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Taspase1 cleaves MLL1 to activate cyclin E for HER2/neu breast tumorigenesis

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Taspase1, a highly conserved threonine protease, cleaves nuclear transcriptional regulators mixed-lineage leukemia (MLL, MLL1), MLL2, TFIIA, and ALF to orchestrate a wide variety of biological processes. *In vitro* studies thus far demonstrated that Taspase1 plays important roles in the proliferation of various cancer cell lines, including HER2-positive breast cancer cells. To investigate the role of Taspase1 in breast tumorigenesis *in vivo*, we deleted Taspase1 from mouse mammary glands by generating *MMTV-neu;MMTV-cre;Tasp1*$^{F/-}$ mice. We demonstrate that initiation of *MMTV-neu*- but not *MMTV-wnt-driven* breast cancer is blocked in the absence of Taspase1. Importantly, *Taspase1* loss alone neither impacts normal development nor pregnancy physiology of the mammary gland. In mammary glands *Taspase1* deficiency abrogates *MMTV-neu*-induced cyclins E and A expression, thereby preventing tumorigenesis. The mechanisms were explored in HER2-positive breast cancer cell line BT474 and HER2-transformed MCF10A cells and validated using knockdown-resistant Taspase1. As Taspase1 was shown to cleave MLL which forms complexes with E2F transcription factors to regulate *Cyclins E*, *A*, and *B* expression in mouse embryonic fibroblasts (MEFs), we investigated whether the cleavage of MLL by Taspase1 constitutes an essential *in vivo* axis for HER2/neu-induced mammary tumorigenesis. To this end, we generated *MMTV-neu;MLL*$^{nc/nc}$ transgenic mice that carry homozygous non-cleavable *MLL* alleles. Remarkably, these mice are also protected from HER2/neu-driven breast tumorigenesis. Hence, MLL is the primary Taspase1 substrate whose cleavage is required for *MMTV-neu*-induced tumor formation. As Taspase1 plays critical roles in breast cancer pathology, it may serve as a therapeutic target for HER2-positive human breast cancer.

***Keywords*:** Taspase1; MLL; cyclin E; HER2; breast cancer
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Introduction

Human breast cancer is a heterogeneous disease comprised of three major subgroups, each encompassing unique molecular signatures, prognoses, and responses to therapies [1]. HER2 is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, which includes EGFR, HER2, HER3, and HER4 [2, 3]. Homo- or hetero-dimerization of these receptors results in the phosphorylation of residues in the intracellular domain and the consequent recruitment of adapter molecules responsible for the initiation of several signaling pathways involved in cell proliferation and survival. HER2-amplified/overexpressed breast cancer is one of the aforementioned subgroups. It accounts for approximately 20% - 30% of all breast cancer cases, and it is characterized by an aggressive phenotype and poor overall survival [4-6]. Despite therapeutic advances brought forth by anti-HER2 agents including monoclonal antibodies such as trastuzumab (Herceptin) and small molecule tyrosine kinase inhibitors, patients with advanced HER2-positive breast cancer frequently experience disease progression and/or recurrence [7-9].

One of the hallmarks of cancer is dysregulated prolif-

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eration [10]. Unsurprisingly, molecular characterization of human tumors reveals that key cell cycle regulators are frequently dysregulated [11]. During cell cycle progression, cyclin-dependent kinases (CDKs) and cyclins constitute the central regulatory apparatus. In mammalian cells, kinase subunits (CDK4, CDK6, CDK2, and CDC2) are expressed alongside cyclins (cyclin D, E, A, and B) sequentially as the cells progress from G1 through mitosis. CDK4 and CDK6 form complexes with one of several D-type cyclins and function early in G1 phase, probably in response to growth factors. CDK2 forms complexes with cyclins E or A and functions in the G1/S phase transition and S phase DNA replication [12-14]. A major molecular consequence of HER2 up-regulation is the increased expression of G_1-to-S cell cycle regulatory proteins cyclins D and E, which leads to aberrant cell proliferation [7, 12-16]. High cyclin E expression is a marker that correlates strongly with poor outcome in patients with breast cancer. Since *Cyclin E* amplification/overexpression leads to trastuzumab resistance, disrupting *Cyclin E* expression could have therapeutic importance for HER2-positive breast cancers [17, 18].

Taspase1 was originally purified as the protease that cleaves MLL (the Mixed-Lineage Leukemia protein; also known as MLL1) for proper regulation of *HOX* gene expression [19, 20]. Other genetically and biochemically proven Taspase1 substrates include MLL2 (also known as MLL4), TFIIAα-β, ALFα-β (TFIIA-Like Factor) and *Drosophila* HCF-1 (Host Cell Factor 1) [20-24]. Interestingly, all confirmed Taspase1 substrates are nuclear transcription factors that play important roles in gene regulation. *Taspase1* encodes a highly conserved 50 kDa α-β proenzyme, which undergoes intramolecular autoproteolysis, producing the mature α28/β22 heterodimeric enzyme that displays an overall α/β/β/α structure [20, 25]. A complete genetic knockout of *Taspase1* in mice resulted in profound early postnatal lethality and the few surviving *Taspase1*$^{-/-}$ mice universally exhibited small body sizes and homeotic transformations at the axial skeleton [22]. *Taspaes1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) displayed cell cycle progression defects with downregulation of *cyclins E*, *A*, and *B* and upregulation of *CDKIs* (cyclin-dependent kinase inhibitors) *p16*, *p21* and *p27* [22]. Importantly, *Taspase1*$^{-/-}$ MEFs were resistant to oncogenic transformation *in vitro* [26]. In MEFs, Taspase1 cleaves MLL that interacts with E2Fs, core transcription factors of the mammalian cell cycle, to activate select *Cyclin* genes [22, 27, 28]. How Taspase1 regulates *CDKIs*, however, remains unclear. Importantly, Taspase1 shows a high level of expression in most human cancer cell lines [22], and knockdown of Taspase1 in many cancer cell lines impairs cancer cell proliferation and even sensitizes brain cancer and melanoma cells to anoikis [26].

Encouragingly, despite the fact that Taspase1 plays an important role in mammalian embryogenesis, acute genetic deletion of Taspase1 in adult mice does not confer discernible toxicities on the mice, which suggests a wide therapeutic index for Taspase1 inhibition in adult cancer patients [29]. Moreover, pharmacological inhibition of Taspase1 has been attempted [29, 30], and a primitive small molecular Taspase1 inhibitor (TASPIN) showed effects on U251 brain tumor xenografts and HER2-driven mouse breast cancers [29]. The latter findings prompted us to hypothesize that Taspase1 could play a critical role in HER2-positive breast cancer and that Taspase1 inhibitors may be developed as a safe treatment option for Taspase1-dependent cancers.

Here, we report the data from preclinical experiments that we conducted by constructing genetically well-defined mouse models, demonstrating that Taspase1 ablation blocks MMTV-neu-driven breast cancer initiation *in vivo*. We further pinpoint that MLL is the key Taspase1 substrate whose cleavage is required for *MMTV-neu*-induced tumor formation. The cleavage of MLL by Taspase1 enables HER2/neu-induced overexpression of *Cyclins E* and *A*, presenting an essential *in vivo* genetic network conferring breast tumorigenesis.

Results

Taspase1 deficiency disrupts the expression of cyclins and proliferation of HER2^{+} *breast cancer cells*

To determine whether Taspase1 is required for HER2-positive breast cancer cell proliferation, we conducted genetic knockdown experiments in two HER2-overexpressing breast cancer cell lines, BT474 and HCC1419. Taspase1 deficiency significantly reduced the cell number in both cell lines (Figure 1A). Cell death assay confirmed that there is no significant difference in cell death between the Taspase1 knockdown cells and the control in either cell line (Figure 1B). On the other hand, cell cycle analysis showed that Taspase1 knockdown significantly decreased the S phase population in both cell lines (Figure 1C). These data suggest that Taspase1 regulates HER2-positive breast cancer cell proliferation through promoting cell cycle progression.

We next investigated the underlying mechanisms by which Taspase1 regulates cell division. Key regulators of the mammalian cell cycle machinery include E2Fs, Rbs, cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs), which form complex positive and negative epistatic regulatory loops to ensure accurate cell cycle progression. In MEFs, following the cleav-

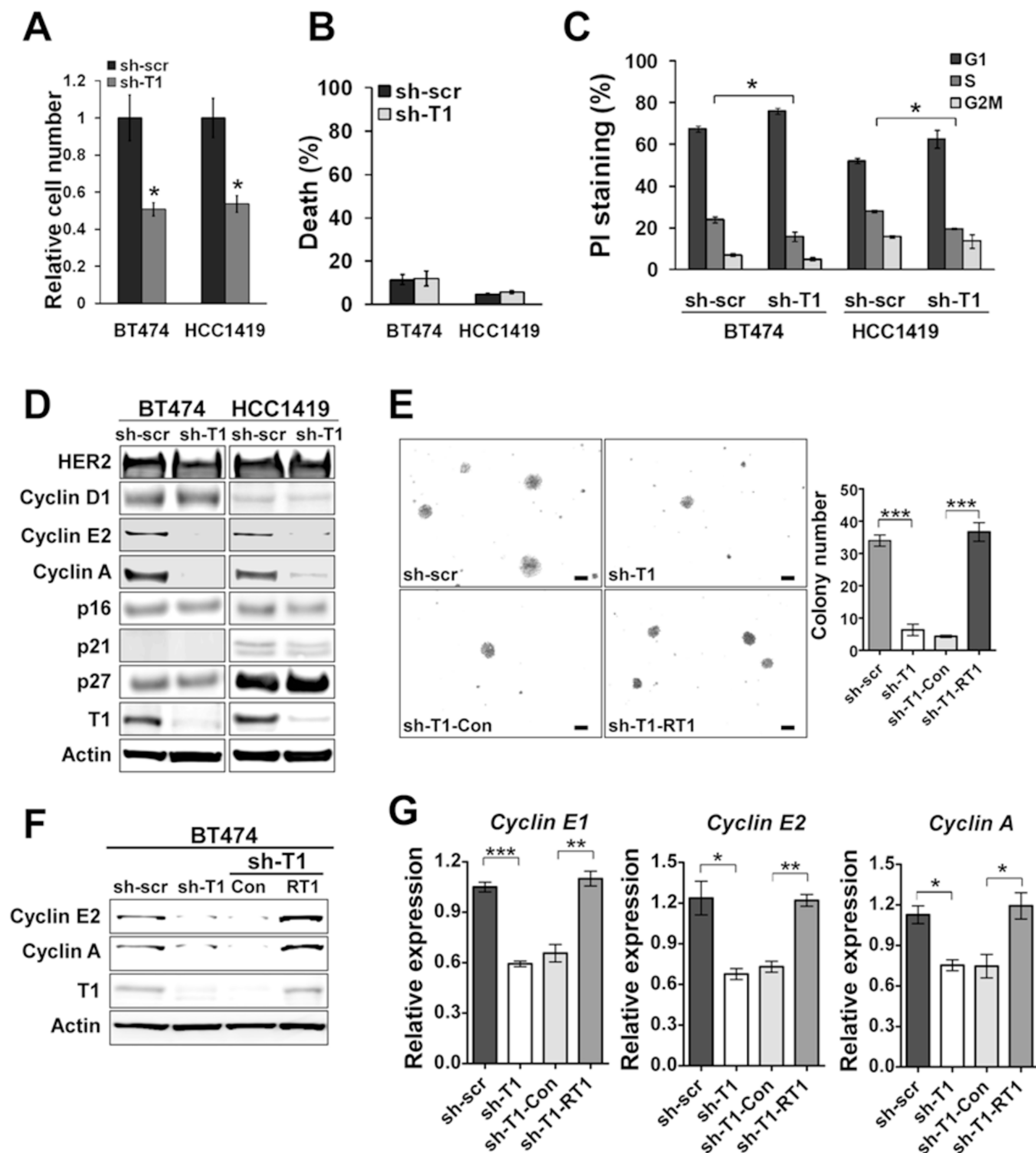


Figure 1 Taspase1 deficiency disrupts the proliferation of HER2-positive breast cancer cells. **(A)** Proliferation of Taspase1 knockdown BT474 and HCC1419 cells. 1×10^5 scramble-control (sh-scr) or Taspase1 (sh-T1) knockdown cells were seeded in triplicate wells and counted at day 4. Data presented are mean ± SD of three independent experiments. *P < 0.001. **(B)** Apoptosis of Taspase1 knockdown BT474 and HCC1419 cells. Cell death of scramble-control (sh-scr) or Taspase1 (sh-T1) knockdown cells was assessed by FACS analysis, following Annexin V staining. Data presented mean ± SD of four independent experiments. **(C)** Cell cycle profiles of Taspase1 knockdown BT474 and HCC1419 cells. The indicated scramble-control (sh-scr) or Taspase1 (sh-T1) knockdown cells were stained with propidium iodide (PI) and analyzed by FACS. Data presented are mean ± SD of four independent experiments. *P < 0.005. (**D**) Western blots of cyclins, CDKIs, and Taspase1 on scramble-control (sh-scr) or Taspase1 (sh-T1) knockdown BT474 and HCC1419 cells. β-Actin serves as loading control. **(E)** Soft agar assays using Taspase1 knockdown BT474 cells expressing either empty vector (sh-T1-Con) or knockdown resistant Taspase1 (sh-T1-RT1). Scale bar, 200 μm. Data presented are the mean ± SD. ***P < 0.0005. **(F)** Western blots of cyclin E2, cyclin A, Taspase1, and β-Actin on scramble-control knockdown (sh-scr), Taspase1 knockdown (sh-T1), Taspase1 knockdown expressing empty vector (sh-T1-Con), and Taspase1 knockdown expressing knockdown resistant Taspase1 (sh-T1-RT1) BT474 cells. **(G)** qRT-PCR analyses of scramble-control knockdown (sh-scr), Taspase1 knockdown (sh-T1), Taspase1 knockdown expressing empty vector (sh-T1-Con), and Taspase1 knockdown expressing knockdown resistant Taspase1 (sh-T1-RT1) BT474 cells. Expression was normalized to human *18S rRNA*. Data presented are mean ± SD of three independent experiments.*P < 0.05; **P < 0.005; ***P < 0.0005.

age by Taspase1, MLL$^{N320/C180}$ targets to *Cyclins E* and *A* promoters through interaction with E2Fs to methylate histone H3 at K4, thereby transactivating *Cyclins E* and *A* for cell proliferation [22, 28]. To gain mechanistic insight into how Taspase1 regulates HER2-positive breast cancer cell proliferation, we examined the expression of several key cell cycle regulators. Western blot analyses of Taspase1-knockdown BT474 and HCC1419 cells revealed a significant decrease in cyclins E2 and A, but not D1 (Figure 1D), consistent with our prior results obtained in MEFs [22]. Altogether, these results indicate that in HER2-positive breast cancer cells Taspase1 assures the proper accumulation of cyclins E and A for proliferation.

The ability of cancer cells to form colonies on soft agar is a stringent *in vitro* surrogate of *in vivo* tumorigenicity. Soft agar assays assess the capacity of tumor cells to not only proliferate but also resist anoikis under three-dimensional culture conditions that imitate the *in vivo* tumor growth environment. We determined the degree to which Taspase1 is required for the colony formation capability of HER2-positive breast cancer cells on soft agar. Knockdown of Taspase1 (sh-T1) in BT474 cells severely compromised their ability to grow as colonies on soft agar (Figure 1E). To validate the specific requirement of Taspase1 for cancer cell growth on soft agar, we engineered a sh-T1 knockdown resistant version of Taspase1 (RT1). Retroviral delivery of RT1 rescued the ability of Taspase1-knockdown (sh-T1) BT474 cells to form colonies (Figure 1E). Western blot analysis confirmed the successful knockdown of Taspase1 in BT474 cells by sh-T1 and the resulting reduced protein levels of cyclins E2 and A (Figure 1F), and the resistance of RT1 to sh-T1 and the restoration of the protein levels of cyclins E2 and A in RT1-reconstituted sh-T1 BT474 cells (Figure 1F). We further interrogated the mechanisms by which Taspase1 sustains cyclins levels. Quantitative real-time PCR (qRT-PCR) assays revealed that the mRNA levels of *Cyclins E1*, *E2*, and *A* were reduced in sh-T1 BT474 cells and were restored to baseline in RT1 sh-T1 BT474 cells (Figure 1G). Similar results were obtained utilizing HER2-transformed MCF10A cells (Supplementary information, Figure S1A and S1B). Altogether, these results indicate that Taspase1 controls the cell division cycle of HER2-positive breast cancer cells largely by conferring proper transcription of the *Cyclins E* and *A* genes upon aberrant receptor tyrosine kinase signaling.

Deletion of Taspase1 in mouse mammary glands blocks MMTV-neu-driven breast cancer formation

To determine whether Taspase1 is required for breast tumorigenesis *in vivo*, we generated *MMTV-neu;MMTV-cre;Tasp1$^{F/-}$* mice by employing the widely adapted *MMTV-neu* mouse model [15, 31]. *MMTV-neu;MMTV-cre;Tasp1$^{+/+}$* female mice were generated as positive controls and monitored for breast cancer formation. All of our *MMTV-neu;MMTV-cre;Tasp1$^{+/+}$* virgin female mice (*n* = 30) developed breast tumors between 30 and 50 weeks of age (Figure 2A, 2B), as did their counterparts in previously published studies [15, 31]. In stark contrast, 26 of 30 *MMTV-neu;MMTV-cre;Tasp1$^{F/-}$* females remained breast cancer free at 60 weeks of age (Figure 2A). Notably, western blot analysis of 12 week-old mammary glands detected similar levels of HER2/neu protein expression in both *MMTV-neu;MMTV-cre;Tasp1$^{+/+}$* and *MMTV-neu;MMTV-cre;Tasp1$^{F/-}$* female virgin mice (Supplementary information, Figure S2). Interestingly, Taspase1 ablation did not block the tumor initiation in *MMTV-Wnt;MMTV-cre;Tasp1$^{F/-}$* female mice, which suggests the specificity of Taspase1 function in HER2-driven breast cancer (Figure 2C-2E). Altogether, these *in vivo* data in conjunction with our *in vitro* assays unequivocally establish an essential role for Taspase1 in *HER2/neu*-induced breast tumorigenesis in mice and probably in humans.

Genetic deletion of Taspase1 impacts neither normal development nor physiological proliferation of mouse mammary glands

We next investigated the mechanisms by which loss of Taspase1 in the mammary gland protects *MMTV-neu* female mice from developing breast cancer by determining whether Taspase1 ablation simply disrupts mammary gland development and/or response to physiological proliferation signals such as pregnancy. To this end, we compared whole mounts of dissected, carmine-stained mammary glands from *MMTV-cre;Tasp1$^{+/+}$* and *MMTV-cre;Tasp1$^{F/-}$* female mice, prepared at 6 weeks of age, day 13 of pregnancy, and day 1 of lactation. No macroscopic differences were detected between MMT*V-cre;Tasp1$^{+/+}$* and *MMTV-cre;Tasp1$^{F/-}$* mice at any of these pre-specified developmental and physiological states (Figure 3 and Supplementary information, Figure S3). Thus, ablation of Taspase1 in mouse mammary tissues using *MMTV-cre* has no effect on normal development or physiological proliferation of mammary glands. Of note, the importance of Taspase1 in breast tumorigenesis (Figure 2A) and its dispensability in normal mammary gland physiology (Figure 3 and Supplementary information, Figure S3) are reminiscent of those reported with *Cyclin D1* knockout mice [15].

Taspase1 deficiency prevents tumor formation of MMTV-neu mammary glands

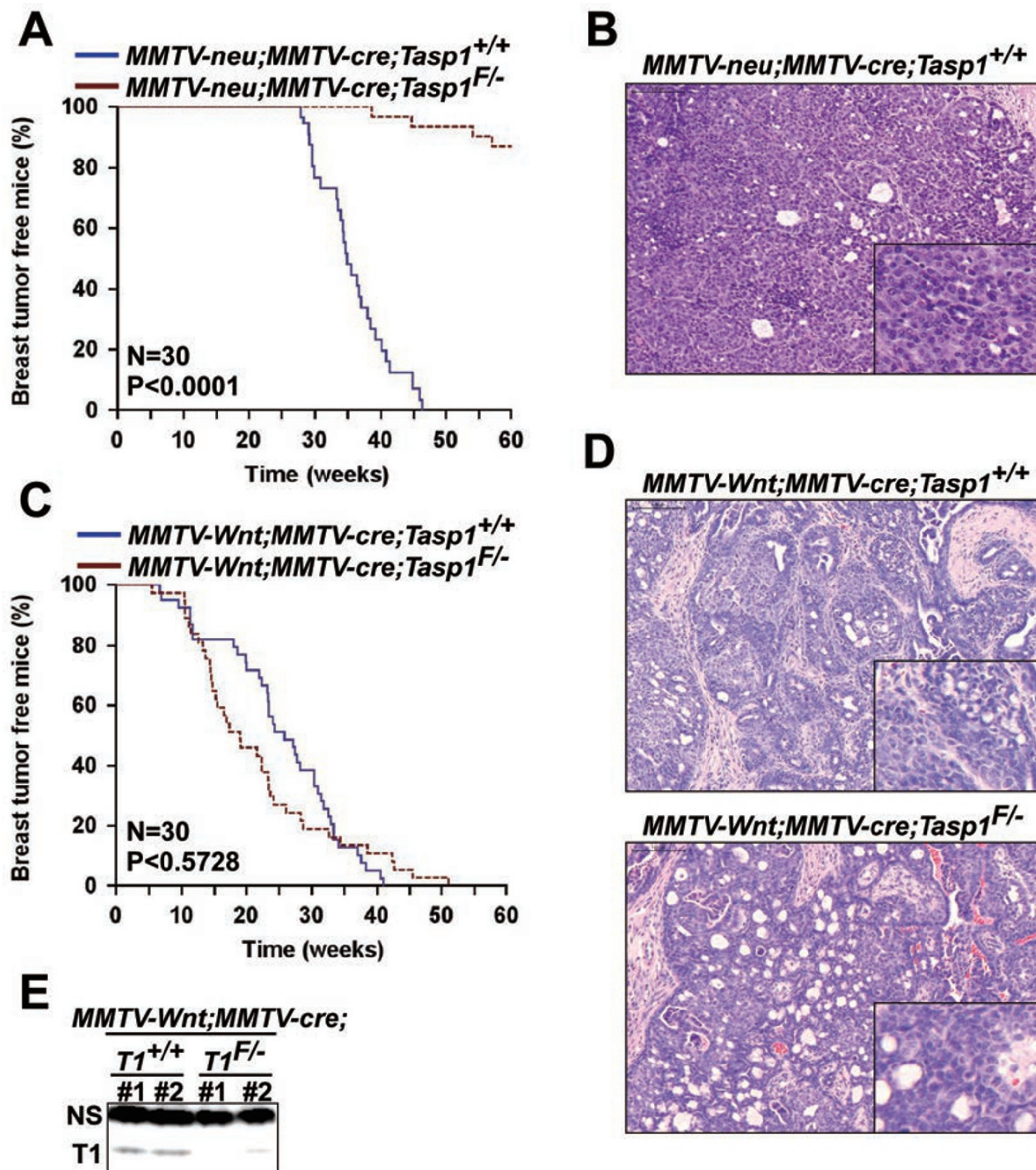


Figure 2 Deletion of Taspase1 in the mammary gland protects mice from developing *MMTV-neu*-driven breast cancer. **(A)** Kaplan-Meier curve of breast cancer incidence of *MMTV-neu;MMTV-cre;Tasp1*$^{F/-}$ female mice. *MMTV-neu;MMTV-cre;Tasp1*$^{+/+}$ female mice were used as control. n = 30 mice per genotype. P < 0.0001. **(B)** H&E staining of mouse mammary tumors for the indicated genotypes. Scale bar, 100 µm. **(C)** Kaplan-Meier curve of breast cancer incidence of *MMTV-Wnt;MMTV-cre;Tasp1*$^{F/-}$ female mice. *MMTV-Wnt;MMTV-cre;Tasp1*$^{+/+}$ female mice were used as control. n = 30 mice per genotype. P = 0.5728. **(D)** H&E staining of mouse mammary tumors for the indicated genotypes. Scale bar, 100 µm. **(E)** Western blot analyses of Taspase1 using female mammary glands of the indicated genotypes. NS indicates non-specific band, serving as control for equal loading.

We analyzed mammary glands of transgenic mice to further dissect the mechanisms by which Taspase1 enables *MMTV-neu*-induced breast tumorigenesis. Whole mounts were performed using mammary glands of 12-week-old wild-type, *MMTV-neu;MMTV-cre;Tasp1*$^{+/+}$, and *MMTV-neu;MMTV-cre;Tasp1*$^{F/-}$ female mice to evaluate glandular structures. In comparison to those of wild-type control mice, mammary glands of *MMTV-neu;MMTV-cre;Tasp1*$^{+/+}$ mice displayed increased densities in ducts and end buds (Figure 4A), similar to what was previously documented for this mouse strain [31]. Interestingly, this overproliferation phenotype induced by the

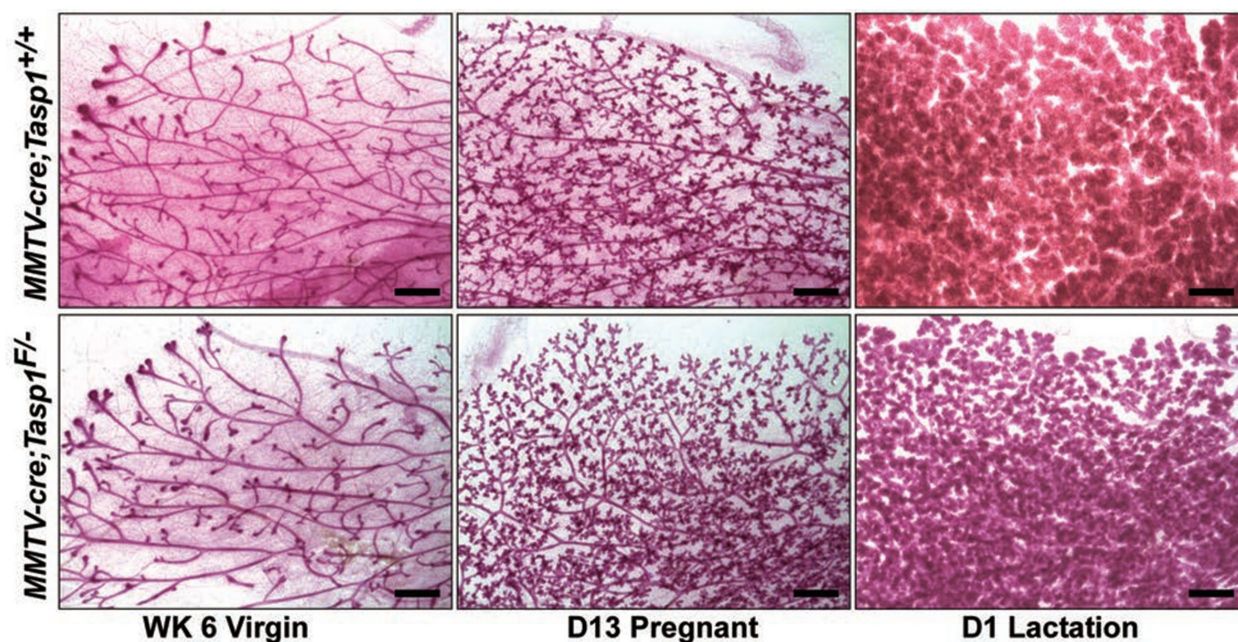


Figure 3 Genetic deletion of Taspase1 does not disrupt mammary gland development or its proliferative response during gestation. Whole mounts of dissected, carmine-stained female mammary glands from mice of the indicated genotypes, age, and gestation status. *n* = 3-4 mice per each genotypes. Representative images are presented. Scale bar, 1 mm.

MMTV-neu transgene was not observed in the mammary glands of *MMTV-neu;MMTV-cre;Tasp1*$^{F/-}$ mice (Figure 4A). Immunohistochemistry for histone H3 serine 10 phosphorylation (pH3S10) which is a cell proliferation marker, showed that mammary glands of 12-week-old *MMTV-neu;MMTV-cre;Tasp1*$^{+/+}$ mice had greater than 10-fold more pH3S10 positive cells than those of Wild-type and *MMTV-neu;MMTV-cre;Tasp1*$^{F/-}$ mice (Figure 4B). Altogether, these genetic data indicate that Taspase1 is required for the *MMTV-neu*-driven aberrant gland proliferation and thus breast carcinogenesis in mice.

MMTV-neu mice can develop multiple microscopic mammary gland tumors at as early as 14 weeks of age [31]. Accordingly, we examined mammary glands of 20-week-old wild-type, *MMTV-neu;MMTV-cre;Tasp1*$^{+/+}$, and *MMTV-neu;MMTV-cre;Tasp1*$^{F/-}$ female virgin mice by whole mounts. The mammary glands of *MMTV-neu;MMTV-cre;Tasp1*$^{+/+}$ mice showed significant glandular proliferation and developed tumor foci of various sizes, whereas those of *MMTV-neu;MMTV-cre;Tasp1*$^{F/-}$ mice displayed neither overproliferation nor tumor foci, which are macroscopically indistinguishable from wild-type (Figure 4C and Supplementary information, Figure S4). Western blot analysis of 10-week-old wild-type, *MMTV-neu;MMTV-cre;Tasp1*$^{+/+}$, and *MMTV-neu;MMTV-cre;Tasp1*$^{F/-}$ mammary glands for cyclins E, A, and D, and CDKIs p16 and p27 detected consistent overexpression of cyclin E in *MMTV-neu;MMTV-cre;Tasp1*$^{+/+}$ mice (Figure 4D). A higher level of Taspase1 expression was also detected in these *MMTV-neu;MMTV-cre;Tasp1*$^{+/+}$mammary glands, which is consistent with our prior observation that Taspase1 is commonly overexpressed in cancer cells [26]. Moreover, examination of *MMTV-neu;MMTV-cre;Tasp1*$^{+/+}$ breast tumors revealed that cyclins E, A, D, and Taspase1 were all expressed at high abundance (Figure 4D). The successful deletion of Taspase1 was observed in the *MMTV-neu;MMTV-cre;Tasp1*$^{F/-}$ mammary glands and remarkably, *MMTV-neu* was no longer able to induce cyclin E overexpression in these glands (Figure 4D). These data support the notion that overexpression of cyclin E and Taspase1 occurs early in *MMTV-neu*-driven breast tumorigenesis and precedes the overexpression of cyclins A and D.

Taspase1-mediated proteolytic cleavage of MLL is required for MMTV-neu-driven breast cancer formation but dispensable for mammary gland development

Our biochemical and genetic data thus far suggest that Taspase1 enables HER2/neu-driven breast tumorigenesis by permitting transcriptional activation of *Cyclin E* (Figures 1-4). Since Taspase1 cleaves nuclear factors MLL, MLL2, TFIIA, and ALF to regulate transcriptional programs, we asked the cleavage of which substrate(s)

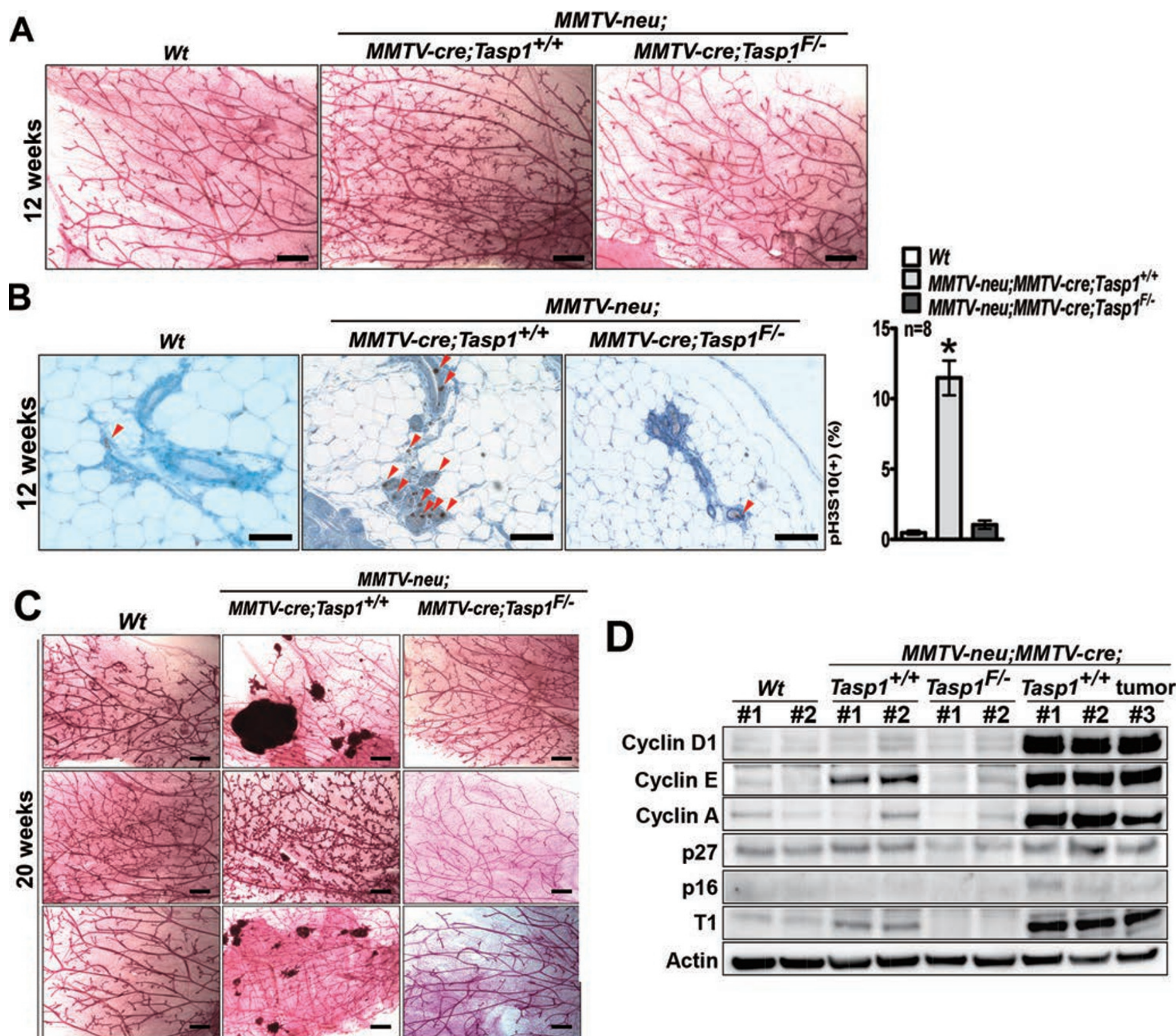


Figure 4 Loss of Taspase1 disrupts the overexpression of cyclin E and the cancer formation in *MMTV-neu* mouse mammary glands. **(A)** Whole mounts of dissected, carmine-stained female mammary glands of the indicated genotypes at 12 weeks of age. n = 5-15 mice per genotype. Representative images are presented. Scale bar, 1 mm. **(B)** pH3S10 staining of female mammary glands of the indicated genotypes at 12 weeks of age. n = 8 mammary glands per group. Representative images are presented. Red arrow head indicates positive cells. Scale bar, 100 µm. $^*P < 0.0001$. **(C)** Whole mounts of female mammary glands of the indicated genotypes at 20 weeks of age. n = 5-15 mice per group. Representative images are presented. Scale bar, 1 mm. **(D)** Western blot analyses of cyclins, CDKIs, and Taspase1 using female mammary glands and tumor extracts of the indicated genotypes. β-Actin serves as loading control.

by Taspase1 is necessary for *MMTV-neu*-induced breast carcinogenesis. Of note, our prior biochemical studies in MEFs revealed that Taspase1 cleaves MLL to transactivate *Cyclin* E [22]. However, MLL plays complex and context-dependent roles in cell cycle control. It can either positively or negatively regulate cell proliferation. For example, contrary to the positive regulation of cell proliferation that is more commonly observed, MLL was shown to negatively regulate pancreatic neuroendocrine cell proliferation. In this context MLL interacts with the tumor suppressor Menin to activate p27, a CDKI for cell cycle inhibition and thus prevent aberrant neoplastic proliferation [32]. Furthermore, an independently generated non-cleavable MLL mouse model showed

no overt proliferation defects in MEFs [33]. Hence, it is of significance to address the role of MLL cleavage, if any, in *MMTV-neu*-driven breast tumorigenesis using genetic models. Our data suggested that specific ablation of Taspase1-mediated MLL cleavage could suppress *MMTV-neu*-driven breast cancer formation. We accordingly generated *MMTV-neu;MLL*$^{nc/nc}$ transgenic mice that harbor homozygous Taspase1 non-cleavable (nc) alleles of *MLL*. Unlike *Taspase1*$^{-/-}$ mice, which died prematurely, *MLL*$^{nc/nc}$ mice were viable and fertile [34]. Like *MMTV-neu;MMTV-cre;Tasp1*$^{F/-}$ mice (Figure 2A), most *MMTV-neu;MLL*$^{nc/nc}$ females (27 of 30) were, remarkably, free of breast cancer at 60 weeks of age (Figure 5A). We then determined whether *MLL*$^{nc/nc}$ females display any mammary gland defects. Whole mounts of *MLL*$^{nc/nc}$ female mammary glands at 6 weeks of age, day 13 of pregnancy, and day 1 of lactation did not reveal any abnormalities (Figure 5B). Altogether, these results indicate that Taspase1-mediated MLL cleavage plays a critical role in *MMTV-neu*-induced breast carcinogenesis *in vivo*.

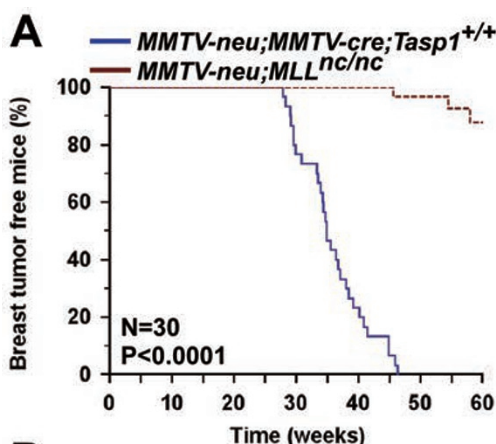
Non-cleavable MLL disrupts MMTV-neu-induced aberrant proliferation and cyclin E overexpression in mammary glands

We next investigated the molecular basis underlying *MLL*$^{nc/nc}$ females' resistance to *MMTV-neu*-induced breast tumorigenesis by comparing the mammary glands of *MMTV-neu;MLL*$^{nc/nc}$ female virgin mice to those of wild-type and *MMTV-neu;MMTV-cre;Tasp1*$^{+/+}$ mice at 12 and 20 weeks of age. Consistently, the increased duct and end bud density induced by the *MMTV-neu* transgene was not observed in mammary glands of *MMTV-neu;MLL*$^{nc/nc}$ mice (Figure 6A). We subsequently performed immunohistochemistry analysis for histone H3 serine 10 phosphorylation (pH3S10) on the mammary glands of 12-week-old *MMTV-neu;MLL*$^{nc/nc}$ mice. Like *MMTV-neu;MMTV-cre;Tasp1*$^{F/-}$ and wild-type mice (Figure 4B), *MMTV-neu;MLL*$^{nc/nc}$ mice had 10-fold fewer pH3S10+ cells in their mammary glands than *MMTV-neu;MMTV-cre;Tasp1*$^{+/+}$ mice (Figure 6B). Finally, we examined the expression of cyclins E, A, D, and CDKIs p16 and p27 in the mammary glands of 10-week-old *MMTV-neu;MLL*$^{nc/nc}$ mice. Non-cleavage of MLL suppressed *MMTV-neu*-induced cyclin E accumulation (Figure 6C), similar to what was observed in *MMTV-neu;MMTV-cre;Tasp1*$^{F/-}$ mice (Figure 4D). Overall, these genetic and biochemical results demonstrate that Taspase1 and thus mature MLL$^{N320/C180}$, generated upon Taspase1-mediated cleavage of the precursor MLL^{500}, are required for *MMTV-neu*-driven breast carcinogenesis, which involves transcriptional activation of *Cyclin E* in the mammary glands.

Discussion

The cloning of Taspase1 (threonine aspartase) founded a novel class of endopeptidases that employ the NH2-terminal threonine of the mature β subunit to cleave protein substrates after P1 aspartate [20]. In this study, using genetically well-defined mouse breast cancer models we show that Taspase1 is required for *MMTV-neu*-driven mammary tumorigenesis, which represents the first *in vivo* study demonstrating that Taspase1 ablation suppresses tumor initiation.

The *mixed-lineage leukemia* (*MLL*) gene encodes an epigenetic transcriptional regulator belonging to the *trithorax* group family [35]. MLL is a confirmed substrate of Taspase1 [22]. In its best-known developmental role, MLL maintains proper expression of *Hox* genes and thus coordinates the segmental body plan of vertebrates [36, 37]. Mice deficient for *MLL* (*MLL*$^{+/-}$ and *MLL*$^{-/-}$) accordingly display homeotic defects in their axial skeleton [37]. In addition to patterning body axis, MLL also regulates hematopoiesis, cell cycle, and cancer cell invasion [24, 37-42]. MLL possesses histone H3 lysine 4 (H3K4) methyl transferase (HMT) activity [43-46]. MLL-catalyzed H3K4 trimethylation (H3K4me3) activates transcription, leading to orchestrated upregulation of key developmental, cell cycle, and cancer cell invasion genes such as *Hox* genes, *Cyclins E* and *A*, and *MMP1* and *MMP3*, respectively [22, 24, 28, 45, 46]. Notably, the activity of MLL can be modulated by post-translational modifications, such as phosphorylation, ubiquitination, and proteolysis [20, 41, 42, 47]. The 500-kDa precursor MLL (MLL^{500}) undergoes Taspase1-mediated proteolytic cleavage, which gives rise to the mature MLL$^{N320/C180}$ heterodimer [20, 25] that binds to *Cyclins E* and *A* promoters [22, 28]. MLL forms complexes with E2Fs to methylate H3K4 at promoters, and thereby transactivates *Cyclins E* and *A* [22, 28]. In the absence of Taspase1, MLL exists as MLL^{500}, a noncleaved precursor with reduced HMT activity [20, 22], and is unable to fully activate the expression of *Cyclins E, A and B* [22]. The *in vivo* significance of MLL cleavage has nonetheless been questioned on the grounds that whereas *Taspase1*$^{-/-}$ mice exhibit diverse developmental defects, *MLL*$^{nc/nc}$ mice are born at Mendelian ratio, fertile, and grossly normal [24, 33]. Our genetic study here unequivocally establishes the *in vivo* significance of the Taspase1-MLL-cyclin E pathway in carcinogenesis by demonstrating that *MMTV-neu;MLL*$^{nc/nc}$ mice are protected from *MMTV-neu*-driven breast cancer formation. Taspase1-mediated cleavage of MLL is therefore required for HER2/neu-induced tumorigenesis. However, whether cleavages of other Taspase1 substrates, such as MLL2, have roles in tumorigenesis



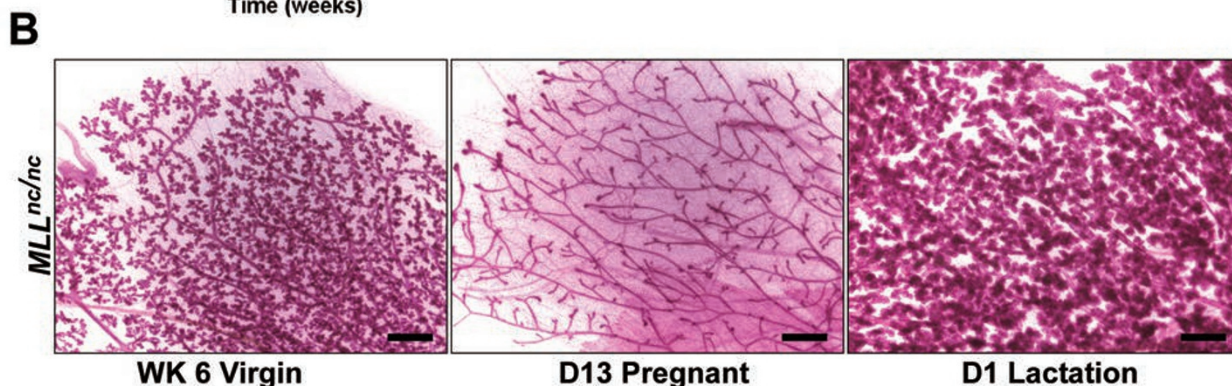


Figure 5 The cleavage of MLL by Taspase1 is required for *MMTV-neu*-driven breast cancer formation but dispensable for mammary gland development. **(A)** Kaplan-Meier curve of breast cancer incidence of *MMTV-neu;MLL*$^{nc/nc}$ mice. The same positive control *MMTV-neu;MMTV-cre;Tasp1*$^{+/+}$ female mice **(Figure 3)** were plotted as control. n = 30 mice per group. P < 0.0001. **(B)** Whole mounts of female mammary glands of *MLL*$^{nc/nc}$ mice at the indicated age and gestation status. n = 3-4 mice per group. Representative images are presented. Scale bar, 1 mm.

remains to be determined.

HER2-amplified/overexpressed breast cancer is characterized by an aggressive phenotype and poor overall survival. Although the application of anti-HER2 therapy has improved the clinical outcome of HER2-positive breast cancers, primary and secondary resistance constitute major obstacles to the further success of such treatment strategy. As cyclin E amplification/overexpression in HER2-positive human breast cancers results in decreased sensitivity to the anti-HER2 agent trastuzumab, high levels of cyclin E in human HER2-positive breast cancer may be predictive of resistance to anti-HER2 therapy [7, 16, 17]. Pharmacological inhibition of cyclin E expression may therefore benefit HER2-positive breast cancer patients by delaying disease progression and/or preventing recurrence while they are receiving anti-HER2 therapy. A series of studies including this current report clearly demonstrate that Taspase1 cleaves MLL to promote cyclin E expression and this Taspase1-MLL-cyclin E axis is required for HER2/neu induced breast tumorigenesis [22, 26]. Therefore, administering Taspase1 inhibitors in conjunction with anti-HER2 agents could produce therapeutic benefits for HER2-positive breast cancer patients. As proteases are drug targets, small-molecule inhibitors of Taspase1 may be developed for cancer therapy.

Lines of evidence indicate the active participation of Taspase1 in tumorigenesis, and thus support the development of small molecule inhibitors of Taspase1 for potential cancer therapy. However, caution should be exercised while exploiting Taspase1 inhibition as a therapeutic means in treating human subjects. First and foremost, the severe perinatal lethality resulting from the embryonic loss of Taspase1 suggests that inactivation of Taspase1 by genetic or pharmacological means is inadvisable in pregnant females and children in order to avoid potential developmental sequelae [22]. Nevertheless, inactivation of Taspase1 in fully developed adult mammals appears to be well-tolerated [29]. Cancer commonly hijacks key developmental pathways during tumorigenesis and thus frequently exhibits unique properties such as stem cell-like and dedifferentiated states [48], which may underlie the preferential therapeutic benefit conferred by targeting Taspase1 to treat cancers.

Primitive Taspase1 inhibitors (TASPINs) were designed and discovered, lending support to developing highly effective, specific Taspase1 inhibitors for cancer therapy [29]. Interestingly, although mammary gland–specific knockout of *Taspase1* disrupts *MMTV-neu*–driven breast tumorigenesis, the same genetic deletion of

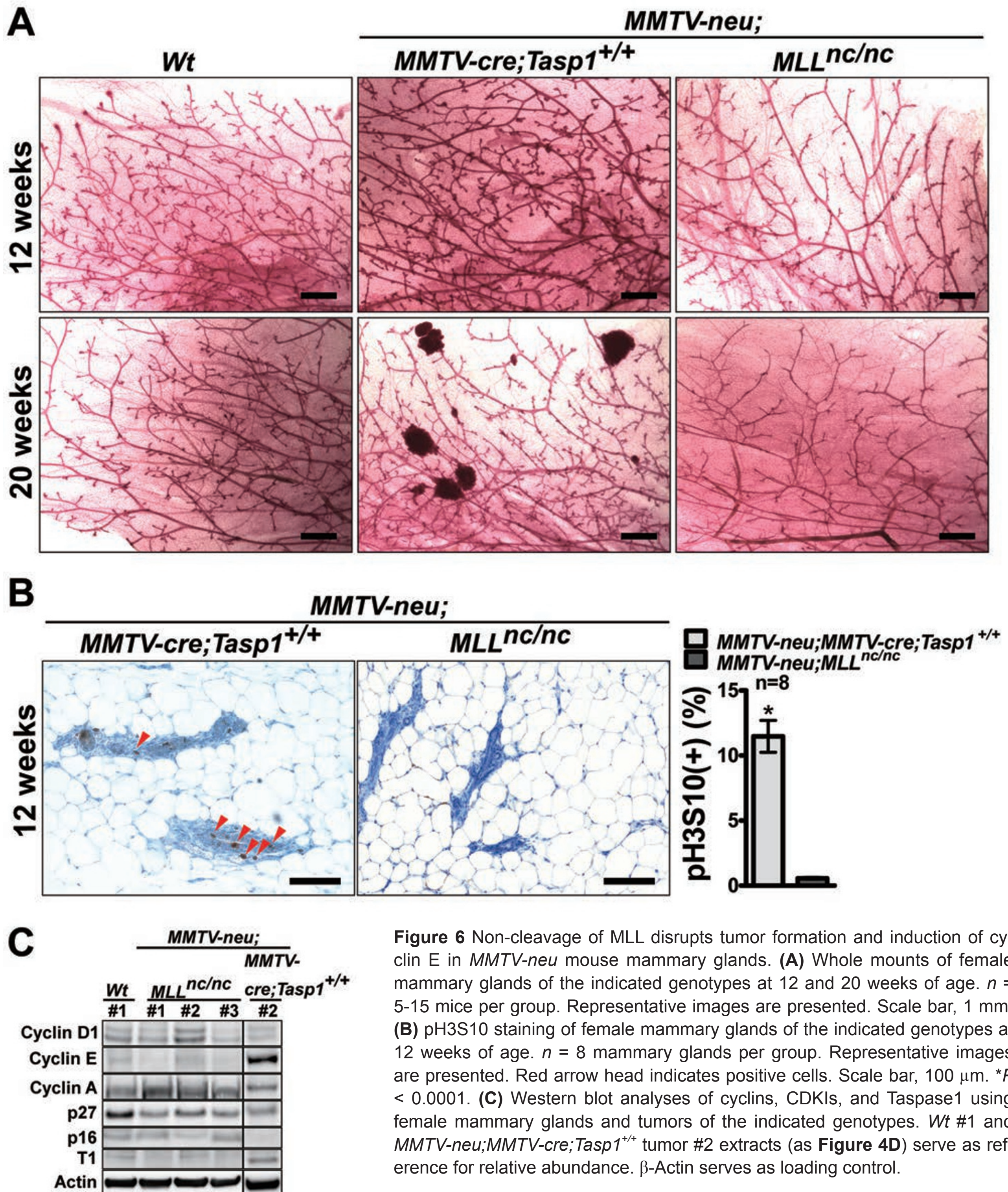


Figure 6 Non-cleavage of MLL disrupts tumor formation and induction of cyclin E in *MMTV-neu* mouse mammary glands. **(A)** Whole mounts of female mammary glands of the indicated genotypes at 12 and 20 weeks of age. n = 5-15 mice per group. Representative images are presented. Scale bar, 1 mm. **(B)** pH3S10 staining of female mammary glands of the indicated genotypes at 12 weeks of age. n = 8 mammary glands per group. Representative images are presented. Red arrow head indicates positive cells. Scale bar, 100 µm. *P < 0.0001. **(C)** Western blot analyses of cyclins, CDKIs, and Taspase1 using female mammary glands and tumors of the indicated genotypes. *Wt* #1 and *MMTV-neu;MMTV-cre;Tasp1*$^{+/+}$ tumor #2 extracts (as **Figure 4D**) serve as reference for relative abundance. β-Actin serves as loading control.

Taspase1 in *MMTV-wnt;MMTV-cre;Tasp1*$^{F/-}$ mice did not deter *MMTV-wnt*–driven breast carcinogenesis (Figure 2C). These data highlight the various mechanisms underlying individual tumorigenesis and the importance of selecting responsive cancers that might benefit from the treatment with TASPINs. Although it is beyond the scope of this study, it is of our interests to discover biomarkers that are predictive of Taspase1 addiction. Based on our unpublished data, Taspase1 has a very long protein half-life and is likely under multilayers of regulations in addition to transcription. Nevertheless, we analyzed the data of Taspase1 expression in HER2-positive and -negative tumors from breast TCGA dataset, and did not observe significant differences in Taspase1 expression (Supplementary information, Figure S5). Since Taspase1 is important in many aspects of cancer biology, Taspase1 inhibitors likely will benefit patients with different cancer types. Further studies with regard to the involvement of Taspase1 in various oncogenic pathways and the pathogenesis of subtypes of cancer could guide selection of cancer patients who would benefit from the inhibition of Taspase1.

Materials and Methods

Animal studies

All animal work was performed in accordance with MSKCC guidelines and IACUC approval. Mice were monitored for tumors by palpation twice a week. Tumor free Kaplan-Meier survival was calculated using MedCalc analysis software.

Cell culture, knockdown, proliferation, cell cycle, cell death, and western blot assays

BT-474 and HCC1419 cell lines were obtained from American Type Culture Collection and cultivated for no more than 2 months after each frozen aliquot was thawed. Amphotropic retrovirus carrying Taspase1 specific knockdown hairpin was generated as described [26, 29]. To assay cell proliferation, 1×10^5 cells were seeded onto each well of a 6-well plate and counted 4 days later. Cell cycle and cell death analyses were performed as described [26]. For western blot, cells and tissues were lysed in standard RIPA buffer. The anti-Taspase1 rabbit polyclonal antibody is as described [22, 26]. Antibodies for cyclin E2 (4132, Cell Signaling), cyclin A (C4710, Sigma), p21 (sc-397, Santa Cruz Biotechnology), p27(sc-528, Santa Cruz Biotechnology), cyclin D1(sc-450, Santa Cruz Biotechnology), p16(554079, BD Pharmingen), and ErbB2(OP-15, Calbiochem) were purchased from indicated companies. Antibodies were detected using the enhanced chemiluminescence method (Western Lightning, PerkinElmer). Immunoblot signals were acquired with the LAS-3000 Imaging system (FujiFilm) and were analyzed with ImageJ software.

Knockdown resistant Taspase1

Knockdown resistant Taspase1 (RT1) was generated by site-directed mutagenesis to create synonymous mutations at amino acids 387 to 392 (GGA AAG GCG AAA ACG CAT) of Taspase1. The cDNA was inserted into an MSCV-neo expression construct.

Soft agar assay

1×10^5 cells were seeded onto a 6 cm dish containing a top layer of 0.3% noble agar and a bottom layer of 0.6% noble agar base. Cells were fed with media every 3 days. After 3 weeks, colonies with diameter larger than 200 μm were scored. Three independent triplicate experiments were performed.

Quantitative RT-PCR

mRNA was isolated using TRIzol (Invitrogen) and further purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription reactions were carried out as described with Superscript II (Invitrogen) and random decamer primers (Ambion) [20, 22, 34]. Quantitative RT-PCR was performed using TaqMan 2× buffer and an ABI Prism 7300 sequence detection system (Applied Biosystems). For cyclin E1 and E2 reactions, the TaqMan Hs01026536 and Hs00180319 probes were used, and for cyclin A, SYBR Green master mix and the following primers were used: CAA AGC ACC ACA GCA TGC ACA AC and GAT TTA GTG TCT CTG GTG GGT TGA GG. All reactions were normalized against 18s rRNA using an 18s rRNA TaqMan probe (Applied Biosystems).

Mammary gland whole-mount assays

Mouse mammary glands were surgically dissected, spread onto a glass slide, and fixed in a 1:3:6 mixture of glacial acetic acid/chloroform/100% ethanol. Following hydration, they were stained overnight in 0.2% carmine and 0.5% $ALK(SO_4)_2$; dehydrated in graded solutions of ethanol, cleared in xylenes; and mounted with Permount.

Immunohistochemistry

Tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 5-μm thickness were prepared. pH3S10 was detected by immunohistochemistry using the antibody from Millipore (06-570).

Statistical analysis

Student's *t*-test was performed to compare means between two groups. Data were expressed as the mean ± SD or ± sem as indicated.

Taspase1 gene expression in human breast cancer

To determine whether Taspase1 (Tasp1) expression differs in HER2 positive and HER2 negative human breast cancer, normalized RNA sequencing (RNA-Seq) data produced by The Cancer Genome Atlas (TCGA) [49] were downloaded from Broad GDAC Firehose. PAM50 subtype classifications were available for 500 of the 526 primary breast invasive carcinoma tumor samples TCGA subjected to mRNA expression profiling using the Illumina HiSeq 2000 RNA Sequencing Version 2 platform. The subtype classifications were obtained through cBioPortal for Cancer Genomics, and the 26 samples lacking classifications were discarded. The Tasp1 mRNA expression values of the remaining samples formed a dataset that was partitioned by HER2 status to form HER2 positive and HER2 negative datasets. Two-tailed Mann-Whitney tests were performed to compare the means of the HER2 positive and HER2 negative datasets.

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