eIF4E Threshold Levels Differ in Governing Normal and Neoplastic Expansion of Mammary Stem and Luminal Progenitor Cells

Svetlana Avdulov, Jeremy Herrera, Karen Smith, Mark Peterson, Jose R. Gomez-Garcia, Thomas C. Beadnell, Kathryn L. Schwertfeger, Alexey O. Benyumov, J. Carlos Manivel, Shunan Li, Anja-Katrin Bielinsky, Douglas Yee, Peter B. Bitterman, and Vitaly A. Polunovsky

Abstract

Translation initiation factor eIF4E mediates normal cell proliferation, yet induces tumorigenesis when overexpressed. The mechanisms by which eIF4E directs such distinct biologic outputs remain unknown. We found that mouse mammary morphogenesis during pregnancy and lactation is accompanied by increased cap-binding capability of eIF4E and activation of the eIF4E-dependent translational apparatus, but only subtle oscillations in eIF4E abundance. Using a transgenic mouse model engineered so that lactogenic hormones stimulate a sustained increase in eIF4E abundance in stem/progenitor cells of lactogenic mammary epithelium during successive pregnancy/lactation cycles, eIF4E overexpression increased self-renewal, triggered DNA replication stress, and induced formation of premalignant and malignant lesions. Using complementary in vivo and ex vivo approaches, we found that increasing eIF4E levels rescued cells harboring oncogenic c-Myc or H-RasV12 from DNA replication stress and oncogene-induced replication catastrophe. Our findings indicate that distinct threshold levels of eIF4E govern its biologic output in lactating mammary glands and that eIF4E overexpression in the context of stem/progenitor cell population expansion can initiate malignant transformation by enabling cells to evade DNA damage checkpoints activated by oncogenic stimuli. Maintaining eIF4E levels below its proneoplastic threshold is an important anticancer defense in normal cells, with important implications for understanding pregnancy-associated breast cancer.

Introduction

Dysregulation of translation initiation is frequent in malignancy (1–3). The cap-binding protein eIF4E is a rate-limiting component of the translation initiation complex eIF4F (4). When constitutively activated, eIF4E confers cells with cancer hallmark capabilities (1, 2). However, important aspects of eIF4E-mediated translational control of cancer remain undefined. Particularly vexing is the role of eIF4E dysfunction in cancer incidence. Although mice with increased amounts of eIF4E available for translation initiation due to lack of the eIF4E antagonists 4E-BP1 and 4E-BP2 are hypersensitive to oncogenic stimuli, they do not develop spontaneous tumors (5, 6). These findings point to upregulated eIF4E as an essential, but passive participant in oncogenesis with eIF4E functioning as an integrator and downstream effector of oncogenic mutations. However, compelling data from several model systems show that enforced overexpression of deregulated eIF4E confers mammalian cells with a neoplastic phenotype in vitro (7, 8) and induces tumorigenesis in vivo (9, 10)—findings consistent with the view that aberrant eIF4E can be a cancer driver.

As a means to define the role of eIF4E overexpression versus eIF4E dysregulation in cancer incidence, it is reasonable to hypothesize that sustained activation of the eIF4E-mediated translational machinery in expanding cell populations, such as the mammary epithelium during gestation, may create a high-risk state in which relatively small increases in eIF4E expression above the physiologic maximum might set the stage for oncogenesis. Pregnancy exerts a bidirectional, age-dependent effect on mammary carcinogenesis: in women older than 25, breast cancer incidence increases immediately after parturition, remains increased for 10 years, and then gradually falls below the level of nulliparous women (11). Breast cancers diagnosed during or soon after pregnancy, designated pregnancy-associated breast cancer (PABC), tend to be highly aggressive (12). Explanations for PABC include aberrations in the postpartum/weaning...
day 15 (Supplementary Fig. S1A). Transgenic mice were generated by the gift of Dr. Jeff Rosen, Baylor College of Medicine, Houston, TX; pWkpbAll plasmid encoding the murine WAP promoter (a kind three hemagglutinin (HA) epitopes at the C-terminus into the ligation of wild-type (WT) human eIF4E sequences in frame with animal experiments were carried out under an IACUC approved.

Carcinogenesis requires cells to breach the multilayered intrinsic cancer defense program (14, 15). One such defense is triggered when oncogenes increase DNA replication stress. Stalled replication forks that collapse into double-strand breaks (DSB) activate the DNA damage response (DDR). However, persistent lesions often lead to apoptotic death or premature senescence (16). Examples include the induction of premature senescence by oncogenic Ras (17) and the activation of apoptosis by oncogenic Myc (18). The apparent exception is overexpressed elf4E, which drives cell proliferation without triggering cell death, counteracts Myc-induced apoptosis (10, 19), and rescues mammary epithelial cells from premature senescence (20). Thus, it is plausible that fluctuations of elf4E levels just above the usual physiologic maximum could drive oncogenesis by promoting excess proliferation, while disabling DNA damage checkpoints.

To test this formulation, we developed a transgenic mouse model in which naturally occurring pregnancy and lactogenic hormones controlled ectopic elf4E expression in mammary luminal progenitor cells and their progeny. Here, we show that increased elf4E abundance during successive cycles of pregnancy and lactation is sufficient to promote pathologic self-renewal of mammary luminal progenitor cells and induce neoplastic breast lesions. In companion in vitro mechanistic studies, we show that elf4E-mediated hyperproliferation of human mammary epithelial cells is accompanied by increased DNA replication stress and an enhanced DDR that rescues cells from otherwise lethal oncogene-induced DNA damage.

**Materials and Methods**

**Transgenic mice**

FVB/N mice were obtained from The Jackson Laboratory. All animal experiments were carried out under an IACUC-approved protocol. The whey acidic protein (WAP) vector was constructed by ligation of wild-type (WT) human elf4E sequences in frame with three hemagglutinin (HA) epitopes at the C-terminus into the pWkpbAll plasmid encoding the murine WAP promoter (a kind gift of Dr. Jeff Rosen, Baylor College of Medicine, Houston, TX; Supplementary Fig. S1A). Transgenic mice were generated by the University of Minnesota Mouse Genetics Laboratory by microinjection of this construct into FVB/N embryos. Transgenic mice were identified by Southern blotting of tail-snip genomic DNA and confirmed by PCR using the following primers: sense sequence 5’-AGGACGCGATTGACCGCTAT-3’; antisense sequence 3’-GGAAAGAATCACGGTCCGAT-5’.

**Cap-affinity binding assay and immunoblotting**

m7GTP-Sepharose chromatography was performed as previously described (19). Primary antibodies are listed in Supplementary Material and Methods.

**Polysome profiling**

See Supplementary Material and Methods.

**Colonies-forming assay**

We followed our previously published procedure (21). Cultures were continued for 12 days (37°C, 5% CO2) and photographed.

**Cell culture and reagents**

Human mammary epithelial cells (HMEC) constitutively expressing human telomerase reverse transcriptase (hTERT) were provided by Robert Weinberg (Whitehead Institute, Cambridge, MA) and cultured as described previously. (See Supplementary Material and Methods for details; ref. 20).

**Statistical analysis**

ANOVA, Wilcoxon rank sum, or the Student t test with the Dunnett multiple comparison test (S-PLUS Guide to Statistical and Mathematical Analysis, Version 4.0, Seattle, WA) was used with two tails and unequal variance expressed as mean ± SE unless otherwise stated.

**Results**

elf4E is activated during pregnancy and lactation

Prior studies indicate that the cap-dependent translation initiation complex elf4E is activated in proliferating cells (22, 23). Immunoblot analysis of mammary tissue lysates obtained during pregnancy-induced mammary morphogenesis revealed a large increase in the scaffold protein elf4GI and a moderate increase in the mRNA cap-binding protein elf4E (representative blot shown in Fig. 1A). To assess elf4F activity, mammary tissue lysates were incubated with agarose-immobilized m7GTP cap analogue to pull down elf4F and its binding partners; and the amounts of cap-captured elf4E, elf4GI, and 4E-BP1 were evaluated by

---

**Figure 1.**

eIF4F is activated during pregnancy and lactation. A, immunoblot analysis showing steady-state expression levels of eIF4GI, eIF4E, and 4E-BP1 in mammary tissue of virgin or parous (gestation 2) WT FVB/N mice. In parous animals, tissue samples were obtained at pregnancy day 5 (n=2) and day 15 (n=3) and at lactation day 15 (n=3). B, cap-affinity analysis of eIF4F integrity. Cap-bound proteins were subjected to SDS-PAGE, transferred to nitrocellulose, and probed for eIF4GI, eIF4E, and 4E-BP1.
immunoblot analysis (Fig. 1B). The striking increase in cap-captured eIF4GI compared with 4E-BP1 during late pregnancy and lactation indicated marked activation of eIF4F. These data indicate that during the physiologic mammary morphogenesis that occurs in pregnancy, large fluctuations in eIF4GI abundance and eIF4E activity occur; whereas oscillations in eIF4E abundance are minimized.

**Generation of transgenic mice expressing increased levels of eIF4E during pregnancy and lactation**

To test whether larger hormone-driven fluctuations of eIF4E abundance might induce neoplastic changes, we developed a transgenic mouse model in which expression of exogenous eIF4E (tagged with hemagglutinin to distinguish it from the endogenous protein, designated HA-eIF4E) is controlled by the mammary-specific WAP promoter (Supplementary Fig. S1A). In this system, transgene expression is activated by lactogenic hormones and directs expression of HA-eIF4E to a subpopulation of hormone-responsive WAP-expressing mammary cells (24). These cells survive post-weaning involution of mammary tissue, self-renew, and form lactating ductal/alveolar units during subsequent gestations (25). We established three lines of hemizygous transgenic mice (designated WAP-4E) expressing different levels of HA-tagged eIF4E (Supplementary Fig. S1B). Line 3 demonstrated the most stable pattern of gene expression in mammary glands, and was used in all experiments. The presence of exogenous HA-eIF4E can be probed with HA antibody (Fig. 2A) or with antibody against eIF4E (Fig. 2D and in Supplementary Fig. S1C). In virgin females (Fig. 2A) and in WT animals (Fig. 2D, left) expression of HA-eIF4E was below the limit of detection, and its expression in transgenic females followed the same temporal pattern as the fluctuations in levels of endogenous eIF4GI and eIF4E during pregnancy and lactation.
eIF4F integrity; turned on during lactation and turned off upon weaning (Fig. 2A and D left, and Supplementary Fig. S1C). To determine whether physiologically patterned overexpression of eIF4E is sufficient to induce breast oncogenesis, transgenic hemizygous females were continuously housed with WT males beginning after puberty, resulting in almost incessant pregnancy/lactation cycles. WAP promoter–driven transgenes typically show a mosaic expression pattern in hemizygous mice (26). Accordingly, we found that transgene expression in multiparous WAP-4E females was organized into clusters of HA-eIF4E–positive and HA-eIF4E–negative ductal/alveolar units (representative image shown in Fig. 2B). Quantification of HA-eIF4E–expressing ductal/alveolar unit frequency during successive gestations (Fig. 2C) revealed two distinct phases: during the initial phase, encompassing gestations 1 to 5, there were few HA-eIF4E–positive ductal/alveolar units (n = 2 for each gestation); whereas during the second phase, which included gestations 6 to 9 (from 3 to 7 months of sustained pregnancy/lactation), the number of HA-eIF4E–positive ductal/alveolar units progressively increased with each successive pregnancy, indicating that transgenic eIF4E may confer PI-MECs with an advantage in self-renewal during subsequent gestations.

A cap pull-down assay confirmed participation of HA-eIF4E in the eIF4F complex (representative blot shown in Fig. 2D, right); and mammary gland lysates from transgenic mice manifested a subtle shift in their polysome profile toward heavier polysomes (representative tracing shown in Fig. 2E), suggesting activation of global translation. Of note, due to the mosaic pattern of HA-eIF4E expression, quantitative analysis of whole-gland lysates underestimates the levels of exogenous eIF4E, total eIF4E, and translational activity in those acini/ducts that expressed the transgene product. Thus, our data indicated that WAP-driven eIF4E overexpression results in physiologically patterned hyperactivation of cap-dependent translation.

Eif4e transgene-ON: overexpressed eIF4E produces neoplastic lesions and mammary tumors

WAP-4E females experienced multiple pregnancies and lactated normally. Histopathologic analysis of mammary glands (Fig. 3A and B) revealed that during gestations 1 to 5—the phase characterized by low numbers of HA-eIF4E–positive ductal/alveolar units (Fig. 2C)—the overall rates of cell proliferation in the mammary epithelium of WAP-4E mice were similar to that in WT females (n = 2 for each gestation; Fig. 3B); however, proliferation was dramatically increased during gestation 6 (100–120 days after beginning joint male/female housing) and later. One hundred percent of ductal/alveolar units in a random sample of mammary glands from transgenic mice (gestations 5 to 9, n = 14

![Figure 3.](image-url)

**Figure 3.** Eif4e transgene-ON: overexpressed eIF4E produces hyperplastic mammary lesions and stimulates tumorigenesis. A, representative images of mammary tissue from WT and WAP-4E females (gestation 9, day 15 of lactation, n = 2) stained for Ki-67 (brown) and counterstained with hematoxylin. B, shown is two-phase kinetics of Ki-67 expression (mean ± SE) during successive pregnancy/lactation cycles (day 15 of lactation) of WT and WAP-4E mice (n = 4 for each gestation). C, Ki-67 expression in HA-positive and HA-negative mammary epithelial cells (mean ± SE, n = 5), *P < 0.001. D, shown are multifocal (top left, gestation 5) and unifocal (bottom left, gestation 6) breast tumors. Tumor histopathology (hematoxylin/eosin) reveals features of high-grade infiltrating ductal carcinoma, including invasion of vessels (top right) and perineural invasion (bottom right). E, Immunoblot analysis showing steady-state levels of eIF4GI, endogenous eIF4E, exogenous eIF4E (HA-eIF4E), and 4E-BP1 in the multifocal breast tumor (T) shown at D (top) and in the contralateral tumor-free mammary gland (NT); bars, 75 μm.
adenocarcinoma with vascular (Fig. 3D, right upper) and neural tumor (Fig. 3D, left upper) and another developed a solitary mammary tumors. By contrast, among 91 transgenic WAP-4E mice of a cohort of 91 mice) manifested signs of diffuse epithelial hyperplasia and anaplasia (Fig. 3A, right); and most hyperproliferation was restricted to cells expressing exogenous HA-eIF4E (n = 5; Fig. 3C).

One limitation of this model is that the long-term analysis of tumor incidence during continuous pregnancy/lactation cycles in FVB/N females is restricted by their short reproductive period (9–11 gestations during the first 8–10 months of life). During this interval, no multiparous WT FVB/N mice (n = 120) developed mammary tumors. By contrast, among 91 transgenic WAP-4E females followed for 6 to 9 months, one developed a multifocal tumor (Fig. 3D, left upper) and another developed a solitary tumor (Fig. 3D, left bottom). These lesions were high-grade adenocarcinoma with vascular (Fig. 3D, right upper) and neural (Fig. 3D, right bottom) invasion. These findings show that overexpressed elf4E triggered development of breast tumors with a long (minimum of 6 months) latency, which is typical for strong oncogenes driven by the WAP promoter in the tumor-resistant FVB/N line of mice (27). Expression of WAP-driven transgenes is not detectable in advanced tumors (28). Accordingly, we did not detect expression of exogenous elf4E in the tumor tissue (Fig. 3E). Instead, tumors overexpressed endogenous elf4E and increased expression levels of elf4E and 4E-BP1—alterations that are typically observed in advanced breast cancers (2, 29). Together, our observations provide direct evidence that physiologically patterned overexpression of elf4E in lactating mammary glands triggered unscheduled proliferation of mammary epithelial cells and development of premalignant lesions, some of which progress to invasive mammary tumors.

**Eif4e transgene-OFF: elf4E-induced premalignant lesions persist but do not progress**

Prior studies indicate that premalignant lesions formed during pregnancy can progress to breast cancer only if they escape post-weaning involution (27). When weaning was initiated after gestation 2, lobules and alveoli underwent normal regression in both WT and transgenic mammary glands (not shown). In contrast, when weaning was delayed until gestations 6 to 10 (high number of HA-elf4E–positive cells), all transgenic mice (n = 28) developed focal neoplastic nodules (range, 2–4/gland; representative images of mammary gland whole mounts at gestation 9 are shown in Fig. 4A). At that time, most ductal/alveolar units outside of neoplastic nodules were eliminated in both WT and transgenic mammary glands, and proliferation rates in areas adjacent to neoplastic nodules returned to a rate that was indistinguishable from rates for mammary glands from WT mice (Fig. 4B). In marked contrast, proliferation in neoplastic nodules remained increased (Fig. 4C and D). Apoptosis frequency in areas adjacent to neoplastic nodules was similar to that in glands from WT mice (Fig. 4E). In sharp contrast, most cells in involution-resistant neoplastic nodules manifested elf4E-dependent acquisition of resistance to apoptosis (Fig. 4F and G). Although neoplastic nodules persisted for at least 45 days after involution, they did not show signs of progression to more advanced stages of neoplasia in the absence of HA-elf4E expression (Supplementary Fig. S2).

Taken together, results from the elf4E-ON and elf4E-OFF states demonstrated that physiologically patterned elf4E overexpression is sufficient for neoplastic conversion of mammary epithelial cells. We observed two types of neoplastic lesions with distinct fates after weaning: (i) multiple hyperproliferative lesions with cells that were eliminated during involution; and (ii) stochastically induced involution-resistant neoplastic foci, which remained benign in involuted mammary glands (Eif4e transgene-OFF) or progressed to invasive mammary tumors in continuously lactating glands (Eif4e transgene-ON).

**Exogenous elf4E is expressed in luminal cells and increases self-renewal**

The adult mammary epithelium is maintained by a population of mammary stem/progenitor cells (MaSPC) that includes bipotent stem cells and committed myoepithelial and luminal progenitors (30). To identify which cell types were targeted by WAP-driven elf4E for aberrant proliferation, we double-stained mammary glands: (i) with anti-HA antibody to identify cells expressing HA-elf4E, and (ii) with either the luminal marker cytokeratin K8 or the basal marker cytokeratin K14. This analysis revealed that only cells expressing luminal K8 expressed HA-elf4E; whereas cells expressing basal K14 were uniformly HA-negative (Fig. 5A). Further analysis to identify the proliferating pool using Ki-67 revealed that predominantly luminal K8–positive cells manifested increased proliferation compared with their WT counterparts (Fig. 5B, Supplementary Fig. S3).

When MaSPCs are targeted by aberrant proliferation-driving stimuli, some combination of self-renewal and differentiation—with or without proliferation of the more differentiated progeny—ensues (31). To evaluate these events in our system, we used a mammosphere assay (32) in which only MaSPCs from disaggregated mammary glands survive and propagate in vitro without adherence to a substratum. We prepared single-cell suspensions from explants of phenotypically normal (gestation 3, low numbers of HA-elf4E–expressing cells) or hyperplastic (gestation 8, high numbers of HA-elf4E–expressing cells) mammary glands of WAP-4E mice and from pregnancy-matched WT controls (n = 3 in each group). When comparing gestation 8 with gestation 3 on post-weaning day 8 (i.e., expression of HA-elf4E is turned off), primary mammospheres from WAP-4E mice were 3-fold larger and secondary mammospheres were 4-fold more abundant during gestation 8; whereas those from WT mice did not vary with parity (Fig. 5C and D). Thus, as the number of HA-elf4E–positive cells increased, the mammary epithelium of multiparous WAP-4E females became enriched with MaSPCs displaying increased self-renewal and retained this property at least for several generations when expression of exogenous elf4E was turned off.

Luminal progenitors can form both luminal and basal-like tumors (33). Histologic analysis demonstrated that the epithelial cores of both tumors only expressed the luminal marker K8 (Supplementary Fig. S4). Together, our findings show that the WAP-driven expression of HA-elf4E in committed luminal progenitors and/or in bipotent mammary stem cells resulted in a population of aberrantly self-renewing cells prone to forming luminal subtypes of premalignant lesions and mammary tumors.

**Increased expression of elf4E orchestrates ATR-mediated signaling and rescues cells from oncogene-induced replication catastrophe**

To become malignant, cells harboring oncogenic elf4E must evade anticancer defenses that are triggered by intense DNA replication stress caused by unrelenting positive proliferation signals from oncogenic proteins. This leads to collapse of replication forks with subsequent cell death (14, 15, 34). Replication stress-induced DSBs are recognized by the meiotic recombination

www.aacrjournals.org Cancer Res; 75(4) February 15, 2015 691
11 (Mre11)/radiation-sensitive 50 (Rad50)/Nijmegen breakage syndrome protein 1 (NBS1) or the MRN complex, which triggers the genome surveillance pathway and activates the ataxia-telangiectasia–mutated (ATM) kinase. After further processing, vulnerable stretches of single-stranded DNA (ssDNA) are coated with replication protein A (RPA), which orchestrates the activation of the ATM- and Rad3-related (ATR) kinase. In concert, ATM and ATR phosphorylate the downstream effectors histone H2AX (pS139), p53 (pS15), and Chk1 (pS345; ref. 35). During oncogene-driven hyperreplication, however, this system typically proceeds to the induction of premature senescence (36). One exception is hyperproliferation driven by increases in eIF4E, which stimulates cell proliferation while counteracting premature senescence (20) and apoptosis (10, 19, 37).

In mammalian cells, activation of the DNA damage checkpoint can be detected by immunostaining of the phosphorylated form of chromatin-bound histone H2AX (γH2AX). WAP-4E mice had a moderate increase in the proportion of luminal cells with high expression of γH2AX (Fig. 6A and B), indicating that eIF4E-mediated hyperproliferation of luminal progenitors was associated with replication stress and extensive DSBs. To examine the mechanism and to determine whether eIF4E-induced activation of the DDR was associated with rescue of mammary cells from oncogene-induced cell death, we took advantage of an in vitro system, in which overexpression of eIF4E is triggered by peptide growth factors routinely used to maintain hTERT-immortalized HMECs (HMEC/hTERT). Similar to our in vivo model, quiescent HMEC/hTERT harboring the gene encoding HA-eIF4E manifested only traces of exogenous eIF4E, whereas the addition of growth factors dramatically increased expression of HA-eIF4E, which peaked in S and G2–M (Fig. 6C and D). Growth factor–induced overexpression of eIF4E accelerated transit of cells into G2 (Fig. 6C),

Figure 4. Eif4e transgene-OFF: eIF4E-induced premalignant lesions persist in post-weaning mammary glands. A, post-weaning mammary glands of multiparous WAP-4E females contain neoplastic foci whole mounted mammary glands of WT and WAP-4E females (gestation 9, day 10 post-weaning) were stained with carmine (1 and 2). Representative gland pictures and photomicrographs are presented (n = 28). Arrows, neoplastic nodules; LN, lymph node. One nodule (shown in the boxed area, 2) was surgically removed, and 5-μm sections were stained with hematoxylin/eosin (3 and 4). Arrows, deformed acinar structures. B, shown are the proliferative indices (mean ± SD) in post-weaning WT (n = 4) and WAP-4E (n = 4) mammary glands (gestation 8, day 20 post-weaning). C, representative image of the focus counterstained with hematoxylin and probed for expression of Ki-67 (brown). D, the relative numbers of Ki-67–positive cells in neoplastic foci and in adjacent areas of mammary glands from WT (n = 3) and WAP-4E (n = 3) females are shown as mean ± SE. *, P < 0.0005. E, time course of apoptotic activity quantified outside of neoplastic foci in post-weaning WT (n = 3) and WAP-4E mice (n = 3). F, representative image shows scattered TUNEL-positive cells (arrows) in the WAP-4E neoplastic focus. G, shown are the relative numbers of TUNEL-positive cells in neoplastic foci and in adjacent areas of post-weaning WAP-4E mammary glands (day 10 post-weaning; n = 3 mice; mean ± SE); #, P < 0.001.
significantly increased expression of the DNA damage sensor Mre11 and intensified ATR/ATM signaling as evidenced by increased phosphorylation of Chk1 and H2AX and increased levels and phosphorylation of p53 (Fig. 6D). Because cells overexpressing eIF4E revealed signs of enhanced replication stress but survived, we examined whether overexpression of eIF4E might aid in preserving cell viability and mediating neoplastic conversion. When we introduced c-Myc or H-RasV12 into HMEC/hTERT cells, we detected massive DNA degradation (Fig. 7A) and profound suppression of colony formation (Fig. 7B). In sharp contrast, HMEC/hTERT/4E cells harboring c-Myc or H-RasV12 displayed predominantly intact DNA and formed multiple colonies (Fig. 7B). Analysis of cells transfected with H-RasV12 (Supplementary Fig. S5A) demonstrated that coexpression of HA-eIF4E decreased Ras-induced accumulation of cells in S–G2–M and efficiently rescued cells from Ras-mediated DNA degradation (Supplementary Fig. S5B).

To determine whether eIF4E-mediated escape from Ras-induced DNA damage was associated with activation of the DDR, we assessed broken DNA by quantification of RPA1 and γH2AX foci in the nucleus (34). The presence of γH2AX foci alone indicates cells harboring DSBs, from which RPA has either been displaced with resultant homologous recombination (16) or broken ends have not been resected to ssDNA and have been repaired by other pathways. In the context of eIF4E expression, we observed an increased number of cells expressing γH2AX alone (both control and those harboring H-RasV12; Fig. 7C and Supplementary Fig. S5C). Thus, overexpressed eIF4E leads to robust DSBs while also activating the repair machinery to promote cell survival, likely at the expense of oncogene-induced neoplastic transformation.

Discussion
Our findings strongly support the hypothesis that mitogen-driven fluctuations of eIF4E abundance above a safe physiologic maximum create a state of vulnerability to subsequent oncogenic events. We show that physiologically patterned eIF4E overexpression in lactating mammary glands leads to aberrant self-renewal of MaSPCs and promotes development of premalignant and malignant mammary lesions. We further demonstrate that growth factor–driven overexpression of eIF4E sets the stage for preserving DNA integrity in oncogene-stressed mammary epithelial cells by activating a prosurvival DDR. Our findings support a model in which increased eIF4E abundance above the physiologic maximum promotes neoplastic transformation through at least two steps (Fig. 7D). During the first step, overexpressed eIF4E is the sole engine of aberrant self-renewal of MaSPCs. This profoundly disturbs the normal balance of cellular subtypes in the mammary epithelium, resulting in aberrantly proliferating MaSPCs, which...
manifest replicative stress and an increased DDR. Continued hyperproliferation and replicative stress create genomic instability causing some cells to accumulate oncogenic mutations. Such cells serve as a reservoir for the second step in neoplastic transformation, in which overexpressed eIF4E mediates escape from oncogene-induced replication catastrophe. This allows cells that may have sustained otherwise lethal oncogenic lesions to survive and continue on the cancer pathway.

During normal cell proliferation, eIF4GI abundance increases, reminiscent of eIF4GI levels in cancer. In contrast, during normal mammary epithelial cell proliferation the intracellular abundance of eIF4E is tightly clamped, whereas relatively modest elevations of eIF4E levels reproducibly resulted in neoplastic lesions. These findings show that distinct threshold levels of eIF4E—within very tight tolerances—mediate either normal or proneoplastic cell proliferation. These results support the idea that eIF4E abundance is a crucial metric used by cells to discriminate between normal and oncogenic translational signals, and that normal cells must strongly restrain changes in eIF4E abundance to avoid neoplastic conversion.

When proliferation is enforced by strong oncogenic signals, there is increased DNA damage during S phase, replication fork collapse and cell death (15, 36). Importantly, recent findings establish that genes governing DNA repair and genome integrity are under strong translational control (38). We found that increased eIF4E expression in mammary luminal progenitor cells in vivo induces DNA replication stress reflected by γH2AX focus formation. Because replication stress typically results in genetic instability and oncogene-activating mutations (39), these findings help explain the occurrence of sporadic oncogenic lesions.

Figure 6.
Overexpressed eIF4E accelerates cell-cycle progression and stimulates a replication stress response. A and B, analysis in vivo. A, representative images of mammary epithelial cells from WT (n = 3) and transgenic (WAP-4E, n = 4) mammary glands (gestation 6). Sections were immunostained for γH2AX (red) and DAPI (blue); bars, 10 μm. B, quantification of γH2AX expression in luminal and nonluminal cells (mean ± SE, n = 4); *, P < 0.001. C and D, analysis ex vivo. C, representative flow cytometric histograms showing cell-cycle progression of HMEC/hTERT cells harboring HA-eIF4E or an empty vector. Cells were growth factor restricted for 48 hours, stimulated to cycle with growth factors, and DNA content was analyzed at the indicated times after growth factor stimulation. D, whole-cell lysates were obtained at the indicated times after growth factor stimulation of cultured cells and immunoblotted for cell-cycle checkpoint regulators (p27, p53, p-p53Ser15, and p21), ATR targets (Chk1, H2AX, and γH2AXSer139), and Mre11. β-Actin was used as a loading control. P-asynchronously proliferating cells. Shown are represented histograms (C) and blots (D) from three independent experiments.
during the second phase of eIF4E-driven oncogenesis. Our in vitro studies using oncogenic Myc or Ras as prototype inducers of the DDR-mediated anticancer barrier (15) documented that overexpressed eIF4E counteracts the adverse effects of oncogene-induced replication stress and rescues mammary epithelial cells from replication catastrophe, enabling them to undergo clonal expansion. This proneoplastic activity of eIF4E is associated with activation of the DDR machinery, including the MRN complex, which is a major sensor of DSBs and is central to the prosurvival function of the DDR (40). One established mechanism of oncogene-driven replication catastrophe is increased replication initiation or origin firing, a condition that can lead to depletion of intracellular nucleotide pools (16, 41). One possible salvage pathway downstream of overexpressed eIF4E is maintenance of the essential nucleotide pool based upon the recently found ability of eIF4E to stimulate nucleotide synthesis in oncogene-expressing cells by translational activation of phosphoribosyl–pyrophosphate synthetase 2 (PRPS2; ref. 42).

One clinical implication of our study relates to how eIF4E oscillations during pregnancy and lactation might create a high-risk state in which relatively subtle changes in eIF4E-mediated translation could potentially initiate oncogenesis. We speculate that increases in eIF4E abundance above the safe range might occur in expanding cell populations of individuals whose eIF4E levels are at the upper end of the normal eIF4E distribution. Such individuals would represent a potentially identifiable high-risk group for PABC or other malignancies that occur in tissues with actively proliferating stem/progenitor cell...
populations such as the gastrointestinal tract and bone marrow. Our findings imply that therapeutic interventions to normalize aberrant eIF4E function might not only be promising for advanced cancers (3, 43–45), but might also be productively studied for cancer prevention or interception of the neoplastic process at its early stages.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Avdulov, J. Herrera, J.R. Gomez-Garcia, D. Yee, P.B. Bitterman, V.A. Polunovsky
Acquisition of data (eIF4E promotes tumor formation and managed patients, provided facilities, etc.): S. Avdulov, J. Herrera, M. Peterson, T.C. Beadnell, K.L. Schwentker, A.O. Benyounov, J.C. Manivel, S. Li, P.B. Bitterman, V.A. Polunovsky
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Avdulov, J. Herrera, J.R. Gomez-Garcia, A.O. Benyounov, S. Li, A.K. Bielinsky, P.B. Bitterman, V.A. Polunovsky
Writing, review, and/or revision of the manuscript: S. Avdulov, J. Herrera, K.L. Schwentker, J.C. Manivel, A.K. Bielinsky, D. Yee, P.B. Bitterman, V.A. Polunovsky

References
10. Wendel HG, De Stanchina E, Manni S, Malini S, Li, P.B. Bitterman, V.A. Polunovsky
11. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 30, 2014; revised November 24, 2014; accepted November 24, 2014, published OnlineFirst December 18, 2014.

Cancer Research

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.B. Bitterman, V.A. Polunovsky

Study supervision: D. Yee, P.B. Bitterman, V.A. Polunovsky

Acknowledgments
The authors thank Naoko Shima for helpful discussions and advice regarding studies addressing the DDR. David Largaespada for consultations related to gene-expression patterns in transgenic mice and for his critical reading of the article. The authors also thank Sandra Wagner from the University of Minnesota Mouse Genetics Laboratory for her expert assistance in developing transgenic mice.

Grant Support
This work was supported by NIH/NCI grant CA-11338-05, Susan G. Komen Breast Cancer Foundation grant BCTR0402671, and the University of Minnesota MCC/Department of Medicine Ridge grant (V. Polunovsky); by NIH/ NHLBI training grant T32 HL07741 (J. Herrera); by NIH/NCI P30 Masonic Cancer Center support grant 2P30CA07598 (D. Yee and P. Bitterman); and NIH GM074917 (A.-R. Bielinsky).

Law and Practice

The use of this article is deferred in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 so as to indicate this fact.

Downloaded from cancerres.aacrjournals.org on July 21, 2017. © 2015 American Association for Cancer Research.


eIF4E Threshold Levels Differ in Governing Normal and Neoplastic Expansion of Mammary Stem and Luminal Progenitor Cells

Svetlana Avdulov, Jeremy Herrera, Karen Smith, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-2571

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/12/18/0008-5472.CAN-14-2571.DC1

Cited articles
This article cites 45 articles, 10 of which you can access for free at:
http://cancerres.aacrjournals.org/content/75/4/687.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/75/4/687.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.