Variable Clonal Repopulation Dynamics Influence Chemotherapy Response in Colorectal Cancer

Antonija Kreso,[1,2]* Catherine A. O'Brien,[3]* Peter van Galen,[1] Olga Gan,[1] Faiyaz Notta,[1,2] Andrew M. K. Brown,[4] Karen Ng,[4] Jing Ma,[5] Erno Wienholds,[1] Cyrille Dunant,[6] Aaron Pollett,[7] Steven Gallinger,[8] John McPherson,[4] Charles G. Mullighan,[5] Darryl Shibata,[9] John E. Dick[1,2]†

[1]Campbell Family Institute, Ontario Cancer Institute, Princess Margaret Cancer Centre, University Health Network, Toronto, Ontario M5G 1L7, Canada. [2]Department of Molecular Genetics, University of Toronto, Toronto, Ontario M5G 1L7, Canada. [3]Department of Laboratory Medicine and Pathobiology and Department of Surgery, University of Toronto, Toronto, Ontario M5L 1F4, Canada. [4]Ontario Institute for Cancer Research, Toronto, Ontario M5G 1L7, Canada. [5]St. Jude Childrens Hospital, Memphis, TN 38105, USA. [6]Department of Civil Engineering, University of Toronto, Toronto, Ontario M5S 1A4, Canada. [7]Deparment of Pathology, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada. [8]Fred Litwin Centre for Cancer Genetics Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario M7H 2B9, Canada. [9]University of Southern California Keck School of Medicine, Los Angeles, CA 90089, USA.

*These authors contributed equally to this work.

†To whom correspondence should be addressed. E-mail: jdick@uhnres.utoronto.ca

Intratumoral heterogeneity arises through evolution of genetically diverse subclones during tumor progression. However, whether cells within single genetic clones are functionally equivalent remains unknown. By combining DNA copy number alteration (CNA) profiling, sequencing, and lentiviral lineage tracking, we followed the repopulation dynamics of 150 single lentivirus-marked lineages from ten human colorectal cancers through serial xenograft passages in mice. CNA and mutational analysis distinguished individual clones, and showed that clones remained stable on serial transplantation. Despite this stability, the proliferation, persistence, and chemotherapy tolerance of lentivirally marked lineages were variable within each clone. Chemotherapy promoted dominance of previously minor or dormant lineages. Thus, apart from genetic diversity, tumor cells display inherent functional variability in tumor propagation potential, a mechanism that contributes both to cancer growth and therapy tolerance.

Cancer is sustained by production of aberrant cells that vary in many morphological and physiological properties. This cellular diversity remains a major challenge to our understanding of the neoplastic process and therapeutic resistance. Genetic and non-genetic processes can generate heterogeneity; however, the degree of coordination between these mechanisms and their relative contribution to tumor propagation remains unresolved.

Tumor cell diversity can arise through accrued genetic changes (1) resulting in single tumors composed of many subclones that develop through complex evolutionary trajectories (2, 3). As well, tumors contain genetic subclones that vary with respect to differential growth in xenograft assays (4–6), recurrence (7), and metastatic potential (8, 9). Likewise, resistance to cancer therapies can arise through genetic mutations (10, 11). These and other studies substantiate the widely accepted view that tumors comprise genetically diverse subclones, some of which survive therapy and contribute to disease recurrence.

In the absence of differences at the level of genetic mutation, heterogeneity within a population of tumor cells can still exist, but the mechanisms remain incompletely understood. For example, the bidirectional interaction between tumor cells and the microenvironment can influence tumor phenotype (12). Other processes have also been proposed, including inter-convertible activation of Rac and Rho GTPases (13), metastable configurations of intracellular networks (14, 15), and altered epigenetic states (16). These studies collectively indicate that in apparently homogeneous in vitro environments, cells of the same genotype can exist in different states that influence their behavior (17). The detection of in vitro cellular diversity, which is not coupled to genetic diversity, underscores the need to investigate the extent of intraclonal functional heterogeneity in vivo and in primary human cancers.

Arguably the most important function of any cancer clone is to maintain long-term tumor propagation. For many tumors only a minority of cells are able to sustain tumor growth, although such cells can comprise the majority of tumor cells for some cancer types (18, 19). Whether these cells are part of one genetic clone or are derived from distinct genetic subclones remains to be determined. Moreover, whether tumor propagating cells of a single genetic clone are all equivalent or whether there is functional variability amongst individual tumor cells is uncertain. Addressing these questions requires genetic analyses combined with functional assays that measure tumor propagation at the resolution of individual clones derived from single cells.

Clonal stability is maintained through serial tumor transplantation. To explore the relative contribution of genetic and non-genetic mechanisms to the functional heterogeneity of single human cancer cells that are capable of long-term clonal propagation, we used an in vivo xeno-transplantation assay. The fates of single cell derived lineages were tracked from 10 primary human colorectal cancers (CRCs, table S1). To facilitate clonal tracking, we transduced patient-derived CRC cells with a green fluorescent protein (GFP)-expressing lentivirus and injected the cells into the renal capsule of immunodeficient mice (supplementary text and tables S2 and S3). Transduced cells efficiently generated xenografts, and GFP expression in the xenografts remained stable over serial transplantation (figs. S1 and S2). The average time to palpable tumor formation (99 ± 18 days) was stable over serial transplants (Fig. 1A) and xenografts maintained patient tumor characteristics (figs. S3 to S5). Genomic profiling of three patient tumors and corresponding xenografts using copy number alteration (CNA) analysis (Fig. 1B, figs. S6 and S7, and table S4) and targeted deep sequencing of 660 mutational hotspots in 42 genes (Fig. 1C and tables S5 to S7) established that xenografts largely retained the genomic profile of the primary tumor sample. Several CNAs and single nuclear variants (SNVs) were enriched in 1° xenografts from some samples, consistent with selection of a subclone from the patient tumor. Genomic analysis of subsequent xenograft transplants, encompassing 393 days (CT38), 341 days (CT54), and 261 days (CT59) of total tumor growth, demonstrated that the majority of genomic lesions detected in 1° xenografts were recapitulated upon serial passage (supplementary text). Exome sequencing of CT38 and its corresponding 1°, 2°, and 4° xenografts supported these findings (supplementary text, figs.

S8 and S9, and tables S8 to S11). Finally, analysis of methylation pattern diversity of patient tumors and their corresponding xenografts indicated that population diversity was maintained over serial passage (supplementary text and fig. S10). Collectively, these data indicate that the CRC clones that are selected in the xenograft remain stable over sequential transplantation.

Variation in clonal dynamics of CRC cells. To evaluate the repopulation kinetics of multiple single cell-derived clones within a primary human tumor, we used lentiviral marking to track the progeny of single CRC cells (henceforth termed 'LV clones' to distinguish them from genetic clones) over serial xenografts. To facilitate qualitative and quantitative characterization, we developed a system for establishing clonal identity and then classifying clonal behavior based on persistence, absence, or emergence across multiple recipients (materials and methods). Importantly, our detection strategy was not PCR-based, but focused on the identification of LV clones that possessed robust clonal expansion capacity. We observed five distinct behaviors. LV clones that were present in all serial transplants were termed Type I or persistent clones (Fig. 2A, purple arrowhead). LV clones that did not persist but exhausted before reaching the final passage had less potent tumor propagating ability compared to Type I clones and were termed Type II or short-term clones (Fig. 2A, blue arrowhead). Finally, we observed LV clones that lacked tumor propagating ability because they were only detected in the 1° recipient and were not detected in 2° and subsequent recipients (Fig. 2A, green arrowhead); these were termed Type III or transient clones. In ten patient samples, out of a total of 150 marked clones, we tracked 34 Type I, 33 Type II, and 31 Type III clones (Fig. 2B), suggesting that there is substantial functional diversity with respect to clonal longevity in successive tumor transplants.

In addition to heterogeneity in longevity, we also observed dynamic behaviors in 34 of the 150 marked clones. These LV clones were initially below detection limit (approximately $<10^4$ cells/tumor) in the 1° recipients but could be identified at later transplants (Fig. 2A, red arrowhead). Since the xenograft assay monitors the output of 'active' CRC cells, these Type IV or resting LV clones were likely produced by CRC cells that initially were dormant or slowly proliferated but became activated in later transplants, resulting in the generation of a measurable clone. Finally, LV clones whose progeny appeared early, then became undetectable in a subsequent transplant, only to re-appear at a later time point (Fig. 2A, orange arrowhead) were termed Type V or fluctuating clones. Such LV clones displayed extensive, but intermittent proliferation, distinct from the continuous rapid proliferation of Type I clones; we observed 18 examples of Type V clones (Fig. 2B). Overall, these results demonstrate that not all CRC cells with the potential for tumor propagation actually function and contribute to tumor growth at any given time. Such cells can become activated at later time points.

It is noteworthy that LV clones can remain undetected for two to four months while being diluted over 100-fold during consecutive transplants and then recur to dominate tumor growth (supplementary text and fig. S11). Whereas random growth dynamics would predict dilution of minor LV clones over multiple transplants, the frequent detection of Type IV and V clones indicates that the behaviors we observe cannot be solely attributed to dilution over time or stochasticity in LV clonal behaviors. Further, mathematical modeling also predicted that LV clonal emergence correlates with changes in tumor structure and that newly appearing clones are functionally distinct from active clones, although it did raise uncertainty as to whether Type I, II, and III are distinct or whether stochastic processes related to the transplantation method may contribute to clonal loss (supplementary text and fig. S12). The distinct proliferative kinetics of the five LV clonal behaviors we observed underscore the functional variability of individual cells (Fig. 2C). Each LV clone type was identified in four or more patient samples (Fig. 2D and fig. S13), establishing that the varied clonal behaviors are reproducibly

found in primary CRC from a spectrum of patients. Given the absence of accompanying changes in CNAs and SNVs with serial transplantation, our data provide evidence for functional heterogeneity between individual tumor propagating cells that share a common genetic lineage.

The genetic analysis across xenografts was carried out on bulk tumor cell populations, where clonal marking tracked the functional behavior of single cells. The experimental design of genomically analyzing bulk tumor does not directly evaluate the genomic properties of each individual functionally distinct LV clone type that is present. To directly compare the genomes of distinct LV clone types from an individual tumor, we used a limiting dilution approach to isolate different LV clones and used deep sequencing of mutational hotspots to compare their mutational load. We compared the mutational hotspots between tumors generated by a Type I and Type V clone from CT38 and a Type I and Type II clone from CT59. For CT38, a linear correlation between the SNV frequencies of the Type I- and Type V-derived tumors was seen (fig. S14A and table S12). Likewise, for CT59, Type I- and Type II-derived tumors also demonstrated similar SNV frequencies in all analyzed SNVs, including the nine somatically acquired SNVs (fig. S14B and table S13). Thus, for both CT38 and CT59, the frequency of analyzed hotspot mutations was congruent between tumors generated by single LV clones, which were derived from distinct clone types (Type I and V for CT38 and Type I and II for CT59). Analyzing tumors generated from single cells enabled us to determine that distinct clone types within a tumor sample share similar mutational patterns as assessed by targeted sequencing. These data, together with the CNA and targeted sequencing analysis of the bulk tumor, provide further evidence for the existence of functionally distinct LV clones within a genetic lineage in CRC.

Variable response of LV clones to oxaliplatin. The existence of functionally heterogeneous CRC cells prompted us to investigate whether these cells might also intrinsically differ in response to therapy. We examined the effect of a commonly used chemotherapy drug, oxaliplatin, on the dynamics of LV clones. We utilized established xenografts of five patient samples for which the LV clone types had already been curated. In parallel with serial transplantation of untreated recipient mice described in the previous section, three to five additional mice were transplanted with tumor cells and systemically treated with oxaliplatin once tumors were established, allowing assessment of the effects of drug treatment on steady-state clonal distribution (fig. S15A). Although oxaliplatin treatment significantly reduced tumor burden (figs. S15B and S16A), there was no apparent change in the absolute number of marked clones (fig. S15C), nor were the proportions of clone types significantly altered (figs. S15D and S17).

To determine whether treatment with oxaliplatin affected CRC cells with tumor propagation capability, we serially transplanted equal numbers of viable cells from both the control and treatment groups into secondary mice that were left untreated. Across five patient samples, a total of 60 secondary recipients were transplanted with tumor cells from oxaliplatin-treated xenografts (31 control and 29 oxaliplatin-treated). We observed a reduction in tumor weight (Fig. 3A and fig. S16B) as well as a trend toward a decrease in the absolute number of clones (Fig. 3B) in untreated secondary recipients transplanted with cells from oxaliplatin-treated xenografts *versus* sham-treated control cells, suggesting that drug treatment altered the growth properties of the regrown tumor. For two samples (CT33 and CT57), GFP-expressing tumor tissue was not detected in the secondary recipients that were transplanted with oxaliplatin-treated cells, which precluded lentiviral insertion site analysis. For the remaining three patient samples (CT17, CT38, and CT54) major changes in the proportion of LV clone types occurred in xenografts derived from the oxaliplatin-treated group compared to control xenografts (Fig. 3C). The proportion of Type I persistent clones was significantly reduced, while LV clones that were below the detection limit in primary recipients appeared in secondary recipients transplanted with oxaliplatin-

treated tumors (Fig. 3D; Fig. 3E, arrowheads to the right). These new LV clones were classified as Type IV, since they were not consistently detected in control or oxaliplatin treated tumors in preceding mice. When considering the absolute numbers of different LV clone types over the complete set of secondary mice, 84 Type I clones were observed in mice transplanted with untreated control cells and 27 Type I clones in mice transplanted with oxaliplatin-treated cells (Fig. 3D). By contrast, 12 Type IV clones were detected in the control group *versus* 40 Type IV clones in the oxaliplatin-treated group. These data indicate that the response of individual CRC cells to standard chemotherapy is markedly variable. Despite eradication of some persistent LV clones, resting or slowly proliferating CRC cells can endure oxaliplatin treatment and re-initiate tumor growth, although such tumors are of smaller size. Interestingly, mathematical modeling predicted that the LV clonal landscape of post-treatment tumors was distinct compared to untreated samples (supplementary text and fig. S12).

To determine whether the altered clonal patterns after oxaliplatin treatment were due to major changes in genetic clones, DNA from control and oxaliplatin treated tumors was profiled by genome-wide CNA analysis (Fig. 3F), targeted deep sequencing (Fig. 3G), and passenger methylation analysis (fig. S10). The results indicated that the CNAs, SNVs, and methylation pattern diversity of the oxaliplatin treated group closely matched the control recipients (Supplementary Text). The absence of a detectable bottleneck or selection for novel genetic clones after chemotherapy treatment indicates that therapeutic tolerance is not always linked to the acquisition of new driver mutations. Instead, variable tumor propagation behavior of individual cells can represent a non-genetic determinant of tumor growth after therapy.

Discussion. Our findings establish that individual tumor cells within a uniform genetic lineage are functionally heterogeneous: they display extensive variation in growth dynamics, persistence through serial transplantation, and response to therapy. Not all functionally important cells contribute continuously to tumor growth; some are held in reserve, while others have the ability to oscillate between periods of dormancy and activity. This distinct intraclonal behavior affected response to conventional chemotherapy, as actively proliferating progeny were preferentially eliminated, while the relatively dormant CRC cells became dominant during tumor re-initiation after chemotherapy. The intraclonal diversity in single cell functional behavior of primary human CRC cells in vivo has the net effect of contributing to tumor growth during both homeostasis and therapy response.

Most tumors are expected to consist of genetically distinct subclones that contain different growth characteristics and will therefore read out differently in xenotransplantation assays (*1*). Similar to published work using CRC cells propagated in immunodeficient mice (*20*), we expanded and sequentially propagated CRC clones from different patient samples that remained stable upon serial transplantation, indicating that xenografting does not select for a significantly different tumor cell population between multiple recipients at each stage of serial transplant. Despite this stability, we observed reproducible differences in the functional fates of single marked CRC cells, indicating that in vivo dynamics of lentivirally tracked CRC clones are not driven by readily detectable genomic changes. This conclusion is supported by the genetic concordance between functionally distinct LV clone types within a single tumor isolated by limiting dilution. Thus, in addition to the widely accepted mechanism of tumor heterogeneity being driven by genetic diversity, other diversity generating processes exist within a genetic clone, which endow cells with robust survival potential, especially during stress. The contribution of diversity-generating mechanisms, such as epigenetic regulation, noise in gene expression or variability in the microenvironment (*21*, *22*), may shed insight into CRC cell heterogeneity.

There is growing evidence of evolutionary selection for diversity-generating mechanisms in other disciplines, such as ecology (*23*, *24*) and

microbiology (*25–27*). For example, genetically homogeneous pools of single-cell prokaryotes display heterogeneity, where a small portion of cells naturally display drug resistance that is not caused by genetic mutation or acquisition of plasmids encoding antibiotic resistance genes. Rather this phenomenon is due to mechanisms that reduce cell proliferation and induce a dormant non-dividing state (*28*). We consistently observe a relatively dormant cell population in CRC, suggesting that cancer cells may take advantage of this 'ancient' mechanism and use dormancy as an adaptive strategy during times of stress. We provide evidence for a relatively dormant or slowly proliferating cell population in primary human CRC cells that still retains potent tumor propagation potential, thereby preferentially driving tumor growth after chemotherapy. These findings may provide a biological basis for recurrent and metastatic disease following standard of care treatment (*29*). Our findings should focus efforts to uncover the molecular mechanisms driving chemotherapeutic tolerance in CRC cells.

The often unstated assumption in considering cellular response to stress is that cells react in a uniform manner to the inducing signal because the classically used techniques employed bulk populations. However data averaged across millions of cells masks any heterogeneity that might exist at the single-cell level. Such conventions are changing as methodological advances (*30*) are fueling a surge of interest in the processes governing cell-to-cell variability (*14*). By coupling genetic analysis to functional tumor growth assays, we find that when cells are tracked at single cell resolution while still being part of a population of genetically stable cancer cells, variable cellular behaviors can be detected. These observations set a precedent for future studies examining the basis of intraclonal behavior of single cells, especially with respect to tumor propagation and other functional properties. In a broader sense, our findings reveal another layer of complexity, beyond genetic diversity, that drives intratumoral heterogeneity of CRC. The prospect of understanding how genetic and non-genetic determinants interact to influence the functional diversity and therapy response for other cancers should drive future cancer research.

References and Notes
1. M. Greaves, C. C. Maley, Clonal evolution in cancer. *Nature* **481**, 306 (2012). doi:10.1038/nature10762 Medline
2. S. Nik-Zainal *et al*.; Breast Cancer Working Group of the International Cancer Genome Consortium, The life history of 21 breast cancers. *Cell* **149**, 994 (2012). doi:10.1016/j.cell.2012.04.023 Medline
3. M. Gerlinger *et al*., Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N. Engl. J. Med.* **366**, 883 (2012). doi:10.1056/NEJMoa1113205 Medline
4. K. Anderson *et al*., Genetic variegation of clonal architecture and propagating cells in leukaemia. *Nature* **469**, 356 (2011). doi:10.1038/nature09650 Medline
5. F. Notta *et al*., Evolution of human BCR-ABL1 lymphoblastic leukaemia-initiating cells. *Nature* **469**, 362 (2011). doi:10.1038/nature09733 Medline
6. E. Clappier *et al*., Clonal selection in xenografted human T cell acute lymphoblastic leukemia recapitulates gain of malignancy at relapse. *J. Exp. Med.* **208**, 653 (2011). doi:10.1084/jem.20110105 Medline
7. C. G. Mullighan *et al*., Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. *Science* **322**, 1377 (2008). doi:10.1126/science.1164266 Medline
8. X. Wu *et al*., Clonal selection drives genetic divergence of metastatic medulloblastoma. *Nature* **482**, 529 (2012). doi:10.1038/nature10825 Medline
9. W. Liu *et al*., Copy number analysis indicates monoclonal origin of lethal metastatic prostate cancer. *Nat. Med.* **15**, 559 (2009). doi:10.1038/nm.1944 Medline
10. M. E. Gorre *et al*., Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* **293**, 876 (2001). doi:10.1126/science.1062538 Medline
11. C. Roche-Lestienne, J. L. Laï, S. Darré, T. Facon, C. Preudhomme, A mutation conferring resistance to imatinib at the time of diagnosis of chronic myelogenous leukemia. *N. Engl. J. Med.* **348**, 2265 (2003). doi:10.1056/NEJMc035089 Medline

12. M. J. Bissell, M. A. Labarge, Context, tissue plasticity, and cancer: Are tumor stem cells also regulated by the microenvironment? *Cancer Cell* **7**, 17 (2005). doi:10.1016/S1535-6108(04)00375-7 Medline

13. V. Sanz-Moreno *et al*., Rac activation and inactivation control plasticity of tumor cell movement. *Cell* **135**, 510 (2008). doi:10.1016/j.cell.2008.09.043 Medline

14. S. L. Spencer, S. Gaudet, J. G. Albeck, J. M. Burke, P. K. Sorger, Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis. *Nature* **459**, 428 (2009). doi:10.1038/nature08012 Medline

15. A. Roesch *et al*., A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. *Cell* **141**, 583 (2010). doi:10.1016/j.cell.2010.04.020 Medline

16. S. V. Sharma *et al*., A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* **141**, 69 (2010). doi:10.1016/j.cell.2010.02.027 Medline

17. P. B. Gupta *et al*., Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. *Cell* **146**, 633 (2011). doi:10.1016/j.cell.2011.07.026 Medline

18. K. Ishizawa *et al*., Tumor-initiating cells are rare in many human tumors. *Cell Stem Cell* **7**, 279 (2010). doi:10.1016/j.stem.2010.08.009 Medline

19. P. N. Kelly, A. Dakic, J. M. Adams, S. L. Nutt, A. Strasser, Tumor growth need not be driven by rare cancer stem cells. *Science* **317**, 337 (2007). doi:10.1126/science.1142596 Medline

20. S. Jones *et al*., Comparative lesion sequencing provides insights into tumor evolution. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 4283 (2008). doi:10.1073/pnas.0712345105 Medline

21. A. Marusyk, V. Almendro, K. Polyak, Intra-tumour heterogeneity: A looking glass for cancer? *Nat. Rev. Cancer* **12**, 323 (2012). doi:10.1038/nrc3261 Medline

22. M. Kærn, T. C. Elston, W. J. Blake, J. J. Collins, Stochasticity in gene expression: From theories to phenotypes. *Nat. Rev. Genet.* **6**, 451 (2005). doi:10.1038/nrg1615 Medline

23. M. Loreau *et al*., Biodiversity and ecosystem functioning: Current knowledge and future challenges. *Science* **294**, 804 (2001). doi:10.1126/science.1064088 Medline

24. D. F. Flynn, N. Mirotchnick, M. Jain, M. I. Palmer, S. Naeem, Functional and phylogenetic diversity as predictors of biodiversity—ecosystem-function relationships. *Ecology* **92**, 1573 (2011). doi:10.1890/10-1245.1 Medline

25. H. B. Fraser, A. E. Hirsh, G. Giaever, J. Kumm, M. B. Eisen, Noise minimization in eukaryotic gene expression. *PLoS Biol.* **2**, e137 (2004). doi:10.1371/journal.pbio.0020137 Medline

26. H. L. True, S. L. Lindquist, A yeast prion provides a mechanism for genetic variation and phenotypic diversity. *Nature* **407**, 477 (2000). doi:10.1038/35035005 Medline

27. J. M. Raser, E. K. O'Shea, Control of stochasticity in eukaryotic gene expression. *Science* **304**, 1811 (2004). doi:10.1126/science.1098641 Medline

28. K. Lewis, Persister cells, dormancy and infectious disease. *Nat. Rev. Microbiol.* **5**, 48 (2007). doi:10.1038/nrmicro1557 Medline

29. A. M. Abulafi, N. S. Williams, Local recurrence of colorectal cancer: The problem, mechanisms, management and adjuvant therapy. *Br. J. Surg.* **81**, 7 (1994). doi:10.1002/bjs.1800810106 Medline

30. J. M. Raser, E. K. O'Shea, Noise in gene expression: Origins, consequences, and control. *Science* **309**, 2010 (2005). doi:10.1126/science.1105891 Medline

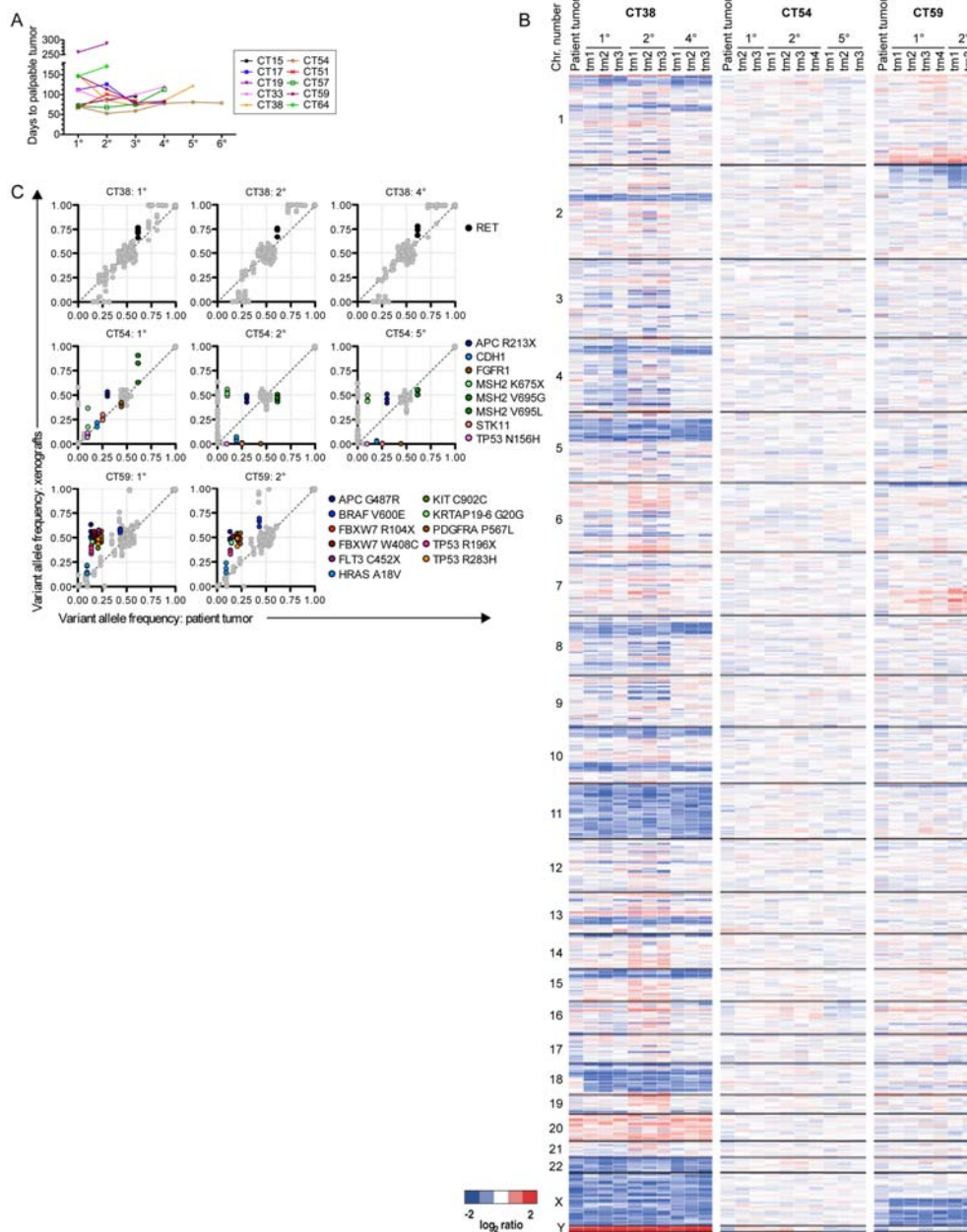**Fig. 1.** Xenograft characterization. (**A**) Diagnostic tumor samples were transduced with a lentiviral vector encoding GFP and 5×10^4 to 2×10^5 viable cells were transplanted into immunodeficient mice. Once tumors formed (1°), an equal number of cells was transplanted into the next passage. Time to tumor formation from previous injection for each transplant is shown. Each point is the mean of all recipients at the indicated passage. (**B**) DNA from diagnostic and matching 1°, 2°, 4°, and 5° tumor transplants was profiled using Affymetrix SNP 6.0 arrays. Raw $\log_2$ ratios are shown (median smoothing format; blue, deletion; white, normal; red, gain). (**C**) DNA from diagnostic and xenograft derived tumors was sequenced using RainDance platform. Frequency of both germline (gray circles) and somatic variants (colored circles) is shown. Each circle is data of one xenograft-derived tumor as compared to the patient tumor sample; there are 3 to 4 xenografts per passage. Data are not normalized for copy number changes and tumor cellularity, although generally >80% of cells are estimated to be tumor cells.
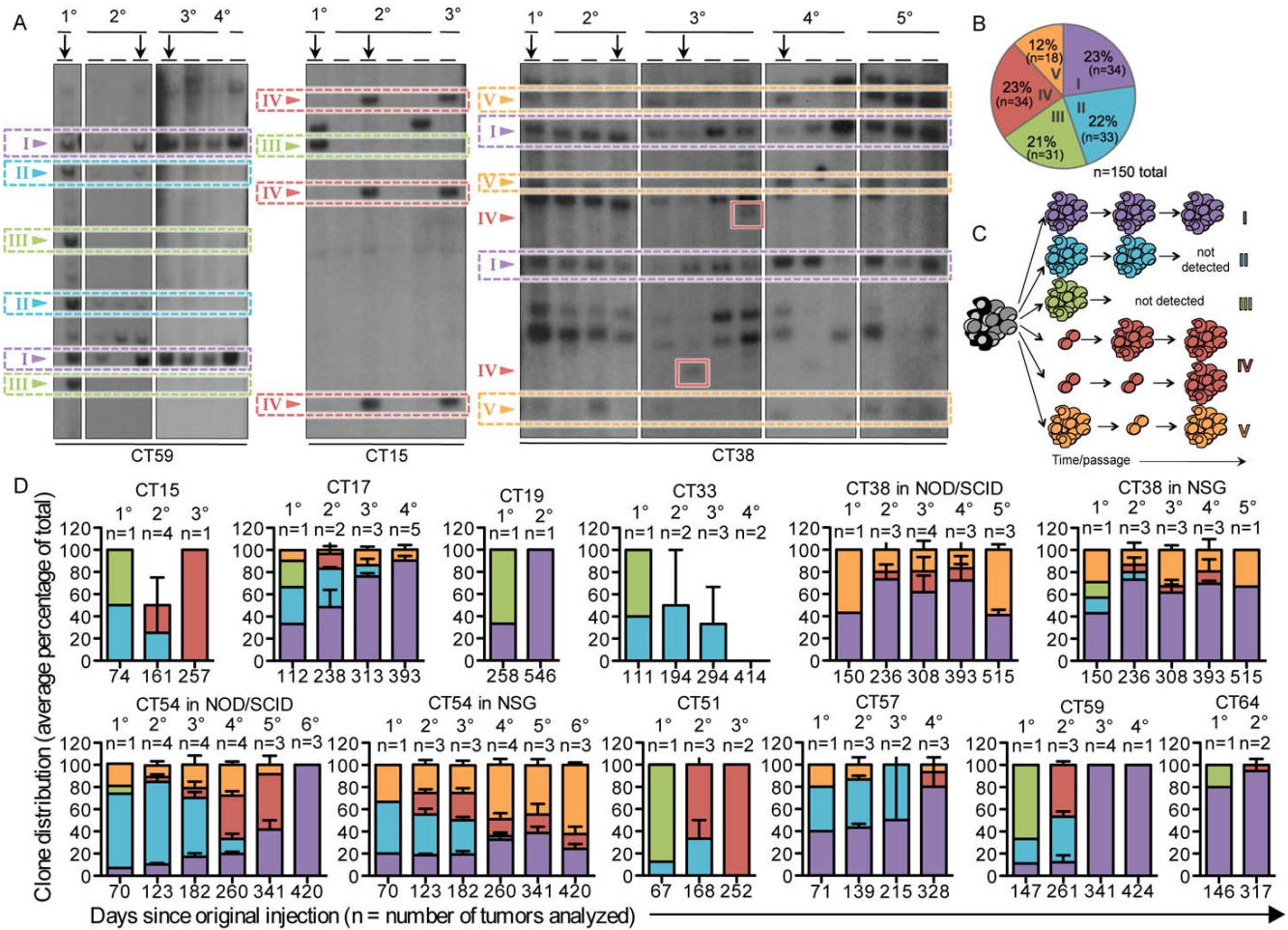
Fig. 2. Variation in repopulation potential of individual lentivirally-marked CRC cells. (**A**) DNA from xenografts (one tumor per lane) of various transplants (denoted by 1°, 2°…5°) was analyzed using Southern blotting with a GFP probe. Arrows above certain lanes indicate the tumor that was re-transplanted into the next set of mice. Colored arrowheads indicate representative examples of different lentivirally marked (LV) clone types. (**B**) Pie chart showing the sum of each of the five LV clone types observed over all experiments. (**C**) Schematic illustrating the different types of LV clonal behaviors. LV clones were classified on the basis of detection in serial transplants; for example, a Type IV clone would be below the detection limit initially and come up in later transplants. (**D**) Charts showing the proportion of LV clone types for each patient sample, displayed as the averages of all mice per transplant. The proportion of each LV clone type was determined in every recipient mouse by dividing the number of times a particular LV clone type was observed by the total number of LV clones detected in that mouse, thereby normalizing for differential marking of samples. Next, the proportion of each LV clone type for all recipients per transplant was averaged, including recipients for which LV clones were not detected. Each bar is representative of the averaged data at a transplant and error bars indicate SEM between the tumors; 'n' represents the number of tumors or recipients analyzed.
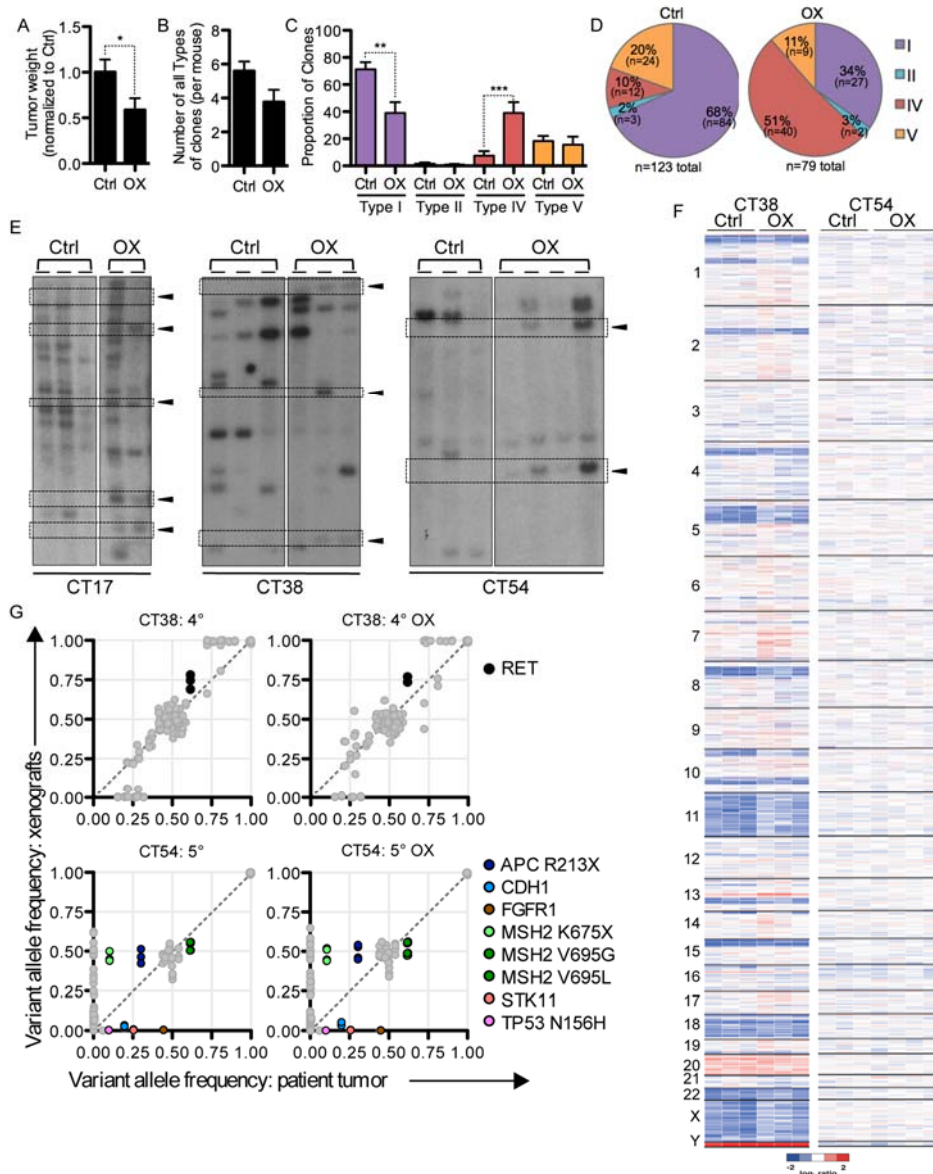
Fig. 3. Variable response of marked clones to oxaliplatin. Mice were either treated with PBS (Ctrl) or oxaliplatin (OX) for 2 to 4 weeks and cells from these tumors were re-injected into mice, which did not receive further treatment. Once new tumors formed (approximately 100 days following re-injection), mice from both groups were sacrificed and tumor weight was measured. (**A**) Previously OX-treated tumor weights were normalized to Ctrl tumor weights and data was pooled from five patient samples, representing 31 Ctrl and 29 previously OX-treated samples. (**B**) Cumulative number of LV clones per mouse following transplantation of OX-treated tumors. (**C**) The proportion of LV clone types in tumors that were generated by re-injecting Ctrl and OX-treated tumors. LV clone types were assigned based on the behavior of individual clones across all transplants over the entire experiment. (**D**) Pie chart showing the number of times the LV clone types were observed, represented as the sum of all experiments. (A-D) Mean±SEM is shown of pooled data from independent OX treatments using different patient samples; p-values were calculated using two-tailed t test. (**E**) Southern blot showing the LV clonal make-up of tumors generated by re-injecting tumor cells from OX-treated recipients. Representative data from three patient samples are shown; solid arrowheads to the right of each experiment identify newly appearing LV clones in the previously OX-treated tumors. Each lane represents the DNA of one mouse. For comparative purposes, the Southern blot for CT38 Ctrl is the same as in Fig. 2A. (**F** and **G**) DNA from tumors that were generated by re-injecting Ctrl or OX-treated samples was analyzed using CNA arrays (F) or targeted deep sequencing (G). (F) Raw $\log_2$ ratio copy number data are shown (median smoothing format; blue, deletion; white, normal; red, gain). (**F, G**) For comparative purposes, data for Ctrl are re-productions of 4° (CT38) and 5° (CT54) transplants from Fig. 1, B and C.