***TCEB3C* a putative tumor suppressor gene of small intestinal neuroendocrine tumors**

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Abstract

Small intestinal neuroendocrine tumors (SI-NETs), formerly known as midgut carcinoids, are rare and slow-growing neoplasms. Frequent loss of one copy of chromosome 18 in primary tumors and metastases has been observed. The aim of the study was to investigate a possible role of *TCEB3C* (Elongin A3), currently the only imprinted gene on chromosome 18, as a tumor suppressor gene in SI-NETs, and whether its expression is epigenetically regulated. Primary tumors, metastases, the human SI-NET cell line CNDT2.5, and two other cell lines were included. Immunohistochemistry, gene copy number determination by PCR, colony formation assay, western blotting, real-time quantitative RT-PCR, RNA interference, and quantitative CpG methylation analysis by pyrosequencing were performed. A large majority of tumors (33/43) showed very low to undetectable Elongin A3 expression and as expected 89% (40/45) displayed one gene copy of *TCEB3C*. The DNA hypomethylating agent 5-aza-2'-deoxycytidine induced *TCEB3C* expression in CNDT2.5 cells, in primary SI-NET cells prepared directly after surgery, but not in two other cell lines. Also siRNA to DNMT1 and treatment with the general histone methyltransferase inhibitor 3-deazaneplanocin A induced *TCEB3C* expression in a cell type-specific way. CpG methylation at the *TCEB3C* promoter was observed in all analyzed tissues and thus not related to expression. Overexpression of *TCEB3C* resulted in a 50% decrease in clonogenic survival of CNDT2.5 cells, but not of control cells. The results support a putative role of *TCEB3C* as a tumor suppressor gene in SI-NETs. Epigenetic repression of *TCEB3C* seems to be tumor cell type-specific and involves both DNA and histone methylation.

Key Words

- carcinoid
- elongation
- epigenetic
- imprinting
- LOH

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Introduction

Small intestinal neuroendocrine tumors (SI-NETs) arise from the enterochromaffin cells which are scattered in the intestinal mucosa. The annual incidence of SI-NETs is about one case per 100 000, but the incidence and prevalence rates are increasing. The tumors are small (often <2 cm) and slow growing (Ki67 proliferation index is often <2%), but still have a lethal outcome

with a 5-year survival rate around 65% (Yao *et al.* 2008, Norlen *et al.* 2012). Many patients have tumor spread to regional lymph nodes and liver at the time of diagnosis. The tumors can produce hormones, e.g. serotonin and tachykinins, which can give rise to the carcinoid syndrome causing flushing, bronchoconstriction, and heart disease (de Herder 2007, Stålberg *et al.* 2009).

NETs from the gastrointestinal tract (GEP-NET; gastroenteropancreatic NET) were previously divided according to their embryologic origin into foregut, midgut, and hindgut carcinoid tumors. GEP-NET usually develops in the small intestine, especially in the distal ileum. According to the WHO classification system, from year 2010, SI-NETs are divided into three grades (Bosman *et al.* 2010); Grade 1 Neuroendocrine neoplasm (Ki67 <3%), Grade 2 Neuroendocrine neoplasm (Ki67 3–20%), and Grade 3 Neuroendocrine carcinoma (NEC; Ki67 >20%).

The knowledge about genetic and epigenetic aberrations in SI-NETs is sparse. The most common aberration is loss on chromosome 18, which have been seen in 43–100% (Kytola *et al.* 2001, Löllgen *et al.* 2001, Tonnies *et al.* 2001, Stancu *et al.* 2003, Wang *et al.* 2005, Kim *et al.* 2008, Kulke *et al.* 2008, Andersson *et al.* 2009, Cunningham *et al.* 2011, Walsh *et al.* 2011), supporting the presence of a crucial tumor suppressor gene. Mutational analyses of several putative tumor suppressor genes located at 18q21–18q22 revealed no alterations (Löllgen *et al.* 2001, Kulke *et al.* 2008, Cunningham *et al.* 2011). Massively parallel exome sequencing of 48 SI-NETs has revealed a low general mutation rate and no recurrently mutated genes. Amplification of genes *AKT1* or *AKT2* was observed in tumors from 16 patients (Banck *et al.* 2013). Recent analysis of a different tumor cohort by exome- and whole-genome sequencing (Francis *et al.* 2013) identified frameshift mutations (7.8%) and hemizygous deletions (14%) in *CDKN1B* on chromosome 12p13.1–p12, which encodes the cyclin-dependent kinase inhibitor p27 (Kip1). Imprinted genes are of particular interest in cancer since only one mutational hit is required to fulfill the Knudson's two-hit hypothesis of tumor suppressor genes. Imprinted genes are epigenetically regulated and have a parental origin-specific differential expression of the two alleles and the epigenetic regulation is often tissue-specific (Ferguson-Smith 2011, Uribe-Lewis *et al.* 2011). Presently, the only known imprinted gene on chromosome 18 (<http://www.geneimprint.com>) is *TCEB3C* (18q21.1), which is maternally imprinted in some tissues (Strichman-Almashanu *et al.* 2002, Li *et al.* 2010) and encompasses a CpG island. Methylation of CpG islands in gene promoter regions often results in repressed gene expression (Jones 2012). The ubiquitously expressed *TCEB3C* encodes for Elongin A3, a polypeptide of 546 amino acids which is 49 and 81% identical to Elongin A and Elongin A2, respectively. Not much is known regarding Elongin A3 function, although it can like Elongin A form a stable complex with Elongin BC and

stimulate the rate of transcription elongation by RNA polymerase II *in vitro*. Like Elongin A, Elongin A3 may be involved in ubiquitin-dependent degradation of proteins (Yamazaki *et al.* 2002).

This study investigated whether *TCEB3C* may present a tumor suppressor gene in small intestinal neuroendocrine cells and whether its expression may be regulated by epigenetic mechanisms.

Materials and methods

Tissue specimens

All 42 patients included in the study were diagnosed with SI-NETs and operated upon at Uppsala University Hospital between years 1995 and 2012. Informed consent was obtained from all patients and the study was approved by the local ethical committee, Uppsala. Twenty-nine patients were classified as NET-G1 and 13 as NET-G2. In total 64 tumors were analyzed: 27 primary tumors, 23 lymph node metastases, and 14 liver metastases. Only nine patients had succumbed making survival analysis inconclusive. Primary tumors and metastases were snap-frozen in liquid nitrogen and kept at -70°C . A human SI-NET cell line, CNDT2.5, developed from a liver metastasis from a patient diagnosed with primary ileal SI-NET (Van Buren *et al.* 2007), was used in the experiments reported here at cell passages 10–30. These cells expressed neuroendocrine markers and somatostatin receptor 2 and responded to synthetic somatostatin analog (octreotide) treatment (Van Buren *et al.* 2007, Li *et al.* 2012), although skepticism regarding the neuroendocrine authenticity of this cell line has also been raised (Ellis *et al.* 2010). The growth medium for CNDT2.5 was DMEM-F12 complemented with 10% fetal bovine serum (Sigma Aldrich), 1% vitamins, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids and 1% penicillin-streptomycin (PEST), and the cells were cultured at 37°C in 5% CO_2 . Two other cell lines were used for comparisons. The human parathyroid tumor cell line sHPT-1 was used at cell passages 18–30 and the cells were routinely tested for parathyroid hormone expression by immunostaining of cultured fixed cells (Björklund *et al.* 2007). The human embryonic kidney cell line HEK293T was obtained from ATCC (LGC, Promochem, Sweden) and used at cell passages 10–30. For extraction and purifying of RNA and DNA the AllPrep DNA/RNA kit (Qiagen) was used according to manufacturer's instructions. DNA, RNA and protein quantities were determined by NanoDrop measurements.

Immunohistochemistry

Paraffin embedded tumor tissue sections (5 µm) were passed through descending alcohol concentrations and distilled water. Background staining was blocked with 3% hydrogen peroxide and heated in citrate buffer. The tissues were treated with normal goat serum and a rabbit polyclonal anti-TCEB3C antibody (ab69873; Abcam, Cambridge, UK). A biotinylated secondary antibody was added to the tissues and then treated with ABC complex. Visualization was done with DAB color reagent. A peptide rabbit polyclonal anti-Elongin A3 antibody was also used (Santa Cruz, sc-84811) and specificity of the antibody was verified using a blocking peptide (sc-84811P). Forty-three different tumors were evaluated: 24 primary tumors, 14 lymph node metastases, five liver metastases, and also normal kidney tissue. Consecutive tissue sections of intestinal mucosa were stained with the anti-TCEB3C antibody (Abcam, ab69873) or a mouse monoclonal anti-chromogranin A antibody (Ab-1, LK2H10; Thermo Fisher Scientific, Pittsburgh, PA, USA).

Drug treatment and transfection

CNDT2.5 (2×10^5), HEK293T (8×10^5), and sHPT-1 (2×10^5) cells were distributed onto six-well plates and treated with 5-aza-2'-deoxycytidine (5-aza-dC), a global hypomethylating agent, or 3-deazaneplanocin A (DZNep), a global histone methylation inhibitor (Miranda et al. 2009). 1.0 µM 5-aza-dC or 5.0 µM DZNep were used and treatments continued for 72 h. Growth medium with additions were changed every 24 h. To further analyze DNA methylation, CNDT2.5 cells (2×10^5) were transfected with siRNA to DNA methyltransferase 1 (DNMT1) (sc-156049; Santa Cruz) and control nonsilencing siRNA (Qiagen). After 24 h, the cells were transfected again and harvested after 72 h of transfection for DNA, RNA, and protein preparations. The transfection reagent INTERFERin (Polyplus transfection) was used with 20 pmol siRNA. Triplicate samples were analyzed and the experiments were repeated twice.

The cells from 11 SI-NETs were prepared directly after surgery according to a procedure described previously (Liu et al. 2001). Ten liver metastases and one lymph node metastasis were cultured and treated with 0.1 µM 5-aza-dC. Treatment continued for 72 h and fresh 5-aza-dC was added every 24 h. RNA was extracted from treated and control cells.

Western blotting analysis

Western blotting analysis were done on protein extracts prepared in Cytobuster Protein Extract Reagent

(Merck Millipore) with Complete protease inhibitor cocktail (Roche Diagnostics GmbH). Primary rabbit polyclonal anti-DNMT1 (ab16632), rabbit polyclonal anti-TCEB3C (sc-84811), and goat polyclonal anti-actin (sc-1616) antibodies were used. Mouse monoclonal anti-DDK (TA50011, Origene, Rockville, MD, USA) antibody was used to detect Elongin A3 expression after transient transfection of the TCEB3C expression vector. After incubation with the appropriate secondary antibodies, bands were visualized using the enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ, USA).

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed after RT of DNA-free RNA with random hexamer primers using the 'First strand cDNA Synthesis kit', according to manufacturer's instructions (Thermo Fisher Scientific). Successful DNase I treatment of all RNA preparations was established by PCR analysis. qRT-PCRs were performed on the Step 1 qRT-PCR system (Applied Biosystems) using TaqMan Gene Expression Master Mix and assays for GAPDH (Hs02758991_g1) and TCEB3C (Hs01015402_s1). All samples were amplified in triplicates, and non-template controls were included. Each sample's mean threshold value was corrected for the corresponding mean value for GAPDH mRNA, used as internal control.

Analysis of gene copy number

Analysis of gene copy number was performed on DNA from the cell lines, normal kidney and 45 tumors; 19 primary tumors, 15 lymph node metastases, and 11 liver metastases. A duplex quantitative real-time PCR was performed with a FAM dye-labeled probe for TCEB3C and with a VIC dye-labeled probe for reference gene detection. Two different assays were used for TCEB3C; CCFARJL and CCD1TDD (Applied Biosystems). RNaseP (Applied Biosystems) was used as reference gene for all samples and for a limited number of tumors, the TERT gene (Applied Biosystems) was used with similar results. PCR conditions were as followed: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Four replicates of each sample were analyzed using 20 ng DNA per well. DNA from placenta and blood from a healthy donor was used as calibrators. Two tumors with known copy numbers from a previous LOH analysis (Löllgen et al. 2001) were included as controls of the quantitative PCR assay and showed the expected results. The data analysis was performed in CopyCaller software (Applied Biosystems).

Pyrosequencing

Promoter methylation status was investigated for seven CpG sites in seven primary tumors and seven lymph node metastases (seven matched pairs), CNDT2.5, sHPT-1, HEK293T, and normal kidney. DNA was treated with bisulfite using EZ DNA methylation Gold Kit (Zymo Research, Irvine, CA, USA) and PCR amplified using HotStarTaq Plus Master Mix kit (Qiagen): 10 pmol of each primer and 2 µl DNA was used as template. PCR was performed with an initial denaturation at 95 °C for 15 min, followed by 45 cycles of 94 °C for 15 s, 51 °C for 20 s, 72 °C for 20 s, and completed with 72 °C for 7 min. Forward primer used was: 5'-GTAAAGGTAGAATTTAGTGGGGTGTG-3', and reverse primer: 5'-CCCCCTAAACCCCTAAACTACC-3'. Sequencing primer: 5'-GGGGTATAGTAGTTTATTTG. Sequence analyzed: 5'-GYGTGGATYGTTTYGATTGGTT-GYGTGGGTTGAYGGAATGAATTTTATYGGGGYGG-3'. The pyrosequencing was carried out with PyroMark Q24 (Qiagen), according to manufacturer's instructions, and 20 µl PCR-product was used.

Colony forming assay

CNDT2.5 cells (1×10^5) were seeded onto six-well plates and transfected in triplicates with 4 µg *TCEB3C* plasmid expression vector (Origene) or empty vector (pcDNA3.1) using 8 µl Lipofectamine 2000 transfection reagent (Life Technologies), according to manufacturer's instructions. Six hours after transfection, a fresh medium was added complemented with 1% PEST and 0.2 mg/ml geneticin (G418, Sigma Aldrich). After 24 h, 2000 cells were distributed onto six-well plates, a fresh medium with 0.2 mg/ml geneticin was added every 72 h. After 8 days in G418 selection, the cells were fixed with 10% acetic acid/10% methanol and stained with 0.4% crystal violet, and the visible colonies were counted. Successful transfections were monitored by RT-PCR for CNDT2.5 and sHPT-1 and by western blotting analysis for HEK293T, after 24 h. sHPT-1 (2×10^5) and HEK293T (1.5×10^6) cells were transfected with the FuGENE 6 transfection reagent (Roche Diagnostics). Twenty four hours after transfection, 1000 sHPT-1 cells and 8000 HEK293T cells were distributed onto six-well plates in triplicates and after additional 24 h a fresh medium with 0.1 mg/ml geneticin was added. A fresh medium with antibiotics were added every 96 h and after 8 days in selection, the cells were fixed, stained, and counted as described above.

Statistical analysis

All data are presented as mean $\pm$ s.d. and unpaired *t*-test was used for statistical analysis. $P < 0.05$ was considered significant.

Results

One *TCEB3C* gene copy and undetectable expression of Elongin A3 for the majority of investigated SI-NETs

TCEB3C is considered an ubiquitously expressed gene and Elongin A3 was clearly immunohistochemically stained in chromogranin A-positive cells of the small intestine using a rabbit polyclonal anti-*TCEB3C* antibody (Fig. 1A). Elongin A3 expression was further analyzed in 43 SI-NETs (Supplementary Table 1, see section on supplementary data given at the end of this article) from 29 patients, 24 primary tumors, 14 lymph node metastases, and five liver metastases (Fig. 1B, C, and D). In 12 tumors more than 90% of the cells stained negatively for Elongin A3, in 21 tumors more than 50% of the cells were negative, and in ten tumors more than 50% of the cells showed positive Elongin A3 immunostaining. One patient presented positive staining in the primary tumor, but mostly negative staining in the lymph node metastasis, and another patient had mostly negative tumor cells in the primary tumor and lymph node metastasis, but mostly positive staining in the liver metastasis. There was no difference in staining patterns between Grade 1 ($n = 28$) and Grade 2 ($n = 15$) tumors. Thirty-two tumors displayed an insular growth pattern in the whole or in some part of the tissue section. For some of these ($n = 14$), the majority of insular cells was negative but showed a strong positive staining in the cells surrounding the islets (Fig. 1D). To control for specificity, a peptide rabbit anti-*TCEB3C* antibody was used and the staining was successfully blocked by the immunizing peptide (Fig. 1E).

Chromosome 18 aberrations are very common (43–100%) in primary and metastatic SI-NETs, the majority of which have lost the entire chromosome (Kytola et al. 2001, Löllgen et al. 2001, Tonnies et al. 2001, Stancu et al. 2003, Wang et al. 2005, Kim do et al. 2008, Kulke et al. 2008, Andersson et al. 2009, Cunningham et al. 2011, Walsh et al. 2011). This was confirmed in the present cohort by determining the gene copy number of *TCEB3C* by quantitative PCR, using two different PCR assays (Supplementary Table 1). One gene copy of *TCEB3C* was detected in 40/45 (89%) of the investigated tumor samples (from 36 patients).

Figure 1

Expression of Elongin A3 as revealed by immunohistochemistry. Representative immunostainings of normal intestinal mucosa and lymph node metastases are shown using an anti-*TCEB3C* rabbit polyclonal antibody (A, B, C, and D). No staining was seen in the absence of primary antibody (data not shown). (A) Consecutive tissue sections of intestinal mucosa stained for Elongin A3 (left panel) and chromogranin

(A) (right panel). (B) Negative staining. (C) Positive staining, insular growth pattern. (D) A tumor with insular growth pattern showing mostly negative staining and positive staining of cells surrounding the islets. (E) A tumor with insular growth pattern was positively stained for Elongin A3 (left panel) and successfully blocked with an excess of immunizing peptide (right panel).

These included 19 primary tumors, 15 lymph node metastases, and 11 liver metastases. One primary tumor, one lymph node metastasis, and three liver metastases displayed two gene copies. Twenty-six of the tumors with one detected *TCEB3C* gene copy were included in the immunostaining analysis and in 13 of these the majority of tumor cells were negative, seven additional samples were completely negative (>90%), and six displayed mostly positive staining for Elongin A3. One primary tumor with two gene copies displayed negative staining, a lymph node metastasis and a liver metastasis displayed two gene copies and in these two tumors the majority of cells were negative but displayed positive areas.

The *TCEB3C* gene is epigenetically repressed in SI-NETs

TCEB3C is known to be imprinted in some tissues (Strichman-Almashanu *et al.* 2002, Li *et al.* 2010), suggesting a possibility of one allele being epigenetically repressed also

in SI-NET cells. In order to investigate this possibility, the human SI-NET cell line CNDT2.5, harboring one *TCEB3C* gene copy, was treated with the DNA hypomethylating agent 5-aza-dC. The treatment strongly induced *TCEB3C* mRNA expression (90-fold) as well as increased Elongin A3 expression from a very low level in CNDT2.5 cells (Fig. 2A and B). A role of DNA methylation was further supported by RNA interference experiments, by knocking down DNMT1. Transient transfection of siRNA to DNMT1 (Fig. 2C), but not of control siRNA, induced both *TCEB3C* mRNA (4.5-fold) and protein expression (Fig. 2D and E). In order to rule out a cell line-specific effect, metastatic SI-NET cells from 11 patients were freshly prepared directly after surgery and cultured *in vitro* in the presence of 5-aza-dC. The tumor cells had either one or two copies of the *TCEB3C* gene. Expression of the *TCEB3C* gene was significantly induced in ten out of the 11 cultures (two- to 16-fold) in the presence of 5-aza-dC (Fig. 2F), strongly supporting an epigenetic repressive mechanism in SI-NET cells.

Figure 2

Effects on *TCEB3C* mRNA and protein (Elongin A3) expression after 5-aza-dC treatment and siRNA transfections. (A, B, C, D, and E) CNDT2.5 cells. (F) Primary SI-NET cell cultures ($n = 11$). (A) *TCEB3C* mRNA after 5-aza-dC treatment. (B) Elongin A3 after 5-aza-dC treatment. (C) DNMT1 protein expression after siControl or siDNMT1 transfection. (D) *TCEB3C* mRNA after siControl or siDNMT1 transfection. (E) Elongin A3 after siControl or siDNMT1 transfection. (F) *TCEB3C* mRNA after $-/+$ 5-aza-dC treatment. * $P < 0.05$.

Next, CNDT2.5 cells were treated with DZNep, a global histone methyltransferase inhibitor. Also this treatment strongly induced *TCEB3C* mRNA expression (80-fold) and detectably increased Elongin A3 expression (Fig. 3A and B). Repressive histone methylations therefore also play a role in repression of *TCEB3C* in this cell type. We also analyzed the effects of the epigenetic drugs in HEK293T and sHPT-1 cells, both harboring one *TCEB3C* gene copy. Elongin A3 could readily be detected in sHPT-1 cells by western blotting and approximately ten times more *TCEB3C* mRNA was expressed in these cells compared with CNDT2.5 cells, and at least ten times more mRNA was expressed in the HEK293T cells (data not shown). 5-aza-dC failed to induce expression of *TCEB3C* in both cell lines. DZNep showed no effect in sHPT-1 cells and a small but significant effect in the HEK293T cells (Fig. 3C). Thus, epigenetic repression of *TCEB3C* seems to be tumor cell type-specific and likely involves both DNA and histone methylation.

Figure 3

Effects on *TCEB3C* mRNA and protein (Elongin A3) expression after DZNep treatment in CNDT2.5 cells (A and B). Effects of 5-aza-dC and DZNep in HEK293T and sHPT-1 cells (C). * $P < 0.05$.

The *TCEB3C* promoter region is CpG methylated

The *TCEB3C* gene consists of one exon and is very CpG-rich with large CpG islands. The presence of CpG methylation in the promoter region was first established by bisulfite sequencing (data not shown). Quantitative pyrosequencing then revealed high level (~90%) of CpG methylation in all 14 investigated SI-NETs (Fig. 4A). As expected, the treatment of CNDT2.5 cells with 5-aza-dC or transfection of siDNMT1 caused a reduction in the mean methylation level, to 56 and 80% respectively (Fig. 4B). Thus, the expected relationship between induced *TCEB3C* expression and decreased promoter DNA methylation was observed in these cells. Somewhat unexpected, the *TCEB3C* promoter was found to be highly methylated (Fig. 4C) also in the *TCEB3C*-expressing cell lines, sHPT-1 (91%) and HEK293T (89%). Furthermore, high methylation (89%) was also detected in normal kidney, displaying two gene copies and prominent expression of Elongin A3 (Fig. 4C). Therefore, the induction of *TCEB3C* by 5-aza-dC in the SI-NET cell line probably involves demethylation of cell type-specific regulatory elements other than the limited promoter region analyzed in this study. It could also be due to indirect effects.

A growth regulatory role of Elongin A3 in SI-NET cells

To investigate whether *TCEB3C* could play a growth regulatory role, a colony formation assay was performed with transfection of a *TCEB3C* expression vector to the CNDT2.5 SI-NET cell line followed by G418 selection of *TCEB3C*-expressing cells. Overexpression of *TCEB3C* resulted in a significant reduced number (50%) of cell colonies (Fig. 5A and B). No growth inhibitory effect was seen in parathyroid sHPT-1 cells (Fig. 5C and D) or HEK293T cells (Fig. 5E and F). These results support a tumor suppressor role of *TCEB3C* in SI-NET cells.

Discussion

Not much is known regarding Elongin A3 function, except that it can form a stable complex with Elongin BC and stimulate elongation by RNA polymerase II *in vitro*, just like Elongin A (Yamazaki et al. 2002). Elongins B (*TCEB2*) and C (*TCEB1*) play an important role in tumor suppression by von Hippel-Lindau (VHL), where the VHL protein binds to the same Elongin BC binding sequence as Elongin A (*TCEB3*) and among other targets also binds to the α subunits of hypoxia-inducible factor and promote their ubiquitination and proteosomal

Figure 4

Quantitative bisulfite pyrosequencing analysis measuring methylation of seven CpGs in the *TCEB3C* promoter region. (A) Mean methylation level in SI-NETs ($n=14$). (B) Methylation level of individual CpGs in untreated, siDNMT1 transfected, and in 5-aza-dC treated CNDT2.5 cells. (C) Mean methylation level in sHPT-1, HEK293T, and normal kidney. Right panel, immunostaining of normal kidney with the anti-*TCEB3C* rabbit polyclonal antibody.

degradation. More than 70% of VHL familial and sporadic mutations affect the VHL Elongin C binding site (Conaway et al. 1998, Zhang & Yang 2012). Elongin A on the other hand can mediate DNA damage-induced ubiquitination and degradation of the largest subunit of RNA polymerase II together with Elongin BC and the Cul5/Rbx2 module (Yasukawa et al. 2008).

Figure 5

Colony formation assay in CNDT2.5 (A and B), sHPT-1 (C and D), and HEK293T (E and F). Cells were transfected with an expression vector for *TCEB3C* or empty vector and were selected by incubation with G418 for 8 days. (B and D) Successful transfection was monitored by expression of

TCEB3C by RT-PCR for CNDT2.5 and sHPT-1. (F) Successful transfection in HEK293T was observed by western blotting analysis with an antibody to the DDK epitope, fused to Elongin A3. **P* < 0.05.

Like Elongin A and VHL, Elongin A3 possesses the Elongin C binding sequence and can form a putative ubiquitylation complex with Elongin BC and Cul5/Rbx2 (Yamazaki *et al.* 2002). Interestingly, Elongin C (TCEB1)

has been shown to be overexpressed and to promote invasion of prostate cancer cells (Jalava *et al.* 2009).

A number of investigations have found frequent (43–100%) aberrations on chromosome 18 in primary and

metastatic SI-NETs, using a variety of techniques, such as LOH, CGH, CGH-array, SNP analysis, and SNP-array (Kytola *et al.* 2001, Löllgen *et al.* 2001, Tonnie *et al.* 2001, Stancu *et al.* 2003, Wang *et al.* 2005, Kim do *et al.* 2008, Kulke *et al.* 2008, Andersson *et al.* 2009, Cunningham *et al.* 2011, Walsh *et al.* 2011). Perhaps the most conspicuous finding is the observation of frequent loss of one copy of the whole chromosome. Smaller deletions not necessarily overlapping have only been found in a few tumors (summarized in Cunningham *et al.* (2011)). Mutational analysis of candidate tumor suppressor genes have been performed for *SMAD4*, *SMAD2*, and 18 additional genes in the 18q21–18q22 region, without identifying any mutations (Löllgen *et al.* 2001, Kulke *et al.* 2008, Cunningham *et al.* 2011). Massively parallel exome sequencing of SI-NETs in one study did not reveal recurrently mutated genes (Banck *et al.* 2013), but recent analysis of a different tumor cohort identified a relatively low frequency of recurrent mutations (~9%), in the cyclin-dependent kinase inhibitor gene *CDKN1B* on chromosome 12p13.1–p12 (Francis *et al.* 2013). Imprinted genes are of particular interest in cancer because only one mutational hit is required to fulfill the Knudson's two-hit hypothesis of tumor suppressor genes. The imprinted allele is normally inactivated by epigenetic mechanisms, in a way that could be tissue specific (Ferguson-Smith 2011, Uribe-Lewis *et al.* 2011). *TCEB3C* is currently the only known imprinted gene on chromosome 18 (<http://www.geneimprint.com>) and is located at 18q21.1. The data presented in this study strongly support a putative role of *TCEB3C* as tumor suppressor in SI-NETs. The majority of tumors had one *TCEB3C* gene copy (in 40/45, 89%), showed prominently reduced expression of Elongin A3 (in 20/26, 77%) and overexpression led to a decrease in clonogenic survival of SI-NET tumor cells and not of control cells. Epigenetic repression of *TCEB3C* expression was found to be SI-NET cell-specific and regulated by DNA and histone methylation. Gene induction after treatment with 5-aza-dC or DZNep could in principle also be due to indirect effects, although perhaps less likely here as both compounds strongly induced expression of *TCEB3C*. 5-aza-dC induced *TCEB3C* expression in the cultured primary SI-NET cells containing either one or two copies of the *TCEB3C* gene, supporting the possibility that the gene is imprinted in this cell type. Heterogenous Elongin A3 expression, including the lining of positive cells around negative insular cells, as was observed for 14 SI-NETs, may be understood by the reversible nature of epigenetic deregulation of gene expression.

Further studies on a putative role of *TCEB3C* (Elongin A3) as a tumor suppressor gene in SI-NET cells

are warranted and it will be of interest to investigate a possible role of Elongin A3 in ubiquitination.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0419>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Andersson E, Sward C, Stenman G, Ahlman H & Nilsson O 2009 High-resolution genomic profiling reveals gain of chromosome 14 as a predictor of poor outcome in ileal carcinoids. *Endocrine-Related Cancer* **16** 953–966. (doi:10.1677/ERC-09-0052)
- Banck M, Kanwar R, Kulkarni A, Boora G, Metge F, Kipp B, Zhang L, Thorland E, Minn K, Tentu R *et al.* 2013 The genomic landscape of small intestine neuroendocrine tumors. *Journal of Clinical Investigation* **123** 2502–2508. (doi:10.1172/JCI67963)
- Björklund P, Åkerström G & Westin G 2007 Activated β -catenin in the novel human parathyroid tumor cell line sHPT-1. *Biochemical and Biophysical Research Communications* **352** 532–536. (doi:10.1016/j.bbrc.2006.11.056)
- Bosman FT, Carneiro F, Hruban RH & Theise ND 2010 *WHO Classification of Tumours of the Digestive System*. Geneva, Switzerland: WHO.
- Conaway JW, Kamura T & Conaway RC 1998 The Elongin BC complex and the von Hippel Lindau tumor suppressor protein. *Biochimica et Biophysica Acta* **1377** M49–M54.
- Cunningham JL, Diaz de Stahl T, Sjöblom T, Westin G, Dumanski JP & Janson ET 2011 Common pathogenetic mechanism involving human chromosome 18 in familial and sporadic ileal carcinoid tumors. *Genes, Chromosomes & Cancer* **50** 82–94. (doi:10.1002/gcc.20834)
- Ellis LM, Samuel S & Sceusi E 2010 Varying opinions on the authenticity of a human midgut carcinoid cell line – letter. *Clinical Cancer Research* **16** 5365–5366. (doi:10.1158/1078-0432.CCR-10-2550)
- Ferguson-Smith AC 2011 Genomic imprinting: the emergence of an epigenetic paradigm. *Nature Reviews. Genetics* **12** 565–575. (doi:10.1038/nrg3032)
- Francis JM, Kiezun A, Ramos AH, Serra S, Pedamallu CS, Qian ZR, Banck MS, Kanwar R, Kulkarni AA, Karpathakis A *et al.* 2013 Somatic mutation of *CDKN1B* in small intestine neuroendocrine tumors. *Nature Genetics* **45** 1483–1486. (doi:10.1038/ng.2821)
- de Herder WW 2007 Biochemistry of neuroendocrine tumours. *Best Practice & Research. Clinical Endocrinology & Metabolism* **21** 33–41. (doi:10.1016/j.beem.2006.12.002)

- Jalava SE, Porkka KP, Rauhala HE, Isotalo J, Tammela TL & Visakorpi T 2009 TCEB1 promotes invasion of prostate cancer cells. *International Journal of Cancer* **124** 95–102. (doi:10.1002/ijc.23916)
- Jones PA 2012 Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Reviews. Genetics* **13** 484–492. (doi:10.1038/nrg3230)
- Kim do H, Nagano Y, Choi IS, White JA, Yao JC & Rashid A 2008 Allelic alterations in well-differentiated neuroendocrine tumors (carcinoid tumors) identified by genome-wide single nucleotide polymorphism analysis and comparison with pancreatic endocrine tumors. *Genes, Chromosomes & Cancer* **47** 84–92. (doi:10.1002/gcc.20510)
- Kulke MH, Freed E, Chiang DY, Philips J, Zahrieh D, Glickman JN & Shivdasani RA 2008 High-resolution analysis of genetic alterations in small bowel carcinoid tumors reveals areas of recurrent amplification and loss. *Genes, Chromosomes & Cancer* **47** 591–603. (doi:10.1002/gcc.20561)
- Kytola S, Hoog A, Nord B, Cedermarck B, Frisk T, Larsson C & Kjellman M 2001 Comparative genomic hybridization identifies loss of 18q22-qter as an early and specific event in tumorigenesis of midgut carcinoids. *American Journal of Pathology* **158** 1803–1808. (doi:10.1016/S0002-9440(10)64136-3)
- Li SS, Yu SL & Singh S 2010 Epigenetic states and expression of imprinted genes in human embryonic stem cells. *World Journal of Stem Cells* **2** 97–102. (doi:10.4252/wjsc.v2.i4.97)
- Li SC, Martijn C, Cui T, Essaghir A, Luque RM, Demoulin JB, Castano JP, Öberg K & Giandomenico V 2012 The somatostatin analogue octreotide inhibits growth of small intestine neuroendocrine tumour cells. *PLoS ONE* **7** e48411. (doi:10.1371/journal.pone.0048411)
- Liu W, Ridefelt P, Åkerström G & Hellman P 2001 Differentiation of human parathyroid cells in culture. *Journal of Endocrinology* **168** 417–425. (doi:10.1677/joe.0.1680417)
- Löflgen RM, Hessman O, Szabo E, Westin G & Åkerström G 2001 Chromosome 18 deletions are common events in classical midgut carcinoid tumors. *International Journal of Cancer* **92** 812–815. (doi:10.1002/ijc.1276)
- Miranda TB, Cortez CC, Yoo CB, Liang G, Abe M, Kelly TK, Marquez VE & Jones PA 2009 DZNep is a global histone methylation inhibitor that reactivates developmental gene not silenced by DNA methylation. *Molecular Cancer Therapeutics* **8** 1579–1588. (doi:10.1158/1535-7163.MCT-09-0013)
- Norlen O, Stalberg P, Öberg K, Eriksson J, Hedberg J, Hessman O, Janson ET, Hellman P & Åkerström G 2012 Long-term results of surgery for small intestinal neuroendocrine tumors at a tertiary referral center. *World Journal of Surgery* **36** 1419–1431. (doi:10.1007/s00268-011-1296-z)
- Stålberg P, Hellman P & Åkerström G 2009 *Carcinoid: Presentation and Diagnosis, Surgical Management*. Springer-Verlag London Limited: Springer Specialist Surgery Series.
- Stancu M, Wu TT, Wallace C, Houlihan PS, Hamilton SR & Rashid A 2003 Genetic alterations in goblet cell carcinoids of the vermiform appendix and comparison with gastrointestinal carcinoid tumors. *Modern Pathology* **16** 1189–1198. (doi:10.1097/01.MP.0000097362.10330.B1)
- Strichman-Almashanu LZ, Lee RS, Onyango PO, Perlman E, Flam F, Frieman MB & Feinberg AP 2002 A genome-wide screen for normally methylated human CpG islands that can identify novel imprinted genes. *Genome Research* **12** 543–554.
- Tonnies H, Toliat MR, Ramel C, Pape UF, Neitzel H, Berger W & Wiedenmann B 2001 Analysis of sporadic neuroendocrine tumours of the enteropancreatic system by comparative genomic hybridisation. *Gut* **48** 536–541. (doi:10.1136/gut.48.4.536)
- Uribe-Lewis S, Woodfine K, Stojic L & Murrell A 2011 Molecular mechanisms of genomic imprinting and clinical implications for cancer. *Expert Reviews in Molecular Medicine* **13** e2. (doi:10.1017/S1462399410001717)
- Van Buren G II, Rashid A, Yang AD, Abdalla EK, Gray MJ, Liu W, Somcio R, Fan F, Camp ER, Yao JC et al. 2007 The development and characterization of a human midgut carcinoid cell line. *Clinical Cancer Research* **13** 4704–4712. (doi:10.1158/1078-0432.CCR-06-2723)
- Walsh KM, Choi M, Oberg K, Kulke MH, Yao JC, Wu C, Jurkiewicz M, Hsu LI, Hooshman SM, Hassan M et al. 2011 A pilot genome-wide association study shows genomic variant enriched in the non-tumor cells of patients with well-differentiated neuroendocrine tumor of the ileum. *Endocrine-Related Cancer* **18** 171–180. (doi:10.1677/ERC-10-0248)
- Wang GG, Yao JC, Worah S, White JA, Luna R, Wu TT, Hamilton SR & Rashid A 2005 Comparison of genetic alterations in neuroendocrine tumors: frequent loss of chromosome 18 in ileal carcinoid tumors. *Modern Pathology* **18** 1079–1087. (doi:10.1038/modpathol.3800389)
- Yamazaki K, Guo L, Sugahara K, Zhang C, Enzan H, Nakabeppu Y, Kitajima S & Aso T 2002 Identification and biochemical characterization of a novel transcription elongation factor, Elongin A3. *Journal of Biological Chemistry* **277** 26444–26451. (doi:10.1074/jbc.M202859200)
- Yao JC, Hassan M, Phan A, Dagohoy C, Leary C, Mares JE, Abdalla EK, Fleming JB, Vauthey JN, Rashid A et al. 2008 One hundred years after "carcinoid": epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States. *Journal of Clinical Oncology* **26** 3063–3072. (doi:10.1200/JCO.2007.15.4377)
- Yasukawa T, Kamura T, Kitajima S, Conaway RC, Conaway JW & Aso T 2008 Mammalian Elongin A complex mediates DNA-damage-induced ubiquitylation and degradation of Rpb1. *EMBO Journal* **27** 3256–3266. (doi:10.1038/emboj.2008.249)
- Zhang Q & Yang H 2012 The roles of VHL-dependent ubiquitination in signaling and cancer. *Frontiers in Oncology* **2** 35. (doi:10.3389/fonc.2012.00035)

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