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Title:

"Hypo-methylation mediates Chromosomal Instability in pancreatic NET"

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Short title:

Methylation and genomic instability in PanNET

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Summary:

DAXX and or ATRX loss occur in 40% of Pancreatic Neuro-endocrine Tumors (PanNETs). PanNETs negative for DAXX or ATRX show an increased risk of relapse. The tumor-associated pathways activated upon DAXX or ATRX loss and how this event may induce Chromosomal Instability (CIN) and Alternative Lengthening Telomeres (ALT) are still unknown. Both DAXX and ATRX are involved in DNA methylation regulation. DNA methylation of heterochromatin and of non-coding sequences is extremely important for the maintenance of genomic stability. We analysed the association of DAXX and or ATRX loss and CIN with global DNA methylation in human PanNET samples and the effect of DAXX knock down on methylation and cell proliferation.

We assessed *LINE1* as well as global DNA methylation in 167 PanNETs and we found that DAXX and or ATRX negative tumors and tumors with CIN were hypo-methylated. DAXX knock-down in PanNET cell lines blocked cells in G1/G0 phase and seemed to increase CIN in QGP-1 cells. However, no direct changes in DNA methylation were observed after DAXX knock down *in vitro*.

In conclusion our data indicate that epigenetic changes are crucial steps in the progression of PanNETs loss and suggest that DNA methylation is the mechanism via which CIN is induced, allowing clonal expansion and selection.

Introduction

The molecular pathways and mechanisms underlying initiation and progression of pancreatic neuroendocrine tumors (PanNET) are still poorly understood. Mutations in *DAXX* (Death Domain Associated Protein) and *ATRX* (ATR-X) with correspondent loss of protein expression in the tumor tissue occur in 40% of PanNETs (Jiao, et al. 2011). DAXX and or ATRX loss correlates with Chromosomal Instability (CIN) and predicts for relapse in low stage patients (Stage I to III in the absence of distant metastasis) (Marinoni, et al. 2014). DAXX and or ATRX negative tumors show Alternative Lengthening of Telomeres (ALT), a telomerase independent mechanism for telomere length maintenance, based on homologous recombination (Heaphy, et al. 2011). The mechanisms and the sequence of events linking *DAXX/ATRX* mutation, ALT and CIN are unknown.

In addition to mutations, the importance of epigenetic changes is increasingly recognized for many cancer types (reviewed in (Das and Singal 2004). DAXX and ATRX participate in maintaining the epigenetic status of the cells by regulating both DNA methylation and H3.3 deposition at telomeric and peri-centromeric regions. DAXX interacts and recruits DNA methyl-transferase enzyme 1 (DNMT1) to specific promoters including *RASSF1* and *RELB* (Puto and Reed 2008). ATRX contains the ADD domain as does DNMT3-DNMT3L, important for establishing and maintaining DNA methylation pattern (Noh, et al. 2016).

Impairment of DNA methylation in PanNETs has been shown in several genes (*VHL, cdkn2a* (p16), *RASSF1*, *DAPK1*, *TIMP3*, *PAX5*, *HIC1*, *CADM1*, *PYCARD*, *ESR1*, *VHL*, *RARB, WT1 and MGMT*). (House, et al. 2003; Malpeli, et al. 2011; Schmitt, et al. 2014; Schmitt, et al. 2009; Stefanoli, et al. 2014). Genome wide methylation profiling of PanNET has shown a difference of methylation pattern between DAXX and ATRX negative tumors, suggesting that mainly DAXX loss is driving DNA methylation changes in PanNETs (Pipinikas, et al. 2015).

DNA methylation not only regulates gene expression, but also genomic stability. Indeed hetero-chromatin and silenced regions are usually hyper-methylated resulting in a highly condensed chromatin structure not accessible to the transcription machinery. Hypo-methylated DNA regions are usually highly transcribed. Repetitive and mobile elements such *LINE1* (Long Interspersed Element-1) and *ALU* (Arthrobacter luteus) sequences are preserved from transcription and activation by DNA hyper-methylation. Once activated, these sequences move within the genome and thereby generate chromosomal instability. Hypo-methylation of *LINE1* and *ALU* sequences occurs in several tumors including PanNETs (Choi, et al. 2007; Stefanoli et al. 2014). It is currently unknown whether *LINE1* and *ALU* hypo-methylation is directly linked to *DAXX* and or *ATRX* mutation. Regulation of transcription of the telomeric sequences TERRA (Telomeric repeat containing RNA) and telomere stability is also dependent on the DNA methylation status. Dnmt1 deficient mouse embryonic stem cells show elongated telomeres, increased recombination at telomeric regions and ALT activation (Gonzalo, et al. 2006).

In the present study we aimed to analyse the effect of DAXX and or ATRX loss on global methylation in ex-vivo human PanNETs samples and *in vitro* in BON-1 and QGP-1 cell line-models. *In vitro* we also assessed genomic instability induction upon DAXX knock down. We found that DAXX and or ATRX negative PanNETs showed global hypo-methylation but not LINE1 hypo-methylation compare to positive tumors. Additionally we were able to find a link between LINE1 hypo-methylation and CIN in PanNETs. DAXX knock down does not induce methylation changes in BON-1 and QGP-1 cells.

Material and Methods

Human samples

167 out of 207 resected well differentiated PanNETs (G1-G2) and 11 matching control were used

in the study based on DNA viability and informative IHC results (Marinoni et al. 2014).

CIN data obtained by Comparative Genomic Hybridization (CGH) were previously reported (Marinoni et al. 2014).

CIN were defined as showing a total number of gains and losses of 8 or more in conventional CGH and of 20 or more in array CGH. The use of patient material was approved by the local ethics committees (Number 105/2015). The composition of samples is described in table 1 and in the supplementary figure 1.

Cell line culture conditions and treatments

The pancreatic neuroendocrine cell line BON-1 (pancreatic carcinoid) was provided by E.J. Speel, Department of Pathology, Maastricht, The Netherlands and metastatic somatostatinoma derived QGP-1 cell line was obtained from Japanese Health Sciences Foundation, Osaka, Japan. Both cell lines authentication was performed by STRs analysis (Mycrosynth, Balgach, Switzerland) upon arrival (2011) and in the present year. QGP-1 cells resulted 100% matching with the QGP-1 profile (GNE586559, Genentech, Roche, Basel, Switzerland). BON-1 cells are not commercially available therefore no comparison is possible, however STRs analysis of these cells revealed no matching with any data available in the ATCC database, excluding any contamination with other cell lines. Additionally the expression of the neuro-endocrine markers Synaptophysin and Chromogranin-A was checked yearly. Both QGP-1 and BON-1 cells express the two markers. Cells were kept in culture for a maximum of 20-25 passages.

Both cell lines were cultured at 37°C under 5% CO_2. BON-1 were cultured in DMEM/Nutrient Mixture F-12 Ham (D6421, Sigma-Aldrich, Buchs SG, Switzerland) and QGP-1 in RPMI 1640 Dutch modification (R7638, Sigma-Aldrich), both media supplemented with 10% FBS (GIBCO,

Thermo Fisher Scientific, Paisley, UK), 2nM L-alanyl-L-glutamin (Sigma-Aldrich), 100µg/ml streptomycin and 100units/ml of penicillin (Sigma-Aldrich).

Immuno-histo-chemistry (IHC)

Four-micrometer sections were taken from a TMA including 207 pNETs derived from patients who underwent surgery at the Inselspital Bern, Switzerland previously described (Marinoni et al. 2014) and stained with an anti-5-methylcytidine antibody BI_MECY_0100 (Eurogentec, Kaneka, Liege, Belgium). The Immunohistochemical staining was performed on an automated staining system (Leica Bond III; Leica Biosystems, Nunningen, Switzerland). Antigen retrieval was performed by heating citrate buffer at 100° for 30 minutes. The primary antibody was incubated for 30 minutes at a dilution of 1:200. Visualization was performed using the avidin-biotin complex method, which yielded a brown staining signal. Normal pancreatic islets show strong positive staining indicating a certain level of DNA methylation thus we scored as high-methylated samples showing similar or stronger staining than normal islets and low-methylated samples showing weaker staining. To exclude false-negative samples, only samples with positive nuclear staining of non-neoplastic cells and negative tumor nuclei were scored as negative (153 of 207 samples remaining informative). 54 Samples with both negative tumor nuclei and non-neoplastic stromal and endothelial cells were scored as non-informative and excluded from further analysis.

DNA extraction, bisulfite conversion and global methylation analysis

DNA was extracted from cell pellet of about $6x10^5$ cells or from paraffin embedded human PanNETs (55 tumor samples with more than 80% tumor content and 11 matching normal pancreatic tissues, Table 1 and supplementary figure 1) using Nucleo Spin kit by Macherey-

Nagel (Düren, Germany) according to the manufacturer's instructions. For bisulfite conversion of gDNA the EZ DNA Methylation-Gold kit by Zymo Research Corporation (Freiburg, Germany) was used following the manufacturer's instructions.

Genomic DNA of 10 tumor samples was analyzed by its global methylation status using EpiSeeker methylated DNA Quantification Kit (ab117128, Abcam, Cambridge, United Kingdom), according to the supplier's instructions. Colorimetric measurements were done on an ELISA reader (Tecan, Männerdorf, Switzerland). Hyper-methylated and hypo-methylated DNAs (Zymo Research) were used as controls.

PCR and pyrosequencing investigation for *LINE-1* for 51 samples (GenBank accession number X58075) methylation analysis was performed using PyroMark kit (Qiagen, Hilden, Germany). To analyse sequence methylation of *LINE-1*, the PyroMark Q24 CpG LINE-1 (4x24) Methylation detection assay was used (Qiagen). Amplifications were performed with Master Mix PyroMark PCR Kit (Qiagen) according to supplier's instructions on a Veriti System gradient apparatus (Applied Biosystems, Thermo Fisher Scientific). PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences, GE Healthcare, Little Chalfont, United Kingdom), purified, washed, denatured and washed again with the PyroMark Q24 Vacuum Workstation 220V (Qiagen). Pyrosequencing was performed using the Qiagen PyroMark Q24 System with the pyromark Q24 Cartridge Method 0011 (Qiagen). The assay setups and analysis were performed with PyroMark Assay Design Software V.1.0.6 (Qiagen).

MGMT (O-6-methylguanine-DNA-methyltransferase) promoter methylation was assessed in 25 tumor samples as previously described (Vassella, et al. 2011). Samples with methylation level higher and equal to 5% were considered hyper-methylated and samples with methylation level <5% were considered hypo-methylated.

The MEN1 gene was analysed in 26 tumor samples by semi-conductive sequencing using an Ion Torrent PGM (Life Technologies). Protein coding exons were amplified by multiplex polymerase chain reaction using 2 primer pools designed by the Ion AmpliSeq Designer (Life Technologies). Library construction, emulsion polymerase chain reaction, and sequencing were performed according to the manufacturer's recommendations. The Torrent Suite 5.0.3 platform was used for sequence alignment with the hg19 human genome reference. Variant calling was performed with the variant caller 5.0.3.5. and the IonReporter 5.0 software (Life Technologies, Grand Island, NY). The average base coverage depth for most samples was more than 2000 reads. MEN1 mutations found are summarized in table 1 in the Supplementary Material.

DAXX knock down

For virus infection $6x10^5$ cells were seeded in 6 wells plate the day before. pLKO.1 lentiviral vectors expressing small hairpin (sh) RNAs targeting DAXX (Sh-DAXX_H2410: and Sh-DAXX_2503) or a nontargeting shRNA control (SHCOO2) were purchased from Sigma-Aldrich Lentiviral production and transduction were done as described in (Tschan, et al. 2003). Transduced cells were selected by puromycin treatment (1.5µg/ml, Invitrogen). For micro-nuclei counting, cells were seeded in 24-well plates on coverslips coated with FBS, $1x10^4$ cells per well. Cells were fixed with 4% Paraformaldehyde for 30 min at room temperature. DAPI was used to stain nuclei. Micronuclei were counted under a fluorescent microscope (Axiophot2, Zeiss).

Viability and apoptosis assays

For MTT assay cells were seeded into 96-well plates at the density of 16'000 cells per well. After 24h medium was replaced by 100ul of medium with 10% MTT (0.5mg/ml final concentration) (Sigma-Aldrich) and incubate 1h at 37°. After incubation the medium was removed and 200 µl of DMSO (Sigma-Aldrich) and 25 µl of Sorensen solution (0.1M Glycine, 0.1M NaCl, pH 10.5 in

water) were added into each well. Absorbance at 570nm was determined on a spectrophotometer (Tecan).

Cell cycle analysis with FACS

Briefly, cells were seeded $5x10^5$ cells in duplicates or triplicates in 6-well plates. The day after cells were treated with Nocodazol. After 16h of treatment cells were fixed in ice cold EtOH overnight. PI staining was performed directly before measurement on BD-FACS LSR SorpII using BD FACSDiva 6.1 software (BD Bioscience, Heidelberg, Germany). Results were analyzed using FlowJo v9.8 software (TreeStar, USA).

Protein extraction and Western blot analysis

For western blotting Cells were cultivated in 6-well plates and lysate in Urea buffer. Forty ng proteins were loaded on 4-20% Mini-PROTEAN® TGX Stain-Free™ Gel (Bio-Rad, Cressier, Switzerland). Transfer was performed using transfer turbo blot system (Bio-Rad). Primary monoclonal antibodies rabbit anti DAXX (clone 25C12, 1:1000 in 5% BSA, Cell signaling, Cambridge, United Kingdom) and mouse anti human α-tubulin (clone B-5-1-2, 1:1000 in 5% BSA, Sigma), GAPDH (clone 6c5, 1:1000 in 5% skimmed milk, Merk Millpore, Darmstadt, Germany) were used. As secondary antibodies goat anti-rabbit DyLight® 650 conjugate (LabForce, Muttenz, Switzerland) and goat anti-mouse DyLight® 550 conjugate (LabForce) both diluted 1:500 in 5% milk were used. Signal was detected with ChemiDoc MP System (Bio-Rad) and analyzed in ImageJ (freeware, 1.48v, Bethesda, USA). Protein levels were normalized to α-tubulin, GAPDH or total protein.

Statistics

Statistical analysis was performed using GraphPAD prism 5 software (La Jolla, CA, USA). Statistical differences were calculated using unpaired two-tailed Student's t test. The χ^2 test or the Fisher exact test was used to calculate contingency tables. *P* values less than .05 were considered statistically significant.

Results

Ex-vivo analysis of human PanNET samples

DAXX and or ATRX negative PanNET are hypo methylated

In order to first assess whether DAXX/ATRX loss had an impact on the DNA methylation pattern we evaluated global DNA methylation on 10 PanNET samples. DNA extracted from 5 PanNET samples positive for DAXX/ATRX IHC staining, negative for ALT activation and CIN negative (DAXX/ATRX+/ALT-/CIN-) and from 5 PanNET samples negative for DAXX/ATRX staining, positive for ALT activation and CIN positive (DAXX/ATRX-/ALT+/CIN+) were analyzed for global DNA methylation with the colorimetric EpiSeeker assay (Methylated DNA Quantification Kit, Abcam). As shown in figure 1-A DAXX/ATRX-/ALT+/CIN+ tumors exhibit a significantly lower level of DNA methylation compared to DAXX/ATRX+/ALT-/CIN- ones ($p<0.01$**), which is comparable to the un-methylated control. To confirm our findings in a larger setting and with an additional method, we stained 2 TMAs, composed as described in the material and methods section and in (Marinoni et al. 2014) with an anti-5-methylcytidine antibody (Eurogentec) that specifically recognizes the methylated base and does not cross-react with the un-methylated one. We obtained results on 153 samples; the characteristic of these samples is reported in table 1.

We observed that strong 5-methylcytidine staining associates with DAXX and ATRX proficient tumors while weak signal correlates with DAXX/ATRX deficient ones $p<0.05$* (Figure 1 B-G and Table 2), suggesting that DAXX/ATRX deficient tumor show global hypo-methylation compared to the positive ones. Normal islets showed a strong 5-methylcytidine staining (Figure 1 B-C).

To examine the DNA methylation level of non-coding regions we evaluated the (Long Interspersed Element) *LINE1* methylation status as a marker of global DNA methylation in 55 PanNET and 11 non-neoplastic pancreatic specimens, by pyro-sequencing (Qiagen). We observed that *LINE1* is hypo-methylated in PanNETs compared to normal pancreas ($p<0.001$***) (Figure 2-A) as previously reported (Choi et al. 2007; Stefanoli et al. 2014). *LINE1* hypo-methylation indeed associates with CIN ($p<0.05$*) (Figure 2-B), as we hypothesized. However, no differences in LINE1 methylation were observed in either DAXX/ATRX positive and negative tumors, ALT positive and negative (Figure 2 C-E). In order to assess if MEN1 mutations impact on LINE1 methylation levels we sequenced MEN1 gene in 26 samples. Eleven samples resulted to be mutated. The mutations found are summarized in Supplementary Material Table 1. No difference in LINE1 methylation status were observed between mutant and wild type tumors (Figure 2 F). Interestingly, *LINE1* hypo-methylation predicts for shorter disease free survival ($p<0.05$*), (figure 3 supplementary material) in agreement with the results reported by Stefanoli et al. (Stefanoli et al. 2014); while no differences in survival are detectable in tumors positive and negative for 5-methylcytidine staining (data not shown).

We performed quantitative methylation specific PCR to assess whether LINE1 hypo-methylation or DAXX/ATRX loss correlate with hyper-methylation of MGMT promoter, due to its clinical relevance. We found 8 samples with MGMT promoter hyper-methylated and 17 samples where MGMT promoter was un-methylated. No correlation between LINE1 hypo-methylation or

DAXX/ATRX loss and MGMT methylation level was found (Supplementary Figure 4), suggesting two independent mechanisms for the two events, as previously proposed (Stefanoli et al. 2014).

In vitro upon DAXX knock-down

Induction of genomic instability and G1 arrest

In order to assess if DAXX loss is sufficient to induce genomic instability, we knocked down DAXX in two PanNET cell lines, QGP-1 and BON-1 by Lentiviral transduction of SH-RNAs. DAXX knock down was evaluated by western blotting 9, 17 and 24 days after lentiviral infection. Two different DAXX specific vectors (1 and 2) and scramble control were used in both cell lines. DAXX expression is highly reduced after 9 days from infections in both cell lines (Figure 3-A and supplementary material Figure 5-A). After 17 days DAXX expression is down-regulated to a lesser extent while both cell lines re-express DAXX after 24 days. Therefore, all experiments were performed within the first 10 days after infection. Of note, the knock down efficiency was higher with vector SH-DAXX-1 than SH-DAXX-2; consistently we observed a stronger phenotype on the cells transduced with the first vector.

Interestingly, we observed that in QGP-1 cells DAXX knock down induced an increased number of micronuclei formation and anaphase bridges while no micro-nuclei formation was observed in BON-1 cells. DAXX knock down QGP-1 cells showed an increased number of micronuclei formation compared to scramble (Figure 3 B-D). This result suggests an increased genomic instability induced by DAXX loss in QGP-1 cells.

DAXX knock down in both QGP-1 and BON-1 does not induce ALT activation as detected by telomere FISH *in vitro* (data not shown), similarly to what has been shown by others in ATRX knock down cells (Napier, et al. 2015).

DAXX knock down reduced cell viability, measured by MTT assay (Figure 3-E and supplementary material Figure 5-B), indicating that DAXX has not a classical tumor suppressor gene function. No significant increase in apoptosis was observed in DAXX knock down samples compared to the scramble control (Supplementary Figure 6). FACS analysis revealed that DAXX knock down induces G1 arrest in both BON-1 cells and QGP-1 (Figure 3-F and Supplementary Figure 7).

Short time DAXX knock down does not affect *LINE1* and global methylation.

Since in human samples we observed that DAXX and or ATRX loss correlates with DNA hypo-methylation we wanted to investigate whether DAXX knock down impairs DNA methylation *in vitro* as well. No difference in *LINE1* methylation as well as global methylation were observed after 10 days of DAXX knock down in both BON-1 and QGP-1 cells (Supplementary Figure 8 and data not shown). Similarly, no difference in MGMT promoter methylation were observed upon DAXX knock down (data not shown).

Discussion

The sequence of the events and the mechanism by which DAXX and or ATRX loss induces ALT and CIN are still unknown. Here we provide evidence, that epigenetic mechanisms could be involved in this process. In detail, we showed by immunohistochemistry and biochemical methods that DAXX and or ATRX negative PanNET are globally hypo-methylated. Hypo-methylation of repeated sequences has been described in several cancers in correlation with progressive increase of the grade of malignancy (Ehrlich 2002). Long interspersed nuclear elements (*LINE1*) are the most abundant mobile DNAs in the human genome and hypo-methylation of these sequences has been shown in different type of cancer including PanNETs

(Choi et al. 2007; Stefanoli et al. 2014). Indeed, we could confirm that *LINE1* is hypo-methylated in PanNET compared to normal pancreas. Here we additionally showed that PanNETs showing CIN have a lower level of *LINE1* methylation than chromosomally stable PanNET. We previously showed that DAXX and or ATRX loss in PanNETs correlates with CIN (Marinoni et al. 2014). We hypothesize that DAXX and ATRX loss in PanNET cells result in a decreased DNA methylation thus promoting CIN. Indeed, we found that DAXX/ATRX negative PanNET show lower level of global methylation assessed by IHC and by a colorimetric assay.

However, we could not find a correlation between DAXX and or ATRX loss, ALT activation and *LINE1* methylation. LINE1 methylation could therefore be mediated by other unknown events. On the other hand, a multi-step model in which DAXX and ATRX loss progressively impairs DNA methylation and chromatin structure would also explain this discrepancy. Indeed, our cell line results could point into this direction: Short time DAXX knock down did not induce any changes in global or *LINE1* DNA methylation. Either DAXX knock down is not sufficient to induce global hypo-methylation without other events, or it is instead a matter of time and additional cell cycles are needed.

Impairment of DNA methylation, particularly at telomeres has been described in glioblastoma with ALT activation as well as in Astrocytoma with low ATRX expression (Cai, et al. 2015; Sturm, et al. 2012). Recently it has been shown that DAXX deficient PanNET showed higher methylation variation compared to ATRX negative ones (Pipinikas et al. 2015). However this report did not focus on global methylation levels, which could largely be influenced by non-coding sequences, such as *LINE1* and telomeric regions.

A reduction of methylation in the subtelomeric regions, allowing DNA recombination, might also promote ALT activation (Gonzalo et al. 2006). Upon DAXX knock-down in PanNET cell lines we did not observed ALT activation, in keeping with other finding showing that DAXX and

ATRX loss does not induce ALT activation *in vitro* in telomerase positive cells (Napier et al. 2015). In QGP-1 cells we observed a higher number of micronuclei upon DAXX knock down compared to control which might be a sign of increased genomic instability already at this short time-point and without ALT phenotype and impairment in DNA methylation. However, the small percentage, of cells showing micro nuclei formation upon DAXX knock out might not be sufficient to detect DNA methylation changes when this is assessed in the whole cell population.

The phenotype of DAXX knock down cells has confirmed the non-conventional tumor suppressor role. DAXX silencing *in vitro* in BON-1 and QGP-1 cells induced G1/G0 cell cycle arrest. Similarly, ATRX knock down in other tumor models reduced cell proliferation and induced genomic instability (Cai et al. 2015; Huh, et al. 2016; Huh, et al. 2012; Lovejoy, et al. 2012). It is important to point out that BON-1 and QGP-1 cells are mainly a model for G3 PanNECs with mutations in genes, which are usually not altered in G1-G2 PanNETs (i.e. *TP53*) (Vandamme, et al. 2015). In this context DAXX knock down may impact on the cell cycle regulation and genomic instability differently than in the slow progressive tumors with a different mutational spectrum. Additionally the difference in the mutations background between QGP-1 and BON-1 may explain the different results in the micronuclei formation. Recently it has been shown that Daxx knock-down in a rat insulinoma cell line increased cell proliferation, suggesting that this model might be more appropriate for Daxx functional studies (Feng, et al. 2016). However rodent cells usually show minor and different genomic instability pattern compared to human cells and they rarely activate ALT mechanism for telomeres lengthening, even in absence of telomerase (Hermsen, et al. 2015) (Argilla, et al. 2004). This suggests that even this model harbours some limitation in the study of DAXX loss impact on genomic instability, ALT activation and DNA methylation which was the main focus of our work.

Our finding very likely only show a part of a more complex situation as other factors than DAXX and ATRX mutation can influence the epigenetic status. DAXX and or ATRX loss is frequently accompanied by *MEN1* mutations and this possibly contributes to the epigenetic status. MEN1 is mutated in almost 44% of sporadic PanNETs (Jiao et al. 2011). *MEN1* gene, encodes the transcription factor menin, which recruits the H3K4me3 histone methyltransferase mixed lineage leukaemia (MLL1) complex that plays an essential role in chromatin remodelling and gene expression (Agarwal, et al. 1999; Agarwal and Jothi 2012; Yang, et al. 2013). However MEN1 mutations in PanNETs are not associated with CIN nor with ALT phenotype. In agreement with a model of epigenetically induced CIN, our results showed no correlation between LINE1 methylation level and MEN1 mutation status. Similarly we did not detect any correlation between global methylation assessed by IHC and MEN1 mutations (data not shown).

In conclusion our data provide evidence that DAXX and ATRX loss impact on global DNA methylation of PanNETs cells, which seem to be involved in chromosomal instability. This could enable PanNET to acquire clonal heterogeneity leading to a selection of more aggressive clones.

Declaration of interest

The authors disclose no potential conflicts of interested.

Author contributions

Ilaria Marinoni: experimental design, supervision and execution of *in vitro* and ex-vivo experiments, manuscript writing.

Astrid Wiederkeher: execution of the *in vitro* experiments

Tabea Wiedmer: execution of the FACS analysis and critical reading of the manuscript

Sophia Pantasis: *LINE1* pyrosequencing.

Rasmus Frank: LINE1 pyrosequencing and MGMT methylation.

Annunziata Di Domenico: DNA extraction and samples collection.

Erik Vassella: MEN1 sequencing and MGMT methylation analysis.

Anja Schmitt: Acquisition of clinical data and critical reading of the manuscript

Aurel Perren: experimental design, data acquisition of ex-vivo experiments, morphological analysis and manuscript writing.

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Figure legends:

Figure 1: Global methylation in PanNETs. A) Global methylation levels of DNA isolated from human PanNETs using EpiSeeker methylated DNA Quantification Kit (Abcam). Highly methylated DNA and unmethylated DNA were used as controls. DAXX/ATRX-/ALT+/CIN+ tumors showed hypo-methylation compared to DAXX/ATRX+/ALT-/CIN- ones ($p<0.01$**). B-G) IHC with anti-5-methylcytidine antibody (Millipore) on human tissues. (B-C) Normal pancreas: (*) pancreatic islets and (**) exocrine pancreas. D-E two PanNETs samples showing low 5-methlycytidine expression and F-G two PanNET samples showing high 5-methylcytidine expression.

Figure 2: ***LINE-1* methylation in PanNETs.** A) PanNET samples showed a significant lower level of methylation compared to normal surrounding tissues ($p<0.001$*). B) PanNETs with high CIN showed a lower level of *LINE1* methylation ($p<0.05$) compared to tumors with no CIN. C-D) DAXX/ATRX- tumors and ALT+ tumors do not show difference in *LINE1* methylation compared to respectively DAXX/ATRX+ and ALT- tumors. E) Tumors which are both DAXX/ATRX- and ALT+ did not show difference in LINE1 methylation level compared to tumors, which are DAXX/ATRX+ and ALT-, F) MEN1 mutated and wild type PanNET do not show differences in LINE1 methylation levels.

Figure 3: DAXX knock-down in QGP-1 cell lines. A**)** Western blotting showing DAXX knock-down after 9, 17 and 24 days upon infection with two different plasmids SH-DAXX-1 and SH-DAXX-2 in QGP-1. Parental (Par.) cells and cells transduced with scramble vector (Scr.) were used as controls. DAXX expression comes back to normal level after 24 days from the infection. Quantification of the knock-down after 9 and 17 days from infection was based on three different experiments. B**)** DAPI staining of QGP-1 cells after DAXX knock-down; arrows indicate an anaphase bridge and micronuclei. C) Fold changes of the number of micronuclei (MN) per cells compare to scramble. D) Number of anaphase bridges compare to mitosis. SH-DAXX-1 and SH-DAXX-2 cells showed an increased on micronuclei and anaphase bridges compared to controls. E) Graphic representation of MTT viability assays after 8 days and after 16 days from infection. DAXX knock-down impairs cell viability. After 16 days when DAXX expression is restored to normal levels, the cells proliferate as the controls, suggesting a direct effect of DAXX on cell viability. F) FACS analysis of QGP1 cells after DAXX knock-down. Cells knocked-down for DAXX showed an increased in the percentage of cells in G1/G0 compared controls. The results are based on at least three repetitions.

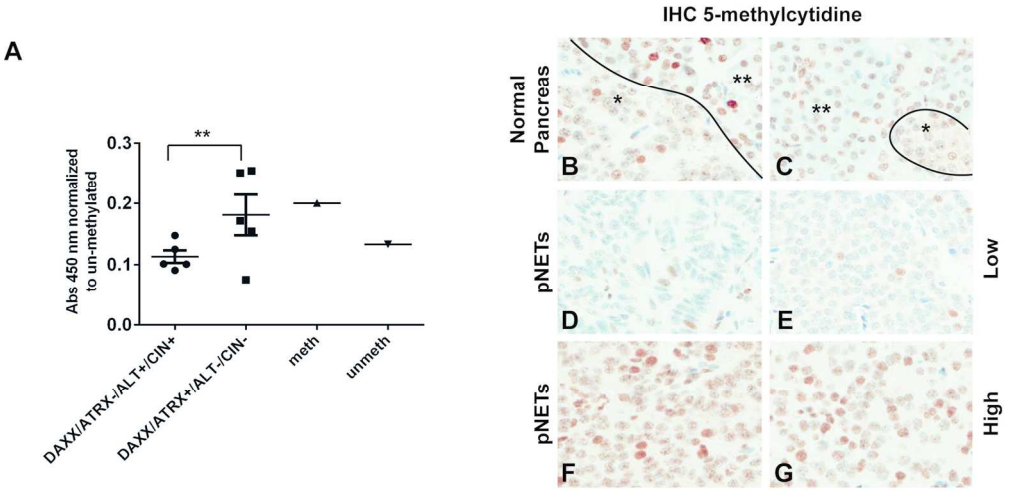

Figure 1

192x97mm (300 x 300 DPI)

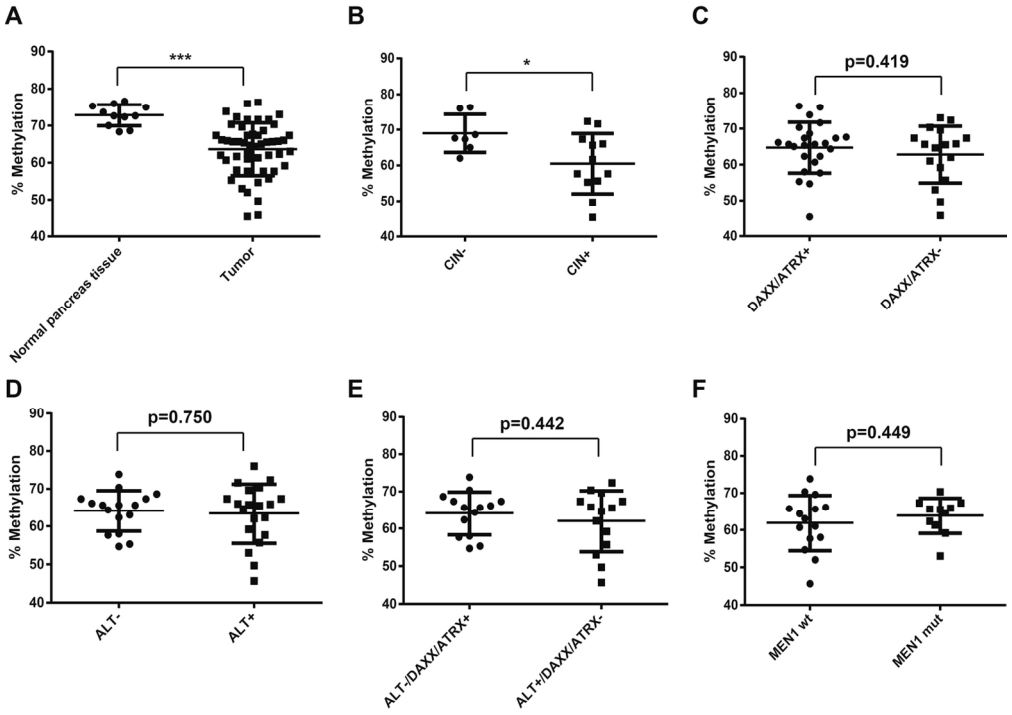

Figure 2

143x101mm (300 x 300 DPI)

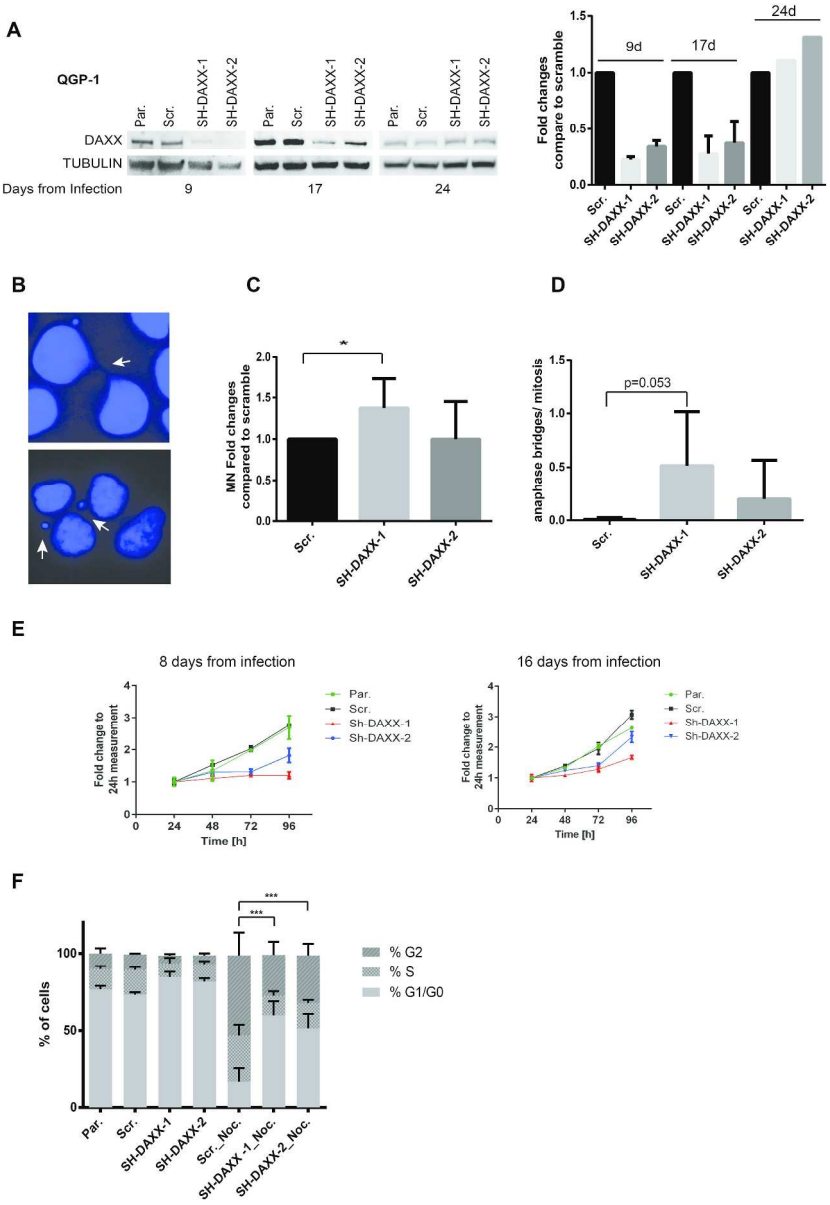

Figure 3

287x417mm (300 x 300 DPI)

Table 1: Characteristics of the human sample collective

Collective	Total patients (IHC plus *LINE1* and Global methylation)	IHC	*LINE1* and global methylation
Tot	**167**	153	55
Female	82	75	26
Male	83	76	28
	2	2	1
Age average	56,66	56,09	55,21
G1	119	107	33
G2	48	46	22
T1	61	53	13
T2	46	44	18
T3-4	45	41	23
n.a.	15	15	1
N0	58	54	15
N1	39	36	24
n.a.	70	63	16
M0	95	85	34
M1	30	30	12
n.a.	42	38	9
DAXX/ATRX +	74	67	13
DAXX/ATRX-	47	44	30
n.a.	46	42	12
DAXX-	23	23	3
DAXX+	106	97	38
n.a.	38	33	14
ATRX-	28	25	13
ATRX+	95	88	33
n.a.	44	40	9
ALT-	59	53	16
ALT+	39	37	20
n.a.	69	63	19
CIN-	22	15	11
CIN+	30	26	11
n.a.	115	112	33
***MEN1* wt**	15	15	15
***MEN1* mut**	11	11	11
n.a.	141	127	29
MGMT hyper			8
MGMT hypo			17
			30
RFS (months)			52 (data based on 44 patients)
TSS (months)			80 (data based on 44 patients)

Table legend: G (Grade), T (Tumor Stage), N (lymph node metastasis), M (Distant Metastasis) ALT (Alternative Lengthening Telomeres), CIN (Chromosomal Instability), RFS (Relapse free Survival), TSS (Tumor Specific Survival).

Table 2: Correlation between DAXX/ATRX expression and 5-methylcytidine staining in human PanNET tissues.

	Low 5-metyl-cytidine	High 5-metyl-cytidine	p value
DAXX/ATRX-	28	15	0.019*
DAXX/ATRX+	28	39	
DAXX-	17	6	0.020*
DAXX+	44	52	
ATRX-	15	13	0.517
ATRX+	40	48	
ALT-	25	28	0.338
ALT+	21	15	