PTEN Enters the Nuclear Age

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Regulation of the PTEN tumor suppressor protein is poorly understood. In this issue, Wang et al. (2007) and Trotman et al. (2007) describe how ubiquitination regulates PTEN stability and its nuclear localization. Additionally, Shen et al. (2007) report that a nuclear pool of PTEN helps to maintain chromosomal stability.

The PTEN (Phosphatase and TENsin homolog) tumor suppressor is one of the most frequently mutated genes in human cancer. Somatic mutations in *PTEN*, which result in its inactivation, occur in multiple sporadic tumor types. Germline mutations of *PTEN* cause the inherited hamartoma and cancer predisposition syndrome Cowden disease (Chow and Baker, 2006). PTEN is the central negative regulator of the Phosphatidylinositol-3-kinase (PI3K) signal transduction cascade. The class I family of PI3 kinases are activated downstream of receptor tyrosine kinases (RTK) or G protein-coupled receptors (GPCR) to catalyze the conversion of phosphatidylinositol 4,5 phosphate (PIP2) to phosphatidylinositol 3,4,5 phosphate (PIP3) leading to the activation of AKT kinase and other downstream effectors (Engelman et al., 2006). PTEN is a lipid phosphatase that dephosphorylates PIP3 at the plasma membrane and thereby inhibits PI3K-mediated signals for growth, proliferation, and survival (Figure 1). Despite its well-defined role in signaling at the plasma membrane, PTEN is found in the nucleus in a number of different normal and tumor cell types. Intriguingly, nuclear localization of PTEN may contribute to its tumor suppressor activity (Lian and Di Cristofano, 2005). Given that many of the main components of the PI3K signaling pathway are found in the nucleus—including PIP2, PIP3, PI3K, PDK1, and AKT (Deleris et al., 2006)—it is possible that PTEN is a PIP3 phosphatase in the nucleus as well. However, a recent study suggests that nuclear PTEN does not dephosphorylate the nuclear pool of PIP3 (Lindsay et al., 2006). Other studies have implicated phosphatase-independent functions of PTEN within the nucleus including protein-protein interactions that modulate the activity and stability of p53 (Li et al., 2006; Lian and Di Cristofano, 2005).

In this issue of *Cell*, three exciting papers (Shen et al., 2007; Trotman et al., 2007; Wang et al., 2007) provide insights into the role of ubiquitination in regulating PTEN stability and its nuclear localization, as well as the function of nuclear PTEN in chromosomal stability (Figure 1).

PTEN Degradation

PTEN contains two PEST motifs, which are frequently found in proteins targeted for degradation by the ubiquitin pathway. In this issue, Wang et al. (2007) investigated the role of ubiquitin-mediated degradation of PTEN. After demonstrating that overexpressed PTEN was polyubiquitinated in cells, they used an in vitro polyubiquitination assay to identify NEDD4-1 (neural precursor cell expressed, developmentally downregulated 4-1) as the E3 ubiquitin ligase that specifically targets PTEN

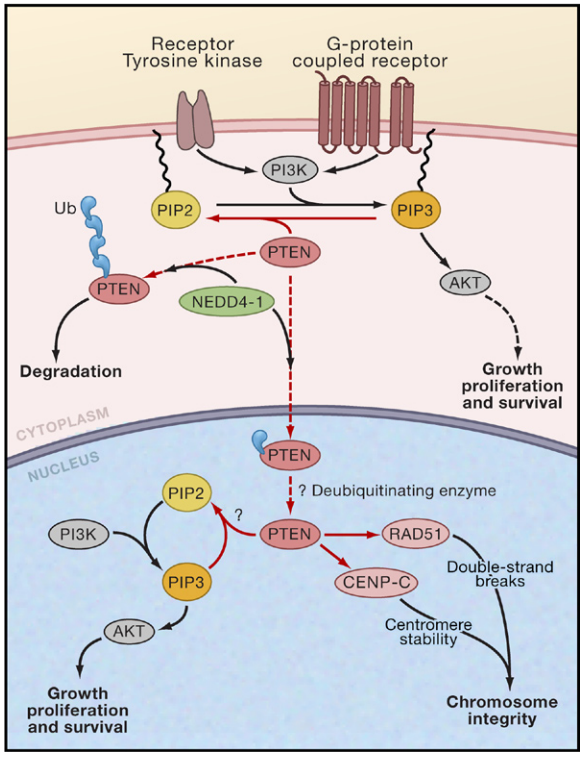

Figure 1. Proposed Nuclear and Cytoplasmic Function and Regulation of PTEN
In the cytoplasm, PI3K is activated downstream of receptor tyrosine kinases and G protein-coupled receptors to convert PIP2 to PIP3, leading to AKT activation and enhanced growth, proliferation, and survival. PTEN phosphatase antagonizes PI3K by converting PIP3 to PIP2. Wang et al. (2007) show that NEDD4-1 polyubiquitinates PTEN, promoting its degradation in the cytoplasm, while Trotman et al. (2007) show that monoubiquitination increases nuclear localization of PTEN. Nuclear PTEN could function as a lipid phosphatase as it does in the cytoplasm but may also be involved in maintaining genomic integrity as shown by Shen et al. (2007).

for ubiquitination. NEDD4-1 and PTEN showed a direct physical interaction, and overexpression of NEDD4-1 resulted in polyubiquitination and proteasomal degradation of endogenous PTEN. Conversely, knockdown of NEDD4-1 by RNAi increased the levels and stability of overexpressed PTEN.

These data suggest that NEDD4-1 may contribute to oncogenesis by inhibiting PTEN tumor suppressor activity. *p53*-deficient MEFs were transformed by *Pten* deletion (Chen et al., 2005) but were not transformed by NEDD4-1 overexpression, most likely because Pten protein, although diminished, remained sufficient for tumor suppression. Although NEDD4-1 was not oncogenic alone, its overexpression enhanced Ras-mediated transformation of p53-deficient mouse embryonic fibroblasts (MEFs). Importantly, this effect was not observed in Pten-deficient cells, indicating that the oncogenic activity of NEDD4-1 is PTEN dependent.

To evaluate NEDD4-1 in a more physiological context, Wang et al. (2007) analyzed a mouse model for prostate cancer in which the onset and rate of prostate tumorigenesis is directly correlated with decreased levels of Pten expression (Trotman et al., 2003). Using immunohistochemistry, they showed that neoplastic lesions in the prostate with decreased Pten levels contained increased amounts of Nedd4-1, thereby associating increased Nedd4-1 levels with oncogenesis. Furthermore, knockdown of NEDD4-1 by RNAi inhibited xenograft growth of a prostate tumor cell line expressing PTEN but had no effect on tumor growth of a PTEN-deficient cell line.

NEDD4-1 may not have oncogenic activity in all contexts, as it may not completely deplete PTEN levels. Are there human tumor types in which partial inactivation of PTEN is sufficient for oncogenesis? Many different types of sporadic tumors show frequent allelic deletion of *PTEN* but retain a wild-type allele. In these tumors, *PTEN* haploinsufficiency may or may not contribute to tumorigenesis. In a large collection of invasive human bladder cancers, there was an association between higher levels of *NEDD4-1* message and decreased PTEN protein. However, there was not a consistent inverse correlation between *NEDD4-1* and PTEN expression as would be predicted from a simple model where NEDD4-1 is the predominant regulator of PTEN steady-state levels.

A precedent for ubiquitin-mediated regulation of tumor suppressor activity is provided by MDM2, which ubiquitinates p53 and promotes its degradation. Amplification of *MDM2* in tumors with wild-type p53 provided compelling genetic evidence that *MDM2* can function as an oncogene in human tumors. Identification of *NEDD4-1* amplification in primary tumors would provide exciting evidence for oncogenic activity of this ubiquitin ligase in human cancer. Additional information about how NEDD4-1 is regulated, which tissues and physiological settings involve appreciable loss of PTEN due to NEDD4-1-mediated ubiquitination, what alternative proteins are also targeted by NEDD4-1-mediated ubiquitination, and whether other ubiquitin ligases also modify PTEN will provide critical insights into whether NEDD4-1 inhibition would be an effective therapeutic approach.

PTEN Ubiquitination: More than Meets the Eye

Also in this issue, Trotman et al. (2007) show that predicting the physiological outcome of NEDD4-1-mediated ubiquitination of PTEN is indeed quite complicated. They investigated a lysine to glutamate mutation in *PTEN* (K289E) identified in a Cowden syndrome family. Previous studies showed that this mutation did not disrupt the phosphatase activity or membrane localization of the mutated PTEN protein (Georgescu et al., 2000). In intestinal polyps obtained from a patient with an inherited K289E mutation, the wild-type *PTEN* allele was deleted, leaving only expression of the mutated allele. Using an intestinal polyp from a patient with this germline mutation, Trotman et al. (2007) showed that the K289E mutant protein was excluded from the nuclei of dysplastic epithelial cells. They next showed that overexpression of a GFP-PTEN K289E fusion protein resulted in predominantly cytoplasmic PTEN localization whereas a wild-type GFP-PTEN fusion protein was found in the nucleus and cytoplasm. This aberrant localization was not dependent on PTEN phosphatase activity but was due to a dramatic decrease in nuclear import, without enhancing nuclear export.

The authors showed that NEDD4-1 was capable of ubiquitinating K289, along with a number of other sites on PTEN. Overexpression of PTEN K289E showed that this mutation resulted in decreased PTEN monoubiquitination, although specific ubiquitination of K289 was not demonstrated directly in cells. Lysine 13 was identified as a second site of ubiquitination in transfected cells. A mutation in this lysine, K13E—originally identified in sporadic brain cancer—also disrupted nuclear localization and decreased the rate of nuclear import. Ubiquitin overexpression increased the rate of PTEN nuclear import, and overexpression of a mutated ubiquitin capable of only monoubiquitination resulted in a much greater increase.

This study shows a strong increase in nuclear PTEN associated with increased ubiquitination, particularly monoubiquitination. However, this effect could be mediated by ubiquitination of other substrates unrelated to PTEN. Therefore, PTEN localization was evaluated in the absence and presence of NEDD4-1. Knockdown of NEDD4-1 redistributed PTEN to the cytoplasm, whereas overexpression of NEDD4-1 increased nuclear PTEN localization. Monoubiquitinated PTEN was enriched in the nuclear fraction; however, the entire population of nuclear PTEN was not monoubiquitinated, suggesting that ubiquitin is removed from PTEN in the nucleus or that a proportion of nuclear PTEN was imported without ubiquitination. Clearly, the identification of PTEN-deubiquitinating enzymes in the nucleus would elucidate another level of regulation in PTEN stability and localization.

Interestingly, MDM2-mediated ubiquitination of p53 enhances its nuclear export and cytoplasmic degrada-

tion. In contrast, monoubiquitination mediated transport into the nucleus and increased PTEN stability (Trotman et al., 2007), whereas polyubiquitination of PTEN by NEDD4-1 enhanced degradation of PTEN (Wang et al., 2007) (Figure 1). Are these two opposing outcomes regulated through control of the ubiquitination process? It will be particularly interesting to determine if ubiquitination works in concert with previously identified mechanisms of PTEN turnover and nuclear localization. For example, phosphorylation of the C-terminal tail influences PTEN stability, although the physiological stimuli that influence this process remain unclear (Okahara et al., 2004; Vazquez et al., 2000). PTEN containing a mutation in K289 was excluded from nuclei, presumably because of decreased monubiquitination of this lysine. However, monoubiquitination of K289 is not required for PTEN nuclear import, as shown by both nuclear and cytoplasmic localization of a number of C-terminal deletion mutants lacking K289 (Shen et al., 2007). Several different modes of regulation for PTEN nuclear import have been described including passive transport by diffusion, active transport via putative nuclear localization signals within the C terminus of PTEN (Lian and Di Cristofano, 2005 and references therein), and Ran-dependent import mediated by an N-terminal nuclear localization domain along with multiple nuclear exclusion motifs (Gil et al., 2006). It is possible that monoubiquitination provides a signal for enhanced transport to the nucleus through some of these mechanisms, or through an independent process.

PTEN and Genomic Integrity

How does PTEN nuclear localization influence tumor suppression? Previous studies showed that PTEN loss contributed to genomic instability through cytoplasmic signaling by causing increased AKT-mediated sequestration of the kinase CHK1, thereby compromising the G2/S phase checkpoint in response to DNA damage (Puc et al., 2005). In this issue, Shen et al. (2007) investigated the association between PTEN deficiency and aneuploidy. They showed that *Pten*-deficient MEFs contained an increased number of chromosomal fragments with breakage at the centromeres, breakage of chromatids, and reciprocal translocations compared to wild-type MEFs. The frequency of chromosomal instability was much lower in *Pten*-deficient embryonic stem cells than in MEFs, suggesting that the contribution of Pten to genomic stability may vary between cell types. The nuclear pool of endogenous Pten protein colocalized with centromeres in MEFs, and Pten could be coimmunoprecipitated with the core centromeric protein Cenp-C, which is required for proper kinetochore assembly and for the metaphase to anaphase transition during mitosis.

Using mutations of *PTEN* that were originally identified in Cowden syndrome patients, Shen et al. (2007) showed that the C terminus, but not the phosphatase domain of PTEN, was required for interaction with Cenp-C and with centromeres. In wild-type cells, expression of C-terminally truncated PTEN proteins abrogated binding of endogenous wild-type Pten to Cenp-C and induced chromosomal abnormalities without affecting levels of phospho-Akt. This suggests that expression of a mutant PTEN lacking the C terminus can interfere with normal function of PTEN at the centromeres without affecting its cytoplasmic activity. Mechanistic experiments are required to determine if and how PTEN interaction influences CENP-C function in kinetochore assembly or metaphase to anaphase transition.

The chromatid breaks and translocations observed in *Pten*-deficient MEFs suggested a potential defect in the repair of DNA damage. Indeed, Shen et al. (2007) found that *Pten*-deficient MEFs showed increased nuclear foci containing phosphorylated histone H2AX and p53-binding protein 1, both of which bind to DNA double-strand breaks (DSBs). This is consistent with previous reports of increased nuclear foci containing phosphorylated H2AX following knockdown of PTEN by RNAi (Kim et al., 2006; Puc and Parsons, 2005). A survey for expression changes in DNA DSB repair genes in *Pten*-deficient MEFs identified a significant decrease in the mRNA and protein for Rad51, an essential component of homologous recombination and repair of DNA DSBs. Enforced expression of Rad51 in *Pten*-deficient MEFs decreased DSBs to levels that were comparable to wild-type MEFs, demonstrating a functional connection between depleted Rad51 levels and DSBs. Similarly, the PTEN null prostate cancer cell line PC-3 expressed very little RAD51, and exogenous PTEN expression induced RAD51 and diminished the occurrence of DNA DSBs in these cells.

A connection between PTEN loss and transcriptional regulation of Rad51 was unexpected. PTEN is known to influence transcription through inhibition of AKT-mediated phosphorylation and sequestration of the FOXO family of transcription factors (Chow and Baker, 2006; Engelman et al., 2006). This requires the phosphatase activity of PTEN and could potentially involve both cytoplasmic and nuclear PTEN. Shen et al. (2007) suggest a more direct impact of PTEN on transcription regulation, as they detected Pten bound to the Rad51 promoter by a chromatin-IP (ChIP) assay. Given the diverse functions already attributed to PTEN, it will be important to demonstrate that PTEN binds to DNA in a sequence-specific manner, as nonspecific binding of PTEN to DNA could give the same result in the ChIP assay. Although PTEN did not directly activate transcription of a Rad51 promoter reporter construct, PTEN expression enhanced E2F-mediated transactivation of this construct. Overexpression of PTEN can affect cell-cycle regulation of cultured cells, with outcome varying based on the cell line and the levels of exogenous expression. It is possible that E2F-mediated changes in Rad51 promoter activation could have been an indirect result of PTEN-mediated cell-cycle effects. Shen et al. (2007) postulated that Pten may cooperate with E2F-mediated transcription of Rad51 by influencing chromatin remodeling of the

Rad51 promoter. This mechanism does not explain the Pten-mediated effect in the transient transfection assay where the DNA reporter construct lacked histones. However, a connection between PTEN and chromatin remodeling is possible. Recent studies have shown interactions between PTEN and the transcriptional coactivators PCAF and p300 that function as histone acetyltransferases. These interactions are likely to result in complex functional outcomes beyond an influence on histone acetylation, as PCAF-mediated acetylation of PTEN can modulate PTEN phosphatase activity, and the interaction between PTEN and p53 stimulates p300-mediated acetylation of p53 (Li et al., 2006; Okumura et al., 2006).

Concluding Remarks

Taken together, the three papers in this issue raise a number of important questions about PTEN regulation and tumor suppression. What are the physiological signals to regulate PTEN stability, activity, and localization? When and how is disruption of this regulation involved in tumorigenesis? What is the relative contribution of nuclear PTEN and genomic stability to tumor suppression compared with its role as a cytoplasmic lipid phosphatase regulating PI3K signaling? Are all functions attributed to PTEN important for tumor suppression in every context, or are distinct activities engaged in particular oncogenic settings?

PTEN can participate in a number of cellular processes relevant to tumorigenesis including regulation of proliferation and survival, cell migration and invasion, angiogenesis, genomic instability, induction of cell-cycle checkpoints in response to DNA damage, and stem cell self-renewal (Chow and Baker, 2006 and references therein). This wide range of activities spans both the nucleus and the cytoplasm. Many but not all of these functions require the lipid phosphatase activity of PTEN that directly regulates PIP3 levels. In tumors with point mutations that selectively inactivate phosphatase activity, the phosphatase-independent activities of PTEN are presumably intact and therefore not involved. The Shen et al. (2007) and Trotman et al. (2007) studies made use of rare mutations found in Cowden syndrome patients. Analysis of these mutations that retained the phosphatase domain of PTEN, but deleted or mutated key residues in the C terminus, revealed novel insights into previously uncharacterized aspects of PTEN function. Are the ubiquitin-mediated regulation of PTEN stability and nuclear localization and the role of PTEN in chromosomal stability relevant for all tumors in which PTEN activity is compromised? Because the Cowden syndrome mutations tested in these studies are quite rare, it is impossible to evaluate patient information to determine whether these mutations could give rise to the full spectrum of disease associated with PTEN deficiency. It is likely that distinct subsets of PTEN functions are essential for tumor suppression in particular tissue types and in response to different combinations of oncogenic stimuli. Mouse models containing knockin mutations of key residues in the C terminus will help to determine whether effects on localization and chromosome stability are observed in all tissues and are associated with a tumor spectrum similar to null mutations studied in *Pten* heterozygous or conditional knockout mice. Understanding the specific involvement of the PTEN pathway in particular tumors, and the cellular players that may be modulated to influence these functions, will provide the necessary foundation for successful development and informed use of pathway inhibitors for cancer therapy.

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