Image-based detection and targeting of therapy resistance in pancreatic adenocarcinoma

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Pancreatic intraepithelial neoplasia is a pre-malignant lesion that can progress to pancreatic ductal adenocarcinoma, a highly lethal malignancy marked by its late stage at clinical presentation and profound drug resistance. The genomic alterations that commonly occur in pancreatic cancer include activation of KRAS and inactivation of p53 and SMAD4 (refs 2–4). So far, however, it has been challenging to target these pathways therapeutically; thus the search for other key mediators of pancreatic cancer growth remains an important endeavour. Here we show that the stem cell determinant Musashi (Msi) is a critical element of pancreatic cancer progression both in genetic models and in patient-derived xenografts. Specifically, we developed Msi reporter mice that allowed image-based tracking of stem cell signals within cancers, revealing that Msi expression rises as pancreatic intraepithelial neoplasia progresses to adenocarcinoma, and that Msi-expressing cells are key drivers of pancreatic cancer: they preferentially harbour the capacity to propagate adenocarcinoma, are enriched in circulating tumour cells, and are markedly drug resistant. This population could be effectively targeted by deletion of either Msi1 or Msi2, which led to a striking defect in the progression of pancreatic intraepithelial neoplasia to adenocarcinoma, and that Msi-expressing cells are key drivers of pancreatic cancer: they preferentially harbour the capacity to propagate adenocarcinoma, are enriched in circulating tumour cells, and are markedly drug resistant. This population could be effectively targeted by deletion of either Msi1 or Msi2, which led to a striking defect in the progression of pancreatic intraepithelial neoplasia to adenocarcinoma, and that Msi-expressing cells are key drivers of pancreatic cancer: they preferentially harbour the capacity to propagate adenocar

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serve as a tool to visualize drug-resistant cells, and identify therapies to target them.

Because Msi expression rose during progression (Extended Data Figs 1f–k and 4a), and marked therapy-resistant cells, we tested if genetic or pharmacological targeting of Msi could eradicate this ‘high risk’ population. Deletion of Msi1 led to a fivefold reduction in tumour volume by magnetic resonance imaging (MRI) (Fig. 2a, b, Extended Data Fig. 4b and Supplementary Videos 2–4). Histologically, adenocarcinoma areas comprised 67% of wild-type (WT)-KPf/fC but less than 10% of Msi1+/−-KPf/fC tumours. Log-rank (Mantel–Cox) survival analysis (*P < 0.001, **P < 0.001, ***P < 0.0001 by Student’s t-test or one-way analysis of variance (ANOVA)). Source data for all panels are available online.

Figure 1 | Msi reporter+ pancreatic cancer cells are enriched for tumour-initiating capacity. a, b, Design of Msi reporter constructs (REM1, Msi1+/++; REM2, Msi2+/++; REM3, Msi2+/–). c, d, Live images of Msi reporter cells in (c) REM1–KPf/fC and (d) REM2–KPf/fC tumours; VE-cadherin (magenta), Hoechst (blue), Msi reporter (green). e, f, Msi1 and Msi2 reporter expression in dissociated tumours (n = 6). g, h, Sphere-forming ability of Msi reporter+ and reporter− cells (g, n = 8; h, n = 6). I, In vivo growth of Msi2 reporter+ tumour cells (n = 8). J, Survival of mice orthotopically transplanted with Msi2 reporter+ and reporter−. KPf/fC tumour cells (n = 6). Log-rank (Mantel–Cox) survival analysis (P < 0.05). K, Reporter frequency in primary tumours (n = 3), and circulating tumour cells from ascites (n = 3) or peripheral blood (n = 4). L, Average frequency of tumour-spheres from Msi2 reporter+ and reporter− circulating tumour cells (n = 2–4 technical replicates). M, N, Reporter frequency in REM2–KPf/fC mice treated with vehicle or 500 mg per kg (body weight) gemcitabine (n = 6). Data are represented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by Student’s t-test or one-way analysis of variance (ANOVA). Source data for all panels are available online.

Figure 2 | Loss of Msi1 or Msi2 impairs tumour initiation and progression in a genetic mouse model of pancreatic cancer. a, Coronal and sagittal MRI images of normal, WT-KPf/fC, and Msi1+/−-KPf/fC mice with three-dimensional volume rendering of tumour mass (red). b, Average volumes of isolated WT-KPf/fC (n = 13) and Msi1−/−-KPf/fC tumours (n = 9). c, d, Histology and (e, f) quantification of PanIN and/or adenocarcinoma areas in WT-KPf/fC and Msi1−/−-KPf/fC tumours. g, Survival of mice orthotopically grafted with Msi1+/−-KPf/fC or WT-KPf/fC tumours (n = 16). Analysis of Msi2+/−-KPf/fC tumours (h) by MRI and (i) after isolation, WT-KPf/fC (n = 5), Msi2+/−-KPf/fC (n = 7). j, m, Histology of WT-KPf/fC and Msi2+/−-KPf/fC pancreatic tumours (×40 magnification); k, adenocarcinoma, liver invasion (green arrows); l, adenocarcinoma (yellow arrows); m, PanINs (blue arrows). n, o, Quantification of PanIN and/or adenocarcinoma areas in WT-KPf/fC and Msi2+/−-KPf/fC tumours (n = 6). p, Survival of autochthonous Msi2+/−-KPf/fC (n = 19) or WT-KPf/fC (n = 32) mice. Log-rank (Mantel–Cox) survival analysis (P < 0.0001). Data represented as mean ± s.e.m. **P < 0.01, ***P < 0.001 by Student’s t-test. Source data for all panels are available online.

No detectable tumour mass in most Msi2−/−-KPf/fC mice (Fig. 2h, i, Extended Data Fig. 4e and Supplementary Videos 2, 5 and 6). Histologically, KPf/fC pancreata were mostly replaced by adenocarcinoma, often accompanied by extracapsular invasion into surrounding structures; in contrast, Msi2−/−-KPf/fC pancreata contained low-grade PanINs with rare high-grade PanINs and microfoci of adenocarcinoma within predominantly normal tissue (Fig. 2j–o). Median survival, tracked in the autochthonous model, was 122 days for Msi2−/−-KPf/fC versus 87 days for WT-KPf/fC mice (Fig. 2p), representing a fourfold decreased risk of death. Collectively, our data show that Msi inhibition markedly improves disease trajectory, leading to an approximate doubling of survival. The fact that the mice ultimately succumbed to disease is probably due to the strong selection for Msi-expressing escaper cells in Msi1 and Msi2 single, or double, knockout mice (Extended Data Fig. 5). Additionally, some redundancy between Msi1 and Msi2, as well as a partial gene fragment present in Msi1−/− mice (data not shown), may also exert compensatory activity.
To understand the molecular basis of the effects of Msi loss, we genomically profiled Msi deficient tumour cells (Extended Data Figs 6 and 7a–d). Msi loss led to downregulation of many key genes, including regulators of stem cell function (Wnt7a, Aldh, Lin28), proto-oncogenes (cMet, Fos, Fyn) and Regenerating (Reg) family genes, linked to gastrointestinal cancers. Among these, analysis of 3’ untranslated regions (UTRs) for Msi binding sites and RNA immunoprecipitation (RIP)–qPCR identified BRD4, cMET, and HMGA2 as potential direct targets (Fig. 3a and Extended Data Fig. 7e). We focused on cMET23, which was diminished in Msi null pancreatic cancer and bound MSI1 in ultraviolet-cross-linked immunoprecipitation followed by sequencing (CLIP-seq) experiments (Fig. 3b–d and Extended Data Fig. 7f, g). cMET could not only be activated molecularly but also effectively complemented Msi loss (Fig. 3e, f and Extended Data Fig. 7h). While these results suggest that cMet is a direct functional target of Msi, it is almost certainly one of many. In fact, the powerful impact of Msi on cancer is probably because of its ability to control a broad range of programs (Extended Data Fig. 6). In this context, BRD4 and HMGA2 may represent a particularly attractive class of targets23,24, as they could act at an epigenetic level with cMet to collectively mediate Msi function. Emphasizing such a potential convergence of epigenetic and oncogenic pathways, inhibitors of both Brd4 and cMet effectively targeted gemicitabine-resistant Msi2+ cells (Fig. 3g, h).

To complement the mouse models, we tested the impact of MSI inhibition on primary patient samples, which harbour more complex mutations, and are uniformly drug resistant. Primary pancreatic cancer cells were infected with Msi short hairpin RNAs (shRNAs) and implanted as xenografts (Extended Data Fig. 8a). While shMsi cells were equivalently present at time of transplant, their ability to contribute to the tumour mass in vivo was reduced by 4.9- to 6.5-fold (Fig. 4a, b and Extended Data Fig. 8b, c), demonstrating that inhibition of either Msi1 or Msi2 results in marked suppression of primary human pancreatic cancer growth. Interestingly, Msi2 expression was more homogeneous in patients than in mouse models (Extended Data Figs 1a, b and 2d, e). This could be a consequence of selection due to treatment and end-stage disease in patients, or because Msi2 patterns differ between mouse models and human disease. However, regardless of the level of heterogeneity, our loss-of-function studies indicate that the mouse and human disease are both highly dependent on Msi signalling.

Given that inhibition of Msi has profound effects on pancreatic cancer progression, we explored its potential as a therapeutic target by developing antisense oligonucleotides (ASOs)35,26 specific for Msi1. Because ASO inhibitors are designed on the basis of target RNA sequences, they can be a powerful approach for inhibiting proteins such as cMet.
as Msi, considered 'undruggable' by traditional approaches. Of 400 candidate MS1-ASOs screened, the two most potent markedly reduced colony formation, as well as human cell line and KEAP1-derived tumour growth in vivo (Fig. 4c–g and Extended Data Fig. 8d, e). The MS1-ASOs have not yet been lead-optimized, a longer-term process designed to maximize therapeutic level efficacy with systemic delivery. To test if a lead-optimized ASO can penetrate the tumour microenvironment, a lead-optimized ASO against Malat1 was delivered intraperitoneally and was effective in knocking down its target both in stem and non-stem cell fractions (Fig. 4h and Extended Data Fig. 8f–j). These studies provide proof-of-principle that deliverable Msi inhibitors can antagonize pancreatic cancer growth in vivo, and suggest that ASOs should be explored further as a new class of therapeutics in this disease.

The Msi reporters we describe here may be broadly applicable for cancer diagnostic and therapeutic studies. Because Msi reporter activity can be visualized through live imaging, these mice can be used to track cancer stem cells in vivo, and provide a dynamic view of cancer growth and dissemination within the native microenvironment. The fact that reporter+ cells are gemcitabine resistant raises the exciting possibility that this could serve as a platform to visualize resistance in vivo. Integration of such reporters during drug development may provide a powerful complement to conventional screens, and allow identification of therapies that can better target drug-resistant disease. Further, the spatially restricted distribution of Msi2+ cells could have important implications for designing strategies to loco-regionally target cells that drive residual disease and relapse.

One of the biggest disappointments in pancreatic cancer therapy has been the failure of targeted agents to make a meaningful impact. Our data demonstrate that Msi function is critical for growth and progression of pancreatic cancer, and Msi therefore represents an attractive therapeutic target. We also show that cell-penetrating ASOs are able to antagonize Msi and inhibit growth of pancreatic cancer. These findings highlight the value of targeting Msi, and suggest that ASOs27–30 and other antagonists should be developed for pancreatic and other cancers highlighted in the preclinical model of pancreatic cancer. Cancer Res. 75, 3480–3492 (2015).

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Author Information Microarray and RNA-seq data have been deposited in the Gene Expression Omnibus under accession numbers GSE73312 and GSE75797. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to A.M.L. (alowy@ucsd.edu) or T.R. (treya@ucsd.edu).
Mice. REM1 (Msi1+GFP−/−) and REM2 (Msi2+GFP−/−) reporter mice were generated by conventional genetic targeting (Genoway; Fig. 1); all of the reporter mice used in experiments were heterozygous for the corresponding Msi allele. The Msi1ΔG (Msi1+GFP+/−/−) mice were generated by conventional genetic targeting by inserting loxP sites around exons 1–4 (Genoway). The Msi2 mutant mouse, B6; CB-Msi1eYFP/−/−; Msi2−/−− was established by gene trap mutagenesis as previously described7. H. Okano provided the Msi1−/− mice as previously described13. M. Sander provided Tp53 tm1Brn (Leica Microsystems) or with a Nikon Eclipse E600 fluorescent microscope. For 1 h at 20–25 °C. 4′,6-diamidino-2-phenylindole (DAPI) ( Molecular Probes) was used to detect DNA and images were obtained with a Confocal Leica TCS SP5 II (Leica Microsystems) or with a Nikon Eclipse E600 fluorescent microscope. For immunohistochemical staining, endogenous peroxidase was blocked by incubating slides in 3% H2O2 for 15 min before primary antibody. Incubation with biotinylated secondary antibodies (Vector Laboratories) was performed for 45 min at 20–25 °C. ImmPACT NovaRED Kit (Vector Laboratories) was used according to the manufacturer’s protocol. Sections were counterstained with haematoxylin. The following primary antibodies were used for human tissue sections: rabbit anti-Msi1 (Abcam, ab52865) 4 μg ml−1; rabbit anti-Msi2 (Abcam, ab76148) 1 μg ml−1; and mouse anti-Keratin (Abcam, ab8068) 1:20. The following primary antibodies were used to stain mouse tissues: rabbit anti-ALDH1 (Abcam, ab24334) 1:200; rabbit anti-c-Met (Abcam, ab5662) 1:250; chicken anti-GFP (Abcam, ab13970) 1:250 (for pancreatic tumours and brain) or 1:200 (for bone marrow); rabbit anti-Msi2 (Abcam, ab76148) 1:500 (for pancreatic tumours and brain) or 1:200 (for bone marrow); rat anti-Ki67 (Ebioscience, 14-5698) 1:1,000; rat anti-Msi1 (Ebioscience, 14-9896-00) 1:1,000; mouse anti-c-Met (Abcam, ab8068) 1:10; and biotinylated DBA (Vector Laboratories, B-1035) 1:1,000.

Pancreatic tumoursphere formation assay. (A) Pancreatic tumoursphere formation assays were performed on freshly isolated mouse pancreatic tumour cells or circulating tumour cells from peripheral blood modified from ref. 33. Brieﬂy, pancreatic tumours from 10- to 13-week-old REM1-KPf/f or REM2-KPf/f mice were dissociated and FACS sorted for YFP+ and YFP− or EPcAM+/GFP− and EPcAM−/GFP− cells, respectively. One hundred to 500 cells were suspended in 100 μl DMEM F-12 (Gibco, Life Technologies) containing 1× B-27 supplement (Gibco, Life Technologies), 3% FBS, 100 μM 3-mercaptoprotoxin (Gibco, Life Technologies), 1× non-essential amino acids (Gibco, Life Technologies), 1× N2 supplement (Gibco, Life Technologies), 20 ng ml−1 EGF (Gibco, Life Technologies), 20 ng ml−1 FGF2 (Gibco, Life Technologies), and 10 ng ml−1 ESGRO mLIF (Millipore). Culture medium for circulating tumour cells also contained 20 ng ml−1 mHGF (R&D Systems). Cells in medium were plated in 96-well ultra-low adherence culture plates (Costar) and incubated at 37 °C for 7 days. Images were collected using a Nikon 80i fluorescence microscope. Spheres size was measured using Image grabbing software version 1.47.

Histological analysis and quantification of PanIN and pancreatic ductal adenocarcinoma. Mouse tumours from 4.5- to 13-week-old Msi1−/−−/−, Msi2−/−−/−, and WT-KPf/f mice were harvested at 11 weeks of age. Tumours were sectioned and total cellular RNAs were purified, labelled, and hybridized onto Affymetrix GeneChip Mouse Genome 430 2.0 arrays and raw hybridization data were collected (VA/NMRF Microarray and NGS Core, University of California San Diego). Expression level data were extracted using R package gplots, and normalized using a multiple-loess algorithm as previously described.66. Probes whose
expression levels exceed a threshold value in at least one sample were considered detected. The threshold value is found by inspecting from the distribution plots of log, expression levels. Detected probes were sorted according to their q-value, which is the smallest false discovery rate at which a probe is called significant\(^7\). A false discovery rate value of \(q \leq \alpha\) false discovery rate was evaluated using significance analysis of microarrays and its implementation in the official statistical package sam\(^6\). The samples were treated as ‘two class paired’ according to the date of RNA extraction. No gene expression level of \(q \leq 0.1\). A 48-h split-labeled genes was created using in-house software. (B) MIA PaCa-2 cells were infected with GFP-tagged or TomatoRed-tagged lentiviral particles containing shRNAs for MSI1, MSI2, MSI1 + MSI2, and a scrambled control. At 72 h after infection, positively infected cells were sorted and total cellular RNAs were isolated using a Qiagen RNeasy mini kit. RNA-seq fastq files were processed into transcript-level summaries using kallisto, an ultrafast pseudo-alignment algorithm with expectation maximization. Transcript-level summaries were processed into gene-level summaries by adding all transcript counts from the same gene. Gene counts were normalized across samples using DESeq normalization\(^9\), and the gene list was filtered on the basis of mean abundance, which left 13,684 detected genes for further analysis. Differential expression was assessed with an R package limma\(^8\) applied to log2-transformed counts. Statistical significance of each test was expressed in terms of posterior error probability \(p^P\) using the limma function eBayes\(^41,42\). Posterior error probability, also called local false discovery rate, is the probability that a particular gene is not differentially expressed, given the prior probabilities of the model. The list of genes sorted by \(p^P\) (in ascending order) was analysed for over-represented biological processes and pathways using a non-parametric version of gene set enrichment analysis\(^43,44\). Denoting \(p^P(1)\) as the probability that a gene is not differentially expressed in the MSI1 knockdown and \(p^P(2)\) the probability that a gene is not differentially expressed in the MSI2 knockdown, the probability that a gene is differentially expressed in both samples was estimated as \([1 − p^P(1) [1 − p^P(2)]\). By the same token, the probability that a gene is differentially expressed in the MSI1 knockdown but not in the MSI2 knockdown was estimated as \([1 − p^P(1)] [1 − p^P(2)]\); likewise with indices 1 and 2 switched.

**Reverse transcription PCR.** RNA was isolated using RNeasy Micro and Mini kits (Qiagen) and converted to cDNA using Superscript III (Invitrogen). Quantitative PCR was performed using an iCycler (BioRad) by mixing cDNAs, iQ SYBR Green Supermix (BioRad), and gene specific primers. Primer sequences are available upon request. All real-time data were normalized to actin or GAPDH.

**In vivo transplantation assay and analysis.** In vivo we focused on the tumorigenic potential of MSI2 reporter cells since MSI1- cells were unable to form tumours in small numbers (100, 1,000), possibly because they are less tumorigenic or more quiescent (data not shown). Pancreatic tumours from 10- to 13-week-old REM2-MSI2/− mice were dissociated and sorted for RNA isolation to compare Malat1 expression in MSI1-bound targets including tags with the binding core sequence ‘rUAG’ prepared into the pLENTI-PGK-PURO DEST vector. MIA PaCa-2 cells were infected with pLENTI-PGK-MET or pLENTI-PGK-EMPTY virus. After the establishment of the stable cell line over-expressing cMET, lentiviruses containing shRNAs for Control, MSI1, or MSI2 were delivered. Cells were sorted for GFP expression and plated into a soft agar colony assay. Colonies were counted 14 days after plating.

**In vivo and in vitro drug therapy.** Nine- to 10-week-old REM2-KP+/- mice were treated with gemcitabine alone or in combination with crizotinib or iBet762 for 6 days. On day 6, tumours were removed, dissociated (as described above), counted for total cellular content, stained with anti-mouse EpCam antibody, and analysed for reporter expression by flow cytometry. Gemcitabine (Sigma, G6423) was resuspended in H2O at 20 mg ml\(^{-1}\) and delivered at 200 mg per kg (body weight) or 500 mg per kg (body weight) by intraperitoneal injection twice over 6 days (on days 0 and 3). Crizotinib (Selleckchem PF-0341066) was resuspended in dimethyl-sulfoxide (DMSO) at 50 mg ml\(^{-1}\), diluted 1:10 in H2O, and delivered at 100 mg per kg (body weight) per day for 6 days by oral gavage. iBet762 (Selleckchem S7189) was resuspended in DMSO at 50 mg ml\(^{-1}\), diluted 1:10 in H2O, and delivered at 30 mg per kg (body weight) per day by intraperitoneal injection for 6 days. For in vitro drug assay, low-passage MSI2 reporter KP+/- cells were loaded with 2µM Dil and imaged continuously for up to 48 h while receiving 10µM gemcitabine treatment.

**ASO inhibitors.** To identify human MSI ASO inhibitors, rapid throughput screens were performed to identify effective ASOs as previously described\(^7,48\). ASOs were tested in full dose–response experiments to determine potency. The top two most effective ASOs were chosen to test free uptake and verify target knockdown in MIA PaCa-2 cells. The sequences of Gen 2.5 MSI1 ASOs used for the study were ASO-1, 5′-ATATGATACAGGACCG-3′ and ASO-2, 5′-TTATCATAATGACAGG-3′, with underlined letters indicating 2′-modified bases. The sequence of Gen 2.5 scrambledd (5′-GGCTTACTAGGGCCGCTACA-3′) ASO with no perfect match for any known transcript was included as a negative control. (A) In vivo: MIA PaCa-2 cells were treated with 0.5–20µM of antisense compound for 24 h, after which cells were lysed and RNA isolated. Gene expression was assessed with Taqman probes for MSI1 and MSI2. Actin was used to normalize all real-time data. For functional testing, MIA PaCa-2 cells were plated in the colony assay as previously described. The growth medium was supplemented with 0.25–10µM of ASO. Cells were supplemented weekly with fresh antisense compound. Colonies were counted 21 days after the first ASO treatment. (B) In vivo: 5 × 10\(^3\) MIA PaCa-2 cells were transplanted into the flank of 5- to 8-week-old NSG recipient mice. Once tumours were measurable at 2 weeks after transplantation, 50µg of either control ASO or MSI1 ASO-1 in PBS was administered intratumorally. ASOs were delivered daily over the course of the study. Tumour measurements were recorded every 3 days. Subcutaneous tumours did not exceed 2 cm in diameter as per the University of California San Diego Institutional Animal Care and Use Committee Policy on Experimental Neoplasia. (C) In vivo: in 8-week-old WT-KP/+ C mice, either control ASO or Malat1 ASO was delivered by intraperitoneal injection at a dose of 50 mg per kg (body weight). ASOs were delivered daily for 14 days. On day 15, mice were killed and the tumour removed. Tumours were harvested and used as follows: (1) flash frozen for RNA isolation and MALAT1 (2) placed into 4% paraformaldehyde for paraffin embedding, sectioning, and in situ hybridization analysis for Malat1; and (3) dissociated and sorted for RNA isolation to compare Malat1 expression in EpCam+ /ALDH+ and EpCam- /ALDH- populations.

**Tumour imaging.** Eleven- to 12-week-old REM-KP+/- mice were anasthetized by intraperitoneal injection of ketamine and xylazine (100/20 mg per kg (body weight)). To visualize blood vessels and nuclei, mice were injected retro-orbitally with Alexa Fluor 647 anti-mouse CD114 (VE-cadherin) antibody and Hoechst 33342 immediately after anaesthesia induction. Pancreatic tumours were removed and placed in HBSS containing 5% FBS and 2 mM EDTA. Images (80-100µm in the flank of 5- to 8-week-old NSG recipient mice) were acquired with an HCX APO L20 objective on an upright Leica SP5 confocal system using Leica LAS AF 1.8.2 software. Videos were generated using Velocity 3D image analysis software and compressed using Microsoft Video 1 compression.

**Cirulating tumour cell analysis.** Ten- to 13-week-old REM2-KP+/- mice were anasthetized and approximately 100–500 µl of peripheral blood and ascites was performed with anti-Flag antibody (Sigma-Aldrich) or control immunoglobulin-G (IgG) using an EZ-Magna RIP kit according to the manufacturer’s protocol (Millipore). Immunoprecipitated RNA was converted to cDNA and analysed for the expression of indicated genes by real-time PCR.

**CLIP-seq.** Briefly, MIA PaCa-2 cells were ultraviolet cross-linked with a Stratalinker (Model 2400, Stratagene). Cells were lysed and supernatant added to Dynabeads conjugated to MSI1 antibody (clone 1H1, ebioscience). CLIP library preparation and sequencing, as well as sample preparation and sequencing, were performed as described\(^5\). A total of 73,329 unique tags were obtained from MSI1-bound targets including tags with the binding core sequence ‘rUAG’ site, as reported previously\(^5\).

**MET rescue assay.** Using gateway technology, pENTR-Human cMET was engineered into the pLENTI-PGK-PURO DEST vector. MIA PaCa-2 cells were infected with pLENTI-PGK-MET or pLENTI-PGK-EMPTY virus. After the establishment of the stable cell line over-expressing cMET, lentiviruses containing shRNAs for Control, MSI1, or MSI2 were delivered. Cells were sorted for GFP expression and plated into a soft agar colony assay. Colonies were counted 14 days after plating.
collected in PBS containing 5 mM EDTA and 2% dextran. Samples were incubated at 37°C and red blood cells were lysed using RBC lysis buffer (eBiosciences). Remaining cells were stained with anti-mouse EpCAM-PE (eBiosciences) and anti-mouse CD45-PE-Cy7 (eBiosciences) antibodies. Analysis was performed on a FACSaria III machine (Becton Dickinson) and data analysed with FlowJo software (Tree Star).

**In situ hybridization.** Msi1 and Msi2 mRNA were detected in tumour samples using RNAscope, an RNA in situ hybridization method that allows signal amplification and background suppression. Human tissue was drop-fixed in RNase-free manner and dried at room temperature overnight. Staining was initiated by baking the slides for 32 min at 60°C, then they were deparaffinized, subjected to antigen retrieval, and treated with protease (two sequential incubations at 65°C and 75°C for 12 min each) to enhance probe penetration, as described by the manufacturer (Advanced Cell Diagnostics). Msi1-specific and Msi2-specific RNA target probe sets were generated and supplied by the manufacturer (Advanced Cell Diagnostics). Sequential amplification steps resulted in a large number of horseradish peroxidase molecules per mRNA. The probe was visualized by incubation with 3,3′ diaminobenzidine (DAB). Sections were counterstained with haematoxylin. All steps of this procedure were performed using a Ventana Discovery Ultra (Roche). Slides were analysed by conventional light microscopy.

**Msi1−/−KPf/fC survival curve.** For the Msi1−/−KPf/fC mice, tracking survival was complicated by the incidence of hydrocephaly observed in the knockout mice reported previously. To avoid confounding the data with deaths due to non-tumorigenic events, we performed orthotopic transplants. Briefly, Msi1−/−KPf/fC and WT KPf/fC mice at 8 weeks of age were killed and tumours collected. Tumours were divided into four equal chunks, and then surgically transplanted into the pancreas of 8-week-old NSG mice. After surgery, the orthotopically transplanted mice were tracked for survival.

**Luciferase assay.** A Lightswitch Luciferase Assay System (Active Motif) was used to assess Msi1 regulation of cMET. Briefly, 1 × 10^5 MIA PaCa-2 cells were plated into 96-well plates and cultured for 24 h. Fifty nanograms of cMET 3′ UTR GoClone (S811259, Active Motif) plasmid DNA and increasing concentrations (0 ng, 50 ng, and 100 ng) of either PGK-GFP or PGK-MSI1 plasmid vector were co-transfected with Msi1-specific and Msi2-specific RNA target probe sets generated and supplied by the manufacturer (Advanced Cell Diagnostics). Sequencl amplification steps resulted in a large number of horseradish peroxidase molecules per mRNA. The probe was visualized by incubation with 3,3′ diaminobenzidine (DAB). Sections were counterstained with haematoxylin. All steps of this procedure were performed using a Ventana Discovery Ultra (Roche). Slides were analysed by conventional light microscopy.

**Caerulein-induced pancreatitis.** Four-week-old C57BL/6 mice received 8 injections of 50 μg per kg (body weight) caerulein (Sigma-Aldrich) or PBS hourly each day for 2 consecutive days (for a total of 16 injections). Pancreata were isolated 2 days after the last injection, fixed in 4% paraformaldehyde, and paraffin embedded according to standard protocols. Sections (7 μm) were obtained, deparaffinized in xylene, and stained as described above.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism software version 6.0d (GraphPad Software). Sample sizes were determined on the basis of the variability of pancreatic tumour models used. Tumour-bearing animals within each group were randomly assigned to treatment groups. The investigators were not blinded to allocation during experiments and outcome assessment. Data are shown as the mean ± s.e.m. Two-tailed unpaired Student’s t-tests with Welch’s correction or one-way ANOVA for multiple comparisons when appropriate were used to determine statistical significance (**P < 0.05, ***P < 0.01, ****P < 0.001).
Extended Data Figure 1 | See next page for caption.
Extended Data Figure 1 | The Musashi genes MSI1 and MSI2 are expressed in human pancreatic adenocarcinoma. a, Top row: representative images of a primary patient pancreatic adenocarcinoma sample stained with anti-keratin (green), DAPI (blue), and anti-MSI1 (red) antibodies. White arrows indicate MSI1− cells; yellow arrow indicates a MSI1+ cell. a, Bottom row: representative images of a primary patient pancreatic adenocarcinoma sample stained with anti-keratin (green), DAPI (blue), and anti-MSI2 (red) antibodies. White dotted regions indicate MSI2− cells while yellow dotted regions indicate MSI2+ cells. b, Top row: representative images of a primary patient pancreatic adenocarcinoma sample stained with anti-keratin (green), DAPI (blue), and anti-MSI1 (red) antibodies. White arrows indicate MSI1− cells; yellow arrow indicates a MSI1+ cell. b, Bottom row: representative images of a primary patient pancreatic adenocarcinoma sample stained with anti-keratin (green), DAPI (blue), and anti-MSI2 (red) antibodies. Yellow dotted region indicates MSI2+ cells. c, Top row: representative images of a matched liver metastasis from a patient with pancreatic adenocarcinoma stained with anti-keratin (green), DAPI (blue), and anti-MSI1 (red) antibodies. White arrows indicate MSI1− cells; yellow arrows indicate MSI1+ cells. c, Bottom row: representative images of a matched liver metastasis from a patient with pancreatic adenocarcinoma stained with anti-keratin (green), DAPI (blue), and anti-MSI2 (red) antibodies. Yellow dotted region indicates MSI2+ cells.

a–c, Total magnification ×200. Source data for all panels are available online.

d, Quantification of MSI1 and MSI2 expression in four patients comparing primary pancreatic adenocarcinoma to the patient-matched liver metastasis; four images analysed per patient.

e, Quantification of the frequency of MSI1− and MSI2+ cells in four patients comparing primary pancreatic adenocarcinoma to the patient-matched liver metastasis; four images analysed per patient.

f, MSI1 and (g) MSI2 expression in normal pancreas (n = 1), PanIN (n = 9), and pancreatic adenocarcinoma samples (n = 9).

h, Quantification of MSI2 expression from a human tissue array comparing grade 1 (well-differentiated, n = 9), grade 2 (moderately differentiated, n = 12), and grade 3 (poorly differentiated, n = 16) adenocarcinoma relative to normal pancreas (n = 14) and normal adjacent pancreas (n = 16).

i, MSI1 and (j) MSI2 expression in well-differentiated, moderately differentiated, and poorly differentiated human pancreatic cancer cell lines (n = 3 independent experiments).

k, Colony formation of well-differentiated, moderately differentiated, and poorly differentiated human pancreatic cancer cell lines (n = 3 independent experiments). Data are represented as mean ± s.e.m.
Extended Data Figure 2 | Validation of Msi1 and Msi2 reporter mice. 

a, FACS analysis of Msi1 expression in haematopoietic stem cells, progenitors, and lineage-positive differentiated cells. b, Representative image of Msi1 expression in FACS-sorted YFP+ neuronal cells; YFP (green), Msi1 (red), and DAPI (blue). c, Representative image of Msi2 expression in FACS-sorted GFP+ haematopoietic cells; GFP (green), Msi1 (red), and DAPI (blue). d, Msi1–YFP reporter (green, white arrows) and keratin (red) staining was performed on tissue sections of REM1-KPf/fC mice; e, Msi2–GFP reporter (green, white arrows) and keratin (red) staining was performed on tissue sections of REM2-KPf/fC mice. Rare cells (<5%) were found to be keratin− (possibly mesenchymal population). f, Immunofluorescence analysis of Msi1 and Msi2 expression overlap in isolated EpCAM+ KPf/fC cells (n = 3, 1,000 total cells analysed from 3 independent experiments). Data are represented as mean ± s.e.m. g, h, Survival of Msi reporter-KPf/fC and WT-KPf/fC mice. Survival curves of (g) Msi1YFP+/−-KPf/fC (REM1-KPf/fC, n = 21) or WT-KPf/fC (n = 18) mice and (h) Msi2GFP+/−-KPf/fC (REM2-KPf/fC, n = 65) or WT-KPf/fC (n = 54) mice. i, Live image of Msi2 reporter cells in REM2-KPf/fC tumour; VE-cadherin (magenta), Hoechst (blue), Msi reporter (green). See also Fig. 1c, d. Source data for all panels are available online.
Extended Data Figure 3 | Analysis of stem cell traits in Msi1 and Msi2 reporter+ KPf/fC populations. a, ALDH expression in reporter+ tumour cells sorted from REM1-KPf/fC (top row) and REM2-KPf/fC (bottom row) mice; ALDH1 (red), DAPI (blue), and GFP or YFP (green). b, Average ALDH expression in bulk or Msi1 and Msi2 reporter+ tumour cells (n = 3 each; 90 total cells analysed from 3 REM1-KPf/fC and 150 total cells analysed from 3 REM2-KPf/fC). c, Average Msi expression in ALDH+ cells from REM1-KPf/fC and REM2-KPf/fC tumours (n = 3 independent experiments for each genotype). d, Representative images of spheres formed from (d) Msi1 and (e) Msi2 reporter+ and reporter− tumour cells. See also Fig. 1g, f, g. In vivo tumour growth of Msi2 reporter+ or Msi reporter− KPf/fC cells at (f) 500 or (g) 1,000 cells (n = 16). See also Fig. 1i. (h) Survival of mice orthotopically transplanted with 10,000 Msi2 reporter+ and reporter− KPf/fC tumour cells (n = 6). See also Fig. 1j. Log-rank (Mantel–Cox) survival analysis (P < 0.05). i, j, Reporter frequency in REM2-KPf/fC mice treated with vehicle or 200 mg per kg (body weight) gemcitabine (n = 3 each). See also Fig. 1m, n for high-dose (500 mg per kg (body weight)) gemcitabine. Data are represented as mean ± s.e.m. ***P < 0.001 by Student’s t-test or one-way ANOVA. k, Msi2 reporter− KPf/fC cells do not turn on Msi2 expression after in vitro gemcitabine treatment, suggesting that Msi-reporter− cells are differentially resistant to gemcitabine. Low-passage Msi2 reporter KPf/fC cells loaded with DiI were live-imaged continuously for up to 48 h. Representative series of images from 10 μM gemcitabine treatment. Reporter− cells (red); GFP reporter+ cells (green); tracking of Msi2 reporter− cells (white arrows); tracking of Msi2 reporter+ cells (yellow arrows) (n = 3 independent experiments). Source data for all panels are available online.
Extended Data Figure 4  | Analysis of tumours from Msi null KPf/fC mice. a, Msi2 (green) and Keratin (red) immunofluorescent staining was performed on tissue sections from WT pancreas (normal, n = 3 samples), KRASG12D/+;Ptf1aCre/+ (PanIN, n = 2 samples), and KRASG12D/+;p53f/f;Ptf1aCre/+ (pancreatic ductal adenocarcinoma, n = 3 samples) mice with quantification of Msi2 fluorescence in keratin + cells. b, Average weights of WT-KPf/fC (n = 13) and Msi1 −/−-KPf/fC tumours (n = 9). See also Fig. 2a, b for tumour volume analysis. c, PAS and Alcian blue stained sections of pancreata isolated from WT-KPf/fC represent areas used to identify the stages of PanINs (yellow boxes) and adenocarcinoma (red box). d, Tumours from 11- to 13-week-old WT-KPf/fC (n = 6), Msi1 −/−-KPf/fC (n = 3), and Msi2 −/−-KPf/fC (n = 3) mice were stained and quantified for percentage of Keratin + tumour cells (red) expressing Ki67 (green); DAPI staining is shown in blue. e, Average weights of WT-KPf/fC (n = 5) and Msi2 −/−-KPf/fC tumours (n = 7). See also Fig. 2h, i for tumour volume analysis. Data are represented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t-test or one-way ANOVA. Source data for all panels are available online.
Extended Data Figure 5 | Selection for escaper Msi-expressing cells in Msi1, Msi2 single and double knockout KPf/fC mice.

a–c, Immunohistochemical staining for (a) IgG control (n = 4) or (b, c, red) Msi2 in 13-week-old WT-KP<sup>f/fC</sup> (n = 4) and Msi2<sup>−/−</sup>-<sup>KP</sup>f/fC (n = 4) mice.

d, Immunohistochemical staining for Msi2 (red) in 22-week-old Msi2<sup>−/−</sup>-<sup>KP</sup>f/fC mouse (n = 1).

e–g, Immunohistochemical staining for (e) IgG control, (f, red) Msi1, and (g, red) Msi2 in a 15-week-old Msi1<sup>f/f</sup>Msi2<sup>−/−</sup>-<sup>KP</sup>f/fC mouse (n = 1).

h, Survival curves of Msi1<sup>f/f</sup>Msi2<sup>−/−</sup>-<sup>KP</sup>f/fC (n = 6) or WT-KP<sup>f/f</sup>C tumours (n = 35). Source data for all panels are available online.
Extended Data Figure 6 | Genome-wide analysis of Msi controlled programs in pancreatic cancer. a, Genome-wide expression analysis of dissociated pancreatic tumours. Microarray analysis was performed on RNA from three pairs of WT-KPf/−C and Msi1−/−-KPf/−C matched littermates. Heat map shows differential expression of selected mRNAs identified as part of a stem-cell-associated gene signature. b, Concordantly (upper right and lower left quadrants) and discordantly (upper left and lower right quadrants) regulated genes (red) in MSI1-knockdown and MSI2-knockdown MIA PaCa-2 cells. c, Gene changes specific to MSI1-knockdown (turquoise) or MSI2-knockdown (purple) in MIA PaCa-2 cells. d, Heat maps indicating concordant, MSI1-specific, and MSI2-specific genes. e, Venn diagram displaying the intersection of probe sets that are differentially regulated in MSI1-knockdown, MSI2-knockdown, and double knockdown of MSI1 and MSI2 in MIA PaCa-2 cells. Within scatterplots, lighter colour corresponds to a probability > 0.5 and the darker colour corresponds to a probability > 0.75. Source data for all panels are available online.
Extended Data Figure 7 | Molecular targets of Msi signalling.

**a, b.** Quantitative PCR analysis of (a) Msi1 and (b) Msi2 expression in MIA PaCa-2 human pancreatic cancer cells relative to normal pancreas ($n = 3$ independent experiments).

**c, d.** Analysis of shRNA knockdown efficiency in GFP$^+$-sorted MIA PaCa-2 cells infected with GFP-tagged lentiviral shRNA against scrambled control sequences, (c) MSI1, or (d) MSI2 ($n = 3$ independent experiments).

**e.** Analysis of direct Msi targets: Msi consensus binding sites in 3′ UTR of BRD4, HMGA2, and cMET transcripts.

**f, g.** Phospho-cMet staining in WT-KPf/fC and (g) Msi2$^{−/−}$-KPf/fC mice; keratin (magenta), phospho-cMet (green), DAPI (blue). See Fig. 3b–c for quantified data.

**h.** Colony formation of MIA PaCa-2 cells infected with empty vector or cMET overexpression vector (three independent experiments) shows no impact of overexpressed cMet on control MIA PaCa-2 (control for cMet-mediated rescue of MSI knockdown in Fig. 3f). Data are represented as mean ± s.e.m. ***$P < 0.001$, ****$P < 0.0001$ by Student’s $t$-test. Source data for all panels are available online.
Extended Data Figure 8 | Analysis of impaired pancreatic cancer growth with shMSI and MSI1-ASOs. a, Schematic for inhibiting MSI in primary patient-derived xenografts. b, c, Frequency of GFP+ patient tumour cells before and after transplantation. See also Fig. 4a, b for patients 1 and 2. d, e, MSI1 expression after free uptake of (d) control ASO or (e) MSI1-ASO2 in human pancreatic cancer line (n = 3 per condition). See also Fig. 4c for impact of MSI1-ASO1. f–j, ASO delivery in vivo. f, Target knockdown efficacy of lead-optimized ASO in KPf/fC stem cells. Malat1 expression in EpCAM+/ALDH+ and EpCAM+/ALDH− cells after systemic delivery of control ASO or lead-optimized Malat1-ASO in autochthonous KPf/fC model (n = 3 independent experiments). See also Fig. 4h for target knockdown in unfractionated EpCAM+ cells. g, h, Analysis of potential toxicity of MSI-ASO: g, cage weight of mice receiving daily treatment of MSI1 ASO-1 (50 mg per kg (body weight)) or vehicle by intraperitoneal injection; four mice per cage; cage weight was measured every 3 days; h, average body weight of mice after 3 weeks of daily treatment with MSI1 ASO-1 (50 mg per kg (body weight)) or vehicle by intraperitoneal injection (n = 4 mice/cohort). In vivo delivery of MSI1 ASOs (50 mg per kg (body weight)) had no deleterious impact on body weight and maintained plasma chemistry markers (AST, ALT, BUN, T.Bil) within 3× upper limit of normal. i, j, Representative images of in situ hybridization for Malat1 (purple) in pancreatic tumours isolated from KPf/fC mice treated by daily intraperitoneal injection with (i) control ASO (50 mg per kg (body weight)) or (j) Malat1-ASO (50 mg per kg (body weight)) for 14 days. Source data for all panels are available online.
Extended Data Figure 9 | Elevated expression of Msi in pancreatitis.
Msi2 expression in a caerulein-induced mouse model of pancreatitis, and in human pancreatitis. a, Msi2 staining and (b) quantification of ten images per group in pancreas from PBS-treated (a, top panels, n = 1) and caerulein-treated mice (a, bottom panels, n = 1). c, Msi2 immunohistochemical staining in islets (black dotted outlines) and acinar cells (blue squares) in caerulein-treated or PBS-treated mice (n = 1 for each group). d, Immunofluorescent staining of Msi2 (green) in DBA^+ ductal cells (red) treated with PBS (left panels) or caerulein (right panels) (n = 1 for each group); DAPI is shown in blue. e, MSI2 expression in human tissue arrays from patients presenting with mild chronic inflammation (n = 4) and chronic pancreatitis (n = 6) compared with normal pancreas (n = 14). Data are represented as mean ± s.e.m. ****P < 0.0001 by Student’s t-test. Source data for all panels are available online.