

# Par-4 Downregulation Promotes Breast Cancer Recurrence by Preventing Multinucleation following Targeted Therapy

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### SUMMARY

Most deaths from breast cancer result from tumor recurrence, but mechanisms underlying tumor relapse are largely unknown. We now report that Par-4 is downregulated during tumor recurrence and that Par-4 downregulation is necessary and sufficient to promote recurrence. Tumor cells with low Par-4 expression survive therapy by evading a program of Par-4-dependent multinucleation and apoptosis that is otherwise engaged following treatment. Low Par-4 expression is associated with poor response to neoadjuvant chemotherapy and an increased risk of relapse in patients with breast cancer, and Par-4 is downregulated in residual tumor cells that survive neoadjuvant chemotherapy. Our findings identify Par-4-induced multinucleation as a mechanism of cell death in oncogene-addicted cells and establish Par-4 as a negative regulator of breast cancer recurrence.

### INTRODUCTION

Most deaths from breast cancer result from disease relapse following treatment of the primary tumor. Although 5-year survival rates for patients with breast cancer are relatively high compared to other cancers, tumors can recur up to 20 years after diagnosis (Saphner et al., 1996). This reflects the fact that residual cancer cells, termed minimal residual disease, often survive surgery, radiation, and adjuvant therapy and serve as a reservoir for breast cancer recurrence. Consistent with this, disseminated tumor cells (DTCs) are present in the bone marrow of up to 40% of patients with breast cancer at diagnosis, and their presence and number following adjuvant therapy are strong independent predictors of recurrence risk (reviewed in Bednarz-Knoll et al., 2011). These observations suggest that the ability of residual cancer cells to survive treatment and persist in a clinically undetectable state is a common precursor to the development of recurrent disease.

Although the molecular and cellular events that contribute to primary tumorigenesis in the breast have been intensively studied, much less is known about pathways that contribute to the survival and recurrence of residual breast cancer cells. This is due, in part, to the difficulty in isolating and analyzing residual tumor cells in humans, the paucity of clinical samples from patients with recurrent disease, and the need for animal models that faithfully recapitulate essential aspects of minimal residual disease and recurrence.

To address this critical gap, our laboratory has developed conditional transgenic mouse models for HER2/neu (*MMTV-rtTA; TetO-HER2/neu*), MYC (*MMTV-rtTA;TetO-MYC*), and Wnt1/p53 (*MMTV-rtTA;TetO-Wnt1;p53<sup>-/+</sup>*)-induced mammary tumorigenesis (D'Cruz et al., 2001; Gunther et al., 2003; Moody et al.,

### Significance

Recurrent breast cancer is typically an incurable disease. Consequently, the propensity of breast cancers to recur following surgery, radiation, and adjuvant therapy is the most important determinant of clinical outcome. Residual neoplastic cells that persist following treatment constitute the reservoir from which recurrent tumors arise and are a major obstacle to the successful treatment of human cancers. Accordingly, understanding the mechanisms by which residual tumor cells survive treatment is a critical research priority. Our findings establish downregulation of the tumor suppressor Par-4 in mice and in women as a mechanism of tumor cell survival and recurrence following targeted therapy and chemotherapy. As such, therapies that target pathways repressed by Par-4—or that restore Par-4 expression—may hold significant clinical promise.



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2002, 2005; Boxer et al., 2004) that permit analysis of the properties of minimal residual disease and the mechanisms of tumor recurrence. These models exhibit key features of breast cancer progression as it occurs in women, including the survival of residual cancer cells following therapy and their eventual spontaneous recurrence.

In each of these models, administration of doxycycline (dox) induces oncogene expression in the mammary epithelium, which in turn results in the formation of invasive primary mammary adenocarcinomas. Moreover, akin to the responses of some human cancers to targeted therapies, the majority of oncogene-induced mammary tumors in mice regress to a nonpalpable state following oncogene downregulation, illustrating the phenomenon of oncogene addiction. However, as is frequently observed in patients with breast cancer, mice in which primary tumors have regressed spontaneously develop recurrent cancers following a variable latency period. Because recurrent tumors in these models occur in the absence of expression of the initiating oncogene, this suggests that mammary tumors escape and recur by activating alternate growth and survival pathways.

Based on these observations, we reasoned that the specific mechanisms by which tumor cells survive oncogene downregulation and recur in mice could provide clues to pathways that contribute to breast cancer relapse in patients. In this regard, we considered the tumor suppressor, prostate apoptosis response 4 (Par-4), to be an attractive candidate. Par-4, encoded by the Pawr gene, is a proapoptotic protein that is upregulated in response to apoptotic stimuli and required for cell death in multiple contexts (Sells et al., 1994, 1997; Díaz-Meco et al., 1996). Par-4 is silenced in a variety of human cancers, and its re-expression induces cell death in some cancer cell lines (reviewed in Ranganathan and Rangnekar, 2005). Consistent with its proapoptotic effects, mice lacking Par-4 are tumor prone and exhibit an increase in spontaneous tumorigenesis as well as increased susceptibility to chemical and hormone-induced cancers (reviewed in Diaz-Meco and Abu-Baker, 2009). Thus, Par-4 is a bona fide tumor suppressor and a critical regulator of tumor cell survival.

Emerging data have implicated Par-4 downregulation as a prognostic factor in breast cancer. Low Par-4 expression has recently been shown to be associated with reduced overall survival in two patient cohorts, raising the possibility that Par-4 downregulation may be associated with an increased risk of recurrence (Méndez-López et al., 2010; Nagai et al., 2010). However, one of these studies examined only a small patient cohort (Méndez-López et al., 2010), and the other found an association between Par-4 and overall survival, but not disease-free survival (Nagai et al., 2010). As such, the relationship between Par-4 downregulation and breast cancer recurrence remains unclear. Moreover, the mechanisms underlying the apparent association between low Par-4 expression and tumor recurrence, as well as whether Par-4 downregulation functionally contributes to breast cancer recurrence, have not been addressed.

### RESULTS

# Par-4 Is Downregulated during Tumor Recurrence in Mice

We reasoned that genetically engineered mouse models for tumor recurrence could provide insight into the functional effects of Par-4 downregulation on breast cancer relapse. We first asked whether Par-4 expression is altered during the recurrence of primary mammary tumors induced by the HER2/neu, MYC, or Wnt1;p53+/- oncogenic pathways. Quantitative reverse-transcription PCR and immunoblotting performed on primary and spontaneous recurrent tumors arising in MTB/TAN, MTB/TOM, and MTB/TWnt1 transgenic mice revealed that Par-4 mRNA and protein were downregulated in recurrent tumors in all three models (Figures 1A-1E). Immunofluorescence staining for Par-4 in HER2/neu-induced tumors confirmed that whereas Par-4 was readily detectable in primary tumors, its expression was markedly downregulated in recurrent tumors (Figure 1F). These results demonstrate that Par-4 is frequently-and spontaneously-downregulated during the process of recurrence in mammary tumors induced by three different oncogenic pathways relevant to human cancer.

### Par-4 Is Downregulated in Tumors that Recur following Chemotherapy

The aforementioned results indicated that Par-4 is downregulated in recurrent tumors that arise spontaneously in *MTB/TAN* mice following primary tumor regression induced by HER2/neu downregulation, which is a surrogate for targeted therapy. However, whereas women with *HER2/neu*-amplified tumors are typically treated with targeted therapies, such as trastuzumab or lapatinib, most patients with breast cancer receive adjuvant chemotherapy. As such, the majority of tumor recurrences in women arise in the context of prior treatment with cytotoxic chemotherapeutic agents. Therefore, we asked whether Par-4 is downregulated in recurrent tumors that arise following chemotherapy.

Mice bearing orthotopic mammary tumors generated by injection of tumor cells from HER2/neu-induced primary tumors arising in *MTB/TAN* mice were treated with Adriamycin and cyclophosphamide (AC) for 2 weeks, followed by paclitaxel (T) for 2 weeks. AC plus T led to marked regression of all tumors, whereas untreated control tumors continued to grow (Figures S1A and S1B available online). Following tumor regression, treatment was stopped, and mice were monitored for relapse. All tumors relapsed within 3 weeks of treatment cessation (Figure S1B), and tumors that relapsed following chemotherapy exhibited a marked reduction in Par-4 expression (Figure S1C). This suggests that Par-4 is downregulated in tumors that relapse following chemotherapy as well as oncogene downregulation.

# Low Par-4 Predicts an Increased Risk of Recurrence in Women with Breast Cancer

In light of our observation that Par-4 is frequently downregulated during tumor recurrence in mice, and given the preliminary finding that low Par-4 expression is associated with poor prognosis in at least some patients with breast cancer (Méndez-López et al., 2010; Nagai et al., 2010), we asked whether low Par-4 expression is associated with an increased risk of recurrence in a broader panel of patients with breast cancer. We obtained gene expression data from publicly available human breast cancer data sets for which clinical outcome was available (Table S1) and examined the relationship between Par-4 expression and recurrence-free survival (RFS). Using both the Cox proportional hazards (PH) model, which treats Par-4 expression as a

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HER2/neu

continuous variable, and an outcome-oriented approach, which divides tumors into two groups based upon Par-4 expression, we found that women with breast cancers expressing low levels of Par-4 exhibited a significantly increased risk of recurrence over a 5-year period compared to women whose breast cancers expressed higher levels of Par-4 (Figures S2A–S2D; Table S2; Cox PH model, hazards ratio [HR] range: 0.21–0.62; outcome-oriented model, HR range: 0.11–0.44).

We next asked whether the association between Par-4 downregulation and decreased RFS is independent of other clinicopathological variables. We first assessed whether Par-4 downregulation is associated with aggressive subtypes of human breast cancer. Indeed, Par-4 expression was lower in estrogen receptor (ER)– compared to ER+ tumors, in basal-like tumors compared to other molecular subtypes, and in high-grade (grade 3) compared to lower-grade tumors in three data sets (Table S3). In contrast, Par-4 expression was similar between HER2+ and HER2– tumors and between tumors with lymph node metastases and those without (data not shown).

Because ER–, basal-like, and high-grade breast cancers are each associated with poor clinical outcome, we wished to determine whether the reduced RFS observed in patients with breast cancer with low Par-4 expression was an indirect reflection of the association between reduced Par-4 expression and these clinicopathological markers or, alternately, whether reduced Par-4 expression might be an independent prognostic factor. After individually adjusting for ER status, basal-like subtype, and tumor grade, the association between low Par-4 expression and decreased RFS remained significant in the majority of data

### Figure 1. Par-4 Is Downregulated in Recurrent Mammary Tumors

(A–D) Quantitative reverse-transcription PCR analysis (A) and western analysis (B–D) showing Par-4 expression in primary (Pri) and recurrent (Rec) HER2/neu, MYC, and Wnt1;p53<sup>+/-</sup> tumors.
(E) Quantification of Par-4 protein levels, normalized to tubulin.

(F) Immunofluorescence (IF) analysis of Par-4 in primary and recurrent HER2/neu tumors. Scale bars, 50  $\mu m.$ 

Error bars denote mean  $\pm$  SEM. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.

See also Figure S1.

sets (Table S4). These findings identify low Par-4 expression as an independent predictor of decreased RFS in women with breast cancer.

We previously demonstrated that Snail expression promotes mammary tumor recurrence in mice, induces epithelialto-mesenchymal transition (EMT) in mouse mammary tumor cells, and is associated with decreased RFS in women with breast cancer. Therefore, we assessed the relationship among Par-4 downregulation, Snail upregulation, EMT, and RFS in the aforementioned human breast cancer data sets. Par-4

and Snail were not significantly correlated in these data sets ion, (r = -0.02; p = 0.65), and the association between low Par-4 expression and decreased RFS remained after correcting for Snail expression (Table S5).

### Low Par-4 Predicts a Decreased Response to Chemotherapy in Women with Breast Cancer

Given the proapoptotic function of Par-4, we reasoned that low Par-4 expression might be associated with an increased ability of tumor cells to survive chemotherapy and that this might underlie the increased risk of recurrence associated with tumors expressing low levels of Par-4. To address this question, we examined Par-4 expression in human breast cancers in women enrolled in the I-SPY 1 TRIAL treated with neoadjuvant chemotherapy (Esserman et al., 2012). In this trial, patients with locally advanced breast cancer underwent tumor biopsy prior to receiving neoadjuvant chemotherapy. Following chemotherapy, residual primary tumors were surgically resected, and gene expression profiles were measured on paired biopsies and residual tumors.

First, we examined the relationship between RFS and Par-4 expression in primary breast cancers prior to the administration of chemotherapy. Consistent with our prior findings, patients in the I-SPY 1 TRIAL whose breast cancers expressed low levels of Par-4 were more likely to relapse over a 5-year period using either a Cox PH model (HR = 0.66, 95% confidence interval [CI] 0.46–0.94; p = 0.02) or an outcome-oriented cut-point approach (HR = 0.43, 95% CI 0.23–0.8; p = 0.028; Figure 2A; Table S2).



### Figure 2. Decreased Par-4 Expression Predicts Poor Response to Neoadjuvant Chemotherapy and Is Selected for following Treatment

(A) Five-year RFS for patients with breast cancer in the I-SPY1 neoadjuvant trial with HR, 95% Cls, and adjusted p value.

(B) Par-4 expression in primary human breast cancers prior to treatment as a function of RCB following neoadjuvant therapy.

(C and D) Par-4 expression in matched pretreatment breast cancer biopsies and residual tumor following neoadjuvant therapy at surgery.

Error bars denote mean  $\pm$  SEM. \*p < 0.05; \*\*p < 0.01.

See also Figure S2 and Tables S1-S5.

tumors harvested at surgery following neoadjuvant chemotherapy. We found that Par-4 expression decreased following treatment in 13 of the 21 patients for whom expression data were available at both pre- and posttreatment

Next, we examined patient responses to neoadjuvant chemotherapy as a function of Par-4 expression in their breast cancers prior to chemotherapy. To quantify tumor response, residual cancer burden (RCB) was assessed at the time of surgery, which takes into account parameters such as residual tumor size, cellularity, and lymph node involvement. We separated tumors into three groups based upon RCB class. The first group contained RCB 0 and I tumors, which have the lowest residual cancer burden. The second group contained RCB II tumors. The third group contained RCB III tumors, which exhibited minimal response or progression in the breast and/or lymph nodes at the time of surgery, and have the highest burden. Because RCB 0 and I tumors are associated with a low risk of recurrence, these classes were combined for our analysis (Symmans et al., 2007).

Analysis of the correlation between Par-4 expression and RCB class revealed that tumors with the poorest response to neoadjuvant chemotherapy (i.e., the greatest amount of residual disease, RCB class III) exhibited substantially lower Par-4 expression prior to therapy than tumors that exhibited a greater response to chemotherapy (RCB class 0 plus I and RCB class II; Figure 2B). This indicates that low Par-4 expression in locally advanced human breast cancers is associated with poor response to chemotherapy, suggesting that the observed relationship between low Par-4 expression and decreased RFS may reflect, at least in part, a decreased response to therapy in breast cancers expressing low levels of Par-4.

# Par-4 Is Downregulated in Human Breast Cancer Cells that Survive Chemotherapy

We next hypothesized that cancer cells with low Par-4 expression might preferentially survive chemotherapy in women with breast cancer. If this were the case, we reasoned that cells surviving chemotherapy would, on average, exhibit lower Par-4 expression than bulk tumor cells prior to treatment.

To test this hypothesis, we compared Par-4 expression in the I-SPY 1 TRIAL in matched primary tumor biopsies and residual time points, and increased in only one (Figures 2C and 2D; p = 0.002). In response to chemotherapy, Par-4 expression decreased  $\sim$ 1.7-fold across all tumors (Figure 2D) and 2.5-fold for those tumors in which Par-4 mRNA levels declined following treatment (data not shown). These results are consistent with a model in which neoadjuvant chemotherapy selects for cancer cells with low Par-4 expression.

### Par-4 Downregulation Promotes Tumor Recurrence

The aforementioned observations raised the possibility that Par-4 downregulation might play a functional role in breast cancer recurrence by promoting tumor cell survival following therapy. To test directly whether Par-4 downregulation promotes tumor recurrence, we used an orthotopic mouse model to determine the impact of Par-4 knockdown on the propensity of primary HER2/neu-induced mammary tumors to recur.

Par-4 expression was suppressed in tumor cells cultured from a primary HER2/neu-induced mammary tumor (Prim 1) using retrovirally delivered shRNAs. Two independent hairpins (Par-4.891 and Par-4.1011) were identified that suppressed Par-4 levels by 70%–80% to levels similar to those found in recurrent HER2/neu tumor cells that had spontaneously downregulated Par-4 (Figure 3A). Control cells expressed an shRNA targeting Renilla luciferase or an empty vector (MLP). Knocking down Par-4 did not cause cells to undergo EMT, as assessed by the expression of epithelial and mesenchymal markers (Figure S3A), further suggesting that Par-4 functions independently of Snail and EMT.

Control and Par-4 knockdown primary tumor cells were injected into the mammary fat pads of recipient *nu/nu* mice maintained on dox, and primary tumors were allowed to form in the presence of HER2/neu expression. Par-4 knockdown and control tumors grew at similar rates (data not shown), suggesting that Par-4 knockdown does not influence primary tumor formation. Once tumors had formed, dox was withdrawn to initiate HER2/neu downregulation and tumor regression. All tumors regressed to a nonpalpable state regardless of Par-4 knockdown

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status. Mice were then palpated twice weekly to monitor for tumor recurrence.

Control tumors from Prim 1 cells recurred with a median latency of 110 days (Figures 3B and 3C). In contrast, tumors expressing either of the two Par-4 shRNAs recurred 3–5 weeks more rapidly than controls, with median latencies of 76 and 90 days (Figures 3B and 3C; HR = 2.4, 95% Cl 1.6–8.7, p = 0.002 for Par-4.891; HR = 2.7, 95% Cl 1.9–14.7, p = 0.002 for Par-4.1011). Notably, as observed in intact *MTB/TAN* mice, Par-4 was spontaneously downregulated in recurrent orthotopic tumors that arose from control cells (Figure 3D).

In women with breast cancer, primary tumors with low Par-4 expression displayed poorer responses to chemotherapy than tumors expressing higher levels of Par-4. To determine whether this was also true in mice, we measured the extent of regression of control and Par-4 knockdown tumors following oncogene downregulation. This revealed that tumors with Par-4 knockdown regressed to a lesser extent than control tumors (Figure S3B).

Finally, to examine the effect of Par-4 knockdown on recurrence at distant sites, we analyzed lungs from these mice because tumors in this model are known to recur in the lung. Mice with recurrent tumors from Par-4 knockdown cells had significantly more metastases than mice with control recurrent tumors, suggesting that Par-4 downregulation also promotes recurrence at distant sites (Figures S3C and S3D). Together, these results provide direct functional evidence that Par-4 downFigure 3. Par-4 Downregulation Is Necessary and Sufficient for Tumor Recurrence

(A) Western analysis showing Par-4 expression in control and Par-4 knockdown primary HER2/neu tumor cells.

(B and C) RFS for mice harboring control tumors or tumors expressing one of the two shRNAs targeting Par-4.

(D) Western analysis showing Par-4 expression in four control (MLP) recurrent orthotopic tumors.(E) RFS for mice harboring control tumors or tu-

mors expressing a cDNA encoding Par-4.

(F) Western analysis showing endogenous (Endog.) and ectopic Par-4 expression in primary and recurrent tumors.

See also Figure S3.

regulation promotes mammary tumor recurrence at both local and distant sites.

### Par-4 Downregulation Is Required for Tumor Recurrence

Our observations in mice that nearly all recurrent mammary tumors downregulate Par-4 suggested that Par-4 downre-gulation might be required for tumor recurrence. To test this hypothesis, we asked whether enforced expression of Par-4 would delay tumor recurrence. A second line of primary HER2/neu-induced tumor cells (Prim 2) cultured from tumor-bearing *MTB/TAN* mice was

transduced with a retrovirus expressing epitope-tagged Par-4 or a control vector, pK1. These cells were used in an orthotopic recurrence assay to test the effect of forced Par-4 expression on tumor recurrence.

Primary tumors expressing ectopic Par-4 grew at rates similar to control tumors (data not shown), suggesting that Par-4 expression does not alter primary tumor growth. In contrast, the latency for recurrence of tumors expressing ectopic Par-4 was delayed by 3 weeks compared to control tumors (Figure 3E, median latency of 92 versus 70 days; HR = 0.34, 95% CI 0.07-1.00; p = 0.05). As before, recurrent tumors arising from control cells spontaneously downregulated endogenous Par-4 (Figure 3F). Strikingly, the majority of recurrent tumors arising from Par-4-transduced primary tumor cells not only downregulated endogenous Par-4 but also extinguished ectopic Par-4 expression (Figure 3F). These findings indicate that Par-4 downregulation is required for mammary tumor recurrence and suggest the existence of a strong selective pressure to downregulate both endogenous and ectopically expressed Par-4 during the process of recurrence.

### Par-4 Is Upregulated following Oncogenic Pathway Inhibition

In the aforementioned mouse models, tumor recurrence occurs in discrete stages. First, tumors regress acutely in response to oncogene downregulation as a consequence of increased apoptosis and decreased cell proliferation. Thereafter, the small



### Figure 4. Par-4 Is Upregulated following Oncogene Inhibition

(A) Western analysis of Par-4 following HER2/neu downregulation in primary tumor cells.

(B) Quantification of Par-4 levels from (A) normalized to tubulin.

(C) IF analysis of Par-4 in primary tumors (on dox; HER2/neu expressing) and tumors 3 or 7 days following HER2/neu downregulation. Scale bars, 50 μm. (D) Western analysis of Par-4 in *HER2*-amplified human breast cancer cells treated with 100 nM lapatinib (Lap.).

(E) Quantification of Par-4 levels from (D) normalized to tubulin.

Error bars denote mean ± SEM. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.

See also Figure S4.

fraction of surviving tumor cells resides in a dormant state in a histologically identifiable residual neoplastic lesion (data not shown). Following a variable latent period, residual tumor cells stochastically reinitiate growth to form recurrent tumors. Par-4 downregulation could occur anywhere along this process, and its downregulation could functionally impact one or more stages of recurrence.

To identify the stage of recurrence at which Par-4 is downregulated, we first examined Par-4 expression shortly after oncogene downregulation in vitro. Prim 1 cells, derived from a primary HER2/neu-induced tumor, were cultured in the presence of dox to maintain oncogene expression. Dox was then removed from the media to initiate HER2/neu downregulation. Surprisingly, HER2/neu downregulation led to a dramatic increase, rather than decrease, in Par-4 expression beginning 48 hr following dox withdrawal (Figures 4A and 4B). Furthermore, Par-4 levels continued to increase for at least 7 days following dox withdrawal. Par-4 expression was also upregulated in vivo following acute HER2/neu (Figure 4C) or MYC downregulation (Figure S4).

We next wished to determine whether Par-4 upregulation might be an evolutionarily conserved response to HER2/neu inhibition in cancer cells. To address this, we treated two

HER2/neu-amplified human breast cancer cell lines, BT-474 and SKBR3, with the dual HER2/EGFR inhibitor lapatinib. In each cell line, HER2/neu inhibition resulted in dramatic increases in Par-4 expression (Figures 4D and 4E). Together, these results indicate that Par-4 levels increase, rather than decrease, following acute oncogene inhibition in both mouse and human cancer cells, and in vivo as well as in vitro.

### Pre-Existing Cells with Low Par-4 Expression Preferentially Survive Oncogene Downregulation

Our findings to this point suggested that oncogene inhibition and chemotherapy might select for pre-existing cells with low Par-4 expression. We therefore asked whether a population of cells with low Par-4 expression existed in primary tumors. Immunofluorescence staining for Par-4 performed on primary HER2/neu and Wnt1;p53<sup>+/-</sup> tumors revealed that whereas Par-4 was expressed at high levels throughout most of each primary tumor, isolated regions exhibited low levels of Par-4 staining that were comparable to levels found in recurrent tumors (Figures S5A and S5B).

We previously reported that some primary Wnt1;p53+/tumors fail to regress completely following Wnt1 downregulation, suggesting that they have become Wnt independent (Gunther et al., 2003). We considered the possibility that these primary tumors have already downregulated Par-4. To address this, we biopsied a cohort of primary Wnt1;p53+/- tumors and then removed dox to induce tumor regression. As previously reported, some tumors failed to regress following Wnt downregulation, whereas others regressed to a nonpalpable state. We then compared Par-4 expression levels in biopsies from primary tumors that ultimately regressed completely following oncogene downregulation with biopsies from primary tumors that did not. As predicted, Par-4 levels were lower in primary tumors that did not regress completely following Wnt1 downregulation compared to primary tumors that did (Figures S5C and S5D). This provides further evidence that primary tumor cells with low Par-4 expression preferentially survive oncogene downregulation.

To test directly whether pre-existing primary tumor cells with low Par-4 expression preferentially survive HER2/neu downregulation, we asked whether cells in which Par-4 had been knocked down exhibit a survival advantage following HER2/ neu downregulation in vivo and are thereby selected for in residual lesions. We performed a cellular competition assay in which GFP-labeled HER2/neu Prim 1 tumor cells expressing a Par-4 shRNA were admixed in a 1:1 ratio with isogenic mCherrylabeled primary tumor cells expressing a control shRNA targeting Renilla luciferase. Cells were injected into the mammary fat pads of recipient nu/nu mice maintained on dox. Primary tumors were harvested from one cohort of mice, and dox was withdrawn from a second cohort to induce tumor regression. Twenty-eight days later, mammary glands bearing residual tumor cells were harvested, and the ratio of GFP:mCherry-labeled tumor cells in residual lesions was assessed by fluorescence microscopy.

In primary tumors, the ratio of GFP-labeled Par-4 knockdown cells to mCherry-labeled control cells was approximately 1:1, confirming that Par-4 knockdown does not confer a selective advantage during primary tumor formation in the presence of HER2/neu expression (Figures 5A and 5B). In contrast, cells

with Par-4 knockdown constituted 80%–90% of the tumor cells present within residual lesions (Figures 5A and 5B).

We next asked whether Par-4 knockdown also confers a survival advantage in vitro following HER2/neu downregulation using a similar cell competition assay. In the presence of HER2/ neu expression, cells maintained an  $\sim$ 1:1 ratio over the course of 10 days (data not shown). In contrast, selection for Par-4 knockdown cells was evident within 5 days following HER2/ neu downregulation, and within 14 days, these cells were predominant (Figure 5C). The ratio of control GFP- to control mCherry-labeled cells did not change in the presence or absence of dox (Figure 5C; data not shown).

Together, these results demonstrate that Par-4 downregulation confers a selective, cell-intrinsic advantage to tumor cells following HER2/neu downregulation. Furthermore, these findings confirmed our prediction that pre-existing primary tumor cells with low Par-4 expression preferentially survive oncogene downregulation. This, in turn, suggests that Par-4 downregulation promotes recurrence by facilitating tumor cell survival following treatment with antineoplastic agents.

# Par-4 Downregulation Promotes Tumor Cell Survival following Oncogene Inhibition

HER2/neu downregulation in mouse mammary tumors results in apoptosis as a consequence of oncogene addiction (Moody et al., 2002). Because the increase in apoptosis following HER2/neu downregulation is accompanied by increased expression of the proapoptotic protein Par-4, and because cells with Par-4 knockdown are selected for following HER2/neu downregulation in vivo, we reasoned that Par-4 upregulation may contribute to cell death following oncogene inhibition.

To address this possibility, we measured the viability of HER2/ neu-dependent primary tumor cells, with or without Par-4 knockdown, 4 days following HER2/neu downregulation. HER2/neu downregulation resulted in a 15% absolute increase in cell death in control cells, whereas only an ~5% increase in cell death was observed in Par-4 knockdown cells generated using two different hairpins (Figure 5D; p < 0.05). Importantly, whereas the level of Par-4 increased following HER2/neu downregulation in cells expressing Par-4 shRNAs, Par-4 levels remained lower than in control cells expressing HER2/neu (Figures S5E and S5F). Because primary tumor cells expressing HER2/ neu tolerate basal levels of Par-4, these findings suggest that Par-4 upregulation above this basal level may contribute to cell death.

To determine the impact of Par-4 downregulation on longerterm measures of cell survival following HER2/neu downregulation, we measured the effect of Par-4 knockdown on clonogenic survival following dox withdrawal. In the presence of dox, control cells and Par-4 knockdown cells formed colonies with similar efficiency (Figure 5E, top row). However, when grown in the absence of dox for 3 weeks, cells expressing a Par-4 shRNA formed 8- to 21-fold more colonies than control cells (Figures 5E and 5F; p < 0.001). Dox was added back to a subset of plates for 1 week to reinduce oncogene expression in any cells that had survived HER2/neu downregulation. Again, cells expressing either of the two Par-4 shRNAs formed a greater number of colonies than control cells, and their mean size was larger (Figures 5E and 5F).



### Figure 5. Par-4 Downregulation Promotes Survival following Oncogene Withdrawal

(A) Fluorescent micrographs from an in vivo competition assay showing primary tumors and residual lesions formed from injecting a 1:1 mixture of GFP- and mCherry-labeled control cells (MLP + MLP-Red) or a 1:1 mixture of GFP-labeled Par-4 knockdown cells and mCherry-labeled control cells (Par-4.891 + MLP Red and Par-4.1011 + MLP-Red). Scale bars, 50  $\mu$ m.

(B) Quantification of the relative proportion of GFP+ and mCherry+ cells in primary tumors or residual lesions.

(C) Control (MLP) or Par-4 knockdown (Par-4.891 or Par-4.1011) cells labeled with GFP were mixed in a 1:1 ratio with control cells labeled with mCherry (MLP-Red), and the relative proportion of green to red cells was measured by flow cytometry following HER2/neu downregulation in vitro.

(D) Percent increase in nonviable cells following HER2/neu downregulation in primary tumor cells expressing a control vector (MLP) or one of the two shRNAs targeting Par-4.

(E) Clonogenic survival of cells of the indicated genotype grown in the presence of HER2/neu (+dox) or in the absence of HER2/neu (-dox) for 3 weeks. Dox was added back to a subset of plates for 1 week to reinduce HER2/neu expression ( $-dox \rightarrow +dox$ ).

(F) Quantification of the surviving colonies in (E).

Error bars denote mean  $\pm$  SEM. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001. See also Figure S5.



# Figure 6. Reexpressing Par-4 in Recurrent Tumor Cells Induces Multinucleation Accompanied by p53 Activation, Growth Arrest, and Apoptosis

(A) Cell viability was measured following retroviral transduction of Par-4 into primary or recurrent cells.

(B) Western analysis showing Par-4 expression following ShId-1 treatment of recurrent tumor cells expressing L106P-Par-4 constructs.

(C) Growth curves of vehicle or Shld-1-treated control cells (L106P-YFP or Luc-L106P) or Par-4-inducible cells (L106P-Par-4).

(legend continued on next page)

We also asked whether Par-4 knockdown protects primary tumor cells from cell death induced by chemotherapeutic agents. Control or Par-4 knockdown cells were treated with etoposide, vincristine, or Adriamycin for 4 days, and cell viability was measured. Par-4 knockdown increased the fraction of cells surviving each treatment by ~20%-40% (Figure S5G), confirming that Par-4 downregulation protects cells from chemotherapy-induced cell death. Together, these results demonstrate that Par-4 downregulation promotes cell survival following antineoplastic therapy, thereby providing a mechanistic explanation for the observations that cells with Par-4 knockdown are selected for following oncogene downregulation and are more likely to recur.

# Par-4 Induces Multinucleation and Cell Death in Recurrent Tumor Cells

Our findings to this point demonstrated that Par-4 is acutely upregulated following HER2/neu inhibition and that pre-existing cells with low Par-4 expression preferentially survive tumor regression induced by oncogene downregulation. These observations suggested that cells in which Par-4 is upregulated in response to HER2/neu inhibition might be selected against during tumor regression and, in a related manner, that elevated Par-4 expression in recurrent tumor cells might be incompatible with cell survival.

To address this question, we investigated the consequences of reexpressing Par-4 in recurrent tumor cells that had spontaneously downregulated endogenous Par-4. Independent cell lines were generated from two recurrent tumors that arose in *MTB/ TAN* mice. As predicted, Par-4 expression was lower in recurrent compared to primary tumor cell lines (Figure S6A). Ectopic expression of Par-4 by retroviral transduction of each of these recurrent cell lines induced high levels of cell death (Figure 6A). In contrast, ectopic expression of Par-4 in primary HER2/neuexpressing tumor cells resulted in only a modest increase in cell death (Figure 6A). These results suggest that high Par-4 expression is incompatible with the survival of recurrent, but not primary, tumor cells and are consistent with a model in which Par-4 downregulation is required for the survival of cells in which the HER2/neu pathway has been inhibited.

To avoid artifacts due to the expression of nonphysiological levels of Par-4, we titrated the levels of ectopically expressed Par-4 in recurrent tumor cells to those observed in primary tumor cells following acute oncogene downregulation using an inducible system comprised of a mutant FKBP domain (L106P) and its synthetic ligand Shld-1 (Banaszynski et al., 2006). Treatment of recurrent tumor cell lines expressing an L106P-Par-4 fusion protein with 1  $\mu$ M Shld-1 yielded Par-4 levels comparable to those observed in primary tumor cells following HER2/neu downregulation (Figures S6B and S6C). Shld-1 induction of Par-4 in L106P-Par-4-expressing recurrent tumor cell lines led

to a profound reduction in their growth rates (Figures 6B and 6C). ShId-1 had no effect on the growth rates of control tumor cells expressing L106P-YFP or Luc-L106P fusion proteins (Figure 6C).

Strikingly, visual examination of recurrent cells following Shld-1 treatment for 24 hr revealed large numbers of multinucleated cells (Figure 6D). Multinucleated cells appeared as early as 6 hr following Shld-1 treatment (data not shown) and constituted 15%–20% of all cells within 24 hr (Figure 6E). Multinucleated cells were also observed in cells transduced with wild-type Par-4 (i.e., not fused to L106P), but not in Shld-1-treated control cells (data not shown). This indicates that multinucleated cells arise as a consequence of Par-4 expression rather than Shld-1 administration or L106P-fusion protein expression.

Multinucleated cells have a tetraploid DNA content, and tetraploid cells reportedly undergo p53-dependent cell-cycle arrest and death (Andreassen et al., 2001; Margolis et al., 2003). To address the fate of multinucleated cells, we treated recurrent tumor cells with Shld-1 for 4 days and analyzed p53 expression, BrdU incorporation, and cleaved caspase-3 staining. Multinucleated cells in Par-4-expressing cultures exhibited robust nuclear p53 staining, whereas control cells did not (Figure 6F), suggesting that Par-4-induced multinucleation engenders a p53 response. Multinucleated cells exhibited a 50% lower rate of proliferation than control cells (Figures 6G and 6H), and multinucleated cells induced by retroviral transduction of Par-4 (Figure 6I, top) or Shld-1 treatment (Figure 6I, bottom) stained strongly positive for cleaved caspase-3, indicating that they were undergoing apoptosis.

To determine whether Par-4-dependent growth arrest and apoptosis are p53 dependent, we knocked down p53 in recurrent cells (Figure S6D) and measured the number of viable cells 4 days following Shld-1 treatment. The decrease in cell viability induced by Par-4 was partially rescued by p53 knockdown (Figure S6E). Similarly, p53 knockdown increased the clonogenic survival of cells following Par-4 induction by Shld1 treatment (Figure S6F). Together, these results indicate that p53 mediates some, but likely not all, of the antiproliferative and proapoptotic effects of restoring Par-4 expression in recurrent tumor cells.

These findings demonstrate that restoring Par-4 expression in recurrent tumor cells results in the formation of multinucleated cells that undergo p53 activation, growth arrest, and apoptosis. This, in turn, suggests a mechanism selecting against cells that have upregulated Par-4 in response to inhibition of an oncogenic pathway to which they had become addicted.

### Par-4 Induces Multinucleation through ZIPK-Mediated MLC2 Phosphorylation and Cytokinesis Failure

To determine the mechanism by which Par-4 causes multinucleation, we performed live-cell imaging on cells treated with

<sup>(</sup>D) Recurrent tumor cells were treated with Shld-1 for 24 hr and visualized by light microscopy or fluorescence microscopy following immunostaining for Par-4. Arrows indicate multinucleated cells.

<sup>(</sup>E) Quantification of multinucleated cells following 24 hr Shld-1 treatment.

<sup>(</sup>F) IF staining for p53 in multinucleated cells following ShId-1 treatment.

<sup>(</sup>G) IF analysis of BrdU incorporation in multinucleated and control cells following Shld-1 treatment.

<sup>(</sup>H) Quantification of percentage of BrdU+ cells corresponding to the experiment shown in (G).

<sup>(</sup>I) IF staining for cleaved caspase-3 in multinucleated cells following ShId-1 treatment or retroviral transduction of Par-4 in recurrent cells.

Scale bars, 50  $\mu m.$  Error bars denote mean  $\pm$  SEM. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001. See also Figure S6.



Figure 7. Par-4 Causes Cytokinesis Failure through ZIPK-Mediated MLC2 Phosphorylation

(A) Stills from live-cell imaging of recurrent tumor cells expressing H2B-mCherry and L106P-Par-4 untreated (top row) or treated with ShId-1 (bottom row). Scale bars, 15 µm.

(B) Western analysis showing p-MLC2 levels following retroviral transduction of Par-4 into recurrent tumor cells.

(C) IF analysis showing p- MLC2 following ShId-1 treatment of recurrent cells expressing L106P-Par-4. Scale bars, 10 µm.

(D) Quantitative reverse-transcription PCR showing ZIPK expression following transfection of recurrent cells with a control pool or an siRNA pool targeting ZIPK.

(E) Western analysis of p-MLC2 levels in ZIPK knockdown cells 24 hr after Par-4 expression.

(F) Quantification of the percentage of multinucleated cells 24 hr after Par-4 expression in control and ZIPK knockdown cells.

Error bars denote mean  $\pm$  SEM. \*p < 0.05.

See also Movies S1 and S2.

Shld-1 to induce Par-4 expression. In untreated cells, mitosis proceeded normally giving rise to two daughter cells with one nucleus each (Figure 7A, top; Movie S1). In contrast, cells treated with Shld-1 underwent aberrant mitoses that often failed during cytokinesis (Figure 7A, bottom; Movie S2). These cells progressed normally through metaphase, anaphase, and initial ingression of the cleavage furrow. However, during later stages of cleavage furrow ingression, Par-4-expressing cells underwent dramatic blebbing that was ultimately accompanied by regression of the cleavage furrow, yielding a binucleated cell (Figure 7A; Movie S2). Cytokinesis failure occurred during the first mitosis following Shld-1 treatment, consistent with Par-4-induced cytokinesis failure being a direct effect rather than a secondary consequence of cell stress or growth arrest.

To elucidate the molecular mechanism by which Par-4 causes cytokinesis failure, we evaluated ZIP kinase (ZIPK) and its substrate myosin light-chain 2 (MLC2). Par-4 has been shown to induce MLC2 phosphorylation through activation of ZIPK, and this pathway has been implicated in the regulation of cell contractility and cell death (Boosen et al., 2009; Page et al., 1999; Vetterkind et al., 2005; Vetterkind and Morgan, 2009). MLC2 phosphorylation is also required for cytokinesis because phosphorylation of MLC2 on threonine 18 and serine 19 activates myosin II and promotes furrow ingression (Matsumura, 2005). Conversely, increased MLC2 phosphorylation, which may occur through depletion of myosin phosphatase (MYPT1), results in cytokinesis defects in *C. elegans* (Piekny and Mains, 2002) as well as mammalian cells (Yamashiro et al., 2008). These observations suggest that cytokinesis requires tightly regulated levels of MLC2 phosphorylation.

We hypothesized that Par-4 upregulation may induce cytokinesis failure by dysregulating MLC2 phosphorylation. Consistent with this possibility, retroviral expression of Par-4 in recurrent tumor cells led to an increase in MLC2 phosphorylation (Figure 7B), and multinucleated cells induced by Shld-1 treatment exhibited high levels of pMLC2 (Figure 7C).

To determine whether Par-4-induced cytokinesis failure requires ZIPK, we examined the effect of ZIPK knockdown on Par-4-induced multinucleation. Transfection of recurrent tumor cells with an siRNA targeting ZIPK led to an ~60% reduction in ZIPK levels compared to control cells (Figure 7D). Control and ZIPK knockdown cells were treated with Shld-1 to induce Par-4 expression, and the fraction of multinucleated cells was determined after 24 hr. ZIPK knockdown abrogated Par-4induced MLC2 phosphorylation (Figure 7E) and led to a dramatic reduction in Par-4-induced multinucleation (Figure 7F). These results suggest that Par-4 induces multinucleation through a ZIPK-dependent increase in MLC2 phosphorylation that disrupts the precise temporal and spatial control of MLC2 phosphorylation that is required for the successful completion of cytokinesis.

### HER2/neu Inhibition Induces Par-4-Dependent Multinucleation in Mouse and Human Cancer Cells

Re-expression of Par-4 in recurrent tumor cells that do not express HER2/neu results in multinucleation, apoptosis, and reduced proliferation. In an analogous manner, HER2/neu down-regulation in primary tumor cells is accompanied by Par-4 upregulation, apoptosis, and growth arrest. This suggested that the increased apoptosis and growth arrest observed in primary tumor cells following acute HER2/neu downregulation might be mediated, in part, by Par-4-induced multinucleation. We therefore asked whether primary tumor cells become multinucleated following HER2/neu downregulation.

Dox withdrawal induced a 2-fold increase in the percentage of multinucleated cells in two independent cultures of primary HER2/neu tumor-derived cells (Figures 8A and 8B). Multinucleation was dependent upon Par-4 because cells expressing either of the two shRNAs targeting Par-4 failed to become multinucleated following dox withdrawal (Figure 8C).

We next examined whether human breast cancer cells also become multinucleated following HER2/neu inhibition. Treatment of *HER2*-amplified BT474 cells with lapatinib led to a dose-dependent increase in polyploid cells (Figure 8D). Moreover, analogous to our observations in mouse tumor cells, the lapatinib-induced increase in polyploid BT474 cells was dependent upon Par-4 because cells expressing an shRNA targeting Par-4 failed to become polyploid following HER2/neu inhibition (Figure 8D).

Finally, we asked whether Par-4 upregulation and multinucleation also occur in response to treatment with chemotherapeutic agents. Treatment with low-dose Adriamycin has been reported to induce multinucleation in certain breast cancer cell lines (Mansilla et al., 2006). Therefore, we determined whether multinucleation in this context was accompanied by Par-4 upregulation. Treatment of MDA-MB-231 cells with Adriamycin induced polyploidy (Figure S7A). Interestingly, whereas total Par-4 levels were unchanged, Adriamycin treatment led to an increase in phosphorylation of T163 of Par-4 (Figure S7B), a phosphorylation event that is required for Par-4-induced apoptosis (Gurumurthy et al., 2005). Our results reveal that a chemotherapeutic agent that induces multinucleation also leads to Par-4 activation through phosphorylation on a conserved threonine residue. In aggregate, our findings suggest that treatment of human and mouse cancer cells with targeted agents or chemotherapeutic agents results in Par-4-dependent multinucleation and cell death.

### DISCUSSION

We have identified the proapoptotic tumor suppressor protein Par-4 as a critical negative regulator of mammary tumor recurrence. Par-4 expression is spontaneously downregulated in recurrent tumors arising in mice bearing fully regressed HER2/ neu, MYC, or Wnt1;p53+/--induced mammary tumors. Par-4 knockdown accelerated tumor recurrence, whereas enforced expression of Par-4 delayed recurrence, indicating that Par-4 downregulation is both necessary and sufficient to promote mammary tumor recurrence. Furthermore, we found that preexisting cells with low Par-4 expression preferentially survive oncogene downregulation in vivo and in vitro. Finally, we defined the mechanistic basis for the preferential survival of low Par-4expressing cancer cells as resulting from the fact that HER2/ neu inhibition results in Par-4 upregulation that is accompanied by Par-4-dependent multinucleation, growth arrest, and apoptosis. These findings provide a mechanistic link between Par-4, oncogene addiction and the suppression of tumor recurrence.

Consistent with our findings in mice, our studies also revealed that low Par-4 expression is associated with an elevated risk of recurrence in women with breast cancer. Although Par-4 expression is associated with aggressive subsets of human breast cancers, including ER–, basal-like, and high-grade tumors, multivariate analysis revealed that the association of low Par-4 expression with increased risk of recurrence is independent of each of these variables, suggesting that Par-4 downregulation may mediate at least some aspects of the aggressive biological behavior of these subtypes.

Additional mechanistic insights into the association between low Par-4 expression and poor prognosis in women with breast cancer were garnered from the I-SPY 1 neoadjuvant chemotherapy trial, which suggested that Par-4 downregulation enhances the ability of tumors cells to survive chemotherapy. Tumors that responded poorly to neoadjuvant chemotherapy exhibited significantly lower Par-4 expression prior to chemotherapy than tumors that responded well, indicating that the level of Par-4 expression in a tumor prior to treatment predicts that tumor's response to chemotherapy. This, in turn, raises the intriguing possibility that Par-4 may be a determinant of that response.

Consistent with the finding that low Par-4 expression is associated with decreased RFS in women with breast cancer, and that low Par-4-expressing cancer cells in mice preferentially survive treatment with chemotherapeutic or targeted agents, we found that residual breast cancer cells in patients treated with neoadjuvant chemotherapy in the I-SPY 1 TRIAL downregulated Par-4. This suggests that chemotherapy selects for pre-existing cells within primary tumors that express low levels of Par-4.

In aggregate, our data suggest a two-step model in which cells that upregulate Par-4 following acute oncogene inhibition are selected against or are otherwise eliminated by Par-4-dependent multinucleation, growth arrest, and



### Figure 8. Oncogene Inhibition Leads to Par-4-Dependent Multinucleation

(A) Photomicrographs of cells following HER2/neu downregulation in primary *MTB/TAN* tumor cells. Arrows indicate multinucleated cells. Scale bars, 50 μm.
(B) Quantification of percentage of multinucleated cells corresponding to the experiment shown in (A).

(C) Percentage of multinucleated control or Par-4 knockdown MTB/TAN cells following HER2/neu downregulation.

(D) Percentage of control or Par-4 knockdown BT-474 cells with polyploid DNA content following lapatinib treatment as measured by flow cytometry.

(E) Model for the role of Par-4 downregulation in tumor recurrence. Oncogene inhibition or chemotherapy results in Par-4-dependent multinucleation, which in turn leads to p53 activation, growth arrest, and apoptosis. Together, these cellular processes contribute to tumor regression, such that cells surviving tumor regression exhibit spontaneous Par-4 downregulation. These cells ultimately give rise to recurrent tumors with low Par-4 expression. Error bars denote mean  $\pm$  SEM. \*p < 0.05; \*\*p < 0.01.

See also Figure S7.

apoptosis; consequently, cells that maintain low Par-4 expression in the face of oncogene inhibition are selected for during tumor regression, and recurrent tumors arising from this population of cells express low levels of Par-4 (Figure 8E). Par-4 expression in primary tumors is heterogeneous, with most cells expressing Par-4 but some expressing lower levels. Treatment of tumors—either through blockade of a dominant oncogenic pathway or treatment with conventional chemotherapeutic agents—results in activation of Par-4 through phosphorylation or upregulation of Par-4 protein levels. Par-4 activation, in turn, results in multinucleation, p53 stabilization, decreased proliferation, and increased apoptosis, which together contribute to tumor regression. In this manner, treatment with antineoplastic agents selects for cancer cells that have downregulated Par-4, as we observed in residual cancer cells in patients with breast cancer following chemotherapy and tumor regression. Because these residual cells ultimately serve as the reservoir from which recurrent tumors arise, recurrent tumors express low levels of Par-4.

As a corollary to this model, we hypothesize that the level of Par-4 expression observed in primary human breast cancers reflects, at least in part, the fraction of Par-4-expressing tumor cells contained within that cancer. As such, breast cancers exhibiting low Par-4 expression may contain a high proportion of cells in which Par-4 has been downregulated, a greater number of cancer cells that survive chemotherapy, a higher RCB, and ultimately, an increased rate of tumor recurrence. This, in turn, would explain the spontaneous downregulation of Par-4 that we observed in recurrent mammary tumors in mice, the ability of Par-4 knockdown to accelerate tumor recurrence in mice, the decrease in Par-4 expression observed in women treated with neoadjuvant chemotherapy in the I-SPY 1 TRIAL, and the association between low Par-4 expression and decreased RFS in women with breast cancer.

Our studies have also identified the induction of multinucleation as a mechanism of cell death following the blockade of a dominant oncogenic pathway in tumor cells. Specifically, our findings reveal that Par-4 re-expression in recurrent cells results in cytokinesis failure through increased MLC2 phosphorylation and that this requires ZIPK activity. Multinucleated cells possess a tetraploid DNA content, and a "tetraploid checkpoint" has been proposed that induces cell-cycle arrest and apoptosis in response to an abnormal complement of chromosomes during the G1 phase of the cell cycle (Andreassen et al., 2001; Margolis et al., 2003). Although the existence of this checkpoint remains controversial, it is clear that mechanisms exist that limit the proliferation and survival of tetraploid cells (Castedo et al., 2006; Ganem and Pellman, 2007; Ganem et al., 2007). Moreover, this response likely requires an intact p53 pathway because the induction of tetraploidy in a p53-deficient background is tumorigenic (Fujiwara et al., 2005). This suggests that an intact p53 pathway limits the survival of tetraploid cells and that induction of tetraploidy in cells with functional p53 may be antitumorigenic, rather than protumorigenic. Consistent with this, we observed p53 stabilization in multinucleated cells following Par-4 expression. These results demonstrate that induction of tetraploidy can serve as a mechanism for eliminating oncogene-addicted cells or, more generally, as a mechanism of tumor suppression.

Our results demonstrate that Par-4-dependent multinucleation induced by HER2/neu inhibition represents a pathway by which oncogene-addicted cells are selected against following oncogene downregulation. Cancer recurrence requires that the tumor-suppressive effects of multinucleation be circumvented via downregulation of Par-4. Therapeutic approaches that restore Par-4 expression in tumors that have downregulated this tumor suppressor protein may enhance the therapeutic response to targeted agents as well as chemotherapy. Furthermore, because Par-4 expression is incompatible with the survival of recurrent cancer cells, restoring Par-4 expression in dormant cancer cells or recurrent cancers may constitute an effective approach to preventing, or treating, this fatal stage of cancer progression.

### **EXPERIMENTAL PROCEDURES**

### **Animals and Orthotopic Recurrence Assays**

Animal care and experiments were performed with the approval of, and in accordance with, guidelines of the University of Pennsylvania IACUC. Details of tumor generation, orthotopic recurrence assays, and competition assays are provided in Supplemental Experimental Procedures.

#### **Human Subjects**

The I-SPY 1 TRIAL was a collaboration of the American College of Radiology Imaging Network (ACRIN), Cancer and Leukemia Group B (CALGB), and the National Cancer Institute (NCI)'s Specialized Programs of Research Excellence. It consisted of two protocols developed to identify markers of response to conventional neoadjuvant chemotherapy: CALGB 150007 (molecular marker component), and ACRIN 6657/CALGB 150012 (imaging component). The protocol was approved by institutional review boards at all participating institutions. Patients signed one combined informed consent form before joining the study, which allowed them to simultaneously enroll in the CALGB and ACRIN protocols.

#### **Tissue Culture, Plasmids, and RNAi**

Primary and recurrent *MTB/TAN* primary tumor cells were grown as described by Moody et al. (2005). Additional information on in vitro assays, plasmids, and RNAi is provided in Supplemental Experimental Procedures.

### Immunoblotting, Immunofluorescence, Quantitative Reverse-Transcription PCR, Microscopy, and Flow Cytometry

Immunoblotting and immunofluorescence were performed as described by Moody et al. (2005). Information on antibodies, and descriptions of experimental procedures for quantitative reverse-transcription PCR, live-cell imaging, and flow cytometry are presented in Supplemental Experimental Procedures.

#### **Human Breast Cancer Data Sets**

Publicly available microarray data for human primary breast cancer data sets and their corresponding clinical annotation were downloaded and, where necessary, converted to log2 scale. Details on data sets and methods for survival and multivariate analysis are provided in Supplemental Experimental Procedures.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, five tables, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2013.05.007.

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