Heterogeneity of Myc expression in breast cancer exposes pharmacological vulnerabilities revealed through executable mechanistic modeling

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Cells with higher levels of Myc proliferate more rapidly and supercompetitively eliminate neighboring cells. Nonetheless, tumor cells in aggressive breast cancers typically exhibit significant and stable heterogeneity in their Myc levels, which correlates with refractoriness to therapy and poor prognosis. This suggests that Myc heterogeneity confers some selective advantage on breast tumor growth and progression. To investigate this, we created a traceable MMTV-Wnt1-driven in vivo chimeric mammary tumor model comprising an admixture of low-Myc- and reversibly switchable high-Myc-expressing clones. We show that such tumors exhibit interclonal mutualism wherein cells with high-Myc expression facilitate tumor growth by promoting protumorigenic stroma yet concomitantly suppress Wnt expression, which renders them dependent for survival on paracrine Wnt provided by low-Myc-expressing clones. To identify any therapeutic vulnerabilities arising from such interdependency, we modeled Myc/Ras/p53/Wnt signaling cross talk as an executable network for low-Myc, for high-Myc clones, and for the 2 together. This executable mechanistic model replicated the observed interdependence of high-Myc and low-Myc clones and predicted a pharmacological vulnerability to coadministration of COX2 and MEK. This was confirmed experimentally. Our study illustrates the power of executable models in elucidating mechanisms driving tumor heterogeneity and offers an innovative strategy for identifying combination therapies tailored to the oligoclonal landscape of heterogenous tumors.

Oncogenic signaling | Myc | cancer heterogeneity | computational modeling | breast cancer

Most solid tumors exhibit extensive intratumoral genetic heterogeneity (1–3) and comprise multiple clones whose identities and prominence shift between primary tumors, metastatic colonies, and relapse after therapy. Such heterogeneity fuels tumor evolution and contributes to the failure of durable therapeutic responses and to subsequent relapse (4, 5). In breast cancers, distant breast cancer metastases often comprise multiple clones from the primary tumor (6), suggesting that certain polyclonal ensembles may be advantageous, and perhaps necessary, for metastatic dissemination, persistence, and outgrowth (7). In line with these observations, murine models of breast cancer have been reported to show mutualism between genetically distinct clones that enhances tumor growth in a concerted fashion (8, 9). Hence, while tumor heterogeneity often confounds successful therapy, interclonal dependencies might yet exist that create novel therapeutic vulnerabilities (9).

The Myc transcription factor is a key coordinator of somatic cellular proliferation and regeneration. In normal somatic cells, Myc activity is tightly controlled and dependent upon mitogenic signals, whereupon it drives cells into proliferation along with metabolic transition to biosynthesis, varying degrees of differentiation, and co-option through signals of stromal, inflammatory, and immune compartments (10). Oncogenic deregulation of Myc, which hijacks this regenerative program, is evident in most, perhaps all, cancers. In breast cancers, Myc is one of the most frequently overexpressed genes (11), especially in higher-grade regions of such tumors. However, high-Myc–expressing tumor cells do not typically dominate the growing tumor mass in breast cancer but are instead interspersed among tumor cells expressing lower levels of Myc (12–16). Such stable and persistent Myc heterogeneity is surprising since Myc is one of several genes reported to elicit supercompetitive behavior. When precociously activated, such supercompetitive genes not only drive cells to outproliferate their neighbors but also to actively induce their neighbors’ demise through, as yet, poorly understood mechanisms that appear to require direct cell contact (17). However, evidence for Myc-driven supercompetition in mammalian cancers remains sparse and so far has only been observed at the boundaries of neoplastic lesions where tumor cells may be killing adjacent healthy tissues (18). The net outcome of high-Myc expression is further complicated by the fact that cells expressing elevated levels of Myc are greatly predisposed to apoptosis, which self-limits their expansion. These 2 antagonistic properties of elevated Myc expression—supercompetition vs. apoptosis—make

Significance

Breast cancer remains a leading cause for cancer-related mortality worldwide. All breast cancers, including the more difficult-to-treat, higher-grade, and triple-negative subtypes of breast cancer, exhibit strong genetic heterogeneity, which hampers treatment and fuels relapse. Our study advances the development of successful treatment approaches by unravelling the mechanistic basis of one form of heterogeneity arising from mutualism between high- and low-Myc-expressing clones in breast cancer. We use this mechanistic understanding to build an executable in silico model of oncogenic Myc/Ras/p53/Wnt signal cross talk for each Myc-expressing clone, separately and together, and then use this model to identify potential therapeutic vulnerabilities, which we then verify experimentally.

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it difficult to predict the fates of high- vs. low-Myc–expressing cancer cells during tumor evolution in vivo.

The heterogeneity of Myc expression observed in breast cancers may indicate some novel mechanism acts to maintain stable clonal variation in Myc levels within the tumor cell population. On the other hand, it could also be a snapshot illusion arising from fluctuating Myc levels in individual cells over time. To explore these possibilities, we constructed a unique estrogen-negative mammary carcinoma mouse model in which tumor cell clones expressing high vs. low preset levels of Myc are tested for tumorigenic efficacy, separately and together, using a combination of experiment and in silico executable modeling of the intracellular oncogenic signaling network.

Results

Generation of Genetically Engineered Mice Allowing for Both Switchable and Heterogeneous Myc Expression in Wnt-Driven Mammary Cancer.

To determine the impacts of different levels of Myc expression on mammary tumors, we used the well-characterized MMTV-Wnt1-driven [B6SlTg(Wnt1)1Hey/J] (19) mouse model of mammary carcinoma. This was crossed into the R26<sup>C</sup>CAG:LSL-MycER<sup>T2</sup> (R26C<sup>LSL-MER</sup>) and R26<sup>B</sup>tm1(Gt(RosA)2Sor<sup>CreERT2<sup>Flx/Flx</sup>EGFP<sup>Lys</sup>Flp<sup>Lys</sup>) reporter backgrounds (20). After Cre-mediated excision of the LSL transgene of TOP<sup>ER</sup> or STOP<sup>ER</sup> allele constitutively expressing the 4-hydroxytamoxifen (4OHT)-dependent allele of Myc, MycER<sup>T2</sup>, at supraphysiological (~6 to 10x physiological) levels. In addition, Cre recombination toggles the constitutive R26<sup>B</sup>tm1 allele from red (Tomato) to green (EGFP) (SI Appendix, Fig. S1A). The genotype of the resultant MMTV-Wnt1; R26<sup>C</sup>LSL-MER; R26<sup>B</sup>T2<sup>GmT</sup> mice was designated WMT.

MMTV-Wnt1 tumors occasionally develop estrogen receptor (ER)-positive tumors. However, these rapidly switch to an ER-negative phenotype in response to sustained tamoxifen treatment (21). Therefore, to obviate any complexities arising from direct action of tamoxifen (used to trigger MycER<sup>T2</sup> activation) on WMT mammary via endogenous estrogen receptors, we first converted all MMTV-Wnt1–induced tumors to ER-negative status by pretreating tumor-bearing MMTV-Wnt1; R26<sup>C</sup>LSL-MER; R26<sup>B</sup>T2<sup>GmT</sup> mice with tamoxifen prior to their deployment in serial transplantation studies. ER negativity of treated mammary tumors was confirmed by immunohistochemistry (IHC) (SI Appendix, Fig. S1B). Furthermore, transplanted tumors exhibited no discernible changes in tumor cellularity, necrosis, proliferation, and incidence of cell death following tamoxifen treatment (SI Appendix, Fig. S1C). ER-negative WMT tumor cells were then infected ex vivo with adenovirus-CRE, which triggered efficient recombination and activation of both R26<sup>C</sup>LSL-MER and R26<sup>B</sup>T2<sup>GmT</sup> alleles (SI Appendix, Fig. S1D). These recombined tumor cells were then flow-sorted into green MycER<sup>T2</sup>–positive (WMT<sup>T</sup>) and red MycER<sup>T2</sup>–negative (WMT<sup>R</sup>) populations and injected, either separately or mixed together into the fat pads of recipient SCID mice. Tumors were then allowed to grow around 1 cm<sup>3</sup> before treating mice with tamoxifen to activate MycER<sup>T2</sup>. Despite a stark reduction in the levels of endogenous Myc upon MycER<sup>T2</sup> activation in WMT<sup>T</sup> tumors, almost every cell in these tumors retained overall Myc levels that are higher than those seen in WMT<sup>R</sup> tumors (SI Appendix, Fig. S1G–I).

Low vs. High Levels of Myc in MMTV-Wnt–Driven Mammary Tumors Exhibit Distinct Behaviors and Dynamics.

To determine the impact of low vs. high Myc expression on mammary tumor dynamics, we first compared the phenotypes of WMT<sup>T</sup> (Myc<sup>high</sup>) and WMT<sup>R</sup> (Myc<sup>low</sup> without tamoxifen, Myc<sup>high</sup> with tamoxifen) tumors. Histologically, Myc<sup>low</sup> (WMT<sup>T</sup>) tumors exhibited a “loose” structure, characterized by low cellularity, and signs of differentiation such as the retention of a recognizable epithelial organization with large luminal spaces separated by sheets of tumor cells (Fig. 1 A and B and SI Appendix, Fig. S1L). Generally, they appear to lack the ability to instruct sufficient supportive stroma for their growth, resulting in large areas of necrosis and hemorrhagic cysts surrounded by hypoxic regions as evidenced by the presence of nuclear HIF1α (Fig. 1 C and D and SI Appendix, Fig. S1 J and K). However, administration of tamoxifen to activate high levels of Myc in transplanted WMT<sup>T</sup> tumors induced profound histological changes. Within 3 d, luminal spaces were completely lost and replaced by tightly packed nests of highly invasive tumor cells (Fig. 1 A and B and SI Appendix, Fig. S1J). Myc activation also rapidly induced a profound switch to angiogenesis, marked by extensive vascular remodeling and highlighted by a smaller average vessel size, which correlated temporally with a fall in active nuclear HIF1α and a profound decrease in hemorrhage and necrosis (Fig. 1 C–E and SI Appendix, Fig. S1K). Nonetheless, despite these ostensibly-protumorigenic stromal changes, persistent elevation of Myc activity actually retarded, and occasionally reversed, net tumor growth (Fig. 1F). Such reduced growth was not associated with any measurable decrease in tumor cell proliferation, whose already high baseline rate was unaffected by MycER<sup>T2</sup> activation (Fig. 1G and SI Appendix, Fig. S1L). Rather, Myc overexpression dramatically increased the incidence of tumor cell apoptosis, as indicated by the presence of cleaved caspase 3 (CC3) (Fig. 1H and SI Appendix, Fig. M and N). Elevated Myc has well-described—proapoptotic activity (22–24) that is, in many instances, facilitated via activation of the p53 tumor suppressor. Both IHC and Western blot (WB) analysis confirmed marked accumulation of p53 in WMT<sup>T</sup> tumor cells, clearly evident by 3 d post-MycER<sup>T2</sup> activation (Fig. U and SI Appendix, Fig. S1O) and accompanied by robust induction of the p53 target genes Puma, Noxa, and Cldn1a (Fig. U). In mice, Myc-dependent activation of p53 is mediated principally through induction of the p19<sup>ARF</sup> protein, encoded by an alternate CDKN2A gene ORF, which acts to inhibit the Mdm2 p53 E3 ubiquitin ligase (25). MycER<sup>T2</sup> activation induced rapid accumulation of p19<sup>ARF</sup> (Fig. 1 J and K). Of note, expression of the BH3-encoding gene BIM, reported elsewhere to be a direct, p53-independent, downstream BH3 apoptotic effector of Myc, was unaffected by MycER<sup>T2</sup> activation (SI Appendix, Fig. S1 P and Q) (26). Taken together, these results implicate engagement of a p19<sup>ARF</sup>→p53→PUMA/NOXA pathway as the likely apoptotic effector mechanism activated by elevated Myc in Wnt-driven mammary tumors.

High- and Low-Myc–Expressing Mammary Tumor Cells Exhibit Mutual Interdependence.

Our data from WMT<sup>T</sup> tumors are consistent with previous studies indicating that apoptotic signaling by Myc at high levels self-limits its overall capacity to drive oncogenesis despite its potent proproliferative effects (27, 28). However, this seems at odds with diverse observations that increased Myc gene expression and/or copy number is associated with later-stage, more aggressive breast cancers. It is therefore noteworthy that Myc overexpression or amplification in breast cancers is usually observed in only a subpopulation of cancer cells within individual tumors and that such chimerism in Myc expression level persists

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Fig. 1. Myc activation leads to rapid reorganization of the tumor and its stroma, while increasing growth through tumor-suppressive pathways. All tumors were analyzed after 3 d of sustained treatment with tamoxifen (100 μg/mouse, twice daily) or oil (vehicle) for the respective controls. Abbreviations in graphs: C, controls; M, MycER+; + tamoxifen. Error bars represent SD. (A) Gross morphological features and histological appearance of WM'T tumors compared to controls (in all following experiments are as follows: WM'T plus tamoxifen and plus vehicle; WM'T plus vehicle). (B) Cellularity of WM'T tumors and controls (n = 6/9, respectively). (C) Quantification of necrosis of WM'T tumors and controls (n = 6/9, respectively). (D) Quantification of hypoxia in WM'T tumors and controls (n = 6/9, respectively). (E) Average area of blood vessel, in WM'T tumors and controls (n = 6/9, respectively). (F) Caliperimetric measurement of tumor growth of WM'T tumors and controls, as percentage change from start of treatment (n = 6 WM'T tumors and 11 controls). (G) Quantification of proliferation, by IDU incorporation, in WM'T tumors and controls (n = 7/14 respectively). (H) Quantification of cell death, by presence of cleaved caspase 3, in WM'T tumors and controls (n = 6/9, respectively). (I) WB analysis of p53 and actin as a loading control in whole-tissue lysates of 2 representative WM'T tumors and controls. Bands were quantified by comparison with the loading control encoded membrane-targeted GFP marker, indicating that Cre staining for p19ARF (red), EGFP (green), and DNA (Hoechst; blue), of WM'T tumors and controls (n = 6/9, respectively). For box-and-whisker plots, the error bars represent min to max values, the box represents the interquartile range, and the horizontal line represents the median. P values are based on Student’s t test: n.s., not significant; *P < 0.05, **P < 0.01, and ***P < 0.001.
expression is likely to be due to a failure to recombine at both the MycER<sup>TM</sup> and GFP allele. To test this hypothesis, we performed genomic DNA analysis on one of the 2 outgrowing Myc<sup>B</sup> tumors and found that it showed a marked presence of unrecombined R26<sup>CLSL-MER</sup> for the GFP allele (Fig. 2L). As these cells usually represent a very minor clone of WMT<sup>T</sup> tumors (SI Appendix, Fig. S1F), we conclude that extended selection of Myc<sup>B</sup>, alone mammary tumors spontaneously regenerates the Myc<sup>low</sup>/Myc<sup>B</sup> biclonal phenotype, as the growth of Myc<sup>B</sup> cells will be hampered until the small Myc<sup>low</sup> population has sufficiently expanded (Fig. 2K, Right, and SI Appendix, Fig. S2H). The failure of 2 out of 4 Myc<sup>B</sup> tumors to grow attests to the necessity of Myc<sup>low</sup>/Myc<sup>B</sup> bicolality for tumor growth. No such selection for an unrecombined R26<sup>CLSL-MER</sup> were observed in biclonal tumors and even after long-term treatment genomic DNA analysis revealed a similar proportion of both alleles in the tumor we analyzed. (Fig. 2 K, Left, and L). This, paired with the observation that Myc<sup>B</sup> cells have a proliferative advantage in mixed clonal tumors (Fig. 2H), implies that admixtures of Myc<sup>low</sup>/Myc<sup>B</sup> clones converge toward an interdependent equilibrium.

Myc-induced apoptosis is mitigated by paracrine survival factors (29). Since suppression of apoptosis in Myc<sup>B</sup> mammary tumor cell was most evident in areas of interface between Myc<sup>B</sup> and Myc<sup>low</sup> cells, we hypothesized that Myc<sup>B</sup> clones secrete a paracrine survival signal that suppresses Myc<sup>low</sup> cell apoptosis. One prominent candidate is Wnt itself, which is a potent survival factor in many developing tissues (30) and has been directly shown to block Myc-induced apoptosis (31). Such a prosurvival role for Wnt is especially germane since, in a separate study, we had noted that activation of high levels of Myc antagonizes Wnt signaling. We thus tested whether Wnt and Wnt-signaling was suppressed by Myc in our model system, which was confirmed by the loss of Wnt and Axin2 expression (Fig. 3 and SI Appendix, Fig. S3 A and B). Wnt1 expression in these cells is promiscuously driven from the heterologous MMTV promoter; however, the transgene retains almost all of the proximal Wnt1 promoter and therefore retains many of the original sites for transcriptional activator and inhibitors. To make sure that the effects on Wnt1 were not due to its expression by the MMTV-promoter, we analyzed the expression of endogenous Wnt1 following MycER<sup>Tm</sup> activation in otherwise normal mammary glands. Wnt1 expression was potently inhibited after only 4 h of tamoxifen administration showing a general negative feedback of Myc

WM<sup>T</sup>/WM<sup>T</sup> tumors. (E) Quantification of hypoxia in mixed WM<sup>T</sup>/WM<sup>T</sup> tumors. (F) Average area of blood vessel in mixed WM<sup>T</sup>/WM<sup>T</sup> tumors. (G) Quantification of cell death, by presence of cleaved caspase 3, in mixed WM<sup>T</sup>/WM<sup>T</sup> tumors. Clones were distinguished via GFP staining. (H) Quantification of proliferation, by IDU incorporation, in mixed WM<sup>T</sup>/WM<sup>T</sup> tumors. Clones were distinguished via GFP staining. (I) Immunofluorescent staining for GFP (WM<sup>T</sup>) and p19<sup>ARF</sup> of intermingled areas in mixed WM<sup>T</sup>/WM<sup>T</sup> tumors and controls (in G–I, n = 8 mixed WM<sup>T</sup>/WM<sup>T</sup> tumors treated with tamoxifen and 5 vehicle controls). (J) Caliperimetric measurement of tumor growth of individual WM<sup>T</sup>, WM<sup>T</sup>, mixed WM<sup>T</sup>/WM<sup>T</sup> tumors, and controls on long-term tamoxifen treatment. (K) Representative images of immunofluorescence staining for Myc, dTomato, and GFP on frozen tissue sections from long-term-treated WM<sup>T</sup> and mixed WM<sup>T</sup>/WM<sup>T</sup> tumors showing loss of Myc in the WM<sup>T</sup> tumors, but no such phenomenon in the mixed WM<sup>T</sup>/WM<sup>T</sup> tumors. (L) Digital droplet PCR on genomic DNA comparing the recombination status of the R26<sup>CLSL-MER</sup> allele in 20% WM<sup>T</sup> and 100% WM<sup>T</sup> tumor after long-term tamoxifen treatment (n = 1 20% WM<sup>T</sup> and 100% WM<sup>T</sup> tumor). For box-and-whisker plots, the error bars represent min to max values, the box represents the interquartile range, and the horizontal line represents the median. P values are based on Student’s t test: *P < 0.05, **P < 0.01, and ***P < 0.001. In (L), the Myc<sup>low</sup> clone is outlined by a dotted line. Areas that are neither GFP positive nor marked up by dotted lines are nontumor tissues such as stroma and necrotic areas.
construct (67NR-Myc-RFP) (Fig. 3B). Again, induction of Myc led to an immediate down-regulation of Wnt1 expression creating paracrine dependency. (4) WB analysis for nuclear β-catenin in most cells, while low expression persisted in a few cells, concomitant with the apoptosis that high-Myc expression elicits, while those with lower Myc expression survived and propagated, as evident from the lower overall fluorescence of the outgrowing population (Fig. 3E). This is consistent with the notion that Wnt signaling protects breast cancer cells from the apoptotic impact of chronic high Myc activity. Collectively, these data show that Myc has a negative feedback on Wnt and that, in turn, Wnt signaling can rescue cells from Myc-mediated apoptosis. Having shown a lack of Wnt signaling in WM'T tumors (Fig. 3A and SI Appendix, Figs. S3 A and B), we set out to analyze the extent of Wnt signaling in the mixed clonal tumors as well. To do so, we used immunofluorescent staining for nuclear β-catenin as a readout of active canonical Wnt signaling. As expected, control (WM'T with or without tamoxifen, WM'T without tamoxifen) tumors exhibited abundant nuclear β-catenin in most cells, while WM'T tumors exposed to tamoxifen did not (Fig. 3 F and G), presumably due to Wnt down-regulation (Fig. 3A and SI Appendix, Fig. S3A). Since MMTV-Wnt1–driven tumors are dependent upon Wnt signaling for their maintenance (8), Myc-induced down-regulation of Wnt1 effectively deprives the tumor cells of their own survival signal. By contrast, mixed WM'T/Wm'T tumors exhibited strong nuclear β-catenin in both Myc-low clones and in a high proportion of the Myc-high cells lying at the interface of the 2 clones (Fig. 3 F and G), although this decreased with distance from the boundary with Myc-low cells (SI Appendix, Fig. S3E). Taken together, these results confirm the notion that high levels of Myc deprive tumors of Wnt survival signaling but that this can then be restored by juxtaposition with Myc-low cells, so providing mechanistic explanation for the stable mutualism between Myc-high and Myc-low tumor cells in mammary cancers.

**Executable Modeling Identifies Pharmacological Vulnerabilities in Heterogeneous My Mammary Tumors.** While it is possible that the obligate mutualism between Myc-low and Myc-high mammary tumor cells create novel vulnerabilities for therapeutic targeting, the complexity, redundancy, and feedback in biological networks make the search for such contextual vulnerabilities both difficult and arduously empirical. We therefore turned to executable mechanistic in silico models, which allow for rapid, systematic simulation and testing of large numbers of signaling network perturbations. We constructed an initial executable model of breast cancer using publicly available data drawn from the literature (Datasets S1 and S2). The network is modeled as a qualitative.
network (32), which is then simulated and analyzed with the BioModelAnalyzer (BMA) tool (33) (http://biomodelanalyzer.org/). The process of building and testing this network model is illustrated in SI Appendix, Fig. S4, and the result is an executable network encompassing proteins and transcription factors that contribute to the overall tumor cell phenotype (Fig. 4). Although not an exhaustive map of all interactions within a cell, the model nonetheless models the key pathways in our system and the fidelity of its iterations may then be rapidly evaluated by in vitro and in vivo experiment. To address Wnt-driven triple-negative breast cancer specifically, we focused on the Wnt1 and EGFR receptor pathways, since these are 2 predominant drivers of oncogenic signaling in ER- and HER2-negative breast cancers that converge downstream on Myc and Ras effector pathways. In addition, since aberrant cross talk and excessive signaling flux across Myc and Ras pathways trigger tumor suppression, we included the p53 signaling pathway in our executable model. Finally, to encompass critical aspects of the interaction between tumor clones and their microenvironment, we simulated responses to hypoxia via the HIF1α pathway and consequent release of signaling molecules such as VEGF. The overall output of the model governs the net balance between cell proliferation and apoptosis.

The genes, proteins, and environmental conditions of a cell in the tumor are each represented by nodes in the executable network model (Fig. 4). Their behaviors are defined by target functions attached to each node. These target functions are mathematical formulae that define how a protein responds to changes in the other proteins with which it interacts. Target functions can therefore model, for example, a series of proteins activated in a signaling cascade, or their change in expression in response to a transcription factor. Mutations, drug treatments, and environmental conditions can be represented in the network model by changing the target functions of nodes. This allows the network model to reproduce the different cells in our mouse model. For example, Myc\textsuperscript{high} conditions were reproduced by fixing the activity of Myc to be a constant value at an arbitrarily maximum level (Dataset S5).

We first verified the behavior of the executable model against published data derived from experiments on breast cell lines with known oncogenic mutations. These were represented in the model by changes to the relevant target functions of the nodes representing the affected genes and proteins, as described above. We then adjusted the levels of various nodes to represent experimental perturbations: For example, fixing a node at zero represents pharmacological inhibition. The resulting behavior of the model is then compared with that observed by experiment (Dataset S3). In this way, we can test whether the model’s behavior is correct under a wide array of perturbations. We further tested the model against each of the monoclonal MMTV-Wnt1 Myc\textsuperscript{low} and Myc\textsuperscript{high} tumors by comparing the predicted activity of nodes in the model with the activity observed experimentally (Dataset S4). We iteratively alternated between testing and refining the model until all of the simulation results reproduced the experimental observations (SI Appendix, Fig. S4). Comparisons were made predominantly against in vitro published experiments, so angiogenesis was not simulated. However, when modeling the in vivo tumors, we introduced an angiogenic node. Since the in vivo tumors were not HER2 driven, these nodes were removed.

We next simulated the effect of treatment on the mixed Myc\textsuperscript{high} and Myc\textsuperscript{low} tumors, including the predicted cross talk between the clones, to generate cell fate predictions and to find the most effective targeted therapies for each clone. As the cooperation of clones was mediated by changes in the microenvironment, we were able to simulate each clone in the mixed tumor separately by modifications to the relevant node level to represent these changes; for example, increasing the activity of the Wnt1 node to represent that there is a source of paracrine Wnts for the Myc\textsuperscript{high} cells from the Myc\textsuperscript{low} cells in the mixed tumors. This was in addition to the changes representing the mutations in each subclone. These node level changes are depicted in Dataset S5. We modeled the effect of targeted therapies by setting the activity of a node to zero, representing inhibition by a drug, and repeated this for all major nodes. This allowed us to model the therapeutic outcome (net proliferation or net apoptosis) of modalities that target one clone or the other.

The model predicted that heterogenous tumors would be more resilient to therapy, with higher proliferation and lower apoptosis than pure Myc\textsuperscript{high} or Myc\textsuperscript{low} clones for the same inhibiting drug (SI Appendix, Fig. S5 A and B). This is consistent with our experimental evidence of mutual benefit for each clone from one another. The model also predicted that most inhibitors would be more effective against one clone than another, with Myc\textsuperscript{high} cells being resistant to cell cycle arrest but more vulnerable to apoptosis (SI Appendix, Fig. S5 A and B), which meant that targets in some pathways were predicted to be effective for one clone but not the other. Because of these differences in the effectiveness of a single inhibitor in treating either of the 2 different clones, as well as the proclivity of neoplastic systems to acquire compensatory or evolutionary resistance to monotherapies, we hypothesized that simultaneous application of 2 inhibitors would be therapeutically more effective. Accordingly, we simulated pairwise combinations of inhibitors across all major nodes (SI Appendix, Figs. S6 A–E and S7 A–E). This generated a striking increase in the proportion of modeled therapies predicted to be successful, many eliciting

![Fig. 4. The executable network model as seen in the BMA tool. The nodes representing genes and proteins are grouped by pathway for ease of interpreting the diagram, while each phenotype is singled out in its own module. Nodes outside any module represent external factors produced by or influencing cell behavior. The arrows represent activating interactions, while the bars represent inhibition.](http://biomodelanalyzer.org/)
marked impacts on both Myc\textsuperscript{high} plus Myc\textsuperscript{low} tumor cell populations (SI Appendix, Fig. S8 A and B). We then further filtered our search on the basis of target druggability and searched for combinatorial synergy by assessing whether the efficacy of one inhibitor was enhanced by addition of a second inhibitor (Fig. 5 A and B and SI Appendix, Fig. S9).

From this analysis, the combination of COX2 and MEK inhibition appeared to be the most effective combination for increasing apoptosis (Fig. 5B and SI Appendix, Fig. S9B). The model predicted that inhibition of MEK alone would induce more apoptosis in the Myc\textsuperscript{high} than the Myc\textsuperscript{low} cells, and that in the Myc\textsuperscript{low} cells, inhibition of MEK and COX2 together would improve the cytotoxic effect over either inhibition of MEK or COX2 applied separately, while still remaining effective against Myc\textsuperscript{high}, and so effectively treat both populations of cells.

To test the predicted therapeutic efficacy of this combination in vivo, we again used our biclonal Myc\textsuperscript{high}\text/-Myc\textsuperscript{low} tumor model: High MycER\textsuperscript{12} was activated in the Myc\textsuperscript{high} subpopulation, and 48 h later, mice were treated with either the COX2 inhibitor celecoxib or the MEK inhibitor PD0325901 alone, or with the 2 inhibitors combined. The tumors were then observed over a further 3 d. Each inhibitor alone offered some therapeutic benefit: COX2 inhibition slowed down overall tumor growth, while MEK inhibition stalled net tumor expansion (Fig. 5C). However, celecoxib and PD0325901 in combination induced rapid tumor regression (Fig. 5C), resulting in residual masses almost devoid of tumor cells and comprising mainly hemorrhagic cysts (Fig. 5D and SI Appendix, Fig. S10 A and B). Detailed histological analysis of the few remaining regions harboring residual tumor cells showed a significant increase in apoptosis, most evident in the Myc\textsuperscript{high} cells, together with a decrease in proliferation, most notably in the Myc\textsuperscript{low} clones (Fig. 5F and SI Appendix, Fig. S10 C–F). These single and combined responses were consistent with our executable model's predictions (Fig. 5 E and F). Last, we decided to test whether combining the more recently developed Cox/Lox inhibitor licofelone with PD0325901 gave any advantage over the single target drug celecoxib. This was not the case, as the response of the tumor to the triple inhibition was indistinguishable to the double inhibition (SI Appendix, Fig. S10 A–F). This result is consistent with a further test of the specific Lox inhibitor zileuton, which did not significantly synergize with MEK inhibition (SI Appendix, Fig. S10 A–F). Due to the relatively high doses of licofelone used (100 mg/kg/d) to observe a combined effect with MEK inhibition compared to those used for celecoxib (20 mg/kg/d) and given that celecoxib has fewer known side effects in humans, the dual-inhibition with PD0325901 and celecoxib seems preferable over licofelone.

Taken together, these results show how computational modeling of solid bclonal tumors allowed us to devise a very potent therapeutic strategy.

Discussion

The component tumor cells of many human breast cancers exhibit persistent heterogeneity in Myc expression (12, 13, 16). Individually, ubiquitously Myc\textsuperscript{high} or Myc\textsuperscript{low} mammary tumor cells exhibit markedly different features, each of which significantly restrains tumorigenic potential. Mammary tumor cells with high levels of Myc enjoy significant potential growth advantages by virtue of their enhanced proliferative rates, invasiveness, and their capacity to instruct an angiogenic microenvironment conducive to rapid tumor spread. However, elevated Myc predisposes Myc\textsuperscript{high} cells to apoptosis. Consequently, Myc\textsuperscript{high} cells are handicapped by a greatly increased reliance on continuous survival signals (29). Since elevated Myc also concomitantly suppresses expression of Wnt1, a key autocrine survival factor for mammary epithelial cells, tumors comprising only Myc\textsuperscript{high} tumor cells effectively starve themselves of autocrine survival signals. Conversely, tumor monocultures of Myc\textsuperscript{low} cells, while enjoying an intrinsically much lower predisposition to apoptosis, are constrained by their inability to instruct significant stromal angiogenic changes, limiting them to indolent, hypovascular, hypoxic, and necrotic lesions. The stability of Myc\textsuperscript{high}/Myc\textsuperscript{low} mixtures of tumor cells therefore appears to derive from the obligate complementarity of their 2, individually self-limiting, biology. While proximity of invasive and angiogenic Myc\textsuperscript{high} cells facilitates both growth and spread of Myc\textsuperscript{low} cells, reciprocal proximity of Myc\textsuperscript{low} cells provides sufficient Wnt1 to keep their more aggressive siblings alive. This relationship becomes particularly clear when trying to grow tumors solely comprising Myc\textsuperscript{high} cells. The observed escape tumors convert to a heterogeneous phenotype through outgrowth of a minor MycER\textsuperscript{12}, negative clone (Fig. 2 J and K). The observation that more than one-half of the cells comprising the escape tumor we analyzed genomically did not express MycER\textsuperscript{12} (Fig. 2L) suggests the need for a significant amount of Myc\textsuperscript{low} cells to support Myc\textsuperscript{high} cells, and is consistent with the idea that Wnt, a heavily palmitoylated and glycosylated ligand, acts at relatively short range (34, 35). Therefore, sufficient Wnt is a prerequisite for any secondary consequences of polyclonality, such as the development of tumor supportive stroma by Myc\textsuperscript{low} clones (Figs. 1 D and E and 2 E and F and SI Appendix, Figs. S1A and S2E).

This mutualism explains why, in human Myc\textsuperscript{high}/Myc\textsuperscript{low} mixed mammary tumors, Myc\textsuperscript{high} clones typically do not rapidly overgrow Myc\textsuperscript{low} clones (13). A similar role for Wnt-secreting supportive niches in tumor evolution and maintenance has recently been identified in lung adenocarcinomas (7), indicating that such mutualism may be a common feature of tumor cells expressing high levels of Myc. Furthermore, when we switched on MycER\textsuperscript{12} in an untransformed mammary gland, we equally observed rapid loss of Wnt1 and inhibition of Wnt signaling (Fig. 3B). This indicated that the mutual exclusivity between expression of high levels of Myc and Wnt ligands is not idiosyncratic for tumors, but rather a general phenomenon. This is most likely part of an inherent tissue organization, where proliferative niches are organized in proliferative (Myc\textsuperscript{high}/Wnt\textsuperscript{low}) cells and supportive (Myc\textsuperscript{low}/Wnt\textsuperscript{high}) cells. Myc-heterogenous mammary tumors appear to retain this reliance on supportive niches and evolve accordingly.

There is clearly a complex interplay between the key growth and survival factors, Myc and Wnt, that is highly contextual. For example, Myc is reported to down-regulate the secreted Wnt inhibitors DKK1 and SFRP1 (36), implying that Myc acts to sensitize cells to Wnt signaling. Myc\textsuperscript{high} inhibitors DKK1 and SFRP1 (36), implying that Myc acts to sensitize cells to Wnt signaling. Myc\textsuperscript{high} inhibitors DKK1 and SFRP1 (36), implying that Myc acts to sensitize cells to Wnt signaling. Myc\textsuperscript{high} in inhibi...
Likewise, it is possible that Myc-driven supercompetition is a significant factor in evolution of tumors in which Myc-induced apoptosis is circumvented by secondary, antiapoptotic mutation. The notion that certain aspects of the oncogenic process might expose novel vulnerabilities in tumor cells underpins the rationale for selective cancer therapies, and the obligation mutualism we observe between Myc\textsuperscript{high} and Myc\textsuperscript{low} mammary cancer cells is one such example. To explore this case, we generated a computational model of the Myc\textup{Ras/p53} signaling network in breast cancer cells. Starting from a general model of breast cancer, we added Wnt as a constant node and overlaid high Myc activity. Our initial simulation predicted levels of proliferation higher than those seen in Myc high tumors in vivo, suggesting that some level of interruption in Wnt signaling was at play in the tumors. This was experimentally confirmed and shown to be due to Myc-dependent suppression of Wnt expression. This was then factored back into the computational model of both Myc\textsuperscript{high}, only tumors and Myc\textsuperscript{low}/Myc\textsuperscript{low} heterogenous tumors, to accurately predict the underlying set of mechanistic rules that indicated a biconal mutualism of the Myc\textsuperscript{high}/Myc\textsuperscript{low} mixed tumors.

A key dividend of such executable models is their ability to screen vast numbers of therapeutic combinations virtually and identify combinatorial regimens that specifically target the obligate biconality of the tumors. Thus, the model predicted that coinhibition of MEK and COX2 would exert a more potent therapeutic impact on both clonotypes than their individual inhibition would on either individual clonotype. The model correctly predicted the augmented response of the individual clones to various inhibitor combinations, including the sharp drop in proliferation of Myc\textsuperscript{low} cells when exposed either to MEK inhibition alone or to coinhibition of MEK and COX2 together, and the strong apoptotic response of Myc\textsuperscript{high} clones exposed to the combination therapy. Intriguingly, our model consistently underestimated the efficacy of the inhibitors, particularly with respect to their impact on the Myc\textsuperscript{high} tumor cell population. However, in its current form, the model considers only initial clonal distributions and does not accommodate clonal dynamics known to occur during the course of treatment. This is a drawback, since we predict that the expected loss of Myc\textsuperscript{low} cells during treatment will progressively curtail the survival of Myc\textsuperscript{high} clones due to loss of Wnt1 signaling. Future development of the model could be extended to accommodate the shifting interactions that follow from changes in clonal composition of the tumor during treatment. A further benefit of our executable modeling approach is that it suggests potential mechanisms by which the combination treatment works. Specifically, it suggests that therapeutic efficacy relies on disrupting the balance between proapoptotic and antiapoptotic signals: MEK inhibition blocks antiapoptotic signaling, and so predominantly affects the Myc\textsuperscript{high} clone, whereas COX2 inhibition increases proapoptotic signaling, other and are removed as this otherwise prevents clustering of the heatmaps. (C) Caliperimetric measurement of tumor growth of mixed WM\textup{T}/WM\textup{F} tumors during 3 d of treatment with tamoxifen (100 \mu g/mouse, twice daily) followed by 4-d treatment with tamoxifen and drug combinations (celecoxib, 20 mg/kg/d; PD0325901, 10 mg/kg/d). Measurements are normalized to the beginning of the drug treatment course as tumors at this stage were at different sizes (n = 4 to 5; error bars represent SD). (D) Representative picture of the gross morphology of mixed WM\textup{T}/WM\textup{F} tumors after the treatment described above. (E) Quantification of cell death in mixed WM\textup{T}/WM\textup{F} tumors as percentage of C3-positive pixels in the individual clones (n = control, PD0325901 = 4; celecoxib, celecoxib plus PD0325901 = 5). For box-and-whisker plots, the error bars represent min to max values, the box represents the interquartile range, and the horizontal line represents the median. P values are based on Student’s t test: *P < 0.05.
thereby reinforcing the impact of MEK inhibition on the Myc<sub>low</sub> cells.

As our understanding of normal tissue organization and its pathogenic equivalent in tumors deepens, we propose that qualitative computational models such as the one we have used in this study will be needed to grasp the totality of iterative and dynamic tumor cell signaling—both in its initial state and as it morphs and adapts to perturbations induced by treatments and spontaneous changes in the genome and epigenome. Only in this way can we stay ahead of drug resistance and disease relapse.

**Materials and Methods**

**Mice and In Vivo Procedures.** All treatments and procedures of mice were conducted in accordance with protocols approved by the home Office UK guidelines under project licenses to G.I.E. (70/7586, 80/2396) at the University of Cambridge. The following mouse strains were used: Rosa26-CAG-lox-STOP-lox-MycER<sup>R3</sup>Rosa26-mTmGMMTV-Wnt1 and Rosa26-CAG-lox-STOP-lox-MycER<sup>R2</sup>. MycER<sup>R3</sup> was activated by administration of tamoxifen at 1 mg/20 g, i.p., twice daily, id administration period <12 h. IDU was administered at 1 mg/20 g. More details are in *SI Appendix*.

**IHC and Immunofluorescence.** Standard protocols were followed for IHC and immunofluorescence. For details, see *SI Appendix*. The following primary antibodies were used: HIF1α (sc-10790; 1:500); CD31 (ab23863; 1:100); cleaved caspase 3 (sc6664; 1:1,000); estrogen receptor α (sc-542; 1:50); IDU (BD347580; 1:100); α-GFP (BD610153; 1:500); β-catenin (BD610153; 1:250); Myc (ab23072; 1:2,000); p-β-catenin (BD610153; 1:500); and α-tubulin (Leica CM55; 1:500). Unless otherwise stated, quantifications were carried out for at least 3 visual field on at least 3 independent biological replicates.

**Quantitative Real-Time PCR.** SBYR Green Master Mix (Thermo Fisher Scientific)-based qRT-PCR was performed after RNA extraction and complementary DNA production following standard protocols. For primer, see *SI Appendix*.

**Genomic DNA Analysis.** Genomic DNA was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen), following the manufacturer’s instructions. Genomic DNA from samples on long-term tamoxifen studies were extracted by the same method from shavings of formalin-fixed freeze-preserved tissues. The presence of CRE recombinase at the R26<sup>M</sup>lox-MycER<sup>R2</sup> allele was tested via quantitative digital droplet PCR on the QX 200 droplet reader (Bio-Rad), following the manufacturer’s standard protocol. For primers and probes, see *SI Appendix*.

**Western Blotting.** Samples were prepared using standard protocols. Proteins were labeled following the manufacturer’s protocol for the Li-Cor Near-Infrared (NIR) Western Blot Detection system, or the Amersham 600 imager. The following primary antibodies were used: p53 (Leica; NCL-L-p53-CMP5; 1:2,000); actin (Santa Cruz; sc-69879; 1:5,000); Wnt1 (Abcam; ab15251; 1:1,000); CC3 (Cell Signaling Technologies; 1:1,000); Myc (ab32072; 1:2,000); p-α-GFP (BD610153; 1:500); and α-tubulin (Leica CM55; 1:500). Unless otherwise stated, quantifications were carried out for at least 3 visual field on at least 3 independent biological replicates.

**Therapeutic Studies.** Tumors were generated as described above at ratios of Myc<sup>high</sup>/Myc<sup>low</sup> clones of 30%/70%. Tamoxifen treatment started at a size of 1 cm<sup>2</sup>, and IDU was injected 3 h after the drug administration. For details on drug administration, see *SI Appendix*.

**Using Qualitative Networks to Model Genetic and Molecular Networks.** We model the system as a qualitative network (32), an extension of the Boolean network formalism, using the BMA tool (*SI Appendix, Methodology*) (33) ([http://biomodelanalyzer.org](http://biomodelanalyzer.org)).

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