

# 1 **Functional Characterisation of the ATOH1 Molecular** 2 **Subtype Indicates a Pro-Metastatic Role in Small** 3 **Cell Lung Cancer**

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29 **ABSTRACT**

30 Molecular subtypes of Small Cell Lung Cancer (SCLC) have been described based  
31 on differential expression of transcription factors (TFs) *ASCL1*, *NEUROD1*, *POU2F3*  
32 and immune-related genes. We previously reported an additional subtype based on  
33 expression of the neurogenic TF *ATOH1* within our SCLC Circulating tumour cell-  
34 Derived eXplant (CDX) model biobank. Here we show that ATOH1 protein was  
35 detected in 7/81 preclinical models and 16/102 clinical samples of SCLC. In CDX  
36 models, ATOH1 directly regulated neurogenesis and differentiation programs  
37 consistent with roles in normal tissues. In *ex vivo* cultures of ATOH1-positive CDX,  
38 ATOH1 was required for cell survival. *In vivo*, ATOH1 depletion slowed tumour  
39 growth and suppressed liver metastasis. Our data validate ATOH1 as a *bona fide*  
40 oncogenic driver of SCLC with tumour cell survival and pro-metastatic functions.  
41 Further investigation to explore ATOH1 driven vulnerabilities for targeted treatment  
42 with predictive biomarkers is warranted.

## 43 INTRODUCTION

44 SCLC is an aggressive neuroendocrine (NE) tumour constituting ~15% of lung  
45 cancers. SCLC is the sixth most common cause of cancer-related deaths,  
46 accounting for ~250,000 diagnoses worldwide each year<sup>1-4</sup>. Most patients with SCLC  
47 present with extensive stage (ES) disease characterised by widespread metastases  
48 and rapidly acquired resistance to initially effective standard-of-care (SoC) platinum-  
49 based chemotherapy<sup>5</sup>. SoC was unchanged for >30 years<sup>6</sup> until the recent addition  
50 of immunotherapy that extends overall survival of a minority of patients, including  
51 rare patients with durable responses<sup>7-10</sup>.

52 SCLC molecular subtypes were recently defined based on expression of master  
53 neurogenic transcription factors (TFs) *ASCL1* (SCLC-A) and *NEUROD1* (SCLC-N)  
54 and a rarer subtype defined by the non-neuroendocrine (Non-NE) Tuft Cell TF  
55 *POU2F3* (SCLC-P)<sup>11,12</sup>. SCLC expressing an immune signature without these TFs  
56 was defined as 'inflamed' (SCLC-I)<sup>13</sup>. Preclinical studies suggest subtype-dependent  
57 therapeutic vulnerabilities<sup>14</sup> heralding potential for stratified therapy in clinical trials,  
58 potentially guided by ctDNA methylation subtyping<sup>15</sup> where serial liquid biopsy could  
59 assess evolving subtype plasticity<sup>16</sup>.

60 Patients with SCLC have prevalent circulating tumour cells (CTCs)<sup>17</sup>, prompting our  
61 establishment of CTC-Derived patient eXplant (CDX) models in immunodeficient  
62 mice to explore SCLC biology and test novel therapeutics<sup>12</sup>. *ASCL1* and/or  
63 *NEUROD1* subtype CDX consist primarily of NE cells with a minority Non-NE  
64 subpopulation<sup>12,18</sup> consistent with the NE to NonNE phenotype switch brought about  
65 by Notch signalling generating intra-tumoral heterogeneity<sup>16,19</sup>. *POU2F3* expressing  
66 CDX13 tumours are exclusively Non-NE<sup>12</sup>. *YAP1*, initially considered a subtype  
67 determinant of SCLC<sup>11</sup>, is expressed in Non-NE cells within *ASCL1* or *NEUROD1*  
68 CDX<sup>18</sup>.

69 We recently described a subset of SCLC CDX lacking expression of *ASCL1* or  
70 *POU2F3*, that instead expressed the neurogenic, basic helix-loop-helix TF *ATOH1*,  
71 which could be co-expressed with *NEUROD1*<sup>12</sup>. *ATOH1* was expressed in 4 CDX  
72 models from 3/31 SCLC patients (9.6%). Two of these CDX were generated from the  
73 same patient pre- and post-treatment and maintained *ATOH1* expression.

74 ATOH1 is homologue of *Drosophila melanogaster Atonal*, first identified in sensory  
75 organs of developing embryos<sup>20</sup>. In mouse models, *Atoh1* (or *Math1*) is critical for  
76 development and differentiation of sensory cell types, including granule cells in the  
77 brain, sensory inner ear hair cells, Merkel cells in the skin, and secretory cells in the  
78 intestine<sup>21-27</sup>. *Atoh1*, like *Ascl1*, engages Notch signalling through lateral inhibition to  
79 avoid aberrant cellular differentiation in brain and intestine<sup>24,28,29</sup>. ATOH1 impact in  
80 cancer is context-dependent, described as a tumour suppressor in colorectal cancer  
81 and an oncogene in medulloblastoma<sup>30,31</sup>. Functional role(s) of ATOH1 in SCLC are  
82 unknown.

83 Here we explore transcriptional programmes and cellular functions(s) regulated by  
84 ATOH1 in SCLC. Although rare in our CDX biobank compared to SCLC-A, we  
85 identified ATOH1 in a subset of patients' tumours and in additional Patient-Derived  
86 eXplants (PDX) models<sup>32</sup>. We show that in SCLC cell lines and/or CDX models,  
87 ATOH1 regulates neurogenesis, maintains cell survival *in vitro* and promotes tumour  
88 growth and liver metastasis *in vivo*. Our study adds to the emerging landscape of  
89 SCLC heterogeneity, highlighting potential for subtype-stratified approaches for  
90 improved treatment outcomes.

## 91 RESULTS

### 92 ATOH1, MYCL and chemosensitivity

93 We suggested ATOH1 as a SCLC subtype determinant after noting its expression in  
94 4/38 CDX models that were distinct upon unsupervised clustering of whole  
95 transcriptomes<sup>12</sup> (Figure 1A-i). Four ATOH1 CDX were derived from three donors:  
96 one sampled prior to chemotherapy (CDX25), one post-chemotherapy (CDX30P)  
97 and one where paired CDX were generated pre- and post-chemotherapy (CDX17,  
98 CDX17P), with maintained ATOH1 expression<sup>12</sup> (Table S1). Whilst ATOH1 can be  
99 co-expressed with NEUROD1 (Figure 1A-i), we confirmed and extended Principal  
100 Component Analysis (PCA) of transcriptomic data from 39 CDX (including SCLC-A  
101 CDX31P<sup>18</sup>) that separated *ATOH1* models from *NEUROD1*-only models and from  
102 models expressing *ASCL1* or *POU2F3* (Figure 1A-ii). As ATOH1 is expressed in  
103 Merkel cells and most Merkel cell carcinomas (MCCs)<sup>33</sup>, we checked whether  
104 ATOH1 CDX were in fact derived from CTCs from mis-diagnosed MCC primary  
105 tumours. MCC is characterised by the presence of oncogenic Merkel cell polyoma  
106 virus (MCPyV) in 80% of cases<sup>34</sup>. We detected MCPyV sequences in MCC patient  
107 samples from a publicly available dataset (PRJNA775071) but not in any ATOH1  
108 SCLC CDX (Figure S1A). Because a minority of MCC expresses neither ATOH1 nor  
109 MCPyV, we performed differential gene expression analysis (DGEA) of ATOH1 CDX  
110 compared to the entire CDX biobank and applied a Merkel cell-specific gene  
111 signature<sup>35</sup> (Table S2), which was not significantly enriched in ATOH1 CDX (Figure  
112 S1B), further supporting that ATOH1 CDX do not have a Merkel cell origin.

113 SCLC subtyping was based predominantly on transcriptomes<sup>11,13,36</sup>. To examine  
114 ATOH1 protein expression we optimised an IHC assay using a commercially  
115 available antibody (from here on referred to as Ptech), that revealed nuclear ATOH1  
116 staining only in ATOH1 subtype CDX (Figure 1A-iii, quantified in 1A-iv). Like *ASCL1*  
117 and *POU2F3* and in contrast to *NEUROD1*, ATOH1 transcript and protein  
118 expression followed a bimodal pattern; ATOH1 was either highly expressed or  
119 undetectable (Figure 1A-i, 1A-iii, 1A-iv). Whilst ATOH1 CDX expressed neither  
120 *ASCL1* nor *POU2F3* (Figure 1A-i), ATOH1 was expressed alone (CDX17P) or in  
121 combination with *NEUROD1* at the transcript (Figure 1A-i) and protein level (Figure

122 1A-iii, CDX25, CDX30P: high *NEUROD1* expression, 78% positive tumour cells;  
123 CDX17: moderate *NEUROD1* expression, 30% positive tumour cells).

124 *MYCL* amplification is often observed in SCLC and MCC<sup>37,38</sup>. ATOH1 expression in  
125 CDX strongly correlates with *MYCL* focal amplification (Figure 1A-v,  $p=2.43 \times 10^{-5}$ ),  
126 resulting in higher levels of *MYCL* transcript (Figure S1C) and *MYCL* protein (Figure  
127 1A-vi, S1D) compared to other subtypes.

128 CDX reflect chemosensitivity profiles of their patient donors<sup>12,39</sup>. We investigated  
129 responses of ATOH1 CDX models to SoC (cisplatin/etoposide) *in vivo* adopting a  
130 modified version of preclinical RECIST (pRECIST) (see methods); tumour growth  
131 data are transformed to progressive disease (PD1, PD2), stable disease (SD) and  
132 partial (PR), complete (CR) and maintained responses (MCR)<sup>40,41</sup>. Compared to  
133 other molecular subtype CDX (31 SCLC-A, 25 patients, 2 SCLC-N, 2 patients) which  
134 displayed variable chemotherapy responses, all 4 ATOH1 CDX (3 patients) were the  
135 most chemoresistant, scoring as PD1 (Figure 1A-vii, Fisher's exact test,  $p = 0.0049$ ;  
136 Table S1). This finding was mirrored in clinical data from the 3 ATOH1 CDX patient  
137 donors who all had chemorefractory disease (Table S1). Whilst a larger number of  
138 ATOH1 models are required, our early findings imply a putative association of  
139 ATOH1 with chemotherapy resistance.

140 ATOH1 was expressed (transcript and protein) in 2/51 SCLC cell lines<sup>42</sup> (Figure 1B)  
141 and 2/42 SCLC PDX<sup>32</sup> (Figure 1C). The PDX and cell lines also exhibited bimodal  
142 ATOH1 expression accompanied by either low (HCC33) or high expression of  
143 *NEUROD1* (CORL24, LX424, LX443) (Figure 1B-C, inserts). *MYCL* amplification  
144 was observed in ATOH1-expressing SCLC cell lines<sup>43</sup> (HCC33 CN ratio ~5, CORL24  
145 CN ratio ~2) and PDX (LX424/443<sup>32</sup>) and all ATOH1 preclinical models express  
146 amongst the highest reported levels of *MYCL* (Figure S1E-F). The ATOH1  
147 expressing PDX were obtained from one chemorefractory donor (Table S1). Overall,  
148 whilst requiring larger sample sizes, these findings indicate that ATOH1 expression  
149 in SCLC CDX, PDX and cell lines, with or without *NEUROD1*, correlates with high  
150 *MYCL* expression and chemoresistance.

151 **ATOH1 in SCLC clinical specimens**

152 *ATOH1* was detected in 1/81 SCLC tumours<sup>36</sup> and in 3/100 small cell NE pulmonary  
153 and extrapulmonary carcinoma (SCNC) biopsies<sup>44</sup>. We detected *ATOH1* in 1/19  
154 SCLC tumours profiled by single cell RNA-Seq (scRNA-Seq)<sup>45</sup>, previously classified  
155 as NEUROD1 subtype with expression of *NEUROD2* and *NEUROD4* (Figure 2A).  
156 We quantified ATOH1 protein in 65 specimens from 11 LS and 54 ES SCLC patients  
157 from the CHEMORES protocol and 37 specimens from LS patients enrolled in the  
158 CONVERT trial (methods, Table S4). ATOH1 was detected in 16/102 (16%) cases  
159 (Figure 2Ai-ii). One patient sample co-expressed ATOH1 and NEUROD1 (1/16, 6%)  
160 (Figure 2A-iii, Table S5) but in contrast to CDX and PDX, 8/16 (50%) ATOH1+  
161 samples also had detectable ASCL1 expression and all three neurogenic TFs were  
162 detectable in 3/16 (19%) cases (Figure 2A-iii). Due to scant biopsies, we could not  
163 investigate cellular co-expression of TFs. ATOH1 expression did not correlate with  
164 altered OS or PFS compared to other SCLC subtypes (data not shown) in this  
165 cohort. Nevertheless, the relatively high prevalence of ATOH1 expression in clinical  
166 samples either alone or combined with ASCL1 and/or NEUROD1 encouraged further  
167 study of ATOH1-driven biology.

### 168 **ATOH1 regulates a neurogenesis program by binding to E-boxes at promoter** 169 **and enhancer regions in SCLC CDX**

170 To interrogate the biological role of ATOH1 in CDX, we developed stable CDX17P  
171 lines carrying doxycycline-inducible (DOX) ATOH1 knock down (KD) ShRNA  
172 constructs (ShATOH1#1, -#3) or a control ShRNA targeting Renilla luciferase<sup>46</sup>  
173 (ShRen) which also expressed GFP following DOX induction (Figure 3A-i). GFP  
174 expression enabled flow cytometric sorting of transduced cells. Maximal ATOH1 KD  
175 was observed after 7 days with both the Ptech antibody (Figure S2A) shown  
176 previously for IHC, as well as a previously in-house generated antibody (SY0287)  
177 (S2B-E, 3A-ii).

178 Transcriptional programs of ATOH1 are unexplored in SCLC. To reveal ATOH1-  
179 specific TF-DNA binding we conducted chromatin immunoprecipitation with  
180 massively parallel sequencing (ChIP-Seq) on ATOH1-competent CDX17P (ShRen, 7  
181 days DOX and untreated ShATOH1#3) and ATOH1-depleted ShATOH1#3 CDX17P  
182 (7 days DOX). Upon ATOH1 KD (Figure 3B-i), samples clustered based on ATOH1  
183 expression (Figure S3A). Whilst ATOH1 ChIP-Seq signal was almost completely lost



184 upon ATOH1 KD using SY0287 (Figure 3B-ii), some ChIP-Seq signal (~50%) was  
185 retained with Ptech (Figure S3B) possibly due to non-specific antibody binding  
186 consistent with immunoblots (Figure S2A, 3B-i). Metagene analysis showed that  
187 ATOH1 peaks were located on the Transcription Start Site (TSS), near H3K4me3  
188 peaks that identify active promoter regions<sup>47</sup> and at intergenic regions mostly  
189 downstream of the gene body (Figure S3C) indicating that ATOH1 could regulate  
190 transcription at both promoter and distal regulatory elements. In support we found  
191 that ATOH1 binds to its own enhancer located downstream and highly conserved  
192 across species<sup>22</sup> (Figure 3B-iii, S3D).

193 To identify high confidence ATOH1 binding peaks, we performed differential binding  
194 analysis between ATOH1 replete and depleted conditions, considering peaks  
195 detected by both antibodies and thus avoiding potential false positives. We found  
196 17,738 ATOH1-specific binding events corresponding to 70% total peaks detected  
197 (25,464) (Figure 3C-i, Table S6). Amongst ATOH1-specific binding events, peaks  
198 are located at promoter regions (25%) and putative enhancer regions, such as distal  
199 intergenic (24%) and intronic regions (41%) (Figure 3C-ii) in accordance with recent  
200 results from MCC lines<sup>48</sup>. The most highly enriched motifs in ATOH1-specific peaks  
201 were basic helix-loop-helix binding motifs, including the reported ATOH1 DNA  
202 binding motif (MA0461.2) and the Atoh1 E-box-associated motif (AtEAM) identified in  
203 murine studies<sup>22,49</sup> (Figure 3C-iii). Compared to the second and third most enriched  
204 motifs (homeodomains and zinc-fingers), E-box and ATOH1-specific motifs were  
205 found at the summit of ATOH1 peaks (Figure 3C-iv) suggesting they are uniquely  
206 present where there is highest ATOH1 signal<sup>50</sup>.

### 207 **ATOH1 target genes in SCLC CDX**

208 We then sought to identify the biological processes in SCLC regulated by ATOH1  
209 and its putative target genes. Consistent with its role as a neurogenic TF, ATOH1-  
210 bound genes were enriched in pathways related to neurogenesis (Figure S3E-F,  
211 Table S7). However, this analysis only considered DNA binding events irrespective  
212 of gene expression changes. To define genes directly regulated by ATOH1, we  
213 performed global transcriptomics (RNA-Seq) of CDX17P cells cultured *ex vivo* in  
214 presence or absence of DOX-induced ATOH1 KD (ShATOH#1, -#3). Genes directly  
215 regulated by ATOH1 should be downregulated after ATOH1 loss. As expected,

216 ATOH1 was the most differentially expressed (DE) gene of ~500 genes (Figure 4A-i,  
217 Table S8). Genes upregulated after ATOH1 KD included those involved in cell  
218 adhesion and migration, whereas downregulated genes play roles in neurogenesis  
219 (Figure 4A-ii, Table S9) and in inner ear hair cell differentiation, corroborated by  
220 decreased expression of independent inner ear hair cell signatures upon ATOH1  
221 KD<sup>51,52</sup> (Figure S4A-B, Table S10-S11). Overall, our findings agree with known  
222 ATOH1 transcriptional programs in murine developmental models whereby *Atoh1* is  
223 required for inner ear hair cell and cerebellar granule cell development and  
224 differentiation<sup>21</sup>, although relevance of these processes to SCLC initiation and  
225 progression is unclear.

226 *ASCL1* and *NEUROD1* are highly expressed in NE subtypes of SCLC<sup>11,53</sup> and drive  
227 a NE transcriptional program. Given that ATOH1 also regulates neurogenesis, we  
228 asked whether NE status was affected by ATOH1 depletion. Whilst a 25-gene NE  
229 signature<sup>54</sup> and *SYP* expression were unchanged upon ATOH1 KD (Figure S4C,E,  
230 Table S10), a 25-gene Non-NE signature was upregulated<sup>54</sup> (Figure S4D, Table  
231 S10) suggesting that ATOH1 may contribute to NE to Non-NE plasticity, albeit  
232 without increased expression of *YAP1* nor *MYC* (Figure S4E).

233 Fewer significant transcriptional changes were seen upon ATOH1 KD relative to the  
234 abundance of ATOH1 binding sites (by ChIP-Seq), suggesting that ATOH1 activity  
235 might be restricted to a subset of ATOH1-bound genes in SCLC CDX. Thus, to infer  
236 direct ATOH1 transcriptional targets in SCLC, we performed an integrated analysis  
237 of ChIP-Seq and RNA-Seq with the Binding and Expression Target Analysis  
238 (BETA)<sup>55</sup>. We found that ATOH1 mainly acts as a transcriptional activator (Figure  
239 4B-i, blue line) and identified 150 genes downregulated upon ATOH1 depletion,  
240 directly downstream of ATOH1 (Table S12). Among these genes were components  
241 of Notch signalling (including *HES6*, *DLL1*, *DLL3*, *DLL4*) consistent with the interplay  
242 between ATOH1 and Notch signalling during brain and intestinal development<sup>24,56</sup>  
243 and genes important for inner ear hair cell development such as *USH2A*, *LHX3* and  
244 *RASD2*<sup>52</sup>. Concordant with transcriptomics analysis (Figure 4A-ii), ATOH1 direct  
245 targets are also involved in neurogenesis and inner ear hair cell differentiation  
246 (Figure 4B-ii, Table S13).

247 This integrated analysis was performed in only CDX17P, so we next asked whether  
248 ATOH1 direct targets were conserved across all ATOH1 expressing CDX. We  
249 performed DGEA between ATOH1 CDX (CDX17, 17P, 25, 30P) and the whole CDX  
250 Biobank (35 CDX) (Figure 4C-i, Table S14), followed by gene set enrichment  
251 analysis (GSEA) for ATOH1 direct targets to demonstrate ATOH1 direct target genes  
252 were conserved (Figure 4C-ii, NES = 2.48,  $p = 1.13 \times 10^{-16}$ ). We also detected high  
253 expression of ATOH1 target genes in the 2 ATOH1 SCLC PDX (Figure 4C-iii, NES =  
254 2.44,  $p = 5 \times 10^{-10}$ ) and an ATOH1 expressing tumour from the MSK SCLC tumour  
255 atlas dataset<sup>45</sup> (Figure 4C-iv). These direct targets comprise the first SCLC-based  
256 ATOH1 gene signature consistently observed in CDX, PDX and tumour biopsies,  
257 indicative of a conserved transcriptional role for ATOH1 in SCLC.

### 258 **Impact of ATOH1 on SCLC CDX cell survival *ex vivo***

259 We examined the biological effects of ATOH1 depletion via DOX-inducible ATOH1  
260 KD in CDX17P cells. Maximal ATOH1 KD was achieved after 7 days of DOX (Figure  
261 S2A) and was maintained for 14 days (the longest duration of *ex vivo* studies).  
262 Withdrawal of DOX restored ATOH1 expression (7 days +DOX, then 7 days -DOX)  
263 (Figure 5A-i, ii). ATOH1 depletion caused >50% decrease in cell viability  
264 (ShATOH1#1,  $p=0.0025$ ; ShATOH1#3,  $p=0.0124$ ), compared to un-induced and  
265 ShRen controls, which was attenuated by restoring ATOH1 expression (Figure 5B-i).  
266 To interrogate the mechanism of decreased cell viability, we established DOX-  
267 inducible ATOH1 KD in CDX30P and HCC33 SCLC cells (Figure S5A-B) and  
268 assessed cell death and cell cycle progression following ATOH1 depletion.  
269 Compared to ShRen DOX-induced controls and un-induced cells, there were no  
270 reproducible changes in cell cycle progression in CDX17P or CDX30P upon ATOH1  
271 depletion for 14 days (Figure 5B-ii, Figure S5C). A modest ~12% decrease in cell  
272 proliferation was evident in HCC33 cells although this did not constitute a complete  
273 proliferation arrest with ~15% cells still cycling (Figure S5D). These slightly different  
274 effects on proliferation in CDX versus HCC33 may result from differences between  
275 cell lines and CDX *ex vivo* cultures. Instead, ATOH1 depletion increased cell death  
276 in CDX17P (55%), CDX30P (42%) and HCC33 (44%) after 14 days of ATOH1  
277 depletion (Figure 5B-iii) via a caspase-3-independent process (Figure 5B-iv). After 7  
278 days of DOX treatment, ATOH1 KD already induced detectable cell death (Figure  
279 5C-i) and a decrease in ATP production, used as a proxy for viable cell number

280 (Figure 5C-ii, iii, in red). Because other types of non-apoptotic, programmed cell  
281 death such as ferroptosis and pyroptosis have been observed in SCLC<sup>57,58</sup>, we  
282 induced ATOH1 KD in CDX17P and CDX30P ShATOH1#1 with DOX, with or without  
283 cell death pathway inhibitors for 7 days. Inhibition of apoptosis, pyroptosis,  
284 necroptosis or ferroptosis (with single or combined inhibitors) did not prevent ATOH1  
285 KD-induced loss of cell viability (Figure 5C-ii, iii). Taken together, these findings  
286 identify ATOH1 as necessary for cell survival in CDX17P, CDX30P and HCC33 cells  
287 as its depletion induces cell death, either via an undefined programmed cell death  
288 pathway or most likely via necrosis.

### 289 **Impact of ATOH1 on tumour growth *in vivo***

290 We next asked whether the role of ATOH1 in maintaining cell survival *ex vivo*  
291 translated to an impact on tumour growth *in vivo*. CDX17P control ShRen or  
292 ShATOH1(#3) cells were implanted subcutaneously (s.c.) in immunocompromised  
293 mice, and KD was induced with DOX-supplemented feed after 19 days (Figure 6A),  
294 when mice had palpable tumours. Once tumours reached 500-800 mm<sup>3</sup> they were  
295 surgically resected and mice kept on study for 28 days to allow time for metastatic  
296 dissemination (based on previous experiments, see methods, Figure 6A).

297 A significantly delayed s.c. tumour growth was observed in mice bearing DOX-  
298 induced ATOH1 KD tumours compared to DOX-induced ShRen controls or un-  
299 induced tumours (Figure 6B-i, ii). This tumour growth delay extended time to reach  
300 the experimental endpoint tumour volume or s.c. tumour surgical resection (22 days  
301 for ShRen, 35 days for ShATOH1,  $p < 0.0001$ , Figure 6C). To interpret the observed  
302 growth delay, we examined persistence of ATOH1 KD throughout the experiment by  
303 performing IHC for ATOH1 and GFP in resected s.c. tumours (mean tumour volume  
304 and time from implant: 603±54 mm<sup>3</sup>, 44±5 days ShRen +DOX; 552±48 mm<sup>3</sup>, 70±13  
305 days ShATOH1 +DOX) (Figure 6B-i). At tumour resection, mice bearing DOX-  
306 induced ATOH1 KD tumours showed a 75% reduction in ATOH1 protein expression  
307 and both DOX-induced controls and KD tumours had high expression of GFP (Figure  
308 S6A-i, ii). However, GFP expression was ~10% lower in DOX-induced ATOH1 KD  
309 tumours (Figure S6A-ii,  $p = 0.008$ ) and expression of GFP and ATOH1 was  
310 heterogeneous in DOX-induced ATOH1 KD tumours, with most tumour presenting  
311 with some GFP-, ATOH1+ regions (Figure S6A-iii).

312 Overall, these data indicate that reduced ATOH1 expression promotes tumour  
313 growth delay *in vivo*, where impact may have been attenuated by outgrowth of  
314 ATOH1 positive cells which are potentially un-transduced wild-type cells or cells that  
315 escaped inducible KD, as reported in other settings<sup>59,60</sup>. These data are consistent  
316 with a selective pressure to re-instate ATOH1 expression in ATOH1 KD tumours  
317 supporting a pro-tumorigenic role for ATOH1.

### 318 **A Role for ATOH1 in liver-metastatic dissemination *in vivo***

319 We previously reported metastasis to multiple organs, including brain and liver,  
320 occurs after resection of s.c. CDX17P tumours<sup>12</sup>. To investigate whether ATOH1  
321 supports metastatic growth, s.c. tumours were resected and mice left on study for 28  
322 days (Figure 6A) before metastasis (defined as >50 tumour cells) were quantified  
323 using a human mitochondria antibody and IHC. Dissemination, predominantly to the  
324 liver, was observed in all cohorts regardless of DOX feed, including single tumour  
325 cells, micro-or macro-metastasis (Figure 6D). Although frequency of liver metastases  
326 between control and DOX-induced ATOH1 KD mice was approximately equivalent,  
327 all liver metastases from DOX-induced ShATOH1 mice were negative for GFP and  
328 expressed similar levels of ATOH1 compared to un-induced tumours (Figure 6E-i, ii),  
329 again implying a selective pressure to retain/re-express ATOH1<sup>59,60</sup> and indirectly  
330 suggesting a role for ATOH1 in promoting liver metastasis.

331 In a more direct approach to investigate the role of ATOH1 in metastasis, we  
332 performed intracardiac injection of tumour cells (Figure 6F), reasoning liver  
333 metastasis would occur faster, allowing less time for outgrowth of cells with high or  
334 re-expressed ATOH1 (Figure 6E). CDX17P control ShRen or ShATOH1 cells were  
335 cultured with or without DOX for 4 days to induce ATOH1 KD *in vitro* and GFP-  
336 positive viable cells were sorted by flow cytometry before intra-cardiac injection. One  
337 group of mice per construct (ShRen and ShATOH1) received DOX-supplemented  
338 feed (N=5 ShRen and N=8 ShATOH1), while control animals were maintained on  
339 standard diet (N=5 ShRen and N=5 ShATOH1). Animals were removed from study  
340 70 days after intracardiac injection (see methods, Figure 6F).

341 Almost all animals (14/15) in control cohorts (standard feed or implanted with DOX-  
342 induced ShRen cells) were removed before study endpoint due to extensive  
343 metastatic liver disease (Figure S6B). In contrast, 8/8 (100%) animals implanted with

344 DOX-induced ShATOH1 cells reached study endpoint (time from implantation:  $53.6 \pm$   
345  $7.9$  ShRen+DOX;  $70 \pm 0$  ShATOH1+DOX; Figure 6G). There was a significant  
346 reduction in metastatic burden in animals with ATOH1 KD compared to control  
347 cohorts (Figure 6H-I) and only one animal in the DOX-induced ShATOH1 group  
348 developed liver metastasis (Figure S6B). Despite showing positive GFP expression  
349 ( $>40\%$  GFP+ cells), the only liver metastasis derived from ATOH1 KD cells also  
350 exhibited ATOH1 positivity in  $>60\%$  of metastatic cells, indicating that ATOH1 KD  
351 was not completely retained in these cells (Figure 6Ji-ii). These data provide more  
352 direct evidence that ATOH1 KD reduced metastasis to the liver and promoted longer  
353 survival.

## 354 **DISCUSSION**

355 Emerging understanding of SCLC subtypes and phenotypic plasticity are considered  
356 key to support rational development of biomarker-directed personalised treatments<sup>14</sup>.  
357 Building upon knowledge of inter- and intra-tumoural heterogeneity<sup>32,44</sup>, we have  
358 characterised the ATOH1 subtype, defining its prevalence and demonstrating pro-  
359 tumour functions of growth and metastasis.

360 ASCL1, NEUROD1 and ATOH1 are all proneural TFs negatively regulated by Notch  
361 signalling<sup>24,28,61</sup>. Whilst expression of ATOH1 is not reported during normal lung  
362 development, its expression has been reported in NE lung cancer<sup>62</sup>, extrapulmonary  
363 high-grade neuroendocrine cancers<sup>44</sup>, Merkel cell carcinoma (MCC)<sup>33</sup>,  
364 medulloblastoma<sup>63,64</sup> and rarely in NSCLC<sup>65</sup> and colorectal cancer (CRC)<sup>30,66,67</sup>.  
365 Whilst mechanistically understudied, in medulloblastoma and MCC ATOH1 is  
366 tumour-promoting<sup>31,68-70</sup>, whereas it is a tumour suppressor in CRC<sup>30,66</sup>. These  
367 opposing context-dependent functions have been attributed to imbalance between  
368 differentiation and proliferation driven by abnormal ATOH1 expression levels<sup>71</sup>.

369 Co-expression of subtype TFs is commonly observed, contributing to SCLC  
370 heterogeneity<sup>12,32,72,73</sup>. ATOH1 was found to be frequently expressed in SCLC  
371 clinical samples, either alone or with ASCL1 and/or NEUROD1 (Figures 1, 2)  
372 extending existing sparse data<sup>62</sup>. In CDX30 where ATOH1 was co-expressed with  
373 NEUROD1, ATOH1 depletion impacted cell survival *ex vivo* (Figure 5), suggesting  
374 that NEUROD1 could not compensate for ATOH1 loss. Furthermore, NEUROD1 was



375 not identified amongst ATOH1 direct targets and there was minimal overlap with  
376 ASCL1 and NEUROD1 target genes (Figure 4, Table S15). Like NEUROD1 and  
377 ASCL1 in their respective subtypes<sup>74-79</sup>, ATOH1 supports cell viability in ATOH1  
378 subtype tumour cells (Figure 5).

379 In SCLC, ATOH1 exerts its function by binding E-box motifs at promoter and  
380 enhancer regions of target genes as in the developing mouse brain<sup>49</sup> and in MCC<sup>80</sup>,  
381 including binding to its own downstream enhancer<sup>22</sup> (Figure 3). In CDX, ATOH1  
382 directly regulates expression of genes involved in neuronal fate development and  
383 mechanoreceptor differentiation (Figure 4) consistent with murine developmental  
384 studies<sup>21,81,82</sup>. This is also consistent with the role of ATOH1 in MCC<sup>33</sup>. The ability of  
385 ATOH1 to regulate neuronal fate determination and Notch ligands (DLL1, DLL3,  
386 DLL4) in mice<sup>24</sup> mirrors the activity of ASCL1 in SCLC<sup>53,74</sup>; in CDX17P, ATOH1  
387 depletion increased expression of Non-NE and cell adhesion genes invoking a  
388 similar role for ATOH1 in NE fate determination in SCLC (Figure S4). However, as  
389 the NE gene expression signature was retained upon ATOH1 depletion (Figure S4),  
390 additional factors, for example, MYC overexpression<sup>16</sup>, are likely required to promote  
391 full NE to Non-NE transition in ATOH1-driven SCLC. The need for additional signals  
392 to fully induce a NE to Non-NE transition is similarly posited in studies of ASCL1 and  
393 NEUROD1 depletion in SCLC, where morphological changes or a NE to Non-NE  
394 transition were not observed<sup>77,78,83,84</sup>.

395 Both ATOH1 and ASCL1 correlate with *MYCL* overexpression (Figure 1). In SCLC,  
396 overexpression/genetic amplification of *MYCL* was often correlated with the SCLC-A  
397 subtype and *MYCL* is a direct transcriptional target of ASCL1<sup>35, 52, 86</sup>. A more  
398 complex relationship was recently revealed by a clinical study whereby *MYCL*  
399 protein was present in only ~30% of ASCL1+ samples<sup>73</sup>. Further adding to this  
400 heterogeneity, we show that all ATOH1-expressing CDX present focal amplification  
401 and overexpression of *MYCL* (Figure 1, S1). A correlation between ATOH1 and  
402 *MYCL* expression was also observed in MCC<sup>37,38</sup>. However, we did not identify  
403 *MYCL* as a direct ATOH1 target (Table S12) and *MYCL* expression was unchanged  
404 upon ATOH1 depletion (Table S8, Figure 4). Combined, these data indicate that  
405 other factors contribute to *MYCL* expression in ATOH1-positive SCLC.

406 The profound impact of metastasis on SCLC patient outcomes drives a pressing  
407 need to understand and target underlying mechanisms. Acquisition of neuronal gene  
408 expression programmes is associated with invasive and metastatic SCLC in cell  
409 lines and GEMMs<sup>59,85,86</sup>. In CDX17P, ATOH1 is pro-metastatic (Figure 6) drawing  
410 parallels with the ATOH1 pro-invasive phenotype in MCC<sup>87</sup> and its pro-metastatic  
411 role in medulloblastoma<sup>88</sup>. ATOH1 downregulation was linked with loss of cell  
412 adhesion (Figure 4A-ii, Table S8), which was also observed in MCC<sup>33,89</sup>.

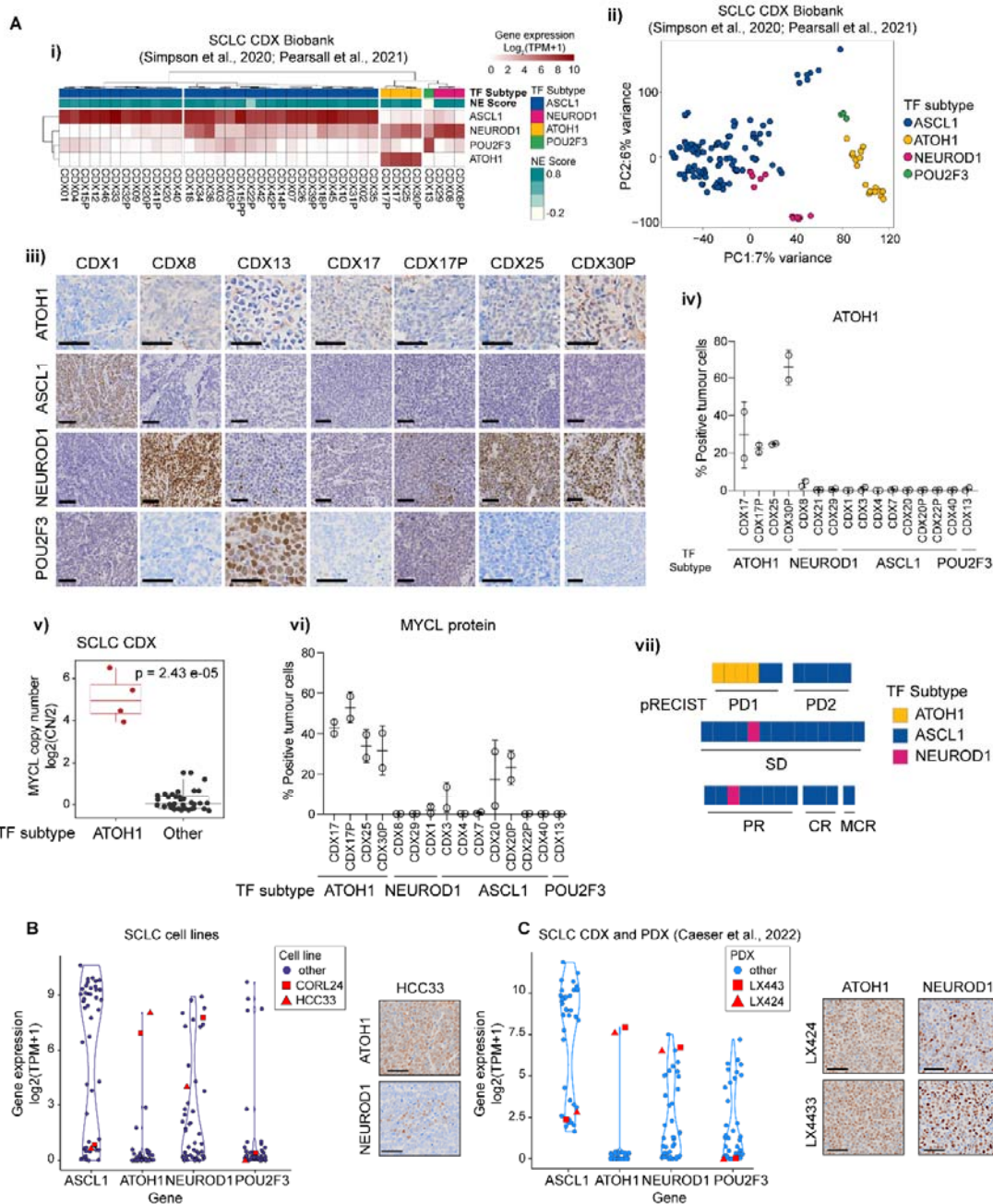
413 SCLC was once considered to derive from pulmonary neuroendocrine cell (PNEC)  
414 precursors<sup>90</sup>. However, elegant studies in SCLC GEMMs describe different potential  
415 cells of origin<sup>59,91-93</sup> with differences only evident at the molecular level<sup>16,45,53</sup>. In this  
416 regard, similarities between MCC and ATOH1-driven SCLC are intriguing. MCC is a  
417 NE skin carcinoma, expressing epithelial and NE markers with morphological,  
418 ultrastructural and immunohistochemical features shared with Merkel cells<sup>91-93</sup> yet  
419 there is no direct histo-genetic link between Merkel cells and MCC with ongoing  
420 debate on cell(s) of origin of MCC<sup>94,95</sup>. Tumour heterogeneity in MCC is attributed to  
421 variant disease aetiologies mediated by either UV exposure or Merkel cell  
422 polyomavirus (MCPyV) integration<sup>95</sup>. Virus-positive MCC has low mutation burden,  
423 whilst virus-negative MCC, like SCLC, have characteristic RB1 and TP53 mutations  
424 in a highly mutated landscape<sup>96,97</sup>. The recent identification of ‘mesenchymal-like’  
425 MCC with an ‘inflamed’ phenotype exhibiting better response to immunotherapy  
426 draws parallels with the SCLC-I subtype<sup>13</sup> and contrasts ‘immune-cold’  
427 immunotherapy resistant MCC with higher expression of neuroepithelial markers  
428 including ATOH1<sup>98</sup>. Altogether, that the ATOH1 subtype of SCLC CDX shares  
429 features with NE SCLC and with MCC, another NE cancer, is perhaps not surprising  
430 and might indicate convergent tumour evolution<sup>94,99</sup>.

431 In summary, here we validate the ATOH1 SCLC subtype (SCLC-AT) where ATOH1  
432 suppresses cell death and promotes tumour growth and metastasis. Further studies  
433 are now needed to deepen our understanding of ATOH1-driven SCLC biology and to  
434 address whether there are therapeutic vulnerabilities of this subtype.



435 **FIGURES AND LEGENDS**

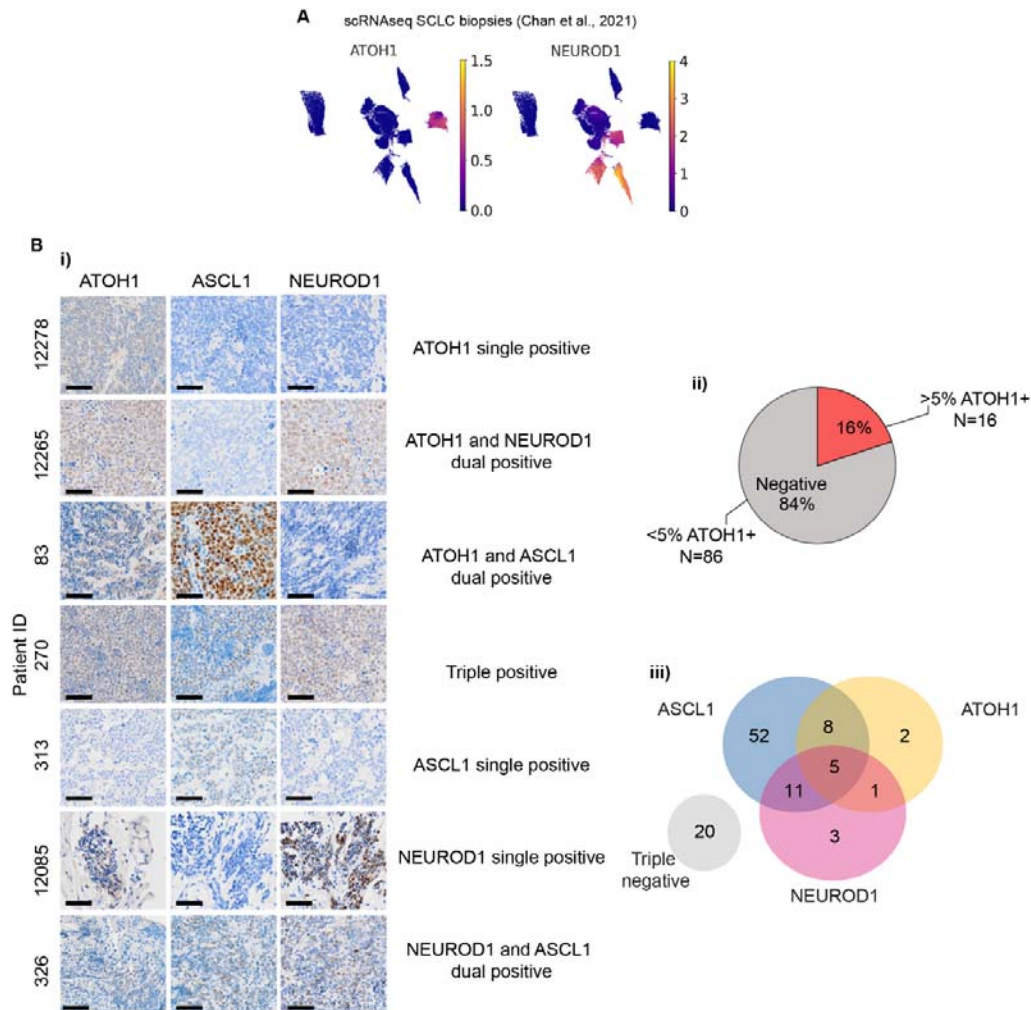
Figure 1



436 **Figure 1. ATOH1 is expressed in a transcriptionally distinct subset of SCLC**  
 437 **CDX, PDX and established cell lines.** (A-i) Heatmap illustrating expression levels  
 438 of *ASCL1*, *NEUROD1*, *ATOH1* and *POU2F3* in the SCLC CDX biobank, annotated  
 439 by SCLC subtype and NE score<sup>12,18</sup>. Gene expression is shown as log<sub>2</sub>(TPM+1). (A-

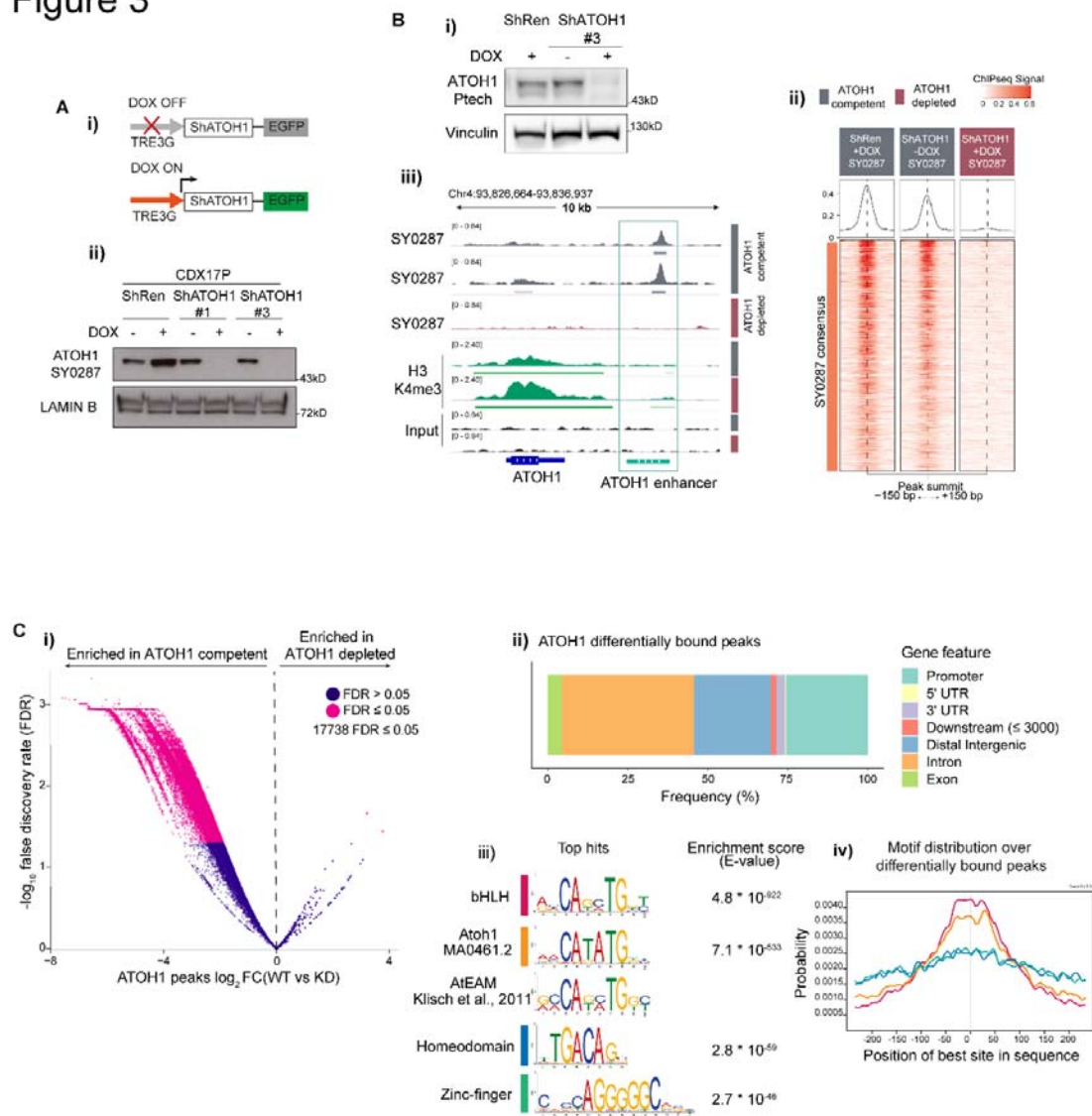
440 ii) Unbiased principal component analysis (PCA) of SCLC CDX in the biobank  
441 annotated by SCLC molecular subtypes. Key: blue, ASCL1; pink, NEUROD1; yellow,  
442 ATOH1; green, POU2F3. (A-iii) Representative IHC images for ATOH1, ASCL1,  
443 NEUROD1 and POU2F3 in a panel of CDX models belonging to different SCLC  
444 molecular subtypes. Scale bars: 50  $\mu$ m. (A-iv) Quantification of ATOH1 expression in  
445 N=2 CDX tumours in a panel of CDX models. (A-v) Boxplot of MYCL copy number  
446 (CN), reported as CN ratio ( $\text{Log}_2(\text{CN}/2)$ ), in CDX grouped by molecular subtype  
447 (ATOH1 or other). Statistics reported as per Wilcoxon rank sum exact test. (A-vi)  
448 Quantification of MYCL expression by IHC in N=2 CDX tumours in a panel of CDX  
449 models belonging to different SCLC molecular subtypes (annotated below). (A-vii)  
450 Chemosensitivity scores of the SCLC CDX biobank according to pRECIST criteria,  
451 coloured by SCLC molecular subtypes. Key: yellow, ATOH1; blue, ASCL1; pink,  
452 NEUROD1. Data are reported after 1 cycle of cisplatin/etoposide treatment and as  
453 average of N>3 mice for N=29 CDX (see methods). Statistical analysis was  
454 performed with a Fisher's exact test between ATOH1 CDX and the remaining CDX;  
455  $p = 0.0049$ . (B-C) Violin plot representing expression of indicated NE and Non-NE  
456 TFs in SCLC established cell lines (B) and the SCLC CDX and PDX biobank<sup>32</sup> (C);  
457 ATOH1-expressing HCC33, CORL24 (B) and LX424, LX443 (C) are highlighted in  
458 red. Gene expression is reported as  $\text{Log}_2(\text{TPM}+1)$ . Inserts are representative images  
459 of ATOH1 and NEUROD1 IHC staining for HCC33 (B) and LX424, LX443 (C).

Figure 2



460 **Figure 2. ATOH1 protein is expressed in SCLC clinical samples.** (A) UMAP plots  
 461 of single cell RNA-Seq (scRNA-Seq) from SCLC biopsies from the publicly available  
 462 MSK SCLC Atlas<sup>45</sup> reporting expression of *ATOH1* (left panel) and *NEUROD1* (right  
 463 panel). Gene expression reported in units of  $\log_2(X + 1)$  where  $X$  = normalized  
 464 counts. (B-i) Representative IHC images for ATOH1, ASCL1 and NEUROD1 in  
 465 SCLC tissue biopsies presenting with single, dual or triple positivity (annotated). (B-  
 466 ii) Pie chart illustrating the prevalence of ATOH1-positive (>5% positive tumour cells)  
 467 clinical specimens (N=16/102). (B-iii) Venn diagram illustrating overlap of ASCL1,  
 468 ATOH1 and NEUROD1 expression in N=102 clinical specimens as detected by IHC.  
 469 Positivity determined as >1.5% positive tumour cells for ASCL1 and NEUROD1;  
 470 positivity for ATOH1 determined as in B-ii.

## Figure 3

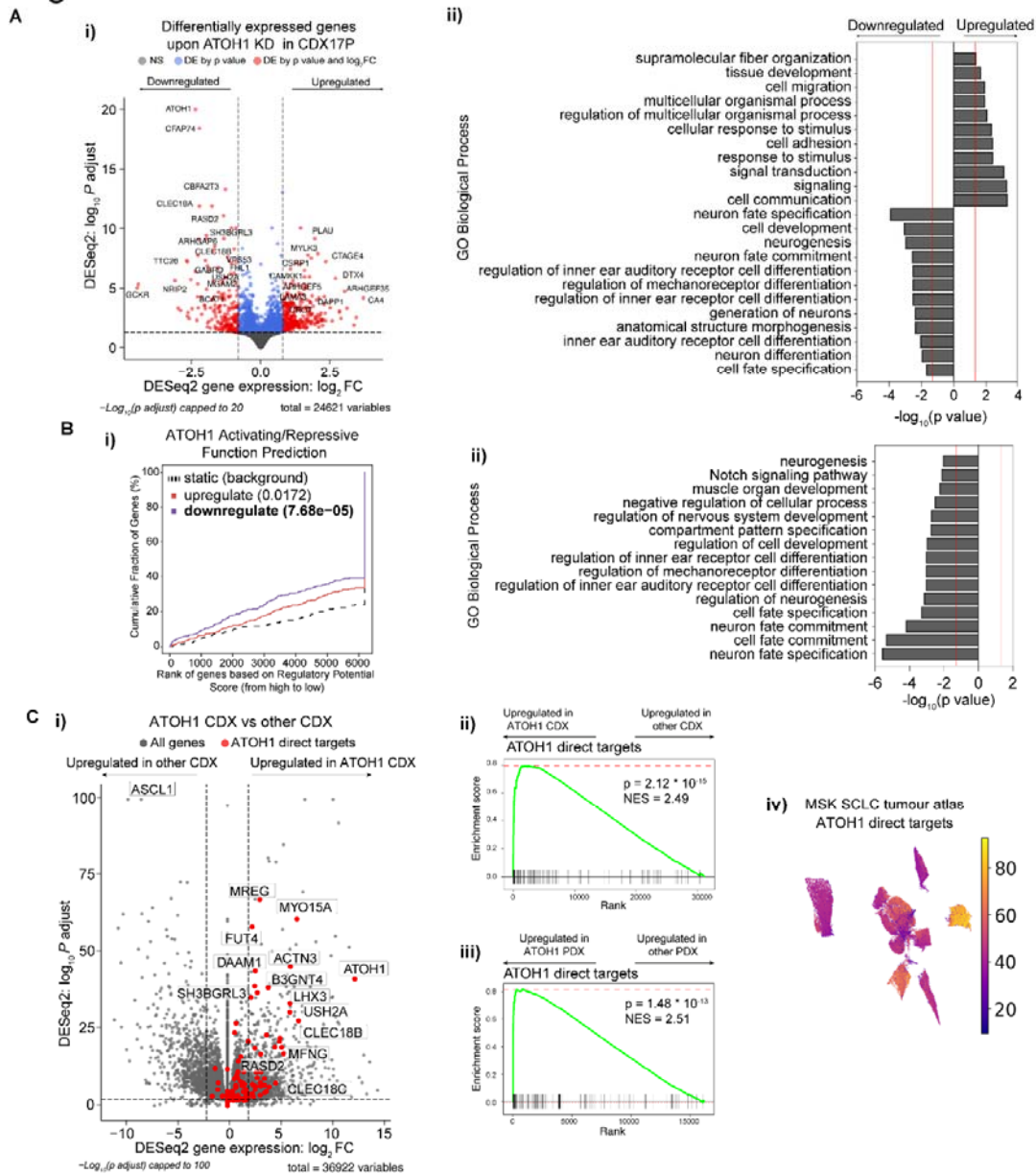


471 **Figure 3. High confidence ATOH1 binding sites are located at promoter and**  
 472 **enhancer regions and are enriched for E-box motifs.** (A-i) Schematic of DOX-  
 473 inducible knock-down (KD) system: without DOX, eGFP and shRNAs targeting  
 474 ATOH1 (ShATOH1) or Renilla Luciferase (ShRen) are not expressed; upon induction  
 475 with DOX, both eGFP and ShATOH1 or ShRen are expressed. (A-ii) (F) Nuclear  
 476 fractionation validating ATOH1 KD with the in-house ATOH1 antibody SY0287 in  
 477 CDX17P ShRen, ShATOH1#1 and ShATOH1#3 upon treatment with DOX for 7  
 478 days. (B-i) Western blot showing ATOH1 expression (detected with the Ptech  
 479 antibody) in the samples processed for ChIP-Seq. (B-ii) Heatmap of ChIP-Seq signal

480 for consensus peak sets SY0287 in ATOH1 competent (grey) and depleted (red)  
481 CDX17P, generated with the generateEnrichedHeatmap function within profileplyr  
482 v1.8.1<sup>100</sup>. (B-iii) ATOH1 binding peaks at ATOH1 locus highlighting ATOH1 binding  
483 peaks at ATOH1 downstream enhancer (light green), which are lost upon ATOH1  
484 depletion. In dark green, ChIP-Seq tracks for H3K4me3 at the ATOH1 locus. The  
485 peaks were visualized with the Integrated Genomics Viewer genome browser. (C-i)  
486 Volcano plot of ATOH1 differentially bound regions (by false discovery rate, FDR <  
487 0.05) in ATOH1 competent vs ATOH1 depleted CDX17P. Significant peaks  
488 highlighted in pink (17,738). (C-ii) Relative frequency of ATOH1 differentially bound  
489 peaks in regulatory genetic regions. (C-iii) Motif enrichment analysis of ATOH1  
490 differentially bound peaks with MEME ChIP<sup>101</sup>. Mouse Atoh1 E-box-associated motif  
491 (AtEAM<sup>49</sup>) reported for comparison with Atoh1 DNA binding motif and bHLH motif.  
492 (C-iv) Centrimo<sup>50</sup> analysis of the location of enriched motifs in ATOH1 differentially  
493 bound peaks.



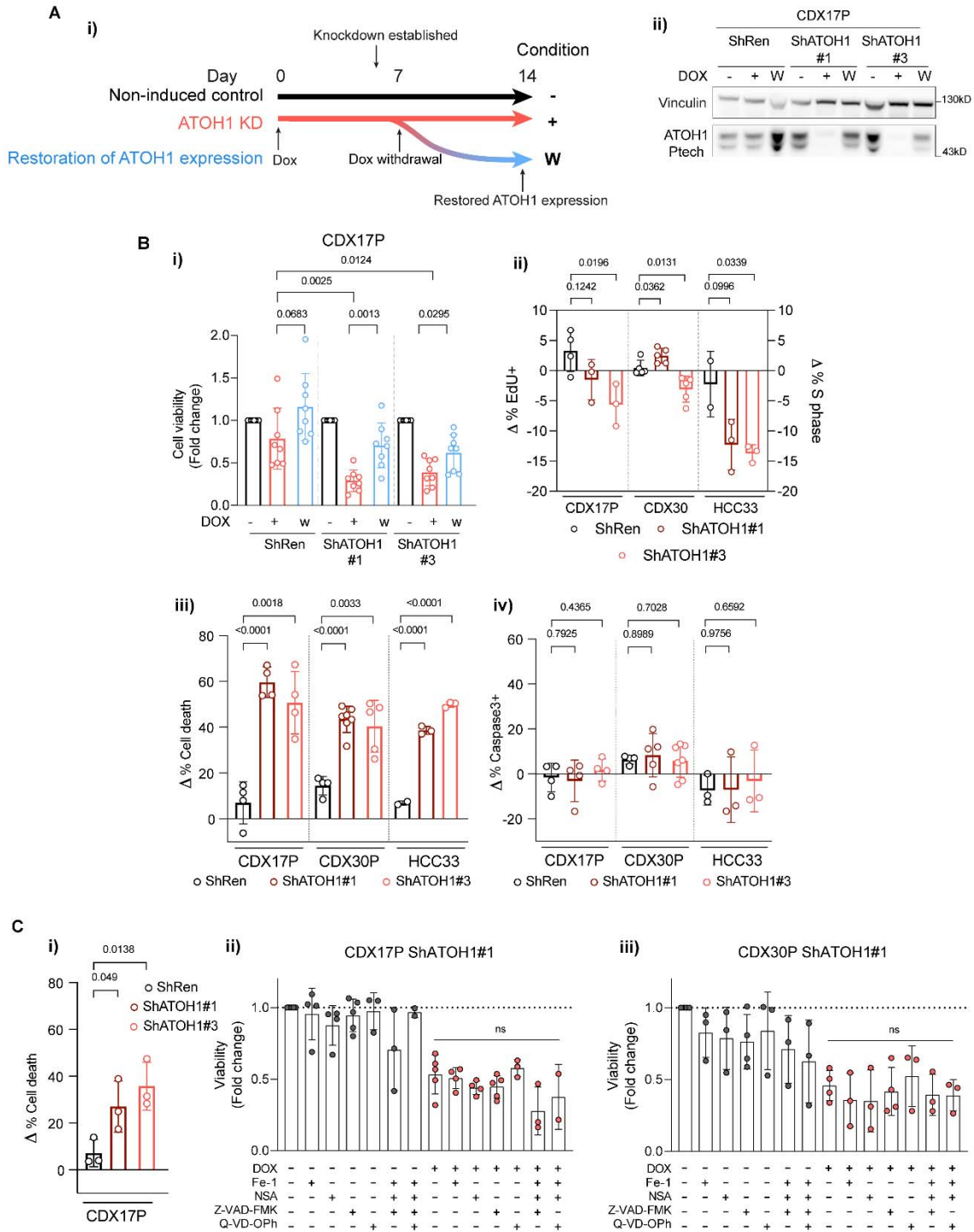
Figure 4



494 **Figure 4. Identification of ATOH1 targetome and gene signature.** (A-i) Volcano  
 495 plot illustrating differentially expressed (DE) genes upon ATOH1 depletion (DOX  
 496 treatment for 6 days) in CDX17P. Key: grey, not significant; blue, significant by p  
 497 value; red, significant by p value < 0.01 and log<sub>2</sub>(fold change) > 0.8 or < -0.8. Dotted  
 498 lines represent the thresholds for determining significant gene expression changes  
 499 (p value < 0.01 and log<sub>2</sub>(fold change) > 0.8 or < -0.8). The most significant DE genes  
 500 are labelled. (A-ii) Bar plot illustrating the top 20 biological processes up- and

501 downregulated upon ATOH1 KD in CDX17P. Analysis was performed with  
502 gProfiler2<sup>102</sup>. (B-i) Prediction of ATOH1 transcriptional function after integration of  
503 ChIP-Seq and RNA-Seq with BETA<sup>55</sup>. ATOH1 KD results in downregulation of genes  
504 with ATOH1 binding sites identified in ChIP-Seq ( $p = 7.68 * 10^{-5}$ ) and is predicted to  
505 have a function in promoting transcription. (B-ii) Bar plot illustrating biological  
506 processes (performed with gProfiler2) associated with ATOH1 target genes identified  
507 in B-i. (C-i) Volcano plot illustrating genes enriched in ATOH1 CDX (N=4) compared  
508 to the whole CDX biobank (N=35). ATOH1 gene signature (i.e. ATOH1 target genes)  
509 highlighted in red. Dotted lines represent the thresholds for determining significant  
510 gene expression changes ( $p$  value  $<0.01$  and  $\log_2(\text{fold change}) >2$  or  $<-2$ ). (C-ii)  
511 Gene set enrichment analysis (GSEA) for ATOH1 direct targets in ATOH1 CDX  
512 (N=4) vs the rest of the biobank (N=35). NES: normalised enrichment score. (C-iii)  
513 GSEA for ATOH1 direct targets in ATOH1 PDX (N=2) vs the rest of the MSK PDX  
514 biobank (N=40). GSEA analysis was performed with Fgsea<sup>103</sup>. (C-iv) UMAP of  
515 cumulative expression of ATOH1 direct targets in scRNA-Seq of SCLC tumour  
516 biopsies<sup>45</sup>. Expression of ATOH1 target genes is highest in the only ATOH1-  
517 expressing tumour (identified in Figure 2A).

## Figure 5

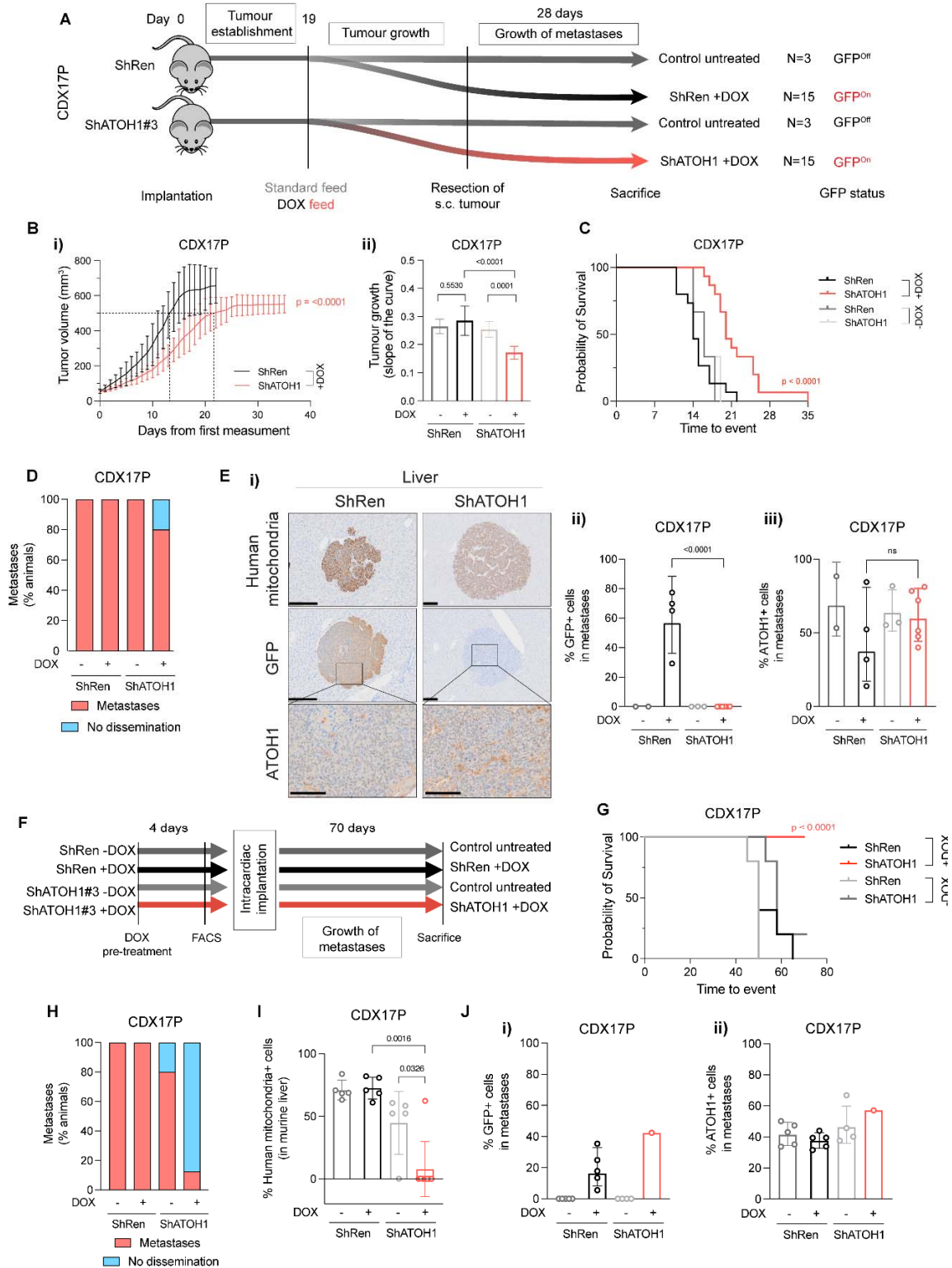




518 **Figure 5. ATOH1 is necessary for SCLC cell survival *in vitro*.** (A-i) Schematic of  
519 induction of ATOH1 KD. ATOH1 KD was established after 7 days induction with 1  
520  $\mu\text{g/ml}$  doxycycline (DOX). Cells were cultured for a total of 14 days with DOX (red  
521 line, +) or without DOX as controls; after the initial 7 days induction with DOX, a part  
522 of cells was plated without DOX to restore ATOH1 expression (blue line, W).  
523 Untreated parental cells served as additional control (black line, -). (A-ii) Western blot  
524 validation of ATOH1 depletion and restoration in the conditions specified in A-i.  
525 ShRen treated with DOX for 14 days and untreated ShRen, ShATOH1#1,  
526 ShATOH1#3 and were used as control. (B-i) Relative cell viability measured with  
527 CellTiter-Glo® (Promega) upon ATOH1 KD (red) and restoration (blue) compared to  
528 un-induced controls (black). N=8 independent experiments. (B-ii) Flow cytometry  
529 quantification of cell cycle progression by EdU (CDX17P, HCC33) and PI  
530 incorporation (CDX30P). Data was normalised to DOX-untreated parental controls  
531 by subtracting the proportion of cells in S phase in untreated cells to that of DOX-  
532 treated cells ( $\Delta \% \text{ S phase} = \% \text{ S phase}_{\text{DOX-treated}} - \% \text{ S phase}_{\text{untreated}}$ ); ShATOH1  
533 conditions were then compared to ShRen controls. CDX17P, N=4 ShRen, N=3  
534 ShATOH1#1 and #3; CDX30P, N=5; HCC33, N=2 ShRen, N=3 ShATOH1#1 and #3  
535 independent experiments. (B-iii) Flow cytometry quantification of cell death after 14  
536 days induction with DOX of ATOH1 KD, normalised as in B-ii. Total cell death is  
537 reported as sum of apoptotic and necrotic cells. CDX17P: N=4; CDX30P: N=4  
538 ShRen, N=7 ShATOH1#1, N=5 ShATOH1#3; HCC33: N=2 ShRen, N=3  
539 ShATOH1#1 and #3 independent experiments. (B-iv) Same as B-iii, reporting total  
540 Caspase-3 positive cells. All statistics in panel B are reported as two-tailed unpaired  
541 *t* tests across indicated conditions. C-i) Flow cytometry quantification of cell death  
542 (as defined in B-iii) after 7 days DOX-induction of ATOH1 KD in CDX17P. N=3  
543 independent experiments. P values are reported in panel B and C-i as per two-tailed  
544 unpaired *t* test. (C-ii, C-iii) ShATOH1#1 CDX17P (C-ii) and CDX30P (C-iii) cells were  
545 treated with (red) or without (black) DOX and with or without ferrostatin-1 (1 $\mu\text{M}$ ),  
546 necrosulfonamide (NSA, 100 nM) or Z-VAD-FMK/Q-VD-OPh (20 $\mu\text{M}$ ) and indicated  
547 combinations for 7 days. Cell viability was measured with CellTiter-Glo®, normalized  
548 to vehicle treated, DOX-untreated cells and reported as fold change. Statistics in C-ii  
549 and C-iii are reported as per one-way ANOVA test with Dunnett's test correction for

550 multiple comparisons between DOX-treated conditions with and without programmed  
551 cell death inhibitors. Data are shown as mean  $\pm$  SD.

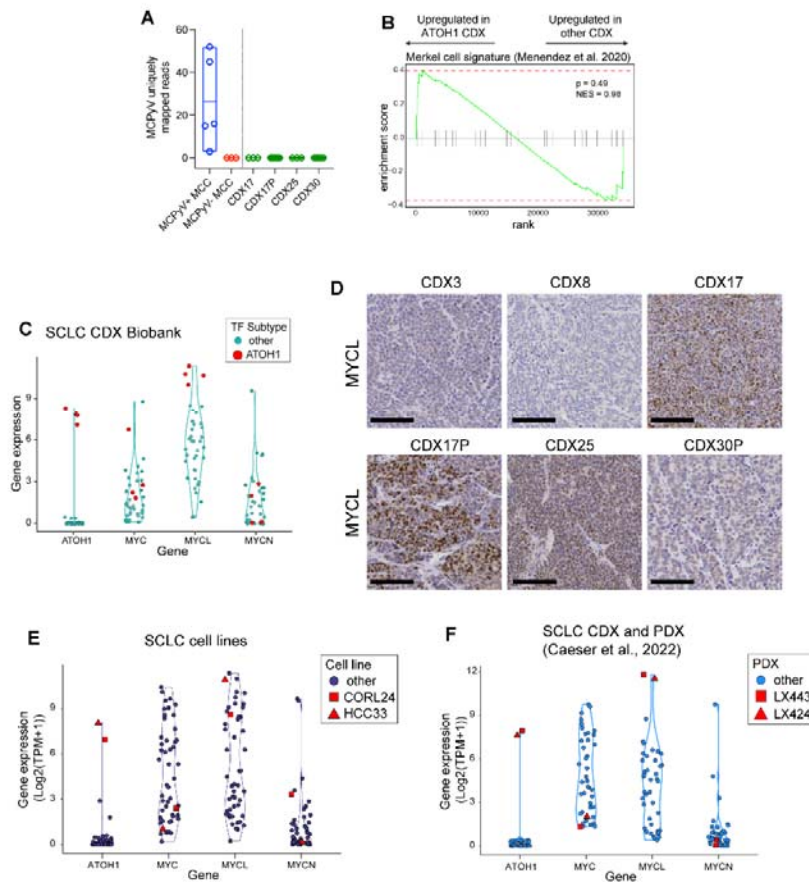
Figure 6



552 **Figure 6. ATOH1 depletion decreases tumour growth kinetics and metastasis**  
553 ***in vivo*.** (A) *In vivo* study design to investigate subcutaneous (s.c.) tumour growth  
554 and metastasis after s.c. tumour resection. CDX17P ShRen and ShATOH1#3  
555 (ShATOH1) were injected s.c. in NSG mice and left for 19 days to allow for tumour  
556 establishment. After 19 days, mice were fed either standard diet (control arms, N=3)  
557 or DOX-supplemented feed (experimental arms, N=15) and s.c. tumour growth was  
558 assessed. S.c. tumours were surgically resected when at 500-800 mm<sup>3</sup> to allow for  
559 metastatic dissemination and mice were kept on study for 28 days or until s.c.  
560 tumour reached maximum size, whichever came first. (B-i) S.c. tumour growth  
561 curves, from day of first tumour measurement to s.c. tumour resection (see  
562 methods), of mice implanted with ShRen and ShATOH1 and fed DOX-supplemented  
563 diet. Key: black, ShRen fed DOX-diet; red, ShATOH1#3 fed DOX-diet. N=15 mice  
564 per cohort; data reported as mean  $\pm$  SD. Dotted lines indicate when tumours from  
565 each cohort reached 500 mm<sup>3</sup>: ShRen, 14  $\pm$  3 days; ShATOH1, 21  $\pm$  5 days. (B-ii)  
566 Quantification of the slope of tumour growth curves in B. Key: same as in B; shades  
567 of grey for control cohort fed standard diet for the duration of the study. P values  
568 were calculated with ANCOVA test and slope of the curve was reported as mean  $\pm$   
569 SD for each cohort. (C) Kaplan-Meier curve of time to surgical resection of s.c.  
570 tumour or maximum 800 mm<sup>3</sup> for inoperable tumours. Control arms, fed a standard  
571 diet, reported in scales of grey. P values were calculated with Log-rank Mantel-Cox  
572 test. (D) Quantification of metastatic dissemination to the liver in N=3 mice fed  
573 standard diet, N=5 ShRen- and N=15 ShATOH1-tumour bearing mice fed DOX-diet  
574 that underwent surgical resection of s.c. tumour and survived on study for at least 22  
575 days after resection. Data is shown as percentage of animals displaying metastatic  
576 dissemination (disseminated tumour cells and micro/macro-metastases, in red) or no  
577 metastatic dissemination in the liver (blue). Metastases were identified based on  
578 human mitochondria staining. (E-i) Representative images of human mitochondria,  
579 GFP and ATOH1 IHC staining in liver from ShRen DOX-fed and ShATOH1#3 DOX-  
580 fed cohort. Scale bars: 200  $\mu$ m for human mitochondria and GFP; 100  $\mu$ m for  
581 ATOH1. (E-ii, E-iii) Quantification of GFP (E-ii) and ATOH1 (E-iii) IHC staining in  
582 metastases from N=2 DOX-untreated ShRen, N=3 DOX-untreated ShATOH1#3,  
583 N=4 ShRen DOX-fed, N=6 ShATOH1#3 DOX-fed mice. Data are shown as  
584 geometric mean  $\pm$  geometric SD. P values are reported as per two-tailed unpaired

585 Mann Whitney U test. (F) *In vivo* study design to investigate development of  
586 metastasis following intracardiac implantation. Prior to cell implantation, ATOH1  
587 depletion was induced by DOX treatment for 4 days *in vitro*, followed by sorting GFP-  
588 positive, viable cells by flow cytometry. Untreated control cells were sorted  
589 exclusively for viable cells. Animals in the DOX treatment cohorts were fed a DOX-  
590 supplemented diet 24 hours prior to implantation and they were kept on that diet until  
591 endpoint. Animals in the uninduced control groups were given a standard diet.  
592 Animals from all 4 cohorts (ShRen +/- DOX and ShATOH1 +/- DOX) were removed  
593 at the onset of symptoms (i.e., distended abdomen, detailed in methods) or after 70  
594 days. (G) Kaplan-Meier curve of time to sacrifice. Control cohorts, fed a standard  
595 diet, reported in scales of grey. P values were calculated with Log-rank Mantel-Cox  
596 test. (H) Quantification of metastatic dissemination to the liver for each cohort. Data  
597 is shown as per Figure 6D. (I) Quantification of metastatic cells in the liver for each  
598 cohort. Metastatic cells were identified based on human mitochondria staining. Data  
599 shown as mean  $\pm$  SD. P values were calculated with a two-tailed unpaired Mann  
600 Whitney U test. (J) Quantification of GFP (J-i) and ATOH1 (J-ii) IHC staining in  
601 metastases from N=5 DOX-untreated ShRen, N=5 DOX-untreated ShATOH1, N=5  
602 ShRen DOX-fed, N=1 ShATOH1#3 DOX-fed mice. Data are shown as geometric  
603 mean  $\pm$  geometric SD. No statistical test could be performed as ShATOH1 contained  
604 only one value.

## Supplementary figure 1 - relative to Figure 1

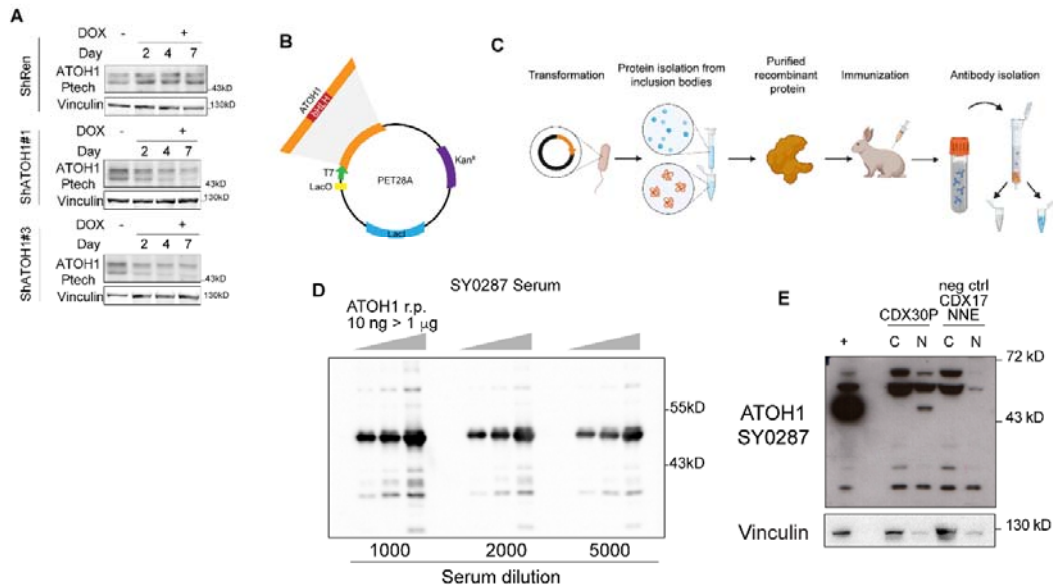


605 **Figure S1. ATOH1 CDX do not have MCC origin and present high expression of**  
 606 **MYCL. Relative to Figure 1.** (A) Detection of Merkel cell polyoma virus (MCPyV)  
 607 transcripts in positive and negative control human Merkel cell carcinoma (MCC)  
 608 samples (PRJNA775071) and ATOH1 CDX. (B) Gene set enrichment analysis  
 609 (GSEA) for a Merkel cell gene signature from Menendez et al.<sup>35</sup> in ATOH1 CDX  
 610 (N=4) compared to the whole biobank (N=35). GSEA was performed with Fgsea<sup>103</sup>.  
 611 (C) Violin plot of expression of indicated *MYC* family genes in the SCLC CDX  
 612 biobank (N=39). ATOH1 subtype samples and preclinical models highlighted in red.  
 613 (D) Representative IHC images for MYCL in SCLC-A CDX3, SCLC-N CDX8 and  
 614 ATOH1 CDX CDX17, 17P, 25 and 30P. (E-F) Violin plot of expression of indicated  
 615 *MYC* family genes in SCLC cell lines<sup>42</sup> (E) and SCLC PDX<sup>32</sup> (F) from publicly



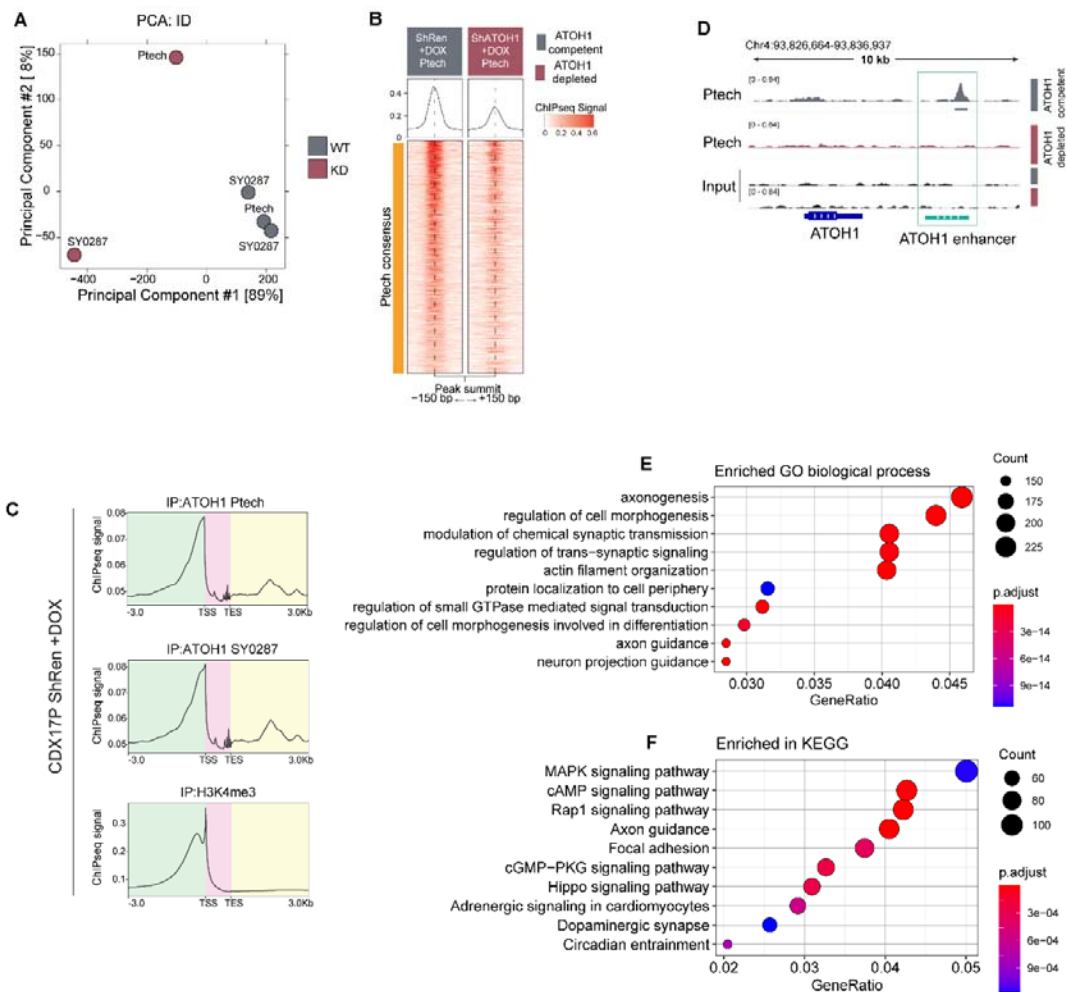
616 available datasets. ATOH1 subtype preclinical models highlighted in red and  
617 annotated by shape as in legend.

## Supplementary figure 2 - relative to Figure 3



618 **Figure S2. ATOH1 antibody production and validation. Relative to Figure 2.** (A)  
619 Western blot showing ATOH1 expression detected by the Ptech antibody over a  
620 time-course (0 to 7 days) of ATOH1 knockdown (KD) induction with doxycycline  
621 (DOX) in CDX17P. ShRen served as control for ATOH1 KD and Vinculin served as  
622 loading control. Western blots are representative of N=2 independent experiments.  
623 (B) Schematic of plasmid construct to express ATOH1 recombinant protein in IPTG-  
624 inducible PET28A system. (C) Workflow to produce the in-house antibody: ATOH1  
625 recombinant protein was purified from bacterial culture and used for immunization of  
626 one rabbit. Polyclonal antibodies were isolated from final bleed serum by affinity  
627 purification. (D) Test of SY0287 serum before affinity purification against increasing  
628 amounts of ATOH1 recombinant protein (10 ng, 100 ng and 1 μg) by western blot.  
629 (E) Validation of ATOH1 detection by nuclear (N) and cytoplasmic (C) fractionation of  
630 CDX30P (positive control) and CDX17 Non-NE cells (Negative control). Transient  
631 ATOH1 overexpression in LentiX 293T cells (indicated as +) served as positive  
632 control for detection.

## Supplementary figure 3 - relative to Figure 3

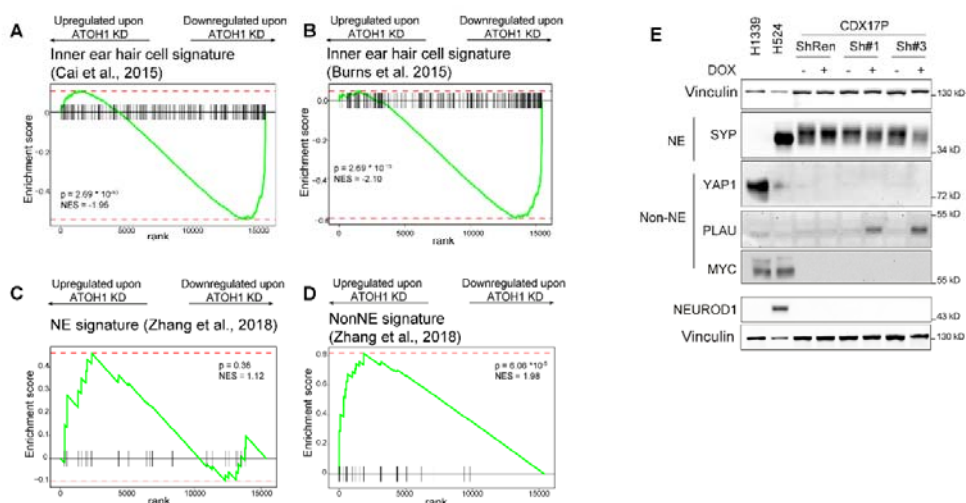


633 **Figure S3. ChIP-Seq samples cluster based on ATOH1 competency and ATOH1**  
 634 **binds to its own enhancer. Relative to Figure 2.** (A) Principal component analysis  
 635 (PCA) of ChIP-Seq samples where ATOH1 competent samples (grey, WT) cluster  
 636 together and away from ATOH1-depleted samples (red, KD). (B) Heatmap of ChIP-  
 637 Seq signal for consensus peak sets of Ptech in ATOH1 competent (grey) and  
 638 depleted (red) CDX17P, generated with the generateEnrichedHeatmap function  
 639 within profileplyr v1.8.1<sup>100</sup>. (C) Metagene analysis of ATOH1 (detected with Ptech  
 640 and SY0287) and H3K4me3 ChIP-Seq signal generated with deepTools<sup>104</sup>. Key:  
 641 green, upstream of gene body; pink, gene body; yellow, downstream of gene body.  
 642 (D) ATOH1 binding peaks at ATOH1 locus as detected by the Ptech antibody at the  
 643 ATOH1 downstream enhancer (light green), which are lost upon ATOH1 depletion.



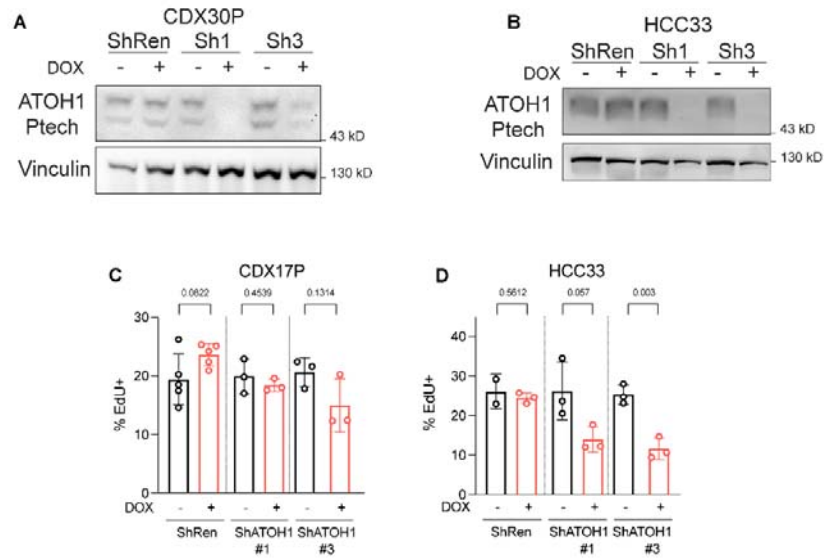
644 The peaks were visualized with the Integrated Genomics Viewer genome browser.  
 645 (E-F) Gene ontology (GO) biological process (E) and KEGG (F) enrichment analysis  
 646 of differentially bound ATOH1 peaks identified Figure 3C-i. Analysis was performed  
 647 with gage<sup>105</sup>.

### Supplementary figure 4 - relative to Figure 4



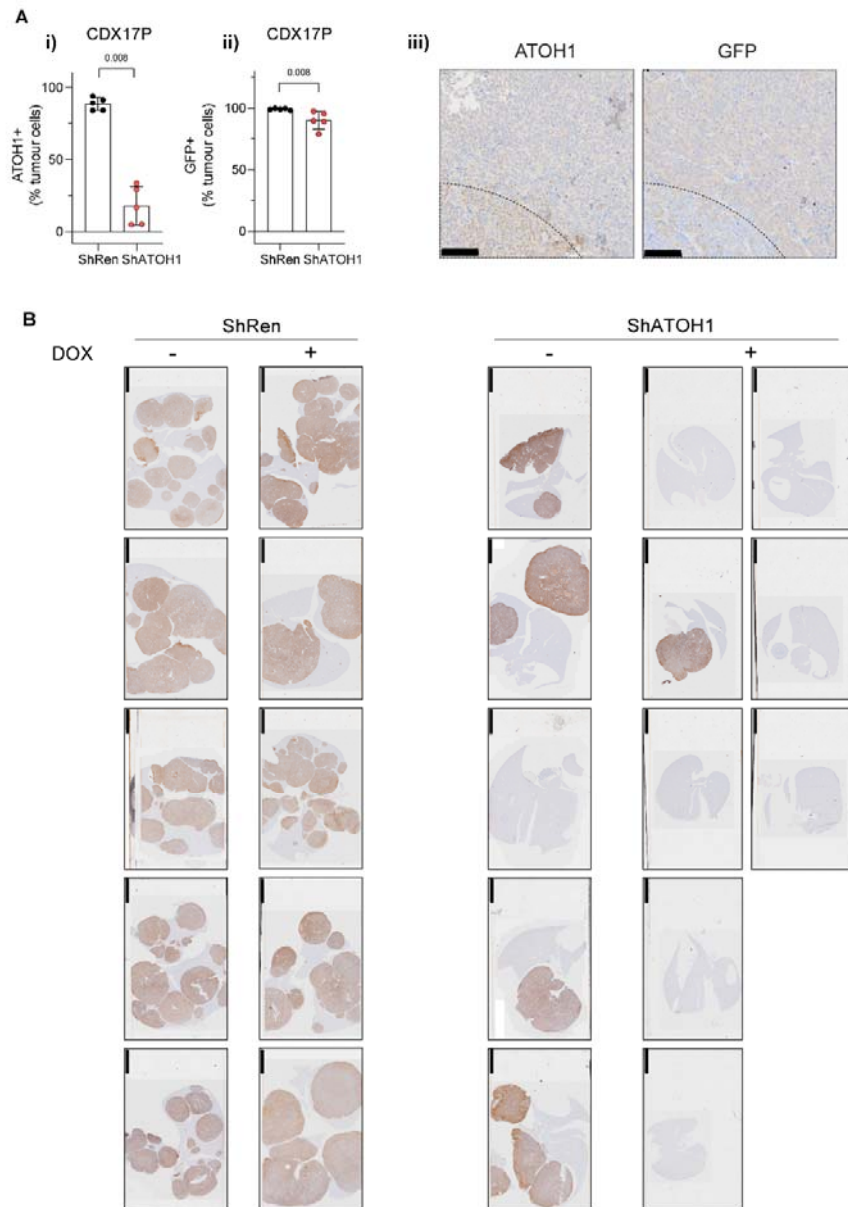
648 **Figure S4. ATOH1 direct targets identified in CDX17P are upregulated in**  
 649 **ATOH1 CDX. Relative to Figure 4.** (A-B) Gene set enrichment analysis (GSEA) for  
 650 inner ear hair cell gene signatures obtained from ref<sup>51</sup> (A) and ref<sup>52</sup> (B) upon ATOH1  
 651 depletion in CDX17P, performed with Fgsea<sup>103</sup>. (C-D) GSEA for NE (C) and Non-NE  
 652 (D) gene signatures obtained from ref<sup>106</sup>. NES: normalized enrichment score. (E)  
 653 Western blot expression of NE marker SYP and NonNE markers YAP1, MYC and  
 654 PLAU after 14 days of ATOH1 knockdown (KD) induction with doxycycline (DOX) in  
 655 CDX17P. ShRen served as control for ATOH1 KD; H1339 and H524 served as  
 656 positive controls for expression of YAP1 and MYC; Vinculin served as loading  
 657 control. Western blots are representative of N=2 independent experiments.

## Supplementary figure 5 - relative to Figure 5



658 **Figure S5. ATOH1 knockdown in CDX17P, CDX30P and HCC33. Relative to**  
659 **Figure 4.** (A-B) Representative western blot for ATOH1 in CDX30P (A) and HCC33  
660 (B) cells transduced with ShRenilla (ShRen) and ShATOH1#1 and #3 and treated  
661 with DOX for 7 days. (C-D) Bar plot of percentage of cells in S phase, as identified  
662 by EdU incorporation, in CDX17P (C) and HCC33 (D) upon ATOH1 depletion.  
663 Statistics are reported as two-tailed unpaired *t* test between DOX untreated and  
664 treated condition.

## Supplementary figure 6 - relative to Figure 6



665 **Figure S6. Heterogeneous GFP and ATOH1 expression in ATOH1 KD**  
666 **subcutaneous tumours. ATOH1 KD cells exhibit reduced metastatic ability.**  
667 **Relative to Figure 6. (A) Quantification of ATOH1 (A-i) and GFP (A-ii) IHC staining**  
668 **in N=5 subcutaneous tumours from mice implanted with either ShRen or ShATOH1**  
669 **cells and fed DOX-supplemented diet. KD cohort highlighted in red. Statistics**  
670 **reported as per two-tailed unpaired Mann Whitney U test. (A-iii) Representative**  
671 **images of ATOH1 and GFP IHC staining in consecutive sections highlighting parts of**

672 tumours negative for GFP and positive for ATOH1 (dotted lines). Scale bars: 100  
673  $\mu\text{m}$ . (B) IHC staining of human mitochondria in livers from animals that underwent  
674 intracardiac implantation of ShRen cells and fed a standard diet (-DOX, N=5) or a  
675 DOX-supplemented diet (+ DOX, N=5) or ShATOH1 cells and fed a standard diet (-  
676 DOX, N=5) or a DOX-supplemented diet (+DOX, N=8). Only one animal in the  
677 ATOH1 KD cohort developed metastasis in the liver. Scale bars: 5  $\mu\text{m}$ .

678

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