Title:

"Hypo-methylation mediates Chromosomal Instability in pancreatic NET"

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Short title:

Methylation and genomic instability in PanNET

Keywords: Pancreatic Neuro-Endocrine Tumors, DNA methylation, Chromosomal Instability, DAXX and ATRX.

Word count: 5720

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.

1 **Introduction**

2 The molecular pathways and mechanisms underlying initiation and progression of pancreatic

3 neuroendocrine tumors (PanNET) are still poorly understood. Mutations in *DAXX* (Death

4 Domain Associated Protein) and *ATRX* (ATR-X) with correspondent loss of protein expression in

5 the tumor tissue occur in 40% of PanNETs (Jiao, et al. 2011). DAXX and or ATRX loss

6 correlates with Chromosomal Instability (CIN) and predicts for relapse in low stage patients

7 (Stage I to III in the absence of distant metastasis) (Marinoni, et al. 2014). DAXX and or ATRX

8 negative tumors show Alternative Lengthening of Telomeres (ALT), a telomerase independent

9 mechanism for telomere length maintenance, based on homologous recombination (Heaphy, et al.

10 2011). The mechanisms and the sequence of events linking *DAXX/ATRX* mutation, ALT and CIN

11 are unknown.

12 In addition to mutations, the importance of epigenetic changes is increasingly recognized for

13 many cancer types (reviewed in (Das and Singal 2004). DAXX and ATRX participate in

14 maintaining the epigenetic status of the cells by regulating both DNA methylation and H3.3

15 deposition at telomeric and peri-centromeric regions. DAXX interacts and recruits DNA methyl-

16 transferase enzyme 1 (DNMT1) to specific promoters including *RASSF1* and *RELB* (Puto and

17 Reed 2008). ATRX contains the ADD domain as does DNMT3-DNMT3L, important for

18 establishing and maintaining DNA methylation pattern (Noh, et al. 2016).

19 Impairment of DNA methylation in PanNETs has been shown in several genes (*VHL, cdkn2a*

20 (p16), *RASSF1, DAPK1, TIMP3, PAX5, HIC1, CADM1, PYCARD, ESR1, VHL, RARB, WT1 and*

21 *MGMT*). (House, et al. 2003; Malpeli, et al. 2011; Schmitt, et al. 2014; Schmitt, et al. 2009;

22 Stefanoli, et al. 2014). Genome wide methylation profiling of PanNET has shown a difference of

23 methylation pattern between DAXX and ATRX negative tumors, suggesting that mainly DAXX

24 loss is driving DNA methylation changes in PanNETs (Pipinikas, et al. 2015).

25   DNA methylation not only regulates gene expression, but also genomic stability. Indeed hetero-

26   chromatin and silenced regions are usually hyper-methylated resulting in a highly condensed

27   chromatin structure not accessible to the transcription machinery. Hypo-methylated DNA regions

28   are usually highly transcribed. Repetitive and mobile elements such *LINE1* (Long Interspersed

29   Element-1) and *ALU* (Arthrobacter luteus) sequences are preserved from transcription and

30   activation by DNA hyper-methylation. Once activated, these sequences move within the genome

31   and thereby generate chromosomal instability. Hypo-methylation of *LINE1* and *ALU* sequences

32   occurs in several tumors including PanNETs (Choi, et al. 2007; Stefanoli et al. 2014). It is

33   currently unknown whether *LINE1* and *ALU* hypo-methylation is directly linked to *DAXX* and or

34   *ATRX* mutation. Regulation of transcription of the telomeric sequences TERRA (Telomeric

35   repeat containing RNA) and telomere stability is also dependent on the DNA methylation status.

36   Dnmt1 deficient mouse embryonic stem cells show elongated telomeres, increased recombination

37   at telomeric regions and ALT activation (Gonzalo, et al. 2006).

38   In the present study we aimed to analyse the effect of DAXX and or ATRX loss on global

39   methylation in ex-vivo human PanNETs samples and *in vitro* in BON-1 and QGP-1 cell line-

40   models. *In vitro* we also assessed genomic instability induction upon DAXX knock down. We

41   found that DAXX and or ATRX negative PanNETs showed global hypo-methylation but not

42   LINE1 hypo-methylation compare to positive tumors. Additionally we were able to find a link

43   between LINE1 hypo-methylation and CIN in PanNETs. DAXX knock down does not induce

44   methylation changes in BON-1 and QGP-1 cells.

45

46   **Material and Methods**

47   **Human samples**

48   167 out of 207 resected well differentiated PanNETs (G1-G2) and 11 matching control were used

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

50   CIN data obtained by Comparative Genomic Hybridization (CGH) were previously reported

51   (Marinoni et al. 2014).

52   CIN were defined as showing a total number of gains and losses of 8 or more in conventional

53   CGH and of 20 or more in array CGH. The use of patient material was approved by the local

54   ethics committees (Number 105/2015). The composition of samples is described in table 1 and in

55   the supplementary figure 1.

56   **Cell line culture conditions and treatments**

57   The pancreatic neuroendocrine cell line BON-1 (pancreatic carcinoid) was provided by E.J.

58   Speel, Department of Pathology, Maastricht, The Netherlands and metastatic somatostatinoma

59   derived QGP-1 cell line was obtained from Japanese Health Sciences Foundation, Osaka, Japan.

60   Both cell lines authentication was performed by STRs analysis (Mycrosynth, Balgach,

61   Switzerland) upon arrival (2011) and in the present year. QGP-1 cells resulted 100% matching

62   with the QGP-1 profile (GNE586559, Genentech, Roche, Basel, Switzerland). BON-1 cells are

63   not commercially available therefore no comparison is possible, however STRs analysis of these

64   cells revealed no matching with any data available in the ATCC database, excluding any

65   contamination with other cell lines. Additionally the expression of the neuro-endocrine markers

66   Synaptophysin and Chromogranin-A was checked yearly. Both QGP-1 and BON-1 cells express

67   the two markers. Cells were kept in culture for a maximum of 20-25 passages.

68   Both cell lines were cultured at 37°C under 5% CO_2. BON-1 were cultured in DMEM/Nutrient

69   Mixture F-12 Ham (D6421, Sigma-Aldrich, Buchs SG, Switzerland) and QGP-1 in RPMI 1640

70   Dutch modification (R7638, Sigma-Aldrich), both media supplemented with 10% FBS (GIBCO,

71   Thermo Fisher Scientific, Paisley, UK), 2nM L-alanyl-L-glutamin (Sigma-Aldrich), 100μg/ml

72   streptomycin and 100units/ml of penicillin (Sigma-Aldrich).

73

74   **Immuno-histo-chemistry (IHC)**

75   Four-micrometer sections were taken from a TMA including 207 pNETs derived from patients

76   who underwent surgery at the Inselspital Bern, Switzerland previously described (Marinoni et al.

77   2014) and stained with an anti-5-methylcytidine antibody BI_MECY_0100 (Eurogentec, Kaneka,

78   Liege, Belgium). The Immunohistochemical staining was performed on an automated staining

79   system (Leica Bond III; Leica Biosystems, Nunningen, Switzerland). Antigen retrieval was

80   performed by heating citrate buffer at 100° for 30 minutes. The primary antibody was incubated

81   for 30 minutes at a dilution of 1:200. Visualization was performed using the avidin-biotin

82   complex method, which yielded a brown staining signal. Normal pancreatic islets show strong

83   positive staining indicating a certain level of DNA methylation thus we scored as high-

84   methylated samples showing similar or stronger staining than normal islets and low-methylated

85   samples showing weaker staining. To exclude false-negative samples, only samples with positive

86   nuclear staining of non-neoplastic cells and negative tumor nuclei were scored as negative (153

87   of 207 samples remaining informative). 54 Samples with both negative tumor nuclei and non-

88   neoplastic stromal and endothelial cells were scored as non-informative and excluded from

89   further analysis.

90

91   **DNA extraction, bisulfite conversion and global methylation analysis**

92   DNA was extracted from cell pellet of about $6\mathrm{x}10^5$ cells or from paraffin embedded human

93   PanNETs (55 tumor samples with more than 80% tumor content and 11 matching normal

94   pancreatic tissues, Table 1 and supplementary figure 1) using Nucleo Spin kit by Macherey-

95   Nagel (Düren, Germany) according to the manufacturer's instructions. For bisulfite conversion of

96   gDNA the EZ DNA Methylation-Gold kit by Zymo Research Corporation (Freiburg, Germany)

97   was used following the manufacturer's instructions.

98   Genomic DNA of 10 tumor samples was analyzed by its global methylation status using

99   EpiSeeker methylated DNA Quantification Kit (ab117128, Abcam, Cambridge, United

100 Kingdom), according to the supplier's instructions. Colorimetric measurements were done on an

101 ELISA reader (Tecan, Männerdorf, Switzerland). Hyper-methylated and hypo-methylated DNAs

102 (Zymo Research) were used as controls.

103 PCR and pyrosequencing investigation for *LINE-1* for 51 samples (GenBank accession number

104 X58075) methylation analysis was performed using PyroMark kit (Qiagen, Hilden, Germany). To

105 analyse sequence methylation of *LINE-1*, the PyroMark Q24 CpG LINE-1 (4x24) Methylation

106 detection assay was used (Qiagen). Amplifications were performed with Master Mix PyroMark

107 PCR Kit (Qiagen) according to supplier's instructions on a Veriti System gradient apparatus

108 (Applied Biosystems, Thermo Fisher Scientific). PCR product was bound to Streptavidin

109 Sepharose HP (Amersham Biosciences, GE Healthcare, Little Chalfont, United Kingdom),

110 purified, washed, denatured and washed again with the PyroMark Q24 Vacuum Workstation

111 220V (Qiagen). Pyrosequencing was performed using the Qiagen PyroMark Q24 System with the

112 pyromark Q24 Cartridge Method 0011 (Qiagen). The assay setups and analysis were performed

113 with PyroMark Assay Design Software V.1.0.6 (Qiagen).

114 MGMT (O⁻6-methylguanine-DNA-methyltransferase) promoter methylation was assessed in 25

115 tumor samples as previously described (Vassella, et al. 2011). Samples with methylation level

116 higher and equal to 5% were considered hyper-methylated and samples with methylation level

117 <5% were considered hypo-methylated.

118   The MEN1 gene was analysed in 26 tumor samples by semi-conductive sequencing using an Ion

119   Torrent PGM (Life Technologies). Protein coding exons were amplified by multiplex polymerase

120   chain reaction using 2 primer pools designed by the Ion AmpliSeq Designer (Life Technologies).

121   Library construction, emulsion polymerase chain reaction, and sequencing were performed

122   according to the manufacturer's recommendations. The Torrent Suite 5.0.3 platform was used for

123   sequence alignment with the hg19 human genome reference. Variant calling was performed with

124   the variant caller 5.0.3.5. and the IonReporter 5.0 software (Life Technologies, Grand Island,

125   NY). The average base coverage depth for most samples was more than 2000 reads. MEN1

126   mutations found are summarized in table 1 in the Supplementary Material.

127   **DAXX knock down**

128   For virus infection $6x10^5$ cells were seeded in 6 wells plate the day before. pLKO.1 lentiviral

129   vectors expressing small hairpin (sh) RNAs targeting DAXX (Sh-DAXX_H2410: and Sh-

130   DAXX_2503) or a nontargeting shRNA control (SHCOO2) were purchased from Sigma-Aldrich

131   Lentiviral production and transduction were done as described in (Tschan, et al. 2003).

132   Transduced cells were selected by puromycin treatment (1.5µg/ml, Invitrogen). For micro-nuclei

133   counting, cells were seeded in 24-well plates on coverslips coated with FBS, $1x10^4$ cells per well.

134   Cells were fixed with 4% Paraformaldehyde for 30 min at room temperature. DAPI was used to

135   stain nuclei. Micronuclei were counted under a fluorescent microscope (Axiophot2, Zeiss).

136   **Viability and apoptosis assays**

137   For MTT assay cells were seeded into 96-well plates at the density of 16'000 cells per well. After

138   24h medium was replaced by 100ul of medium with 10% MTT (0.5mg/ml final concentration)

139   (Sigma-Aldrich) and incubate 1h at 37°. After incubation the medium was removed and 200 µl of

140   DMSO (Sigma-Aldrich) and 25 µl of Sorensen solution (0.1M Glycine, 0.1M NaCl, pH 10.5 in

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

142   (Tecan).

143   **Cell cycle analysis with FACS**

144   Briefly, cells were seeded $5x10^5$ cells in duplicates or triplicates in 6-well plates. The day after

145   cells were treated with Nocodazol. After 16h of treatment cells were fixed in ice cold EtOH

146   overnight. PI staining was performed directly before measurement on BD-FACS LSR SorpII

147   using BD FACSDiva 6.1 software (BD Bioscience, Heidelberg, Germany). Results were

148   analyzed using FlowJo v9.8 software (TreeStar, USA).

149   **Protein extraction and Western blot analysis**

150   For western blotting Cells were cultivated in 6-well plates and lysate in Urea buffer. Forty ng

151   proteins were loaded on 4-20% Mini-PROTEAN® TGX Stain-Free™ Gel (Bio-Rad, Cressier,

152   Switzerland). Transfer was performed using transfer turbo blot system (Bio-Rad). Primary

153   monoclonal antibodies rabbit anti DAXX (clone 25C12, 1:1000 in 5% BSA, Cell signaling,

154   Cambridge, United Kingdom) and mouse anti human α-tubulin (clone B-5-1-2, 1:1000 in 5%

155   BSA, Sigma), GAPDH (clone 6c5, 1:1000 in 5% skimmed milk, Merk Millpore, Darmstadt,

156   Germany) were used. As secondary antibodies goat anti-rabbit DyLight® 650 conjugate

157   (LabForce, Muttenz, Switzerland) and goat anti-mouse DyLight® 550 conjugate (LabForce) both

158   diluted 1:500 in 5% milk were used. Signal was detected with ChemiDoc MP System (Bio-Rad)

159   and analyzed in ImageJ (freeware, 1.48v, Bethesda, USA). Protein levels were normalized to α-

160   tubulin, GAPDH or total protein.

161   **Statistics**

162   Statistical analysis was performed using GraphPAD prism 5 software (La Jolla, CA, USA).

163   Statistical differences were calculated using unpaired two-tailed Student's t test. The χ^2 test or the

164   Fisher exact test was used to calculate contingency tables. *P* values less than .05 were considered

165   statistically significant.

166

167   **Results**

168   **Ex-vivo analysis of human PanNET samples**

169   **DAXX and or ATRX negative PanNET are hypo methylated**

170   In order to first assess whether DAXX/ATRX loss had an impact on the DNA methylation

171   pattern we evaluated global DNA methylation on 10 PanNET samples. DNA extracted from 5

172   PanNET samples positive for DAXX/ATRX IHC staining, negative for ALT activation and CIN

173   negative (DAXX/ATRX+/ALT-/CIN-) and from 5 PanNET samples negative for DAXX/ATRX

174   staining, positive for ALT activation and CIN positive (DAXX/ATRX-/ALT+/CIN+) were

175   analyzed for global DNA methylation with the colorimetric EpiSeeker assay (Methylated DNA

176   Quantification Kit, Abcam). As shown in figure 1-A DAXX/ATRX-/ALT+/CIN+ tumors exhibit

177   a significantly lower level of DNA methylation compared to DAXX/ATRX+/ALT-/CIN- ones

178   (p<0.01**), which is comparable to the un-methylated control. To confirm our findings in a

179   larger setting and with an additional method, we stained 2 TMAs, composed as described in the

180   material and methods section and in (Marinoni et al. 2014) with an anti-5-methylcytidine

181   antibody (Eurogentec) that specifically recognizes the methylated base and does not cross-react

182   with the un-methylated one. We obtained results on 153 samples; the characteristic of these

183   samples is reported in table 1.

184   We observed that strong 5-methylcytidine staining associates with DAXX and ATRX proficient

185   tumors while weak signal correlates with DAXX/ATRX deficient ones $p<0.05$* (Figure 1 B-G

186   and Table 2), suggesting that DAXX/ATRX deficient tumor show global hypo-methylation

187   compared to the positive ones. Normal islets showed a strong 5-methylcytidine staining (Figure 1

188   B-C).

189   To examine the DNA methylation level of non-coding regions we evaluated the (Long

190   Interspersed Element) *LINE1* methylation status as a marker of global DNA methylation in 55

191   PanNET and 11 non-neoplastic pancreatic specimens, by pyro-sequencing (Qiagen). We

192   observed that *LINE1* is hypo-methylated in PanNETs compared to normal pancreas ($p<0.001$***)

193   (Figure 2-A) as previously reported (Choi et al. 2007; Stefanoli et al. 2014). *LINE1* hypo-

194   methylation indeed associates with CIN ($p<0.05$*) (Figure 2-B), as we hypothesized. However,

195   no differences in LINE1 methylation were observed in either DAXX/ATRX positive and

196   negative tumors, ALT positive and negative (Figure 2 C-E). In order to assess if MEN1

197   mutations impact on LINE1 methylation levels we sequenced MEN1 gene in 26 samples. Eleven

198   samples resulted to be mutated. The mutations found are summarized in Supplementary Material

199   Table 1. No difference in LINE1 methylation status were observed between mutant and wild type

200   tumors (Figure 2 F).   Interestingly, *LINE1* hypo-methylation predicts for shorter disease free

201   survival ($p<0.05$*), (figure 3 supplementary material) in agreement with the results reported by

202   Stefanoli et al. (Stefanoli et al. 2014); while no differences in survival are detectable in tumors

203   positive and negative for 5-methylcytidine staining (data not shown).

204   We performed quantitative methylation specific PCR to assess whether LINE1 hypo-methylation

205   or DAXX/ATRX loss correlate with hyper-methylation of MGMT promoter, due to its clinical

206   relevance. We found 8 samples with MGMT promoter hyper-methylated and 17 samples where

207   MGMT promoter was un-methylated. No correlation between LINE1 hypo-methylation or

208   DAXX/ATRX loss and MGMT methylation level was found (Supplementary Figure 4),

209   suggesting two independent mechanisms for the two events, as previously proposed (Stefanoli et

210   al. 2014).

211

212   **In vitro upon DAXX knock-down**

213   **Induction of genomic instability and G1 arrest**

214   In order to assess if DAXX loss is sufficient to induce genomic instability, we knocked down

215   DAXX in two PanNET cell lines, QGP-1 and BON-1 by Lentiviral transduction of SH-RNAs.

216   DAXX knock down was evaluated by western blotting 9, 17 and 24 days after lentiviral

217   infection. Two different DAXX specific vectors (1 and 2) and scramble control were used in both

218   cell lines. DAXX expression is highly reduced after 9 days from infections in both cell lines

219   (Figure 3-A and supplementary material Figure 5-A). After 17 days DAXX expression is down-

220   regulated to a lesser extent while both cell lines re-express DAXX after 24 days. Therefore, all

221   experiments were performed within the first 10 days after infection. Of note, the knock down

222   efficiency was higher with vector SH-DAXX-1 than SH-DAXX-2; consistently we observed a

223   stronger phenotype on the cells transduced with the first vector.

224   Interestingly, we observed that in QGP-1 cells DAXX knock down induced an increased number

225   of micronuclei formation and anaphase bridges while no micro-nuclei formation was observed in

226   BON-1 cells. DAXX knock down QGP-1 cells showed an increased number of micronuclei

227   formation compared to scramble (Figure 3 B-D). This result suggests an increased genomic

228   instability induced by DAXX loss in QGP-1 cells.

229   DAXX knock down in both QGP-1 and BON-1 does not induce ALT activation as detected by

230   telomere FISH *in vitro* (data not shown), similarly to what has been shown by others in ATRX

231   knock down cells (Napier, et al. 2015).

12

232   DAXX knock down reduced cell viability, measured by MTT assay (Figure 3-E and

233   supplementary material Figure 5-B), indicating that DAXX has not a classical tumor suppressor

234   gene function.   No significant increase in apoptosis was observed in DAXX knock down

235   samples compared to the scramble control (Supplementary Figure 6). FACS analysis revealed

236   that DAXX knock down induces G1 arrest in both BON-1 cells and QGP-1 (Figure 3-F and

237   Supplementary Figure 7).

238

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

240   Since in human samples we observed that DAXX and or ATRX loss correlates with DNA hypo-

241   methylation we wanted to investigate whether DAXX knock down impairs DNA methylation *in*

242   *vitro* as well. No difference in *LINE1* methylation as well as global methylation were observed

243   after 10 days of DAXX knock down in both BON-1 and QGP-1 cells (Supplementary Figure 8

244   and data not shown). Similarly, no difference in MGMT promoter methylation were observed

245   upon DAXX knock down (data not shown).

246

247   **Discussion**

248   The sequence of the events and the mechanism by which DAXX and or ATRX loss induces ALT

249   and CIN are still unknown. Here we provide evidence, that epigenetic mechanisms could be

250   involved in this process. In detail, we showed by immunohistochemistry and biochemical

251   methods that DAXX and or ATRX negative PanNET are globally hypo-methylated. Hypo-

252   methylation of repeated sequences has been described in several cancers in correlation with

253   progressive increase of the grade of malignancy (Ehrlich 2002). Long interspersed nuclear

254   elements (*LINE1*) are the most abundant mobile DNAs in the human genome and hypo-

255   methylation of these sequences has been shown in different type of cancer including PanNETs

256 (Choi et al. 2007; Stefanoli et al. 2014). Indeed, we could confirm that *LINE1* is hypo-methylated

257 in PanNET compared to normal pancreas. Here we additionally showed that PanNETs showing

258 CIN have a lower level of *LINE1* methylation than chromosomally stable PanNET. We

259 previously showed that DAXX and or ATRX loss in PanNETs correlates with CIN (Marinoni et

260 al. 2014). We hypothesize that DAXX and ATRX loss in PanNET cells result in a decreased

261 DNA methylation thus promoting CIN. Indeed, we found that DAXX/ATRX negative PanNET

262 show lower level of global methylation assessed by IHC and by a colorimetric assay.

263 However, we could not find a correlation between DAXX and or ATRX loss, ALT activation and

264 *LINE1* methylation. LINE1 methylation could therefore be mediated by other unknown events.

265 On the other hand, a multi-step model in which DAXX and ATRX loss progressively impairs

266 DNA methylation and chromatin structure would also explain this discrepancy. Indeed, our cell

267 line results could point into this direction: Short time DAXX knock down did not induce any

268 changes in global or *LINE1* DNA methylation. Either DAXX knock down is not sufficient to

269 induce global hypo-methylation without other events, or it is instead a matter of time and

270 additional cell cycles are needed.

271 Impairment of DNA methylation, particularly at telomeres has been described in glioblastoma

272 with ALT activation as well as in Astrocytoma with low ATRX expression (Cai, et al. 2015;

273 Sturm, et al. 2012). Recently it has been shown that DAXX deficient PanNET showed higher

274 methylation variation compared to ATRX negative ones (Pipinikas et al. 2015). However this

275 report did not focus on global methylation levels, which could largely be influenced by non-

276 coding sequences, such as *LINE1* and telomeric regions.

277 A reduction of methylation in the subtelomeric regions, allowing DNA recombination, might also

278 promote ALT activation (Gonzalo et al. 2006). Upon DAXX knock-down in PanNET cell lines

279 we did not observed ALT activation, in keeping with other finding showing that DAXX and

14

280   ATRX loss does not induce ALT activation *in vitro* in telomerase positive cells (Napier et al.

281   2015). In QGP-1 cells we observed a higher number of micronuclei upon DAXX knock down

282   compared to control which might be a sign of increased genomic instability already at this short

283   time-point and without ALT phenotype and impairment in DNA methylation. However, the small

284   percentage, of cells showing micro nuclei formation upon DAXX knock out might not be

285   sufficient to detect DNA methylation changes when this is assessed in the whole cell population.

286   The phenotype of DAXX knock down cells has confirmed the non-conventional tumor

287   suppressor role. DAXX silencing *in vitro* in BON-1 and QGP-1 cells induced G1/G0 cell cycle

288   arrest. Similarly, ATRX knock down in other tumor models reduced cell proliferation and

289   induced genomic instability (Cai et al. 2015; Huh, et al. 2016; Huh, et al. 2012; Lovejoy, et al.

290   2012). It is important to point out that BON-1 and QGP-1 cells are mainly a model for G3

291   PanNECs with mutations in genes, which are usually not altered in G1-G2 PanNETs (i.e. *TP53*)

292   (Vandamme, et al. 2015). In this context DAXX knock down may impact on the cell cycle

293   regulation and genomic instability differently than in the slow progressive tumors with a different

294   mutational spectrum. Additionally the difference in the mutations background between QGP-1

295   and BON-1 may explain the different results in the micronuclei formation. Recently it has been

296   shown that Daxx knock-down in a rat insulinoma cell line increased cell proliferation, suggesting

297   that this model might be more appropriate for Daxx functional studies (Feng, et al. 2016).

298   However rodent cells usually show minor and different genomic instability pattern compared to

299   human cells and they rarely activate ALT mechanism for telomeres lengthening, even in absence

300   of telomerase (Hermsen, et al. 2015) (Argilla, et al. 2004). This suggests that even this model

301   harbours some limitation in the study of DAXX loss impact on genomic instability, ALT

302   activation and DNA methylation which was the main focus of our work.

303   Our finding very likely only show a part of a more complex situation as other factors than DAXX

304   and ATRX mutation can influence the epigenetic status. DAXX and or ATRX loss is frequently

305   accompanied by *MEN1* mutations and this possibly contributes to the epigenetic status. MEN1 is

306   mutated in almost 44% of sporadic PanNETs (Jiao et al. 2011). *MEN1* gene, encodes the

307   transcription factor menin, which recruits the H3K4me3 histone methyltransferase mixed lineage

308   leukaemia (MLL1) complex that plays an essential role in chromatin remodelling and gene

309   expression (Agarwal, et al. 1999; Agarwal and Jothi 2012; Yang, et al. 2013). However MEN1

310   mutations in PanNETs are not associated with CIN nor with ALT phenotype. In agreement with a

311   model of epigenetically induced CIN, our results showed no correlation between LINE1

312   methylation level and MEN1 mutation status. Similarly we did not detect any correlation between

313   global methylation assessed by IHC and MEN1 mutations (data not shown).

314   In conclusion our data provide evidence that DAXX and ATRX loss impact on global DNA

315   methylation of PanNETs cells, which seem to be involved in chromosomal instability. This could

316   enable PanNET to acquire clonal heterogeneity leading to a selection of more aggressive clones.

317

318   **Declaration of interest**

319   The authors disclose no potential conflicts of interested.

320

321   **Author contributions**

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

324   Astrid Wiederkeher: execution of the *in vitro* experiments

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

326   Sophia Pantasis: *LINE1* pyrosequencing.

327   Rasmus Frank: LINE1 pyrosequencing and MGMT methylation.

328   Annunziata Di Domenico: DNA extraction and samples collection.

329   Erik Vassella: MEN1 sequencing and MGMT methylation analysis.

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

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

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343

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421 **Figure legends:**

422

423 **Figure 1: Global methylation in PanNETs. A)** Global methylation levels of DNA isolated from

424 human PanNETs using EpiSeeker methylated DNA Quantification Kit (Abcam). Highly

425 methylated DNA and unmethylated DNA were used as controls. DAXX/ATRX-/ALT+/CIN+

426 tumors showed hypo-methylation compared to DAXX/ATRX+/ALT-/CIN- ones (p<0.01**). B-

427 G) IHC with anti-5-methylcytidine antibody (Millipore) on human tissues. (B-C) Normal

428 pancreas: (*) pancreatic islets and (**) exocrine pancreas. D-E two PanNETs samples showing

429 low 5-methlycytidine expression and F-G two PanNET samples showing high 5-methylcytidine

430 expression.

431

432 **Figure 2**: *LINE-1* **methylation in PanNETs.** A) PanNET samples showed a significant lower

433 level of methylation compared to normal surrounding tissues (p<0.001*). B) PanNETs with high

434 CIN showed a lower level of *LINE1* methylation (p<0.05) compared to tumors with no CIN. C-

435 D) DAXX/ATRX- tumors and ALT+ tumors do not show difference in *LINE1* methylation

436 compared to respectively DAXX/ATRX+ and ALT- tumors. E) Tumors which are both

437 DAXX/ATRX- and ALT+ did not show difference in LINE1 methylation level compared to

438 tumors, which are DAXX/ATRX+ and ALT-, F) MEN1 mutated and wild type PanNET do not

439 show differences in LINE1 methylation levels.

440

441 **Figure 3: DAXX knock-down in QGP-1 cell lines.** A**)** Western blotting showing DAXX knock-

442 down after 9, 17 and 24 days upon infection with two different plasmids SH-DAXX-1 and SH-

443 DAXX-2 in QGP-1. Parental (Par.) cells and cells transduced with scramble vector (Scr.) were

444 used as controls. DAXX expression comes back to normal level after 24 days from the infection.

445 Quantification of the knock-down after 9 and 17 days from infection was based on three different

446 experiments. B**)** DAPI staining of QGP-1 cells after DAXX knock-down; arrows indicate an

447 anaphase bridge and micronuclei. C) Fold changes of the number of micronuclei (MN) per cells

448 compare to scramble. D) Number of anaphase bridges compare to mitosis. SH-DAXX-1 and SH-

449 DAXX-2 cells showed an increased on micronuclei and anaphase bridges compared to controls.

450 E) Graphic representation of MTT viability assays after 8 days and after 16 days from infection.

451 DAXX knock-down impairs cell viability. After 16 days when DAXX expression is restored to

452 normal levels, the cells proliferate as the controls, suggesting a direct effect of DAXX on cell

453 viability. F) FACS analysis of QGP1 cells after DAXX knock-down. Cells knocked-down for

454 DAXX showed an increased in the percentage of cells in G1/G0 compared controls. The results

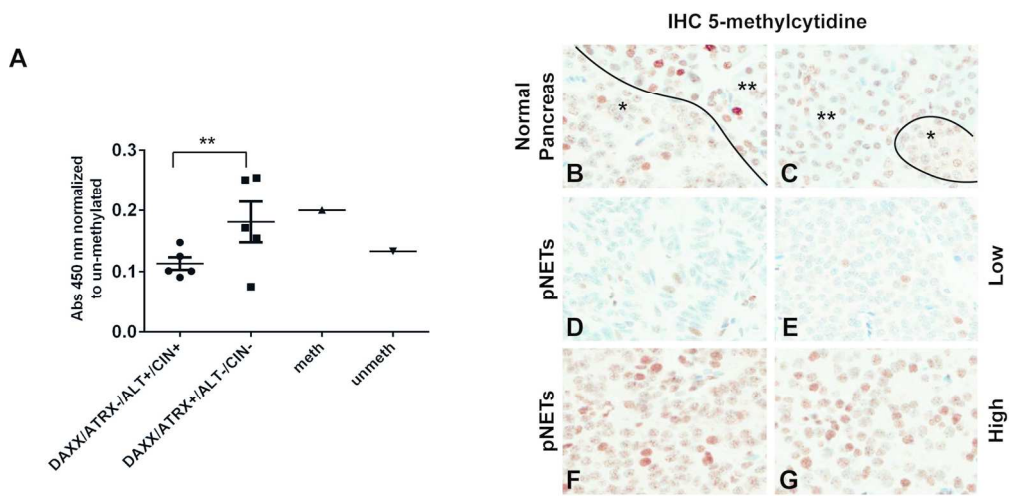455 are based on at least three repetitions.
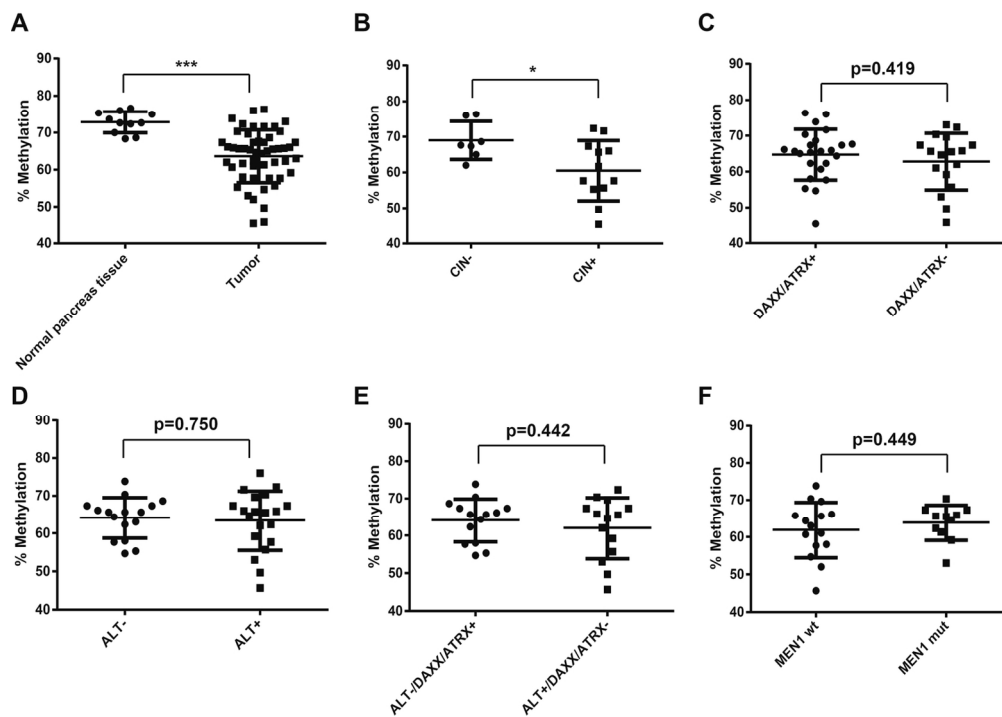
456

Figure 1

192x97mm (300 x 300 DPI)

Figure 2
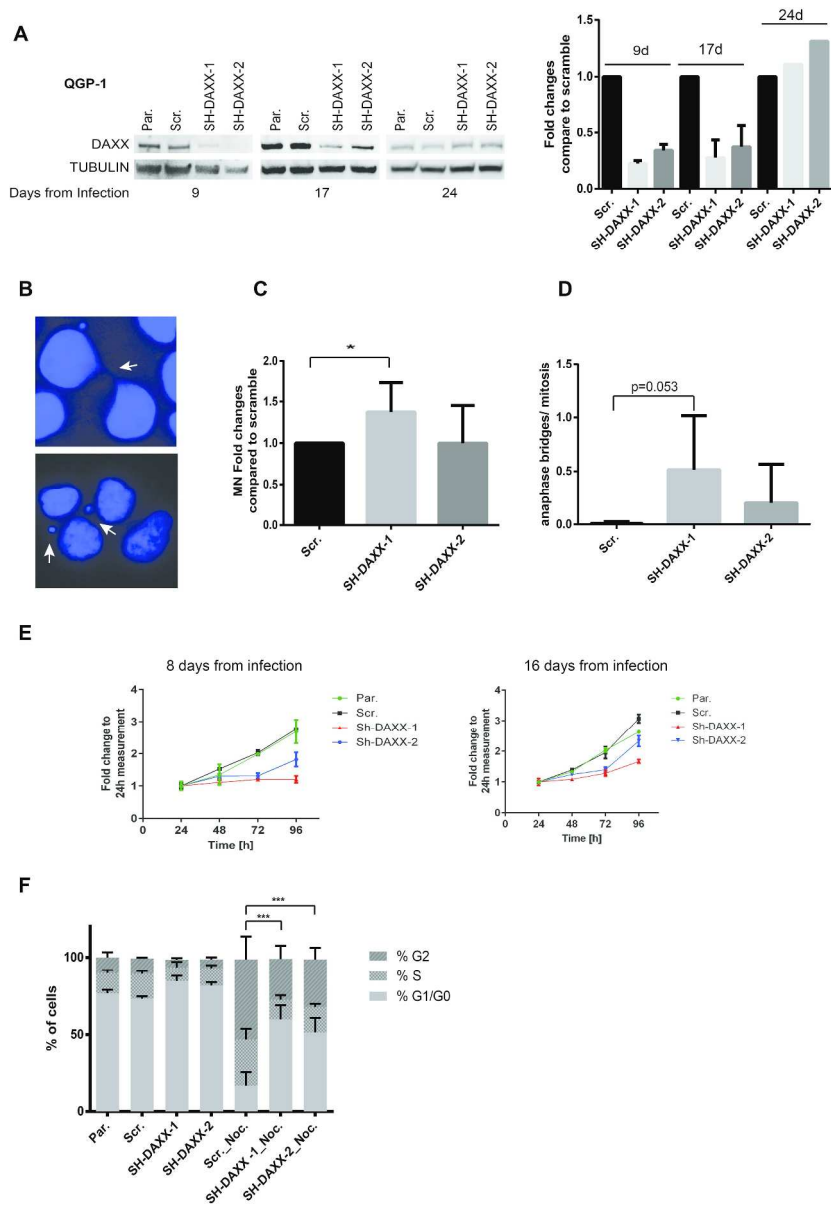
143x101mm (300 x 300 DPI)

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	