SUPPLEMENTAL MATERIALS AND METHODS

Mice: MMTV/Notch1IC Tg mice (48) were maintained in viral-free conditions on FVB background. Eight month-old mice bearing visible tumors were used for preparation of single cell suspensions. Three-week-old CD1-nude or FVB mice were used as recipients.

Working medium: It contains DME/HAMF12 (1:1) supplemented with 10% bovine calf serum (FBS), 1mM glutamine, 5 µg/ml insulin, 500 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor and 20 ng/ml cholera toxin (all from Sigma).

Mammary Tumor Single Cell preparation: Solid mammary tumors were collected from 8-10 month-old donor mice and were cut into small pieces. These pieces were then minced by passing 5 times through a 18 gage needle in 10 ml of sterile working medium to yield 1mm³ pieces which were then filtered through 40 µm mesh (Facol) and rinsed 3 times with 10 ml of serum-free HBSS to eliminate debris and blood cells. Tissues retained in the sieve were digested with 300 U/ml of collagenase and 100 U/ml of hyaluronidase (Stem Cell Technology Inc) in working medium for 3 h at 37°C. The resultant cells were sequentially resuspended in 0.25% trypsin-EGTA for 2 min, and 0.1 mg/ml DNase (Roche) for 5 min, and 0.64% NH₄Cl for 3 min before filtration through a 70-µm mesh (Facol). The single cell suspension obtained was washed twice in working medium before transplantation and/or labelling.

Antibodies: PE-labeled anti-CD24, biotinylated anti-CD31, CD45, TER119, FITC-labelled anti-CD29 and Streptavidin-APC were purchased from BD Pharmingen. Anti-cytokeratin (CK) 5, 6 and 14 were from Covance; anti-cytokerating 18 and cyclin D1 (H-295, sc-753) were from Santa Cruz; Cytokeratin 19 were from the Developmental Studies Hybridoma Bank; Smooth muscle actin (SMA) was from
Sigma. Fluorochrome-conjugated secondary antibodies included anti-rabbit and anti-mouse Ig-Alexa 594, anti-goat Ig-Alexa 488 and 633 and anti-rabbit Ig-Alexa 488 (Molecular probes).

**FACS Based Tumor Cell Sorting:** Single cell suspension was first incubated with 1 μg/ml of primary antibodies (CD24-PE, CD29-FITC, and biotin conjugated –CD3; -CD45.2; -Ter-119) for 45 min at RT, followed by 1 hr staining with Streptavidin-APC (1:1000 dilution). The stained cells were then washed once in 10 ml of Hanks with 2% of FBS and subjected to MoFlo cell sorter (Dako, Fort Collins, Colorado) for sorting. The gates were set to exclude CD31, CD45, TER119-positive and dead cells.

**Tumor Transplantation and Analysis:** For fat pad transplantation, young 3-week-old FVB or CD1- nude female mice were anesthetised with avertin and cleared fat pads were obtained by surgically removing the endogenous epithelium of the area between nipple and the lymph node. For some experiments, tumor cells were inoculated subcutaneously on the back. Un-sorted or sorted cells were resuspended in the working medium and injected in 10 μl volumes subcutaneously or into inguinal cleared fat pads of recipient mice. Mice were monitored for signs of tumor growth and euthanized for gross and histological analysis of tumors or other outgrowths.

**Mammosphere culture:** Single cells were plated in ultra-low attachment plates (Corning, Acton, MA, USA) at a density of 1000 sorted or un-sorted viable cells/60mm plates in a culture medium containing DME/HAMF12 (1:1), 1 mM glutamine, 5 μg/ml insulin, 50 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor and 20 ng/ml cholera toxin for 72 hr or until small cell clumps had formed. These cell masses were then collected by gentle centrifugation (500 rpm) and transferred into a new plate, and cultured in the same medium now containing 5% of pregnant mouse serum. For counting mammospheres, the content of all dishes was collected by gentle centrifugation (500 rpm) after 7-10 days and transferred on a matrigel-coated dish in the culture medium supplemented with 10% of FBS.
Mammospheres adhered to the bottom of the dishes in these conditions in approximately 48 hr, after which they were stained with methyl blue and counted under low magnification.

**Mammosphere differentiation condition:** Primary mammospheres were collected by gentle centrifugation (500 rpm) and resuspended at 100 mammospheres per 60 mm ultra-low attachment plates in 5 ml of differentiation media (1:1 DME/HAMF12, 1 mM glutamine, 5 μg/ml insulin, 50 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor and 20 ng/ml cholera toxin supplemented with 10% of pregnant mouse serum). Cultures dishes were shaken twice a day. These cultures were maintained for 8 weeks with medium change once a week.

**Establishment of transplantable tumor cell line:** Primary tumor cells were seeded at limiting dilution into 96-well plates. Microscopy evaluation were preformed to ensure one cell per well. Cells were allowed to grow in low serum condition for one week and transferred to ultra-low attachment plates for mammosphere formation. Three mammospheres from each donor were selected to establish tumor cell lines.

**Immunostaining:** Cultures containing 3D structures were collected by gentle centrifuge and treated with 10% and 20% D-sucrose for 30 min of each before being embedded in Shandon cryomatrix (Thermo Scientific). Serial sections were made for immunofluorescence staining. The sections were fixed in 10% formaldehyde-PBS for 60 min at 4°C. Antigen retrieval was performed by boiling the fixed section in 10 mM citrate buffer (pH 6) for 30 min. The treated sections were then washed with 1% Triton X-100/PBS three times before blocking with 5% mouse serum in 1% Triton X-100 PBS. Sections were then incubated with primary antibodies against CK5, CK6, CK14, SMA, CK18 and CK19 overnight at 4°C at 1:100 dilutions and then washed with PBS three times for 5 min at RT.
Alexa Fluor®-488 (1:4000), Alexa-594 (1:500) and Alexa-633 (1:1000)-conjugated secondary antibodies (Molecular Probes) were added for 60 min in the dark at RT. The slides were washed repeatedly in 1× PBS and mounted with Vectashield containing 1.5 μg of 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc). Digital fluorescence images were taken on An Axiovert S100TV microscope.

**shRNA**<sub>**cyclinD1**</sub>: Mouse cyclin D1 mission shRNA vectors containing bacteria were purchased from Sigma (NM_007631) which included shRNA-pLKO.1-puro-1511, shRNA-pLKO.1-puro-1608. The insert sequences are:

CCGGGCATCTACACTGACAACTCTACTCGAGTAGAGTTGTCAGTGTAGATGCTTTTT for 1511 and

CCGGCCACGATTTCATCGAACACTTCTCGAGTAGTTGTCAGTGTAGATGCTTTTT for 1608. The puromycin resistant gene was replaced by the gene of resistance to hygromycin. The negative controls shRNA-pLKO.1-puro GFP vector was a gift from Dr. Cayouette’s lab from our Institute. Production of pseudotyped infectious viruses was done by co-transfecting 293 cells with pspPAX2-Gag, and SV-E-MLV-Env plasmids with calcium phosphate. The virus was titered by an endpoint-limiting dilution assay. N1<sup>1C</sup>- induced mammary tumor cells were infected and stable expressing clones were selected with 250 μg/ml hygromycin. After 2 weeks of selection, cyclin D1 expression was tested by Western blot and further experiments were carried out.
LEGENDS TO SUPPLEMENTAL FIGURES

Figure S1. Tumor-derived CD24^{+}CD29^{high} (R4) cells do not significantly transit into CD24^{int} CD29^{int} (R5) cells

GFP^{+} cell-sorted purified CD24^{+}CD29^{high} (R4) N1^{IC} tumor cells from an established line (A) were mixed (1:1) with cell-sorted purified CD24^{int}CD29^{int} R5 N1^{IC} tumor cells not expressing GFP and 1 X 10^{4} cells were transplanted into cleared fat pads of normal FVB mice. Transplanted tumors (n=7) were evaluated by FACS after 6 weeks (B). Note the very small percentage (0.0012%) of GFP^{+} R5 cells, in total transplanted tumor cell population.

Figure S2. Tumor-initiating cells in N1^{IC}-induced tumors.

A) Frequency of tumor formation after transplantation of unselected tumor cells from 10 different primary tumors into FVB mice. (*) Data from these individual tumors (Group 1, Fig.S5) were pooled.

B) Frequency of tumor-initiating cells within the CD24^{int} CD29^{int} (R5) cell subset of primary M209326 tumor. The R5 cells were purified by cell sorting and transplanted at the indicated numbers sub-cutaneously (s.c.) or into clear fat pads (FP) of 3 week-old syngeneic FVB or nude mice.

C) Volume of tumors after 12 weeks in relation to number of unselected M212057 tumor cells transplanted sub-cutaneously into nude mice.

Figure S3. Serial transplantation of N1^{IC}-induced tumors enhances the number of tumor-initiating and mammosphere-forming cells and selects for tumor cells with higher N1^{IC} expression.

A) Frequency of tumor formation (number of tumors/number of transplanted sites) after serial transplantation of unselected cells from 5 tumors (M197872, M197874, M197054, M228925, M228927) in nude mice. Latency in weeks. Data from individual tumors were pooled.
B) Northern blot analysis of N1IC expression at different stages of tumor transplantation.

C) Frequency of mammosphere-forming cells during serial transplantation of tumor cells from 5 (unsorted) or 7 (sorted) independent tumors. The lower number of mammospheres obtained with the unsorted primary tumors in this experiment relative to that shown in Fig 4F may be related to unknown variable of cultures run months apart, or to the fact that primary tumors shown here were freshly prepared, while those shown in Fig 4 were frozen, thawed and plated overnight before the assay.

D) Mammosphere formation with mixed tumor cells. Equal numbers of GFP-expressing and wild-type tumor cells were mixed and allow to form mammospheres. Note that all mammospheres are either GFP-positive or GFP-negative, strongly suggesting that each originates from a single cell.

Figure S4. Frequency of tumor formation of CD24\textsuperscript{int} CD29\textsuperscript{int} (R5) cells from tumors originating in cyclin D1\textsuperscript{+/−} N1IC Tg mice.

Sorted R5 cells from the indicated primary tumors were transplanted into syngeneic FVB mice. Results from all tumors of each group were pooled. Cyclin D1\textsuperscript{+/−} (M197874, M197872, M197054) and cyclin D1\textsuperscript{+/−} (M228747, M248174, M248176) were analyzed; FP, fat pads; s.c., sub-cutaneously.

Figure S5. Presence of N1IC-expressing pre-malignant mammary cells do not promote the differentiation of N1IC-expressing tumor cells.

The same GFP-positive N1IC-expressing tumor cells used in Fig 6 were mixed with pre-malignant mammary cells from virgin 8 week-old N1IC T mice at 1:5 ratio and transplanted into cleared fat pads of normal FVB mice. Outgrowths were counted and analyzed 6 weeks after transplantation.

Figure S6. MMTV-driven expression of GFP reporter gene in mammary tissue of newborn mice
MMTV/rtTA Tg mice generated in our laboratory (to be described) were bred with tetO/GFP reporter mice (Jackson Laboratory). Pregnant females were provided with doxycycline in drinking water from day 14 of pregnancy until delivery. Pups were typed and mammary glands from double Tg mice analyzed for GFP expression by microscopic examination. The mammary trees were visualized with carmine staining.

Figure S7. List of tumors used for experiments

A) Groups of tumors used for specific experiments:

B) Summary of all tumors.