Heterogeneity of Myc Expression in Breast Cancer Exposes New Susceptibilities Revealed through Executable Mechanistic Modelling

Peter Kreuzaler[1,4]^, Matthew A. Clarke[1]^, Elizabeth J. Brown[1], Catherine H. Wilson[1], Roderik M. Kortlever[1], Nir Piterman[2], Trevor Littlewood[1], Gerard I. Evan[1]* and Jasmin Fisher[1, 3]*

[1]Department of Biochemistry, University of Cambridge, Cambridge CB2 1GA, UK; [2]Department of Computer Science and Engineering, Chalmers University of Technology, Gothenburg, SE-41296, Sweden; [3]UCL Cancer Institute, University College London, WC1E 6DD, UK, [4]The Francis Crick Institute, London NW1 1AT, UK

^ These authors contributed equally to this work

* Correspondence should be addressed to Jasmin Fisher jf416@cam.ac.uk or Gerard Evan gie20@cam.ac.uk

1 Abstract

Cells with higher levels of Myc proliferate more rapidly and super-competitively eliminate neighbouring cells. Nonetheless, tumour 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 tumour growth and progression. To investigate this, we created a traceable *MMTV-Wnt1*-driven *in vivo* chimeric mammary tumour model comprising an admixture of low Myc and reversibly switchable high Myc-expressing clones. We show that such tumours exhibit interclonal mutualism wherein cells with high Myc-expression facilitate tumour growth by promoting pro-tumourigenic 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 modelled Myc/Ras/p53/Wnt signalling crosstalk as an executable network for low Myc for high Myc clones, and for the two together. This executable mechanistic model replicated the observed interdependence of high-Myc and low-Myc clones and predicted a novel pharmacological vulnerability to co-inhibition of COX2 and MEK. This was confirmed experimentally. Our study illustrates the power of executable models in elucidating mechanisms driving tumour heterogeneity and offers an innovative strategy for identifying novel combination therapies tailored to the oligoclonal landscape of heterogenous tumours.

Keywords: Oncogenic signalling, Myc, cancer heterogeneity, breast cancer, combination therapy, executable biology, mechanistic modelling, computational modelling, *in silico*.

2 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 a novel class of executable *in silico* models of oncogenic Myc/Ras/p53/Wnt signal crosstalk for each Myc-expressing clone, separately and together, and then use this model to identify potential novel therapeutic vulnerabilities, which we then verify experimentally.

3 Introduction

Most solid tumours exhibit extensive intratumoural genetic heterogeneity (1–3) and comprise multiple clones whose identities and prominence shift between primary tumours, metastatic colonies and relapse after therapy. Such heterogeneity fuels tumour 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 tumour (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 tumour growth in a concerted fashion (8, 9). Hence, while tumour 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 de-differentiation, and co-option through signals of stromal, inflammatory and immune compartments (10). Oncogenic deregulation of Myc, which hijacks this regenerative programme, 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 tumours. However, high Myc-expressing tumour cells do not typically dominate the growing tumour mass in breast cancer but are instead interspersed among tumour 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 super-

competitive behaviour. When precociously activated, such super-competitive genes not only drive cells to out-proliferate their neighbours but also to actively induce their neighbours' demise through, as yet, poorly understood mechanisms that appear to require direct cell contact (17). However, evidence for Myc-driven super-competition in mammalian cancers remains sparse and so far has only been observed at the boundaries of neoplastic lesions where tumour 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 two antagonistic properties of elevated Myc expression – supercompetition versus apoptosis – make it difficult to predict the fates of high versus low Myc-expressing cancer cells during tumour 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 tumour cell population. On the other hand, it could also be a snap-shot illusion arising from fluctuating Myc levels in individual cells over time. To explore these possibilities, we constructed a unique oestrogen-negative mammary carcinoma mouse model in which tumour cell clones expressing high versus low pre-set levels of Myc are tested for tumourigenic efficacy, separately and together, using a combination of experiment and *in silico* executable modelling of the intracellular oncogenic signalling network.

4 Results

4.1 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 tumours, we used the well-characterized *MMTV-Wnt1*-driven (*B6SJL-Tg(Wnt1)1Hev/J*) (19) mouse model of mammary carcinoma. This was crossed into the $R26^{CAG\text{-}LSL\text{-}MycERT2}$ ($R26C^{LSL\text{-}MER}$) and $R26^{mTmG}$ ($Gt(ROSA)26Sor^{tm4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$) reporter backgrounds (20). After Cre-mediated excision of the *LSL* transcriptional STOP element, $R26C^{MER}$ allele constitutively expresses the 4-hydroxytamoxifen (4OHT)-dependent allele of Myc, $MycER^{T2}$, at supraphysiological (~6-10X physiological) levels. In addition, Cre recombination toggles the constitutive $R26^{mTmG}$ allele from red (Tomato) to green (EGFP) (SI Appendix Fig. S1a). The genotype of the resultant *MMTV-Wnt1;* $R26C^{LSL\text{-}MER}$*;* $R26^{mTmG}$ mice was designated *WMT.*

MMTV-Wnt1 tumours occasionally develop oestrogen receptor (ER) positive tumours. 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 $MycER^{T2}$ activation) on *WMT* mammary via endogenous oestrogen receptors, we first converted all *MMTV-*

Wnt-induced tumours to ER negative status by pre-treating tumour-bearing *MMTV-Wnt1; R26C$^{LSL\text{-}MER}$; R26^{mTmG}* mice with Tamoxifen prior to their deployment in serial transplantation studies. ER negativity of treated mammary tumours was confirmed by immunohistochemistry (IHC) (SI Appendix Fig. S1b). Furthermore, transplanted tumours exhibited no discernible changes in tumour cellularity, necrosis, proliferation and incidence of cell death following Tamoxifen treatment (SI Appendix Fig. S1c). ER negative *WMT* tumour cells were then infected *ex vivo* with Adenovirus-CRE, which triggered efficient recombination and activation of both *R26C$^{LSL\text{-}MER}$* and *R26^{mTmG}* alleles (SI Appendix Fig. S1d). These recombined tumour cells were then flow-sorted into green MycER^{T2}-positive (*WM^{+}T*) and red MycER^{T2}-negative (*WM^{-}T*) populations and injected, either separately or mixed together into the fat pads of recipient SCID mice. Tumours were then allowed to grow to around 1 cm^3 before treating mice with Tamoxifen to activate MycER^{T2}.

Our tumour model depends on two concurrent Cre-mediated recombinations in each targeted cell – one to activate MycER^{T2} and the other to induce the switch from (red) Tomato to (green) EGFP. Analysis via flow cytometry of outgrown tumours revealed a small population of GFP/TdTomato double positive cells in *WM^{+}T* tumours, which are the likely result of a monoallelic recombination on the *R26C$^{LSL\text{-}MER}$* only (SI Appendix Fig S1e). Conversely, genomic analysis on the respective tumours, showed that *WM^{+}T* tumours had very small amounts of unrecombined *R26C$^{LSL\text{-}MER}$* (SI Appendix Fig. S1f). As expected, *WM^{-}T* tumours, did not show recombination at any allele of either of the two transgenic loci (SI Appendix Fig S1e, f). We also compared the expression levels of MycER^{T2} driven by the recombinant *R26C$^{LSL\text{-}MER}$* allele to that of endogenous Myc. Only around a quarter of cells in *WM^{-}T* tumours had detectable levels of Myc. Despite a stark reduction in the levels of endogenous Myc upon MycER^{T2} activation in *WM^{+}T* tumours, almost every cell in these tumours retained overall Myc levels that are higher than those seen in *WM^{-}T* tumour cells (SI Appendix Fig. S1g-i).

4.2 Low versus high levels of Myc in MMTV-Wnt-driven mammary tumours exhibit distinct behaviours and dynamics

To determine the impact of low versus high Myc expression on mammary tumour dynamics, we first compared the phenotypes of *WM^{-}T* (Myc^{low}) and *WM^{+}T* (Myc^{low} without Tamoxifen, Myc^{high} with Tamoxifen) tumours. Histologically, Myc^{low} (*WM^{-}T*) tumours exhibited a "loose" structure, characterized by low cellularity, and signs of differentiation such as the retention of a recognizable epithelial organisation with large luminal spaces separated by sheets of tumour cells (Fig. 1 a-b, SI Appendix Fig. S1j). Generally, they appear to lack the ability to instruct sufficient supportive stroma for their growth, resulting in large areas of necrosis and haemorrhagic cysts surrounded by hypoxic regions as evidenced by the presence of nuclear HIF1α (Fig. 1c-d, Appendix Fig. S1j, k). However,

administration of Tamoxifen to activate high levels of Myc in transplanted *WM^{+}T* tumours induced profound histological changes. Within 3 days, luminal spaces were completely lost and replaced by tightly packed nests of highly invasive tumour cells (Fig. 1a, b, SI Appendix Fig. S1j). Myc activation also rapidly induced a profound switch to angiogenesis, marked by extensive vascular remodelling and highlighted by a smaller average vessel size, which correlated temporally with a fall in active nuclear HIF1α and a profound decrease in haemorrhage and necrosis (Fig. 1 c-e, SI Appendix Fig. S1k). Nonetheless, despite these ostensibly pro-tumourigenic stromal changes, persistent elevation of Myc activity actually retarded, and occasionally reversed, net tumour growth (Fig. 1f). Such reduced growth was not associated with any measurable decrease in tumour cell proliferation, whose already high baseline rate was unaffected by MycER^{T2} activation (Fig. 1g, SI Appendix Fig. S1l). Rather, Myc over-expression dramatically increased the incidence of tumour cell apoptosis, as indicated by the presence of cleaved caspase 3 (CC3) (Fig 1h, SI Appendix Fig. m-n). Elevated Myc has well described pro-apoptotic activity (22–24) that is, in many instances, facilitated via activation of the p53 tumour suppressor. Both IHC and Western Blot (WB) analysis confirmed marked accumulation of p53 in *WM^{+}T* tumour cells, clearly evident by 3 days post MycER^{T2} activation (Fig. 1i, SI Appendix Fig. S1o and accompanied by robust induction of the p53 target genes *Puma*, *Noxa*, and C*dkn1a* (Fig. 1j). In mice, Myc-dependent activation of p53 is mediated principally through induction of the p19^{ARF} protein, encoded by an alternate *CDKN2A* gene ORF, which acts to inhibit the Mdm2 p53 E3 ubiquitin ligase (25). MycER^{T2} activation induced rapid accumulation of p19^{ARF} (Fig. 1j-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^{T2} activation (SI Appendix Fig. S1p, q (26). Taken together, these results implicate engagement of a p19^{ARF}→p53→PUMA/NOXA pathway as the likely apoptotic effector mechanism activated by elevated Myc in Wnt-driven mammary tumours.

4.3 High and Low Myc expressing mammary tumour cells exhibit mutual interdependence

Our data from *WM^{+}T* tumours are consistent with previous studies indicating that apoptotic signalling by Myc at high levels self-limits its overall capacity to drive oncogenesis despite its potent pro-proliferative 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 over-expression or amplification in breast cancers is usually observed in only a sub-population of cancer cells within individual tumours and that such chimaerism in Myc expression level persists through tumour evolution (12, 13, 16). We therefore

hypothesised that some selective advantage or mutualism exists to maintain co-existence of Myc-high with Myc-low tumour cells. To investigate this idea, we generated bespoke bi-clonal Wnt-driven mammary tumours comprising both Myc high and Myc low tumour cells, by co-injecting a mixture of floxed *WM^{+}T* (20-30%) and unfloxed *WM^{-}T* (80-70%) clones into the same fat pad. Tumours were then allowed to develop and MycER^{T2} in the *WM^{+}T* cells then activated acutely by Tamoxifen administration to generate Myc^{high} tumour cells. After 3 days of Tamoxifen or vehicle treatment, mixed tumours showed a wide range of variation in the ratios of the two clones, and exhibited a spectrum of chimaerism – in some regions one or the other clone predominated, while elsewhere we saw convoluted interfaces between the Myc^{high} and Myc^{low} populations as well as mixing of the Myc^{high} and Myc^{low} clones (Fig. 2a, SI Appendix Fig S2a-d, f). Importantly, none of these heterogeneous Myc^{high}/Myc^{low} Tamoxifen-treated tumours showed the self-limitation of growth characteristic of Myc^{high}-only tumours (Fig. 2b). However, like Myc^{high}-only tumours, and quite unlike Myc^{low} only tumours, Myc^{high}/Myc^{low} chimeric tumours were invasive, angiogenic and predominantly normoxic, exhibiting little necrosis and displaying a strong trend towards increased cellularity (p=0.054) (Fig. 2c, d, e, f, SI Appendix Fig. S2b, e). Focussing on areas of Myc^{high}/Myc^{low} clonal intermingling where any interclonal cooperation is likely to be most relevant, both tumour necrosis (Fig. 2c, SI Appendix Fig S2b) and tumour cell apoptosis (Fig. 2g, SI Appendix Fig S1n,Fig. S2e (bottom panels)) appeared profoundly suppressed to the low background levels characteristic of Myc^{low}-only lesions. Such suppression of necrosis was evident only in tumours of mice in which MycER^{T2} had been activated with Tamoxifen (SI Appendix S2b). Moreover, suppression of apoptosis in areas of Myc^{high}/Myc^{low} intermingling coincided spatially with suppressed expression of p19^{ARF} (Fig. 2i) as well as increased proliferation of the Myc^{high} cells over Myc^{low} ones, evident from increased numbers of IDU positive Myc^{high} nuclei (Fig. 2h, SI Appendix Fig. S2g). Hence, chimeric regions of Myc^{high}/Myc^{low} mammary tumours selectively exhibit the combined pro-tumourigenic attributes of each clone - the aggressive tumour stromal features, angiogenesis, low necrosis and high cellularity associated with Myc^{high} cells and the greatly reduced apoptosis exhibited by Myc^{low} tumour cells.

To investigate whether selection over the long-term can drive evasion of apoptosis in Myc^{high} tumours, we implanted tumour cells into recipient fat pads. 10 days later, at which time tumours are not yet palpable, we activated MycER^{T2} continuously for over 30 days, by which stage Myc^{low} and Myc^{low}/Myc^{high} tumours had grown to a size greater than 1.5cm^{3}. From the outset, Myc^{low} and Myc^{low}/Myc^{high} bi-clonal tumours grew progressively, with kinetics similar to vehicle-treated tumours. Moreover, the mixed clonal tumours retained polyclonality throughout, highlighting the fact that even over a long period of expansion neither Myc^{low}- nor Myc^{high} clones out-competed the other (Fig. 2j,2k left panel, SI Appendix Fig. S2h). By contrast, Myc^{high}-alone tumours either failed to grow at all or

showed a marked delay in growth (Fig. 2j, SI Appendix Fig. S2f). All those Myc^{high} tumours that did eventually grow out after prolonged Myc activation presented as mixtures of cells with and without MycER^{T2} expression (Fig. 2k (right panel), SI Appendix Fig. S2h). Notably, all the MycER^{T2} negative cells in these escaping tumours expressed the *Rosa26*^{mTmG} encoded membrane-targeted GFP marker, indicating that Cre-recombination had occurred in those cells: hence, absence of MycER^{T2} expression is likely to be due to a failure to recombine at both the *MycER*^{T2} and *GFP* allele. To test this hypothesis, we performed genomic DNA analysis on one of the two outgrowing Myc^{high} tumours and found that it showed a marked presence of unrecombined *R26C*$^{LSL\text{-}MER}$ allele (Fig. 2l). As these cells usually represent a very minor clone of *WM*$^{+}$*T* tumours (SI Appendix Fig. S1f), we conclude that extended selection of Myc^{high}-alone mammary tumours spontaneously regenerates the Myc^{low}/Myc^{high} bi-clonal phenotype, as the growth of Myc^{high} cells will be hampered until the small Myc^{low} population has sufficiently expanded (Fig. 2k, right panels, SI Appendix Fig S2h). The failure of two out of four Myc^{High} tumours to grow attests the necessity of Myc^{low}/Myc^{high} biclonality for tumour growth. No such selection for an unrecombined *R26C*$^{LSL\text{-}MER}$ allele was observed in bi-clonal tumours and even after long term treatment genomic DNA analysis revealed a similar proportion of both alleles in the tumour we analysed. (Fig. 2k (left panel), l). This, paired with the observation that Myc^{high} cells have a proliferative advantage in mixed clonal tumours (Fig. 2h), implies that admixtures of Myc^{low}/Myc^{high} clones converge towards an interdependent equilibrium.

Myc-induced apoptosis is mitigated by paracrine survival factors (29). Since suppression of apoptosis in Myc^{high} mammary tumour cells was most evident in areas of interface between Myc^{high} and Myc^{low} cells, we hypothesised that Myc^{low} clones secrete a paracrine survival signal that suppresses Myc^{high} 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 pro-survival role for Wnt is especially germane since, in a separate study we had noted that activation of high levels of Myc antagonises Wnt signalling. We thus tested if Wnt and Wnt-signalling was suppressed by Myc in our model system, which was confirmed by the loss of Wnt and Axin2 expression (Fig. 3a, SI Appendix Fig S3a-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 analysed the expression of endogenous Wnt1 following MycER^{T2}activation in otherwise normal mammary glands. *Wnt1* expression was potently inhibited after only four hours of Tamoxifen administration showing a general negative feedback of Myc signalling on Wnt1 expression (Fig 3b). To test whether these observations were also true in an unrelated breast cancer cell line, we generated

67NR cells that expressed a doxycycline-inducible *TRE-Myc* construct (67NR-Myc-RFP) (SI Appendix Fig S3c). Again, induction of Myc lead to an immediate down regulation of both Wnt1 and Axin2 (Fig. 3c). We thus decided to use this cell line to test the hypothesis that Wnt can counteract Myc-mediated apoptosis. We compared the behaviour of 67NR-Myc-RFP cells on media conditioned by L-cells expressing recombinant Wnt3a (L3-CM) versus media conditioned by control L-cells (L-CM). Upon induction of Myc on control media (L-CM), we saw, in addition to the loss of Wnt signalling, robust induction of the p53 stabilising protein $p19^{ARF}$ and the p53 target gene *Noxa* as well as widespread apoptosis, recapitulating our observations of Myc activation in $WM^{+}T$ $MycER^{T2}$ tumours (Fig. 3c-d). Conversely, Myc-induced apoptosis of 67NR-Myc-RFP cells was rescued by addition of media conditioned by L-cells expressing recombinant Wnt3a (L3-CM) (Fig. 3d). Further, Wnt-mediated rescue of 67NR-Myc-RFP cells on L3-CM correlated with markedly reduced expression of $p19^{ARF}$ and Noxa, consistent with general inhibition of p53-dependent Myc induced apoptosis by Wnt signalling (Fig. 3c). Hence, cell-free recombinantly expressed Wnt effectively suppresses programmed cell death in mammary tumour cells expressing high levels of Myc.

The maximum level of Myc a cell can tolerate without apoptosis is not an absolute value but highly dependent on cellular context, integrating intracellular stresses with extracellular survival signals. To gain better insight into the levels of Myc tolerated by breast cancer cells over the long-term with and without the addition of an exogenous survival signal, we took advantage of the fact that our 67NR-Myc-RFP cell population exhibits a broad range of Myc expression levels because the conditional Myc transgene (SI Appendix Fig. S3c) inserts in varied locations and copy numbers in the genomes of the recipient population of 67NR cells. Moreover, the level of Myc in each transfected 67NR-Myc-RFP cell will correlate with that cell's co-induced RFP fluorescence intensity. Doxycycline mediated induction of Myc caused quantitative loss of the high-RFP/high-Myc expressing cells, consistent 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, L-CM (control) vs L-CM + Dox). L3-CM Wnt3a-conditioned media mitigated selection against the high-Myc/high-RFP-expressing cells in a dose dependent manner (50/50 mix of LC-CM/L3-CM vs L3-CM alone), while L-CM did not (Fig. 3e). This is consistent with the notion that Wnt signalling 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-signalling can rescue cells from Myc-mediated apoptosis. Having shown a lack of Wnt-signalling in $WM^{+}T$ tumours (Fig 3a, SI Appendix Fig S3a-b), we set out to analyse the extent of Wnt-signalling in the mixed clonal tumours as well. To do so, we used immunofluorescent staining for nuclear β-catenin as a readout of active canonical Wnt-signalling. As expected, control ($WM^{-}T$ with or without

Tamoxifen, *WM^{+}T* without Tamoxifen) tumours exhibited abundant nuclear β-catenin in most cells, while *WM^{+}T* tumours exposed to Tamoxifen did not (Fig. 3f-g), presumably due to Wnt1 down-regulation (Fig. 3a, SI Appendix Fig S3a). Since *MMTV-Wnt1* driven tumours are dependent upon Wnt signalling for their maintenance (8), Myc-induced downregulation of Wnt1 effectively deprives the tumour cells of their own survival signal. By contrast, mixed *WM^{+}T/WM^{-}T* tumours 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 two clones (Fig. 3f-g), although this decreased with distance from the boundary with Myc^{low} cells (SI Appendix Figure S3e). Taken together these results confirm the notion that high levels of Myc deprive tumours of obligate Wnt survival signalling 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} tumour cells in mammary cancers.

4.4 Executable modelling identifies novel pharmacological vulnerabilities in heterogenous Myc mammary tumours

While it is possible that the obligate mutualism between Myc^{low} and Myc^{high} mammary tumour 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 signalling network perturbations. We constructed an initial executable model of breast cancer using publicly available data drawn from the literature (see SI Appendix Dataset S1 and S2). The network is modelled as a Qualitative Network (32), which is then simulated and analysed with the BioModelAnalyzer (BMA) tool (33) (http://biomodelanalyzer.org/). The process of building and testing this network model is illustrated in Fig. S4 and the result is an executable network encompassing proteins and transcription factors that contribute to the overall tumour 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 EGF receptor pathways, since these are two predominant drivers of oncogenic signalling in ER- and HER2- negative breast cancers that converge downstream on Myc and Ras effector pathways. And since aberrant crosstalk and excessive signalling flux across Myc and Ras pathways triggers tumour suppression, we included the p53 signalling pathway in our executable model. Finally, to encompass critical aspects of the interaction between tumour clones and their microenvironment, we simulated responses to hypoxia via the HIF1α pathway and consequent release of signalling 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 tumour are each represented by nodes in the executable network model (Fig. 4). Their behaviours 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 signalling 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^{high} conditions were reproduced by fixing the activity of Myc to be a constant value at an arbitrarily maximum level (see SI Appendix Dataset S5).

We first verified the behaviour 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 behaviour of the model is then compared with that observed by experiment (SI Appendix Dataset S3). In this way, we can test whether the model's behaviour is correct under a wide array of perturbations. We further tested the model against each of the monoclonal *MMTV-Wnt1* Myc^{low} and Myc^{high} tumours by comparing the predicted activity of nodes in the model with the activity observed experimentally (SI Appendix Dataset S4). We iteratively alternated between testing and refining the model until all the simulation results reproduced the experimental observations (SI Appendix Fig. 4). Comparisons were made predominantly against *in vitro* published experiments, so angiogenesis was not simulated. However, when modelling the *in vivo* tumours, we introduced an angiogenic node. Since the *in vivo* tumours were not HER2 driven, these nodes were removed.

We next simulated the effect of treatment on the mixed Myc^{high} and Myc^{low} tumours, including the predicted crosstalk 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 tumour separately by modifications to the relevant nodes level to represent these changes. For example, increasing the activity of the Wnt1 node to represent that there is a source of paracrine Wnt1 for the Myc^{high} cells from the Myc^{low} cells in the mixed tumours. This was in addition to the changes representing the mutations in each sub-clone. These node level changes are depicted in SI Appendix Dataset S5. We modelled 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 tumours would be more resilient to therapy, with higher proliferation and lower apoptosis than pure Myc^{high} or Myc^{low} clones for the same inhibiting drug (SI Appendix Fig. S5a, 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^{high} cells being resistant to cell cycle arrest but more vulnerable to apoptosis (SI Appendix Fig. S5a, 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 two different clones, as well as the proclivity of neoplastic systems to acquire compensatory or evolutionary resistance to monotherapies, we hypothesized that simultaneous application of two inhibitors would be therapeutically more effective. Accordingly, we simulated pairwise combinations of inhibitors across all major nodes (SI Appendix Fig. S6a-e, Fig. S7a-e). This generated a striking increase in the proportion of modelled therapies predicted to be successful, many eliciting marked impacts on both Myc^{high} plus Myc^{low} tumour cell populations (SI Appendix Fig. S8 a, 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. 5a, b, 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, SI Appendix Fig. 9b). The model predicted that inhibition of MEK alone would induce more apoptosis in the Myc^{high} than the Myc^{low} cells, and that in the Myc^{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^{high}, and so effectively treat both populations of cells.

To test the predicted therapeutic efficacy of this combination *in vivo*, we again used our bi-clonal Myc^{high}/Myc^{low} tumour model: high $MycER^{T2}$ was activated in the Myc^{high} subpopulation and 48 hrs later mice were treated with either the COX2 inhibitor Celecoxib or the MEK inhibitor PD0325901 alone, or with the two inhibitors combined. The tumours were then observed over a further 3 days. Each inhibitor alone offered some therapeutic benefit: COX2 inhibition slowed down overall tumour growth, while MEK inhibition stalled net tumour expansion (Fig. 5c). However, Celecoxib and PD0325901 in combination induced rapid tumour regression (Fig. 5c), resulting in residual masses almost devoid of tumour cells and comprising mainly haemorrhagic cysts (Fig. 5d, SI Appendix Fig. S10a, b). Detailed histological analysis of the few remaining regions harbouring residual tumour cells

showed a significant increase in apoptosis, most evident in the Myc^{high} cells, together with a decrease in proliferation, most notably in the Myc^{low} clones (Fig. 5f, Fig. S10c-f). These single and combined responses were consistent with our executable model's predictions (Fig. 5e, f). Lastly, 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 tumour to the triple inhibition was indistinguishable to the double inhibition (SI Appendix Fig. S10). This result is consistent with a further test of the specific Lox inhibitor Zileuton, which did not significantly synergise with MEK inhibition. (SI Appendix Fig. S10a-f). Due to fewer known side effects for Celecoxib compared to Licofelone the combination of PD0325901 and Celecoxib thus seems preferable.

Taken together these results show how computational modelling of solid biclonal tumours allowed us to devise a very potent therapeutic strategy.

5 Discussion

The component tumour cells of many human breast cancers exhibit persistent heterogeneity in Myc expression (12, 13, 16). Individually, ubiquitously Myc^{high} or Myc^{low} mammary tumour cells exhibit markedly different features, each of which significantly restrains tumourigenic potential. Mammary tumour 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 tumour spread. However, elevated Myc predisposes Myc^{high} cells to apoptosis. Consequently, Myc^{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, tumours comprising only Myc^{high} tumour cells effectively starve themselves of autocrine survival signals. Conversely, tumour monocultures of Myc^{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^{high}/Myc^{low} mixtures of tumour cells therefore appears to derive from the obligate complementarity of their two, individually self-limiting, biologies. While proximity of invasive and angiogenic Myc^{high} cells facilitates both growth and spread of Myc^{low} cells, reciprocal proximity of Myc^{low} cells provides sufficient Wnt1 to keep their more aggressive siblings alive. This relationship becomes particularly clear when trying to grow tumours solely comprising Myc^{high} cells. The observed escapee tumours convert to a heterogeneous phenotype through outgrowth of a minor $MycER^{T2}$ negative clone (Fig. 2j, k). The observation that more than half of the cells comprising the escapee tumour we analysed genomically did not express $MycER^{T2}$ (Fig. 2l) suggests the need for a significant amount of Myc^{low} cells to support Myc^{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 tumour supportive stroma by Myc^{high} clones (Fig. 1d-e, Fig. 2e-f, SI Appendix Fig. S1k, S2e). Such mutualism explains why in human Myc^{high}/Myc^{low} mixed mammary tumours, Myc^{high} clones typically do not rapidly overgrow Myc^{low} clones (13). A similar role for Wnt-secreting supportive niches in tumour evolution and maintenance has recently been identified in lung adenocarcinomas (7), indicating that such mutualism may be a common feature of tumour cells expressing high levels of Myc. Furthermore, when we switched on $MycER^{T2}$ in an untransformed mammary gland, we equally observed rapid loss of Wnt1 and inhibition of Wnt signalling (Fig. 3b). This indicated that the mutual exclusivity between expression of high levels of Myc and Wnt ligands is not idiosyncratic for tumours, but rather a general phenomenon. This is most likely part of an inherent tissue organisation, where proliferative niches are organised in proliferative (Myc^{high}/Wnt^{low}) cells and

supportive (Myc^{low}/Wnt^{high}) cells. Myc-heterogenous mammary tumours 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 downregulate the secreted Wnt inhibitors DKK1 and SFRP1 (36), implying that Myc acts to sensitize cells to Wnt signalling. Myc^{high} cells are thus dependent on Wnt signalling, but create a more permissive environment for this very signalling to occur. We noted a similar complexity in the interplay between Myc and Wnt is on the level of the apoptotic machinery. We observed a clear quenching of the $p19^{Arf}$→ p53 axis in Myc-expressing cells when treated with Wnt3a conditioned media (L3-CM) but recorded an unexpected upregulation of PUMA. However, this was inconsequential in terms of inducing apoptosis (Fig. 3d, SI Appendix Fig. S3d). This shows that the downstream signalling of large signalling hubs such as Wnt and Myc is highly contextual, as is the resulting phenotypic outcome, such as cell death or proliferation.

In both *Drosophila* and mouse development, cells expressing high levels of Myc are reported to exhibit super-competition – they not only outgrow their Myc^{low} neighbours but also actively eliminate them. In the case of mixed Myc^{high}/Myc^{low} mammary tumours, such aggressive supercompetition would, of course, be expected to expeditiously eradicate the Wnt1-producing Myc^{low} cells that are the principal source of Wnt1 survival signals keeping the Myc^{high} cells alive. However, we see no evidence of such super-competition in our mixed Myc^{high}/Myc^{low} mammary tumours: rather, the principal determinant of Myc^{high} cell fate appears not to be their innate competitiveness but their increased dependency on Myc^{low}-generated survival signals. It may be that reported instances of super-competition in mice and *Drosophila* development arise in situations where the proclivity of Myc^{high} cells to undergo apoptosis is abrogated, for example by abundant survival factors. Likewise, it is possible that Myc-driven super-competition is a significant factor in evolution of tumours in which Myc-induced apoptosis is circumvented by secondary, anti-apoptotic mutation.

The notion that certain aspects of the oncogenic process might expose novel vulnerabilities in tumour cells underpins the rationale for selective cancer therapies and the obligate mutualism we observe between Myc^{high} and Myc^{low} mammary cancer cells is one such example. To explore this case, we generated a computational model of the Myc/Ras/p53 signalling network in breast cancer cells. Starting from a general executable model of breast cancer, we added Wnt1 as a constant node and overlaid high Myc activity. Our initial simulation predicted levels of proliferation higher than those seen in Myc high tumours *in vivo*, suggesting that some level of interruption in Wnt signalling was at play in the tumours. This was experimentally confirmed and shown to be due to Myc-dependent suppression of Wnt expression. This was then factored back in to the computational model of both

Myc^{high}-only tumours and Myc^{high}/Myc^{low} heterogenous tumours, to accurately predict the underlying set of mechanistic rules that indicated bi-clonal mutualism of the Myc^{high}/Myc^{low} mixed tumours.

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 bi-clonality of the tumours. Thus, the model predicted that co-inhibition of MEK and COX2 would exert a more potent therapeutic impact on both clonatypes than their individual inhibition would on either individual clonatype. The model correctly predicted the augmented response of the individual clones to various inhibitor combinations, including the sharp drop in proliferation of Myc^{low} cells when exposed either to MEK inhibition alone or to co-inhibition of MEK and COX2 together, and the strong apoptotic response of Myc^{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^{high} tumour 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^{low} cells during treatment will progressively curtail the survival of Myc^{high} clones due to loss of Wnt1 signalling. Future development of the model could be extended to accommodate the shifting interactions that follow from changes in clonal composition of the tumour during treatment. A further benefit of our executable modelling 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 pro and anti-apoptotic signals: MEK inhibition blocks anti-apoptotic signalling, and so predominantly affects the Myc^{high} clone, whereas COX2 inhibition increases pro-apoptotic signalling, thereby reinforcing the impact of MEK inhibition on the Myc^{low} cells.

As our understanding of normal tissue organisation and its pathogenic equivalent in tumours 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 tumour cell signalling – 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.

6 Materials and Methods

Mice and *in vivo* procedures

All treatments and procedures of mice were conducted in accordance with protocols approved by the by 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^{T2}/ Rosa26-mTmG/ MMTV-Wnt1, Rosa26-CAG-lox-STOP-lox-MycER^{T2}.* MycER^{T2} was activated by administration of tamoxifen at 1mg/20 g *i.p.* twice daily, id administration period <12h. IDU was administered at 1 mg/20 g. More details in the SI Appendix.

Immunohistochemistry and Immunofluorescence

Standard protocols were followed for immunohistochemistry and immunofluorescence. For details see SI Appendix. The following primary antibodies were used: HIF1α - SC10790, 1:50; CD31 - ab28364, 1:100; Cleaved Caspase 3 - cs9664, 1:1000; oestrogen receptor alpha - SC-542, 1:50; IDU – BD347580, 1:100; p19^{ARF} - sc-32748, 1:100, β-catenin - BD610153, 1:250, Myc - ab32072, 1:1000; GFP - ab6556, 1:500; p53 – Leica CM5p, 1:500. Unless otherwise stated, quantifications were carried out for at least three visual field on at least three independent biological replicates.

Quantitative real-time PCR

SBYR^{TM} green master mix (Thermo Fisher Scientific) based qRT-PCR was performed after RNA extraction and cDNA 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. gDNA 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 recombination at the R26C^{MER} allele was tested via quantitative Digital Droplet PCR on the QX 200 Droplet Reader (BioRad), following the manufacturer's standard protocol. For primers and probes see SI Appendix.

Western Blotting

Samples were prepared using standard protocols. Proteins were labelled 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-CM5p, 1:2.000; actin, Santa

Cruz sc-69879, 1:5.000; Wnt1, AbCam ab15251, 1:1.000: CC3, Cell Signalling Technologies, 1:1.000; Myc, ab32072, 1:1000, secondary antibody: goat-anti rabbit, sc-2301, 1:7.500.

Therapeutic studies

Tumours were generated as described above at ratios of Myc^{high}/Myc^{low} clones of 30%:70%. Tamoxifen treatment started at a size of ca. 0.5 cm^3 and from day 3 drugs were administered via oral gavages. Tumours were measured daily and IDU was injected 2h prior to culling. 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 BioModelAnalyzer (BMA) tool (SI Appendix, Methods) (33) (http://biomodelanalyzer.org/).

The network (Fig. 4) consists of nodes representing the decision-making machinery of the cell; the genes, proteins and transcription factors e.g. Myc, and those representing the overall cell phenotype; proliferation and apoptosis. Corresponding genes for each node are given in SI Appendix Dataset S6. The interactions between these components are represented by edges, in the network model.

Each node has an associated Target Function, that determines the value it takes, based on the values of nodes that connect to it (see SI Appendix Dataset S2). These connections, and the Target Functions that describe their interactions, are drawn from the literature (see SI Appendix Dataset S1) of experiments on breast cancer cell lines.

Most nodes use the default BMA Target Function, $avg(pos) - avg(neg)$, which compares the average state of all the nodes that connect to the current node via an activating, i.e. positive edge, to the average state of all the nodes that connect via an inhibitory, i.e. negative edge. In cases where there were no positive inputs, we assumed some constitutive activity of the node by setting the target value to be some constant value minus the average negative inputs. More complex target functions were used when the literature evidence around the interaction demanded it (see SI Appendix Dataset S2).

The model was built and tested using literature data, and literature supporting the network is collated in SI Appendix Dataset S1, Target Functions in SI Appendix Dataset S2, and experiments used to test the model in SI Appendix Dataset S3 & S4, with more details about this process in SI Appendix Methods. The process of building and testing the network model is illustrated in SI Appendix Fig. S4. We use BMA to predict cell behaviour by searching for stable states, or attractors. These capabilities

can be accessed through the BMA GUI at http://biomodelanalyzer.org or through the command-line with a local instance https://github.com/Microsoft/BioModelAnalyzer.

Exhaustive computational search for effective drug inhibitor combinations

To assess efficacious combinations of targeted therapies, we performed single and pairwise inhibition (set Target Function to zero) of every single node or pair of nodes in the network to represent the effects of drug inhibition, in addition to the background of existing mutations shown in SI Appendix Dataset S5. These perturbations are shown in the axes of the heatmaps in SI Appendix Figures S5a, S5b, S6, S7. Ranking our various potential targets for perturbation by the number of available inhibitors according to the Drug-Gene Interaction Database (37) accessed using the package rDGIdb (38) (SI Appendix Fig. S9c), we considered only those nodes for which there was at least one drug known to interact with it. For each perturbation, we find the stable state of the network, or the smallest range to which we can constrain each node. In order to compare different tumour clones, we also added a background set of mutations that are unaffected by the above mutations. If BMA could not restrict the value of the node to a single value, for example if there is an oscillation between two levels of activity, we used the mean of the minimum and maximum value. Visualisation for SI Appendix Fig. S5, S6, S7, S8 and S9 was performed using R (39) and ggplot2 (40). The BMA network model (JSON file) can be found at http://www3.bioc.cam.ac.uk/fisher/.

7 Funding

This work was generously supported by Cancer Research UK to GIE (programme grant number A12077), the Francis Crick Institute which receives its core funding from Cancer Research UK (FC001223), the UK Medical Research Council (FC001223), and the Wellcome Trust (FC001223) to PK, Trinity College Cambridge to PK, the Kay Kendall Leukaemia Fund KKL1045 to GIE, the Wellcome Trust Mathematical Genomics and Medicine Program (102274/Z/13/Z), the Glover Research Fund hosted at the Department of Biochemistry, University of Cambridge to MC and Microsoft Research to JF.

8 Acknowledgements

We thank Alessandra Perfetto for help with animal husbandry, Ben Hall and David Shorthouse for valuable discussions on analysing Qualitative Network models, Victoria Wang for help with modelling the Ras pathway and Dan Lu for helpful discussions. Jasmin Fisher is a member of the Mark Foundation Institute for Integrated Cancer Medicine at the University of Cambridge.

9 References

1. Liu W, et al. (2009) Copy number analysis indicates monoclonal origin of lethal metastatic prostate cancer. *Nat Med* 15(5):559–565.
2. Gerlinger M, et al. (2012) Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing. *N Engl J Med* 366(10):883–892.
3. Navin N, et al. (2011) Tumour evolution inferred by single-cell sequencing. *Nature* 472(7341):90–95.
4. Kreso A, et al. (2013) Variable Clonal Repopulation Dynamics Influence Chemotherapy Response in Colorectal Cancer. *Science (80-)* 339(6119):543–548.
5. Sun C, et al. (2014) Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma. *Nature* 508(7494):118–122.
6. Bruna A, et al. (2016) A Biobank of Breast Cancer Explants with Preserved Intra-tumor Heterogeneity to Screen Anticancer Compounds. *Cell* 167(1):260-274.e22.
7. Tammela T, et al. (2017) A Wnt-producing niche drives proliferative potential and progression in lung adenocarcinoma. *Nature* 545(7654):355–359.
8. Cleary AS, Leonard TL, Gestl SA, Gunther EJ (2014) Tumour cell heterogeneity maintained by cooperating subclones in Wnt-driven mammary cancers. *Nature* 508(7494):113–117.
9. Marusyk A, et al. (2014) Non-cell-autonomous driving of tumour growth supports sub-clonal heterogeneity. *Nature* 6(2):54–58.
10. Kortlever RM, et al. (2017) Myc Cooperates with Ras by Programming Inflammation and Immune Suppression. *Cell* 171(6):1301–1315.
11. Vita M, Henriksson M (2006) The Myc oncoprotein as a therapeutic target for human cancer. *Semin Cancer Biol* 16(4):318–330.
12. Glöckner S, Buurman H, Kleeberger W, Lehmann U, Kreipe H (2002) Marked intratumoral heterogeneity of c-myc and cyclinD1 but not of c-erbB2 amplification in breast cancer. *Lab Invest* 82(10):1419–1426.
13. Heselmeyer-Haddad K, et al. (2012) Single-cell genetic analysis of ductal carcinoma in situ and invasive breast cancer reveals enormous tumor heterogeneity yet conserved genomic imbalances and gain of MYC during progression. *Am J Pathol* 181(5):1807–1822.
14. Pavelic ZP, Pavelic K, Carter CP, Pavelic L (1992) Heterogeneity of c-myc expression in histologically similar infiltrating ductal carcinomas of the breast. *J Cancer Res Clin Oncol* 118(1):16–22.
15. Gupta N, et al. (2017) High Myc expression and transcription activity underlies intra-tumoral heterogeneity in triple-negative breast cancer. *Oncotarget* 8(17):28101–28115.
16. Yang F, et al. (2017) Intratumor heterogeneity predicts metastasis of triplenegative breast cancer. *Carcinogenesis* 38(9):900–909.
17. Johnston LA (2014) Socializing with MYC: Cell competition in development and as a model for premalignant cancer. *Cold Spring Harb Perspect Med* 4(4):1–15.
18. Suijkerbuijk SJE, Kolahgar G, Kucinski I, Piddini E (2016) Cell competition drives the growth of intestinal adenomas in Drosophila. *Curr Biol* 26(4):428–438.

19. Tsukamoto AS, Grosschedl R, Guzman RC, Parslow T, Varmus HE (1988) Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell* 55(4):619–625.

20. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L (2007) A global double-fluorescent Cre reporter mouse. *genesis* 45(9):593–605.

21. Zhang X, et al. (2005) Estrogen receptor positivity in mammary tumors of Wnt-1 transgenic mice is influenced by collaborating oncogenic mutations. *Oncogene* 24(26):4220–31.

22. Evan GI, et al. (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 69(1):119–28.

23. Evan GI, Vousden KH (2001) Proliferation, cell cycle and apoptosis in cancer. *Nature* 411(May):342–348.

24. Evan G, Littlewood TD (1998) A Matter of Life and Cell Death. *Science (80-)* 281(5381):1317–1322.

25. Zindy F, et al. (1998) Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev* 12:2424–2433.

26. Muthalagu N, et al. (2014) BIM Is the Primary Mediator of MYC-Induced Apoptosis in Multiple Solid Tissues. *Cell Rep* 8(5):1347–53.

27. Pelengaris S, Khan M, Evan GI (2002) Suppression of Myc-induced apoptosis in beta cells exposes multiple oncogenic properties of Myc and triggers carcinogenic progression. *Cell* 109(3):321–34.

28. Lowe SW, Cepero E, Evan G (2004) Intrinsic tumour suppression. *Nature* 432(7015):307–315.

29. Harrington EA, Bennett MR, Fanidi A, Evan GI (1994) c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO J* 13(14):3286–3295.

30. Pećina-Šlaus N (2010) Wnt signal transduction pathway and apoptosis: a review. *Cancer Cell Int* 10(1):22.

31. You Z, et al. (2002) Wnt signaling promotes oncogenic transformation by inhibiting c-Myc-induced apoptosis. *J Cell Biol* 157(3):429–440.

32. Schaub MA, Henzinger TA, Fisher J (2007) Qualitative networks: a symbolic approach to analyze biological signaling networks. *BMC Syst Biol* 1:4.

33. Benque D, et al. (2012) BMA: Visual Tool for Modeling and Analyzing Biological Networks. *Comput Aided Verif*:686–692.

34. Willert K, et al. (2003) Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 423(6938):448–452.

35. Farin HF, et al. (2016) Visualization of a short-range Wnt gradient in the intestinal stem-cell niche. *Nature* 530(7590):340–343.

36. Cowling VH, D'Cruz CM, Chodosh LA, Cole MD (2007) c-Myc Transforms Human Mammary Epithelial Cells through Repression of the Wnt Inhibitors DKK1 and SFRP1. *Mol Cell Biol* 27(14):5135–5146.

37. Wagner AH, et al. (2016) DGIdb 2.0: Mining clinically relevant drug-gene interactions. *Nucleic Acids Res* 44(D1):D1036–D1044.

38. Thurnherr T, Singer F, Stekhoven DJ, Beerenwinkel N (2016) Genomic variant annotation

workflow for clinical applications. *F1000Research* 5(0):1963.

39. R Core Team (2017) R: A Language and Environment for Statistical Computing. Available at: https://www.r-project.org/.

40. Wickham H (2016) *ggplot2: Elegant Graphics for Data Analysis* (Springer-Verlag New York) Available at: http://ggplot2.org.

10 Figure Legends

Figure 1. Myc activation leads to rapid reorganization of the tumour and its stroma, while limiting growth through tumour suppressive pathways.

All tumours were analysed after three days of sustained treatment with Tamoxifen (100 μg/mouse twice daily) or oil (vehicle) for the respective controls. Abbreviations in graphs: C=controls, M=Mycer^{T2}+Tamoxifen. Error bars represent standard deviation.

a) Gross morphological features and histological appearance of *WM^{+}T* tumours compared to controls (controls in all following experiments are: *WM^{-}T* + Tamoxifen and + vehicle, *WM^{+}T* plus vehicle).
b) Cellularity of *WM^{+}T* tumours and controls (n = 6/9 respectively).
c) Quantification of necrosis of *WM^{+}T* tumours and controls (n = 6/9 respectively).
d) Quantification of hypoxia in *WM^{+}T* tumours and controls, (n = 6/9 respectively).
e) Average area of blood vessel, in *WM^{+}T* tumours and controls (n = 6/9 respectively).
f) Caliperimetic measurement of tumour growth of *WM^{+}T* tumours and controls, as percentage change from start of treatment. (n = 6 *WM^{+}T* tumours and 11 controls).
g) Quantification of proliferation, by IDU incorporation, in *WM^{+}T* tumours and controls (n = 7/14 respectively).
h) Quantification of cell death, by presence of cleaved caspase 3, in *WM^{+}T* tumours and controls (n = 6/9 respectively).
i) Western Blot analysis of p53 and actin as a loading control in whole tissue lysates of two representative *WM^{+}T* tumours and controls. Bands were quantified by comparison with the loading control and are represented as fold change relative to average of the control tumours.
j) Quantitative realtime PCR of whole tissue mRNA extracts from *WM^{+}T* tumours and controls for indicated genes and GAPDH as a housekeeping gene (n = 6/11 respectively).
k) Representative immunofluorescent staining for p19^{ARF} (red), EGFP (green) and DNA (Hoechst, blue), of *WM^{+}T* tumours 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 the median. p-values are based on Student's t-test, n.s. = not significant, * p<0.05, **p<0.01, ***p<0.001

Figure 2. Myc^{High} and Myc^{Low} cells exhibit clonal mutual interdependence.

Unless otherwise indicated, analysis of mixed *WM^{+}T/ WM^{-}T* tumours was carried out after three days of treatment with Tamoxifen (100 μg/mouse twice daily) or oil (vehicle) for the respective controls. Abbreviations in graphs: C= mixed tumours + vehicle, T= mixed tumours + Tamoxifen. Error bars represent standard deviation.

a) Representative picture of the gross morphology of mixed (in this case 30%/70%) *WM^{+}T/ WM^{-}T* tumours and representative fluorescent image of mixed tumour tissue.
b) Caliperimetic measurement of tumour growth of mixed *WM^{+}T/ WM^{-}T* tumours and oil controls.
c) Necrotic burden of mixed *WM^{+}T/ WM^{-}T* tumours.
d) Quantification of the cellularity of mixed *WM^{+}T/ WM^{-}T* tumours.
e) Quantification of hypoxia in mixed *WM^{+}T/ WM^{-}T* tumours.
f) Average area of blood vessel in mixed *WM^{+}T/ WM^{-}T* tumours.
g) Quantification of cell death, by presence of cleaved caspase 3, in mixed *WM^{+}T/ WM^{-}T* tumours. Clones were distinguished via GFP staining.
h) Quantification of proliferation, by IDU incorporation, in mixed *WM^{+}T/ WM^{-}T* tumours. Clones were distinguished via GFP staining
i) Immunofluorescent staining for GFP (*WM^{+}T*) and p19^{ARF} of intermingled areas in mixed *WM^{+}T/ WM^{-}T* tumours and controls (In panels d-i: n = 8 mixed *WM^{+}T/ WM^{-}T* tumours treated with tamoxifen and 5 vehicle controls).
j) Caliperimetic measurement of tumour growth of individual *WM^{+}T, WM^{-}T*, mixed *WM^{+}T/ WM^{-}T* mixed tumours and controls on long term tamoxifen treatment.
k) Representative images of immunofluorescence staining for Myc, dtTomato, and GFP on frozen tissue sections from long-term treated *WM^{+}T* and mixed *WM^{+}T/ WM^{-}T* tumours showing loss of Myc in the *WM^{+}T* tumours, but no such phenomenon in the mixed *WM^{+}T/ WM^{-}T* tumours.
l) Digital Droplet PCR on genomic DNA comparing the recombination status of the *R26C^{MER}* allele in 20% *WM^{+}T* and 100% *WM^{+}T* tumour after long-term tamoxifen treatment (n = 1 20% *WM^{+}T* and 1 100% *WM^{+}T* tumour)

For Box and Whisker plots, the error bars represent min. to max values, the box represents the interquartile range and the horizontal line the median. p-values are based on Student's t-test: * p<0.05, **p<0.01, ***p<0.001. In panel i) the Myc^{low} clone is outlined by a dotted line. Areas that are

neither GFP positive nor marked up by dotted lines are non-tumour tissues such as stroma and necrotic areas.

Figure 3. Myc overexpression reduces Wnt expression creating paracrine dependency.

a) Western Blot analysis for Wnt1 and actin protein levels in two representative *WM^{+}T* and *WMT* control tumours. Bands were quantified by comparison with the loading control and are represented as fold change relative to average of the control tumours.
b) qRT-PCR for *Axin 2* in normal mammary glands of *ZP3-Cre;RCAG-MycER^{T2}* mice or *ZP3-Cre* control mice (n = 5/5).
c) Quantitative real time PCR for indicated targets in cellular extracts from 67NR-Myc-RFP cell lines treated for 24h with Doxycycline and either L-CM or L3-CM both normalised to respective untreated controls with L-CM alone (n = 3).
d) Flow cytometric analysis of cell death in 67NR-Myc-RFP cells after a 24h treatment with Doxycycline and either L-CM or L3-CM both normalised to respective untreated controls with L-CM alone (n = 3).
e) Representative flow cytometric analysis showing a dose dependent retention of high-RFP/high-Myc expressing 67NR-Myc-RFP clones during a 72h treatment of Doxycycline and mixtures of L-CM and L3-CM as indicated in the figure.
f) Quantification of β-catenin positive nuclei on *WM^{+}T*, *WM^{-}T* control tumours and mixed *WM^{+}T/ WM^{-}T* tumours stained for β-catenin (n: Myc^{low} = 4, Myc^{high} and mixed = 3).
g) Immunofluorescence for nuclear ß-catenin in *WM^{+}T*, *WM^{-}T* control tumours and mixed *WM^{+}T/ WM^{-}T* tumours.

For Box and Whisker plots, the error bars represent min. to max values, the box represents the interquartile range and the horizontal line the median. p-values are based on Student's t-test: * p<0.05, **p<0.01. For bar charts error bars represent standard deviation.

Figure 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 behaviour. Arrows represent activating interactions, while bars represent inhibition.

Figure 5. Change in effect of therapy when adding a second inhibitor predicted by the computational model, and successful MEK and COX2 inhibition combination therapy *in vivo*.

a-b) Rows and columns of heatmaps are coloured by pathway to which nodes which are treated belong, the pathway categories are laid out in Dataset S7. Mean change in proliferation (a) or apoptosis (b) across both clones when adding a second inhibitor to an inhibitor already shown to be effective in monotherapy. In the heatmap y-axis shows first inhibitor, x-axis the inhibitor which is added in combination. Grey boxes are combinations which are nonsensical (two different inhibitions of the same node), or cause apoptosis above 3 in the healthy cells. Inhibition of PHD2 or VHL cause high apoptosis with every partner other than each other and are removed as this otherwise prevents clustering of the heat maps.

c) Caliperimetic measurement of tumour growth of mixed *WM^{+}T/ WM^{-}T* tumours during three days of treatment with Tamoxifen (100 μg/mouse twice daily) followed by four days treatment with Tamoxifen and drug combinations (Celecoxib, 20 mg/kg/day, PD0325901 10 mg/kg/day). Measurements are normalised to the beginning of the drug treatment course as tumours at this stage were at different sizes. (n = 4-5, error bars represent standard deviation).

d) Representative picture of the gross morphology of mixed *WM^{+}T/ WM^{-}T* tumours after the treatment described above

e) Quantification of cell death in mixed *WM^{+}T/ WM^{-}T* tumours as percentage of CC3 positive pixels in the individual clones (n: Control, PD0325901, = 4, Celecoxib, Celecoxib + PD0325901 = 5).

f) Quantification of proliferation in mixed *WM^{+}T/ WM^{-}T* tumours by automated counting of IDU positive nuclei over total nuclei in the individual clones. (n: Control, PD0325901, = 4, Celecoxib, Celecoxib + 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 the median. p-values are based on Student's t-test: * $p<0.05$.