AXL Is a Driver of Stemness in Normal Mammary Gland and Breast Cancer

HIGHLIGHTS
- AXL + mammary epithelial cells have multipotent activity conserved in women and mice
- AXL allows accesses to epithelial-to-mesenchymal transition genes and prevents differentiation into luminal cells
- Deletion of Axl reduced incidence of Wnt1-driven tumors in mice
- Provides a rationale explaining the advantage to cancer cells that co-opt AXL signaling
AXL Is a Driver of Stemness in Normal Mammary Gland and Breast Cancer

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SUMMARY
The receptor tyrosine kinase AXL is associated with epithelial plasticity in several solid tumors including breast cancer and AXL-targeting agents are currently in clinical trials. We hypothesized that AXL is a driver of stemness traits in cancer by co-option of a regulatory function normally reserved for stem cells. AXL-expressing cells in human mammary epithelial ducts co-expressed markers associated with multipotency, and AXL inhibition abolished colony formation and self-maintenance activities while promoting terminal differentiation in vitro. Axl-null mice did not exhibit a strong developmental phenotype, but enrichment of Axl+ cells was required for mouse mammary gland reconstitution upon transplantation, and Axl-null mice had reduced incidence of Wnt1-driven mammary tumors. An AXL-dependent gene signature is a feature of transcriptomes in basal breast cancers and reduced patient survival irrespective of subtype. Our interpretation is that AXL regulates access to epithelial plasticity programs in MaSCs and, when co-opted, maintains acquired stemness in breast cancer cells.

INTRODUCTION
Phenotypic plasticity, the capacity of a single genotype to exhibit variable phenotypes in different environments, is a key feature of epithelial homeostasis. The multi-lineage potential of epithelial stem cells is thought to be maintained by specific niche microenvironments and elicited by regenerative cues from tissue wounding and inflammation (Blanpain and Fuchs, 2014). Co-option of these homeostatic mechanisms by carcinoma cells facilitates transition between stem-like mesenchymal and differentiated epithelial states in response to tumor microenvironment dynamics and therapeutic challenge and is associated with poor clinical outcome (Nieto et al., 2016).

The adult human mammary gland is a bilayer epithelium with basal-located myoepithelial (MEP) cells that surround luminal epithelial cells (LEPs) that are thought to be maintained by mammary stem cells (MaSCs), which continue to elude a consensus definition (Fridriksdottir et al., 2017; Petersen and Polyak, 2010; Villadsen et al., 2007; Eirew et al., 2008). The breast epithelium exhibits remarkable organ-scale remodeling during puberty and multiple lactation cycles that requires a renewable reservoir of stem and committed progenitor cells (Visvader and Stingl, 2014). Stem cell-related gene expression programs are utilized by breast cancer cells during malignant progression – here also referred to as co-option (Lawson et al., 2015; Billaud and Santoro, 2011). Transcription factors (e.g. SNAI1/2) that regulate the epithelial-to-mesenchymal transition (EMT) gene program during early development influence MaSC state transitions, and their dysregulation causes luminal compartment expansion (Phillips et al., 2014; Ye et al., 2015). The AXL receptor tyrosine kinase (RTK) is associated with malignant progression and poor patient survival in several malignancies including breast cancer (Davidsen et al., 2017). AXL is activated by a single ligand, GAS6, that activates a unique RTK signaling network in cancer cells (Meyer et al., 2013). AXL expression is correlated with epithelial-mesenchymal transition, immune evasion, increased metastatic potential, as well as therapeutic resistance in several tumor types (Gjerdrum et al., 2010; Ludwig et al., 2018; Zhang et al., 2012).
AXL is therefore an important therapeutic target, and AXL kinase inhibitors are currently in clinical trials (Davidsen et al., 2017).

In contrast to the prominence of AXL in cancer progression, the role of AXL signaling in normal physiology is comparatively unknown, limiting our understanding of the consequences of AXL activation in malignant progression. We hypothesize that AXL is a gatekeeper to the signaling cascades and gene programs that enable epithelial plasticity, and that its principle role in normal epithelia is to regulate access to programs that are permissive for stem or progenitor cell states. Herein we identify AXL as a conserved mediator that governs human and mouse MaSC activity, providing a conserved marker of adult stem cells that is correlated with epithelial plasticity.

RESULTS

AXL Expression Is a Feature of Rare Adult Human Breast Epithelial Cells

AXL knockout mice are viable and do not show a developmental phenotype (Lu and Lemke, 2001); thus there is no evidence linking AXL to developmental EMT. This prompted us to investigate whether AXL is a heretofore unappreciated feature of adult human mammary epithelia. Immunofluorescence staining for AXL expression in normal human breast tissue specimens detected infrequent AXL-expressing cells. A subset of the AXL-expressing cells in human mammary gland overlap with the K14/K19 double-positive population, a phenotype associated with multipotency (Shimoto et al., 2009; Huo and Macara, 2014; Dravis et al., 2015; Lilja et al., 2018; Wuidart et al., 2018; Spike et al., 2012; Giraddi et al., 2018; Chen et al., 2017) (Figures 1A–1D). Small clusters of AXL+ epithelial cells co-expressing of luminal (K19) and basal (K14) markers were also detected in lobular acini (Figures 1E–1H). In ductal structures, AXL-expressing cells constitute a rare population of cells with strong AXL staining (Figure 1I). RNA in situ hybridization (RNA-ISH) analysis of the ductal structures verified this expression pattern (Figure 1J (AXL, brown)). Epithelial cells with >15 AXL mRNA transcripts comprised a minority of cells of the mammary epithelium, consistent with a low Histo-score of 63 (Figure 1L). In contrast, RNA-ISH for the AXL-ligand GAS6 (Figure 1K), revealed a more widely distributed expression among most LEPs, and this heterogeneous expression pattern is correspondingly reflected in the higher Histo-score of 141 (Figure 1M). Dual RNA-ISH detected cells with GAS6 transcripts in LEP that were adjacent to the AXL-expressing cells in the same FFPE tissue sections (Figure 1N; RNA-ISH controls are shown in Figure S1). Collectively, these results suggested that AXL-expressing cells constitute a rare population of cells in the mammary epithelium. Some of the weaker staining AXL-positive cells in the ductal and lobular epithelium are K14+/K19+ positive, and based on RNA-ISH the GAS6 ligand is produced mainly by luminal cells.

Phenotypic Characterization of AXL-Expressing Cells in Human Breast Epithelium

To further characterize the population of AXL+ cells in mammary epithelia, we analyzed primary uncultured human mammary epithelial cells (HMECs) from reduction mammoplasty specimens by flow cytometry using AXL antibody in addition to a panel of LEP, MEP, and stem/progenitor cell markers: CD227, CD10, EP-CAM, CD49f. AXL-expressing HMEC represented 1–6% of the total HMEC population from different individuals (n = 5), which partitioned into an EP-CAM+/CD49f+ subpopulation that is discrete from differentiated LEP (CD227+/CD10+) and MEP (CD227+/CD10+) populations (Figures 2A–2D). The EP-CAM/CD49f/CD227/CD10/AXL FACs-analyzed HMECs were evaluated by spanning-tree progression analysis of density-normalized events (SPADE) to generate a putative hierarchical tree. The SPADE technique organizes groups of similar cells next to each other based on expression similarity, creating tree-like structures. AXL expression was highest in the node located at the apex of separate LEP (CD227 biased) and MEP (CD49f biased) radiations (Figure 2E). Next, we evaluated the expression of AXL, KIT, K14, and K19, among 25 other proteins, in a 29-marker mass cytometry (CyTOF) data set derived from dissociated primary breast epithelia of 57 women (Pelissier Vatter et al., 2018). Non-linear dimensionality reduction by t-distributed stochastic neighbor embedding projection and unsupervised clustering of intra-lineage subpopulations identified distinct LEP, MEP, and progenitor cells (Figure 2F). This high-dimensional analysis showed that AXL was primarily associated with KIT/K14/K19 expressing progenitor cells (Figures 2G and 2H). Notably, K14 was expressed relatively more than K19, which is anecdotally consistent with our immunofluorescence observations that K19 exhibits consistently lower, yet detectable, expression. Previously EpCAM+/CD49f+ epithelia that express K14 and K19, and shared properties of LEP and MEP, were shown to be enriched for multipotent activity in human mammary epithelia (Villadsen et al., 2007). Epithelial cells expressing KIT were shown to be enriched for multipotent epithelial cell activity (Garbe et al., 2012; Lim et al., 2009; Pelissier et al., 2014). The finding that AXL also is expressed in epithelial cells bearing this
Figure 1. AXL Expression Is a Feature of Rare Adult Human Breast Epithelial Cells
AXL is expressed in rare epithelial cells in normal breast epithelium.

(A–D) (A) Multi-color immunofluorescence analysis of normal breast epithelium biopsies (n = 20). The ducts shown in (A–D) are formalin-fixed paraffin-embedded (FFPE) tissue sections stained with monoclonal antibodies against AXL (MAB10C9, white), MEP-specific cytokeratin 14 (K14, red), and luminal epithelial-specific cytokeratin 19 (K19, green); nuclear counterstain (DAPI, blue).

(E–H) Cryosections of normal human breast epithelium stained with monoclonal antibodies against: (E) AXL (ab21965, green), (F) K19 (magenta), (G) K14 (red). Overlay (H) reveals the areas of co-localized expression of AXL, K19, and K14 (white) in the lobular acini.

(I) Chromogenic IHC of AXL (ab21965, HRP-DAB brown) on FFPE sections of human mammary tissue. Counterstain by hematoxylin (blue).

(J–M) RNA in situ hybridization (RNA-ISH) on FFPE breast tissue specimens reveal the localization and distribution of (J) AXL mRNA transcripts, and (K) mRNA transcripts of the AXL-ligand GAS6. Each dot (brown, HRP-DAB) represents a single RNA molecule. (L and M) Histo-score analysis was performed to quantify the heterogeneity of the AXL and GAS6 mRNA transcripts within the breast epithelium at the single cell level. The Histo-score of transcript expression was determined by categorizing epithelial cells in five predefined bins based on the number of transcripts per cell. Distribution (% of cells/bin) as well as Histo-scores provided on a range of 0-400 is given.
same constellation of markers suggests that AXL may be a marker and regulator of multipotent cells in the human mammary epithelium.

**KIT+/AXL+ Primary HMECs Display a Unique Transcriptional Profile**

We next examined the distribution of AXL expression relative to KIT in situ. Immunofluorescence staining of normal breast tissue revealed that KIT+/AXL+ breast epithelial cells were predominantly positioned basally relative to KIT-/AXL- staining cells that were positioned adjacent to the lumen (Figure 3A). Primary HMEC were enriched by FACS based on expression of KIT, and AXL was found to be expressed by 16% of the KIT+ epithelial population, thus representing about 1% of the total primary mammary epithelial cell population (Figure 3B). To further delineate the difference between these epithelial populations, we sorted KIT+AXL+ and KIT+/AXL- cells, and CD227+/CD10- LEP and CD227-/CD10+ MEP subpopulations from primary HMEC at fourth passage (from two different women) and performed whole-genome expression analysis. Unsupervised hierarchical clustering identified distinct KIT+/AXL+, KIT+/AXL-, LEP, and MEP populations (Figure 3C). Principal component analysis of gene expression data revealed strong separation of KIT+/AXL+, LEP and MEP populations, whereas the KIT+/AXL- HMEC showed greater similarity to differentiated LEPs (Figure 3D). These results are consistent with the hypothesis that KIT+/AXL+ cells represent a distinct breast epithelial progenitor population compared to the KIT+/AXL- population.

**AXL Kinase Activity Is Required for Self-Renewal and Acini Formation in Primary HMECs**

Ex Vivo Assays

To address the function of AXL signaling during differentiation, we cultured HMEC in the presence of an AXL-specific small molecule tyrosine kinase inhibitor (TKI) and monitored changes in the percentage of LEPs during subsequent passages. Standard in vitro culture of HMEC does not maintain the LEP population effectively, with an observed half-life of 3.8 ± 0.2 days due to the favored expansion of cells with basal properties, such as MEP (Figure 3E) (Garbe et al., 2012). Addition of a selective AXL TKI (bemcentinib, 600 nM) (Holland et al., 2010) counteracted LEP loss in culture (observed LEP half-life of 11.9 ± 5.9 days; p = 0.43), consistent with a pro-luminal differentiation effect, whereas a KIT TKI (imatinib, 1 μM) did not significantly alter LEP half-life (5.8 ± 0.5 days) (Figure 3E). Next, we assessed the requirement of AXL signal transduction for in vitro secondary mammosphere formation by HMEC enriched for KIT expression through two passages. Secondary mammosphere formation assays are frequently used a measure of self-maintenance activity. Secondary mammosphere formation was not affected by treatment with imatinib, but was reduced more than 4-fold by treatment with the AXL-inhibitor bemcentinib (Figure 3F). Formation of multi-lineage acini in 3D laminin-rich ECM (lECM) was also significantly inhibited by treatment with bemcentinib, and only a few single K19-staining cells were observed in culture post-treatment (Figures 3G and 3H). Functional ex vivo assays showed that AXL kinase activity is required for self-renewal and maintenance of multi-lineage differentiation potential of KIT+/AXL+ HMECs. Taken together, in situ, multiparameter cytometry, gene expression, and functional cell-based ex vivo analyses of primary human mammary epithelia align and suggest that AXL+/KIT+ cells exhibit multipotent activity. AXL-/KIT+ cells reside in the luminal compartment and exhibit activity consistent with luminal-biased progenitors. Our data do not establish a direct hierarchy between the two cell types.

**AXL Is Required for Regeneration of Mouse Mammary Glands upon Transplantation**

To explore the role of AXL in mouse mammary epithelia, we examined the mammary glands from a mouse strain that carries a targeted LacZ gene knock-in mutation that disrupts AXL protein expression (B6.129P2-AxklacZ/LacZ; Figures S2A and S2B). Analysis of the mammary glands of nulliparous adult expressing Axl+/+ (wild type) mice and the functional knockout Axl+/-/LacZ mice showed no statistically significant difference in estrogen receptor alpha (ESR1), progesterone receptor, or proliferation marker Ki67 (Figures 4A, S3B, and S3D). These results indicated that differentiated lineages present in the luminal compartment of Axl+/LacZ/LacZ glands were similar to wild type (Figures S3B and S3D). Morphometric analysis of H&E stained FFPE tissue sections revealed a significant increase in the average caliber (cross-sectional area) of ducts from Figure 1. Continued

(N) Dual RNA ISH on paraffin-embedded section of normal human breast specimen reveal the spatial distribution and juxtaposition of AXL (AP-based Fast Red) and GAS6 (HRP-based Green) mRNA transcripts within the normal human mammary epithelium. Counterstain by hematoxylin (blue). The ducts shown in (A–D), (I), and (M–K), and (N), and the lobular acini shown in (E–H) are obtained from tumor biopsies from different patients. iScience, November 20, 2020
Figure 2. Phenotypic Characterization of AXL-Expressing Cells in Human Breast Epithelium

Analysis of AXL expression by flow cytometry of primary human breast epithelial cells (HMECs) isolated from patient reduction mammoplasty tissue samples. (A) Total surface AXL staining of human breast epithelial cells isolated from epithelial-enriched preparations of reduction mammoplasty samples (range: 1–6% n = 5 different patient biopsies; 500,000 events collected for each flow cytometry experiment displayed).

(B–E) (B) EPCAM/CD49f staining pattern of AXL-expressing cells (blue; gate shown in (E) within total human breast epithelial cell population (red topography map) (C) CD227/CD10 staining pattern and gating of LEP (green box) and MEP (red box) populations in epithelial-enriched preparations from reduction mammoplasty samples. (D) EPCAM/CD49f staining pattern and resolution of epithelial hierarchy cell types in enriched human breast epithelial and residual stromal cells isolated from reduction mammoplasty samples show enrichment of AXL-expressing cells in the stem/progenitor subpopulation (E) Analysis of HMEC EPCAM/CD49f/CD227/CD10/AXL flow cytometry data (from Figures 1C and 1D; 500,000 events; two different patients) using spanning-tree progression analysis of density-normalized events (SPADE), a computational approach to determine cell hierarchies from multiparametric data (Qiu et al., 2011). The SPADE-generated HMEC hierarchical tree comprises a continuum of distinct cell subpopulations depicted as circles with radii corresponding to cell number. The predicted HMEC hierarchy shows common origin at the apex with two radiations, MEP biased and LEP biased populations, respectively. Relative expression of a surface marker on cells within the hierarchy is shown on a blue (low expression) to red (high expression) scale. LEP-biased cells that express CD227, MEP-biased cells that express CD49f, and AXL-expressing cells are shown superimposed onto the HMEC hierarchy. Differentiated LEP and MEP cell populations occupy the left and right lineage radiations respectively, while AXL is expressed primarily in the putative bipotent epithelial/stem/progenitor subpopulations found at the apex of the hierarchical tree.

(F) High-dimensional mass cytometry-based analysis of primary human mammary epithelia reveals a progenitor population expressing AXL, KIT K14 and K19. Non-linear dimensionality reduction, t-distributed stochastic neighbor embedding (tSNE) (Amir El et al., 2013) created a projection of 29 marker expression in 2D. Each point depicts a single cell of dissociated uncultured breast epithelium from women <30 years old (merged and subsampled at 50,000 cells, n = 7) (Pelissier Vatter et al., 2018). The raw data have been transformed with arcsinh with the cofactor of 5. Intra-lineage subpopulations were identified as distinct clusters of cells with shared phenotypes using PhenoGraph software (Levine et al., 2015). Unsupervised clustering identified four distinct phenotypes of LEP (LEP1–4), seven types of MEP (MEP 1–7), a progenitor subpopulation and a low-expressing cell phenotype. The LEP and MEP clusters were merged and the tSNE projection of the PhenoGraph clusters is represented.

(G) Heatmaps of Z-score of KIT, AXL, K14 and K19 expression in PhenoGraph clusters of uncultured breast epithelia from women <30 years old (merged, n = 7).

(H) tSNE projection (generated as described in detail for (F)), showing the relative expression of AXL, KIT, K19, and K14, respectively. The relative expression of these markers is shown on a violet-blue (low expression) to red (high expression) rainbow scale representing ion-counts from 0-27,500/cell.
Figure 3. AXL Is Required for Self-Renewal and Generation of Differentiated Acini from Human Breast Epithelial Cells Ex vivo

(A) AXL is co-expressed with KIT on breast epithelial cells. Immunofluorescence of normal breast epithelial ducts in normal human breast biopsy FFPE sections (n = 6) show AXL (red), KIT (green), and AXL+/KIT+ double-positive cells (yellow). Nuclear counterstain by DAPI (blue). Scalebar: 30 μm.

(B) AXL expression defines a subpopulation of KIT-expressing breast epithelial progenitors. Flow cytometry-based quantification of KIT and AXL surface expression levels in human breast epithelial cells isolated from reduction mammoplasty samples (>100,000 sorted events; n = 3 patient samples). Quadrant gates and percentages of total events/gate are shown.

(C) AXL defines a distinct breast epithelial KIT-expressing progenitor subpopulation. Unsupervised hierarchical clustering based on whole-genome gene expression analysis (Illumina Bead Array) of FACS-isolated KIT+/AXL+, KIT+/AXL+, CD227+ luminal (LEP), and CD10+ myoepithelial (MEP) subpopulations of HMEC cells (independent FACS analysis of HMEC strains 240L and 122L at passage 4). Weighted average linkage (WPGMA); Distance metric: Pearson’s Correlation.

(D) KIT+ progenitors lacking AXL show gene expression evidence of luminal commitment. Principal component correspondence plot derived from whole-genome gene expression analysis of KIT+/AXL+, KIT+/AXL+, CD227+ luminal (LEP), and CD10+ myoepithelial (MEP) FACS-isolated cells. X axis component variance: 22.387%; Y axis component variance: 17.091%; Total variance retained: 39.478%.
Figure 3. Continued

(E) Serial passage analysis of the percentage of CD237+/CD10- luminal cells (LEP) in the HMEC strains 240L and 122L seeded at passage 4 in the presence of DMSO (control), KIT tyrosine kinase inhibitor (TKI) (imatinib, 1 µM) or AXL TKI (bemcentinib, 600 nM). %LEP was normalized to passage 0. Observed half-life values + - SD for each treatment condition (insert) were calculated using the formula: t = ln(2)/ln(N0/Nt) (p < 0.05). (F) AXL activity is required for breast progenitor cell self-renewal as analyzed by secondary mammosphere formation generated by flow cytometry-enriched KIT + human mammary epithelial cell progenitors treated with DMSO (control), KIT TKI (imatinib, 1 µM), or AXL TKI (bemcentinib, 600 nM). Y axis represents total number of secondary mammospheres formed per well (mean ± S.E.M., n = 72, **p = 0.0073, t test). (G) AXL is required for efficient formation of bilayered epithelial organoids in laminin-rich ECM assays. Image analysis of phase contrast images of mammary acini-like colonies formed from flow cytometry-enriched KIT + human mammary epithelial cell progenitor cells in 3D embedded laminin-rich ECM (intregel) treated with DMSO (control), KIT TKI (imatinib, 1 µM), and AXL TKI (bemcentinib, 600 nM), respectively. Number of acini formed per 50,000 sorted KIT+ progenitor cells (mean ± S.E.M., n = 72; p values derived from t test) is shown by size distribution (Acini area: mm²). (H) Size distribution of organoids formed under the conditions described i (G) (Area: mm²). Brightfield images and immunofluorescence analysis of representative organoids (inserts) display cytokeratin 14 (K14, red); cytokeratin 19 (K19, green); and nuclear counterstain: DAPI (blue). Scalebar 50 µM.

AxlLe/+/LacZ mice (n = 9) compared to ducts from wild type mice (n = 7, p < 0.0001) (Figure 4C). Image analysis of ductal diameters in carcin al al um stained mammary gland whole mounts from 16-week-old AxlLe/+/LacZ (n = 9) and Axl+/+ wild type mice (n = 8) confirmed differences in the overall size distributions (Figures 4B and S3A). β-galactosidase histochemistry revealed basal localization of LacZ-expressing cells in adult ducts (Figure 4D), sites where mouse mammary multipotent epithelial stem cells are thought to reside (Joshi et al., 2012). Mammary ducts from AxlLe/+/LacZ glands stained by immunofluorescence for detection of transgene product Beta-galactosidase in combination with MEP-marker K5 and LEP marker K8 confirmed the basal localization of the cells harboring the LacZ transgene (Figure 4E).

The observed phenotype of AxlLacZ/LacZ mammary glands is consistent with the viability of Axl-knockout animals; however, we speculated that forced postnatal regeneration of the mammary epithelia would exert sufficient stress to overcome compensatory mechanisms and reveal a more penetrant Axl-null phenotype. Thus, we evaluated the effect of AXL on mammary gland reconstitution upon transplantation of sorted cells in serial dilution. Lineage-negative (i.e. CD45, CD31, CD11b−) AXL-expressing cells were enriched from dissociated AxlLe/+/LacZ and AxlLe/+/LacZ adult mammary glands by FACS using the fluorogenic β-galactosidase substrate fluorescein Di-ß-D-galactopyranoside (FDG). An FDGhigh gate (highest 5%) was used to enrich for AXL-expressing cells, while AXL-negative cells were sorted using an FDGlow gate (lowest 65%) (Figure 4F). Dermal injections of cells sorted on the basis of FDG (from 10,000 to 100 cells) were implanted into cleared fat pads of recipient prepubescent nude mice. Eight weeks post-transplantation, the mammary glands were harvested, fixed, and whole mount stained with carmine alum to visualize epithelial outgrowths. Mammary repopulating units (MRUs) frequencies were estimated by serial passage and transgene product β-galactosidase substrate fluorescein Di-ß-D-galactopyranoside (FDG). An FDG-high gate (highest 5%) was used to enrich for AXL-expressing cells, while AXL-negative cells were sorted using an FDG-low gate (lowest 65%). %LEP was normalized to passage 0. Observed half-life values + /- SD for each treatment condition (insert) was calculated using the formula: t = ln(2)/ln(N0/Nt) (p < 0.05). (E) Serial passage analysis of the percentage of CD237+/CD10- luminal cells (LEP) in the HMEC strains 240L and 122L seeded at passage 4 in the presence of DMSO (control), KIT tyrosine kinase inhibitor (TKI) (imatinib, 1 µM) or AXL TKI (bemcentinib, 600 nM). %LEP was normalized to passage 0. Observed half-life values + - SD for each treatment condition (insert) were calculated using the formula: t = ln(2)/ln(N0/Nt) (p < 0.05). (F) AXL activity is required for breast progenitor cell self-renewal as analyzed by secondary mammosphere formation generated by flow cytometry-enriched KIT + human mammary epithelial cell progenitors treated with DMSO (control), KIT TKI (imatinib, 1 µM), or AXL TKI (bemcentinib, 600 nM). Y axis represents total number of secondary mammospheres formed per well (mean ± S.E.M., n = 72, **p = 0.0073, t test). (G) AXL is required for efficient formation of bilayered epithelial organoids in laminin-rich ECM assays. Image analysis of phase contrast images of mammary acini-like colonies formed from flow cytometry-enriched KIT + human mammary epithelial cell progenitor cells in 3D embedded laminin-rich ECM (intregel) treated with DMSO (control), KIT TKI (imatinib, 1 µM), and AXL TKI (bemcentinib, 600 nM), respectively. Number of acini formed per 50,000 sorted KIT+ progenitor cells (mean ± S.E.M., n = 72; p values derived from t test) is shown by size distribution (Acini area: mm²). (H) Size distribution of organoids formed under the conditions described i (G) (Area: mm²). Brightfield images and immunofluorescence analysis of representative organoids (inserts) display cytokeratin 14 (K14, red); cytokeratin 19 (K19, green); and nuclear counterstain: DAPI (blue). Scalebar 50 µM.

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A number of previous reports show that luminal-biased progenitor cells cultured in 3D IrECM form hollow acini, whereas isolated MRU-competent MaSC form dense, pleomorphic structures (Lim et al., 2009; Shackleton et al., 2006; Stenfl et al., 2006; Guo et al., 2012). FACS-isolated FDG-high mammary epithelial cells from Axl+/−/LacZ mice formed solid basal/stem-like colonies in IrECM characteristic of regenerative MaSC (Figures 4H and 4J upper row), whereas the FACS-isolated FDG-high mammary epithelial cells from AxlLe/+/LacZ mice formed significantly less colonies, and the colonies were predominantly well differentiated bilayered acini with basal K5 expressing and luminal K8 expressing compartments of cells (Figures 4H and 4J lower row).

Transcriptome of Sorted AXL + Mammary Cells Are Enriched in Genes Characteristic of Sorted MaSC Populations

Transcriptomes from FACS sorted FDG-high and FDG-low cells isolated from Axl+/−/LacZ adult mammary glands were then compared to previously published gene expression signatures from sorted mammary epithelial populations (Lim et al., 2009). Consistent with the functional analyses described above, the sorted FDG-high cell population isolated from Axl+/−/LacZ mammary glands displayed significant transcriptional similarity with...
### Table 1: Repopulating frequency of mammary cell subpopulations

| Genotype          | Mammary cell subpopulation | No. cells injected | No. positive outgrowths | Repopulating frequency (95% CI) |
|-------------------|-----------------------------|--------------------|-------------------------|---------------------------------|
| \(Axl^{+}\)/LacZ | \(FDG^{\text{high}}\)       | 1000               | 8/8                     | 8/8                             | 1 (1/86 – 1)                    |
|                   |                             | 100                | 8/8                     |                                 |                                |
|                   | \(FDG^{\text{low}}\)       | 10000              | 4/7                     | 3/8                             | 1/7569 ** (1/17406 – 1/3292)    |
|                   |                             | 1000               | 3/8                     |                                 |                                |
|                   |                             | 100                | 0/8                     |                                 |                                |
| \(Axl^{+}\)/LacZ/LacZ | \(FDG^{\text{high}}\)       | 10000              | 8/8                     | 1/2245 ** (1/5373 – 1/938)      |
|                   |                             | 1000               | 2/8                     |                                 |                                |
|                   |                             | 100                | 1/8                     |                                 |                                |
|                   | \(FDG^{\text{low}}\)       | 10000              | 6/8                     | 1/7388 *** (1/16209 – 1/3368)    |
|                   |                             | 1000               | 1/8                     |                                 |                                |
|                   |                             | 100                | 0/8                     |                                 |                                |

### Notes

- **Note:** Specific details about the methods and experimental conditions are not provided in the image. For a comprehensive understanding, please refer to the original research article.
AXL MaSC Regulation Is Associated with Breast Cancer

We next examined whether gene signatures of normal AXL+ MaSC were an underlying feature of mammary glands of nulliparous adult Axl−/− animals (Figure 5D). Quantification of ductal diameter based on whole mounts shown in Figure S3A. Scalebar: 250 μm.

Figure 4. AXL Is Required for Regeneration of Mouse Mammary Glands upon Transplantation

(A) Immunostaining of FFPE sections of mammary glands from adult heterozygous Axl+/LacZ and homozygous functional AXL knockout AxlLacZ/LacZ of the B6.129P2-Axltm1OgenJ mice revealed insignificant difference in estrogen alpha receptor (ESR1) expression. Quantification is shown in Figure S3A. Scalebar: 100 μm.

(B) Carmine alum stained whole mounts of mammary glands from adult Axl+/LacZ and AxlLacZ/LacZ mice. Quantification of ductal diameter based on whole mounts are shown in Figure S3A. Scalebar: 250 μm.

(C) Morphometric histological analysis of HE stained mammary epithelial ducts from 16-week-old Axl+/+/ (n = 7) and AxlLacZ/LacZ (n = 9) mice. Quantification of the epithelial ducts were evaluated from HE stained FFPE sections. Both inguinal glands were harvested and included in the analysis, and area of epithelial structures from 10 separate fields/gland were included in the analysis (Mann-Whitney p < 0.0001).

(D) AXL promoter drives β-galactosidase expression in the two AxlLacZ alleles of homozygous AxlLacZ/LacZ mice. Whole mount beta-galactosidase histochemistry reveal the transgene expression from Axl promoter activity of mammary epithelial ducts. Glands were FFPE embedded post-staining and sections counterstained by eosin. Scalebar: 50 μm.

(E) Immunofluorescent staining of β-galactosidase (white), K8 (red), and KS (green) reveal the localization of transgene expressing cells relative to the luminal (K8+) and basal (K5+) population in mammary epithelial ducts of homozygous AxlLacZ/LacZ mice. Image without DAPI (blue) nuclear stain is displayed the right to allow better visual inspection of the transgene expression (white) relative to K8 (red) and KS (green). Scalebar: 30 μm.

(F and G) (F) FACS sorted cells expressing high (5% upper gate) and low (65% lower gate) cells were used for subsequent in vitro assays and limiting dilution transfection assays (G) Representative whole mount image of cleared mammary fat pad repopulated by 100 FDG+/+/ cells from heterozygous Axl+/LacZ mammary gland (left panel). Repopulating mammary epithelial outgrowths were found in 8/8 animals in this group (filled black circles). Representative whole mount image of mammary gland of animal reconstituted with 100 FDG+ cells from homozygous Axl+/+/ mammary glands (right panel). Repopulating mammary epithelial outgrowths were observed in 1/8 animals in this group (filled black, and empty circles, respectively). Data from one of three representative experiments are shown. Scalebars: 0.5 mm.

(H) Quantification of solid (black shaded bars) and acinar (gray shaded bars) in vitro colonies per 1000 seeded cells. Solid organoids were significantly reduced in the cultures derived from Axl+/+/ mice (t test: p = 0.0023).

(I) Limiting dilution in vivo mammary transplantation assay of FDG high and FDG low cell populations. The number of mammary outgrowths per transplantation group were quantified 8 weeks post-implantation, and the stem cell frequency and confidence intervals were calculated by limiting dilution analysis using Extreme Limiting Dilution Analysis (ELDA) software (http://bioinf.wehi.edu.au/software/elda/) (Hu and Smyth, 2009).

(J) Images of mammary epithelial cells in 3D embedded organoids derived from Axl+/LacZ cells (upper row) and AxlLacZ/LacZ cells in 3D laminin-rich ECM (lrECM) culture (lower row) reveal the undifferentiated solid organoids derived from Axl+/LacZ and the differentiated acini formed by AxlLacZ/LacZ cells. As indicated, images represent (from left to right) brightfield images of live organoid cultures, HE stained organoids in formalin-fixed paraffin embedded (FFPE) sections, and immunofluorescence (IF) of FFPE sections with the markers K5 (green), K8 (red) and K5/K8 co-staining (yellow). The pictures to the right in the panel show beta-gal (green), K8 (red). Counterstain by DAPI (blue). Scalebars: 50 μm.

MaSC populations enriched using previously described markers (CD49fhi CD29hi CD24+ Sca1-subset) (Figures 5A and 5B). Indeed, CD61/CD49f flow cytometry analysis of dissociated mammary glands showed an increase in the luminal progenitor and LEP populations, and a slight reduction of the basal/stem populations in cells from Axl+/LacZ and the differentiated acini formed by Axl+/LacZ and the differentiated acini formed by Axl+/LacZ cells. As indicated, images represent (from left to right) brightfield images of live organoid cultures, HE stained organoids in formalin-fixed paraffin embedded (FFPE) sections, and immunofluorescence (IF) of FFPE sections with the markers K5 (green), K8 (red) and KS/K8 co-staining (yellow). The pictures to the right in the panel show beta-gal (green), K8 (red). Counterstain by DAPI (blue). Scalebars: 50 μm.

We hypothesized that AXL regulation of MaSC luminal potency in adult mammary gland regeneration could be governed by juxtacline interactions between GAS6-producing LEP and AXL-expressing MaSC. Warfarin has previously been shown to be a well-tolerated inhibitor of GAS6-AXL signaling (Kirane et al., 2015). Thus, to test this model, we treated adult mice (12 weeks old) for 5 months with warfarin administered ad libitum in the drinking water to inhibit post-translational glutamic acid gamma-carboxylation of GAS6. Gamma-carboxylation of GAS6 has been shown to be required for a pro-differentiation phenotype of mammary glands of nulliparous adult Axl−/− animals (Figure 5D). We next examined whether gene signatures of normal AXL+ MaSC were an underlying feature of mammary tumors. Gene set enrichment analysis was used to detect the most differentially expressed genes between sorted FDG+ and Axl+/LacZ/LeucZ mammary cells. A signature comprising 33 downregulated and 37 upregulated genes was identified (rank product test, Table S1). Genes in this set have not been previously associated with MaSC, and thus may represent an unprecedented facet of the
regenerative adult MaSC cell state (Figure 6A and Table S1). Notably, the AXL MaSC gene signature (AXL-stem) is not significantly enriched for core EMT genes. The generic EMT score captures the universal features of EMT based on expression of 315 EMT-related genes, and computed EMT scores range from $1_0$ (epithelial) to 1 (mesenchymal) (Tan et al., 2014). The FDG high sorted cell population of $Axl^+/LacZ$ mice has a mean generic EMT score of 0.24, while FDG high sorted cells of $Axl^LacZ/LacZ$ mice have a mean generic EMT score of 0.21. Thus, these comparable intermediate mesenchymal EMT scores support a heretofore unappreciated role for AXL signaling in epithelial plasticity more generally, rather than maintaining EMT per se.

We and others have previously showed that AXL protein and mRNA expression is frequently detectable in basal-like breast cancer subtypes (Gjerdrum et al., 2010; Blick et al., 2010). The AXL-stem signature correlated with the degree of basal-like gene expression in the Metabric breast cancer patient cohort (Curtis et al., 2012) (Figure 6B) and was significantly elevated in basal-like breast tumor subtypes (PAM50 and IC10 subtypes; Figures 6C and S5). Furthermore, the AXL-stem score was
significantly associated with breast cancer-specific patient survival in a univariate model and in a multivariate model correcting for grade and lymph node status (Figure 6D). Hence, the AXL-dependent gene expression signature from murine MaSC correlates with clinical outcome in human breast cancer.

**Figure 6. AXL MaSC Regulation Is Associated with Breast Cancer**

(A) Volcano plot of significance versus fold gene expression change between the FDGhigh populations from Axl+/LacZ and AxlLacZ/LacZ adult mouse mammary epithelial cells. The most highly differentially expressed genes (cutoff: 1.5-fold change) are shaded.

(B) The Metabric breast cancer patient cohort (n = 1,980 patients) (Curtis et al., 2012) was interrogated with the AXL MaSC gene signature (AXL-stem, Table S1) to access the distribution correlated with the degree of basal-like gene expression in breast cancer (\( \rho = 0.181, p = 5.5 \times 10^{-16} \)).

(C) The Metabric breast cancer patient cohort (n = 1,980) (Curtis et al., 2012) was interrogated with the AXL MaSC gene expression signature (AXL-stem, Table S1) to assess the influence of the AXL stem gene expression signature on clinical endpoints. AXL-stem score was significantly elevated in the core basal subtype of integrative cluster 10 (IC10) subtyped tumors from the Metabric breast cancer patient cohort (n = 1,980) (Curtis et al., 2012) (\( p = 5.2 \times 10^{-33} \), Kruskal-Wallis rank test).

(D) AXL-stem score was associated with breast cancer-specific outcome in a univariate model, stratified for hospital (\( p = 0.00496; \text{hazard ratio: } 1.026 \)) and in a multivariate model correcting for grade and lymph node status (\( p = 0.00899; \text{hazard ratio: } 1.024 \), Cox proportional hazards regression, Breitling et al., 2004).
Figure 7. AXL Is Required for Breast Cancer Cell Phenotypic Plasticity, and Genetic Ablation of AXL Significantly Reduces Mammary Tumor Incidence in the MMTV-Wnt1 Mice

(A) AXL expression is required for SNAI2/Slug-induced EMT. MCF10a cells transduced with control or SNAI2/Slug retroviral expression vectors (GFP), and AXL-targeting shRNA (shAXL) or luciferase targeting control shRNA vectors

(B) MCF10a/Slug shLuc and MCF10a/Slug shAXL 2D and 3D morphology

(C) MCF10a/Slug shLuc and MCF10a/Slug shAXL tumor incidence

(D) MCF10a/Slug shLuc and MCF10a/Slug shAXL 3D morphology

(E) Cells injected Tumor incidence

| Cells injected    | 10^5 | 10^4 | 10^3 |
|-------------------|------|------|------|
| HMLER/vector      | 6/10 | 4/10 | 1/10 |
| HMLER/shAXL2      | 3/10 | 1/10 | 0/10 |
| HMLER/Slug/shLuc  | 9/10 | 6/10 | 6/10 |
| HMLER/Slug/shAXL2 | 3/10 | 2/10 | 1/10 |

(F) Tumorphere formation

| Treatment          | Tumorsphere formation |
|--------------------|-----------------------|
| Vehicle            | 1.0                   |
| 3 μM Warfarin      | 1.0                   |
| 900 nM bemcentinib | 1.0                   |

(G) In vitro pretreatment Tumor incidence

| Pretreatment       | 10^4 | 10^3 |
|--------------------|------|------|
| 3 nM pacilitaxel   | 3/5  | 3/5  |
| 3 nM pacilitaxel 600nM bemcentinib | 0/5  | 0/5  |

(H) 4T1-Luc tumor growth (total photon count)

| Treatment          | 4T1-Luc tumor growth (total photon count) |
|--------------------|-----------------------------------------|
| Vehicle            | 72,100                                  |
| 50 μg/l Warfarin    | 23,624                                  |
| 100 μg/l Warfarin   | 16,273                                  |
| AXL TKI (bemcentinib) | 4,388                                   |

(I) MMTV-Wnt1 background Mammary tumor incidence

| MMTV-Wnt1 background | Mammary tumor incidence |
|----------------------|-------------------------|
| MMTV-Wnt1: Axl^{lox/lox} | 4/16                    |
| MMTV-Wnt1: Axl^{lsl/lsl}   | 7/8                     |
| MMTV-Wnt1: Axl^{+/+}          | 15/16                   |

(J) Survival curve of MMTV-Wnt1 mice

- MMTV-Wnt1: Axl^{lox/lox} (red)
- MMTV-Wnt1: Axl^{+/+} (green)
- MMTV-Wnt1: Axl^{lsl/lsl} (blue)

(K) Log (fold change)

- **P < 0.001
- *P < 0.01
- +P < 0.05
Figure 7. Continued

(shLuc) (RFP). Transduced cells were sorted based on GFP and RFP expression, and analyzed for AXL (140 kDa), SNAI2/Slug (30 kDa), and epithelial markers E-cadherin (135 kDa), β-catenin (92 kDa) and mesenchymal markers vimentin (55 kDa), and N-cadherin (100 kDa) by Western blotting. Loading control: β-actin (42 kDa).

(B) AXL-dependent loss of cell-surface glycoprotein CD44 shown by flow cytometric analysis of MCF10a/Slug/shAXL and MCF10a/Slug/shLuc cells.

(C) AXL expression is required for self-renewal activity. Quantification of mammosphere formation by MCF10a/Slug/shAXL and MCF10a/Slug/shLuc cells. Y axis represents total number of mammospheres formed per well (mean ± S.D., n = 5; *p < 0.05, t test).

(D) Phase contrast images of MCF10a/Slug/shAXL and MCF10a/Slug/shLuc cells grown as monolayer (2D, upper) and colony formation in 3D embedded laminin-rich ECM (IrECM) (matrigel, lower) reveal that Slug-mediated mesenchymal cell morphology and invasiveness are AXL-dependent.

(E) AXL is required for tumor initiation in vivo. Tumor incidence of HMLER/shLuc, HMLER/shAXL and HMLER/Slug/shAXL and HMLER/Slug/shLuc cells injected s.c. into recipient NOD-SCID mice at limiting dilutions (between 106-103 cells). HMLER/Slug/shAXL versus HMLER/Slug/shLuc, p = 0.0002, Fisher’s exact test.

(F) Inhibition of AXL signaling using warfarin (3 uM) or AXL tyrosine kinase inhibitor (TKI) bemcentinib (900 nM) blocks 4T1 tumorsphere formation. Tumorsphere formation (day 7) was scored as Total Area (pixels²)/20,000 cells using ImageJ Analysis. Data plotted relative to vehicle (DMSO) treated cells (mean ± S.D., n = 6; ***p < 0.0005, t test).

(G) Reduced tumor incidence of 4T1 cells pretreated in vitro with 3 nM paclitaxel in the presence of 600 nM bemcentinib prior to injection injected into syngeneic host mice at limiting dilution versus in vitro treatment with 3 nM paclitaxel alone (p = 0.0108, Fisher’s exact test).

(H) Inhibition of AXL kinase activity reduces mammary tumor formation. Bioluminescence (total photon counts) from tumors formed from orthotopically-implanted 4T1-luciferase (4T1Luc) cells at Day 7 treated with bemcentinib (50 and 100 mg/kg QD) (mean ± S.D., n = 6; *p < 0.05, **p < 0.005, one-way ANOVA).

(I) Spontaneous mammary tumor incidence in genetically modified animals carrying MMTV-Wnt1 and the AXL.LacZ knock-in allele. Comparison of tumor incidence between female AXL wild type MMTV-Wnt1:AXL+/+ and AXL wild type MMTV-Wnt1:AXL+/− and AXL-null MMTV-Wnt1:AXL−/− mice revealed that Wnt1-induced mammary tumor incidence (within 14 months) was significantly reduced in the AXL-null background (p = 0.0002, Fisher’s exact test, two-tailed).

(J) Kaplan-Meier survival analysis of MMTV-Wnt1 mice (Log rank (Mantel-Cox) test, Chi square: 14.98, p = 0.0001).

(K) Relative mRNA expression levels of a selection of significantly deregulated genes associated with EMT, CSC and WNT signaling. Data are reported as mean fold changes ± SEM after normalization to the levels of housekeeping genes (ACTB, B2M, GAPDH, GUSB, HSP90AB1) in the panel. *p < 0.05, **p < 0.01 (Unpaired Student’s t test).

AXL Signaling Is Required for Breast Cancer Cell Phenotypic Plasticity

Expression of the AXL-stem genes in breast cancer may reflect a co-option of MaSC-related regenerative activity that drives epithelial plasticity in breast cancer. SNAI2/Slug expression in immortalized human breast epithelial cell lines such as MCF10A engages an EMT-related stem cell program that results in expression of the AXL RTK (Gjerdrum et al., 2010; Vuoriluoto et al., 2011; Jokela et al., 2018). AXL knockdown were shown to reverse the SNAI2/Slug-dependent mesenchymal phenotype, restoring epithelial morphology and molecular marker expression, and blocking sphere-forming activity, without affecting SNAI2/Slug protein levels (Figures 7A–7D). Ectopic expression of AXL did not drive EMT in MCF10a (Figure S6). These results indicated that AXL signaling is required for SNAI2/Slug-dependent regulation of the EMT program. HMLER is a cell line generated by oncogenic transformation of HMECs that displays increased tumorigenicity upon overexpression of SNAI2/Slug (Mani et al., 2008). AXL knockdown significantly decreased tumor incidence independent of SNAI2/Slug overexpression in this model (Figure 7E).

The 4T1 murine mammary tumor model enables tumor-forming experiments in isogenic, immune-competent mice. Tumorsphere formation with 4T1 cells was nearly abolished by treatment with warfarin (3uM) or with bemcentinib (900 nM) (Figure 7F). Pretreatment of 4T1 with paclitaxel (3 nM) reduced tumor incidence following implantation, and pretreatment with paclitaxel and bemcentinib (600 nM) completely prevented tumor formation (Figure 7G). Furthermore, bemcentinib administered in vivo (both 50 and 100 mg/kg) decreased 4T1 tumor growth in syngeneic host mice (Figure 7H). These results support the notion that AXL is required to sustain epithelial plasticity traits that facilitate tumor formation in mammary glands.

AXL Is Required for Efficient MMTV-Wnt1 Mammary Tumorigenesis

Mammary tumors arise in the MMTV-Wnt1 model within an expanded MaSC pool and aberrant multipotent progenitor cells (Lim et al., 2009; Vaillant et al., 2008). Finally, in order to investigate the role of AXL in Wnt1-induced malignant transformation, we crossed the AxlLacZ knock-in and MMTV-Wnt1 mouse strains. As expected, female MMTV-Wnt1:AXL+/+ and MMTV-Wnt1:AXL−/− animals developed mammary tumors with
high penetrance (Figure 7I). Strikingly, the incidence of mammary tumor development was reduced in the functional Axl-null knockout MMTV-Wnt1:Axl^LacZ/LacZ animals (Figure 7I), reflected also by the significantly increased survival of these animals (Figure 7J). Thus, AXL expression supported efficient Wnt1-mediated tumorigenesis, and comparison of gene expression in mammary tumor cells isolated from MMTV-Wnt1 animals revealed significantly reduced expression of WNT, EMT, and cancer stem cell genes in rare tumors from the Axl^LacZ/LacZ background (Figures 7K and S8).

**DISCUSSION**

AXL is expressed in multiple cancer types and is associated with poor clinical outcome and resistance to a number of therapies, including immune checkpoint inhibitors (Davidsen et al., 2017; Hugo et al., 2016; Goyette et al., 2018). While the presence of AXL in pathological contexts is increasingly well documented, our understanding of the role of AXL in normal physiology is lacking, with the current study providing evidence of a conserved role for AXL in human and murine mammary gland outside of innate immune regulation.

Supported by analysis of healthy human breast tissue, genetic mouse models, and patient tumor gene expression, our results are consistent with the interpretation that AXL is expressed by epithelial cells that are in a stem cell state. Our data suggest that AXL is so far a singular example of a putative MaSC marker that is conserved in human and mouse mammary epithelia. Data support the role of AXL as a regulator of a cellular plasticity program that endows a rare proportion of HMECs with the ability to transition between multipotent and differentiated states. Our multiparametric gene and marker expression data sets as well as functional ex vivo analyses are consistent with the interpretation that AXL is required for conditional expansion of a multipotent MaSC population in the adult mammary gland of both humans and rodents. In particular, AXL was found to regulate a unique gene set, AXL-stem, in regenerative MaSC that was inclusive of ARTN, a GDNF-family/syndecan-3 ligand, a gene family associated with stem cell self-renewal (Merrell and Stanger, 2016), and linked to EMT and drug resistance traits in breast cancer (Kang et al., 2009; Ding et al., 2014). The AXL-stem signature identified in murine MaSC correlated with clinical outcome in the human Metabric breast cancer patient cohort. Significantly elevated in basal-like breast tumor subtypes (PAM50 and IC10 subtypes), the AXL-stem signature was correlated with poorer overall survival across all breast cancer subtypes. AXL may allow breast cancer cells the capacity to transition between distinct phenotypic states with different functional traits that support tumorigenesis (Visvader and Stingl, 2014). More work is necessary to determine if AXL signaling is a characteristic of long-lived multipotent MaSC or whether it is necessary for expansion and acquired multi-lineage differentiation potential during certain regenerative conditions (Rios et al., 2014; Van Keymeulen et al., 2011).

AXL was shown to be required for the efficient formation of bilayered acini in lrECM assays from FACS enriched KIT+ HMECs, and AXL inhibition prevented LEP loss in 2D HMEC culture and significantly decreased self-renewal capacity in secondary mammosphere assays. Although we cannot rule out the possibility that AXL⁺ MaSC are luminal-biased, both mammary transplantation in cleared murine fat pads and acinus formation assays with primary human cells showed a capacity for self-renewal and multi-lineage differentiation at least ex vivo. Multi-color immunofluorescence staining of mammary tissues showed that AXL⁺ cells expressed also the intermediate filament proteins K14 and K19 that were located in a basal or suprabasal position, in ducts. FACs staining showed that AXL⁺ cells were enriched among EpCAM⁺/CD49f⁺ cells. Our previous work showed that EpCAM⁺/CD49f⁺ HMECs that exhibited stem cell activity co-expressed K14 and K19 (Villadsen et al., 2007). EpCAM⁺/CD10⁺ high epithelial cells also were enriched for stem cell activities and were reported to express K14 and K18, but not K19 (Bachelard-Cascales et al., 2010). Taken together, we interpreted these findings such that cells in MaSC states have shared properties of MEP and luminal cells. From the human high-dimensional expression data, it is evident that the KIT⁺-AXL⁺ HMEC are more luminal lineage-biased, and their localization adjacent to the lumen, and the similarity of gene expression to CD227⁺/CD10⁻/KIT⁻ luminal cells supported this interpretation. However, more work and lineage tracing experiments are needed to determine definitively the lineage potential of the AXL + mammary epithelial population.

A conundrum raised by our results is that Axl-null animals are able to develop functional mammary glands, yet upon ex vivo transplantation into cleared fat pads of recipient mice the sorted Axl-null epithelial cells showed a significant reduction in the ability to reconstitute the mammary tree. Relatedly, the GAS6-null mouse also develops functional mammary glands (Mills et al., 2018). Knockout of the TAM family member Mer did show a developmental phenotype during post-lactational involution as it was shown necessary for
mediating epithelial efferocytosis of apoptotic cells, but AXL and Tyro3 were explicitly not required for that process (Sandahl et al., 2010). The importance of the mammary gland for mammals implies strong evolutionary pressure to favor gland development with much redundancy. Indeed, we are not aware of any reports of gene knockouts that reach adulthood and have no mammary gland. AXL is an imprinted gene, which rather suggests that fine-tuned temporal and spatial regulation of AXL expression during embryonic development is crucial. AXL + epithelial cells were enriched for their ability to form mammary glands in the cleared fat pad assay, which may be a better representation of regeneration or even wound repair. Historically, a number of gene knockout animals have shown no overt phenotype until stressors were applied, and this may be an example in which AXL was essential for allowing the mammary epithelial cells to survive in, or even to establish, the regenerative niche. Furthermore, as we have noted above, the role of AXL in cancer progression is being increasingly appreciated and the regenerative or wound healing microenvironment has also been used to characterize the tumor microenvironment. Even though cell transfer and tissue regeneration assays are considered a gold standard for stem cell biology, it is worth recognizing that the assays are prone to disrupting solid tissues in a way that might be orthogonal to development.

A reasonable interpretation of our data is that AXL allows access to gene expression programs that repress the luminal phenotype. Hence, removal of signaling through the AXL pathway is sufficient to allow cells to access the luminal differentiation program. Indeed lineage-priming occurs in the expanded stem cell population during pregnancy prior to commitment along the alveolar lineage (Pal et al., 2013). Interfering with AXL expression in double-positive epithelial cells may push the progenitor profile into a different compartment, or the cells lose self-renewal abilities in favor of terminal differentiation due to a transition from a symmetric to an asymmetric mode of stem cell division. Although the mode of stem cell division was not directly addressed in this study, the increased mammary tumor incidence in the MMTV-Wnt model as well as the impact of AXL inhibition in in vivo assays could support a model where AXL act as a mediator of symmetric self-renewing stem cell division, and the differentiation induced upon AXL inhibition is due to a switch to an asymmetric or symmetric differentiating stem cell division which is less advantageous for stem cell expansion and neoplastic transformation.

The striking reduction of Wnt1-driven mammary tumors in the Axl-null background was associated with a reduced expression of WNT-signaling genes (e.g. Wnt5a, Wnt10a, Wnt1, Fzd5, Mycn), genes associated with EMT (e.g. Twist1, Snai2, Vim), and K19 and Tgfb1/Tgfbr1 expression. It is tempting to speculate that AXL may support Wnt1-induced multipotent stem-like properties (Vaillant et al., 2008). Further characterization of the MMTV-Wnt1 tumor data is required; more mechanistic insights should be gathered to determine if Axl depletion leads to an impairment of Wnt signaling, or whether the effect is independent of Wnt.

We recently found that warfarin-use is associated with lower cancer risk in a large retrospective epidemiology study (Haaland et al., 2017). The vitamin K antagonist warfarin selectively inhibits gamma-carboxylation of Gla domain proteins, of which there are only 14 encoded in the human genome, most representing coagulation factors predominantly expressed in the liver. GAS6 is an outlier in this group with expression in several tissues and whose only known function is as the sole ligand for AXL (and a shared lower affinity ligand for MERTK/TYRO3). Of note, GAS6 is an estrogen inducible gene in mammary epithelial cells, and by bridging the kinase ectodomain and externalized phosphatidylserine (PS) displayed e.g on apoptotic cells, gamma-carboxylation of the GAS6 Gla domain is required for strong AXL activation, but not for MERTK/TYRO3 activation (Lew et al., 2014). Thus, “Gla-less” GAS6 is a specific AXL antagonist (Lew et al., 2014). Relevant to our warfarin experiments, and unaccounted for so far, is the possibility that other Gla domain proteins (peristin and osteocalcin) that are mainly expressed in the stroma were affected in a way that alters mammary epithelial activity.

Based on the data presented here we propose a working model where juxtracrine GAS6 and AXL interactions support microenvironmental PS-sensing and adult mammary gland stem cell expansion in the context of tissue damage and regeneration that deserves to be further explored in relevant models. Collectively, the results support further exploring aberrant AXL signaling as a therapeutic target in the treatment as well as chemoprevention of breast cancers.

Limitations of the Study
The study of human breast epithelial stem cells is hampered by a lack of experimental models and thus our current understanding of mammary gland biology is derived largely from rodent models. Although a
A number of flow cytometric markers are used to enrich for MaSC, a consensus set of markers that identifies multipotent MaSC in both human and mouse mammary remain elusive. This lack of common markers and mechanistic understanding governing human and mouse multipotent MaSC has brought into question how congruent MaSC biology is between these two species, casting doubt on the relevance of results determined in mouse studies, particularly for breast cancer. Furthermore, it remains to be elucidated whether regeneration of adult mammary tissue from endogenous stem cells exploits the same molecular pathways used to establish the mammary epithelium during development. As the ability of sorted cells to form mammary glands in the cleared fat pad assay may be a better representation of regeneration, and the role of GAS6-AXL signaling in murine mammary gland development have not been addressed. From emerging high-dimensional data, one can speculate that a continuum of cells along the differentiation axis is far more complex than previously anticipated and remains to be explored further.

Resource Availability

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, James Lorens (jim.lorens@uib.no).

Materials Availability
All unique/stable reagents generated in this study are available from the Lead Contact upon request pending completed Materials Transfer Agreement.

Data and Code Availability
The CyTOF data reported in this paper is accessible through Mendeley: https://doi.org/10.17632/j7mrbg3thh.1. The BeadChip array data of sorted cells from B6.129P2-Axltm1Dgen/J mice are accessible through GEO database, accession number: GSE156662: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE156662.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101649.

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AUTHOR CONTRIBUTIONS
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DECLARATION OF INTERESTS
J.B.L. is founder and shareholder of BerGenBio ASA. K.W.L. and G.G. and J.B.L. are former or current employees of BerGenBio ASA. J.P.T. is scientific founder and CSO of Biocheetah Pte Ltd Singapore and consultant/shareholder Biosygen Pte Ltd Limited and ACTgenomics Taipei Taiwan. S.C. and R.A.B. signed Sponsored Research Agreements with BerGenBio ASA related to a separate research project. The remaining authors declare no conflict of interest.

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AXL Is a Driver of Stemness in Normal Mammary Gland and Breast Cancer

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Table S1. A unique 70-gene AXL MaSC gene signature

| Mu Gene | Dir | Illumina ID | Hu Gene |
|---------|-----|-------------|---------|
| Axl     | 1   | ILMN_2651715 | AXL    |
| Supt3h  | -1  | ILMN_1213872 | SPUT3H |
| Elp2    | 1   | ILMN_1216985 | ELP2   |
| Fbxo3   | 1   | ILMN_1228298 | FBXO3  |
| Anp32a  | 1   | ILMN_1230271 | ANP32A |
| Selp    | 1   | ILMN_1236889 | SELP   |
| Tmem154 | 1   | ILMN_1237114 | TMEM154 |
| LOC100047935 | 1 | ILMN_1239211 | RPL5   |
| Bhlhb9  | 1   | ILMN_1251595 | BHLHB9 |
| Artn    | 1   | ILMN_1254114 | ARTN   |
| Mrps12  | 1   | ILMN_1254734 | MRPS12 |
| Aifm2   | 1   | ILMN_1259418 | AIFM2  |
| Tnc     | 1   | ILMN_2463180 | TNC    |
| Pbx2    | 1   | ILMN_2599858 | PBX2   |
| Rab27a  | 1   | ILMN_2614966 | RAB27A |
| Oas1g   | 1   | ILMN_2628822 | OAS1   |
| Ing4    | 1   | ILMN_2639665 | ING4   |
| Mrpl3   | 1   | ILMN_2643264 | MRPL3  |
| P2ry1   | 1   | ILMN_2684316 | P2RY1  |
| Arfgap1 | 1   | ILMN_2700126 | ARFGAP1|
| Fbxo32  | 1   | ILMN_2715893 | FBXO32 |
| Ss18l1  | 1   | ILMN_2726159 | SS18L1 |
| Cdc42ep3| 1   | ILMN_2733185 | CDC42EP3|
| Coasy   | 1   | ILMN_2741236 | COASY  |
| Chchd10 | 1   | ILMN_2749037 | CHCHD10|
| Asb2    | 1   | ILMN_2765759 | ASB2   |
| Pxdn    | 1   | ILMN_2828896 | PXDN   |
| Actg2   | 1   | ILMN_2839313 | ACTG2  |
| B230339M05Rik | 1 | ILMN_2894574 | RALGAPB|
| Atp2a3  | 1   | ILMN_2900462 | ATP2A3 |
| Acta2   | 1   | ILMN_2923445 | ACTA2  |
| Ubac2   | 1   | ILMN_2949605 | UBA2   |
| Ccr1l   | 1   | ILMN_2983624 | CCR1L  |
| Trub2   | 1   | ILMN_2983686 | TRUB2  |
| Tnrc18  | 1   | ILMN_3003152 | TNRC18 |
| Stk4    | 1   | ILMN_3004142 | STK4   |
| Dyrk1b  | 1   | ILMN_3053158 | DYRK1B |
| Fam173a | -1  | ILMN_1215218 | FAM173A|
| Apose   | -1  | ILMN_1216042 | APOE   |
| Taf5    | -1  | ILMN_1218205 | TAF5   |
| Pcdh10  | -1  | ILMN_1228833 | PCDH10 |
| 150001SO10Rik | -1 | ILMN_1249000 | C2orf40|
| Slc19a2 | -1  | ILMN_1250531 | SLC19A2|
| Mal2    | -1  | ILMN_1252628 | MAL2   |
| Cdk5r1  | -1  | ILMN_1259339 | CDK5R1 |
| Akir1c8 | -1  | ILMN_1260323 | AKR1C3 |
| Tnfrsf12a | -1  | ILMN_2424299 | TNFRSF12A|
| Tmem2   | -1  | ILMN_2430220 | TMEM2  |
| Ccar1   | -1  | ILMN_2516266 | CCA1   |
| Lmna    | -1  | ILMN_2597710 | LMANA  |
| Ell3    | -1  | ILMN_2627179 | ELL3   |
Figure S1. Controls for RNA in situ experiments

A

B
Figure S2. Description of the AXL-targeting mutation in B6.129P2-Axltm1Dgen/J mice

A

Exon:     1     2     3     4    5     6     7    8     9   10   11   12   13  14   15   16   17  18   19   20

NH2

Ig-Like Domains     FNIII Domains     Transmembrane Domain     Kinase Domain     COOH

5’  3’

Exon:  1  2  3  4  5  6  7  8  9  10  11  12  13  14  15  16  17  18  19  20

5’  3’

Targeting Vector

Endogenous Locus

B

Axl+/+  Axl+/LacZ  Axl+/+LacZ

AXL

Actin

C

Axl+/+  Axl+/LacZ  Axl+/+LacZ

AXL
Figure S3. Characterization of AXL null mammary glands

A

\[\text{Ax}l^{+/+}\quad \text{Ax}l^{\text{LacZ}/\text{LacZ}}\]

\[
\begin{array}{c}
\text{Ductal diameter (μm)} \\
0 \quad 20 \quad 40 \quad 60 \quad 80 \quad 100 \quad 120 \quad 140 \quad 160 \quad 180 \quad 200 \quad 220
\end{array}
\]

- \# ducts

B

\[
\begin{array}{c}
\text{Ax}l^{+/+}\quad \text{Ax}l^{\text{LacZ}/\text{LacZ}}
\end{array}
\]

- n.s

\[
\begin{array}{c}
\% \text{ ERα} \\
0 \quad 20 \quad 40 \quad 60 \quad 80 \quad 100
\end{array}
\]

C

\[
\begin{array}{c}
\text{Ax}l^{+/+}\quad \text{Ax}l^{\text{LacZ}/\text{LacZ}}
\end{array}
\]

- n.s

\[
\begin{array}{c}
\% \text{ PR} \\
0 \quad 10 \quad 20 \quad 30 \quad 40 \quad 50
\end{array}
\]

D

\[
\begin{array}{c}
\text{Ax}l^{+/+}\quad \text{Ax}l^{\text{LacZ}/\text{LacZ}}
\end{array}
\]

- n.s

\[
\begin{array}{c}
\% \text{ Ki67} \\
0 \quad 10 \quad 20 \quad 30 \quad 40
\end{array}
\]

- Ductal diameter (μm)

- n.s
Figure S4. Analysis of mammary gland whole mounts from pubescent warfarin-treated mice
Figure S5. The AXL-stem gene expression signature is elevated in basal-like tumors in the Metabric breast cancer patient cohort.
Figure S6. AXL overexpression is not sufficient to induce EMT in MCF10a cells
Figure S7. AXL is required for Slug-mediated epithelial plasticity
Figure S8. Deregulated signaling pathways of AXL null WNT induced tumors

A: EMT

B: CSC

C: WNT

-6 -4 -2 0 2 4 6

Ax1 Bmip1 Bmp7 Cald1 Camk2n1 Il1r1 Igfbp4 Jag1 Krt7 Krt19 Mif Ocln Plek2 Sna12 Sox10 Tgfb1 Tmeff1 Twist1 Vim

-6 -4 -2 0 2 4 6

Alcam Atm Atxn1 Axl Bmi1 Cd24a Chek1 Dach1 Hdac1 Id1 Jag1 Mam11 Mycn Plaur Prom1 Tgfbr1 Taz

-6 -4 -2 0 2 4 6

Bcl9 Btrc Ctbp1 Ctbp1 Dvl1 Fzd5 Pygo1 Rhou Tcf7l1 Wnt1 Wnt10a Wnt5a
Supplemental Table and Figure Legends

Table S1. A unique 70-gene AXL MaSC gene signature (related to Figure 5A-B, Figure 6A-C)
The top 70 differentially expressed genes (33 down-regulated and 37 up-regulated by rank product test) determined by Illumina MouseWG-6 v2.0 Expression BeadChip Array gene expression analysis of mRNA isolated from FACS-sorted FDG\textsuperscript{high} AXL\textsuperscript{+/LacZ} and FDG\textsuperscript{high} AXL\textsuperscript{LacZ/LacZ} cell populations. Mu=murine, Hu= human. Dir= direction (1=up, -1=down).

Figure S1. Controls for RNA in situ experiment (related to Figure 1 J-N)
(A) Appropriate controls for RNA in situ experiment for single RNA in situ approach with chromogen DAB (brown). Left image shows positive control probe targeting the housekeeping gene PPIB, and right image shows the negative control probe targeting the bacterial DapB gene. Counterstain hematoxylin. (B) Appropriate controls for the dual RNA in situ approach. Left image shows positive control probes targeting the housekeeping gene PPIB (C2: chromogens AP-based Fast Red), as well as POLR2A (C1: chromogen HRP-based green) for the dual approach. Right image show hybridization with negative control probes targeting the bacterial gene DapB, each detection channel (C1 and C2) has its own negative control probe, Counterstain hematoxylin. Scalebar 50 µm.

Figure S2. Description of the AXL-targeting mutation in B6.129P2-AXL\textsuperscript{tm1Dgen/J} mice (related to mice described in Figure 4. Gene signatures derived from these mice are further described in Table S1, Figure 5 and Figure 6. These mice has been crossed with MMTV-Wnt1 mice as shown in Figure 7I-J)
(A) Schematic illustration of the LacZ-Neo cassette insertion site in exon 11 of the murine AXL gene, disrupting AXL protein expression. A 5′ splice acceptor ensures that the LacZ open reading frame is spliced into the endogenous AXL mRNA under control of the murine AXL promoter. (B) Western blot control analysis of murine AXL expression in dissociated lung tissue from wildtype (AXL\textsuperscript{+/+}), heterozygous (AXL\textsuperscript{+/LacZ}), and homozygous (AXL\textsuperscript{LacZ/LacZ}) mice of the B6.129P2-AXL\textsuperscript{tm1Dgen/J} strain (The Jackson Laboratory, Bar Harbour, ME). AXL protein expression from LacZ-insertion allele is undetectable. (C) PCR product (http://jaxmice.jax.org/protocolsdb/) from different genotypes of B6.129P2-AXL\textsuperscript{tm1Dgen/J} mice (wildtype (AXL\textsuperscript{+/+}), heterozygous (AXL\textsuperscript{+/LacZ}), and homozygous (AXL\textsuperscript{LacZ/LacZ}). Larger sized band represents LacZ-insertion allele.

Figure S3. Characterization of AXL null mammary glands (related to Figure 4 A-C)
(A) Mammary gland whole-mounts from adult (16 week-old) AXL\textsuperscript{+/+} (wildtype) and AXL\textsuperscript{LacZ/LacZ} (AXL-null) mice were harvested and processed for characterization by carmine alum staining. Ductal diameters were quantified with MATLAB using image segmentation analysis of the carmine-alum stained glands. The Kolmogorov–Smirnov test rejected the hypothesis that ductal diameters of wildtype AXL\textsuperscript{+/+} and AXL\textsuperscript{LacZ/LacZ} are derived from the same distribution (p= 9.23x10-7). (B-D) Histopathologic quantification of (B) ER\textalpha, (p= 0.35), (C) PR (p= 0.96) and (D) Ki67, (p= 0.24) immunohistochemistry of mammary duct FFPE tissue sections of adult AXL\textsuperscript{+/+} (wildtype) AXL\textsuperscript{LacZ/LacZ} mammary glands. n.s= non-significant by Mann-Whitney, non-parametric test.

Figure S4. Analysis of mammary gland whole mounts from pubescent warfarin-treated mice (related to Figure 5E)
(A) β-galactosidase activity is shown in terminal end buds (TEB) of mammary gland whole-mounts of 6-week old prepubescent AXL\textsuperscript{+/LacZ} mice stained by β-galactosidase histochemistry and counterstain by carmine alum. β-galactosidase activity was prominent in cells located to the cap region of TEBs. Scalebar: 100µm. (B) Schematic overview of warfarin treatment regimen. Peroral warfarin administration were initiated at in 3 week-old mice post-weaning. Representative composite images of carmine alum stained mammary gland whole-mounts from mice treated for 5 weeks with either pure or warfarin (1 mg/L) containing drinking water. (C) Quantification of terminal end buds (TEB) in carmine alum stained mammary gland whole-mounts harvested from 8 week-old control and warfarin treated animals (t-test, p=0.0004). Duration of treatment: 5 weeks.
Figure S5. The AXL-stem gene expression signature is elevated in basal-like tumors in the Metabric breast cancer patient cohort (related to Figure 6C)
The Metabric breast cancer patient cohort (n=1,980) (Curtis et al., 2012) was interrogated with the AXL MaSC gene expression signature (AXL-stem, Table S1) to assess the influence of the novel AXL stem gene expression signature on clinical endpoints. The AXL-stem score correlated with tumor subtype of PAM50 subtyped tumors is shown; the AXL score is significantly elevated in the basal-like tumors (P = 7.5x10^{-46}, Kruskal-Wallis rank test).

Figure S6. AXL overexpression is not sufficient to induce EMT in MCF10a cells (related to Figure 7 A-D)
MCF10a cells transduced with control vector or retroviral expression vector encoding AXL, Slug or Ha-Ras(G12V) as well as GFP reporter-gene. Post transduction, cells were FACS sorted based on their GFP expression, and protein expression of Vimentin were measured by Western blotting (upper). Cell-surface expression of AXL were quantified by flow cytometry (middle) (>100,000 events) and displayed as geometric mean. Phase contrast images displaying cell morphologies of control and AXL-, Slug- and Ha-Ras(G12V)-expressing MCF10a cells are shown below.

Figure S7. AXL is required for Slug-mediated epithelial plasticity (related to Figure 7 A-F)
(A) AXL is induced by hypoxia. Western blot analysis of CD44, N-cadherin (CDH2), and AXL protein expression in MCF10a/control and MCF10a/Slug cells grown under normoxic and hypoxic (1% O2) conditions. (B) AXL is induced by TGFb. Western blot analysis of AXL, CD44, epithelial (E-cadherin, β-catenin) and mesenchymal (vimentin, N-cadherin) marker expression. (C) Flow cytometric analysis of CD44 and AXL surface levels; and (D) Phase contrast images of MCF10a cell morphology, after culture with TGFb (10 ng/ml) or vehicle for 7 days. (E) AXL is required for Slug-mediated mesenchymal and stem cell traits in HMLER cells. HMLER transduced with control or Slug retroviral expression vectors (with GFP reporter gene), as well as AXL-targeting shRNA (shAXL2) or control luciferase shRNA (shLuc) vectors (with RFP reporter gene), were sorted for GFP and RFP, and analyzed for by western blot for expression of AXL, Slug (SNAI2), epithelial (E-cadherin, β-catenin) and mesenchymal (vimentin, N-cadherin). β-actin was used as a loading control. (F) AXL is required for EMT-induced stellate colony formation. Phase contrast images of HMLER/shLuc, HMLER/shAXL2 and HMLER/Slug/shAXL2 and HMLER/Slug/shLuc cell colonies in 3D embedded Ir/ECM (matrigel). (G) AXL is necessary for EMT-induced mammosphere formation. Quantification of mammosphere formed by HMLER/shLuc, HMLER/shAXL2 and HMLER/Slug/shAXL2 and HMLER/Slug/shLuc cells. Y-axis represents total number of mammospheres formed per well (mean ±S.D., n=6; *** p<0.0001, t-test). (H) AXL signaling is required for EMT-induced drug resistance. Multicolor flow cytometry analysis of MCF10a cells transduced with control or Slug retroviral vector following 24 hour treatment with pacilitaxel (30 µg/ml). The percentage of non-apoptotic cells represents the % viable cells post-treatment (of >100,000 total events) within the AnnexinV negative, Sytox Blue negative gate for each cell line.

Figure S8. Deregulated signaling pathways of AXL null WNT induced tumors (related to Figure 7 I-K)
Pathway focused RT2 Profiler TM PCR arrays demonstrate alterations in (A) EMT signaling pathway, (B) CSC pathway and (C) WNT pathway analysed in 6 Axl^{+/+} wild-type and 4 AXL^{LacZ/LacZ} (Axl-null) Wnt1- induced tumors. The gene expression levels of the Axl-null (n=4) samples were quantified relative to the expression levels for WT samples (n=6) after normalization to the levels of the suitable housekeeping genes (ACTB, B2M, GAPDH, GUSB, HSP90AB1) in the panel. Data in heatmaps are reported as mean fold changes.
Transparent Methods

Reagents
Anti-hAXL MAB10C9 (Lorens laboratory), MAB154, AF154 (R&D Systems), Anti-mAXL (sc-1097, Santa Cruz), mouse anti-human Slug (L40Cb, Cell Signaling), rabbit anti-human E-cadherin (24E10, Cell Signaling), rabbit anti-human N-cadherin (ab18203, Abcam), α-actin (A5060, Sigma), mouse anti-human β-catenin (L54E2, Cell Signaling), rabbit anti-human β-catenin (Cell Signaling), mouse rat anti-human Vimentin (MAB2105, R&D Systems), anti-CD227-FITC (Becton Dickinson, clone HMPV), anti-CD10-PE (BioLegend, clone Hi10a), anti-CD117- Alexa647 (BioLegend, clone 104D2), EPCAM-BV421 (BioLegend, clone 9C4), anti-CD49f-PE (Chemicon, clone CBL-458P), anti-K14 (Covance, polyclonal rabbit), anti-K19 (Developmental Studies Hybridoma Bank, clones Troma-II and Troma-III), anti-CD44 (Cell Signaling, 3570), anti-CD31-PE (17-0311), anti-CD45-PE (17-0451), and anti-CD11b (11-0112; ebioscience). Imatinib (LC laboratories l-5508) and bencentinib (also known as BGB324 and R428, BerGenBio AS) (Holland et al., 2010) were prepared in DMSO.

Immunohistochemistry (IHC-P, IHC-F)
IHC-P, fluorescent detection: Paraffin-embedded normal human breast tissue sections (generously provided by Dr. A. Borowsky) were prepared for immunofluorescence and stained as previously described (Garbe et al., 2012) using anti-K14 (Covance, polyclonal rabbit), anti-K19 (Developmental Studies Hybridoma Bank, clone Troma-III), anti-CD117 (BioLegend, clone 104D2), anti-hAXL MAB10C9 (BerGenBio ASA) overnight at 4oC, visualized with fluorescent secondary antibodies (Invitrogen) and 4,6-diamidino-2-phenylindole (DAPI) nuclear stain at room temperature for 1 hour, and imaged using a 710LSM microscope (Carl Zeiss).

IHC-P, chromogenic detection: Paraffin-embedded normal human breast tissue sections were prepared for staining as described (Garbe et al., 2012). Anti-Axl antibody were incubated o/n at 1:6000 dilution (clone EPR19880, Abcam). Peroxidase stain was performed by Ultravision ONE Detection System (Thermo Scientific). Sections were counterstained with hematoxylin and mounted with Entellan (Merck) prior to imaging.

IHC-F: Mammary tissue specimens were embedded in OCT and snap frozen in liquid nitrogen. Cryosections (6µm thickness) were stained with anti-Axl antibody (clone EPR19880, 1:100, Abcam); anti-K14 (clone LL002, 1:100, Monosan); anti-K19 (clone A53-B/A2, 1:100, BioLegend). Alexa Fluor conjugated secondary antibodies (1:500, Invitrogen) and 4,6-diamidino-2-phenylindole (DAPI) nuclear stain.

RNA in situ hybridization
In situ detection of AXL and GAS6 mRNA in human mammary FFPE tissue were performed using the RNA Scope technology. Probes and reagents were provided by Advanced Cell Diagnostics (ACD, Hayward, CA). Briefly, human archival mammary gland tissue sections of 5 um thickness were deparaffinized in xylene, followed by dehydration in ethanol. Tissue sections were then incubated in citrate buffer (10 nM/ L, pH 6) maintained at boiling temperature (100°C to 103°C) using a hot plate for 15 minutes, rinsed in deionized water, and immediately treated with 10 µg/ mL protease (Sigma-Aldrich, St. Louis, MO) at 40°C for 30 minutes in a HybEZ hybridization oven (Advanced Cell Diagnostics, Hayward, CA). Hybridization with target probes, preamplifier, amplifier, label probe and chromogenic detection were performed according to ACD’s recommendations. Sections were counterstained with hematoxylin and mounted with EcoMount prior to imaging. Assays using archival FFPE specimens were performed in parallel with positive and negative control probes, to ensure interpretable results (Supplementary Figure 4). Histo score analysis were performed to evaluate the heterogeneity in marker expression. Cells were scored and grouped in 5 bins based on the number of dots/ cell (Bin 0= 0 dots/cell, Bin 1= 1-3 dots/ cell, Bin 2= 4-9 dots/ cell, Bin 3= 10-15 dots/cell (<10% dots in clusters), Bin 4= >15 dots/cell (>10% dots in clusters). Each sample was manually scored, and the percentage of cells in each bin recorded. Histo score was calculated by totaling the percentage of cells in each bin according to the given formula. Histo score= 0*(% of cells in bin 0) +1*(% of cells in bin 1) +2*(%of cells in bin 2) +3*(% of cells in bin 3) +4*(% of cells in bin 4). Histo score is provided on the range 0-400.
Human mammary epithelial cells (HMEC) isolation and culture

Pre-stasis human mammary epithelial cells (HMEC) were derived from reduction mammoplasties (n=60) were established and maintained as described (Labarge et al., 2013) in low-stress M87A medium with oxytocin and cholera toxin (Garbe et al., 2012). HMEC of 4th passage or lower were used.

Flow cytometry and FACS sorting

Flow cytometry analysis of MCF10a cells was conducted as described (Gjerdrum et al., 2010). For flow analysis of isolated human breast epithelial organoids and pre-stasis HMEC, cells were recovered following trypsin treatment and resuspended in their media. For enrichment or identification of luminal epithelial and myoepithelial lineages, anti-CD227-FITC (Becton Dickinson, clone HMPV, 1:50) or anti-CD10-PE, -PE-Cy5 or -APC (BioLegend, clone HI10a, 1:50), were used, respectively. Other analysis was carried out with EPCAM-BV421 (BioLegend, clone 9C4), anti-CD49f-PE (Chemicon, clone CBL-458P, 1:50), anti-CD117-Alexa647 (BioLegend, clone 104D2, 1:200) and anti-hAXL MAB10C9 conjugated to Alexa647 (1:200). MABs were added to the media at 1:50 for 25 minutes on ice, washed in PBS, and sorted or analyzed. Kit-expressing HMEC progenitors were isolated by staining with anti-CD117-PE (BioLegend, clone 104D2, 1:200) and were added to cells in media for 25 min on ice, washed in PBS and sorted on a FACS Vantage SE DIVA (Becton Dickinson). β-galactosidase flow cytometry was done using FluoReporter® lacZ Flow Cytometry Kit (Molecular Probes F-1930). For transplantation assays and organoid assays of murine cells the following sorting strategy were used: freshly dissociated and labeled cells were discriminated from the debris and gated in the forward scatter (FSC-A) and side scatter (SSC-A) plots. Subsequently, the forward scatter Width (FSC-W) versus FSC-A plots were used for doublet cell exclusion. Propidium iodide (PI) staining was used for dead cell discrimination and the APC channel was used as a dump channel for the APC conjugated lineage markers used. Thus, only single, viable, PI-negative, and APC-negative cells were included in the final sort of FDG-high and FDG-low cells used for subsequent experiments. A post-sort analysis was performed to verify purity of the sorted cells and sort numbers were obtained from each sort, however, the cell counts used for subsequent experiments were determined by visualizing and enumerating live healthy single cells. T Data analysis was carried out on 500 000 events using the FlowJo software (Tree Star, Inc., Ashland, OR, USA). SPADE analysis was performed with Cytoscape (www.cytoscape.org) and R (Foundation for Statistical Computing).

High-dimensional Mass cytometry

Mass cytometry analysis of dissociated primary human breast epithelia was described in great detail in (Pelissier Vatter et al., 2018). Primary HMEC strains were generated and maintained as described (Labarge et al., 2013). All tissues were obtained with proper oversight from the Lawrence Berkeley National Laboratory institutional review board. Breast tissue from reduction mammaplasty was manually dissected to enrich for gland-containing material. Stromal tissue was separated from epithelial fragments using a brief treatment with collagenase. The uncultured breast epithelia samples were dissociated as single cells with trypsin. Cells were incubated with cisplatin (WR International, Cat# 89150- 634, 25 mM) for 1 min to assess cell viability (Fienberg et al., 2012), fixed in 1.6% PFA for 10 min at RT, and washed once with Cell Staining Media (CSM, PBS with 0.5% BSA and 0.02% NaN3 with 0.03% saponin). The cells were then resuspended in PBS, and DMSO stocks of the barcoding reagent were added as described (Bodenmiller et al., 2012, Zivanovic et al., 2014). The cells were incubated at RT for 30 min, washed three times with CSM, and then pooled into a single FACS tube for staining with metal-labeled antibodies for 1 hr at RT. After antibody staining, the cells were washed twice with CSM and once with PBS, and then incubated for 20 min at RT or overnight at 4°C with an iridium-containing intercalator (DVS Sciences) in PBS with 1.6% PFA. The cells were then washed three times with CSM and once with PBS, diluted with water to ~106 cells/mL, and filtered through a 40-mm membrane just before analysis by mass cytometry. The scale used before analysis is the arcsinh with the cofactor of 5 (x_transf = asinh(x/5)). After gating out viable and iridium-labeled events, the data were analyzed by applying TSNE. This non-linear dimensionally reduction technique is implemented via Barnes-Hut approximations in the MATLAB toolbox cyt (Amir el et al., 2013). We used the default parameters (initial dimensions, 110; perplexity, 30; and theta, 0.5). The
unsupervised PhenoGraph algorithm in cyt has been used to group cells that are
phenotypically similar and cluster these subpopulations using modularity optimization (Levine
et al., 2015). tSNE and PhenoGraph were performed only on surface markers. A number of
neighbors of 800 was selected. The heatmap was obtained with MATLAB. The accession
number for the CyTOF data reported in this paper is Mendeley Data:
https://doi.org/10.17632/j7mrbgt3hh.1.

3D-embedded laminin-rich ECM assay
MCF10a or KIT-enriched HMEC were resuspended in media (50000 cells/ µL) and 200 µL of
matrigel (BD Sciences 356234) were added to the cells and transferred to a 24-well plate pre-
coated with 50 µL of Matrigel, then cultured for 10-12 days prior to microscopy analysis. For
immunofluorescence analysis of HMEC colonies, Matrigel smears were fixed in methanol/acetone 1:1 at -20°C for 20 min, incubated with blocking buffer overnight at 4°C, incubated with anti-K14 and anti-K19 overnight at 4°C, extensively washed with PBS, then
incubated with fluorescent secondary antibodies overnight at 4°C, and washed overnight at
4°C before mounting coverslips with Fluormount G. Insert Keratin 5 (murine).

Mammosphere formation assay
Mammosphere culture of MCF10a was performed as previously described(Dontu et al.,
2003). Single cells were plated in ultra-low attachment plates (Corning, Acton, MA, USA)
20,000 viable cells/ ml. For mammosphere assays of HMEC, flow cytometry sorted KIT+
enriched HMEC (p4) were resuspended in mammosphere media (MammoCult human
medium kit (StemCell 05620, enriched with heparin and hydrocortisone) at 25,000 cells/ mL in
polyHema (0.133 mL at 12 mg/ ml in 95% EtOH) treated 24-well plates (in triplicate) and
cultured for 10 days. Large (≥70 µm) and hollow mammospheres were identified by
microscopy of each well. Total mammospheres per well were quantified using ImageJ.
Secondary mammospheres were prepared by trypsinizing and resuspending for cell from first
passage mammospheres. For the immunofluorescence, mammospheres were fixed in 4%
paraformaldehyde for 5 min, blocked with PBS, 5% normal goat serum, 0.1% Triton
X-100, and incubated with anti-K14 (1:1000, Covance, polyclonal) and anti-K19 (1:10, Developmental Studies Hybridoma Bank, clone Troma-III) overnight at 4°C, then visualized
with fluorescent secondary antibodies (Invitrogen) incubated with sections for 2 hours at room
temperature.

Gene expression analysis
Global gene expression analysis of HMEC lineage was performed on FACS sorted (FACS
Vantage SE DIVA, Becton Dickinson) pre-stasis HMEC strains 240L and 122L cells (4th
passage). Total RNA from FACS-enriched primary culture cells was isolated with TRizol
(Invitrogen) and RNeasy Mini column (Qiagen) and evaluated using Bioanalyzer (Agilent
Technologies). Gene expression levels were measured using the Illumina HumanHT-12 v4
Expression BeadChip whole-genome expression array. The Illumina Bead Array data were
quality controlled in Genome Studio and both probe level and gene level data were imported
into J-Express Pro (http://jexpress.bioinfo.no) for analysis. After quantile normalization both
datasets were log2 transformed. Correspondence Analysis (Fellenberg et al., 2001) was
performed on the datasets, together with Hierarchical Clustering of the samples using a
Pearson correlation measure on a per gene mean centered version of the data.

Gene expression analysis of FDG FACS-sorted 16 week old nulliparous AXL<sup>LacZ</sup> and
AXL<sup>LacZ,LacZ</sup> (B6.129P2-AXL<sup>em10gen</sup> strain, Jackson Labs) was conducted using the Illumina
MouseWG-6 v2.0 Expression BeadChip (BD-201-0202, BD-201-0602). Log2 quantile
normalized of the gene expression data were used for unsupervised hierarchical clustering,
gen gene signature analysis and differentially expressed genes. Hierarchical clustering was
performed using Pearson’s correlation as distance measurement. Gene signatures among
cell population were determined by comparing the gene expression levels and available
molecular signatures of the mammary cell subpopulations (Lim et al., 2009) using GSEA
software package (Subramanian et al., 2005). Differentially expressed genes between
AXL<sup>LacZ</sup> FDC<sup>high</sup> and AXL<sup>LacZ,LacZ</sup> FDC<sup>high</sup> cell groups were identified using Rank Product
method. The genes with a p-value <0.01 and fold change >= 1.5 were considered as
differentially expressed genes. All analyses were performed using R version 3.2.2. To assess
the influence of AXL and its downstream targets on survival of breast cancer patients, we
derived a score capturing the expression of these genes. The score is the sum of the top 70 genes from the rank product test, of which 33 genes were downregulated and 37 upregulated, adjusted for expected directionality. For genes represented by multiple probes, mean signal intensity was used. The influence on breast cancer-specific survival and putative difference between molecular subtypes was investigated in the Metabric cohort, composed of 1980 breast cancer patients enrolled at five different hospitals in the UK and Canada (Curtis et al., 2012). Gene expression was assessed using the Illumina HT-12 v3 microarray and normalized data was downloaded from the European Genome-phenome Archive (EGA) data portal. Missing values were imputed using the impute.knn function as implemented in the R library ‘impute’ with default settings (R package version 1.46.0.). The data was batch adjusted for hospital effect using the pamr.batchadjust function in the ‘pamr’ library with default settings. Association between the score and molecular subtypes was tested using Kruskal-Wallis rank test, and correlations were estimated with Spearman’s rank correlation. Survival analyses were performed using Cox proportional hazards regression model as implemented in the R library ‘rms’. The generic EMT 315 gene expression signature were used to compute the EMT score of the FDG high AxI<sup>LacZ</sup> and AxI<sup>LacZ</sup>/LacZ populations as previously described (Tan et al., 2014).

**Retroviral vectors**
MCF10a (American Type Culture Collection, Rockville, MD) were cultured as described (Gjerdrum et al., 2010). The CRU5-IRES-hSlug retroviral vector prepared as described (Gjerdrum et al., 2010). Retroviral production and infections were conducted using Phoenix A retroviral packaging cells as described (Swift et al., 2001).

**Immunoblotting**
MCF10A cells were lysed using NP40 Cell Lysis Buffer (40 mM HepesNAOH, 75 mM NaCl, 2 mM EDTA, 1% NP40, phosphatase inhibitor cocktail tablet, protease inhibitor cocktail tablet (Roche)). Running of SDS/PAGE gel and immunoblotting were carried out according to standard procedures.

**Animal studies**
Animal experiments were approved by the Institutional Animal Care Research Authority and in accordance with The European Convention for the Protection of Vertebrates Used for Scientific Purposes. Animals were housed in a germ-free environment in filter top cages. Environmental parameters were monitored by the Laboratory Animal Facility of UIB and followed the institutional SOP. Animals were provided certified laboratory feed and sterile drinking water ad libitum. Clinical observation of animal appearance were recorded daily. At study termination the animals were anesthetized by Sevoflurane and euthanized by cervical dislocation euthanized following Institutional SOP.

**Mammary transplantation assay**
Mammary glands were dissected from adult (12-16 week old) B6.129P2-AXL<sup>Im1Dgen</sup> heterozygous and homozygous mice (Jackson Labs), washed in cold PBS, minced and incubated overnight at 37°C in dissociation medium containing EpiCult-B Basal Mouse Medium (05611, STEMCELL Technologies) supplemented with 5% fetal bovine serum and
1.7 mg/ml collagenase XI (C9697, Sigma). After dissociation and centrifugation, cells were suspended in a 1:4 mixture of cold Hanks Balanced Salt Solution Modified (37250, STEMCELL Technologies) supplemented with 2% FBS and ammonium chloride (07850, STEMCELL Technologies) in order to lyse red blood cells. Dissociation was ended by proteolysis using pre-warmed 0.25% trypsin-EDTA followed by incubation in pre-warmed 5 mg/mL dispase with 0.1 mg/mL DNase I solution (07900, STEMCELL Technologies). FluoReporter® LacZ Flow Cytometry Kit (F-1930, Molecular Probes) was used for fluorescent β-galactosidase detection followed by FACS. Protocol provided by manufacturer was followed, with minor modifications. Briefly, isolated mammary epithelial cells were suspended in staining medium containing Hanks Balanced Salt Solution Modified (37250, STEMCELL Technologies) supplemented with 2% FBS and 300 µM chloroquine to inhibit lysosomal β-galactosidase activity. The same volumes of 2 mM FDG working solution and cell suspension were pre-warmed at 37°C for 10 or 20 minutes, respectively. Cell suspension was loaded with FDG working solution, mixed and incubated at 37°C for exactly one minute. The reaction was stopped by adding ice cold staining medium. Cells were analyzed and sorted immediately by FACS ARIA (Becton Dickinson) cell sorter. Gates were set following intensity of fluorescein signal and there were selected distinct positive and negative populations.

Recipient 4 week old athymic Nude-Foxn1nu mice (Harlan Laboratories) were prepared by clearing the epithelium of the inguinal mammary glands on both sides to avoid interference from the host’s gland. Distinct fluorescein positive cells in limiting dilution series (10 000, 1000, 100) were implanted in 25 µl of BD Matrigel (BD Biosciences) on the one side and limiting serial dilution (10 000, 1000, 100) of distinct fluorescein negative cells on the other side as a control. The wound was closed using fine sutures. After 8 weeks restored mammary glands were dissected, spread on the glass slide and submerged in 2% paraformaldehyde in PBS for 3 hours. The mammary glands were then rehydrated in a series of ethanol dilutions (70%, 50%, 25%) and distilled water. Rehydrated glands were stained overnight in Carmin alum solution containing 1 g Carmin (C1022, Sigma), 2.5 g aluminium potassium sulphate (A7210, Sigma) per 500 ml of distilled water. Next, glands were dehydrated in a series of ethanol dilutions (70%, 95%, 100%) and bleached in xylenes for 48 hours. At the end mounted with Organo/Limonene Mount (O8015, Sigma). Samples were let dry overnight before imaging by light microscopy. Repopulating frequency and confidence intervals were calculated using limiting dilution analysis using Extreme Limiting Dilution Analysis (ELDA) software (http://bioinf.wehi.edu.au/software/elda/; (Hu and Smyth, 2009). ELDA uses a general linear model approach to calculate a maximum likelihood estimate from which the stem cell frequency is derived. Pairwise tests for differences in stem cell frequencies are determined by likelihood ratio tests using the asymptotic chi-square approximation to the log-ratio.

**Mammary gland phenotype studies**

Heterozygous and homozygous mice of B6.129P2-AXLtmed/gen strain (Jackson Labs) were sacrificed at 16 weeks, and mammary gland tissue harvested for Carmin alum whole-mount staining. Separate glands where harvested for formalin fixation and parafin embedded for subsequent H&E staining, immunofluorescence and IHC-P as described. Paraffin-embedded tissue sections from B6.129P2-AXLtmed/gen reporter mice were de-parafinized, antigen-retrieved (citrate buffer), and incubated 45 minutes in blocking buffer (5% goat serum in PBS w. 0.1% Triton X-100). Sections were then stained with primary antibodies chicken anti-beta galactosidase (1:100, ab9361, abcam), rat anti-Cytokeratin 8 (1:100, clone TROMA-1), and rabbit anti-Keratin 5 (1:400, PRB-160P-100, BioLegend) overnight at 4°C. To avoid cross-reactivity between the secondary antibodies, the samples were first incubated 45 minutes with AF647 goat anti-rabbit (A21244, Invitrogen) together with AF546 goat anti-rabbit (1:200, A11081, Invitrogen), then washed 3 times with PBS-T before a second round of 45 minutes incubation with AF488 rabbit anti-chicken IgY (IgG)Fc fragment specific (2:100, Jackson ImmunoResearch). Samples were then washed and mounted with ProLong Diamond Antifade Mountant with DAPI nuclear stain (P36962, Molecular Probes/Invitrogen) and imaged the next day with a Leica TCS Sp8 confocal microscope (Leica microsystems, Germany) using a 93x glycerin objective (NA = 1.3, WD = 0.30 mm, HC PL APO motCORR STED white). Images displayed in the figure are presented as a maximum projection of 4 z-stack images.
Warfarin treatment studies
12 week-old female C57BL/6 mice (UT Southwestern breeding core) were administered 1mg/L warfarin (Coumadin, Bristol-Myers Squibb Company) in drinking water. After five months of treatment mice were sacrificed and the mammary glands were dissected, fixed in 2% paraformaldehyde in PBS, stained with Carmin alum solution (Sigma C1022), cleared in xylene, mounted with Organo/Limonene (Sigma O8015) and imaged using brightfield tile scan with a Nikon TE2000. Treatment of pre-pubescent mice were initiated in 3 week old mice post-weaning. Mice were treated with either pure water or warfarin (1 mg/L) containing drinking water for 5 weeks prior to harvest of mammary glands and carmine alum staining as described above.

Generation of MMTV-Wnt1:AXL+/+ and MMTV-Wnt1:AXL+/-AxLlacZlacZ transgenic animals
MMTV-Wnt1 male mice (Jackson Laboratory) (Donehower et al., 1995; Tsukamoto et al., 1988) (kindly provided by Prof. Stein Ove Døskeland) were crossed with females of the AXLLacZ knock-in strain (B6.129P2-AXLtm1Dgen, figure S5) to generate MMTV-Wnt1-positive females that develop spontaneous mammary tumors in a wildtype AXL+/- (MMTV-Wnt1:AXL+/+) and AXL-null (MMTV-Wnt1:AXL+/- AxLlacZlacZ) background. Tumor formation was monitored by weekly palpation of the mammary glands from 1 month to 14 months of age. Previous studies showed that all MMTV-Wnt1 females developed tumors within 1 year (Donehower et al., 1995, Tsukamoto et al., 1988). Mice that did not develop tumors within the duration of the 14-month observation period were scored as tumor-free.

Mammary tumor incidence studies
Female NOD/SCID b2mnull and Balb/c mice (6–8 weeks old; Gades Institute, University of Bergen) were used for tumor studies. The orthotopic 4T1-GFP-Luc mouse mammary carcinoma model was conducted as previously described (Gjerdrum et al., 2010). Cells were suspended in MEM/ EBSS medium/Matrigel (1: 1) (1x 10^6 cells in 50 µl) and were subsequently injected into the mammary fat pad of female BALB/c mice. Tumor growth was monitored and imaging of the ventral view were performed by the eXplore Optix Imaging System 10–15 min after D-luciferin (Biosynth) injection.

Xenograft tumor-initiation studies
Xenograft tumor-initiation studies were conducted as described by (Gupta et al., 2009). HMLER cells stably transduced by retroviral vectors (Slug, shLuc, shAXL) were suspended in DMEM/Matrigel (1:1) in a total volume of 50 µl and injected subcutaneously into the flank of NOD-SCID mice. Tumor incidence was monitored for up to 60 days after injection. For syngeneic tumor seeding studies, 4T1 cells were pretreated for 4 days with paclitaxel (3 nM) and bencentsinib (600 nM), and allowed to recover in the absence of drug for 1 week prior to injection. Tumor formation was assayed by palpation and caliper measurement between 7-9 days post implantation.

Histology and morphometry
The ductal morphology of mammary glands from 16 week old nulliparous Axl+/+ and Axl+/-AxLlacZlacZ of the B6.129P2-AXLtm1Dgen strain (The Jackson Laboratory, Bar Harbour, ME) mice were examined by hematoxylin/eosin (HE) stained FFPE tissue sections and carmine alum stained whole-mount specimens. Quantification of ductal area (µm2) per epithelial structure were performed on HE stained FFPE sections from Axl+/+ (n= 7) and Axl+/-AxLlacZlacZ (n= 9) animals. Images were obtained using the NikonTE2000 microscope and area (µm2/ structure) were recorded using the Nikon software from 10 separate fields/ gland (Mann Whitney p< 0.0001). Carmine alum stained mammary gland whole-mounts of 16-week old nulliparous Axl+/+ (n= 8) and Axl+/-AxLlacZlacZ (n= 9) (B6.129P2-AXLtm1Dgen Strain, The Jackson Laboratory) mice were prepared and imaged as described. Both inguinal glands were included in the analysis. Ducts were partitioned from the background using image thresholding with MATLAB. The binary image obtained was sliced vertically pixel-wise. Segment lengths from the same duct were measured and averaged. FFPE sections from the mouse mammary fat pad tissue were stained by IHC-P for detection of ERAlpha (ESR1) (ab37453, Abcam), Progesterone receptor (ab2765), and Ki67 (ab15580, Abcam). DAKO EnVisionTM System-HRP (DAB) for Rabbit primary antibodies (K4011, DAKO) were applied according to the manufacturer’s instructions. Antibodies were diluted in antibody-diluent with
background reducing components (S3022, DAKO). Stained sections were counterstained with haematoxylin, prior to mounting using Faramount Aqueous Mounting Medium (S3225, DAKO). FFPE sections from the mouse mammary fat pad tissue were assessed for progesterone and estrogen receptor expression (scored strong or weak versus negative by trained pathologists), and proliferation rate by Ki-67 expression in luminal cells in the glands and duct structures. We used 25 high-power fields (x 400) and counted 20 luminal cells in each field (total of 500 luminal cells per case). Cells with cytoplasmic staining were not considered positive, and myoepithelial cells were not evaluated. All intensities of nuclear positivity were recorded as positive. The percentage of positive cells in each case was then calculated.

**Statistical analysis**
Where not otherwise stated, Graphpad Prism 5.0 for PC and Graphpad Prism 6.0 for Mac and MATLAB were used for statistical analysis using tests specified in the Figure Legends. The following symbols are shown to report established statistical significance: NS = P > 0.05, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

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