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# Combined Wnt/β-Catenin, Met, and CXCL12/CXCR4 Signals Characterize Basal Breast Cancer and Predict Disease Outcome

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## SUMMARY

**Prognosis for patients with estrogen-receptor (ER)-negative basal breast cancer is poor, and chemotherapy is currently the best therapeutic option. We have generated a compound-mutant mouse model combining the activation of β-catenin and HGF (Wnt-Met signaling), which produced rapidly growing basal mammary gland tumors. We identified the chemokine system CXCL12/CXCR4 as a crucial driver of Wnt-Met tumors, given that compound-mutant mice also deficient in the *CXCR4* gene were tumor resistant. Wnt-Met activation rapidly expanded a population of cancer-propagating cells, in which the two signaling systems control different functions, self-renewal and differentiation. Molecular therapy targeting Wnt, Met, and CXCR4 in mice significantly delayed tumor development. The expression of a Wnt-Met 322 gene signature was found to be predictive of poor survival of human patients with ER-negative breast cancers. Thus, targeting CXCR4 and its upstream activators, Wnt and Met, might provide an efficient strategy for breast cancer treatment.**

## INTRODUCTION

Breast cancer (BC) is a heterogeneous disease divided into therapeutic groups, based on the estrogen receptor (ER) and HER2/ErbB2 status (Figure S1A; van 't Veer et al., 2002; Weigelt and Reis-Filho, 2009). ER-negative basal breast cancers represent the most aggressive subtype with poor prognosis and a lack of targeted therapies. The majority of ER-negative basal breast cancers (70%) are triple negative for the ER, progesterone receptor (PR), and HER2 (Carey et al., 2010). An additional risk factor for developing basal breast cancer is the *BRCA1* germline mutation (Foulkes et al., 2003). More recently, human basal breast cancers have been shown to display high frequencies of specific gene alterations, such as *TP53*, *RB*, and *PTEN* mutations, overexpression of *PI(3)KCA*, *WNT* signaling components, and *MYC,* as well as activating mutations of receptor tyrosine kinases, such as the *EGFR*, *FGFR*, *IGFR1*, and *MET* (Carey et al., 2006; Shah et al., 2012; Cancer Genome Atlas Network, 2012).

The relevance of Wnt/β-catenin signaling in breast cancer has long been controversial. However, following molecular subclassification, it is now clear that high expression of nucleocytoplasmic β-catenin, the hallmark of canonical Wnt signaling, is an important clinical feature of basal breast cancers and is predictive for poor overall survival (Geyer et al., 2011; Khramtsov et al., 2010; López-Knowles et al., 2010). Mutations of β-catenin encoding the amino-terminal domain have been observed in 92% of patients with metaplastic carcinomas, a distinct group of basal breast cancers (Hayes et al., 2008). Knockdown of the gene for the Wnt receptor *FZD7* in basal breast cancer cell lines reduced the expression of Wnt target genes and inhibited tumor growth in mice (Yang et al., 2011). Ectopic expression of genes encoding inhibitory Wnt ligands, such as *sFRP1*, in breast cancer cell lines, can bind and effectively competed with FZD receptors, reducing the ability of these cells to form mammary gland tumors in mice (Matsuda et al., 2009). The tyrosine kinase receptor Met and its ligand, hepatocyte growth factor/scatter factor (HGF/SF), have also been described to be associated with basal breast cancers and correlate with poor patient outcome (Gastaldi et al., 2010; Gherardi et al., 2012). High Met overexpression was consistently associated with coexpression of basal markers, cytokeratin 5, cytokeratin 6, caveolin 1, c-Kit, and p63, thus assigning Met as an additional constituent of the basal breast cancer phenotype (Garcia et al., 2007).

Breast cancer can be modeled in mice carrying mutations or transgenes. For instance, conditional mutations of *Brca1* and/or *p53* in mammary gland epithelial cells induced mammary gland

tumors in mice that displayed characteristics of human basal breast cancer (Bouwman et al., 2010; Shafee et al., 2008). Remarkably, a genome-wide screen revealed amplifications of the *Met* locus in 73% of Brca1/p53-deficient mouse tumors, indicating that Met signaling is crucial for the generation of basal mammary gland tumors (Smolen et al., 2006). Expression of activating mutations of MET in mice, or overexpression of HGF induced histologically diverse mammary gland tumors, among them tumors that resemble human basal breast cancer (Graveel et al., 2009; Ponzo et al., 2009; Knight et al., 2013; Gallego et al., 2003). Similarly, activation of the Wnt signaling system, either by overexpression of Wnt ligands or by gain-of-function mutations of *β-catenin* and *APC* mutations resulted in formation of mammary gland neoplasias. The type of tumors ranged from squamous metaplasias to adenocarcinomas depending on the particular model, which also varied in the degree or the time point of activation of the signals (Monteiro et al., 2013; Imbert et al., 2001; Nusse and Varmus, 1982; Michaelson and Leder, 2001; Miyoshi et al., 2002). Moreover, Wnt/β-catenin signals can cooperate with other oncogenic signaling systems to promote the development of aggressive carcinomas (Malanchi et al., 2008; Vermeulen et al., 2010), but in breast cancer models such cooperation has not been assessed by genetic means.

In the present study, we modeled rapid basal breast cancer formation in mice by combining activating mutations of Wnt/β-catenin and HGF/Met in mammary gland epithelial cells using the pregnancy-induced Whey Acidic Protein (WAP) promoter. We identified a gene signature of murine Wnt-Met tumors, which was found to predict poor survival of human patients harboring ER-negative, basal breast cancer types. Furthermore, by gene expression profiling and genetic means, the chemokine system CXCL12/CXCR4 was found to be controlled by Wnt and Met in an intricate manner. Our study also suggests that combination therapies targeting CXCR4 and its upstream activators, Wnt and Met, might thus be beneficial for patients with basal breast cancer.

## RESULTS

### Activation of Wnt/β-Catenin and HGF/Met Signaling with the WAP Promoter in Mice Induces Basal Mammary Gland Tumors

To assess the potential role of the cooperation of Wnt/β-catenin and HGF/Met signaling during mammary gland tumorigenesis, we generated compound-mutant mice coexpressing activating mutations in these signaling systems. Gain-of-function *β-catenin* mutant mice (carrying a floxed allele of exon 3 of *β-catenin*, recombined by WAP-cre, which is expressed in the mammary gland of pregnant and postpartum mice) were crossed with mice that overexpressed the Met ligand HGF under the control of the WAP promoter (*WAP-cre;β-catenin$^{ex3/+}$;WAP-HGF* or *WAP-cre;β-catn$^{ex3/+}$;HGF*; referred to as compound Wnt-Met mutant mice; see the Experimental Procedures and Supplemental Experimental Procedures) (Harada et al., 1999; Gallego et al., 2003; Miyoshi et al., 2002). Protein expression of WAP and β-catenin was strongly induced during pregnancy, and cre recombinase was detected in early to late postpartum animals (Figures S1B and S1C). Transgene and cre expression was almost absent in virgin mice; however, the combined activation of *β-catenin* and *HGF* in mammary glands induced precocious lobuloalveolar hyperplasia (Figure S1D, bottom, marked by arrows). Two weeks postpartum, the majority of compound Wnt-Met mutant mice presented with palpable tumors in each mammary gland (Figures 1A and 1B), and in few cases virgin mice also developed small tumors (Figure 1C). Tumor onset in the compound Wnt-Met mutants was rapid compared to single-mutant mice, which remained tumor free 30 days postpartum or longer (Figure 1B). Histological analysis revealed that compound-mutant tumors appearing after 2 weeks postpartum exhibited a mixed phenotype; areas resembling basaloid hyperplasia and squamous metaplasia (Figure 1D). Single *HGF* and *β-catenin* mutant mice have been previously described to develop adenosquamous carcinomas and squamous metaplasia, respectively, but these tumors were only observed 4 and 12 months postpartum (see also Gallego et al., 2003; Miyoshi et al., 2002). Analysis of tumor tissue from Wnt-Met compound-mutant mice demonstrated strong expression of nucleocytoplasmic β-catenin, relative to single mutants or wild-type mice (Figure S1E, left, bottom, marked by arrows). Furthermore, production of the milk protein β-casein after pregnancy could not be detected in Wnt-Met compound-mutant mice, indicating a block in normal mammary gland differentiation (Figure S1E, right, bottom). Next, we performed transcription profiling of compound-mutant tumors and compared the gene signature with signatures of tumors in previously analyzed mouse models (Herschkowitz et al., 2007). In unsupervised cluster analysis, the expression profile of β-catn$^{ex3}$-HGF mutant tumors grouped closely with the profiles of basal tumors induced by *Wnt1* overexpression, *Brca1$^{+/-}$;Trp53$^{+/-}$* mutations, or dimethylbenzanthracene treatment, but were more distinct from luminal tumors induced by PyMT, Neu, and Myc (Figure 1E). The transcription profile also revealed that compound-mutant tumors were triple negative for HER2/ErbB2, ER, and PR. Genes encoding *keratin 5*, *14*, *keratin 17*, and collagens, i.e., components of basal tumors, were highly upregulated, whereas expression levels of *Claudin 3* and *7* and the luminal genes *Notch*, *Hey1*, and *Xpa1* were low. Moreover, quantitative RT-PCR (qRT-PCR) and immunofluorescence analyses confirmed that compound Wnt-Met mutant tumors exhibited basal characteristics; i.e., high levels of basal and myoepithelial markers (*K5*, *K14*, and *SMA*) were expressed throughout the tumor, whereas luminal cell markers (*K8*, *K18*, and *E-cadherin*) were present at low levels (Figure 1F, top; Figure 1G). Further, the expression of Wnt target genes and stem cell markers *Lrp6*, *Lrp5*, and *Axin2* were increased (Figure 1F, middle) (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes) (Holland et al., 2013). The expression of several metastasis-associated genes such as *Twist1*, *Cxcr4*, and *Postn* were upregulated in Wnt-Met compound tumors, indicating that these tumors display invasive properties and may have the potential to metastasize (Figure 1F, bottom). Altogether, combined activation of Wnt and Met in the adult murine mammary gland induces rapid formation of aggressive tumors that exhibit a morphological, gene, and protein expression profile characteristics of basal breast cancers.

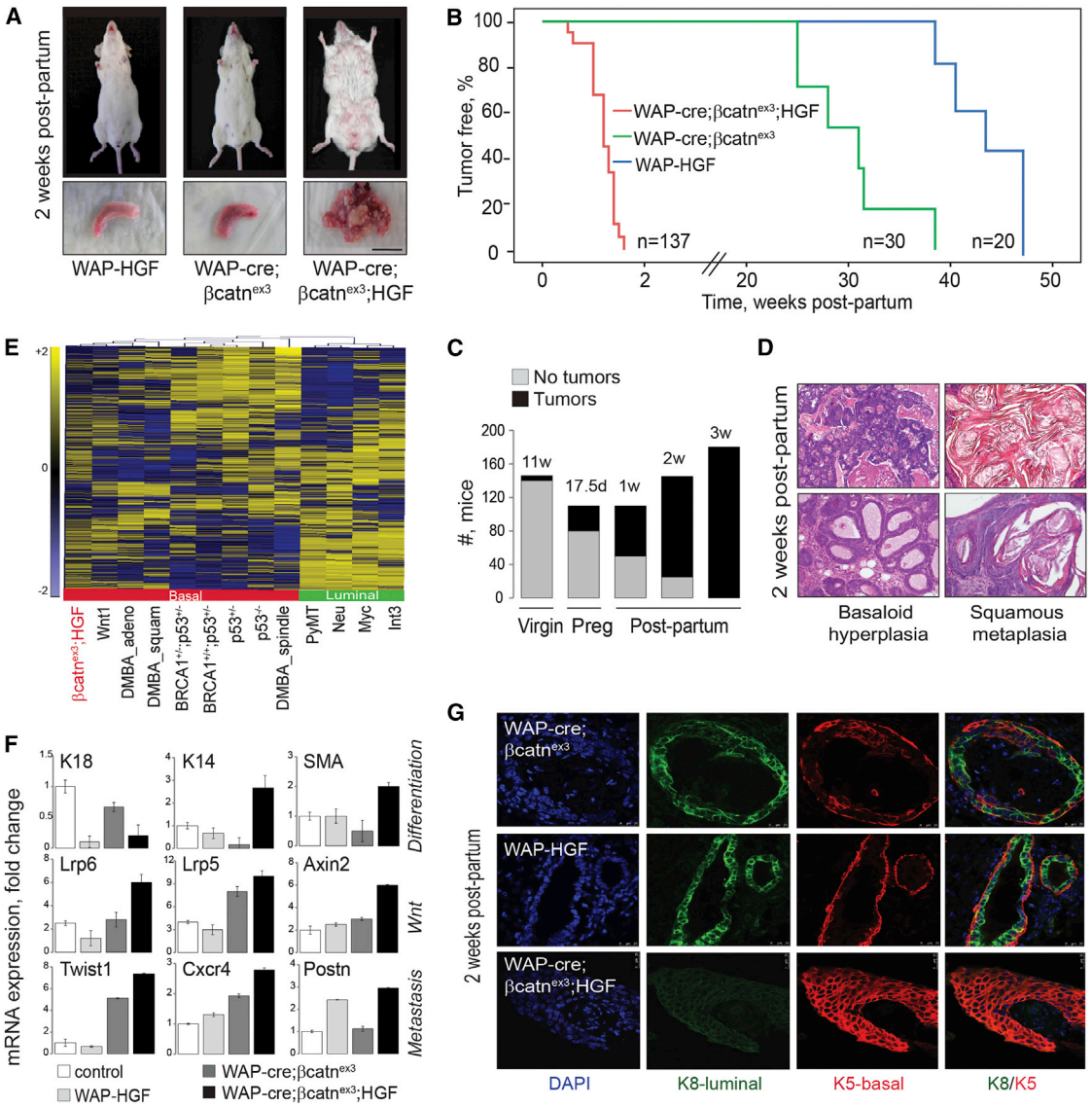



**Figure 1. Combined β-Catenin and HGF Activation by the WAP Promoter Drives Rapid Mammary Gland Tumor Formation in Mice**

(A and B) Visual examination and quantification of tumor development in single and compound (*WAP-cre*;*β-catn*$^{ex3/+}$;*HGF*) mutants. (A) Single- and compound-mutant mice and explanted tumors examined at 2 weeks postpartum (the scale bar represents 1 cm).

(B) Kaplan-Meier curve showing tumor-free survival period for single- and compound-mutant mice.

(C) Numbers of compound-mutant mice displaying tumors (black) during mammary gland development.

(D) Hematoxylin and eosin (H&E)-stained sections of compound-mutant tumors 2 weeks postpartum.

(E) Heatmap depicting a hierarchical clustering of gene expression of compound-mutant tumors (red) compared to previously reported basal and luminal mouse tumors (Herschkowitz et al., 2007).

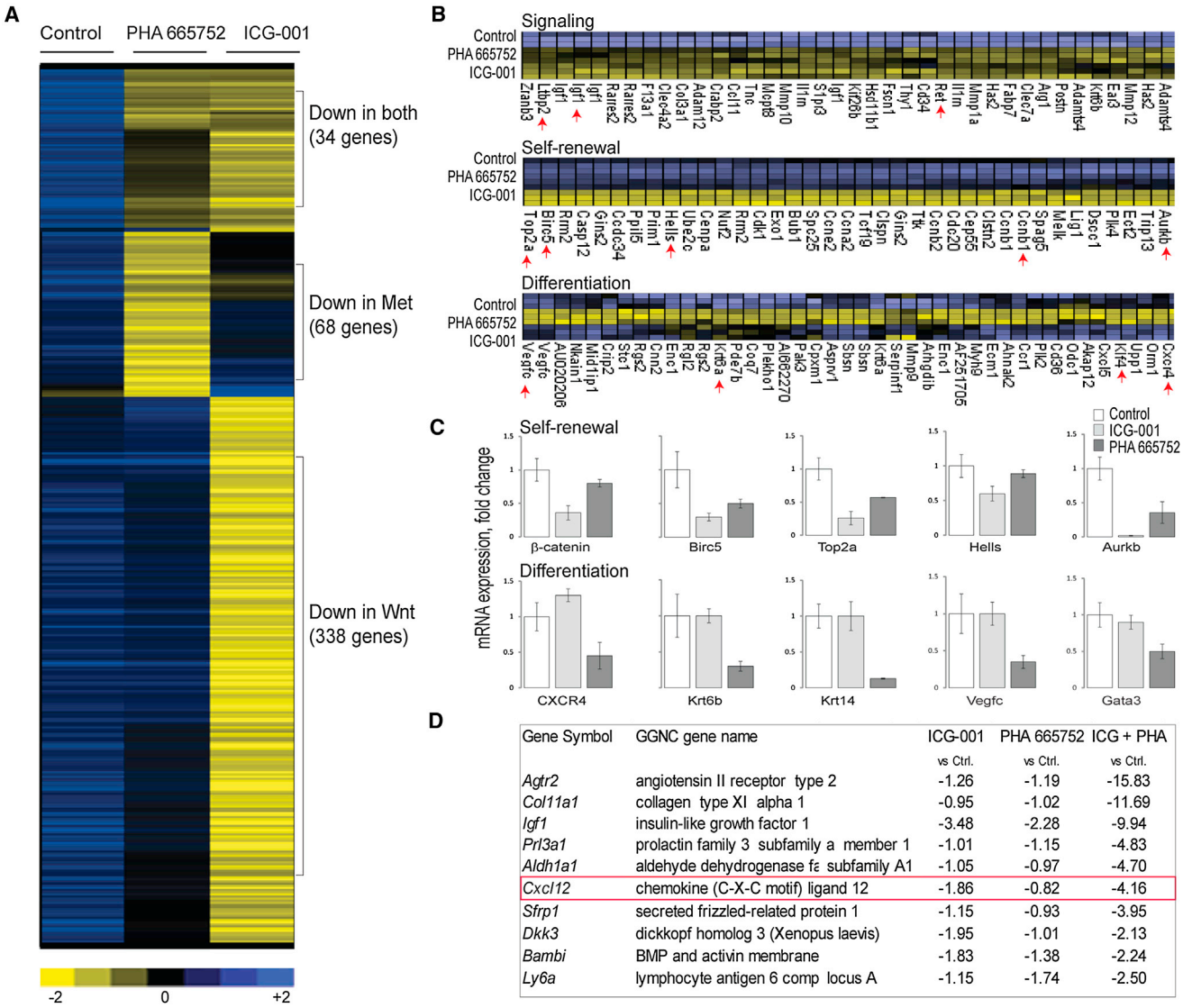
(F) qRT-PCR analysis of compound-mutant tumors for differentiation markers (top), canonical Wnt target genes (middle), and metastasis-inducing genes (bottom). Data are shown as fold change compared to control (error bars represent ±SEM, n = 3).

(G) Immunofluorescent staining for K8 luminal and K5 basal cell markers in single- versus compound- mutant tumor tissue.

Magnification, 40×.

## Canonical Wnt Signaling Controls Self-Renewal, whereas Met Signaling Suppresses Differentiation in Tumor Cells

To assess the individual contribution of Wnt and Met signaling to mammary gland tumorigenesis, isolated epithelial cells from compound Wnt-Met mutant tumors were treated with small molecular weight inhibitors, and gene expression profiling was performed with Affymetrix microarrays (Figure 2A). The compound ICG-001 interferes with binding of β-catenin to the CBP cofactor, which is important for Wnt-dependent proliferation and target gene expression (Emami et al., 2004), whereas PHA 665752 acts as ATP-competitive inhibitor that blocks the tyrosine




| Gene Symbol | GGNC gene name | ICG-001 vs Ctrl. | PHA 665752 vs Ctrl. | ICG + PHA vs Ctrl. |
|---|---|---|---|---|
| *Agtr2* | angiotensin II receptor type 2 | -1.26 | -1.19 | -15.83 |
| *Col11a1* | collagen type XI alpha 1 | -0.95 | -1.02 | -11.69 |
| *Igf1* | insulin-like growth factor 1 | -3.48 | -2.28 | -9.94 |
| *Prl3a1* | prolactin family 3 subfamily a member 1 | -1.01 | -1.15 | -4.83 |
| *Aldh1a1* | aldehyde dehydrogenase fa subfamily A1 | -1.05 | -0.97 | -4.70 |
| *Cxcl12* | chemokine (C-X-C motif) ligand 12 | -1.86 | -0.82 | -4.16 |
| *Sfrp1* | secreted frizzled-related protein 1 | -1.15 | -0.93 | -3.95 |
| *Dkk3* | dickkopf homolog 3 (Xenopus laevis) | -1.95 | -1.01 | -2.13 |
| *Bambi* | BMP and activin membrane | -1.83 | -1.38 | -2.24 |
| *Ly6a* | lymphocyte antigen 6 comp locus A | -1.15 | -1.74 | -2.50 |

**Figure 2. Self-Renewal of Tumor Cells Is Regulated by Activated β-Catenin, whereas Dedifferentiation depends on Activated Met**

(A) Compound-mutant tumor cells treated with PHA 665752 (1 μM), ICG-001 (25 μM) or a control, and changes in gene expression analyzed by Affymetrix arrays. Shown is a heatmap depicting the expression of genes regulated by Wnt/β-catenin or HGF/Met signaling; yellow represents downregulation.

(B) Detailed heat map showing regulated genes implicated in signaling, self-renewal, and differentiation after PHA 665752 or ICG-001 treatment (genes marked by red arrows were further characterized in C).

(C) qRT-PCR validation of regulated genes after inhibitor treatment. Data are shown as fold change compared to control (error bars represent ±SEM, n = 3).

(D) Compound-mutant tumor cells were treated with a combination of PHA 665752 (1 μM) and ICG-001 (25 μM), or a control, and changes in gene expression were analyzed with Affymetrix arrays. Shown are the top synergistically regulated gene set following combined inhibitor Wnt and Met treatment (Cxcl12 is boxed in red) (see also Figure S2D).

kinase activity of the Met receptor (Christensen et al., 2003) (see Figure S2A for chemical structures of the compounds). A heatmap of unsupervised cluster analysis identified genes downregulated individually by ICG-001 (338 genes) or PHA 665752 (68 genes), and common genes downregulated by the two inhibitors (34 genes) (Figures 2A and S2B). Remarkably, Wnt signaling controlled the expression of genes essential for proliferation and self-renewal, e.g., *Birc5*, *Top2a*, *Hells*, and *Aurkb* (DiMeo et al., 2009), whereas Met signaling regulated the expression of genes important for epithelial differentiation, *Krt6b*, *Krt14*, *Vegfc*, *Cxcr4*, and *Gata3* (Lim et al., 2010) (Figure 2B, marked by red arrows; confirmation by qRT-PCR is in Figure 2C; summarized in Figure S2C). The genes regulated by both Wnt and Met included components of TGF-β, insulin, and receptor tyrosine kinase signaling, for instance, *Ltbp2*, *Igf1*, and *Ret* (Figure 2B). These data suggest that the two individual signaling pathways drive independent biological programs: Wnt signaling may regulate self-renewal, whereas Met may control differentiation.

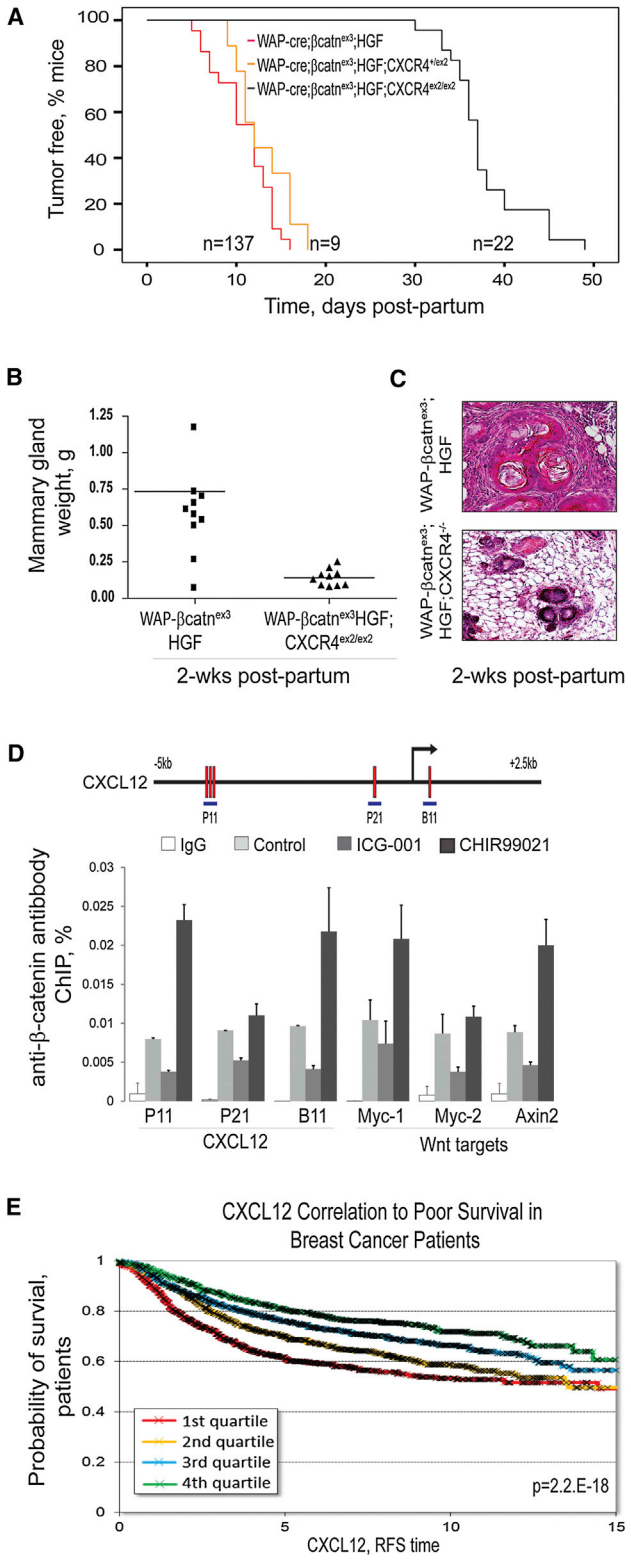



**Figure 3. Wnt-Met Mammary Gland Tumors Require CXCR4/CXCL12 Signaling**

(A) Kaplan-Meier curve depicting tumor-free survival period in triple compound-mutant mice lacking CXCR4 (*WAP-cre*; *β-catn*$^{ex3}$*;HGF;CXCR4*$^{ex2/ex2}$) shown in black, compared to compound-mutant mice with a heterozygous allele of CXCR4 (*WAP-cre*; *β-catn*$^{ex3}$*;HGF;CXCR4*$^{+/ex2}$) shown in orange, compared to compound-mutant mice containing the wild-type allele of CXCR4 (*WAP-cre;β-catn*$^{ex3/+}$*;HGF*) shown in red.

(B) Mammary gland tumor weight from Wnt-Met compound- and triple-mutant mice 2 weeks postpartum.

(C) H&E staining of compound double- and triple-mutant tumors examined 2 weeks postpartum.

(D) Structure of the 5′ end of the *CXCL12* gene containing three predicted LEF-TCF binding sites, P11 being composite, and ChIP in MDA MB431 cells using these DNA sequences and anti-β-catenin. ICG-001 and CHIR99021 treatments were performed to show specificity of canonical Wnt signaling. *Myc* and *Axin2* sequences are confirmed binding sites. Error bars represent ±SEM, n = 3.

(E) Kaplan-Meier survival plots showing four distinct *CXCL12* expression groups across 3,547 breast cancer patients with relapse-free survival (RFS) data.

We also examined the effect of combining ICG-001 and PHA 665752 inhibitor treatment in the Wnt-Met mammary gland tumor cells by gene expression profiling. Genes that were minimally or not affected by single inhibitors (<2-fold change; $p < 0.005$) but responded synergistically (>2.5-fold change; $p < 0.005$) to the inhibitor combination are displayed in a heatmap (Figure S2D; see also a gene ontology-term enrichment analysis using DAVID) (Figure S2E) (Huang et al., 2009). Remarkably, 322 genes clustered into a "synergistic" group, i.e., they were deregulated more strongly by the combination of inhibitors compared to the single inhibitors, hereafter referred to as the "Wnt-Met gene signature" (Figure S2D). Among the genes with significant synergistic changes in gene expression, many have been implicated in mammary gland biology and breast cancer, like *Agtr2*, *Col11a1*, *Igf1*, *Prl3a1*, *Aldh1a1*, and *Cxcl12* (Figure S2D, marked in red on the right) (Maxwell, 2010), and others are known to function in Wnt signaling, like *Sfrp1*, *Dkk3*, and *Bambi* (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes). *Agtr2* and *Col11a1* are regulated specifically by the combination of inhibitors, whereas *Cxcl12* was mildly regulated by the Wnt inhibitor and more strongly by the combination of Wnt and Met inhibitors (Figure 2D, *Cxcl12* is highlighted in red). Because the Cxcl12 receptor Cxcr4 is regulated by Met (see Figures 2B, 2C, and S3A), the changes in expression of the chemokine and its receptor suggest a mode of autocrine signaling during Wnt-Met-driven basal mammary gland tumor formation.

## Wnt-Met Mammary Gland Tumors Require CXCR4/CXCL12 Signaling

To investigate the potential role of CXCL12/CXCR4 chemokine/receptor signaling on Wnt-Met-driven tumor formation, we used mouse genetics to conditionally ablate the *Cxcr4* gene, i.e., through deletion of the essential exon 2 (Nie et al., 2004) in the mammary gland tissue in compound-mutant mice (*WAP-cre*; *β-catn*$^{ex3/+}$*;HGF;CXCR4*$^{ex2/ex2}$, referred to as triple-mutant mice) (Figure S3A). Strikingly, the appearance of tumors was significantly delayed in triple-mutant mice, compared to Wnt-Met mutants, whereas tumor growth in triple mutants with only one floxed *Cxcr4* allele was unaffected (Figure 3A). The weight of mammary gland tissues, an indication of tumor size, was 6-fold lower in triple than in double mutant mice 2 weeks postpartum (Figure 3B).

Remarkably, the histology of triple-mutant mammary glands at 2–5 weeks postpartum contained areas that resembled normal mammary glands (Figure 3C, bottom). At 7 weeks, triple-mutant mice developed tumors; however, these late stage tumors re-expressed CXCR4, indicating a subset of tumor cells that had escaped cre recombination (Figure S3B). Overall, these genetic findings highlight the importance of CXCR4 signaling in Wnt-Met-driven mammary gland tumor formation.

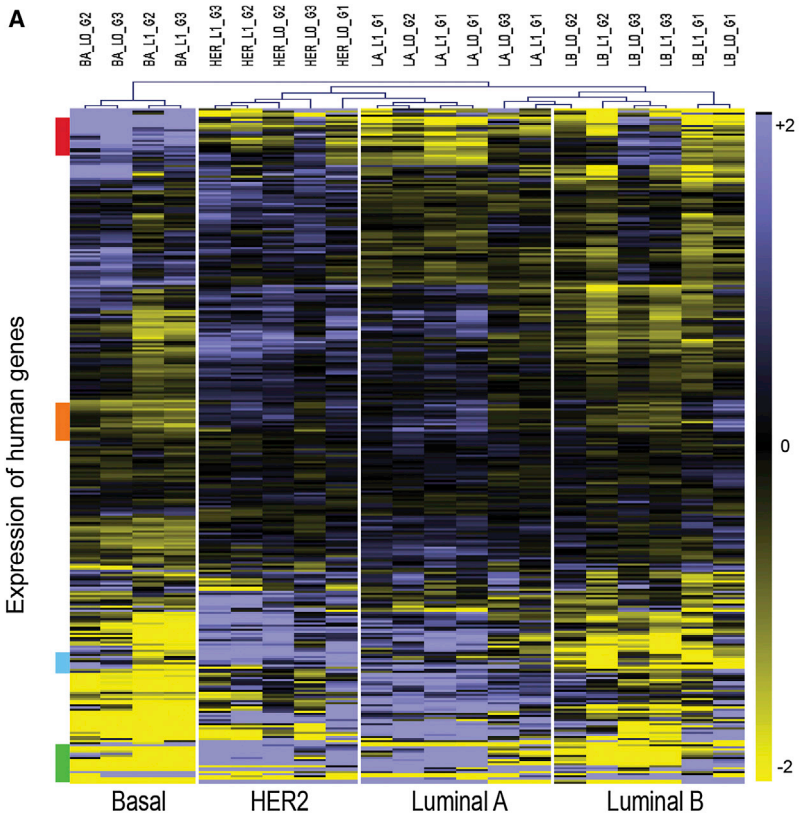
Gene expression data indicated that *CXCL12* is a Wnt target and moreover is controlled synergistically by Wnt and Met (Figure 2D). We performed in silico analysis of potential TCF/LEF binding sites on the promoter and 5′ regions of the *CXCL12* gene using the PATCH 1.0 program, which was followed by chromatin immunoprecipitation (ChIP) in human breast cancer MDA-MB-231 cells. Three potential TCF/LEF binding regions were found in the *CXCL12* gene, and one of them contained three consecutive TCF/LEF consensus sequences (scheme in Figure 3D, top). ChIP was performed to confirm these as potential binding sites for β-catenin/TCF/LEF complexes. Both anti-β-catenin and anti-LEF1 could enrich the three TCF/LEF binding sites, and the enrichment was comparable to those observed with TCF/LEF binding sites in the promoters of *c-Myc* and *Axin2*, two well-characterized canonical Wnt target genes (Figure 3D, bottom; Figure S3C) (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes). ICG-001 reduced the abundance of β-catenin on these TCF/LEF binding sites, whereas the Wnt activator CHIR99021 led to enhancement. Neither ICG-001 nor CHIR99021 treatment influenced LEF1-binding to these sites. These results demonstrate that *CXCL12* is a direct Wnt/β-catenin target gene in mammary gland cancer cells. We also compared the expression levels of *CXCL12* with the survival of human breast cancer patients. Kaplan-Meier analysis was performed on data obtained from 3,597 patient tumors from 21 public data sets, which revealed that *CXCL12* expression levels significantly correlated with patients survival (analyzed in four different quartiles, p = 2.2E-18) (Figure 3E and Table S1). We conclude from these data (1) that the compound Wnt-Met-driven mammary gland tumors are dependent on CXCR4 signaling, (2) that *CXCL12* is a direct Wnt target gene, which (3) is further synergistically regulated by Wnt and Met, and (4) the expression levels of the *CXCL12* gene can predict disease outcome in breast cancer patients.

## The Mouse Wnt-Met Gene Signature Predicts Disease Outcome in Human Breast Cancer Patients

We analyzed whether the mouse Wnt-Met gene signature consisting of 322 genes (see Figure S2D) can predict clinical outcome in human breast cancer patients. Data obtained from 3,597 patient tumors from 21 public data sets were grouped based on breast cancer subtypes and analyzed by hierarchical clustering using the expression of the Wnt-Met gene signature (see also Experimental Procedures and Supplemental Experimental Procedures) (Györffy and Schäfer, 2009). The Wnt-Met gene signature could be used to subgroup the main subtypes of breast cancers, i.e., basal, HER2/ErbB2, and luminal A and B subtypes (Figure 4A; colored area enlarged in Figure S4). Next, retrospective Kaplan-Meier survival analyses were performed from patient data of basal (n = 624), luminal A (n = 1,609), luminal B (n = 757), and HER2/ErbB2 (n = 607) breast cancers, using the expression of the murine Wnt-Met gene signature. Remarkably, high expression of the Wnt-Met signature correlated to a shorter relapse-free survival (RFS) time in human patients with ER-negative basal and HER2/ErbB2 breast cancers (Figure 4B, left, p = 3.6E-06 and p = 2.3E-05, respectively; note that 31% of HER2/ErbB2 tumors were ER negative), but not in luminal A and B cancer types (Figure 4B, right). We conclude from these data that high expression level of the mouse 322 Wnt-Met signature genes predicts poor survival of human patients with ER-negative basal breast cancers.

## Combinations of Wnt, Met, and CXCR4 Inhibitors Delay Tumor Formation in Mice

We performed therapy experiments to test whether targeting Wnt, Met, and CXCR4 in the genetic mouse model of basal breast cancer could reduce tumor burden. The combination of Wnt and Met inhibitors ICG-001 and PHA 665752 or the small molecule inhibitor AMD3100, a competitive antagonist of the CXCR4 receptor (Nimmagadda et al., 2010), were administered in Wnt-Met mutant mice postpartum at regular intervals over 24 days (Figure 5A). The compounds were tested at several concentrations (PHA 665752 at 25 and 50 mg/kg; ICG-001 at 200 and 100 mg/kg; and AMD3100 at 1, 5, and 10 mg/kg), which displayed little effect on body weight, indicating minimal toxicity (Figures S5A–S5C, left graphs; see also the Experimental Procedures). Animals treated with either ICG-001 or PHA 665752 exhibited a moderate decrease in mammary gland tumor volume; however, treatment with the combination of ICG-001 and PHA 665752 or with AMD3100 strongly suppressed tumor onset up to 16 days (Figure 5B, red and blue curves; Figures S5A–S5C, right graphs). Tumor relapse was observed following prolonged treatments, although this was significantly delayed in mice treated with the combination of ICG-001 and PHA 665752. Further inhibitor combinations were examined revealing the strongest inhibition in tumor size after triple treatment using AMD3100, ICG-001 plus PHA 665752 (Figure 5C). The use of AMD3100 in combination with ICG-001 and PHA 665752 also revealed the most significant delay in tumor onset (Figure 5D), with the smallest tumors (0.1 $cm^3$) palpable at 24 days of treatment. Histological analyses of mammary gland tumors in untreated animals revealed basaloid hyperplasia and squamous metaplasia (see also above); however, treatment with PHA 665752 affected squamous differentiation, which was more pronounced with ICG-001 treatment because mammary glands structures remained largely hyperplastic (Figure 5E, top, quantified in the graph below). Strikingly, the combination of the inhibitors ICG-001 plus PHA 665752, AMD3100 and triple combinations resulted in the appearance of alveolar glandular structures, indicating epithelial cell differentiation (Figure 5E, bottom pictures, graph below; Figure S5D). Tumor histology revealed distinct areas resembling normal mammary gland tissue in mice treated with all three compounds, which could be confirmed by K8/K5 and β-casein staining (Figures S5E and S5F). Bromodeoxyuridine (BrdU) pulse-labeling experiments revealed decreased proliferation in K5-positive epithelial cells after treatment with ICG-001, ICG-001 plus PHA 665752, AMD3100, or AMD3100 plus ICG-001 plus PHA 665752 (Figure 5F, quantified below).



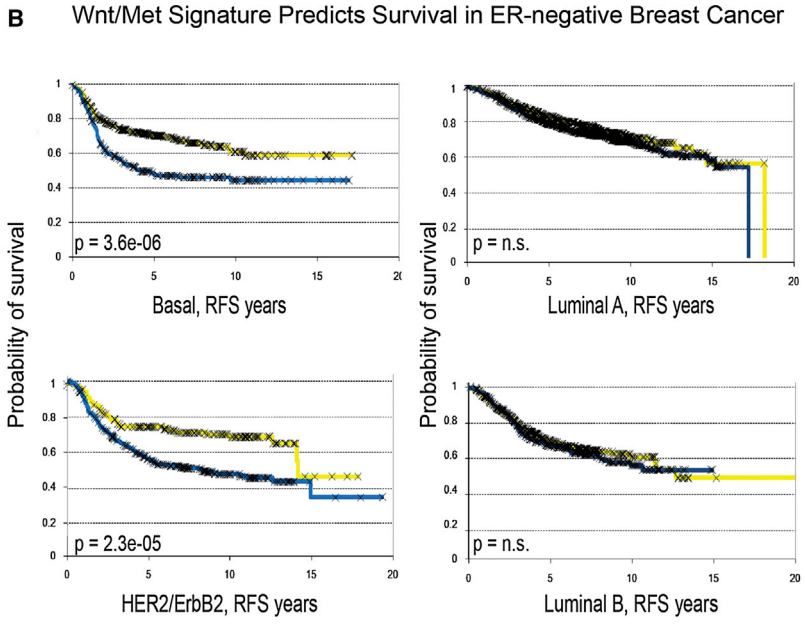




**Figure 4. The Wnt-Met Gene Signature Predicts Survival of Human Breast Cancer Patients**

(A) The mouse 322 intrinsic Wnt-Met gene set shows predictive power for the molecular subtypes of human breast cancer, shown in a heatmap. Molecular subtypes of breast cancer, which have been described previously, were divided into cohorts according to their clinical characteristics (lymph node status and grade) and molecular subtypes. Red represents a basal cell gene cluster, orange represents a HER2/ErbB2 gene cluster, blue represents a luminal A gene cluster, and green represents a luminal B gene cluster.

(B) Kaplan-Meier survival plots of four subgroups of breast cancer patients with RFS time, using the 322 Wnt-Met mouse gene set (BA, basal; HER, HER2; LA, luminal; A, LB, luminal B; LO, lymph node negative; L1, lymph node positive; G, grade).

## Wnt and Met Signaling Cooperate to Maintain Cancer-Propagating Cells

We wanted to confirm on the cellular level that Wnt, Met, and CXCR4 signaling regulate distinct biological functions in the adult mammary gland tumor cells, as suggested by gene expression profiling (see Figures 2B and 2C). Mammary gland tissues from the different mouse model genotypes were enzymatically digested to obtain single cells and subjected to endothelial and hematopoietic cell depletion for the enrichment of epithelial cells (Figure S6A). Flow cytometric analysis using antibodies against CD24 and CD29 surface markers (Shackleton et al., 2006; Stingl et al., 2006) separated three major cell populations in single-mutant tissues: a $CD24^{+}/CD29^{high}$ enriched mammary stem cell (MaSC) subpopulation, a $CD24^{high}/CD29^{+}$ luminal progenitor and mature cell population, and a $CD24^{low}/CD29^{+}$ fraction containing a stromal cell/mixed compartment (Figure 6A, top). In contrast, compound Wnt-Met tumors at 1 week postpartum displayed expansion of a cell population that we define as $CD24^{+}/CD29^{medium}$ cells (37.7%, encircled in red) (Figure 6A, lower left). When $CD24^{+}/CD29^{medium}$ and $CD24^{low}/CD29^{+}$ cells were cultured for 7 days as mammospheres, $CD24^{+}/CD29^{medium}$ cells were highly enriched, and the $CD24^{low}/CD29^{+}$ cells that correspond to a stromal population were largely depleted (Figure 6A, lower right). Cultured tumor cells of compound Wnt-Met tumors maintained the expression of both transgenes after 14 days in culture, as confirmed by YFP-Cre and HGF expression (Figure S6B). In addition, tumor cells were profiled using the combination of CD24 and CD49f surface markers, which revealed an expansion of $CD24^{+}/CD49f^{hi}$ subpopulation in the Wnt-Met compound-mutant tissue (Figure 6B, lower left). Analysis using other cell-surface markers such as $Sca1^{+}$, ALDH, or CD61 did not reveal

Taken together, treatment with a combination of Wnt and Met inhibitors, with the CXCR4 inhibitor, or with triple treatments significantly delayed tumor onset, suppressed proliferation and induced differentiation of the Wnt-Met tumors.

heterogeneity of the CD24$^{+}$/CD29$^{medium}$ tumor cell populations. These data demonstrate that combined activation of Wnt and Met signaling promotes the expansion of a population of cells with progenitor and stem cell characteristics, which is further supported by mammosphere culture assays (see also below). qRT-PCR analysis could confirm transgene expression in isolated tumor cells from the different mouse models (Figure 6C).

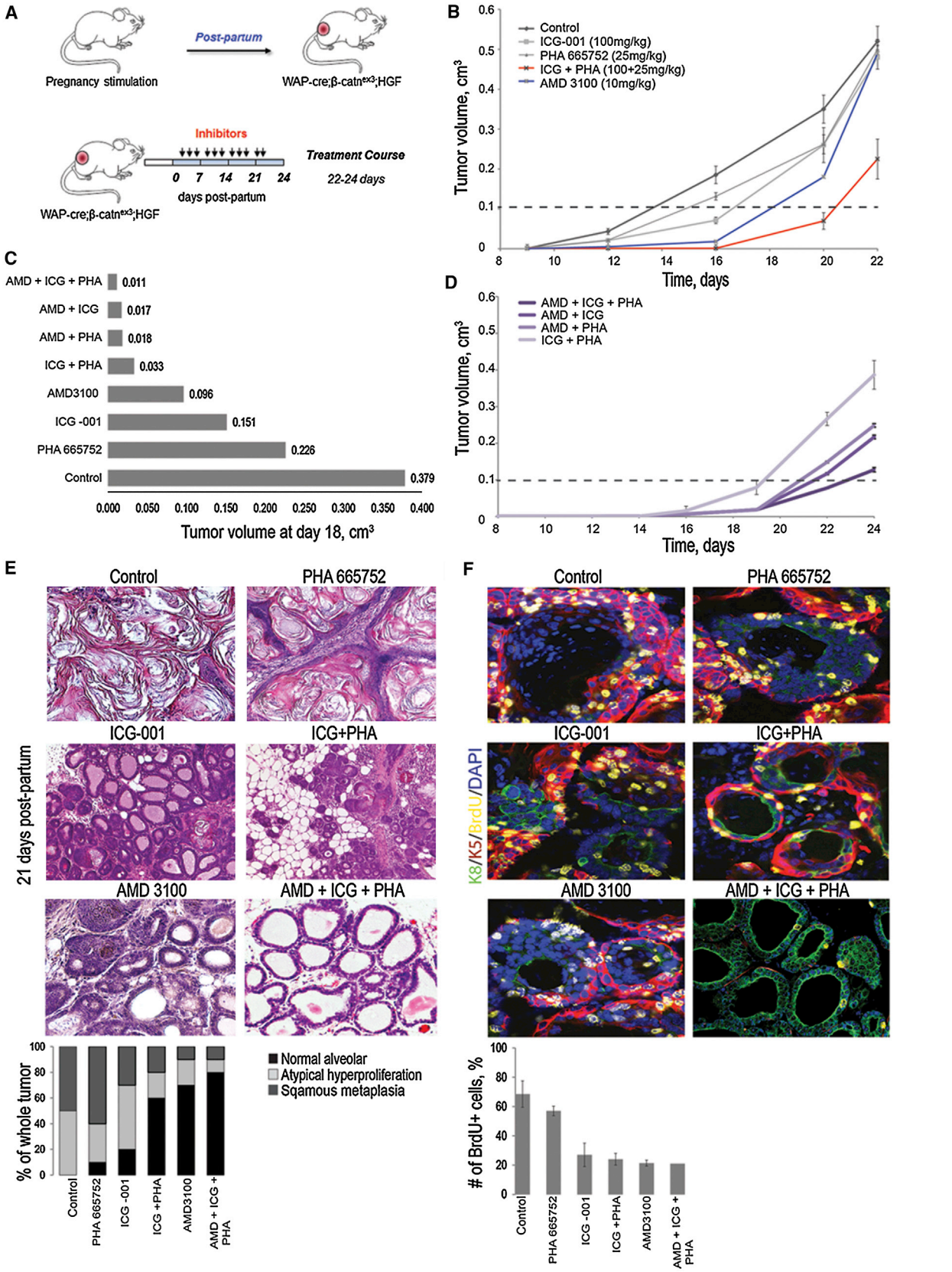
To assess the tumorigenic potential of CD24$^{+}$/CD29$^{medium}$ cells isolated from 1 week postpartum Wnt-Met mammary glands, compared to CD24$^{+}$/CD29$^{high}$ mammary gland cells from 1 week postpartum single mutants, the cell populations at different dilutions were transplanted into cleared mammary gland fat pads of *NOD/SCID/IIR2*$^{-/-}$ mice (Figure S6A, right) (Quintana et al., 2008). Remarkably, as few as 100 CD24$^{+}$/CD29$^{medium}$ cells from compound Wnt-Met mutant mice were able to generate tumor outgrowths 4 weeks posttransplantation, indicating that these cells harbor cancer-propagating capacity (Figure 6D, bottom left). In contrast, 50,000 of unsorted cells were required for tumor formation (Figure 6D, upper left). Transplantation of CD24$^{+}$/CD29$^{high}$ cells from single-mutant mice, even at the highest injection numbers, did not result in tumor outgrowths (Figure 6D, right). Histological analysis revealed that tumors derived from transplanted CD24$^{+}$/CD29$^{medium}$ cells were indistinguishable from the original tumors, i.e., were mixed tumors, demonstrating that this pool of cancer-propagating cells retained their ability to form complex tumors (Figure 6E, compare with Figure 1D). Additionally, we used a fluorescent reporter mouse expressing YFP to perform lineage tracing of transplanted tumor cells and could show that YFP$^{+}$ cells gave rise to tumors, which expressed luminal and basal differentiation markers, K8 and K5, respectively (Figure S6C). These results demonstrate that combined Wnt-Met signaling expands a population of cells that demonstrate a strong cancer-propagating ability.

The self-renewal properties of mammary gland epithelial cells from mice with different genotypes were measured by mammosphere formation in culture, i.e., growth in suspension in serum-free media (Figure S6A, left) (Dontu et al., 2003). After 7 days in culture, the number and size of mammospheres generated from Wnt-Met compound-mutant tumors were increased 5- to 6-fold in comparison to controls (Figure S6D, quantified in Figure S6E). To further address the function of Wnt, Met, and CXCR4 signaling on self-renewal, mammosphere formation was assessed in the presence of pharmacological inhibitors. Remarkably, mammospheres treated with the Wnt inhibitor ICG-001 or β-catenin siRNAs were significantly reduced in size and number (Figure 6F, left; quantifications in Figures 6G, S6F, and S6G). In contrast, suppression of Met or CXCR4 by pharmacological inhibitors or siRNAs had minor effects on mammosphere formation (Figure 6F, left; quantifications Figures 6G, S6F, and S6G). Next, we examined the differentiation capacities of cells derived from the different genotypes by growth in semi-solid 3D Matrigel (Figure S6A, middle) (Lee et al., 2007). CD24$^{+}$/CD29$^{high}$ cells derived from wild-type and single-mutant mammary glands produced hollow acini-like structures composed of a polarized layer of epithelial cells encapsulating a lumen, whereas CD24$^{+}$/CD29$^{medium}$ cells from compound Wnt-Met mutants produced filled structures, which continued to expand rapidly (Figure S6H; quantification in Figure S6I). Remarkably, treatment of the CD24$^{+}$/CD29$^{medium}$ cells from double mutants with the Met or CXCR4 inhibitors (PHA 665752 or AMD 3100) for 14 days transformed filled structures into hollow acini with a polarized outer epithelial layer that resembled structures formed by control and single-mutant CD24$^{+}$/CD29$^{high}$ cells (Figure 6F, middle; quantification in Figure 6H). Differentiation induced by blocking Met and CXCR4 signaling could be confirmed by the expression of the epithelial cell markers K8/K5 (Figure 6F, right). In contrast, the Wnt inhibitor ICG-001 had no major effect on the morphology of the filled structures formed in 3D Matrigel, although sphere size was moderately reduced. Thus, the two signaling systems Wnt and Met indeed control distinct properties of mammary gland tumor cells, self-renewal by Wnt on one side, and block of differentiation and morphogenesis by Met on the other. The chemokine receptor CXCR4 regulates similar cellular properties as Met, i.e., induces block of differentiation and morphogenesis.

## DISCUSSION

In an adult murine model of breast cancer, we could show that genetic activation of both Wnt/β-catenin and HGF/Met signaling using the pregnancy-induced WAP promoter, induced fast-growing basal mammary gland tumors. We found that enhanced chemokine/receptor CXCL12/CXCR4 signaling is an important driver of tumor formation, because mutation in the *Cxcr4* gene induced resistance to tumorigenesis. In Wnt-Met mutant mice, combination therapies with small molecule Wnt, Met, and CXCR4 inhibitors yielded significant reduction in tumor development. Furthermore, we could show that a mouse Wnt-Met signature of 322 genes predicted disease outcome in human basal breast cancer patients. Thus, our data suggest that Wnt, Met, and CXCR4-regulated genes are valuable biomarkers in patients with basal breast cancer, and these patients may benefit from treatment using combinations of Wnt, Met, and CXCR4 inhibitors.

The coactivation of Wnt/β-catenin and HGF/Met signaling in mouse adult mammary gland epithelium was performed under the control of the WAP promoter. The WAP promoter is active in stem cells and luminal cells present during pregnancy and lactation, i.e., in lobuloalveolar progenitor cells (Wagner et al., 1997). The activation of β-catenin and HGF in mouse mammary glands produced hyperplastic alveolar nodules, which formed tumors resembling features described for other basal mammary gland tumors (Herschkowitz and Lubet, 2010); however, compared to the other mouse models, the compound Wnt-Met mice developed tumors extremely rapidly. Thus, our data indicate that the combination of Wnt and Met signaling in the adult mammary gland rapidly expands a population of stem or lobuloalveolar progenitor cells that display cancer-propagating cell properties. The two signaling systems in mouse mammary gland tumors control distinct biological functions that cooperate in tumor formation, promotion of self-renewal and inhibition of differentiation. Gene expression profiling supported the concept that Wnt signaling contributes mainly to proliferation and self-renewal, because important cell-cycle checkpoint, proliferation, and self-renewal genes were deregulated. Further, Wnt signaling strongly promoted self-renewal in the mammosphere assay,

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which enriches for cancer-propagating cells (Dontu et al., 2003). This is in line with other reports, which demonstrate that Wnt1, Wnt3a, and Wnt10b can support self-renewal and regenerative capacities of mammary gland stem cells and cancer stem cells (Shackleton et al., 2006; Zeng and Nusse, 2010; Holland et al., 2013; Wend et al., 2013). A role for Wnt signaling in the self-renewal of cancer-propagating/cancer stem cells has been proposed for several other tumor types (Clevers, 2011; Holland et al., 2013). In contrast, high Met activity in mammary gland tumor cells affected mainly differentiation. This was notable in 3D Matrigel cultures, where tumor cells reformed alveolar structures subsequent to Met inhibition. Gene expression profiling supported the role of Met in differentiation, because genes modulating epithelial differentiation were controlled by Met activity. In other cellular contexts, Met can also control growth and migration of tumor cells (Gherardi et al., 2012). The question arises as to which cells may represent the cell of tumor origin in compound Wnt-Met mice. Previous lineage-tracing experiments using WAP-cre identified luminal cells capable of resisting apoptosis during involution and clonally expand upon the succeeding pregnancy to give rise to luminal and alveolar cells. These cells were therefore described as alveolar progenitors or parity-induced cells (Wagner et al., 2002). More recently, lineage tracing of the mammary gland using inducible cre expressed either in myoepithelial cells (K14- or K5-expressing cells) or in luminal cells (K8- or K18-expressing cells) demonstrated that the mammary gland initially develops from multipotent embryonic K14-expressing progenitors, which give rise to both myoepithelial cells and luminal cells. However, postnatal mammary gland development that occurred during puberty, as well as mammary gland expansion that accompanied pregnancy, confirmed the presence of two types of long-lived stem cells (Van Keymeulen et al., 2011). Our experiments therefore indicate that in the Wnt-Met compound-mutant model lobuloalveolar progenitor cells are expanded and, when transplanted into fat pads of immune-deficient mice, can give rise to complex tumors displaying cancer-propagating cell characteristics. Thus, our data support the notion that a multi- or bipotent progenitor cell population represents the target of oncogenic transformation driven by Wnt and Met in the adult mammary gland.

Compound Wnt-Met-driven tumors express the chemokine receptor CXCR4, which is required for Met-dependent suppression of differentiation. During metastasis, CXCR4 and the chemokine CXCL12 control the migration of tumor cells from sites of primary growth (Holland et al., 2006; Zlotnik et al., 2011). Our data provide genetic evidence that CXCR4 signaling is a crucial step downstream of Wnt and Met activation during mammary gland tumorigenesis, as tumor development does not occur when *CXCR4* is mutated. Moreover, inhibition of CXCR4 signaling using the AMD3100 small molecule antagonist inhibited tumor growth in therapy experiments of mice and strongly induced tumor cell differentiation in mice and Matrigel cultures. The fact that ablation and inhibition of CXCR4 suffice to reverse many biological effects of activated Wnt and Met signaling highlights the importance of CXCL12/CXCR4 as functional targets. CXCR4 has been described as a Met target in breast cancer cells in recent studies using pharmacological interference (Huang et al., 2012; Matteucci et al., 2007), which is in accordance to our genetic and expression data. Moreover, Wnt and Met activity synergistically activated *CXCL12* expression, and the ChIP experiments on cultured human breast cancer cells indicated that *CXCL12* is a direct Wnt/β-catenin target (see model in Figure S7). Overall, our study thus demonstrates that production of CXCL12 and autocrine activation of its receptor CXCR4 are hallmarks of Wnt-Met-driven adult mammary gland cancers.

Wnt-Met compound-mutant mice were treated with Wnt, Met, and CXCR4 inhibitors or a combination of inhibitors. Remarkably, combined treatment with all three inhibitors strongly suppressed the emergence of mammary gland tumors in compound mutants, and in major areas the presence of differentiated normal ductal structures was observed. In vivo, all inhibitors and inhibitor combinations affected tumor morphology, although to varying extents. Differences seen on self-renewal/differentiation are clearly distinguishable in vitro; however, this is more difficult to distinguish in vivo, which may be due to additional factors, e.g., tumor-stroma interaction. Intense efforts are made worldwide to develop potent inhibitors of Wnt/β-catenin signaling, but only few of these are already assessed in clinical trials (Takahashi-Yanaga and Kahn, 2010). An improved version of the ICG-001 used here is presently in clinical trial for leukemia (Wend et al., 2013). Similarly, strategies to inhibit HGF/Met signaling are currently being evaluated in clinical trials, with promising results (Gherardi et al., 2012). A few of these inhibitors have been suggested to preferentially target cancer-propagating cells, which are often resistant to conventional cancer treatments like chemotherapy and radiation (Gherardi et al., 2012; Holland et al., 2013; Zhang et al., 2010). We found that the combination of Wnt and Met inhibitors had profound effects on endogenous tumors, and our experiments on sorted tumor cells indicate that these inhibitors targeted not only the bulk of the tumor but preferentially cancer-propagating cells. Combinations of Wnt and Met inhibitors can be examined in the future, for instance, in therapy experiments of human tumor xenografts

**Figure 5. Therapy with a Combination of Wnt and Met or CXCR4 Inhibitors Delays Tumor Formation in Compound-Mutant Mice**

(A) Scheme showing the time course of inhibitor treatment in Wnt-Met compound-mutant mice.

(B) Tumor volume in compound-mutant mice after inhibitor treatment with 25 mg/kg PHA 665752 (i.v.), 100 mg/kg ICG-001 (i.p.), the combination of the two, or 10 mg/kg AMD 3100 (s.c.). n = 10 mice per group.

(C) Comparison of Wnt-Met compound tumor volume at day 18 after the different inhibitor treatment combinations.

(D) Tumor volume in compound-mutant mice after combined inhibitor treatment with PHA 665752 plus ICG-001 (25 mg/kg, 100 mg/kg), AMD 3100 plus PHA 665752 (10 mg/kg, 25 mg/kg), AMD 3100 plus ICG-001 (10 mg/kg, 100 mg/kg), and AMD 3100 plus PHA 665752 plus ICG-001 (10 mg/kg, 25 mg/kg, 100 mg/kg). n = 6 mice per group.

(E) H&E staining of tumors treated with the indicated inhibitors; bottom left, quantification of tumor morphology in compound mutants after different treatments.

(F) BrdU staining in tumors treated with different inhibitors; BrdU (yellow), K5 (red), K8 (green), and DAPI (blue); bottom left, quantification of BrdU incorporation at day 16. (ip, intraperitoneal injection; iv, intravenous injection; sc, subcutaneous injection).

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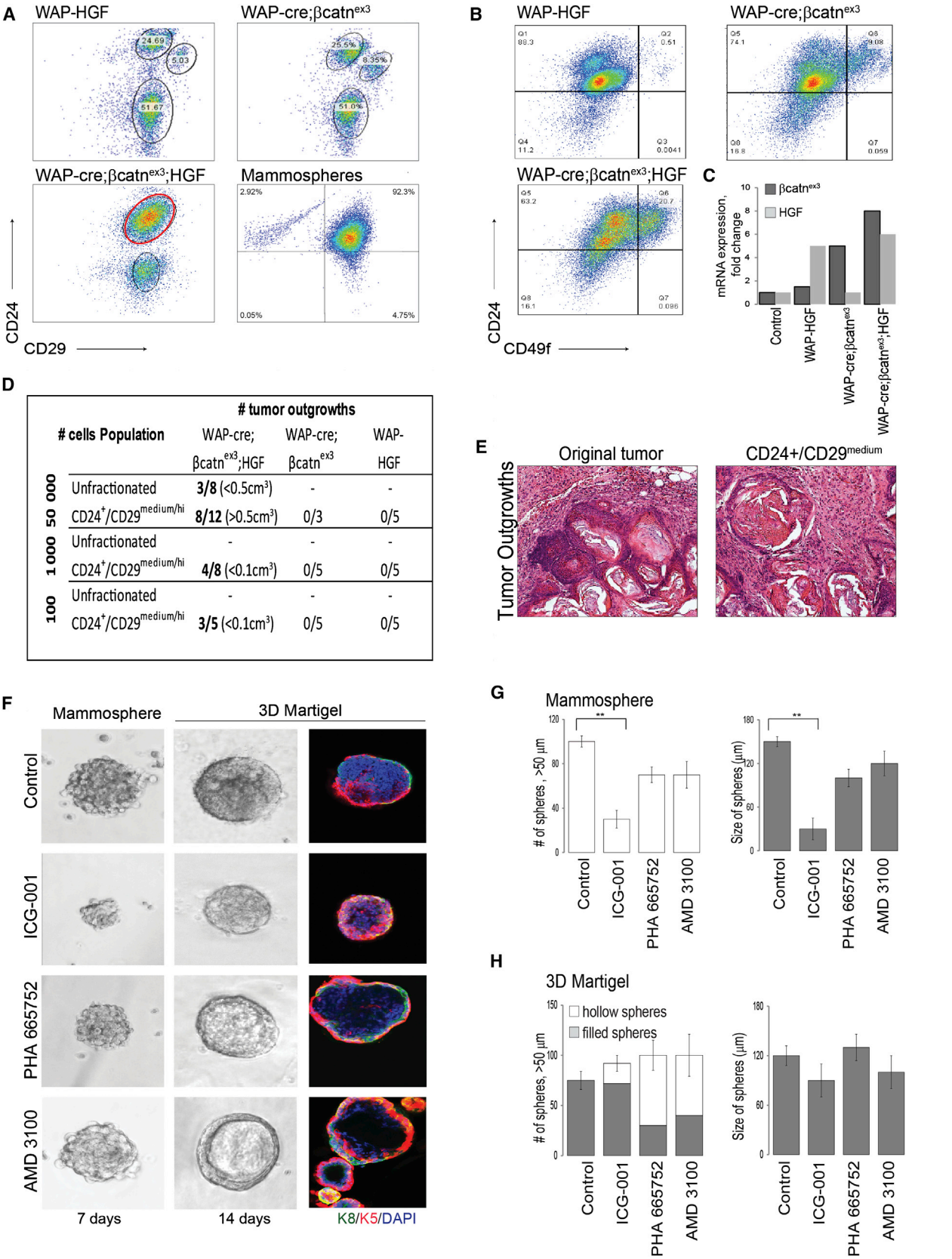


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in animal models. AMD3100, a compound that targets the Wnt-Met-driven CXCR4/CXCL12 signaling system, has been examined clinical trials in patients with lymphomas or multiple myelomas (Keating, 2011) but not in trials for solid tumors. We have also found that combinations of the three inhibitors, against Wnt, Met, and CXCR4, are the most effective in delaying tumor onset. Overall, our findings encourage the use of combinations of Wnt, Met, and CXCR4 inhibitors for therapies directed against cancer-propagating cells of solid tumors.

We have also shown that the mouse Wnt-Met gene signature can be used to distinguish human breast cancer subtypes in several aspects. Remarkably, the overall expression of 322 signature genes identified in the mouse tumors is predictive for poor survival of patients with ER-negative breast cancers, i.e., in basal and HER2/ErbB2 but not in luminal A and B subtypes. The data therefore suggest that the mouse Wnt-Met signature may be used as prognostic indicator in cancer patients. We also demonstrate here that high expression of one of the synergistically regulated Wnt-Met target gene, *CXCL12*, correlates with poor prognosis in human breast cancer patients. Other gene expression tools like Mammaprint and Oncotype Dx are now used worldwide in clinical settings, and their application improved treatment strategies (Oakman et al., 2010). It is conceivable that the Wnt-Met signature described here, or a number of selected genes thereof, can be used to further improve stratification of breast cancers, in particular, those cancers that do not respond to hormonal therapy. Moreover, such an analysis could identify patients that might benefit from a treatment with a combination of Wnt and Met inhibitors, or treatment combinations with CXCR4 inhibitors. Collectively, our results suggest that the analysis of Wnt and Met expression and target genes in basal breast cancer may serve as useful biomarkers to predict patient prognosis and suggest new therapeutic options.

## EXPERIMENTAL PROCEDURES

### Mouse Strains

All animal experiments were conducted in accordance with national, European, and internal MDC regulations. *WAP-cre*, *β-catn*$^{ex3}$/*β-catn*$^{+}$, *WAP-HGF*, and *CXCR4*$^{flox/flox}$ mice have been described (Gallego et al., 2003; Harada et al., 1999; Nie et al., 2004). All crossings were performed in FVB/N mice for at least ten generations. After one round of pregnancy, phenotypes were analyzed in each of the models.

### Mammosphere and 3D Matrigel Assays and Inhibitor Treatments

Mammosphere assays are described in the Supplemental Information. For 3D Matrigel assays, cells were plated in 25% Matrigel in MAM-media on top of an agarose layer. Pharmacological inhibitors ICG-001 (10 μM), PHA665752 (1 μM), and AMD 3100 (1 μM) were added to either Mammosphere or 3D Matrigel cultures and were supplemented every 3 days. Mice were administration with 25 mg/kg PHA 665752 (intravenously [i.v.]), 100 mg/kg ICG-001 (intraperitoneally [i.p.]), the combination of the two, or 10 mg/kg AMD 3100 (subcutaneously [s.c.]) three times per week for 4 weeks.

### Mammary Fat Pad Transplantation Assays

Transplantations of cells into cleared mammary gland fat pads were carried out as described (Deome et al., 1959) using 3-week-old NOD/SCID/ILR2$^{-/-}$ immunodeficient mice.

### In Vivo Treatment of Wnt-Met Compound-Mutant Mice with Inhibitors

Early postpartum animals were injected with single doses or combinations of PHA 665752 (25 mg/kg i.v.), ICG-001 (100 mg/kg i.p.), AMD 3100 (i.v., 10 mg/kg), or control for 22–24 days, three times per week. Tumor burden was monitored and measurements were taken several times per week. Individual tumor volumes (*V*) were calculated using the formula $V = (\text{length} \times [\text{width}]^2)/2$.

### Microarray and Bioinformatic Analysis

For details, see the Supplemental Information.

## ACCESSION NUMBERS

The GenBank accession number for the microarray expression data is GSE35899.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.11.001.

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**Figure 6. Wnt and Met Signaling Cooperate in Distinct Functions in Order to Maintain Cancer-Propagating Cells**

(A) Flow cytometric analysis of CD24/CD29 surface marker expression in cells prepared from single- and compound-mutant mammary glands and in unsorted cells from compound mutants 1 week postpartum that were grown 7 days in mammosphere culture. Encircled in red is the expansion of the CD24$^{+}$/CD29$^{medium}$ population of cells from compound-mutant tumors.

(B) Flow cytometric analysis of CD24/CD49f surface expression from single- and compound-mutant mammary glands.

(C) qRT-PCR analysis for transgene expression (β-catenin$^{ex3}$ and HGF) in isolated CD24$^{+}$CD29$^{+}$ cells from mammary gland control, single-mutant, and double-mutant mice.

(D) Generation of tumors following transplantation of sorted and unsorted cells into mammary gland fat pads of *NOD/SCDID/Il2*$^{-/-}$ mice: different numbers of CD24$^{+}$/CD29$^{medium/hi}$ and unfractionated cells of single- and double-mutant tumors were examined (in brackets, the size of the tumor outgrowths; –, no tumors formed).

(E) H&E stainings showing histological images from the original tumor and from tumor from transplanted CD24$^{+}$/CD29$^{medium}$ double-mutant cells.

(F) Mammosphere (left) and 3D Matrigel (middle) aggregate formation using isolated CD24$^{+}$/CD29$^{medium}$ cells (5,000 cells were plated) of compound-mutant mammary glands, in the presence and absence of inhibitors. Matrigel cultures (right) stained by immunofluorescence for the differentiation marker K8 and K5, and with DAPI. Error bars represent ±SEM, n = 6.

(G) Quantification of the number and size of mammospheres from the experiment in (F). Error bars represent ±SEM, n = 4.

(H) Quantification of the number of filled and hollow 3D Matrigel organoids from the experiment in (F). Error bars represent ±SEM, n = 3.

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