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HER2/Neu tumorigenesis and metastasis is regulated by E2F activator transcription factors

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Abstract

HER2 / Neu is amplified and overexpressed in a large proportion of human breast cancers, but the signaling pathways that contribute to tumor development and metastatic progression are not completely understood. Using gene expression data and pathway signatures we predicted a role for activator E2F transcription factors in Neu induced tumors. This was genetically tested by interbreeding Neu transgenics with knockouts of the three activator E2Fs. Loss of any E2F delayed Neu induced tumor onset. E2F1 loss accelerated tumor growth while E2F2 and E2F3 loss did not. Strikingly, it was observed that loss of E2F1 or E2F2 significantly reduced the metastatic capacity of the tumor and this was associated with a reduction in circulating tumor cells in the E2F2 knockout. Gene expression analysis between the tumors in the various E2F mutant backgrounds revealed that there was extensive compensation by other E2F family members in the individual knockouts, underscoring the importance of the E2Fs in HER2 / Neu induced tumors. Extension to HER2 positive human breast cancer revealed a number of HER2+ subtypes based on E2F activity with differences in relapse free survival times. Taken together these data demonstrate that the E2F transcription factors are integral to HER2+ tumor development and progression.

Keywords

MMTV-Neu; metastasis; E2F transcription factors; gene expression; signatures

Introduction

ErbB2 (HER2, Neu) is amplified and overexpressed in 20-30% of human breast cancer (1, 2) and is correlated with a poor clinical prognosis (3). ErbB2 is an orphan receptor in the Epidermal Growth Factor Receptor (EGFR) family of receptor tyrosine kinases. Together with the other members of the EGFR family, including EGFR, ErbB3 and ErbB4, ErbB2 is able to activate a number of key signaling pathways after the receptors dimerize and phosphorylate key tyrosine residues in the carboxy terminus. These major signaling pathways include Ras (4), phosphoinositide 3-kinase (PI3K), AKT (5, 6) and STAT (7). Activation of these various pathways culminates in proliferation, cell cycle progression and survival signals (8). Importantly, these events can regulate CyclinD1, which can serve to

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regulate key events in cell cycle progression, including Rb phosphorylation and release of the activator E2Fs (9).

There are a number of mouse models that mimic human HER2+ breast cancer. This included the original description of mammary overexpression of a point mutated activated form of Neu that caused rapid tumor development in the mammary gland (10) where mammary specific expression was directed by the Mouse Mammary Tumor Virus (MMTV) promoter / enhancer. Subsequently, breast cancer was observed in mice with overexpression of the wild type Neu allele with a longer latency (11). Importantly, by interbreeding these MMTV-Neu mice with the cyclinD1 knockout strain it was observed that CyclinD1 was required for tumor formation in the Neu model (12). When the requirement for CyclinD1 in Neu tumors is considered along with the role it plays in cell cycle progression, the possibility emerges that other cell cycle regulation genes are critical for Neu tumor development and progression, including genes such as the activator E2F transcription factors.

E2F transcription factors have been reviewed in detail (13), and are well known to regulate cell cycle progression after release from sequestration upon Rb phosphorylation. E2Fs have also been demonstrated to have other critical activities including the regulation of apoptosis (14, 15), metastasis (16) and development of many tissues, including the mammary gland (17). Indeed, through examination of knockouts for E2F1 (18), E2F2 (19) and E2F3 (20), the role of the E2Fs in diverse aspects of development was established. When considering a potential role for these transcription factors in breast cancer, it is essential to consider their role in the development of the normal mammary gland. The characterization of knockouts of E2F1, 2 and 3 for mammary gland development revealed unique roles for the E2Fs in mammary gland outgrowth, lactation and involution (17). Importantly, these findings served to validate genomic signaling predictions that had predicted a role for the E2Fs in mammary gland development.

Recently predictive gene signatures have been developed from gene expression data using Bayesian regression models (21) in conjunction with training data where E2F expression was induced (22, 23). In short, this generates a signature by inducing expression of a given genetic pathway and tests for activation of the pathway in other samples. By applying these methods to mouse tumor models, the involvement of E2Fs has been predicted in a variety of tumors (24) and a subset of these predictions have been genetically tested (25). For example, interbreeding mice overexpressing Myc with the E2F knockout mice revealed that loss of E2F1 accelerated tumor formation by reducing the apoptosis present in Myc tumors. Conversely, mutant E2F2 or E2F3 backgrounds resulted in a significant delay in tumor onset for Myc tumors due to defects in the early proliferation induced by Myc. When this work was extended to human breast cancer, a link was noted with relapse and human breast cancer for E2F2 using predictive gene signatures (25). Together, this illustrates the efficacy of using genomic signatures in conjunction with genetic tests, demonstrating that E2Fs have a key role in certain cancers.

While E2Fs have been implicated in several cancers, no studies have demonstrated that activator E2Fs regulate HER2 positive breast cancer. However, in addition to the previously discussed demonstration that CyclinD1 was required for MMTV-Neu mediated

tumorigenesis, additional work has demonstrated similar findings showing that CyclinD1 was required for Neu mediated transformation (26). In addition to CyclinD1 requirements, they demonstrated that dominant negative E2F1 blocked Neu mediated transformation *in vitro*. Given that E2F1 dominant negative expression can block transformation and that CyclinD1 is required in MMTVNeu mediated tumors (12), these data strongly argue that E2Fs are critical in HER2/Neu breast cancer. However, in contrast to the Myc studies where we demonstrated a role for the E2Fs in breast cancer (25), there are distinct signaling events and transcriptional patterns and one might expect that the E2Fs may mediate different transcriptional programs. Indeed, given the wide variety of genes with putative E2F binding sites in their promoters (27) (28), it may be that the E2Fs are able to mediate specific activities beyond simple cell cycle control depending upon the signaling context. In the present study we tested the premise that E2Fs were involved in MMTV-Neu mediated breast cancer, demonstrating a role for the E2Fs in breast cancer development and progression. Importantly, these mouse model findings were extended to human HER2 positive breast cancer where a combination of E2F activation statuses predict relapse.

Results

In order to investigate the signaling pathways that are critical in Neu tumor biology we have compared cell signaling pathway signatures across several key mouse models. We applied several cell signaling pathway signatures (23) to gene expression data from MMTV transgenic Neu, Myc and Ras breast cancer models as well as Rb knockout tumors. This generated predictions of pathway activation in the four tumor types. These results, ordered by model system, reveal numerous insights into key pathways involved in tumor biology (Figure 1A). Focusing on MMTV-Neu predictions, we observe that Stat3 and E2F1 had strikingly elevated predicted activity. The demonstration in mouse models that Stat3 was required for metastasis of Neu initiated tumors (29) serves as an additional independent genetic validation of these predictions. Given that this survey of tumor models utilized few samples, we downloaded several datasets that included MMTV-Neu gene expression data. In this merged Neu dataset the probability of activation of the activator E2Fs in Neu tumors relative to other breast cancer tumor models was predicted (Figure 1B). This demonstrated a high probability of E2F1 activation in the majority of the Neu induced tumor samples. In contrast, E2F2 and E2F3 had elevated predicted activity in only a small subset of the Neu tumor samples relative to other tumor types. Taken together this data has allowed the generation of the hypothesis that the E2Fs are involved in Neu induced tumor development.

To directly test the hypothesis that the activator E2F transcription factors regulate Neu tumor development and progression we initiated a genetic cross using mouse model systems. To test this hypothesis we interbred MMTV-Neu transgenic mice (11) with knockouts of E2F1 (18), E2F2 (19) and E2F3 (20) in the FVB background. Monitoring these mice for tumor development revealed that all activator E2F mutant backgrounds delayed tumor onset compared to the wild type E2F background. As predicted by the signaling pathway signatures, loss of E2F1 significantly delayed tumor onset by 84 days, from 227 days in 50% of the control mice to 311 days in the E2F1 knockout ($p<0.0001$) (Figure 2A). This difference was greater than the latency changes associated with E2F2 or E2F3 mutant backgrounds. However, an E2F2 null background did delay tumor onset in 50% of mice

from 227 days in the control to 260 days in the E2F2 knockout (p=0.0089) (Figure 2B). Moreover, loss of one copy of E2F3 delayed tumor onset in 50% of mice from 227 days in the control to 293 days in the E2F3 mutant strain (p<0.0001)(Figure 2C). Together these data demonstrated that loss of any of the activator E2Fs delayed Neu induced tumor onset, with the loss of E2F1 resulting in the greatest delay. At necropsy we noted the total number of tumors was significantly reduced in both the E2F1 and E2F2 knockout backgrounds (Figure 1D). On average the E2F wildtype control was noted to have 2.33 tumors per mouse while the knockout of E2F1 had 1.4 tumors and E2F2 nulls had 1.61 tumors, a significant reduction for both (p=0.0102 and 0.0397 respectively). E2F3 mutant mice had a similar tumor burden reduction but was not quite statistically significant (p=0.1075). Given the latency delay and reduction in tumor burden we then examined the growth rate of the tumors. The time required to progress from initial palpation to 2500 mm^3 (Figure 2E) was recorded. This demonstrated that Neu tumors required 46.1 days to reach endpoint with E2F2 and E2F3 mutant backgrounds not significantly altering this growth rate. Conversely, loss of E2F1 resulted in a significant acceleration of tumor growth with tumors only requiring 28.3 days to reach endpoint, nearly a twofold reduction in time (p=0.001). Together these data illustrate that the activator E2Fs play unique roles in tumor development and growth.

To examine whether loss of E2Fs affected Neu mediated tumor characteristics we examined the histology of the tumors that arose. However, we did not note any major changes in histological patterns, PCNA or TUNEL staining with the loss of E2Fs (data not shown). To then test how loss of E2Fs has affected progression of the tumors, we examined lungs of mice at endpoint for the presence of metastatic lesions. In Neu initiated tumors in the control background metastases were readily observed (Figure 3A). In contrast, Neu tumors in both the E2F1 null and E2F2 null background often did not result in metastatic lesions (Figure 3B and 3C respectively). Unlike the other activator E2Fs, E2F3 mutant mice did develop metastases (Figure 3D). To quantitate this phenotype we examined the histological sections of the lung and counted the number of metastatic lesions. Neu tumors in the control wild type background resulted in metastasis in 86% of mice while loss of E2F1 significantly reduced this to 46% of mice (p=0.0097) (Figure 3E). E2F2 had an even more significant reduction of metastasis with only 35% of mice developing lung metastases (p=0.0055). E2F3 mutant mice do not result in a significant reduction in the proportion of mice that have developed metastasis. In addition to the number of mice developing metastatic lesions, we looked at the number of metastases within an individual section of the lung and scored for mice with 0, 1 to 5 or greater than 5 metastases. This clearly illustrated that there were far fewer E2F1 knockout or E2F2 knockout mice with greater than 5 metastases in a section of the lung relative to the control or E2F3 mutant backgrounds. In addition to examining metastatic frequency, we sought to determine the extent of metastatic spread. For only those mice with metastasis the average area of the metastatic tumor as a function of the area of the entire lung section was quantitated (Figure 3F). This demonstrated that in mice with metastasis, there was no significant difference between control and E2F1 or E2F2 knockout mice. In contrast, E2F3 mutant mice have an increased area of metastatic tumor relative to the lung, but this effect is driven by two animals with extensive metastasis, resulting in the

large standard deviation. When these data are considered together, it is readily apparent that loss of E2F1 or E2F2 inhibits metastasis.

To begin to determine how E2F1 and E2F2 impact metastasis we cultured the circulating tumor cells (CTC) in a colony forming assay from animals at endpoint. A large number of colonies were detected in the control background, a similar number in the E2F1 null background and a reduced number in the E2F2 null background (Figure 4A-C). Quantification of the number of colonies revealed an average of 30 colonies in the control background, 17.3 in the E2F1 knockout and 9.1 in the E2F2 knockout, a significant reduction for E2F2 ($p=0.0031$) (Figure 4D). Considered with the number of metastases observed in Figure 3, this indicates that there are tumor cells within the circulation but that they fail to colonize the lung. To test if metastasis was also linked to microenvironment, CD31 staining for vasculature was completed. This revealed significant differences in vasculature between control and E2F knockout tumors (Figure 4E-F). To directly test if the metastatic effects were cell autonomous, transplantation of Neu induced tumors from E2F1 and E2F2 knockout were orthotopically implanted into wild type recipients. This resulted in tumors with vasculature that closely resembled original tumors for both control and knockout backgrounds (Figure 4G-H). At endpoint we observed metastases in the wild type tumors transplanted into the wild type background, albeit at a vastly reduced rate relative to the spontaneous tumors (Figure 4I). Importantly, no metastatic lesions were observed in the tumors from E2F1 or E2F2 knockout backgrounds (Figure 4J) indicating cell autonomous effects.

In order to elucidate how E2Fs are regulating tumor development and metastasis control background and E2F mutant backgrounds tumors were placed on microarray. Unsupervised hierarchical clustering of this data did not result in stratification of the data by genotype as the various genotypes were equally distributed through the various clusters (data not shown). The gene expression data was then assessed using gene signature methods which revealed significant differences in a number of pathways (Figure 5A). Interestingly, this analysis revealed that the E2F1, E2F2 and RB knockout pathways were upregulated in tumors from the E2F knockout background. Indeed, when the cluster with low levels of E2F1 and E2F2 was examined we noted that the majority of samples were from the wild type E2F background. Conversely, when the cluster with elevated levels of E2F1 and E2F2 were examined we only noted one sample from the E2F wild type background. These data clearly indicate that with the loss of an E2F transcription factor the other family members compensate for the loss. In addition, we noted loss of TGFβ and a slight increase of Myc and β-catenin with the knockout of the E2Fs. To determine if the E2F compensation effects from Figure 5A were significant we compared E2F signatures in the individual genotypes. This revealed significant compensation by the E2Fs, including compensation by E2F1 in the E2F3 mutant line ($p=0.0202$) (Figure 5B) and by E2F2 in the E2F1 knockout line ($p=0.0226$) (Figure 5C). To extend this investigation to other signaling pathways where we had not developed training data, we compared the various genetic backgrounds using Gene Set Enrichment Analysis (GSEA). This analysis reinforced our findings where we observed a significant activation of the Rb pathway with the knockout of E2F2 in comparison to the wild type phenotype (Figure 5C). In addition, by examining the use of transcription factors in the data we noted a significant enrichment of genes containing E2F transcription factor

binding elements within their promoter in the E2F2 knockout genetic background relative to the wild type background (Figure 5D). In a similar fashion, we noted that the E2F3 mutants background tumors had upregulated E2F target genes (data not shown). Together these data indicate that the E2F transcription factor are compensating for the loss of other E2F family members during tumor formation and progression.

Given that these experiments have described a central role for the E2F transcription factors in Neu mediated tumors in a mouse model system, we then tested the hypothesis that the E2Fs were involved in HER2+ human breast cancer. To address this we downloaded several human breast cancer datasets and merged them after removing batch effects. Clinical HER2 status was not reported for all samples and we therefore used a signature to then predict HER2 status for each sample. In addition, we predicted E2F1, E2F2 and E2F3 status for these human samples. These results were clustered and are shown in Figure 6A. There were four major clusters present in the data with large number of samples and patterns emerged for E2F status in these tumors. For instance, cluster A in general had low predicted levels of E2F1 activity and high levels for E2F2 activity while cluster C had the opposite pattern. When these clusters of human patients were examined for relapse free survival, we noted striking differences between the various clusters (Figure 6B). Indeed, the relapse free survival differences between clusters A and C were striking with a p value of 0.0183, indicating that the E2Fs are clearly involved in select subtypes human HER2+ breast cancer. The reported clinical data is shown for the patients in the various clusters (Supplementary Table 1), revealing no major differences between clusters. This analysis was repeated for HER2- samples and the similar clusters for E2F1 / E2F2 expression did not result in striking relapse free survival differences (Supplementary Figure 1). Together, this demonstrates that E2Fs are able to differentiate human HER2+ breast cancer.

Discussion

Using predictive signatures that were trained for the activation of a number of key signaling pathways we surveyed a number of mouse tumor model systems. This analysis predicted that E2F1 was activated in a mouse model of HER2 +ve breast cancer. To directly test this bioinformatically derived hypothesis, we then interbred MMTV-Neu transgenic mice with E2F1, E2F2 and E2F3 mutant mice. The loss of any activator E2F resulted in the delay of tumor onset, with E2F1 loss having the greatest effect. In addition there were specific results for each of the individual E2Fs. Notably, E2F1 loss resulted in tumors that grew significantly more quickly than the controls. E2F2 and E2F3 loss delayed onset but tumors grew at the same rate as controls. The results of these genetic tests clearly validated the bioinformatically generated hypothesis that E2F activation was critical for tumor development and progression. Further examination of the pathway predictions for Neu induced tumors revealed a strong probability for Stat3 being activated in Neu mediated tumors. This prediction has previously been tested in an experiment where Neu induced tumors were generated in a Stat3 deficient background. In agreement with the pathway signaling predictions, Stat3 was found to have a critical role in the progression of Neu mediated tumor metastasis (29). Together, our genetic test and this previous experiment illustrate the importance and utility of predicting pathway activation status in uncovering the roles of key pathways in tumor development and progression.

The most striking data from this study was the observation that specific E2Fs were of critical importance to the metastatic process. Indeed, loss of E2F1 or E2F2 but not E2F3 vastly reduced the metastatic capacity of the tumors. For E2F1 knockouts, the average onset of Neu mediated tumors was delayed, but once the tumors developed they grew at a faster rate than the wild type E2F background controls. Despite these tumors growing more rapidly, they did not metastasize. While seemingly contradictory, the E2F1 knockout both slowed tumor onset and accelerated tumor growth. This may result from accumulation of additional mutations but may also reflect the microenvironment. Indeed, loss of E2F1 affected the micro-vessel density, in agreement with previous studies demonstrating that E2F1 could affect vascularization through p53 dependent control of VEGF transcription (30). Importantly, both the metastatic effects and the vascular effects were cell autonomous where Neu E2F1 and E2F2 knockout tumors did not metastasize and had altered vasculature in wild type recipient mice. Together, it is clear that E2F1 loss can impact many facets of tumor development and progression. In addition, E2F2 null tumors grew at the same rate as control tumors but also did not metastasize, despite having a tumor for the same duration of time as control mice. Conversely, the E2F3 mutant background resulted in tumors that were delayed in onset but that metastasized just as well as tumors from the E2F wild type background. Taken together these data demonstrate that the individual E2Fs have distinct roles in mediating metastasis. In support of this, when we examined the number of circulating tumor cells we only noticed that there was only a significant reduction in the CTCs in the circulation in the E2F2 knockout background. In addition, we noted that this reduction was not a complete absence of CTC, rather it reflected a fourfold reduction of the number of detected CTCs in the E2F2 knockout background. Considered together, these data suggest that the E2F transcription factors are subsequently differentially regulating genes and pathways mediating the metastatic effects.

The importance of E2Fs in tumor progression and metastasis is reinforced by the compensatory activity of the other E2F genes. Indeed, through GSEA analysis we noted that in each of the knockouts there was a significant upregulation of E2F target genes relative to the wild type E2F background, indicating compensation by the other family members which was consistent with previous reports of functional redundancy in the E2F family (31). Given that the E2Fs are released from Rb after phosphorylation by Cyclin D (32) and that Cyclin D is essential for Neu mediated tumors (12), the importance of the combination of E2F alleles becomes apparent. Indeed, our results indicated that individual E2Fs played a role in Neu mediated tumors but the compensation and previous Cyclin D results suggest that a Neu transgenic lacking all of the activator E2Fs may recapitulate the Cyclin D experiment. However, our compensatory results illustrate the importance of multiple E2F family members to Neu induced tumors. Importantly, despite this compensation, E2F loss still resulted in phenotypic effects with differential effects for the unique E2F knockouts. This suggests that despite compensation there are certain genes that are uniquely regulated by individual E2Fs.

While we demonstrated that signaling networks regulated by the individual activator E2Fs are unique based on the differences in phenotypes between E2F knockout backgrounds, it is also instructive to compare the role of the E2Fs between breast cancer induced by different oncogenes. Previously we had predicted and tested a role for the E2Fs in Myc mediated

breast cancer (25) where E2F1 loss accelerates tumor onset and growth rates, in stark contrast to the delayed onset of Neu mediated tumors presented here. However, unlike the Neu tumors, the Myc tumors are highly apoptotic and E2F1 loss reduced the amount of apoptosis observed in the resulting tumors. In addition, E2F2 and E2F3 loss in the Myc model resulted in a similar reduction in tumor latency but also reduced the penetrance of tumor formation. In contrast to the Myc model, E2F2 or E2F3 loss in tumors induced by Neu consistently resulted in tumors. In addition to these gross changes, there were key differences with E2F loss at the signaling pathway level. Indeed, loss of E2Fs in the Myc model activated Ras signaling (25), but we did not observe alterations to Ras levels in the Neu model. Clearly the E2F transcription factors are able to regulate different pathways depending on the signaling context they are placed in. Together, these data demonstrate the ability of the E2Fs to differentially regulate the specific oncogenic signals emanating from major oncogenes.

Importantly, we also extended the analysis of the E2Fs to human breast cancer. While it has previously been demonstrated that basal breast cancer has a poor prognosis (33), recent work has demonstrated that subsets of basal cancer can be distinguished on the basis of pathway activation and that these subclasses have very different overall survival characteristics (23). While E2Fs have previously been implicated in regulation of triple negative breast cancer (34), our mouse data implicate E2Fs in HER2+ tumors. Accordingly, we predicted the HER2 status of a number of human breast cancers and then predicted and clustered E2F activity in the resulting HER2 positive tumors. This resulted in identification of four subtypes of HER2 positive breast cancer and we noted that in one of these subtypes there was significantly improved relapse free survival. These data clearly indicate that E2Fs are involved in human HER positive breast cancer and can be used to predict relapse free survival.

Taken together our results have predicted and then demonstrated a role for the activator E2Fs in HER2 / Neu mediated breast cancer in both mouse model and human breast cancer. Importantly, these results also demonstrate a role for E2Fs in mediating metastasis. While clinically targeting the E2Fs is not feasible due to the large number of putative E2F transcriptional targets, the pathways that they regulate may well be eventually considered as targets for therapy.

Materials and Methods

Animal Work

Animal use and husbandry was in accordance with institutional and federal guidelines. MMTV-Neu mice (11) were purchased from Jackson Laboratories (Bar Harbor, Maine) and were interbred with E2F1 knockout (18), E2F2 knockout (19) and E2F3 knockout mice(20). Mice were monitored twice weekly for tumor development by palpation and visible tumors were measured twice weekly using calipers. Mice were euthanized when the primary tumor reached 20mm in the largest dimension. Kaplan-Meier curves for tumor latency were generated using GraphPad Prism (www.graphpad.com). At the time of necropsy, 200 ul of blood was collected to culture circulating tumor cells in a colony forming assay as

previously described (35). Injections of viably frozen tumor pieces (1 mm^3) were implanted into the inguinal mammary gland to determine cell autonomy of metastasis.

Microarray Processing and Gene Signatures

RNA from flash frozen tumors was collected with the RNeasy Midiprep kit from Qiagen. RNA was used with Affymetrix 430A 2.0 arrays to generate gene expression data. This data was submitted to GEO as GSE42533. Publicly available datasets for microarray data were downloaded from GEO Datasets. Initial mouse model predictions were made using GSE3158. Predictions for human breast cancer datasets were made using a series of datasets including GSE11121, GSE14020, GSE2034, GSE2603, GSE3494, GSE4922 (Singapore cohort), GSE6532, and GSE7390. Datasets were normalized using the MAS5 or RMA algorithm depending on the application or signature being employed. Datasets were then merged after performing Bayesian Factor Regression Modelling (BFRM) to eliminate batch effects between the various datasets (36). Cell signaling signatures were applied to the data as previously described (17, 22, 23, 25) and were depicted as a heat map using MATLAB. Clustering and image analysis was performed with Cluster 3.0 and JavaTreeview.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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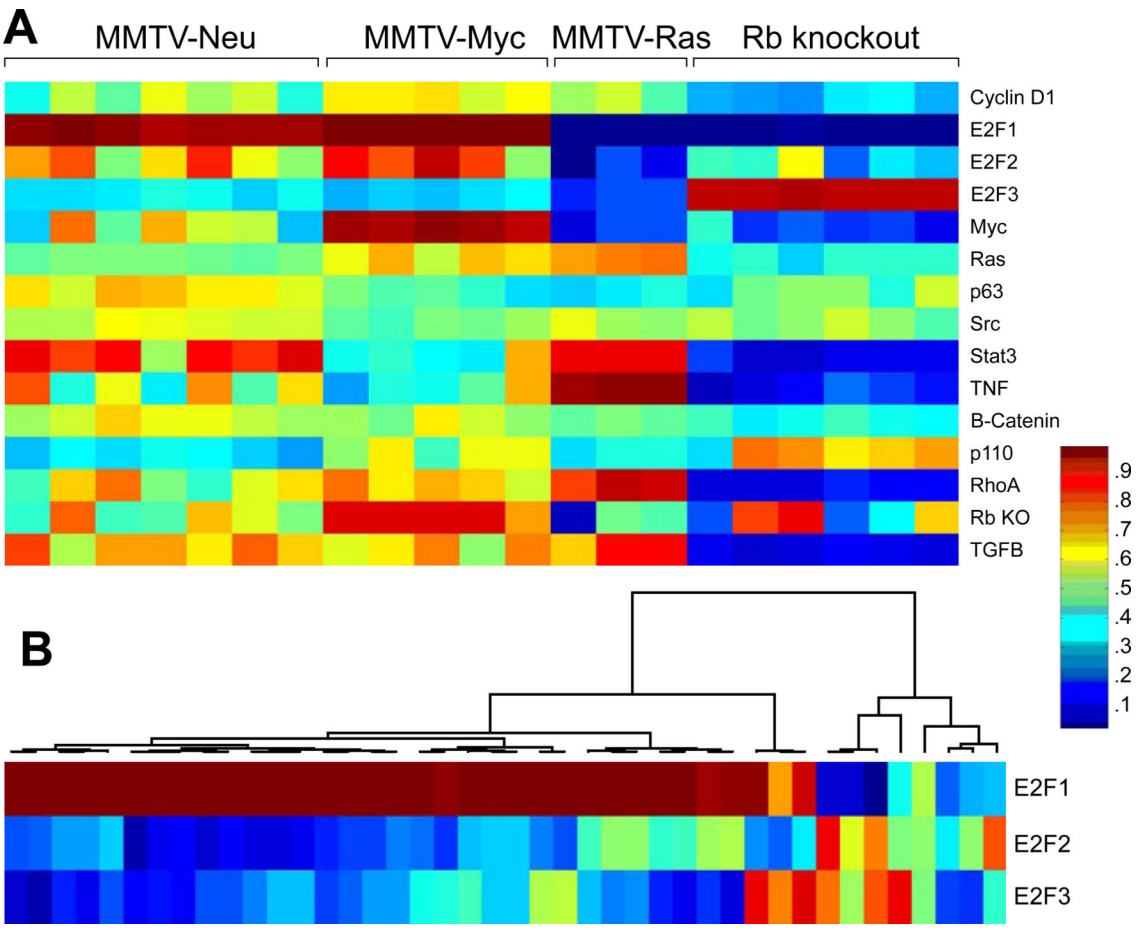

Figure One. Genomic Predictions of E2F Activity in Neu tumors

Signatures for key cell signaling pathways listed on the right were applied to the four tumor types noted on the top of the heat map (A). Probability of pathway activation is illustrated by the heat map with red being a high probability of activation and blue being a low probability of pathway activation. E2F transcription factor signatures were then applied to a larger dataset of Neu mediated tumors revealing cell signaling activation status for the E2Fs (B).

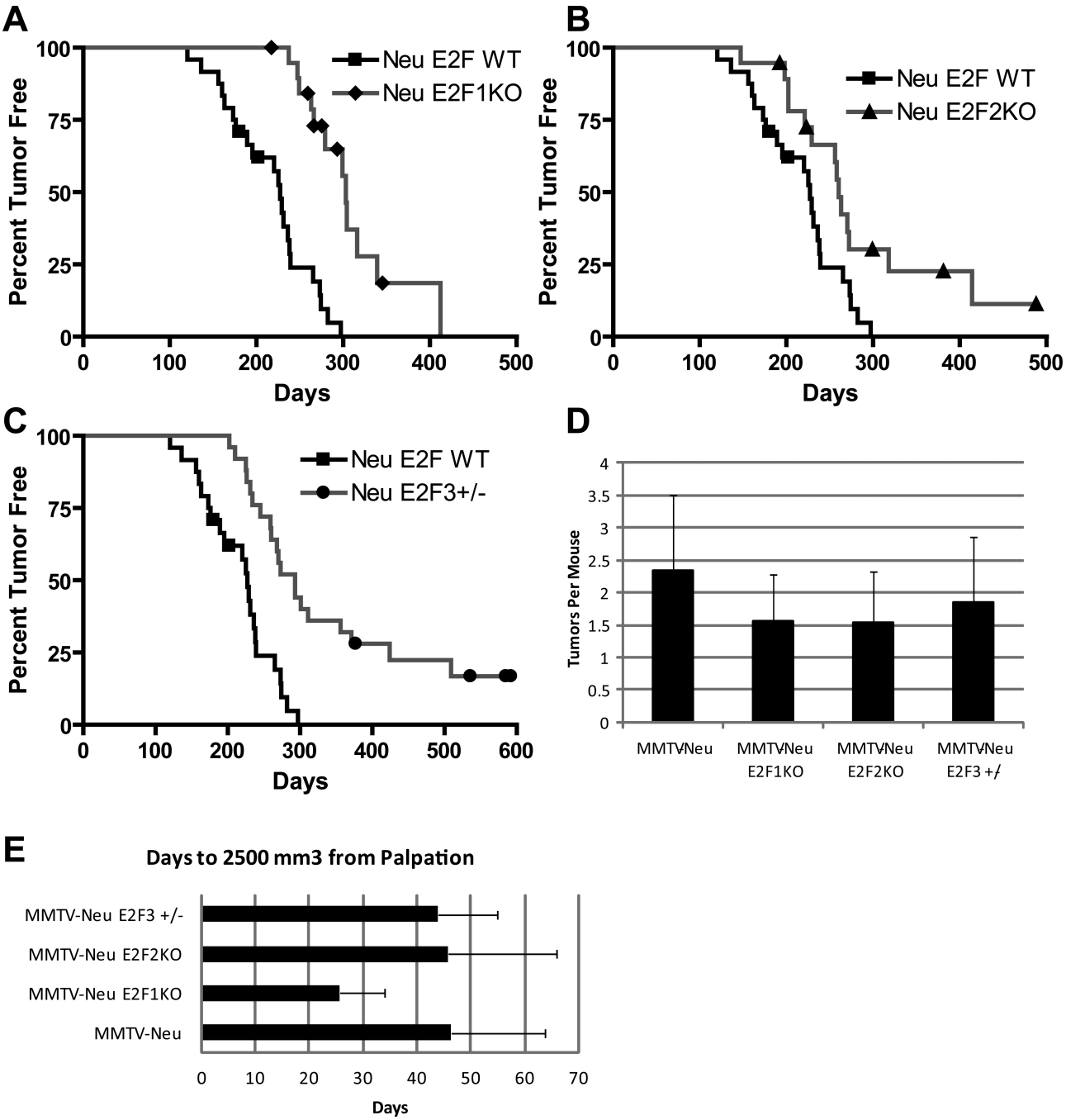

Figure Two. E2F loss delays tumor development and alters growth

MMTV-Neu mice were interbred with FVB E2F1, E2F2 and E2F3 knockout mice and tumor development was monitored. Tumor incidence is shown in the Kaplan-Meier plots for MMTVNeu in a wild type E2F background (black line) compared to MMTV-Neu in a E2F1 knockout (A), E2F2 knockout (B) and E2F3 heterozygous background (grey lines) (C). Tumor latency was significantly delayed in all genotypes ($p<0.0001$, $p=0.0089$ and $p<0.0001$ respectively). Tumor burden was assessed by examining the number of tumors per mouse. The average number of tumors per mouse and the standard deviation is plotted (D),

revealing significant reductions in E2F1 (p=0.0102) and E2F2 knockout backgrounds (p=0.0397). Tumor growth rates were assessed by measuring time to grow from the initial tumor palpation to 2500 mm^3 (E). This revealed a significant acceleration of growth in the E2F1 knockout (p=0.001).

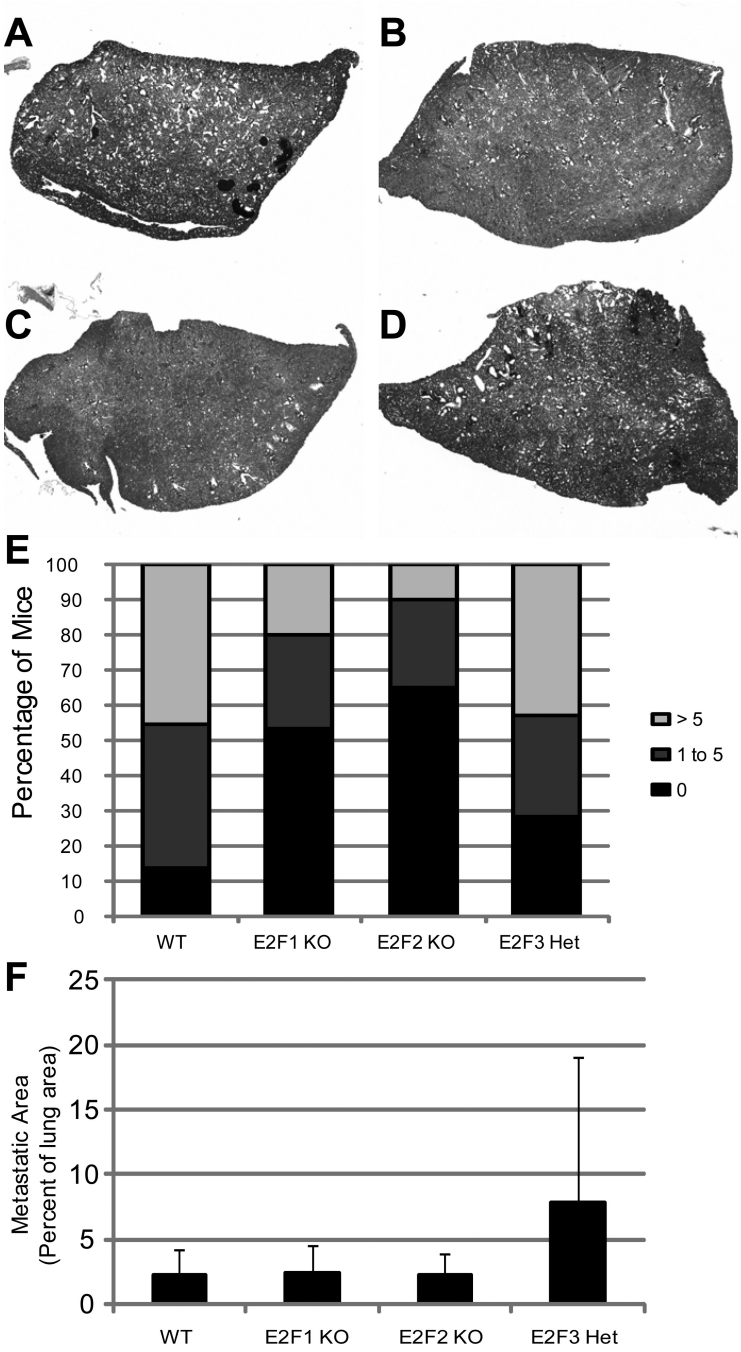

Figure Three. E2F1 or E2F2 loss reduces metastatic potential of Neu induced tumors

Pulmonary sections were examined for lung metastasis of MMTV-Neu induced tumors in the wild type E2F background (A) as well as the E2F1 knockout (B), E2F2 knockout (C) and E2F3 heterozygous background (D). The extent of metastasis was examined (E), and median samples were chosen for the histological images (A-D). The percentage of mice with no metastases (black bar), 1-5 metastases (dark grey bar) and greater than 5 metastases (light grey bar) in a pulmonary section are shown (E) revealing a significant reduction of

metastasis with E2F1 and E2F2 loss. For those mice that did develop metastasis the area of metastasis relative to the lung was determined (F).

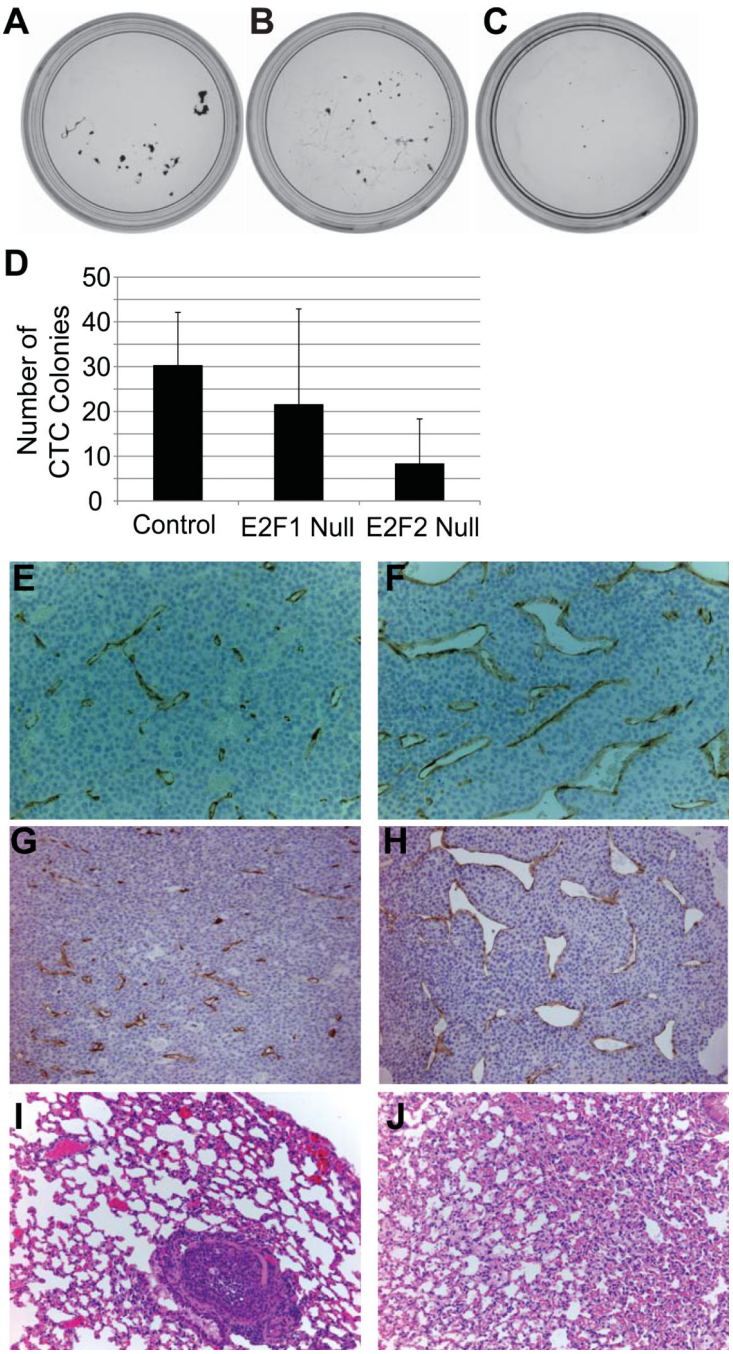

Figure Four. E2F loss is associated with a reduction in the number of circulating tumor cells, altered vasculature and metastatic effects are cell autonomous

Mice at endpoint were assayed for circulating tumor cells through a colony forming assay. The results of the colony forming assay for the MMTV-Neu line in a wild type E2F background (A), an E2F1 knockout background (B) and an E2F2 knockout background (C) are shown. The number of colonies were quantitated (D), with average number of colonies and the standard deviation shown. This revealed a significant ($p=0.0031$) reduction in the number of colonies in the E2F2 knockout background relative to the control background. CD31 staining of tumors from MMTV-Neu (E) and MMTV-Neu E2F1 knockout mice (F)

reveals differences in microvessel structure. These microvessel effects were maintained when MMTV-Neu (G) and MMTV-Neu E2F1 knockout (H) tumors were transplanted into a wild type recipient. Metastasis was observed in lungs from wild type recipients transplanted with Neu (I) but not E2F1 knockout tumors (J) indicating cell autonomy.

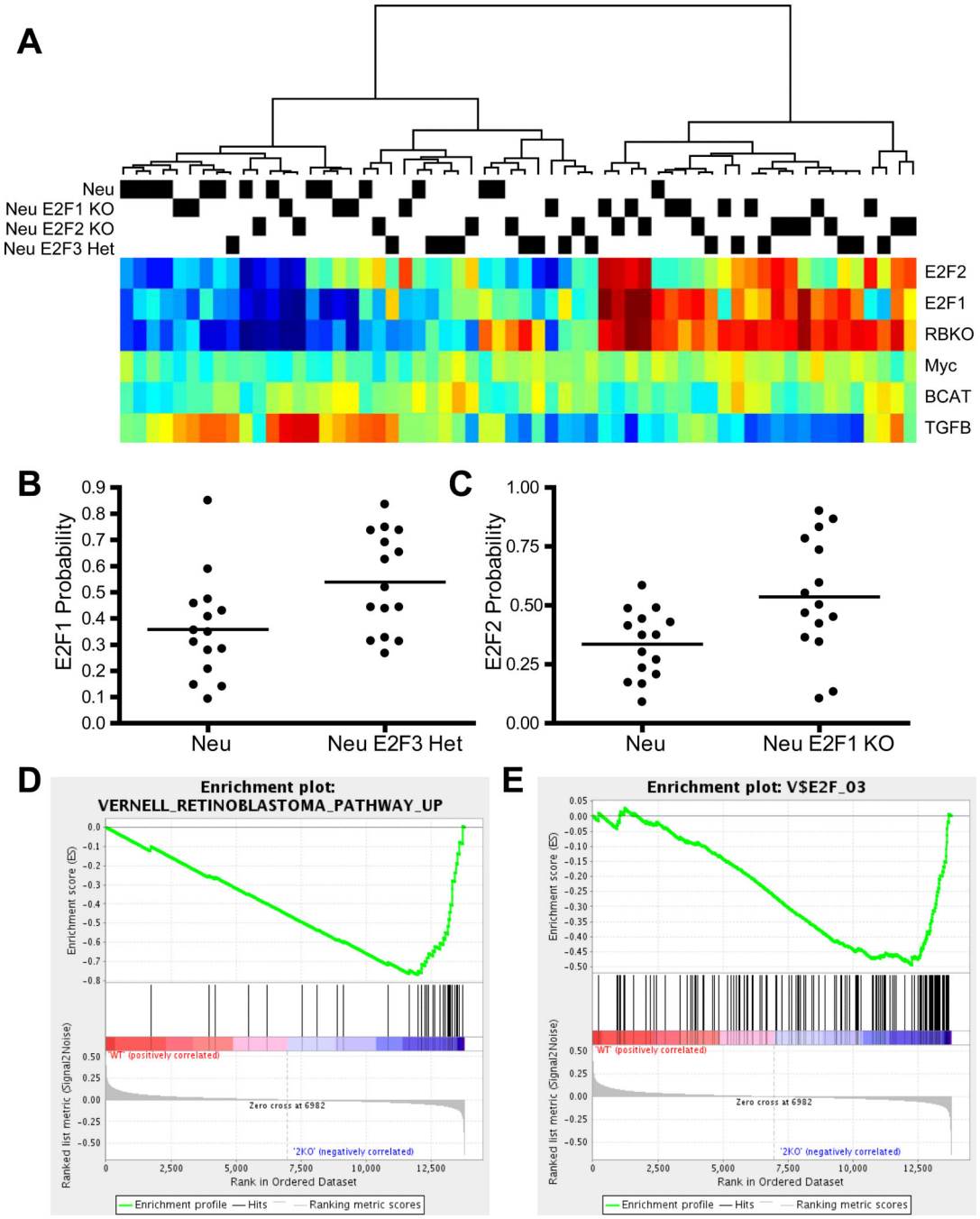

Figure Five. Gene Expression Analysis Reveals Compensation for E2F Knockout

Gene expression data from the MMTV-Neu tumors in the wild type and E2F knockout backgrounds was examined for cell signaling pathway signature activation and were clustered (A). For pathways listed on the right, pathway activation status was calculated. Red denotes high level of predicted activity while blue denotes a low level of predicted activity. Genotypes for the samples are indicated with a black tick mark for each genotype shown on the left. This analysis demonstrated differences in the signaling in the various genetic backgrounds (A). To determine whether there was a significant effect by background

on pathways, the probabilities of the E2F pathways from panel A were tested for significant differences between genotypes. This revealed significant differences for E2F1 probabilities in E2F3 heterozygous mice relative to wild type controls (B) as well as E2F2 probabilities in E2F1 knockout mice (C). Further examination of compensation through GSEA was completed with example plots of the Retinoblastoma pathway (D) and genes containing an E2F site in their promoter (E) being shown after comparing Neu induced tumors in a wild type background to the E2F2 knockout background.

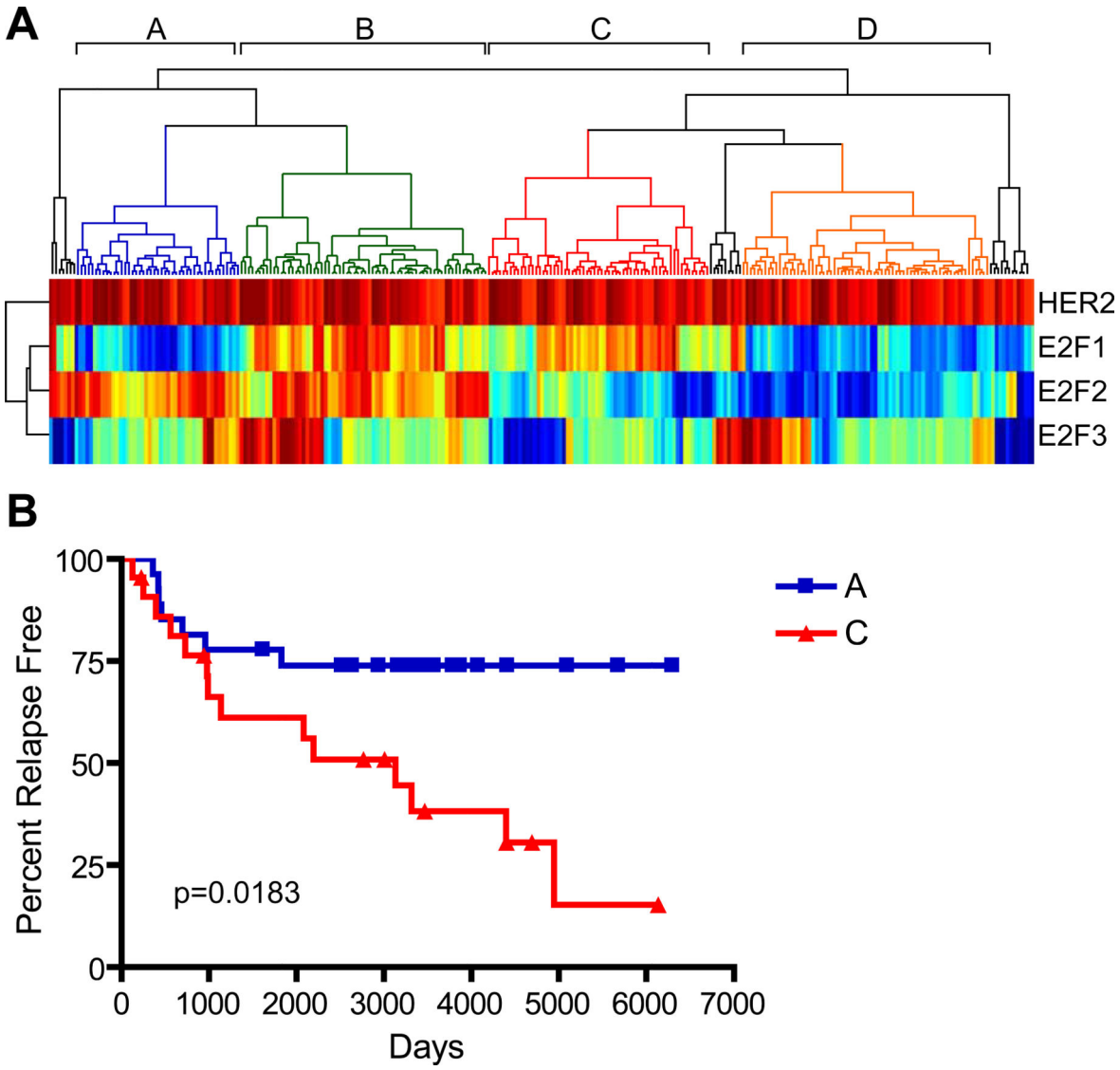

Figure Six. E2F Status Clusters Human HER2+ tumors into Subtypes with Different Relapse Free Survival Times

A dataset of human breast cancer samples was generated and HER2 status was predicted. For HER2+ samples, E2F pathway status was predicted and the results were clustered revealing four distinct clusters with large numbers of samples (A). The dendrograms for these major clusters were color coded and labeled A-D (top of dendrogram). Comparing the relapse free survival times of clusters A and C revealed a significant difference (p=0.0183) in a Kaplan-Meier plot (B).