Loss of p120-catenin induces metastatic progression of breast cancer by inducing anoikis resistance and augmenting growth factor receptor signaling

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Precis: Based on conditional mouse models of metastatic breast cancer that are immunocompetent and clinically relevant, the current study provides an alternate rationale for therapeutic intervention of p120-catenin negative invasive breast cancer.
Abstract

Metastatic breast cancer remains the chief cause of cancer-related death among women in the Western world. While 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 hyper-sensitization of growth factor receptor 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 growth factor receptor signals to treat p120-negative metastatic breast cancers.
Introduction

Adherens junctions (AJ) 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 AJ inactivation has considerable impact on tumor etiology and cellular biochemistry. Whereas mutational inactivation of E-cadherin is an initiating and causal event in the development of invasive lobular carcinoma (ILC) (3-5), late epigenetic silencing may underlie the progression of invasive ductal carcinoma (IDC) (6) in a process called epithelial to mesenchymal transition (EMT) (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 physiological 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 (CBLLI1) (11), presenilin-1 (PSEN1) (12) and/or Numb (NUMB) (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 AJ 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 chemoo-attractants (17, 18, 20). Interestingly, recent data showed that p120 can function as a bona fide tumor suppressor in the upper gastro-intestinal 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 cancers 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, 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 signaling due to inactivation of the AJ. Finally, we show that loss of p120 results in secretion of inflammatory cytokines, which may promote formation of a prometastatic tumor microenvironment.
Results

Loss of p120 expression in invasive breast cancer

To extend previous findings on loss of p120 expression in breast cancer, we performed immunohistochemistry on a panel of 298 invasive ductal breast cancers and determined their clinicopathological variables (Table S1). Membranous p120 was scored (absent/low versus medium/high) in three independent tissue cores per tumor. Since 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 (Figure S1A), as has been used for E-cadherin (25). Using these parameters, we observed that 34% of the IDC samples showed absent/low p120 staining. Upon correlation of p120 expression levels to clinicopathological 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) (Table S2). Although p120 expression levels did not associate with tumor size, lymph node status or the other clinicopathological variables tested (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 AJ through somatic inactivation of E-cadherin is causal to the development of ILC (5). In E-cadherin mutant breast cancer, p120-catenin (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 (Ctnnd1f/f; (17)) onto the Wcre;Trp53f/f (4) noninvasive mammary carcinoma model to produce Wcre;Ctnnd1f/+;Trp53f/f and Wcre;Ctnnd1f/f;Trp53f/f mice. To correct for differences in genetic background between the Ctnnd1f/f (17) and the Wcre;Trp53f/f (FVB/N; 129P2/OlaHsd) mice, a control cohort was bred using Wcre;Ctnnd1f/+;Trp53f/f litter mates to produce Wcre;Trp53f/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 $\text{Wcre;Ctnnd1}^{1/2};\text{Trp53}^{+/+}$ ($T_{50}=223$ days) or $\text{Wcre;Ctnnd1}^{1/2};\text{Trp53}^{+/+}$ female mice ($T_{50}=214$ days) when compared to $\text{Wcre;Trp53}^{+/+}$ females ($T_{50}=213$ days) ($p=0.5006$ and 0.5859 and respectively) (Figure 1A and Table 1). Mammary tumors from $\text{Wcre;Trp53}^{+/+}$ 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 $\text{Wcre;Ctnnd1}^{1/2};\text{Trp53}^{+/+}$ and $\text{Wcre;Ctnnd1}^{1/2};\text{Trp53}^{+/+}$ females showed a shift from expansive to invasive growth as compared to $\text{Wcre;Trp53}^{+/+}$ animals ($p=0.026$ and $p=0.006$ 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 CK8 or CK14, reminiscent of an EMT (Figure 1B, top panels Table S3 and Figure S1B). Tumors developed with high incidence multifocally in different mammary glands and were characterized by a more abundant and dense stromal microenvironment. Mammary tumors from $\text{Wcre;Ctnnd1}^{1/2};\text{Trp53}^{+/+}$ females did not show loss of heterozygosity of the $\text{Ctnnd1}$ locus, as tumors expressed membrane-localized p120 and E-cadherin (Figure 1B middle panels and Table S3). Mammary tumors from $\text{Wcre;Ctnnd1}^{1/2};\text{Trp53}^{+/+}$ females showed large cells with pleomorphic nuclei, coarsely clumped chromatin and sparse cytoplasm. As expected, all tumors from $\text{Wcre;Ctnnd1}^{1/2};\text{Trp53}^{+/+}$ female mice lacked expression of membranous p120 and E-cadherin (Figure 1B right panels and Table S3).

When compared to human breast cancer, tumors that developed in $\text{Wcre;Trp53}^{+/+}$ 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 (Figure S1B and Table S3). Also, these tumors show a relatively good prognosis with infrequent formation of distant metastases. The highly infiltrative tumors arising in $\text{Wcre;Ctnnd1}^{1/2};\text{Trp53}^{+/+}$ 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, expression of Cytokeratin 14 (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 (Figure S1C and Table S3). Human metaplastic carcinoma corresponds with poor prognosis and rapid onset of distant metastases (26).

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 (Figure 2A). CIS lesions showed loss of p120, but occasionally retained low levels of E-cadherin expression (Figure 2B and 2C), 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 gastro-intestinal tract or skin (17, 18, 20, 21). Metastases phenotypically resembled the primary tumor and localized to regional or distant lymph nodes and lungs (Figure 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, 27). 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, while 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 (Figure 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 AJ, transition to a mesenchymal phenotype and anoikis resistance

Since we observed anoikis resistance in primary tumor cells derived from Wcre;Ctnnd1\textsuperscript{F/F};Trp53\textsuperscript{F/F} female mice (Figure 2D), we examined if p120 inactivation was causal to this anchorage-independent survival phenotype. To this end, we made use of Trp53\textsuperscript{Δ/Δ} tumor cell lines previously generated from adenocarcinomas that developed in either K14Cre;Trp53\textsuperscript{F/F} (28) or Wcre;Trp53\textsuperscript{F/F} female mice (4). Two independent Trp53\textsuperscript{Δ/Δ} cell lines were transduced using a doxycycline (Dox)-inducible lentiviral construct targeting p120 (p120-iKD). Dox administration resulted in a strong reduction of p120 protein expression (Figure 3A), which was accompanied by a decrease in both E-cadherin and β-catenin levels and a slight decrease in α-catenin protein expression (Figure S2A and S2B). Upon p120-iKD, cells lost their typical epithelial appearance and transitioned towards a mesenchymal and motile phenotype (Figure S2F). To confirm the specificity of the RNAi sequence used, we reconstituted cells with a nontargetable p120 cDNA to near endogenous levels, which completely reverted the Dox-induced EMT phenotype (Figure 3A, Figure S2E and S2F).

We next cultured two independent Trp53\textsuperscript{Δ/Δ};p120-iKD cell lines in suspension and assayed anoikis resistance in the presence and absence of Dox. While the majority of untreated Trp53\textsuperscript{Δ/Δ};p120-iKD cells underwent anoikis, administration of Dox induced anoikis resistance (Figure 3B), which could be fully reverted upon expression of a non-targetable p120 construct (Figure 3B). To substantiate these findings, we employed two E-cadherin expressing and anchorage-dependent human breast cancer cell lines (T47D and MCF7) in which we performed 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 AJ members and functionally induced anoikis resistance (Figure S2C and S2D, Figure 3C and 3D). In conclusion, our data show that p120 knockdown results in loss of AJ function,
and suggests that this underlies acquisition of anoikis resistance. Since these features are well known hallmarks of malignancy (29), our findings suggest that loss of p120 may lead to metastasis through acquisition of anchorage independence.

Loss of p120 potentiates growth factor receptor 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) (9). While we could confirm that active Rac1 (RAC1) levels increased in MCF7 upon p120-iKD, we did not detect activation of Rac1 in either Trp53Δ/Δ;p120-iKD cell lines or human T47D;p120-iKD cells after Dox administration (Figure S3). Also, no changes were observed in the levels or activity of the other Rho family members RhoA (RHOA) and Cdc42 (CDC42) (Figure S3C), indicating that loss of p120 in these cell systems does not result in aberrant Rho GTPase activation.

Formation or functional disruption of AJs can affect epidermal growth factor receptor (EGFR) activity (30). Whether AJ disruption results in activation or inhibition of EGFR appears to be largely cell type dependent (31, 32). Because p120 knockdown resulted in a loss of AJ formation through downregulation of E-cadherin, we wondered whether growth factor receptor (GFR) signaling was affected. To this end, we stimulated Dox-treated Trp53Δ/Δ;p120-iKD cells with EGF and assayed EGFR phosphorylation. While 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 (Figure 4A, compare lanes 3 and 4; quantification of 3 independent experiments in Figure 4B, non-stimulated in Figure S5A). In line with these findings, we observed that the downstream PI3K/AKT and MAP kinases (MAPK) pathways displayed a 3-5-fold increased EGF-induced phosphorylation upon knockdown of p120 (Figure 4A and 4B). To substantiate these findings, we made use of the Ctnnd1Δ/Δ;Trp53Δ/Δ cell lines. Similarly to the GFR sensitization after inducible p120 knockdown, Ctnnd1Δ/Δ;Trp53Δ/Δ cells showed a robust increase in EGF-induced phosphorylation of EGFR and...
MAPK and a modest induction of phosphorylated AKT as compared to Trp53Δ/Δ cells (Figure S6A). Furthermore, EGF stimulation of MCF7;p120-iKD cells also showed a sensitization of EGFR, AKT and MAPK phosphorylation upon knockdown of p120 (Figure 4C and 4D). The increased EGFR signaling following p120 knockdown was not due to increased EGFR levels or EGF binding at the plasma membrane (Figure 4A and Figure 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 (Figure 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 (Figure S4A-S4C), indicating that p120-controlled growth factor receptor 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;Trp53F/F;Ctnnd1F/F and Wcre;Trp53F/F female mice. Indeed, loss of p120 induced activation of AKT, and to a lesser extend MAPK signals (Figure S6C). In conclusion, our data imply that loss of p120 leads to an increased sensitization of GFR signaling in breast cancer cells.

**Growth factor receptor sensitization stimulates anchorage independent viability**

Since 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 Trp53Δ/Δ;p120-iKD and Ctnnd1Δ/Δ;Trp53Δ/Δ cells under anchorage-independent conditions and assayed anoikis resistance. In control cells, EGF stimulation did not mediated survival. However, addition of EGF led to an additional increase in anoikis resistance upon knockdown of p120 (Figure 4E) and in Ctnnd1Δ/Δ;Trp53Δ/Δ cell lines (Figure S6B). Although less prominent, EGF stimulation also induced a significant increase in anoikis resistance of MCF7;p120-iKD cells (Figure 4F). Because AJ-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 (Figure 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

Based on 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. Immunohistochemistry indeed revealed an abundant presence of Vimentin-expressing cells surrounding p120 negative tumor cells (Figure 5A, middle panel). We next analyzed p120 negative carcinomas for influx of macrophages, a renowned source of EGF (33). p120 negative tumors from mouse and human samples contained a large macrophage-dense microenvironment (Figure 5A and 5B). Moreover, a combination of immunohistochemistry and RNA in situ hybridization revealed that the tumor-associated macrophages expressed EGF mRNA (Figure 5C), indicating that a paracrine source of EGF is present in the tumor microenvironment.

In the GI-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^p/p;p120-iKD, Ctnnd1^p/p;Trp53^p/p and control Trp53^p/p 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 like IL1α (IL1A) (34), IL11
(IL11) (35) and IL12 (IL12A) (36) (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 growth factor receptors signaling pathways.
Discussion

p120 loss leads to anoikis resistance and metastasis

In breast cancer, disruption of AJ 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 AJ 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), gastro-intestinal 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 gastro-intestinal tract resulted in the formation of invasive squamous esophageal carcinomas (21).

In order to determine a possible role for p120 during breast cancer progression we crossed the conditional p120 allele (37) onto the Wcre;Trp53F/F mouse model of noninvasive breast cancer. Using this compound mouse model we observed that –while 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;Trp53F/F and Wcre;Ctnnd1F/+;Trp53F/F female mice was a rare event. Since E-cadherin remained expressed and derivate 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;Ctnnd1F/+;Trp53F/F mice. Also, we have shown previously that low E-cadherin expression levels are sufficient to induce anchorage dependency in anoikis resistant 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 MRIP-dependent regulation of the Rho, Rock and the cytoskeleton (8, 38). Moreover, in contrast to E-cadherin inactivation, we did not observe metastases to typical ILC dissemination sites such as bone marrow and gastro-intestinal 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 AJ inactivation and metastasis, p120 loss leads to a biochemically and functionally different breast cancer phenotype.

**p120 loss sensitizes cells to growth factor receptor signaling**

How does p120 loss regulate anoikis resistance? Our data indicate that p120 loss results in a decrease of E-cadherin dependent pro-apoptotic 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 (39). Depending on cellular context, E-cadherin expression may influence EGFR activation (31, 32). Indeed, studies in gastric cancer showed that germ-line and somatic (in-frame deleterious) mutations in the E-cadherin extracellular domain lead to increased activation of EGFR signaling (40). We show here that inactivation of the AJ through loss of p120 leads to increased sensitization of GFR signaling, a phenomenon that is likely dependent on E-cadherin turnover. While 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 (Figure 4A and Figure 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 like Gab1 (GAB1), Grb2 (GRB2) or Sos (SOS1) to sites of tyrosine kinase phosphorylation (41, 42), 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 (43). 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 growth factor receptor 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 AJ and growth factor receptors.

**Loss of p120 results in a prometastatic microenvironment**

While 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 (44) and are correlated with poor prognosis in breast cancer (45, 46). 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 (47, 48).

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 (49-51). This, and because of the fact that homozygous p120 inactivation in mice does not 
induce the formation of mouse ILC, leads us to hypothesize that inactivation of p120 probably occurs 
during late progression of invasive breast cancer. While 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. Since 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 Figure 6.

We think that our findings may have substantial clinical ramifications. In our model GFR signaling can 
be enhanced independent of growth factor receptor 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.
Materials and Methods

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

Patient material

298 cases of IDC were collected and histologically examined as described in the supplemental experimental procedures.

Antibodies and cytokine array

pig (Jackson ImmunoResearch). Biotin Label-based Mouse Antibody Array, was used according to the manufactures 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) (17) were a kind gift from Al Reynolds (Vanderbilt University Medical Center, Nashville, TN) and crossed onto the Wcre;Trp53f/f mouse model (FVB/N;Ola129/sv) (4, 5). Cohorts of Wcre;Ctnnd1f/f;Trp53f/f and Wcre;Ctnnd1f/f;Trp53f/f mice were bred from the resulting offspring. Wcre;Ctnnd1f/f;Trp53f/f mice were subsequently used to generate Wcre;Trp53f/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 previously described (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 10mm. Full autopsies were performed 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;Ctnnd1f/f;Trp53f/f animals were extracted, minced by hand using scalpel blades and plated onto regular culture dishes in DMEM-F12 medium as described (5).

**Plasmids**

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

**Virus production and Rho GTP pulldown**

Lentivirus production and transductions were done as described (5). In short, 10⁶ Cos-7 cells were seeded onto 10 cm petri dishes and transiently transfected after 24 hrs with third-generation
packaging constructs (52) and the indicated viral construct using X-tremeGENE 9 reagent (Roche). Pulldown assays for GTP-loaded RhoGTP family members were performed as described (8, 53).

**Cell culture**

Mouse Trp53Δ/Δ-7 (WP6) and Trp53Δ/Δ-3 (KP6) cell lines were generated from primary tumors that developed in Wcre;Trp53F/F and K14cre;Trp53F/F female mice respectively. Cells were cultured as described (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 µg/ml streptomycin. T47D and MCF7 cell lines were obtained from ATCC. Authenticity was tested on 16-05-2012 by means of STR profiling (lgc standards), after which large quantities of cells were frozen separately.

**Immunohistochemistry and fluorescence**

Tissues and cells were isolated, fixed and stained as described in the supplemental experimental procedures. In situ hybridisation–IHC double staining experiments were performed using labeled PCR products to identify EGF mRNA as described in the supplemental 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.

**Western blot**

Western blotting was done as described (54).

**Anoikis resistance and FACS analysis**

Anoikis resistance was analyzed by seeding cells at a density of 20,000 cells per well (in 500 µl) in a 24-well ultra-low cluster polystyrene culture dish (Corning). After 4 days, cells were harvested and resuspended in 75µl of Annexin-V buffer supplemented with Annexin-V (IQ Products) and Propidium

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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 1x10^5 trypsinized ice-cold cells in 100µl PBS. Cells were washed with PBS to remove unbound EGF-647 and subjected to 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 (5ng/ml; Sigma) or HGF (25ng/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 performed using GraphPad Prism 5 (GraphPad Software). Analyses on human samples were performed as previously described (55). For analysis of growth pattern and metastasis formation, Fisher’s 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’s T-test (2-tailed), showing measurements of at least three independent experiments. Error bars in all experiments represent standard deviation or standard error of the mean 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. Use of anonymous or coded leftover material for scientific purposes is part of the standard treatment contract with patients in our hospitals.
Acknowledgements

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References


Figure legends

Figure 1

p120 is a metastasis suppressor in mammary carcinoma. A) Conditional mammary-specific inactivation of p120 does not influence tumor-free latency. Kaplan-Meier tumor-free survival curves for Wcre;Trp53^{f/+} (red line) versus Wcre;Ctnnd1^{f/+};Trp53^{f/+} (black line) versus Wcre;Ctnnd1^{f/f};Trp53^{f/f} (green line). Mice were killed when tumors reached an average diameter of 10 mm. NS = not significant. B and C) Loss of p120 induces a switch from nonmetastatic to metastatic mammary carcinoma. (B) Histopathology of consecutive sections from mammary tumors derived from Wcre;Trp53^{f/f} (left panels), Wcre;Ctnnd1^{f/+};Trp53^{f/f} (middle panels) and Wcre;Ctnnd1^{f/f};Trp53^{f/f} (right panels). Shown are H&E staining and immunohistochemistry for p120 and E-cadherin. Arrow in right panels point to a pre-existing hyperplastic mammary duct. (C) Homozygous loss of p120 leads to metastasis. Examples of distant metastases from Wcre;Ctnnd1^{f/f};Trp53^{f/f} female animals showing disseminated tumor cells in a lymph node (left) and lungs (right). Bars: 100 μm.

Figure 2

Somatic inactivation of p120 leads to development of carcinoma in situ (CIS). A) CIS formation in premalignant Wcre;Ctnnd1^{f/+};Trp53^{f/f} female mice. Shown are two separate H&E stainings. B and C) Residual E-cadherin expression in CIS-type lesions in Wcre;Ctnnd1^{f/+};Trp53^{f/f} female mice. (B) Shown are H&E staining and immunohistochemistry for p120 and E-cadherin on consecutive sections (B) and IF stainings for E-cadherin (green) and p120 (red) in a normal mammary duct (upper panel) and a CIS-type mammary lesion (lower panel) from a Wcre;Ctnnd1^{f/+};Trp53^{f/f} female mouse (C). Note the presence of E-cadherin (arrows) in the absence of p120 expression. Bars 10 μm. D) Metastatic capacity correlates to anoikis resistance. Trp53^{A/A} cell line (white bar) and primary cultures derived from tumors that developed in Wcre;Ctnnd1^{A/A};Trp53^{A/A} (gray bars) and Wcre;Ctnnd1^{A/A};Trp53^{A/A} (black bars) female mice were subjected to 4 days of anchorage independent culturing and subsequent anoikis resistance analysis using FACS.
Figure 3
Loss of p120 results in anoikis resistance of E-cadherin expressing breast cancer cells. **A** Two independent Trp53Δ/Δ cell lines were transduced with viruses carrying Dox-inducible p120 shRNAs (p120-iKD) and a non-targetable 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** Trp53Δ/Δ;Control-iKD, p120-iKD and p120-1A expressing mammary carcinoma cells were cultured in the presence or absence of Dox 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 Dox for 4 days and subjected to non-adherent culturing. Anoikis resistance was analyzed after 4 days. Lower panels show the extent of p120 knockdown (upper blot). AKT was used as loading control (lower blot). *=p<0.005. Error bars represent standard deviation of triplicate experiments.

Figure 4
Loss of p120 sensitizes cells to growth factor receptor-mediated anoikis resistance. **A and B** p120 knockdown sensitizes EGF signaling. Two independent Trp53Δ/Δ cell lines expressing control-iKD or p120-iKD constructs were treated with Dox for 4 days, serum starved, stimulated with EGF and subjected to western blot analysis using phospho-specific antibodies against EGFR (upper panels), AKT (middle panels) and MAPK (lower panels). Total EGFR, AKT and MAPK were used as loading controls. Quantification is shown in (B). **C and D** Dox 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 Trp53Δ/Δ;p120-iKD cells in the presence or absence of EGF and Dox 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 Dox 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.

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;Ctnnd1F/F;Trp53F/F (top panels) and Wcre;Ctnnd1F/F;Trp53F/F (bottom panels) mouse mammary carcinomas were analyzed by immunohistochemistry. Shown are representative stainings for p120, Vimentin and F4/80. Bars: 100 μm. B) Human IDC samples were stained for p120 (left panels) and CD68 (Macrophage marker; right panels). Shown are representative examples of IDC expressing high p120 (upper panels) and low/absent p120 expression (lower panels). Bars 20μm. C) Tumor-associated macrophages produce EGF. Wcre;Ctnnd1F/F;Trp53F/F tumors were stained for macrophages using immunofluorescence (green, left panel) and EGF mRNA using RNA in situ hybridization (red, middle panel). DNA was visualized using DAPI (blue, right panel). Bars 20μm.

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 AJs. The AJ inhibits growth factor (GF) induced growth factor receptor (GFR) activation through currently unknown mechanisms. B) Upon loss of p120 the AJ 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 micro-environment. 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 appear to involve increased growth factor receptor expression, autocrine growth factor receptor activation or increased growth factor binding.
Table 1. Mammary tumor spectrum of Wcre;Trp53<sup>F/F</sup>, Wcre;Ctnnd1<sup>F/+</sup>;Trp53<sup>F/F</sup> and Wcre;Ctnnd1<sup>F/F</sup>;Trp53<sup>F/F</sup> female mice.

<table>
<thead>
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<th>genotype</th>
<th>number of mice</th>
<th>median latency (days)</th>
<th>metastasis</th>
<th>local invasion</th>
<th>AC</th>
<th>SC/CS</th>
<th>mILC</th>
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<tbody>
<tr>
<td>Wcre;Trp53&lt;sup&gt;F/F&lt;/sup&gt;</td>
<td>7</td>
<td>213</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>3 (43%)</td>
<td>0 (0%)</td>
<td>7 (100%)</td>
</tr>
<tr>
<td>Wcre;Ctnnd1&lt;sup&gt;F/+&lt;/sup&gt;;Trp53&lt;sup&gt;F/F&lt;/sup&gt;</td>
<td>20</td>
<td>223</td>
<td>1 (5%)</td>
<td>10 (50%)</td>
<td>1 (5%)</td>
<td>19 (95%)</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>Wcre;Ctnnd1&lt;sup&gt;F/F&lt;/sup&gt;;Trp53&lt;sup&gt;F/F&lt;/sup&gt;</td>
<td>21</td>
<td>214</td>
<td>9 (43%)</td>
<td>14 (67%)</td>
<td>3 (14%)</td>
<td>18 (86%)</td>
<td>1 (5%)</td>
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</table>

If tumors were composed of 2 separate histological types, both were counted separately. AC: adenocarcinoma: glandular-type mammary carcinoma. SC/CS: solid carcinoma/carcinosarcoma: tumor consisting of epithelial and mesenchymal cell types. mILC: mouse invasive lobular carcinoma.
Figure 1

A

Genotype

% Tumor free

LATENCY (days)

Wcre;Trp53^{+/+}

Wcre:Ctnnd1^{+/+};Trp53^{+/+}

Wcre;Ctnnd1^{+/+};Trp53^{+/+}

B

Wcre;Trp53^{+/+}

Wcre:Ctnnd1^{+/+};Trp53^{+/+}

Wcre;Ctnnd1^{+/+};Trp53^{+/+}

H&E

p120

E-cadherin

C

Wcre;Ctnnd1^{+/+};Trp53^{+/+}

Lymph node

Lung

H&E

ns

ns

ns

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Figure 2

A

H&E

B

H&E

p120

E-cadherin

C

Normal Duct

CIS

p120

E-cadherin

Merge

D

Relative anoikis resistance

Relative anoikis resistance

0

0.25

0.50

0.75

1.00

1.25

*
Figure 3

(A) Western blot analysis showing the expression of p120 and AKT in Trp53\(\Delta/\Delta\)-7; p120-iKD and Trp53\(\Delta/\Delta\)-3; p120-iKD cells with and without Dox and p120-1A. The blots are normalized to the control-iKD condition.

(B) Bar graphs showing the relative anoikis resistance of Trp53\(\Delta/\Delta\)-7 and Trp53\(\Delta/\Delta\)-3 cells treated with Dox and p120-iKD with and without p120-1A.

(C) Bar graph showing the relative anoikis resistance of T47D; p120-iKD cells treated with Dox.

(D) Bar graph showing the relative anoikis resistance of MCF7; p120-iKD cells treated with Dox.
Figure 6

**p120 expressed**

- Tumor microenvironment
- GFR
- GF
- Actin
- ERK
- Akt
- p120

**p120 lost**

- Tumor microenvironment
- GFR
- GF
- Actin
- ERK
- Akt
- p120

Anoikis resistance/metastasis

Cytokine release
Loss of p120–catenin induces metastatic progression of breast cancer by inducing anoikis resistance and augmenting growth factor receptor signaling


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