrafish Ptenb, and open conformation PTEN, PTEN QMA-mCherry, were derived (**Fig 1A**). PTEN QMA contains five mutations of phosphorylation sites (S370A, S380A, T382A, T383A, S385A). All five phosphorylation sites and surrounding sequences are absolutely conserved in zebrafish Ptena as well as Ptenb. Nevertheless, we chose to use human PTEN and PTEN QMA for these experiments, to exclude the possibility that the conformational change in PTEN QMA was not reproduced in mutant zebrafish Ptenb with the homologous five substitutions, due to small differences in the sequence of Ptenb away from the five phosphorylation sites.

To characterize and compare subcellular localizations of the zebrafish and human PTEN-mCherry fusions, we microinjected Tg(*H2A-eGFP*) zebrafish embryos at the one-cell stage with synthetic mRNA encoding the above mentioned constructs and performed confocal live imaging on the zebrafish embryos at 6hpf (**Fig 1B**). Ptenb-mCherry and PTEN-mCherry both localized predominantly to the cytoplasm with only weak fluorescence in the nucleus. Ptenb-mCherry C124S was evenly distributed over cytoplasm and nucleus and showed clearly enhanced membrane localization. PTEN QMA-mCherry localized away from the cytoplasm, towards the cell membrane and to the nucleus, consistent with the open conformation of PTEN QMA.

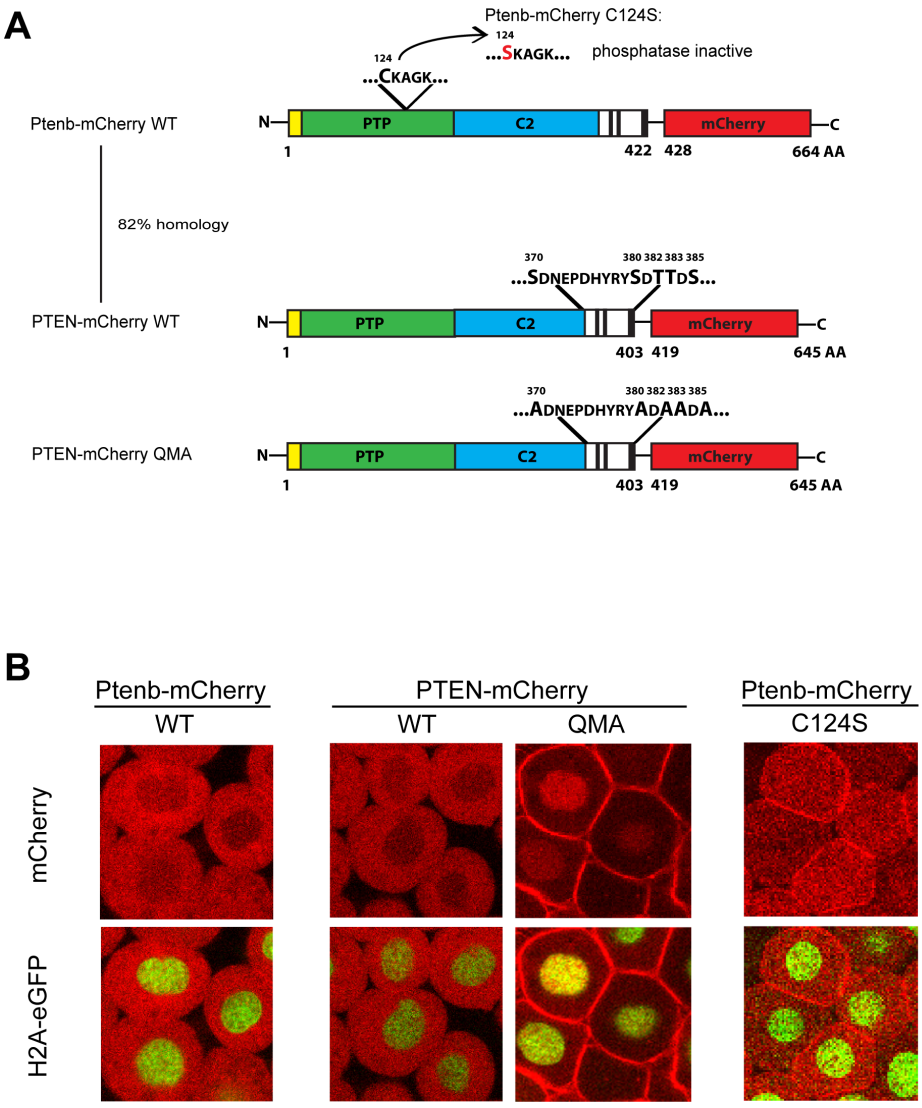

Fig 1. PTEN QMA localizes to the cell membrane and nucleus in zebrafish embryos.
(A) Wild type Ptenb (long splicing variant, 422 amino acids) and human PTEN share a homology of 82%, both consisting of an N-Terminus (yellow), the PTP-domain (green), the C2 domain (blue) and the C-terminus (black and white). Mutation of the catalytic cysteine 124 to serine, C124S, results in catalytically inactive Ptenb. PTEN QMA contains five point mutations in its C-terminal phosphorylation sites, resulting in an open conformation of PTEN [19]. The red fluorescent protein mCherry (red) is C-terminally tagged in frame to all constructs used in these experiments. **(B)** Subcellular localization of Ptenb-mCherry, PTEN-mCherry, PTEN-mCherry QMA and Ptenb-mCherry C124S was assessed in tg(*H2A-eGFP*) zebrafish embryos, microinjected with 300pg synthetic mRNA at the one-cell stage and submitted to confocal live imaging at 6hpf. Top panels: mCherry expression; bottom panels: overlay of mCherry expression with H2A-GFP, which localizes to the nucleus.

PTEN QMA rescues hyperbranching in *pten* mutants and induces stalled intersegmental vessels

Angiogenesis, the formation of new blood vessels from the existing vasculature, is a process tightly controlled by PI3K signaling and therefore a good read-out to evaluate the rescue capacity of PTEN mutants in *pten* double homozygous zebrafish embryos. In order to functionally characterize PTEN and PTEN QMA in angiogenesis, we microinjected embryos from a Tg(*kdrl:eGFP*) *ptena*+/–*ptenb*–/– incross at the one-cell stage with synthetic mRNA, encoding Ptenb-mCherry, PTEN-mCherry or PTEN-mCherry QMA. At 3dpf, we monitored the vasculature by confocal live imaging (**Fig 2A-2J**). According to Mendelian law, 25% of the offspring of a *ptena*+/–*ptenb*–/– incross was expected to be double homozygous and develop the hyperbranching phenotype (**Fig 2K**). This percentage was reflected in the non-injected control (NIC) embryos. Expression of Ptenb, PTEN or PTEN QMA significantly decreased the percentage of embryos with the characteristic hyperbranching phenotype (**Fig 2D, 2F, 2H** and **2K**), whereas the phosphatase inactive Ptenb C124S did not have this effect (**Fig 2J** and **2K**). Surprisingly, we discovered a second phenotype in the *ptena*–/–*ptenb*–/– embryos and siblings expressing PTEN QMA (**Fig 2G'**). This phenotype consisted of the occurrence of intersegmental vessels (ISV) that did not connect to the dorsal longitudinal anastomotic vessel (DLAV) but instead stalled somewhere along the way. This stalled vessel phenotype might be caused by excessive activity of PTEN towards PIP3, leading to diminished VEGFR signaling. In order to test this hypothesis, we assessed the levels of phosphorylated Akt, p-Akt (**Fig 2L**). Our data confirmed that PTEN QMA, as well as PTEN WT, reduced the elevated p-Akt levels in 4dpf *ptena*–/–*ptenb*–/– embryos at least as effectively as Ptenb WT. Ptenb C124S lacks phosphatase activity and did not reduce p-Akt levels.

Lysine 13 mutations modulate Ptenb subcellular localization

In all other experimental conditions, the number of fish displaying the stalled vessel phenotype was remarkably low, indicating that it is specifically caused by microinjection of PTEN in its open conformation, PTEN QMA. Next, we investigated whether it was rather the nuclear localization or the membrane accumulation of open conformation PTEN that induced the stalled vessel phenotype and whether the phosphatase activity of PTEN played a crucial role in this process. Some mutations in the N-terminus of Pten, such as mutations of the putative mono-ubiquitination site lysine 13 (K13) (**Fig 3A**), alter its subcellular localization and shift it either towards the cell membrane (Ptenb-mCherry K13R, Ptenb-mCherry K13A) or to the nucleus (Ptenb-mCherry K13E) (**Fig 3B**). We assessed the altered subcellular localization of these constructs by microinjecting Tg(*H2A-eGFP*) zebrafish embryos with the synthetic mRNAs at the one-cell stage and at 6hpf, the em-

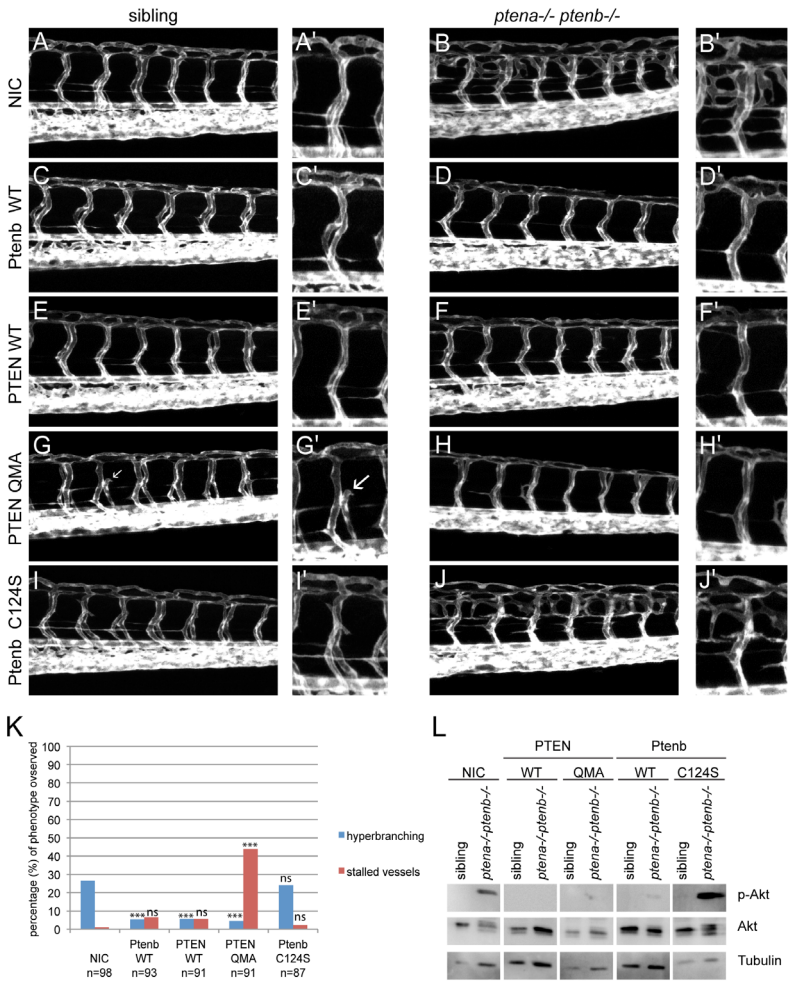

Fig 2. Ptenb, PTEN and PTEN QMA, but not Ptenb C124S rescued the hyperbranching vasculature phenotype.
(A-J) Zebrafish embryos from a tg(*kdrl:eGFP*) *ptena*+/– *ptenb*–/– incross were microinjected at the one-cell stage with 300 pg synthetic mRNA encoding wild type PTEN-mCherry, PTEN-mCherry QMA, Ptenb-mCherry WT or Ptenb-mCherry C124S. At 3dpf the embryos were analyzed for the hyperbranching vessel phenotype by confocal live- imaging on a Leica TCS-SPE microscope (anterior to the left, 20x objective, 2µm z-stacks). Pictures show the trunk region distal from the urogenital opening of representative, genotyped embryos. Non-injected control embryos (control) were included for reference. **(A'-J')** A close-up is added on the right side of each image. **(G)** Remarkably, we found a second phenotype in the PTEN-mCherry QMA injected embryos, consisting of a significantly increased number of lacking or stalled intersegmental vessels (indicated with white arrows). **(K)** Quantification of the embryos showing the typical *ptena*–/–*ptenb*–/– hyperbranching vessel phenotype at 3dpf (blue bars). In the non-injected control (NIC), approximately 25% of the embryos showed the characteristic phenotype (Mendelian segregation). The percentage of embryos showing the stalled vessel phenotype at 3dpf is also indicated (red bars). The statistical significance of each of the conditions compared to the non-injected control was determined using two-tailed Fisher's exact test and is indicated in the bar graph (ns = not significant, * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001).
(L) Embryos from a *ptena*+/–*ptenb*–/– incross were microinjected at the one-cell stage with wild type PTEN, PTEN QMA, wild type Ptenb or Ptenb C124S. At 4dpf, single embryos were cut in half. The trunk region was used for genotyping and the anterior half was lysed and processed for immunoblotting. Lysates from siblings and *ptena*–/–*ptenb*–/– embryos were run side by side on gels and blotted. The membranes were probed with phosphospecific anti-pAkt antibody (directed against pSer473), stripped and probed with Akt-specific antibody, stripped and probed with a Tubulin-specific antibody as a loading control. Representative blots are shown.

bryos were screened for Pten subcellular localization by confocal live imaging. The charge conserving mutation of Ptenb-mCherry K13R enhanced the accumulation of Ptenb at the cell membrane. The same localization pattern was observed for the positive to neutral charge mutation of Ptenb-mCherry K13A. Mutation of positively charged lysine 13 to negatively charged glutamic acid, Ptenb-mCherry K13E, resulted in accumulation in the nucleus (**Fig 3B**). These Ptenb mutants allowed us to assess whether subcellular localization affected Pten function.

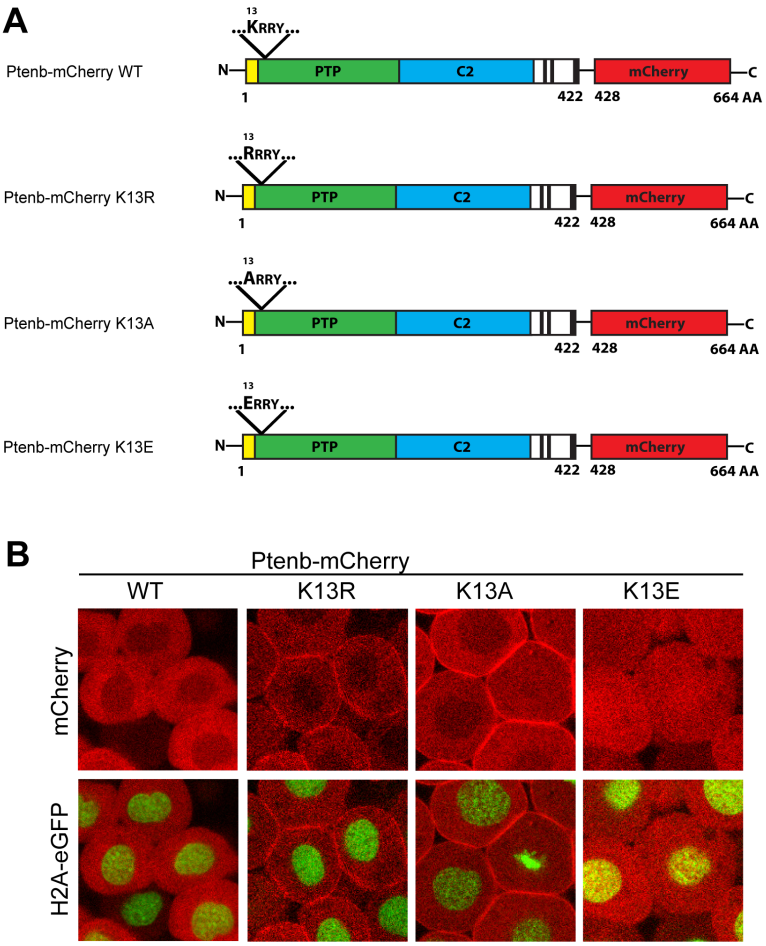

Fig 3. Mutation of Pten K13 alters Pten subcellular localization in 6hpf zebrafish embryos.
(A) Point mutations were introduced into the N-terminus of Ptenb (long splicing variant, 422 amino acids) via site-directed mutagenesis. K13 was replaced by either arginine (K13R, charge conserving), alanine (K13A, change to neutral charge) or glutamate (K13E, charge inverting) to address the importance of lysine 13 for the subcellular localization of Pten. The red fluorescent protein mCherry (red) is C-terminally tagged in frame to all constructs used in these experiments. **(B)** Subcellular localization of Ptenb-mCherry K13R, K13A and K13E was assessed in tg(*H2A-eGFP*) zebrafish embryos, microinjected with 300pg synthetic mRNA and submitted to confocal live imaging at 6hpf. Top panels: mCherry expression; bottom panels: overlay of mCherry expression with H2A-GFP, which localizes to the nucleus.

Accumulation of phosphatase-active Pten at the membrane induces stalled intersegmental vessels

We investigated whether subcellular localization of Ptenb affected the rescue capacity of the hyperbranching defect on the one hand and induced stalled vessels on the other. To this end, we microinjected Tg(*kdrl:eGFP*) *ptena*+/–*ptenb*–/– incross embryos at the one-cell stage with synthetic mRNA, encoding Ptenb WT, Ptenb K13R, K13A or K13E. At 3dpf, we analyzed the embryonic vasculature by confocal live imaging (**Fig 4A-4J**). We detected the characteristic hyperbranching vessel phenotype in about 25% of the Ptenb-mCherry K13E and Ptenb-mCherry K13A injected embryos, like in the non-injected control (NIC) embryos (**Fig 4K**). This finding is consistent with published data that PTEN K13E and K13A mutants exhibit diminished or even abolished phosphatase activity [12]. Ptenb-mCherry K13R on the other hand significantly rescued the hyperbranching phenotype of *pten* double homozygous embryos (**Fig 4F** and **4K**). Furthermore, similar to the PTEN QMA injected embryos (**Fig 2G**), we observed a dramatic increase of the amount of stalled vessel phenotypes in the Ptenb-mCherry K13R injected embryos. Besides Ptenb-mCherry K13R, none of the other lysine 13 mutants significantly induced the stalled vessel phenotype.

At 4dpf, the embryos were genotyped, lysed for immunoblotting and probed for p-Akt-levels (**Fig 4L**). Ptenb-mCherry WT, as well as Ptenb-mCherry K13R drastically decreased p-Akt levels in *ptena*–/–*ptenb*–/– zebrafish embryos, whereas neither Ptenb-mCherry K13A or K13E had this effect. A trivial explanation for the lack of effects of Ptenb K13A and K13E might be that these proteins are not stable and are rapidly degraded. Unfortunately, due to low expression levels of all mutant Pten-mCherry proteins, we were not able to monitor protein expression in the zebrafish embryos. However, transfection of the constructs encoding the (mutant) Pten proteins in human embryonic kidney 293 cells revealed similar expression levels of all mutant Pten proteins, suggesting that there are no big differences in protein stability (**S1 Fig**). Therefore, we infer in concordance with data obtained for human PTEN [12], that zebrafish Ptenb K13E and K13A both significantly lack phosphatase activity towards phospholipids. The charge conserving K13R mutation, which caused enhanced accumulation of Ptenb at the cell membrane but not in the nucleus, induced stalled vessels with a similar incidence as PTEN QMA. Increased accumulation of phosphatase inactive Pten at the cell membrane, such as Ptenb-mCherry C124S or K13A, however, did not result in this phenotype. Altogether this led us to conclude that it is the excessive membrane accumulation of phosphatase active PTEN/Ptenb that caused the stalled intersegmental vessels during angiogenesis.

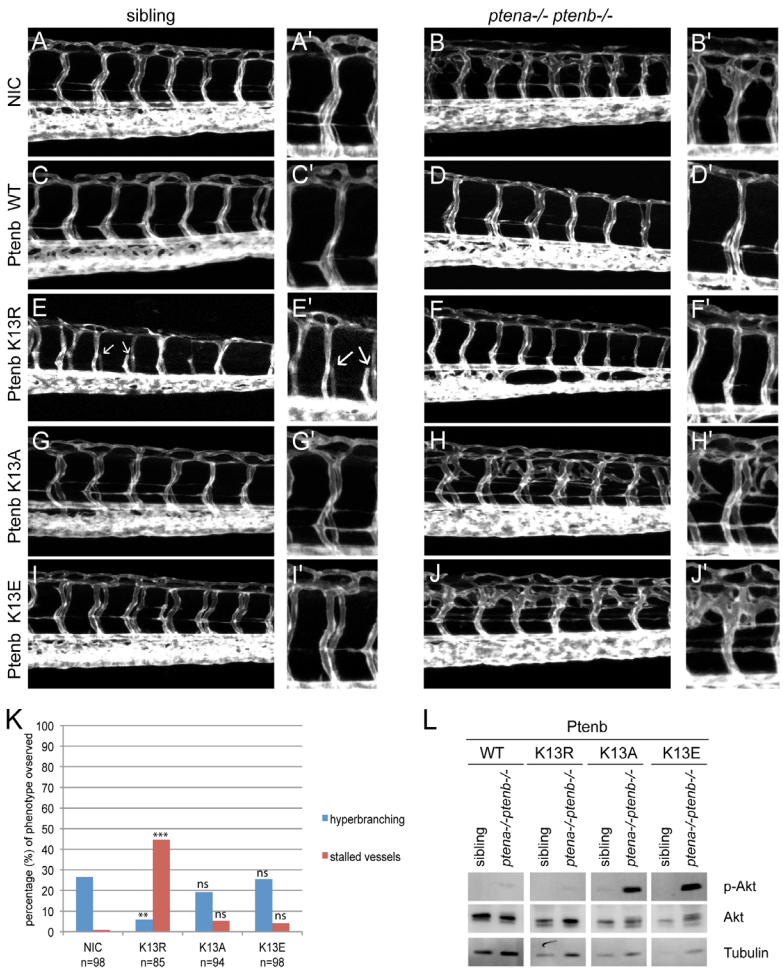

Fig 4. Enhanced membrane accumulation of phosphatase-active Pten induces stalled intersegmental vessels. **(A-J)** Zebrafish embryos from a tg(*kdrl:eGFP*) *ptena*+/–*ptenb*–/– incross were microinjected at the one-cell stage with 300 pg synthetic mRNA encoding either Ptenb-mCherry WT, K13R, K13A or K13E. At 3dpf the embryos were analyzed for the hyperbranching vessel phenotype by confocal live imaging on a Leica TCS-SPE microscope (anterior to the left, 20x objective, 2µm z-stacks). Pictures show the trunk region distal from the urogenital opening of representative, genotyped embryos. Non-injected control embryos (NIC) were included for reference. **(A'-J')** A close-up is added on the right side of each image. Stalled vessels are indicated with white arrows. **(K)** Quantification of the embryos expressing the indicated Ptenb mutants, showing the typical *ptena*–/–*ptenb*–/– hyperbranching vessel phenotype (blue bars) or the stalled vessel phenotype (red bars) at 3dpf. In the non-injected control (NIC), approximately 25% of the embryos showed the characteristic hyperbranching phenotype (Mendelian segregation). The statistical significance of each of the conditions compared to the non-injected control was determined using two-tailed Fisher's exact test and is indicated in the bar graph (ns = not significant, * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001). **(L)** Embryos from a *ptena*+/–*ptenb*–/– incross were microinjected at the one-cell stage with wild type Ptenb or Ptenb K13 mutants as indicated. At 4dpf, single embryos were cut in half. The trunk region was used for genotyping and the anterior half was lysed and processed for immunoblotting. Lysates from siblings and *ptena*–/–*ptenb*–/– embryos were run side by side on gels and blotted. The membranes were probed with phosphospecific anti-pAkt antibody (directed against pSer473), stripped and probed with Akt-specific antibody, stripped and probed with Tubulin-specific antibody as a loading control. Representative blots are shown.

Dominant effects of Ptenb K13R and PTEN QMA on angiogenesis in wild type fish

To address the question whether excessive membrane accumulation of phosphatase active PTEN might induce angiogenesis defects in *pten* wild type fish, we microinjected Tg(*kdrl:eGFP*) embryos at the one-cell stage with synthetic RNA encoding PTEN-mCherry, PTEN-mCherry QMA or Ptenb-mCherry, K13R, K13A or K13E and subsequently performed confocal live imaging of their trunk vasculature (**Fig 5A**). As expected, the non-injected control (NIC) embryos did not display any of the two vasculature phenotypes, whereas microinjection of synthetic Pten mRNA occasionally induced stalled vessels in a small subset of embryos (around 3-5%). This background however is not statistically significant, thus neither Ptenb-mCherry, or Ptenb-mCherry K13E or K13A induced significant angiogenesis defects. PTEN QMA and Ptenb K13R, both with enhanced membrane localization, on the other hand induced stalled or even missing intersegmental vessels in 31-33% of the injected embryos (**Fig 5A** and **5B**). We conclude that localization of phosphatase-active Pten to the cell membrane induced stalled vessel defects.

Morphological defects upon expression of membrane-localized active Pten

The overall morphology of embryos expressing (mutant) Ptenb was assessed by bright-field microscopy at 4dpf. We chose this time point because it is the developmental stage at which the pleiotropic phenotype of *ptena–/–ptenb–/–* embryos becomes apparent. Surprisingly, Ptenb K13R and PTEN QMA induced phenotypes similar to the *pten* double homozygous phenotype (**Fig 5C**) [28,31]. Common features of the phenotypes include massive edemas, craniofacial defects, shorter body axis and aberrant pigmentation. Expression of wild type PTEN or mutant Ptenb K13A and Ptenb K13E did not induce morphological defects (**Fig 5C**). These data indicate that expression of mutant Pten with enhanced function phenocopied embryos that lack functional Pten.

6

Discussion

PTEN subcellular localization is regulated by protein conformation, which in turn is largely dependent on the phosphorylation status of the PTEN C-terminal tail, thus regulating PTEN membrane and nuclear localization [23]. PTEN catalytic activity is also regulated by conformational changes within the protein's tertiary structure [12,41].We were interested in studying the physiological consequences of expressing open conformation PTEN, PTEN QMA, in a multicellular organism. Zebrafish is a good model for our purposes,

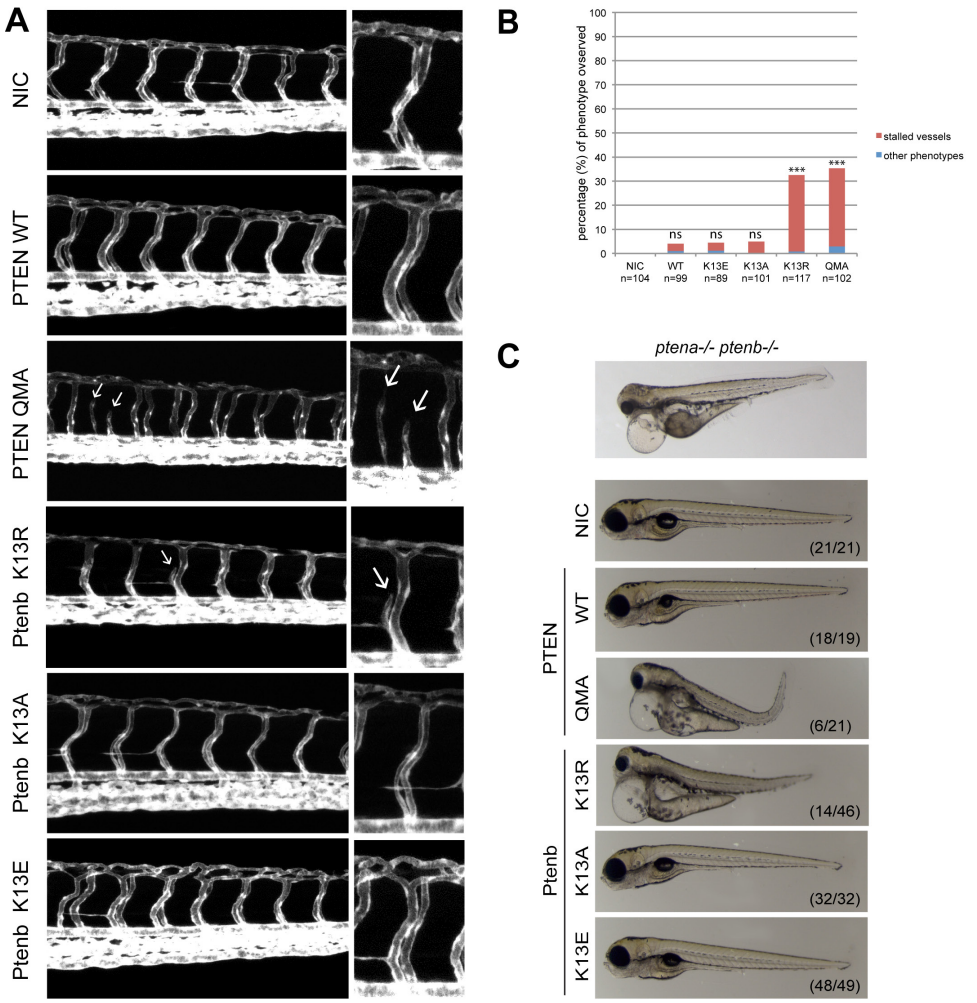

Fig 5. Ptenb K13R and PTEN QMA induce stalled vessels and developmental defects in wild type embryos. **(A)** Tg(*kdrl:eGFP*) zebrafish embryos were microinjected at the one-cell stage with 300 pg synthetic mRNA encoding either PTEN-mCherrry WT, PTEN-mCherry QMA, Ptenb-mcherry K13R, Ptenb-mCherry K13A or Ptenb-mCherry K13E. At 3dpf the embryos were analyzed for the hyperbranching or stalled vessel phenotype by confocal live- imaging using a Leica TCS-SPE microscope (anterior to the left, 20x objective, 2µm z-stacks). Pictures show the trunk region distal to the urogenital opening of representative embryos; non-injected control embryos (NIC) were included for reference. Stalled vessels are indicated with white arrows. **(B)** Quantification of the number of embryos showing either stalled intersegmental vessels (red bars) or other phenotypes, such as hyperbranching (blue bars) (percentage of total number of embryos). The statistical significance was determined using two-tailed Fisher's Exact test. (ns = not significant, * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001). **(C)** At 4dpf, brightfield images of single embryos were taken to assess gross morphological defects in response to expression of PTEN, Ptenb or mutants. A picture of a mutant *ptena–/–ptenb–/–* embryo (top panel) is provided as a reference. Note the massive edemas, short body axis and craniofacial abnormalities in the *ptena–/–ptenb–/–* embryo as well as the embryos expressing PTEN QMA and Ptenb K13R. The numbers in the bottom right corner represent the number of embryos showing the depicted phenotype/ total number of embryos.

due to the extra-uterine development and the transparency of the embryos [30]. The similar localization of both zebrafish and human PTEN in our model was indicative of the similarity of the two highly conserved homologues at the functional level. Therefore, we further characterized the human open conformation PTEN QMA in our functional rescue assay, taking along PTEN WT, Ptenb WT and Ptenb subcellular localization mutants.

Our results confirmed that human PTEN compensates for lack of zebrafish Pten during angiogenesis in *pten* double homozygous embryos. Remarkably, microinjection of phosphatase active Pten with enhanced membrane recruitment, like PTEN QMA and Ptenb K13R, rescued the hyperbranching phenotype and in addition induced a stalled vessel phenotype. Since the hyperbranching phenotype emerges upon lack of Pten, we hypothesized that the stalled vessel phenotype might be induced by enhanced Pten function. This enhanced Pten function might be a consequence of the increased recruitment to the cell membrane, a feature that both PTEN QMA and K13R share. It has been previously reported that PTEN K13R displayed an increased ability to counteract PI3K activity in yeast [24]. To date, only few studies have focused on the effect of overexpression of wild type PTEN. For instance, expression of wild type PTEN [42] or the longer variant, PTEN-L, *in vivo* have been reported, but no defects in angiogenesis or other serious adverse effects were reported in these models. Also in our study, overexpression of wild type PTEN or Ptenb did not increase the occurrence of stalled blood vessels at 3dpf. Lack of Pten and its consequences on angiogenesis, by contrast, have been more extensively studied *in vivo* and have been related to increased microvascular density and VEGF expression in gastric cancer biopsies [37]. The hyperbranching phenotype in developing zebrafish embryos is associated with increased PIP3 signaling, Akt signaling and *vegfaa* expression [29]. A recent study has further unraveled that Notch signaling induces enhanced transcription levels of Pten to suppress proliferation of the stalk cells within the sprouting front during angiogenesis in a cell-autonomous manner [32]. Moreover, that gain and loss of PTEN induces similar proliferation phenotypes in postnatal mouse retinas is consistent with our observations that zebrafish embryos that express mutant Pten or lack functional Pten display similar morphological defects. We believe that both the cell-autonomous and the paracrine functions of Pten are likely underlying the two opposing phenotypes, hyperbranching and stalled vessels. Membrane-associated, phosphatase active Pten may be hyperfunctional due to an increased PIP3 dephosphorylation rate, leading to decreased downstream Akt signaling and hence suppression of endothelial cell proliferation and migration, as well as reduced *vegfaa* transcription and secretion in the surrounding stroma cells. *Serra et al.* [32] further discovered that Pten exerts its anti-proliferative functions in angiogenesis by facilitating the APC/C-Fzr1/Cdh1 complex activity in the nucleus, independently of its phosphatase activity.

Our results suggest that this function of Pten has a minor role during the suppression of pro-angiogenic signals, since Ptenb K13E, a phosphatase inactive mutant, shows a shift in subcellular localization pattern towards the nucleus, yet, did not rescue the hyper-branching phenotype in *pten* double homozygous embryos. However, we cannot completely rule out that enhanced nuclear localization of PTEN QMA contributes to the stalled vessel phenotype. Altogether, our results indicate that Pten K13R and PTEN QMA localize to the cell membrane and are potent and dominant inhibitors of Akt signaling. The enhanced membrane localization of Pten K13R and PTEN QMA could potentially be caused by an increased substrate binding affinity or an altered binding mechanism of both mutants in comparison to wild type PTEN or Ptenb. It has been recently discovered that the catalytic kinetics of PTEN-L and PTEN greatly differ from each other. Whereas PTEN dephosphorylates PIP3 in a "hopping" mode that allows for the diffusion between separate membranes, PTEN-L seems to dephosphorylate PIP3 in a "scooting" mode [43]. A similar change in membrane binding kinetics could be underlying the potential functional hyperactivity of PTEN QMA and Ptenb K13R. It is also likely that these two PTEN mutants deregulate other signaling pathways, for example signal transduction via focal adhesion kinase (FAK) [44-46]. Increased PTEN functionality may have direct or indirect effects on FAK, either by dephosphorylation of FAK by PTEN, or by transcriptional downregulation of FAK via the PI3K/Akt/NFκB-axis [47], respectively. In either case, suppression of FAK activity would inhibit proliferation, migration, invasion and tube formation of vascular endothelial cells into the surrounding tissue during angiogenesis [48-50]. From our experiments, we conclude that PTEN function and the level of PIP3 signaling need to be tightly controlled for normal embryonic angiogenesis.

Acknowledgements

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Author Contributions

Conceived and designed the experiments: MS JdH. Performed the experiments: MS SBF. Analyzed the data: MS SBF JdH. Contributed reagents/materials/ analysis tools: MS JdH. Wrote the paper: MS JdH.

Materials and Methods

Ethics statement

All procedures involving experimental animals described here were approved by the local animal experiments committee (Koninklijke Nederlandse Akademie van Wetenschappen-Dierexperimenten commissie KNAW-DEC protocol HI12.0701) and performed according to local guidelines and policies in compliance with national and European law.

Fish line

Zebrafish were maintained and the embryos were staged according to standard protocols [38]. The lines tg(*H2A-eGFP*) and tg(*kdrl:eGFP*) have been previously described [39,40]. The *ptena*–/– and *ptenb*–/– zebrafish lines were created by target-selected gene inactivation (TSGI). Both the *ptena*^{hu1864} and the *ptenb*^{hu1435} allele result from non-sense mutations that lead to a premature stop codon upstream of the catalytic site [28].

Constructs, mRNA synthesis and Microinjections

The PTEN-mCherry and Ptenb-mCherry fusion constructs were obtained by PCR amplification of *ptenb* or *PTEN*, respectively, from the vectors described in [28]. The PTEN-mCherry QMA construct was obtained by amplification of PTEN QMA [22]. All PCR products were subsequently ligated into the pCS2+mCherry vector. The point mutations in the PTP domain and the N-terminus were introduced by site-directed mutagegesis. The constructs were linearized with NotI and to synthesize 5' capped sense mRNA, the mMessage mMachine SP6 kit (Ambion) was used. mRNA injections were performed at the one-cell stage as described using a total of 300 pg of mRNA.

Genotyping

Zebrafish embryos (4dpf) were anesthesized with 16mg/ml 3-amino benzoic acid ethylesther (MS-222) and cut in half (just below the yolk sack extension). The tail was lysed for genotyping and the head/trunk region was subjected to a protein lysis protocol for subsequent immunoblotting (see below). For genotyping, the genomic target sequence was amplified by two subsequent tilling PCRs, using the following primer pairs: Tilling 1: Ptena and Ptenb #1 and #4. Tilling 2: Ptena and Ptenb #2 and #3. For sequencing, diluted Tilling2 product and primer Ptena #2 or Ptenb #3 was submitted to Macrogen. The sequencing data was analyzed with Lasergene software.

Primer sequences:

Ptena #1 GCGCTAGTTTCTTGTTTAGATTG
Ptena #2 TGTTAACCTGGTGTACAGTGC
Ptena #3 TGGGCAAAATTAAAGAGACC
Ptena #4 CAGACTATTATTTCCCCCAAAC

Ptenb #1 AAAGAACAGAAATCCAGTTCCA
Ptenb #2 TGTTGAGCTTTTGTTGGATGA
Ptenb #3 TGCCAAAACCAACAGAACAA
Ptenb #4 TGCTTAGAACTTTGCACCAA

Immunoblotting

The trunks of 4dpf zebrafish embryos were isolated (see above) and lysed in 25mM HEPES (pH7,4), 125mM NaCl, 0,25% Deoxycholate, 10mM MgCl2, 1mM EDTA, 1% Triton X-100, 10% Glycerol buffer containing proteinase and phosphatase inhibitors. Samples were lysed for 30min and subsequently subjected to sonication with the Bioruptor (settings: high intensity, 15min, 1min on/off cycles). Samples were run on a 10% SDS-PAGE gel, transferred to a PVDF membrane and stained with Coomassie Blue to verify equal loading. The blots were probed with antibodies specific to pAkt (1:2000), Akt (1:1000) (all Cell Signaling) and Tubulin (1:3000); (Calbiochem). For signal detection, enhanced chemiluminescence (ThermoScientific kit) was diluted 1:2 in home-made ECL.

Confocal microscopy and Analysis

Zebrafish embryos were anesthetized at 3dpf and laterally mounted on glass bottom dishes (Greiner bio one) in 0.5% agarose (type V, Sigma Aldrich) in E3 embryo medium containing 16mg/ml 3-amino benzoic acid ethylesther (MS-222) to block contractile movements. Confocal microscopy was performed using a Leica TCS SPE, 20x objective. Z-stacks (step size 2µm) of the vasculature in the tail region, right below the yolk sack extension, were acquired for every embryo at 28.5°C. Image J software (http://rsb.info.nih.gov./ij/) was used to generate z-projection images of the zebrafish embryonic vasculature.

Statistics

Significance of the frequency of occurrence of the "hyperbranching" and "stalled vessel" phenotypes in the different experimental conditions compared to the non-injected control was assessed using two-tailed Fisher's exact test. Results were considered significant when $p<0.05$ (p values * $p<0.05$, ** $p<0.01$, *** $p<0.001$.)

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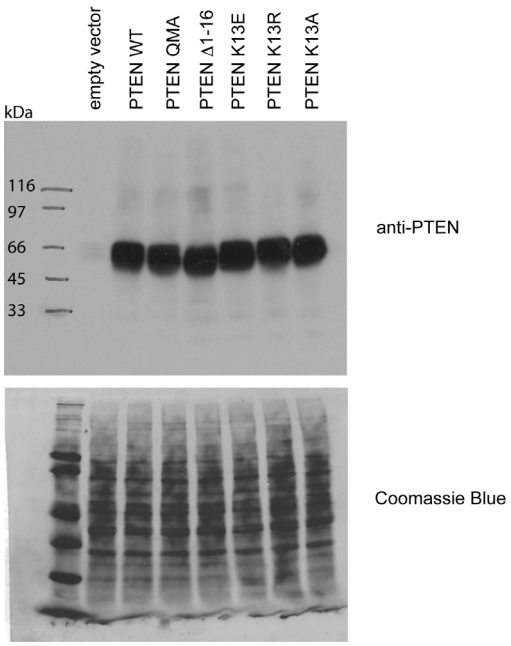
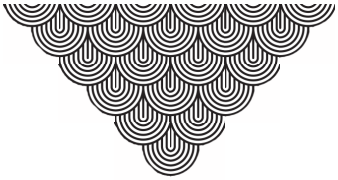
Supporting information



S1 Fig. Similar expression levels of PTEN mutants.
Human embryonic kidney 293 cells were transfected with empty vector, CMV-promoter driven expression vectors for human PTEN, PTEN QMA, a deletion mutant of PTEN lacking the N-terminal 16 residues (not relevant here), PTEN K13E, PTEN K13R and PTEN K13A. The cells were lysed and the lysates were run on an SDS-PAGE gel. The gels were blotted and the blots were probed with PTEN-specific antibodies and developed using enhanced chemiluminescence. Coomassie staining of the blot is provided as a loading control. Similar levels of (mutant) PTEN protein were detected, suggesting there are no major differences in stability between the mutant PTEN proteins.



7

Summarizing discussion

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PTEN and its role in human health and disease have been extensively studied since the identification of *PTEN* as the second most frequently mutated tumor suppressor gene in human cancers. Yet it seems that for every research question concerning PTEN that is answered, ten new ones emerge. PTEN is a lipid- and dual-specificity protein phosphatase that autodephosphoylates itself and forms homo- and heterodimers, it localizes to the cell membrane, to microtubuli- bound endosomes in the cytoplasm, to the ER, to the nucleus, even to nucleoli and is involved in almost every cellular process known. The aim of this thesis is to contribute to the understanding of the complex interplay of PTEN subcellular localization and catalytic activities *in vivo*.

Characterizing the PTEN N-Terminus and PTEN *point mutations*

The PTEN N-terminus is frequently found mutated in tumor biopsies (15% of samples with mutated PTEN show mutations in residues 1–40; about 40% of these being missense mutations; COSMIC database; http://cancer.sanger.ac.uk/cosmic). Over the decades, different functional regions within the PTEN N-terminus have been identified, such as the PIP2-binding motif (PBM; residues 6-15), which mediates binding to phospholipid containing membranes and allosteric activation of PTEN [1-4], the nuclear localization-like signal (NLS; residues 8-32) [5, 6] and a cytoplasmic localization signal (CLS; residues 19-25) [7]. Together with the C-terminal phosphorylation-regulated region of PTEN, the N-terminus controls the PTEN open/closed conformational status, which dictates the phosphatase's subcellular localization, function and protein stability [8-12].
In **Chapter 3**, we have performed a comprehensive functional analysis of the PTEN N-terminal region (residues 2 to 43), using in parallel a humanized *S. cerevisiae*-based system and human cancer cell lines to monitor PTEN PIP3- phosphatase activity and subcellular localization. The heterologous galactose-inducible p110α-CAAX expression system in yeast is a valuable tool for rapid and reliable assessment of PTEN activity towards PIP3 *in vivo* [13-15] because co-expression of wild type PTEN rescues the highly toxic activity of the constitutively active PI3K p110α subunit. In combination with our results from immunofluorescence experiments in mammalian cells, we demonstrated that most tumor-related N-terminal PTEN mutations that lead to a loss of PIP3-phosphatase activity also showed impaired nuclear localization, especially A34D and L42P. Besides the tumor-related mutations, we further characterized experimentally engineered PTEN mutations in a full Ala-scanning mutagenesis of PTEN residues 2-43, spanning the N-terminal tail and the three β-strands at the phosphatase domain. Also in this approach, we identified many mutations that abrogated both nuclear accmulation and PIP3 phosphatase activity of PTEN, indicating that the relationship between these two properties extends beyond the NLS- and the PBM-residues.

Among other mutated residues across the N-terminus, especially mutations at the basic residues of the N-terminal NLS-like region inhibited nuclear localization of PTEN (K13A, R14A, R15A). Upon differential mutational analysis of the motif, we found that Arg15, but neither Lys13 nor Arg14, was essential for dephosphorylation of PIP3 by PTEN *in vivo* while Arg15 was not essential for PTEN nuclear accumulation. These results coincide with the requirement of Arg15 for targeting PTEN to the cell membrane [16]. However, a basic amino acid residue at position 13 was essential for dephosphorylation of PIP3 by PTEN (**Chapter 3, Chapter 6** and [17]). Whereas a charge conserving mutation of Lys13 (K13R) did not compromise PTEN phosphatase activity (on the contrary, it even seemed to enhance it), mutation of Lys13 to neutral (K13A) or negatively charged (K13E) amino acid residues rendered catalytically inactive PTEN (**Chapter 3, Chapter 6** and [1, 2, 16, 17]). Remarkably, monoubiquitination of Lys13 has been shown to be required for PTEN nuclear entry [18], as well as for PTEN secretion via exosomes [19]. In our zebrafish model, however, we could not confirm the necessity of monoubiquitination of Lys13, since Pten K13E did not show any impaired nuclear accumulation (**Chapter 6**). Together, our results reveal a differential and partially complementary involvement of PTEN Lys13, Arg14 and Arg15 in PTEN nuclear localization and phosphatase activity that might be mediated by direct interactions with nuclear transporters or PIP2 (**Chapter 4, Chapter 6** and [1, 4, 5, 20]).

Furthermore, we identified some mutations that predominantly affected one of the two features, lipid phosphatase activity or subcellular localization *in vivo*, indicating a distinctive control of both features. Mutation of residues within a previously mapped CLS signal [7] (D19A, G20A and F21A), for example, shifted PTEN localization to the nucleus, indicating that the CLS signal might function similarly to the nuclear exclusion signals (NES) identified in other PTEN regions [5]. The lipid phosphatase activity of PTEN, however, was not affected by these mutations. On the other hand, reduced activity towards PIP3 but no changes in PTEN subcellular localization were observed with higher frequency upon mutation of the first amino acid residues within the highly unstructured region of the N-terminal tail [21] (A3V, K6A, S10A), suggesting the existence of additional PTEN regulatory elements in this region. It is not surprising that mutations that only affect lipid phosphatase activity could be positively selected for during tumor formation, given that PTEN lipid phosphatase activity is essential for certain biological processes, including for example angiogenesis (**Chapter 5, Chapter 6** and [22]).

Our results from soft agar colony formation assays, using PTEN 1-375/L23F (impaired activity towards PIP3) and 1-375/N31A (impaired nuclear accumulation) mutations, further indicated that both the PTEN PIP3 phosphatase activity and the PTEN capacity to accumulate in the nucleus were important for the full tumor suppression capacity of PTEN. These findings are supported by the observation that lack of nuclear PTEN is associated with a poor prognosis in many human cancers [23]. Taking into account recent studies

on the role of PTEN subcellular localization for its tumor suppressor capacity [5, 7, 11, 12, 16, 17, 24], our results described in **Chapter 3** and our *in vivo* results described in **Chapter 6**, we suggest to widen this hypothesis and propose that the capacity of PTEN to adopt the open conformation (and localize to either the cell membrane or the nucleus) is essential for its full functionality. It would be very interesting to characterize mutant PTEN from human tumors that is excluded from the nucleus for its ability to bind to the cell membrane and for its phosphatase activity.
Taken together, we propose that the PTEN N-terminus comprises regulatory motifs for PTEN cataytic activity and subcellular localization that, despite of a partial overlap, each underlie specific molecular determinants. As a result, PTEN N-terminal mutations can be categorized in three subgroups: a group involved in nuclear accumulation, a group affecting lipid phosphatase activity and a large third group affecting both features. Better understanding of the N-terminal properties of PTEN (and of the extended N-Terminus of PTEN-L) might be relevant for personalized therapies of cancer patients or preventive measures for PHTS patients in the future.

PTEN nuclear translocation: Involvement of importin α3 and possible other mechanisms

PTEN exerts its most prominent function, the dephosphorylation of PIP3 to PIP2, at the cell membrane [25]. A vast body of evidence indicates that PTEN also has phosphatase-dependent and –independent functions in the nucleus [5, 26-32] and concerted activity of PTEN at both subcellular compartments has been shown to be required for its full tumor suppressor activity (**Chapter 3**). Although various models for PTEN translocation to the nucleus have been proposed during the past decade, from passive diffusion [33] and a MVP (major vault protein)-mediated transport [34, 35] to Ran-GTPase-dependent nuclear import of PTEN [5], many unanswered questions remain. Is there one main mechanism of PTEN nuclear translocation or are there multiple mechanisms that are being used depending on the exact conditions? In **Chapter 4**, we focused on the classical protein import pathway as a possible major nuclear transport mechanism for PTEN, given the previously reported involvement of the small GTPase Ran in this process [5] and the broad spectrum of known cargo proteins that this pathway transports between cytoplasm and nucleus in eukaryotic organisms [36-38]. We studied subcellular localization of PTEN by immunofluorescence and confocal microscopy, and found that co-expression of importin α3 (and α7, data not shown), but not importin α6 nor any of the other importin alpha isoforms tested (α1, α4, α5) enhanced nuclear accumulation of PTEN in COS-7 cells (african green monkey fibroblast-like kidney cell line). All PTEN constructs used in this study have also been expressed in U87MG cells (glioblastoma multiforme grade IV cell line; Pten-null status), where they showed a similar subcellular

localization pattern. Published data from *in vitro* import assays suggest that most substrates are imported by all importin α isoforms with similar efficiencies but that each importin α isoform has different substrate preferences that could regulate import efficiency *in vivo* [39]. Moreover, these preferences seem to be to a certain extent dynamic and dependent on the substrate, as reported for the influenza A virus polymerase PB2, which shifts specifity from importin α3 to importin α7 during cross-species virus transfer [40]. Therefore it is not unusual that PTEN nuclear import seems to be enhanced by respective co-expressionof either importin α3 or α7. For simplicity, in **Chapter 4**, we focused on the characterization of the role of importin α3 in PTEN nuclear import. We mapped the required regions for importin α3- mediated nuclear import of PTEN to the N-terminal region of PTEN and the minor binding pocket of importin α3. The N-terminal region (residues 1-32) of PTEN was sufficient for increased nuclear accumulation of PTEN when co-expressed with importin α3, which supports our notion that this region could mediate nuclear accumulation of PTEN through interactions with nuclear transporters (**Chapter 3**, [5]). We have tried to address importin-mediated nuclear translocation of PTEN biochemically, using co-immunoprecipitations and GST-pulldown assays, but failed due to technical reasons. It is not uncommon that importin α substrate specificity is not recapitulated *in vitro*. For instance, PB2 binds all importin α isoforms with comparably high affinity *in vitro*, but is highly selective in living cells [40]. Interestingly, the importin-cargo binding affinity does not necessarily reflect transport efficiency, because also the dissociation rate and the conformation of the import complex contribute to nuclear transport. Nuclear import of a cargo protein can be either promoted or blocked, depending on the importin α isoform that it binds to [41]. Therefore, binding assays may not be the most reliable way to study nuclear transport mechanisms.

Mutagenesis of both the minor and the major binding pocket of importin α3 finally revealed that the minor binding pocket of importin α3 is indispensable for enhanced nuclear localization of PTEN. In GST-Pulldown assays performed with either wild type or mutant importin α3, however, we did not observe a loss of PTEN binding. Again, as previously discussed, the results of this *in vitro* binding assay do not necessarily have to reflect the *in vivo* transport efficiency.

Interestingy, it has recently been suggested that importin α isoforms of particular subfamilies, including importin α3 and α7 might provide a nuclear import "fast-track" of important cellular cargos by forming an import pre-complex without importin β, which provides them a kinetic advantage over classical NLS-cargos that form an import complex only in the presence of importin β [40]. Importin α3 and α7 might represent such a fast-track way to import PTEN, especially in cellular contexts like genotoxic stress [29, 42], that require an immediate exertion of PTEN's nuclear functions in DNA damage repair [32, 43] or induction of apoptosis [5, 44, 45]. In this context it is worth to mention that many proteins functionally associated with PTEN are also imported to the nucleus by

importin α3, for example p53 [46], Notch [47], STAT3 [48], VEGF [49] and NFκB [50-52]. An as yet unknown regulatory importin α3-dependent mechanism may exist for certain „cell function programs", for example „apoptosis", „hypoxia" or „inflammation" that promotes or blocks nuclear import of a group of cellular cargos in a coordinated way, for example via importin α3 expression levels [52]. Hypotheses apart, several other mechanisms have been proposed to regulate PTEN nuclear translocation, such as ATM-mediated phosphorylation [43], caspase3-cleavage [5], monoubiquitination [18, 53-55] and SUMOylation [32], which could be involved in regulating importin α- dependent or -independent import mechanisms of PTEN [5, 34, 35]. Further, PTEN nuclear accumulation has been reported to be impaired by protein-protein interactions with $p27^{Kip}$ [44] and PNUTS [56], respectively. We believe that any of the currently known mechanisms, though some more likely than others, might be employed in a given cellular context to promote or prevent nuclear accumulation of PTEN and that they are not mutually exclusive with our findings.

From our own experiments, we conclude that PTEN is transported to the nucleus via a mechanism particularly involving the N-terminal region of PTEN and the classical protein import pathway and that the minor binding pocket of importin α3 mediates PTEN nuclear import. To further confirm these results, subcellular fractionation and subsequent immunoblotting analysis should be performed. Another experimental approach to do so could be treatment of the co-transfected cells with small peptidomimetic inhibitors of importin α, once a specific inhibitor for importin α3 exists [57]. Alternatively, a targeted knockdown of importin α3 (and α7) with siRNAs (small interfering RNA) or shRNAs (short hairpin RNA), according to the standards of the field, and subsequent assessment of PTEN subcellular localization (eventually upon stimulation of the cells with TNF-α), could be performed to further confirm our results. In addition, it would be interesting to validate whether mutation of the predicted NLS sequence of PTEN, KRRY, disrupts the enhanced nuclear localization of the 32-GST-GFP fusion protein in the presence of importin α3. Especially the effect of the K13R mutation could possibly clarify the role of monoubiquitination in importin-mediated PTEN nuclear transport (**Chapter 3** and **Chapter 6**). Future research on the mechanisms of PTEN nuclear translocation and their regulation may provide new insights into potential therapeutic strategies in cancer, PHTS and autism-spectrum disorders.

Differential requirement for Pten lipid- and protein- phosphatase activity during development

Though initially identified as a dual-specificity phosphatase [58, 59], the main focus in the emerging field of PTEN research has long time been on PTEN's lipid phosphatase activity. In spite of the identification of a number of putative PTEN protein targets, such as FAK (focal adhesion kinase) [60-62], Shc (SHC-transforming protein 1) [63], CREB (Cyclic AMP-responsive element-binding protein 1) [30], IRS1 (insuline receptor substrate 1) [64], Dishevelled [65] and MCM2 (DNA replication licensing factor MCM2) [66], certain doubts persist about the role of PTEN protein phosphatase activity *in vivo*.

In **Chapter 5**, we studied the requirement of Pten lipid- and protein-phosphatase activities during zebrafish embryonic development and we found that Pten lipid phosphatase activity was necessary and sufficient for correct angiogenesis. For correct embryonic development on the other hand, both catalytic activites of Pten were required. Given the complexity of processes involved in embryogenesis, it is not surprising that not only Pten lipid phosphatase activity but also protein phosphatase activity is essential. Protein phosphatase activity of PTEN has previously been shown to be required in order to suppress cell migration, spreading and invasion [60, 62, 67-69], important processes for embryonic development, *in vitro*. We are the first to demonstrate the requirement of both Pten phosphatase activities for its full functionality *in vivo*. Our results further support the notion that both phosphatase activities might have to be localized in the same molecule, as suggested by *Tibarewal et al.* [69]. Recent data on PTEN autodephosphorylation at Thr366 (and Ser370) enhancing PTEN lipid phosphatase activity [69] has brought up the question whether PTEN itself could be its physiologically most important protein target. The debate about this issue [70, 71] has been newly fuelled this year by the finding that dephosphorylation of Thr366 and Ser370 leads to a partial exposure of the active site which allows cytosolic PTEN to dephosphorylate soluble but not membrane-bound substrates [72]. Combined with the growing number of newly-discovered PTEN protein substrates, such as MCM2 [66] and Dishevelled [65], this discovery sheds a new light on the relevance of PTEN protein dephosphorylation *in vivo*. In spite of this, Thr366 and Ser370 dephosphorylation could still mainly occur in an auto-regulatory manner [69] and also enhance dephosphorylation of soluble protein-bound PIP3 (for example SF-1-bound PIP3 in the nucleus [73]), as suggested by Masson et al. [72].

Taken together, our results suggest that certain developmental processes, such as angiogenesis, mainly require PTEN lipid phosphatase activity (**Chapter 5** and **Chapter 6**), while embryogenesis as a whole requires both PTEN catalytic activities in order to occur flawlessly. The G129E mutation, that we characterized here, which renders lipid phosphatase inactive Pten, has first been identified in Cowden syndrome patients [74].

It was initially shown to possess intermediate tumor suppressor capacity *in vivo* without significantly affecting the apoptotic or proliferative rate. However, Pten G129E failed to suppress tumor angiogenesis [22], which is consistent with our results presented in **Chapter 5**. Recent studies have revealed that the (partially) lipid phosphatase inactive mutants C124S and G129E have dominant negative effects on wild type Pten that lead to more severe tumor spectra than just a loss of heterozygosity *in vivo*. Homozygous knock-in of these alleles, just as homozygous knock-out of Pten, is embryonically lethal [75]. Mutations in all three residues, Pten Cys124, Gly129 and (to a lesser extent) in Tyr138 have been found in biopsies of spontaneous cancer (COSMIC database). It is imaginable though, that germline heterozygous loss of PTEN protein phosphatase activity (as suggested for other PTEN partial loss of function mutations [76]) could lead to milder phenotypes than a similar loss of PTEN lipid phosphatase activity. To our knowledge, however, point mutations in Tyr138 have not been associated with PHTS or autism-spectrum disorders yet. As discussed in **Chapter 3**, this study and future characterization of point mutations differentially affecting PTEN lipid- and protein- phosphatase activity *in vivo* will improve our understanding of genotype-phenotype correlations in patients and of the requirement of each of the two catalytic activities of PTEN in human health and disease.

Role of Pten catalytic activity and subcellular localization during angiogenesis

Dephosphorylation of PIP3 to PIP2 by PTEN occurs, for the greatest part [73], at the cytoplasmic face of the cell membrane [21, 25, 77]. For this reason, the mechanism that targets PTEN to the cell membrane has been extensively studied in the past [1, 3, 4, 11, 12, 20, 21, 72, 78]. In a great effort, a membrane-targeted and functionally more active PTEN protein, ePTEN (enhanced PTEN) has been engineered in order to study the causal relation of PTEN membrane association and lipid phosphatase activity in the amoeba *Dictyostelium* [16]. As reported and discussed in **Chapter 3**, the PTEN N-terminus, more specifically the PIP2-binding motif [4], is crucial for membrane recruitment. Other important regions for efficient membrane binding within the PTEN structure are the C2 domain [21] and the C-terminal tail, whose phosphorylation status determines PTEN protein conformation [5, 8, 11, 17]. In fact, recent data suggests that C-terminal fully phosphorylated PTEN is completely inactive and incapable of binding membranes [72, 79]. In **Chapter 6**, we study the functional consequences of expressing open conformation PTEN, PTEN QMA, *in vivo*. In agreement with published data on the subcellular distribution of open conformation PTEN in cells [5, 17], PTEN QMA showed enhanced nuclear localization and recruitment to the cell membrane at early embryonic stages in our zebrafish model. Furthermore, expression of open conformation PTEN not only res-

cued the hyperbranching phenotype and the increased p-Akt levels typical for *pten* double homozygous embryos in our functional rescue assay, but it induced stalled intersegmental vessels, which failed to connect with the dorsal longitudinal anastomotic vessel, independently of the embryo's *pten* status. A similar vasculature phenotype has been reported to occur in murine postnatal retina upon a Notch-induced increase in PTEN expression levels [80]. The functional hyperactivity of open conformation PTEN in comparison to PTEN wild type in our model seemed to result predominantly from its enhanced recruitment to the cell membrane, given that Ptenb K13R, which predominantly localized to the cell membrane but not to the nucleus, induced the same stalled vessel phenotype. The reason why Ptenb K13R accumulated at the cell membrane in zebrafish embryos while PTEN K13R showed cytoplasmic localization in mammalian cells might be that Pten predominantly exists in an open conformation in these fast-cycling embryonic cells and that Lys13 seems to play a specifically important role for determining the subcellular localization of open conformation PTEN (**Chapter 3** [17]).
Interestingly, the anti-angiogenic effect of high PTEN expression levels observed in the mouse retina model has been attributed to both phosphatase-dependent (via dephosphorylation of PIP3) and -independent (suppression of cell cycle progression via the PTEN-APC/C-Fzr1/Cdh1 axis [31]) functions of PTEN [80]. The data from our functional *in vivo* rescue assays, however, do not support the observation of a relevant role for PTEN phosphatase-independent functions during angiogenesis (**Chapter 5** and **Chapter 6**), since none of the Pten mutants lacking catalytic activity towards PIP3 rescued the hyperbranching vessel phenotype at 3dpf. Enhanced recruitment of phosphatase inactive mutants to the membrane, such as Ptenb C124S or K13A, did not significantly induce the stalled vessel phenotype, indicating that PTEN phosphatase activity is indispensable for its regulatory function during angiogenesis.
In conclusion, in **Chapter 6** we demonstrate that enhanced membrane localization of phosphatase active PTEN dramatically increased its biological function in suppression of angiogenic sprouting. Tight temporal and spatial regulation of PTEN activity during angiogenesis might therefore play an essential role. These data, in combination with the recent data concerning the regulatory mechanisms controlling PTEN (and PTEN-L) substrate binding and phosphatase activity states [72] and the recruitment of PTEN (via PI(3)P) to PIP3-containing endosomes for signal termination [81], shed a new light on the importance of a coordinated „how, when and where" of PIP3 dephosphorylation by PTEN *in vivo*.

Conclusion

Overall, we demonstrate that various domains of the PTEN molecule are required to regulate its catalytic activity and subcellular localization, whose syntony is essential for normal embryonic development and to maintain adult health. In a comprehensive approach, we identified three subgroups of point mutations within the PTEN N-terminus: one affecting PTEN catalytic activity, one affecting PTEN subcellular localization and a third one that affects both features, indicating that the N-terminus contains two partially overlapping but functionally independent regulatory regions (**Chapter 3**). We further unraveled that nuclear translocation of PTEN is enhanced by an importin α3-dependent mechanism that involves the PTEN N-terminus and the importin α3 minor binding pocket (**Chapter 4**). Using zebrafish as a model organism, we demonstrate that Pten lipid phosphatase activity is required and sufficient to control angiogenesis *in vivo* but that embryogenesis requires concerted activity of PTEN towards phospholipids and –proteins (**Chapter 5**). Finally, we discovered that enhanced membrane recruitment of catalytically active Pten during zebrafish development, as a consequence of a constitutively open conformation, suppresses angiogenic sprouting and results in a stalled vessel phenotype, irrespectively of the endogenous *pten* status (**Chapter 6**). Taken together, our results emphasize the importance of the temporal and spatial control of PTEN activities *in vivo*.

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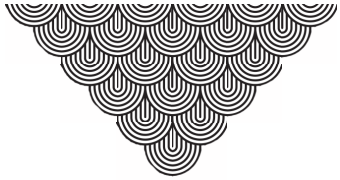

Addendum

Abbreviation list

Summary

Nederlandse Samenvatting

List of publications

Acknowledgements

Curriculum vitae

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Abbreviation list

αIBB	Importin-β binding domain of importin α
AKT/PKB	RAC-alpha serine/threonine-protein kinase, Protein Kinase B
Arm	Armadillo repeat
BRRS	Bannayan-Riley-Ruvalcaba syndrome
CAAX	Cysteine (C), aliphatic amino acid (A), any amino acid (X)- sequence
CHT	Caudal Hematopoietic Tissue
CMV	Cytomegalovirus
COS-7	african green monkey fibroblast-like kidney cell line
CS	Cowden Syndrome
CLS	Cytoplasmic Localization Signal
CNS	Central Nervous System
DA	Dorsal Aorta
DLAV	Dorsal Longitudinal Anastomotic Vessel
dpf	days post fertilization
eGFP	enhanced Green Fluorescent Protein
Fig/Figs	Figure/Figures
GDP	Guanosine Diphosphate
GST	Glutathion-S-Transferase
GTP	Guanosine Triphosphate
H2A	Histone 2A
HEK 293T	Human Embryonic Kidney cell line
hpf	hours post fertilization
HIV	Human Immunodeficiency Virus
HSPCs	Hematopoietic Stem and Progenitor Cells
ISV	Intersegmental Vessel
kDa	Kilo-Dalton
kdrl	vascular endothelial growth factor receptor kdr-like
LOH	Loss Of Heterozygosity
μm, μl, μM	micrometer, microliter, micromolar
NIC	Non-Injected Control
NES	Nuclear Exclusion Signal
NLS	Nuclear Localization Signal
nm	nanometer
NPC	Nuclear Pore Complex
PAGE	Polyacrylamide Gel Electrophoresis
PBD	Phospholipid Binding Domain
PCV	Posterior Cardinal Vein
PDZ	Domain present in PSD-95, Dlg, and ZO-1/2
PEST	Proline (P), glutamic acid (E), serine (S) and threonine (T) -rich motif
pg	picogram
PHTS	PTEN Hamartoma Tumor Syndrome
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol(3, 4, 5)-trisphosphate
PTEN	Phosphatase and Tensin Homolog
PTEN-L	Phosphatase and Tensin Homolog-Long
PTEN QMA	Phosphatase and Tensin Homolog; open conform. (S370A, S380A, T382A, T383A, S385A)
PTP	Protein Tyrosine Phosphatase
RTK	Receptor Tyrosine Kinase
SDS	Sodium Dodecyl Sulfate
SV40	Simian Virus 40
T-ALL	T-cell Acute Lymphoblastic Leukemia
TSGI	Target Selected Gene Inactivation
U2OS	Osteosarcoma cell line
U87MG	Glioblastoma multiforme grade IV cell line; Pten-null status
VEGF	Vascular Endothelial Growth Factor
WT	wild type

Summary

This thesis describes the use of mammalian cells, *S. cerevisiae* and *D. rerio* to unravel the complex interplay of PTEN subcellular localization and catalytic activities. In the first two chapters, we provide a background for the following four chapters.

In **Chapter 1** we provide a general introduction to signal transduction, the PI3K/Akt (PKB)/PTEN axis, PTEN phosphatase-dependent and –independent functions and the regulation of those functions through changes in protein conformation and subcellular localization. Last, we give a short résumé on the role of PTEN in human health and disease.

The use of zebrafish to study cancer and developmental biology is discussed in **Chapter 2**. To this end, we give an overview of developmental and pathological processes that have been successfully studied in zebrafish *pten* models. Further, we introduce techniques that we and others have developed for modulation of Pten expression *in vivo* and for the establishment of zebrafish cell lines from tumors. The chapter concludes with an outlook on what we can expect to learn about the function of Pten from using the zebrafish model system.

In **Chapter 3** we performed a comprehensive mutational and functional analysis of the PTEN N-terminus. To analyze the contribution of this region to PTEN tumor suppressor function *in vivo*, we studied a panel of tumor-related mutations, employing *S.cerevisiae* and mammalian cells. We found that most tumor-related N-terminal PTEN mutations that lead to a loss of PIP3-phosphatase activity, especially A34D and L42P, also showed impaired nuclear localization. This suggests that PIP3 catalytic activity and nuclear localization of PTEN are coordinated by the PTEN N-terminus in an overlapping manner. We further confirmed this notion via an anlanine (Ala) scanning approach, which helped us to identify three different categories of mutations: the ones that only affect PIP3 phosphatase activity (A3V, K6A, S10A), the ones that only affect nuclear accumulation properties of PTEN (D19A, G20A and F21A) and some mutations that affect both characteristics (R15A, Y16A, D24A, I32A, I33A, M35A, A39V and L42A), confirming that there exists a certain overlap in the regulation of both properties within the N-terminal sequence but also indicating that each of them underlie specific molecular determinants. Further, we picked apart the role of the single amino acids constituting the N-terminal KRR-motif of PTEN for the protein's lipid phosphatase activity and subcellular localization. Arg15, but neither Lys13 nor Arg14, was essential for dephosphorylation of PIP3 by PTEN *in vivo* while Arg15 was not essential for PTEN nuclear accumulation. Our results from soft agar colony formation assays, using PTEN 1-375/L23F

(impaired activity towards PIP3) and 1-375/N31A (impaired nuclear accumulation) mutations, further indicated that both the PTEN PIP3 phosphatase activity and the capacity to accumulate in the nucleus were important for the full tumor suppressor capacity of PTEN.

In **Chapter 4** we focused on the classical protein import pathway as a possible major nuclear transport mechanism for PTEN and, by immunofluorescence and confocal microscopy performed in mammalian cells, we identified importin alpha3 as a factor involved in PTEN nuclear translocation. We found that co-expression of importin α3 (and α7, data not shown), but not importin α6 nor any of the other importin alpha isoforms tested (α1, α4, α5) enhanced nuclear accumulation of PTEN in COS-7 cells. All PTEN constructs used in this study have also been expressed in U87MG cells (glioblastoma multiforme grade IV cell line; Pten-null status), where they showed a similar subcellular localization pattern. Further, we found that the PTEN N-terminal region (residues 1-32) was sufficient for increased nuclear accumulation of PTEN when co-expressed with importin α3, which supports our notion that this region could mediate nuclear accumulation of PTEN through interactions with nuclear transporters. Systematic introduction of point mutations in the importin α3 substrate binding pockets and subsequent co-expression with PTEN allowed us to further narrow down the region of importin α3 that is important for nuclear accumulation of PTEN to the minor binding pocket.

Using the zebrafish as a model organism, in **Chapter 5** we unveil a differential requirement of Pten lipid and protein phosphatase activity during embryonic development. To this end, we performed rescue assays and screened the Pten lipid phosphatase inactive mutant Ptenb G129E and the protein phosphatase inactive mutant Ptenb Y138L via confocal microscopy and immunoblotting for their capacity to prevent the typical angiogenesis and pleiotropic phenotype of *pten* double homozygous embryos when microinjected at the one-cell stage. Ptenb Y138L rescued the hyperbranching vessel phenotype at 3dpf with similar efficiency as wild type Ptenb, while Ptenb G129E did not. The pleiotroic phenotype at 4dpf was not rescued by either of the two phosphatase-deficient Ptenb mutants. We propose that the role of Pten during angiogenesis mainly consists of suppressing PI3K signaling via its lipid phosphatase activity, whereas the complex process of embryonic development requires lipid and protein phosphatase activity of Pten.

In **Chapter 6** we characterize the subcellular localization and the functional consequences of the expression of open conformation PTEN, PTEN QMA, *in vivo* during zebrafish development. PTEN QMA showed enhanced nuclear localization and recruitment to the cell membrane at early embryonic stages and expression of open

conformation PTEN not only rescued the hyperbranching phenotype and the increased p-Akt levels typical for *pten* double homozygous embryos in our functional rescue assay, but it induced stalled intersegmental vessels, which failed to connect with the dorsal longitudinal anastomotic vessel, independently of the embryo's *pten* status. The functional hyperactivity of open conformation PTEN in comparison to PTEN wild type in our model seemed to result predominantly from its enhanced recruitment to the cell membrane, given that Ptenb K13R, which predominantly localized to the cell membrane but not to the nucleus, induced the same stalled vessel phenotype. Enhanced recruitment of phosphatase inactive mutants to the membrane, such as Ptenb C124S or K13A, did not significantly induce the stalled vessel phenotype, indicating that PTEN phosphatase activity is indispensable for its regulatory function during angiogenesis. In conclusion, we show that enhanced membrane localization of phosphatase active PTEN dramatically increased its biological function in suppression of angiogenic sprouting. The findings of this chapter demonstrate the requirement of tightly regulated and equilibrated Pten activity during zebrafish development and angiogenesis.

Finally, **Chapter 7** provides a summarizing discussion of the work presented in each previous chapter in the context of the latest publications in the field and the implications of our findings for future research.

Nederlandse Samenvatting

Dit proefschrift beschrijft het gebruik van zoogdiercellen, *S. cerevisiae* en *D. rerio* bij het ontrafelen van de complexe wisselwerking tussen de subcellulaire lokalisatie en katalytische activiteit van PTEN. De eerste twee hoofdstukken vormen de achtergrond voor de daaropvolgende vier hoofdstukken.

In **hoofdstuk 1** geven we een algemene introductie over signaaltransductie, de PI3K/Akt (PKB)/PTEN-signaaltransductieroute, fosfatase-afhankelijke en –onafhankelijke functies van PTEN en de regulatie van deze functies door middel van veranderingen in eiwitconformatie en subcellulaire lokalisatie. Ten slotte geven we een kort overzicht van de rol van PTEN in de mens.

Het gebruik van de zebravis bij het onderzoek naar kanker en ontwikkelingsbiologie wordt besproken in **hoofdstuk 2**. We geven een overzicht van ontwikkelinggerelateerde en pathologische processen die succesvol zijn onderzocht met behulp van het zebravis/*pten*-model. Daarnaast introduceren we technieken, die door ons en anderen zijn ontwikkeld, voor de verandering van Pten-expressie *in vivo* en het verkrijgen van zebraviscellijnen uit tumoren. Het hoofdstuk besluit met een vooruitblik van wat we verwachten te leren over de functie van Pten met behulp van het zebravismodelsysteem.

In **hoofdstuk 3** hebben we uitgebreide mutatie- en functionele analyses van de N-terminus van PTEN uitgevoerd. De bijdrage van dit domein aan de tumoronderdrukkende functie van PTEN hebben we geanalyseerd aan de hand van diverse tumorgerelateerde mutaties, gebruikmakend van *S.cerevisiae* en zoogdiercellen. Hieruit blijkt dat de meeste tumorgerelateerde PTEN-mutaties, die leiden tot het verlies van PIP3-fosfataseactiviteit, daarnaast ook verminderde lokalisatie in de celkern vertonen, in het bijzonder A34D- en L42P-mutaties. Dit suggereert dat de katalytische activiteit van PIP3 en de lokalisatie van PTEN in de celkern beiden worden gecoördineerd door de N-terminus van PTEN. Deze vaststelling hebben we bevestigd door middel van een alaninescanningsmethode. Hiermee hebben we drie verschillende mutatieklassen kunnen identificeren: degene die enkel de PIP3-fosfataseactiviteit aantasten (A3V, K6A, S10A), degene die enkel de accumulatie van PTEN in de celkern aantasten (D19A, G20A en F21A) en mutaties die beide genoemde eigenschappen aantasten (R15A, Y16A, D24A, I34A, I33A, M35A, A39V en L42A). Dit bevestigt dat er een zekere overlap bestaat in de regulatie van beide eigenschappen binnen het N-terminale domein, maar dat er daarnaast aan elke eigenschap ook specifieke moleculaire oorzaken ten grondslag liggen. Vervolgens hebben we de rol van de afzonderlijke

aminozuren, die het N-terminale KRR-motief van PTEN vormen, onderzocht met betrekking tot de lipidefosfatase-activiteit en de subcellulaire lokalisatie van het eiwit. Onze resultaten van zogenaamde soft agar colony formation assays, gebruikmakend van PTEN 1-375/L23F- (verlies van PIP3 activiteit) en 1-375/N31A- (verminderde accumulatie in de celkern) mutaties, geven verder aan dat zowel de PIP3-fosfataseactiviteit als de capaciteit tot accumulatie van PTEN in de celkern belangrijk zijn voor de volledige tumoronderdrukkende functie van PTEN.

In **hoofdstuk 4** richten wij ons op de klassieke route voor eiwitimport als mogelijk belangrijk transportmechanisme voor PTEN van en naar de celkern. Met behulp van immunofluorescentie en confocale microscopie van zoogdiercellen hebben we importine alfa3 geïdentificeerd als factor betrokken bij het transport van PTEN van en naar de celkern. Co-expressie van importine α3 (en α7) verhoogt de accumulatie van PTEN in de celkern in COS-7 cellen, in tegenstelling tot importine α6 of elke andere geteste importine alfa isovorm (α1, α4, α5). Alle PTEN-constructen gebruikt voor deze studie zijn ook tot expressie gebracht in U87MG cellen (glioblastoma multiforme grade IV cell line; PTEN-nul status), waarin een vergelijkbaar subcellulair lokalisatiepatroon waarneembaar is. Daarnaast blijkt dat co-expressie van het N-terminale domein van PTEN (residuen 1-32) en importine α3 voldoende is voor verhoogde accumulatie van PTEN in de celkern. Dit bevestigt de vaststelling dat het N-terminale domein betrokken is bij de accumulatie van PTEN in de celkern door middel van interacties met nucleaire transporteurs. Systematische introductie van puntmutaties in de substraatbindingsdomeinen van importine α3 en daaropvolgende co-expressie met PTEN beperkt de regio van importine α3 dat belangrijk is voor de accumulatie van PTEN in de celkern tot de kleine bindingsplaats.

Met behulp van de zebravis als modelorganisme tonen we in **hoofdstuk 5** een differentiële afhankelijkheid van lipide- en eiwitfosfatase-activiteit van Pten tijdens embryonale ontwikkeling aan. Hiervoor zijn er zogenaamde reddingsexperimenten uitgevoerd, waarbij de lipidefosfatase-inactieve mutant Pten G129E en de eiwitfosfatase-inactieve mutant Ptenb Y138L zijn geïnjecteerd in dubbel homozygote *pten*-embryo's. Vervolgens is de capaciteit van deze mutanten om het typische fenotype van verstoorde angiogenese en het pleiotrope fenotype van de dubbel homozygote *pten*-embryo's te voorkomen geanalyseerd met behulp van confocale microscopie en immunoblotting. Ptenb Y138L is in staat het angiogenesefenotype op 3 dpf te redden, in tegenstelling tot Ptenb G129E. Het pleiotrope fenotype op 4 dpf kon niet worden gered door één van beide fosfatasedeficiënte Ptenb mutanten. We suggereren dat de rol van Pten gedurende angiogenese voornamelijk bestaat uit het onderdrukken van PI3K-signalering door middel van de lipidefosfatase-activiteit, terwijl

het complexe proces van embryonale ontwikkeling zowel lipide- als eiwitfosfatase-activiteit van Pten vereist.

In **hoofdstuk 6** karakteriseren we de subcellulaire lokalisatie en de functionele gevolgen van expressie van PTEN met een open conformatie, PTEN QMA, *in vivo* tijdens de ontwikkeling van de zebravis. PTEN QMA vertoonde verhoogde lokalisatie in de celkern en celmembraan in de eerste stadia van embryonale ontwikkeling. Daarnaast is expressie van PTEN QMA niet alleen in staat het angiogenesefenotype en de verhoogde p-Akt niveaus, kenmerkend voor *pten* dubbel homozygote embryo's, te redden, maar induceert het ook onvolledige intersegmentale bloedvaten, die geen contact maken met het dorsale longitudinale anastomotisch vat, ongeacht de *pten* status van het embryo. In ons model lijkt de functionele hyperactiviteit van PTEN QMA, in vergelijking met wild type PTEN, het resultaat van verhoogde lokalisatie aan het celmembraan, zeker gezien Ptenb K13R, welke voornamelijk gelokaliseerd is aan het celmembraan en niet in de celkern, hetzelfde fenotype met onvolledige intersegmentale vaten veroorzaakt. Verhoogde translocatie van fosfatase-inactieve mutanten, zoals Ptenb C124S en K13A, naar het celmembraan veroorzaakte niet het onvolledige intersegmentale vaten fenotype, wat aangeeft dat fosfatase-activiteit van PTEN onmisbaar is voor zijn regulerende functie gedurende angiogenese. Tot besluit laten we zien dat verhoogde lokalisatie in het celmembraan van fosfatase-actieve PTEN zijn functie in het onderdrukken van angiogenese sterk verhoogt. De bevindingen in dit hoofdstuk laten de noodzaak zien van strak gereguleerde en gebalanceerde Pten-activiteit tijdens de ontwikkeling van de zebravis en angiogenese.

Ten slotte geven we **in hoofdstuk 7** een samenvattende discussie van de resultaten van elk voorgaand hoofdstuk in de context van recente publicaties in het veld. Daarnaast geven we implicaties van onze bevindingen ten behoeve van toekomstig onderzoek.

List of publications

Stumpf M., Blokzijl-Franke S., den Hertog J. (2016)
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Gil A., Rodríguez-Escudero I., **Stumpf M.**, Molina M., Cid V.J., Pulido R. (2015)
A functional dissection of PTEN N-terminus: implications in PTEN subcellular targeting and tumor suppressor activity, PLoS One. 2015 Apr 15;10(4):e0119287

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There we go, I'm getting emotional, sorry! Well, while we're on it:
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Finally, I thank **my family** for everything they have done and do for me! I know that without this happy home and childhood that I was privileged to enjoy, I would not be the same person that I am today! Danke, **Oma Ruth**, für 31 Jahre voller Fürsorge, Liebe und Aufmerksamkeit und für deine Unterstützung in allen Lebenslagen! Ich wünsch dir ganz viel Gesundheit und Zufriedenheit!!!
Liebe **Mama**, lieber **Papa**, thanks for the unconditional love and all the time you gave me literally from day one until today! Goethe once said „There are two things children should get from their parents: roots and wings." Thanks for both! I couldn't have asked for better parents, love you mum and dad!!!

Miriam

Curriculum vitae

Miriam Stumpf was born on the 6th of April 1985 in Oberhausen, Germany. She grew up with her parents Jutta and Klaus in Mülheim an der Ruhr, where she completed her secondary education, specially focusing on English and Biology, and obtained her „Abitur" in 2004. In that same year, she moved to Marburg to study Human Biology (Biomedical Science) at the Philipps University Marburg, where she graduated and obtained her diploma title, Dipl.-Humanbiologin, in 2009. During her diploma thesis she investigated the role of the protein arginine methyl-transferase PRMT6 in cell cycle, at Prof. Dr. Uta-Maria Bauer's lab at the IMT (Insitute of Molecular Biology and Tumor Research) in Marburg.

In 2010 she moved to Valencia, Spain, where she started her PhD thesis at the CIPF (Centro de Investigación Prince Felipe) at Dr. Rafael Pulido's lab, investigating the role of PTEN nuclear localization and its mechanisms. During this period she further obtained her MSc in Molecular approximations in health sciences from the University of Valencia. By the end of 2011, Dr. Pulido's lab and various other research groups were forced to close down their laboratories at the CIPF due to massive budget cuts inflicted by the spanish Ministry of Health.

In april 2012 Miriam continued her PhD thesis at the Hubrecht Institute in Utrecht, The Netherlands, with Prof. Dr. Jeroen den Hertog, using zebrafish as a model to study the interplay of Pten subcellular localization and catalytic activities *in vivo*. The results of these studies are presented in this thesis.