Mitotic polarization of transcription factors during asymmetric division establishes fate of forming cancer cells

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A model of K-Ras-initiated lung cancer was used to follow the transition of precancerous adenoma to adenocarcinoma. In hypoxic, Tgf-β1-rich interiors of adenomas, we show that adenoma cells divide asymmetrically to produce cancer-generating cells highlighted by epithelial mesenchymal transition and a CD44/Zeb1 loop. In these cells, Zeb1 represses the Smad inhibitor Zeb2/Sip1, causing Pten loss and launching Tgf-β1 signaling that drives nuclear translocation of Yap1. Surprisingly, the nuclear polarization of transcription factors during mitosis establishes parent and daughter fates prior to cytokinesis in sequential asymmetric divisions that generate cancer cells from precancerous lesions. Mutation or knockdown of Zeb1 in the lung blocked the production of CD44hi, Zeb1hi cancer-generating cells from adenoma cells. A CD44/Zeb1 loop then initiates two-step transition of precancerous cells to cancer cells via a stable intermediate population of cancer-generating cells. We show these initial cancer-generating cells are independent of cancer stem cells generated in tumors by p53-regulated reprogramming of existing cancer cells.

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A small population of cells, termed cancer stem cells (CSC) or tumor-initiating cells, have been identified in many tumors, including lung adenocarcinoma (AC)\(^1\). These cells can divide asymmetrically to generate cancer cells, while maintaining their numbers in the tumor. CSC were thought to arise from the transformation of adult stem cells or progenitor population persisting in tissues, and these cells, in turn, were responsible for the generation of initial cancer cells. But, recent studies demonstrate that existing cancer cells undergo reversible reprogramming to generate CSC, which are then thought to be critical for maintaining cancer cell numbers in tumors and generating new cancer cells following therapy\(^1\). Thus, a relationship between CSC generated from reprogramming of existing cancer cells and the pathway leading to initial cancer cell generation are still being unraveled.

Although CSC display normal stem cell properties such as asymmetric division, there are key differences in pathways and gene expression patterns in CSC vs. stem cells. Perhaps, the foremost among these differences is tissue stems cells display an epithelial-like phenotype, and induced pluripotent stem cells (iPS) reprogramming to generate stem-like cells requires a mesenchymal-to-epithelial transition\(^1\), whereas CSC are characterized by an opposing epithelial mesenchymal transition (EMT), which can be driven by induction of EMT transcription factors such as Zeb1\(^2,5\). This EMT in CSC is linked to high expression of CD44, which marks CSC in tumors including breast and lung cancers\(^6\), and a positive CD44/Zeb1 loop has been shown to drive EMT and reprogramming of existing cancer cells to CSC phenotype\(^10,11\). This loop can be initiated by Tgf-β induction of Zeb1 in cell culture\(^2\), but it is unclear if such a loop is present or functional in vivo.

We utilized a K-Ras-initiated model of lung AC\(^12\) to search for a CD44/Zeb1 loop in vivo, and address its potential role in cancer cell generation. Ras pathway mutations, including K-Ras itself and EGFR, have been widely utilized in mouse models of human lung AC\(^13\). These mutations are mutually exclusive in human lung AC, suggesting that they are redundant and thus equivalent in Ras pathway activation in the lung\(^14\). Mutations such as Pten or p53 affect tumor progression in this K-Ras model, and they have been widely utilized with K-Ras to evaluate their roles in tumors. Notably, Pten is not mutated in K-Ras-initiated tumors such as lung and pancreatic AC, but instead, its expression is somehow repressed as these tumors progress, accounting for Pten mutation accelerating tumor progression in these mice\(^15-17\). Compound mutation of p53 does not affect cancer cell generation or their expansion into tumors\(^18,19\). Instead, its mutation allows K-Ras-initiated tumors to transition to metastasis, implying p53 is acting later to promote cancer cell metastasis in this model.

As opposed to compound mutations generated simultaneously in mouse models, mutations are thought to arise sequentially over a long period in patients. In this regard, it is of note that K-Ras mutation alone initiates a pathway leading to lung AC in mice, but with this single mutation, the process is highlighted by a protracted period of precancerous lesion expansion\(^12,20\). In these mice, precancerous subpleural adenomas form around bronchial airways (Fig. 1a). AC cells appear later in these adenomas, and they expand into large tumors that invade airways.

Elegant lineage mapping in the lung has shown both Clara and ATII cells are the targets of K-Ras transformation responsible for precancerous adenoma generation\(^21,22\). In the absence of concurrent p53 mutation, transformed ATII cells are the only lineage that transitions to AC, thus AC in this model are derived from ATII cells. But, as noted above, steps involved in the transition of ATII-derived adenoma cells to AC are still being unraveled. We focused our studies on such sites of initial cancer cell generation within expanding adenomas in the lungs of mice expressing mutant K-Ras. We found an evidence that a positive CD44/Zeb1 loop is initiated in small clusters of cells derived from asymmetrically dividing adenoma cells at sites of hypoxia, EMT, and Tgf-β1 accumulation in the interiors of expanding adenomas. Zeb1 repression of the Smad inhibitor Zeb2/Sip1 launches Tgf-β1 signaling in these cell clusters, and they activate transcription factors and pathways known to be important for CSC formation. We show these adenoma-derived cluster cells are initial cancer-generating cells (CGC), and they, in turn, divide asymmetrically to produce AC cells. These two sequential asymmetric divisions that generate cancer cells from precancerous lesions both show early nuclear polarization of key transcription factors during mitosis, and resulting asymmetric expression of their target genes in opposite cell poles then establishes parental and daughter fates prior to cytokinesis. Mutation or knockdown of Zeb1 in the lung blocked the production of these Zeb1\(^15\), CD44\(^16\)–\(^17\) cancer-generating cluster cells from adenoma cells, in turn inhibiting cancer cell formation. These results provide evidence that a CD44/Zeb1 loop is activated in vivo at sites of hypoxia and Tgf-β1 accumulation in expanding precancerous adenomas, and this loop then initiates a two-step pathway in which precancerous cells transition to cancer cells via a stable intermediate population of CGC. We provide evidence that this population of initial CGC is independent of CSC that are generated in tumors via p53-regulated reprogramming of existing cancer cells.

**Results**

**An Intermediate Population Arises in the Hypoxic Interior of Precancerous Adenomas.** In response to K-Ras mutation in the mouse lung, atypical adenomatous hyperplasia (AAH) develops around bronchial airways, and these cells expanded to form benign subpleural precancerous adenomas by postnatal day (P) \(126\)\(^1\) (Fig. 1a). Inflammatory infiltrate and Tgf-β1 accumulation was evident at these sites of AAH and adenoma outgrowth (Fig. 1a, b). Although Tgf-β1 is a known driver of EMT transcription factor expression and thus EMT, cells in AAH and adenomas, as well as adjacent normal lung epithelium, continued to express the epithelial specification protein E-cadherin, and they failed to induce the mesenchymal marker Vimentin, indicating they had not undergone EMT (Fig. 1c). By P150, the interior of expanding adenomas showed evidence of hypoxia, detected by immunostaining for EF5 (EF5\(^+\)) (Fig. 1d). Within these regions, small sites of intense EF5 immunostaining (EF5\(^++\)) appeared (Fig. 1e). These sites consisted of cell clusters at sites of Tgf-β1 accumulation that had undergone EMT, as evidenced by the loss of E-cadherin and induction of Vimentin (Fig. 1f–i). These cluster cells displayed the decondensation of nuclear heterochromatin, and in this way, were also distinct from surrounding adenoma cells, which contained small nuclei with densely packed chromatin (Fig. 1f–i). By P170, the clusters had expanded, and although blood vessels were evident in adenoma at the border of the clusters, these hypoxic clusters themselves were devoid of vessels (Fig. 1j, j'). At this age, cancer cells, characterized by large euchromatic vesicular nuclei with an irregular contour and nuclear enlargement, were scattered through the clusters (Fig. 1j–j'). The clusters at P170 then contained cells with three different nuclear morphologies: AD cells with small nuclei containing densely packed heterochromatin, cancer cells with large euchromatic nuclei, and intermediate cells (IC) with nuclear size heterochromatin content between adenoma and cancer cells (Fig. 1j'). Clusters per adenoma are quantified in Fig. 1k, and IC are the predominant cells within these clusters at P170 (Fig. 1k). As the number of cancer cells increased, they formed dense foci and began to display papillary structure (Fig. 1j, l–n). As opposed
miR-200 repression in clusters (Fig. 2i, j). Consistently, in cell culture heterozygous mutation of Bmi123 resulted in the lack of E-cadherin and expression of Zeb1, which has been shown to target mRNAs including CD44 and the CSC transcription factor Bmi123–28. We reasoned that the transcriptional repressor Zeb1, might be important for CD44 expression via its repression of miR-200. In situ hybridization showed that miR-200 is expressed in AAH, adenomas, and surrounding normal lung (Fig. 2g), but it was repressed with Zeb1 induction in clusters (Fig. 2h). As with CD44, Bmi1 was also induced with miR-200 repression in clusters (Fig. 2i, j). Consistently, in cell culture heterozygous mutation of Zeb1 in primary cultures of mouse embryo fibroblasts (MEFs), to reduce its level, or Zeb1
knockdown was sufficient for induction of miR-200 and repression of CD44 and Bmi1 mRNAs (Fig. 2k).

**Zeb1 Repression of Zeb2 Removes a Block in Tgf-β1 Signaling.**

Tgf-β classically induces Zeb1 to repress epithelial specification genes such as E-cadherin during EMT\(^{25,29}\), implying Tgf-β1 accumulating at sites of tumor formation might be important for driving EMT. Other EMT transcription factors, such as Snail, are also induced by Tgf-β, but they rapidly decline and only Zeb1 maintains repression of epithelial genes during EMT induced by Tgf-β.\(^{30}\) Beyond repression of epithelial genes, mesenchymal genes must be induced for full EMT, and mesenchymal genes, such as Vimentin, are directly activated by Smads in response to Tgf-β, demonstrating roles for Tgf-β signaling in both Zeb1-mediated repression of epithelial genes and direct induction of mesenchymal genes in EMT. Despite the widespread accumulation of Tgf-β1, AAH, adenoma, and surrounding normal lung continued to display an epithelial expression pattern (Fig. 1c), implying Tgf-β1 signaling might somehow be blocked in these cells, restricting Tgf-β1-driven EMT to cells in clusters.

Zeb1 and Zeb2 are related genes containing a common repressor domain\(^{31}\), leading to the notion that Zeb2 functionally overlaps with Zeb1 in cancer. However, Zeb2 contains a Smad-binding domain that blocks R-Smad activity, and it was first identified as Smad-interacting protein1 (Sip1)\(^{32}\). A switch from Zeb2 to Zeb1 highlights malignant transition in melanoma, and Zeb1 acted as an oncogene, whereas Zeb2 was a tumor suppressor in xenographic transplants of melanoma cell lines\(^{33-35}\). These results highlight opposing expression patterns and suggest different roles for Zeb1 and Zeb2 in melanoma, but such a transition from Zeb2 to Zeb1 has not been evaluated in lung cancer, and it is unclear if any functional differences in melanoma might be linked to Zeb2-mediated Smad inhibition. Nevertheless, mutation of Zeb2 revealed it has a critical role in the inhibition of Tgf-β family signaling to prevent competing mesoderm differentiation during neuroectoderm formation\(^{36,37}\), demonstrating its Smad inhibition is important in vivo during development.

As opposed to Zeb1, we found immunostaining for Zeb2 in AAH, adenomas, and normal lung, and it was downregulated with Zeb1 induction in hypoxic cell clusters forming in the interior of adenomas (Fig. 3a, b). These results demonstrate a Zeb2-to-Zeb1 switch linked to EMT, and they raised the possibilities, first that elevated Zeb2 might be responsible for blocking Tgf-β1 signaling in normal epithelium, AAH, and adenomas, and thereby preventing EMT, and, second Zeb1 repression of Zeb2 might be responsible for their switch in expression.

We turned to cell culture to examine these possibilities. Knockdown or mutation of Zeb1 led to the induction of Zeb2 mRNA (Fig. 3c–e). But, knockdown of Zeb2 did not affect the level of Zeb1 mRNA. Consistent with Zeb1 directly repressing Zeb2, we found Zeb1 bound to the Zeb2 promoter in vivo using chromatin immunoprecipitation assays, but Zeb2 was not detected at the Zeb1 promoter in the cells (Fig. 3f). We concluded that Zeb1 is directly repressing Zeb2.

Knockdown of Zeb1 led to the de-repression of E-cadherin mRNA, and Tgf-β1 was then unable to repress its expression (Fig. 3g). Tgf-β1 induction of Vimentin mRNA was also blocked by Zeb1 knockdown. By contrast, knockdown of Zeb2 augmented
Tgf-β1 induction of Zeb1 and Vimentin mRNAs. We concluded that the level of Zeb2 accumulating in the absence of Zeb1 is sufficient to block Tgf-β1 signaling, and thus Zeb1 repression of Zeb2 is acting to relieve the inhibition of Tgf-β1 signaling.

Zeb2 Smad Inhibition Blocks Yap1 Nuclear Translocation. Yap1 is a key target in regulating cell–cell contact inhibition\(^1\,26,38,39\). In this capacity, it is required during the development to block progenitor differentiation allowing outgrowth of forming organs, and in cancer, it is oncogenic, critical for CSC generation and it transitions tumor cells to Ras pathway independence\(^40-42\). Activation of the Hippo pathway, for example, in response to cell–cell contact, results in Rassf1a binding to Yap1, blocking its interaction with Smad co-transcription factors that mediates Yap1 nuclear translocation and function\(^43\). In response to Tgf-β signaling, Rassf1a is degraded by Itch and it has been shown this is sufficient for Yap1 association with Smads and nuclear translocation of a functional Yap1–Smad transactivation complex.

Nuclear Yap1 was not evident in AAH, adenomas, or surrounding normal lung, but nuclear translocation occurred specifically in hypoxic clusters where a Zeb2-to-Zeb1 switch occurs in a Tgf-β1-rich environment (Fig. 4a–c). IL-6 is a key downstream effector of Yap1 in progenitor cell expansion, and it is directly targeted by Yap1 for induction in vivo\(^44\). IL-6 marks sites of hypoxia in tumors, and its induction in early lesions is necessary for unrestricted cancer cell outgrowth\(^45\). Consistent with nuclear translocation of Yap1 in cell clusters, IL-6 induction was evident in these cells (Fig. 4d).

In cell culture, as reported previously\(^38\), we found that Yap1 became sequestered in the cytoplasm as cells reached confluence, and Tgf-β1 was unable to drive its nuclear translocation in these confluent cells (Fig. 4e). In these confluent cells, expression of Zeb1 mRNA was repressed and Zeb2 mRNA was induced (Fig. 4f). But, with Zeb2 knockdown, Yap1 persisted in the nucleus of confluent cells, and the cells lost contact inhibition allowing their outgrowth into foci (Fig. 4e, g). We conclude that Zeb2 is blocking nuclear translocation of Yap1. With Zeb1 knockdown, and the resulting elevation of Zeb2, Yap1 became sequestered in the cytoplasm of subconfluent cells, and these subconfluent cells stopped proliferation (Fig. 4h–j). Taken together, our results suggest that Zeb1 repression of Zeb2 is a key focal point in the regulation of Tgf-β1 signaling, and nuclear translocation of Yap1 is linked to cell proliferation.

Repression of Zeb2 Leads to Loss of Pten and Constitutive Akt Phosphorylation. Zeb2 has been shown to act as a competitive endogenous RNA that sequesters miRNAs normally targeting Pten mRNA\(^33\). In this way, Zeb2 expression was shown to maintain Pten. Consistent with these findings, we found Pten diminished with Zeb2 repression in hypoxic clusters (Fig. 5a–c).

![Figure 3](image-url) Zeb1 represses Zeb2 to launch Tgf-β1 signaling capacity. a Immunostaining showing Zeb2 is high in normal lung, AAH, and AD. b Zeb2 is repressed as Zeb1 is induced in clusters at P170. c Real-time PCR showing mutation of Zeb1 in cell culture leads to induction of Zeb2 and Pten mRNAs. d Western blot showing that Zeb1 protein expression parallels mRNA levels with knockdown of Zeb2 causes loss of Pten mRNA in cell culture. e Lentivirus shRNA knockdown of Zeb1 leads to induction of Zeb2 mRNA, and IL-6 induction of Zeb1 and Vimentin mRNAs. We concluded that the level of Zeb2 accumulating in the absence of Zeb1 is sufficient to block Tgf-β1 signaling, and thus Zeb1 repression of Zeb2 is acting to relieve the inhibition of Tgf-β1 signaling.
In culture, Zeb1 mutation or knockdown led to induction of Pten mRNA (Fig. 3c, e). Conversely, Zeb2 knockdown led to the loss of Pten mRNA (Fig. 3e). We then concluded that de-repression of Zeb2 following Zeb1 loss is responsible for this induction of Pten. Pten inhibits PI3K activity, preventing downstream PI3K-Zeb2 following Zeb1 loss is responsible for this induction of Pten. mRNA (Fig. 3c, e). Conversely, Zeb2 knockdown led to the loss of Pten mRNA (Fig. 3e). We then concluded that de-repression of Pten mRNA (Fig. 3e). We then concluded that de-repression of Zeb2, leading to loss of Pten in culture, caused constitutive pS473Akt upon Zeb2 knockdown, these cells showed increased survival in suspension (Fig. 5f).

**Nuclear Polarization of Transcription Factors in Mitosis Establishes Parent and Daughter Fates Prior to Cytokinesis as Adenoma Cells Divide Asymmetrically to Generate IC.** The appearance of IC in the interior of expanding adenomas suggested that they might be derived from adenoma cells. Dividing cells in hypoxic clusters at P170 were confirmed by Tubulin immunostaining of mitotic spindles, and cells displaying patterns of mitosis or cytokinesis were included (Fig. 5g). We identified mitotic cells where one forming pole displayed decondensed chromatin indicative of IC, whereas the opposite pole retained condensed chromatin of adenoma cells (Fig. 5g). In these mitotic cells, Zeb1, Bmi1, and Yap1 were restricted to decondensed chromatin, whereas Zeb2 was associated exclusively with compacted chromatin already showed an E-cadherin+ pattern of parent adenoma cells and daughter IC, in opposite poles of the dividing cells with Zeb2+. 

**Fig. 4 Zeb2 blocks nuclear translocation of Yap1.** a Immunostaining showing Yap1 is not concentrated in the nucleus in normal lung, AAH, and AD. b A cluster forming in the interior of an AD at P170. Arrows show the cluster border. c Yap1 is translocated to the nucleus in clusters. d The downstream target of Yap1, IL-6, is induced with nuclear translocation of Yap1 in clusters. e Cells in culture were allowed to reach confluency, and were then treated with 75 pM Tgf-β1 for 12 h. Note the block in Yap1 nuclear translocation at confluence. Similar cytoplasmic retention of Yap1 was evident at confluence in the absence of Tgf-β1. Zeb2 shRNA knockdown maintains nuclear Yap1 at confluence. f Real-time PCR showing Zeb1 mRNA is repressed and Zeb2 mRNA is induced when cells are allowed to become confluent. g Control cells remain growth-arrested at confluence, but knockdown of Zeb2 allows continued proliferation and cell outgrowth into foci. h Knockdown of Zeb1 leads to loss of proliferation and senescent morphology29. i-j′ Yap1 becomes sequestered in the cytoplasm of subconfluent cells. j-j″ Yap1 is nuclear in subconfluent control cells.

In culture, Zeb1 mutation or knockdown led to induction of Pten mRNA (Fig. 3c, e). Conversely, Zeb2 knockdown led to the loss of Pten mRNA (Fig. 3e). We then concluded that de-repression of Zeb2 following Zeb1 loss is responsible for this induction of Pten. Pten inhibits PI3K activity, preventing downstream PI3K-Zeb2 phosphorylation of Akt on S473 (pS473Akt)15. Consistent with the loss of Pten in hypoxic clusters, pS473Akt accumulated in these cells (Fig. 5g). And, knockdown of Zeb2, leading to loss of Pten in culture, caused constitutive pS473Akt (Fig. 5d–e”). pS473Akt inhibits apoptosis, and it can prevent cell death when adhesion-dependent cells are placed in suspension culture (anoikis)46. Consistent with constitutive pS473Akt upon Zeb2 knockdown, these cells showed increased survival in suspension (Fig. 5f).
symmetrically dividing stem cells and CSC. Recent evidence demonstrates that asymmetric division is regulated by miRNAs targeting Numb miRNA, and miR-34a directly targets Numb miRNA, and repression of miR-34a by inflammation induces Numb to promote asymmetric division. In addition to miR-34a, Zeb1 represses miR-34a, and this loss of miR-34a was shown to drive cytoskeletal changes. We did not detect Numb in Zeb1 cells (Fig. 5g). We identified other dividing cell clusters in culture that did not display nuclear polarization, and showed uniform chromatin decondensation in both poles. These dividing cells maintained the N-cadherin−, Zeb1−, CD44−, Zeb2−, Pten−, pS473Akt− pattern of daughter IC. Notably, Numb, which was not evident in adenoma cells, was induced in the dividing cells and co-localized asymmetrically with E-cadherin during cytokinesis. Outside of EF5+ regions in AD, AD cells divide symmetrically to produce AD daughters. We identified other dividing cell clusters that did not display nuclear polarization, and showed uniform chromatin decondensation of IC in both poles. Accordingly, these dividing cells maintained the E-cadherin−, Zeb1−, CD44−, Zeb2−, Pten−, pS473Akt− pattern of daughter IC. These results demonstrate symmetric division of IC to produce additional daughter IC accounting at least in part for the increase in cluster size from P150 to P170 (Fig. 1f, j, k).

**IC are Initial Cancer-generating Cells (CGC).** We then examined clusters for evidence that IC might be dividing to produce cancer cells. A second form of nuclear polarization was evident in dividing cells in clusters at P170. Partially decondensed chromatin representative of IC was evident in one pole of these cells in mitosis, whereas euchromatin indicative of cancer cells was present in the opposite pole (Fig. 6a). Zeb1 was enriched in decondensed chromatin, and this pole of the cell maintained the E-cadherin+ and CD44hi pattern of IC. The opposite pole of the cell with Zeb1lo euchromatin displayed the E-Cadherin+ and CD44lo patterns of mitosis or cytokinesis, as well as the morphology of dividing cells, were examined. We noted mitotic cells where one pole displayed dense heterochromatin of AD cells, whereas the opposite pole showed decondensed chromatin of IC. Immunostaining showed that Zeb1, Yap1, and Bmi1 co-localized with decondensed chromatin, whereas Zeb2 was restricted to dense chromatin. This transcription factor polarization persisted in cytokinesis. The pole of the dividing cells with Zeb2+ compact chromatin showed an E-cadherin+, CD44+, pS473Akt+ pattern of parent adenoma cells during cytokinesis, whereas the opposite pole with Zeb1+, Bmi1+, Yap1+ decondensed chromatin had transitioned to an E-cadherin−, CD44−, pS473Akt− pattern of daughter IC. Consistent with the anti-apoptotic properties of pS473Akt, Zeb2 knockdown causes pS473Akt of tumorigenic CD44hi pattern of IC in both poles. Although Numb was maintained in these dividing IC, it was asymmetrically distributed. Cells type and number of dividing cells in clusters is quantified in Fig. 1k.
pattern of cancer cells. By contrast to Zeb1, Bmi1 and Yap1 were equally distributed in the decondensed chromatin and euchromatin of both poles, and both poles maintained a Zeb2−, Pten−, pattern (Figs. 5g, h and 6a). Numb expression was asymmetric in these dividing cells, where it co-localized with E-cadherin. By contrast to Zeb1, Bmi1 and Yap1 were equally distributed in the decondensed chromatin and euchromatin of both poles, and both poles maintained a Zeb2−, Pten−, and pS473Akt+ pattern. We conclude parent IC are dividing to produce daughter AC, and they are therefore cancer-generating cells (CGC). We identified symmetrically dividing cancer cells that displayed euchromatin as well as the same Zeb1+, CD44lo, E-Cadherin+, Zeb2−, Pten−, pS473Akt+, Bmi1+, nuclear Yap1+ pattern in both poles. Quantification of different cell divisions occurring in clusters. AD, CGC, and AC cells were identified in clusters by nuclear morphology and expression patterns. AD = Zeb2+, E-cad−; CGC = CD44hi; AC = Zeb2−, E-cad+. n = 10 clusters were averaged at P170. Expanding tumors display papillary morphology and are E-cadherin (E-cad)+. These E-cad+ tumors remain Zeb150. Tumor cells are Pten− and Zeb2−. g CD44 expression is low on the tumor cells. h In situ hybridization shows that tumors cells are miR-200−. Bars are 50 μm.

Invading Cancer Cells Display Plastic EMT, but Fail to Induce CD44 or Display Properties of CGC. We reasoned one explanation for the Zeb150, CD44lo pattern, and lack of EMT in the cancer cells (Fig. 6a–g) might be they are moving away from sites of hypoxia and Tgf-β1 accumulation as they expand into tumors. Notably, subsequent invasion of expanding tumors into airways occurs at sites of inflammation and Tgf-β1 accumulation (Fig. 7a, b), and we found that immunostaining for Zeb1 was increased in these invading cells and the cells had undergone EMT as evidenced by loss of E-cadherin (Fig. 7c–e). Based on our findings in cell culture, we suggest that ongoing repression of Zeb2 in the cancer cells allows the cells to respond to Tgf-β1 concentrated around airways and induce Zeb1 and EMT to facilitate invasion. However, despite this induction of Zeb1 and EMT and continued repression of miR200, the invading cancer cells remained CD44lo (Fig. 7f), they did not show evidence of EF5++ clusters, a decondensed chromatin pattern resembling CGC, and we did not observe asymmetric distribution of Numb in any of the invading cells undergoing division (Fig. 7g). We conclude that EMT in cancer cells is plastic and linked to inflammatory infiltrate, Tgf-β1 accumulation, and resulting Zeb1 induction at sites of invasion.
But, this induction of Zeb1 does not restore a CD44hi CGC-like population among the invading cancer cells. Thus, it appears that the Zeb1/CD44 loop evident in CGC is disrupted by stable Zeb1-independent repression of CD44 that is initiated as cancer cells are generated.

Zeb1 is Required for CGC Generation. We have shown that Zeb1 is important for tumor progression in K-Ras-initiated lung cancer\(^20\). But, the mechanism was unknown, and we concluded that the effects were likely due to a classic role for EMT in cancer cell behavior (e.g., migration and invasion). However, our results above raised the possibility that Zeb1 in the context of a CD44/Zeb1 loop might be critical for CGC formation and in turn cancer cell generation. Knockout of Zeb1 leads to late embryonic lethality, and its loss causes changes in developing epithelial field borders\(^29,52\). Heterozygous mutation of Zeb1 does not affect viability, but the mice develop posterior corneal dystrophy, demonstrating that simply lowering the level of Zeb1 is impactful in vivo\(^53\). And, we show here that heterozygous mutation or Zeb1 knockdown causes repression of CD44 and induction of Zeb2, suggesting this threshold level of reduction in Zeb1 is sufficient to deregulate genes marking the transition to CGC.

K-Ras mice were crossed into a Zeb1\(^{+/−}\) background (Methods). The reduced level of Zeb1 had no effect on adenoma formation, or generation of hypoxia in interiors of expanding adenomas (EF5\(^+\)) at P170 (Fig. 8a). But, small intensely EF5\(^++\) hypoxic sites indicative of cluster cells were inhibited, and accordingly, clusters of cells displaying decondensed chromatin failed to form (Fig. 8a). In turn, CD44 was not induced, Zeb2 was not repressed, and cells displaying an E-cadherin\(^−\), Pten\(^−\) pattern of EMT were diminished in adenomas. Consistent with this inhibition in the formation of CGC, cancer cell number was inhibited as was AC outgrowth at P220 (Fig. 8a, b).

Lentivirus co-expressing GFP and Zeb1 shRNA, or a control sequence (Methods), was delivered intratracheally every 2 weeks into K-Ras mice beginning at P30 and ending at P90. Mice were then analyzed at P170, and using GFP as a marker, virus efficiently infected normal lung tissue (Fig. 8c). We did not detect a difference in GFP\(^+\) adenoma formation or transition of the interiors of these expanding adenomas to hypoxia (EF5\(^+\)), but EF5\(^++\) clusters were inhibited as was cancer cell generation (Fig. 8d). We concluded that a threshold of Zeb1 induction is necessary to drive adenoma cells to produce CGC, and in the absence of CGC, initial cancer cells are not generated.
Characterization of CD44hi Cells from Lung Tumors. We attempted to isolate and compare adenoma, CD44hi CGC, and cancer cells from K-Ras initiated tumors. First, we noted that neither adenoma cells at P150 nor cancer cells at P225 survived when lungs were dissociated and placed in cell culture. And, we found that CD44hi cells in these primary cultures, or following lungs were dissociated and placed in cell culture. And, we neither adenoma cells at P150 nor cancer cells at P225 survived cancer cells from K-Ras initiated tumors. First, we noted that in vivo, we hypothesized that its induction under the stress of cancer cell generation or proliferation in K-Ras tumors for cell senescence. Even though p53 does not appear to affect induction in primary rodent cell culture is classically responsible for blocking CD44 expression and viability in culture.

Notably, a small subset of cells was shown previously to expand in mouse lung tumors and cells in culture. Consistent with the findings in the mouse, both ZEB2 and PTEN mRNAs were downregulated in human lung AC with a Ras pathway mutation (Fig. 10a), and there was a linear correlation between their expression in the tumors (Fig. 10b), implying the same linkage in their expression we found in mouse lung tumors and cells in culture. Consistent with the importance of ZEB2 and PTEN, their co-expression was a remarkable predictor of patient survival (Fig. 10c).
Discussion

Mutation of K-Ras leads to a prolonged period of precancerous adenoma outgrowth in the mouse lung, followed by cancer cell generation in the hypoxic, Tgf-β-rich interiors of these expanding lesions (Fig. 10d–f). These cancer cells, in turn, proliferate into large AC that invade airways (Fig. 10g). We identify a two-step pathway where precancerous cells divide asymmetrically to produce an intermediate population of CGC, which in turn, divide asymmetrically to produce cancer cells. Studies of mamalian asymmetric division mechanism have largely focused on cell polarization causing the mitotic plane in driving cells to unequally partition cytoplasmic contents into daughter cells in a Numb- and Par-mediated process54. However, studies of asymmetric division in flies and worms have shown differences in heterochromatin on the two DNA strands arising during the S phase, linking fate of the two daughter cells to differential segregation of these sister chromatids based on heterochromatin content55–57. A DNA replication-mediated process highlighted by differential loading of new unmodified histones on leading vs. lagging DNA strands was proposed to drive the heterochromatin differences in sister chromatids, and these differences in histone modifications in turn allowed mother and daughter centrosomes to segregate sister chromatids based on heterochromatin content into the two daughter cells. The implication being that resulting differences in heterochromatin content would be expected to regulate differential transcription factor binding, gene expression, and thus phenotype in the daughter cells. Although our studies lack the mechanistic detail found to drive differential heterochromatin assembly on sister chromatids in asymmetrically dividing flies and worm stem cells, we provide consequential and complimentary evidence that similar asymmetric heterochromatin assembly in sister chromatids actually drives mitotic polarization of key transcription factors including Zeb1 in cancer cell initiation. And, we provide linked evidence that this mitotic polarization of transcription factors has already produced asymmetric expression of their key target genes in opposite poles of the cell before cytokinesis.

CGC arise at hypoxic sites in expanding adenomas and are marked by EMT and a CD44hi loop. Disruption of this loop by mutation or knockdown of Zeb1 eliminates CGC formation from adenoma cells, thereby inhibiting cancer cell production. Notably, hypoxia induces expression of Zeb158, and Zeb1 repressor activity and its function in EMT are mediated through interaction with the NADH-dependent co-repressor CtBP59. Hypoxia-driven aerobic glycolysis, leading to NADH generation, then allows CtBP, and in turn Zeb1, to act as an oxygen/metabolic sensor40–42. Zeb1 repression of Zeb2 in these cells leads to the repression of Pten expression that is a hallmark of K-Ras driven
AC, and it releases Smad inhibition allowing Tgf-β signaling to drive nuclear translocation of the CSC transcription factor Yap1. Division of these CD44hi, Zeb1hi cells generates CD44lo, Zeb1lo cancer cells. The reduced level of Zeb1 in the cancer cells is not sufficient to maintain repression of E-cadherin, and EMT is reversed. However, the level of Zeb1 was still sufficient to maintain repression of Zeb2, allowing the cancer cells to respond to Tgf-β1 accumulating at sites of inflammation and airway invasion to re-establish Zeb1hi and EMT in the invading cells. We suggest that this constitutive repression of Zeb2 leads to plastic EMT in the cancer cells dictated by inflammation and Tgf-β1 accumulation concentrated around airways.

Despite the restoration of a Zeb1hi level in invading cancer cells, the cells remained CD44lo and we failed to detect cells with properties of CGC within the tumors. We interpret these findings to mean that cells with properties of CSC are not being generated from existing cancer cells in this model. Notably, migrating cells with properties of CSC are also linked to metastasis, and despite the formation of large invasive AC, no metastasis was evident in this K-Ras model. As noted above, attempts to isolate CD44hi CGC by flow or simply observing the cells after tumor dissociation in cell culture led to rapid loss of CD44 expression and failure of the cells to expand in culture. In contrast to the K-Ras model, a small subset of cells could be cultured from K-Ras, p53 compound mutant tumors, and these cells were highly tumorigenic when delivered intrathecally into the lung21. Here, we demonstrate these cells correspond to a CD44hi subset of the tumors. p53 represses CD44, and we conclude that p53 induction in culture, which is classically responsible for senescence in primary rodent cell cultures, is likely causing the loss of CD44 expression and failure of expansion of the cells in culture. Evidence suggests that CSC in tumors are derived from existing cancer cells that undergo reversible p53-regulated reprogramming, and they are important for ongoing tumor expansion and tumor renewal following therapy. Indeed, p53 mutation is required for a CSC pool in mouse breast cancer63. p53 triggers stable repressive epigenetic changes64, and we suspect p53 in expanding cancer cells in the K-Ras model is responsible for maintaining CD44 silencing, even as Zeb1 is re-induced during the invasion (Fig. 10f, g). In this regard, Numb sustains high p53 activity65, and notably it was expressed in cancer cells, where it was symmetrically distributed along with E-cadherin in the dividing cells (Fig. 6b). We suggest this inability to induce CD44 in the cancer cells prevents their reprogramming to CSC until p53 is mutated. Although it remains to be seen how the CD44hi, Zeb1hi CGC we identify as initial CGC within adenomas might be related to CSC, we propose CSC generation from existing cancer cells reflects reprogramming aimed at restoring the cancer cell-generating phenotype of CGC.
Methods

RNA Extraction and Real-time PCR. RNA was extracted using TRIzol, and cDNA was synthesized using the Invitrogen RT kit (Invitrogen), and SYBR Green real-time PCR was performed using a Stratagene Mx3000P Real-time PCR system. PCR primer sequences and annealing conditions are shown in Supplementary Table 1. Three independent samples, each in triplicate, were analyzed for each real-time PCR condition. The detection of miRNAs was described previously. Briefly, polyadenylation of at least 5 µg of total RNA was completed by poly(A) polymerase kit (PAP, Ambion) in 20 µl of reaction volume according to manufacturer’s instruction. The polyadenylated RNA was then treated with protease K. The probes were hybridized to the sections at 57 °C overnight. Images were captured with a Zeiss Axioscope.2 microscope equipped with AxiosCam IC3 and AxioCam MRm cameras. Images in the Zeiss Zen imaging program were transferred to Powerpoint for figure assembly. Only brightness and contrast were modified. Then a PDF was created. Immunostaining and In Situ Hybridization. Images were captured with a Zeiss Axios Cam and by an experienced pathologist (M.C.). p53 mutant mice in a C57BL/6 background to yield K-RasLA1, Zeb1shRNA Knockdown. We have described lentiviral shRNA knockdown of Zeb1 and Zeb2 antibodies and for chromatin immunoprecipitation using Zebs1 and Zebs2 antibodies were described previously. Cell Culture. Primary cultures of Zeb1 mutant embryonic fibroblasts were isolated as described. Cells were cultured in Dulbecco’s modified eagle media (DMEM) with 10% heat-inactivated fetal bovine serum, or serum starved for 12 h prior to β-treatment. When lung tumors from K-Ras, p53 compound mutants were placed in culture, a small subset of these tumor cells survived and proliferated in culture, and these cells were highly tumorigenic when delivered intratracheally back into WT lungs. These cells were grown in DMEM with 10% heat-inactivated fetal bovine serum. We found that this population was CD44hi. shRNA Knockdown. We have described lentiviral shRNA knockdown of Zeb1 and Zeb2 protein and miRNAs in detail previously. Three shRNA lentiviruses with different Zeb1 and Zeb2 shRNA sequences from Open Biosystems were used for knockdowns with similar effects. Lentivirus with a scrambled shRNA sequence 5′-CACAGATGAGCAACCACTTCTTTAGATGGCGCTTCTCATGTGG-3′ was used as a control—this control sequence was blasted against all mouse chromosome sequences to ensure that it did not target an miRNA.

Mutant Mice. Housing and handling of all mice were in accordance with procedures approved by the University of Louisville Institutional Animal Care and Use Committee (IACUC). K-rasLAI mice in a C57BL/6 background were obtained from Jackson Laboratory. These mice were crossed with Zeb1−/− mice also in a C57BL/6 background to yield K-RasLA1, Zeb1−/−, and K-RasLAI, Zeb1−/− mice. Lung tumors were harvested for histological analysis of tumors at the indicated ages. PCR genotyping was described as previously. Tumor pathology was evaluated blindly by an experienced pathologist (M.C.). p53 mutant mice in a C57BL/6 background were also crossed to the latent K-rasLAI mice above and lung tumors were trypsinized and placed in cell culture in DMEM with 10% fetal bovine serum. A small subset of cells survived, and RNA was isolated from these cells and starting lung tumor tissue for real-time PCR analysis of CD44 mRNA expression. For lentiviral Zeb1 shRNA or control shRNA treatment, K-RasLA1 mice at 1 month of age were anesthetized by IP injection of a combination of ketamine, and a 0.5 mg/kg dose of atropine. A small fraction of cells survived, and RNA was isolated from these cells and analyzed by PCR using a universal primer (5′-GCGAGCAACCAATAATACGACTTATAAGAG-3′) in 40 µl of reaction volume. Real-time quantitative PCR was performed using a universal primer (5′-GCGAGCAACCAATAATACGACTTATAAGAG-3′) and a miRNA-specific primer (Supplementary Table 2). Tgfβ1 was from BioSource, Camarillo, CA.

Immunostaining and In Situ Hybridization. Antibodies and conditions are described in Supplementary Table 3. EF5 staining for hypoxia was described previously. Immunohistochemistry was performed as described previously. Antibodies and conditions are described. Then a PDF was created. Immunostaining and In Situ Hybridization. Sources, Camarillo, CA.

Statistical Analysis. Significance was determined by Student’s t-test. Error bars in figures represent standard deviations. For survival curves, a logrank test was used to generate p values with the GraphPad Prism program. Data availability. All relevant data are available from the authors.

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Author contributions

Y.L., L.S., and K.D. performed experiments; M.C. analyzed pathology and immunostaining; and Y.L., A.P., and D.D. planned experiments, analyzed results, and wrote the paper.

Additional information

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