Published in final edited form as:

Gene. 2010 October 1; 465(1-2): 53–60. doi:10.1016/j.gene.2010.06.011.

Characterization of the human *PANK2* promoter

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Abstract

Pantothenate kinase 2 (*PANK2*) is an essential regulatory enzyme in coenzyme A biosynthesis. *PANK2* mutations cause pantothenate kinase-associated neurodegeneration (PKAN), which leads to pigmentary retinopathy, progressive dystonia and other abnormalities. Two nearly identical *PANK2* isoforms have been described: short *PANK2* and mature *PANK2*, which are processed from a precursor isoform. Since the biological relevance of these isoforms remains unclear, we sought to explore their transcriptional regulation. Here we show that their regulation is distinct and describe a promoter for the short isoform of *PANK2*. Moreover, we identify potential regulators of *PANK2* expression, including NF-Y, FOXN4 and the human heterogeneous nuclear ribonucleoprotein A/B family. These findings validate expression of the short *PANK2* isoform and enable predictions about potentially deleterious sequence variants in the regulatory region of this human disease gene.

Keywords

Pantothenate kinase; hnRNPA/B; FOXN4; NF-Y; PKAN

1. Introduction

Pantothenate kinase (*PANK*) is the rate limiting enzyme in the biosynthesis of coenzyme A. The pantothenate kinase gene family includes *PANK1*, *PANK2*, *PANK3* and *PANK4*, which give rise to five functional isoforms; *PANK1a*, *PANK1b*, *PANK2*, *PANK3* and *PANK4* (Zhou et al., 2001a; Rock et al., 2002). *PANK1*, *PANK2* and *PANK3* are functional kinases encoded from six conserved core exons preceded by differing initiation exons (Zhou et al., 2001a). Also, *PANK2* differs from other pantothenate kinases in its mitochondrial localization and its regulation by acyl-CoA and palmitoylcarnitine (Rock et al., 2000; Hortnagel et al., 2003; Johnson et al., 2004; Leonardi et al., 2007a).

We investigated the transcriptional regulation of *PANK2*, the causative gene for pantothenate kinase-associated neurodegeneration (PKAN), an autosomal recessive disease characterized by dystonia and retinal degeneration (Zhou et al., 2001a). A critical question in PKAN pathogenesis is why disruption of a ubiquitously expressed gene causes a neurological-specific disease. Thus we sought to identify regulatory factors controlling *PANK2* expression.

Multiple *PANK2* isoforms have been identified (Zhou et al., 2001b; Hortnagel et al., 2003; Kotzbauer et al., 2005). Initially, we described a mitochondrial 50.6 kDa *PANK2* isoform with a leucine initiation codon (CTG) (Zhou et al., 2001a). This protein has become known as the

PANK2 short form (sPANK2) (Zhang et al., 2006). Subsequently, a larger precursor isoform (pPANK2) was identified that begins translation 330 nucleotides upstream with a methionine initiation codon (ATG) (Hortnagel et al., 2003). This precursor isoform is sequentially cleaved at two sites by the mitochondrial processing peptidase, yielding intermediate (iPANK2) and mature (mPANK2, 48 kDa) isoforms (Kotzbauer et al., 2005). sPANK2 and mPANK2 both localize to mitochondria, although they utilize different localization signals and may be subject to distinct transcriptional, translational and post-translational regulation (Hortnagel et al., 2003; Johnson et al., 2004; Kotzbauer et al., 2005).

In this work, we sought to characterize the transcriptional regulation of *PANK2*. These studies advance our understanding of the regulation of short and mature PANK2 isoforms and allow us to test, for the first time, whether sequence variants in the region affect transcription.

2. Materials and methods

2.1. Database searches

Human *PANK2* genomic organization was obtained from the UCSC Human Genome Browser (Kent et al., 2002) and sequence comparisons were performed using the ECR Browser (Ovcharenko et al., 2004), Mulan (Ovcharenko et al., 2005) and ClustalW2 (Larkin et al., 2007). The transcription factor binding sites in the promoter region were analyzed using MatInspector (Cartharius et al., 2005). Prediction of CpG islands and TATA-dependent transcription initiation sites were generated by CpG Island Searcher and CorePromoter (Takai and Jones, 2003; Zhang, 1998).

2.2. Plasmid construction

We employed three methods for constructing plasmids. The *PANK2* promoter fragments in plasmids -2351/-301, -2351/+13, -1332/+13, -1332/-301, -633/+13, -327/+13, -102/+13, -327/-76, -180/-76, -327/-144, -327/-217 and -247/-144 were amplified from human genomic DNA by PCR using primers (Table 1) containing *NheI* (forward primers) or *HindIII* (reverse primers) recognition sites. The promoter fragments were cloned into a promoter-less luciferase reporter vector, pGL3-Basic (Promega). We created additional reporter plasmids (-262/+13, -216/+13, -162/+13) using the Erase-a-Base Kit (Promega). Briefly, the -633/+13 plasmid was linearized with *NheI* and *SacI* and digested with Exonuclease III. The digestion was stopped by S1 Nuclease, treated with Klenow DNA polymerase, ligated and transformed into competent cells. To create the -254/-76, -237/-76, and -211/-76 truncation plasmids, we generated novel *NheI* restriction sites (GCTAGC) in the -327/-76 plasmid with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Briefly, we designed oligonucleotides with our desired nucleotide change (Table 2), annealed the oligonucleotides to the plasmid template (-327/-76), transcribed with *PfuTurbo* polymerase, treated with *DpnI* endonuclease and transformed into competent cells. *NheI* digestion and re-ligation of the plasmids generated the truncated plasmids. We also used the QuikChange Site-Directed Mutagenesis Kit to alter putative transcription factor binding sites (Ets1, IRF4, NF-Y, FOXN4, NFkB) with the -327/-76 plasmid.

2.3. RNA ligase-mediated rapid amplification of cDNA ends (RACE)

Total RNA from human brain (Ambion) was used to identify the transcriptional start points with the GeneRacer Kit (Invitrogen). Briefly, 5 mg RNA was treated with calf intestine alkaline phosphatase and tobacco acid pyrophosphatase, ligated with an RNA adapter oligonucleotide using T4 RNA ligase, and random-primed for reverse transcription. cDNA was amplified by nested PCR. The PCR products were cloned into pCRII TOPO TA cloning vector (Invitrogen) and sequenced.

2.4. Cell culture, transient transfections and luciferase assays

Human neuroblastoma (SH-SY5Y) cells were cultured in RPMI with 10% FBS, 10 ng/ml human neuron growth factor, non-essential amino acids and penicillin/streptomycin antibiotics at 37 °C and 5% CO₂. Cells were seeded in 12-well plates, grown to 90% confluency, and transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Each well was transfected with 0.4 µg CMV-β-galactosidase and 0.4 µg of pGL3-Basic or luciferase reporter plasmids containing the 5'flanking fragments of human *PANK2* gene. Cells were harvested in 100 µl Passive Lysis Buffer (Promega) 24 h after transfection. To assay for β-galactosidase activity, 50 µl of cell lysate was combined with 60 µl Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM 2-Mercaptoethanol, pH 7.5) and 40 µl ONPG substrate (Sigma) (4 mg/ml in 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, pH 7.5 buffer) and incubated at 30 °C for 30 min. The reaction was quenched by adding 100 µl of 1 M Na₂CO₃ and measured at A420. To assay for luciferase activity, 20 µl of cell lysate was combined with 300 µl of GlyGly-ATP (25 mM Glycylglycine, 15 mM MgSO₄, 5 mM ATP) and 100 µl of luciferin (Promega) (300 µg/ml) and measured on a luminometer. The luciferase values were normalized to β-galactosidase activity. Each transfection was performed in triplicate and repeated at least 3 times.

2.5. Electrophoresis mobility shift assay (EMSA)

Nuclear extracts were prepared from SH-SY5Y cells. Briefly, $\sim 2 \times 10^7$ cells were washed with ice cold PBS, harvested in CE buffer (1 mM HEPES KOH, 6 mM KCl, 100 µM EDTA, 100 µM DTT, 100 µM PMSF, Complete Protease Inhibitors EDTA-free (Roche)) plus 0.5% NP-40 and centrifuged to pellet the nuclei. The pellet was washed in CE buffer, resuspended in 200 µl NE buffer (250 µM Tris HCl, 60 µM KCl, 1 mM DTT, 1 mM PMSF, Complete Protease Inhibitors EDTA-free (Roche)), lysed by freeze/thaw cycles in liquid nitrogen, and centrifuged. The final supernatant was frozen in liquid nitrogen and stored at -80 °C.

Single stranded probes labeled with IRDye-700 were designed to contain the putative binding sites for predicted transcription factors within the *PANK2* promoter (Table 3). The probes were annealed at 95 °C, cooled to room temperature, and used in DNA-protein binding reactions according to the Odyssey Infrared EMSA Kit (LiCor Biosciences). Briefly, the labeled oligonucleotides were incubated with 3 µl SH-SY5Y nuclear extract containing 10 µg protein at room temperature for 20 min and resolved on a 4% TBE native acrylamide gel at 4 °C, avoiding light exposure. For competitor assays, oligonucleotides with identical sequence to EMSA1, EMSA2, EMSA3, EMSA4 and EMSA5 forward and reverse oligonucleotides were ordered from Invitrogen. Additional competitor oligonucleotides were designed (Table 3) to contain putative or mutated binding sites for NF-Y and FOXN4 (Schlake et al., 1997; Zhu et al., 2004). The oligonucleotides were annealed at 95 °C, cooled to room temperature, and added in to the DNA-protein binding reaction in 200-fold excess of IRDye-700 labeled probes. For antibody binding, the reaction mixture was incubated with 2 µg of hnRNPA/B (Santa Cruz Biotechnology, cs-15385) or STAT3 (kind gift from Dr. David Farrell, OHSU) antibody prior to addition of labeled oligonucleotides. As a control for binding conditions, the reaction was also incubated with labeled oligonucleotides prior to the addition of 2 µg of antibody. The gels were imaged at 700 nm using the LiCor Odyssey Imaging System.

2.6. Proteomic analysis

We submitted native 4% acrylamide TBE gel slices to the OHSU Proteomics Shared Resource for analysis. The samples were digested with trypsin and analyzed by LC-MS using an Agilent 1100 series capillary LC system (Agilent Technologies) to elute the peptides from a Zorbax SB-C18 column (Agilent Technologies, cat. No. 5064-8258) into an LTQ linear ion trap mass spectrometer (ThermoFisher). Electrospray ionization was performed with an ion max source fitted with a 34 gauge metal needle (ThermoFisher, cat. No. 97144-20040). Peptides were

identified by comparing the observed MS/MS spectra to theoretical MS/MS spectra of peptides using the program Sequest (Version 27, rev. 12, ThermoFisher). Lists of identified proteins were assembled using the program Scaffold (version 2_06_00, Proteome Software).

3. Results

3.1. In silico analysis of PANK2

28 out of 39 *PANK2* spliced expressed sequence tags (ESTs) correspond to the initiating exon of *PANK2* transcript variant 1 (NM_153638) in the UCSC Human Genome Browser (Fig. 1A). This transcript gives rise to the mitochondrial *PANK2* isoforms sPANK2 and pPANK2. The less abundant *PANK2* transcript variants 2 (NM_153640) and 3 (NM_024960) are predicted to encode a non-functional cytosolic *PANK2* isoform (Zhang et al., 2006). The dominance of variant 1 expression is supported by the mitochondrial localization of *PANK2* in human brain sections and tissue culture models (Hortnagel et al., 2003; Johnson et al., 2004; Kotzbauer et al., 2005). *In silico* analysis of the *PANK2* 5' region reveals the presence of a large CpG island spanning the initiating exons for *PANK2* transcript variants 1, 2 and 3, suggesting that *PANK2* expression is driven by a TATA-less promoter (Takai and Jones, 2003).

In addition, there is a lack of sequence conservation in the 5' end of *PANK2* transcript variant 1 (Fig. 1A) (Ovcharenko et al., 2004). Overall, *PANK2* variant 1 has 87% identity to mouse *Pank2* cDNA, although the sequence conservation ends downstream of the pPANK2 translation initiation site. In fact, mouse, cat, dog and cow *PANK2* transcripts do not contain the pPANK2 mitochondrial localization signal and are predicted to initiate translation near the 5' end of exon 1 (Leonardi et al., 2007b).

3.2. Identification of a promoter for sPANK2 expression

In search of a promoter, we analyzed the transcriptional activity of DNA fragments from the 5' end of *PANK2* in the human neuroblastoma cell line, SH-SY5Y. We designated nucleotide numbering with +1 corresponding to the cytosine in the sPANK2 translation initiation codon (CTG) (chr20:3870078 on Human GRCh37). We found that the -2351/+13 and -1332/+13 fragments showed strong promoter activity, while -2351/-301 and -1332/-301 fragments did not demonstrate promoter activity (Fig. 1B).

To further characterize this region, we tested truncations of the 2.3 kb fragment (Fig. 1B). The -2351/+13 and -1332/+13 fragments produced half the transcriptional activity of -633/+13 fragments, indicating that the DNA upstream of -633 may contain negative regulatory elements. In contrast, 5' truncations of the region downstream of -327 resulted in decreasing promoter activity, suggesting that the sequence contains essential positive regulatory elements. We also assayed the luciferase activity of a 3' truncation construct (-327/-76), which demonstrated similar promoter activity to the -327/+13 fragment. To identify transcription initiation sites for the *PANK2* gene, 5'RACE was performed. We identified three sites (-78, -87, -122) upstream of the sPANK2 translational start site (Fig. 2). The -327/-76 promoter region has 62.9% identity to mouse, with the highest sequence conservation centering around the transcription initiation sites and sPANK2 leucine initiation codon (Fig. 2) (Ovcharenko et al., 2005).

3.3. Analysis of PANK2 promoter for potential transcription factors

We performed additional truncation analysis of the -327/-76 *PANK2* region, which revealed several potential regulatory elements within the promoter. The -180/-76, -247/-144 and -327/-217 fragments displayed less than 50% of the transcriptional activity of -327/-76 (Fig. 3A). We used MatInspector to identify factors that may interact with these promoter elements (Cartharius et al., 2005). Putative ETS1 and IRF4 sites (disrupted in -180/-76), NF-Y, FOXN4

and NF- κ B sites (eliminated in -327/-217) and a second NF-Y (disrupted in -247/-144) were identified (Figs. 2,3B). The FOXN4 site was described as a WHN (FOXN1) binding site in MatInspector; however, we were able to detect *FOXN4*, but not *FOXN1*, mRNA in SH-SY5Y cells (data not shown). FOXN1 and FOXN4 share a similar DNA binding domain, which is distinct from the rest of the FOX family members (Kato, 2004). Two NF-Y sites were identified and we reference them as the 5' or 3' NF-Y sites within the promoter. The NF-Y sites are highly conserved in mammals, although the binding sites for ETS1, IRF4, FOXN4 and NF- κ B are less conserved (Fig. 2).

We created constructs with point mutations in these predicted regulatory elements and assayed the effect on promoter activity. Disruption of the ETS1 and NF- κ B binding sites caused an increase in promoter activity, while disruption of IRF4, FOXN4 and the 5' residing NF-Y binding sites caused a decrease in promoter activity (Fig. 3C).

Next, we performed electrophoresis mobility shift assays (EMSA) to investigate whether SH-SY5Y nuclear proteins could bind to the regulatory elements in the promoter. We designed probes to contain the predicted transcription factor binding sites within the *PANK2* promoter (Fig. 4A) and found that the probes containing IRF4/ETS1 sites (EMSA3), 3'NF-Y site (EMSA4) and FOXN4 site (EMSA5) form DNA-protein complexes and cause a band shift upon gel electrophoresis (Supplemental Fig. 1).

We chose to further examine the EMSA4 and EMSA5 regions because NF-Y is a common regulator of CG rich promoters and FOXN4 plays a role in neuronal tissues. We found that 200-fold excess of unlabeled oligonucleotides containing NF-Y or FOXN4 binding sites competes with the EMSA4 and EMSA5 probes, respectively (Fig. 4B, C). We investigated the specificity of this competition by adding 200-fold excess of unlabeled oligonucleotides containing mutated NF-Y or FOXN4 binding sites, and found they did not compete with the EMSA4 and EMSA5 probes (Fig. 4B, C). These results suggest that NF-Y and FOXN4 are responsible for the shifted EMSA4 and EMSA5 bands upon electrophoresis, and may bind the *PANK2* promoter.

Interestingly, upon attempting to confirm FOXN4 binding to the EMSA5 probe, we identified hnRNPA/B as an additional potential regulator of the *PANK2* promoter. The addition of a FOXN4 antibody to the DNA-protein binding reaction did not result in additional shifting of the EMSA5 band (data not shown). Therefore, we excised the shifted EMSA5 band following gel electrophoresis and analyzed the protein content by mass spectrometry; FOXN4 was not found in the sample. However, one protein was identified in two replicate EMSA5 analyses: the heterogeneous Nuclear Ribonucleoprotein A/B (hnRNPA/B) family. The binding of hnRNPA/B to the EMSA5 oligo was confirmed by addition of hnRNPA/B antibody to the DNA-protein binding reaction, which caused a supershift of the EMSA5 band upon electrophoresis (Fig. 5). Of note, the STAT3 antibody negative control for the EMSA5 supershift inhibits protein binding to the EMSA5 probe when added after lysates. Therefore, the antibody may interfere with protein-DNA binding, either non-specifically or by disrupting STAT3 protein associations.

3.4. Predicting the effect of *PANK2* sequence variants on promoter activity

We also investigated the effects of two *PANK2* sequence variants found in patients with brain iron accumulation. One sequence variant located -55 relative to the leucine initiation codon (276G>A in NM_153639) was found in a PKAN patient with only one identified *PANK2* mutant allele (785-788del in exon 3)(Zhou et al., 2001a). A second variant located at -193 (138A>T in NM_153639) was identified in a patient with a related disorder being tested for PKAN. *In silico* analysis reveals that these variants do not reside in *PANK2* promoter regulatory

elements and are not conserved in mouse and rat. Indeed, neither variant had a demonstrable effect on *PANK2* promoter activity (data not shown).

4. Discussion

Our results describe a GC-rich *PANK2* promoter that drives expression of the sPANK2 isoform. Most of the *PANK2* spliced ESTs listed in the UCSC Genome Browser overlap with the sPANK2 transcript and support the presence of a promoter in the region we identified. The -327/-76 promoter region has 62.9% identity to mouse, which is typical for core promoter elements conserved in human and mouse orthologs (Jin et al., 2006). In contrast, PANK2 transcript has ~87% identity to mouse PANK2 cDNA.

Although an alternate promoter for pPANK2 may exist, we were unable to identify one within the 2-kb region we studied. TATA sites are predicted further than 2 kb upstream of the pPANK2 transcript, suggesting the presence of alternate promoters (Takai and Jones, 2003; Zhang, 1998). The presence of an additional TATA-dependent *PANK2* promoter would provide an intriguing mechanism for tissue specific expression of the pPANK2 isoform. This idea is not unprecedented, as recent studies have suggested that over half of RefSeq genes are regulated by alternative promoters, and in 17% of those genes a tissue specific alternative promoter was observed (Kimura et al., 2006). Interestingly, the long *PANK2* transcript encoding pPANK2 has been identified in human brain tissues, and a single 48 kDa PANK2 isoform (mPANK2) was detected in cortical brain extracts, raising the possibility of tissue specific expression (Hortnagel et al., 2003; Kotzbauer et al., 2005). In contrast, the 50.6 kDa sPANK2 isoform has only been reported in HeLa cell extracts (Johnson et al., 2004). A direct comparison of pPANK2 and sPANK2 expression across tissues has not been published, and further investigation would be necessary to clarify the biological significance of two putative mitochondrial PANK2 isoforms.

Our results suggest that the *PANK2* promoter may be regulated by NF-Y. MatInspector predicted two highly conserved CCAAT boxes in the *PANK2* minimal promoter, although only one site affected promoter activity and was able to bind SH-SY5Y nuclear lysates (Fig. 4). This CCAAT-box is located 58–100 nucleotides upstream of the *PANK2* transcriptional start sites, which is typical for a functional NF-Y binding site (Fig. 3)(Bucher, 1990). NF-Y is composed of three subunits (NF-YA, NF-YB and NF-YC) and is implicated in the regulation of 30% of eukaryotic promoters (Bucher, 1990; Sinha et al., 1995). CCAAT boxes are found in the promoters of several types of genes, including those that are developmentally controlled, tissue specific, housekeeping, inducible and cell cycle regulated (Mantovani, 1999). Although NF-Y is a major CCAAT box recognizing protein, numerous other transcription factors can also regulate gene expression through this common regulatory element (Mantovani, 1998). Therefore, further investigation is necessary to characterize the regulation of *PANK2* through the CCAAT box.

We also identified FOXN4 as a potential regulator of *PANK2* transcription. FOXN4 is a forkhead/wing helix transcription factor with a role in the specification of cells in retinogenesis and spinal neurogenesis in mouse (Gouge et al., 2001; Li et al., 2005). Interestingly, recent studies have also shown that Foxn4 regulates the expression of Nr4a2, a nuclear receptor that is associated with familial Parkinson's disease (Jiang and Xiang, 2009). We were unable to confirm FOXN4 binding to the *PANK2* promoter by EMSA supershift or mass spectrometry. In addition, the FOXN4 core binding site is not well conserved in mouse and rat. However, these results do not eliminate FOXN4 as a potential *PANK2* transcriptional regulator and further investigation is warranted.

While not predicted *in silico*, our proteomic analysis revealed that the hnRNPA/B family may regulate *PANK2* expression. The hnRNPA/B family of proteins play a role in RNA processing/trafficking, telomere maintenance, and transcriptional regulation (He and Smith, 2009). hnRNPA1 has been identified by mass spectrometry as a transcriptional regulator for several genes, including gamma-fibrinogen, thymidine kinase, apoE and vitamin D receptor (Lau et al., 2000; Campillos et al., 2003; Chen et al., 2003; Xia, 2005). Recently, hnRNPA2 was found to function as a transcriptional activator in response to mitochondrial respiratory stress (Guha et al., 2009). A possible link between mitochondrial stress and *PANK2* expression is intriguing in light of current hypotheses of PKAN pathogenesis (Zhou et al., 2001a; Johnson et al., 2004).

Finally, characterization of the *PANK2* promoter enables prediction of the consequences of *PANK2* sequence variants on transcriptional activity. We did not detect any consequence of two *PANK2* sequence variants that lie outside key promoter regulatory elements. However, we predict that sequence variants disrupting key regulatory elements or a transcriptional start site would alter *PANK2* expression and could cause PKAN.

5. Conclusion

Our work provides the first description of *PANK2* transcriptional regulation. We conclude that sPANK2 and pPANK2 are controlled by distinct promoters. The sPANK2 promoter may be regulated by NF-Y, FOXN4 and hnRNPA/B. These findings validate the biological relevance of sPANK2, serve as a reference for interpreting regulatory sequence variants, and provide insight to the regulation of this disease-associated gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

PKAN	pantothenate kinase-associated neurodegeneration
PANK	pantothenate kinase
EMSA	electrophoresis mobility shift assay

Acknowledgments

This work was supported by the National Institute of Child Health and Human Development, National Eye Institute, NBIA Disorders Association and Huebner Family Pediatric Neurobiology of Disease Fellowship. This work was also supported by the Proteomics Shared Resource generously funded by the Oregon Opportunity, and NIH center grants 5P30CA069533 and 5P30EY010572. We are grateful to Dr. Richard Mauer's laboratory for use of their luminometer, and members of the Hayflick laboratory, Dr. Shawn Westaway and Dr. Jayme Gallegos for critical reading of the manuscript.

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Fig. 1. *PANK2* structure and transcriptional activity (A) *PANK2* genomic structure, RefSeq mRNA variants, human spliced ESTs and sequence conservation. The sPANK2 leucine initiation codon (arrow) and pPANK2 methionine initiation codon (triangle) are designated. The enhanced region is adapted from the UCSC Human Genome Browser (<http://genome.ucsc.edu>) and corresponds to chr20:3869450–3870650 on Human GRCh37 Assembly (hg19)(Kent et al., 2002). The conservation summary is adapted from the ECR Browser (ecrbrowser.dcode.org) (Ovcharenko et al., 2004). (B) Luciferase activity in SH-SY5Y cells driven by serial truncation of *PANK2*. Luciferase activity was normalized to β -

galactosidase activity and results are shown as fold change from the empty pGL3 vector. The error bars represent standard error of three independent experiments.

Fig. 2.

Mammalian conservation of *PANK2* 5' genomic sequence. The cytosine of the sPANK2 leucine initiation codon (CTG) is labeled as +1. The sPANK2 leucine initiation (arrow) and the pPANK2 methionine initiation codon (triangle) are designated. Transcriptional start sites identified by 5'RACE are marked by diamonds and boundaries of the -327/-76 promoter region are indicated with brackets. The locations of putative binding sites for transcription factors are boxed and the core binding regions are underlined. *PANK2* sequence variants are highlighted in grey. The sequence corresponds to chr20:3869748-3870083 on Human GRCh37 Assembly (hg19). The sequence alignment was created with Mulan (mulan.dcode.org) and ClustalW2.

Fig. 3.

Deletion and mutational analysis of the *PANK2* minimal promoter. (A) Luciferase activity in SH-SY5Y cells driven by fragments of the -327/-76 minimal promoter region. (B) Diagram of predicted transcription factor binding sites within the *PANK2* minimal promoter. (C) Luciferase activity in SH-SY5Y cells driven by constructs containing mutant transcription factor binding sites. Luciferase activity was normalized to β -galactosidase activity and results are shown relative to -327/-76 activity. Error bars represent standard error of three independent experiments.

Fig. 4.

Binding of SH-SY5Y nuclear proteins to DNA probes EMSA4 and EMSA5 (A) Diagram of predicted transcription factor binding sites within the *PANK2* minimal promoter and relative location of EMSA probes. (B, C) Binding of SH-SY5Y nuclear proteins to DNA probes EMSA4 and EMSA5. Unlabeled oligonucleotide competitors were added to the reactions, as indicated. The reactions were resolved on a 4% TBE native acrylamide gel at 4 °C.

Fig. 5.

Binding of SH-SY5Y nuclear proteins to EMSA5 probe and supershift by hnRNPA/B antibody. 2 μ g of antibody was added before or after (*) the binding reaction. A STAT3 antibody was included as a control and unlabeled oligonucleotide competitors were added to the reactions, as indicated. The reactions were resolved on a 4% TBE native acrylamide gel at 4 °C.

Table 1

PCR primer sequences for plasmid construction.

Name	Sequence
Hp2prom5F (-2351)	AGGCTAGCGATTACAGGCACGCACCACTACGCCCAGCTAAG
Hp2prom6F (-1332)	AGGCTAGCCACAAGCGAAGCTCAACCTTTTGTTCAGCC
Hp2prom3F (-633)	AGGCTAGCTCTCTCCAACGCAGGCGGAAAGGAGG
Hp2prom2F (-327)	AGGCTAGCAGGAGGCTCGGGCCCTTCCACCCACG
Hp2prom9F (-247)	AGGCTAGCCCTCCGCGGAACCCGGATCCCTCCTCCACCACCC
Hp2prom10F (-180)	AGGCTAGCGACGGAGGCACGGTCAATCCTCCTCGAGTTAGGGAG
Hp2prom1F (-102)	AGGCTAGCGATTGGCTTCCTGCGCGTTGGCGCAAC
Hp2prom7R (-217)	GCAAGCTTGGGTGGAGGAGGGGATCCGGGTTCGCGGAGG
Hp2prom8R (-144)	GCAAGCTTGCTCCCTAACTCGAGGAGGATTGACCGTGCCTCCGTC
Hp2prom4R (-76)	GCAAGCTTGGTTGCGCCAACGCGCAGGAAGCCAATC
Hp2prom3R (-301)	GCAAGCTTGGCGTGGGTGGAAGGGCCCGAGCCTCCTCAT
Hp2prom1R (+13)	GCAAGCTTGCAAGCCCCCAGCGTCGGATTCTTCTCGCC

Table 2

Oligonucleotides for QuikChange site-directed mutagenesis.

Plasmid	Primer	Sequence ^a
-254/-76	hp2promqct3F	CCATCACTCTCTTCTGGGCTACGCTAGCTTCTCTCCTCCGC
	hp2promqct3R	GCGGAGGAAGAGAAGCTAGCGTAGCCCAGAAGAGAGTGATGG
-237/-76	hp2promqct4F	GGGCTACACCGCCTTCTCTCCTCGCTAGCACCCGGATCCCC
	hp2promqct4R	GGGGATCCGGGTGCTAGCGAGGAAGAGAAGGCGGTGTAGCCC
-211/-76	hp2promqct6F	CGGATCCCCTCCTCCACGCTAGCCTCCCCGCCCCGTCAC
	hp2promqct6R	GTGACGGGGCGGGGAGGCTAGCGTGGAGGAGGGGATCCG
Ets1	hp2promETS_mt1f	CTACACCGCCTTCTCttaaTCCGCGGAACCCGG
	hp2promETS_mt1r	CCGGGTTCCGCGGAttaagAGAAGGCGGTGTAG
IRF4	hp2promIRF4_mt1f	CTCTTCCTCCGCGaatCCGGATCCCCTCCTC
	hp2promIRF4_mt1r	GAGGAGGGGATCCGGattCGCGGAGGAAGAG
5' NF-Y	hp2promNFY_mt1f	CACGATAGCCTCTCcatgtACGGAGGCACGGTC
	hp2promNFY_mt1r	GACCGTGCCTCCGTccatGAGAGGCTATCGTG
FOXN4	hp2promWHN_mt1f	GTAGGGAGCCGACTGGaatGAGGCCTTTGGG
	hp2promWHN_mt1r	CCCAAAGGCCTCgattCCAGTCGGCTCCCTAAC
NfκB	hp2promNFκ_mt1f	GGCCTTTGGGCCGtaacCAGCCTCGTCGG
	hp2promNFκ_mt1r	CCGACGAGGCTGgttaCGGCCCAAAGGCC
3' NF-Y	hp2promNFY2_mt1f	CAGCCTCGTCGGatgtCTTCCTGCGCGTTG
	hp2promNFY2_mt1r	CAACGCGCAGGAAGccatCCGACGAGGCTG

^a Nucleotide changes are underlined and putative transcription factor binding sites are in lowercase italics.

Table 3

Oligonucleotides for EMSA.

EMSA probe	Oligonucleotide	Sequence
EMSA1	hp2promEMSA1F	GACTGGACGCGAGGCCTTGGGCGTCCCCAGCCTCGTCG
	hp2promEMSA1R	CGACGAGGCTGGGGACGGCCAAAGGCCTCGCGTCCAGTC
EMSA2	hp2promEMSA2F	TCGTCCGATTGGCTTCCTGCGCGTTGGCGCAAC
	hp2promEMSA2R	GTTGCGCCAACGCGCAGGAAGCCAATCCGACGA
EMSA3	hp2promEMSA3F	CGCCTTCTCTCCTCCGCGGAACCCGGATC
	hp2promEMSA3R	GATCCGGGTTCCGCGGAGGAAGAGAAGGCG
EMSA4	hp2promEMSA4F	AGCCTCTCATTGGACGGAGGC
	hp2promEMSA4R	GCCTCCGTCCAATGAGAGGCT
EMSA5	hp2promEMSA5F	GGGAGCCGACTGGACGCGAGGCCTTTGGGC
	hp2promEMSA5R	GCCCAAAGGCCTCGCGTCCAGTCGGCTCCC
FOXN4	FOXN1siteF	ATAGGGCGAATTGGGTACCAAAGGGACGCTATCGAGCTCCAGCTTT
	FOXN1siteR	AAAGCTGGAGCTCGATAGCGTCCCTTTGGTACCCAATTGCGCCCTAT
NF-Y	NFYsiteF	AGACCGTACGTGATTGGTTAATCTCTT
	NFYsiteR	AAGAGATTAACCAATCACGTACGGTCT
FOXN4 mt	FOXN1mt1F	ATAGGGCGAATTGGGTACCAAAGGGAGCCTATCGAGCTCCAGCTTT
	FOXN1mt1R	AAAGCTGGAGCTCGATAGGCTCCCTTTGGTACCCAATTGCGCCCTAT
NF-Y mt	NFYmt1F	AGACCGTACGTGATCAGTTAATCTCTT
	NFYmt1R	AAGAGATTAACGTATCACGTACGGTCT