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Pretreatment Mitochondrial Priming Correlates with Clinical Response to Cytotoxic Chemotherapy

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females was 3 to 35 times larger than expected if the sex ratio bias of the offspring produced by each female was as precise as possible given her brood size (table S2), suggesting that females could have been more precise, even in the context of their small brood size.

We have thus tested the three main predictions of LMC theory and found that (i) populations evolving under higher LMC exhibited a greater mean female bias in their sex ratios and (ii) individual females could adjust their sex ratios to different intensities of LMC through phenotypic plasticity. However, (iii) females did not produce precise sex ratios that exhibit lower than binomial variance. We could reach these conclusions only because our setup allowed us to vary both the LMC conditions under which the populations evolved and the conditions in which they were tested. Therefore, we leveraged the advantage of both plasticity and comparative tests and added analytical power to this approach that could not be reached with correlative or observational approaches.

We found that the norm of reaction for sex ratio evolved differently in selection lines evolving under different LMC intensities. We hypothesize that LMC+ lines evolved under more constant LMC levels compared to LMC– and Panmixia lines. In LMC– and Panmixia lines, several females were present on each leaf, so that the populations may have been spatially structured, leading to more varying levels of LMC. Moreover, in some generations, one or few females may have died before laying any egg; hence, the intensity of LMC may have varied across generations. Therefore, conditions under LMC– and Panmixia lines were probably heterogeneous, which is predicted to favor adaptive phenotypic plasticity (*23*, *24*). In contrast, in LMC+ lines, each generation of selection was initiated by a single foundress per leaf fragment; thus, the level of LMC never varied. Such homogeneous selection is predicted to favor a loss of phenotypic plasticity, particularly if phenotypic plasticity is costly to maintain (*25*). These results are consistent with a comparative study on fig wasps, which showed that adaptive plasticity of sex ratio was higher in species subject to more variable LMC in nature, suggesting that the selective regime shapes the evolution of sex ratio adjustment (*26*, *27*).

It is also possible that inbreeding depression occurred in our LMC+ lines, reducing phenotypic plasticity. Inbreeding increases with the LMC level; hence, females from LMC+ lines might suffer from a reduced fitness, expressed here by a reduced ability to adaptively adjust their behavior. There is evidence for some level of inbreeding depression in female fitness components in *T. urticae* (*28*). Whether such inbreeding depression also affects the ability of females to precisely control their brood sex ratio remains to be studied.

Hamilton's theory of LMC has strongly influenced the field of evolutionary biology. Our results suggest that this theory is correct and that evolutionary theories can make precise predictions, both qualitatively and quantitatively.

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Supporting Online Material

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Pretreatment Mitochondrial Priming Correlates with Clinical Response to Cytotoxic Chemotherapy

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Cytotoxic chemotherapy targets elements common to all nucleated human cells, such as DNA and microtubules, yet it selectively kills tumor cells. Here we show that clinical response to these drugs correlates with, and may be partially governed by, the pretreatment proximity of tumor cell mitochondria to the apoptotic threshold, a property called mitochondrial priming. We used BH3 profiling to measure priming in tumor cells from patients with multiple myeloma, acute myelogenous and lymphoblastic leukemia, and ovarian cancer. This assay measures mitochondrial response to peptides derived from proapoptotic BH3 domains of proteins critical for death signaling to mitochondria. Patients with highly primed cancers exhibited superior clinical response to chemotherapy. In contrast, chemoresistant cancers and normal tissues were poorly primed. Manipulation of mitochondrial priming might enhance the efficacy of cytotoxic agents.

Cancers that respond well to one cytotoxic agent often respond well to other cytotoxic agents even when these agents act through very different mechanisms (as in acute lymphoblastic leukemia). Conversely, cancers that respond poorly to one type of cytotoxic agent often respond poorly to all types of chemotherapy (as in pancreatic cancer or renal cell carcinoma).

One important determinant of chemosensitivity is cellular proliferation rate (*1*). However, the observation that some rapidly dividing tumors are resistant to chemotherapy and that some slowly dividing tumors are chemosensitive suggests that additional factors play a role (*2*–*6*). Although there are probably agent-specific mechanisms underlying chemosensitivity, such as drug uptake and metabolism, we hypothesized that there might also be a central signaling node engaged by many different forms of chemotherapy and that variation in this node might contribute to differences in drug response. Because many chemotherapeutic agents kill cells through the mitochondrial apoptosis pathway, we focused on this pathway and investigated whether tumor cells show pretreatment variation in the propensity to undergo apoptosis and whether this variation correlates with clinical response to cytotoxic chemotherapy.

Death signaling from chemotherapy ultimately results in the activation of proapoptotic or inactivation of antiapoptotic BCL-2–family proteins. If the changes are of sufficient magnitude, the proapoptotic proteins BAX and BAK are activated and oligomerize at the mitochondrion, causing mitochondrial outer membrane permeabilization (MOMP) and commitment to programmed cell death (*7*–*9*). Preconditions of the mitochondrial apoptotic pathway rely on at least a dozen members of the BCL-2 family (*10*).

To measure MOMP, we developed a functional assay called BH3 profiling, which uses peptides derived from the BH3 domains of proapoptotic BH3-only proteins of the BCL-2 family (*11*–*13*). In this assay, test mitochondria in whole cells are exposed to BH3 peptides and the resulting MOMP is measured (fig. S1) (*13*). The peptides gain access by diffusion through a plasma membrane that has been permeabilized with low concentrations of digitonin. MOMP is measured indirectly by the fluorescent dye JC-1, which measures potential across the mitochondrial inner membrane. This potential rapidly degrades in response to MOMP. We previously demonstrated how the pattern of response to selectively interacting peptides such as BAD BH3 and NOXA BH3, which selectively interact with BCL-2 and MCL-1, respectively, can indicate BCL-2 or MCL-1 dependence (*11*, *12*, *14*, *15*). In this study, to measure overall "priming" for death, irrespective of dependence on individual antiapoptotic proteins, we instead used the PUMA, BMF, and BIM BH3 peptides, which interact more promiscuously with the five main antiapoptotic proteins (*11*, *16*, *17*). All promiscuous peptides gave similar results when used at concentrations that provided a useful dynamic range (fig. S2, A to F). The assay was reproducible when repeated with the same sample on different days (fig. S2, G to I). We use the term priming here simply to describe proximity to the apoptotic threshold, as revealed by mitochondrial depolarization induced by promiscuously interacting BH3 peptides, without making any comment on the molecular biology underlying this state in any individual cell.

To investigate whether the pretreatment state of the mitochondrial apoptotic apparatus of human cancers correlates with the response to chemotherapy in the clinical setting, we studied 85 total patient tumors, 51 with individual clinical follow-up. The spectrum of cancer types studied—multiple myeloma, acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), and ovarian cancer—was based on the availability of viable pretreatment primary cancer specimens. ALL and AML samples were obtained from a local tissue bank, whereas multiple myeloma and ovarian cancer samples were acquired prospectively. All BH3 profiling was performed by investigators who were blinded to individual clinical outcomes.

For the analysis of multiple myeloma, we obtained pretreatment bone marrow samples from 17 different patients with the disease (table S1) (*18*–*21*) and performed BH3 profiling on CD138-positive myeloma cells in these samples (Fig. 1A and figs. S1A and S3). In most cases of multiple myeloma, serum levels of the monoclonal protein (M-protein) secreted by the malignant clone can be used as a marker of total tumor burden (*22*). We found a tight correlation between priming and maximal M-protein decrease after treatment whether priming was measured by BMF BH3 ($r = 0.80$, $P = 0.00005$, Fig. 1B) or PUMA BH3 ($r = 0.78$, $P = 0.0001$, fig. S4A). We also divided patients into responders, who experienced partial responses or better, and nonresponders, who had stable disease or inferior responses. (*22*). Again, strong mitochondrial response to either BMF ($P = 0.001$, Fig. 1C) or PUMA BH3 ($P = 0.01$, fig. S4B) peptides before treatment was closely associated with better clinical response to chemotherapy. Finally, when we classified the myeloma specimens (from the 15 patients who did not go to bone marrow transplant) into low versus high depolarization response (Fig. 1D), we found that the patients with highly primed myeloma had a longer progression-free survival. On the basis of a comparison of samples from previously treated versus untreated patients, prior treatment with chemotherapy was strongly associated with reduced priming in cancer cells (fig. S4E).

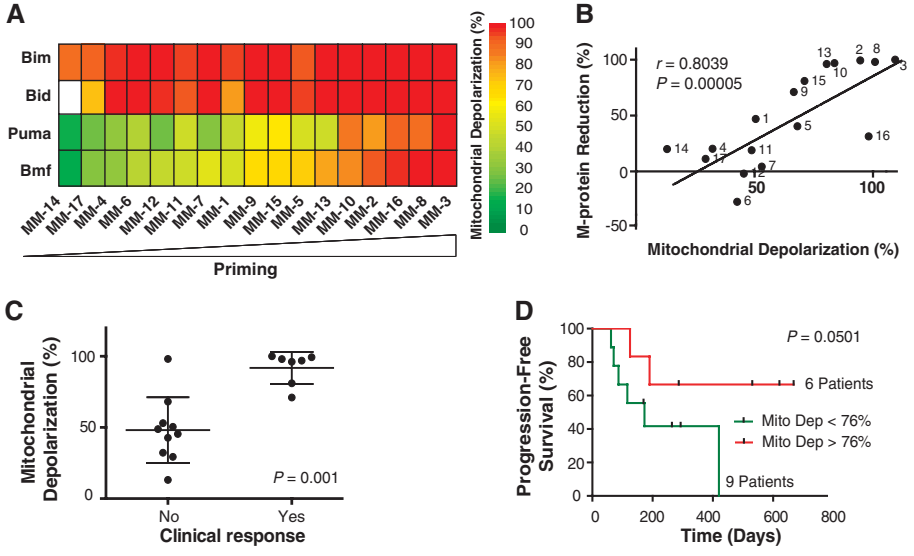

Fig. 1. Mitochondrial priming correlates with clinical response to chemotherapy in multiple myeloma. (**A**) Heat map of mitochondrial depolarization caused by the BIM, BID, PUMA, and BMF peptides (measurements of priming) in bone marrow samples from 17 patients with multiple myeloma. Individual patient codes are shown along the *x* axis, and samples are ordered according to increasing depolarization by the BMF peptide. Unless indicated otherwise, the concentration of peptide in the assays was 100 μM. Data shown are the mean of two or three replicate wells for each peptide. (**B**) Mitochondrial depolarization caused by the BMF peptide in myeloma patient samples compared with the percent reduction in serum level of M-protein, a biomarker of disease burden in multiple myeloma. Each point is labeled with a patient code. (**C**) Mitochondrial depolarization (Mito Dep) caused by BMF peptide in myeloma patients that were grouped according to their clinical response [see methods in supporting online material (SOM)]. (**D**) Patients with highly primed myeloma exhibited superior progression-free survival.

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We next studied AML, a leukemia primarily of adults that is curable by intensive chemotherapy alone in approximately 40% of patients (*23*). We performed flow cytometry–based BH3 profiling (*12*) (fig. S1B) on malignant myeloblasts from banked, pretreatment bone marrow specimens from 15 adult AML patients (Fig. 2A and table S2). Myeloblasts from patients who achieved a complete remission were more primed than those from patients who did not ($P = 0.0027$, Fig. 2B and fig. S5, A and B).

A different leukemia type, ALL, carries a different prognosis in children and adults. Pediatric ALL responds better to therapy, and patients have a better long-term survival rate than those with adult ALL (80 to 90% versus 40%) (*24*). Examination of 15 adult and 17 childhood ALL samples by BH3 profiling revealed that the adult ALL samples were less primed than the pediatric samples ($P = 0.007$, Fig. 2C). We were able to obtain individual clinical response information for eight of the adult samples in Fig. 2C, which we could use to compare with mitochondrial priming. Because complete response is common in ALL, we compared patients who responded and subsequently relapsed to those who responded and did not relapse (Fig. 2D and table S3). Again, superior clinical outcome was closely associated with increased mitochondrial priming ($P = 0.0012$, Fig. 2E and fig. S5, C to F).

To complement these studies of hematological malignancies, we studied a solid tumor—ovarian cancer—using normalization of CA125, an ovarian tumor marker, as an index of response. We performed BH3 profiling on 18 ovarian cancers (Fig. 3A, fig. S6, and table S4). Of these, 10 cases had persistent elevation of CA125 a month after surgery and before chemotherapy, so that response to chemotherapy could be scored by normalization of CA125. We found that pre-chemotherapy mitochondrial priming was associated with clinical normalization of CA125 ($P = 0.033$, Fig. 3B). We examined progression-free survival in 14 cases; of the 18, 4 had no follow-up CA125 values and thus could not be evaluated. Using a CA125 value that is abnormal and rising after initiation of chemotherapy as an index of progression, we found that highly primed ovarian cancer mitochondria were correlated with superior progression-free survival (Fig. 3C, $P = 0.0003$). The results when the PUMA peptide in Fig. 3, A to C, was used were similar to those when the BMF or BIM peptides were used (fig. S2, C to F, and fig. S7, A to D). Thus, in multiple myeloma, AML, ALL, and ovarian cancer, pretreatment mitochondrial priming was a consistent correlate of better response and clinical outcome, suggesting a fundamental relationship between mitochondria and response to cytotoxic chemotherapy in vivo.

The series of experiments described above revealed interesting correlations between pretreatment priming of tumor cells and clinical response. We found similar correlations in related in vitro experiments (figs. S7, E and F, and S8 to S10). To explore whether priming was not only a correlate but perhaps a determinant of chemosensitivity, we tested whether modulation of priming would alter chemosensitivity. We used ABT-737, a BH3-mimetic drug that binds to antiapoptotic proteins BCL-2, BCL-XL, and BCLw (*25*), to increase priming in the myeloid leukemia cell line K562 (Fig. 3D). This resulted in an increase in sensitivity to the chemotherapeutic agents doxorubicin, vincristine, and etoposide (Fig. 3E). This result, in agreement with additional priming modulation experiments in fig. S11, supports the hypothesis that mitochondrial priming may be a determinant of chemosensitivity.

The clinical success of chemotherapy depends not only on the sensitivity of cancer cells but also on the relative insensitivity of normal cells in order to provide a therapeutic index. Differences in proliferation may play a role, because one of the most rapidly proliferating tissues, the bone marrow, is also the most consistently sensitive to chemotherapy. However, many chemosensitive tumors divide more slowly than tissues such as the epidermis and gut epithelium (*26*–*28*) and yet experience vastly greater cytoreduction, even elimination, by chemotherapy. This discrepancy suggests that differences in proliferation alone may not fully explain the chemotherapeutic index.

It has been proposed that cancer cells are more sensitive than normal cells to chemotherapy because they are inherently more sensitive to apoptosis, presumably because of their oncogenic lesions (*29*). To test this model and the possible role of mitochondrial priming as its mechanistic basis, we examined normal tissues of mouse and human origin. We found that relatively chemoresistant murine normal tissues (heart, liver, testes, brain, and kidney) indeed

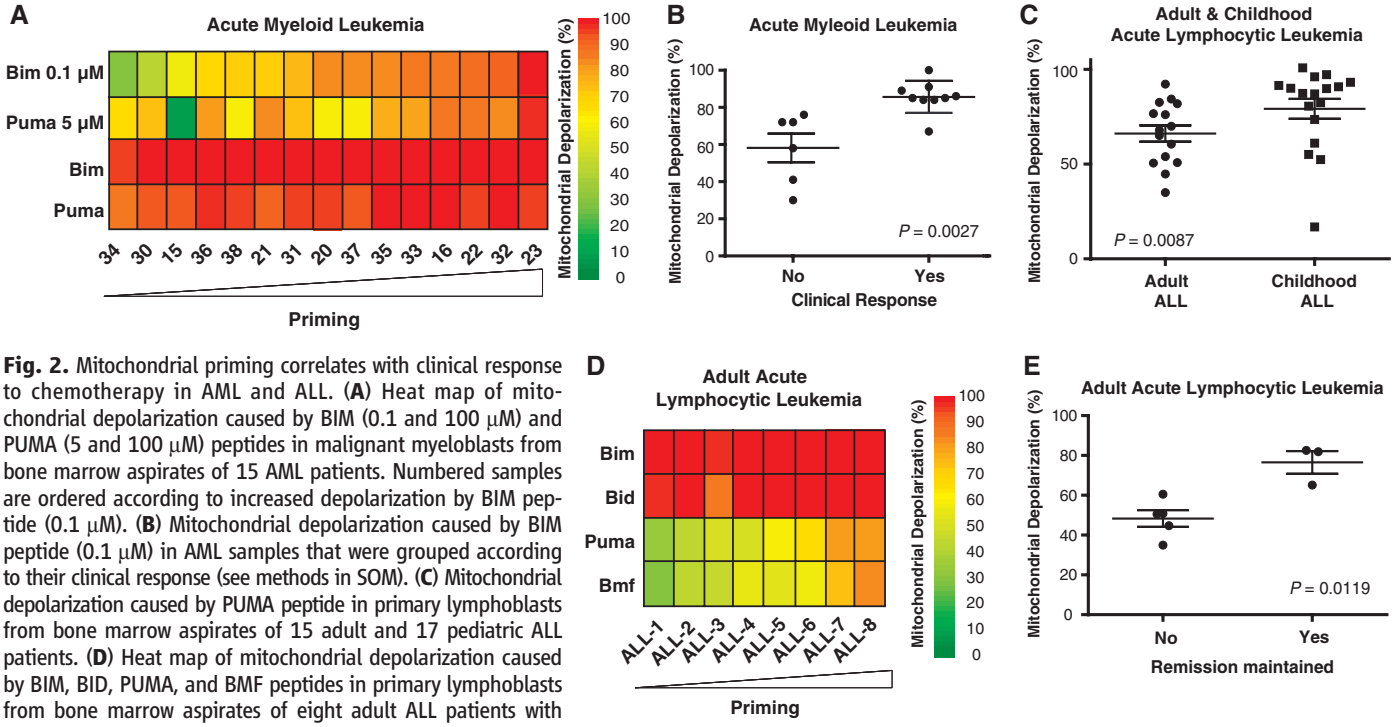

Fig. 2. Mitochondrial priming correlates with clinical response to chemotherapy in AML and ALL. (**A**) Heat map of mitochondrial depolarization caused by BIM (0.1 and 100 μM) and PUMA (5 and 100 μM) peptides in malignant myeloblasts from bone marrow aspirates of 15 AML patients. Numbered samples are ordered according to increased depolarization by BIM peptide (0.1 μM). (**B**) Mitochondrial depolarization caused by BIM peptide (0.1 μM) in AML samples that were grouped according to their clinical response (see methods in SOM). (**C**) Mitochondrial depolarization caused by PUMA peptide in primary lymphoblasts from bone marrow aspirates of 15 adult and 17 pediatric ALL patients. (**D**) Heat map of mitochondrial depolarization caused by BIM, BID, PUMA, and BMF peptides in primary lymphoblasts from bone marrow aspirates of eight adult ALL patients with clinical follow-up. Samples are ordered according to increased depolarization by PUMA peptide. (**E**) Mitochondrial depolarization caused by PUMA peptide in primary lymphoblasts from bone marrow aspirates of eight adult ALL patients grouped according to whether clinical remission was maintained.

were poorly primed (Fig. 4A and fig. S12). Likewise, relatively chemoresistant normal human tissues [kidney (both cortex and medulla), ovary, myometrium, foreskin, cervix, and endometrium] were poorly primed (Fig. 4B and fig. S13). In Fig. 4B we compare the priming of the primary human normal and cancer tissues we studied. Additionally, we present results from the typically chemosensitive childhood ALL and typically chemoresistant endometrial cancer, serous borderline ovarian tumor, and renal cell carcinoma samples for which individual clinical chemotherapeutic response data were not available. This comparison shows that mitochondria from chemosensitive cancers are consistently

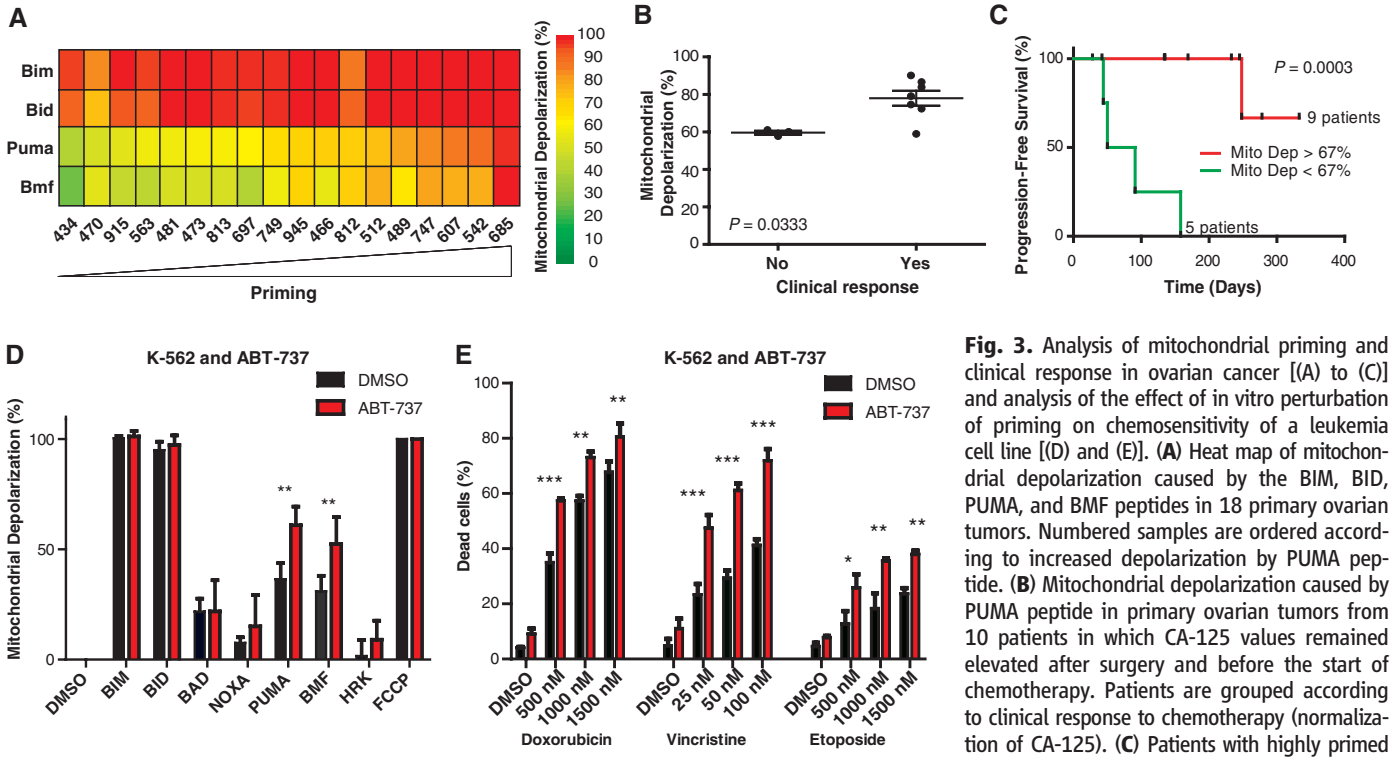

Fig. 3. Analysis of mitochondrial priming and clinical response in ovarian cancer [(A) to (C)] and analysis of the effect of in vitro perturbation of priming on chemosensitivity of a leukemia cell line [(D) and (E)]. (**A**) Heat map of mitochondrial depolarization caused by the BIM, BID, PUMA, and BMF peptides in 18 primary ovarian tumors. Numbered samples are ordered according to increased depolarization by PUMA peptide. (**B**) Mitochondrial depolarization caused by PUMA peptide in primary ovarian tumors from 10 patients in which CA-125 values remained elevated after surgery and before the start of chemotherapy. Patients are grouped according to clinical response to chemotherapy (normalization of CA-125). (**C**) Patients with highly primed ovarian tumors exhibited superior progression-free survival. (**D**) Mitochondrial depolarization response to BH3 peptides (BH3 profile) for the K-562 leukemia cell line with and without pretreatment with 2 μM ABT-737 for 16 hours. Dimethyl sulfoxide (DMSO) is a solvent negative control, and the ionophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) is a positive control for depolarization. (**E**) Assessment of cell viability (annexin V/propidium iodide staining) in the K-562 cell line after pretreatment with 2 μM ABT-737 for 16 hours and 48 hours of treatment with the indicated compounds (mean ± SD, n = 2 replicates).

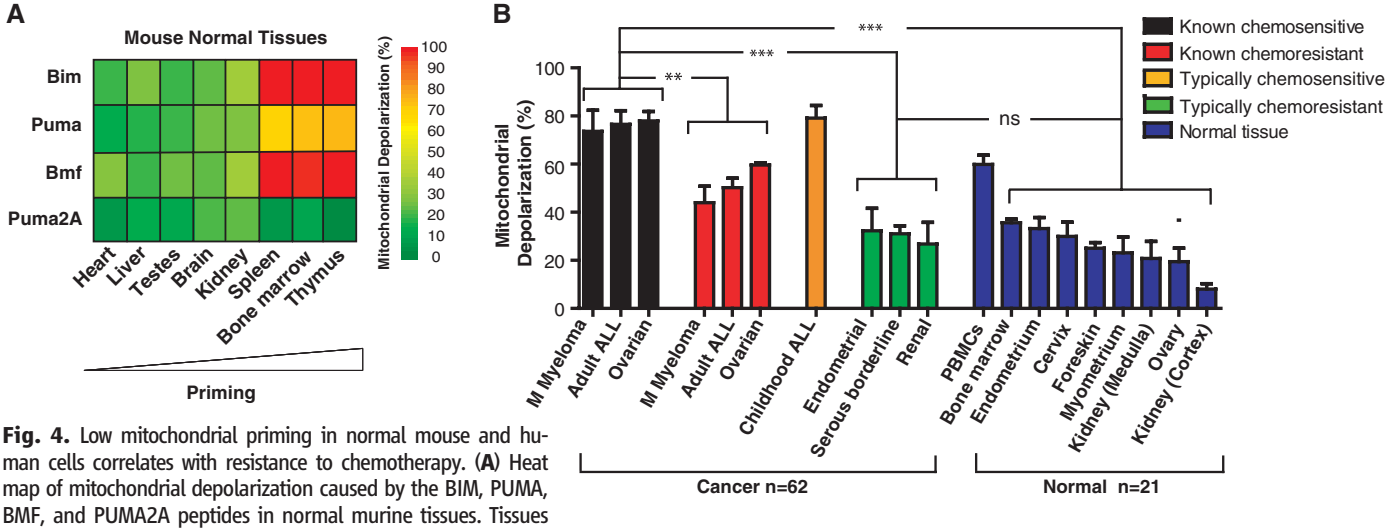

Fig. 4. Low mitochondrial priming in normal mouse and human cells correlates with resistance to chemotherapy. (**A**) Heat map of mitochondrial depolarization caused by the BIM, PUMA, BMF, and PUMA2A peptides in normal murine tissues. Tissues are ordered according to increased depolarization by PUMA peptide. PUMA2A is a double alanine–substituted (loss-of-function) PUMA peptide serving as a negative control to establish background signal. (**B**) Comparison of mitochondrial priming among all primary human cancers and normal tissues described in this paper. The cancers with clinical follow-up were classified as known chemosensitive or known chemoresistant. Cancers classified as typically chemoresistant (serous borderline, n = 3; endometrial, n = 3; and renal, n = 3) (fig. S14) or typically chemosensitive (childhood ALL, n = 17) lacked individual clinical follow-up data. Data shown are mean ± SD across all specimens tested. Analysis of variance was used to demonstrate statistical significance between the different categories with a Tukey's multiple comparison post test. ns, P value > 0.05; **P value < 0.01; and ***P value < 0.001.

more primed than those from chemoresistant cancers and chemoresistant normal tissues. Priming levels were similar among typically chemoresistant cancers and chemoresistant normal tissues, which may explain why we are unable to find conventional chemotherapy regimens of tolerable toxicity to successfully treat these cancers. The chemosensitive hematologic tissues, including murine bone marrow, thymus, and spleen, and human bone marrow and peripheral blood mononuclear cells are the most highly primed normal tissues tested. Prior work has demonstrated that these are also the most radiosensitive tissues in mice and the tissues most sensitive to p53 activation (*30*, *31*), suggesting that the apoptotic death observed after these insults may also be regulated by the mitochondrial preset measured by BH3 profiling.

Cytotoxic chemotherapy has been the mainstay of clinical cancer therapy for the past 60 years despite an incomplete understanding of why it works—why it is more toxic to some cancer cells than others and, most importantly, why it is more toxic to tumors than to normal tissues. Here we show that differential mitochondrial priming correlates with, and may be a determinant of, differential chemosensitivity. One implication of these results is that agents that selectively increase priming in cancer cells, even if they do not cause cell death by themselves, might enhance the response of tumors to conventional chemotherapy (Fig. 3, D and E). A tool such as BH3 profiling, which can detect changes in priming, might be useful in identifying such agents.

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Supporting Online Material

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Transplanted Hypothalamic Neurons Restore Leptin Signaling and Ameliorate Obesity in db/db Mice

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Evolutionarily old and conserved homeostatic systems in the brain, including the hypothalamus, are organized into nuclear structures of heterogeneous and diverse neuron populations. To investigate whether such circuits can be functionally reconstituted by synaptic integration of similarly diverse populations of neurons, we generated physically chimeric hypothalami by microtransplanting small numbers of embryonic enhanced green fluorescent protein–expressing, leptin-responsive hypothalamic cells into hypothalami of postnatal leptin receptor–deficient (db/db) mice that develop morbid obesity. Donor neurons differentiated and integrated as four distinct hypothalamic neuron subtypes, formed functional excitatory and inhibitory synapses, partially restored leptin responsiveness, and ameliorated hyperglycemia and obesity in db/db mice. These experiments serve as a proof of concept that transplanted neurons can functionally reconstitute complex neuronal circuitry in the mammalian brain.

To functionally repair complex circuitry in the central nervous system (CNS), newly incorporated neurons should be electrophysiologically and functionally integrated, whether transplanted or derived from endogenous progenitors. Newly integrated neurons might optimally be a single neuronal subtype in some "point-to-point" sensorimotor CNS systems, or instead a diverse and nucleus-specific population of neuronal subtypes for some critical, homeostatic systems organized into distributed nuclear structures. These systems often signal polymodally via neuropeptides and are regulated by paracrine and endocrine mechanisms via multiple neuronal subtypes operating in parallel. One such critical homeostatic system is that of leptin signaling in the hypothalamus, regulating energy balance, glucose, food intake, and body weight.

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