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1 **The EMT-activator Zeb1 is a key factor for cell plasticity and promotes metastasis in**
2 **pancreatic cancer**

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34 **ABSTRACT**

35 Metastasis is the major cause of cancer-associated death. Partial activation of the epithelial-
36 to-mesenchymal transition (partial EMT) program was considered a major driver of tumour
37 progression from initiation to metastasis. However, the role of EMT in promoting metastasis
38 was recently challenged, in particular concerning effects of the Snail and Twist EMT
39 transcription factors (EMT-TFs) in pancreatic cancer. In contrast, we show here that in the
40 same pancreatic cancer model driven by Pdx1-cre-mediated activation of mutant *Kras* and
41 *p53* (KPC-model) the EMT-TF Zeb1 is a key factor for the formation of precursor lesions,
42 invasion and notably metastasis. Depletion of *Zeb1* suppresses stemness, colonisation
43 capacity and particularly phenotypic/metabolic plasticity of tumour cells, likely causing the
44 observed *in vivo* effects. Accordingly we conclude that different EMT-TFs have
45 complementary and tissue-specific sub-functions in driving tumours towards metastasis.
46 Consequently, therapeutic strategies directed at EMT-TFs, should consider such specificities
47 and target those factors simultaneously.

48

49 Metastasis is still the major cause of cancer-associated death. Partial activation of the
50 embryonic epithelial-to-mesenchymal transition (partial EMT) program was considered as a
51 major driver of tumour progression from initiation to metastasis¹⁻³. Most of the studies
52 involved manipulation of different EMT-inducing transcription factors (EMT-TFs), such as
53 Snail, Slug, Twist and ZEB1 in cell-culture or xenograft mouse models. Particularly, the EMT
54 activator ZEB1 was shown to be important for tumourigenicity and metastasis, by triggering
55 combined activation of cell motility and stemness properties⁴⁻⁶. However, the role of EMT in
56 invasion and metastasis was challenged by two recent publications using genetic mouse
57 models for breast and pancreatic cancer^{7, 8}. Particularly, genetic depletion of the EMT-
58 activators *Snai1* or *Twist1* had no effect on tumour initiation, invasion or metastasis in
59 pancreatic cancer (PDAC) driven by Pdx1-cre-mediated activation of mutant *Kras* and *p53*
60 (KPC-model)⁸. Therefore the authors claimed that EMT is dispensable for metastasis.
61 We here used the same KPC-mouse model for pancreatic cancer and conditionally ablated
62 the EMT-activator *Zeb1* in tumour cells. In contrast to *Snai1* and *Twist1*, depletion of *Zeb1*
63 strongly affected formation of precursor lesions, tumour grading, invasion and notably
64 metastasis during PDAC progression. In summary we conclude that EMT is important for
65 metastasis, but there is considerable variability and tissue specificity (and not redundancy) in
66 the role and function of different EMT-TFs.

67

68 **RESULTS**

69 ***Zeb1* depletion reduces grading, invasion and distant metastasis in PDAC**

70 KPC-mice develop metastatic pancreatic cancers with an almost 100% penetrance⁹. Of note,
71 a fraction of cancer cells and cells in precursor lesions (PanINs) express the EMT-TF Zeb1,
72 which was considered to be important for disease progression¹⁰, which we could confirm
73 (Supplementary Fig. 1a and b). To prove the role of Zeb1 in the progression towards
74 metastasis, we generated a conditional knockout allele of *Zeb1* (*Zeb1^{fl}*) (Fig.1a). Cre-

75 mediated zygotic deletion of *Zeb1* phenocopied the described developmental defects of a
76 conventional *Zeb1* knockout¹¹, thereby confirming its loss-of function¹². We crossed the
77 floxed *Zeb1* allele homozygously into KPC mice (*Pdx1-cre;Kras^{LSL.G12D/+};Tp53^{LSL.R172H/+}*) to
78 generate KPC;*Zeb1^{fl/fl}* mice (termed KPCZ) (Fig. 1a). Progeny were born in expected ratios
79 and showed no obvious functional defects of the pancreas. Like KPC mice, all KPCZ mice
80 developed pancreatic cancer. Notably, no significant differences to KPC were detected for a
81 heterozygous *Zeb1* loss (KPC;*Zeb1^{fl/+}*) (KPCz) (Supplementary Fig. 1c), therefore KPCz
82 mice were merged with *Zeb1* wild type genotypes (KPC) for all analyses. Loss of *Zeb1*
83 expression in KPCZ tumour cells was confirmed by immunohistochemistry (Supplementary
84 Fig. 1b and 2). It was associated with a reduced expression of the EMT activators *Zeb2*,
85 *Slug*, and tentatively also *Snail*, but the expression frequency of *Twist* was maintained
86 (Supplementary Fig. 3a). Depletion of *Zeb1* did not delay the onset and only insignificantly
87 reduced the growth rate of primary tumours (Fig. 1b). In line with this, the number of Ki67⁺
88 proliferating tumour cells, as well as the spontaneous apoptotic rate and the blood vessel
89 density did not significantly differ (Supplementary Fig. 2). However, *Zeb1* deletion strongly
90 influenced tumour differentiation. Whereas KPC tumours were often high grade and showed
91 a high intra- and intertumourous heterogeneity, the number of high-grade tumours in KPCZ
92 animals was strongly reduced and the tumours displayed homogenous, mostly differentiated
93 phenotypes (Fig. 1c,d, and Supplementary Fig. 1b and 2). Better differentiation was also
94 associated with a significantly higher *Gata6* expression (Supplementary Fig. 3b), which is a
95 marker for higher differentiation and better clinical prognosis of human PDAC¹³. KPCZ mice
96 showed an increased deposition of extracellular matrix (Supplementary Fig. 2). Future work
97 will address this aspect, since the different composition of the stroma in pancreatic cancer
98 can be associated with increased^{14, 15} or reduced^{16, 17} aggressiveness.

99 Next we analysed whether depletion of *Zeb1* affects malignant tumour progression. Primary
100 KPCZ tumours showed markedly lower local invasion (Fig. 1d). Of note, differentiated KPC

101 tumours also often underwent a de-differentiation associated with upregulation of *Zeb1*
102 expression in invasive tumour cells. This was not detected in KPCZ tumours, a first sign for
103 reduced plasticity in *Zeb1*-depleted cancer cells (Fig. 1e). A major finding was that the
104 capacity for distant metastasis was strongly reduced in KPCZ tumours (Fig. 1f,
105 Supplementary Table 1). Thereby the corresponding metastases showed a histology and
106 *Zeb1* expression state similar to that of the primary tumor (Fig. 1g and Supplementary Fig.
107 3c). In summary, *Zeb1* depletion strongly reduced progression towards highly malignant,
108 metastatic pancreatic tumours. This is in stark contrast to depletion of *Snai1* or *Twist1* in the
109 same model, which did not affect malignant tumour progression⁸.

110

111 ***Zeb1* depletion reduces stemness, tumourigenic and colonisation capacities**

112 To further investigate the consequences of *Zeb1* depletion, we isolated primary tumour cells
113 from KPC and KPCZ mice. In agreement with the strong heterogeneity of the KPC primary
114 tumours, corresponding tumour cells displayed highly variable phenotypes from
115 mesenchymal, to mixed and epithelial. This was evident from the growth patterns, as well as
116 the expression of epithelial and mesenchymal marker genes (Fig. 2a-d and Supplementary
117 Fig. 4a). In contrast all tumour lines derived from KPCZ mice were fixed in an epithelial state
118 with strongly reduced mesenchymal gene expression. However, despite the strong
119 phenotypical differences between KPC and KPCZ-derived cancer cell lines, we detected no
120 consistent difference in proliferation (Fig. 2e). Accordingly, the sensitivity to the
121 chemotherapeutic agent gemcitabine, which targets proliferating cells, was variable, but not
122 consistently changed between KPC and KPCZ cancer cells. This was also the case for two
123 pancreatic cancer cell lines isolated from KPC tumours with depletion of *Snai1* (KPCS)
124 (Supplementary Fig. 4b). KPCZ cells were tentatively more resistant to the EGFR inhibitor
125 erlotinib, but we did not detect a significant difference between KPC and KPCS cells. Upon
126 s.c. grafting into syngeneic mice at high injection dose (1×10^5 cells), all KPC and KPCZ cell

127 lines gave rise to tumours mimicking the differentiation state of the cell line and the growth
128 pattern of the corresponding primary tumour, supporting the *in vitro* data on differentiation
129 and proliferation (Supplementary Fig. 4a,c,d).

130 Strikingly, although all tumour cell lines did not show significant changes in proliferation and
131 were able to grow subcutaneously, the lung colonisation capacity after intravenous injection
132 was almost completely eradicated for all KPCZ cell lines (Fig. 3a). This was not due to
133 differences in the capability to reach the lung, since there was no significant reduction of
134 disseminated cancer cells in the lung (Fig. 3b and Supplementary Fig. 5a). Notably, in
135 comparison to KPCZ lines, genetic depletion of *Snai1* (KPCS cells) had no effect on lung
136 colonisation capacity (Fig. 3c), confirming data by Zheng et al.⁸. This goes along with
137 considerably high, albeit varying levels of Zeb1 expression in the KPCS lines, which might
138 explain the maintained colonisation capacity. The relevance of Zeb1 expression even at
139 reduced levels was further demonstrated in KPC cells after partial depletion of Zeb1 to 30-
140 50% of the original levels, which did not significantly affect the lung colonisation capacity
141 (Fig. 3d).

142 Since crucial traits for distant colonisation include stemness and tumourigenicity, we tested
143 these features. Tumourigenicity of the cell lines was significantly reduced in KPCZ cell lines,
144 particularly when compared to the KPC cell lines with a similar epithelial phenotype
145 (Supplementary Fig. 5b). Interestingly within the KPC cell lines the epithelial differentiated
146 cells had a higher tumourigenic capacity compared to mesenchymal type cell lines. This is in
147 agreement with data showing that the plasticity of re-epithelialisation is important to some
148 degree for tumourigenic and colonisation capacity and that non-plastic mesenchymal cells do
149 not efficiently metastasize¹⁸⁻²⁰. In addition, depletion of *Zeb1* almost completely reduced the
150 sphere forming capacity, a surrogate test for stemness competence (Fig. 3e and
151 Supplementary Fig. 5c). Analysis of established marker combinations²¹ for human pancreatic
152 cancer stem cells displayed no significant differences for CD24/CD44 and CD133. Epcam,

153 another marker was not applicable, since it is a direct target of *Zeb1* repression²² and thus
154 strongly upregulated in KPCZ cells (Supplementary Fig. 5d). This is in line with data showing
155 that human PDAC stemness markers are not applicable in the KPC model²³. However, the
156 stem cell marker *Sox2* turned out to be completely absent in KPCZ cell lines and s.c. grafted
157 tumours in comparison to KPC cell lines (Fig. 3f,g). Strongly reduced *Sox2* expression upon
158 *Zeb1* depletion was also reflected in the primary KPC tumours (Supplementary Fig. 2). *Sox2*
159 expression was proposed to be stabilized by *Zeb1*, through its reciprocal feedback loop with
160 miR-200 family members²⁴. We confirmed this hypothesis by showing that miR-200c, which
161 is strongly upregulated in KPCZ cell lines (Fig. 2c), suppressed both *Zeb1* and *Sox2*
162 expression in KPC cell lines (Fig. 3h). These data are of particular relevance since *Sox2*
163 expression is enhanced in aggressive subtypes of human PDACs²⁵⁻²⁷. Together our data
164 indicate that *Zeb1* increases the tumourigenic capacity and is crucial for colonisation of
165 distant organs. Moreover, depletion of *Zeb1* is again in stark contrast to a depletion of *Snai1*
166 or *Twist1*, which did not affect the tumourigenic and colonisation capacity.

167 According to this data we wondered, why we did not see an effect on the primary tumour-free
168 survival in KPCZ mice (Fig. 1b). It is known that mutant *p53* boosts tumour progression by
169 inducing a mutator phenotype^{28, 29}. In addition it was shown that mutant *p53* overcomes a
170 growth arrest in pancreatic cancer³⁰. Thus we speculated that once a precursor lesion is
171 formed, the progression towards a highly proliferating tumour is too fast to detect changes in
172 the initial tumourigenic capacity. Therefore we analysed mutant *Kras* mice without the *p53*
173 mutant allele (*Pdx1-cre;Kras^{LSL.G12D/+}*, termed KC). These mice develop slowly progressing
174 acinar-ductal metaplasia (ADM)- as well as PanIN-precursor lesions, which also express
175 *Zeb1*¹⁰. In contrast to KPCZ, KC mice with homozygous deletion of *Zeb1* (termed KCZ)
176 showed a strongly reduced number and grading of PanIN and ADM lesions (Fig. 4a,b and
177 Supplementary Fig. 6a). This data further indicates that *Zeb1* triggers the tumourigenic
178 capacity in pancreatic cancer from initial development till late stage metastasis.

179

180 **Zeb1 is crucial for cancer cell plasticity**

181 Zeb1 does not affect expression of single genes or small gene clusters but thousands of
182 genes, leading to a complete reprogramming of cells³¹ and we have shown that Zeb1 exerts
183 pleiotropic effects on many different programs and pathways³¹⁻³³. Therefore we performed a
184 global gene expression analysis to examine the impact of Zeb1 on cell plasticity. A principal
185 component analysis (PCA) showed a clear separation of KPC- and KPCZ-cell lines and a
186 separation of the epithelial and mesenchymal phenotype along the first (PC1) and second
187 principal component (PC2), respectively (Fig. 5a). The latter verified the initial findings that a
188 depletion of *Zeb1* fixes the cells in a homogenous epithelial state, indicating that Zeb1 is a
189 critical factor underlying cell heterogeneity and potentially also plasticity. In line with the PCA,
190 a gene set enrichment analysis (GSEA) confirmed that *Zeb1* depletion shifts the cells
191 towards an epithelial phenotype (Supplementary Fig. 6b). Moreover, loss of *Zeb1* expression
192 enriches for genes associated with addiction to *Kras* expression³⁴, reduced metastatic
193 competence³⁵, as well as the “classical” subtype of human PDACs, which have the best
194 clinical prognosis³⁶ (Fig. 5b). We further analysed the expression of genes strongly
195 associated with metastatic progression, including *Pdgfrb*, which is essential to drive
196 metastasis in pancreatic cancer together with mutant p53³⁷. All of the analysed genes were
197 expressed in KPC cell lines, but strongly downregulated upon *Zeb1* depletion (Fig. 5c).
198 However, in agreement with the heterogeneous phenotypes, these pro-metastatic genes
199 were expressed only at low levels in KPC tumour cells with epithelial differentiation, although
200 these cell lines had the highest lung colonisation capacity. We hypothesized that epithelial
201 KPC cells possess enough plasticity to adapt their gene expression.
202 Enhanced plasticity of cancer cells is considered an important driving force of malignant
203 tumour progression by allowing continuous adaptations to the demanding conditions in the
204 changing tumour environment^{1, 38, 39}. We have previously demonstrated that ZEB1,

205 particularly through its feedback loop with miR-200 family members, is a motor of cellular
206 plasticity in response to extracellular cues⁴. Thus, we hypothesized that the presence of
207 Zeb1 allows adaptations of gene expression patterns and that loss of cellular plasticity is an
208 important consequence of *Zeb1* depletion in cancer cells. We tested this hypothesis by
209 treating KPCZ cells with TGFβ1, a driver of malignant tumour progression and prominent
210 inducers of EMT^{40, 41}. As expected, upon TGFβ treatment KPC cells with an epithelial
211 phenotype underwent an EMT. However, even after long-term TGFβ treatment KPCZ cells
212 maintained their epithelial phenotype (Fig. 6a,b and Supplementary Fig. 7a). Thus without
213 Zeb1, the cells were locked in their phenotypic state and lost plasticity. Loss of plasticity was
214 also reflected in TGFβ-induced changes in global gene expression, where in contrast to KPC
215 cell lines with an epithelial phenotype, the epithelial KPCZ cell lines displayed a strongly
216 reduced responsiveness to TGFβ (Fig. 6c). The PCA showed an induction of a mesenchymal
217 phenotype only of the KPC cell lines under TGFβ stimulation along the first principal
218 component (PC1). Among the 20,052 analysed genes, 1514 were significantly regulated
219 upon long-term TGFβ treatment (Fig. 6c and Supplementary Table 2), however, 1,377 (91%)
220 of them depended on the genetic presence of *Zeb1*. The genes associated with metastatic
221 progression including *Pdgfrb*, which were not present in epithelial KPC cells, were also
222 upregulated by TGFβ in a Zeb1-dependent manner (Fig. 6d). These data also indicate that
223 Zeb1 is important for a large fraction of TGFβ induced changes. The Zeb1-dependent TGFβ
224 induced genes also included genes, which we recently identified as common Zeb1/Yap
225 target genes upregulated in aggressive cancer types (Supplementary Fig. 7b)³¹. The high
226 Zeb1 dependent plasticity was further indicated by the fact that Zeb1 associated phenotypic
227 and gene expression changes were reversible after withdrawal of TGFβ (Fig. 6e-g).

228 Another important aspect in cancer cell biology is metabolics. Tumour cells show a high
229 metabolic plasticity in reacting to environmental changes on their way to metastasis⁴². We
230 exemplified this by modulating the two basic energy consumption pathways: glycolysis and

231 oxidative phosphorylation (OxPhos). As measured in a mito stress test, KPCZ cells have a
232 lower basal respiration and respiration-related ATP production as indication of reduced
233 OxPhos (Fig.7a), which is also visible in a glycolysis stress test (Fig. 7b). Blocking of OxPhos
234 by oligomycin in a glycolysis stress test forces cells to exploit their glycolytic capacity for
235 fulfilling energy demands and demonstrates a considerable glycolytic reserve in KPC cells
236 (Fig. 7b). However, this glycolytic switch was no longer possible in KPCZ cells owing to a
237 complete lack of a glycolytic reserve. Thus, also the plasticity in switching between basic
238 energy pathways and adapting to different oxygen availability was strongly dependent on the
239 expression of *Zeb1*.

240 Finally, high phenotypic plasticity of epithelial KPC cells was also detected *in vivo* after
241 grafting into syngeneic mice. Although they displayed a differentiated phenotype in central
242 tumour regions, KPC tumour cells underwent a de-differentiation associated with an
243 upregulation of *Zeb1* at the invasive front. In contrast grafted KPCZ cell lines displayed no
244 phenotypic plasticity, but were fixed in their differentiated state (Fig. 7c and Supplementary
245 Fig. 7c). Altogether, the data indicate that *Zeb1* is very important for cellular plasticity in
246 cancer cells.

247

248 **DISCUSSION**

249 Here, we describe a key role for the EMT-TF *Zeb1* in the *in vivo* progression of pancreatic
250 cancer from early precursor lesions towards metastasis. Genetic depletion of *Zeb1* in the
251 pancreas reduces formation of ADM and PanIN precursor lesions, undifferentiated (high
252 grade) carcinomas, invasion and metastasis. In isolated primary cancer cell lines *Zeb1*
253 ablation leads to loss of cellular plasticity and fixation in an epithelial phenotype, a likely
254 cause of reduced stemness, tumourigenicity and colonisation capacities (Table 1).

255

256 Our data demonstrate that Zeb1 acts in strong contrast to the EMT-TFs Snail and Twist in
257 pancreatic cancer. *Snai1* or *Twist1* depletion in the same KPC-model did not affect formation
258 of PanINs, tumour differentiation, invasion, colonisation and importantly metastasis⁸. Based
259 on their results, Zheng et al. claimed that EMT is dispensable for metastasis. However, our
260 data favour a different interpretation and allow a more comprehensive picture of the effect of
261 EMT-TFs in tumours. Our results point to functional differences of EMT-TFs and demonstrate
262 that Zeb1 stimulates pancreatic tumour progression from formation of precursor lesions to
263 late stage metastasis.

264

265 What could be the critical functions of Zeb1? Its regulatory potential is not limited to effects
266 on a few crucial downstream target genes, but rather leads to a global reprogramming of
267 gene expression patterns³¹ and does not only control EMT but also other programs and
268 pathways. One of the most striking consequences of *Zeb1* depletion was the almost
269 complete inhibition of lung colonisation. We postulate two major effects of *Zeb1* inactivation
270 as the underlying molecular mechanism: the block in cellular plasticity, considered as a major
271 driving force of tumour progression towards metastasis and the reduction of stemness, a
272 crucial property underlying tumourigenicity and colonisation. Enhanced plasticity of cancer
273 cells impresses as ongoing transitions between an undifferentiated/(partial) mesenchymal
274 and a differentiated/epithelial phenotype^{1, 38, 39, 43, 44}. We here describe a central role of Zeb1
275 in exerting different aspects of cellular plasticity, particularly the response to TGF β , but also
276 to metabolic changes and changes in the *in vivo* intratumourous heterogeneity. Differentiated
277 KPC as well as KPCZ cancer cells only expressed low levels of metastasis-associated
278 genes. However, only KPC cells, but not KPCZ cells, were able to activate their expression
279 upon TGF β treatment. These genes include *Pdgfrb*, which was recently shown to be
280 absolutely required for metastasis in *p53*-mutant pancreatic cancer³⁷. As a side effect, our
281 finding that absence of *Zeb1* strongly reduces the number of TGF β -regulated genes

282 indicates that Zeb1 is important for a large part of the TGF β response (Supplementary Table
283 2). Furthermore, Zeb1-linked plasticity is exemplified by its impact on central metabolic
284 pathways. The plasticity in switching between basic energy pathways is strongly
285 compromised in *Zeb1*-depleted cells, displaying both a reduced OxPhos and reduced
286 glycolytic reserve, which might also be critical for the colonisation step. In addition *Zeb1*
287 inactivation affects stemness and tumourigenic properties, supporting the view that EMT-
288 MET dynamics also reflects the plasticity between stemness and a differentiated state^{45, 46}.
289 Particularly the strong reduction of the stem cell factor *Sox2* in KPCZ tumours and derived
290 cell lines is of high relevance, since its expression was correlated with stemness, plasticity
291 and progression in pancreatic and other cancer types²⁵⁻²⁷. Together, our data indicate that
292 Zeb1 is crucial for cellular plasticity and stemness/tumourigenic properties in pancreatic
293 cancer cells.

294

295 There are several potential reasons, why particularly Zeb1 is associated with cellular
296 plasticity. Firstly, Zeb1 is linked in a reciprocal double-negative feedback loop with members
297 of the mir-200 family, which controls a switch between an undifferentiated/stemness and a
298 differentiated phenotype⁴. Secondly, the *Zeb1* gene itself has a poised, bivalent chromatin
299 configuration, allowing a rapid switch between high expression in cancer stem cells (CSCs)
300 and low expression in non-CSCs⁴⁷. Moreover, we are beginning to understand functional
301 differences between Zeb1 and other EMT-TFs at the biochemical level. For instance, we
302 have described a direct interaction of ZEB1 with the Hippo-pathway effector YAP1, which is
303 crucial for activating a common ZEB1/YAP1 target gene set important for tumour
304 progression³¹. Genes of this target set can be activated by TGF β in epithelial KPC cells, but
305 not in KPCZ cells. Notably, as demonstrated here for Zeb1, also Yap1 was shown to be
306 important for the progression through ADM towards pancreatic carcinoma^{48, 49}.

307

308 *Zeb1*-dependent gene expression signatures also point to a clinical relevance of our findings.
309 *Zeb1* ablation associates with tumours of the 'classical subtype' of pancreatic cancer, which
310 has the best clinical prognosis compared to other subtypes^{36, 50}. These data fit to the reduced
311 aggressiveness of KPCZ tumours and further support data showing that *Zeb1* expression
312 correlates with more aggressive precursor lesions and poor outcome in human pancreatic
313 cancer^{24, 51, 52}. Moreover, KPCZ cells show enrichment of a gene signature associated with
314 KRAS-addiction. Notably, in this study absence of ZEB1 was already a determinant of
315 KRAS-dependency^{34, 53}. Thus, although KRAS bears the key mutation in pancreatic cancer⁵⁴,
316 expression of *Zeb1* might render cancer cells independent of mutant KRAS.

317

318 However, our findings also raise additional questions. Firstly, why did we not observe a
319 significant effect of *Zeb1* depletion on primary tumour-free survival in KPCZ mice (Fig. 1b)?
320 When we omitted the mutant p53 allele, *Zeb1* was critical for the formation of Kras-driven
321 ADM and PanIN lesions as its depletion strongly reduced their occurrence. Similar data were
322 recently shown in the MMTV-PyMT model of breast cancer, where Snail was important for
323 tumour initiation and progression in a p53 wild type but not p53 mutant context⁵⁵. Thus our
324 data support the hypothesis that in the context of mutant p53 the progression towards a
325 highly proliferating tumour is too fast to allow detection of changes in initial tumourigenicity.
326 Secondly, why did we detect metastases in KPCZ animals at all? The fact that *Zeb1* loss
327 reduces the metastatic competence to approximately 30% shows that *Zeb1*-associated EMT
328 and plasticity is strongly supporting metastasis. Nevertheless, it also indicates a *Zeb1*-
329 independent, albeit less efficient metastasis formation, which might include a potential partial
330 redundancy with remaining EMT-TFs, although at a significantly lower efficacy. Another
331 explanation could be different routes to metastasis, which likely cooperate with EMT-TF
332 dependent mechanisms to various extents. As already postulated, different routes may
333 emerge by acquisition of additional genetic alterations driving metastasis independent of

334 cellular plasticity-associated traits^{1, 56}. Again, mutated p53 might enhance the generation of
335 such a genetically driven metastasis³⁰. In this light, the fact that *Zeb1* depletion efficiently
336 reduces plasticity, colonisation and metastasis even in the context of mutant p53 is
337 remarkable and further supports the importance of *Zeb1* as a crucial driver of tumour
338 progression.

339

340 In conclusion we demonstrated that the EMT-TF *Zeb1* is a key driver of pancreatic tumour
341 progression from early tumourigenesis to late stage metastasis, underscoring the important
342 role of EMT-activation in these processes. By contrast, *Snail* and *Twist* were shown to be
343 dispensable for metastasis in this cancer type, indicating that EMT-TFs have specific sub-
344 functions, which are not redundant but complementary. Non-redundant sub-functions of
345 EMT-TFs were already described, e.g. for *Zeb1* and *Zeb2* in melanoma^{57, 58}, for *Snail* and
346 *Slug* in breast cancer⁵⁹, as well as for *Sox4*⁶⁰ and *Prrx1*¹⁹. Moreover sub-functions can be
347 tissue specific, as demonstrated by the different roles of *Snail* in metastasis of breast⁶¹ and
348 pancreatic cancer⁸. Consequently, therapeutic strategies directed at EMT-TFs, should
349 consider these specificities and target such factors simultaneously.

350

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361

362 **AUTHOR CONTRIBUTIONS**

363 A.M.K. planned and performed experiments and wrote the manuscript. J.M performed mouse
364 experiments. M.L.L. performed drug studies. O.S. generated the floxed Zeb1 allele. M.B. and
365 H.B. performed bioinformatics analyses. M.B. and D.M. performed metabolic tests. W.R.
366 performed MRI analyses. P.B. performed histological analyses. V.G.B. established mouse
367 models. C.P. generated cell lines. T.H.W. performed mouse experiments. S.B. generated the
368 floxed Zeb1 allele, planned and performed experiments. M.P.S. generated the floxed Zeb1
369 allele, planned and performed mouse experiments, was involved in coordination and wrote
370 the manuscript. T.B. planned and coordinated the project, analysed data and wrote the
371 manuscript.

372

373 **COMPETING FINANCIAL INTEREST**

374 The authors declare no competing financial interest.

375

376 **REFERENCES**

- 377 1. Brabletz, T. To differentiate or not — routes towards metastasis. *Nat Rev Cancer*
378 **12**, 425-436 (2012).
- 379 2. Kalluri, R. & Weinberg, R.A. The basics of epithelial-mesenchymal transition. *J*
380 *Clin Invest* **119**, 1420-1428 (2009).
- 381 3. Nieto, M.A., Huang, Ruby Y.-J., Jackson, Rebecca A. & Thiery, Jean P. EMT:
382 2016. *Cell* **166**, 21-45 (2016).
- 383 4. Brabletz, S. & Brabletz, T. The ZEB/miR-200 feedback loop--a motor of cellular
384 plasticity in development and cancer? *EMBO Rep* **11**, 670-677 (2010).
- 385 5. Vandewalle, C., Van Roy, F. & Berx, G. The role of the ZEB family of
386 transcription factors in development and disease. *Cell Mol Life Sci* **66**, 773-787
387 (2009).
- 388 6. Zhang, P., Sun, Y. & Ma, L. ZEB1: at the crossroads of epithelial-mesenchymal
389 transition, metastasis and therapy resistance. *Cell Cycle* **14**, 481-487 (2015).
- 390 7. Fischer, K.R. *et al.* Epithelial-to-mesenchymal transition is not required for lung
391 metastasis but contributes to chemoresistance. *Nature* **527**, 472-476 (2015).

- 392 8. Zheng, X. *et al.* Epithelial-to-mesenchymal transition is dispensable for
393 metastasis but induces chemoresistance in pancreatic cancer. *Nature* **527**, 525-
394 530 (2015).
- 395 9. Hingorani, S.R. *et al.* Trp53R172H and KrasG12D cooperate to promote
396 chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma
397 in mice. *Cancer Cell* **7**, 469-483 (2005).
- 398 10. Rhim, Andrew D. *et al.* EMT and Dissemination Precede Pancreatic Tumor
399 Formation. *Cell* **148**, 349-361 (2012).
- 400 11. Higashi, Y. *et al.* Impairment of T Cell Development in deltaEF1 Mutant Mice.
401 *The Journal of Experimental Medicine* **185**, 1467-1480 (1997).
- 402 12. Brabletz, S. *et al.* Generation and characterization of mice for conditional
403 inactivation of Zeb1. *Genesis*, doi: 10.1002/dvg.23024 (2017).
- 404 13. Martinelli, P. *et al.* GATA6 regulates EMT and tumour dissemination, and is a
405 marker of response to adjuvant chemotherapy in pancreatic cancer. *Gut*, doi:
406 10.1136/gutjnl-2015-311256. (2016).
- 407 14. Laklai, H. *et al.* Genotype tunes pancreatic ductal adenocarcinoma tissue tension
408 to induce matricellular fibrosis and tumor progression. *Nat Med* **22**, 497-505
409 (2016).
- 410 15. Erkan, M. *et al.* The Activated Stroma Index Is a Novel and Independent
411 Prognostic Marker in Pancreatic Ductal Adenocarcinoma. *Clinical*
412 *Gastroenterology and Hepatology* **6**, 1155-1161 (2008).
- 413 16. Özdemir, Berna C. *et al.* Depletion of Carcinoma-Associated Fibroblasts and
414 Fibrosis Induces Immunosuppression and Accelerates Pancreas Cancer with
415 Reduced Survival. *Cancer Cell* **25**, 719-734 (2014).
- 416 17. Rhim, Andrew D. *et al.* Stromal Elements Act to Restrain, Rather Than Support,
417 Pancreatic Ductal Adenocarcinoma. *Cancer Cell* **25**, 735-747 (2014).
- 418 18. Tsai, Jeff H., Donaher, Joana L., Murphy, Danielle A., Chau, S. & Yang, J.
419 Spatiotemporal Regulation of Epithelial-Mesenchymal Transition Is Essential for
420 Squamous Cell Carcinoma Metastasis. *Cancer Cell* **22**, 725-736 (2012).
- 421 19. Ocaña, Oscar H. *et al.* Metastatic Colonization Requires the Repression of the
422 Epithelial-Mesenchymal Transition Inducer Prrx1. *Cancer Cell* **22**, 709-724
423 (2012).
- 424 20. Korpala, M. *et al.* Direct targeting of Sec23a by miR-200s influences cancer cell
425 secretome and promotes metastatic colonization. *Nat Med* **17**, 1101-1108 (2011).
- 426 21. Raj, D., Aicher, A. & Heeschen, C. Concise Review: Stem Cells in Pancreatic
427 Cancer: From Concept to Translation. *STEM CELLS* **33**, 2893-2902 (2015).
- 428 22. Vannier, C., Mock, K., Brabletz, T. & Driever, W. Zeb1 Regulates E-cadherin and
429 Epcam (Epithelial Cell Adhesion Molecule) Expression to Control Cell Behavior in
430 Early Zebrafish Development. *Journal of Biological Chemistry* **288**, 18643-18659
431 (2013).
- 432 23. Dosch, J.S., Ziemke, E.K., Shettigar, A., Rehemtulla, A. & Sebolt-Leopold, J.S.
433 Cancer Stem Cell Marker Phenotypes Are Reversible and Functionally
434 Homogeneous in a Preclinical Model of Pancreatic Cancer. *Cancer Research* **75**,
435 4582-4592 (2015).
- 436 24. Wellner, U. *et al.* The EMT-activator ZEB1 promotes tumorigenicity by repressing
437 stemness-inhibiting microRNAs. *Nat Cell Biol* **11**, 1487-1495 (2009).
- 438 25. Herreros-Villanueva, M. *et al.* SOX2 promotes dedifferentiation and imparts stem
439 cell-like features to pancreatic cancer cells. *Oncogenesis* **2**, e61. doi:
440 10.1038/oncsis.2013.1023 (2013).
- 441 26. Sanada, Y. *et al.* Histopathologic Evaluation of Stepwise Progression of
442 Pancreatic Carcinoma with Immunohistochemical Analysis of Gastric Epithelial

- 443 Transcription Factor SOX2: Comparison of Expression Patterns between
444 Invasive Components and Cancerous or Nonneoplastic Intraductal Components.
445 *Pancreas* **32**, 164-170 (2006).
- 446 27. Singh, S.K. *et al.* Antithetical NFATc1–Sox2 and p53–miR200 signaling networks
447 govern pancreatic cancer cell plasticity. *The EMBO Journal* **34**, 517-530 (2015).
- 448 28. Muller, Patricia A.J. & Vousden, Karen H. Mutant p53 in Cancer: New Functions
449 and Therapeutic Opportunities. *Cancer Cell* **25**, 304-317 (2014).
- 450 29. Rivlin, N., Koifman, G. & Rotter, V. p53 orchestrates between normal
451 differentiation and cancer. *Seminars in Cancer Biology* **32**, 10-17 (2015).
- 452 30. Morton, J.P. *et al.* Mutant p53 drives metastasis and overcomes growth
453 arrest/senescence in pancreatic cancer. *Proceedings of the National Academy of
454 Sciences* **107**, 246-251 (2010).
- 455 31. Lehmann, W. *et al.* ZEB1 turns into a transcriptional activator by interacting with
456 YAP1 in aggressive cancer types. *Nat Commun* **7**, doi: 10.1038/ncomms10498
457 (2016).
- 458 32. Mock, K. *et al.* The EMT-activator ZEB1 induces bone metastasis associated
459 genes including BMP-inhibitors. *Oncotarget* **6**, 14399-14412 (2015).
- 460 33. Brabletz, S. *et al.* The ZEB1/miR-200 feedback loop controls Notch signalling in
461 cancer cells. *EMBO J* **30**, 770-782 (2011).
- 462 34. Singh, A. *et al.* A Gene Expression Signature Associated with "K-Ras Addiction"
463 Reveals Regulators of EMT and Tumor Cell Survival. *Cancer Cell* **15**, 489-500
464 (2009).
- 465 35. Nakamura, T., Fidler, I.J. & Coombes, K.R. Gene Expression Profile of
466 Metastatic Human Pancreatic Cancer Cells Depends on the Organ
467 Microenvironment. *Cancer Research* **67**, 139-148 (2007).
- 468 36. Collisson, E.A. *et al.* Subtypes of pancreatic ductal adenocarcinoma and their
469 differing responses to therapy. *Nat Med* **17**, 500-503 (2011).
- 470 37. Weissmueller, S. *et al.* Mutant p53 Drives Pancreatic Cancer Metastasis through
471 Cell-Autonomous PDGF Receptor β Signaling. *Cell* **157**, 382-394 (2014).
- 472 38. Diepenbruck, M. & Christofori, G. Epithelial–mesenchymal transition (EMT) and
473 metastasis: yes, no, maybe? *Current Opinion in Cell Biology* **43**, 7-13 (2016).
- 474 39. Ye, X. & Weinberg, R.A. Epithelial–Mesenchymal Plasticity: A Central Regulator
475 of Cancer Progression. *Trends in Cell Biology* **25**, 675-686 (2015).
- 476 40. Bellomo, C., Caja, L. & Moustakas, A. Transforming growth factor [beta] as
477 regulator of cancer stemness and metastasis. *Br J Cancer* **115**, 761-769 (2016).
- 478 41. Korpala, M. & Kang, Y. Targeting the transforming growth factor- β signalling
479 pathway in metastatic cancer. *European Journal of Cancer* **46**, 1232-1240
480 (2010).
- 481 42. Lehuédé, C., Dupuy, F., Rabinovitch, R., Jones, R.G. & Siegel, P.M. Metabolic
482 Plasticity as a Determinant of Tumor Growth and Metastasis. *Cancer Research*
483 **76**, 5201-5208 (2016).
- 484 43. Nieto, M.A. Epithelial Plasticity: A Common Theme in Embryonic and Cancer
485 Cells. *Science* **342**, doi: 10.1126/science.1234850. (2013).
- 486 44. Brabletz, T. *et al.* Variable beta-catenin expression in colorectal cancer indicates
487 a tumor progression driven by the tumor environment. *Proc Natl Acad Sci U S A*
488 **98**, 10356-10361 (2001).
- 489 45. Chaffer, C.L. *et al.* Normal and neoplastic nonstem cells can spontaneously
490 convert to a stem-like state. *Proceedings of the National Academy of Sciences*
491 **108**, 7950-7955 (2011).
- 492 46. Puisieux, A., Brabletz, T. & Caramel, J. Oncogenic roles of EMT-inducing
493 transcription factors. *Nature cell biology* **16**, 488-494 (2014).

- 494 47. Chaffer, Christine L. *et al.* Poised Chromatin at the ZEB1 Promoter Enables
495 Breast Cancer Cell Plasticity and Enhances Tumorigenicity. *Cell* **154**, 61-74
496 (2013).
- 497 48. Gruber, R. *et al.* YAP1 and TAZ Control Pancreatic Cancer Initiation in Mice by
498 Direct Up-regulation of JAK–STAT3 Signaling. *Gastroenterology* **151**, 526-539
499 (2016).
- 500 49. Zhang, W. *et al.* Downstream of Mutant KRAS, the Transcription Regulator YAP
501 Is Essential for Neoplastic Progression to Pancreatic Ductal Adenocarcinoma.
502 *Science Signaling* **7**, ra42-ra42 (2014).
- 503 50. Moffitt, R.A. *et al.* Virtual microdissection identifies distinct tumor- and stroma-
504 specific subtypes of pancreatic ductal adenocarcinoma. *Nat Genet* **47**, 1168-
505 1178 (2015).
- 506 51. Galvan, J.A. *et al.* Expression of E-cadherin repressors SNAIL, ZEB1 and ZEB2
507 by tumour and stromal cells influences tumour-budding phenotype and suggests
508 heterogeneity of stromal cells in pancreatic cancer. *Br J Cancer* **112**, 1944-1950
509 (2015).
- 510 52. Bronsert, P. *et al.* Prognostic significance of Zinc finger E-box binding homeobox
511 1 (ZEB1) expression in cancer cells and cancer-associated fibroblasts in
512 pancreatic head cancer. *Surgery* **156**, 97-108 (2014).
- 513 53. Singh, A. & Settleman, J. EMT, cancer stem cells and drug resistance: an
514 emerging axis of evil in the war on cancer. *Oncogene* **29**, 4741-4751 (2010).
- 515 54. Eser, S., Schnieke, A., Schneider, G. & Saur, D. Oncogenic KRAS signalling in
516 pancreatic cancer. *Br J Cancer* **111**, 817-822 (2014).
- 517 55. Ni, T. *et al.* Snail1-dependent p53 repression regulates expansion and activity of
518 tumour-initiating cells in breast cancer. *Nat Cell Biol* **18**, 1221-1232 (2016).
- 519 56. Turajlic, S. & Swanton, C. Metastasis as an evolutionary process. *Science* **352**,
520 169-175 (2016).
- 521 57. Caramel, J. *et al.* A Switch in the Expression of Embryonic EMT-Inducers Drives
522 the Development of Malignant Melanoma. *Cancer Cell* **24**, 466-480 (2013).
- 523 58. Denecker, G. *et al.* Identification of a ZEB2-MITF-ZEB1 transcriptional network
524 that controls melanogenesis and melanoma progression. *Cell Death Differ* **21**,
525 1250-1261 (2014).
- 526 59. Ye, X. *et al.* Distinct EMT programs control normal mammary stem cells and
527 tumour-initiating cells. *Nature* **525**, 256-260 (2015).
- 528 60. Tiwari, N. *et al.* Sox4 Is a Master Regulator of Epithelial-Mesenchymal Transition
529 by Controlling Ezh2 Expression and Epigenetic Reprogramming. *Cancer Cell* **23**,
530 768-783 (2013).
- 531 61. Tran, H.D. *et al.* Transient SNAIL1 Expression Is Necessary for Metastatic
532 Competence in Breast Cancer. *Cancer Research* **74**, 6330-6340 (2014).

534

535 FIGURE LEGENDS

536

537 **Figure 1: Zeb1 depletion reduces invasion and metastasis in pancreatic cancer.**

538 **(a)** Scheme of the genetic mouse models for pancreatic cancer. The colour code (blue
539 KPC, red KPCZ) is used for all results. **(b)** Tumour-free survival (n= 28 KPC, 18 KPCZ;
540 Log-rank (Mantel-Cox) test), tumour volume (0 = start of MRI measurements; n=23 KPC,

541 27 KPCZ; error bars show mean \pm S.E.M.; multiple t-tests with correction for multiple
542 comparison using the Holm-Sidak method). n.s. = not significant. **(c)** Representative HE-
543 stained sections for the grading of the respective tumours. Scale bar, 250 μ m and 125
544 μ m for higher magnifications. (n=48 KPC, 29 KPCZ independent tumours) **(d)** Grading
545 and local invasion of the respective tumours (n=48 KPC, 29 KPCZ independent tumours;
546 error bars show mean \pm S.D.; Mann-Whitney test (two-tailed), Chi-square test (two-tailed)
547 for grade3/4 tumours), ****p<0.0001. **(e)** Representative immunohistochemical stainings
548 of consecutive sections showing nuclear Zeb1 in tumour cells (arrows) of invasive
549 tumour regions in KPC, but not in KPCZ mice. Asterisks mark Zeb1 expression in
550 stromal cells, cen (central) and inv (invasive tumour regions). n= 15 KPC, 13 KPCZ
551 independent tumours, Scale bar, 75 μ m. **(f)** Numbers and grading of metastasized
552 tumours (n=52 KPC, 29 KPCZ independent tumours; error bars mean \pm S.D.; Chi-square
553 test (two-tailed) for metastasis, Mann-Whitney test (two-tailed) for grading). **(g)**
554 Representative images of differentiated (KPC and KPCZ) and undifferentiated (KPC)
555 primary tumours (PT) and corresponding metastases (Met) with the same phenotype (L=
556 liver). n= 19 KPC, 4 KPCZ independent tumours and corresponding metastases. Scale
557 bar, 150 μ m.

558

559 **Figure 2: Depletion of *Zeb1* affects phenotypic variability of tumour cells.**

560 **(a)** Anti-E-cadherin and anti-vimentin immunofluorescence stainings showing variable
561 expression in KPC cell lines and homogeneous E-cadherin and lack of vimentin
562 expression in all KPCZ cell lines. Scale bar, 100 μ m. **(b)** Relative mRNA expression
563 levels of indicated marker genes in the isolated tumour cells. **(c)** Relative mRNA
564 expression levels for EMT transcription factors and epithelial microRNAs. mRNA levels
565 of the cell line 661 was set to 1. n=3 biologically independent experiments, error bars
566 mean \pm S.E.M. *p<0.05, **p<0.01, n.s. = not significant, Mann-Whitney test (one-tailed)
567 (b-c). **(d)** Immunoblots of indicated marker genes (unprocessed scans of immunoblots
568 are shown in Suppl. Fig. 8). **(e)** BrdU proliferation assay for the isolated tumour cell lines.
569 n=3 biologically independent experiments, error bars mean \pm S.E.M. The colour code for
570 the isolated cell lines as depicted in b) is valid for all corresponding results.

571

572 **Figure 3: Depletion of *Zeb1* affects stemness, tumourigenic and colonisation**
573 **capacities.**

574 **(a)** Representative images of macroscopic and HE-stained lungs, 18 days after i.v.
575 injection of tumour cells in syngeneic mice. Quantification of lung colonies (left, cell lines
576 grouped by genotype; right, individual cell lines (for a,b,c,e), normalised to 20 mm² lung
577 area). n=3mice/cell line, n=4 mice for line 524, error bars mean \pm S.D.; ****p<0.0001,
578 Mann-Whitney test (two-tailed), Scale bar, 200 μ m. **(b)** No. of GFP+ cells per visual field
579 2 h after i.v. injection. n=3 mice/cell line, error bars mean \pm S.D. Mann-Whitney test (two-
580 tailed). **(c)** Quantification after i.v. injection of KPC, KPCS and KPCZ tumour cells in
581 nude mice; n=13 mice for KPC, n=8 for KPCS, n=6 for KPCZ- 4 mice/cell line, Mann-
582 Whitney test (two-tailed), **p<0.01, n.s. = not significant. Relative mRNA expression
583 levels in KPCS cell lines; mRNA levels of KPC661 (expressing low levels of Snail) set to
584 1; average of n=2 biologically independent experiments, error bars mean \pm S.D.
585 Immunoblot for the indicated proteins with KPC701 as control expressing high Snail
586 levels. **(d)** Number of lung colonies after i.v. injection of KPC shcontrol (ctr) and KPC
587 shZeb1 tumour cells in nude mice (normalized to 20 mm² lung area). n= 3 mice/cell line,
588 error bars mean \pm S.D.; Mann-Whitney test (two-tailed), n.s = not significant.
589 Immunoblots and corresponding quantifications, showing shRNA-mediated partial
590 reduction of Zeb1. n=3 biologically independent experiments, error bars mean \pm S.E.M.;
591 unpaired Student's t-test (two-tailed), **p<0.01. **(e)** Quantification of sphere forming
592 capacity. n=3 biologically independent experiments, error bars mean \pm S.D.; *p<0.05,
593 Mann-Whitney test (two-tailed). **(f)** Relative mRNA expression levels and immunoblots of
594 stem cell genes. mRNA levels of the line 661 set to 1. n=3 biologically independent
595 experiments, error bars mean \pm S.E.M. *p<0.05, Mann-Whitney test (one-tailed). **(g)** HE
596 and immunohistochemical staining for Sox2 in tumours grown subcutaneously (s.c.)
597 (n=51) or in the lung (n=36) after i.v. injection (l.c.) of indicated cell lines. Scale bar, 100
598 μ m. **(h)** Immunoblot for indicated proteins upon overexpression of *Mir200c*. Source data
599 for Fig. 3c, d, f see Supplementary Table 5; unprocessed scans of immunoblots are
600 shown in Suppl. Fig. 8.

601

602 **Figure 4: Depletion of *Zeb1* reduces ADM and PanIN precursor lesions.**

603 **(a-b)** Consecutive sections of representative HE and PAS stained sections showing
604 precancerous PanIN (a) and ADM lesions (b) in the pancreas of 6 month old KC and
605 KCZ mice. Specific dark blue/purple PAS staining indicates the mucin-rich PanIN
606 lesions, arrows indicate ADMs. Squares mark the magnified regions; Scale bars 1 mm
607 and 150 μ m for higher magnifications in (a) and 75 μ m in (b). Quantification of the ADM

608 and PanIN areas and PanIN grading is given. n=12 KC and 7 KCZ independent mice,
609 error bars mean \pm S.D.; **p<0.01, ****p<0.0001 unpaired Student's t-test (two-tailed) with
610 Welch's correction for ADM and PanIN areas and Mann-Whitney test (two-tailed) for
611 grading.

612

613 **Figure 5: Depletion of *Zeb1* reduces phenotypic variability**

614 **(a)** Principal component analysis (PCA) of the KPC and KPCZ cell line transcriptomes.
615 The plot depicts the first two principal components using all samples accounting for
616 ~44%, ~17% of the variance, respectively. **(b)** Gene set enrichment analyses (GSEA) of
617 transcriptome data from KPCZ vs. KPC cells reveal enrichment of gene signatures
618 associated with *Kras* dependency and the classical type of pancreatic cancer, as well as
619 a reduction of genes associated with metastasis in KPCZ cell lines. NES=normalized
620 enrichment score; FDR=false discovery rate. **(c)** Relative mRNA expression levels (qRT-
621 PCR) and immunoblots of indicated genes associated with metastasis in the isolated
622 tumour cells. mRNA levels of the cell line 661 was set to 1. n=3 biologically independent
623 experiments, error bars mean \pm S.E.M. *p<0.05, **p<0.01, Mann-Whitney test (one-
624 tailed). Unprocessed scans of immunoblots are shown in Suppl. Fig. 8.

625

626

627 **Figure 6: Depletion of *Zeb1* reduces TGF β -induced cellular plasticity.**

628 **(a)** E-cadherin and vimentin immunofluorescence staining of two epithelial KPC and two
629 KPCZ cancer cell lines treated with TGF β 1 for 3 and 21 days. Scale bar, 100 μ m. **(b)**
630 Immunoblots for indicated marker genes of the same lines as in a). Unprocessed scans
631 of immunoblots are shown in Suppl. Fig. 8. **(c)** PCA of transcriptome signatures of the
632 KPC and KPCZ cell lines upon TGF β treatment. TGF β -induced shifts in expression of
633 the cell lines shown in a) are marked with coloured boxes (microarrays performed in
634 duplicates, referred to as TGF β _1 and TGF β _2). Note, a great shift for KPC cell lines
635 towards a mesenchymal pattern but not for KPCZ lines(upper panel). Venn diagram
636 showing number of significantly up-or downregulated genes (cut-off: adj. p-value<0.05
637 and log₂FC>0.5) by 14 days of TGF β treatment of cell lines shown in a). Moderated t-
638 test (lower panel). **(d)** Relative mRNA expression levels (qRT-PCR) of indicated genes
639 (including the metastasis set in Fig. 5c) in KPC and KPCZ cell lines treated for different
640 times with TGF β (time points: 0, 6 h, 1, 3, 7, 14, 21 days). mRNA levels of the cell line
641 661 at day 0 were set to 1. n=3 biologically independent experiments, error bars mean

642 ±S.E.M. Statistical analysis is shown for the comparison of TGFβ treated and untreated
643 samples (grey bars) of each individual cell line *p<0.05, **p<0.01, unpaired Student's t-
644 test (one-tailed). **(e)** Anti-E-cadherin and anti-vimentin immunofluorescence staining of
645 two epithelial KPC and two KPCZ cancer cell lines treated with TGFβ for more than 21
646 days followed by 14 days TGFβ withdrawal. Scale bar, 100 μm. **(f-g)** Immunoblots (f)
647 and relative mRNA expression levels (qRT-PCR) (g) of indicated marker genes of the
648 same cell lines as in e). mRNA levels of the cell line 661 at day 0 were set to 1. n=3
649 biologically independent experiments, error bars mean ±S.E.M.; *p<0.05, **p<0.01,
650 ***p<0.001, unpaired Student's t-test (one-tailed). Source data for Fig. 5d,f see
651 Supplementary Table 5; unprocessed scans of immunoblots are shown in Suppl. Fig. 8.
652

653 **Figure 7: Depletion of *Zeb1* reduces metabolic and phenotypic plasticity.**

654 **(a)** Mito stress test (MST) showing the oxygen consumption rate (OCR) as indicator for
655 oxidative phosphorylation and deduced levels for basal respiration and ATP production.

656 **(b)** Glycolysis stress test (GST) showing the extracellular acidification rate (ECAR) as
657 indicator for glycolysis and the OCR after glucose stimulation, blocking of oxidative
658 phosphorylation with oligomycin and blocking of glycolysis with 2-deoxy-glucose (2DG),
659 as well as deduced glycolytic capacity and glycolytic reserve. Note a complete lack of a
660 glycolytic reserve (upper arrow) after blocking oxidative phosphorylation (lower arrow) in
661 KPCZ cells. KPC661 and 792 as well as all KPCZ cell lines were used. n=7 biologically
662 independent experiments; error bars ±S.E.M. for MST and GST and ±S.D. for other
663 parameters; for MST and GST a multiple t-test with correction for multiple comparison
664 using the Holm Sidak method was used; for other parameters an unpaired Student's t-
665 test (two-tailed) was used; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **(c)**

666 Representative images of consecutive sections of immunohistochemical stainings for
667 Ck19 and Zeb1 comparing the plasticity of Zeb1 expression in central and invasive
668 tumour regions. Shown are tumours derived from one KPC and one KPCZ cell line.
669 Asterisks label Zeb1 expression in stromal cells, arrows indicate Zeb1-positive tumour
670 cells at the invasive front. Ck19 expression is shown to identify cancer cells. n= 15 KPC,
671 13 KPCZ independent tumours, Scale bars, 50 μm and 150 μm for higher
672 magnifications.

673

674 **Table 1: Summary of the differential behaviour of KPC vs. KPCZ cell lines**
675 **concerning crucial traits for tumour progression towards metastasis.**

676 Table summarizing the experimental results of the differential behaviour of KPC vs.
677 KPCZ cell lines concerning crucial traits for tumour progression towards metastasis. For
678 experimental data on sphere formation see Figs. 3e, Suppl. Fig. 5c; tumorigenicity see
679 Suppl. Fig. 5b; plasticity see Figs. 6 and 7, Suppl. Fig. 7a,c; lung colonisation see Fig.
680 3a; lung dissemination see Fig. 3b, Suppl. Fig. 5a. (-, no capacity; +, weak capacity; ++,
681 moderate capacity; +++, strong capacity; na, not analysed).

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1 METHODS

2

3 Ethic statement

4 Animals were kept on a 12:12 h light-dark cycle and provided with food and water ad libitum.

5 Animal husbandry and all experiments were performed according to the European Animal

6 Welfare laws and guidelines. The protocols were approved by the committee on ethics of

7 animal experiments of the states Baden-Württemberg and Bavaria (Regierungspräsidium

8 Freiburg and Regierung Unterfranken, Würzburg).

9 Mice

10 The *Pdx1-Cre* transgene (Tg(Pdx1-cre)6Tuv), the conditional *Kras*^{LSL.G12D} (*Kras*^{tm4Tyj}),

11 *Tp53*^{LSL.R172H} (*Trp53*^{tm2Tyj}) and GFP (Z/EG; Tg(CAG-Bgeo/GFP)21Lbe) alleles and the KPC

12 mouse model have been described^{9, 62-66} and were kept on a C57BL/6 background. The

13 generation of the conditional *Zeb1* knockout allele (*Zeb1*^{fl}) is described elsewhere¹². In brief,

14 exon6 was flanked by loxP sites to remove sequences coding for large parts of the protein

15 and to induce a premature translational stop. Tumour mice were generated by breedings of

16 *Pdx1-Cre* with *Kras*^{LSL.G12D/+}; *Tp53*^{LSL.R172H/+} mice (KPC) and *Pdx1-Cre;Zeb1*^{fl/fl} with

17 *Kras*^{LSL.G12D/+}; *Tp53*^{LSL.R172H/+}; *Zeb1*^{fl/fl} mice (KPCZ). KPC and KPCZ offspring was palpated

18 weekly for tumour initiation and enrolled for MRI measurements when tumours were

19 identified. KC and KCZ mice (*Tp53*^{+/+} genotype) were analysed with 6 months of age. Once

20 the tumour reached a maximum tolerated size (tumour diameter of 1 cm), mice were

21 sacrificed, perfused and organs, tumour and macroscopic metastases were isolated.

22 Animals, which died or were sacrificed due to non-pancreatic tumour reasons (mainly growth

23 of skin papilloma) were excluded from the analyses. Tissue was fixed in 4%

24 paraformaldehyde (PFA) or snap frozen in TissueTek. A summary of basic tumour mice data

25 is shown in Supplementary Table 1.

26 MRI

27 Mice were analysed with a Bruker Bio Spin 94/20, 9.4Tesla – 400MHz – 20cm small animal
28 MR using coronal and transverse scans with a spatial resolution of 117 μm x 117 μm /pixel
29 and a 256 x 256 matrix. Slice distance was set to 0.5 mm. Measurements were repeated
30 weekly. Tumour volume was approximated by $\pi/6 \times l \times w \times d$. Initial detection of a tumour
31 after a series of tumour-free MRI measurements was defined as time-point of tumour
32 initiation. For analysis of tumour growth curves all mice were adjusted to a tumour size of 50
33 mm^3 .

34 **Histology, histopathology and immunohistochemistry**

35 PFA-fixed tissues were embedded into paraffin, sectioned at 4-5 μm and stained with Mayer's
36 Haematoxylin and Eosin solution G (HE). For histopathological scoring, tumours were
37 classified using the standard pathological grading scheme into either well differentiated
38 (grade 1), moderately differentiated (grade 2), poorly differentiated (grade 3) and anaplastic
39 or sarcomatoid (grade 4). The histological invasion score was scored from no invasion (0) to
40 high invasion (2), with invasion defined as number and distance of tumour cells disseminated
41 from the main tumour mass. Masson's trichrome staining (MTS) was performed according to
42 the manufacturer's instructions (Sigma-Aldrich, HT15) and counterstained by Weigert's Iron
43 Haematoxylin. Tumour stroma composition was scored either based on MTS or HE staining
44 for intensity of extracellular matrix deposition on a scale from 0-4. KC and KCZ pancreata
45 were stained by alcian blue-periodic acid/Schiff's (PAS) reagent. Scoring for CD31 and
46 Gata6 was done according to staining intensity with no (0), low (1), medium (2) and high (3)
47 expression. PanINs were classified using the standard pathological grading score from 1-3.
48 The complete numbers of PanINs and ADMs was counted on at least four independent
49 tumour sections and normalized to a tissue area of 20 mm^2 . In addition to macroscopic
50 metastases, lungs and livers were screened for metastases identified by screening four
51 series of HE stained sections separated by at least 200 μm .

52 Immunohistochemical analysis was performed as previously described³¹. Primary antibodies
53 against the following proteins were used: polyclonal rabbit anti-Zeb1 (Novus Biological,
54 NBP1-05987, 1:250); polyclonal rabbit anti-Zeb2 (Novus Biological, NBP1-82991, 1:200);
55 monoclonal rabbit anti-Snail (Cell Signaling, #3879, Clone C15D3, 1:200); monoclonal rabbit
56 anti-Slug (Cell Signaling, CS9585, Clone C19G7, 1:150); polyclonal goat anti-Twist (Abcam,
57 ab50581, 1:500); polyclonal goat anti-Gata6 (R&D, AF1700, 1:1500); monoclonal mouse
58 anti-E-Cadherin (BD Transduction Laboratories, 610182, Clone 36, 1:350); monoclonal rabbit
59 anti-CD31 (Santa Cruz, sc-1506, Clone M-20, 1:50); monoclonal rabbit anti-Ki67 (Abcam,
60 ab16667, Clone SP6, 1:300); monoclonal rabbit anti-cleaved Caspase 3 (Cell Signaling,
61 CS9664, Clone 5A1E, 1:1,000); monoclonal rat anti-KRT19 (TROMA-3 hybridoma
62 supernatant, 1:20, a kind gift from Rolf Kemler); polyclonal rabbit anti-Sox2 (Abcam, ab97959,
63 1:1,000) and counterstained with Mayer's Haematoxylin. For Zeb1 immunofluorescence
64 staining, cryosections were fixed in 4% PFA for 10 min, then permeabilised for 10 min in
65 0.25% Triton-X100/PBS. After blocking in 3% BSA/PBS, tissue was incubated with anti-Zeb1
66 antibody (Sigma, HPA027524, 1:100) followed by Alexa594-conjugated secondary antibody
67 (Life technologies). All images were acquired on a Leica DM5500B microscope and a 2D
68 deconvolution was performed when appropriate. No statistical method was used to
69 predetermine sample size and the experiments were not randomized. Histological analyses
70 were performed by two independent pathologists. The Investigators were not blinded to
71 allocation during experiments and outcome assessment. Each demonstrated IHC and IF
72 image was representative for minimum five or more cases (tumours) of indicated subtype.

73 **Primary cell lines**

74 A small piece of primary tumour was dissected, minced with a scalpel and plated on 6-well
75 plates in DMEM (Gibco, 31966)/ 10%FBS (Gibco, 10500)/ 1%P/S (Gibco, 15140) at 37°C/5%
76 CO₂ in a humidified incubator. Tumour cells that attached to the plate and grew out were
77 passaged for generation of cell lines. Successful and complete recombination of cell line

78 deprivation was confirmed by PCR. KPCS cells were obtained from Dieter Saur (Dept. of
79 Internal Medicine, TU Munich, Germany) and generated from the same KPC mouse model
80 that additionally carried a homozygous *Snai1* deletion⁶⁷. For partial knockdown of *Zeb1*, cells
81 were infected with lentivirus containing a pGIPZ shZeb1 knockdown (V2LMM_18639) or a
82 pGIPZ non-silencing shRNA control construct. Puromycin resistant GFP medium/high cells
83 were used. Zeb1 protein expression was normalized to β -actin levels using BioRad
84 ImageLab Software to calculate knockdown efficiencies. Induction of EMT in primary tumour
85 cell lines was performed by adding 5 ng/ml TGF β 1 (PeproTech, 100-21) and replacing the
86 medium daily for the duration of the experiment. miRNA overexpression was performed as
87 previously described³¹. For FACS analysis of cancer stem cells markers 1×10^6 cells were
88 incubated with a combination of monoclonal rat anti-CD24-PE (BD, 553262, Clone M1/69,
89 1:200), monoclonal rat anti-CD44-APC (BD, 561862, Clone IM7, 1:100) and monoclonal rat
90 anti-Epcam-FITC (ebioscience, 11-5791, Clone G8.8, 1:200) antibodies and analysed in a
91 BD Cytoflex using CytExpert software. A total of 10,000 vital cells were counted. All studies
92 were performed on cells cultured for less than 30 passages. All experiments using primary
93 cells in vitro were done at least in triplicates (n=3). Only primary cells from mouse tumours
94 were used and these were not further authenticated nor tested for mycoplasma
95 contamination.

96 **Immunoblotting, RNA isolation and quantitative RT-PCR**

97 Protein was extracted with RIPA buffer and Western blotting was carried out as described³¹,
98 ³² with the exception that protein detection on the nitrocellulose membrane was done by
99 incubation in Western Lightning Plus-ECL (Perkin Elmer, NEL103001EA) or SuperSignal
100 West Femto Maximum Sensitivity Substrate (Thermo Scientific, 34095) and a ChemiDoc
101 imaging system (BioRad). Antibodies against the following proteins were used: polyclonal
102 rabbit anti-Zeb1 (Sigma, HPA027524, 1:5000); monoclonal rabbit anti-Snail (Cell Signaling,
103 #3879, Clone C15D3, 1:1000); monoclonal mouse anti-E-Cadherin (BD Transduction

104 Laboratories, 610182, Clone 36, 1:5000); monoclonal mouse anti-N-Cadherin (BD
105 Transduction Laboratories, 610920, Clone 32, 1:1,000); monoclonal rabbit anti-Vimentin (Cell
106 Signaling, CS5741, Clone D21H3, 1:5,000); monoclonal mouse anti- β -actin (Sigma, A5441,
107 Clone AC-15, 1:10,000); polyclonal rabbit anti-Sox2 (Novus Biological, NB110-37235,
108 1:3,000); monoclonal mouse anti-Bmi1 (Millipore, 05-673, Clone F6, 1:300); monoclonal
109 rabbit anti-PDGFR β (Cell Signaling, CS3169, Clone 28E1, 1:1,000); monoclonal rabbit anti-
110 Sparc (Cell Signaling, CS8725; Clone D10F10, 1:1,000); monoclonal mouse anti- α -tubulin
111 (Sigma, T6199, Clone DM1A, 1:5000). Western blots were done for at least three individual
112 experiments and one representative blot is shown.

113 Total RNA was isolated and reversely transcribed using the RNeasy Plus Mini Kit (Qiagen,
114 74136) and the RevertAid First Strand cDNA Synthesis Kit (Thermo, K1622) for mRNA and
115 the miRCURY universal cDNA synthesis kit II (Exiqon, 203301) for miRNA. mRNA transcripts
116 were detected by using cDNA from 7.5 ng total RNA with 300 nM gene-specific primers, the
117 Universal Probe Library (Roche, 04869877001) and the TaqMan Universal Master Mix
118 (4440040, Applied Biosystems) in a 12 μ l volume. miRNAs were analysed with the
119 miRCURY ExiLENT SYBR Green Kit (Exiqon, 203421) with specific primer sets (Exicon)
120 according to the manufacturer's instructions. All samples were run in a LightCycler 480
121 (Roche) and values were normalised to *Gapdh* and *Mir16-1* levels where appropriate and
122 expressed relative to controls. For primer sequences and miR primer set details see
123 Supplementary Table 3.

124 **Cell viability (MTT) and BrdU cell proliferation assays**

125 Cell viability upon gemcitabine (Sigma, G6423; ranging from 0.78 to 1000 nM) and erlotinib
126 treatment (Cell Signaling, 5083 or Selleckchem, S1023, ranging from 0.2 to 51.2 μ M) was
127 analysed by plating 6,000 cells in 96- or 48-well plates and measured after 72 h of treatment
128 using 5 mg/ml MTT (methylthiazolyldiphenyl-tetrazolium bromide; Sigma, M2128) as
129 described⁶⁸. IC50 values were calculated with GraphPad Prism using logarithmic transformed

130 data and nonlinear regression. For proliferation analysis 1,000 cells were plated in 96-well
131 plates and BrdU incorporation was measured after a 2-h pulse with BrdU using the Cell
132 Proliferation ELISA Kit (Roche, 11647229001) according to the manufacturer's instructions.

133 **Sphere assay**

134 For detecting sphere forming capacity, cells were resuspended as single cell suspension in
135 serum-free DMEM/F12 medium (Gibco, 31331), containing 1% methylcellulose (Sigma,
136 M0512), 20 ng/ml human EGF (R&D Systems, 236-EG), 20 ng/ml human FGF (BD
137 biosciences, 354060), B27 supplement (1:50, Invitrogen, 17504), N2 supplement (1:100,
138 Gibco, 17502), and 1% P/S. 500 single cells were seeded into individual wells of a poly(2-
139 hydroxyethylmetacrylate)-coated (Sigma, P3932) 96-well plates. Colonies with a diameter of
140 >80 µm were counted after 12 days.

141 **Immunofluorescence staining**

142 Immunofluorescence labelling was performed as described previously³¹. Cells were seeded
143 on coverslips and fixed with 4% PFA, followed by permeabilization with 0.1% Triton X-
144 100/PBS. After blocking in 3% BSA/PBS, cells were incubated with primary antibodies
145 overnight at 4°C (polyclonal rabbit anti-Zeb1 (Sigma, HPA027524, 1:300); monoclonal
146 mouse anti-E-Cadherin (BD Transduction Laboratories, 610182, Clone 36, 1:200), followed
147 by appropriate Alexa594- and Alexa488-conjugated secondary antibodies (Life technologies)
148 for 1 hour at RT. All images were acquired with a Leica DM5500B microscope and the LAX
149 software (Leica). All IF experiments were performed in at least three individual experiments
150 and one representative image is shown.

151 **Lung colonization/tumourigenicity**

152 Tumour cell colonisation and metastasising capacities to the lung were analysed by tail vein
153 injections into syngeneic mice or NMRI-*Foxn1^{nu/nu}* mice. Primary tumour cell lines were
154 trypsinised and resuspended in appropriate volumes of PBS to inject 200,000 tumour cells in
155 a 200 µl volume using a 27G needle. Mice were sacrificed after 18 days and analysed for

156 lung metastasis by HE staining. For each cell line three mice were injected and the number
157 or lung metastases were counted on 2 independent sections separated by at least 200 μm .
158 For short-term colonisation analysis cells were infected with pCDH-MSCV-LUC_EF1-GFP-
159 T2A-Puro, selected by puromycin and sorted for medium to high levels of GFP expression.
160 After tail vein injection mice were sacrificed after 2 h. For calculating tumourigenicity and
161 analysis of tumour growth upon subcutaneous engraftment 500, 2,500, 12,500 and 100,000
162 cells were injected into flanks of C57BL/6 mice. Tumour size was measured 3 times per
163 week and mice were sacrificed if tumours exceeded the size of 500 mm^3 or ulcerated.
164 Tumour initiating frequencies were calculated using the ELDA software
165 (<http://bioinf.wehi.edu.au/software/elda/>).

166 **Microarray analysis, pre-processing, GSEA and data availability**

167 Gene expression of three epithelial, three mesenchymal KPC, six KPCZ, two TGF β -treated
168 epithelial KPC and two TGF β -treated KPCZ cell lines was measured using Illumina Mouse
169 WG6 v2 beadarrays (Illumina, San Diego, CA, USA). Total RNA was isolated, labelled and
170 hybridised according to the manufacturer's protocol in two separate experiments. Raw
171 microarray data were processed and quantile normalised using the Bioconductor R package
172 beadarray⁶⁹ and subsequently batch corrected according to their chip identity via ComBat⁷⁰
173 as implemented in the R Bioconductor sva package. Illumina probes were mapped to Entrez
174 IDs using the IlluminaMousev2 annotation (v. 1.26) from Bioconductor. If several probes
175 mapped to the same Entrez ID, the one having the largest interquartile range was retained,
176 which resulted in 20,052 uniquely annotated genes. Gene Set Enrichment analysis (GSEA)
177 was performed using the Broad Institute platform
178 (<http://www.broadinstitute.org/gsea/index.jsp>; Version 2.2.2). A total of 189 gene sets of the
179 oncogenic signature C6 from the Molecular Signatures database
180 (<http://www.broadinstitute.org/gsea/msigdb/genesets.jsp?collection=C6>) were used for the
181 analysis with default settings and 1,000 gene set permutations. Additionally 36 gene sets,

182 related to pancreatic cancer, Zeb1 or metastasis were selected from MSigDB and also
183 analysed (Supplementary Table 4). Gene Sets from classical, quasi-mesenchymal and
184 exocrine-like PDAC subtypes were obtained from Collisson et al. 2011³⁶.

185 **Metabolic parameters**

186 Bioenergetics of epithelial KPC and KPCZ cell lines was determined using the XFe96
187 Extracellular Flux Analyzer (Seahorse Bioscience/Agilent Technologies, North Billerica, MA).
188 Cells were seeded in specialised cell culture microplates at a density of 15,000 /well and
189 cultured for 18 h. 1 h before the measurement cells were incubated at 37°C in a CO₂-free
190 atmosphere. For the determination of glycolytic parameters a glucose stress test was
191 performed: basal extracellular acidification rate (ECAR; indicative of glycolysis) was first
192 determined under glucose-free conditions. Secondly, the rate of glycolysis was calculated
193 using the ECAR after glucose supplementation (10 mM). Finally, glycolytic capacity and
194 glycolytic reserve were calculated after inhibition of mitochondrial respiration via oligomycin
195 (Sigma, 75351, 1 µM) and hexokinase activity via 2-deoxy-glucose (2DG, Sigma, D6134, 100
196 mM).). For the determination of respiratory parameters a mito stress test was performed:
197 basal oxygen consumption rate (OCR, indicator for mitochondrial respiration) was measured.
198 Next, responses toward the subsequent addition of oligomycin (1 µM), FCCP (Sigma, C2920,
199 1 µM) and the combination of antimycin A (Sigma, A8674, 3 µM) and rotenone (Sigma,
200 R8875, 3 µM) were evaluated allowing for the calculation of basal and maximal respiration as
201 well as respiration-related ATP production. All experiments were performed in heptaplicates.

202 **Statistics and Reproducibility**

203 Statistical analysis was performed using GraphPad Prism software (Version 6.07). Data are
204 represented by means ±SD unless otherwise indicated. For survival analysis the log-rank
205 Mantel-Cox test was used. Tumour/PanIN grading, ECM deposition, local invasion, CD31
206 and Gata 6 staining, Ki67-positive tumour cell counting, cleaved Casp3-positive tumour cell
207 amounts, PanIN areas, lung colonisation assay and sphere forming capacity analysis were

208 tested for significance with a two-tailed Mann-Whitney test or an unpaired two-tailed t-test as
209 indicated. A Welch's correction was performed where appropriate. Chi-square analysis was
210 performed to compare frequencies of metastases and number of tumour-initiating cells as
211 well as frequency of Zeb1, Snail, Slug, Twist Zeb2, E-cad and Sox2 positive tumours.
212 Tumour growth, ECAR and OCR were tested for significance at individual time points by a t-
213 test with Holm-Sidak test for multiple comparison. qPCR data were tested for significance
214 with a one-tailed Mann-Whitney test or an unpaired one-tailed t-test as indicated. p-values of
215 statistical significance are represented as: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

216 **Data Availability**

217 Microarray data generated in this study have been deposited in the Gene Expression
218 Omnibus (GEO) under accession code GSE87472. The 189 publically available gene sets
219 reanalysed here were from of the oncogenic signature C6 available from the Molecular
220 Signatures database (<http://www.broadinstitute.org/gsea/msigdb/genesets.jsp?collection=C6>,
221 Broad Institute, 741 MSigDB, Version 5.1.). The 36 publically available gene sets related to
222 pancreatic cancer, Zeb1 or metastasis were selected from MSigDB and reanalysed here
223 (see also Supplementary Table 4). Gene Sets from classical, quasi-mesenchymal and
224 exocrine-like PDAC subtypes re-analysed here were obtained from Collisson et al. 2011³⁶.
225 Source data for Fig. 3c,d,f; Fig. 6d,g and Supplementary Fig. 5d, 7a have been provided as
226 Supplementary Table 5. All other data supporting the findings of this study are available from
227 the corresponding author on reasonable request.

228

229 **Additional references for methods**

- 230 62. Olive, K.P. *et al.* Mutant p53 gain of function in two mouse models of Li-Fraumeni
231 syndrome. *Cell* **119**, 847-860 (2004).
232 63. Jackson, E.L. *et al.* Analysis of lung tumor initiation and progression using
233 conditional expression of oncogenic K-ras. *Genes Dev* **15**, 3243-3248 (2001).
234 64. Hingorani, S.R. *et al.* Preinvasive and invasive ductal pancreatic cancer and its
235 early detection in the mouse. *Cancer Cell* **4**, 437-450 (2003).

236 65. Chan, I.T. *et al.* Conditional expression of oncogenic K-ras from its endogenous
237 promoter induces a myeloproliferative disease. *J Clin Invest* **113**, 528-538
238 (2004).

239 66. Novak, A., Guo, C., Yang, W., Nagy, A. & Lobe, C.G. Z/EG, a double reporter
240 mouse line that expresses enhanced green fluorescent protein upon Cre-
241 mediated excision. *Genesis* **28**, 147-155 (2000).

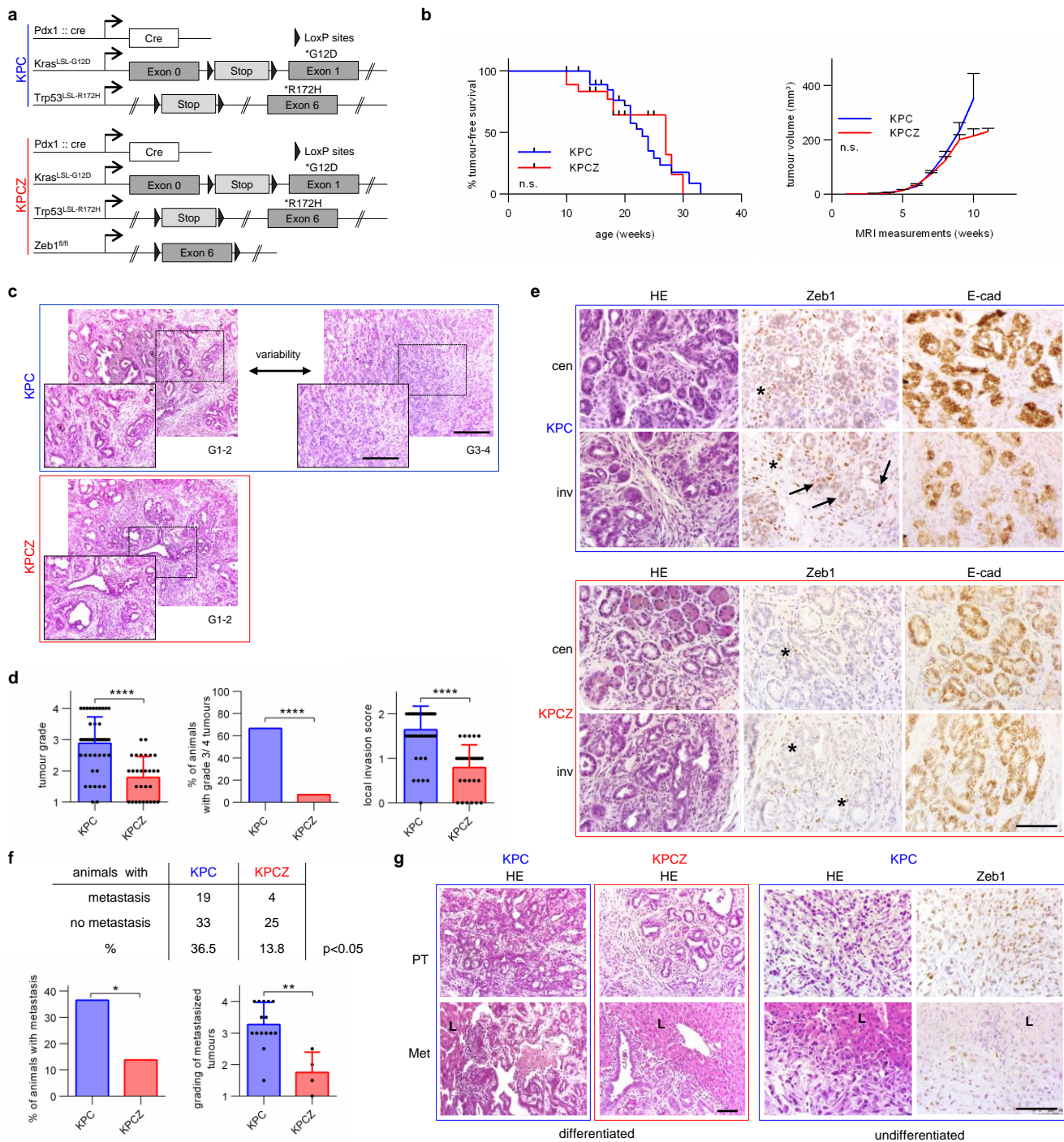
242 67. Murray, S.A., Carver, E.A. & Gridley, T. Generation of a Snail1 (Snai1)
243 conditional null allele. *Genesis* **44**, 7-11 (2006).

244 68. Meidhof, S. *et al.* ZEB1-associated drug resistance in cancer cells is reversed by
245 the class I HDAC inhibitor mocetinostat. *EMBO molecular medicine* **7**, 831-847
246 (2015).

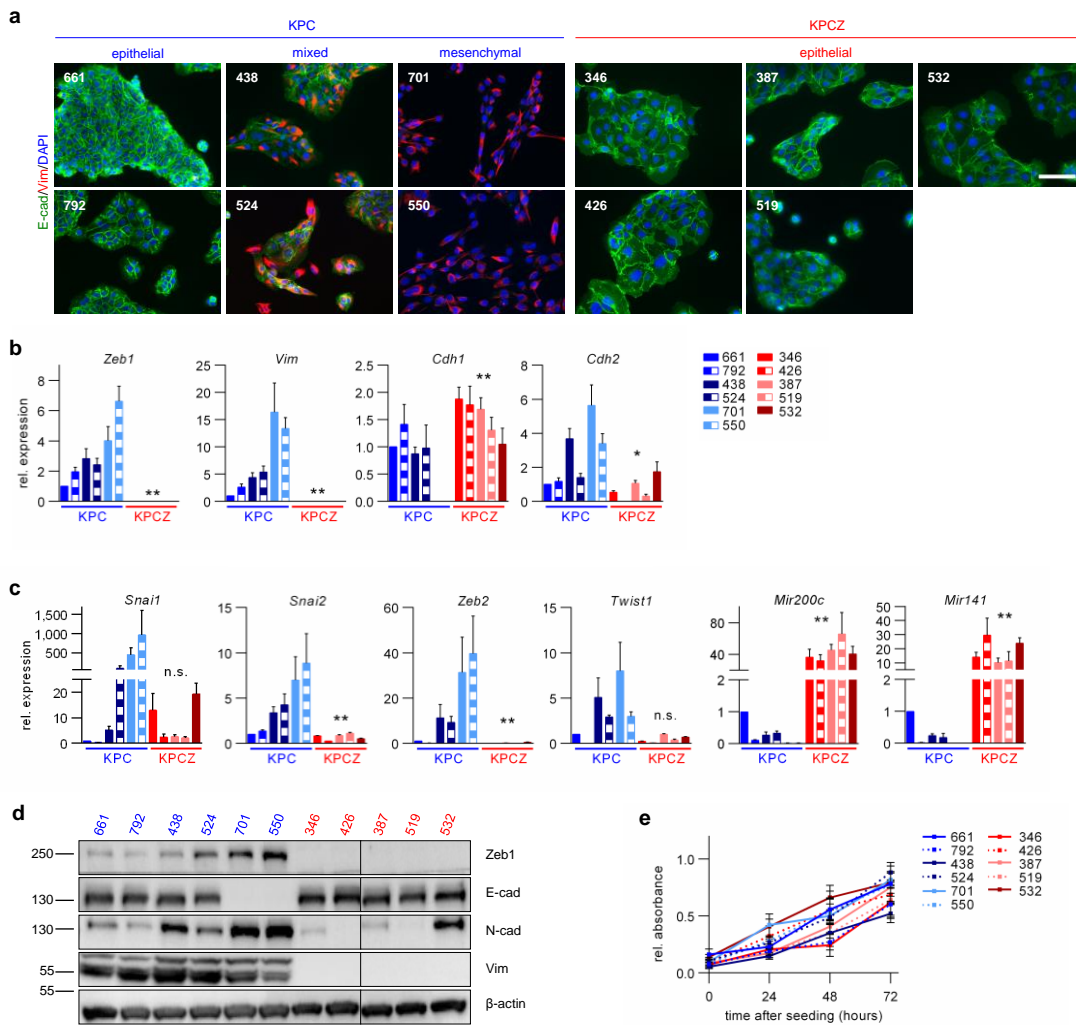
247 69. Dunning, M.J., Smith, M.L., Ritchie, M.E. & Tavare, S. beadarray: R classes and
248 methods for Illumina bead-based data. *Bioinformatics* **23**, 2183-2184 (2007).

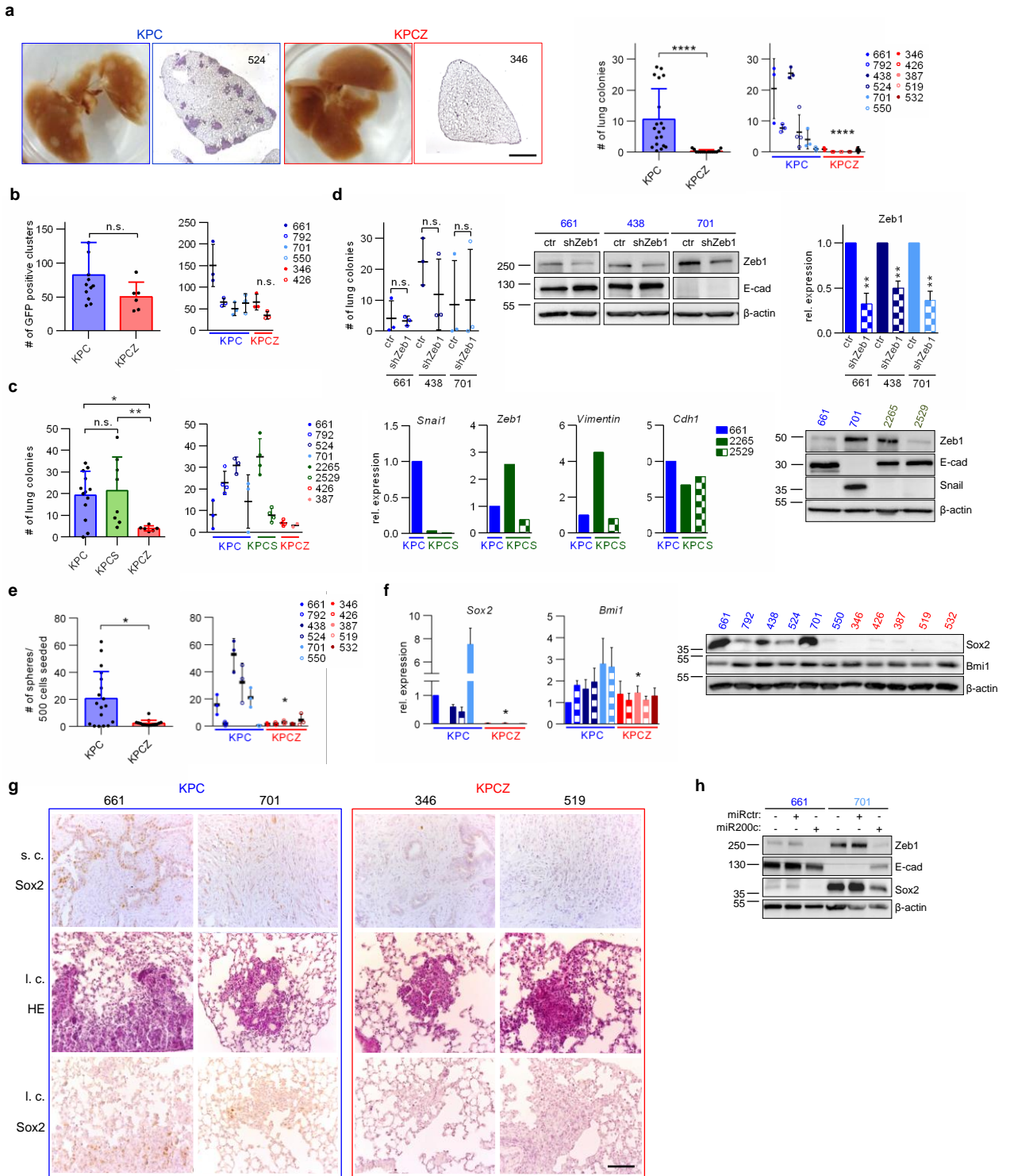
249 70. Johnson, W.E., Li, C. & Rabinovic, A. Adjusting batch effects in microarray
250 expression data using empirical Bayes methods. *Biostatistics* **8**, 118-127 (2007).

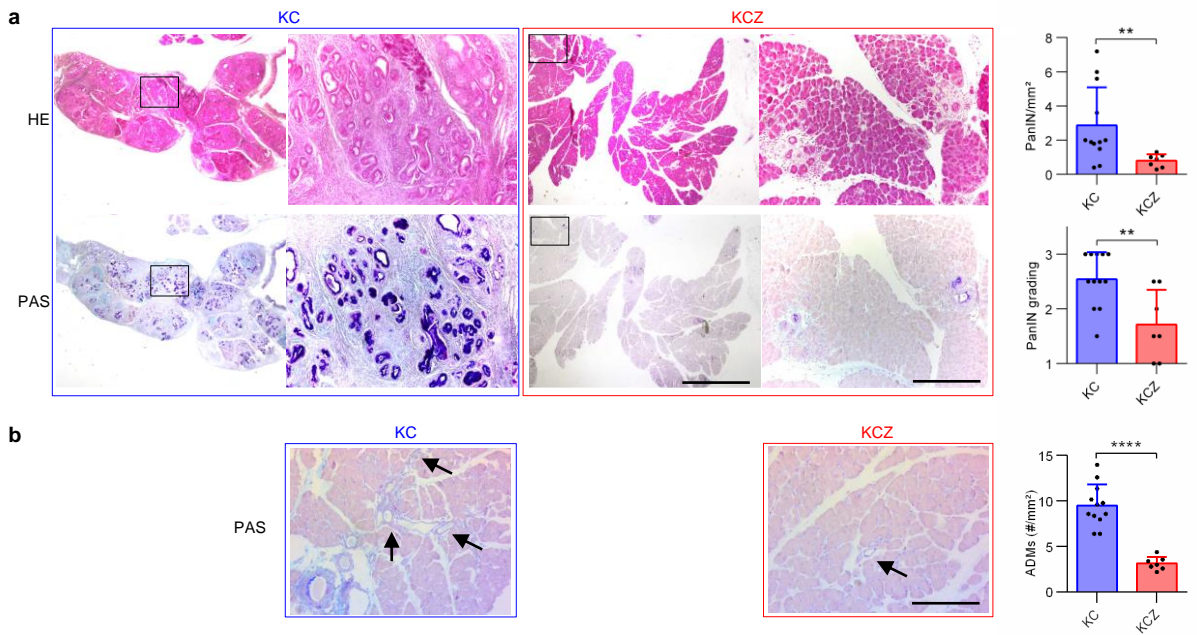
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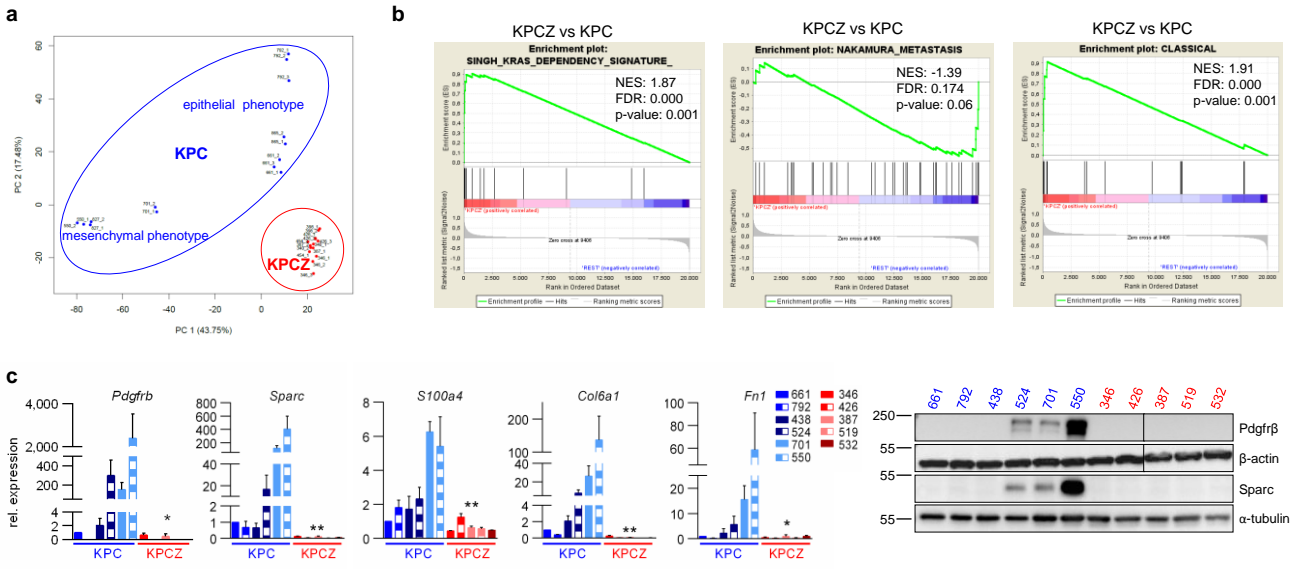


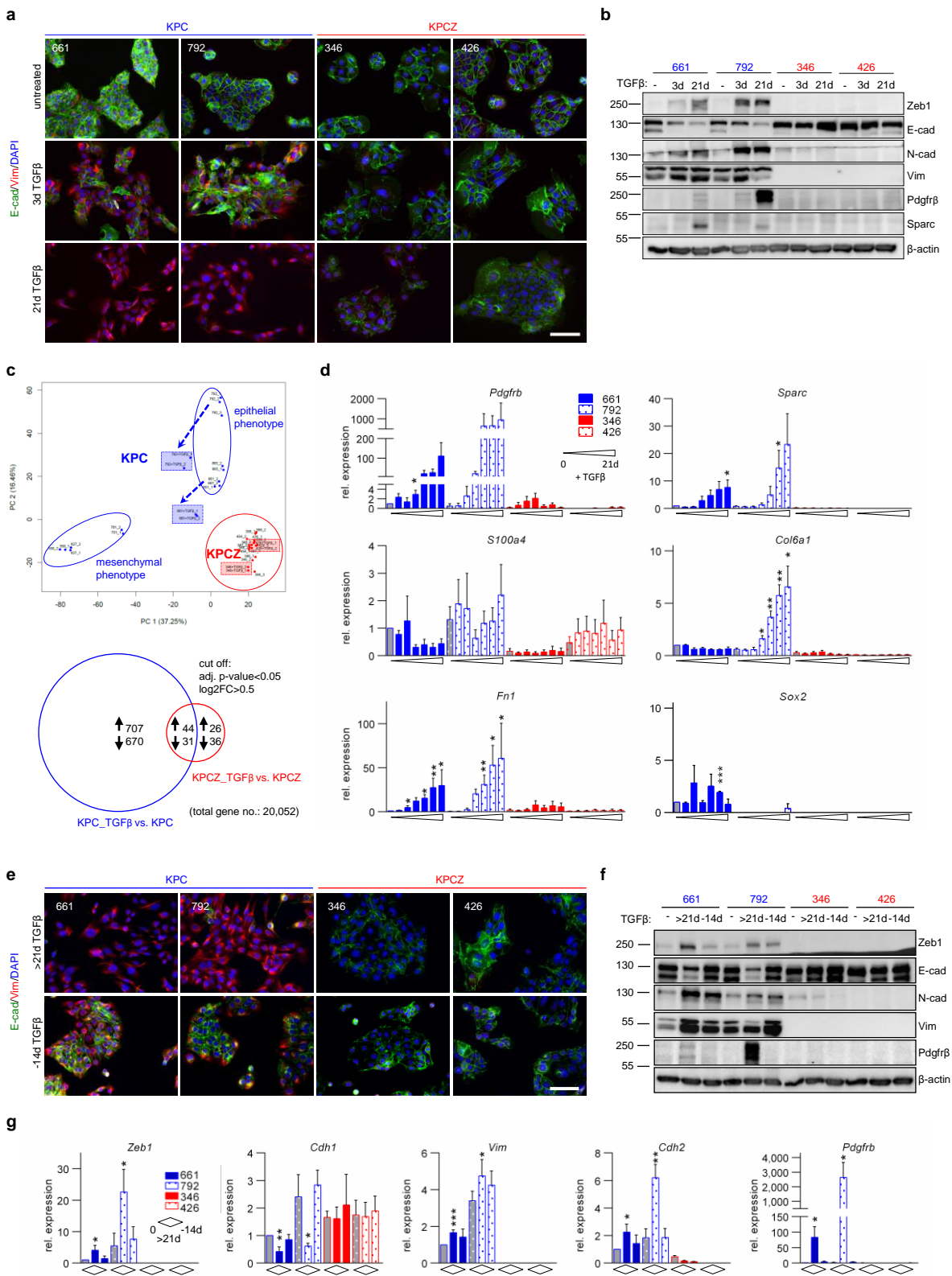
Krebs et al. Fig. 1

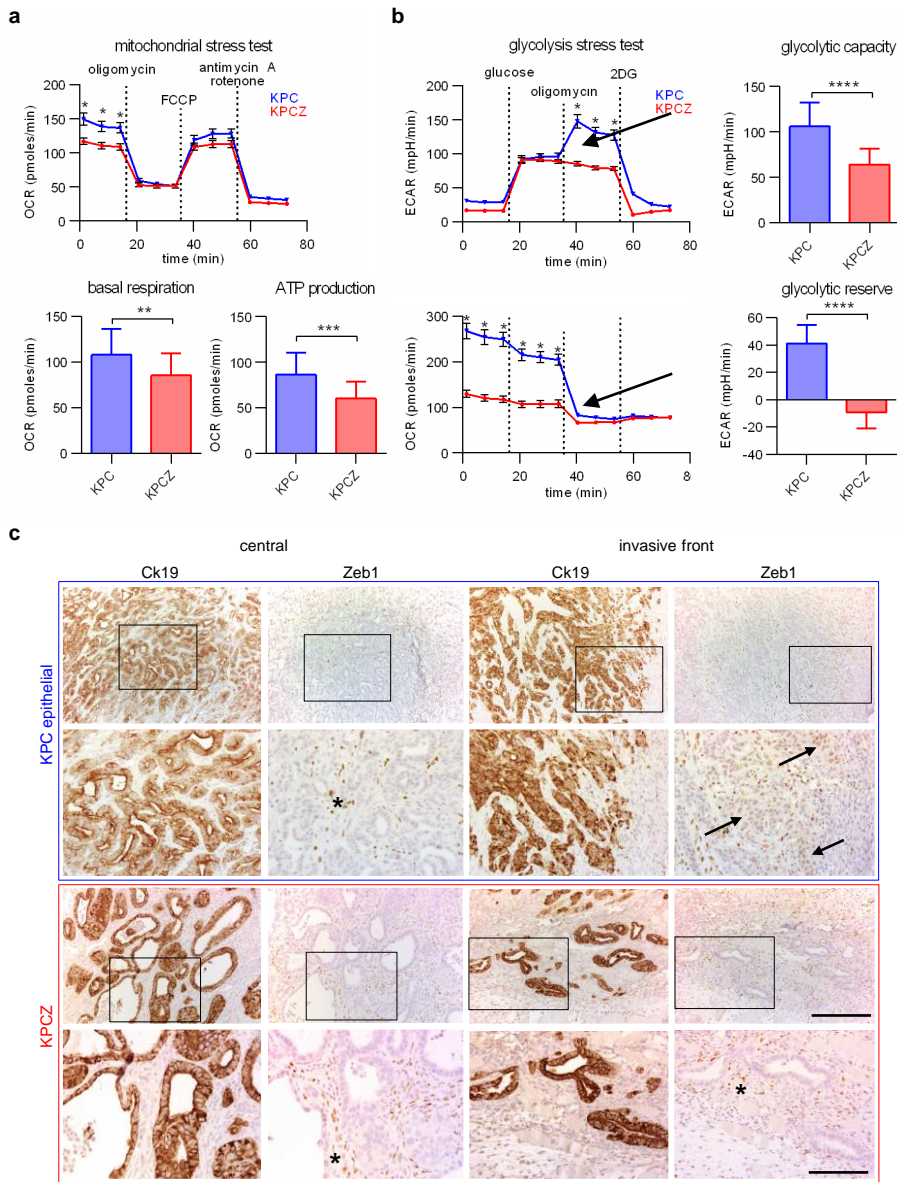












| genotype | phenotype | cell line | sphere formation | tumourigenicity | plasticity | lung colonisation | lung dissemination |
|----------|-------------|-----------|------------------|-----------------|------------|-------------------|--------------------|
| KPC | epithelial | 661 | ++ | +++ | +++ | +++ | +++ |
| | | 792 | + | +++ | +++ | + | ++ |
| | mesenchymal | 701 | ++ | ++ | na | + | ++ |
| | | 550 | - | + | na | - | ++ |
| KPCZ | epithelial | 346 | - | + | - | - | ++ |
| | | 426 | - | + | - | - | ++ |

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