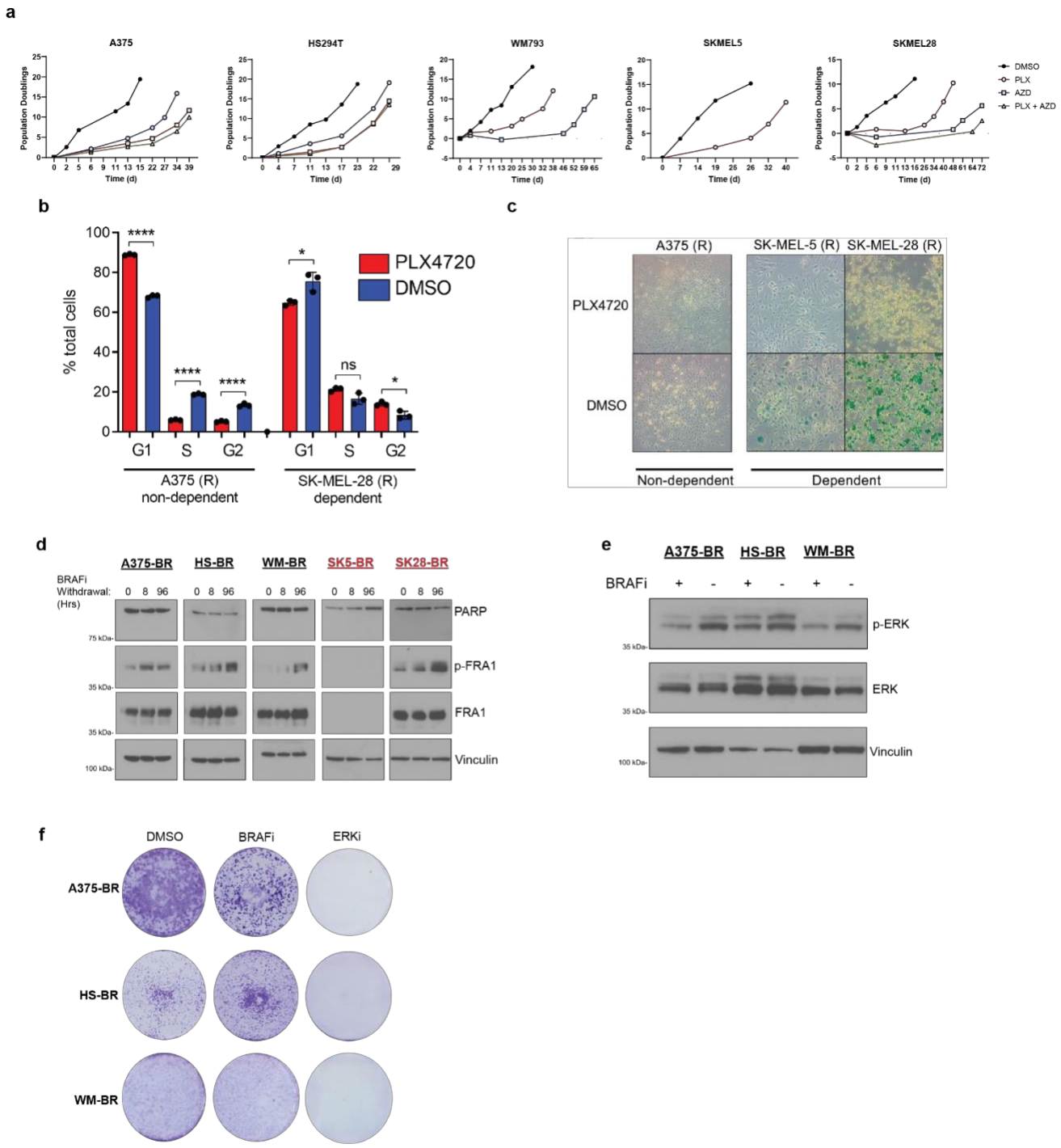
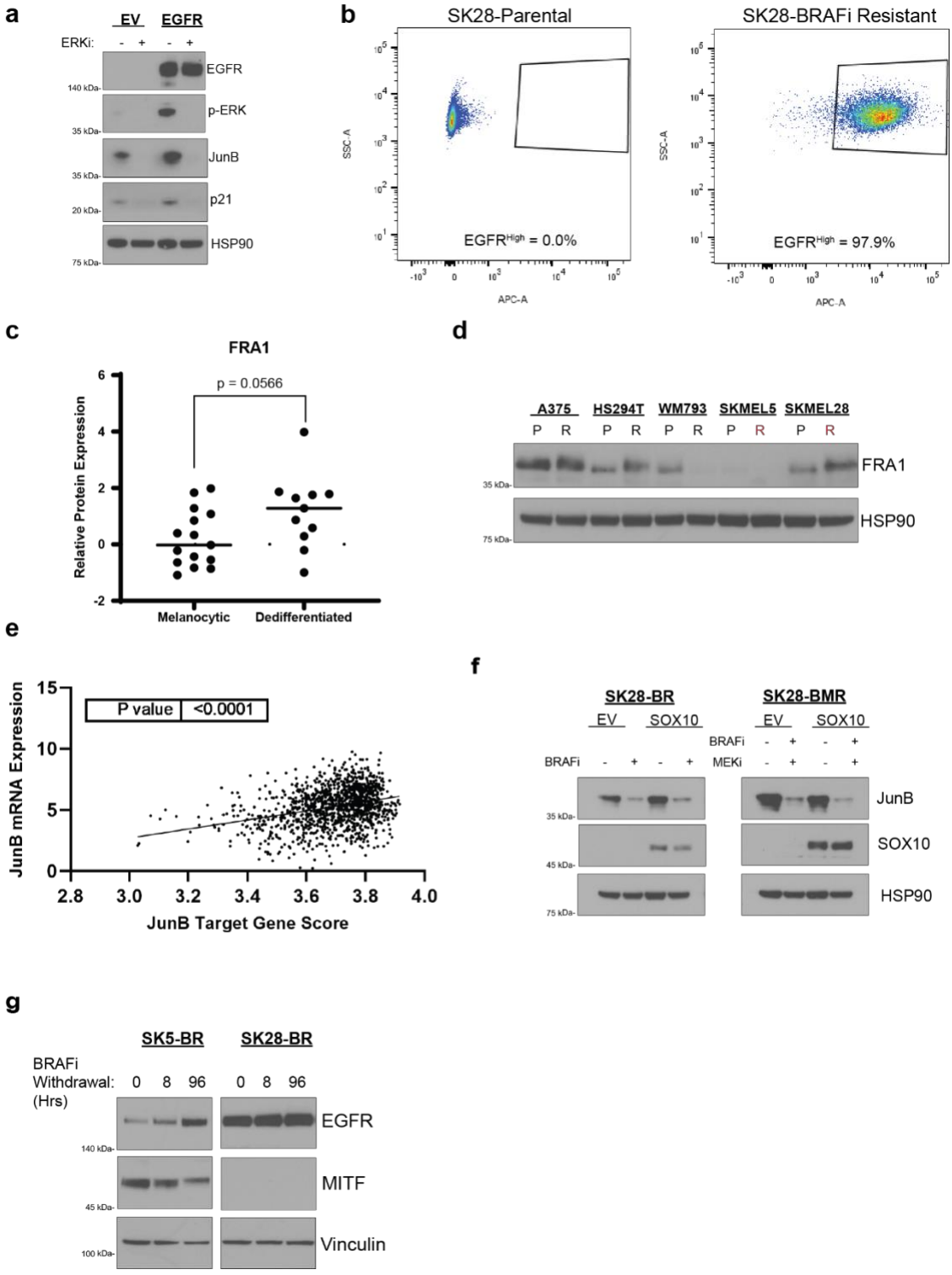
Supplementary Information

Supplementary Figures 1-4

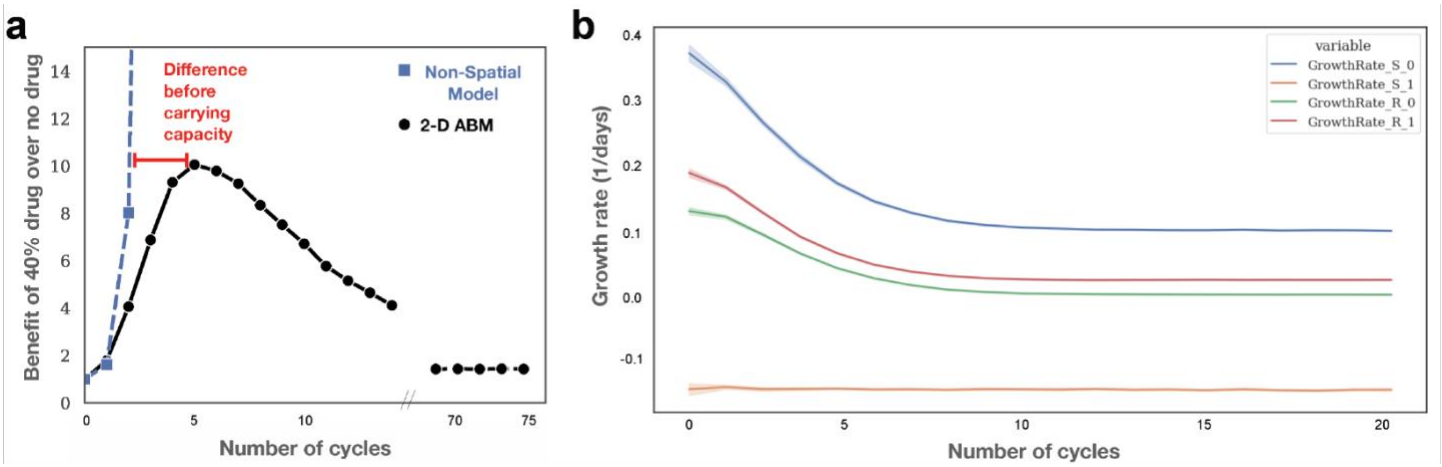
Discussion on Mutational Rates



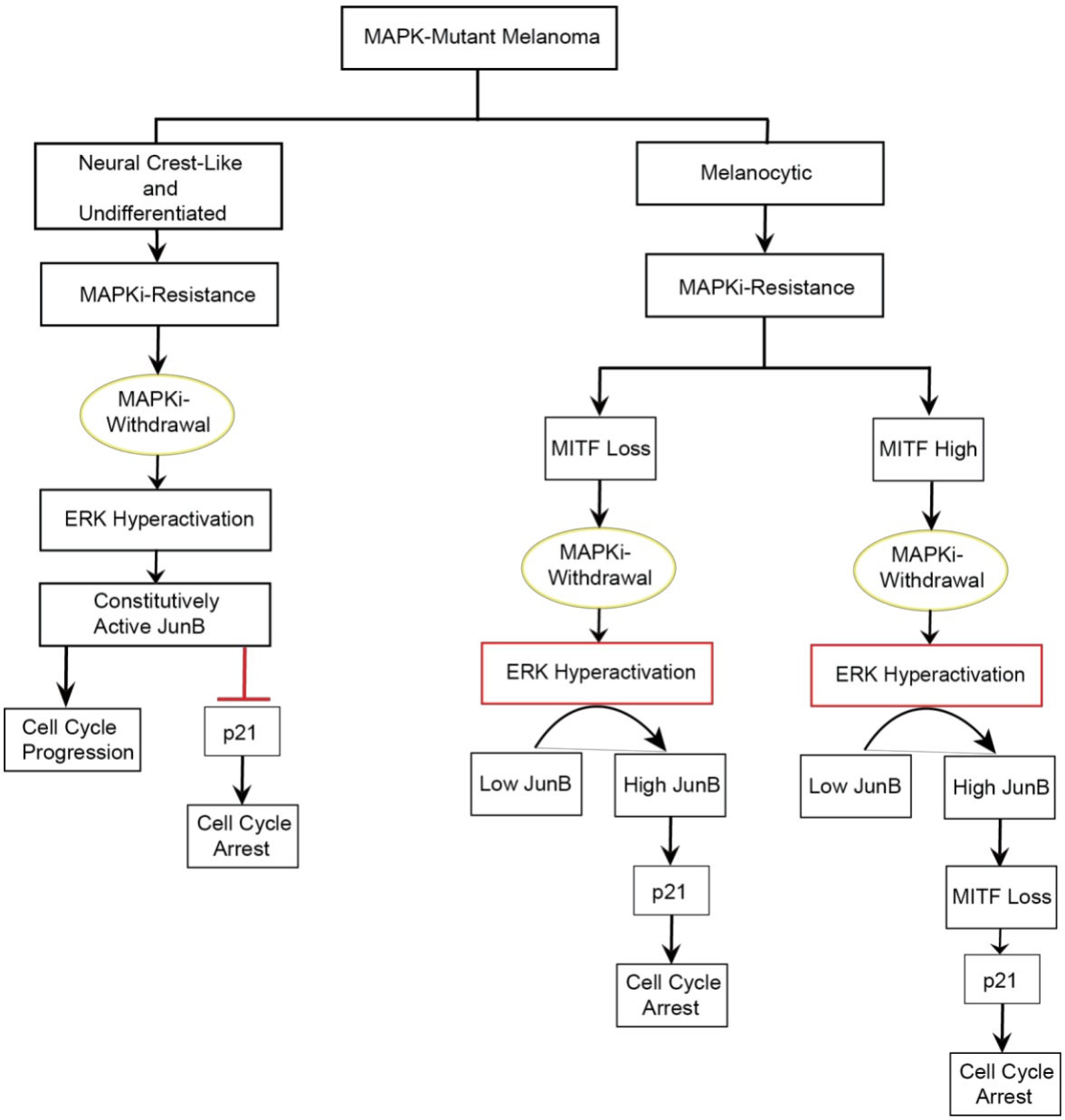
Supplemental Fig 1: a, Population doublings of indicated cell lines following continuous treatment with DMSO, 3 μM BRAFi (PLX4720), 3 μM MEKi (AZD6244), or 3 μM BRAFi + 3 μM MEKi . **b,** A375 and SKMEL28 BRAFi-resistant lines were treated with DMSO or PLX4720 for 3 days and cell cycle analysis was performed as described in the Material and Methods. Two-tailed unpaired t-test, p-values; * <0.05, ****<0.0001. The percent of total cells in each phase is shown. The data represent three independent experiments (*n* =3). Data are presented as mean ± SEM. **c,** A375, SKMEL-5 and SKMEL-28 PLX4720-resistant lines were plated at low density and treated with PLX4720 or DMSO the following day. After 14 days, cells were stained for SA b-galactosidase activity as described in Materials and Methods. **d,** Continuation of western blot experiment seen in Fig. 2d. **e,** Western blot of indicated proteins in BRAFi-resistant (BR) A375, HS294T, and WM793 cells treated with DMSO or 3 μM BRAFi (PLX4720) for 4 hrs. **f,** BRAFi-resistant A375 (A375-BR), HS294T (HS-BR), and WM793 (WM-BR) cells were seeded at 10,000 cells/ml and treated with DMSO, 3 μM BRAFi (PLX4720) , or 3 μM ERKi (SCH772984). Drug was refreshed every 72 hrs. Crystal violet staining was performed when the DMSO treatment group reached confluency. **c-f,** The data are representative of three biologically independent experiments (*n* = 3)



Supplemental Fig 2: a, SKMEL28 cells transduced with empty (EV) or EGFR (EGFR) expression constructs were treated with DMSO or 50 nM SCH772984 (ERKi) for 96 hrs before western blot analysis was performed for the indicated proteins. **b,** Cell surface expression of EGFR in parental or BRAFi-resistant SKMEL28 cells was detected with an APC-conjugated EGFR antibody and analyzed via flow cytometry. **c,** FRA1 protein level analysis of melanocytic (n = 17) and dedifferentiated (n = 9) MAPK-mutant melanoma cell lines using the DepMap Proteomics dataset. Two-tailed unpaired t-test, p-values included in figure. **d,** Continuation of western blot experiment seen in Fig. 3c. **e,** Depmap correlation analysis of the JunB Target gene score and JunB mRNA for every cell line represented in the Expression dataset. **f,** SKMEL28 cells were transduced with an empty (EV) or SOX10 (SOX10) expression vector before being treated with DMSO or 3 μM of the indicated MAPKi treatment for 96 hrs in the presence of 100 ng/ml doxycycline. Western blots were performed on the cell lysates to probe for the indicated proteins. **g,** Continuation of western blot experiment seen in Fig. 2d. **a, d, f, g** The data are representative of three biologically independent experiments (*n* = 3).



Supplemental Fig 3: a, Benefit of applying 40 percent time in drug (Number of cells treated with no drug / Number of cells treated with 40% time in drug) in the well-mixed model (blue line) compared to the spatial ABM (black line). The divergence between well-mixed prediction and spatial ABM simulation occurs well before carrying capacity effects play a role. **b**, Growth rate of sensitive and resistant populations in (1) and out (0) of drug as competition for space increases with further dosing cycles. This effect is asymmetric across cell lines and environments, such that the optimal dose itself changes as seen in the main text. Data is presented as such; mean is center solid line and the 95% confidence interval is the shaded band above and below the mean.



Supplemental Fig 4. Diagram of proposed mechanism underlying ERK-JunB-p21-induced drug dependence

A SIMPLE MODEL OF N SUBPOPULATIONS

Consider a simple model with N different cell populations. The i^{th} cell population grows exponentially. The per capita growth rate of population i is given by $k_{i,OFF}$ in the absence of drug and by $k_{i,ON}$ in the presence of drug, which we assume to be at a fixed concentration when present. The entire metapopulation evolves according to

$$\dot{n} = G_d n, \tag{S1}$$

where n is a vector with components $n_i(t)$ (the number of cells of type i at time t) and G_d is an N-by-N square matrix whose diagonal entries are $k_{i,ON}$ (or $k_{i,OFF}$) for $i = 1, 2, ...N$ and whose off-diagonal entries correspond to interconversions between different cell types. The solution to Equation S1 is given by $n(t) = A(t)n(0)$, where the matrix $A(t) \equiv e^{G_d t}$ is a time-dependent matrix and e denotes the standard matrix exponential. In the presence of drug, we refer to the matrix $A(t)$ as $A(t_{ON})$; in the absence of drug, we refer to it as $A(t_{OFF})$.

If the cell population is exposed to a treatment of duration t_{OFF} without drug followed by a treatment of duration t_{ON} with drug (with $T \equiv t_{OFF} + t_{ON}$), the solution is given by

$$n(T) = M(t_{OFF}, t_{ON})n(0), \tag{S2}$$

where $M(t_{ON}, t_{off}) \equiv A(t_{ON})A(t_{OFF})$. If we imagine repeating the periodic dosing protocol a total of q times, the solution is simply

$$n(qT) = M^q n(0), \tag{S3}$$

where we've dropped the explicit dependence of M on t_{ON} and t_{OFF} for economy of notation.

Long-time solution. We are primarily interested in the long-time behavior of Equation S3; that is, we are interested in solutions in the limit $q >> 1$ such that the periodic dosing protocol has been applied many times. In this regime, the growth will be dominated by the largest eigenvalue of M, which we denote by λ_{max}, and the population will grow exponentially at a per-capita growth rate of g given by (77)

$$g = \frac{\ln \lambda_{\text{max}}}{T}. \tag{S4}$$

The long-term population will be comprised of j sub-populations when the dominant eigenvector of M has j nonzero entries.

Wild-type and Mutants. Consider now a case where population $i = 1$ corresponds to a "wild-type" progenitor cell. These cells can mutate to each of the remaining $N - 1$ cell types ("mutants") with some rate ϵ. We consider these mutations to be irreversible and neglect reversion to wild-type cells or interconversion between different mutant cells. Hence, the matrix G_d is given by

$$(G_d)_{i,j} = \delta_{ij}(k_{i,ON} - \delta_{11}(N-2)\epsilon) + \delta_{i1}\epsilon, \tag{S5}$$

where δ_{ij} is the usual Kronecker delta. In the limit $\epsilon \to 0$, the matrix G_d (and in turn, the matrix M) is diagonal with off-diagonal terms of order ϵ. If we assume $\epsilon \to 0$, G_d is a diagonal matrix whose (i,i)$^{\text{th}}$ entry is $k_{i,ON}$. In turn, the matrix M is also diagonal with eigenvalues given by

$$\lambda_i = e^{k_{i,OFF}t_{OFF} + k_{i,ON}t_{ON}} = e^{T(k_{i,OFF}(1-f_{ON}) + k_{i,ON}f_{ON})}, \tag{S6}$$

where $f_{ON} \equiv t_{ON}/T$ is the fraction of time spent in drug, and eigenvectors are given by the standard basis vectors for $\mathbb{R}^N$. The different cell populations are uncoupled,

and the dominant population is the one with the largest value of λ_i. In this limit, the temporal ordering of drug and no-drug regimens does not matter; the outcome is determined entirely by f_{ON}, the fraction of time spent in drug.

Coexistence of N=2 subpopulations. If we choose $t_{OFF} = 0$ (drug always present) or $t_{ON} = 0$ (drug never present), the population will eventually be dominated by the cell type with the fastest per capita growth rate in the presence or absence of drug, respectively. On the other hand, there may be cases where allowing multiple cell types to co-exist decreases the total population growth. To maintain coexistence of two cell types, i and j, one requires that $\lambda_i = \lambda_j$, which yields

$$(k_{i,OFF} - k_{j,OFF})(1 - f_{ON}) + (k_{i,ON} - k_{j,ON})f_{ON} = 0. \tag{S7}$$

Solving for f_{ON}, we have

$$f_{ON} = \frac{1}{1+\gamma}, \tag{S8}$$

with $\gamma \equiv \frac{k_{i,ON}-k_{j,ON}}{k_{j,OFF}-k_{i,OFF}}$. Note that $0 < f_{ON} < 1$ if and only if $\gamma > 0$ and finite. In other words, one cell type must be favored in the presence of drug, while the other cell type must be favored in the absence of drug. Choosing $t_{ON} = f_{ON}T$ will conserve the ratio of cell types i and j, and the long-term per capita growth of the population is

$$g = \frac{k_{i,OFF}k_{j,ON} - k_{j,OFF}k_{i,ON}}{(k_{i,OFF} - k_{i,ON}) - (k_{j,OFF} - k_{j,ON})}. \tag{S9}$$

Coexistence of N>2 subpopulations. It is not possible, in general, to choose f_{ON} such that more than two cell types coexist asymptotically. Doing so would require $\lambda_i = \lambda_j = \lambda_k$, or equivalently,

$$Kt = 0, \tag{S10}$$

where t is a column vector with entries $t = (t_{OFF}, t_{ON})$ and K is a matrix of growth rate differences given by

$$K \equiv \begin{pmatrix} k_{i,OFF} - k_{j,OFF} & k_{i,ON} - k_{j,ON} \\ k_{i,OFF} - k_{k,OFF} & k_{i,ON} - k_{k,ON} \end{pmatrix}. \tag{S11}$$

Equation S10 has a nontrivial solution only when $\det K = 0$–that is, a non-trivial solution exists only when the growth rates satisfy a particular condition (specifically, when the columns of K are linearly dependent). Aside from this special case, it is not possible to maintain co-existence between more than two cell types in the long time limit.

A brief exploration of nonzero mutation rates. Similar results also apply if we consider cell type $i = 1$ (wild-type) and take $N\epsilon << 1$ but non-zero. In that case, the eigenvalue corresponding to wild-type cells is given by Equation S6 with $k_{i,OFF} \to k_{i,OFF} - (N-1)\epsilon$ and $k_{i,ON} \to k_{i,ON} - (N-1)\epsilon$. The other eigenvalues remain unchanged. Similarly, the eigenvector corresponding to wild-type cells is given by $v = (1 - O(\epsilon), b_1, b_2, ..., b_N)$, where $b_i \sim O(\epsilon)$. In words, this means that when f_{ON} is chosen such that λ_1 dominates, the long-term population maintains a small fraction ($O(\epsilon)$) of all mutants. On the other hand, if f_{ON} is chosen such that λ_1 and λ_j dominate, the population will consist of co-existing populations of wild-type cells and type j cells as well as a diminishing fraction ($O(\epsilon)$) of all additional mutants. Of course, as ϵ approaches the size of the other growth rates in the problem, mutation (and cell interconversion more generally) can have a significant effect on the overall dynamics (see, for example, (78)).

Optimality of coexisting subpopulations. It is possible to maintain heterogeneity in a population as long as $\gamma > 0$. When is it optimal to adopt such a periodic dosing strategy? That is, when does this heterogeneous population exhibit a lower average growth rate than each of the corresponding homogeneous populations? To answer this question, we first assume $\gamma > 0$ such that the long-term population is heterogeneous. Without loss of generality, we consider a population consisting of two subpopulations i and j and take $k_{i,OFF} > k_{j,OFF}$ and $k_{j,ON} > k_{i,ON}$ to ensure $\gamma > 0$. That is, we assume that population i grows faster than j in the absence of drug and j grows faster than i in the presence of drug. In order for this heterogeneous population to represent the optimal (slowest-growing) population, we require that g (given by Equation S9) is smaller than the per capita growth rate of the fastest growing population in each condition alone. Specifically, we have

$$g < k_{i,OFF} \text{ and } g < k_{j,ON} \tag{S12}$$

By combining Equation S9 with Equation S12, we have

$$\begin{aligned} \frac{(k_{j,ON} - k_{i,ON})(k_{j,ON} - k_{j,OFF})}{(k_{i,OFF} - k_{i,ON}) - (k_{j,OFF} - k_{j,ON})} > 0, \\ \frac{(k_{i,OFF} - k_{i,ON})(k_{i,OFF} - k_{j,OFF})}{(k_{i,OFF} - k_{i,ON}) - (k_{j,OFF} - k_{j,ON})} > 0. \end{aligned} \tag{S13}$$

Excluding the singular case where the denominator is zero, we are therefore led to two optimality conditions:

$$\begin{aligned} k_{j,ON} - k_{j,OFF} > 0 \\ k_{i,OFF} - k_{i,ON} > 0 \end{aligned} \tag{S14}$$

In words, cell type j must grow faster with drug than without, while the opposite must be true for cell type i. Hence, for population heterogeneity to be optimal in the long-time limit, we must have one subpopulation of drug-sensitive cells and one population of drug-addicted cells.

Exact solution for N=2. In the simple case where $N = 2$ and $\epsilon = 0$, it is possible to write down an analytic expression for the fractional time in drug (f_{ON}) required to minimize population size. The expression is valid for all times, not merely in the long time limit. To do so, consider that the total population size at the end of period T is governed by Equation S2. Because M is diagonal, one can trivially write the solution for $P = n_1 + n_2$ as

$$P = n_{01} \exp\left(T(k_{1,OFF}(1 - f_{ON}) + k_{1,ON} f_{ON})\right) + n_{02} \exp\left(T(k_{2,OFF}(1 - f_{ON}) + k_{2,ON} f_{ON})\right), \tag{S15}$$

where n_{0i} is the initial size of population i. It is straightforward to show that $\partial^2 P / \partial f_{ON}^2 < 0$, so this is a convex function with a unique minimum on $f_{ON} = [0, 1]$. One can solve for the minimum according to $\partial P / \partial f_{ON} = 0$, which gives

$$f_{ON} = \frac{1}{1+\gamma} + \frac{1}{T} \log\left(\frac{n_{01}(k_{1,OFF} - k_{1,ON})}{n_{02}(k_{2,ON} - k_{2,OFF})}\right) \tag{S16}$$

with $\gamma \equiv \frac{k_{1,ON} - k_{2,ON}}{k_{2,OFF} - k_{1,ON}}$. In the long-time limit ($T \to \infty$), Equation S16 reduces to Equation S8. However, when the addiction criteria (Equation S14) is not met, the second term in Equation S16 is imaginary. As a result, the long-time limit (Equation S8) corresponds to an optimal solution only when $T = \infty$; for any finite time, the long-time limit will, in general, not be optimal and cycling is not beneficial, as f_{ON} is complex with a nonzero imaginary part. On the other hand, when the addiction criteria is satisfied, the solution will converge continuously to the long-time limit.

Summary of Cycling, Heterogeneity, and Optimality. To maintain population heterogeneity in the long-time limit, one must cycle between drug and no-drug conditions with f_{ON} (the time spend in drug) given by Equation S8. In practice, one cell type must be favored in the presence of drug and the other cell type must be favored in the absence of drug. If f_{ON} does not satisfy Equation S8, the population will eventually be dominated by one population or the other. Specifically, it will be dominated by the population corresponding to the largest eigenvalue λ_i, even if a cycling protocol is used. It is not, in general, possible to asymptotically maintain finite fractions of more than 2 cell types by switching between 2 environments. The exception occurs when K (Equation S11) has linearly dependent columns. In the limit where cells do not interact or interconvert, the total fraction of time spent in drug (f_{ON}) determines the outcome, independent of the temporal ordering of the drug and no-drug regimens.

Maintaining heterogeneity through cycling is optimal only when one cell type grows faster with drug than without ("addiction"), while the other cell type grows faster without drug than with drug ("drug sensitive"). It is worth noting that it is possible to maintain population heterogeneity but *not* achieve optimality. This situation occurs when the above condition for optimality is not met, but $0 < f_{ON} < 1$ is chosen according to Equation S8. In this situation, the average growth of the heterogeneous population is larger than the growth of the dominating population in at least one of the two conditions (either with or without drug). Of course, it is also possible under some conditions to achieve optimality without maintaining heterogeneity. This situation occurs, for example, when $\gamma < 0$, meaning that one cell type is favored in both conditions. In that case, one simply chooses the condition in which that cell type grows most slowly. It can also occur when $\gamma > 0$ but addiction is not present (e.g. mixture of a drug sensitive population and a drug-resistant, but not addicted, population with a small fitness cost). In that case, the mutant can be favored over the sensitive cells in the presence of drug, while the wild-type can be favored over mutant in the absence of drug. However, both cell types grow more slowly in drug. In that case, it is possible to maintain both cell types, but the optimal solution is to apply drug always, eventually leading to only resistant mutants.