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Loss of p120-Catenin Induces Metastatic Progression of Breast Cancer by Inducing Anoikis Resistance and Augmenting Growth Factor Receptor Signaling

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Abstract

Metastatic breast cancer remains the chief cause of cancer-related death among women in the Western world. Although loss of cell–cell adhesion is key to breast cancer progression, little is known about the underlying mechanisms that drive tumor invasion and metastasis. Here, we show that somatic loss of p120-catenin (p120) in a conditional mouse model of noninvasive mammary carcinoma results in formation of stromal-dense tumors that resemble human metaplastic breast cancer and metastasize to lungs and lymph nodes. Loss of p120 in anchorage-dependent breast cancer cell lines strongly promoted anoikis resistance through hypersensitization of growth factor receptor (GFR) signaling. Interestingly, p120 deletion also induced secretion of inflammatory cytokines, a feature that likely underlies the formation of the prometastatic microenvironment in p120-negative mammary carcinomas. Our results establish a preclinical platform to develop tailored intervention regimens that target GFR signals to treat p120-negative metastatic breast cancers. *Cancer Res*; 73(15): 4937–49. ©2013 AACR.

Introduction

Adherens junctions are required to maintain epithelial tissue integrity. They are established by homotypic interactions between E-cadherin (*CDH1*) molecules, which in turn control binding of cytosolic catenins that provide linkage to and regulation of the microtubule and actin cytoskeleton (1). Loss or temporal downregulation of E-cadherin is strongly linked to tumor development and progression of several cancer types (2). In breast cancer, timing of adherens junction inactivation has considerable impact on tumor etiology and cellular biochemistry. Although mutational inactivation of E-cadherin is an initiating and causal event in the development of invasive lobular carcinoma (ILC; refs. 3–5), late epigenetic silencing may underlie the progression of invasive ductal carcinoma (IDC; ref. 6) in a process called epithelial-to-mesenchymal transition (EMT;

ref. 7). In conjunction with this is the finding that while translocation of p120-catenin (p120; *CTNND1*) upon E-cadherin inactivation controls Rock-dependent metastasis of ILC, IDC cells do not show dependency on this pathway (8, 9). Thus, p120 may play context-dependent roles in the development and progression of breast cancer.

Under physiologic conditions, p120 binds to the intracellular juxtamembrane domain of E-cadherin (10). Here, p120 controls E-cadherin stability and turnover in a process mediated by Hakai (*CBLI*), (11), presenilin-1 (*PSEN1*; ref. 12), and/or Numb (*NUMB*; ref. 13). Others and we have shown that genetic ablation of E-cadherin or p120 in mouse mammary epithelial cells results in the induction of apoptosis, indicating that inactivation of adherens junction function is not tolerated in the mammary gland (4, 14, 15). In contrast, genetic inactivation in other organ systems does not induce cell death, but instead induces impaired tissue homeostasis and hyperplasia (16–19). Also, p120 inactivation in mice seems to result in inflammation, which may be caused by a loss of barrier function and the production of inflammatory chemoattractants (17, 18, 20). Interestingly, recent data showed that p120 can function as a *bona fide* tumor suppressor in the upper gastrointestinal tract. Here, somatic p120 inactivation induced the development of squamous cell carcinoma, which was accompanied by autocrine production of monocyte/macrophage attractants, thus promoting a proinvasive tumor microenvironment (21).

Several studies have indicated that p120 may be lost or inactivated in approximately 10% of IDC breast cancer cases. Loss was defined as absence of expression in more than 10% of the tumor cells, and correlated to absence of progesterone receptor (PGR) expression and poor prognosis (22–24). Here,

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we have analyzed p120 expression in a comprehensive set of human invasive breast cancer samples and studied the consequences of inactivation of p120 in mammary tumor development and progression in the context of p53 (*Trp53*) loss using conditional mouse models. Mammary-specific p120 loss in mice resulted in a switch from noninvasive to metastatic mammary carcinoma that phenotypically resembled human metaplastic breast cancer. Furthermore, inactivation of p120 resulted in anoikis resistance, which was exacerbated by a sensitization to growth factor receptor (GFR) signaling due to inactivation of the adherens junction. Finally, we show that loss of p120 results in secretion of inflammatory cytokines, which may promote formation of a prometastatic tumor microenvironment.

Materials and Methods

Additional experimental procedures are described in detail in the Supplementary Experimental Procedures.

Patient material

Of note, 298 cases of IDC were collected and histologically examined as described in the Supplementary Experimental Procedures.

Antibodies and cytokine array

The following antibodies were used: mouse anti-p120 [Western blot (WB), 1:2,000; immunofluorescence (IF), 1:500; IHC, 1:500; clone 98/pp120; BD Biosciences], tetramethyl-rhodamin-conjugated p120 (immunofluorescence, 1:150; BD Biosciences) mouse anti-E-cadherin (WB, 1:2,000; clone 36/E-cadherin; BD Biosciences), fluorescein isothiocyanate (FITC)-conjugated E-cadherin (IF, 1:150; BD Biosciences), mouse anti-E-cadherin (IHC, 1:200; clone 4A2C7; Zymed, Invitrogen), rat anti-CK 8 (Troma-1; IHC, 1:125; Developmental Studies Hybridoma Bank products), rabbit anti-CK14 (IHC, 1:10,000; BabCo), guinea pig anti-vimentin (IHC, 1:400; RDI), rabbit anti-SMA (IHC, 1:350; Lab Vision), rat anti-mouse F4/80 (IHC, 1:300; Serotec), mouse anti- β -catenin (WB, 1:2,000; BD Biosciences), rabbit anti- α E-catenin (WB, 1:2,000; Sigma) goat anti-AKT1 (C-20; WB, 1:1,000; Santa Cruz Biotechnology), rabbit anti-p-AKT1^{s473} (WB, 1:1,000; Cell Signaling Technology), rabbit anti-ERK2 (C-14; Santa Cruz Biotechnology), rabbit anti-p-MAPK (p44/42; WB, 1:2,000; Cell Signaling Technology), mouse anti-RhoA (WB, 1:250; Santa Cruz Biotechnology), mouse anti-Rac1 (WB, 1:1,000; Upstate), rabbit anti-Cdc42 (WB, 1:250; Santa Cruz Biotechnology), rabbit anti-EGFR (WB, 1:500; Cell Signaling Technology), rabbit anti-p-EGFR^{T1068} (WB, 1:1,000; Cell Signaling Technology), and sheep anti-digoxigenin (Roche). Secondary antibodies: horseradish peroxidase (HRP)-conjugated rabbit anti-goat, goat anti-mouse, (1:2,000; Dako), HRP-conjugated goat anti-rabbit (1:2,000; Cell Signaling Technology), rabbit anti-sheep antibodies (Dako), Alexa Fluor 561-conjugated anti-mouse antibodies (Molecular Probes), goat anti-rat Alexa Fluor 488 (Invitrogen), goat anti-rabbit Alexa Fluor 555 (Invitrogen), biotin-conjugated anti-guinea pig (Jackson ImmunoResearch). Biotin label-based mouse antibody array was used according to the manufacturer's recommendations (RayBiotech AAM-BLM-1-4).

Mouse crossings genotyping and generation of cell lines

p120 conditional mice containing *loxP* sites in intron 2 and 8 of *Ctnnd1*, (Black-swiss;129SvEvTac; ref. 17) were a kind gift from Al Reynolds (Vanderbilt University Medical Center, Nashville, TN) and crossed onto the *Wcre;Trp53^{F/F}* mouse model (FVB/N;Ola129/sv; refs. 4, 5). Cohorts of *Wcre;Ctnnd1^{F/+}*; *Trp53^{F/F}* and *Wcre;Ctnnd1^{F/+};Trp53^{F/F}* mice were bred from the resulting offspring. *Wcre;Ctnnd1^{F/+};Trp53^{F/F}* mice were subsequently used to generate *Wcre;Trp53^{F/F}* mice to control for the introduction of Black-swiss;129SvEvTac genetic material. Mice were bred and maintained on a mixed background of (FVB/N;Ola129/sv). Genotyping was done by PCR as described previously (4, 17). Mice were monitored for the development of mammary tumors by palpation and euthanized by CO₂ inhalation when mammary tumor size reached a diameter of 10 mm. Full autopsies were conducted for the analysis of tumor histology and the detection of metastases. Age at the time of euthanasia was used to generate the tumor-free survival curves. For the generation of tumor cell lines, tumors from *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* animals were extracted, minced by hand using scalpel blades, and plated onto regular culture dishes in Dulbecco's Modified Eagle Medium (DMEM)-F12 medium as described previously (5).

Plasmids

For stable knockdown of p120, previously described sequences were cloned into a doxycycline-inducible lentiviral expression system (8). For p120 reconstitution experiments, a lentiviral p120-1A cDNA expression construct was generated as described in the Supplementary Experimental Procedures.

Virus production and Rho GTP pulldown

Lentivirus production and transductions were done as described previously (5). In short, 10⁶ Cos-7 cells were seeded onto 10-cm Petri dishes and transiently transfected after 24 hours with third-generation packaging constructs (25) and the indicated viral construct using X-tremeGENE 9 reagent (Roche). Pull-down assays for GTP-loaded RhoGTP family members were conducted as described previously (8, 26).

Cell culture

Mouse *Trp53 Δ/Δ -7* (WP6) and *Trp53 Δ/Δ -3* (KP6) cell lines were generated from primary tumors that developed in *Wcre;Trp53^{F/F}* and *K14cre;Trp53^{F/F}* female mice, respectively. Cells were cultured as described previously (5). Human breast cancer cell lines T47D and MCF7 were cultured in DMEM-F12 (Invitrogen) containing 6% fetal calf serum, 100 IU/mL penicillin, and 100 μ g/mL streptomycin. T47D and MCF7 cell lines were obtained from American Type Culture Collection. Authenticity was tested on May 16, 2012 by means of short-tandem repeat (STR) profiling (LGC Standards), after which large quantities of cells were frozen separately.

Western blot analysis, immunohistochemistry, and fluorescence

Western blotting was conducted as described previously (27). For immunohistochemistry and fluorescence, tissues and cells were isolated, fixed, and stained as described in the

Supplementary Experimental Procedures. *In situ* hybridization–IHC double staining experiments were carried out using labeled PCR products to identify EGF mRNA as described in the Supplementary Experimental Procedures. Samples were analyzed using a DeltaVision RT system (Applied Precision), equipped with a CoolSnap HQ camera and SoftWorx software. Maximum projections were taken from a stack of deconvolved images.

Anoikis resistance and FACS analysis

Anoikis resistance was analyzed by seeding cells at a density of 20,000 cells per well (in 500 μ L) in a 24-well ultra-low cluster polystyrene culture dish (Corning). After 4 days, cells were harvested and resuspended in 75 μ L of Annexin V buffer supplemented with Annexin V (IQ Products) and propidium iodide (Sigma-Aldrich). The percentage of anoikis-resistant cells was defined as the Annexin V and propidium iodide–negative population analyzed on a BD FACSCalibur. To determine cellular EGF-binding ability, 400 ng of Alexa Fluor 647–conjugated EGF (Invitrogen) was incubated with 1×10^5 trypsinized ice-cold cells in 100 μ L PBS. Cells were washed with PBS to remove unbound EGF-647 and subjected to fluorescence-activated cell sorting (FACS) analysis.

Growth factor stimulation assays

Cells were seeded at 400,000 cells per 6-well in growth factor–free medium for 4 hours, subsequently washed and serum-starved overnight. Next, cells were stimulated with EGF (5 ng/mL; Sigma) or HGF (25 ng/mL; R&D Systems) for 10 minutes. Cells were placed on ice and washed twice with ice-cold PBS containing Ca^{2+} and Mg^{2+} , and directly lysed in lysis buffer.

Statistical analyses

Statistical analyses were conducted using GraphPad Prism 5 (GraphPad Software). Analyses on human samples were conducted as described previously (28). For analysis of growth pattern and metastasis formation, Fisher exact test was used. For metastasis-free survival analysis, the log-rank test was used. For anoikis assays, statistical significance was calculated using the Student *t* test (two-tailed), showing measurements of at least three independent experiments. Error bars in all experiments represent SD or SEM as indicated, of at least triplicate measurements. We considered *P* values less than 0.05 as statistically significant.

Ethics statement

All animal experiments were approved by the University Animal Experimental Committee, University Medical Center Utrecht (Utrecht, the Netherlands). Use of anonymous or coded leftover material for scientific purposes is part of the standard treatment contract with patients in our hospitals.

Results

Loss of p120 expression in invasive breast cancer

To extend previous findings on loss of p120 expression in breast cancer, we conducted immunohistochemistry (IHC) on

a panel of 298 invasive ductal breast cancers and determined their clinicopathologic variables (Supplementary Table S1). Membranous p120 was scored (absent/low vs. medium/high) in three independent tissue cores per tumor. Because we hypothesized that loss of p120 may be linked to increased tumor progression, we defined expression as absent/low if more than 10% of the tumor cells were negative for p120 (Supplementary Fig. S1A), as has been used for E-cadherin (29). Using these parameters, we observed that 34% of the IDC samples showed absent/low p120 staining. Upon correlation of p120 expression levels to clinicopathologic variables, a significant association was obtained between p120 loss, high tumor grade (*P* = 0.007), mitotic index (MAI; *P* = 0.002), and overall absence of hormone receptor expression (*P* = 0.039; Supplementary Table S2). Although p120 expression levels did not associate with tumor size, lymph node status, or the other clinicopathologic variables tested (Supplementary Table S2), these data suggest that loss of p120 expression coincides with breast cancer aggressiveness.

Somatic inactivation of p120 results in the formation of metastatic mammary carcinoma

Functional inactivation of the adherens junction through somatic inactivation of E-cadherin is a causal event in the development of ILC (5). In E-cadherin–mutant breast cancer, p120 translocates to the cytosol where it exerts a key oncogenic role by regulating anchorage-independent tumor growth and metastasis (8). To study the effect of p120 loss on tumor development and progression of breast cancer, we introduced a conditional p120 allele (*Ctnnd1^F*; ref. 17) onto the *Wcre*; *Trp53^{F/F}* (4) noninvasive mammary carcinoma model to produce *Wcre*; *Ctnnd1^{F/+}*; *Trp53^{F/F}* and *Wcre*; *Ctnnd1^{F/F}*; *Trp53^{F/F}* mice. To correct for the differences in genetic background between the *Ctnnd1^F* (17) and the *Wcre*; *Trp53^{F/F}* (FVB/N; 129P2/OlaHsd) mice, a control cohort was bred using *Wcre*; *Ctnnd1^{F/+}*; *Trp53^{F/F}* litter mates to produce *Wcre*; *Trp53^{F/F}*. Next, females were monitored for spontaneous tumor development. In contrast to conditional inactivation of E-cadherin (4, 5), we observed that the median tumor-free latency (*T*₅₀) did not significantly change in either *Wcre*; *Ctnnd1^{F/+}*; *Trp53^{F/F}* (*T*₅₀ = 223 days) or *Wcre*; *Ctnnd1^{F/F}*; *Trp53^{F/F}* female mice (*T*₅₀ = 214 days) when compared with *Wcre*; *Trp53^{F/F}* females (*T*₅₀ = 213 days; *P* = 0.5006 and 0.5859, respectively; Fig. 1A; Table 1). Mammary tumors from *Wcre*; *Trp53^{F/F}* females were morphologically typed as adenocarcinomas and carcinosarcomas, with expansive growth patterns, dense cellular sheets, and irregular bundles of polygonal to plump spindle-shaped cells (Table 1). Tumors that developed in *Wcre*; *Ctnnd1^{F/+}*; *Trp53^{F/F}* and *Wcre*; *Ctnnd1^{F/F}*; *Trp53^{F/F}* females showed a shift from expansive to invasive growth as compared with *Wcre*; *Trp53^{F/F}* animals (*P* = 0.026 and 0.006, respectively; Table 1). In contrast to somatic E-cadherin inactivation and the development of ILC, both heterozygous and homozygous loss of p120 resulted in metaplastic tumor cells displaying a spindle cell morphology, often expressing vimentin with focal expression of cytokeratin (CK)8 or CK14, reminiscent of an EMT (Fig. 1B, top; Supplementary Table S3; Supplementary Fig. S1B). Tumors developed

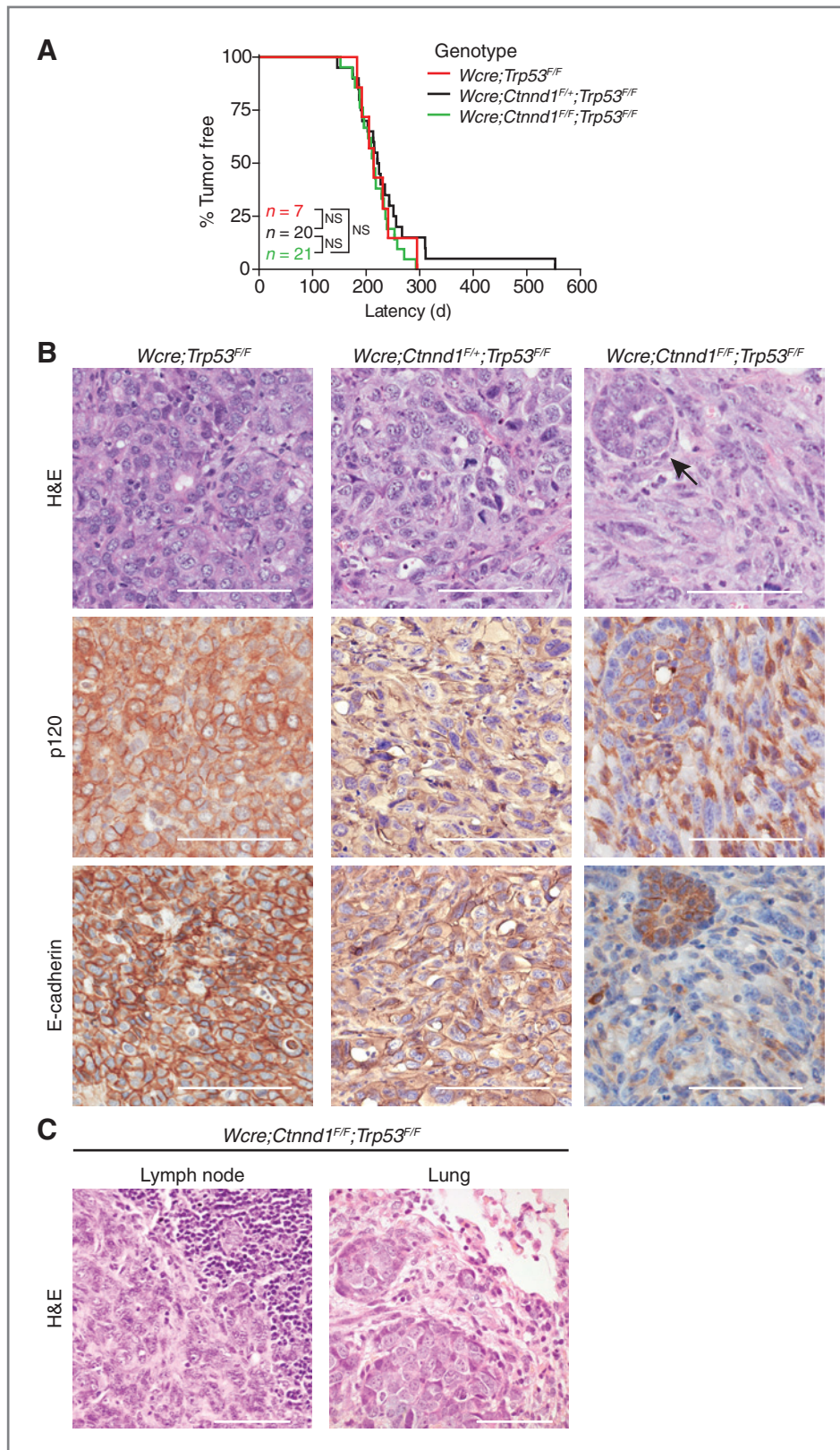


Table 1. Mammary tumor spectrum of *Wcre;Trp53^{F/F}*, *Wcre;Ctnnd1^{F/+};Trp53^{F/F}* and *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* female mice

Genotype	Number of mice	Median latency, d	Metastasis	Local invasion	AC	SC/CS	mILC
<i>Wcre;Trp53^{F/F}</i>	7	213	0 (0%)	0 (0%)	3 (43%)	7 (100%)	0 (0%)
<i>Wcre;Ctnnd1^{F/+};Trp53^{F/F}</i>	20	223	1 (5%)	10 (50%)	1 (5%)	19 (95%)	1 (5%)
<i>Wcre;Ctnnd1^{F/F};Trp53^{F/F}</i>	21	214	9 (43%)	14 (67%)	3 (14%)	18 (86%)	1 (5%)

NOTE: If tumors were composed of two separate histologic types, both were counted separately.

Abbreviations: AC, adenocarcinoma, glandular-type mammary carcinoma; SC/CS, solid carcinoma/carcinosarcoma, tumor consisting of epithelial and mesenchymal cell types.

with high incidence multifocally in different mammary glands and were characterized by a more abundant and dense stromal microenvironment. Mammary tumors from *Wcre;Ctnnd1^{F/+};Trp53^{F/F}* females did not show LOH of the *Ctnnd1* locus, as tumors expressed membrane-localized p120 and E-cadherin (Fig. 1B, middle and Supplementary Table S3). Mammary tumors from *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* females showed large cells with pleomorphic nuclei, coarsely clumped chromatin, and sparse cytoplasm. As expected, all tumors from *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* female mice lacked expression of membranous p120 and E-cadherin (Fig. 1B, right and Supplementary Table S3).

When compared with human breast cancer, tumors that developed in *Wcre;Trp53^{F/F}* mice corresponded to well-differentiated "luminal type" IDC, with low proliferation and expansive growth patterns. They predominantly express luminal CK8, showing little expression of basal CK14 (Supplementary Fig. S1B and Supplementary Table S3). Also, these tumors show a relatively good prognosis with infrequent formation of distant metastases. The highly infiltrative tumors arising in *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* females corresponded to poorly differentiated "basal type" tumors. As such, they presented phenotypic features resembling human metaplastic IDC that are characterized by a high proliferation rate, strong nuclear atypia, and expression of CK14 (*KRT14*) and vimentin (*VIM*). In contrast to ILC, mouse and human metaplastic tumors displayed decreased p120 levels and a punctate/mislocalized E-cadherin expression pattern (Supplementary Fig. S1C and Supplementary Table S3). Human metaplastic carcinoma corresponds with poor prognosis and rapid onset of distant metastases (30).

Interestingly, and in contrast to dual somatic inactivation of E-cadherin and p53 (4, 5), we observed abundant formation of precursor carcinoma *in situ* (CIS) lesions in *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* females (Fig. 2A). CIS lesions showed loss of p120, but occasionally retained low levels of E-cadherin expression (Fig. 2B and C), indicating that upon p120 inactivation residual E-cadherin remains expressed in a temporal manner, which may underlie the initial noninvasive nature of the CIS-type structures.

Next, we examined whether the acquisition of invasive behavior upon p120 ablation resulted in an increased metastatic rate. We observed a significant increase in tumor cell dissemination

in *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* versus *Wcre;Ctnnd1^{F/+};Trp53^{F/F}* and *Wcre;Trp53^{F/F}* female mice (both $P < 0.05$; Table 1), a phenotype not observed in previously published models where inactivation was targeted to the gastrointestinal tract or skin (17, 18, 20, 21). Metastases phenotypically resembled the primary tumor and localized to regional or distant lymph nodes and lungs (Fig. 1C). Because tumor-free latency was identical in *Wcre;Ctnnd1^{F/+};Trp53^{F/F}* versus *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* mice, but only *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* showed metastatic dissemination, we generated primary cultures from both tumor models and assayed anoikis resistance, a hallmark of metastatic cells (5, 31). In agreement with the *in vivo* metastatic behavior, we observed that tumor cells derived from *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* female mice (*Ctnnd1^{Δ/Δ};Trp53^{Δ/Δ}*) were anoikis resistant, whereas neither cells derived from *Wcre;Ctnnd1^{F/+};Trp53^{F/F}* nor *Wcre;Trp53^{F/F}* mammary tumors (*Ctnnd1^{Δ/+};Trp53^{Δ/Δ}* and *Trp53^{Δ/Δ}*, respectively) survived under these conditions (Fig. 2D). These results suggested that homozygous inactivation of p120 is necessary for the acquisition of anchorage independence and subsequent dissemination of mammary tumor cells. In conclusion, we show that conditional loss of p120 induces a transition to highly invasive and metastatic mammary carcinoma.

Loss of p120 results in loss of the adherens junction, transition to a mesenchymal phenotype, and anoikis resistance

Because we observed anoikis resistance in primary tumor cells derived from *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* female mice (Fig. 2D), we examined if p120 inactivation was the causal event in this anchorage-independent survival phenotype. To this end, we made use of *Trp53^{Δ/Δ}* tumor cell lines previously generated from adenocarcinomas that developed in either *K14Cre;Trp53^{F/F}* (32) or *Wcre;Trp53^{F/F}* female mice (4). Two independent *Trp53^{Δ/Δ}* cell lines were transduced using a doxycycline-inducible lentiviral construct targeting p120 (p120-iKD). Doxycycline administration resulted in a strong reduction of p120 protein expression (Fig. 3A), which was accompanied by a decrease in both E-cadherin and β -catenin levels and a slight decrease in α -catenin protein expression (Supplementary Fig. S2A and S2B). Upon p120-iKD, cells lost their typical epithelial appearance and transitioned toward a mesenchymal and motile phenotype (Supplementary Fig. S2F). To confirm the specificity of the RNA interference (RNAi) sequence used, we

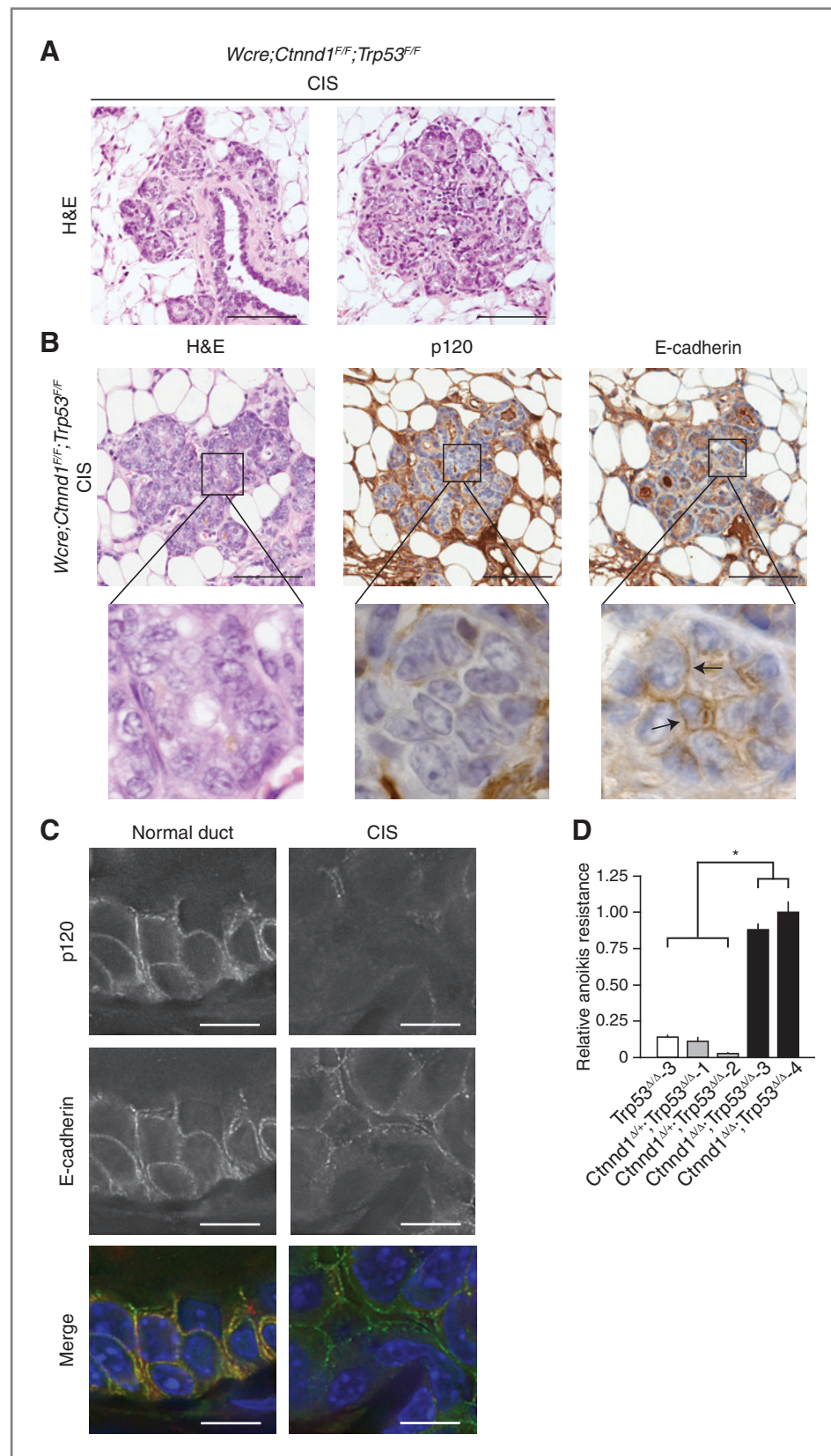


Figure 2. Somatic inactivation of p120 leads to development of CIS. A, CIS formation in premalignant *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* female mice. Shown are two separate hematoxylin and eosin (H&E) stainings. B and C, residual E-cadherin expression in CIS-type lesions in *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* female mice. B and C, H&E staining and IHC for p120 and E-cadherin on consecutive sections (B) and IF stainings for E-cadherin (green) and p120 (red) in a normal mammary duct (top) and a CIS-type mammary lesion (bottom) from a *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* female mouse (C). Note the presence of E-cadherin (arrows) in the absence of p120 expression. Bars, 10 μ m. D, metastatic capacity correlates to anoikis resistance. Trp53^{Δ/Δ} cell line (white bar) and primary cultures derived from tumors that developed in *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* (gray bars) and *Wcre;Ctnnd1^{F/F};Trp53^{Δ/Δ}* (black bars) female mice were subjected to 4 days of anchorage independent culturing and subsequent anoikis resistance analysis using FACS. *, $P < 0.005$. Error bars represent SD of triplicate experiments.

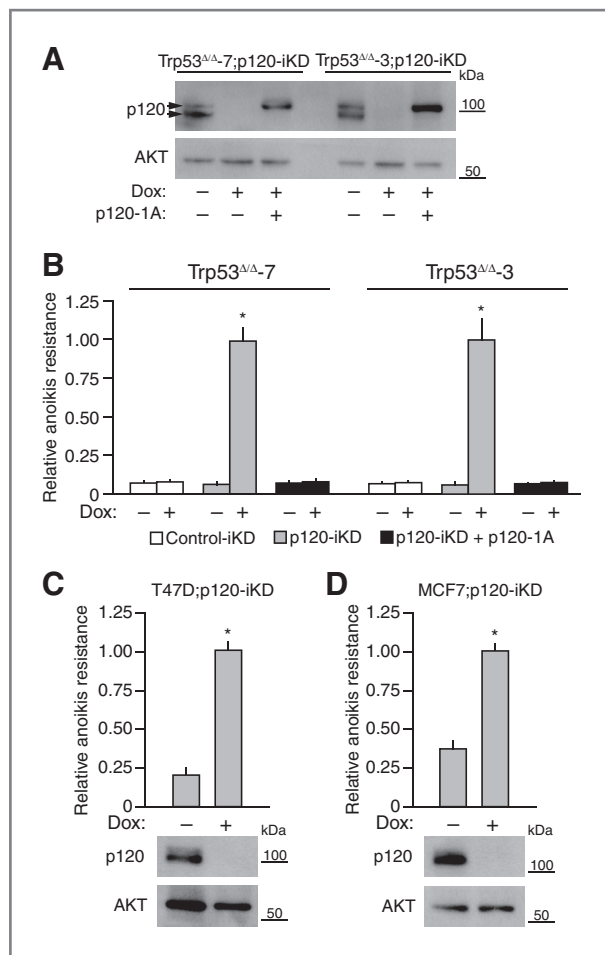


Figure 3. Loss of p120 results in anoikis resistance of E-cadherin-expressing breast cancer cells. **A**, two independent Trp53^{ΔΔ} cell lines were transduced with viruses carrying doxycycline (Dox)-inducible p120 short hairpin RNAs (shRNA; p120-iKD) and a nontargetable p120 isoform1A (p120-1A). Shown is the extent of p120 knockdown and p120-1A expression levels. Arrows indicate p120 isoforms. AKT was used as a loading control. **B**, Trp53^{ΔΔ};Control-iKD-, p120-iKD-, and p120-1A-expressing mammary carcinoma cells were cultured in the presence or absence of doxycycline for 4 days before subjecting cells to 4 days of anchorage-independent culturing and subsequent anoikis resistance analysis using FACS. **C** and **D**, T47D (**C**) and MCF7 (**D**) were transduced with viruses carrying p120-iKD constructs targeting human p120, treated with doxycycline for 4 days, and subjected to nonadherent culturing. Anoikis resistance was analyzed after 4 days. Bottom, the extent of p120 knockdown (top blot). AKT was used as loading control (bottom blot). *, $P < 0.005$. Error bars represent SD of triplicate experiments.

reconstituted cells with a nontargetable p120 cDNA to near endogenous levels, which completely reverted the doxycycline-induced EMT phenotype (Fig. 3A and Supplementary Fig. S2E and S2F).

We next cultured two independent Trp53^{ΔΔ};p120-iKD cell lines in suspension and assayed anoikis resistance in the presence and absence of doxycycline. Although the majority of untreated Trp53^{ΔΔ};p120-iKD cells underwent anoikis, administration of doxycycline-induced anoikis resistance (Fig. 3B), which could be fully reverted upon expression of a non-

targetable p120 construct (Fig. 3B). To substantiate these findings, we used two E-cadherin-expressing and anchorage-dependent human breast cancer cell lines (T47D and MCF7) in which we conducted knockdown of p120 and assayed anchorage independence. MCF7 was previously reported to acquire anchorage independence in soft agar upon constitutive p120 KD (9). Indeed, as for the mouse mammary carcinoma cells, p120-iKD induced downregulation of the adherens junction members and functionally induced anoikis resistance (Supplementary Fig. S2C and S2D and Fig. 3C and D). In conclusion, our data show that p120 knockdown results in loss of adherens junction function, and suggests that this underlies acquisition of anoikis resistance. Because these features are well-known hallmarks of malignancy (33), our findings suggest that loss of p120 may lead to metastasis through acquisition of anchorage independence.

Loss of p120 potentiates GFR signaling

Upon knockdown of p120 in MCF7, cells acquire Rac-dependent anchorage-independent survival, which is probably due to relieve of E-cadherin-mediated inhibition of Ras (*KRAS*; ref. 9). Although we could confirm that active Rac1 (*RAC1*) levels increased in MCF7 upon p120-iKD, we did not detect activation of Rac1 in either Trp53^{ΔΔ};p120-iKD cell lines or human T47D; p120-iKD cells after doxycycline administration (Supplementary Fig. S3). Also, no changes were observed in the levels or activity of the other Rho family members RhoA (*RHOA*) and Cdc42 (*CDC42*; Supplementary Fig. S3C), indicating that loss of p120 in these cell systems does not result in aberrant Rho GTPase activation.

Formation or functional disruption of adherens junctions can affect EGF receptor (EGFR) activity (34). Whether adherens junction disruption results in activation or inhibition of EGFR seems to be largely cell type-dependent (35, 36). Because p120 knockdown resulted in a loss of adherens junction formation through downregulation of E-cadherin, we wondered whether GFR signaling was affected. To this end, we stimulated doxycycline-treated Trp53^{ΔΔ};p120-iKD cells with EGF and assayed EGFR phosphorylation. Although EGF stimulation in control-iKD cells induced a modest EGFR tyrosine phosphorylation, knockdown of p120 resulted in a 1.5- to 2.0-fold increased phosphorylation (Fig. 4A, compare lanes 3 and 4; quantification of three independent experiments in Fig. 4B; nonstimulated in Supplementary Fig. S5A). In line with these findings, we observed that the downstream PI3K/AKT and mitogen-activated protein kinases (MAPK) pathways displayed a 3- to 5-fold increased EGF-induced phosphorylation upon knockdown of p120 (Fig. 4A and B). To substantiate these findings, we made use of the Ctnnd1^{ΔΔ};Trp53^{ΔΔ} cell lines. Similarly to the GFR sensitization after inducible p120 knockdown, Ctnnd1^{ΔΔ};Trp53^{ΔΔ} cells showed a robust increase in EGF-induced phosphorylation of EGFR and MAPK and a modest induction of phosphorylated AKT as compared with Trp53^{ΔΔ} cells (Supplementary Fig. S6A). Furthermore, EGF stimulation of MCF7;p120-iKD cells also showed a sensitization of EGFR, AKT, and MAPK phosphorylation upon knockdown of p120 (Fig. 4C and D). The increased EGFR signaling following p120 knockdown was not due to increased EGFR

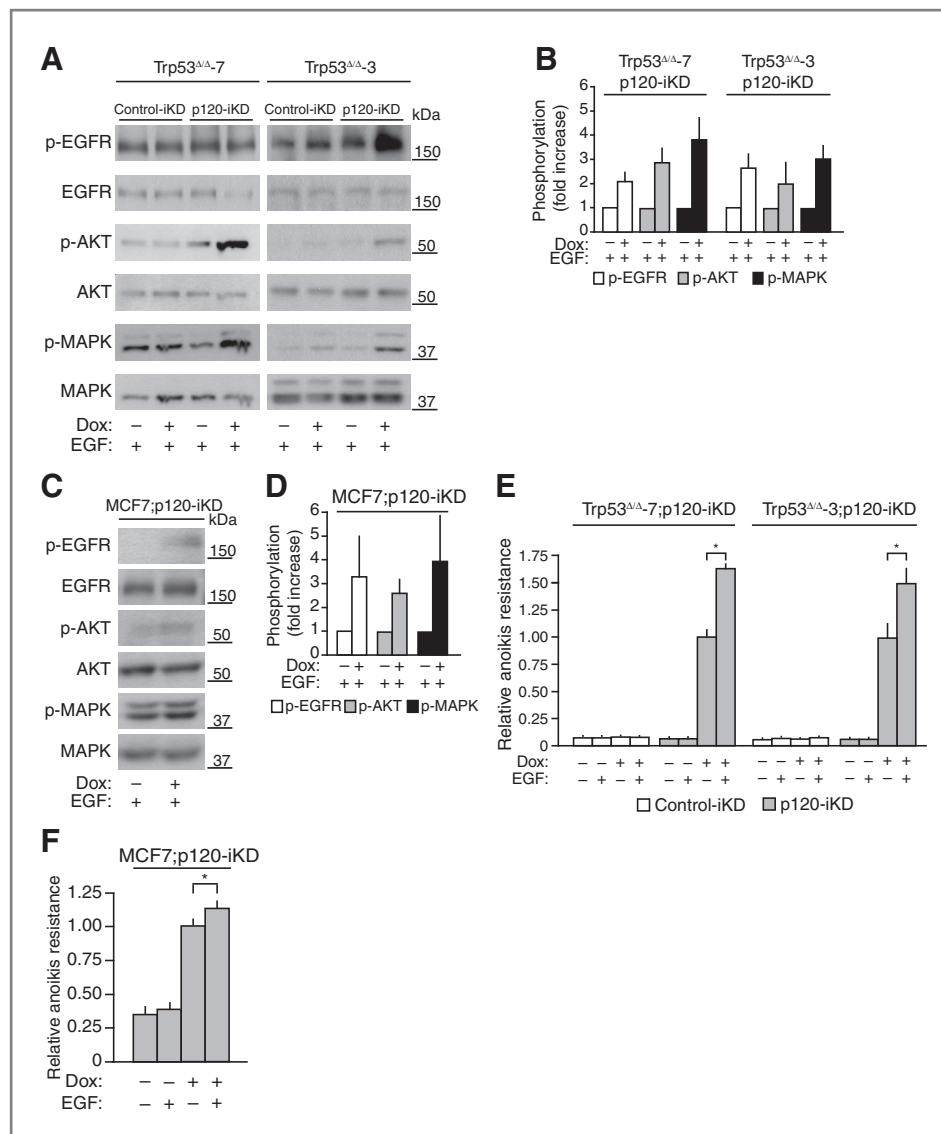


Figure 4. Loss of p120 sensitizes cells to GFR-mediated anoikis resistance. A and B, p120 knockdown sensitizes EGF signaling. Two independent Trp53^{ΔΔ} cell lines expressing control-iKD or p120-iKD constructs were treated with doxycycline (Dox) for 4 days, serum starved, stimulated with EGF, and subjected to Western blot analysis using phospho-specific antibodies against EGFR (top), AKT (middle), and MAPK (bottom). Total EGFR, AKT, and MAPK were used as loading controls. Quantification is shown in B. C and D, doxycycline-treated, serum-starved MCF7;p120-iKD cells were stimulated with EGF and analyzed as in A. Quantification is shown in D. E, EGF promotes anchorage-independent survival upon p120 loss. Anoikis resistance was analyzed in Trp53^{ΔΔ};p120-iKD cells in the presence or absence of EGF and doxycycline as indicated. Error bars represent the SD of triplicate experiments. F, EGF promotes anchorage-independent survival upon p120 loss in human breast cancer cells. Anoikis resistance of MCF7;p120-iKD cells was assayed in the presence or absence of doxycycline and EGF as indicated. *, $P < 0.05$. Error bars in B and D represent SEM of at least triplicate experiments. Error bars in E and F represent the SD of triplicate experiments.

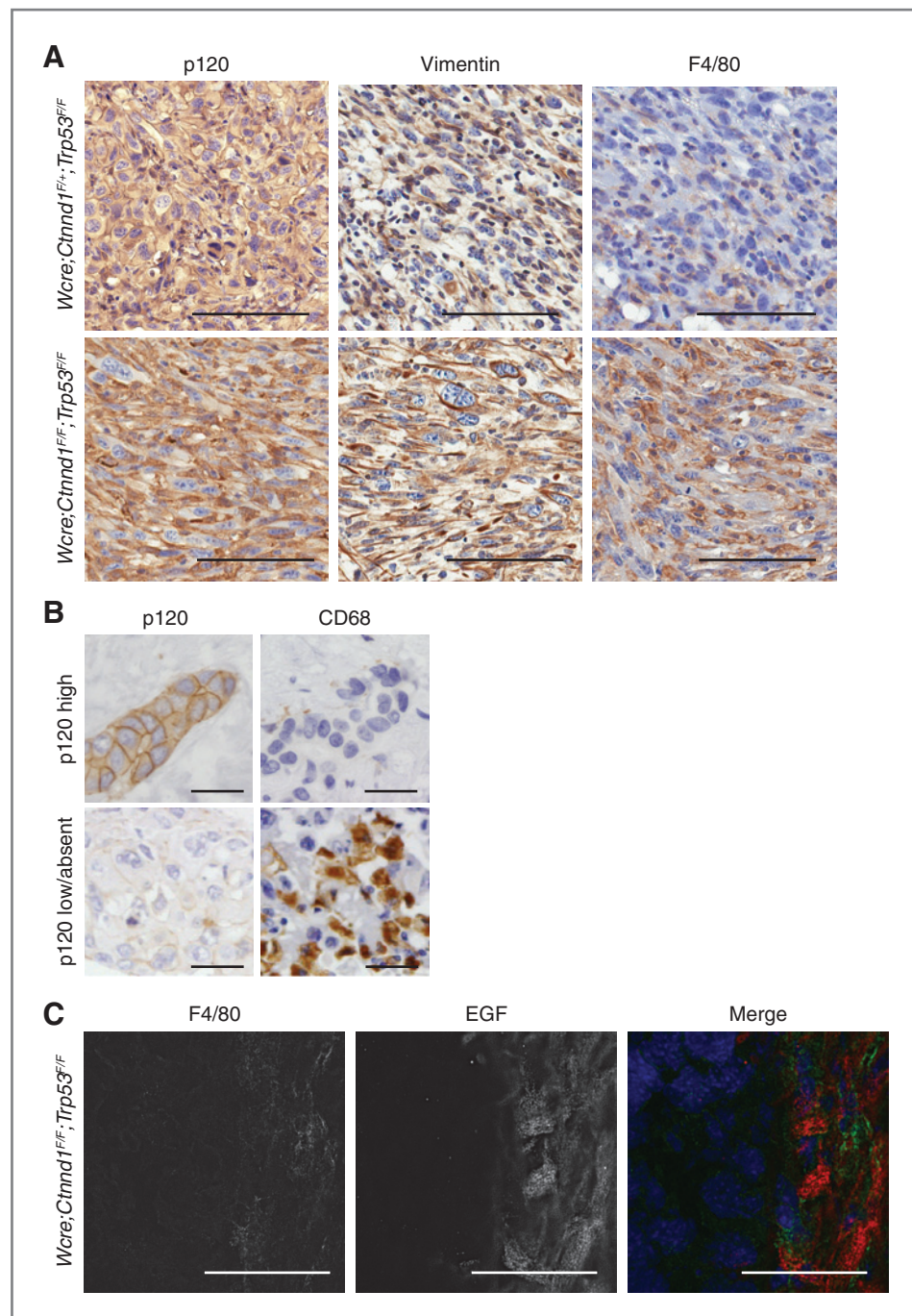
levels or EGF binding at the plasma membrane (Fig. 4A and Supplementary Fig. S5C). However, we cannot exclude that p120 knockdown induced autocrine activation of GFR signaling in the presence of anchorage, as serum starvation resulted in low levels of activated EGFR, AKT, and MAPK signaling (Supplementary Fig. S5A and S5B). To determine whether the sensitized GFR signaling was EGFR-specific, we also stimulated the cells with hepatocyte growth factor (HGF) to activate the receptor tyrosine kinase MET. In line with the effects on EGF-dependent signaling, we observed a marked increase of AKT and MAPK phosphorylation upon p120 knockdown and subsequent HGF treatment of mouse and human cells (Supplementary Fig. S4A–S4C), indicating that p120-controlled GFR signaling is a general mechanism. To validate whether the increased GFR sensitivity also occurred *in vivo*, we investigated the expression of p-AKT and p-MAPK in tumors from *Wcre; Trp53^{E/F};Ctnnd1^{E/F}* and *Wcre;Trp53^{E/F}* female mice. Indeed, loss of p120 induced activation of AKT, and to a lesser extent MAPK

signals (Supplementary Fig. S6C). In conclusion, our data imply that loss of p120 leads to an increased sensitization of GFR signaling in breast cancer cells.

GFR sensitization stimulates anchorage-independent viability

Because knockdown of p120 resulted in increased growth factor sensitization of pathways implicated in growth and survival, we used EGF stimulation to examine whether this mechanism could increase anchorage-independent growth and survival of breast cancer cells. We therefore plated Trp53^{ΔΔ};p120-iKD and Ctnnd1^{ΔΔ};Trp53^{ΔΔ} cells under anchorage-independent conditions and assayed anoikis resistance. In control cells, EGF stimulation did not mediate survival. However, addition of EGF led to an additional increase in anoikis resistance upon knockdown of p120 (Fig. 4E) and in Ctnnd1^{ΔΔ};Trp53^{ΔΔ} cell lines (Supplementary Fig. S6B). Although less prominent, EGF stimulation also induced a significant increase

Figure 5. Loss of p120 induces cytokine production and leads to the development of a prometastatic tumor microenvironment. **A**, p120-deficient mammary tumors are characterized by an abundant tumor microenvironment. The presence of stromal cells/macrophages in *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* (top) and *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* (bottom) mouse mammary carcinomas were analyzed by IHC. Shown are representative stainings for p120, vimentin, and F4/80. Bars, 100 μ m. **B**, human IDC samples were stained for p120 (left) and CD68 (macrophage marker; right). Shown are representative examples of IDC expressing high p120 (top) and low/absent p120 expression (bottom). Bars, 20 μ m. **C**, tumor-associated macrophages produce EGF. *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* tumors were stained for macrophages using immunofluorescence (green; left) and EGF mRNA using RNA *in situ* hybridization (red; middle). DNA was visualized using 4',6-diamidino-2-phenylindole (DAPI; blue; right). Bars, 20 μ m.



in anoikis resistance of MCF7:p120-iKD cells (Fig. 4F). Because adherens junction-dependent relieve of GFR inhibition is not specific for a given receptor and MCF7 responds more prominent to HGF than EGF, we also stimulated MCF7:p120-iKD cells with HGF. In line with the EGF-dependent findings in our mouse cell lines, we observed that activation of the MET receptor induced a prominent p120-dependent increase in anoikis resistance of MCF7 (Supplementary Fig. S4E).

In conclusion, we find that loss of p120 enhances anoikis resistance through increased sensitization of GFR signaling

pathways, a mechanism that may stimulate metastasis in p120-negative breast cancer.

Loss of p120 leads to a proinvasive microenvironment

On the basis of the prominent presence of stroma in the metaplastic p120 null mammary carcinomas and our finding that loss of p120 induces sensitization of GFR signaling, we examined a potential source of growth factors in metastatic tumors from *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* female mice. IHC indeed revealed an abundant presence of vimentin-expressing cells

surrounding p120-negative tumor cells (Fig. 5A, middle). We next analyzed p120-negative carcinomas for influx of macrophages, a renowned source of EGF (37). p120-Negative tumors from mouse and human samples contained a large macrophage-dense microenvironment (Fig. 5A and B). Moreover, a combination of IHC and RNA *in situ* hybridization revealed that the tumor-associated macrophages expressed EGF mRNA (Fig. 5C), indicating that a paracrine source of EGF is present in the tumor microenvironment.

In the gastrointestinal tract and skin, conditional p120 loss resulted in cytokine secretion and subsequent attraction of immune cells (18, 20, 21). To determine whether p120 loss would also control production of inflammatory cytokines in breast cancer, we assayed culture supernatant from Trp53^{Δ/Δ}; p120-iKD, Ctnnd1^{Δ/Δ};Trp53^{Δ/Δ}, and control Trp53^{Δ/Δ} cells using an antibody-based cytokine array. Using these tools, we observed that loss of p120 indeed increased secretion of numerous cytokines. Several of these factors are responsible for macrophage and lymphocyte attraction/maturation and stimulation of cancer-associated fibroblasts such as IL-1α (IL1A; ref. 38), IL-11 (IL11; ref. 39), and IL-12 (IL12A; ref. 40; Supplementary Table S4). These results suggest that p120-negative tumor cells are a paracrine source for the influx of inflammatory cells and subsequent formation of a prometastatic microenvironment.

In conclusion, we show that loss of p120 results in the excretion of cytokines that may control influx of macrophages and other stromal cells that can promote progression of p120-negative tumor cells through the induction of sensitized GFRs signaling pathways.

Discussion

p120 Loss leads to anoikis resistance and metastasis

In breast cancer, disruption of adherens junction complex formation through early mutational inactivation of E-cadherin leads to the development of ILC (3–5). Upon inactivation of E-cadherin, p120 translocates to the cytosol, where it functions as an oncogene controlling Rock-mediated anoikis resistance of ILC cells (8). In contrast to ILC, most IDC cases retain expression of a functional adherens junction complex that may be inactivated through epigenetic mechanisms at later stages of breast cancer progression (6). Interestingly, and in contrast to the salivary gland (17), gastrointestinal tract (20, 21), dental enamel (16), ocular tissue (19), and skin (18), somatic inactivation of p120 in the mammary gland is not tolerated (15). These tissue-specific differences are further exemplified by the fact that conditional p120 ablation in the upper gastrointestinal tract resulted in the formation of invasive squamous esophageal carcinomas (21).

To determine a possible role for p120 during breast cancer progression, we crossed the conditional p120 allele (41) onto the *Wcre;Trp53^{F/F}* mouse model of noninvasive breast cancer. Using this compound mouse model, we observed that—whereas onset and incidence of primary tumor development were unaffected—homozygous p120 loss resulted in metastatic disease. Interestingly, metastatic spread was not reported in other conditional p120 tumor models (17,

18, 20). Metastatic spread of tumors from *Wcre;Trp53^{F/F}* and *Wcre;Ctnnd1^{F/+};Trp53^{F/F}* female mice was a rare event. Because E-cadherin remained expressed and derivative cell lines functionally displayed anchorage dependence, it indicated that E-cadherin expression is the rate-limiting factor preventing initial tumor cell invasion and metastasis upon p120 loss. Supporting this assumption is our observation that E-cadherin is temporally retained on the cell surface upon p120 inactivation in preinvasive CIS-type lesions from *Wcre;Ctnnd1^{F/F};Trp53^{F/F}* mice. Also, we have shown previously that low E-cadherin expression levels are sufficient to induce anchorage dependency in anoikis-resistant mouse ILC (mILC) cells (4, 5).

Interestingly, and in contrast to mutational E-cadherin loss, we observed that somatic p120 inactivation did not result in the formation of ILC, but instead resulted in metaplastic carcinoma. Differences may be explained by the fact that p120 plays a key oncogenic role in ILC progression through myosin phosphatase Rho interacting protein MRIP-dependent regulation of the Rho, Rock, and the cytoskeleton (8, 42). Moreover, in contrast to E-cadherin inactivation, we did not observe metastases in typical ILC dissemination sites such as bone marrow and gastrointestinal tract in the current p120 conditional knockout model. Finally, dual inactivation of p120 and p53 did not accelerate tumor development as seen for combined E-cadherin and p53 loss (4, 5). These phenotypic and functional differences between p120 and E-cadherin suggest that although the overall consequence is adherens junction inactivation and metastasis, p120 loss leads to a biochemically and functionally different breast cancer phenotype.

p120 Loss sensitizes cells to GFR signaling

How does p120 loss regulate anoikis resistance? Our data indicate that p120 loss results in a decrease of E-cadherin-dependent proapoptotic signals in the absence of anchorage, resulting in anoikis. In this scenario, heterozygous p120 loss will not result in metastasis, because residual membranous E-cadherin expression will lead to anoikis in cells that escape from the primary tumor. An additional scenario may be provided by GFR signaling. Membrane receptors can directly influence the activity of unrelated neighboring receptors (43). Depending on cellular context, E-cadherin expression may influence EGFR activation (35, 36). Indeed, studies in gastric cancer showed that germline and somatic (in-frame deleterious) mutations in the E-cadherin extracellular domain lead to increased activation of EGFR signaling (44). We show here that inactivation of the adherens junction through loss of p120 leads to increased sensitization of GFR signaling, a phenomenon that is likely dependent on E-cadherin turnover. Although it is still controversial whether this mechanism is ligand dependent, our data indicate that the GFR hypersensitivity upon p120 loss is not caused by upregulation of EGFR expression levels or increased EGF binding at the cell surface (Fig. 4A and Supplementary Fig. S5C). Given our observation that E-cadherin-dependent inhibition of GFR signaling is not specific for a given receptor, it seems unlikely that specific posttranslational modifications or a specific phosphatase/kinase regulates this process.

A possible scenario may therefore be that E-cadherin elicits a general inhibitory effect on the recruitment of proximal activating molecules and docking adaptors such as Gab1 (*GAB1*), Grb2 (*GRB2*), or Sos (*SOS1*) to sites of tyrosine kinase phosphorylation (45, 46), resulting in a dampening of pathway activation downstream of the GFR. Alternatively, the extracellular domain of E-cadherin could play a role in preventing activation of EGFR, because shedding of E-cadherin has been implicated in EGF-dependent activation of the PI3K/AKT and MAPK pathways and subsequent cell survival (47). Thus, although further research is needed to delineate the exact mechanism that controls the sensitization of GFR signaling upon p120 loss, we propose that increased sensitization of GFR signaling may be caused by a relieve of direct or indirect mechanical restriction, or inhibition of ligand binding as a result of steric hindrance between the adherens junction and GFRs.

Loss of p120 results in a prometastatic microenvironment

Although the precise nature of the influx of inflammatory cells may depend on the model system studied, several groups reported that p120 ablation may lead to inflammation (18, 20, 21). In line with this, we found that loss of p120 results in the development of stromal dense and macrophage-rich mammary tumors. Furthermore, we show that loss of p120 leads to secretion of several cytokines, which may control the recruitment of inflammatory cells (e.g., macrophages) that have been implicated in inflammation-associated cancer initiation and promotion (48) and are correlated with poor

prognosis in breast cancer (49, 50). Moreover, it has been well established that mammary tumor cells may instigate a paracrine loop that involves the production of chemoattractants and subsequent recruitment of EGF-producing macrophages, resulting in activation of prometastatic pathways in tumor cells (51, 52).

In closing, we propose that p120 inactivation and subsequent E-cadherin loss is a late event in IDC tumor progression. To our knowledge, there have been no reports on mutational p120 inactivation in breast cancer. Although such mutations could have easily been missed using past sequencing techniques and the fact that p120 loss is often observed in only a small percentage of the tumor, *CTNND1* mutations are most probably a rare event. Conversely, this could also indicate that p120 inactivation in IDC may be caused by epigenetic or other indirect mechanisms, as has been shown for E-cadherin (53–55). This, and because of the fact that homozygous p120 inactivation in mice does not induce the formation of mILC, leads us to hypothesize that inactivation of p120 probably occurs during late progression of invasive breast cancer. Although the exact mechanism that regulates p120 inactivation remains to be resolved, our data show that E-cadherin-dependent inhibition of GFR signaling is relieved upon inactivation of p120. As a consequence, anoikis-resistant cells are sensitized to prometastatic growth factors. Because others and we show that p120 loss also facilitates concomitant formation of a prometastatic microenvironment, inactivation of p120 induces multiple hallmarks of metastatic cancer. A simplified model of our findings is presented in Fig. 6.

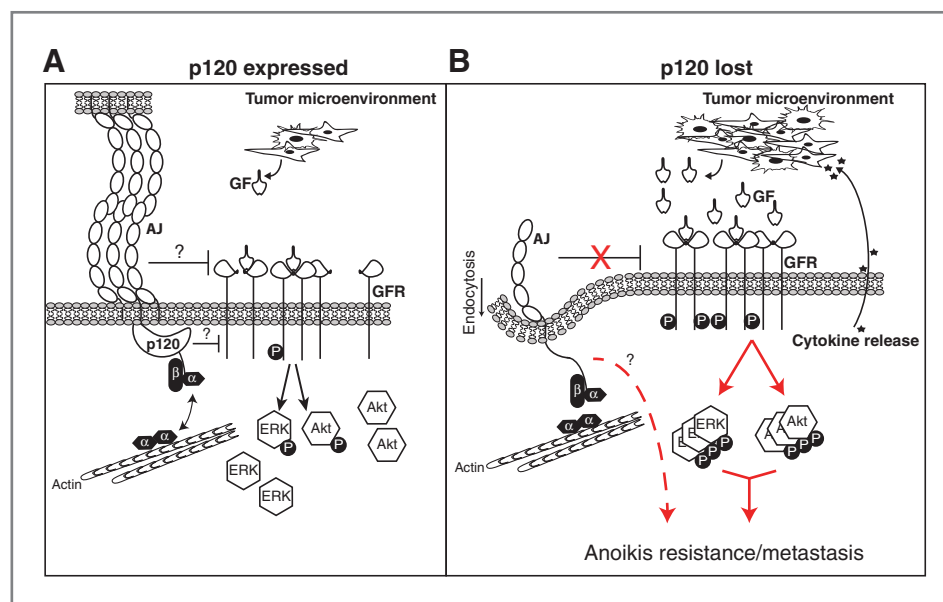


Figure 6. A model for p120 as a breast cancer metastasis suppressor. A, in the presence of p120, E-cadherin is stabilized at the plasma membrane leading to the formation of adherens junctions. The adherens junction inhibits growth factor (GF)-induced GFR activation through currently unknown mechanisms. B, upon loss of p120 the adherens junction is dismantled and E-cadherin and β -catenin are degraded. As a result, anoikis resistance is induced, and subsequently enhanced by hypersensitized GFR signaling due to the relief of E-cadherin-dependent GFR inhibition. In addition, loss of p120 induces cytokine secretion, resulting in stimulation of the tumor microenvironment. This may facilitate a paracrine loop that activates sensitized GFR signaling in p120-negative tumor cells. The exact mechanism behind this increased sensitization is currently unknown, but does not seem to involve increased GFR expression, autocrine GFR activation, or increased growth factor binding. ERK, extracellular signal-regulated kinase.

We think that our findings may have substantial clinical ramifications. In our model, GFR signaling can be enhanced independent of GFR expression levels. Thus, our data imply that the mere presence of certain GFRs is of clinical importance, provided that breast cancer cells are p120 negative. Accordingly and depending on the GFRs expressed, patients suffering from p120-negative IDC may be eligible for treatment with GFR inhibitors targeting the expressed receptors. We have thus uncovered a tumor-promoting role for p120 in breast cancer progression that provides an alternate rationale for therapeutic intervention of p120-negative metastatic breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Development of methodology: R.C.J. Schackmann, T. Peeters, P.J. van Diest, P.W.B. Derksen

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.C.J. Schackmann, S. Klarenbeek, E.J. Vlug, S. Stelloo, M. Tenhagen, J.F. Vermeulen, P.J. van Diest, J. Jonkers

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.C.J. Schackmann, S. Klarenbeek, S. Stelloo, J.F. Vermeulen, P.J. van Diest, J. Jonkers, P.W.B. Derksen

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References

- Pokutta S, Weis WI. Structure and mechanism of cadherins and catenins in cell-cell contacts. *Annu Rev Cell Dev Biol* 2007;23:237-61.
- Jeanes A, Gottardi CJ, Yap AS. Cadherins and cancer: how does cadherin dysfunction promote tumor progression? *Oncogene* 2008; 27:6920-9.
- Berx G, Cleton-Jansen AM, Nollet F, de Leeuw WJ, van de Vijver M, Cornelisse C, et al. E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. *EMBO J* 1995;14: 6107-15.
- Derksen PW, Braumuller TM, van der Burg E, Hornsveid M, Mesman E, Wesseling J, et al. Mammary-specific inactivation of E-cadherin and p53 impairs functional gland development and leads to pleomorphic invasive lobular carcinoma in mice. *Dis Model Mech* 2011; 4:347-58.
- Derksen PW, Liu X, Saridin F, van der Gulden H, Zevenhoven J, Evers B, et al. Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. *Cancer Cell* 2006;10:437-49.
- Gould Rothberg BE, Bracken MB. E-cadherin immunohistochemical expression as a prognostic factor in infiltrating ductal carcinoma of the breast: a systematic review and meta-analysis. *Breast Cancer Res Treat* 2006;100:139-48.
- Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009;119:1420-8.
- Schackmann RC, van Amersfoort M, Haahrhuis JH, Vlug EJ, Halim VA, Roodhart JM, et al. Cytosolic p120-catenin regulates growth of metastatic lobular carcinoma through Rock1-mediated anoikis resistance. *J Clin Invest* 2011;121:3176-88.
- Soto E, Yanagisawa M, Marlow LA, Copland JA, Perez EA, Anastasiadis PZ. p120 catenin induces opposing effects on tumor cell growth depending on E-cadherin expression. *J Cell Biol* 2008;183: 737-49.
- Reynolds AB, Daniel J, McCrea PD, Wheelock MJ, Wu J, Zhang Z. Identification of a new catenin: the tyrosine kinase substrate p120cas associates with E-cadherin complexes. *Mol Cell Biol* 1994; 14:8333-42.
- Fujita Y, Krause G, Scheffner M, Zechner D, Leddy HE, Behrens J, et al. Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. *Nat Cell Biol* 2002;4:222-31.
- Baki L, Marambaud P, Efthimiopoulos S, Georgakopoulos A, Wen P, Cui W, et al. Presenilin-1 binds cytoplasmic epithelial cadherin, inhibits cadherin/p120 association, and regulates stability and function of the cadherin/catenin adhesion complex. *Proc Natl Acad Sci U S A* 2001; 98:2381-6.
- Sato K, Watanabe T, Wang S, Kakeno M, Matsuzawa K, Matsui T, et al. Numb controls E-cadherin endocytosis through p120 catenin with aPKC. *Mol Biol Cell* 2011;22:3103-19.
- Boussadia O, Kutsch S, Hierholzer A, Delmas V, Kemler R. E-cadherin is a survival factor for the lactating mouse mammary gland. *Mech Dev* 2002;115:53-62.
- Kurley SJ, Bierie B, Carnahan RH, Lobdell NA, Davis MA, Hofmann I, et al. p120-catenin is essential for terminal end bud function and mammary morphogenesis. *Development* 2012;139:1754-64.
- Bartlett JD, Dobeck JM, Tye CE, Perez-Moreno M, Stokes N, Reynolds AB, et al. Targeted p120-catenin ablation disrupts dental enamel development. *PLoS ONE* 2010;5:e12703.
- Davis MA, Reynolds AB. Blocked acinar development, E-cadherin reduction, and intraepithelial neoplasia upon ablation of p120-catenin in the mouse salivary gland. *Dev Cell* 2006;10:21-31.
- Perez-Moreno M, Davis MA, Wong E, Pasolli HA, Reynolds AB, Fuchs E. p120-catenin mediates inflammatory responses in the skin. *Cell* 2006;124:631-44.
- Tian H, Sanders E, Reynolds A, van Roy F, van Hengel J. Ocular anterior segment dysgenesis upon ablation of p120 catenin in neural crest cells. *Invest Ophthalmol Vis Sci* 2012;53:5139-53.
- Smalley-Freed WG, Efimov A, Burnett PE, Short SP, Davis MA, Gumucio DL, et al. p120-catenin is essential for maintenance of barrier function and intestinal homeostasis in mice. *J Clin Invest* 2010;120: 1824-35.
- Stairs DB, Bayne LJ, Rhoades B, Vega ME, Waldron TJ, Kalabis J, et al. Deletion of p120-catenin results in a tumor microenvironment with inflammation and cancer that establishes it as a tumor suppressor gene. *Cancer Cell* 2011;19:470-83.
- Dillon DA, D'Aquila T, Reynolds AB, Fearon ER, Rimm DL. The expression of p120ctn protein in breast cancer is independent of alpha- and beta-catenin and E-cadherin. *Am J Pathol* 1998;152:75-82.
- Nakopoulou L, Gakiopoulou-Givalou H, Karayiannakis AJ, Giannopoulou I, Keramopoulos A, Davaris P, et al. Abnormal alpha-catenin

- expression in invasive breast cancer correlates with poor patient survival. *Histopathology* 2002;40:536–46.
24. Talvinen K, Tuikkala J, Nykanen M, Nieminen A, Anttinen J, Nevalainen OS, et al. Altered expression of p120catenin predicts poor outcome in invasive breast cancer. *J Cancer Res Clin Oncol* 2010;136:1377–87.
 25. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, et al. A third-generation lentivirus vector with a conditional packaging system. *J Virol* 1998;72:8463–71.
 26. Reid T, Furuyashiki T, Ishizaki T, Watanabe G, Watanabe N, Fujisawa K, et al. Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhophilin in the rho-binding domain. *J Biol Chem* 1996;271:13556–60.
 27. Derksen PW, Tjin E, Meijer HP, Klok MD, MacGillavry HD, van Oers MH, et al. Illegitimate WNT signaling promotes proliferation of multiple myeloma cells. *Proc Natl Acad Sci U S A* 2004;101:6122–7.
 28. Vermeulen JF, van de Ven RA, Ercan C, van der Groep P, van der Wall E, Bult P, et al. Nuclear Kaiso expression is associated with high grade and triple-negative invasive breast cancer. *PLoS ONE* 2012;7:e37864.
 29. Birchmeier W, Hulsken J, Behrens J. E-cadherin as an invasion suppressor. *Ciba Found Symp* 1995;189:124–36.
 30. Luini A, Aguilar M, Gatti G, Fasani R, Botteri E, Brito JA, et al. Metaplastic carcinoma of the breast, an unusual disease with worse prognosis: the experience of the European Institute of Oncology and review of the literature. *Breast Cancer Res Treat* 2007;101:349–53.
 31. Douma S, Van Laar T, Zevenhoven J, Meuwissen R, Van Garderen E, Peepers DS. Suppression of anoikis and induction of metastasis by the neurotrophic receptor TrkB. *Nature* 2004;430:1034–9.
 32. Liu Y, Xu HT, Dai SD, Wei Q, Yuan XM, Wang EH. Reduction of p120 (ctn) isoforms 1 and 3 is significantly associated with metastatic progression of human lung cancer. *APMIS* 2007;115:848–56.
 33. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–74.
 34. Takahashi K, Suzuki K. Density-dependent inhibition of growth involves prevention of EGF receptor activation by E-cadherin-mediated cell-cell adhesion. *Exp Cell Res* 1996;226:214–22.
 35. Qian X, Karpova T, Sheppard AM, McNally J, Lowy DR. E-cadherin-mediated adhesion inhibits ligand-dependent activation of diverse receptor tyrosine kinases. *EMBO J* 2004;23:1739–48.
 36. Reddy P, Liu L, Ren C, Lindgren P, Boman K, Shen Y, et al. Formation of E-cadherin-mediated cell-cell adhesion activates AKT and mitogen activated protein kinase via phosphatidylinositol 3 kinase and ligand-independent activation of epidermal growth factor receptor in ovarian cancer cells. *Mol Endocrinol* 2005;19:2564–78.
 37. Pollard JW. Macrophages define the invasive microenvironment in breast cancer. *J Leukoc Biol* 2008;84:623–30.
 38. Nozaki S, Sledge GW Jr, Nakshatri H. Cancer cell-derived interleukin 1alpha contributes to autocrine and paracrine induction of pro-metastatic genes in breast cancer. *Biochem Biophys Res Commun* 2000;275:60–2.
 39. Musashi M, Clark SC, Sudo T, Urdal DL, Ogawa M. Synergistic interactions between interleukin-11 and interleukin-4 in support of proliferation of primitive hematopoietic progenitors of mice. *Blood* 1991;78:1448–51.
 40. Trinchieri G. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv Immunol* 1998;70:83–243.
 41. Davis MA, Ireton RC, Reynolds AB. A core function for p120-catenin in cadherin turnover. *J Cell Biol* 2003;163:525–34.
 42. Shibata T, Kokubu A, Sekine S, Kanai Y, Hirohashi S. Cytoplasmic p120ctn regulates the invasive phenotypes of E-cadherin-deficient breast cancer. *Am J Pathol* 2004;164:2269–78.
 43. Chen X, Gumbiner BM. Crosstalk between different adhesion molecules. *Curr Opin Cell Biol* 2006;18:572–8.
 44. Bremm A, Walch A, Fuchs M, Mages J, Duyster J, Keller G, et al. Enhanced activation of epidermal growth factor receptor caused by tumor-derived E-cadherin mutations. *Cancer Res* 2008;68:707–14.
 45. Holgado-Madruga M, Emlen DR, Moscatello DK, Godwin AK, Wong AJ. A Grb2-associated docking protein in EGF- and insulin-receptor signalling. *Nature* 1996;379:560–4.
 46. Yamasaki S, Nishida K, Yoshida Y, Itoh M, Hibi M, Hirano T. Gab1 is required for EGF receptor signaling and the transformation by activated ErbB2. *Oncogene* 2003;22:1546–56.
 47. Inge LJ, Barwe SP, D'Ambrosio J, Gopal J, Lu K, Ryazantsev S, et al. Soluble E-cadherin promotes cell survival by activating epidermal growth factor receptor. *Exp Cell Res* 2011;317:838–48.
 48. Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* 2005;7:211–7.
 49. Murri AM, Hilmy M, Bell J, Wilson C, McNicol AM, Lannigan A, et al. The relationship between the systemic inflammatory response, tumour proliferative activity, T-lymphocytic and macrophage infiltration, microvessel density and survival in patients with primary operable breast cancer. *Br J Cancer* 2008;99:1013–9.
 50. Ueno T, Toi M, Saji H, Muta M, Bando H, Kuroi K, et al. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clin Cancer Res* 2000;6:3282–9.
 51. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 2006;124:263–6.
 52. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell* 2010;141:39–51.
 53. Battle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* 2000;2:84–9.
 54. Graff JR, Herman JG, Lapidus RG, Chopra H, Xu R, Jarrard DF, et al. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Res* 1995;55:5195–9.
 55. Hajra KM, Chen DY, Fearon ER. The SLUG zinc-finger protein represses E-cadherin in breast cancer. *Cancer Res* 2002;62:1613–8.