Enhancers regulate polyadenylation site cleavage and control 3′UTR isoform expression

Buki Kwon, Neil D. Patel*, Shih-Han Lee*, Jihye Lee, Weirui Ma, and Christine Mayr[1,†]

Cancer Biology and Genetics Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

*, These authors contributed equally

[1]Lead Contact

[†]Correspondence: mayrc@mskcc.org

Christine Mayr
Sloan Kettering Institute
1275 York Ave, Box 303
New York, NY 10065
Phone: 646-888-3115

Summary

Enhancers are DNA elements that increase gene expression. mRNA production is determined by transcript production and polyadenylation site (PAS) cleavage activity. We established an assay to measure enhancer-dependent PAS cleavage activity in human cells because PAS cleavage may control alternative 3′UTR isoform expression. We found that enhancers are widespread regulators of PAS cleavage and consistently increase cleavage of proximal and weak PAS. Half of tested transcription factors exclusively regulated PAS cleavage without affecting transcript production, whereas co-activators changed both parameters. Deletion of an endogenous enhancer of *PTEN* did not change gene-level mRNA or protein abundance but affected expression of alternative mRNA transcripts, thus preventing 3′UTR shortening. Our data reveal that in addition to controlling transcript production, enhancers also regulate PAS cleavage, thus changing 3′UTR isoform usage and protein activity, as PTEN proteins translated from the alternative 3′UTR isoforms differ in intrinsic lipid phosphatase activity despite having identical amino acid sequences.

Introduction

Comparative genome analyses showed that organismal complexity scales with gene regulation [1]. It is thought that the increased gene regulatory capacity is largely accomplished by enhancers [1]. According to the original definition, enhancers are sequences that increase the expression of a reporter gene [2,3]. Currently, it is thought that enhancers mostly affect transcript production [3], but mRNA processing is essential for the generation of mature mRNAs and this step includes 3′ end cleavage and polyadenylation. Therefore, when disregarding the contribution of mRNA stability, mRNA production of unspliced transcripts is largely determined by the number of produced transcripts and by the cleavage activity of the polyadenylation site (PAS).

Although PAS cleavage activity is difficult to measure directly, its contribution to mRNA expression levels is revealed by single point mutations that occur in PAS or in their surrounding sequence elements. Such mutations were found in the genes encoding α- and β-globin, p53, and prothrombin. These mutations are associated with disease phenotypes, including α- or β-thalassemia, cancer predisposition syndrome, and thrombophilia, despite causing only a 1.5-fold difference in steady-state mRNA levels [4-7].

In addition to *cis*-elements, differential expression of *trans*-acting polyadenylation factors will influence PAS cleavage. As weak elements are often prone to upregulation by *trans*-acting factors, it is likely that weak PAS are the most regulated PAS. This mode of regulation may be especially important for genes that use alternative cleavage and polyadenylation to generate mRNA isoforms with alternative 3′UTRs as the majority of proximal PAS in 3′UTRs are regarded to be intrinsically weak [8]. Here, we used genes that generate alternative 3′UTRs as experimental system to study PAS cleavage activity as a change in cleavage activity of proximal PAS would manifest at the mRNA level as a change in alternative 3′UTR isoform usage.

Approximately half of human genes generate mRNA isoforms with alternative 3′UTRs [9]. These genes have especially long 3′UTRs that are on average more than four-times longer than genes with constitutive 3′UTRs [9]. Similar to the higher number of enhancers found in more complex organisms also 3′UTR length correlates with the number of cell types present in an organism which is often used as a measure for organismal complexity [10,11]. The expansion of sequence elements in 3′UTRs may provide more post-transcriptional gene regulatory capacity.

3′UTRs control several aspects of mRNA metabolism including mRNA localization, mRNA stability and translational efficiency [11,12]. Although a change in 3′UTR isoform usage does not affect the protein sequence, alternative 3′UTRs were shown to regulate protein multifunctionality

by controlling alternative protein complex assembly [13-15]. 3′UTRs also enable translation of proteins within defined subcellular compartments, which may be another way to influence protein functions [16]. These data indicate that functional diversity of proteins can be encoded by genomic sequence elements that are transcribed into 3′UTRs, thus providing one explanation of how 3′UTRs can contribute to increased organismal complexity.

As changes in alternative 3′UTR isoform usage can have important functional consequences, it is critical to understand how alternative 3′UTRs are controlled. Through knock-down (KD) experiments of RNA-binding proteins, including polyadenylation and splicing factors, it was shown that these factors often induce global shifts in 3′UTR isoform usage [17-24]. However, genome-wide analyses of 3′UTR isoform usage across cell types and various conditions did not show global shifts, but instead revealed gene- and condition-specific changes in 3′UTR ratios, meaning that the same stimulus in different cell types changed the usage of different PAS [9].

In addition to RNA-binding proteins mRNA processing can also be influenced by promoters. For example, promoters may recruit polyadenylation and splicing factors or may influence transcription elongation [25-28], reviewed in [29,30]. Moreover, promoters were reported to influence several aspects of mRNA metabolism, including mRNA decay, mRNA localization, and translation [31-34], reviewed in [35].

As cell type- and condition-specific regulation is often mediated by transcription factors that bind to enhancers [3,36], we established a reporter assay to investigate if promoters and enhancers regulate PAS cleavage activity in mammalian cells. Whereas promoters did not influence PAS cleavage activity in our system, we found that enhancers predominantly increased cleavage activity of proximal PAS. The increase is mediated by transcription factors and co-activators. Deletion of an endogenous enhancer revealed that enhancers are required for a switch in alternative 3′UTR isoform usage. For *PTEN*, this mode of regulation is biologically important as the enhancer-mediated 3′UTR isoform change regulates the intrinsic enzymatic activity of PTEN protein in a manner that is independent of overall protein abundance.

Results

Promoters do not regulate PAS cleavage activity

The *PTEN* gene generates alternative 3′UTR isoforms that encode the same protein (Figure 1A) [9]. A change in cultivation conditions, including media acidification through prolonged culture or

the addition of hydrochloric acid, induces 3′UTR shortening of *PTEN* in MCF7 cells (Figures 1B and S1A).

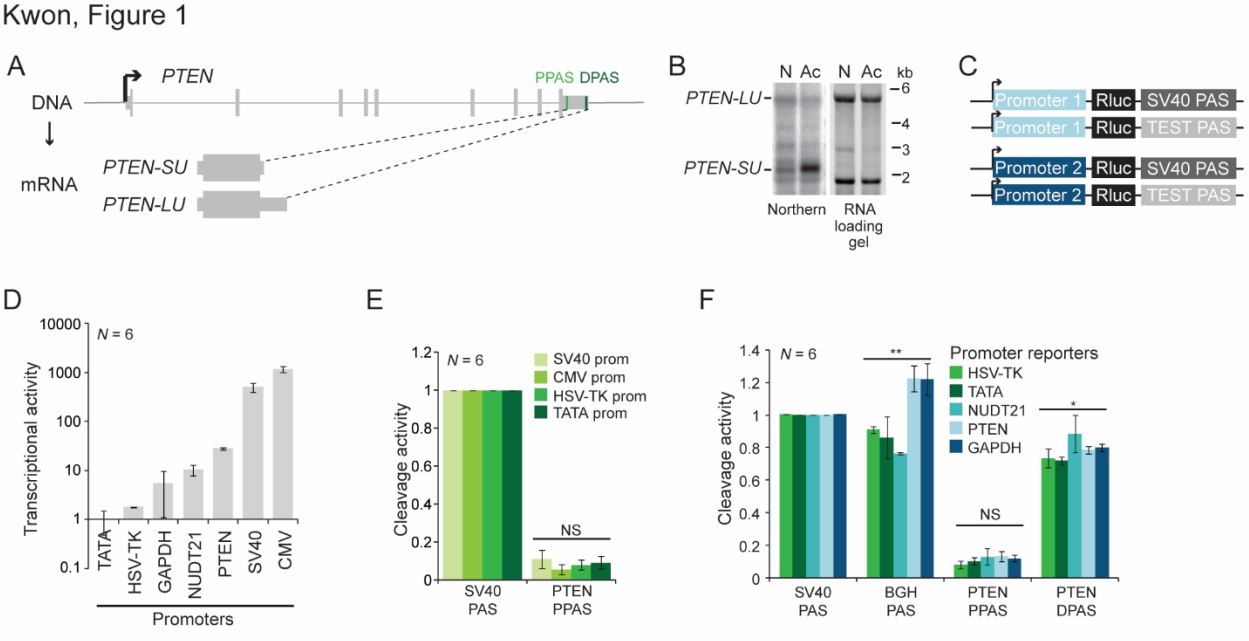

Figure 1. Promoters do not regulate PAS cleavage activity.
(A) Gene model of PTEN. The gene is processed into alternative mRNA isoforms that differ in their 3′UTRs but encode proteins with identical sequence. Transcript cleavage at the proximal PAS (PPAS) generates PTEN-SU, whereas transcript cleavage at the distal PAS (DPAS) generates PTEN-LU.
(B) Northern blot showing PTEN 3′UTR isoform expression in MCF7 cells cultivated in normal (N) or acidified media (Ac; pH = 6.5). The shortest (PTEN-SU) and longest (PTEN-LU) 3′UTR isoforms are indicated. Cultivation in acidified media switches 3′UTR isoform ratios and results in 3′UTR shortening of PTEN.
(C) Schematic of luciferase reporter constructs used to investigate promoter-dependent PAS cleavage activity. The reporter construct with the SV40 PAS provides transcriptional activity of the promoter. Cleavage activity of a test PAS is obtained by the ratio of the luciferase activities obtained from the test PAS reporter over the SV40 PAS reporter when transcribed from the same promoter. The transcription start site is indicated by the arrow. Rluc, Renilla luciferase. See Fig. S1B for more extensive description.
(D) Transcriptional activities of the indicated reporters in MCF7 cells. Transcriptional activity corresponds to luciferase activity of each reporter when terminated by the SV40 PAS. The promoter sequences are reported in Table S1.
(E) Cleavage activity of the PTEN PPAS is independent of transcriptional activity. PTEN PPAS cleavage activity is similar when the reporter is expressed from four different viral promoters despite a 500-1,000-fold difference transcriptional activity, shown in (D). One-way ANOVA was performed. NS, not significant.
(F) Promoter-dependent PAS cleavage activity of different PAS. Bovine growth hormone (BGH) PAS, PTEN PPAS (proximal PAS of PTEN), PTEN DPAS (distal PAS of PTEN). The PAS sequences are reported in Table S1. One-way ANOVA was performed. **, P = 3 x 10^-9; *, P = 0.04.

We set out to investigate how the switch in 3′UTR isoform expression is regulated and hypothesized that promoters may regulate PAS usage in living cells. To assess promoter-dependent PAS cleavage activity, we established a luciferase reporter system that measures

cleavage activity of individual PAS relative to the SV40 virus PAS (Figures 1C, S1B, and Table S1A). As the SV40 PAS is one of the strongest known PAS, it results in cleavage of basically all produced transcripts [37,38]. Therefore, luciferase activity correlates with transcriptional activity of the promoter when the reporter is terminated by the SV40 PAS, a system that has been widely used to measure transcriptional activity [39]. To obtain cleavage activity of a specific test PAS, we generated a similar reporter that instead of the SV40 PAS uses the test PAS for termination. The ratio of luciferase activities obtained from the reporter terminated by the test PAS over the SV40 PAS represents the cleavage activity of the test PAS (Figures 1C and S1B).

We examined promoter-dependent PAS cleavage activity of several promoters derived from viruses or human genes that differ 1000-fold in transcriptional activity (Figure 1D and Tables S1B and S1C). In the context of viral promoters, the proximal PAS (PPAS) of *PTEN* was weak compared to the strong SV40 PAS (Figure 1E). In addition to the SV40 PAS that was used as normalization control, we tested cleavage activity of the strong bovine growth hormone (BGH) PAS that is used in many expression vectors as well as the distal (DPAS) of *PTEN*. We observed a large difference in PAS cleavage activity between the weak PPAS and strong DPAS of *PTEN,* however, different promoters had only a small influence (less than 1.7-fold) on PAS cleavage activity (Figure 1F).

The PTEN enhancer increases cleavage activity of weak PAS

Next, we investigated if enhancers influence PAS cleavage activity and tested the promoter-proximal *PTEN* enhancer (Penh) in the context of the *PTEN* promoter (Pprom; Figures 2A, 2B, and Table S1D). The region of the Penh shows high levels of acetylated H3K27 (H3K27ac) in MCF7 cells and contains a large number of transcription factor binding sites (Figures 2A and S2). Although typical enhancers increase transcriptional activity, the Penh modestly reduced transcriptional activity (< 1.6-fold) in the context of the Pprom but increased transcriptional activity in the context of a weak core promoter (Figures 2C and S1C). Addition of the enhancer had little influence on the cleavage activity of strong PAS, such as the BGH PAS or the PTEN DPAS (Figure 2D). However, strikingly, the enhancer increased cleavage activity of the PTEN PPAS by 3.7-fold (Figure 2D).

In this reporter system, relative PAS cleavage activity corresponds to luciferase activity if the different PAS do not influence stability of the reporters. To minimize the elements that affect mRNA stability we used minimal PAS that only contained 100 base pairs of surrounding

sequence to ensure proper recognition of PAS (Table S1A). We also measured mRNA stability of the reporters and did not observe enhancer or PAS-dependent differences in stability (Figure S1D). To evaluate if presence of the enhancer changed the transcription start site of the reporter, we performed 5′RACE (rapid amplification of cDNA ends) and observed usage of the annotated transcription start site in the presence or absence of the enhancer (Figure 2E). This indicates that the mature mRNAs produced from the *PTEN* promoter, in the presence or absence of the *PTEN* enhancer, are identical.

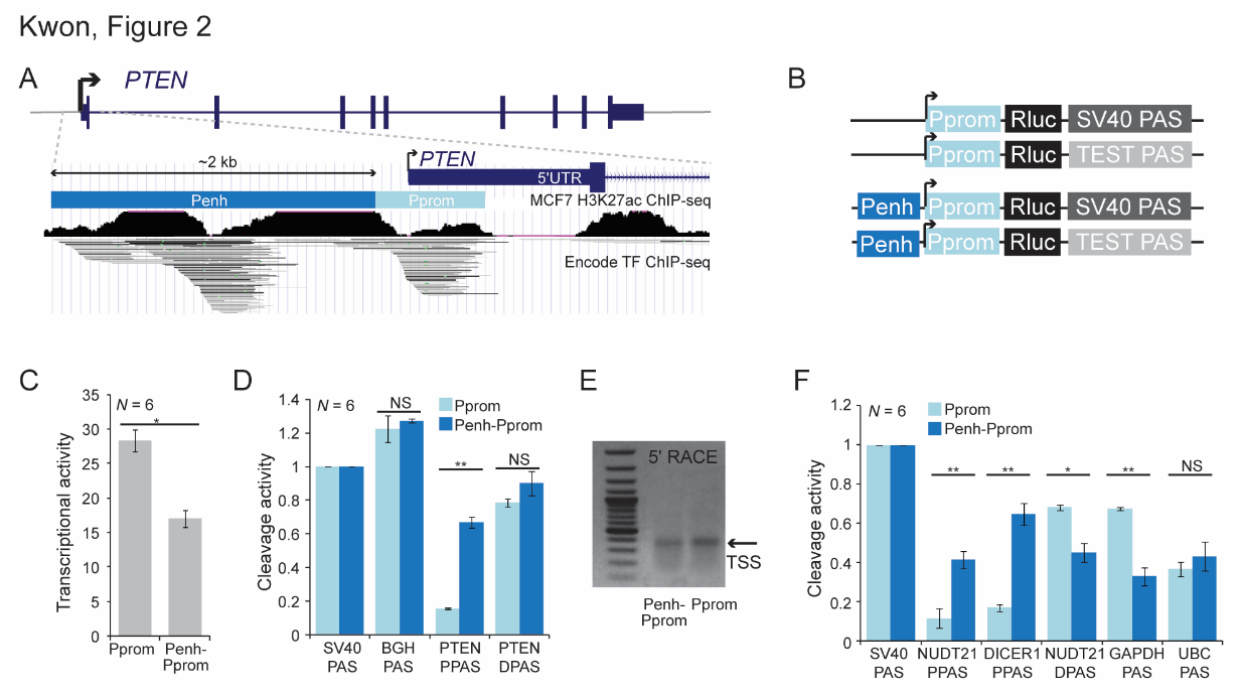

Figure 2. Enhancers upregulate cleavage activity of proximal and weak PAS.
(A) UCSC genome browser snapshot showing the PTEN genomic locus and a zoom into the vicinity of the transcriptional start site (arrow). Also shown are Encode tracks for H3K27ac in MCF7 and transcription factor (TF) ChIP-seq binding sites. Penh, PTEN enhancer; Pprom, PTEN promoter.
(B) Schematic of reporter constructs to investigate enhancer-dependent PAS cleavage activity. As in Fig. 1C, but instead of comparing the influence of two promoters on PAS cleavage activity, the presence or absence of the Penh in the context of the Pprom is examined.
(C) Transcriptional activity of the Pprom reporter in the presence (Penh-Pprom) or absence (Pprom) of the PTEN enhancer obtained by luciferase activity of the reporters when terminated by the strong SV40 PAS. T-test for independent samples was performed; P = 0.002.
(D) Enhancer-dependent PAS cleavage activity of different PAS. The Penh increases PTEN PPAS cleavage activity. T-test for independent samples was performed; **, P = 1 x 10^-8; NS, not significant.
(E) The transcription start sites of the Pprom reporters in the presence or absence of Penh was determined by 5′RACE. The Penh is a true enhancer and does not change the transcription start site. The canonical transcription start site (TSS) is used in both reporters and is indicated by the arrow.
(F) As in (D), but additional PAS are shown. T-test for independent samples was performed; **, P = 1 x 10^-6; *, P = 0.001.

Enhancers are known to regulate transcription independently of their orientation and can be located up- or downstream of genes [3,36]. We cloned the reverse complement of the enhancer downstream of the PAS and measured PAS cleavage activity. This revealed that the capacity of the enhancer to regulate transcriptional and PAS cleavage activity was largely retained when located downstream and in the opposite orientation (Figures S1E and S1F). Taken together, these data suggest that regulation of PAS cleavage activity is a *bona fide* enhancer activity.

Next, we assessed if the enhancer controls cleavage activity of additional PAS. The cleavage activity of the PPAS from *NUDT21* and *DICER1* increased 3.6-fold in the presence of the enhancer, whereas cleavage activity of non-proximal PAS (NUDT21 DPAS, GAPDH PAS, UBC PAS) did not change in a coordinated manner (Figure 2F). These observations suggest that in the absence of an enhancer cleavage activity largely depends on the intrinsic strength of a PAS, but intrinsically weak PAS can have high *in vivo* cleavage activity when transcribed from promoters with active enhancers.

The *PTEN* enhancer is required for a switch in 3′UTR isoform expression of *PTEN* at the endogenous locus

To assess if the *PTEN* enhancer regulates PTEN PPAS usage at the endogenous gene locus, we used MCF7 cells that express wild-type *PTEN* [40] and deleted the promoter-proximal enhancer using a pair of guide RNAs (Figure 3A). Two clones (delta enhancer 1 (dE1) and dE2) with a heterozygous deletion in the region of the *PTEN* enhancer were used for follow-up experiments (Figures S3A and S3B). Deletion of the enhancer did not affect steady-state *PTEN* mRNA or protein level (Figures 3B and 3C). As the reporter assay suggested that the enhancer is required for increased PPAS usage, we performed a Northern blot of the parental wild-type (WT) and the enhancer deletion clones after cultivating them in normal and acidic media conditions. Under these conditions, WT cells induce the expression of the short *PTEN* 3′UTR isoform at the expense of the long 3′UTR isoform (Figure 3D). In contrast, cells with enhancer deletion did not upregulate the expression of the short *PTEN* 3′UTR isoform. These results indicate that the *PTEN* enhancer is required for *PTEN* 3′UTR shortening.

The switch in 3′UTR isoform expression of *PTEN* increases PTEN protein activity without affecting overall *PTEN* mRNA or protein abundance

Interestingly, the pH-induced 3′UTR ratio change did not affect overall PTEN protein levels (Figure 3E), suggesting that the alternative 3′UTR isoforms are translated with equal efficiency into PTEN protein.

Kwon, Figure 3

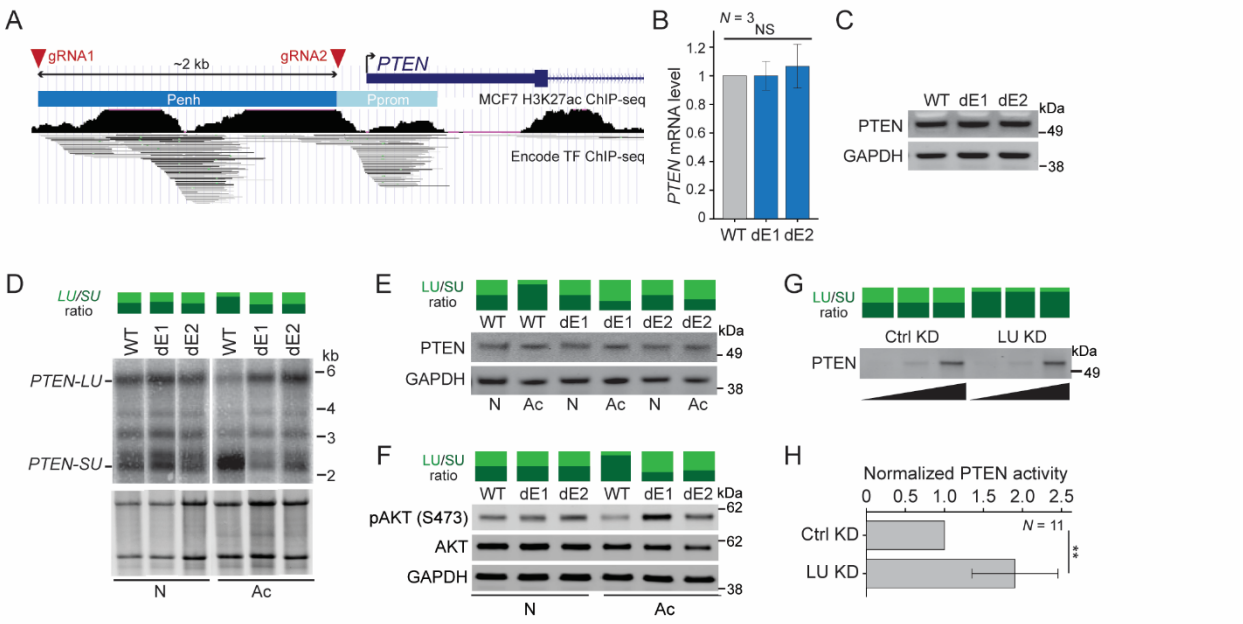

Figure 3. The PTEN enhancer is required for 3′UTR shortening of PTEN at the endogenous gene locus and regulates PTEN protein activity through a 3′UTR isoform change.
(A) As in Fig. 2A but indicated are the positions of the guide RNAs (gRNA) used for heterozygous deletion of the PTEN enhancer in MCF7 cells.
(B) qRT-PCR in wild-type (WT) and enhancer deletion cells (dE1, dE2) shows that steady-state PTEN mRNA level are not affected by heterozygous PTEN enhancer deletion. RPL19 mRNA was used for normalization. T-test for independent samples was performed
(C) Western blot of PTEN in WT and enhancer deletion cells was performed in steady-state cultivation conditions. GAPDH was used as loading control.
(D) Northern blot of endogenous PTEN from WT and enhancer deletion cells cultivated in normal (N) or acidified (Ac, pH = 6.5) media. A low pH induces 3′UTR shortening of PTEN which is prevented upon lack of the enhancer. The RNA gel is shown as loading control. The colored boxes indicate the contribution of PTEN-SU and PTEN-LU to total PTEN mRNA levels.
(E) Western blot of PTEN in WT and enhancer deletion cells after cultivation in normal or acidified media. GAPDH was used as loading control. The colored boxes indicate the contribution of PTEN-SU and PTEN-LU to total PTEN protein levels.
(F) Western blot of pAKT (S473) and total AKT in WT and enhancer deletion cells after cultivation of the cells in normal or acidified media. GAPDH is shown as additional loading control. The colored boxes indicate the contribution of PTEN-SU and PTEN-LU to total PTEN protein levels. Increased expression of PTEN-SU correlates with higher PTEN phosphatase activity, corresponding to lower pAKT level. Low amounts of PTEN-SU in enhancer deletion cells correlate with lower PTEN activity and higher pAKT level.
(G) Western blot showing PTEN protein amount that was used as input for PTEN activity assessment by ELISA. To better judge protein levels, increasing amounts of protein were loaded. Ctrl KD cells were obtained by stably expressing a ctrl shRNA, whereas LU KD cells were obtained by stably expressing an shRNA against the long 3′UTR isoform of *PTEN*. The colored boxes indicate the contribution of PTEN-SU and PTEN-LU to total PTEN protein levels.
(H) Enzymatic PTEN lipid phosphatase activity was measured by ELISA with PIP3 as substrate. LU KD cells predominantly generate PTEN-SU which correlates with a higher enzymatic activity. N = 11 biological replicates; T-test for independent samples was performed; **, P = 0.003.

As the alternative 3′UTR isoforms do not change the sequence or size of PTEN, PTEN protein encoded by the short or long 3′UTR isoforms cannot be distinguished by western blot. We call the alternative PTEN protein products encoded from the mRNA isoforms with the short or long

3′UTRs PTEN-SU or PTEN-LU, respectively. The relative contribution of PTEN-SU and PTEN-LU to total PTEN protein is indicated by the colored boxes (Figure 3E).

Next, we investigated the biological significance of a *PTEN* 3′UTR ratio change. As PTEN is a phosphatase, we examined if the alternative 3′UTR isoforms influence PTEN activity. PTEN is a major negative regulator of the PI3K pathway and high PTEN activity corresponds to low levels of phosphorylated AKT (pAKT) [41,42]. We observed that PTEN activity correlates with the abundance of PTEN-SU. Under normal cultivation conditions cells generate similar amounts of the short and long 3′UTR isoform of *PTEN,* corresponding to an intermediate PTEN phosphatase activity (Figure 3F). The pH-induced increase in short 3′UTR isoform expression in WT cells was associated with higher PTEN phosphatase activity, whereas lack of 3′UTR shortening in enhancer deletion cells correlated with lower PTEN activity, indicated by higher levels of pAKT (Figure 3F). To investigate if the difference in PTEN activity is caused by 3′UTR-dependent regulation of intrinsic enzymatic activity, we performed an ELISA and measured PTEN lipid phosphatase activity using PIP3 as substrate. In order to avoid pH-mediated PTEN activity regulation, we obtained MCF7 cells that predominantly express the short *PTEN* 3′UTR isoform using shRNA-mediated KD of the long *PTEN* 3′UTR (LU KD; Figure S3C). Control (Ctrl) KD cells express similar amounts of *PTEN-SU* and *PTEN-LU*. We immunoprecipitated PTEN protein from Ctrl KD and LU KD cells and used equal amounts of total PTEN protein as input for the ELISA (Figure 3G). Intriguingly, we observed higher PTEN lipid phosphatase activity towards PIP3 with PTEN-SU compared with PTEN-LU (Figure 3H). Taken together, these results indicate that PTEN protein translated from the mRNA isoform containing the short 3′UTR has a higher intrinsic activity than PTEN protein translated from the long 3′UTR isoform. As short vs long 3′UTR isoform expression is regulated by the promoter-proximal *PTEN* enhancer, our data indicate that the enhancer regulates protein activity independently of overall protein abundance.

A distal enhancer also regulates PAS cleavage activity

We then asked if PAS cleavage activity is regulated by other enhancers. The *NUDT21* gene encodes an important polyadenylation factor and generates alternative 3′UTRs [9]. We tested if the enhancer of the *NUDT21* gene regulates PAS cleavage activity. As high levels of H3K27ac are characteristic of enhancers [3,36], we searched for increased H3K27ac levels in the vicinity of the *NUDT21* gene. We detected a ChIP-seq peak with high H3K27ac levels approximately 80 kb downstream of the *NUDT21* gene which is suggestive of a distal enhancer (Figure 4A). We cloned 2 kb of this region that we called the *NUDT21* enhancer (Nenh) and placed it upstream

of the Pprom or the *NUDT21* promoter (Nprom) into the luciferase reporters (Figure 4B). In the context of the reporter, the Nenh did not significantly change transcriptional activity of the two promoters (Figure 4C). However, it increased PTEN PPAS cleavage activity between 3.4 and 5.3-fold without affecting cleavage activity of a stronger PAS (Figures 4D and 4E). This suggests that enhancer-mediated regulation of PAS cleavage activity is widespread.

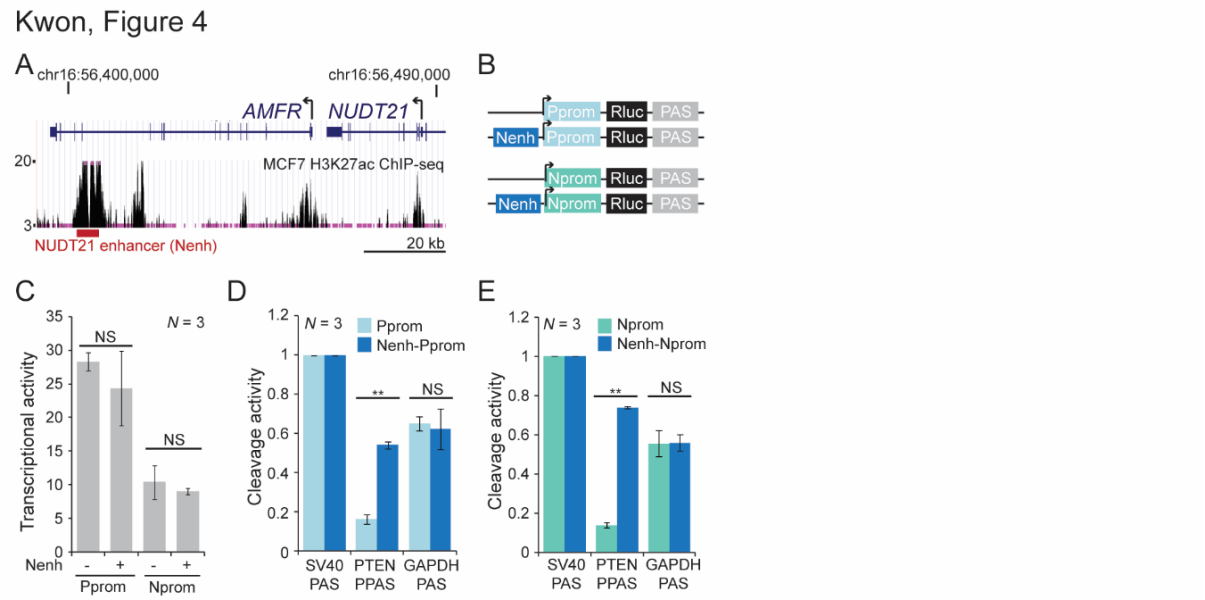

Figure 4. PAS cleavage is regulated by an additional enhancer.
(A) UCSC genome browser snapshot showing the NUDT21 gene locus with Encode ChIP-seq data of H3K27ac. The region that was used as NUDT21 enhancer (Nenh) is indicated. See Table S1 for details.
(B) Schematic of reporter constructs used to investigate Nenh-dependent PAS cleavage activity in the context of two promoters (Pprom and NUDT21 promoter (Nprom)), shown as in Figure 1C.
(C) Transcriptional activity of the reporters in the presence (Nenh-Pprom or Nenh-Nprom) or absence (Pprom or Nprom) of the Nenh. Transcriptional activity corresponds to luciferase activity of the reporters when terminated by the strong SV40 PAS. T-test for independent samples was performed.
(D) Nenh-dependent PAS cleavage activity in the context of the Pprom. T-test for independent samples was performed; **, P = 1 x 10^-8.
(E) Nenh-dependent PAS cleavage activity in the context of the Nprom. T-test for independent samples was performed; **, P = 1 x 10^-5.

Transcription factors regulate PAS cleavage activity without affecting transcriptional activity

Next, we set out to identify transcription factors and co-activators that are responsible for the regulation of PAS cleavage activity. We used the *PTEN* enhancer and performed a small-scale shRNA screen. We stably knocked-down individual factors that are known to bind to the Penh in MCF7 cells and that are involved in transcription regulation (Figure S4 and Table S2). We compared the effect of Ctrl KD and transcription factor KD on transcriptional activity and PAS

cleavage activity in the context of the Penh-Pprom reporter (Figure 5A). As before, we used the reporter with the SV40 PAS to assess transcriptional activity and the reporter with the PTEN PPAS to determine PAS cleavage activity (Figures S5A and S5B).

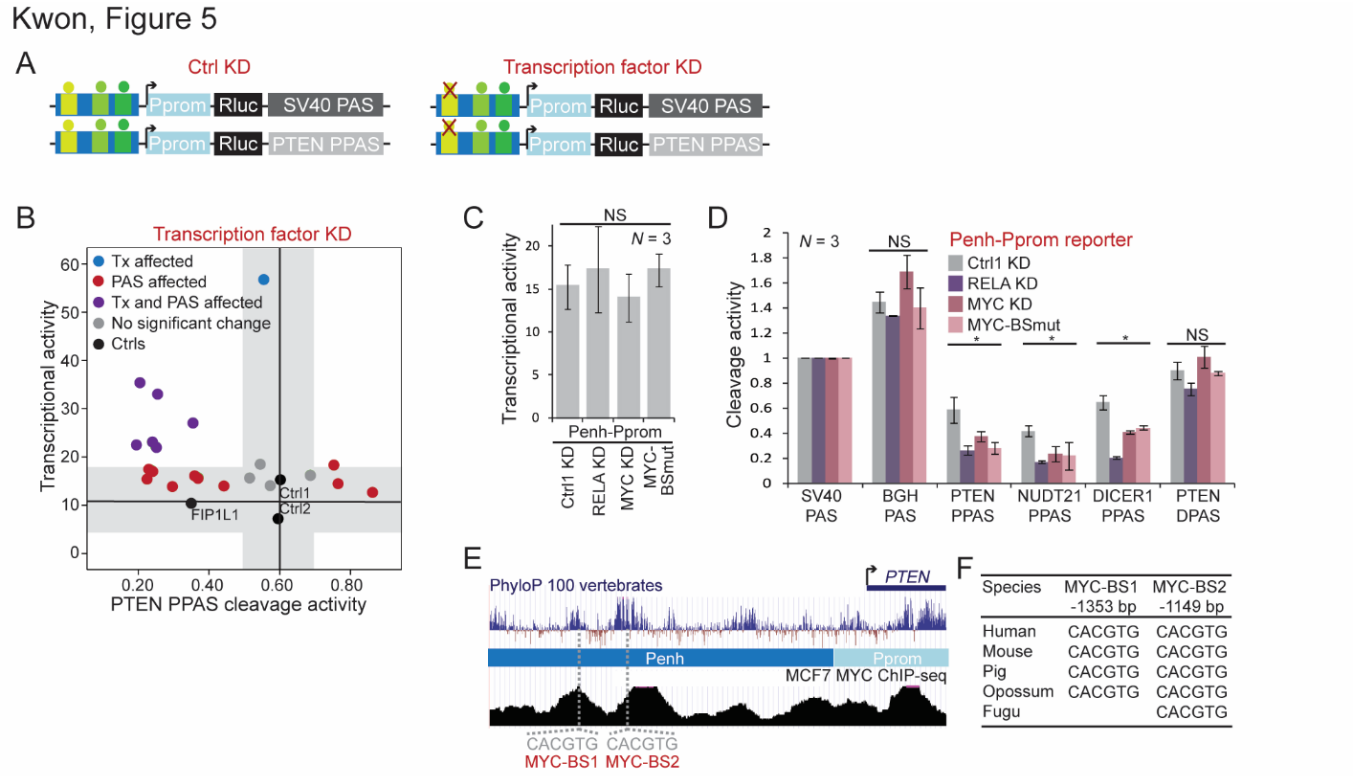

Figure 5. PAS usage is regulated by transcription factors that bind to the PTEN enhancer.
(A) Schematic of reporter constructs to investigate transcription factor-dependent PAS cleavage activity. As in Figure 1C, but the effect of knock-down (KD) of individual transcription factors known to bind to the Penh on PAS cleavage activity is compared to the effect observed by shRNA controls (Ctrl KD).
(B) Summary of the results of the transcription factor shRNA screen. Shown is the effect of KD of individual transcription factors on transcriptional activity and on PTEN PPAS cleavage activity. Tx, transcription. The grey area on the x-axis denotes no significant change in transcriptional activity, whereas the grey area on the y-axis denotes no significant change in PAS cleavage activity. See Figure S5 and Table S2 for complete data.
(C) Transcriptional activity upon KD of a subset of transcription factors is shown, as in Fig. 2C. T-test for independent samples was performed.
(D) Transcription factor-dependent cleavage activity of additional PAS is shown after KD of specific transcription factors. T-test for independent samples was performed; *, $P < 0.03$.
(E) UCSC genome browser snapshot showing the vicinity of the transcription start site (arrow) of the PTEN gene together with ChIP-seq data for MYC and the sequence conservation track of 100 vertebrates. The positions of the two conserved canonical E-boxes (binding sties for MYC, MYC-BS) are indicated.
(F) Sequence conservation of the two MYC-BS located in the PTEN enhancer in different organisms.

In Ctrl KD cells, PTEN PPAS usage was 0.60 (Figure 5B, black dots). As positive control, we knocked-down the polyadenylation factor FIP1L1, which was shown previously to be required for PPAS usage [18]. As expected, KD of FIP1L1 decreased PTEN PPAS usage from 0.6 to 0.36 without affecting transcriptional activity (Figure 5B, black dot). Strikingly, KD of 10/21 tested transcription factors had a similar effect (Figure 5B, red dots). These transcription factors included RELA (NF-κB p65), MYC, and FOXA1 and they regulated PAS cleavage activity without having a strong (less than 1.7-fold) effect on transcriptional activity of the reporter (Figure 5C and Tables 1 and S2).

Mutation of MYC binding sites in the enhancer regulates PAS cleavage activity

We tested the effect of RELA or MYC KD on cleavage activity of additional PPAS and again observed decreased cleavage activity in the context of the Penh reporter (Figures 5D and S5C). MYC binding sites (MYC-BS) are E-boxes [43] and the *PTEN* enhancer contains two conserved MYC-BS (Figures 5E and 5F). We next evaluated the influence of MYC-BS mutation on transcriptional and PAS cleavage activity in the context of the reporter. Mutation of the MYC-BS had no influence on transcriptional activity (Figure 5C). It decreased cleavage activity of weak PPAS and did not affect cleavage activity of strong DPAS, thus phenocopying the effect of MYC KD (Figures 5D). These results suggest that transcription factor binding to conserved motifs in the *PTEN* enhancer regulates cleavage activity of proximal PAS.

Co-activators simultaneously regulate transcriptional activity and PAS cleavage activity

As transcription factor binding to enhancers recruits diverse co-activators to promoters, we also tested the effects of co-activator KD on transcriptional and PAS cleavage activity of the reporter (Figure 6A). In contrast to transcription factors, the majority of tested co-activators (26/35) changed transcriptional activity and PAS cleavage activity at the same time (Figures 6B, purple dots and Table 1). The co-activators included Mediator, general transcription factors, transcription elongation factors, and several histone acetyltransferases (Figures S5A, S5B, and Table S2). KD of the general transcription factor TFIIF or the histone acetyl transferases TIP60 (KAT5) and PCAF (KAT2B) also reduced cleavage activity of additional PPAS (Figure 6C). Moreover, the KD of histone acetyltransferases (PCAF, TIP60, EP300) also decreased PTEN PPAS cleavage activity in the context of the Nenh (Figures 6D and 6E) but had no effect on cleavage activity in reporters that lack the enhancer (Figures S5D and S5E). These results suggest that active enhancers are responsible for increased cleavage activity of weak PAS.

Kwon, Figure 6

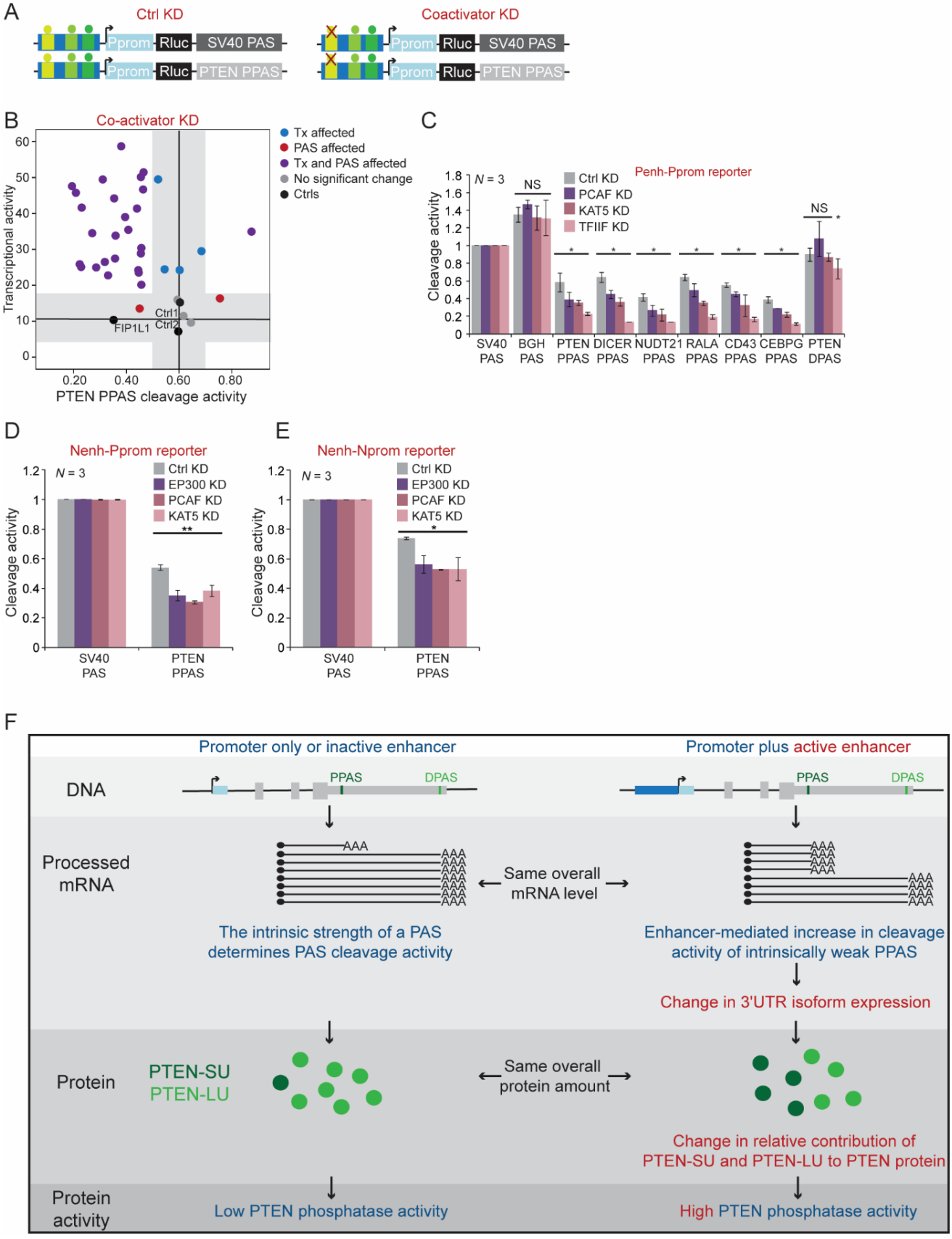

Figure 6. PAS usage is regulated by co-activators and histone acetyltransferases.
(A) As in Fig. 5A, but co-activators were knocked-down.
(B) Summary of the results of the co-activator shRNA screen, shown as in Figure 5B.
(C) Co-activator-dependent cleavage activity of additional PAS is shown after KD of specific co-activators and histone acetyl transferases. T-test for independent samples was performed; *, $P < 0.03$.
(D) Cleavage activity of the PTEN PPAS in the context of the Nenh-Pprom reporter comparing the effect of KD of specific histone acetyl transferases with a ctrl KD. T-test for independent samples was performed. **, $P < 0.001$.
(E) As in (D), but cleavage activity of the PTEN PPAS in the context of the Nenh-Nprom reporter was assessed. KD of histone acetyl transferases reduces PTEN PPAS cleavage activity. T-test for independent samples was performed; *, $P < 0.03$.
(F) Model of enhancer-mediated control of PTEN protein activity through regulation of 3′UTR isoform usage (see text).

Model of enhancer-mediated control of PTEN protein activity through regulation of 3′UTR isoform usage

In summary, our results obtained from reporter assays and the CRISPR-mediated deletion of an endogenous *PTEN* enhancer indicates that enhancers, in addition to regulating transcript production also control PAS cleavage. In the absence of an enhancer or when the enhancer is inactive, the produced transcripts are cleaved and polyadenylated at their 3′ ends based on the intrinsic strength of the PAS (Figure 6F, left panel). In the presence of an active enhancer, PAS cleavage activity of proximal and weak PAS increases, resulting in a change in 3′UTR isoform expression with upregulation of the short 3′UTR isoform (Figure 6F, right panel). This mode of regulation did not affect gene-level mRNA or protein levels. However, the relative contribution of PTEN-SU and PTEN-LU to overall PTEN protein has changed with an increased fraction of PTEN-SU. As PTEN-SU has a higher intrinsic phosphatase activity than PTEN-LU, enhancer activation ultimately changes protein activity. We speculate that the change in 3′UTR-dependent protein activity may be due to conformational changes and/or post-translational modifications (see Discussion).

Discussion

We established a new assay that allows us to assess promoter and enhancer-dependent PAS cleavage activity in living cells. This assay revealed that promoters that differ 1000-fold in transcriptional activity had little effect on PAS cleavage activity. In contrast, we observed that enhancers regulate PAS cleavage activity substantially. The effect of enhancer activation on strong PAS – that usually are located at the 3′ ends of transcription units – was variable and

usually resulted in no change or in a slight downregulation of cleavage activity. However, for six out of six weak PAS that are located at the beginning of 3′UTRs, we found that the presence of an active enhancer caused a substantial upregulation of cleavage activity. These results were observed in the context of different reporters (Figures 2 and 4) as well as at the endogenous genomic context (Figure 3) and were recapitulated in transcription factor or co-activator KD experiments (Figures 5 and 6). In the future, this assay can also be applied to assess the influence of signaling pathways or chromatin states on PAS cleavage activity.

Transcription factors regulate PAS cleavage

Surprisingly, half of the transcription factors that were knocked-down only affected PAS cleavage activity without having a strong (less than 1.7-fold) effect on overall transcriptional activity in the context of the reporter (Figure 5B, Tables 1 and S2). For most transcription factors, we only achieved a partial knock-down, suggesting that we may even underestimate the effects on PAS cleavage activity. Our results indicate that the regulation of PAS cleavage activity is a major function of transcription factors.

Although the KD of co-activators and chromatin regulators also regulated PAS cleavage activity, we observed that these factors influenced both transcriptional and PAS cleavage activity simultaneously (Figure 6B, Tables 1 and S2). In our analysis, we have largely disregarded the influence of mRNA stability on transcript abundance and have focused on disentangling enhancer-dependent transcript production from transcript processing. The two reporters that measure transcriptional activity and cleavage activity only differ in 100 base pairs of sequence surrounding the PAS. Moreover, for the reporters that were used in the majority of the experiments, mRNA stability of the individual reporters was measured and was not the cause of differential cleavage activity (Figure S1D). We present here strong evidence that PAS cleavage activity is a major contributor to mRNA isoform abundance. Our study represents the first step in the development of tools that will be able to measure cleavage activity transcriptome-wide without the need for reporters. Such tools have already been developed to measure transcript production or mRNA stability on a large scale [44,45].

Enhancers are known to integrate cell type- and condition-specific signals to regulate gene expression [1,3]. Our data suggest that enhancers could be major regulators of cell type- and condition-specific expression of alternative mRNA transcripts. We currently do not know how transcription factors mediate the regulation of PAS cleavage activity. However, it was previously shown that transcription factors that bind to promoters are able to recruit RNA-binding proteins, including polyadenylation factors [25-28]. These factors then bind to the C-terminal domain of RNA

polymerase II and may be deposited onto the nascent mRNA in a co-transcriptional manner to regulate mRNA processing [29,30,46,47].

To regulate expression of individual mRNA isoforms, we propose that enhancers recruit specific RNA-binding proteins to promoters that travel with RNA polymerase II and when present result in increased cleavage at weak PAS. Such a mechanism was also proposed for promoter-dependent regulation of post-transcriptional processes in yeast, including the regulation of mRNA stability, cytoplasmic localization, and translation [31-33,35,48].

Enhancers regulate expression of alternative mRNA isoforms

Enhancers are viewed as elements that regulate gene expression [3]. However, our understanding of genes is still evolving and in recent years it has been found that a large fraction of genes produces alternative mRNA isoforms that only differ in their 3′UTRs [9,18-22]. Still, most analysis tools only quantify gene-level mRNA abundance and are not designed to detect alternative mRNA transcript or isoform abundance [49]. Gene-centric analyses equate transcriptional activity with mRNA production. Only when analyzing alternative 3′UTR isoform abundance regulatory processes that occur at the level of PAS cleavage become evident. For *PTEN*, CRISPR-mediated deletion of the enhancer did not change gene-level mRNA levels. Therefore, this enhancer would be missed according to the current enhancer definition [3]. In a recent large-scale study that used CRISPRi to repress enhancers only 10% of tested enhancers showed any evidence of enhancer-mediated regulation of mRNAs levels [50]. One potential reason for this low number of functional enhancers may be the focus on measuring gene-level abundance instead of isoform abundance. This illustrates the need for better tools to assess transcript-level changes and the resulting functional changes in protein activity of these highly regulated factors.

An enhancer controls PTEN protein activity via regulation of alternative 3′UTR isoform expression

For PTEN, we showed that the regulation of PAS cleavage activity did not change overall mRNA or protein levels (Figure 3B-E). However, importantly, differential 3′UTR isoform expression resulted in a difference in PTEN protein activity (Figure 3F). We observed a higher intrinsic enzymatic activity measured as lipid phosphatase activity against PIP3 with PTEN-SU compared with PTEN-LU (Figure 3H). The difference in protein activity is best explained by 3′UTR-dependent effects on protein maturation that either happen co-translationally or right after the protein has been synthesized but when it is still in the proximity of the ribosome [15]. We

showed previously that 3′UTRs recruit proteins to the site of translation that interact with the newly made proteins, thus establishing 3′UTR-dependent protein-protein interactions [13,14]. Alternatively, 3′UTRs can determine translation in specific local environments such as TIS granules which can also affect protein complex assembly [16].

Currently, we do not know how the alternative 3′UTRs of *PTEN* regulate the enzymatic activity of PTEN. We envision several possible scenarios: It has been shown that phosphorylation of PTEN in the C-terminus can abrogate PTEN activity as it induces a closed and inactive protein conformation that is no longer able to associate with membranes and is unable to regulate downstream signaling pathways [51]. It is possible that the long 3′UTR of *PTEN* recruits enzymes that post-translationally modify PTEN protein, thus inducing a conformational change. Moreover, PTEN protein activity is redox-sensitive as the cysteine at the active site can be oxidized [52]. The alternative 3′UTRs may promote translation in local environments that differ in redox state or the 3′UTRs may recruit proteins that either increase or decrease the oxidative state of the cysteine in the active site.

Half of genes generate mRNA transcripts with constitutive 3′UTRs [9]. These genes regulate protein activity through the regulation of mRNA and protein abundance. The majority of genes that generate alternative 3′UTR isoforms encode regulatory factors, including transcription factors, RNA-binding proteins, chromatin regulators, kinases, and ubiquitin enzymes [9]. These genes have an additional way to regulate protein activity through the regulation of 3′UTR isoform abundance and 3′UTR-dependent activity regulation (Figure 6F). This has the added benefit that different functions of a single protein can be regulated separately. For example, protein abundance of the ubiquitin ligase BIRC3 regulates cell death, but BIRC3-LU has additional functions that include the regulation of B cell migration through 3′UTR-dependent protein complex assembly [14]. This implies that a change in protein abundance regulates cell death, but a 3′UTR isoform change regulates migration. It is likely that also PTEN-SU and PTEN-LU have different functions. We showed that PTEN-SU has higher enzymatic activity, but PTEN also has functions that do not require its lipid phosphatase activity [51], and we speculate that PTEN-LU may accomplish these functions. Nevertheless, these examples illustrate how increased enhancer regulation and increased control of protein functions by alternative 3′UTR isoforms may cooperate with each other to accomplish highly sophisticated gene expression regulation observed in complex organisms.

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B.K. designed and performed all experiments regarding endogenous PTEN, N.P. and J.L. cloned the reporter constructs and performed the reporter assays, J.L. evaluated the knock-down efficiency of the shRNAs, S.H.L. performed the western blot for pAKT and immunoprecipitated PTEN for ELISA, W.M. performed the mRNA stability experiment of the reporter. C.M. conceived and supervised the project, designed and analyzed the reporter experiments, and wrote the manuscript with input from all authors.

The authors declare no competing interests.

Table 1. Transcription factors and co-activators that regulate PAS cleavage activity.

Transcription factors that regulate PAS cleavage
FOS
FOXA1
GABPA
IRF1
JUN
JUND
MYC
NFYB
RELA
TCF12
Transcription factors that regulate PAS cleavage and transcription
EGR1
ELF1
RFX5
RXRA
TFAP2A
YY1
Co-activators that regulate PAS cleavage
ING3
SMC3
Co-activators that regulate PAS cleavage and transcription
ACTL6A
BARD1
BRCA1
BRD8
CDC73
CDK9
EP300
EP400
GTF2F1
KAT2B
KAT5
MED1
MORF4L1
NELFB
NELFE
NPM1
SIN3A
SMARCC1
SSRP1
SUPT16H
SUPT4H1
SUPT5H
TAF1
TBP
TCERG1
USP22

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