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

3 Angela M. Krebs<sup>1,2,3</sup>, Julia Mitschke<sup>4</sup>, María Lasierra Losada<sup>1</sup>, Otto Schmalhofer<sup>4</sup>, Melanie  
4 Boerries<sup>3,5</sup>, Hauke Busch<sup>3,5</sup>, Martin Boettcher<sup>6</sup>, Dimitrios Mougiakakos<sup>6</sup>, Wilfried Reichardt<sup>3,7</sup>,  
5 Peter Bronsert<sup>3,8</sup>, Valerie G. Brunton<sup>9</sup>, Christian Pilarsky<sup>10</sup>, Thomas H. Winkler<sup>11</sup>, Simone  
6 Brabletz<sup>1</sup>, Marc P. Stemmler<sup>1,\*</sup>, Thomas Brabletz<sup>1,\*</sup>

7

8 <sup>1</sup> Dept. of Experimental Medicine 1, Nikolaus-Fiebiger-Center for Molecular Medicine, FAU  
9 University Erlangen-Nürnberg, Glückstr. 6, 91054 Erlangen, Germany

10 <sup>2</sup> University of Freiburg, Faculty of Biology, Schaezlestr. 1, 79104 Freiburg, Germany

11 <sup>3</sup> German Cancer Consortium (DKTK), Freiburg, Germany; German Cancer Research Center  
12 (DKFZ), 69121 Heidelberg, Germany.

13 <sup>4</sup> Dept. of General and Visceral Surgery, University of Freiburg Medical Center, Hugstetter  
14 Str. 55, 79106 Freiburg, Germany

15 <sup>5</sup> Systems Biology of the Cellular Microenvironment Group, Institute of Molecular Medicine  
16 and Cell Research (IMMZ), Albert-Ludwigs-University Freiburg, Germany, Stefan-Meier-Str.  
17 17, 79106 Freiburg, Germany

18 <sup>6</sup> Dept. of Internal Medicine 5, Hematology and Oncology, Universitätsklinikum, FAU  
19 University Erlangen-Nürnberg, Ulmenweg 18, 91054 Erlangen, Germany

20 <sup>7</sup> Dept. of Radiology Medical Physics, University Medical Center, Freiburg, Breisacher Str.  
21 60a, 79106 Freiburg, Germany

22 <sup>8</sup> Inst. of Pathology and Comprehensive Cancer Center, University Medical Center, Freiburg,  
23 Breisacher Str. 115a, 79106 Freiburg, Germany

24 <sup>9</sup> Edinburgh Cancer Research Centre, Institute of Genetics & Molecular Medicine, University  
25 of Edinburgh, Crewe Road South, Edinburgh EH4 2XR, UK

26 <sup>10</sup> Dept. of Surgery, Universitätsklinikum, FAU University Erlangen-Nürnberg, Ulmenweg 18,  
27 91054 Erlangen, Germany

28 <sup>11</sup> Chair of Genetics, Nikolaus-Fiebiger-Center for Molecular Medicine, Dept. Biology, FAU  
29 University Erlangen-Nürnberg, Glückstr. 6, 91054 Erlangen, Germany

30

31 \*both authors contributed equally and share senior authorship

32 \*Correspondence should be addressed to T.B. (e-mail: [thomas.brabletz@fau.de](mailto:thomas.brabletz@fau.de)) or M.P.S.  
33 (e-mail: [marc.stemmler@fau.de](mailto:marc.stemmler@fau.de))

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 metastasis<sup>1-3</sup>. 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 properties<sup>4-6</sup>. 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<sup>7, 8</sup>. 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)<sup>8</sup>. 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% penetrance<sup>9</sup>. 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 progression<sup>10</sup>, 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<sup>fl</sup>*) (Fig.1a). Cre-

75 mediated zygotic deletion of *Zeb1* phenocopied the described developmental defects of a  
76 conventional *Zeb1* knockout<sup>11</sup>, thereby confirming its loss-of function<sup>12</sup>. We crossed the  
77 floxed *Zeb1* allele homozygously into KPC mice (*Pdx1-cre;Kras<sup>LSL.G12D/+</sup>;Tp53<sup>LSL.R172H/+</sup>*) to  
78 generate KPC;*Zeb1<sup>fl/fl</sup>* 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<sup>fl/+</sup>*) (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<sup>+</sup>  
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 PDAC<sup>13</sup>. 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<sup>14, 15</sup> or reduced<sup>16, 17</sup> 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 progression<sup>8</sup>.

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 ( $1 \times 10^5$  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.<sup>8</sup>. 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 metastasize<sup>18-20</sup>. 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 combinations<sup>21</sup> 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* repression<sup>22</sup> 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 model<sup>23</sup>. 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 members<sup>24</sup>. 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 PDACs<sup>25-27</sup>. 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<sup>28, 29</sup>. In addition it was shown that mutant *p53* overcomes a  
170 growth arrest in pancreatic cancer<sup>30</sup>. 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<sup>LSL.G12D/+</sup>*, termed KC). These mice develop slowly progressing  
174 acinar-ductal metaplasia (ADM)- as well as PanIN-precursor lesions, which also express  
175 *Zeb1*<sup>10</sup>. 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 cells<sup>31</sup> and we have shown that Zeb1 exerts  
183 pleiotropic effects on many different programs and pathways<sup>31-33</sup>. 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* expression<sup>34</sup>, reduced metastatic  
193 competence<sup>35</sup>, as well as the “classical” subtype of human PDACs, which have the best  
194 clinical prognosis<sup>36</sup> (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 p53<sup>37</sup>. 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<sup>1, 38, 39</sup>. 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 cues<sup>4</sup>. 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 TGF $\beta$ 1, a driver of malignant tumour progression and prominent  
210 inducers of EMT<sup>40, 41</sup>. As expected, upon TGF $\beta$  treatment KPC cells with an epithelial  
211 phenotype underwent an EMT. However, even after long-term TGF $\beta$  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 TGF $\beta$ -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 TGF $\beta$  (Fig. 6c). The PCA showed an induction of a mesenchymal  
217 phenotype only of the KPC cell lines under TGF $\beta$  stimulation along the first principal  
218 component (PC1). Among the 20,052 analysed genes, 1514 were significantly regulated  
219 upon long-term TGF $\beta$  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 TGF $\beta$  in a Zeb1-dependent manner (Fig. 6d). These data also indicate that  
223 Zeb1 is important for a large fraction of TGF $\beta$  induced changes. The Zeb1-dependent TGF $\beta$   
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)<sup>31</sup>. 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 TGF $\beta$  (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 metastasis<sup>42</sup>. 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 metastasis<sup>8</sup>. 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 patterns<sup>31</sup> 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<sup>1, 38, 39, 43, 44</sup>. We here describe a central role of Zeb1  
275 in exerting different aspects of cellular plasticity, particularly the response to TGF $\beta$ , 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 $\beta$  treatment. These genes include *Pdgfrb*, which was recently shown to be  
280 absolutely required for metastasis in *p53*-mutant pancreatic cancer<sup>37</sup>. As a side effect, our  
281 finding that absence of *Zeb1* strongly reduces the number of TGF $\beta$ -regulated genes

282 indicates that *Zeb1* is important for a large part of the TGF $\beta$  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<sup>45, 46</sup>.  
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 types<sup>25-27</sup>. 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 phenotype<sup>4</sup>. 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-CSCs<sup>47</sup>. 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 progression<sup>31</sup>. Genes of this target set can be activated by TGF $\beta$  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<sup>48, 49</sup>.

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<sup>36, 50</sup>. 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<sup>24, 51, 52</sup>. 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<sup>34, 53</sup>. Thus, although KRAS bears the key mutation in pancreatic cancer<sup>54</sup>,  
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 context<sup>55</sup>. 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<sup>1, 56</sup>. Again, mutated p53 might enhance the generation of  
335 such a genetically driven metastasis<sup>30</sup>. 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<sup>57, 58</sup>, for *Snail* and  
346 *Slug* in breast cancer<sup>59</sup>, as well as for *Sox4*<sup>60</sup> and *Prrx1*<sup>19</sup>. Moreover sub-functions can be  
347 tissue specific, as demonstrated by the different roles of *Snail* in metastasis of breast<sup>61</sup> and  
348 pancreatic cancer<sup>8</sup>. 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

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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  $\mu$ m and 125  
544  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ 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 mm<sup>2</sup> 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  $\mu$ 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 mm<sup>2</sup> 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  $\mu$ 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  $\mu$ m for higher magnifications in (a) and 75  $\mu$ 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 $\beta$ -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 $\beta$ 1 for 3 and 21 days. Scale bar, 100  $\mu$ 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 $\beta$  treatment. TGF $\beta$  -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 $\beta$ \_1 and TGF $\beta$ \_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 log<sub>2</sub>FC>0.5) by 14 days of TGF $\beta$  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 $\beta$  (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  
665 t-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*<sup>LSL.G12D</sup> (*Kras*<sup>tm4Tyj</sup>),

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

12 mouse model have been described<sup>9, 62-66</sup> and were kept on a C57BL/6 background. The

13 generation of the conditional *Zeb1* knockout allele (*Zeb1*<sup>fl</sup>) is described elsewhere<sup>12</sup>. 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*<sup>LSL.G12D/+</sup>; *Tp53*<sup>LSL.R172H/+</sup> mice (KPC) and *Pdx1-Cre*; *Zeb1*<sup>fl/fl</sup> with

17 *Kras*<sup>LSL.G12D/+</sup>; *Tp53*<sup>LSL.R172H/+</sup>; *Zeb1*<sup>fl/fl</sup> 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*<sup>+/+</sup> 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 Brucker Bio Spin 94/20, 9.4Tesla – 400MHz – 20cm small animal  
28 MR using coronal and transverse scans with a spatial resolution of 117  $\mu\text{m}$  x 117  $\mu\text{m}$ /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  $\text{mm}^3$ .

#### 34 **Histology, histopathology and immunohistochemistry**

35 PFA-fixed tissues were embedded into paraffin, sectioned at 4-5 $\mu\text{m}$  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  $\text{mm}^2$ . 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  $\mu\text{m}$ .

52 Immunohistochemical analysis was performed as previously described<sup>31</sup>. 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 CO<sub>2</sub> 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* deletion<sup>67</sup>. 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  $\beta$ -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 $\beta$ 1 (PeproTech, 100-21) and replacing the  
86 medium daily for the duration of the experiment. miRNA overexpression was performed as  
87 previously described<sup>31</sup>. For FACS analysis of cancer stem cells markers  $1 \times 10^6$  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 described<sup>31</sup>,  
98 <sup>32</sup> 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- $\beta$ -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 $\beta$  (Cell Signaling, CS3169, Clone 28E1, 1:1,000); monoclonal rabbit anti-  
110 Sparc (Cell Signaling, CS8725; Clone D10F10, 1:1,000); monoclonal mouse anti- $\alpha$ -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  $\mu$ 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  $\mu$ 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 described<sup>68</sup>. 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 previously<sup>31</sup>. 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*<sup>nu/nu</sup> 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  $\mu\text{m}$ .  
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  $\text{mm}^3$  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 $\beta$ -treated  
168 epithelial KPC and two TGF $\beta$ -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 beadarray<sup>69</sup> and subsequently batch corrected according to their chip identity via ComBat<sup>70</sup>  
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. 2011<sup>36</sup>.

#### 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 CO<sub>2</sub>-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**

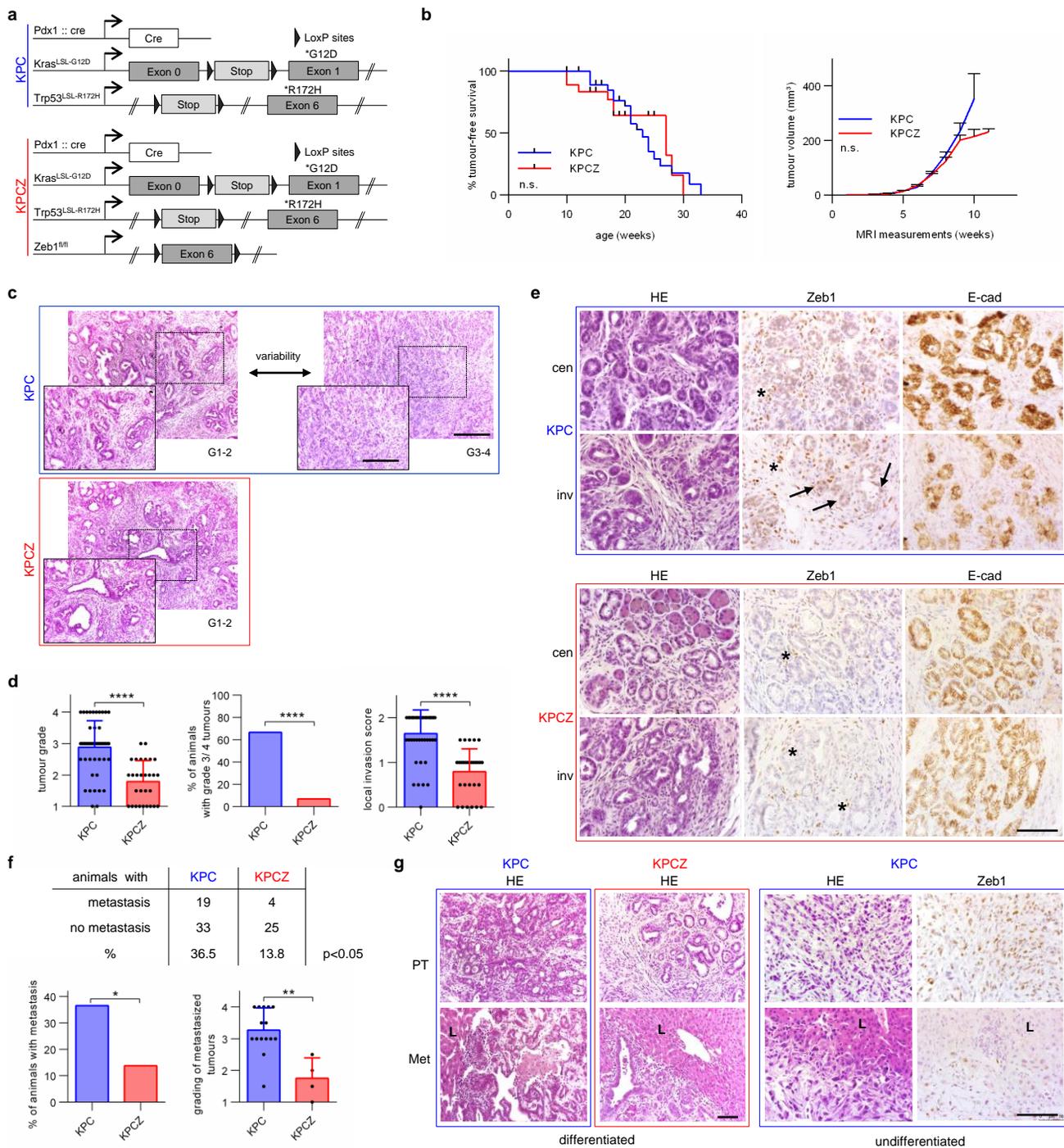
217 Microarray data generated in this study have been deposited in the Gene Expression  
218 Omnibus (GEO) under accession code GSE87472. The 189 publically available gene sets  
219 reanalysed here were from of the oncogenic signature C6 available from the Molecular  
220 Signatures database (<http://www.broadinstitute.org/gsea/msigdb/genesets.jsp?collection=C6>,  
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. 2011<sup>36</sup>.  
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

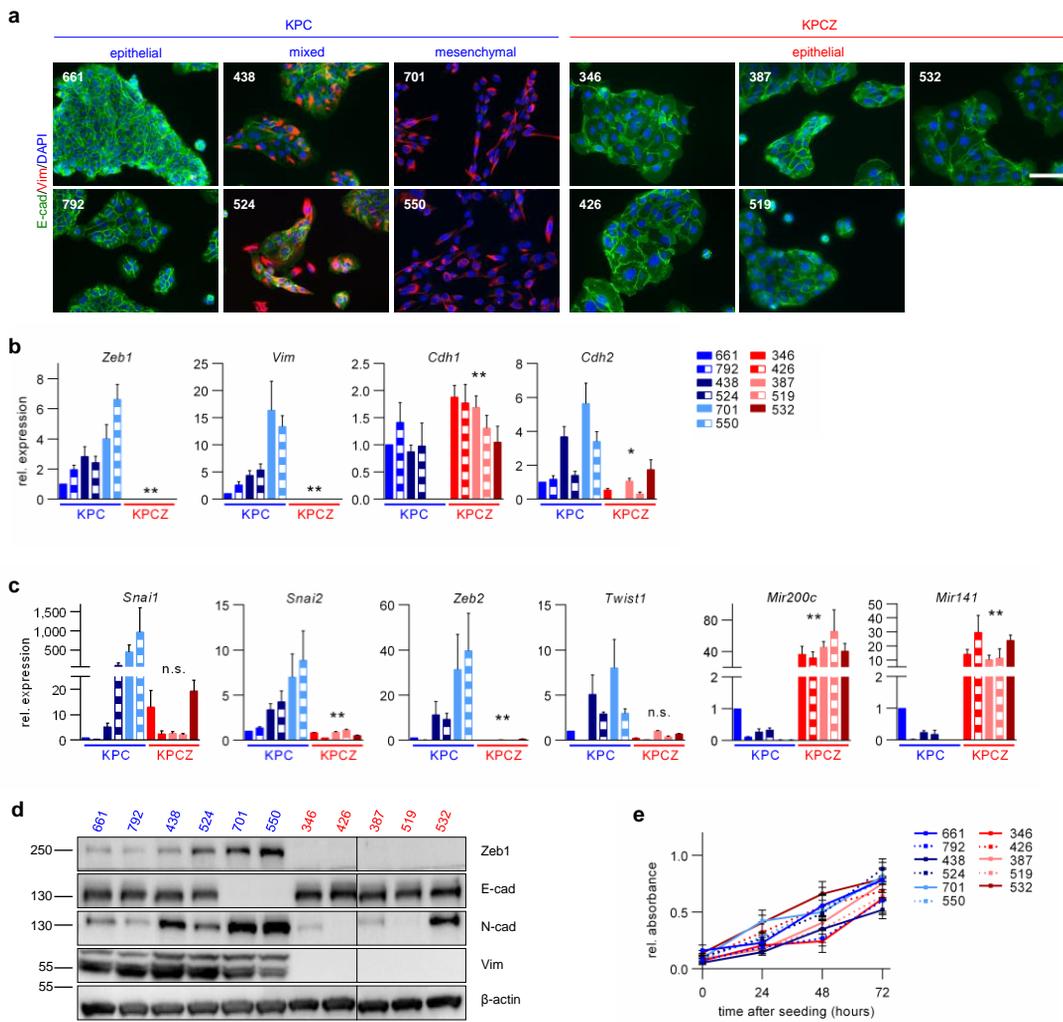
## 229 **Additional references for methods**

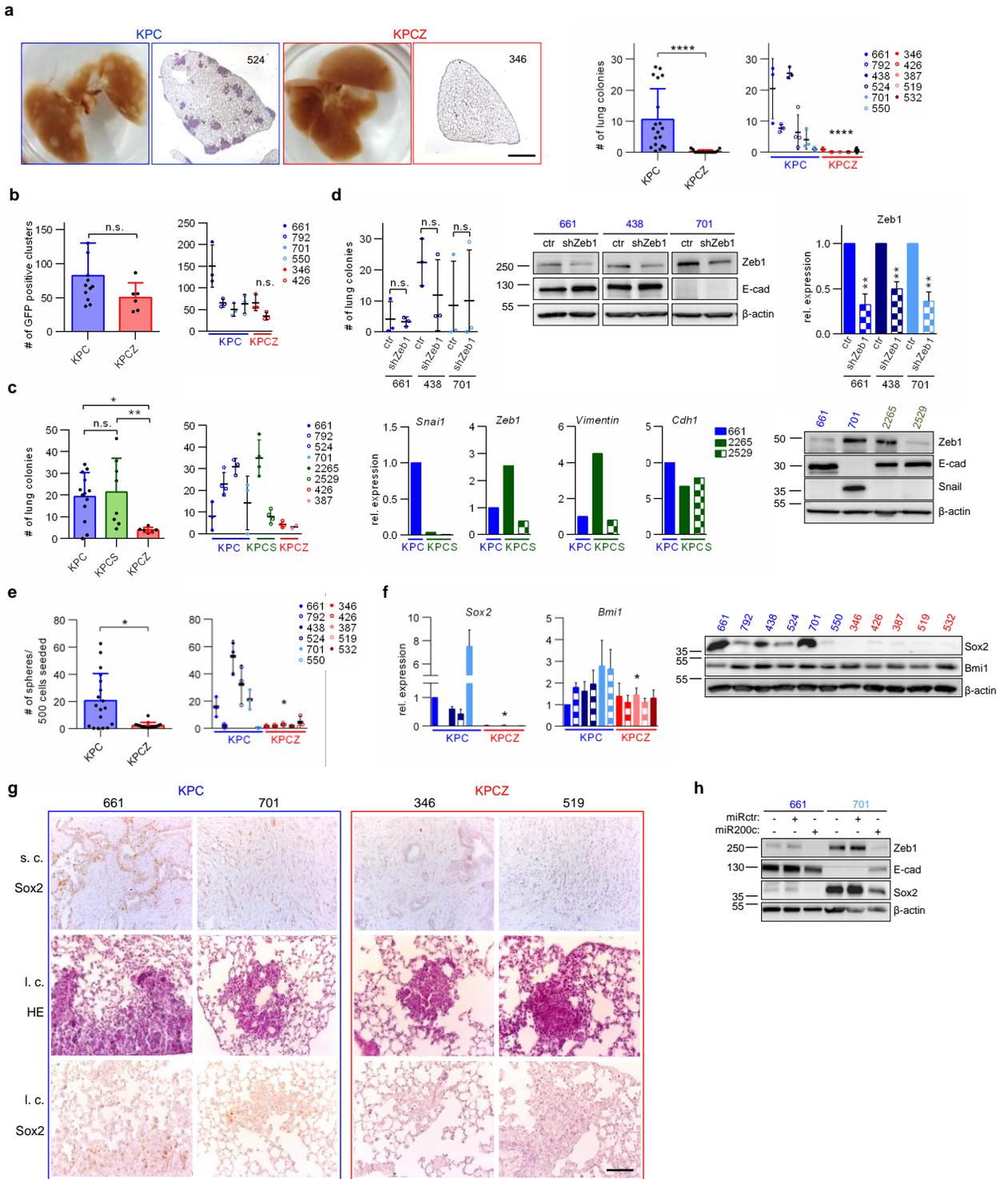
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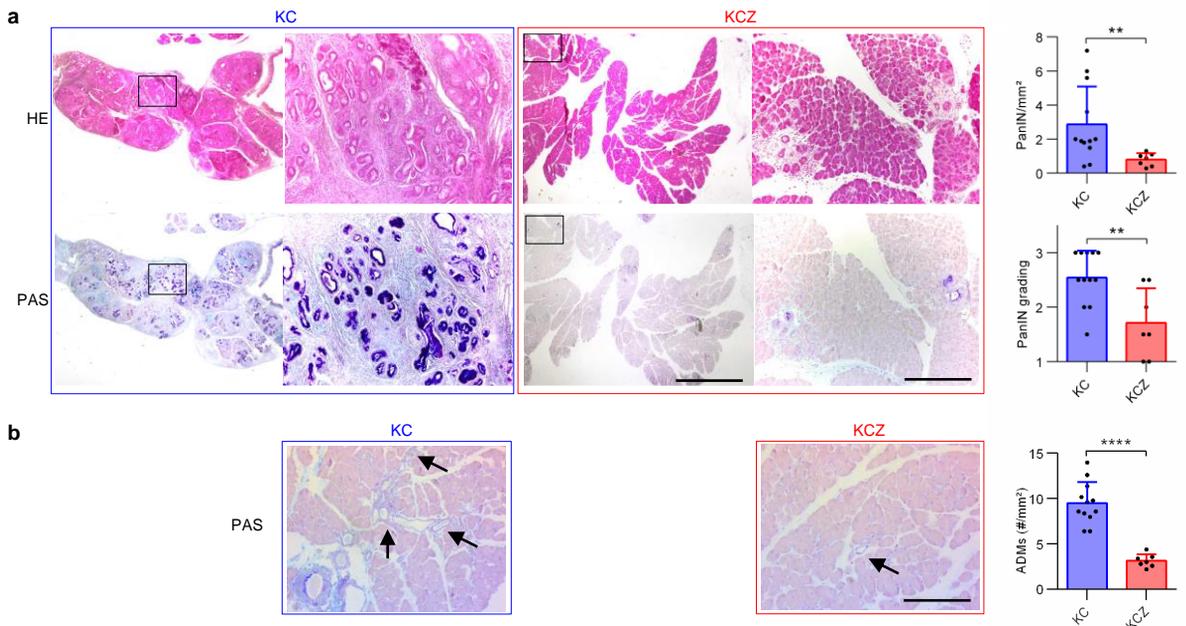
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251

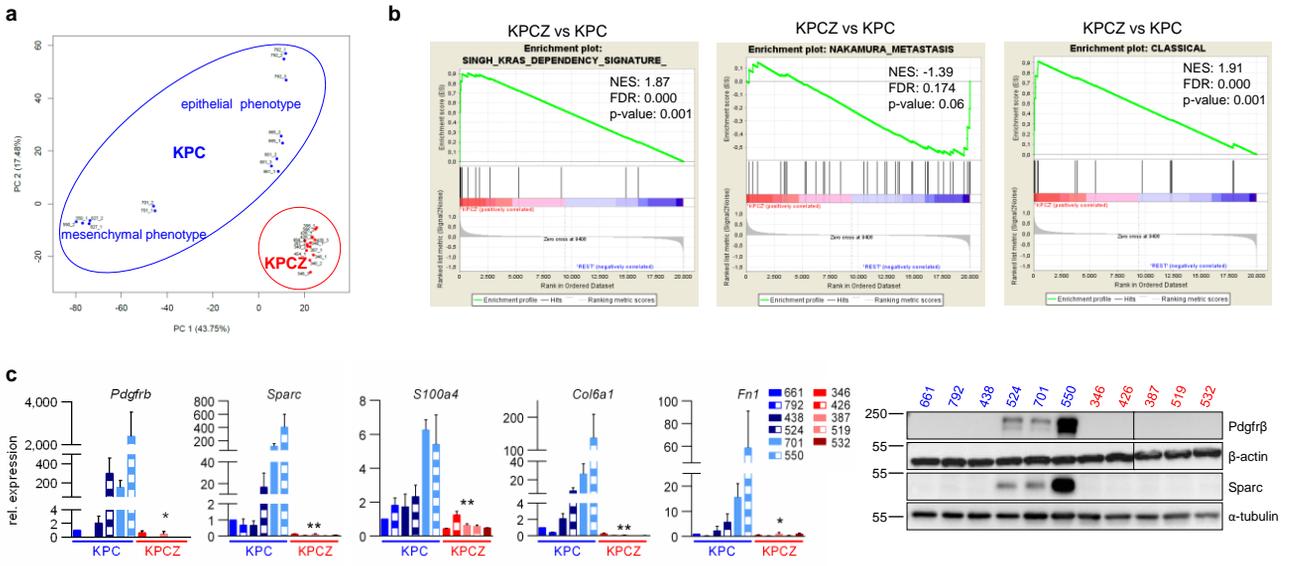


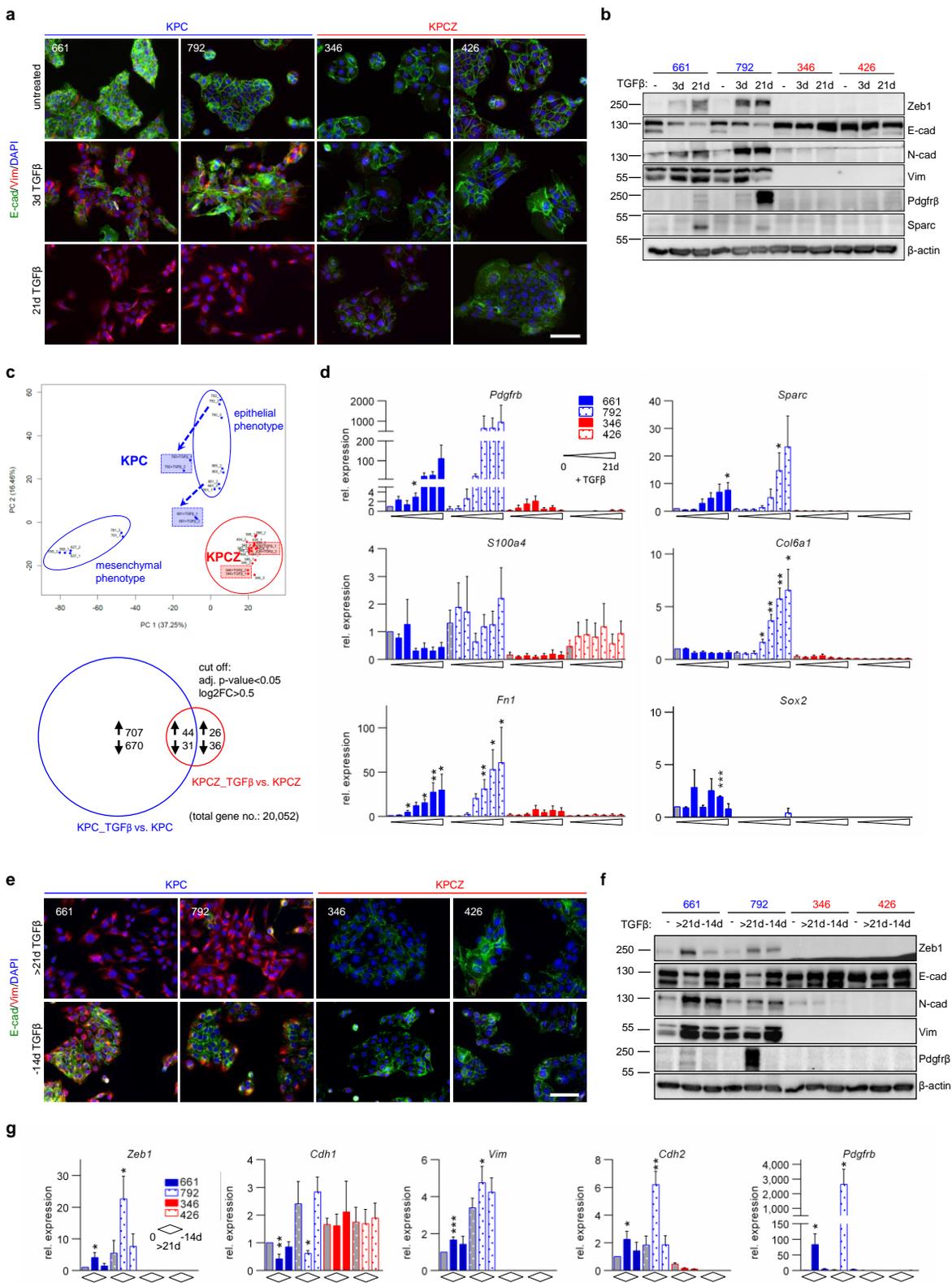
Krebs et al. Fig. 1

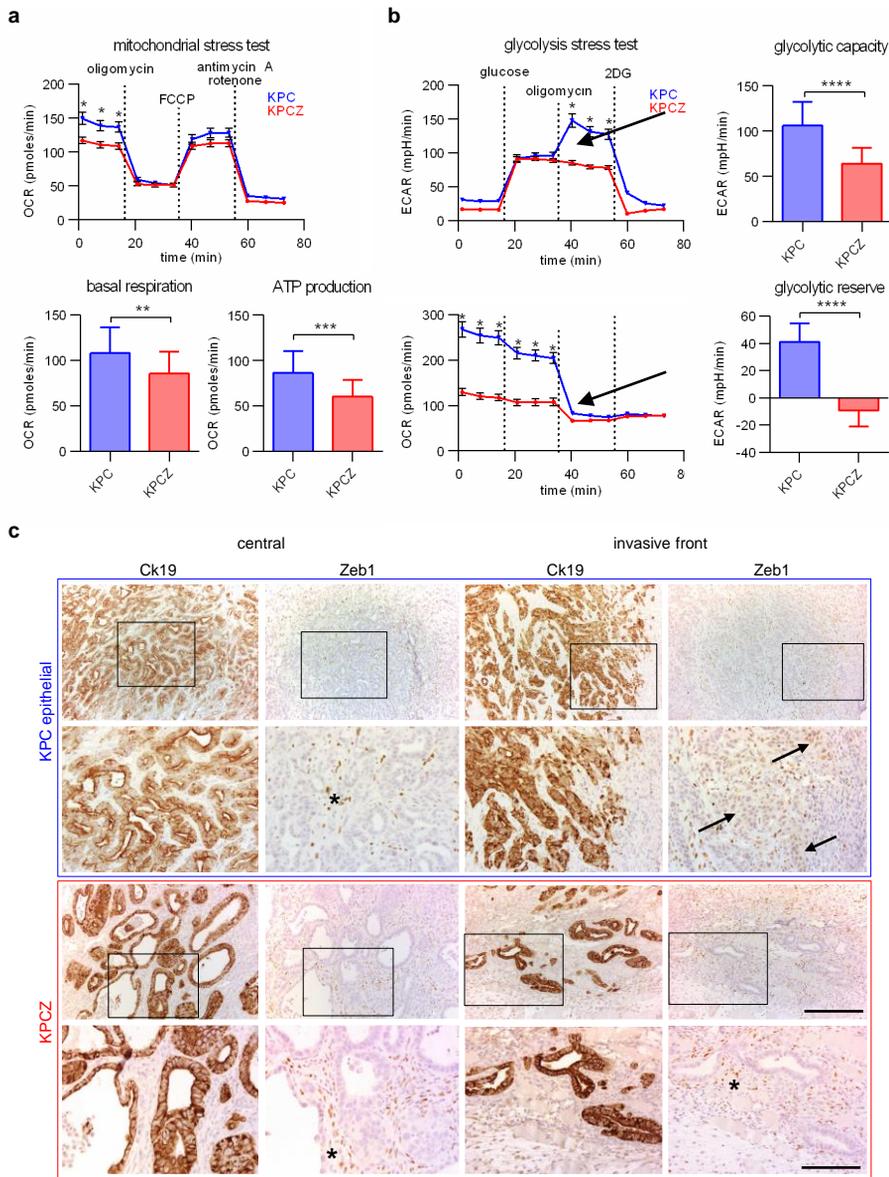












| genotype | phenotype   | cell line | sphere formation | tumourigenicity | plasticity | lung colonisation | lung dissemination |
|----------|-------------|-----------|------------------|-----------------|------------|-------------------|--------------------|
| KPC      | epithelial  | 661       | ++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

Krebs et al. Table 1