Tumour cell heterogeneity maintained by cooperating subclones in Wnt-driven mammary cancers

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Cancer genome sequencing studies indicate that a single breast cancer typically harbours multiple genetically distinct subclones. As carcinogenesis involves a breakdown in the cell–cell cooperation that normally maintains epithelial tissue architecture, individual subclones within a malignant microenvironment are commonly depicted as self-interested competitors. Alternatively, breast cancer subclones might interact cooperatively to gain a selective growth advantage in some cases. Although interclonal cooperation has been shown to drive tumorigenesis in fruitfly models, definitive evidence for functional cooperation between epithelial tumour cell subclones in mammals is lacking. Here we use mouse models of breast cancer to show that interclonal cooperation can be essential for tumour maintenance. Aberrant expression of the secreted signalling molecule Wnt1 generates mixed-lineage mammary tumours composed of basal and luminal tumour cell subtypes, which purportedly derive from a bipotent malignant progenitor cell residing atop a tumour cell hierarchy. Using somatic Hras mutations as clonal markers, we show that some Wnt tumours indeed conform to a hierarchical configuration, but that others unexpectedly harbour genetically distinct basal Hras mutant and luminal Hras wild-type subclones. Both subclones are required for efficient tumour propagation, which strictly depends on luminally produced Wnt1. When biclonal tumours were challenged with Wnt withdrawal to simulate targeted therapy, analysis of tumour regression and relapse revealed that basal subclones recruit heterologous Wnt-producing cells to restore tumour growth. Alternatively, in the absence of a substitute Wnt source, the original subclones often evolve to rescue Wnt pathway activation and drive relapse, either by restoring cooperation or by switching to a defector strategy. Uncoupling similar modes of interclonal cooperation in human cancers may inform efforts aimed at eradicating tumour cell communities.

Cancer progression is known to depend on cooperation between tumour cells and neighbouring host cells in the microenvironment. Some have suggested that cooperation between distinct tumour cell subclones may also contribute to the malignant phenotype. Favouring this possibility, genetically distinct subclones cooperatively enhanced tumour growth in models engineered to recapitulate a form of tumour cell heterogeneity identified in brain cancers. Similarly, phenotypically distinct tumour cell subclones cooperatively enhanced tumour invasion in a murine lung cancer model. In the case of human breast cancer, recent studies highlight the phenotypic and genetic diversity present locally within individual tumours, but whether this heterogeneity is a cause or a consequence of tumour progression remains unclear. Accordingly, we sought definitive evidence for functional cooperation between tumour cell subclones in mouse models of human breast cancer.

Mammary cancers arising in the classic mouse mammary tumour virus (MMTV)-Wnt1 transgenic mouse model display tumour cell heterogeneity that is widely attributed to malignant transformation of a bipotent mammary progenitor cell. Concordantly, MMTV-Wnt1 tumour cells partition into basal and luminal subclones which comingle, recalling the corresponding basal and luminal lineages found in the normal mammary gland (Fig. 1a, b). Although mutations in Wnt pathway components are rare in human breast cancers, the transcriptional profile of Wnt1-initiated tumours resembles that of other mammary cancer models that commonly show mixed-lineage histopathology, including chemical carcinogen-induced rodent mammary cancers.

While studying cooperating oncogenic mutations in the MMTV-Wnt1 model, we found evidence suggesting that some Wnt tumours harbour unexpected genetic heterogeneity. About half of all Wnt-initiated mammary tumours spontaneously acquire somatic Hras mutations that encode an activated oncoprotein. Because Hras mutations act dominantly, Hras mutant allele fractions (MAFs) of approximately 0.5 are expected, barring copy number changes at the Hras locus. Instead, when tumour-derived Hras alleles were amplified by PCR and subjected to DNA sequencing, chromatogram peak heights often indicated smaller Hras MAFs, with fractions <0.3 detected in four out of ten tumours. Notably, tumours maintained their small Hras MAFs as a stable property when explanted onto the flanks of syngeneic host mice (Fig. 1c). This discrepancy could not be explained by contamination of samples with normal (non-tumour) cells as tumour cell content assessed by histopathology consistently exceeded 80%. Moreover, copy number variations leading to either Hras wild-type (Hraswt) allele gain or Hras mutant (Hrasmut) allele loss seemed unlikely driver events. Instead, we considered whether some Wnt tumours might harbour distinct Hraswt and Hrasmut subclones, noting that biclonal tumours would adopt a mixed-lineage phenotype provided each subclone was committed to a distinct lineage.

To search for lineage-restricted Hraswt and Hrasmut subclones, dissociated cells prepared from Hraswt Wnt tumours were sorted into basal and luminal subclones (Extended Data Fig. 1), then Hras MAFs were determined for each subset and for corresponding samples of unsorted cells. Half of the Hrasmut Wnt tumours analysed (five out of ten) showed negligible subset-specific enrichment in Hrasmut alleles, a pattern consistent with a hierarchical configuration (Fig. 1d, e). In these cases, basal and luminal cells from the same tumour always harboured identical Hrasmut alleles (Fig. 1e), suggesting that they descended from a common bipotent Hraswt progenitor. By contrast, for the remaining half of tumours analysed, Hrasmut alleles were highly enriched within the basal tumour cell subset, a pattern consistent with a biclonal configuration (Fig. 1e). Basal Hrasmut allele enrichment correlated with a lower overall Hras MAF, further suggesting the presence of a private, subclone-restricted mutation. Regardless of whether the distribution of Hrasmut alleles fits a hierarchical or biclonal pattern, tumours showed classic mixed-lineage histopathology (Extended Data Fig. 2), and luminal tumour cells were invariably the main source of Wnt1 expression as reported previously (Fig. 1f). Therefore, some Wnt tumours appeared to harbour distinct basal Hrasmut Wntlow and luminal Hraswt Wnthigh subclones, implicating interclonal cooperation in tumour maintenance. These findings recall early reports in which MMTV-associated mammary tumours initiated by activation of endogenous Wnt genes were sometimes noted to be oligoclonal.
Figure 1 | Evidence for distinct basal Hrasmut and luminal Hraswt subclones within some MMTV-Wnt1 tumours. a, Immunostaining for smooth muscle actin (SMA) and keratin 8 performed on serial sections of a representative MMTV-Wnt1 mammary tumour. Scale bar, 50 μm. b, Separation of MMTV-Wnt1 tumour cells into basal (CD49f<sup>high</sup> EpCAM<sup>low</sup>) and luminal (CD49f<sup>low</sup> EpCAM<sup>high</sup>) cell subpopulations (where EpCAM denotes epithelial cell adhesion molecule) by flow cytometry. Percentages depict mean ± s.e.m. for n = 10 tumours. c, DNA sequencing chromatograms depicting an Hras<sup>CAA61CGA</sup> mutation (top panels) and an Hras<sup>CAA61CTA</sup> mutation (bottom panels) detected in representative Wnt1 tumours whose basal Hrasmut allele enrichment fit a hierarchical pattern or biclonal pattern, respectively. d, Tumour cell populations analysed by DNA sequencing and by quantitative reverse-transcriptase PCR (qRT–PCR) for Wnt1 expression relative to Gapdh. Histograms on the left show Hras MAFs determined from chromatogram peak heights. Histograms on the right show relative Wnt1 expression with values from unsorted tumour cells set at 1. B, basal; L, luminal; Un, unsorted. Data represent mean ± s.e.m. for n = 5 tumours of each pattern.

Seeking stringent proof that some Wnt tumours are biclonal and require interclonal cooperation for maintenance, we attempted to rescue growth of basal Hrasmut Wnt<sup>low</sup> subclones from Wnt1 deprivation by providing access to replacement Wnt1-producing cells. For these experiments, the original MMTV-Wnt1 model (hereafter termed cWnt, denoting constitutive Wnt1 expression) was used in combination with a closely related model engineered for doxycycline (Dox)-dependent transgene expression (MMTV-rtTA/Tet-O-Wnt1; hereafter termed iWnt, denoting inducible Wnt1 expression). During chronic Dox treatment, iWnt mice and mammary tumours phenocopy their cWnt counterparts, but iWnt tumours regress following Dox withdrawal owing to abrogation of Wnt1 transgene expression<sup>26</sup>. To enable tracking of cell lineages in tumour reconstitution experiments, iWnt mice were crossed with a monomeric red fluorescent protein (mRFP) reporter line, generating iWnt/mRFP<sup>+</sup> mice. As expected, a subset of Dox-dependent iWnt/mRFP<sup>+</sup> mammary tumours appeared to be biclonal, as they harboured a basally restricted Hras<sup>mut</sup> subclone. After dissociating these tumours into cell suspensions, 10<sup>5</sup> unsorted cells were injected orthotopically into the mammary fat pads of two sets of Dox-treated, mRFP-reporter-negative female host mice (Fig. 2a). Control hosts lacked a transgene capable of rescuing tumour cells from Wnt1 deprivation by flow cytometry. Percentages depict mean ± s.e.m. for n = 10 tumours. c, DNA sequencing chromatograms depicting an Hras<sup>CAA61CGA</sup> mutation (top panels) and an Hras<sup>CAA61CTA</sup> mutation (bottom panels) detected in representative Wnt1 tumours whose basal Hrasmut allele enrichment fit a hierarchical pattern or biclonal pattern, respectively. d, Tumour cell populations analysed by DNA sequencing and by quantitative reverse-transcriptase PCR (qRT–PCR) for Wnt1 expression relative to Gapdh. Histograms on the left show Hras MAFs determined from chromatogram peak heights. Histograms on the right show relative Wnt1 expression with values from unsorted tumour cells set at 1. B, basal; L, luminal; Un, unsorted. Data represent mean ± s.e.m. for n = 5 tumours of each pattern.

During chronic Dox treatment, both control and rescue hosts developed mammary tumours in most glands injected with iWnt/mRFP<sup>+</sup> tumour cells (Fig. 2b and Extended Data Table 1). As expected, these reconstituted tumours usually regressed when iWnt transgene expression was switched off via Dox withdrawal. On control hosts, tumour regression was always complete, and mice remained relapse-free during 6 weeks of monitoring. Interestingly, subclinical disease often persisted, as most control hosts subsequently relapsed after Dox retreatment (Fig. 2b). By contrast, on cWnt rescue hosts, most reconstituted tumours only partially regressed, then relapsed spontaneously (Fig. 2b and Extended Data Fig. 3a). On control hosts, primary tumours were reconstituted almost exclusively from donor mRFP<sup>+</sup> cells, and relapses triggered by Dox treatment remained mRFP<sup>+</sup> as expected (Fig. 2c). By contrast, on rescue hosts primary tumours showed varying degrees of chimeraism due to incorporation of mRFP<sup>−</sup> (host-derived) luminal cells (Extended Data Fig. 3b), and relapses arising during Dox withdrawal always showed pronounced lineage-restricted chimeraism, resulting in mRFP<sup>+</sup>/basal and mRFP<sup>−</sup>/luminal subpopulations (Fig. 2c). To confirm that donor basal subclones recruit host luminal epithelium to serve as a replacement Wnt1 source at relapse, we turned to northern hybridization analysis of tumour RNAs. Notably, tumours reconstituted on rescue hosts typically expressed the larger iWnt transgene before Dox withdrawal (pertinent exceptions discussed in Extended Data Fig. 3), then switched to expressing the smaller cWnt transgene at relapse, indicating heterologous rescue (Fig. 2d).

Furthermore, the biclonal configuration evident in parental tumours was maintained in all reconstituted tumours in that basal cells were Hras<sup>mut</sup> Wnt<sup>low</sup>, whereas luminal cells were Hras<sup>wt</sup> Wnt<sup>high</sup> (Extended Data Fig. 4). We repeated these rescue experiments twice, beginning each time with an independent, iWnt/mRFP<sup>−</sup> tumour harbouring a distinct, basally restricted Hras<sup>mut</sup> mutation. In all cases, we observed rescue of donor-derived basal Hras<sup>mut</sup> Wnt<sup>low</sup> tumour cells by cWnt host-derived luminal Hras<sup>wt</sup> Wnt<sup>high</sup> cells (Fig. 3a, b and Extended Data Fig. 5). Moreover, the Hras<sup>mut</sup> allele detected in relapsed tumours was always identical to that detected in parental tumours, confirming that basal subclones found at relapse were descended from donor-derived tumour cells and were not novel clones. Examination of tumour sections by fluorescence microscopy revealed pervasive intermingling of basal mRFP<sup>+</sup> and luminal mRFP<sup>−</sup> tumour cells within chimeraic relapses, consistent with the prevailing notion that secreted Wnt provides a short-range signal to neighbouring cells (Fig. 3c). Prospective analysis of a larger set of independent Wnt tumours will be required to precisely estimate the overall fraction of Wnt tumours that is biclonally configured. Notably, we cannot yet determine clonal configurations for the half of Wnt tumours that lack an Hras mutation.

Whereas biclonal iWnt/mRFP<sup>+</sup> tumours were readily reconstituted from unsorted (fluorescence-activated cell sorting (FACS)-naive) cells, sorted basal and luminal cells each reconstituted tumours inefficiently when injected into mammary glands (Extended Data Fig. 6a), perhaps
owing in part to loss of cell viability during FACS. Notably, tumours that did arise after injecting a single sorted subtype were always biclonal, comprised of both basal Hras<sup>mut</sup> Wnt1<sup>low</sup> and luminal Hras<sup>wt</sup> Wnt<sup>high</sup> subsets (seven out of seven tumours analysed, Extended Data Fig. 6b). Given the imperfect separation achieved by FACS (95–98% purity), rare cells cross-contaminating each subset presumably sufficed to permit interclonal cooperation during tumour reconstitution. Consistent with this notion, the relative sizes of the basal and luminal cell populations within these tumours approximated that found in parental tumours and did not reflect the lineage enrichment achieved by sorting. We confirmed this result in an experimental context where the putative cooperating subclones were differentially labelled by the mRFP transgene.

Here, tumour cells derived from chimaeric relapses generated in our rescue experiment were studied prospectively. Again, neither the basal (mRFP<sup>+</sup>/Hras<sup>mut</sup> Wnt1<sup>low</sup>) nor the luminal (mRFP<sup>+</sup>/Hras<sup>wt</sup> Wnt<sup>high</sup>) subsets reconstituted tumours efficiently, whereas a 1:1 admixture of both sorted populations reliably reconstituted biclonal tumours (Extended Data Fig. 7). Notably, every tumour reconstituted in these experiments faithfully restored the subclonal composition of the source tumour, pointing to strong selection favouring propagation of both subclones in tandem.

Figure 2 | Rescue of basal Hras<sup>mut</sup> Wnt tumour cells from Wnt withdrawal by heterologous luminal cWnt. a, Schematic of experimental design. b, Growth curves of tumours reconstituted on wild-type or cWnt hosts following injection of iWnt/mRFP<sup>+</sup> tumour cells. c, Representative FACS plots showing contributions by donor-derived mRFP<sup>+</sup> cells and host-derived mRFP<sup>−</sup> cells to reconstituted tumours. Percentages depict mean ± s.e.m.

Figure 3 | Lineage-restricted subclones recapitulate mosaifrom heterogeneity in chimaeric cWnt/iWnt tumours. a, Schematic summarizing the experiment culminating in chimaeric tumour relapse. b, DNA sequence chromatograms depicting matching Hras<sup>mut</sup> MAFs detected in sorted and sorted tumours from primary and relapsed tumours as indicated. c, Immunostaining of basal (SMA, top panels) and luminal (keratin 8, bottom panels) tumour cells within a Dox-independent relapse arising on a cWnt host. Red fluorescence marks donor-derived iWnt/mRFP<sup>+</sup> cells intermingled with mRFP<sup>−</sup> host-derived cells. Scale bar, 50 μm.

For i tumour explants as indicated. Colours indicate events within the basal (blue; CD49<sup>f</sup>EpCAM<sup>low</sup>) and luminal (green; CD49<sup>f</sup>EpCAM<sup>high</sup>) gates. d, Northern hybridization analysis of tumour RNA with Wnt1 probe. The larger bicistronic iWnt transcript encodes both Wnt1 and firefly luciferase transgene.
Figure 4 | Relapse of biclonal tumours through the evolution of either subclone. a, DNA sequencing chromatograms depicting matching \( H\text{ras} \) mutations detected in primary and relapsed tumours, with an increased MAF detected in the setting of a \( \beta \text{cat} \) mutation. Panels on the right depict analysis of unsorted and sorted cells at relapse showing unipotent or bipotent mutant subclones, depending on the mode of Wnt pathway reactivation. b, Primary tumours showed increased MAFs for a series of primary and relapsed tumours derived from a parental biclonal tumour. Top, corresponding gene expression patterns for each tumour by northern hybridization analysis. c, DNA sequencing chromatograms depicting matching \( H\text{ras} \) mutations detected in primary and relapsed tumours, with an increased MAF detected in the setting of a \( \beta \text{cat} \) mutation. d, Histogram showing Wnt1 expression levels relative to \( \text{Gapdh} \) in unsorted and sorted tumour cells from a \( \beta \text{cat} \) relapse versus a \( \beta \text{cat} \) relapse with the value measured in unsorted cells from the latter relapse set at 1.

(Extended Data Fig. 8), implicating copy-number-neutral loss-of-heterozygosity events.

To further examine the clonal configuration of relapsed tumours, an iWnt/mRFP\(^+\) tumour previously confirmed as biclonal in our rescue experiments (Fig. 2) was propagated as above to derive DITs, then relapse-derived tumour cells were separated into basal and luminal subsets and analysed. One DIT that relapsed via Wnt1 transgene re-expression was biclonal with a luminally restricted rTA mutation (Fig. 4c). Trophic support from this luminal rTA\(^{\text{mut}}\) subclone probably rescued growth of its basal rTA\(^{\text{wt}}\) counterpart, providing a plausible cellular mechanism whereby this rescue mutation was maintained at a low MAF. By contrast, our previous analysis indicated that \( \beta \text{cat} \) rescue mutations originate within basal tumour cells and obviate the need to cooperate with Wnt-producing luminal cells. Nonetheless, \( \beta \text{cat} \) relapses consistently harboured abundant luminal tumour cells (Fig. 4c and Extended Data Fig. 9). We proposed that acquired \( \beta \text{cat} \) mutations endow basally restricted subclones with novel bipotent differentiation potential, thereby converting them to hierarchically configured clones at relapse. Two \( \beta \text{cat} \) DITs analysed as above showed comparable \( \beta \text{cat} \) allele prevalence in the basal and luminal subsets (Fig. 4c), consistent with a scenario in which \( \beta \text{cat} \) relapse clones acquired bipotency. (An alternative scenario in which each subclone independently acquired \( \beta \text{cat} \) mutations cannot be formally excluded, but seems less likely.) In our previous experiments (Fig. 2 and Extended Data Fig. 4) and in the rTA\(^{\text{mut}}\) relapse profiled above, this same subclone invariably behaved in a unipotent manner, remaining basally restricted when partnered with a Wnt1-expressing luminal subclone in the context of primary and relapsed tumours.

Efforts to explain how some cancers stably maintain intratumoral lineage diversity typically invoke tumour cell hierarchies. Here we show that cooperation between lineage-restricted subclones provides an alternative mechanism for maintaining tumour cell heterogeneity. In our Wnt models, we found evidence for both hierarchically and biclonally configured tumours, yet differently configured tumours were indistinguishable by histopathology, acquired equivalent cooperating \( H\text{ras} \) alleles (albeit with differences in tumour cell compartmentalization), and were comparably Wnt1 dependent. Thus, although distinct clonal configurations evolved, they converged towards analogous malignant phenotypes. These findings highlight the difficulties associated with inferring the clonal architecture of cancers from histopathology, even in the ‘simplified’ context of mouse models. Notably, the Wnt models described here provide an experimentally tractable system for exploring whether and how a tumour’s clonal configuration determines its clinical behaviour and curability.

Our study does not define when distinct subclones emerge in the course of tumour progression. Interclonal cooperation may be particularly prevalent in tumours initiated by aberrant expression of secreted signalling molecules, such as Wnt1 and platelet-derived growth factor.\(^30\)

In principle, germline mutations that impart a cancer predisposition also might bias tumours towards a biclonal configuration, as any subsequent cooperation-enabling mutations would necessarily accrue in a cell with mutant neighbours. As such, it will be important to determine whether interclonal cooperation can arise when initiating events originate in somatic cells or act primarily in a cell-intrinsic manner. If cooperation emerges as a common mechanism for maintaining subclone diversity in malignancies, this scenario would counter a key assumption made when interpreting cancer genome sequences. Specifically, certain mutations detected at low allelic fractions and commonly assumed to be late events in tumour progression instead may be early events that enable interclonal cooperation.

**METHODS SUMMARY**

The MMTV-Wnt1 (FVB.Cg-Tg(Wnt1)1Hev/J; stock no. 002934) and mRFP (B6.Cg-Tg(CAG-mRFP1)1F1Hadj/J; stock no. 005884) transgenic lines were obtained from Tg(CAG-mRFP1)1F1Hadj/J; stock no. 005884) transgenic lines were obtained from Tg(CAG-mRFP1)1F1Hadj/J; stock no. 005884) transgenic lines were obtained from Tg(CAG-mRFP1)1F1Hadj/J; stock no. 005884) transgenic lines were obtained from
the Jackson Laboratories. The MMTV-rtTA and tet-O-Wnt1 transgenic lines were a gift from L. Chodosh. All mice either were generated in an inbred FVB/N back- 
ground or were backcrossed ten or more generations with FVB/N breeders before 
initiating experiments. To determine Hras MAFs, we measured peak height (PH) 
attributable to mutant and wild-type alleles on DNA sequencing chromatograms 
using ImageJ 1.46 software, then calculated Hras MAF values using the following 
formula: MAF = PHmutant / (PHmutant + PHwild-type). Mammary tumours were 
dissociated into single-cell suspensions and processed for immunostaining and 
FACS as previously described. Tumour cells were re-suspended in a 50% solution 
of Matrigel in PBS before mammary fat pad injection.

Online Content Any additional Methods, Extended Data display items and Source 
Data are available in the online version of the paper; references unique to these 
sections appear only in the online paper.

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METHODS

Transgenic mice. Mice were housed under pathogen-free conditions in the Pennsylvania State University College of Medicine rodent facility with access to water and chow ad libitum. All experimental protocols were approved by the Pennsylvania State University College of Medicine’s Institutional Animal Care and Use Committee. The MMTV-Wnt1 (FVB.Cg-Tg(Wnt1)1Hev/J; stock no. 002934) and mRFP (B6.Cg-Tg(CAG-mRFP1)1F1Hadj/J; stock no. 005884) transgenic lines were obtained from the Jackson Laboratories. The MMTV-vtTA and tetO-Wnt1 transgenic lines were a gift from L. Chodosh. All mice were either generated in an inbred FVB/N background or backcrossed ten or more generations with FVB/N breeders before initiating experiments. Dox was administered by replacing standard mouse chow with chow containing 2 g kg\(^{-1}\) Dox (Bio-Serv). Genotyping was performed by PCR using genomic DNA isolated from tail clips and transgene specific primers (available upon request).

Cell sorting. Mammary tumours were dissociated into single-cell suspensions through mechanical separation and enzymatic digestion as described\(^2\). Dissociated tumour cells were enriched for Lin\(^{-}\) (CD45\(^{-}\) CD31\(^{-}\) TER119\(^{-}\) BP-1\(^{-}\)) mammary epithelial cells with StemCell Technologies EasySep Mouse Epithelial Cell Enrichment Kits per the manufacturer’s instructions. Lin\(^{-}\) cells were then incubated on ice for 20 min with anti-CD49f (66 integrin) (BD Biosciences 555734) together with Alexa Fluor 647 (Invitrogen A21247) in PBS. Cells were spun down for 5 min at 550g, then incubated with EpCAM-FITC-conjugated antibody (Biolegend 118208) in PBS. Tumour cells were sorted on a BD FACS Aria cell sorter machine equipped with Diva software into their luminal (Lin\(^{-}\) CD49f\(^{\text{low}}\) EpCAM\(^{\text{high}}\)) and basal (Lin\(^{-}\) CD49f\(^{\text{high}}\) EpCAM\(^{\text{low}}\)) subpopulations. Sorted cells were collected into 15-ml conical tubes containing PBS. Genomic DNA was collected from sorted cell populations using Qiagen Blood and Tissue DNAeasy spin column kit. Total RNA was collected from sorted cell populations using Qiagen RNeasy spin column kit. RNA was reverse transcribed using Invitrogen Superscript II First Strand Synthesis kit. We used Taqman Gene Expression Assay mix containing unlabelled PCR primers and a FAM-labelled Taqman probe to detect expression of the following genes: Wnt1 transgene (Applied Biosystems Mm00449604_m1), keratin 8 (Krt8) (Applied Biosystems Mm01333430_m1). Relative qPCR (DD\(_{\text{act}}\)) method was performed in triplicate using Agilent Technologies Stratagene Mx3005P detection system and analysed using Stratagene MxPro software. Gene expression levels in sorted cell populations were amplified by RT-qPCR. Primer pairs for probes are as follows: Wnt1 forward 5'-TGGGTTCTGTTATGGTCG-3' and reverse 5'-TGATTCCCTTGGGCAGAGG-3'; Axin2, forward 5'-CCGAG CTCACTTCCAGGC-3' and reverse 5'-GGACAGGAGGAAGGACCT-3'; β-actin (Actb), forward 5'-TGGACCTCTCAACACCCCCAG-3' and reverse 5'-TGAGACCTTCAAGACCCAGC-3'. After subcloning, the identity of each probe was confirmed by DNA sequence analysis.

DNA sequencing. Genomic DNA or copy DNA isolated from tumour specimens or sorted tumour cell populations was amplified by PCR using gene-specific primers (available upon request). PCR product was run out on an agarose gel, cut out and isolated using Qiagen QIAquick Gel Isolation spin column kit. Samples were subjected to Sanger sequencing using gene-specific primers on an ABI 3130XL Capillary sequencer machine. Sequence traces were analysed using AB DNA Sequencing Analysis Software v5.2 and AB Sequence Scanner v1.0. Peak height (PH) on sequencing chromatograms was measured using ImageJ 1.46 software and Hras MAF was calculated using the following formula: MAF = PH\(_{\text{mutant}}\)/(PH\(_{\text{mutant}}\) + PH\(_{\text{wild-type}}\)).

Immunofluorescence. Tissue samples were fixed in 4% paraformaldehyde on ice for 2 h before being paraffin embedded. Paraffin sections (5 μm) were stained with antibodies for smooth muscle actin (SMA) and keratin 8, which label basal and luminal epithelial cells, respectively. Primary antibodies used were: rabbit anti-SMA (ABCAM 5694, 1:250) and rat anti-keratin 8 (Troma-I) (Developmental Studies Hybridoma Bank, University of Iowa, 1:250). Secondary antibodies were: biotinylated rabbit-anti-rat IgG\(^{\text{+}}\) (Dako Cytomation 20468) and biotinylated rabbit IgG\(^{-}\) (Vector BA-1000). The fluorophore was a streptavidin fluoroescein (Vector SA-5001). Hoechst-33342 dye (Invitrogen H1399) was used for nuclear DNA counterstaining, and slides were visualized using a Zeiss wide-field fluorescent microscope equipped with Axiosvision 4.8 software.

qRT–PCR. RNA was reverse transcribed using Invitrogen Superscript II First Strand Synthesis kit. We used Taqman Gene Expression Assay mix containing unlabelled PCR primers and a FAM-labelled Taqman probe to detect expression of the following genes: Wnt1 transgene (Applied Biosystems Mm00449604_m1), keratin 8 (Krt8) (Applied Biosystems Mm00484683_m1), keratin 5 (Krt5) (Applied Biosystems Mm0049604_m1), keratin 5 (Krt5) (Applied Biosystems Mm0053549_m1), keratin 14 (Krt14) (Applied Biosystems Mm00516876_m1), P-cadherin (Cdh3) (Applied Biosystems Mm01294209_m1) and vimentin (Vim) (Applied Biosystems Mm01333430_m1). Relative qPCR (ΔΔC\(_{\text{act}}\) method) was performed in triplicate using Agilent Technologies Stratagene Mx3005P detection system and analysed using Stratagene MxPro software. Gene expression levels in sorted cell populations were normalized to Gapdh transcript levels (Applied Biosystems 4352339E) and compared to the unsorted sample (relative expression = 1).

Northern hybridization. Total RNA was isolated from snap-frozen bulk tumour pieces by CsCl Density Gradient Centrifugation. Northern hybridization was performed as described previously\(^4\) using cDNA probes generated by RT–PCR. Primer pairs for probes are as follows: Wnt1, forward 5'-TGGGTTCTGTTATGGTCG-3' and reverse 5'-TGATTCCCTTGGGCAGAGG-3'; Axin2, forward 5'-CCGAG CTCACTTCCAGGC-3' and reverse 5'-GGACAGGAGGAAGGACCT-3'; β-actin (Actb), forward 5'-TGGACCTCTCAACACCCCCAG-3' and reverse 5'-TGAGACCTTCAAGACCCAGC-3'.
Extended Data Figure 1 | FACS gating strategy for resolving basal and luminal subsets from mammary tumours. Mammary tumours were mechanically and enzymatically dissociated into single-cell suspensions. 

**a**, Negative selection against Lin⁻ cells using Stem Cell Technologies EasySep Mouse Epithelial Cell Enrichment Kit. Resulting Lin⁺ (CD45⁻ CD31⁻ TER119⁻ BP-1⁻) cells were then immunostained with antibodies for CD49f (α6 integrin) and EpCAM and analysed by FACS.

**b**, Exclusion of cell debris and dead/dying cells. Dead/dying cells collect as a band along the bottom of a forward scatter (FSC-A) versus side scatter (SSC-A) two-parameter plot, and these were gated out when defining population (P)1.

**c**, Cell doublets were discarded when defining P2.

**d**, Basal and luminal mammary epithelial cell populations were separated by immunophenotype. Basal epithelial cells are CD49f⁺⁺⁺ EpCAM⁺⁺⁺ (P3) and luminal epithelial cells are CD49f⁺⁺⁺ EpCAM⁺⁺⁺ (P4).

**e**, Gating tree showing gating strategy for FACS analysis as well as parent and total cell percentages within each of the gates for a representative MMTV-Wnt1 tumour.
Extended Data Figure 2  | Hierarchical and biclonal MMTV-Wnt1 tumours are histologically indistinguishable.  

a, Haematoxylin and eosin-stained sections from a series of MMTV-Wnt1 mammary tumours whose Hras<sup>mut</sup> allele distribution pattern suggests hierarchical or biclonal configuration, as indicated. Scale bar, 50 μm.  
b, Both hierarchical and biclonal MMTV-Wnt1 tumours display mixed-lineage character. Serial sections from a hierarchical and biclonal MMTV-Wnt1 mammary tumours immunostained for smooth muscle actin (SMA) or keratin 8 (K8), which recognize basal and luminal epithelial cells, respectively. For both, brown pigment is positive staining. Sections were counterstained with haematoxylin. Scale bars, 50 μm.
Extended Data Figure 3 | Tumour regression following Dox withdrawal.

a, Tumours reconstituted on wild-type or cWnt hosts following injection of iWnt/mRFP
tumour cells were subjected to Dox withdrawal and monitored for regression. *Shown as number of tumour regressions per number of tumours subjected to Dox withdrawal.

b, Northern hybridization analysis of tumour RNA with Wnt1 probe. Tumours were reconstituted on Dox-treated cWnt hosts following injection of iWnt/mRFP tumour cells. Depicted below are the corresponding FACS plots showing the range of contributions by donor-derived mRFP and host-derived mRFP cells to reconstituted tumours before Dox withdrawal. Colours indicate events within the basal (blue; CD49f EpCAMlow) and luminal (green; CD49f EpCAMhigh) gates. On rescue hosts, primary tumours that arose during Dox treatment incorporated a variable number of cWnt luminal cells, indicating that the crosstalk between heterologous cells required to seed relapse sometimes occurs early in tumour reconstitution. For one of three primary tumours analysed, the conversion to lineage-restricted chimaerism and cWnt transgene expression was essentially complete, meaning that cWnt-producing cells had replaced iWnt-producing cells despite ongoing Dox treatment. Analysis of this tumour required necropsy of the host, precluding determination of its clinical response to Dox withdrawal, which we propose would have been negligible. Concordantly, in rare cases the growth of sibling primary tumours propagated on rescue hosts continued unimpeded by Dox withdrawal, and these tumours always showed pronounced, lineage-restricted chimaerism at necropsy. Elucidating mechanisms whereby host cWnt cells compete with luminal iWnt tumour cells to become the predominant Wnt-producing subclone may offer new insights into evolutionary forces shaping tumour microenvironments.
Extended Data Figure 4 | Biclonal configuration of reconstituted iWnt/mRFP* tumours. a, DNA sequencing chromatograms depicting a basally enriched \( \text{Hras}^{\text{G12A/G12A}} \) mutation detected in the parental tumour. b, Evidence for distinct basal \( \text{Hras}^{\text{mut}} \text{Wnt1}^{\text{low}} \) and luminal \( \text{Hras}^{\text{wt}} \text{Wnt1}^{\text{high}} \) tumour subclones. Sorted tumour cell subsets were analysed by DNA sequencing and by qRT–PCR for \( \text{Wnt1} \) expression relative to \( \text{Gapdh} \). Histograms on the left show \( \text{Hras} \) MAFs determined from chromatogram peak heights. Histograms on the right show relative \( \text{Wnt1} \) expression with values from unsorted tumour cells set at 1. B, basal; L, luminal; Un, unsorted. Data represent mean ± s.e.m. for (from left to right) \( n = 2, 4, 3, 4, 1, 2, 6 \) or 12 explants. c, For each condition, sorted tumour cell subsets were analysed by qRT–PCR for expression of several epithelial lineage-specific genes relative to \( \text{Gapdh} \), with values for unsorted tumour cells set at 1. Grey bars, unsorted; blue bars, basal; green bars, luminal. Data represent mean ± s.e.m. for (from left to right) \( n = 4, 4, 3 \) or 12 explants.
Extended Data Figure 5 | Basal subclones from two additional iWnt/mRFP* tumours rescued from Dox withdrawal by heterologous cWnt host cells. 

a, Growth curves of tumour outgrowths derived from an iWnt/mRFP* tumour harbouring a basally restricted Hras<sup>OGA12GGA</sup> mutation. Curves depict regression and relapse of tumours reconstituted on cWnt rescue hosts following Dox withdrawal. 

b, c, Top, representative FACS plots showing contributions from donor-derived mRFP<sup>1</sup> cells and host-derived mRFP<sup>2</sup> cells during tumour reconstitution. Colours indicate events within the basal (blue; CD49f<sup>high</sup> EpCAM<sup>low</sup>) and luminal (green; CD49f<sup>low</sup> EpCAM<sup>high</sup>) gates. 

Bottom, DNA sequencing chromatograms showing matching, basally restricted Hras mutations present in both primary Dox-dependent tumours and chimeraic Dox-independent relapses. 

d–f, Data panels presented as in a–c, showing similar results for an independent iWnt/mRFP* tumour harbouring a distinct, basally restricted Hras<sup>CAA61CGA</sup> mutation. For both tumours shown here, northern hybridization analysis confirmed expression of donor-derived iWnt transgene before Dox withdrawal, followed by a switch to expression of host-derived cWnt transgene at relapse (data not shown).
Extended Data Figure 6 | Biclonal configuration of tumours reconstituted from sorted iWnt/mRFP<sup>+</sup> tumour cell subsets. a, Sorted tumour cell subsets inefficiently reconstitute tumours. Three independent iWnt/mRFP<sup>+</sup> biclonal tumours were resolved into component basal and luminal tumour cell subsets by FACS. Each tumour harboured a different basally restricted Hras mutation, as indicated. 10<sup>5</sup> sorted tumour cells were injected orthotopically into intact, post-pubertal mammary glands of wild-type host mice maintained on chronic Dox treatment. *Shown as number of reconstituted tumour outgrowths per injected gland. b, Tumour cells from a parental iWnt/mRFP<sup>+</sup> tumour harbouring a basally restricted \textit{Hras}\textsuperscript{GGA12GAA} mutation were resolved into basal and luminal cell subsets by FACS. When these isolated tumour cell subsets were injected orthotopically into the mammary glands of Dox-treated wild-type hosts, few tumours were reconstituted. However, tumours that did arise always were comprised of basal \textit{Hras}\textsuperscript{mut} \textit{Wnt1}\textsuperscript{low} and luminal \textit{Hras}\textsuperscript{wt} \textit{Wnt1}\textsuperscript{high} subsets, implicating interclonal cooperation in tumour reconstitution (\(n = 3\) tumours reconstituted from the basal cell-enriched subset; \(n = 4\) tumours reconstituted from the luminal cell-enriched subset).
**Extended Data Figure 7** | Both sorted basal and sorted luminal cell populations are required to reconstitute biclonal tumours. Chimeric tumour relapses generated by injecting iWnt/mRFP^+^ tumour cells onto cWnt rescue hosts were resolved into their component basal (mRFP^+^/Hras<sup>wt</sup> Wnt1<sup>low</sup>) and luminal (mRFP^-/Hras<sup>wt</sup> Wnt1<sup>high</sup>) cell subsets by FACS. Each sorted population was injected separately (10⁵ basal cells per injection or 10⁵ luminal cells per injection) or as a 1:1 admixture (5 × 10⁴ basal cells + 5 × 10⁴ luminal cells per injection) onto wild-type, Dox-naive hosts. All reconstituted tumours faithfully recapitulated the biclonal configuration of the source tumour. Depicted are FACS plots from parental and reconstituted tumours showing both mRFP<sup>+</sup> and mRFP<sup>-</sup> subclonal populations. Colours indicate events within the basal (blue; CD49<sub>f</sub>high EpCAM<sub>low</sub>) and luminal (green; CD49<sub>f</sub>low EpCAM<sub>high</sub>) gates. Percentages depict mean ± s.e.m. for n = 5 clonally related parental tumour outgrowths and n = 11 tumour outgrowths reconstituted from injection of admixed cells.
Increased Hras MAFs in βcatmut DITs is not due to gross copy changes at the Hras locus. Histogram depicts Hras allele copy number relative to β-actin (Actb) determined for a cohort of clonally related Wnt tumour outgrowths. Independent relapse samples are presented in the same order depicted in Fig. 4b. Copy number values were obtained by performing qPCR on genomic DNA from tumour samples and from normal tail, with tail values set at 1. As a positive control, we included a p19Arf-deficient Wnt tumour sample (~10× amplification) previously found to have approximately tenfold Hras copy number gain as determined by Southern hybridization. As Hras MAFs reproducibly exceeded βcat MAFs by approximately twofold across the βcatmut relapse set (Fig. 4b), increased Hras MAFs may reflect duplication of the Hrasmut allele (for example, via a gene conversion event) sometime in the life history of βcatmut subclones.
Extended Data Figure 9 | Mixed-lineage character of DITs. Serial sections of representative Wnt1-transgene-re-expressing and βcatmut-relapsed tumours immunostained for smooth muscle actin or keratin 8, which recognize basal and luminal epithelial cells, respectively. For both, brown pigment indicates positive staining. Sections were counterstained with haematoxylin. Scale bar, 50 μm.
Extended Data Table 1 | Unsorted tumour cells efficiently reconstitute tumours

| HRas mutation | No. of tumors*, wild-type hosts | No. of tumors*, cWnt hosts |
|---------------|---------------------------------|---------------------------|
| Tumor 1       | GGA12GAA                        | 8/12 (67%)                | 20/24 (83%)               |
| Tumor 2       | GGA12AGA                        | 4/4 (100%)                | 4/4 (100%)                |
| Tumor 3       | CAA61CGA                        | 4/4 (100%)                | 3/4 (75%)                 |
| Totals        |                                 | 16/20 (80%)               | 27/32 (84%)               |

Unsorted (FACS naive) tumour cells from three independent iWnt/mRFP* tumours were injected orthotopically into intact, post-pubertal mammary glands of wild-type control hosts or cWnt rescue hosts. Host mice were maintained on chronic Dox treatment. Each tumour harboured a different basally restricted Hras mutation, as indicated. 10^5 tumour cells were injected into each gland. *Shown as number of reconstituted tumour outgrowths per injected gland.