Clonal dynamics following p53 loss of heterozygosity in Kras-driven cancers

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Although it has become increasingly clear that cancers display extensive cellular heterogeneity, the spatial growth dynamics of genetically distinct clones within developing solid tumours remain poorly understood. Here we leverage mosaic analysis with double markers (MADM) to trace subclonal populations retaining or lacking p53 within oncogenic Kras-initiated lung and pancreatic tumours. In both models, p53 constrains progression to advanced adenocarcinomas. Comparison of lineage-related p53 knockout and wild-type clones reveals a minor role of p53 in suppressing cell expansion in lung adenomas. In contrast, p53 loss promotes both the initiation and expansion of low-grade pancreatic intraepithelial neoplasia (PanINs), likely through differential expression of the p53 regulator p19ARF. Strikingly, lineage-related cells are often dispersed in lung adenomas and PanINs, contrasting with more contiguous growth of advanced subclones. Together, these results support cancer type-specific suppressive roles of p53 in early tumour progression and offer insights into clonal growth patterns during tumour development.

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Cancer cells within developing tumours exhibit significant genetic and phenotypic heterogeneity mediating tumour growth, metastasis and therapy resistance2-3. This intratumoral heterogeneity is thought to arise from the sequential accumulation of genetic or epigenetic changes that favour the growth of distinct subclonal populations. Indeed, construction of genetic hierarchies from genomic sequencing data reveals the presence of subclonal populations within individual tumours that propagate throughout progression from early to advanced primary tumours and metastases4-7. Studies in transplant models have underscored the functional importance of specific genetic variants in modulating growth dynamics of different subclones within tumours8-9. Unfortunately, similar analyses in physiologically relevant, autochthonous cancer models during tumour progression are lacking10 due to technical challenges in inducing sequential mutations in subclonal populations and unambiguously tracing them at single-cell resolution.

We have previously developed autochthonous models of lung and pancreatic cancer by simultaneous Cre recombinase-mediated activation of oncogenic Kras (KrasG12D) and biallelic inactivation of p53 in cells residing in the tissues of origin11-13. These models faithfully recapitulate certain prevalent genetic alterations, histologic tumour progression, metastatic behaviour and treatment response of the human diseases. By comparing LSL-KrasG12D/KrasWT; p53flox/flox and LSL-KrasG12D/KrasWT; p53flox/fox mice infected with inhaled adenovirus carrying Cre recombinase, our laboratory revealed a role of p53 in limiting tumour progression from low-grade lung adenomas to advanced adenocarcinomas11. Furthermore, reactivation of p53 in advanced lung tumours led to selective loss of adenocarcinoma cells14,15, consistent with a specific role of p53 mutation in regulating late-stage lung tumour progression. Finally, exome-sequencing analyses of murine lung adenocarcinomas derived from LSL-KrasG12D/KrasWT; p53flox/fox mice revealed no recurrent mutations beyond Kras and p53 (ref. 16), suggesting that p53 loss is the main genetic driver of tumour progression in this model.

Previous studies have also suggested that p53 principally plays a role late in pancreatic tumorigenesis. Similar to what is seen in human lung tumours17, p53 mutations are primarily observed in more advanced human pancreatic lesions, including pancreatic ductal adenocarcinoma (PDAC) or precursor PanINs of high-grade histology18,19. Moreover, p53 mutation shortens the latency and increases the frequency of PDAC formation in mouse pancreatic tumour models in which p53 is simultaneously mutated at the time of oncogenic Kras activation13,20.

In this study, we adapt these models to permit sequential and sporadic p53 loss of heterozygosity (LOH) following oncogenic Kras-mediated tumour initiation. We more faithfully model clonal evolution during tumorigenesis and perform high-resolution tracing of subclones lacking or retaining p53 during tumour progression. We demonstrate that sporadic p53 loss promotes progression to advanced lung and pancreatic tumours. Moreover, we confirm that p53 primarily plays a role late in lung tumorigenesis. In contrast, we determine that p53 suppresses both the initiation and expansion of early pancreatic tumours, which correlates with expression of the p53 regulator p19ARF. Finally, we show surprisingly significant intratumoral cell dispersion of subclones in early lung and pancreatic tumours.

Results

Induction of p53 LOH using MADM in mice. To generate sporadic p53 LOH in Kras-initiated tumours, we took advantage of mosaic analysis with double markers (MADM), which permits simultaneous fluorescence cell labelling and mutagenesis through a single Cre-mediated inter-chromosomal recombination event in mice21. MADM has been used to study the consequence of tumour suppressor gene LOH on tissue development and cancer initiation at single-cell resolution22-25. We crossed LSL-KrasG12D mice with MADM11-GT,p53WT/IMADM11-TG,p53KO mice to generate LSL-KrasG12D/KrasWT; MADM11-GT,p53WT/MADM11-TG,p53KO mice (K-MADM-p53) (Methods). On Cre expression, oncogenic Kras is efficiently induced via intra-chromosomal Cre-mediated recombination permitting tumour initiation (Fig. 1a). Sporadic p53 LOH occurs by subsequent stochastic and inefficient Cre-mediated inter-chromosomal recombination between homologous chromosomes. Mitotic recombination and X segregation (G2-X) of the MADM cassettes is predicted to result in the generation of two genotypically and phenotypically distinct daughter cells from a colourless p53KO/WT parent cell: GFP+/tdTomato− (green) p53KO/WT and GFP−/tdTomato+ (red) p53WT/WT (Fig. 1a,b and Supplementary Fig. 1). In contrast, G2-Z, G0 or G1 recombination results in the generation of GFP+/tdTomato+ (yellow) and GFP−/tdTomato− (colourless) p53KO/WT cells (Fig. 1b,c and Supplementary Fig. 1). As the fluorescent markers are genetically encoded, the MADM system affords tracing of lineage-related green p53KO/WT and red p53WT/WT subclones, allowing for the induction and monitoring of intratumoral heterogeneity in autochthonous tumours.

Sporadic p53 LOH promotes progression to lung adenocarcinoma. To determine whether sporadic and sequential (following Kras mutation) p53 LOH promotes lung tumour progression, we administered lentiviral Cre via the trachea to adult K-MADM-p53 mice to induce stable Cre expression in lung epithelial cells24. Infected mice exhibited multiple small lung tumours containing fluorescently labelled cells, although green (p53KO/WT) cells did not predominate at early time points (Fig. 2a). Mice analysed at later time points, however, displayed an increase in the overall size of tumours and the development of larger and more numerous green tumours. Histologic analysis of these large tumours revealed high-grade lesions (adenocarcinomas) consisting of densely packed green cells (Fig. 2b). We also observed mixed-grade tumours (mixed adenoma–adenocarcinomas) in which the adenocarcinoma component was entirely green (Fig. 2c). These data suggest that the sequential loss of p53 is a driver of tumour progression to adenocarcinoma in oncogenic Kras-initiated lung tumours.

We confirmed these findings using an alternative, less efficient MADM model in which Kras-initiated lung tumours spontaneously arise through a Cre-independent stochastic recombination event (KrasLA2 model)25 and MADM-labelled clones are thereafter generated by tamoxifen-induced Cre activation (CreERT2) (Fig. 3a). This method ensures sequential mutation of p53 following tumour initiation by oncogenic Kras. From eight KrasLA2,Rosa26-CreERT2/KrasWT; MADM-p53 mice dissected following the development of tumour-related morbidity, we observed two fluorescently labelled tumours on whole mount analysis (Fig. 3b). These tumours were green (p53KO/WT) and displayed histologic features of adenocarcinoma (Fig. 3c). In addition, a small number of low-grade adenomas harboured rare yellow p53KO/WT cells (Fig. 3d), supporting the inefficient nature of MADM recombination in this model and the clonality of the green tumour cells. No fluorescently labelled tumours were observed on whole mount analysis of lungs from ten KrasLA2,Rosa26-CreERT2/KrasWT; MADM mice (lacking p53 mutation). Together, these data are consistent with a role of p53 in constraining lung tumour progression to adenocarcinoma.

p53 loss does not greatly impact early lung tumorigenesis. To further evaluate whether p53 also suppresses cell expansion in
early lung tumours, we classified lung adenomas based on their green or red cell predominance in tissue sections of K-MADM-p53 mice at 10–16 weeks post infection (p.i.) (Fig. 4a). As green and red cells are produced at 1:1 stoichiometry following a single G2-X recombination event (Fig. 1a), green cells should out-number red cells in these tumours (green-dominant) if p53 loss promotes cell expansion during early tumorigenesis. In contrast, a plurality of tumours (51 of 132) showed no colour dominance on qualitative analysis of random cross-sections of adenomas. Given the possibility for stochastic differences in individual daughter cell expansion following G2-X recombination, we did observe tumours (69 of 132) that showed green or red cell predominance. However, the proportions of green-dominant and red-dominant tumours were not statistically different, suggesting that green p53KO/KO cells did not have a selective growth advantage at this stage (Fig. 4b). To more rigorously characterize the ratio of green to red cells (green-to-red ratio) within individual tumours, we quantified labelled cells across serial sections through entire lung adenomas derived from mice dissected 10 or 16 weeks p.i. Again, we observed no difference in the intratumoral proportions of p53KO/KO and p53WT/WT cells at 10 weeks p.i. and only a small difference at 16 weeks p.i. (Fig. 4c,d). These results indicate that p53 loss does not significantly affect tumour cell expansion in lung adenomas.

Given the stochastic nature of mitotic recombination events leading to MADM labelling, we were unable to definitively determine the timing of p53 loss in K-MADM-p53 mice. To circumvent this limitation, we used the sum of all red and green single-labelled cells in a tumour as a surrogate for timing of G2-X recombination with the assumption that increased overall cell

Figure 1 | Schematic of MADM system. (a) Schematic of MADM-mediated LOH of p53. Efficient Cre-mediated intra-chromosomal recombination deletes the transcriptional/translational STOP cassette inducing oncogenic Kras activation. Less efficient Cre-mediated inter-chromosomal recombination following DNA replication (during G2 phase) leads to reconstitution of GFP and tdTomato on separate chromosomes before cell division. This diagram was adapted with permission from the original MADM schematic21. (b) X segregation of chromosomes following mitotic recombination (G2-X) results in genetically distinct daughter cells: p53KO/KO (green, GFP+/tdTomato−) and p53WT/WT (red, GFP−/tdTomato+) cells. Z-segregation (G2-Z) leads to the generation of yellow (GFP+/tdTomato+) and colourless (GFP−/tdTomato−) p53WT/WT cells. (c) Cre-mediated inter-chromosomal recombination during G0 or G1 phase results in the production of yellow p53KO/WT from colourless p53KO/WT cells. The MADM system affords faithful correlation between the expression of a specific genetically encoded fluorescence marker and genotype.
labelling indicates earlier time points of p53 LOH. If the duration of p53 loss altered cell expansion, we would expect a positive correlation between the total number of single-labelled cells and the green-to-red ratio. Interestingly, there was no association between these two parameters in lung adenomas (Fig. 4e). Together, these data confirm earlier work11,14,15 demonstrating that p53 loss does not have a significant impact on early lung tumorigenesis.

**p53 LOH drives tumour progression to PDAC.** To evaluate the effect of p53 LOH on pancreatic tumour progression using MADM, we crossed K-MADM-p53 mice with Pdx1-Cre mice to direct Cre expression to the developing pancreas12. Pdx1-Cre-MADM-p53 mice (lacking LSL-Kras<sup>G12D</sup>) exhibited green, red and yellow acinar, ductal and islet cells but no overt cellular phenotypes due to p53 loss (Fig. 5a and Supplementary Fig. 2). In contrast, Pdx1-Cre-K-MADM-p53 mice developed the full spectrum of pancreatic tumour progression from low-grade (Fig. 5b) and high-grade PanINs (Fig. 5c) to advanced PDAC (Fig. 5d) and occasionally distant metastases (Fig. 5e,f). Interestingly, Pdx1-Cre-K-MADM-p53 mice exhibited a median survival of ~11 weeks, falling in between that observed in Pdx1-Cre; LSL-Kras<sup>G12D</sup>/Kras<sup>WT</sup> (KC) mice harbouring homozygous p53 mutation (~6 weeks) and heterozygous p53 mutation (~16 weeks) (Fig. 5g), supporting p53 LOH as an important driver of tumour progression in this model. Consistent with p53 constraining progression to advanced disease, high-grade PanINs and PDACs were predominantly or completely green at an intermediate time point (6 weeks) (Fig. 6a,b). We confirmed this green predominance of advanced lesions in intact pancreata using CLARITY tissue clearing26 (Fig. 6c,d).

**p53 suppresses PanIN initiation and expansion.** To determine the consequence of p53 loss on tumour initiation in pancreatic cancer, we took advantage of the fact that G2-X recombination and labelling could occur during pancreatic development (as Pdx1-Cre is expressed as early as E8.5 (ref. 12)) before transformation, resulting in PanINs comprising cells of uniform colour. We first confirmed that there was no difference in the proportions of green and red normal duct cells, the putative cell-of-origin for PanINs19. Next, we quantified the number of low-grade PanINs harbouring all green or all red cells in 6-week-old Pdx1-Cre-K-MADM-p53 mice (Fig. 7a). Interestingly, we observed a greater frequency of all-green PanINs (Fig. 7b), suggesting that p53 loss promoted pancreatic tumour initiation by oncogenic Kras. We also evaluated the role of p53 on cell expansion in low-grade tumours by analysing the proportion of incompletely labelled low-grade PanINs (G2-X recombination occurring after tumour initiation) containing predominantly green or red cells (Fig. 7c). Unlike our observations in early lung tumours, we found increased numbers of green-dominant compared with red-dominant low-grade PanINs (Fig. 7d), consistent with enhanced cell expansion following p53 loss in early pancreatic tumours. Overall, these findings suggest a potential tumour suppressive role of p53 throughout oncogenic Kras-mediated pancreatic tumorigenesis, contrasting with mainly late functions during lung tumour progression.

To explore the mechanism behind p53-mediated suppression of cell expansion, we assessed proliferation (pulse EdU incorporation) and apoptosis (cleaved caspase-3) by immunostaining tissue sections of pancreatic and lung tumours. The percentage of p53<sup>KO/WT</sup> cells exhibiting EdU incorporation was significantly increased compared with p53<sup>WT/WT</sup> cells in low-grade PanINs (Supplementary Fig. 3a). In contrast, apoptosis was rare, largely limited to cells detached into the lumen and not related to cells of a particular p53 genotype (Supplementary Fig. 3b). As low-grade lung tumours displayed low levels of overall proliferation, few p53<sup>WT/WT</sup> and p53<sup>KO/WT</sup> cells were co-labelled with EdU with no...
obvious difference in the percentages of labelled cells (Supplementary Fig. 3c). High-grade p53KO/KO tumours showed much greater EdU incorporation (Supplementary Fig. 3d,e), whereas apoptosis was not observed in lung tumours (Supplementary Fig. 3f). Together, these data suggest that p53 loss promotes cell cycle progression in early pancreatic tumours.

**Figure 3 | p53 constrains lung tumour progression in the Kras<sup>L42D</sup>-MADM model.** (a) Schematic of MADM-mediated LOH of p53 in Kras<sup>L42D</sup>, Rosa26-Cre<sup>ERT2</sup>/Kras<sup>WT</sup>; MADM-p53 mice. Stochastic recombination results in removal of one of two duplicate copies of mutant Kras exon1 (Kras<sup>G12D</sup>) and an intervening neo cassette permitting expression of mutant Kras expression and tumour initiation<sup>25</sup>. G2-X MADM recombination, resulting in p53<sup>KO/KO</sup> (green, GFP<sup>+</sup>/tdTomato<sup>-</sup>/C<sup>0</sup>) and p53<sup>WT/WT</sup> (red, GFP<sup>-</sup>/tdTomato<sup>+</sup>/C<sup>0</sup>) cells, is initiated through tamoxifen activation of Cre<sup>ERT2</sup>, permitting localization of Cre to the nucleus. This diagram was adapted with permission from the original MADM schematic<sup>21</sup>. (b) Two green tumours (black arrows) were observed on whole-mount analysis of lungs from Kras<sup>L42D</sup>, Rosa26-Cre<sup>ERT2</sup>/Kras<sup>WT</sup>; MADM-p53 mice (n = 8), whereas none were observed in Kras<sup>L42D</sup>, Rosa26-Cre<sup>ERT2</sup>/Kras<sup>WT</sup>; MADM mice (not harbouring p53 mutation, n = 10). White arrows denote tumors without fluorescence labelling. We did not detect any red or yellow tumours in either cohort of mice by whole-mount analysis. Merged fluorescence images of green and red filters are shown. (c) Histologic section of a tumour in b showed green adenocarcinoma cells adjacent to colourless adenoma cells (predominantly to the right of the line). Some green adenocarcinoma cells (arrow) are intercalating in the adenoma area. Blue, DAPI-stained nuclei. Scale bar, 100 µm. (d) Kras<sup>L42D</sup>, Rosa26-Cre<sup>ERT2</sup>/Kras<sup>WT</sup>; MADM-p53 adenoma harbouring rare yellow cells. Blue, DAPI-stained nuclei. Scale bar, 100 µm.

**Differential expression of p19ARF-p53 during tumorigenesis.** We hypothesized that differences in the timing of induction or stabilization of p53 protein expression may account for the functional differences observed between the tumour types. As wild-type p53 is difficult to detect by immunohistochemistry (IHC) on tissue sections with currently available antibodies,
we took advantage of oncogenic Kras-driven lung and pancreatic cancer models harbouring a p53<sup>R172H</sup> mutant allele (LSL-Kras<sup>G12D</sup>; LSL-p53<sup>R172H</sup>), which demonstrate similar histologic progression to the MADM models<sup>11,20</sup>. In these mice, mutant p53 is stabilized, due in part to loss of feedback inhibition, and serves as a marker of endogenous p53 expression<sup>27</sup>. Consistent with our hypothesis, we observed p53 protein expression in pancreatic but not lung cells during all stages of tumorigenesis from acinar-to-ductal metaplasia and low-grade PanINs to advanced disease (Fig. 8).

Previous work from our laboratory has suggested that tissue-specific expression of p19ARF, a positive upstream regulator of p53, could alter the response to oncogenic Kras in tumour initiation<sup>28</sup>. Using LSL-Kras<sup>G12D</sup>; LSL-p53<sup>R172H</sup> mice, we observed expression of p19ARF in early- and late-stage pancreatic lesions in similar pattern to p53 expression (Fig. 9a). In contrast, lung adenocarcinomas, but not adenomas, expressed p19ARF (Fig. 9b). As p53 mutant cells may induce p19ARF by loss of negative feedback<sup>28</sup>, we verified that p19ARF expression...
was observed in early pancreatic tumours even in the context of wild-type p53 (Fig. 9c). These data suggest that the p19ARF-p53 axis may play a role in suppressing early pancreatic tumorigenesis. This observation is compatible with the decreased capacity of oncogenic Kras to initiate pancreatic tumours compared with lung tumours in mice. When we examined the lungs and pancreata of KrasLA2 mice, which undergo stochastic somatic activation of oncogenic Kras throughout the mouse, 48/48 (100%) mice harboured lung tumours, but only 1/48 (2%) had PanIN lesions. **Extratumoral invasion of PanIN cells.** In addition to pursuing quantitative analyses of tumour cell expansion, we also used the MADM models to monitor the spatial relationships between tumours, but only 1/48 (2%) had PanIN lesions.
lineage-related cells during tumour progression. Using MADM, we observed green mesenchymal-like cells nearby but dispersed from green high-grade PanINs, some of which had lost expression of the epithelial marker cytokeratin 19 (Fig. 10a), in 6-week-old Pdx1-Cre-K-MADM-p53 mice. Given the low frequency of high-grade PanINs or PDACs at this time point (~2.2 lesions per pancreas section per mouse), these invasive cells are likely to be derived from the PanIN epithelial cells. These findings are consistent with extratumoral invasion and epithelial-to-mesenchymal transition during putative preinvasive PanIN stages and corroborate previous work using LSL-KrasG12D/KrasWT; p53flox/WT mice harboring a LSL-YFP reporter. Moreover, as MADM induces G2-X recombination sporadically at low efficiency, it offers a much more stringent evaluation of clonally related cells than traditional Cre/loxP-based reporters that may prone to aberrant reporter expression from leaky transgenic Cre lines.

**Intratumoral cell dispersion in early tumours.** Aside from the extratumoral spread of cells from putative preinvasive tumours in the pancreas, we also observed remarkable intratumoral dispersion of lineage-related cells in early lung and pancreatic tumours. Rather than exclusively displaying adjacent clusters of expanding green and red cells as would be expected with the stationary growth of epithelial tumour cells, lung adenomas and low-grade PanIN lesions often displayed subclones in which cells were non-contiguous in tissue sections (Fig. 10b–d). We confirmed this cell dispersal in three dimensions by analysing intact tissues using multi-photon microscopy (Fig. 10e,f and Supplementary Movies 1–4).

To quantitatively assess cell dispersal, we calculated the mean distance between different green or red cells in tissue sections of lung adenomas from K-MADM-p53 mice at 10-16 weeks p.i. Although labelled cells of the same colour were separated by an average of ~8.7 cell diameters (range 2.47–27.76, n = 31 tumours), p53KO/KO cells surprisingly exhibited significantly decreased disperval than p53WT/WT cells (7.2 versus 10.2 cell diameters, P = 0.013, two-tailed paired Student’s t-test). Indeed, as p53KO/KO tumour cells progress, they appear to form more densely packed clones (Figs 2b,c and 3c). Together, these data support a model in which early tumours display subclonal dispersed growth, whereas tumour progression afforded by p53 loss promotes more localized growth.

**Discussion**

In this study, we have used MADM to trace genetically distinct subclones within the same tumours to parse specific roles of p53 during different stages of lung and pancreatic tumorgenesis and to elucidate dynamic subclonal growth patterns in early epithelial tumours. MADM has several advantages over pre-existing mouse models of cancer. Unlike early models that induced simultaneous cooperating mutations in large numbers of cells, MADM permits sporadic and sequential mutagenesis events. Mutagenesis events can be coupled to recombination-dependent fluorescent reporters, to permit tracing of genetically distinct clones. By titrating down the dose and altering the timing of recombinase expression, sporadic genetic modulation can be induced. However, given that mutagenesis and fluorescent labelling occur through separate recombination events, decreased recombinase activity enhances the likelihood.
of uncoupling of these events. In contrast, MADM-dependent labelling and mutagenesis occur through a single recombination event, maintaining the fidelity of the correlation of fluorescence protein expression and genotype. Moreover, MADM generates two genetically distinct subclonal population, permitting the tracing of lineage-related tumour suppressor gene wild-type and knockout clones within the same tumour.

By exploiting this feature of MADM, we have been able to define when p53 functions to suppress lung and pancreatic tumorigenesis. Genetic analyses on human cancers would suggest that p53 functions late during tumour progression, as p53 mutations are principally identified in lung adenocarcinomas and high-grade PanINs or PDAC rather than their lower-grade precursors. Our prospective evaluation of p53 wild-type and knockout clones suggest that this is indeed true during lung tumorigenesis and are consistent with experiments using p53 reactivation models. In contrast, p53 suppresses both the initiation and early expansion of pancreatic tumours. This conclusion would not have been drawn from existing genomic data from human tumours and validates the use of in vivo models to understand the molecular and cellular features that govern tumour progression.

The mechanisms that lead to differential induction of the p19ARF-p53 axis in early lung and pancreatic tumours remain unclear. In lung cancer, it is thought that enhanced oncogenic stress through Kras amplification and hyperactivation of the mitogen-activated protein kinase pathway may trigger p19ARF expression. In contrast, Kras amplification is rarely

Figure 8 | p53 expression in various stages of lung and pancreatic tumour progression. (a) IHC for p53 in LSL-Kras<sup>G12D/Kras</sup><sup>WT</sup>; p53<sup>LSL-R172H/flx</sup> low-grade adenomas and mixed-grade adenocarcinomas revealed p53 staining only in high-grade lung tumour cells. (b) IHC for p53 in Pdx1-Cre; LSL-Kras<sup>G12D/Kras</sup><sup>WT</sup>; p53<sup>LSL-R172H/WT</sup> adult pancreas revealed increased p53 expression in higher-grade pancreatic lesions. Arrows show low-grade PanINs. (c) A subset of acinar-to-ductal metaplasia (ADM) cells expressed p53 (arrows). (d) A subset of low-grade PanIN cells expressed p53 (arrowheads). Scale bars, 100 μm (all).
observed in pancreatic cancer. Instead, activation of the phosphatidylinositol 3-kinase pathway downstream of oncogenic Kras may play a greater role in activating p19ARF during pancreatic tumorigenesis. Indeed, mutant PIK3CA^{H1047R} expression in the pancreas phenocopies oncogenic Kras in terms of tumour progression and p19ARF expression\(^{38}\). In addition, Cre-mediated deletion of the PI3K effector Pdk1 in Kras-initiated pancreatic tumours reduces the induction of p19ARF\(^{38}\).

Alternatively, tissue-specific mediators of ARF induction could also explain the differences in ARF expression between Kras-driven lung and pancreatic tumours. These include additional signalling pathways (for example, Notch\(^{39}\)), epigenetic modifiers (for example, Bmi1 (ref. 28)) and transcription factors (for example, Dmp1 (ref. 40), AP-1 (ref. 41) and STAT3 (ref. 42)). Nonetheless, the capacity of Pdx1-Cre; LSL-Kras\(^{G12D}/\)Kras\(^{WT}\) mice to initiate tumours and develop high-grade pancreatic

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**Figure 9 | p19ARF is expressed in early lesions during pancreatic but not lung tumorigenesis.** (a) IHC for p19ARF in Pdx1-Cre; LSL-Kras\(^{G12D}/\)Kras\(^{WT}\); p53\(^{LSL-R172H}/\)WT tumours showed expression throughout pancreatic tumorigenesis from early acinar-to-ductal metaplasia (ADM) and PanIN lesions to PDAC. (b) IHC for p19ARF exhibited expression in LSL-Kras\(^{G12D}/\)Kras\(^{WT}\); p53\(^{lox/lox}\) lung adenocarcinomas but not adenomas. (c) IHC for p19ARF in Pdx1-Cre; LSL-Kras\(^{G12D}/\)Kras\(^{WT}\) (KC) tumours, lacking p53 mutation, displayed expression throughout pancreatic tumorigenesis. These data suggest that p53 mutation in a does not induce p19ARF by feedback upregulation, as has been previously described\(^{28}\). Scale bars, 100 μm (all).
lesions despite wild-type p53 and intact p19ARF expression (Fig. 9c) supports the existence of non-genetic mechanisms to evade p19ARF-p53-mediated tumour suppression. A better understanding of these mechanisms may offer novel therapeutic approaches to reinforce this tumour suppressive pathway to prevent tumour initiation and progression.

In addition to our p53-related findings, our results offer the first in vivo experimental evidence of spontaneous intratumoral dispersion of genetically distinct subclones during solid tumour progression. Genomic studies have revealed that most cells within a tumour harbour multiple truncal mutations with subclonal genetic heterogeneity occurring late during tumorigenesis1,4–6. How these truncal mutations propagate throughout a tumour remains poorly understood, although a recent computational model predicted intratumoral cell dispersion during tumour growth as a potential mechanism43 and is consistent with our findings. The mechanism that leads to this dispersion phenomenon is unknown. Based on the overall low rates of proliferation in low-grade tumours (Supplementary Fig. 3), we hypothesize that labelled low-grade tumour cells divide at rates comparable to neighbouring unlabelled cells permitting intermingling after several rounds of cell division of both cell types. In contrast, p53KO/KO cells that have progressed to higher grade divide faster than surrounding cells, allowing them to form more localized clusters. Alternative potential mechanisms for subclonal cell dispersion in early lesions include intratumoral cell migration, competition with neighbouring cells or immune cell clearance. The development of new approaches that permit long-term live in vivo imaging of these tumours could aid in exploring these explanations using these models.

**Methods**

**Animal studies.** Animal studies were approved by the MIT Institutional Animal Care and Use Committee. All animals were maintained on a mixed background. MADM11-GT (Stock #013749), MADM11-TG (Stock #013751), Pdx1-Cre (Stock #014647) and p53flox/flox (Stock #008462) mice were obtained from the Jackson Laboratory. MADM11-TG,p53WT/MADM11-TG,p53KO mice were crossed with p53KO/WT mice, which carry a p53-null allele lacking exons 2–6 (ref. 44). Intercrossing the progeny MADM11-TG,p53WT/p53KO mice permitted recombination of the p53-null mutation onto the same chromosome as the MADM cassette. These MADM11-TG,p53WT/p53KO mice were subsequently

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**Figure 10 | Extra- and intra-tumoral dispersal of early lung and pancreatic tumours.**

(a) p53KO/KO (green, GFP+/tdTomato−) high-grade PanIN with extra-tumoral dispersal of CK19-negative cells (arrows) in a 6-week-old Pdx1-Cre-K-MADM-p53 mouse. (b) Stitched confocal image of an entire lung lobe from a 10-week-p.i. K-MADM-p53 mouse reveals multiple low-grade tumours, some harbouring fluorescently labelled cells (arrowheads) and others not (arrows). Most tumours showed dispersed labelling of green and red cells (white arrowheads), whereas some showed clusters of cells (yellow arrowhead). Scale bar, 500 μm. (c) Lung adenoma from a 16-week-p.i. K-MADM-p53 mouse shows dispersed green, red and yellow cells. (d) Low-grade PanIN from a 6-week-old Pdx1-Cre-K-MADM-p53 mouse shows dispersed green, red and yellow cells. (e) Three-dimensional (3D) rendering of multi-photon imaging of a lung adenoma from a 16-week-p.i. K-MADM-p53 mouse showing non-contiguous green (arrows) and red (arrowheads) cells. (f) 3D rendering of multi-photon imaging of a low-grade PanIN from a 6-week-old Pdx1-Cre-K-MADM-p53 mouse showing dispersed green (arrows) and red (arrowheads) cells. Blue, DAPI-stained nuclei. Scale bars, 100 μm (all, unless otherwise noted).
crossed to MADM11-TG,pg3W7/MADM11-TG,pg3W7 mice, to generate MADM11-TG,pg3W7/MADM11-TG,pg3W7. These mice were crossed with LSL-KrasG12D (ref. 32) mice to create LSL-KrasG12D/KrasWT, MADM11-TG,pg3W7/MADM11-TG,pg3KO breeders. Pdx1-Cre or KrasLA2,Rosa26-CreERT2/KrasWT, Rosa26-CreERT2 mice (5,25) were crossed to MADM11-GT/MADM11-GT mice to produce Pdx1-Cre; MADM11-GT/+ and KrasLA2,Rosa26-CreERT2/KrasWT, MADM11-GT/+ mice, which were thereafter intercrossed to generate Pdx1-Cre-MADM11-GT/MADM11-GT and KrasLA2,Rosa26-CreERT2/KrasWT, Rosa26-CreERT2 mice. LSL-KrasG12D/LSL-KrasG12D; MADM11-TG,pg3W7 (K-MADM-p53), Pdx1-Cre; LSL-KrasG12D/LSL-KrasG12D; MADM11-TG,pg3W7; Pdx1-Cre-K-MADM-p53 and control mice lacking LSL-KrasG12D (Pdx1-Cre-MADM-p53) or pg3W7 mutation (Pdx1-Cre-K-MADM) were generated by crossing the above breeders. KrasLA2,Rosa26-CreERT2/KrasWT, MADM11-GT,pg3W7/MADM11-TG,pg3KO (KrasLA2,Rosa26-CreERT2/KrasWT, MADM11-GT,pg3W7; Pdx1-Cre; KrasLA2,Rosa26-CreERT2/KrasWT, MADM11-p53KO), MADM11-p53KO, MADM11-p53KO, and control mice carrying LSL-KrasG12D (Pdx1-Cre-MADM-p53) or pg3W7 mutation were generated by asphyxiation and perfusion with cold 4% paraformaldehyde (PFA; Electron Microscopy Sciences) in PBS. For proliferation studies, mice were intraperitoneally injected with EdU (Setareh Biotech) 1.5 h before sacrifice. Tissues were dissected, fixed in the same solution for 72 h at 4 °C, embedded in OCT (Tissue-Tek). For whole-mount multi-photon imaging, fixed tissues were injected with EdU (Setareh Biotech) 1.5 h before sacrifice. Tissues were dissected, fixed in the same solution for 72 h at 4 °C and CLARITY samples were prepared as previously described26. Briefly, MADM cells were dissociated using a protease cocktail (lacking collagenase (Worthington), dispase (Roche) and trypsin-EDTA) and red cell distances were compared by two-tailed paired Student’s t-test. CLARITY samples were imaged at a depth of up to 0.5 mm using a Nikon A1R Ultra-Fast Spectral Scanning Confocal Microscope. Multi-photon imaging was performed on an Olympus FV1000 MP inverted microscope (Olympus) with argon laser (458 nm) and Co2 laser (840 nm) excitation as green-dominant (lacking pg3W7) or red-dominant (lacking pg3W7) as level of significance for all statistical analyses.

**Quantification methods and statistical analyses.** All lung adenomas (n = 132 total) from a single section from each of five lungs derived from K-MADM-p53 mice infected at 10–16 weeks p.i. with pg3W7 were classified for color dominance as green-dominant (n = 38), red-dominant (n = 31) or no dominance (n = 51). An additional 12 tumours contained no labelled cells or only yellow cells without evidence of G2-X recombination. p2-test of green-dominant and red-dominant tumours was performed with the null hypothesis being an expected ratio of 1:1. Green- and red-dominant cells from a random subset of tumours (31 sections) or clones were counted by two-tailed Student’s t-test. For analyses of PanIN initiation and early expansion, all low-grade PanINs (n = 92) from a single section from each of five pancreata derived from 6-week-old Pdx1-Cre-K-MADM-p53 mice were classified for colour dominance as all green (n = 23), red-dominant (n = 10) or no dominance (n = 2) (Fig. 7). P-value was calculated for all tumours and K and P-values were calculated by linear regression. For quantification of EdU incorporation, lung adenomas (n = 28) and lung adenocarcinomas (n = 3) were imaged using a ×10 objective and the number of DAPI + and EdU + nuclei were quantified using ImageJ for a single image per lung adenoma and a total of eight images from lung adenocarcinomas (n = 3 mice). The percentage of EdU +/DAPI + cells from each image was calculated, averaged for all tumours of each genotype, compared by two-tailed Student’s t-test. Quantification of PanINs, ×10 objective images were obtained (n = 2 mice) and the number of total GFP +, EdU +/GFP +, total tdTomato + and EdU +/tdTomato + were manually counted. The percentage of EdU+/GFP + and EdU+/tdTomato + cells were calculated and compared by two-tailed Student’s t-test. p-value was calculated for all tumours and K and P-values were calculated by linear regression. For analyses of PanIN initiation and early expansion, all low-grade PanINs (n = 92) from a single section from each of five pancreata derived from 6-week-old Pdx1-Cre-K-MADM-p53 mice were classified for colour dominance as all green (n = 23), red-dominant (n = 10) or no dominance (n = 2) (Fig. 7).
The authors declare that the data that support the findings from this study are available within the article and its Supplementary Information files or available from the corresponding author upon request.

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Data availability
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Author contributions
M.D.M. and T.J. designed the study. M.D.M., K.J.D., K.M.C. and T.T. performed experiments. T.T. assisted with CLARITY experiments; N.R. Kerper for help with confocal and multi-photon imaging; A. Berns, A. Lowy, L. Luo and H. Zong for mice. This work was supported by the Lustgarten Foundation, the Howard Hughes Medical Institute and, in part, by a Cancer Center Support (core) grant P30-CA14561 from the National Cancer Institute. M.D.M. is supported by a KL2/Catalyst Medical Research Investigator Training award (an appointed KL2 award) from Harvard Catalyst | The Harvard Clinical and Translational Science Center (National Center for Research Resources and the National Center for Advancing Translational Sciences, National Institutes of Health Award KL2 TR001100). The content is solely the responsibility of the authors and does not necessarily represent the official views of Harvard Catalyst, Harvard University and its affiliated academic healthcare centres, or the National Institutes of Health. T.T. is supported by a NIH Pathway to Independence Award (K99). V.G. is supported by a Jane Coffin Childs Memorial Fund Postdoctoral Fellowship. C.M.L. is supported by the Ludwig Center for Molecular Oncology Fund. T.J. is a Howard Hughes Medical Institute Investigator, the David H. Koch Professor of Biology and a Daniel K. Ludwig Scholar.

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