CDKN1B mutation and copy number variation are associated with tumor aggressiveness in luminal breast cancer

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Supplementary materials and methods

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Supplementary materials and methods

Ethical statement

Solid tumor specimens from breast, ovarian and head and neck cancer patients and liquid biopsies from metastatic breast cancer patients were collected upon signing a written informed consent, in accordance with recognized ethical guidelines and following approval by the Institutional Review Board of CRO Aviano, National Cancer Institute (Aviano, Italy) and University of Rome 'Sapienza' Sant'Andrea Hospital (Rome, Italy). All studies were performed in compliance with the 1975 Declaration of Helsinki, as revised in 1983.

DNA purification and NGS analyses

Maxwell® 16 Tissue DNA Purification Kit (Promega, Madison, WI, USA) was used for automated purification of DNA from fresh solid tumor samples, following the manufacturer's instruction. AllPrep DNA/RNA mini kit and AllPrep DNA/RNA FFPE kit (Qiagen, Hilden, Germany) were used for manual purification of DNA from cryosections or FFPE from solid tumor samples, respectively, following the manufacturer's instruction. Maxwell® RSC ccfDNA plasma kit (Promega) was used for automated purification of circulating cell-free DNA (ccfDNA) from plasma of metastatic LBC patients. Plasma was obtained by centrifuging whole blood from EDTA tubes 1× for 10 min at 2000 × g and 2× for 10 min at 4000 × g.

DNA quantification was performed using QuantiFluor® ONE dsDNA System (Promega). 25 ng DNA from solid biopsies and a pre-established volume of 7 µl DNA from liquid biopsies were used for subsequent mutational analysis by next-generation sequencing (NGS). *CDKN1B* mutational status was investigated by NGS with an amplicon-based strategy. Specific primers covering the complete open reading frame (ORF) of human *CDKN1B* gene were designed and modified adding specific adapter sequences (Merck Life Science, Darmstadt, Germany). Amplicon libraries were generated using a modified Illumina protocol. Briefly, multiplex PCR

products were generated using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and subsequently tagged with specific Index primers (Integrated DNA Technologies, IDT, Coralville, IA, USA), according to manufacturer's instructions. Purified libraries were pooled, and paired-end sequenced in a MiSeq instrument (Illumina, San Diego, CA, USA). Primer sequences and multiplex composition are available upon reasonable request. The sequencing coverage was at least 1000× for DNA from tumor tissue and at least 4000× for ccfDNA.

Droplet digital PCR (ddPCR)

Copy number variation was evaluated using droplet digital PCR (ddPCR) analysis. DNA digestion was performed using the restriction endonuclease HindIII (Promega). Before generation of droplets, ddPCR reactions were prepared following the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). In brief, 2.5 ng DNA was mixed with ddPCR Supermix for Probes (No dUTP) 2× (Bio-Rad) and the appropriate Taqman hydrolysis probes (Bio-Rad). Within each sample, we evaluated the copy number state of *CDKN1B* locus respect to reference locus, in our case *RPP30* and *CRCP* (Bio-Rad, dHsa-CDKN1B #CNS264106317; dHsa-RPP30 #CP2500350; dHsa-CRCP #CP2506271).

Droplet generation was performed in a QX200 Droplet Generator (Bio-Rad) using Droplet Generation Oil for Probes (Bio-Rad), according to the manufacturer's protocol.

PCR was performed using a C1000 touch thermocycler (Bio-Rad) with the following cycles: 95 °C 10 min (1 cycle); 94 °C 30 s and 57 °C 1 min (40 cycles); 98 °C 10 min (1 cycle); then 4 °C. Droplets were analyzed using the QX200 Droplet Reader (Bio-Rad) and data analyzed with QuantaSoft software (Bio-Rad).

Cell culture and generation of MCF-7 p27 KO and KI cell clones

MCF-7 and T47D cells were obtained from ATCC (LGC Standards, Manassas, VA, USA) and grown in DMEM (Merck Life Science) supplemented with 10% FBS (Carlo Erba, Milano, Italy). 293FT cells (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) were used for lentiviral production and grown in DMEM supplemented with 10% FBS (Carlo Erba). All cell lines are grown at standard conditions of 37 ºC and 5% CO_2 and authenticated by STR analysis, according to PowerPlex® 16 HS System (Promega) protocol and using GeneMapper™ software 5 (Thermo Fisher Scientific) to identify DNA STR profiles. All cell lines are routinely tested to exclude Mycoplasma contamination (MycoAlert™, Lonza, Basel, Switzerland).

For lentiviral system, shRNA constructs (pLKO) encoding for two different target sequences on human p27 (sh1 TRC0000039930 and sh2 TRC0000356318) were purchased from Merck (Darmstadt, Germany). 293FT cells were transfected with Gag-Pol and VSV-G (recombinant lentivirus producing system, Invitrogen, Thermo Fisher Scientific) by standard calcium phosphate transfection protocol (Promega). After 48 and 72 h, conditional medium containing lentiviral particles was harvested and used to transduce target cells. Cells were selected in complete culture medium supplemented with 0.75 µg/ml puromycin.

MCF-7 p27 KO cell clones were described previously [23]. In brief, p27 KO cell clones were obtained by Nucleofection of a custom-designed Zinc Finger Nucleases (ZFNs) pair for p27 genomic sequence (Merck Life Science) using the AMAXA V kit for nucleofection (Lonza), following the manufacturer instruction. Following electroporation, cells were maintained at 30 °C for 2–3 days, then at 37 °C and seeded as single-cell into 96-well plates. To confirm the ZFN activity in the clones, CEL-I assay (Transgenomics Surveyor Nuclease Kit, IDT) was performed. Positive clones were analyzed by Next Generation Sequencing (MiSeq V2 Kit, Illumina) and Sanger sequencing to confirm the KO of *CDKN1B* gene. Then, analysis of the protein lysate by western blotting was performed to confirm the KO at the protein level. To obtain the MCF-7 p27 KI clones, the coding DNA sequence of GFP-p27 WT, GFP-p27 K134fs and GFP-p27 T171* were cloned downstream the *CMV* promoter of the vector pZDonor-

AAVS1 (Merck Life Science), a donor plasmid for transgene integration into the human AAVS1 locus (Adeno-Associated Virus integration Site 1), considered a safe harbor locus, located in the human chromosome 19. The donor vector was co-transfected by electroporation into p27 KO MCF-7 clone #17, along with the mRNA coding for the ZFN specific for AAVS1 locus (Merck Life Science). The single-cell clones were screened by PCR using hp27 KI primers (hp27 KI FW: 5'-CATATGTCAAACGTGCGAGTG-3', hp27 KI RV: 5'-AAGCTTTTACGTTTGACGTCTTCT-3') to verify the integration of p27 coding sequence and by western blotting to verify the protein expression.

Multiple cell clones were always tested in each assay, in duplicates or triplicates as appropriate.

Growth curve and FACS analysis of the cell cycle

For growth curves, 5×10^4 cells/well were seeded in 6-well plates in complete medium (DMEM 10% FBS) in triplicate. Fresh medium was added every other day. At the indicated times, cells were detached using trypsin-EDTA and counted in a Trypan Blue exclusion test.

Cell cycle distribution was analyzed by FACS analysis, as described previously [23]. Cells were serum starved for 18 h and then released in complete medium for the indicated times. At each time point, cells were collected and fixed in ice-cold 70% ethanol and maintained at -20 °C until ready for the FACS analysis. Cells were then washed and resuspended in propidium iodide staining solution (50 µg/ml propidium iodide and 0.1 mg/ml RNaseA, in PBS 1×). Stained cells were subjected to flow cytometry analysis (FACS) with a FACScan instrument (BD Biosciences, San Jose, CA, USA). Data were analyzed using WinMDI2.8 software.

Anchorage-independent cell growth

To evaluate cell ability to grow in an anchorage independent manner, soft agar assay was performed. 1×10^4 cells were suspended in 2 ml top agar medium (DMEM 10% FBS, containing 0.4% Low Melting Agarose, Merck Life Science). The cell suspension was then

layered on top of 2 ml of pre-jellified bottom agar layer (DMEM 10%, containing 0.6% Low Melting Agarose in 6-well tissue culture plate, in duplicate. DMEM 10% FBS was added to the plates every 3 days, as a feeder layer. After 2 weeks, pictures of the wells were taken and the number and size of colonies were quantified in at least 9 randomly selected fields/well, at 10× magnification. Colony areas were measured using ImageJ software.

Three dimensional (3D) mammary epithelial cell culture

Three-dimensional cell culture was performed as described previously [24,25,28]. In brief, MCF-7 (3×10^3 cells) or T47D (4×10^3 cells) were embedded as single cells in Cultrex® Growth Factor Reduced Basement Membrane Extract (GFR-BME, 2%) (Trevigen, Gaithersburg, MD, USA), mixed with the appropriate medium (DMEM, 0.1%FBS, 3%Wound Fluid, WF, collected from breast cancer patients after surgery [27] or BMI-P) and layered on top of a pre-polymerized bottom layer of GFR-BME (8.5 mg/ml) (Trevigen) (50 µl/well), of a 12-well Labtek chamber slide (Thermo Fisher Scientific). Embedded cells were incubated at 37 °C for approximately 7 days (MCF-7), or 15 days (T47D). The appropriate medium was replaced every 3 days till the end of the experiment. At the end point, number of acini was counted and images were collected to calculate colony areas, using Volocity® (PerkinElmer, Waltham, MA, USA) software or ImageJ software. If morphological assessment was required, mammary acini were fixed in PFA overnight (ON) at 4 °C and then processed for immunofluorescence.

Immunofluorescence and immunohistochemistry

For immunofluorescence (IF) in 2D cell culture, MCF-7 p27 clones were seeded on coverslips and fixed in PBS 4% paraformaldehyde for 20 min at RT, then washed three times with PBS 1×, permeabilized in PBS 1× with 0.2% Triton X-100 and blocked for at least 1 h in PBS 1× with 1% BSA. Incubation with primary antibodies was performed ON at 4 °C, followed by 1 h at RT with secondary antibody. Nuclear staining was performed with 5 µg/ml Propidium Iodide (PI) in

PBS 1× for 20 min at RT protected from light. Coverslips were mounted in Mowiol 488 (Millipore, Merck) containing 2.5% (w/v) 1,4-diazabicyclo (2,2,2) octane (DABCO, Merck). The primary antibody used was anti-$p27^{kip1}$, cat #610242, 1:200 (Transduction Laboratories, BD). Imaging was performed using a confocal laser-scanning microscope (TSP8, Leica, Wetzlar, Germany) interfaced with a Leica fluorescent microscope.

IF on mammary acini grown in 3D cultures was performed as described previously [24,25,28]. Incubation with primary antibodies was performed ON at 4 °C, followed by 1 h at RT with secondary antibody, 1:400 (AlexaFluor® 488- or 568-conjugated, Invitrogen, Thermo Fisher Scientific). Nuclei were counterstained using TO-PRO-3 (Invitrogen, Thermo Fisher Scientific) for 30 min at RT. Primary antibodies used were: ZO-1, 1:100 cat #8193 (Cell Signaling, Danvers, MA, USA), Ki67, 1:200 cat #ab15580 (Abcam, Cambridge, UK) and Cytokeratin 8, 1:500 cat #904804 (BioLegend, San Diego CA, USA). 3D-mammary acini from the different clones were analyzed using a confocal laser-scanning microscope (TSP8, Leica) interfaced with a Leica fluorescent microscope. Collected images were analyzed using LAS (Leica) or Volocity® (PerkinElmer) software.

For immunohistochemistry (IHC) analysis, to enhance antigen retrieval slides were processed with ULTRA CC1 cat. # 95-224 (Ventana, Roche, Basel, Switzerland), according to the manufacturer's protocol. p27 detection was performed using the primary antibody cat. # 610241, 1:5000, 1h at 37°C (Transduction Lab, BD), followed by the Ultraview Detection Kit cat. #760-500 (Ventana), according to the manufacturer's protocol.

The IHC intensity score was calculated based on a combination of signal intensity (score 0–3) and percent of positive cells (score 0–4).

Preparation of cell lysates and western blotting

Protein lysates and western blot were performed essentially as described previously [23–28]. To extract total proteins, cells were washed in PBS 1× and then scraped on ice into cold RIPA

buffer (150 mM NaCl, 50 mM Tris HCl pH8, 0.1% SDS, 1% Igepal, 0.5% sodium deoxycholate) plus a protease inhibitor cocktail (cOmplete™ Protease Inhibitor Cocktail, Merck Life Science) and supplemented with 1 mM Na_3VO_4 (Merck Life Science), 10 mM NaF (Merck Life Science) and 1 mM DTT (Merck Life Science). Protein concentration was evaluated using a Protein Assay Dye Reagent Concentrate (Bio-Rad). Proteins were separated in 4–20% SDS-PAGE (Criterion Precast Gel, Bio Rad) and transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL, USA). Membranes were cut into horizontal strips, in order to probe the membrane with multiple primary antibodies recognizing targets of different molecular weights. Membrane strips were blocked at RT for 1 h with 5% non-fat dried milk in TBS-0.1% Tween 20 or in Odyssey Blocking Buffer (Li-Cor, Lincoln, NE, USA) and incubated ON at 4 °C with primary antibodies. Then, membranes were washed in TBS-0.1% Tween20 and incubated for 1 h at RT with secondary antibodies IR-conjugated (Alexa Fluor 680, 1:3000, Invitrogen, Thermo Fisher Scientific; IRDye 800, 1:5000, Rockland, Limerick, PA, USA) for infrared detection (Odyssey Infrared Detection System, Li-Cor) or with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) for ECL detection (Clarity Western ECL Substrate, Bio-Rad). Primary antibodies were purchased from Santa Cruz (Dallas, TX, USA): CDK2 (1:700 #sc-6248), Vinculin (1:1000 #sc-73614), CCNE1 (1:200 #sc377100); or from Transduction Laboratories (BD): $p27^{kip1}$ (1:500 #610242); from Merck: GFP (1:500 #11814460001), GAPDH (1:1000 #cb1001). The Re-Blot Plus Strong Solution (Merck Life Science) was used to strip the membranes, when re-blotting was needed.

For the analysis of protein stability, cycloheximide (CHX, Merck Life Science) was added to cell culture medium at 10 µg/ml and cells were collected at the indicated times and processed to extract proteins and western blotting analysis, as described above.

Immunoprecipitation and kinase assays

Immunoprecipitation (IP) experiments were performed using 0.5 mg of total protein lysate in HNTG buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, protease inhibitor cocktail, 1 mM Na_3VO_4, 10 mM NaF and 1 mM DTT) with the specific primary antibodies, gently rocking ON at 4 °C. Protein G Sepharose 4 Fast Flow (GE Healthcare) was added for the last 2 h of incubation. IPs were then washed several times in HNTG buffer and resuspended in Laemmli Sample Buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue and 125 mM β-mercaptoethanol).

For kinase assays, protein lysates were immunoprecipitated using anti-CDK2 or control antibody, as described above. After several washes in HNTG buffer, the IP was resuspended in kinase buffer (20 mM TrisHCl pH 6.8,10 mM $MgCl_2$). One fifth was stored for kinase reactions and the remaining 4/5 pelleted, resuspended in Laemmli sample buffer and loaded for immunoblotting analysis. A kinase reaction solution, containing the sample plus 50 μM ATP, 1 μCi of γ-P32 ATP and 1 μg of H1- Histone as substrate in kinase buffer solution, was prepared. The reaction was carried out at 30 °C for 30 min and then 3× Laemmli sample buffer and 50 mM DTT were added. After denaturation at 95 °C for 10 min, proteins were separated by SDS-PAGE (4–20% gel). The gel was dried and an autoradiographic film exposed (Hyperfilm MP, GE Healthcare) at RT and developed after different time intervals. Band quantification was performed using Image Lab™ Software (Bio-Rad).

RT-qPCR

Total RNA was extracted resuspending the cell pellets in TRIzol reagent following the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific). Total RNA was quantified using the QuantiFluor RNA System (Promega). RNA was reverse transcribed (RT) using the GoScript Reverse Transcriptase (Promega) and RT reactions were run in an Opticon qRT-PCR Thermocycler (Bio-Rad). cDNAs were amplified using SYBR green dye□containing reaction buffer SSoFast (Bio-Rad) and the CFX384 Touch Real□time PCR Detection System (Bio-Rad).

Normalization of the data was performed using two different reference genes (ACTB and GAPDH). The following primers (Merck Life Science) were used:

p27: FW 5'-AGATGTCAAACGTGCGAGTG-3'; REV 5'-TCTCTGCAGTGCTTCTCCAA-3'
GFP: FW 5'-AGATCCGCCACAACATCGAG-3'; REV 5'-AACTCCAGCAGGACCATGTG-3'
ACTB: FW 5'-CCAGAGGCGTACAGGGATAG-3'; REV 5'-CCAACCGCGAGAAGATGA-3'
GAPDH:FW5'-GAAGGTGAAGGTCGGAGTC-3';REV5'-GAAGATGGTGATGGGATTTC-3'
Relative expression was calculated using the comparative Ct method.

Statistical analyses

Statistical significance, mean, median and standard deviation were determined by using GraphPad PRISM software (version 6.01) (GraphPad Inc., San Diego, CA, USA), using the most appropriate test, as specified in each figure. A minimum of three biologically independent experiments was used for statistical significance. The number and type of replicates used in each experiment is specified in the figure legends. When not otherwise specified, mean and standard deviation are shown in all graphs. Significance was calculated by Student's *t*-test or Mann–Whitney two-sided or ANOVA test, as appropriate, and indicated in each figure legend. Differences were considered significant when $p < 0.05$.

Supplementary Figure 1

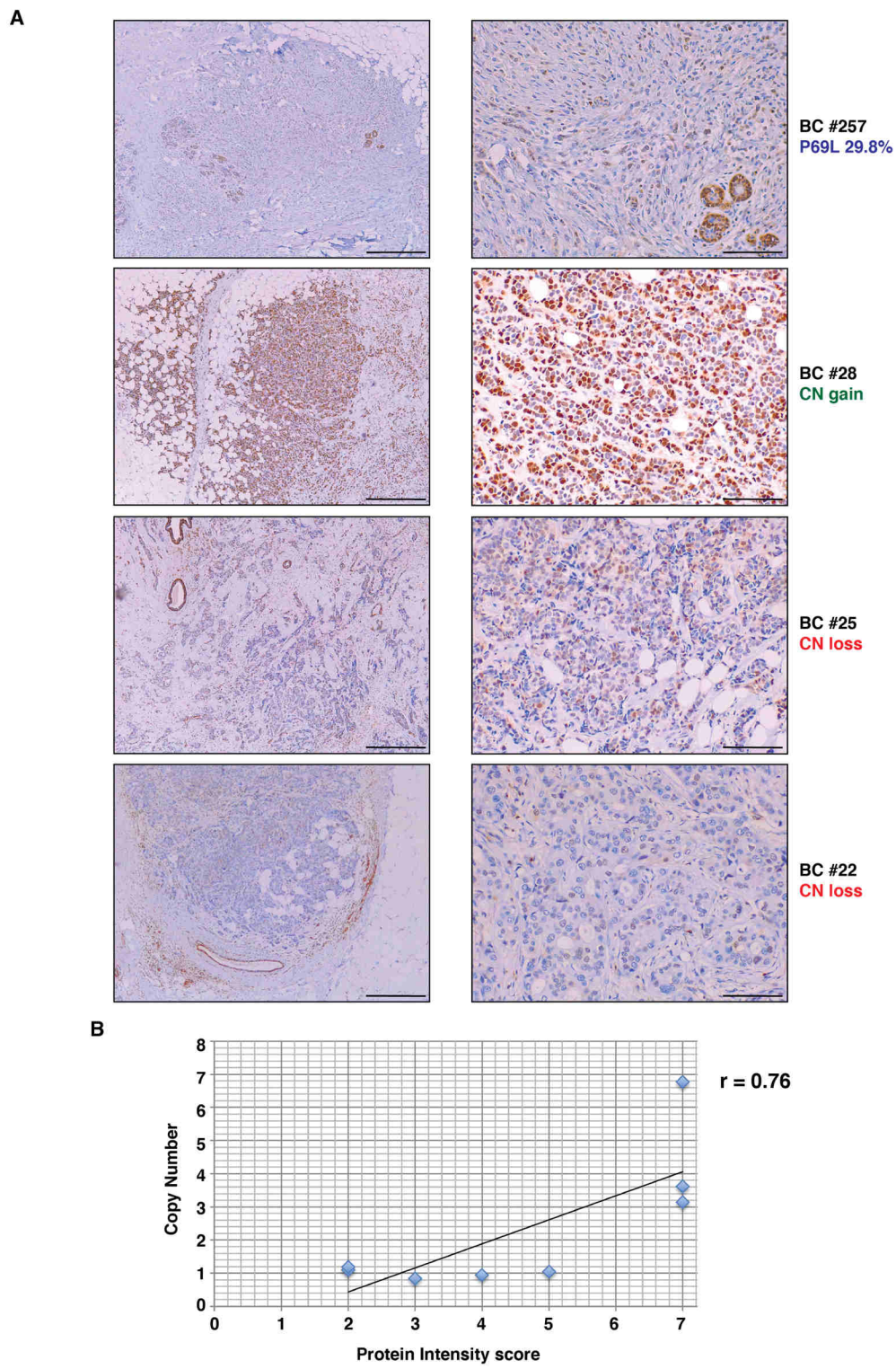

Figure S1. p27 protein expression correlates with CDKN1B copy number in breast cancer samples. (A) Representative pictures of the IHC analysis of p27 in breast cancer samples with *CDKN1B* mutation (upper panels) or copy number variation (gain, middle panels; loss, lower panels), as indicated. 5× (left panels; scale bar, 500 μm) and 20× (right panels; scale bar, 125 μm) magnifications are shown. (B) Graph shows the correlation between *CDKN1B* CN and p27 IHC intensity score, in 8 tumor samples displaying CNV (5 CN loss and 3 CN gain). The IHC intensity score was calculated based on a combination of signal intensity (score 0–3) and percent of positive cells (score 0–4).

Supplementary Figure 2

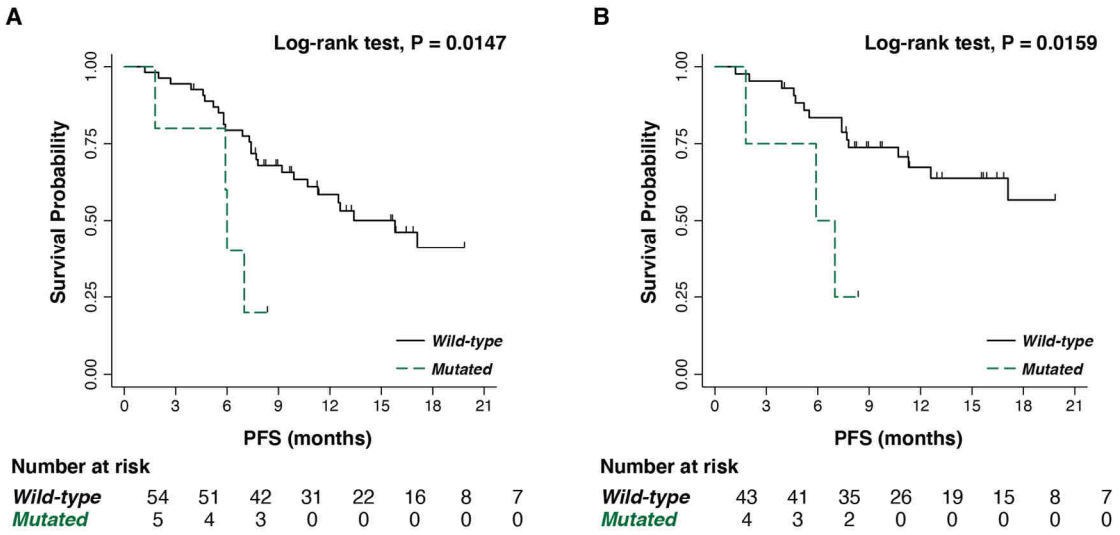

Figure S2. p27 mutations in liquid biopsies of metastatic breast cancer patients correlates with worse progression-free survival. (A,B) Graphs show progression-free survival according to *CDKN1B* mutational status. Total luminal population (A, n = 59; p = 0.0147), or patients treated with endocrine therapy (B, n = 47; p = 0.0159) were analyzed.

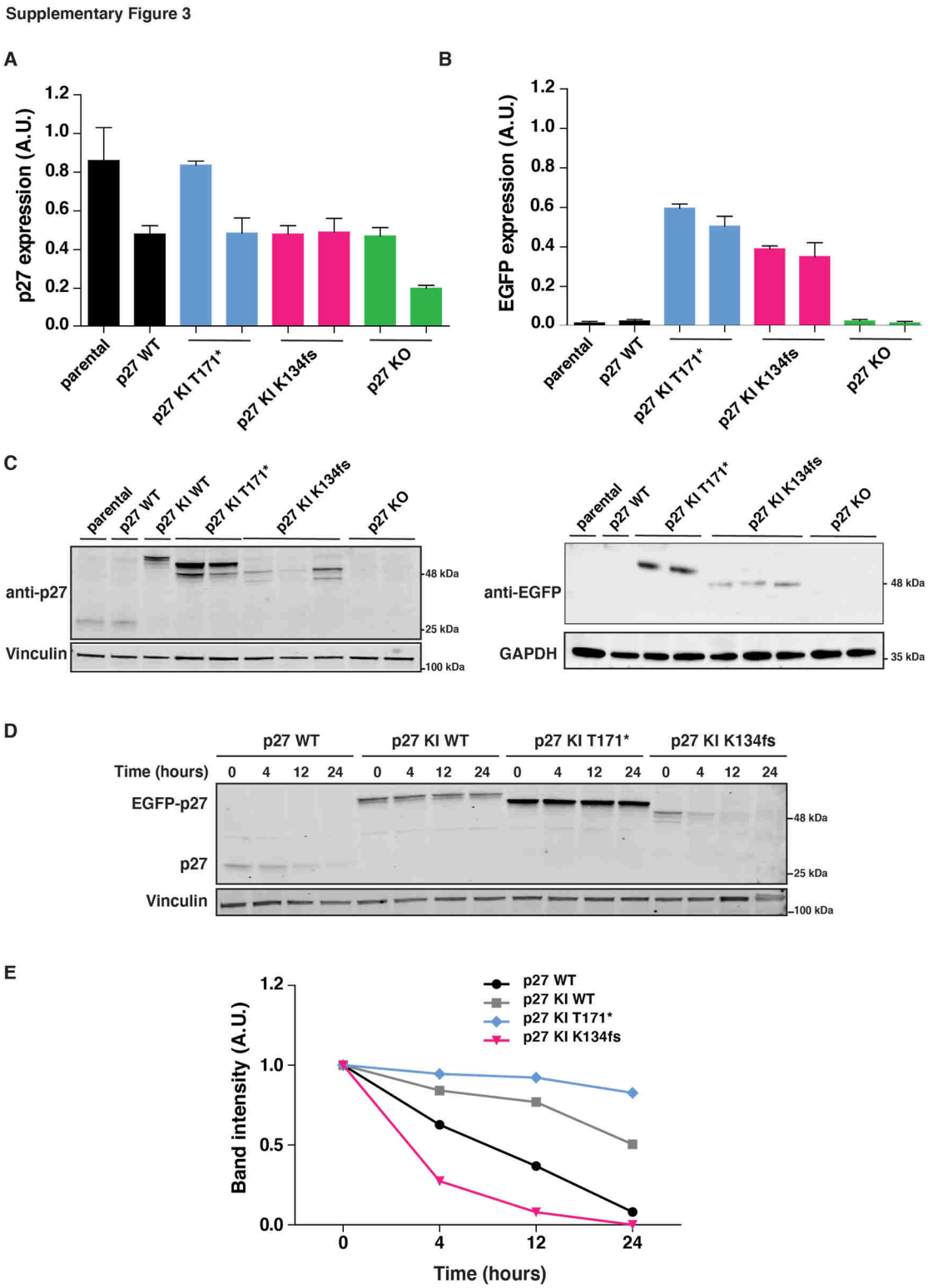

Figure S3. *CDKN1B* truncation mutants display altered protein stability compared with the WT form. (A) Graph shows data from RT-qPCR analysis of normalized p27 expression (in arbitrary units, A.U.) in MCF-7 p27-modified clones. (B) Graph shows the data from RT-qPCR analysis of normalized EGFP expression (in arbitrary units, A.U.) in MCF-7 p27 modified clones. (C) Western blotting analyses for the p27 proteins in lysates from MCF-7 p27 modified

clones. On the left, proteins were blotted with an anti-p27 antibody; on the right, proteins were blotted with an anti-EGFP antibody. (D) Western blotting for p27 proteins of MCF-7 p27 modified clones, harvested at 0, 4, 12, 24 h after CHX treatment. (E) Graph shows the amount of p27 at different times obtained by quantification of western blot bands of the experiment described in (D), normalized to time 0. Band quantification was performed using Image Lab™ Software.

Supplementary Figure 4

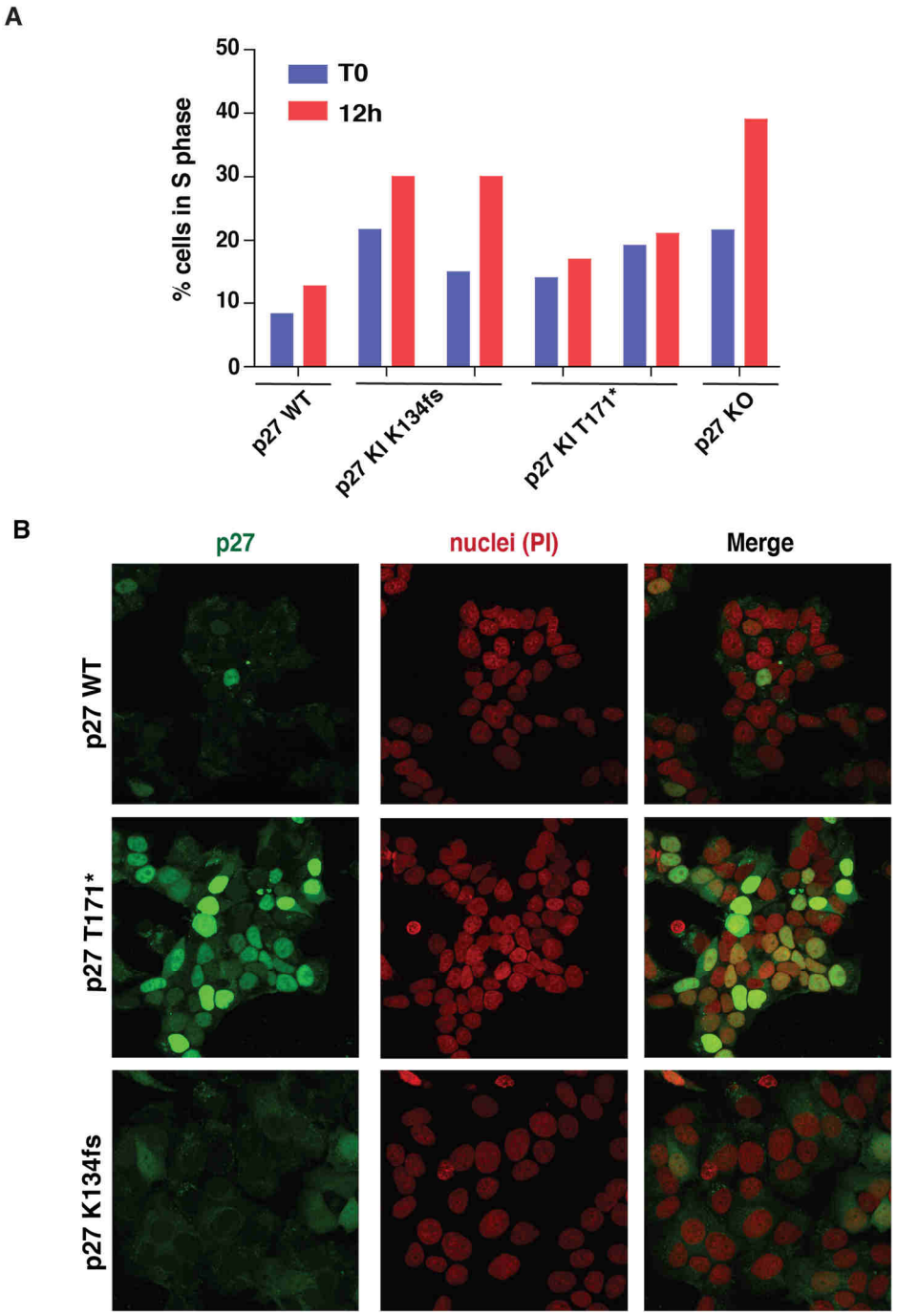

Figure S4. Loss of *CDKN1B* and expression of C-terminal deletion mutants alter S-phase entry and cytonuclear localization in LBC cells. (A) FACS analysis after PI staining of the DNA of indicated MCF-7 clones. Graph shows the S-phase fraction after starvation (T0) and release in complete medium for 12 h. (B) Immunofluorescence analysis for p27 in exponentially growing MCF-7 clones. Nuclei were counterstained with propidium iodide (PI).

Supplementary Figure 5

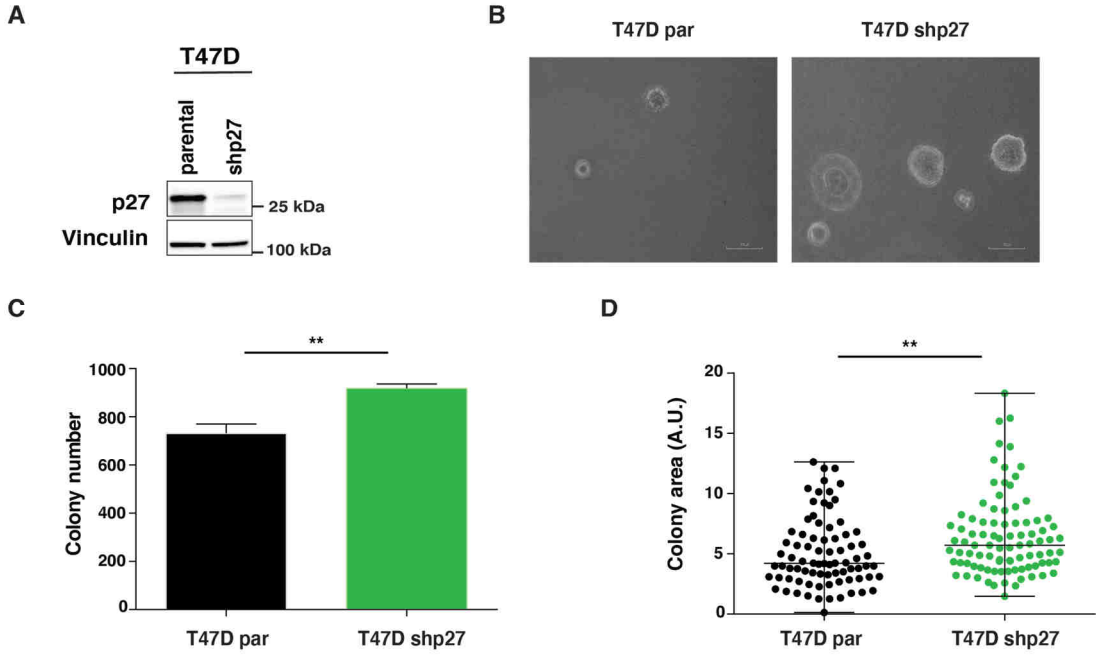

Figure S5. Silencing of p27 induces increased anchorage independent growth in LBC cells. (A) Western blotting analysis for p27 in T47D parental and p27-silenced (shp27) cells. Vinculin expression was used as loading control. (B) Representative bright field images of the colonies grown in soft agar, as described in (C) and (D). (C,D) Graphs show (C) colony number and (D) area of T47D parental (par) and p27 silenced (shp27) cells grown in soft agar for 20 days. Colony size was measured using ImageJ software. Mann–Whitney test and Student's *t*-test were used for statistical analysis. $^{**}p \leqslant 0.01$.

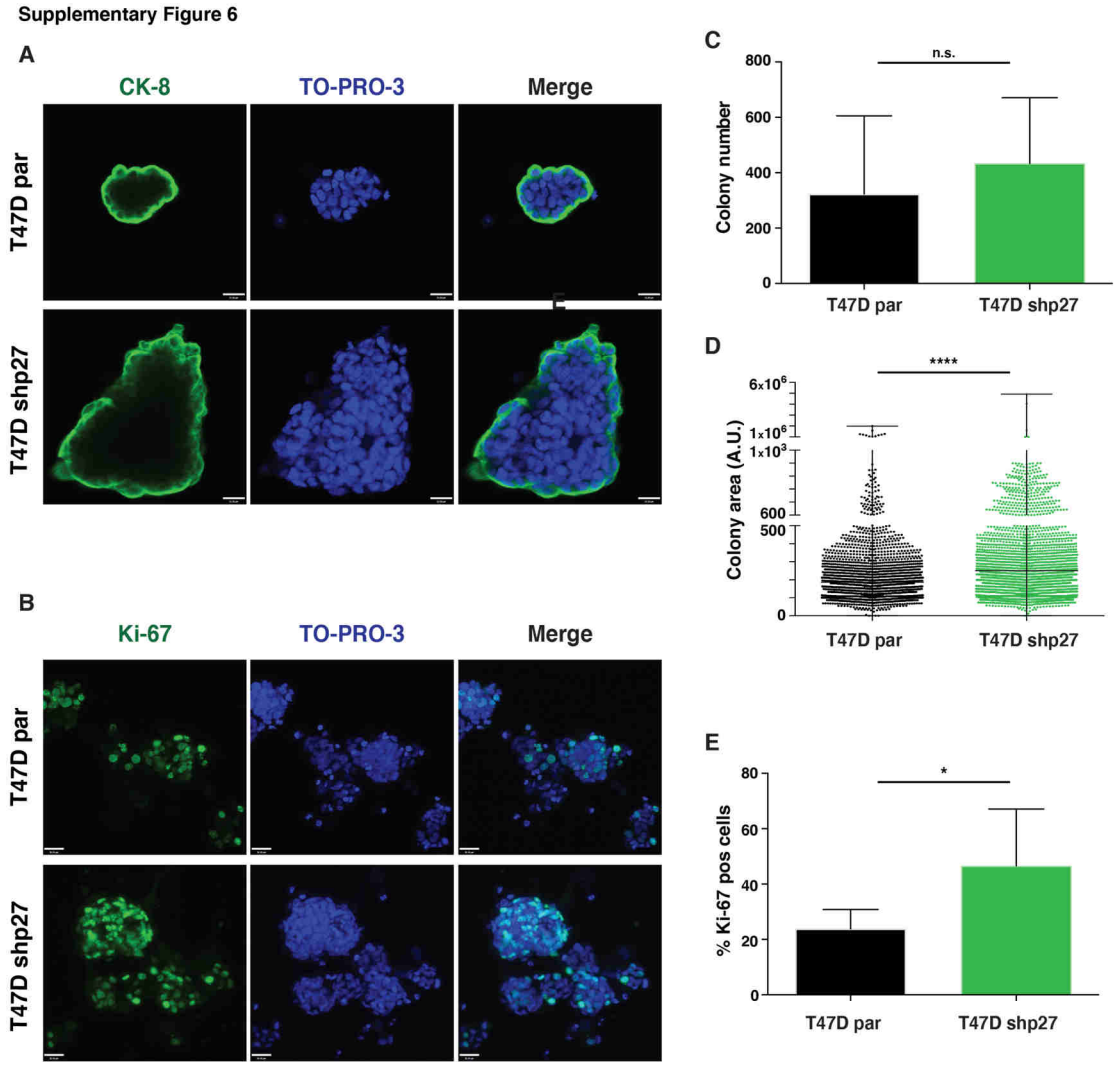

Figure S6. Loss of CDKN1B alters the growth of LBC cells in 3D-Matrigel. (A,B) Representative confocal images of immunofluorescence analyses of T47D parental (par) and p27-silenced (shp27) cells grown in 3D-Matrigel to form mammary acini. In (A), acini were immunostained for CK-8 (green, left panels); in (B), acini were immunostained for Ki-67 (green, left panels). Nuclei were counterstained with TO-PRO-3 (blue, central panels). (C–E) Graphs report (C) the number of colonies and (D) the area and (E) the percentage of Ki-67 positive cells in mammary acini of the experiment described in (A). Colony size was measured using ImageJ software. Ki-67 positive cells were counted in each colony and normalized by the number of nuclei, using Volocity software. Mann–Whitney test and Student's *t*-test were used for statistical analysis, as appropriate. Asterisks indicate significant differences. n.s. not significant; *$p \leqslant 0.05$; **** $p \leqslant 0.0001$.

Table S1. Clinicopathological features of young (<45 years old) premenopausal breast cancer patients (*n* = 227)

Characteristic	Distribution
Age	
Median	40 y
Range	25 -45 y
Subtype	
HER2- HR+ Luminal A	49 (22%)
HER2- HR+ Luminal B	146 (64%)
HER2+ HR+	14 (6%)
HER2+ HR-	4 (2%)
TNBC	12 (5%)
Not Available or Specified	2 (1%)
Tumor Grade	
G1	21 (9%)
G2	76 (33%)
G3	124 (55%)
Not Available or Specified	6 (3%)
Tumor Stage	
I	115 (51%)
II	71 (31%)
III	29 (13%)
IV	0 (0%)
Not Available or Specified	12 (5%)
Nodal Status	
N0	122 (54%)
N+	96 (42%)
Not Available or Specified	9 (4%)
Samples Type	
Primary	220 (97%)
Recurrence	1 (0.5%)
Lymph Metastasis	6 (2.5%)
Not Available or Specified	0 (0%)

HR = Hormone Receptors
Luminal A = HR+, Ki67 <20%
Luminal B = HR+, Ki67 >20%
HER2 = Human Epidermal Growth Factor Receptor 2
TNBC = Triple Negative Breast Cancer

Table S2. Clinicopathological features of breast cancer patients analyzed by liquid biopsy (*n* = 62)

	N	%
Age at diagnosis	Median: 50	IQR: 42 – 60
<45	24	39
45-65	33	53
>65	5	8
Age at blood draw	Median: 60	IQR: 50 – 67
<45	9	15
45-65	36	58
>65	17	27
Subtype		
Luminal	59	95
TNBC	3	5
Histotype		
Ductal	51	82
Lobular	8	13
Mucinous	3	5
Treatment		
CT	15	24
ET	47	76

IQR: interquartile range
TNBC: triple negative breast cancer
CT: chemotherapy
ET: endocrine therapy

Table S3. Clinicopathological features of ovarian cancer patients (*n* = 110)

Characteristic	Distribution
Age-Year	
Median	61 y
Range	34 -83 y
Histotype	
Serous	94 (85%)
Endometrioid	5 (5%)
Clear cell	5 (5%)
Mucinous	3 (3%)
Undifferentiation	3 (3%)
Tumor Grade	
Low (1-2)	5 (5%)
High (3)	90 (82%)
Not Available or Specified	15 (13%)
Tumor Stage	
I or II	19 (17%)
III or IV	80 (73%)
Not Available or Specified	11 (10%)
Samples Type	
Primary	95 (86%)
Recurrence	13 (12%)
Not Available or Specified	2 (2%)

Table S4. Clinicopathological features of head and neck cancer patients (*n* = 202)

Characteristic	Distribution
Age (Years)	
Median	63 y
Range	36 – 91 y
Cancer site	
Oral cavity/Tongue	71 (35%)
Oro-/Hypo-/Pharynx	52 (26%)
Larynx	25 (12%)
Others*	4 (2%)
Not Available or Specified	50 (25%)
Tumor Grade	
G1	13 (6%)
G2	76 (38%)
G3-G4	53 (26%)
Not Available or Specified	60 (30%)
Histology	
SCC **	150 (74%)
Others***	4 (2%)
Not Available or Specified	48 (24%)
Tumor size	
T1-T2	111 (55%)
T3-T4	55 (27%)
Not Available or Specified	36 (18%)
Nodal status	
N0	96 (48%)
N1-N2	73 (36%)
Not Available or Specified	33 (16%)

* Includes Tonsils and Lymph nodes
** SCC = Squamous Cell Carcinoma
*** It includes adenocarcinoma, basal cell carcinoma and undifferentiated Head and Neck cancers

Table S5. Mutations of *CDKN1B* detected by multi-gene panel and not confirmed by targeted sequencing in breast cancer patients

BC ID #	Age	Histotype	Subtype	Stage	Protein position	Amino acid	Maf	Consequence
344	**31**	**Ductal**	**Lum B**	IIIA	**147**	**Q/***	**3.01**	stop_gained
7	**38**	**Ductal**	**TN**	IA	**3**	**N/K**	**37.25**	missense_variant
8	**38**	**Ductal**	**Lum B**	IIA	**48**	**H/Q**	**6.11**	missense_variant
321	**41**	**Lobular**	**Lum B**	IIIA	**179**	**P/L**	**2.68**	missense_variant
292	**44**	**Ductal**	**Lum B**	IA	**40**	**E/D**	**44.56**	missense_variant
320	**44**	**Ductal**	**Lum A**	IA	**194**	**R/I**	**7.99**	missense_variant
41	**49**	**Ductal**	**Lum B**	IIA	**185**	**E/***	**7.55**	stop_gained
31	**54**	**Ductal**	**Lum B**	IIA	**195**	**R/I**	**8.84**	missense_variant
33	**65**	**Lobular**	**Lum A**	IIA	**179**	**P/L**	**9.95**	missense_variant
303	**80**	**Lobular**	**Lum B**	IIB	**147**	**Q/***	**4.05**	stop_gained

Patients (BC ID#) have been ordered by their age (increasing).
Pink colored BC ID# are those <45 years of age.