Evolution of Relapse-Proficient Subclones Constrained by Collateral Sensitivity to Oncogene Overdose in Wnt-Driven Mammary Cancer

Highlights

- Wnt-driven mammary cancers maintain dependence on sub-maximal Wnt signaling
- Rescue mutations destined to subvert targeted therapy carry a fitness cost
- Oncogene overdose imposes negative selection, leading to turnover of rescue subclones

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In Brief
Keller and Gunther show that Wnt-driven mammary cancers challenged with simulated targeted therapy (Wnt withdrawal) undergo clonal evolution, which stringently selects for mutations that restore a “just right” level of oncogenic signaling. Therefore, cancer relapses emerge from rare subclones that are encumbered by an untapped vulnerability to oncogene overdose.
Evolution of Relapse-Proficient Subclones Constrained by Collateral Sensitivity to Oncogene Overdose in Wnt-Driven Mammary Cancer

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SUMMARY

Targeted cancer therapeutics select for drug-resistant rescue subclones (RSCs), which typically carry rescue mutations that restore oncogenic signaling. Whereas mutations underlying antibiotic resistance frequently burden drug-naive microbes with a fitness cost, it remains unknown whether and how rescue mutations underlying cancer relapse encounter negative selection prior to targeted therapy. Here, using mouse models of reversible, Wnt-driven mammary cancer, we uncovered stringent counter-selection against Wnt signaling overdose during the clonal evolution of RSCs. Analyzing recurrent tumors emerging during simulated targeted therapy (Wnt withdrawal) by multi-region DNA sequencing revealed polyclonal relapses comprised of multiple RSCs, which bear distinct but functionally equivalent rescue mutations that converge on sub-maximal Wnt pathway activation. When superimposed on native (i.e., undrugged) signaling, these rescue mutations faced negative selection, indicating that they burden RSCs with a fitness cost before Wnt withdrawal unmasks their selective advantage. Exploiting collateral sensitivity to oncogene overdose may help eliminate RSCs and prevent cancer relapse.

INTRODUCTION

Even when targeted therapy results in cancer remission, relapse can emerge from rare drug-resistant cells (Bozic et al., 2013; Hughes and Andersson, 2015; McGranahan and Swanton, 2017). Indeed, mathematical modeling predicts that clinically detectable, treatment-naive cancers nearly always harbor one or more rescue subclones (RSCs) equipped to seed relapse (Bozic et al., 2013; Diaz et al., 2012). In practice, RSC populations can be vanishingly small, precluding routine RSC detection within clinical specimens until relapse is well underway. Moreover, the detection and enumeration of unique RSCs at relapse can be confounded by parallel evolution. For example, because RSCs typically are identified by their rescue mutations, RSCs falsely appear clonally related when they independently acquire identical, highly recurrent rescue mutations. As such, how evolutionary pressures act in the pre-treatment setting to influence the number, size, and turnover of discrete RSC populations remains obscure.

Targeted therapies typically exploit a phenomenon termed oncogene addiction, wherein cancer cells show exquisite dependence on an aberrantly activated signaling pathway for survival and/or proliferation (Weinstein, 2002). Inversely, diverse basic and clinical research findings indicate that cancer cells become impaired not only when challenged by oncogene withdrawal but also when confronted by oncogene overdose. Overexpressing potent oncogenes in untransformed cells paradoxically triggers proliferation arrest or cell death in cell culture and in mouse models (Nieto et al., 2017; Sarkisian et al., 2007; Serrano et al., 1997). Similarly, certain cancers almost never acquire two driver mutations that potently activate the same oncogenic signaling pathway, perhaps because concerted action of strong activating events triggers overdose (Ambrogio et al., 2017; Unni et al., 2015). In the clinic, sensitivity to excessive oncogenic signaling likely explains why pharmacologic doses of gonadal hormones paradoxically served as effective treatment for hormone-dependent breast cancers before the advent of modern anti-hormonal drugs (Haddow et al., 1944; Jordan and Ford, 2011). More recently, oncogene overdose has been invoked to explain why some cancers, after adapting to potent targeted therapy, paradoxically depend upon continued drug treatment for maintenance and growth (Amin et al., 2015a, 2015b; Das Thakur et al., 2013; Sun et al., 2014). Intriguingly, preclinical models of melanoma, lymphoma, and prostate cancer have led to clinical trials aimed at exploiting oncogene overdose for therapeutic gain in patients (Amin et al., 2015a; Schweizer et al., 2015). Despite these advances, it remains unclear whether and how oncogene overdose shapes the evolution of incipient RSCs prior to treatment.

To model targeted therapy of breast cancer, we previously engineered mice for reversible activation of oncogenic Wnt signaling. In female inducible Wnt1 (iWnt) mice, doxycycline (Dox)-induced expression of a Wnt1 transgene leads to the stochastic onset of Wnt-driven mammary carcinomas. iWnt tumors
regress upon simulated targeted therapy (withdrawal of inducer-dependent Wnt1 expression) and then relapse later with rescue mutations that restore Wnt signaling (Debies et al., 2008; Gunther et al., 2003). Most iWnt relapses acquire one of two rescue mutations: either an activating mutation in the downstream Wnt transducer β-catenin (encoded by Ctnnb1; hereafter β-Cat) or a mutation corrupting the rTA gene switch (enabling Dox-independent expression of the Wnt1 transgene; Cleary et al., 2014). These rescue mutations are highly recurrent, which limits detection of discrete subclones evolving in parallel (Gerlinger et al., 2012; Juric et al., 2015). To render clonal evolution in the iWnt model more amenable to genetic analysis, we introduced a germline Apc\(^{\text{Min}}\) allele (Moser et al., 1993), thereby inactivating one copy of the Adenomatous polyposis coli (Apc) tumor suppressor gene that normally restrains Wnt signaling.

We reasoned that iWnt/Apc\(^{\text{Min/+}}\) tumors should be sensitized to relapse via somatic, inactivating second-hit Apc mutations, which might inform on RSC evolution in two key ways. First, selection for Apc second hits ought to broaden the range of functionally equivalent rescue mutations with which to distinguish independent RSCs. Second, the precise location of second-hit Apc mutations ought to provide a crucial readout of the level of Wnt signaling favored at relapse. In human colorectal cancers, second-hit Apc mutations are contingent upon the first Apc hit (whether inherited or sporadic) in a manner that selects for maintaining some residual Apc-mediated restraint on Wnt signaling (Albuquerque et al., 2002; Rowan et al., 2000). Similarly, studies employing either allelic series of targeted Apc mutations or inducible Apc knockdown in mice consistently show that genotypes conferring graded reductions in Apc function specify graded increases in Wnt signaling (Buchert et al., 2010; Dow et al., 2015; Kielman et al., 2002; Premssrirut et al., 2011). Most notably, hypomorphic mutations that create premature stop codons in the Apc mammary tumor mutation cluster region (Apc\(^{\text{mmcr}}\), ranging from Apc codons 1,512–1,580; Figure 1A), when paired with a near null Apc allele (e.g., Apc\(^{\text{Min}}\)), specify a sub-maximum level of Wnt pathway activation that is uniquely suited to initiating mammary carcinogenesis (Bakker et al., 2013; Gaspar et al., 2009; Kuraguchi et al., 2009; Toki et al., 2013). Whether such cancers, once established, are constrained to maintain sub-maximum Wnt signaling during tumor progression remains unknown.

## RESULTS

### Relapse after Wnt Withdrawal Selects for Hypomorphic Apc Mutations and Sub-maximum Wnt Signaling

To test whether a germline Apc\(^{\text{Min}}\) allele promotes relapse, primary mammary tumors arising on iWnt/Apc\(^{\text{Min/+}}\) and iWnt/Apc\(^{\text{+/+}}\) mice were explanted onto wild-type, syngeneic hosts. Thereafter, mice bearing established outgrowths were subjected to Wnt withdrawal and monitored through regression and relapse. As expected, relapses arose more quickly from iWnt/Apc\(^{\text{Min/+}}\) tumor explants than from their iWnt/Apc\(^{\text{+/+}}\) counterparts (Figure 1B). Sequencing of Apc alleles from iWnt/Apc\(^{\text{Min/+}}\) tumor biopsy samples collected prior to Wnt withdrawal failed to uncover Apc\(^{\text{mmcr}}\) mutations or loss-of-heterozygosity (LOH) events, consistent with negligible selection against Apc function in this context (0 of 14 tumor biopsies; 0%). However, relapses arising after Wnt withdrawal nearly always acquired a hypomorphic, second-hit Apc\(^{\text{mmcr}}\) nonsense mutation (31 of 34 tumors; 94%; Figure 1C). Underscoring selective pressure favoring acquisition of a specific hypomorphic allele, these Apc\(^{\text{mmcr}}\) mutations co-localized within a 200-bp gene segment that comprises only 2.4% of the Apc coding region. Moreover, these relapses rarely underwent Apc LOH (i.e., somatic loss of the germline Apc\(^{\text{Min}}\)-type allele; only 1 of 24 tumors; 4%; Figure S1A), indicating stringent selection for an Apc\(^{\text{mmcr}}\) tumor cell genotype. By contrast, Apc\(^{\text{Min/+}}\) intestinal adenomas routinely underwent LOH as previously described (Haigis and Dove, 2003), consistent with selection for complete loss of the Apc-mediated restraint on Wnt signaling in intestinal tumor cells (Figure S1B).

Next, we tested whether rescue mutations generating an Apc\(^{\text{Min/+}}\) tumor cell genotype confer sub-maximum Wnt pathway activation by performing β-catenin immunohistochemistry on tissue and tumor sections. Stabilization of β-catenin culminates in cytosolic accumulation and nuclear translocation, which serve as classic hallmarks of activated Wnt signaling. In the intestine, weak, membrane-associated β-catenin expression in normal epithelium contrasted with strong nuclear-localized expression in adenomas, as others have described (Figure S2). In both normal mammary gland and tumors arising in the MMTV-Myc transgenic mouse model (Stewart et al., 1984), only weak membrane-associated β-catenin was detected, consistent with negligible Wnt pathway activation (Figure 1E). By comparison, mammary tumors from iWnt/Apc\(^{\text{Min/+}}\) mice consistently stained more strongly for β-catenin, with evidence for modest cytosolic accumulation as well as weak nuclear-localized staining in some cells. Notably, despite the cell-to-cell variability evident within each tissue section, the overall level and pattern of β-catenin expression was remarkably conserved across primary and relapsed mammary tumors, suggesting selection for a preferred Wnt pathway activation level. Confirming that this preferred signaling level resides below that which is conferred by complete Apc loss of function, the lone mammary tumor relapse in our set that underwent Apc LOH showed strong, nuclear-localized β-catenin expression on par with that observed in intestinal adenomas (Figures 1E and S2). Why this relapse was unique in evading selection for the recurring Apc\(^{\text{Min/+}}\) tumor cell genotype is unclear. Nonetheless, in sum, our findings underscore strong selection for a sub-maximum Wnt signaling window during mammary tumorigenesis, as others have proposed. Extending this concept, we find that selection for this signaling window is conserved across diverse Wnt pathway-driven mammary tumor models, because relapses arising in iWnt/Apc\(^{\text{Min/+}}\) mice resemble primary mammary tumors arising in carcinogen-exposed Apc\(^{\text{Min/+}}\) mice (Keller et al., 2016) by converging on the same Apc\(^{\text{Min/+}}\) tumor cell genotype. Moreover, our modeling shows that the constraint to maintain sub-maximum...
Wnt signaling persists during later stages of tumor progression, because invasive tumor explants maintain selection for the Apc<sup>Min/mmcr</sup> tumor cell genotype, even as they undergo clonal evolution to spawn relapse.

Polyclonal Relapse and Turnover of RSCs during Serial Passage of Wnt-Driven Tumors

In pilot studies, we often detected two distinct Apc<sup>mmcr</sup> nonsense mutations in a single relapse sample, suggesting some iWnt/Apc<sup>Min/+</sup> relapses may derive from multiple distinct RSCs. To assess polyclonality in depth, we adopted a multi-region sequencing (MRS) protocol in which relapses routinely were divided into 18 segments prior to targeted re-sequencing of Apc alleles (Figure 2). Mapping Apc<sup>mmcr</sup> mutations onto tumor segments revealed that most iWnt/Apc<sup>Min/+</sup> relapses harbored multiple distinct RSCs, which typically occupied contiguous, non-overlapping territories. Even in polyclonal relapses, each individual tumor segment usually harbored a single predominant Apc<sup>mmcr</sup> rescue mutation detectable by Sanger sequencing, suggesting that RSC outgrowth following Wnt withdrawal primarily involves local clone expansion, with relatively little clone intermixing via local micro-invasion or tumor self-seeding (Figure 2). To enable more sensitive detection of low-prevalence mutant alleles, representative relapse segments underwent further analysis of the Apc<sup>mmcr</sup> region by high-throughput re-sequencing (hereafter, HTS) on the Illumina platform. Again, most segments...
derived from polyclonal relapses harbored a single predominant Apc<sup>mmcr</sup> rescue mutation, indicating that polyclonal relapses tended to be conglomerates of macroscopic, primarily monoclonal RSCs (Figure S3). However, we cannot rule out the possibility that low-prevalence RSCs lacking Apc<sup>mmcr</sup> mutations sometimes contribute as admixed subclones.

To determine whether RSCs derive from stable subpopulations of tumor cells lurking within primary tumor explants prior to Wnt withdrawal, RSC maps were generated for a set of relapses arising within two independent iWnt/Apc<sup>Min/+</sup> tumor lineages, each propagated through multiple generations of serial passage on Dox-treated hosts (Figure 3A). For each lineage analyzed, the number of RSCs per relapse detected after Wnt withdrawal remained stable through serial passage, arguing for little or no accumulation of RSCs over time. Concordantly, for the first tumor lineage (hereafter lineage A), nearly all of the RSC-defining Apc<sup>mmcr</sup> rescue mutations identified across passage generations were unique (18 of 20; 90%), indicating that RSCs likely arose de novo during tumor outgrowth at each generation and were not propagated host to host during tumor passage (Figure 3B, left panels). Incidentally, we noted multiple gross lung lesions at necropsy in three mice bearing iWnt/Apc<sup>Min/+</sup> relapses from lineage A. In each case, all distant lung lesions carried an Apc<sup>mmcr</sup> somatic mutation matching a single clone within the local relapse, indicating monophyletic spread of metastases during relapse (Figures 3B and S3). Analysis of a second independent tumor lineage (hereafter lineage B) likewise failed to reveal pervasive, long-lived RSCs (Figure 3B, right panels). Again, most rescue mutations identified in lineage B were unique (17 of 25; 68%), and novel rescue mutations continued to appear in late-passage tumors. Moreover, comparing rescue mutations across explant generations showed that a subset of mutations that recurred went absent from one or more intervening passages, suggesting they likely arose independently (Figure 3B).

Compared with explants from lineage A, explants from lineage B on average showed reduced relapse-free survival following Wnt withdrawal as well as increased polyclonality at relapse (i.e., more RSCs per relapse) detected by MRS analysis (Figure 3C). The mechanism(s) underlying these differences remain unknown but presumably stem from traits acquired during the clonal evolution of each ancestral primary tumor. In any event, the distinct behavior of these lineages offers an intriguing link between increased polyclonality and more rapid onset of tumor escape.

**Rapid Turnover of RSCs Generated by Mutagen Exposure**

Next, we devised a strategy for detecting turnover of RSCs during growth of iWnt/Apc<sup>Min/+</sup> tumors. When exploring the range of rescue mutations capable of subverting targeted therapy,
researchers often employ accelerated mutagenesis protocols, wherein treatment of a drug-sensitive tumor cell population is preceded by exposure to a potent mutagen. Although mutations are introduced genome-wide, subsequent drug treatment strongly selects for rescue mutations that drive RSC outgrowth (Brammeld et al., 2017). If RSCs are short lived prior to targeted therapy, we reasoned that the number of RSCs available to seed relapse ought to rise in the immediate aftermath of a mutagen exposure capable of generating rescue mutations and then quickly decline due to RSC turnover. To test this model prospectively, mice bearing sibling, iWnt/ApcMin/+ tumor explants either remained unexposed (mutagen-naive throughout Wnt withdrawal and relapse) or received a one-time dose of a fast-acting chemical mutagen, N-ethyl-N-nitrosourea (ENU), either two days or seven days prior to Wnt withdrawal (Figure 4A).

Compared with unexposed control tumors, ENU exposure 2 days before Wnt withdrawal (day−2 ENU) dramatically has-tened relapse onset and increased the number of RSCs per relapse detected via MRS, as expected (Figure 4B). Indeed, the number of RSCs arising after day−2 ENU exceeded the number of tumor segments analyzed, meaning that individual tumor segments themselves typically were polyclonal (Figure S4).

Paradoxically, highly polyclonal tumor segments comprised of multiple Apcmmcr mutant RSCs appeared wild-type when initially assessed by Sanger sequencing, because this low-sensitivity technique only detects an allelic variant that reaches a minimum prevalence of about 10%–15%, which corresponds to an RSC that reaches a minimum prevalence of 20%–30% of the cells analyzed (assuming Apcmmcr mutations are heterozygous and unperturbed by copy number variation). By contrast, analyzing Apcmmcr alleles by HTS permitted detection of mutant alleles above a threshold of about 0.3% prevalence, which enabled detection of as many as 9 distinct, low-prevalence Apcmmcr mutant RSCs residing in a single relapse segment (Figure S4). We confirmed that these day−2 ENU relapse segments were indeed highly polyclonal by two additional means. In one strategy, relapse segments, which typically measured 3–5 mm in diameter, were first subdivided into sub-millimeter fragments before preparing genomic DNA (gDNA) templates for analysis. Subsequent Sanger sequencing of PCR-amplified Apcmmcr alleles detected multiple rescue mutations distributed among the subdivided tumor fragments (Figure S5). In a second strategy, bulk gDNA templates from putative polyclonal segments were used to generate PCR-amplified Apcmmcr alleles detected multiple rescue mutations distributed among the subdivided tumor fragments (Figure S5). In a second strategy, bulk gDNA templates from putative polyclonal segments were used to generate PCR-amplified Apcmmcr alleles detected multiple rescue mutations distributed among the subdivided tumor fragments (Figure S5). In a second strategy, bulk gDNA templates from putative polyclonal segments were used to generate PCR-amplified Apcmmcr alleles detected multiple rescue mutations distributed among the subdivided tumor fragments (Figure S5).

Figure 3. No Accumulation of Apcmmcr Rescue Mutations on Serial Passage of iWnt/ApcMin/+ Tumors
(A) Schematic. Illustration depicts serial passage of iWnt/ApcMin/+ tumor lineages and detection of RSCs at relapse across multiple generations. Expansion of tumor subclones at each passage is depicted by Muller diagrams.
(B) Tiling plots. Relapsed tumors (T) analyzed at the indicated passage (P) were analyzed by MRS to elucidate subclone composition. Apcmmcr rescue mutations are arrayed along the x axis. Subclone maps for representative relapses are depicted to the right of each tiling plot, with 3-by-6 grids depicting bivalve views of individual relapses. An unfilled (white) box indicates no Apcmmcr mutation was detected by Sanger DNA sequencing, whereas an unbounded area indicates a missing segment owing to irregular tumor shape or a pocket of necrosis.
(C) Dot plots depicting time to relapse onset (left) and RSCs per relapse (right) for each tumor lineage.
*p < 0.015; ***p < 0.001 by unpaired, two-tailed t test.
survival and RSCs per relapse, consistent with rapid dropout of short-lived, ENU-initiated RSCs during the added days preceding Wnt withdrawal (Figure 4B).

In a parallel arm of this experiment, an additional set of tumors was subjected to an interrupted Wnt withdrawal schedule to simulate a drug-free break in the midst of targeted therapy (Figure 4A). Here, after day \( /C_0 \) ENU exposure, Wnt withdrawal was maintained during two weeks of tumor regression and then transiently reversed until tumors regrew to their original size, upon which Wnt withdrawal was reinstated for the remainder of the experiment. Interrupted Wnt withdrawal yielded a trend toward prolonged relapse-free survival and generated significantly fewer RSCs per relapse (Figure 4B), consistent with RSC dropout resuming during Wnt pathway reactivation and tumor re-growth.

Examination of \( Apc^{mmcr} \) mutation spectra confirmed that ENU exposure seeded relapse by generating second-hit \( Apc \) rescue mutations. Unexposed relapses yielded a mutation spectrum dominated by indels and single nucleotide variants (SNVs) at \( G:C \) base pairs, mirroring the spectrum of spontaneous, somatic \( Apc^{mmcr} \) mutations described previously (Kuraguchi et al., 2009). By contrast, ENU exposure shifted the spectrum away from indels toward SNVs at \( A:T \) base pairs (Figure 4C), matching known ENU mutation patterns (Jansen et al., 1994b; Keller et al., 2016). Concordantly, replacing ENU with the related mutagen \( N\)-methyl-\( N\)-nitrosourea (MNU) similarly hastened relapse and increased RSCs per relapse while generating a distinct, MNU-specific mutation spectrum (Figures 4B and 4C). Notably, despite MNU’s known propensity to induce \( G:C \) to \( A:T \) base substitutions (Jansen et al., 1994a; Westcott et al., 2015), sense-strand \( G > A \) SNVs were completely absent from our \( Apc^{mmcr} \)-derived mutation spectrum, reflecting a genetic coding constraint. Specifically, the \( Apc^{mmcr} \) lacks a \( TGG \) codon, the only codon capable of converting to a stop codon via a \( G > A \) SNV. We hypothesized that this coding constraint, coupled with the constraint to restore sub-maximum Wnt signaling, might channel sense-strand \( G > A \) SNVs toward rescue mutations in alternative target genes. Indeed, whereas ENU-exposed relapses harbored an \( Apc^{mmcr} \) rescue mutation in nearly every tumor segment (Figure 4D), fully one-third of MNU-exposed relapse segments lacked an \( Apc^{mmcr} \) mutation (35 of 105; 33%), and a subset of these segments instead harbored \( \beta\)-Cat or \( rtTA \) rescue mutations, which invariably were sense-strand \( G > A \) SNVs (Figure 4D).

Relapses Converge on Sub-maximum Wnt Reactivation in the Absence of a Sensitizing \( Apc^{Min} \) Mutation

Surprisingly, acquisition of \( Apc^{mmcr} \) rescue mutations did not strictly require a sensitizing germline \( Apc^{Min} \) mutation. Whereas mutagen-naive \( iWnt/Apc^{+/+} \) tumors were slow to relapse and acquired dominantly acting \( \beta\)-Cat rescue mutations in line with our previous work, ENU-exposed \( iWnt/Apc^{+/+} \) tumors relapsed
quickly and invariably acquired Apc\textsuperscript{mmcr} rescue mutations instead of \(\beta\)-Cat mutations (Figures 5A and 5B), as did a subset of MNU-exposed tumors (see below). Because a single intact Apc allele normally suffices to restrain Wnt signaling, we searched for collaborating second-hit Apc mutations. Remarkably, these Apc\textsuperscript{mmcr} mutant relapses nearly always carried a second somatic inactivating mutation further upstream in Apc (15 of 16 tumors; 94%; Figure 5C). We refer to these somatic upstream Apc mutations as ‘‘Min-like,’’ because they encode truncated proteins similarly devoid of all (or nearly all) of the seven 20-amino-acid repeats implicated in \(\beta\)-catenin downregulation.

Several observations suggest that mutagen exposure may generate biallelic Apc inactivation through a complex mutational process involving quasi-synchronous Apc hits, rather than sequential, metachronous Apc hits. First, neither Apc hit on its own is expected to provide a sizable selective advantage that drives clonal expansion, making sequential acquisition of Apc hits statistically unlikely. Second, four relapses were found to be comprised of two distinct RSCs, each carrying a unique Apc\textsuperscript{Min-like/mmcr} genotype. Subclone maps from these relapses showed that neighboring tumor segments never shared one Apc hit, but not the other, as might be expected to occur if hits accrued metachronously (Figure S6A). Third, when the mutations that generated Apc\textsuperscript{mmcr} versus Apc\textsuperscript{Min-like} alleles were analyzed separately, both mutation sets yielded spectra attributable to ENU exposure, despite ENU’s short-lived mutagenic activity \textit{in vivo} (Probst and Justice, 2010; Figure S6B). In any case, stringent selection for sub-maximum Wnt signaling yielded a recurring Apc\textsuperscript{Min-like/mmcr} tumor cell genotype in mutagen-exposed iWnt/Apc\textsuperscript{Min} relapses. Crucially, these relapses converge on Apc allele pairs that, in sum, retain 3 or 4 \(\beta\)-Cat degradation domains (Figure 5C), again indicating robust selection for a preferred Wnt signaling level conducive to mammary tumor maintenance (Bakker et al., 2013). Concordantly, relapses that acquired biallelic mutations culminating in the Apc\textsuperscript{Min-like/mmcr} tumor cell genotype showed sub-maximum \(\beta\)-cat immunostaining on par with their antecedent primary tumors (Figure S2).

Whereas MNU-exposed iWnt/Apc\textsuperscript{+/+} tumors relapsed even faster than their ENU-exposed counterparts (Figure 5A), they only rarely relapsed with biallelic Apc rescue mutations (3 of 15 tumors; 20%), likely due to the coding constraints discussed above that disfavor acquisition of MNU-induced nonsense mutations within the Apc\textsuperscript{mmcr}. Instead, MNU-exposed relapses often acquired \(\beta\)-Cat or rtTA rescue mutations (Figure 5B), which invariably were sense-strand G \(\rightarrow\) A SNVs, as before (Figure 5D). Together, these findings underscore how exposure-specific mutation spectra, when filtered through narrow signaling requirements and coding constraints, can bias rescue mutations toward
“preferred” driver genes with remarkable specificity (Keller et al., 2016).

Negative Selection of RSCs under Conditions Predicted to Drive Wnt Signaling Overdose

Returning to iWnt/Apc<sup>Mmcr</sup> tumors, we considered whether the rapid dropout of mutagen-initiated RSCs prior to Wnt withdrawal might occur by neutral drift rather than by counter-selection against Wnt signaling overdose. To test whether parental tumor cells compete neutrally with RSC cells, we compared their relative clonal expansion in a competitive tumor reconstitution assay. Single-cell suspensions were prepared concurrently from three freshly harvested, sibling iWnt/Apc<sup>Mmcr</sup> tumor specimens (schematized in Figure 6). P cells (denoting parental) were prepared from a Wnt-dependent primary tumor explant. R1 and R2 cells (denoting relapse) were prepared from distinct segments of a putative polyclonal relapse, which arose from a sibling explant during Wnt withdrawal. R1 and R2 cells were generated from antipodal segments to maximize the likelihood of sampling distinct RSC populations. Then tumors were reconstructed in the mammary fat pads of syngeneic, Dox-treated host mice by injecting each cell population individually (P-only, R1-only, and R2-only control injections) or by co-injecting admixed populations. Specifically, R2 cells were competed against both R1 cells (R1 + R2 injections) and P cells (P + R2 injections). Due to limiting tumor cell numbers, we did not compete R1 cells against P cells (i.e., P + R1 injections were not performed). Injections of all individual and admixed tumor cell populations yielded tumors that arose promptly and grew synchronously, permitting all necropsies and tumor harvests to be performed on the same day. Finally, MRS was performed on each reconstituted tumor to assess the contribution of individual subclones.

P-only injection yielded tumor segments lacking detectable Apc<sup>mncr</sup> mutations, as expected. By contrast, R1-only injection yielded a monoclonal tumor bearing an Apc<sup>1540C>T</sup> mutation in all segments. Interestingly, R2-only injection yielded a tumor comprised of both Apc<sup>1568T</sup> and Apc<sup>1522A>T</sup> mutant segments, indicating R2 cells (unbeknownst to us at the time of injection) derived from a polyclonal tumor segment (Figure 6). Consistent with neutral competition among the distinct RSCs, R1 + R2 co-injections yielded polyclonal tumors comprised of all three component RSCs, and the contribution from each RSC matched their relative clonal expansion in a prospective lineage tracing experiment (see STAR Methods). By contrast, in P + R2 co-injections, R2-derived RSC cells contributed little to tumor reconstitution, consistent with parental cells outcompeting RSCs (Figure 6).

We also sought direct evidence for oncogene overdose-mediated clone dropout in a prospective lineage tracing experiment. Previously, we showed that ENU-initiated primary mammary tumors arising in Apc<sup>Mmcr</sup> mice invariably carry second-hit Apc<sup>mncr</sup> mutations, yielding an Apc<sup>Mmcr/mmcr</sup> tumor cell genotype (Keller et al., 2016). We reasoned that converting hypomorphic Apc<sup>mncr</sup> to a null allele ought to abolish the residual Apc<sup>mncr</sup>-mediated restraint on Wnt signaling, triggering oncogene overdose and clone dropout in mammary tumor cells, but not intestinal tumor cells (which routinely undergo LOH to create an Apc<sup>Mmcr/mmcr</sup> tumor cell genotype; Haigis and Dove, 2003). To test this model, first we modified Apc<sup>Mmcr</sup> mice by introducing transgenes that enable tamoxifen (Tam)-inducible, Cre-mediated multi-color lineage tracing (UBC-Cre<sup>ERT</sup> and R26R-Confetti; hereafter Confetti; Figure S7A; Ruzankina et al., 2007; Snippert et al., 2010). Next, for comparison against Apc<sup>Mmcr/Mmcr</sup>-mediated control mice, we generated experimental mice that additionally carry a conditional knockout allele of Apc (hereafter Apc<i>CreKO</i>; Kuraguchi et al., 2008). In summary, as schematized in Figure 7A, Tam treatment...
initiates lineage tracing in both \( Apc^{\text{Min}+/+} / \text{Confetti control mice} \) and \( Apc^{\text{Min}/\text{CKO}} / \text{Confetti experimental mice} \). However, only experimental mice undergo Cre-mediated recombination at the \( Apc \) locus, thereby converting the \( Apc^{\text{CKO}} \) allele (which functionally approximates wild-type \( Apc \)) to \( Apc^{\Delta580} \) (which functionally approximates near-null \( Apc^{\text{Min}} \); Kuraguchi et al., 2006).

After confirming that the Confetti transgene combination permits Tam-inducible lineage tracing in mammary epithelium (Figure S7), we generated ENU-initiated mammary tumors in both \( Apc^{\text{Min}/\text{CKO}} / \text{Confetti experimental mice} \) and \( Apc^{\text{Min}+/+} / \text{Confetti controls} \). Because of the stringent selection for \( Apc^{\text{mmcr}} \) second-hit mutations, control mammary tumors acquired a “Cre-indifferent” \( Apc^{\text{Min}+/+} \) tumor cell genotype, whereas experimental mammary tumors acquired a “Cre-modifiable” \( Apc^{\text{Min}/\text{CKO-mmcr}} \) tumor cell genotype, which can convert to \( Apc^{\Delta580} \) upon recombination (Figure 7A). Tumor-bearing mice then were dosed with Tam to initiate tracing of both mammary and intestinal tumor cell clones while simultaneously inactivating the \( Apc^{\text{CKO}} \) allele. In control mice, clones residing in both mammary tumors and intestinal tumors at day 2 of the trace comprised mostly solitary labeled cells, which typically expanded to monochrome cell clusters at day 7, as expected. In experimental mice, tracing of intestinal tumor clones was unperturbed by the \( Apc^{\text{CKO}} \) allele, but tracing of mammary tumor clones showed a marked reduction in labeled cells at day 2 and a near absence of labeled cells at day 7, indicating dropout of mammary, but not intestinal, tumor cell clones (Figures 7B and 7C). Concordantly, recombined \( Apc^{\text{CKO}} \) alleles were detectable in these mammary tumors using a PCR-based assay (Kuraguchi et al., 2006) at day 2, but not at day 7 (Figures S7D and S7E).
was withheld in both tumor reconstitution assays (Figure 6) and treatment-resistant RSCs when simulated targeted therapy outcompeted sensitive parental tumor cells consistently over days and weeks. Notably, adaptive therapy hinges on rescue mutations, which are essential for recovery during breaks in treatment. Wnt-dependent mammary cancers meet this requirement, because treatment-naive cancers may help to explain why rescue mutations are inefficient and biased toward readily transduced subsets. Future work will address which cellular mechanism(s) act downstream of Wnt signaling to limit outgrowth of RSCs. Previous studies have implicated either cell death pathways (Kong et al., 2017; Unni et al., 2015; Varmus et al., 2016) or proliferation arrest pathways (Das Thakur et al., 2013; Nieto et al., 2017; Sun et al., 2014) in restricting outgrowth of subclones challenged by oncogene overdose. Which cellular mechanisms come into play may vary depending on the tumor type examined and/or which tumor suppressor mechanisms have remained intact during tumor progression.

Our study has several important limitations that preclude direct translation to clinical settings. Because all of our observations derive from mouse models, species differences must be considered when extrapolating our findings to human cancers. In addition, it is worth noting that mutations activating the Wnt pathway have been observed only rarely in human breast cancer. Thus, dropout of recombined tumor cells was highly context dependent, consistent with the lower Wnt signaling level window conducive to mammary versus intestinal tumor growth. Together, our clone competition and lineage-tracing studies strongly reinforce the causal link between oncogene overdose and clone dropout.

DISCUSSION

Potent targeted therapy triggers regression of oncogene-addicted cancers, but most patients relapse within months due to selection for rare tumor cells, which frequently carry rescue mutations that restore oncogenic signaling. Although rescue mutations are common, pre-existing drug treatments (Bhang et al., 2015; Diaz et al., 2012; Hata et al., 2016; Misale et al., 2012; Turke et al., 2010), the evolutionary dynamics shaping drug-resistant RSC populations prior to targeted therapy remain obscure. Here, by mapping the clonal evolution of relapse in mouse models of breast cancer, we show that rescue mutations can impose collateral sensitivity to oncogene overdose, thereby driving potent negative selection prior to oncogene withdrawal. To make this observation, we developed mouse modeling strategies that enabled the detection of dozens of distinct, yet functionally equivalent, Apc


desmocid rescue mutations, which unequivocally subverted Wnt withdrawal by acting as drivers of mammary cancer relapse. Notably, we defined experimental conditions in which simulated targeted therapy reliably culminated in polyclonal relapse, creating opportunities to model a commonly encountered clinical scenario (Burrell and Swanton, 2014). Our overall methodology offers an alternative to performing tumor cell lineage tracing using barcoded retroviruses. Although barcoding offers a powerful means of monitoring subclonal lineages as they expand and contract through time (Bhang et al., 2015; Hata et al., 2016), the tracing experiments are conducted using cell culture, and they require ex vivo labeling that may be inefficient and biased toward readily transduced subsets of tumor cells (Bystriykh and Belderbos, 2016).

Our analysis of the evolutionary dynamics underlying tumor growth suggests Wnt-driven mammary cancers may be useful for preclinical modeling of adaptive therapy. Whereas traditional cancer treatment protocols are designed to maximize tumor cell killing, adaptive therapy protocols are tailored to forestall tumor growth by exploiting sustained competition between treatment-sensitive and treatment-resistant tumor cell populations (Gatebny et al., 2009). The concept has shown promise in both preclinical breast cancer models (Enriquez-Navas et al., 2016) and a small-scale clinical trial for prostate cancer (Zhang et al., 2017). Notably, adaptive therapy hinges on rescue mutations imposing a fitness cost during breaks in treatment. Wnt-dependent mammary cancers meet this requirement, because treatment-sensitive parental tumor cells consistently outcompeted treatment-resistant RSCs when simulated targeted therapy was withheld in both Wnt reconstitution assays (Figure 6) and in situ lineage-tracing studies (Figure 7). Intriguingly, we saw preliminary evidence that simulating a “treatment holiday” in the midst of Wnt withdrawal offers clinical benefit. Compared with continuous Wnt withdrawal, interrupted Wnt withdrawal led to RSC dropout and a trend toward prolonged relapse-free survival (Figures 4A and 4B). Although the clinical benefit of this interrupted Wnt withdrawal protocol was modest, it should be noted that we applied it against cancers with elevated RSC burden (owing to prior ENU exposure) and tested only a single treatment break, arbitrarily scheduled to last two weeks. Future studies will test whether more durable control of Wnt-driven mammary cancers can be achieved by incorporating multiple treatment breaks, scheduled with evolutionary principles in mind. Our modeling approach ought to permit changes in RSC prevalence to be quantified during response to different adaptive therapy schedules. Monitoring competing tumor subsets through time may reveal strategies for directing clonal evolution along preferred trajectories that most effectively eradicate RSCs and/or block disease progression.

Our study illustrates how targeted therapy, by imposing precise selective pressure, can favor rescue mutations affecting a narrow range of genes, thereby rendering the evolutionary path to relapse predictable. That said, we found that subtle changes in exposure history led to dramatic shifts favoring rescue mutations in specific genes. In our accelerated mutagenesis studies, rescue mutations were induced by two mutagens, ENU and MNU, which bear striking structural similarity yet generate distinct patterns of DNA base substitutions (Jansen et al., 1994a, 1994b). When overlaid on genetic coding constraints, these mutagen-specific mutation patterns channeled rescue mutations to “preferred” driver genes with remarkable specificity (Figures 4D and 5B). These findings underscore how a cancer’s own exposure history can inform efforts to predict the most favored genetic routes to relapse (Keller et al., 2016). Our modeling sets the stage for dissecting mechanisms whereby clinically important mutagenic exposures, such as prior treatments with radiation or chemotherapy, dictate which rescue mutations emerge during subsequent targeted therapy.

Future work will address which cellular mechanism(s) act downstream of Wnt signaling to overdose to limit outgrowth of RSCs. Previous studies have implicated either cell death pathways (Kong et al., 2017; Unni et al., 2015; Varmus et al., 2016) or proliferation arrest pathways (Das Thakur et al., 2013; Nieto et al., 2017; Sun et al., 2014) in restricting outgrowth of subclones challenged by oncogene overdose. Which cellular mechanisms come into play may vary depending on the tumor type examined and/or which tumor suppressor mechanisms have remained intact during tumor progression.
often reside within low-prevalence subclones prior to targeted therapy and why rescue subclone prevalence can drop during breaks in treatment (Siravegna et al., 2015). In addition, our modeling predicts that some highly recurrent rescue mutations observed in the clinic may be common precisely because they elevate oncogenic signaling only modestly in the pre-treatment setting, thereby evading oncogene overdose. Whether clinically important rescue mutations likewise sensitize human cancer cells to oncogene overdose remains to be determined. If so, treatment strategies designed to exploit this vulnerability may help eliminate cancer subclones capable of seeding drug-resistant relapse.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.12.096.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.R.K. and E.J.G.; Methodology, R.R.K. and E.J.G.; Investigation, R.R.K.; Writing – Original Draft, R.R.K. and E.J.G.; Writing – Review and Editing, R.R.K. and E.J.G.; Visualization, R.R.K. and E.J.G.; Funding Acquisition, E.J.G.
Dow, L.E., O’Rourke, K.P., Simon, J., Tschaharganeh, D.F., van Es, J.H., Clevers, H., and Lowe, S.W. (2015). Apc restoration promotes cellular differentiation and reestablishes crypt homeostasis in colorectal cancer. Cell 161, 1539–1552.

Enriquez-Navas, P.M., Kam, Y., Hassan, S., Silva, A., Foroutan, P., Ruiz, E., Martinez, G., Minton, S., Gillies, R.J., and Gatenby, R.A. (2016). Exploring evolutionary principles to prolong tumor control in preclinical models of breast cancer. Sci. Transl. Med. 8, 327ra24.

Gaspar, C., Franken, P., Molenaar, L., Breukel, C., van der Valk, M., Smits, R., and Fodde, R. (2009). A targeted constitutive mutation in the APC tumor suppressor gene underlies mammary but not intestinal tumorigenesis. PLoS Genet. 5, e1000547.

Gatenby, R.A., Silva, A.S., Gillies, R.J., and Frieden, B.R. (2009). Adaptive therapy. Cancer Res. 69, 4894–4903.

Gerlinger, M., Rowan, A.J., Horswell, S., Math, M., Larkin, J., Endesfelder, D., Gronroos, E., Martinez, P., Matthews, N., Stewart, A., et al. (2012). Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N. Engl. J. Med. 366, 883–892.

Gunther, E.J., Moody, S.E., Belka, G.K., Hahn, K.T., Innocent, N., Dugan, K.D., Cardiff, R.D., and Chodosh, L.A. (2003). Impact of p53 loss on reversal and recurrence of conditional Wnt-induced tumorigenesis. Genes Dev. 17, 468–501.

Haddow, A., Watkinson, J.M., Paterson, E., and Koller, P.C. (1944). Influence of breast cancer. Sci. Transl. Med. 5, 327ra24.

Hata, A.N., Niederst, M.J., Archibald, H.L., Gomez-Caraballo, M., Siddiqui, F.M., Mulvey, H.E., Maruva, Y.E., Ji, F., Bhang, H.E., Krishnamurthy Radhakrishna, V., et al. (2016). Tumor cells can follow distinct evolutionary paths to become resistant to epidermal growth factor receptor inhibition. Nat. Med. 22, 262–269.

Hughes, D., and Andersson, D.I. (2015). Evolutionary consequences of drug resistance: shared principles across diverse targets and organisms. Nat. Rev. Genet. 16, 459–471.

Janssen, J.G., Mohn, G.R., Vrieling, A., van de Wetering, M., Lohman, P.H., and van Zeeland, A.A. (1994a). Molecular analysis of hprt gene mutations in skin fibroblasts of rats exposed in vivo to N-methyl-N-nitrosourea or N-ethyl-N-nitrosourea. Proc. Natl. Acad. Sci. USA 91, 893–905, January 22, 2019.

Kuraguchi, M., Ohene-Baah, N.Y., Sonkin, D., Bronson, R.T., and Kucherlapati, R. (2009). Genetic mechanisms in Apc-mediated mammary tumorigenesis. PLoS Genet. 5, e1000367.

McGranahan, N., and Swanton, C. (2017). Clonal heterogeneity and tumor evolution: past, present, and the future. Cell 168, 613–628.

Misale, S., Yager, R., Hober, S., Scala, E., Janakiraman, M., Liska, D., Valtorta, E., Schiavo, R., Buscarino, M., Siravegna, G., et al. (2012). Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature 486, 532–536.

Mozer, A.R., Mattes, E.M., Dove, W.F., Lindstrom, M.J., Haag, J.D., and Gould, M.N. (1993). ApcMin, a mutation in the murine Apc gene, predisposes to mammary carcinomas and focal alveolar hyperplasias. Proc. Natl. Acad. Sci. USA 90, 8977–8981.

Nieto, P., Ambrogio, C., Esteban-Burgos, L., Gómez-López, G., Blasco, M.T., Yau, Z., Marais, R., Rosen, N., Chiarle, R., Pisansky, D.G., et al. (2017). A Braf kinase-inactive mutant induces lung adenocarcinoma. Nature 548, 239–243.

Nik-Zainal, S., Davies, H., Ståf, J., Ramakrishna, M., Glodzik, D., Zou, X., Martincorena, I., Alexandrov, L.B., Martin, S., Wedge, D.C., et al. (2016). Landscape of somatic mutations in 560 breast cancer whole-genome sequences. Nature 534, 47–54.

Premrsirur, P.K., Dow, L.E., Kim, S.Y., Camiolo, M., Malone, C.D., Miething, C., Scuppo, C., Zuber, J., Dickens, R.A., Kogan, S.C., et al. (2011). A rapid and scalable system for studying gene function in mice using conditional RNA interference. Cell 145, 145–158.

Probst, F.J., and Justice, M.J. (2018). Mouse mutagenesis with the chemical supermutagen ENU. Methods Enzymol. 477, 297–312.

Rowan, A.J., Lamlum, H., Ilyas, M., Wheeler, J., Straub, J., Papadopoulou, A., Bicknell, D., Bodmer, W.F., and Tomlinson, I.P. (2000). Apc mutations in sporadic colorectal tumors: a mutational “hotspot” and interdependence of the “two hits”. Proc. Natl. Acad. Sci. USA 97, 3352–3357.

Ruzankina, Y., Pinzon-Guzman, C., Asare, A., Ong, T., Pontano, L., Cotsarelis, G., Zediar, V.P., Velez, M., Bhandoola, A., and Brown, E.J. (2007). Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. Cell Stem Cell 1, 113–126.

Sarkisian, C.J., Keister, B.A., Stairs, D.B., Boxer, R.B., Moody, S.E., and Chodosh, L.A. (2007). Dose-dependent oncogene-induced senescence in vivo and its evasion during mammary tumorigenesis. Nat. Cell Biol. 9, 493–505.

Schweizer, M.T., Antonarakis, E.S., Wang, H., Aiboye, A.S., Spitz, A., Cao, H., Luo, J., Haffner, M.C., Yegnasubramanian, S., Carducci, M.A., et al. (2015). Effect of bipolar androgen therapy for asymptomatic men with castration-resistant prostate cancer: results from a pilot clinical study. Sci. Transl. Med. 7, 269ra2.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88, 593–602.

Siravegna, G., Mussolin, B., Buscarino, M., Corti, G., Cassingena, A., Crisafulli, G., Ponzetti, A., Cremonili, C., Amatu, A., Lauricella, C., et al. (2015). Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat. Med. 21, 827.

Snipper, H.J., van der Flier, L.G., Sato, T., van Es, J.H., van den Born, M., Kroon-Veenboer, C., Barker, N., Klein, A.M., van Rheenen, J., Simons, B.D., and Clevers, H. (2010). Intestinal crypt homeostasis results from neutral selection of beta-catenin signaling. Nature 467, 113–126.

Stewart, T.A., Pattengale, P.K., and Leder, P. (1984). Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell 38, 627–637.

Sun, C., Wang, L., Huang, S., Heynen, G.J., Phrahallad, A., Robert, C., Haenen, J., Blank, C., Wesseling, J., Willems, S.M., et al. (2014). Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma. Nature 508, 118–122.
Toki, H., Inoue, M., Motegi, H., Minowa, O., Kanda, H., Yamamoto, N., Ikeda, A., Karashima, Y., Matsui, J., Kaneda, H., et al. (2013). Novel mouse model for Gardner syndrome generated by a large-scale N-ethyl-N-nitrosourea mutagenesis program. Cancer Sci. 104, 937–944.

Turke, A.B., Zejnullahu, K., Wu, Y.L., Song, Y., Dias-Santagata, D., Lifshits, E., Toschi, L., Rogers, A., Mok, T., Sequist, L., et al. (2010). Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. Cancer Cell 17, 77–88.

Unni, A.M., Lockwood, W.W., Zejnullahu, K., Lee-Lin, S.Q., and Varmus, H. (2015). Evidence that synthetic lethality underlies the mutual exclusivity of oncogenic KRAS and EGFR mutations in lung adenocarcinoma. eLife 4, e06907.

Varmus, H., Unni, A.M., and Lockwood, W.W. (2016). How cancer genomics drives cancer biology: does synthetic lethality explain mutually exclusive oncogenic mutations? Cold Spring Harb. Symp. Quant. Biol. 81, 247–255.

Weinstein, I.B. (2002). Cancer. Addiction to oncogenes--the Achilles heal of cancer. Science 297, 63–64.

Westcott, P.M., Halliwill, K.D., To, M.D., Rashid, M., Rust, A.G., Keane, T.M., Deirosario, R., Jen, K.Y., Gurley, K.E., Kemp, C.J., et al. (2015). The mutational landscapes of genetic and chemical models of Kras-driven lung cancer. Nature 517, 489–492.

Zhang, J., Cunningham, J.J., Brown, J.S., and Gatenby, R.A. (2017). Integrating evolutionary dynamics into treatment of metastatic castrate-resistant prostate cancer. Nat. Commun. 8, 1816.
## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti-
\(\beta\)-catenin | BD Transduction Laboratories | Cat# 610153 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| \(N\)-methyl-\(N\)-nitrosourea (MNU) | Sigma Aldrich | N4766 |
| \(N\)-ethyl-\(N\)-nitrosourea (ENU) | Sigma Aldrich | N3385 |
| Tamoxifen | Sigma Aldrich | T5648-1G |
| **Critical Commercial Assays** | | |
| PCR purification kit | Qiaquick | 28104 |
| ExoSAP-IT | Affimix | P/N: 75001 |
| Taqman Gene Expression Assay Mix | Agilent | Catt#600880 |
| One Shot TOP 10 chemically competent cells | Invitrogen | C404004 |
| TOPO XL PCR Cloning Kit | Invitrogen | 45-0008LT |
| Envision ARK kit | DAKO | Cat#3954 |
| XT index kit | Nextera | FC-131-1001 |
| **Experimental Models: Organisms/Strains** | | |
| Mouse: C57BL/6J-Apc\(^{Min}\)/J | Jackson Laboratories | stock no. 002020 |
| Mouse: B6.Cg-Tg(UBC-cre/ERT2)1Ejb/1J | Jackson Laboratories | stock no. 007001 |
| Mouse: (Gl(ROSA)26Sortm1(CAG-Brainbow2.1)Cle/J | Jackson Laboratories | stock no. 137331 |
| Mouse: MMTV-Myc [a.k.a., Tg(MMTV-Myc)141-3Led] | National Cancer Institute Mouse Repository | strain no. 01XAA |
| Mouse: MMTV-rtTA [a.k.a., MTB] | Chodosh Laboratory University of Pennsylvania | Gift |
| Mouse: TetO-Wnt1 [a.k.a., TWNT] | Chodosh Laboratory University of Pennsylvania | Gift |
| **Oligonucleotides** | | |
| \(\beta\)-catenin PCR and Sanger Sequencing: 5\(^{\prime}\)- GCGTGGACAATGGCTACT CAA | This Paper | N/A |
| \(\beta\)-catenin PCR and Sanger Sequencing: 5\(^{\prime}\)- GCGTCAAACTGCGTGGA TGG | This Paper | N/A |
| rtTA PCR and Sanger Sequencing: 5\(^{\prime}\)-GCCCAAAACCTAGGTGTAAGTAG | This Paper | N/A |
| rtTA PCR and Sanger Sequencing: 5\(^{\prime}\)-CGAATAAGAAGGCTGGCTCTGC | This Paper | N/A |
| Apc PCR and Sanger Sequencing: See STAR Methods | This Paper | N/A |
| Apc\(^{mmcr}\) High Throughput Sequencing: 5\(^{\prime}\)-TCGTCGGCAGCGTCAGAT GTGTTATAAGAAGCAGACGAATAATCTCAGACGGA | This Paper | N/A |
| Apc\(^{mmcr}\) High Throughput Sequencing: 5\(^{\prime}\)-GTCTCGTGGGCTCGGAGAT GTGTTATAAGAAGCAGACGAATAATCTCAGACGGA | This Paper | N/A |
| Gapdh qRT-PCR probe | Applied Biosystems | 435239E |
| Wnt1 qRT-PCR probe | Applied Biosystems | Mm_01300555_g1 |
| **Other** | | |
| Growth Factor Reduced Matrigel Mix | BD Biosciences | Cat#356231 |
| DRAQ5 | Cell Signaling | 40845 |
| Collagenase | Sigma | C9891 |
| Hyaluronidase | Sigma | H3406 |
| Dispase / Neutral Protease | Roche | 0492078001 |
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed and will be fulfilled by the Lead Contact, Edward Gunther (ejg12@psu.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Mice were housed at the Pennsylvania State University College of Medicine and permitted unrestricted access to food and water. All experimental protocols were approved by the Pennsylvania State University College of Medicine’s Institutional Animal Care and Use Committee. Apc\textsuperscript{Min}, UBC-Cre\textsuperscript{ERT}, and Confetti mice were obtained from Jackson Laboratories (C57BL/6J-Apc\textsuperscript{Min}/J stock no. 002020), (B6.Cg-Tg(UBC-cre/ERT2)1Ejb/1J stock no. 007001), (Gt(Rosa)26Sor\textsuperscript{tm1(CAG-Brainbow2.1)Cle/J stock no. 137331). Apc\textsuperscript{Cre/Cre} was obtained from the National Cancer Institute Mouse Repository (B6.Cg-Apc\textsuperscript{Min}Nci strain no. 01XAA). MMTV-rtTA and TetO-Wnt1 transgenic lines were maintained in a FVB/N background. All experimental mice were female. Experimental MMTV-rtTA/TetO-Wnt1/Apc\textsuperscript{Min} mice were obtained by crossing male Apc\textsuperscript{Min} to female MMTV-rtTA/TetO-Wnt1. Experimental MMTV-rtTA/TetO-Wnt1 mice were obtained by crossing male C57BL/6J to female MMTV-rtTA/TetO-Wnt1 for a consistent F1 FVB/N-C57BL/6J genetic background. For doxycycline (dox) treatment, standard mouse chow was replaced with chow containing Gunther (ejg12@psu.edu).

Further information and requests for resources and reagents should be directed and will be fulfilled by the Lead Contact, Edward Gunther (ejg12@psu.edu).

METHOD DETAILS

Primary tumor initiation, explants, and relapse

MMTV-rtTA/TetO-Wnt1 primary tumors and MMTV-rtTA/TetO-Wnt1/Apc\textsuperscript{Min} primary tumors were initiated via N-ethyl-N-nitrosourea (ENU) (described below) exposure 1 week after beginning dox treatment with the exception of lineage A and B (Figure 3), which were spontaneous after ~6 months dox treatment. Explants were performed under isoflurane-induced anesthesia when a mammary tumor reached 2 x 2 cm (measured using calipers). Approximately 1/3 of the primary tumor was harvested and divided into ~2mm x 2mm fragments. Two fragments were used for biopsy histopathology and sequencing analysis. The rest were inserted via a small incision onto both flanks of a number of on-dox, female F1 FVB/N-C57BL/6J (FVB/B6) host mice. Incisions were closed using surgical clips. All explant tumors were grown on dox until reaching 2 cm (measured using calipers). Approximately 1/3 of the primary tumor was harvested and divided into ~2mm x 2mm fragments. Two fragments were used for biopsy histopathology and sequencing analysis. The rest were inserted via a small incision onto both flanks of a number of on-dox, female F1 FVB/N-C57BL/6J (FVB/B6) host mice. Incisions were closed using surgical clips. All explant tumors were grown on dox until reaching 2 x 2 cm (~30-40 days). One mouse was taken for another generation of explants, and the remaining were removed from dox and monitored twice weekly for regression and relapse. The process was repeated over indicated generations. Time-to-relapse was noted as time between dox withdrawal and first post-withdrawal growth. When relapses reached 2 x 2 cm, explant hosts were necropsied, and relapses were cut using a razorblade into 18 segments. Each segment was stored separately at ~80 C until sequencing.

DNA Preparation and Sanger Sequencing

Promega Maxwell 16 Tissue DNA Purification Kit (Promega AS1030) was used to isolate genomic DNA from tail snips and tumor fragments. Primers specific for Apc, \(\beta\)-catenin, and rtTA were used for PCR amplification. Apc primers listed in STAR Methods, \(\beta\)-catenin: 5’-

| GCGTGGACATGGCTACTCAA, 5’- GCCGTCAAAACTGCGTGGATGG, rtTA: 5’- GCCCAAGAAGCTAGGTGTAG, 5’- CGAATAAGAA GGCTGGCTCTGC. | PCR products were purified using a Qiagen PCR purification kit (28104) or Affimetrix ExoSAP-IT (P/N: 75001). Purified products were Sanger sequenced at Genewiz LLC, South Plainfield, NJ, USA. |

Chemical Carcinogenesis

N-methyl-N-nitrosourea (MNU) or N-ethyl-N-nitrosourea (ENU) were given via intraperitoneal (i.p.) injection (150mg/kg) at 7 weeks of age for select primary mice. For explants, injections were administered when tumors reached 1.5 x 1.5 cm. MNU (Sigma Aldrich N4766) was dissolved in 10mg/mL 0.9% saline immediately prior to injection, and 1g ENU (Sigma Aldrich N3385) was dissolved in 10 mL 95% ethanol and 90mL phosphocitrate buffer (Sigma Aldrich P4809) immediately prior to injection.

High throughput sequencing

An Apc\textsuperscript{Cre/Cre} PCR product was amplified using primers designed to add the following adapters. 5’-TCGTCGGCAGCGTCAGATGTG TATAAGA GACAGACAGAAAGTACTCCAGACGGG-3’, and 5’-GTCTCGTGGGCTCGAGATGTG TATAAGA GACAGACAGAAAGTACTCCAGACGGG-3’. Followed Illumina 16S Metagenomic Sequencing Library Preparation Protocol for clean-up, quantification and normalization. Indices were added using Nextera XT index kit # FC-131-1001. Samples were sequenced using the Illumina Miseq platform, 250 x 250 paired end. Targeted deep sequencing of the amplicon resulted in an average of 287,000 reads per base pair between Apc codons 1492 and 1580. Reads were converted to percentages by dividing the number of reads per base pair
at each site by the total number of reads at each site. For example, if a specific site with reference G produced the following: A: 1450 reads, T: 6000 reads G: 242,430 reads, C: 120 reads, it was converted to: A: 0.58%, T: 2.4%, G: 97.0%, C: 0.05%. Next, we estimated error at each site by analyzing the read percentages from the average of two samples known to harbor no mutations, and subtracted that percentage from the experimental samples. Thus, supposing the "no mutations" sample at the above hypothetical G site read the following: A: 0.56%, T: 0.64%, G: 98.73%, C: 0.07%, we subtracted those percentages from the experimental sample to estimate the true variant allele frequency as: A: 0.02%, T: 1.76%, G: −1.73%, C: −0.02%. The hypothetical data here indicate a G > T variant in 1.76% of the sample. In this way, we were able to detect mutants with a mutant allele fraction > 0.3%. Ability to detect these low frequency mutants was corroborated by the fact that the mutants identified were nearly always nonsense mutations and consistent with the known ENU mutation pattern.

**Quantitative Real Time PCR**

RNA was purified from mammary glands or tumor fragments using Promega Maxwell 16 Tissue RNA Purification Kit (Promega AS1050) and reverse transcribed using Invitrogen Superscript First Strand Synthesis kit (15080051). We used Taqman Gene Expression Assay Mix (Agilent, Cat#600880) to measure expression of Wnt1 (Applied Biosystems, Mm_01300555_g1). The mix contained unlabeled PCR primers and FAM-labeled probes. Relative quantification PCR (ΔΔCt method) was performed with duplicate reactions using Agilent Technologies Mx3005P detection system and analyzed using Stratagene MxPro Software. Expression levels of Wnt1 were normalized to Gapdh transcript levels (Biosystems 435239E).

**Bacterial subcloning**

PCR product was purified using a Qiaquick PCR purification kit (28104) and subcloned using One Shot TOP 10 chemically competent cells (Invitrogen C404004) and TOPO XL PCR Cloning Kit (Invitrogen 45-0008LT). Multiple individual colonies positive for the insert were harvested and lysed using 10μl water. Second PCR amplification from plasmid DNA was done using the same primers above and purified again. Products were sequenced at Genewiz LLC, South Plainfield, NJ, USA.

**Cell suspensions and tumor reconstitution**

A tumor relapse (composed of R1 and R2 cell populations), as well as the parental iWnt/ApcMin tumor were made to single cell suspensions. First, ~10×10⁶ fragments of mammary tumor tissue were finely chopped and scraped into a sterile tube. To each, we added 1mL 10X collagenase (diluted in 1X PBS, 63.29mg/5mL) (Sigma C9891), 1mL Hyaluronidase (diluted in 1X PBS, 44.36mg/10mL) (Sigma H3506), and 8 mL DMEM/F12 media. Tissue was incubated for 1 hour at 37°C while slowly shaking, then centrifuged at 550×g for 5 minutes. The pellet was re-suspend in 7μL 0.25% Trypsin-EDTA (GIBCO 15000-054) and shaken vigorously by hand for 1-3 minutes until tissue chunks were broken. Next, the tissue was centrifuged again and the supernatant removed. The pellet was re-suspended in 2mL pre-warmed, sterile-filtered 1X Dispase / Neutral Protease (10mg/mL) (Roche 0492078001) + 0.4 mL 10X DNase (Worthington DNase-DPRF LS006331) + 1.6 mL 1X PBS and shaken by hand for 1-3 minutes. Cells were filtered into 5mL tubes with 40μm filter caps (Falcon cat#352235) and then centrifuged for 5 minutes at 550×g. Finally, pellet was re-suspend in 0.64% NHCl, incubated at room temperature for 3 minutes, then centrifuged at 550×g for 5 minutes and re-suspend in 1X PBS. Cells were counted a hemocytometer, and re-suspend in 50% PBS, 50% Matrigel (BD Biosciences Growth Factor Reduced Matrigel Mix, Cat#356231) at 1,000 cells/μL for mammary gland injections. Mammary glands were exposed via surgical incision, and 10⁴ cells in 100μL Matrigel/PBS were injected into intact 3 or 4 mammary fat pads of on-dox, host FVB/B6 female mice. In mixed population injections, cells were injected in a 2:1 admixture (R1:R2 or P:R2, respectively). Growth occurred synchronously and tumors were harvested at 31 days post injection, when multiregion sequencing was feasible for all tumors.

**Tamoxifen**

Tamoxifen (Sigma T5648-1G) was dissolved in corn oil (Sigma C8267) at 20mg/mL. 3 mg was injected intraperitoneally for Cre-mediated recombination.

**Confocal Imaging**

Snap frozen sections of tumors (mammary or intestine) (~20μm) were cut and mounted on slides. DRAQ5 (Cell Signaling 4084S) was used as a counterstain for far-red fluorescence. Slides were visualized using a Leica SP8 Inverted Confocal Microscope and analyzed using IMARIS Floresent imaging processing software.

**Immunohistochemistry**

Mammary glands, mammary tumors, and intestinal tumors were fixed with 4% paraformaldehyde for at least 2 hours, imbedded in paraffin, and sectioned. Samples were heated to 65°C for 20 minutes in PBS and cooled for 20 minutes at room temperature. They were cleared with 3x 5 minutes incubation in xylene and rehydrated with 2x 1 minute 100% ethanol, 2x 2 minutes 95% ethanol, and 1x 1 minute 70% ethanol, then placed in PBS for 10 minutes. Antigen retrieval was accomplished via incubation in Sodium Citrate Buffer pH 6.0 for 1 hour at 80°C. Samples were then cooled for 10 minutes prior to being washed in PBS at room temperature while slowly shaking. The DAKO envision ARK kit (#3954) was utilized for β-catenin immunohistochemistry. Briefly, endogenous peroxidase activity was quenched for 5 minutes at room temperature with peroxidase block. The primary antibody (mouse anti-β-catenin, BD
Transduction Laboratories, 610153) was conjugated to a biotinylated secondary antibody for 15 minutes at room temperature. Then, the blocking reagent containing mouse serum was added to bind residual Biotinylation Reagent. The mixture containing the primary antibody, biotinylated secondary antibody, and mouse serum was diluted to a final primary antibody concentration of 1:50 with PBS 0.1% Triton X and incubated overnight at 4C in the dark. The following day, samples were washed for 10 minutes in PBS and incubated with streptavidin peroxidase for 15 minutes at room temperature then washed for 10 minutes in PBS and developed with 3’3’-diaminobenidine (DAB) + substrate-chromagen for 10 minutes at room temperature. Finally, samples were washed in water for 5 minutes, 95% ethanol 2x 2 minutes, 100% ethanol 2x 2 minutes, and xylene 3x 1 minutes. Samples were then coverslipped and visualized.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Student’s t test and Log Rank Test were performed using GraphPad Prism 6.04. Fisher’s Exact Probability Tests (2x2 and 2x3 tables) were performed using VassarStats: Website for Statistical Computation. [http://vassarstats.net/](http://vassarstats.net/). n values are reported in figure legends.
Supplemental Information

Evolution of Relapse-Proficient Subclones
Constrained by Collateral Sensitivity to Oncogene Overdose in Wnt-Driven Mammary Cancer

Ross R. Keller and Edward J. Gunther
Fig S1. iWnt/Apc\textsuperscript{Min/+} mammary tumors retain the germline Apc\textsuperscript{wild-type} allele. Related to Figure 1. Tumor-derived genomic DNA samples derived from mammary tumors (A) and intestinal tumors (B) were analyzed by Sanger sequencing of PCR-amplified Apc alleles. Chromatogram segments depict codon 850 sequence from individual tumors. The asterisk (*) denotes a mammary tumor relapse that uniquely shows Apc LOH. By contrast, Apc LOH was detected in most intestinal tumors. Lower panels depict intestinal tumor chromatograms ordered by their degree of Apc LOH, from prominent LOH (left) to undetectable LOH (right).
Fig S2. Submaximal Wnt pathway activation in mammary tumors revealed by β-cat immunohistochemistry. Related to Figures 1, 2, and 5. A. Representative images from Apc\textsuperscript{Min/+} intestine. Nuclear-localized β-cat immunostaining is largely confined to the crypt base in normal intestine, whereas almost all epithelial tumor cells show strong, nuclear-localized β-cat staining. B. Representative images from mammary tissue and tumors. Panels include wider fields of view for the IHC depicted in Fig. 1E, as well as β-cat immunostaining performed on additional representative relapsed mammary tumors. Primary Wnt-driven mammary tumors, as well as descendant relapses arising from a variety of rescue mutations are shown to converge on submaximal β-cat immunostaining (compared against that observed in the context of Apc LOH). Representative specimens include a relapse carrying an activating β-catenin mutation, two independent relapses harboring second-hit Apc\textsuperscript{mmcr} rescue mutations (descended from a “sensitized” iWnt/Apc\textsuperscript{Min/+} tumor, culminating in an Apc\textsuperscript{Min/mmcr} tumor cell genotype), and a relapse carrying biallelic, somatic Apc rescue mutations (descended from an “unsensitized” iWnt/Apc\textsuperscript{+/+} tumor, culminating in an Apc\textsuperscript{Min-like/mmcr} tumor cell genotype; see Fig. 5 for further details).
Fig. S3. Rescue mutation analysis of the $Apc^{mmcr}$ region by HTS. Related to Figure 2. To detect rescue mutations within segments from iWnt/$Apc^{Min/+}$ relapsed tumors, template gDNA was prepared from tumor regions as indicated. PCR-amplified products containing the $Apc^{mmcr}$ region then were subjected to both Sanger sequencing and HTS. Subclone maps are depicted linked to relevant region-specific DNA sequencing data. Boxes rendered in the same color mark segments bearing an identical rescue mutation, which presumably indicates repeat sampling of the same RSC. HTS analysis of the upper left segment identified the 1535 G>T rescue mutation detected by Sanger with read counts indicating a high mutant allele fraction (MAF) of 45%. HTS identified no additional mutant alleles, consistent with this segment being predominantly monoclonal. By contrast, whereas Sanger sequencing of the upper right segment detected only a 1538 C>A rescue mutation, HTS analysis detected not only this mutation (44% MAF), but also a minority 1535 G>T allele (1.2% MAF), which likely reflects invading tumor cells from the neighboring subclone. The 1535 G>T allele also was detected by Sanger in all three lung metastases present at relapse. HTS analysis of a representative metastasis detected the 1535 G>T allele, but no additional mutant alleles, consistent with monophyletic seeding of distant disease. Photomicrograph at lower right shows gross appearance of the lung metastases (indicated by arrows) identified at necropsy. L, lung; H, heart.
Fig. S4. Highly polyclonal tumor relapse segments detected by HTS analysis of the \textit{Apc}^{mmcr}. Related to Figure 4.

A subclone map for a representative ENU\textsubscript{(d-2)} relapse is depicted at left. Colored boxes mark segments harboring rescue mutations detected by Sanger sequencing of the \textit{Apc}^{mmcr}. Hatched boxes mark segments that appear \textit{Apc}^{mmcr} wild-type by Sanger. Blow-ups show relevant portions of HTS read count data and Sanger chromatograms for each indicated segment.

For the blue segment (upper box), both Sanger and HTS sequencing detected only a single mutant allele present at a high MAF, consistent with sampling of a predominantly monoclonal RSC. For the lower segment (hatched box), no mutant alleles were detected by Sanger, whereas 8 distinct nonsense rescue mutations were detected by HTS (MAFs ranging from 0.8-2.1%), consistent with sampling of a polyclonal admixture of RSCs.
**A**
- Sanger Sequencing
- Illumina NGS Sequencing
- Subcloning, Sanger Sequencing
- Putative Polyclonal Segment
  (Wild-type by Sanger Sequencing)

**B**
- Primary Tumors
  - Biopsy Samples
    - Wnt On
  - Sanger: wt \( \rightarrow \) wt
  - NGS: wt \( \rightarrow \) wt

**C** Day -2 ENU

**MRS Analysis of Tumor Sub-Segments**
1. 1540 C>T
2. 1543 C>T
3. 1528 C>T
4. 1516 C>T
5. 1539 G>T
6. 1562 T>G
7. 1573 T>A
8. 1538 C>A
9. 1521 T>A
10. 1544 A>T
11. 1576 A>T
12. 1554 A>T
13. 1561 T>G
14. 1550 A>T
15. 1561 T>A
16. 1570 G>T
17. 1521 T>G
18. 1533 A>A
19. 1537 G>T
20. 1546 T, 0.01bp

**D** Day -7 ENU

**Estimated 16 Additional RSCs**
1. 1516 C>T
2. 1579 C>A
3. 1568 G>G
4. 1562 T>A
5. 1599 A>T
6. 1544 C>A
7. 1573 T>A
8. 1529 G>T
9. 1512 G>G
10. 1561 T>A
11. 1576 A>T
12. 1521 T>A
13. 1562 T>G
14. 1558 C>T
15. 1540 C>T

**Estimated 12 Additional RSCs**
1. 1540 C>T
2. 1539 C>A
3. 1530 C>A
4. 1535 G>T
5. 1521 T>A
6. 1535 A>A
7. 1537 G>T
8. 1521 T>A
9. 1539 G>T
10. 1550 A>T
11. 1537 G>T
12. 1550 A>T
13. 1562 T>G
14. 1522 A>T
15. 1546 A>A

**Estimated 18 Additional RSCs**
1. 1579 C>A
2. 1576 T>A
3. 1516 C>T
4. 1538 C>A
5. 1521 T>A
6. 1535 G>T
7. 1573 T>A
8. 1550 A>T
9. 1544 C>A
10. 1538 C>A
11. 1561 T>A
12. 1546 C>T
13. 1571 T>G
14. 1501 T>A

**E** Day -7 ENU; Interrupted Wnt Withdrawal

**Estimated 16 Additional RSCs**
1. 1538 C>A
2. 1528 C>T
3. 1516 C>T
4. 1521 T>A
5. 1517 A>T
6. 1546 G>T
7. 1573 T>A
8. 1550 A>T
9. 1544 C>A
10. 1538 C>A
11. 1561 T>A
12. 1537 G>T
13. 1540 D>T

**Estimated 12 Additional RSCs**
1. 1540 C>T
2. 1539 C>A
3. 1530 C>A
4. 1535 G>T
5. 1521 T>A
6. 1535 A>A
7. 1537 G>T
8. 1521 T>A
9. 1539 G>T
10. 1550 A>T
11. 1537 G>T
12. 1550 A>T
13. 1562 T>G
14. 1522 A>T
15. 1546 A>A

**Estimated 18 Additional RSCs**
1. 1579 C>A
2. 1576 T>A
3. 1516 C>T
4. 1538 C>A
5. 1521 T>A
6. 1535 G>T
7. 1573 T>A
8. 1550 A>T
9. 1544 C>A
10. 1538 C>A
11. 1561 T>A
12. 1546 C>T
13. 1571 T>G
14. 1501 T>A

1. 1538 C>A
2. 1528 C>T
3. 1516 C>T
4. 1521 T>A
5. 1517 A>T
6. 1546 G>T
7. 1573 T>A
8. 1550 A>T
9. 1544 C>A
10. 1538 C>A
11. 1561 T>A
12. 1537 G>T
13. 1540 D>T

1. 1538 C>A
2. 1528 C>T
3. 1516 C>T
4. 1521 T>A
5. 1517 A>T
6. 1546 G>T
7. 1573 T>A
8. 1550 A>T
9. 1544 C>A
10. 1538 C>A
11. 1561 T>A
12. 1537 G>T
13. 1540 D>T

1. 1538 C>A
2. 1528 C>T
3. 1516 C>T
4. 1521 T>A
5. 1517 A>T
6. 1546 G>T
7. 1573 T>A
8. 1550 A>T
9. 1544 C>A
10. 1538 C>A
11. 1561 T>A
12. 1537 G>T
13. 1540 D>T

1. 1538 C>A
2. 1528 C>T
3. 1516 C>T
4. 1521 T>A
5. 1517 A>T
6. 1546 G>T
7. 1573 T>A
8. 1550 A>T
9. 1544 C>A
10. 1538 C>A
11. 1561 T>A
12. 1537 G>T
13. 1540 D>T
**Fig S5. Subclonal architecture of iWnt/Apc<sup>Min/+</sup> relapses. Related to Figure 4.**

A. Key. b. Representative subclone maps for unexposed (mutagen-naïve) primary tumors and relapses (from Figure 3B). When these tumor segments were subjected to HTS, no additional mutations beyond those identified by Sanger sequencing were detected, consistent with these relapses deriving from only a few RSCs C, D, E. Subclone maps for mutagen-exposed relapses. For ENU<sub>day-2</sub> relapses, segments found to be Apc<sup>mmcr</sup> wild-type by Sanger sequencing routinely were polyclonal when assessed by more sensitive mutation detection methods. For example, when tumor segments were themselves subjected to MRS (left-most panels of C), Sanger sequencing of sub-segments yielded numerous additional rescue mutations. Likewise, both NGS analysis and Sanger sequencing of subcloned alleles permitted detection of Apc<sup>mmcr</sup> mutant subclones that eluded detection by standard Sanger sequencing. When estimating total RSCs-per-relapse, we made a conservative estimate that each uninterrogated Apc<sup>mmcr</sup> wild-type segment from the ENU<sub>day-2</sub> condition harbors two additional RSCs. Therefore, the actual RSCs-per-relapse numbers for ENU<sub>day-2</sub> likely exceed those reported in Fig. 4B.
Fig. S6. Biallelic Apc mutations in ENU-exposed iWnt/Apc\textsuperscript{+/+} relapses. Related to Figures 4 and 5. A. Subclonal architecture of ENU-exposed relapses 8, 9, 10, and 11 from Fig. 5B. Each relapse is comprised of two RSCs. Each individual RSC occupies a contiguous area and harbors a distinct pair of Apc\textsuperscript{Min-like} and Apc\textsuperscript{mmcr} mutations, which invariably co-occur. B. Mutation spectra for upstream Apc\textsuperscript{Min-like} and downstream Apc\textsuperscript{mmcr} hits. Biallelic rescue mutations from iWnt/Apc\textsuperscript{+/+} relapses were used to generate mutation spectra for each hit. The mutation spectrum derived from unexposed relapses reported in Figure 4C is included here for comparison. Evidence for an ENU mutation signature is evident for both Apc hits (compare with Figure 4C), suggesting both mutations are ENU-induced.
Fig. S7. Dropout of $Apc^\text{Min/CKO-mmcr}$ mammary tumor clones after Cre-mediated conversion to $Apc^\text{Min/\Delta580}$. Related to Figure 7. A. Transgenes enabling 4-color Confetti lineage tracing. B. Schematic of Confetti lineage tracing of mammary epithelial cells (MECs). C. Confetti-based lineage tracing in the normal mammary gland. Representative confocal microscopy images of mammary gland sections at various trace time points, measured from the time of Tam exposure. Scale bar, 50 µm. D. Strategy for PCR-based detection of unrecombined $Apc^{\text{CKO}}$ and recombined $Apc^{\Delta580}$ alleles in tumor tissue. Schematic depicts $Apc$ alleles and approximate sites of primer (red arrows) annealing as well as predicted PCR product sizes, as indicated. Primers flanks exon 14 in wild-type, $Min$ or mmcr alleles. For unrecombined $Apc^{\text{CKO}}$ alleles, the amplified product includes two LoxP sites (and one FRT site, not shown), making it the largest. Upon recombination, exon 14 is excised, generating the $\Delta580$ truncation mutation via a frame-shift. E. PCR analysis of $Apc$ alleles. Left four lanes are control samples with germline $Apc$ genotypes as indicated. Tumors that arose in an UBC-Cre$_{\text{ER}}^{\text{ERT}}$/Apc$^{\text{CKO}+}$ mouse serve as positive controls for detecting the recombined $Apc^{\Delta580}$ allele (lanes 3 and 4). Treatment of this mouse with Tam followed by ENU generated a mammary tumor (MT) and a liver tumor (LT), whose onset presumably depended on $Apc^{\text{CKO}}$ to $Apc^{\Delta580}$ conversion. For lanes 5-8, gDNA templates derive from four representative mammary tumor samples whose analysis is reported in the lineage tracing experiment shown in Fig. 7. Note that the PCR product corresponding to $Apc^{\Delta580}$ becomes undetectable between 2 and 7 days after initiating the trace with Tam, which coincides with dropout of Confetti labeled cells evident by confocal microscopy. NTC, no template control.