The deubiquitylase Ataxin-3 restricts PTEN transcription in lung cancer cells

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The phosphatidylinositol-3-kinase (PI3K) pathway is commonly hyperactivated in cancer. One mechanism by which this occurs is by silencing of the phosphatase and tensin homolog (PTEN), a tumor suppressor and major antagonist of the pathway, through genetic, epigenetic or posttranscriptional mechanisms. Here, we used an unbiased siRNA screen in non-small-cell lung cancer cells to identify deubiquitylases (DUBs) that have an impact on PI3K signaling by regulating the abundance of PTEN. We found that PTEN expression was induced by depleting any of three members of the Josephin family DUBs: ataxin 3 (ATXN3), ataxin 3-like (ATXN3L) and Josephin domain containing 1 (JOSD1). However, this effect is not mediated through altered PTEN protein stability. Instead, depletion of each DUB increases expression of both the PTEN transcript and its competing endogenous RNA, PTENP1. In ATXN3-depleted cells, under conditions of transcriptional inhibition, PTEN and PTENP1 mRNAs rapidly decay, suggesting that ATXN3 acts primarily by repressing their transcription. Importantly, the PTEN induction observed in response to ATXN3 siRNA is sufficient to downregulate Akt phosphorylation and hence PI3K signaling. Histone deacetylase inhibitors (HDACi) have been suggested as potential mediators of PTEN transcriptional reactivation in non-small-cell lung cancer. Although PTEN exhibits a very limited response to the broad-spectrum HDACi Vorinostat (SAHA) in A549 cells, we find that combination with ATXN3 depletion enhances PTEN induction in an additive manner. Similarly, these interventions additively decrease cell viability. Thus, ATXN3 provides an autonomous, complementary therapeutic target in cancers with epigenetic downregulation of PTEN.

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Akt phosphorylation and enhance the ability of histone deacetylase inhibitors (HDACi) to induce PTEN expression and decrease cell viability, suggesting ATXN3 inhibition may be of therapeutic benefit in cancers with nongenetic inactivation of PTEN.

RESULTS AND DISCUSSION

We undertook an unbiased screen to identify members of the DUB superfamily that influence the levels of PTEN expression in a non-small-cell lung cancer cell line. We utilized A549 cells as they retain sufficient wild-type PTEN expression to restrict basal Akt phosphorylation.
phosphorylation despite a homozygous activating KRAS mutation but exhibit phosphorylation of Akt in response to EGF stimulation. A DUB siRNA library consisting of pools of four oligonucleotides for each of 92 human DUBs was designed in collaboration with Qigian (Manchester, UK; library details available on request). Cells were transfected with these siRNA pools or with control reagents and nontargeting siRNAs. Libraries of cellular proteins were prepared 72 h later by sequential extraction of cytosol, nucleoplasm and the residual pellet, which were arrayed into 96-well plates. PTEN was only detectable in the cytosol fraction (data not shown), which was utilized for this siRNA screen. The effect of depleting each DUB on PTEN abundance is shown in Figure 1c.

In view of the potential therapeutic applications for reactivating PTEN, we concentrated on the DUBs whose depletion elevated PTEN levels most markedly. Interestingly, among these were three of the four members of the Josephin sub-family, also known as the Machado Joseph Disease sub-family: ATXN3, ATXN3L and JOSD1 (Figure 1b). Each of these ranked above USP7 (HAUSP), a DUB that was previously implicated in the regulation of PTEN monoubiquitylation and sub-cellular localization. Of note, the Josephin DUBs did not significantly or coordinately increase the expression of PTEN, we concentrated on the DUBs whose depletion elevated PTEN. Estimates of PTEN protein half-life vary widely in different cell lines and conditions, ranging from less than 10 h to more than 72 h. We found that the half-life of PTEN was greater than 20 h in A549 cells, and the half-life of ATXN3 was of a similar order (Figure 2c). Although ATXN3 depletion elevated PTEN levels (Figure 2c), it did not influence the rate of PTEN turnover (Figure 2d), suggesting that its role is independent of direct PTEN ubiquitylation.

Many DUBs have been shown to act at the level of RNA processing and ATXN3 has an established role as a transcriptional repressor. We next investigated whether ATXN3 might influence the levels of the PTEN transcript. As some reduction in cell numbers was apparent after 72 h, transcripts were analyzed after 48 h ATXN3 depletion. We found that PTEN mRNA was significantly increased compared with control cells (Figure 3a). The effect of ATXN3 depletion on PTEN protein level was also apparent after 48 h, and strongly correlated with the PTEN RNA level in parallel samples (Figure 3b). Thus, ATXN3 represses PTEN expression.
ATXN3 depletion leads to increased PTEN transcript, which in turn leads to elevated cellular PTEN protein. However, the regulation of PTEN mRNA is highly complex, in part due to co-regulation with ceRNAs. Networks of ceRNAs act as microRNA sponges, so that when a given transcript is overexpressed, cellular concentrations of certain microRNA recognition elements are increased and can result in the de-repression of other transcripts containing the same microRNA recognition elements. The first proven ceRNA, and that most tightly linked to PTEN, is the highly homologous pseudogene PTENP1. We therefore assessed whether the PTENP1 transcript was also responsive to ATXN3 depletion and found that it closely mirrored the response of the PTEN mRNA (Figure 3a). Therefore, ATXN3 may directly regulate the PTEN transcript, or indirectly affect it through regulation of a ceRNA such as PTENP1.

Interestingly, the three DUBs that were validated by deconvolution from the initial screen (Figure 1) are all members of the Josephin/Machado Joseph Disease family, which consists of only four cysteine proteases that share a catalytic Josephin domain. We tested whether these three DUBs function towards PTEN in a similar manner, and found that the individual depletion of ATXN3, ATXN3L or JOSD1 in each case induced both PTEN and PTENP1 mRNA levels by twofold or more (Figure 3c). This suggests that, whether by direct or indirect mechanisms, the regulation of PTEN transcription may be a conserved function of this enzyme family.

To establish whether ATXN3 primarily influences the rate of transcription or the rate of transcript degradation, we performed an actinomycin D chase. The PTENP1 transcript was markedly less stable than that for PTEN (Figure 3d), consistent with a role for elevated PTENP1 in sustaining PTEN expression. In ATXN3-depleted cells, the twofold increase in PTEN/PTENP1 transcript levels was not accompanied by an increase in PTEN/PTENP1 mRNA stability; in fact, both transcripts exhibited an increase in their initial turnover and a reduced half-life (Figure 3e). This rapid re-equilibration in the absence of new transcription is consistent with a transcriptional mechanism for ATXN3. Notably in ATXN3-deficient cells, PTENP1 was again least stable, rapidly degrading during the first 30 min of transcriptional arrest, whereas PTEN turnover commenced after 30 min. Despite a pool of PTEN mRNA that exhibits a shorter half-life in ATXN3-depleted cells, a second more stable pool persisted for at least 6 h, perhaps indicating a secondary effect of ATXN3 on PTEN transcript stability.

As PTEN reactivation may have therapeutic applications in cancer, we next asked whether ATXN3-dependent PTEN
induction has an impact on PI3K signaling. To this end, we depleted ATXN3 and monitored Akt phosphorylation in response to acute EGF stimulation. In this experiment, PTEN depletion increased Akt phosphorylation, demonstrating that PTEN is functional and partially restricts PI3K signaling in A549 cells (Figure 4a). In contrast, the two individual ATXN3 siRNAs (siATXN3_3 and _5) that caused the most profound PTEN induction (Figures 2a and 3a) blunted Akt T308 phosphorylation in response to EGF (Figure 4a).

As PTEN expression may be restricted by histone deacetylation, HDACi have been proposed as therapeutic tools to reactivate PTEN.42,43 We investigated the efficacy of this approach in A549 cells using the broad-spectrum HDACi Vorinostat (SAHA), which is licensed for clinical use in cutaneous T-cell lymphoma.44 SCG3, a transcript that is tightly repressed by REST45 in an HDAC-dependent manner, shows a robust dose-dependent response over a low micromolar range of Vorinostat in A549 cells (Figure 4b). In contrast, PTEN and PTENP1 transcripts exhibit a non-monotonic dose–response curve, with a maximal response at 2 μM of threefold increased PTEN and 1.5-fold increased PTENP1 (Figure 4b). Thus, in isolation, ATXN3 depletion or Vorinostat stimulation can achieve comparable PTEN mRNA induction. Importantly, however, combining the two treatments had an additive effect, resulting in an almost sixfold increase in the PTEN transcript (Figure 4c). At the protein level, ATXN3 siRNA was in fact a more potent inducer of PTEN than Vorinostat and, in combination, an additive 3.5-fold increase in PTEN protein was reached (Figure 4d).

We did not observe cytotoxicity during 16 h Vorinostat treatment. To ascertain the lethal concentration, we extended the period of drug exposure to 48 h; the effective LC50 for...
Figure 4. Reactivating PTEN expression and PI3K pathway inhibition through ATXN3 depletion and HDAC inhibition. (a), ATXN3 depletion limits Akt phosphorylation. A549 cells were transfected with siRNAs as indicated (NT1, nontargeting control). After 48 h cells were stimulated with 0.5 ng/ml EGF (Preprotech) for 5 min and lysed in ice-cold NP40 buffer (0.5% NP40, 25 mM Tris pH 7.9, 100 mM NaCl, 50 mM NaF, with protease and phosphatase inhibitors (Roche)). Samples were processed for immunoblotting with rabbit anti-phospho-Akt (T308, Cell Signaling, 9275) and other antibodies as before. An asterisk indicates nonspecific bands. (b), PTEN transcription shows a limited biphasic response to HDAC inhibition. A549 cells were treated with increasing concentrations of Vorinostat (Selleck) for 16 h prior to preparation of total mRNA. Transcript levels were determined by qRT-PCR and are shown normalized to the vehicle control (below). SCG3 is included as an example of a transcript that is highly responsive to HDAC inhibition. A549 cells were transfected with increasing concentrations of Vorinostat (Selleck) for 16 h prior to preparation of total mRNA. Transcript levels were determined by qRT-PCR and are shown normalized to the vehicle control (below). SCG3 is included as an example of a transcript that is highly responsive to HDAC inhibition. (c and d), ATXN3 depletion enhances the PTEN response to HDAC inhibition. A549 cells were transfected for 48 h and treated with vehicle or 2 μM Vorinostat for the final 16 h prior to preparation of total mRNA or protein extracts. (c), Transcripts were quantified by qRT-PCR and are shown normalized to the level in mock-transfected cells. Bars show the mean values obtained from two independent experiments. (d), In a parallel experiment to that shown in panel c, protein was extracted in NP40 buffer and immunoblotted for ATXN3 and PTEN. (e and f), ATXN3 depletion and HDAC inhibition are additive with respect to cell viability. A549 cells were transfected as indicated with siRNA for 72 h and treated with vehicle (0.04% DMSO) or Vorinostat for the final 48 h prior to analysis. Viable cell numbers were determined based on proportionality with the amount of ATP present, measured using the CellTitre-Glo assay (Promega). Mean values are derived from three independent experiments and normalized to vehicle controls; the error bars show standard deviation. (e) In siC-transfected cells the LC50 for Vorinostat is 2.4 μM. (f), ATXN3 depletion reduces viable cell numbers and works additively with HDAC inhibition. In each case, Vorinostat significantly reduced cell viability relative to the vehicle control, and ATXN3 siRNAs significantly reduced cell viability relative to siC (P < 0.05).
Vorinostat in A549 cells was 2.4 μM (Figure 4e), similar to the optimal concentration for PTEN induction (Figure 4b). We therefore assessed the phenotypic effects of ATXN3 depletion on cell viability by depleting ATXN3 for 72 h, either alone or in conjunction with 48 h in 2 μM Vorinostat. Either treatment reduced the viable cell number by approximately 50%. However, combining ATXN3 depletion with HDAC inhibition decreased the number of residual viable cells to less than 25% (Figure 4f), in line with the additive effects we observed for induction of PTEN expression (Figures 4c and 4d).

Through unbiased siRNA screening we have identified, for the first time, DUBs that regulate the expression of PTEN. These closely related Josephin DUBs restrict PTEN transcription. ATXN3 is by far the best characterized of the three, although JOSD1 was recently shown to be membrane-associated and to influence both cellular motility and endocytosis.3 to date, no specific function has been ascribed to ATXN3L, although its depletion decreases the viable cell number by approximately 50%. However, combining ATXN3 depletion with HDAC inhibition decreased the number of residual viable cells to less than 25% (Figure 4f), in line with the additive effects we observed for induction of PTEN expression (Figures 4c and 4d).

Interference, which can effectively knockdown ATXN3 in mice,30 could mediate their specific repressor activity towards PTEN and/or PTENP1. In fact, ATXN3 interacts with and inhibits CREB-binding protein are limiting, so that sequestration of CREB-binding protein is limiting, so that sequestration of CREB-binding protein could prevent its recruitment to specific promoters, as previously described for suppression of PTEN expression by p53/ReI.52 Interestingly, in the context of interplay between ATXN3 depletion and HDAC inhibition, somatic mutations of CREB-binding protein sensitize diffuse large B-cell lymphoma to Vorinostat.53

Importantly, as PTEN is frequently silenced in cancer through non genomic mechanisms and even small changes in PTEN expression affect outcome, its pharmaceutical reactivation is likely to prove a valuable therapeutic strategy. Targeting the Josephin DUBs provides a novel mechanism by which this may be achieved. ATXN3 is a key therapeutic target in neurodegeneration, as polyglutamine expansion of ATXN3 causes Machado-Joseph Disease.54 Strategies to target ATXN3 include the use of in vivo RNA interference, which can effectively knockdown ATXN3 in mice, and a β-hydroxy-β-ketoacid inhibitor reported to retard progression of polyglutamine-expanded ATXN3.55 Combination therapy, with ATXN3 inhibitors and HDACi, is thus a realistic proposition for PTEN-repressed cancers including lung adenocarcinoma.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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ATXN3 represses PTEN expression
JJ Sacco et al

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