

S100A10 has a Critical Regulatory Function in Mammary Tumor Growth and Metastasis: Insights using MMTV-PyMT Oncomice and Clinical Patient Sample Analysis.

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1 **Full Title:** S100A10 has a critical regulatory function in mammary tumor growth and metastasis:
2 Insights using MMTV-PyMT oncomice and clinical patient sample analysis.

3 **Short Title:** S100A10 (p11) and breast cancer progression

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14 metastasis, carcinoma, mammary gland, triple negative

15

16 **Abstract**

17 **Background:** Breast cancer is one of the leading causes of cancer deaths in women worldwide.
18 Significant advances have been made in the diagnosis and treatment of breast cancer, treatment of
19 triple-negative and metastatic breast cancer poses significant challenge. Metastasis is a multi-step
20 cascade that involves activation of proteases such as plasmin to facilitate the invasive escape of
21 tumor cells to distant organs. The rate-limiting step in plasmin generation requires the interaction
22 of plasminogen with cell surface plasminogen binding sites. Our laboratory first demonstrated
23 that the plasminogen receptor, S100A10 (p11) was upregulated in many cancer cells and was
24 responsible for much of their plasmin generation. Recently, it was reported that p11 is one of a
25 few genes that are activated when human breast cancer cells metastasize from the primary tumor
26 into the blood and is upregulated during the conversion of breast cancer cells to invasive
27 phenotype. In the current study we have investigated the role of p11 in breast cancer tumor
28 progression.

29 **Methods:** We have used MMTV-PyMT a mouse transgenic mammary tumor model to investigate
30 the effects of loss of p11 on spontaneous tumor initiation, growth and progression to invasive
31 carcinoma and metastasis. We used experimental metastasis assays to ascertain the role of stromal
32 p11 in tumor cell extravasation and lung colonization. Genes and cytokines regulated by p11 in
33 the PyMT tumors were assessed by microarray analysis and RT-qPCR. Finally, we employed gene
34 profiling analysis and immunohistochemical staining of breast cancer patient tumors to correlate
35 p11 expression to human breast cancer progression.

36 **Results:** Genetic deletion of p11 resulted in significantly decreased tumor onset, growth rate, and
37 spontaneous pulmonary metastatic burden in the PyMT/p11-KO mice. This phenotype was
38 accompanied by substantial reduction in Ki67 positivity, macrophage infiltration, decreased
39 vascular density in the primary tumors and decrease in invasive carcinoma and pulmonary
40 metastasis. Surprisingly, immunohistochemical analysis of wild-type MMTV-PyMT mice failed
41 to detect p11 expression in the tumors or metastatic tumor cells and loss of p11 did not decrease
42 plasmin generation in the PyMT tumors and cells. Furthermore, tumor cells expressing p11
43 displayed dramatically reduced lung metastasis when injected into p11-depleted mice, further
44 strengthening the stromal role of p11 in tumor growth and metastasis. Transcriptome analysis of
45 the PyMT tumors from p11-KO mice showed marked reduction in genes involved in breast cancer
46 development, progression, and inflammation such as AREG, MUC1 and S100A8. The PyMT/p11-
47 KO tumors displayed a remarkable increase in inflammatory cytokines such as IL-6, IL-10 and
48 IFN- γ . Gene expression profiling from 176 primary breast cancer samples obtained through the
49 CBCF tumor bank showed that p11 mRNA levels were significantly higher in tumors compared
50 to normal tissues. P11 mRNA expression was significantly associated with poor patient prognosis
51 (hazard ratio – 3.34) and significantly elevated in high grade, triple negative (TN) tumors, and
52 tumors with high proliferative index. Evaluation of p11 protein expression in a NSHA cohort of
53 patients revealed substantial upregulation of p11 in cancer tissues compared to normal controls.

54 **Conclusions:** This is the first study demonstrating the crucial role of p11 in breast tumor
55 development and metastasis. The results emphasize the potential of p11 as a diagnostic and
56 prognostic biomarker in breast cancer.

57

58 **Introduction**

59

60 Breast cancer is the leading cause of cancer death among women worldwide. The vast number of
61 cancer-associated deaths are due to metastases rather than primary disease alone. Currently,
62 strategies to treat and eliminate metastasis are limited and challenged by resistance to therapies.
63 Thus, discovering the underlying molecular mechanisms that promote breast cancer metastasis and
64 therapy resistance is critical for identifying novel treatment strategies.

65 Extracellular proteases promote degradation of the extracellular matrix and form a key component
66 of the cascade of events that contribute to cancer cell invasion and metastasis. (1). (2,3). Increasing
67 evidence have shown elevated expression of proteases plasmin and matrix metalloproteases
68 (MMPs) in breast cancer progression and have been extensively studied with mouse tumor models
69 (4). Plasminogen (Plg), synthesized by the liver is activated to plasmin (Pm) by tissue plasminogen
70 activator (tPA) and urokinase plasminogen (uPA) activator, (reviewed in (5,6)). This process,
71 normally slow, is accelerated by plasminogen receptors (PgR) on the cell surface. Our laboratory
72 identified S100A10 (p11) as a plasminogen receptor that forms a complex with both plasminogen
73 and plasminogen activator (7–10).

74 P11 is a multifunctional protein and a member of the S100 proteins. Several extracellular and
75 intracellular functions have been identified for p11; the plasminogen receptor function is the most

76 well studied so far (5). P11, present on the extracellular surface, is complexed with its binding
77 partner annexin A2 (p36). We have reported that p11 regulates plasminogen activation on the cell
78 surface of many cancer cells including fibrosarcoma, colorectal, lung and pancreatic cells (8,11–
79 16). We have also reported that p11 regulates the plasmin production of stromal cells including
80 macrophages and endothelial cells (17–19) and that p11-dependent plasmin generation is necessary
81 for macrophage infiltration to the site of inflammation in subcutaneous tumor growth.

82 Several studies have shown correlations between p11 gene expression and poor prognosis and
83 overall survival in lung (20–22), colorectal, ovarian, kidney, gastric cancers, anaplastic thyroid
84 carcinoma, melanoma and acute lymphoblastic leukemia (reviewed in (12)), and pancreatic ductal
85 adenocarcinoma (PDAC)(16). P11 is upregulated in basal-type breast cancer (23) and during the
86 process of intravasation and epithelial mesenchymal transition (24).

87 Although we have shown that p11 is important for ectopic tumor growth in both NOD/SCID and
88 syngeneic mouse models for various cancer cell lines such as colorectal, fibrosarcoma, and
89 pancreatic cancer, these studies have limitations. Firstly, they do not reproduce the complex
90 multistep landscape of human oncogenesis. Secondly, the growth, invasion and metastasis of
91 cancer also depends on its interaction with the tumor microenvironment and it is also difficult to
92 distinguish the individual contribution of tumor and stromal cells to cancer progression. To
93 circumvent these experimental challenges, and to advance our understanding of the role of p11 in
94 oncogenesis we have established the MMTV-PyMT (Polyoma Middle T) transgenic breast
95 cancer model in wild-type and p11 knockout mice and have used this double transgenic model to
96 investigate the role of p11 in breast cancer malignancy.

97 In the MMTV-PyMT transgenic mouse model, mammary gland specific expression of the
98 oncogene PyMT under the MMTV promoter, results in widespread tumor growth in all ten
99 mammary glands and spontaneous metastasis to the lymph nodes and lungs. This occurs with a
100 mean latency of 92 days with high penetrance and almost a 100% incidence of metastasis. This
101 mouse model is very similar to human breast cancer in that the tumors display histological and
102 molecular characteristics mirroring the progression of human breast cancer (25–27)and have a
103 reactive stroma. This model has been widely used to establish the role of proteolytic activity in
104 cancer cell malignancy. Proteases that affect metastasis in this model system include plasminogen
105 (28,29), uPA (30), MMP-9 and MMP-3 (4,31,32), cathepsin B(33,34) and ADAMTS1(35).
106 Another important feature of the MMTV-PyMT model is that the increased metastatic potential is
107 largely dependent on the presence of macrophages in the primary tumor (36,37). Thus, the MMTV-
108 PyMT model is a pertinent model to investigate the functional role of p11.

109 In the current study, we conducted a systematic, functional, and correlative analysis of the role of
110 p11 in breast cancer oncogenesis using the MMTV-PyMT transgenic mouse model. We have also
111 performed gene expression profiling and protein expression analysis of human breast cancer
112 tissues. The studies described herein indicate that p11 plays a complex and multifunctional role in
113 breast tumor growth, progression, and metastasis.

114 **Materials and Methods**

115 **Mice**

116 All animal experiments were performed according to protocol approved by University Committee
117 on Laboratory Animals, Dalhousie University, Canada. The C57Bl6 MMTV-PyMT mice were
118 obtained from Dr. Mak (OCI, Canada). The p11 WT and p11 KO mice were obtained from P.
119 Svensson (Karolinska Institutet, Sweden). We employed a sequential breeding strategy to
120 generate PyMT/p11-KO homozygous mice. We crossed the male PyMT/p11-WT mice with the
121 female p11 KO mice to obtain heterozygotes for p11. The resultant F1 (PyMT/ p11^{+/−}) males were
122 bred with the female (p11^{+/−}) mice. This crossbreeding produced F2 females PyMT/p11-WT,
123 (PyMT/p11^{+/−} and PyMT/p11-KO. Virgin female C57/BL6 mice heterozygous for the MMTV-
124 PyMT transgene and homozygous KO (PyMT/p11-KO) or homozygous wild-type (PyMT/p11-
125 WT) for the p11 gene were used in our studies. Genotyping of mice was performed by PCR of
126 genomic DNA derived from ear clippings using published primers for PyMT and p11 (Supplement
127 table 1).

128 **Cell culture and reagents**

129 Py8119 cells were obtained from ATCC and maintained in F12 Kaign's medium (Hyclone, Logan,
130 UT, USA) with 5% Fetal Clone II serum (Thermofisher Scientific), 1% penicillin and
131 streptomycin. Py8119 cells were tested for pathogen (Charles River Laboratory) before injecting
132 into the mice. All cells were tested for mycoplasma (Lonza Mycoalert kit) and only mycoplasma-
133 free cells were used.

134 **Whole mount analysis**

135 The harvested tissues were stretched and placed onto a cassette matrix and fixed overnight in
136 Carnoy's fixative. The tissues were sequentially hydrated with 70, 50, 25% ethanol followed by
137 a final water wash. They were stained with carmine alum solution for four hours, followed by
138 sequential dehydration in 70, 95 and 100% ethanol and defatting of the tissues was carried out in
139 two xylene washes for 30 minutes and overnight. The dehydrated and defatted tissues were then
140 mounted on slides using Cytoseal (Richard-Allan Scientific, Thermo Scientific, Waltham, MA,
141 USA) mounting medium. The slides were digitally imaged using a stereomicroscope under
142 similar lighting and contrast conditions (Zeiss microscope).

143 **Tumor measurements**

144 For spontaneous PyMT tumor model, all the mammary glands were palpated once a week for 20-
145 25 weeks to monitor tumor latency and progression. For evaluating the kinetics of tumor growth,
146 the tumors were measured using a Vernier calipers, and tumor volume was determined using the
147 standard calculation for a hemi-ellipsoid; $0.5ab^2$ where a is the smaller and b is the larger diameter.
148 Tumor burden was calculated at the endpoint (20 weeks) by determining the total tumor
149 weight/body weight. We determined the percentage of tumor-free mice using Kaplan Meier
150 analysis (Graph pad prism) in the two PyMT mice groups. Animals were considered tumor-free
151 until a palpable mass (>4.0 mm) persisted for longer than 4 days. All tumor growth and kinetics
152 data were obtained from three independent experiments. Only virgin female mice were used for
153 all the experiments.

154 **Spontaneous and experimental metastasis quantification**

155 For evaluation and assessment of lung metastasis, lungs were harvested at 20 weeks, fixed in 10%
156 buffered formalin for 48 hours and embedded in paraffin. The lungs were cut at 5µm thickness
157 and 3-6 sections from each lung at 100 µm apart were stained with H&E. The stained slides were
158 scanned using Aperio microscope (Leica Biosystems, Concord, Ontario) and number of metastatic
159 foci were counted using Imagescope/Image J. The total metastatic burden was determined using
160 Imagescope/Image J by tracing the metastatic area as a ratio of the total lung area. For experimental
161 metastasis assay, Py8119 cells were injected via the tail vein at a density of 2.5×10^5 cells. The
162 lungs were harvested 14 days after injection and analyzed for metastatic burden and foci
163 calculation as described for spontaneous metastasis quantification.

164 **Tissue Processing and Immunohistochemistry**

165 Tumors, mammary glands, and lungs were harvested and fixed in neutral 10% buffered formalin
166 for 48 hours and stored in 70% ethanol before embedding in paraffin. The embedded tissues were
167 cut into 5µm thick sections, stained with H&E for pathological analysis, and adjacent sections
168 were used for immunohistochemistry. The tissue sections were deparaffinized with sequential
169 washes in xylene, 100%, 95% and 70% ethanol followed by water wash. The details of the
170 antibody dilution, method of chromogen and stain development are provided in the Supplement
171 table 2.

172 Antigen retrieval was performed by heat treatment using a pressure cooker in either Tris-EDTA
173 (pH 9.0) or citrate buffer (pH 6.0). The slides were rinsed in Tis-buffered-saline (TBS) or
174 Phosphate Buffered Saline (PBS) depending on the antibody and blocked with Rodent Block M
175 (Biocare). The primary antibodies CD31, Ki67, smooth muscle α-actin (α-SMA), p11, F4/80,
176 were incubated overnight at room temperature followed by secondary antibody and chromogen
177 steps. For anti-rat, the slides were washed with TBS after primary incubation, followed by 15
178 minutes incubation with rat probe, subsequently the slides were washed and incubated with Rat
179 on mouse HRP polymer for 15 minutes (Biocare), followed by three washes with TBS and
180 stained with DAB (Biocare) and counter stained with hematoxylin. For anti-rabbit secondary
181 antibodies, the slides were incubated with goat anti-rabbit secondary (Envision, DAKO) for 30
182 minutes, followed by washes with TBS and counterstained with hematoxylin. For anti-mouse
183 antibody, we used mouse-on- mouse HRP polymer kit (Biocare) and anti-goat antibodies we
184 used rabbit-anti goat HRP polymer (DAKO), followed by staining with DAB and
185 counterstaining with hematoxylin. All stained sections were washed in water and mounted with
186 aqueous mounting medium (Vectamount). The immunostained sections were imaged using
187 Zeiss Axio Imager Z1 W/ color and monochrome camera (p11, Ki67, CD31, F4/80, α-SMA,
188 H&E, CD3, and TUNEL staining) using 40X (for manual counting – F4/80, Ki67, CD3) and
189 20X for representation. To count the number of CD31 positive vessels, we scanned the slides
190 using Aperio Scanning system, following which CD31-positive vessels were counted from 7-10
191 snapshots of random fields. To quantify number of Ki67 positive, F4/80, CD3-positive, TUNEL
192 positive, we captured images using Zeiss Axio Imager Z1 microscope, and manually counted the
193 positive cells using the Zen software in 7-10 fields. The average of 7-10 fields for Ki67, CD31,
194 F4/80, was used for each mouse in the quantification bar graph. For human p11 staining we

195 followed the same procedure as for mouse tissues, but blocking was performed with Background
196 Terminator (Biocare) for 10 minutes before incubating with primary antibody.

197 **Gene expression profiling (mouse tumors)**

198 Sample preparation, amplification, and hybridization to the Affymetrix Mouse gene 2.0 ST array,
199 and data collection were performed by The Centre for Applied Genomics at the Hospital for Sick
200 Children (Toronto, Canada). The Transcriptome Analysis Console software (ThermoFisher
201 Scientific) was used to normalize data and calculate fold changes in expression (GSE151579).
202 Genes up- or downregulated by more than 2-fold ($\log_2 = 0.678$) at a significance level of $p < 0.05$
203 were considered differentially expressed.

204 **Gene expression profiling (human tumors)**

205 The gene expression microarray dataset was generated from a human breast cancer cohort
206 consisting of 176 treatment-naïve primary tumor samples as previously described (58)
207 (GSE22820). Patient material and clinical information were collected under Research Ethics Board
208 Protocol ETH-02-86-17. Patients received standardized guideline-based chemo- and hormone
209 therapies: i.e., hormone therapy for all patients with ER-positive tumors, trastuzumab for those
210 with HER2-overexpression tumors, anthracycline chemotherapy for high risk node-negative
211 disease and anthracycline plus taxane chemotherapy for node-positive disease. The median follow-
212 up time for surviving patients was 4.5 years.

213 **Quantitative PCR (mouse tumors)**

214 Tumors from PyMT/p11-WT and PyMT/p11-KO mice at 20 weeks of age were excised, snap
215 frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted using Trizol (Invitrogen)
216 and the PureLink RNA kit (Invitrogen) with DNase treatment. Equal amounts of RNA (0.5µg)
217 were reverse transcribed using iScript (BioRad); quantitative real-time PCR (qPCR) reactions
218 were performed with SsoAdvanced Universal SYBR Supermix (BioRad) and gene-specific
219 mouse primers (Supporting information Table S2) on a CFX96 or CFX384 Touch Real-Time
220 PCR Detection system (BioRad). Standard curves for each primer set were generated, and primer
221 efficiencies were incorporated into the CFX Manager software (BioRad). mRNA expression of
222 all samples was calculated using the $\Delta\Delta ct$ method, with gene of interest made relative to two
223 reference genes (rlp10 and PyMT) and an indicated control sample. Relative mRNA expression
224 was log-2 transformed prior to plotting and statistical analysis.

225 **Human patient breast tissue collection, p11 immunohistochemistry and H-score assignment**

226 Anatomical pathology electronic files (Cerner Millennium) for the Queen Elizabeth II Health
227 Sciences Centre, Nova Scotia Health Authority (NSHA) were retrospectively searched for a cohort
228 of invasive and *in situ* breast carcinomas beginning January 1, 2011. One formalin-fixed paraffin-
229 embedded (FFPE) tumor block and one FFPE block of normal breast tissue were selected from
230 each patient of an identified cohort of 119 patients. For this study, FFPE breast tissues were used
231 with approval from the Nova Scotia Health Authority Research Ethics Board, and Materials
232 Transfer and Collaboration Agreement between the NSHA and Dalhousie University. P11

233 immunostaining on patient tissue sections was performed as described earlier (16), using anti-
234 rabbit polyclonal antibody to human p11 (Proteintech, Rosemont, IL, USA).

235 The stained sections were evaluated and assigned a semiquantitative score by a pathologist (PJB)
236 in a blinded fashion based on percent of positive cells and intensity of staining. The percentage of
237 cells were identified as negative, weak, moderate, and strong for membrane staining in tumor cells.
238 An H-score of less than 10% was considered negative. The H-score was determined using the
239 formula (% negative × 0 + % weak × 1 + % moderate × 2 + % strong × 3). The semiquantitative
240 H-score values ranged at 5-250. Nottingham grade 1 and 2 tumors were combined as ‘low grade’
241 and Nottingham grade 3 tumors were labeled ‘high grade’. A scatter plot was generated based on
242 tumor grade (high and low grade, including samples from the molecular subtype category), tumor
243 type (ductal carcinoma *in situ* (DCIS), variants of invasive ductal carcinoma and invasive lobular
244 carcinoma, also including samples from the molecular subtype categories), and molecular subtype
245 (estrogen receptor positive (ER+), Her2+, triple negative (TN)).

246 **Isolation of mouse PyMT tumor cell lines**

247 PyMT tumors from 20-week mice were excised and rinsed in (PBS), minced in RPMI with 5%
248 FBS with penicillin and streptomycin containing 1mg/ml of collagenase (Sigma). The tumor
249 preparation was then incubated at 37°C for 1-2 hours, followed by straining through a 70-micron
250 mesh strainer. The cells were washed with DMEM with 10% FBS, passaged once and used in
251 plasmin generation assays as described below. In some cases, isolated cells were directly plated
252 in 96-well plate without passaging and plasmin generation assay was performed as described.

253 **Plasmin generation assay**

254 Plasmin generation assay was determined in PyMT tumor homogenates. We employed fresh and
255 frozen 20-week old PyMT tumors from PyMT/p11-WT and PyMT/p11-KO mice to prepare tumor
256 homogenates. Briefly the tumors were homogenized in electric homogenizer (Pro Scientific) in
257 Dulbecco's phosphate-buffered saline (DPBS) with 1% Triton. We used 30-60 µg for the assay.
258 Plasmin generation assay was conducted as described in (44) (19).

259 **Immunoblotting**

260 Immunoblotting was performed as described in (59) and anti-mouse p11 antibody (R and D
261 systems) and β-tubulin (Sigma) was used for immune staining (details in Supplemental table 2).

262 **Statistical Analysis**

263 Three independent experiments with varying mice numbers in each were performed. For
264 evaluation of total palpable tumors (tumor onset), tumor-free mice (survival), tumor growth
265 (volume) and metastasis, we pooled the data/measurements from the three independent
266 experiments. For determination of end-point tumor, and metastatic burden and foci we used data
267 from one independent experiment (20-week endpoint). For time course measurements (whole
268 mounts, tumor progression) we employed 3-12 mice at each endpoint. All statistical analyses were
269 performed using Graph pad prism 5 software (La Jolla, CA, USA). Unless indicated in the figure
270 legends, statistical significance was determined using the Mann Whitney non-parametric test. A
271 significance threshold of *P*value < 0.05 was used.

272 For human patient samples, all statistical analyses for gene expression profiling were performed
273 using MedCalc Version 14.12.0 (MedCalc Software). Gene expression microarray signal intensity
274 values were log-transformed to better fit the normal distribution assumption. One-way ANOVA
275 was employed to test the statistical significance for the difference in *S100A10* mRNA levels among
276 molecular subtypes, tumor histologic grades or Ki67 immunoreactivity classifications. Prognostic
277 significance was analyzed using log rank test on Kaplan-Meier survival probabilities.

278

279

280 RESULTS

281 **Loss of p11 results in delayed appearance of multifocal dysplastic lesions**

282 The fourth inguinal mammary gland from PyMT/p11-WT and PyMT/p11-KO mice was isolated
283 at 8, 10 and 12 weeks of age, fixed and stained with carmine alum (37). We observed the
284 appearance of early hyperplastic lesion at a single focus or in some cases multiple foci beneath the
285 nipple in the older zone of the ductal tree in the PyMT/p11-WT as early at 6 weeks of age. At 8
286 weeks, we observed the formation of multiple small nodules in the distal newer ducts, which then
287 spread extensively to the entire length of mammary gland by 12 weeks of age. In contrast,
288 PyMT/p11-KO mice showed complete absence of hyperplastic tumor foci at 6 weeks, which
289 appeared first at 8 weeks of age, and were restricted to only a fraction of the older ductal structures
290 even at 12 weeks. The most dramatic difference was the complete absence of multiple tumor foci
291 throughout the mammary gland of the PyMT/p11-KO mice at 12 weeks (Fig 1).

292

293 **P11 plays a role in mammary tumor growth and progression**

294 We employed a cohort of 27 PyMT/p11-WT and 28 PyMT/p11-KO mice and monitored the time
295 of appearance and size of palpable tumors. A fraction of the mice was sacrificed at 20 weeks and
296 the total tumor burden was determined. The remaining mice were monitored further until a
297 combined tumor volume of 4,000 mm³ was attained, the humane endpoint of the experiments.

298 The first palpable tumor appeared at 9 weeks in the PyMT/p11-WT mice compared to 11 weeks
299 in the PyMT/p11-KO mice (Fig 2A). At 10 weeks 77% of PyMT/p11-WT and 42% of PyMT/p11-
300 KO mice developed palpable tumors. All the PyMT/p11-WT mice developed palpable tumours by
301 105 days of age (15 weeks), whereas at 20 weeks only 89% of PyMT/p11-KO mice developed
302 tumours (Fig 2B). The tumor volume was dramatically decreased in the PyMT/p11-KO mice with
303 a 4 and 6-fold decrease at 15 and 20 weeks respectively (Fig 2D).

304 Histopathological progression to late carcinoma stage was delayed in PyMT/p11-KO mice (Fig
305 2E). By 10 weeks of age, 83% of the PyMT/p11-WT mice and only 25% of the PyMT/p11-KO
306 had progressed to the early carcinoma stage. By 20 weeks, 82% of the PyMT/p11-WT mice and
307 6.25% of the PyMT/p11-KO had progressed to late carcinoma stage. Interestingly, at this endpoint
308 37.5% of PyMT/p11-KO mice showed normal mammary gland histopathology.

309

310 **Loss of p11 reduces tumor cell proliferation, vascular density, and macrophage infiltration.**

311 The proliferative index of PyMT/p11-WT and PyMT/p11-KO tumors at endpoint (20 weeks) were
312 measured using Ki67 positive immunoreactivity (38). We observed a 2.9-fold reduction in mean
313 Ki67 positive-cells in tumors from PyMT/p11-KO mice suggesting that p11 might function in
314 modulation of tumor cell proliferation (Fig 3A). We did not observe a significant difference in
315 tumor cell apoptosis in the PyMT/p11KO mice (Supplemental Fig 2A). We observed a 6-fold
316 reduction in endothelial cell staining (CD31-positive) in the PyMT/p11-KO tumors compared to
317 PyMT/p11-WT tumors (Fig 3B). In both groups of PyMT mice, the macrophages (F4/80-positive)
318 were restricted to the peripheral area of the tumors. However, there was a significant (11.5-fold)
319 decrease in the macrophage density in the tumors from PyMT/p11-KO mice compared to the
320 PyMT/p11-WT mice (Fig 3C). We also observed a qualitative increase in CD3+ (total T cells)
321 staining in the tumors, but this difference was not statistically significant (Supplemental Fig 2B).

322

323 **Loss of p11 reduced spontaneous and experimental metastasis**

324 We harvested the lungs from 20-week old mice and performed histochemical analysis. We
325 observed a significant 18-fold decrease in metastatic burden and a 14-fold decrease in number of
326 metastatic foci (Fig 4A) in the PyMT/p11-KO mice. Furthermore, the presence of metastatic foci
327 was observed only in 3 of 17 (17.6%) in the PyMT/p11-KO mice compared to 10 of 19 (53%) in
328 the PyMT/p11-WT mice, suggesting an important role for p11 in spontaneous metastasis. Next,
329 we injected the PyMT transformed cell line, Py8119 with WT p11 levels, intravenously (lateral
330 tail vein) in p11-WT and p11-KO mice. We observed a dramatic decrease in the metastatic burden
331 (6-fold) and number of metastatic foci (2.5-fold) in the p11-KO mice (Fig 4B). This result suggests
332 that stromal p11 is important for the extravasation process and establishment of metastases by
333 these breast cancer cells.

334

335 **P11 expression is restricted to the stromal compartment in mammary and pulmonary**
336 **metastatic tumors**

337 To further investigate the expression and localization of p11 in the PyMT tumor and stromal
338 compartment, we performed immunohistochemical analysis of tumors from both PyMT/p11-WT
339 and PyMT/p11-KO cohorts. Interestingly, we observed that the majority of the p11 staining was
340 localized to the stromal compartment in the PyMT/p11-WT mice. Only a fraction (33%) of tumors
341 examined presented with diffuse p11 staining in the tumor cells (Fig 5A).

342 To further evaluate if p11 expression was induced in the metastatic tumor cells, we immunostained
343 lung sections from PyMT/p11-WT mice and observed that the lung tissue but not the metastatic
344 foci showed immunoreactivity for p11. (Fig 5B). This suggested that the tumor cells in the PyMT
345 tumors do not express detectable levels of p11 whereas it is highly expressed in the stromal cells

346 surrounding the tumors. Furthermore, there did not appear to be an induction of p11 in the cancer
347 cells that have left the tumor and metastasized to the lungs.

348 We also examined the lung tumors obtained after tail vein injection of Py8119 cells in p11-WT
349 and p11-KO mice. Surprisingly, we observed that the metastatic foci obtained from the lungs of
350 both genotypes showed strong membranous expression of p11. This was more evident in the p11-
351 KO mice, where expression was restricted to the small tumor nodules and completely absent in the
352 surrounding lung tissues (Fig 5C). We further evaluated p11 expression in both the whole tumor
353 homogenates and Py8119 cells by immunoblotting (Fig 5D and 5F). As anticipated, whole tumor
354 homogenates from these groups also showed p11 expression in the PyMT/p11-WT tumors
355 consistent with some expression in the stroma. Unlike the PyMT cancer cells, we observed that
356 Py8119 tumor cells showed robust p11 expression (Fig 5F).

357 **Loss of p11 does not affect plasminogen activation (or plasmin generation) in PyMT tumors**

358 We previously observed that plasminogen activation is substantially reduced in p11-depleted
359 cancer cells such as colorectal (14), fibrosarcoma (13), pancreatic (16), and lung (15). We
360 measured plasmin generation in tumor homogenates and cell lines isolated from PyMT tumors and
361 did not observe any difference (Fig 5E and Supporting Fig 3B). However, PyMT tumors from p11-
362 WT and p11-KO mice showed a marked increase in plasminogen activation compared to normal
363 mammary glands (Supplemental Fig 3C). These data suggest that p11 does not play a significant
364 role in tumor cell plasmin generation in the PyMT mammary tumors.

365

366 **P11 regulates expression of genes and cytokines affecting tumor progression**

367 To examine how p11 might regulate tumor growth and metastasis genes, we performed microarray
368 gene expression profiling on mammary tumors from PyMT/p11-WT and PyMT/p11-KO mice
369 ($n=3$ per group). We observed that 891 transcripts were elevated and 269 were reduced by more
370 than 1.5-fold (P value < 0.05) in the PyMT/p11-KO tumors (Fig 6A). As anticipated, S100A10
371 expression was decreased by 3.08-fold in the PyMT/p11-KO tumors, supporting the validity of the
372 gene expression data set. Of the 891 transcripts, 331 were annotated genes: 144 genes were
373 downregulated and approximately 187 genes were upregulated in the PyMT/p11-KO mice
374 (Supplemental file1). We chose to validate seven downregulated genes (Aldh1a2, Areg, Ctse,
375 Muc1, S100a8, Thbs1, and Tnc) and two upregulated genes (Cdh19 and Cpm) using reverse
376 transcriptase quantitative PCR (RT-qPCR) ($n=11$ mice per group). We confirmed that
377 amphiregulin (Areg), mucin 1 (Muc1), and S100a8, were downregulated whereas cadherin 19
378 (Cdh 19) was upregulated by qPCR. (Fig 6B). In contrast to the microarray data, Aldh1A2 and
379 Tnc were shown to be upregulated by qPCR (Fig 6B and Supplement Fig 4A).

380 qPCR showed no significant change in the expression of cytokines associated with macrophage
381 recruitment including *Csf1*, *Ccl5*, *Ccr5*, and *Ccl2* (Supplement Fig 4B). Comparison of expression
382 of inflammatory cytokines between the two groups showed significant increases in *Ifn-γ* (3.36-
383 fold), *Il-10* (3.7-fold), *Il-6* (3.7-fold), but no significant changes in *Il-12α*, *Il-4* and *Tnf-α* (Fig 6C,

384 Supporting Fig 4B). Interestingly we observed a 3-fold increase in *Csf2* expression in the
385 PyMT/p11-KO tumors (Fig 6C).

386

387 **P11 is associated with poor clinical outcomes**

388 We employed a well-defined breast cancer patient cohort (n=176) and stratified the patient
389 population into *S100A10* high and low mRNA levels. Kaplan-Meier survival analysis showed that
390 high levels of *S100A10* were significantly associated with both shorter overall survival (HR=3.34,
391 p<0.0001) and recurrence-free cancer (HR=2.27, p<0.001) (Fig 7A-B). We further found that
392 *S100A10* levels were significantly increased in high histologic grade tumors compared with normal
393 mammary tissues (p<0.01) and low grade (p<0.05) tumors (Fig 7C) Among the molecular
394 subtypes, *S100A10* mRNA levels were significantly higher in TN and HER2-enriched breast
395 tumors compared to normal breast tissues. TN tumors showed significantly higher *S100A10* levels
396 than luminal (ER+) tumors (Fig 7D). In addition, tumors with high Ki67 immunoreactivity
397 (percentage of positive cells >15%) showed significantly higher *S100A10* levels than tumors with
398 low Ki67 immunoreactivity ($\leq 15\%$) (Fig 7E).

399 Immunostaining of the NSHA patient cohort followed by semiquantitative scoring of the staining
400 showed no p11 immunostaining (100% negative, H-score = 0) of epithelial cells that formed the
401 normal mammary ducts but observed expression in the stromal areas surrounding the normal duct
402 (Supplement Fig 5A). Based on H-score, we found 25% of low grade (LG), 29.3% of high grade
403 (HG), 37.5% of DCIS, 33% of IDC, 23.8% of ER+, 37.5% Her2+ and 52% of TN tissues/samples
404 were positive for p11 expression. We observed a significant increase in H-score values between
405 normal and LG, and normal and HG, but the difference between LG and HG was not significant
406 (Fig 7F). Similarly, DCIS and IDC tissues/samples/patients showed significantly higher H-score
407 values compared to normal tissues. But there was no difference in p11 expression between DCIS
408 and IDC patients, suggesting that p11 expression does not correlate with invasive progression (Fig
409 7G and supplemental Fig 5). We observed the most dramatic increase in p11 staining between
410 normal tissue and TN tumors (p-value<0.0001), consistent with the highest mean H-score value
411 for the TN tumors. We also noted significant increase between normal and ER+, and normal and
412 Her2+ tumors, but there was no significant increase between ER+ and Her2+ tumors. Interestingly,
413 statistical comparison of ER+ and TN tumors showed increase in TN with respect to ER+ although
414 the *P value* (0.0511) was only approaching significance (Fig 7H and supplemental Fig 5).

415

416 **Discussion**

417 The loss of components of the plasmin-plasminogen system decreased pulmonary metastasis but
418 had no effect on tumor onset or growth in the PyMT model (reviewed in (4)). Interestingly, in this
419 study we observed a dramatic delay in tumor onset, growth, and progression to malignancy in the
420 PyMT/p11KO mice. These results suggest a complex role for p11 in breast cancer oncogenesis
421 beyond the regulation of plasmin generation.

422 We observed a dramatic decrease in metastatic burden (18-fold) and metastatic foci (14-fold) in
423 PyMT/p11-KO mice. The histopathological progression to late carcinoma stage was delayed in
424 PyMT/p11KO mice (Fig 2E), potentially attributing the decrease in metastases to the delayed
425 development of malignancy. However, pulmonary metastasis in the PyMT model is an early event
426 and independent of tumor size (39,40). Interestingly, metastases formed by injection of the PyMT
427 transformed cell line, Py8119, into the p11-KO mice were dramatically reduced, suggesting that
428 the p11-deficient stroma is a less favorable environment for the establishment of metastases.

429 Our results are consistent with our previous work showing that the growth of Lewis lung
430 carcinomas or T241 fibrosarcomas were dramatically reduced in p11-deficient mice compared
431 with wild-type mice (19) and that the tumor growth deficit corresponded with a decrease in
432 macrophage and endothelial cell density. Tumor-associated macrophages (TAMs) significantly
433 contribute to tumor progression, angiogenic switch, and metastasis (41,42), with loss of
434 peritumoral TAMs resulting in delayed tumor progression (43). Therefore, it is likely that a portion
435 of the requirement of p11 for PyMT-driven tumor growth and metastasis is due to the function of
436 p11 in macrophage recruitment.

437 We have previously reported that p11 is responsible for majority of the plasmin generation in
438 various cancer cell lines (5,15,16,44). However, in the present study we were unable to detect any
439 differences in plasmin generation between cancer cells or tumor homogenates isolated from the
440 PyMT/p11-KO and PyMT/p11-WT mice. However, we observed that PyMT tumors from p11-
441 WT and p11-KO mice showed a marked increase in plasmin generation compared to normal
442 mammary glands (Supplemental Fig 3D), suggesting compensation by other plasminogen
443 receptors in this model. It is likely that distant peripheral macrophages utilize plasminogen
444 receptors such as p11 to migrate and invade the tumors (19,45,46). Furthermore, we detected weak
445 p11 expression in some of the tumor cells and no expression in metastatic nodules of PyMT/p11-
446 WT tumors. In contrast, lung tumors obtained after tail vein injection of Py8119 cells in p11-WT
447 and p11-KO mice showed robust p11 expression. Overall, the exclusive expression of p11 in the
448 stroma *in vivo* parallels the stromal localization of other components of the plasminogen activation
449 system including uPA, uPAR, tPA and PAI-1 in mouse (PyMT) and human models (4). The lack
450 of p11 expression in the PyMT-expressing tumor cells and restriction of the majority of p11
451 expression to the stromal compartment were striking observations in the current study. The middle
452 T (MT) region of PyMT is an effective oncogene and activates the PI3 kinase signaling pathway
453 (47,48). We recently showed that active PI3K signaling decreased p11 expression in several tumor
454 cells via the FOXC2 transcription factor (15). In addition *in vitro* observations indicate a distinct
455 rewiring of signaling in 2-D cell cultures resulting in increased p11 expression potentially due to
456 derepression of tumor-stroma signaling events observed *in vivo* (49,50). Furthermore, the retention
457 of p11 expression in the Py8119 tumor cells in the lung metastatic nodules, suggests the absence
458 of inhibitory signals in the lung microenvironment.

459 Whole mount analysis of mammary glands indicated a decrease in multifocal dysplastic lesions as
460 early as 6–8 weeks in the PyMT/p11-KO mice (Fig 1). An interesting observation was the
461 reduction in length of the ductal branches in the PyMT/p11-KO mammary glands at the early time
462 points of 6 and 8 weeks, and was comparable to the WT at 10 and 12 weeks (Supplemental Fig 1).

463 Other groups have shown that infiltration of macrophages is required for early mammary gland
464 development (51). It is likely that defective macrophage migration observed with p11-KO mice
465 causes early defects in ductal branching in the mammary gland. This suggests that p11 may play a
466 potential role during early stages of mammary gland development. More recently, another
467 plasminogen receptor, Plg-Rkt, was shown to be critical for lactogenesis and mammary
468 lobuloalveolar development (52).

469 qPCR validation of microarray gene data confirmed the significant downregulation of AREG,
470 MUC1, and S100A8 genes in PyMT/p11-KO tumors. AREG (amphiregulin), a player in breast
471 cancer proliferation, is abundant in the pubertal mammary gland and loss of AREG in mice results
472 in stunted ductal morphogenesis. Our observation of decreased early mammary ductal
473 morphogenesis might be due to the loss of AREG expression (53). Loss of transmembrane
474 glycoprotein MUC1 results in significant delay in tumor progression and metastasis (54),
475 consistent with delayed progression to malignancy displayed by PyMT/p11-KO tumors. S100A8
476 is elevated in ER- and Her2+ subtypes of breast cancer and contributes towards cancer cell survival
477 and metastasis (55), thereby contributing to delayed progression of PyMT/p11-KO tumors. It is
478 unclear whether p11 plays a direct or indirect role in regulating these genes *in vivo*, or whether the
479 alteration of these genes are markers of tumor growth and progression.
480

481 We also compared the levels of inflammatory modulators in PyMT/p11WT and PyMT/p11-KO
482 tumors that might impact macrophage infiltration. The CSF-1 gene, implicated in the proliferation,
483 differentiation and recruitment of macrophages in breast cancer (37) was unchanged. We saw a
484 significant increase in CSF-2 expression in PyMT/p11-KO tumors but CSF-2 has not been shown
485 to correlate with macrophage infiltration in breast tumors. We also observed a significant increase
486 in cytokines such as *Ifn-γ*, *Il-6* and *Il-10*. *Il-6* positively correlates with breast cancer progression
487 and development of metastasis (56). Studies on the role of *Il-10* in breast cancer showed
488 contradicting results (56). *Ifn-γ* plays an important role in antitumor immunity and tumor
489 suppressive phenotype via the JAK/STAT pathway. It is possible that the tumor suppressive
490 phenotype observed in the PyMT/p11-KO mice is ascribed to the increase in tumor suppressive
491 cytokines such as *Il-10* and *Ifn-γ*.

492 Previous gene expression studies showed that p11 correlated to poor overall survival in basal-like
493 breast cancer (reviewed in (57)). In elucidating the prognostic role of p11 in human breast cancer,
494 we found that p11 mRNA was overexpressed in human breast tumors, correlated positively with
495 overall survival and recurrence-free survival, and was increased in high grade tumors compared to
496 low grade tumors and normal tissue. P11 was highly expressed in TN breast cancer, ER+ and
497 HER2+ suggesting a role in breast cancer progression. Interestingly, expression of p11 protein was
498 significantly elevated in tumor cells compared to normal mammary epithelium, but we did not
499 observe any correlation between high p11 expression and clinical and pathological tumor grade or
500 with molecular subtype in human breast cancer samples. The lack of consistency between our gene
501 expression profiling and immunohistochemistry data could be due to two reasons. First, microarray
502 analysis was performed in tumors containing stroma making the increased p11 expression a
503 contribution of the tumor microenvironment. Second, we conducted p11 immunostaining only in
504 a small cohort of patient samples with a small sample size for ER+ and HER2+ tumors. Future
505 studies will be aimed at increasing the sample size for IHC or by using a Tissue Microarray

506 analysis and evaluating the expression pattern in the stroma. Overall p11 expression was
507 completely restricted to the stromal cells in the PyMT mouse model, whereas expression was seen
508 in human tumor cells. This difference can potentially be attributed to the nature of oncogene and
509 mutations between the murine and human breast tumors.

510 **Conclusion**

511 Our current study is the first comprehensive study using a transgenic mouse model to examine the
512 role of p11 in breast cancer. These results demonstrated that p11 plays a causal, complex, and
513 definitive role in breast tumor development, progression, and metastasis, possibly via p11-
514 dependent macrophage migration and tumor infiltration. Surprisingly, our results suggest that
515 stromal and not cancer cell p11 is crucial for mediating these effects specifically in the PyMT
516 model. Since the majority of p11 was expressed by the stromal cells in the tumor periphery, our
517 study also highlights the importance of tumor-stroma interactions and signaling for breast tumor
518 progression. Further studies are required to conclusively demonstrate that p11 can be employed as
519 a biomarker with diagnostic and prognostic value in breast cancer.

520 **Availability of data and materials**

521 All data collected has been presented in the manuscript and supporting information and is available
522 upon request.

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555 **Contributions**

556 Conceptualization: AGB and DMW. Methodology: AGB, MLD, RZL, PC, RWH, LNT, PAM,
557 CKLT, PJB, RG, PM and DMW. Formal analysis: AGB, MLD, RZL and DMW. Investigation:
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559 Funding acquisition: DMW, PAM, CKLT, PJB, RG, PM. The author(s) read and approved the
560 final manuscript.

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562 **Ethics declarations**

563 All animal experiments were performed according to protocol approved by University
564 Committee on Laboratory Animals, Dalhousie University, Canada.

565 **Ethics approval and consent to participate**

566 Patient material and clinical information were collected under Research Ethics Board Protocol
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568 **Consent for publication**

569 Not applicable

570 **Competing interests**

571 The authors declare that they have no conflicts of interest with the contents of this article.

572

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734 **Figure Legends**

735 **Fig 1: Loss of p11 affects formation of hyperplastic lesions in PyMT driven tumors.** Whole
736 mammary glands from PyMT/p11-WT (n=3) and PyMT/p11-KO mice at (8, 10, 12 weeks) were
737 excised, fixed, and stained with Carmine Alum as per standard protocols (*See Materials and*
738 *Methods*). Representative images from the 4th abdominal mammary gland from each group and
739 time point is shown.

740 **Fig 2: Tumor initiation, growth, burden, and progression are decreased and delayed in**
741 **PyMT/p11-KO mice.** (A) Appearance of palpable tumors was monitored weekly. (B) Percent
742 tumor-free mice was calculated using Kaplan-Meir analysis (Hazard ration – 2.24, *P value* –
743 0.0004). (C) Tumor volume was measured weekly using calipers. Total volume was plotted to
744 represent tumor growth rate. (D) Total tumor weight at endpoint (20 weeks) was determined as a
745 percentage of body weight. Data is a compilation of three independent experiments, except (D)
746 which is data from one independent experiment. Significance was determined by non-parametric
747 t-test (Mann Whitney U test – unpaired, non-parametric). (E) Mammary glands and tumors from
748 PyMT/p11-WT and PyMT/p11-KO mice were harvested at 20 weeks, formalin-fixed, embedded,
749 and sectioned at 5 μm thickness. The H&E and smooth muscle α -actin-stained sections were
750 classified into histopathological stages by a pathologist in a blinded manner. (n= 6 to 12 mice in
751 each group). Statistical analysis was performed by χ^2 - test (*P value* < 0.0001).

752 **Fig 3: PyMT/p11-KO mice tumors show reduced proliferation vascular density and**
753 **macrophage infiltration.** Formalin-fixed, paraffin-embedded and sectioned tissues from
754 PyMT/p11-WT (n=11) and PyMT/p11-KO (n=10) (end-point, 20 weeks) mice were
755 immunostained for (A) Ki67 (proliferation marker), (B) CD31 (endothelial marker) and (C) F4/80
756 (mouse macrophage marker). (A) Immunostaining using anti-rabbit Ki67 antibody (Abcam).
757 Stained sections were imaged using Zeiss Zeiss Axio Imager Z1 W/color and monochrome camera
758 at 10X magnification. *Left panel:* The number of Ki67-positive cells was manually counted using
759 Zen (2012) software in 7-10 random fields per tissue section/mouse. Significance was determined
760 using Mann Whitney U test (unpaired, non-parametric), *P value* is 0.0021. *Right panel:* The
761 representative image was captured at 20X magnification. Scale bar – 100 μm . (B) Immunostaining
762 using anti-rabbit CD31 antibody (Abcam). Stained sections were imaged using Aperio Scanning
763 system (Leica Biosystems, Concord, Ontario) at 40X magnification. *Left panel:* The number of
764 CD31-positive cells were manually counted using Imagescope software in 7-10 random fields per
765 tissue section/mouse. Mann Whitney U test (unpaired, non-parametric) shows *P value* < 0.0001.
766 *Right panel:* Representative image at 20X magnification. Scale bar – 100 μm . (C) Immunostaining
767 using anti-rat F4/80 antibody (BM8, ThermoFisher Scientific). Stained sections were imaged as in
768 (A), but at 40X magnification. *Left panel:* The number of F4/80-positive cells were manually
769 counted as in (A). Mann Whitney U test (unpaired, non-parametric) shows *P value* < 0.0001. *Right*
770 *panel:* Representative image at 20X magnification. Scale bar – 100 μm .

771 **Fig 4: Metastasis is diminished in PyMT/p11-KO mice.** (A) We evaluated pulmonary metastasis
772 at 20-week end point in (spontaneous model) PyMT/p11-WT and PyMT/p11-KO mice by
773 microscopic examination of formalin-fixed, H&E-stained lung sections (5 μm). Three lung
774 sections with each 100 μm apart were used for staining. Quantification was performed by Aperio
775 image analysis software (Imagescope). Mean values for three sections were used to calculate the
776 metastatic burden and foci values. (A) Metastatic burden – calculated the total metastatic area and
777 normalized to total lung area (A- *upper left panel*). Mann Whitney U test show statistical
778 significance with *P value* of 0.0062. The number of metastatic foci per mouse lung section was
779 determined by manual counting of imaged sections (A- *upper right panel*). Mann Whitney U test
780 show *P value* of 0.0069. *Lower panels:* representative lung images from WT and KO mice. (B)
781 Experimental metastasis assay. We injected 2.5×10^5 Py8119 (p11-WT levels) cells into p11-WT

782 and p11-KO mice (n=12 mice per group). The lungs were harvested after 14 days, formalin-fixed,
783 and sectioned at 5 μ m as described above. Mann Whitney non-parametric t- test for statistical
784 significance was performed. Metastasis was pooled and combined from two independent
785 experiments (n=6 each). *P value* < 0.0001. *Lower panels*: representative lung images from WT
786 and KO mice.

787 **Fig 5: Expression of p11 in mammary and pulmonary metastatic tumors is restricted to the**
788 **stromal compartment in the PyMT/p11-WT tumors.** Immunohistochemical staining was
789 performed using anti-rabbit p11 antibody (Proteintech), on 5 μ m sections from 20-week end-
790 point PyMT/p11-WT (n=10) and PyMT/p11-KO tumors (n=3), and lungs from spontaneous and
791 experimental metastasis assay. (A) Representative images of p11 immunostained sections of
792 PyMT mammary tumors. As anticipated the tumors from PyMT/p11-KO mice showed no
793 staining, validating the specificity of the antibody. (B) PyMT spontaneous lung metastasis from
794 PyMT/p11-WT and PyMT/p11-KO mice, and (C) experimental metastasis of Py8119 cells
795 injected in p11-WT (n=4) and p11-KO (n=4) mice are shown. Scale bar is 100 μ m. (D) Western
796 blot of p11 expression in total cell tumor homogenates from PyMT/p11-WT (n=3) and
797 PyMT/p11-KO mice (n=3). (E) Fresh and frozen tumors from both PyMT/p11-WT (n=6 mice)
798 and PyMT/p11-KO (n=5 mice) were homogenized and equal protein (30-60 μ g) were used for
799 plasmin generation assay as described in *Materials and Methods and supplemental section*.
800 Mann Whitney U (unpaired, non-parametric) t- test show *P value* of 0.3290). NS, not significant
801 (F) Western blot for p11 expression in total cell lysates in Py8119 cells obtained from ATCC
802 (n=4) using goat anti-mouse antibody (R and D systems).

803 **Fig 6: Tumor transcriptome of PyMT/p11-KO mice suggest the downregulation of tumor**
804 **promoting genes.** Gene expression profiling of PyMT/p11-WT and PyMT/p11-KO tumors were
805 performed (n=3 mice per group). (A) Volcano plot of significantly *P value* < 0.05) upregulated and
806 downregulated genes in the PyMT/p11 KO mice (n=3 mice in each group). The top 6 genes are
807 highlighted. Genes which were up- or down-regulated more than 2-fold ($\log_2 = 0.678$) at a
808 significance level of *P value* < 0.05 were considered differentially expressed. (B) Validation of the
809 top 9 differentially expressed genes using quantitative RT-PCR (10-11 mice per group).
810 Significance was determined by unpaired t- test. Only the genes significantly altered are shown.
811 (C) **Loss of p11 in PyMT tumors results in differential cytokine expression profile.** We
812 performed quantitative RT-PCR on tumors isolated from PyMT/p11-WT and PyMT/p11KO mice
813 (10-11 mice per group), using a CFX96 or CFX384 Touch Real-Time PCR Detection system
814 (BioRad). Relative mRNA expression was log-2 transformed prior to plotting and statistical
815 analysis. Significance was determined by unpaired, t- test. Only those cytokines significantly
816 altered are shown.

817

818 **Fig 7: Expression of S100A10 (p11) expression in breast cancer patients.** Treatment-naïve
819 primary breast cancer samples (n=176) were obtained through the CBCF Tumor Bank and used
820 for gene expression analysis. *S100A10* high and low mRNA levels were based on the signal
821 intensity from our gene expression microarray profile using the Receiver Operating Characteristic
822 (ROC) curve analysis. Correlation of *S100A10* (p11) mRNA with (A) overall survival probability.
823 High mRNA levels of p11 gene (p11) are significantly associated with poor patient overall survival

824 (hazard ratio of 3.34). (B) Recurrence free survival, with hazard ratio 2.27. (C) For histological
825 tumor grade, p11 is significantly upregulated in high grade tumors compared to normal breast and
826 low-grade tumor tissues. (D) For molecular subtype, p11 is significantly up-regulated in HER2+
827 and triple negative breast cancers compared to normal breast tissues. (E) In breast cancer patients,
828 p11 mRNA levels are significantly higher in tumors with high Ki67 immunoreactivity. **S100A10**
829 (**p11**) **protein is overexpressed in breast tumors compared to normal mammary tissues.** (F,
830 G, H) Immunohistochemical staining of p11 in normal breast tissues and in low grade (LG), high
831 grade (HG), ductal carcinoma in situ (DCIS), IDC/Invasive, ER+, Her2+ and Triple negative (TN)
832 tumors was performed using anti-p11 antibody (Proteintech). These human tissues were obtained
833 from Queen Elizabeth II Health Sciences Centre, Nova Scotia Health Authority (*see Materials and*
834 *Methods*). The stained sections were scored (semi-quantitative) based on percent positive tumor
835 cells and intensity of staining by a pathologist (blinded). H score was determined based on the
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837 clinical/histological grade, (G) pathological grade, and (H) molecular subtype. Statistical analysis
838 was performed by Mann Whitney U test (unpaired, non-parametric t-test).

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Figures

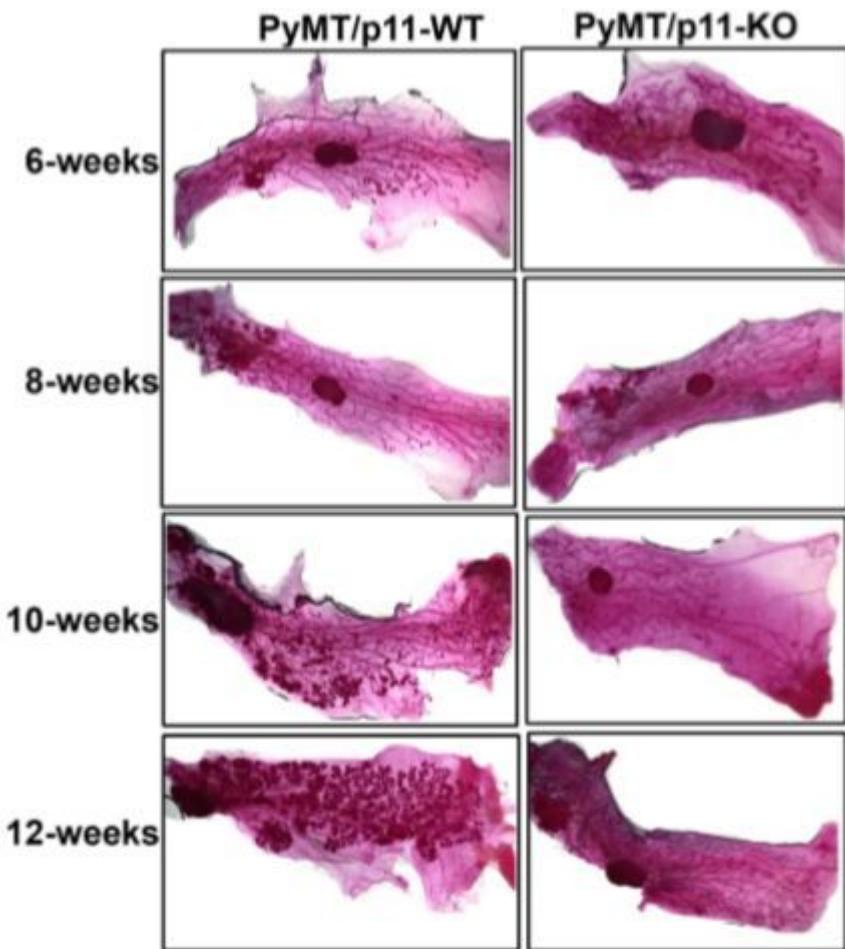


Figure 1

Loss of p11 affects formation of hyperplastic lesions in PyMT driven tumors. Whole mammary glands from PyMT/p11-WT ($n=3$) and PyMT/p11-KO mice at (8, 10, 12 weeks) were excised, fixed, and stained with Carmine Alum as per standard protocols (See Materials and Methods). Representative images from the 4th abdominal mammary gland from each group and time point is shown.

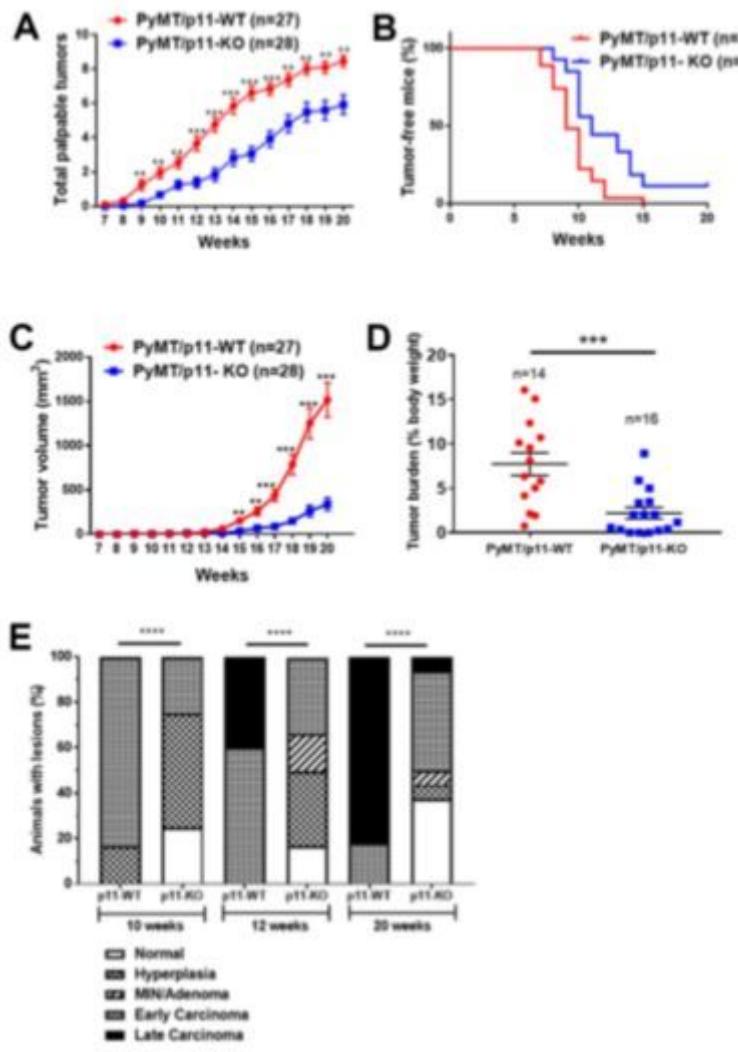


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Tumor initiation, growth, burden, and progression are decreased and delayed in PyMT/p11-KO mice. (A) Appearance of palpable tumors was monitored weekly. (B) Percent tumor-free mice was calculated using Kaplan-Meir analysis (Hazard ration – 2.24, P value –0.0004). (C) Tumor volume was measured weekly using calipers. Total volume was plotted to represent tumor growth rate. (D) Total tumor weight at endpoint (20 weeks) was determined as a percentage of body weight. Data is a compilation of three independent experiments, except (D) which is data from one independent experiment. Significance was determined by non-parametric t-test (Mann Whitney U test – unpaired, non-parametric). (E) Mammary glands and tumors from PyMT/p11-WT and PyMT/p11-KO mice were harvested at 20 weeks, formalin-fixed, embedded, and sectioned at 5 μ m thickness. The H&E and smooth muscle α -actin-stained sections were classified into histopathological stages by a pathologist in a blinded manner. (n= 6 to 12 mice in each group). Statistical analysis was performed by χ^2 - test (P value < 0.0001).

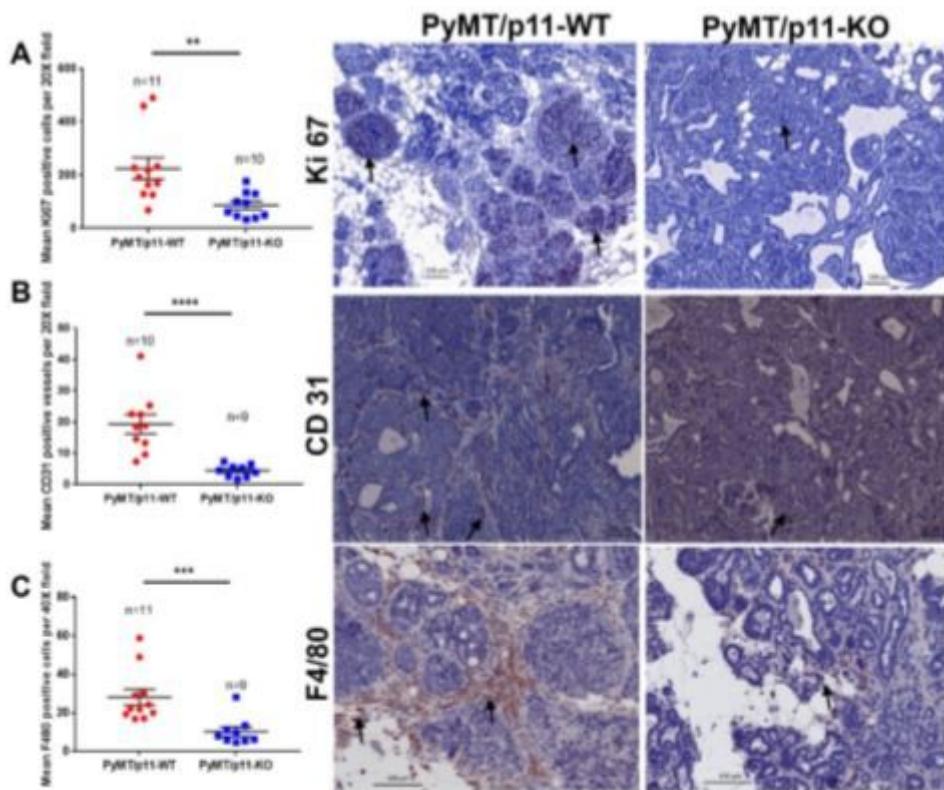


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PyMT/p11-KO mice tumors show reduced proliferation vascular density and macrophage infiltration. Formalin-fixed, paraffin-embedded and sectioned tissues from PyMT/p11-WT ($n=11$) and PyMT/p11-KO ($n=10$) (end-point, 20 weeks) mice were immunostained for (A) Ki67 (proliferation marker), (B) CD31 (endothelial marker) and (C) F4/80 (mouse macrophage marker). (A) Immunostaining using anti-rabbit Ki67 antibody (Abcam). Stained sections were imaged using Zeiss Axio Imager Z1 W/color and monochrome camera at 10X magnification. Left panel: The number of Ki67-positive cells was manually counted using Zen (2012) software in 7-10 random fields per tissue section/mouse. Significance was determined using Mann Whitney U test (unpaired, non-parametric), P value is 0.0021. Right panel: The representative image was captured at 20X magnification. Scale bar – 100 μ m. (B) Immunostaining using anti-rabbit CD31 antibody (Abcam). Stained sections were imaged using Aperio Scanning system (Leica Biosystems, Concord, Ontario) at 40X magnification. Left panel: The number of CD31-positive cells were manually counted using Imagescope software in 7-10 random fields per tissue section/mouse. Mann Whitney U test (unpaired, non-parametric) shows P value < 0.0001. Right panel: Representative image at 20X magnification. Scale bar – 100 μ m. (C) Immunostaining using anti-rat F4/80 antibody (BM8, ThermoFisher Scientific). Stained sections were imaged as in (A), but at 40X magnification. Left panel: The number of F4/80-positive cells were manually counted as in (A). Mann Whitney U test (unpaired, non-parametric) shows P value < 0.0001. Right panel: Representative image at 20X magnification. Scale bar – 100 μ m.

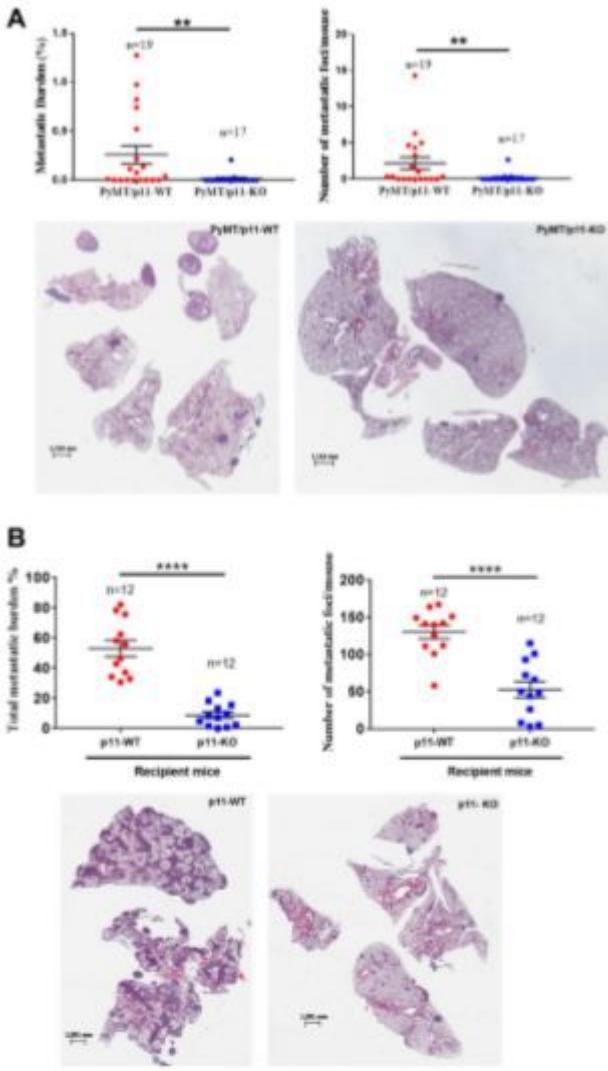


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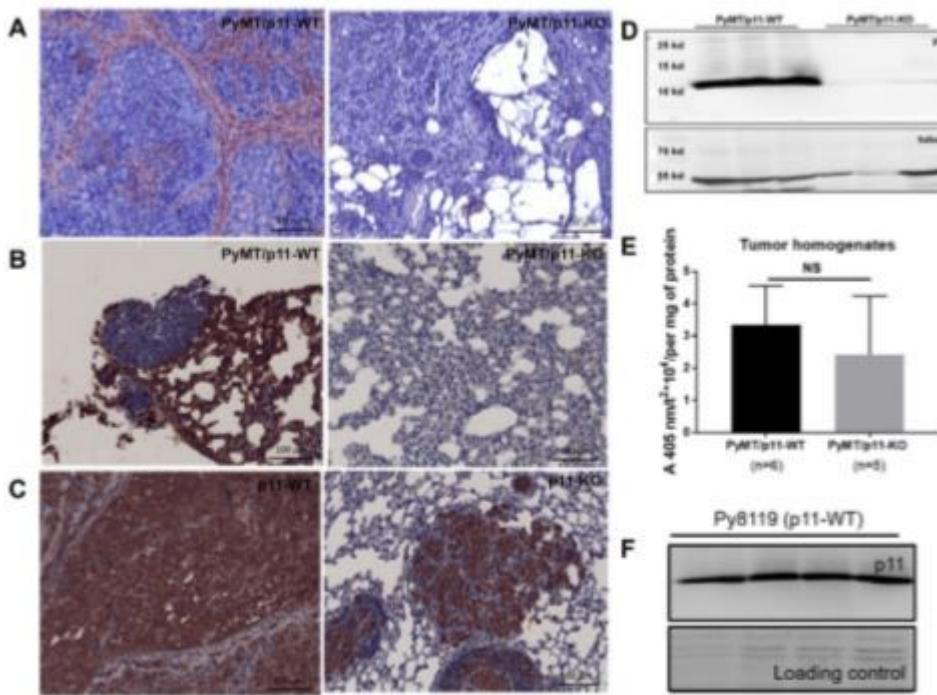


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Expression of p11 in mammary and pulmonary metastatic tumors is restricted to the stromal compartment in the PyMT/p11-WT tumors. Immunohistochemical staining was performed using anti-rabbit p11 antibody (Proteintech), on 5 μ m sections from 20-week end-point PyMT/p11-WT (n=10) and PyMT/p11-KO tumors (n=3), and lungs from spontaneous and experimental metastasis assay. (A) Representative images of p11 immunostained sections of PyMT mammary tumors. As anticipated the tumors from PyMT/p11-KO mice showed no staining, validating the specificity of the antibody. (B) PyMT spontaneous lung metastasis from PyMT/p11-WT and PyMT/p11-KO mice, and (C) experimental metastasis of Py8119 cells injected in p11-WT (n=4) and p11-KO (n=4) mice are shown. Scale bar is 100 μ m. (D) Western blot of p11 expression in total cell tumor homogenates from PyMT/p11-WT (n=3) and PyMT/p11-KO mice (n=3). (E) Fresh and frozen tumors from both PyMT/p11-WT (n=6 mice) and PyMT/p11-KO (n=5 mice) were homogenized and equal protein (30-60 μ g) were used for plasmin generation assay as described in Materials and Methods and supplemental section. Mann Whitney U (unpaired, non-parametric) t-test show P value of 0.3290). NS, not significant (F) Western blot for p11 expression in total cell lysates in Py8119 cells obtained from ATCC (n=4) using goat anti-mouse antibody (R and D systems).

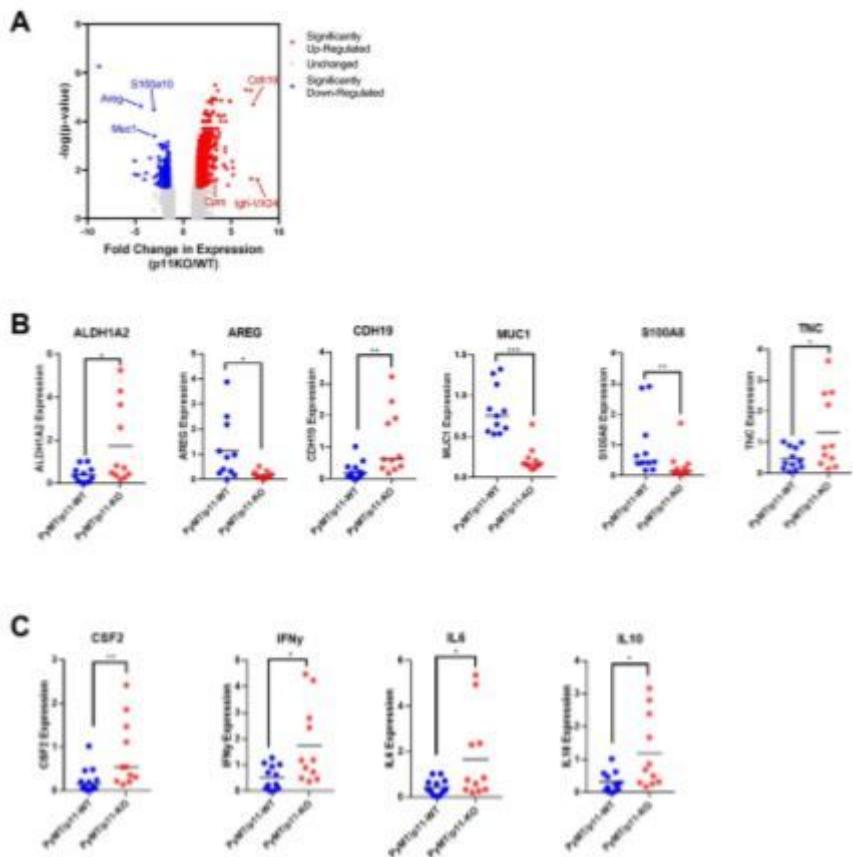


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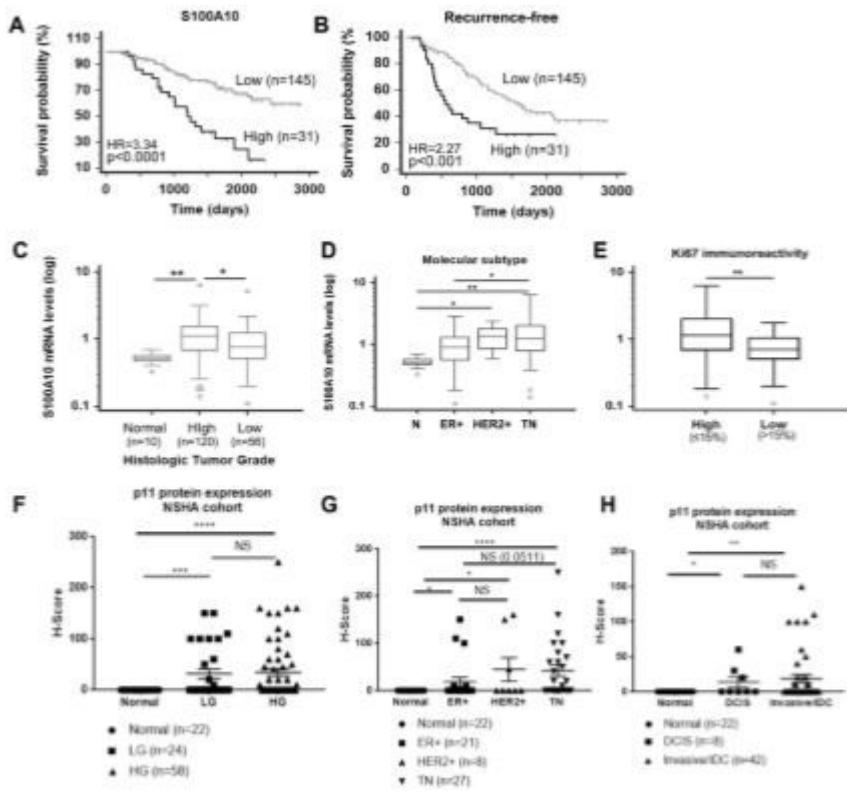


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