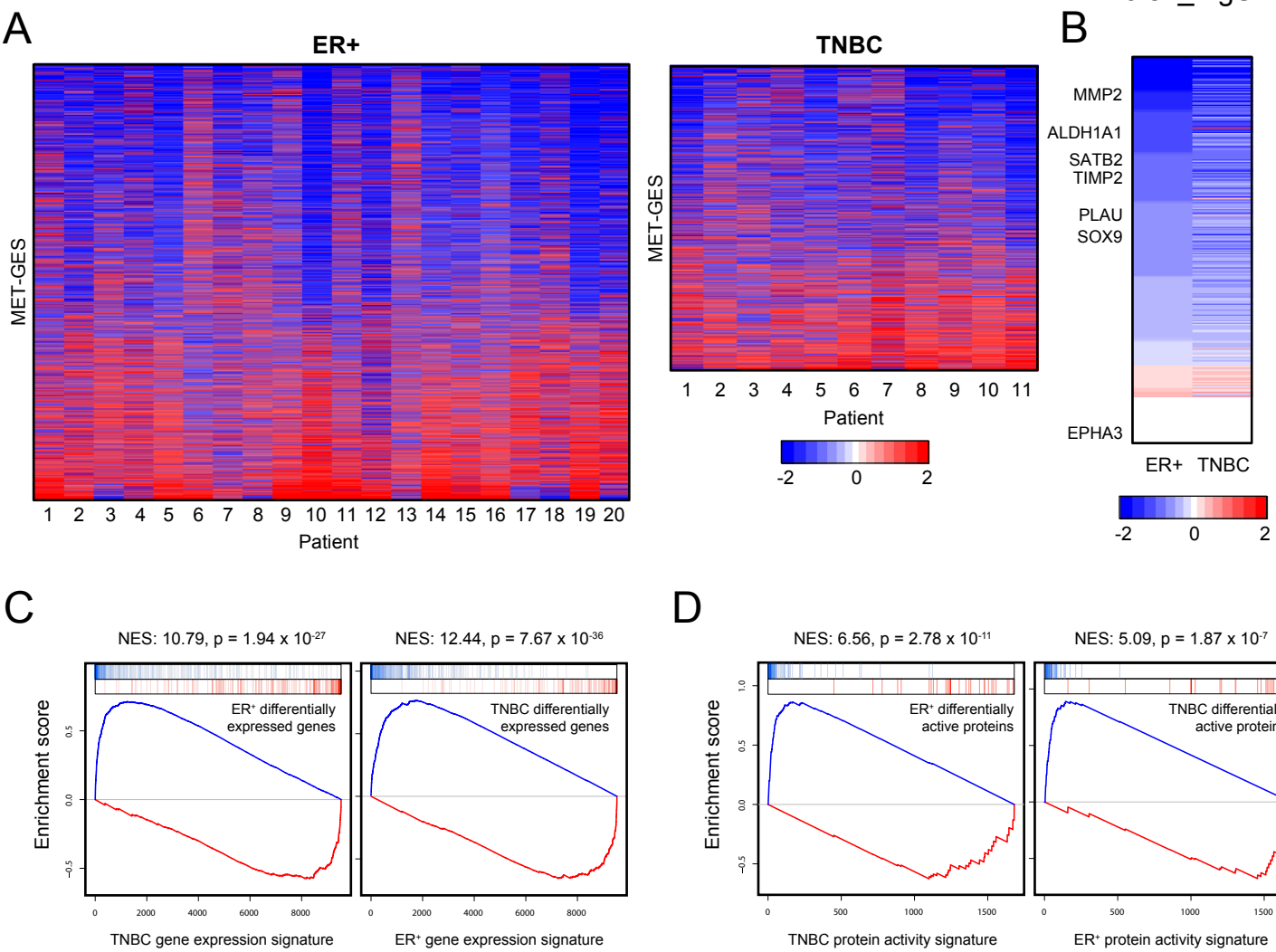
Walsh_FigS1


A
ER+
TNBC
MET-GES
Patient
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1 2 3 4 5 6 7 8 9 10 11
-2 0 2
B
MMP2
ALDH1A1
SATB2
TIMP2
PLAU
SOX9
EPHA3
ER+ TNBC
-2 0 2
C
NES: 10.79, p = 1.94 x 10-27
NES: 12.44, p = 7.67 x 10-36
Enrichment score
ER+ differentially expressed genes
TNBC differentially expressed genes
TNBC gene expression signature
ER+ gene expression signature
D
NES: 6.56, p = 2.78 x 10-11
NES: 5.09, p = 1.87 x 10-7
ER+ differentially active proteins
TNBC differentially active proteins
TNBC protein activity signature
ER+ protein activity signature

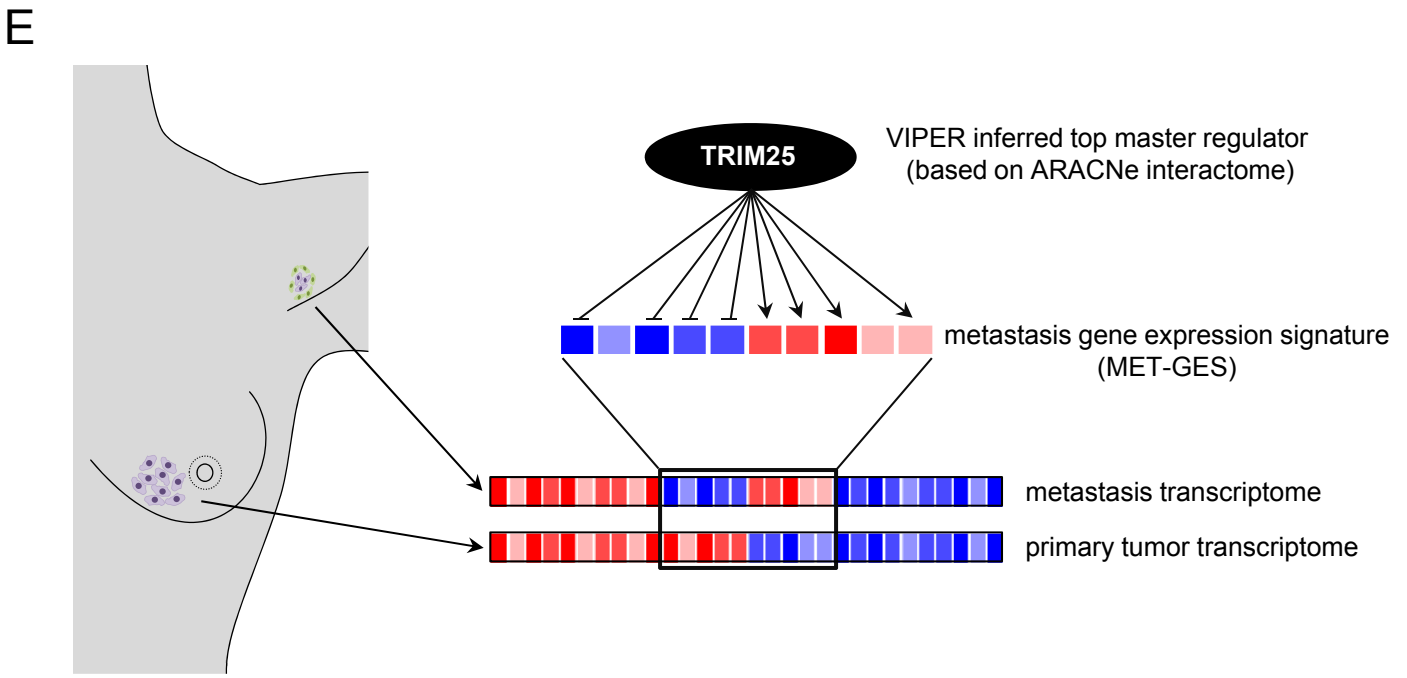

E
TRIM25
VIPER inferred top master regulator
(based on ARACNe interactome)
metastasis gene expression signature
(MET-GES)
metastasis transcriptome
primary tumor transcriptome

Figure S1. MET-GES are similar in ER+ and TNBC tumors. Related to Figure 1.

(A) Heatmap of individual ER+ and TNBC patients showing the relative expression for the top respective MET-GES genes ($p<0.05$). Shown is the fold change in lymph-node metastases compared with matched primary tumors. (B) Heatmap comparing average fold change of overlapping differentially expressed genes between luminal and basal metastasis signatures ($p<0.05$). Some known metastasis genes are highlighted. (C) Two tail GSEA for the enrichment of the top differentially expressed genes in the ER+ subtype ($p<0.05$) over the triple-negative subtype genome-wide signature (left), and for the top differentially expressed genes in the triple-negative subtype ($p<0.05$) over the ER+ subtype genome-wide signature (right). DEG, differentially expressed genes. (D) Two tail GSEA for the enrichment of the top differentially active TFs in the ER+ subtype ($p<0.05$) over the triple-negative subtype TF activity signature (left), and for the top differentially active TFs in the triple-negative subtype ($p<0.05$) over the ER+ subtype TF activity signature (right). DAP, differentially active proteins. (E) Schematic representation of the VIPER inferred top master regulator of the metastasis gene expression signature (MET-GES) based on the ARACNe breast cancer interactome.

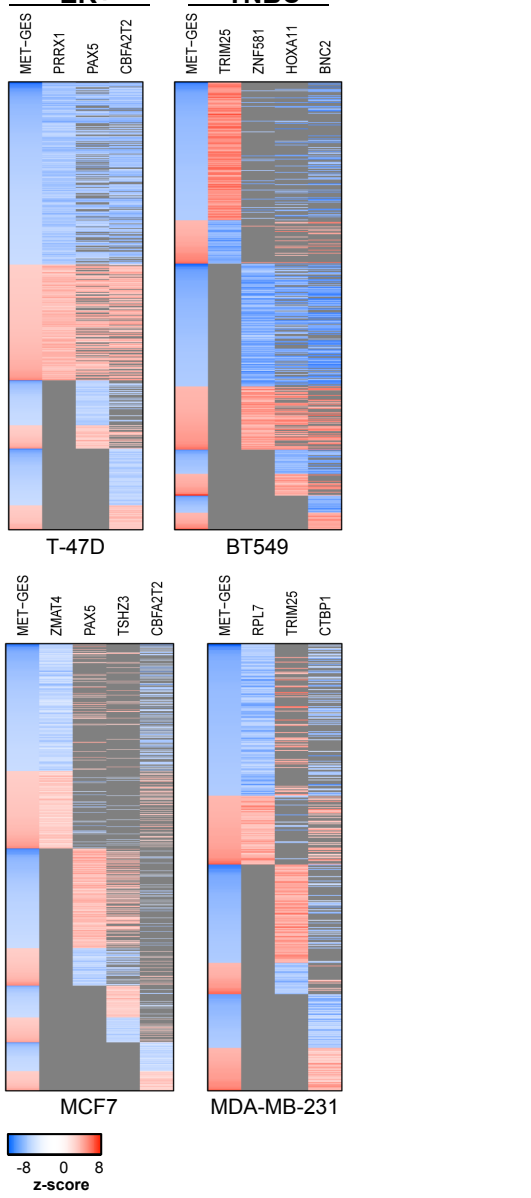
Walsh_FigS2

A

MR	Rank	MR-signature	BRCA Rank	ER+ Rank	TNBC Rank	Integration
TRIM25	1	BRCA	1	1	1	1
ZMAT4	1	ER+	-30	-1	-255	-1
FOXQ1	1	TNBC	-42	-43	-1	-1
HOXA11	1	BRCA	-1	-2	-7	-1
PAX5	2	BRCA	2	2	45	2
ZNF589	3	TNBC	7	37	3	3
ZNF253	3	BRCA	3	4	116	3
CBFA2T2	3	ER+	9	3	170	3
IRX6	3	TNBC	-70	-104	-3	-3
FOXD4L6	4	BRCA	4	10	11	4
CNBP	4	TNBC	20	154	4	4
PRRX1	4	ER+	-5	-4	-31	-4
ZNF142	5	ER+	6	5	126	5
YBX1	5	TNBC	552	773	5	5
ZNF581	6	TNBC	23	310	6	6
RPL7	7	TNBC	60	436	7	7
TSHZ3	7	BRCA	-7	-14	-38	-7
BNC2	7	ER+	-9	-7	-25	-7
CTBP1	8	TNBC	21	142	8	8
NKX6-1	8	BRCA	-8	-21	-18	-8

positive regulators of the MET-GES
negative regulators of the MET-GES

B



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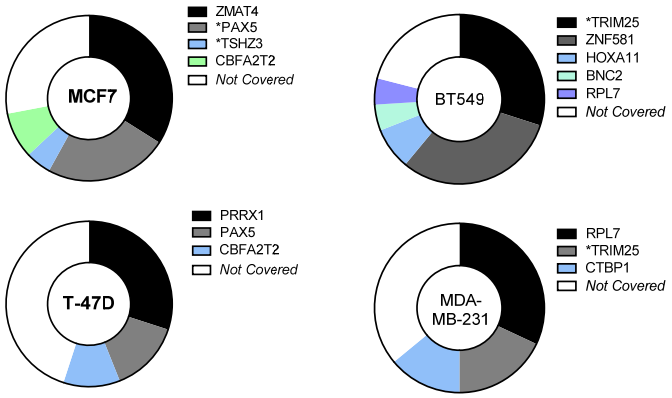
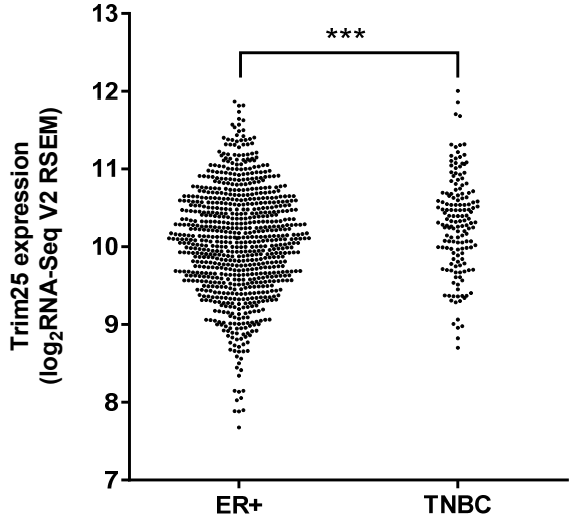
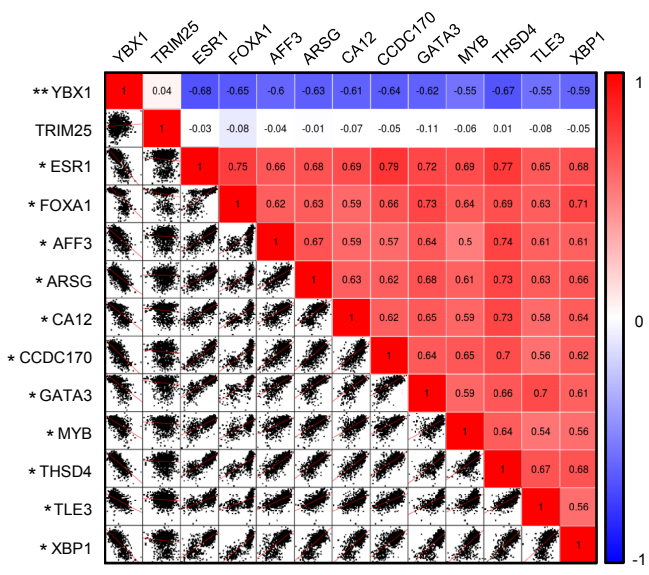


D



E



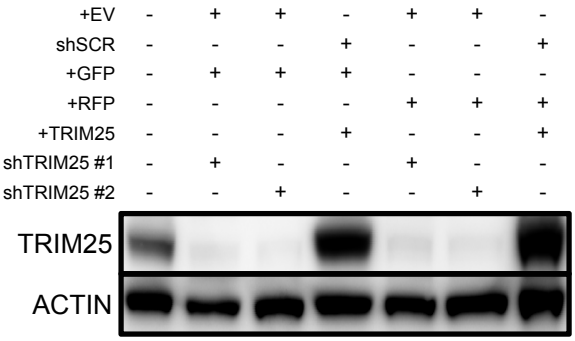
** enriched in estrogen receptor-negative tumors
* estrogen responsive gene

Figure S2. Master regulators of the breast cancer metastasis signature. Related to Figure 1.

(A) Ranking of candidate MRs selected for functional validation. "+" and "-" refer to positive and negative regulators of the MET-GES respectively. (B) Heatmaps showing the Z-score for differentially expressed genes in the MET-GES, either in human patient-matched primary and metastatic tumors, or in multiple cells lines following knockdown of each candidate MR, as identified by the linear regression and GSEA analysis (Figure S2A, S2C and Table S3). Genes not identified by regression/GSEA analysis are shown in grey. Cell lines are labelled below each heatmap. Candidate master regulators are labeled above. (C) Cumulative coverage of the MET-GES by the sequential addition of the MRs best explaining the MET-GES as inferred by the multiple lineal model regression analysis of the ER+ (left) and TNBC (right) cell lines. * denotes gene for which we obtained a negative coefficient in the regression analysis. (D) TRIM25 expression (log2 RNA-seq v2 RSEM from TCGA) in ER+ vs. TNBC tumors; n=1100. (E) Spearman's correlation matrix of established estrogen responsive genes (RNA-seq V2 RSEM) in 1100 breast cancer tumors from TCGA. The correlation coefficient is indicated as a heatmap in the top-right triangle. The normalized expression level for each of the genes is indicated by the scatter-plots on the bottom-left triangle.

Walsh_FigS3

A



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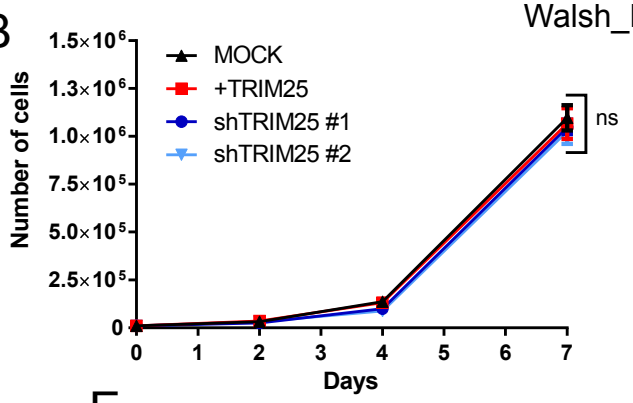
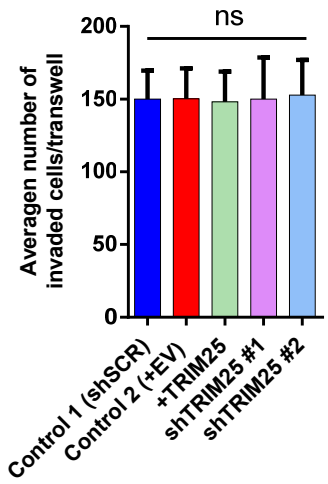
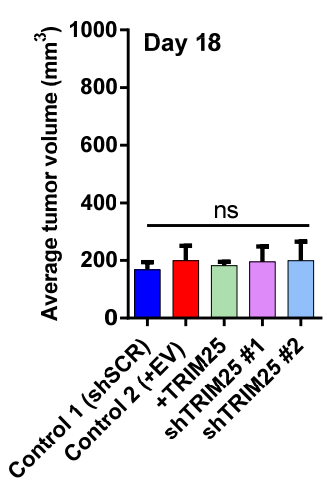


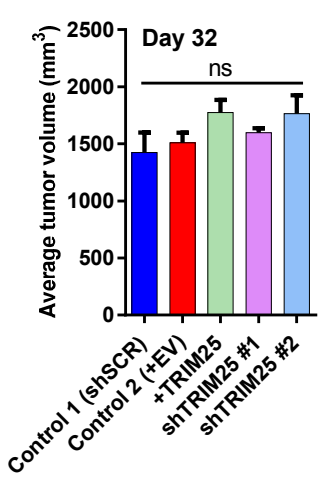
C



D



E



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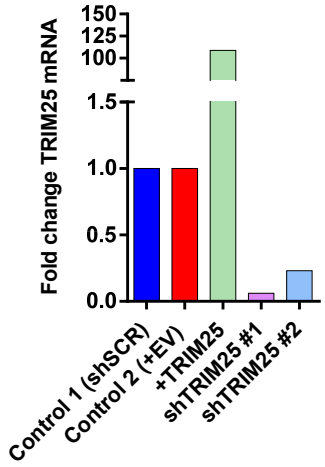
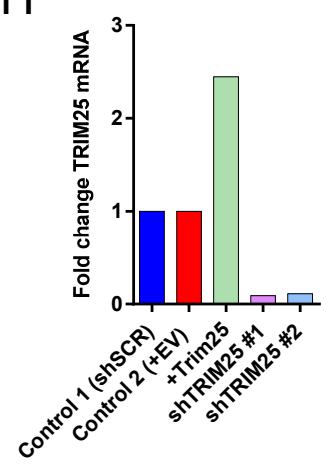
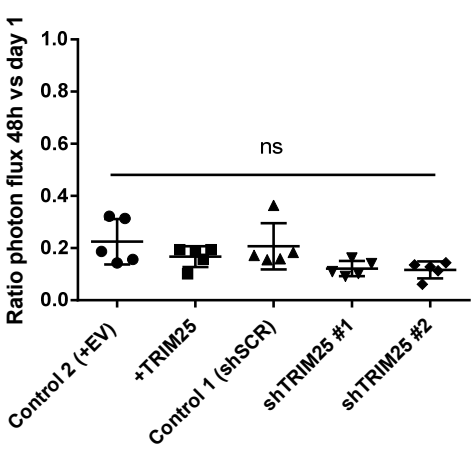


H



I



G

	1x10^5	1x10^4	1x10^3
+EV	8/8	8/8	8/8
+TRIM25	8/8	8/8	8/8
shSCRAMBLE	8/8	8/8	8/8
shTRIM25 #1	8/8	8/8	1/8
shTRIM25 #2	8/8	8/8	2/8

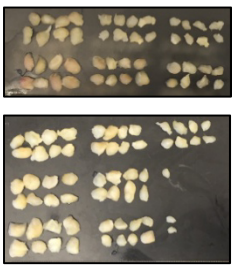


Figure S3. TRIM25 affects tumorigenicity but not primary tumor growth *in vivo*. Related to Figure 2.
(A) Immunoblot of MDA-MB-231 cells infected with indicated constructs. (B) *In vitro* growth curve of MDA-MB-231 cells after TRIM25 perturbation. Data are mean ± s.e.m., n=3; ns, not significant. (C) *In vitro* invasion of MDA-MB-231 cells after TRIM25 perturbation. Data are mean ± s.e.m., n=4; ns, not significant. (D-E) Primary tumor volume 18 (D) or 32 (E) days after injection of 1x106 PDX cells into the mammary fat pad of NSG mice. Data are mean ± s.e.m., n=5; ns, not significant. (F) qRT-PCR quantification of TRIM25 mRNA levels in PDX cells prior to injection into NSG mice. (G) Limiting dilution assay of MDA-MB-231 cells infected with indicated constructs injected into the mammary fat pad of athymic nude mice. Number of mice injected and tumor take is indicated. (H) qRT-PCR quantification of TRIM25 mRNA levels in CAL-51 cells prior to injection into mice. (I) Extravasation assay (BLI quantification) of CAL-51 cells 48h after injection into nude mice. Data are mean ± s.e.m., n=5; ns, not significant.

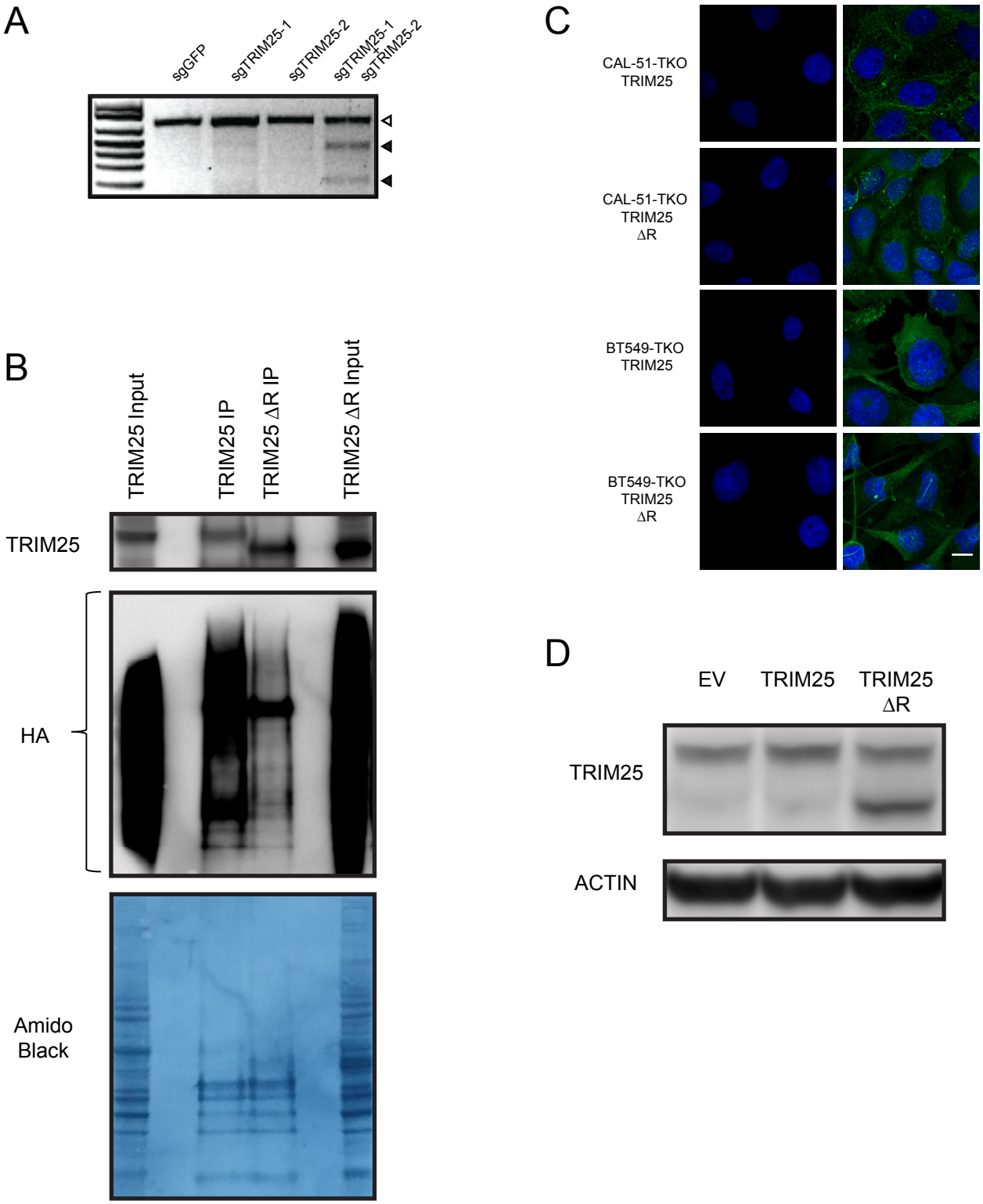

A
sgGFP
sgTRIM25-1
sgTRIM25-2
sgTRIM25-1
sgTRIM25-2
B
TRIM25 Input
TRIM25 IP
TRIM25 ΔR IP
TRIM25 ΔR Input
TRIM25
HA
Amido Black
C
CAL-51-TKO TRIM25
CAL-51-TKO TRIM25 ΔR
BT549-TKO TRIM25
BT549-TKO TRIM25 ΔR
D
EV
TRIM25
TRIM25 ΔR
TRIM25
ACTIN

Figure S4. E3 Ubiquitin ligase activity of TRIM25. Related to Figure 3.

(A) Surveyor analysis with sgRNAs used in CRISPR-mediated knock-out of TRIM25. A 689bp fragment (open arrowhead) surrounding the targeted sites was first amplified from HEK cells that were co-transfected with the indicated constructs. T7 endonuclease I digestion released two bands (closed arrowheads) corresponding to the predicted size upstream and downstream of the paired target sites, as indicated by the 1Kb Plus DNA ladder (B) Immunoblot and amido black staining of TRIM25 IP in BT549-TKO cells transfected with ubiquitin-HA and infected with indicated constructs. (C) TRIM25 immunoflorescence in BT549-TKO and CAL-51-TKO cells infected with indicated constructs. Scale bar represents 10μm. (D) Immunoblot of MDA-MB-231 cells infected with indicated constructs.

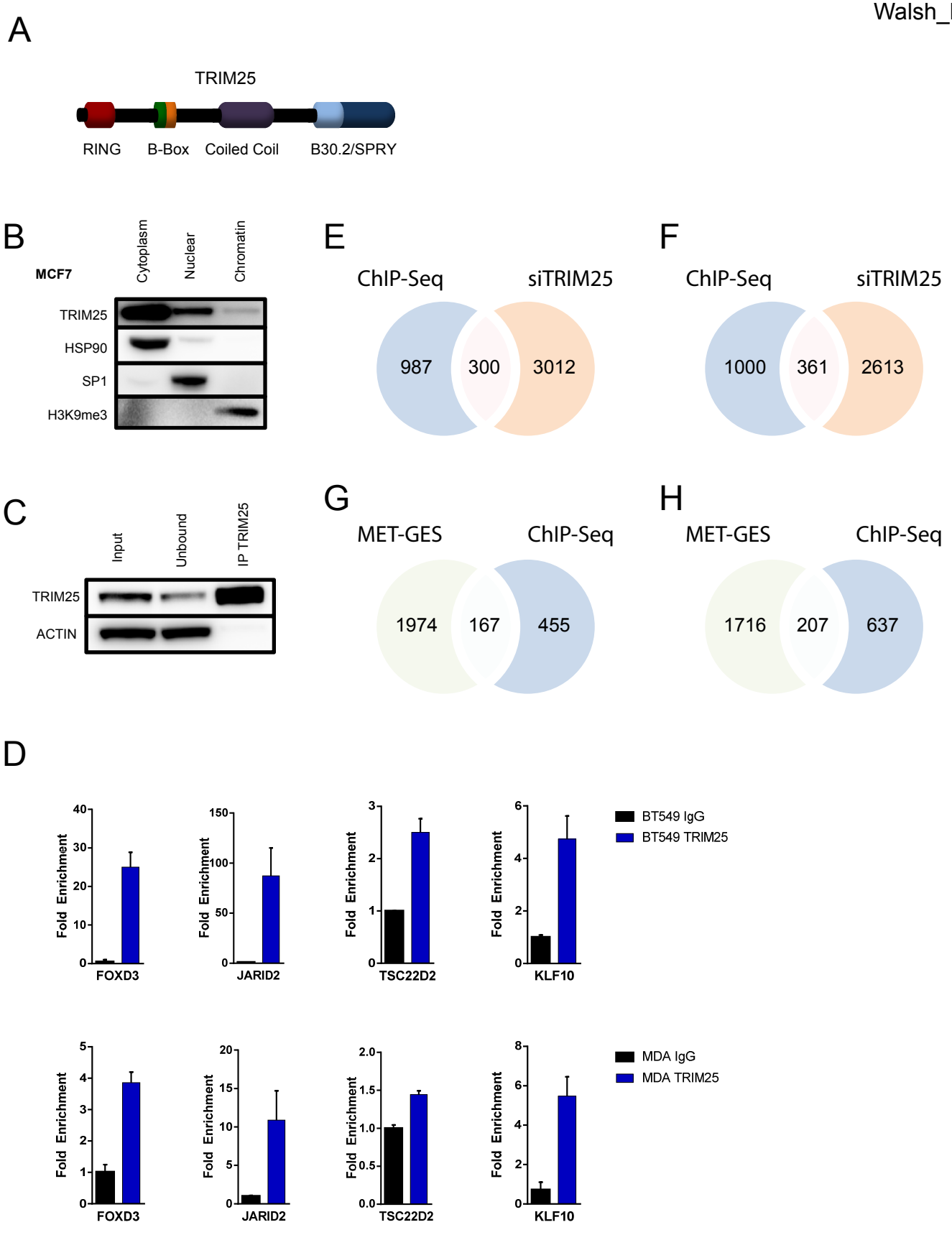
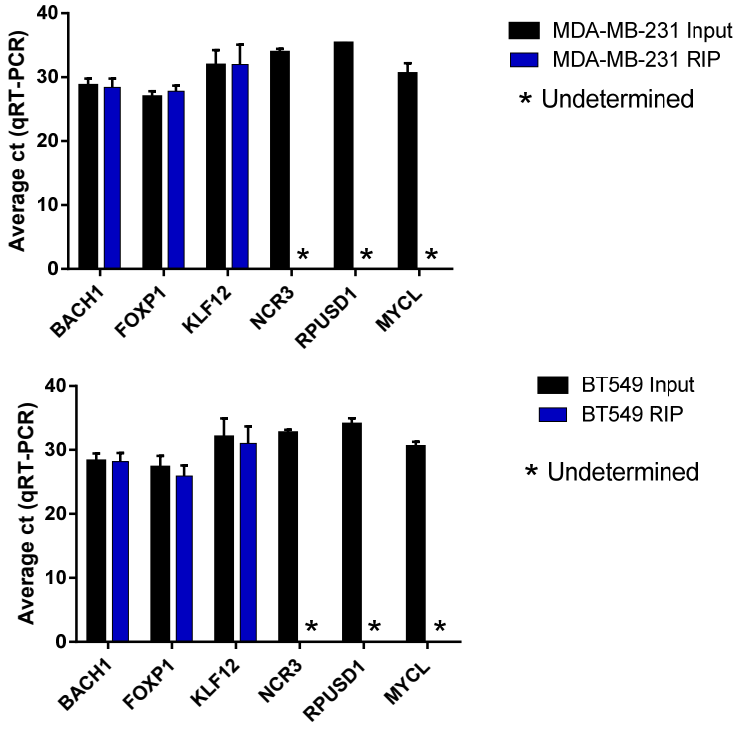

A
TRIM25
RING
B-Box
Coiled Coil
B30.2/SPRY
B
MCF7
Cytoplasm
Nuclear
Chromatin
TRIM25
HSP90
SP1
H3K9me3
C
Input
Unbound
IP TRIM25
TRIM25
ACTIN
D
Fold Enrichment
FOXD3
JARID2
TSC22D2
KLF10
BT549 IgG
BT549 TRIM25
MDA IgG
MDA TRIM25
E
ChIP-Seq
siTRIM25
987
300
3012
F
ChIP-Seq
siTRIM25
1000
361
2613
G
MET-GES
ChIP-Seq
1974
167
455
H
MET-GES
ChIP-Seq
1716
207
637

Figure S5. TRIM25 is localized to the cytoplasm, nucleus and chromatin. Related to Figure 4.

(A) Schematic representation of TRIM25 protein domains. (B) Immunoblot for TRIM25 in MCF-7 cells that have been fractionated into cytoplasmic, nuclear and chromatin fractions. HSP90, SP1 and H3K9me3 were used as control of fractionation efficiency. (C) Representative immunoblot of TRIM25 immunoprecipitation in TNBC cells. (D) Validation of TRIM25 ChIP-seq peaks by qPCR. (E) Venn diagram illustrating the overlap of ChIP-seq TSS peaks in BT549 cells with siTRIM25 (from Figure 4J). (F) Venn diagram illustrating the overlap of ChIP-seq TSS peaks in MDA-MB-231 cells with siTRIM25 (from Figure 4K). (G) Venn diagram illustrating the overlap of ChIP-seq TSS peaks in BT549 cells and the MET-GES signature (from Figure 4L). (H) Venn diagram illustrating the overlap of ChIP-seq TSS peaks in MDA-MB-231 cells and the MET-GES signature (from Figure 4M).

A



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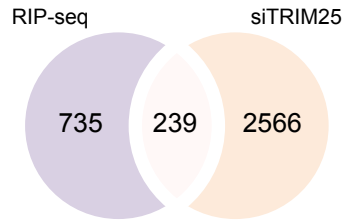
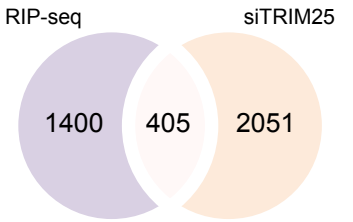


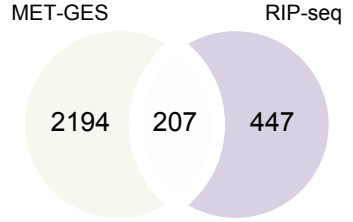
Odds ratio: 1.33, p-value = 1.7×10^{-4}

C



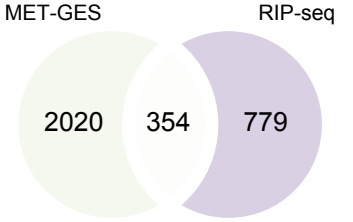
Odds ratio: 1.40, p-value = 3.81×10^{-8}

D



Odds ratio: 1.39, p-value = 1.2×10^{-4}

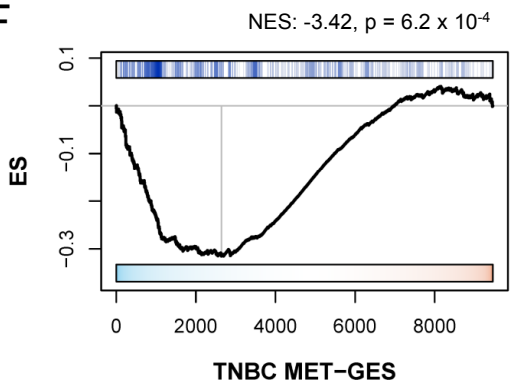
E



Odds ratio: 1.42, p-value = 4.01×10^{-7}

Transcriptional

F



Post-transcriptional

G

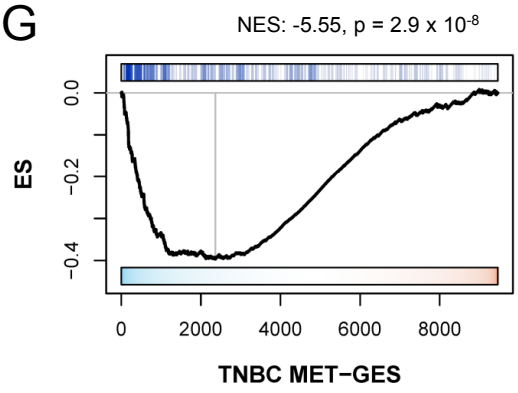


Figure S6. Core genes transcriptionally and post-transcriptionally regulated by TRIM25. Related to Figure 5. (A) Validation of TRIM25 RIP-seq peaks by qRT-PCR. (B) Venn diagram illustrating the overlap of RIP-seq genes in BT549 cells with siTRIM25 (from Figure 5B). (C) Venn diagram illustrating the overlap of RIP-seq genes in MDA-MB-231 cells with siTRIM25 (from Figure 5C). (D) Venn diagram illustrating the overlap of RIP-seq genes in BT549 cells and the MET-GES signature (from Figure 5D). (E) Venn diagram illustrating the overlap of RIP-seq genes in MDA-MB-231 cells and the MET-GES signature (from Figure 5E). (F) GSEA of the core set of genes transcriptionally regulated by TRIM25 on the MET-GES. (G) GSEA of the core set of genes post-transcriptionally regulated by TRIM25 on the MET-GES.

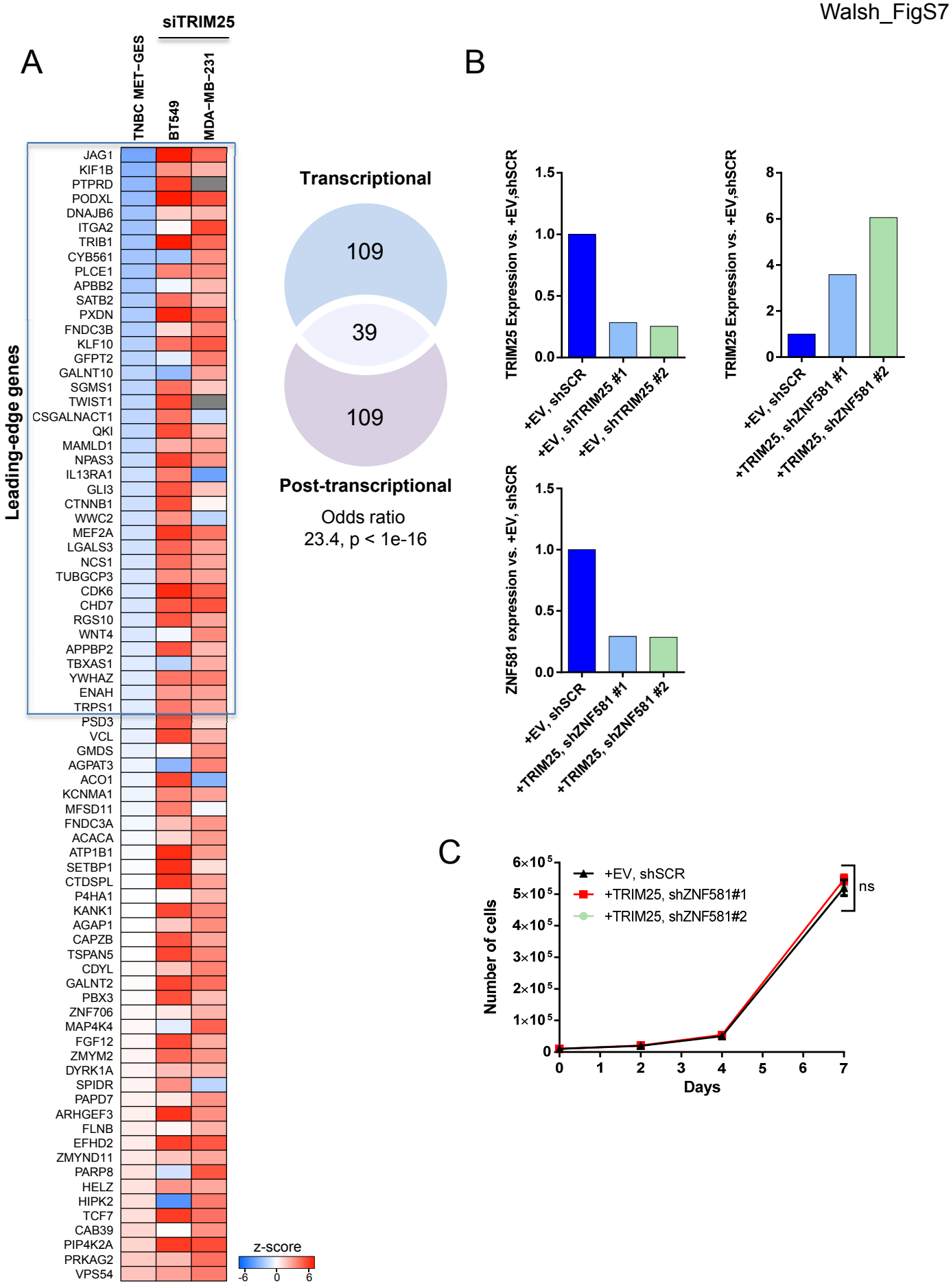

A
siTRIM25
TNBC MET−GES
BT549
MDA−MB−231
Leading-edge genes
JAG1
KIF1B
PTPRD
PODXL
DNAJB6
ITGA2
TRIB1
CYB561
PLCE1
APBB2
SATB2
PXDN
FNDC3B
KLF10
GFPT2
GALNT10
SGMS1
TWIST1
CSGALNACT1
QKI
MAMLD1
NPAS3
IL13RA1
GLI3
CTNNB1
WWC2
MEF2A
LGALS3
NCS1
TUBGCP3
CDK6
CHD7
RGS10
WNT4
APPBP2
TBXAS1
YWHAZ
ENAH
TRPS1
PSD3
VCL
GMDS
AGPAT3
ACO1
KCNMA1
MFSD11
FNDC3A
ACACA
ATP1B1
SETBP1
CTDSPL
P4HA1
KANK1
AGAP1
CAPZB
TSPAN5
CDYL
GALNT2
PBX3
ZNF706
MAP4K4
FGF12
ZMYM2
DYRK1A
SPIDR
PAPD7
ARHGEF3
FLNB
EFHD2
ZMYND11
PARP8
HELZ
HIPK2
TCF7
CAB39
PIP4K2A
PRKAG2
VPS54
z-score
-6
0
6
Transcriptional
109
39
109
Post-transcriptional
Odds ratio
23.4, p < 1e-16
B
TRIM25 Expression vs. +EV,shSCR
1.5
1.0
0.5
0.0
+EV, shSCR
+EV, shTRIM25 #1
+EV, shTRIM25 #2
TRIM25 Expression vs. +EV,shSCR
8
6
4
2
0
+EV, shSCR
+TRIM25, shZNF581 #1
+TRIM25, shZNF581 #2
ZNF581 expression vs. +EV, shSCR
1.5
1.0
0.5
0.0
+EV, shSCR
+TRIM25, shZNF581 #1
+TRIM25, shZNF581 #2
C
Number of cells
6×10^5
5×10^5
4×10^5
3×10^5
2×10^5
1×10^5
0
+EV, shSCR
+TRIM25, shZNF581#1
+TRIM25, shZNF581#2
ns
0
1
2
3
4
5
6
7
Days

Figure S7. Core MET-GES genes regulated by TRIM25. Related to Figure 6.

(A) Core MET-GES genes regulated by TRIM25 transcriptionally or post-transcriptionally. The heatmap includes the genes identified to be regulated both at the transcriptional and post-transcriptional levels in each TNBC cell line (indicated in the plot). Their enrichment on the TNBC MET-GES was computed by GSEA and the genes in the leading-edge are highlighted by a blue box. The Venn diagram shows the overlap between the MET-GES genes transcriptionally and post-transcriptionally regulated by TRIM25 in either TNBC cell line. (B) qRT-PCR quantification of BT549 cells treated with indicated shRNA prior to injection into the mammary fat pad of NSG mice. (C) Growth curve of BT549 cells infected with indicated constructs. Data represents mean ± s.e.m., n=3.

Supplemental Experimental Procedures

Cell lines

BT549, MDA-MB-231, MCF7 and T-47D cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to ATCC's recommended conditions. CAL-51 cells were a generous gift from Dr. Jose Baselga. Cell lines are routinely tested for potential mycoplasma contamination.

Patient Derived Xenograft (PDX) of human breast cancer

Patient derived triple-negative breast cancer xenograft is one of our broad ongoing efforts to routinely generate the PDXs from freshly resected human tumor tissues obtained by core needle biopsies and/or surgeries from our pre-consented patients as approved under the Institutional Review Board (IRB) protocol (97-094A). Clinical breast tumor specimen was quickly minced into $1mm^3$ fragments with sterile stainless steel disposable scalpels in the serum free minimal essential medium. Minced tumor fragments were transplanted subcutaneously in the flank of the NSG mice. Mice are stationed in our Institutional Animal Care and Use Committee (IACUC) approved specifically categorized bio-rooms under our institutional RARC facility and followed for the growth of the human tumor xenografts (PDXs). All the mice were treated and maintained in accordance with the care and animal use committee of laboratory experimental animals under the NIH guidelines and in the form of IACUC approved animal protocol (11-10-026). Palpable PDX tumors are typically variable and detectable around 2-3 weeks following xeno-transplantation and tumors are typically harvested when the tumor volume exceeds $1cm^3$ and the minced tumor tissues are re-transplanted as a new passage. Cell passaging is restricted to no more

than 5-6 times. The PDX derived cells were transduced with indicated lentiviral vectors before re-injection (1x10^6 cells) into the mammary fat pad of randomized 5-7 week old NOD-scid IL2Rgnull (NSG) mice obtained from Jackson Laboratories (Bar Harbor, ME, USA). Tumors were measured using calipers.

CRISPR mediated TRIM25 knock-out cell lines

TRIM25 was knocked out in CAL-51 cells and BT549 cells by co-transfecting with two lentiCRISPR v2 plasmids (Sanjana et al., 2014) modified to express the nickase Cas9 D10A (Al-Ahmadie et al., 2016; Jinek et al., 2012) one encoding sgTRIM25-1 (5′-CACCGTCGCGCCTGGTAGACGGCG-3′), and another encoding sgTRIM25-2 (5′-CACCGAACACGGTGCTGTGCAACG-3′). The cells were selected in puromycin for two days and then single cell clones were sorted into 96-well plates and expanded. Individual clones were validated for frameshift mutations by sequencing TOPO cloned PCR amplicons of the *TRIM25*-targeted genomic region (TRIM25 PCR Primers Forward 5′-TCGCGAGGATGCTCTAAATC-3′, Reverse 5′- TCGCGAGGATGCTCTAAATC-3′; TOPO TA, Invitrogen; M13F and M13R), and by immunoblotting. T7 Endonuclease I (SURVEYOR) analysis was performed using flanking TRIM25 primers (Forward 5′-GGGTGCAGCAGTTGTGTCC-3′ Reverse 5′- CCTCTGCACCACCCATCAG-3′). Cells were infected to express either full-length TRIM25 (Lv151-TRIM25, GeneCopoeia, Rockville, MD, USA), or a modified TRIM25ΔR that lacks aa 11-81, and selected in G418.

ESC Culture

H1 hESCs were grown on Matrigel-coated 6-well plates in mTeSR1 medium (STEM CELL Technologies, Vancouver, Canada) and passaged every five days using dispase (Invitrogen, Grand Island, NY, USA). Medium was changed every day. Cells were maintained at 37°C with 5% CO_2. H1 cells were disassociated and split for experiments using dispase (Invitrogen, Grand Island, NY, USA) onto Matrigel-coated 12 well plates. 24 hours after plating (day 0), media was changed to mTeSR1 supplemented with lentivirus carrying shRNA against TRIM25 or scramble shRNA and 1:2500 dilution of polybrene (EMD Millipore Billerica, MA, USA). The cells were spun at 1000 RPM for a half hour to increase the infection efficiency. On 48 hours after infection (day 2), hESC cells were then changed to mTeSR1 media with 0.5 μg/mL puromycin (Invitrogen, Grand Island, NY, USA) for 2 days to select the infected cells. On day 4, total RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to instructions by the manufacturer.

Virus production and generation of stable cell lines

HEK 293T cells were transfected with lentiviral expression constructs using Fugene 6 (Promega, Madison, WI, USA) in combination with **pCMV-dR8.2** (gag/pol)and **VSV-G** (env) expression vectors using Fugene 6 (Promega, Madison, WI, USA). Viral stocks were collected 48 h post transfection, filtered (0.45 μm) and placed on target cells for 8 h in the presence of 8 μg/ml polybrene. Forty-eight hours post infection, cells were selected in the presence of puromycin or neomycin, as determined by kill curve.

RNA interference

Knockdown using scramble and/or gene-specific siRNA (ON-TARGET SMARTpool, Thermo Scientific, Pittsburgh, PA, USA) was performed using Lipofectamine RNAiMAX (Invitrogen, Grand Island, NY, USA). Stable knockdown was achieved using scramble or gene-specific shRNA constructs: **Lv015** Empty Vector (GeneCopoeia, Rockville, MD, USA), **Lv105** TRIM25 Vector (GeneCopoeia, Rockville, MD, USA), shSCRAMBLE (**RHS6848**) (Sigma, St Louis, MO, USA), shTRIM25#1 (**TRCN0000003445**) (Sigma, St Louis, MO, USA), shTRIM25#2 (**TRCN0000003446**) (Sigma, St Louis, MO, USA), shZNF581#1 (**TRCN0000108101**) (Sigma, St Louis, MO, USA), shZNF581#2 (**TRCN0000108102**) (Sigma, St Louis, MO, USA).

Cell proliferation assay

Cells were plated at indicated concentrations in a 6-cm plate in triplicate, and cell numbers were counted at the indicated days using a ViCELL cell counter (Beckman, Indianapolis, IN, USA).

Cell invasion assay

Ten thousand cells were seeded in serum-free media on Matrigel-coated transwell inserts (8 μm, BD Biosciences). Complete media was placed below transwell inserts to encourage cell invasion. After 24h, cells that did not invade were removed. Membranes were fixed with methanol and stained with DAPI. The number of invaded cells was determined by manual counting using an inverted Nikon fluorescent microscope.

Immunoblotting

Cell lysates were prepared with CellLytic M (Sigma, St Louis, MO, USA) containing protease and phosphotase inhibitor (Thermo Scientific, Pittsburgh, PA, USA). Debris was removed via centrifugation for 10 min at 14 000 r.p.m. in an Eppendorf Centrifuge 5424C (Eppendorf, Hauppauge, NY, USA) at 4 °C. Protein concentrations were determined by BCA protein assay kit (Thermo Scientific, Pittsburgh, PA, USA), and equal amounts of protein samples were loaded on NuPAGE Bis-Tris 4–12% sodium dodecyl sulfate gels (Invitrogen, Grand Island, NY, USA) and blotted onto polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Imaging was performed on a FujiFilm LAS4000 (GE Healthcare, Pittsburgh, PA, USA). For TKO cell lines expressing TRIM25ΔR, we used anti-TRIM25 mouse monoclonal antibody (LifeSpan Biosciences, 5C3). Control antibodies used: Actin A2066 (Sigma, St Louis, MO, USA), SP1 9389S (Cell Signaling Technology, Danvers, MA, USA), H3K93me ab8898 (Abcam, San Francisco, CA, USA).

Cell Fractionation

Subcellular Protein Fractionation was achieved using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific, Pittsburgh, PA, USA) according to manufacturer's instructions.

Immunohistochemistry

The immunohistochemical detection of indicated antibodies was performed at Molecular Cytology Core Facility of Memorial Sloan Kettering Cancer Center using Discovery XT

processor (Ventana Medical Systems). The tissue sections were deparaffinized with EZPrep buffer (Ventana Medical Systems), antigen retrieval was performed with CC1 buffer (Ventana Medical Systems) and sections were blocked for 30 minutes with Background Buster solution (Innovex). Antibodies (COX4, Cell Signaling Cell Signaling Technology, Danvers, MA, USA, EMD Millipore Billerica, MA, USA) were applied and sections were incubated for 6 hours, followed by 60 minutes incubation with biotinylated goat anti-rabbit IgG (Vector labs, cat#PK6101) or (biotinylated horse anti-mouse IgG (Vector Labs, cat# MKB-22258) at 1:200 dilution. The detection was performed with DAB detection kit (Ventana Medical Systems) according to manufacturer instruction. Slides were counterstained with hematoxylin and coverslipped with Permount (Fisher Scientific).

Immunofluorescence

Cells were grown as monolayer cultures on pre coated Millicell EZ slides (Millipore) and were fixed with 4% PFA. After washing 3 times with PBS-T (PBS 0.1% Triton X-100, cells were blocked for 30mins with 10% goat serum and incubated overnight at 4°C with primary antibodies anti-TRIM25 mouse monoclonal antibody (LifeSpan Biosciences, 5C3). Cells were then stained with Alexa Fluor 488 secondary antibody (1:200, Invitrogen) for 1 hr at room temperature. Stained sections and cells were mounted in ProLong Gold™ antifade reagent (Invitrogen) with DAPI. Images were taken on a Leica TCS SP5 confocal microscope.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation was performed using the Magna ChIP™ G - Chromatin Immunoprecipitation Kit according to manufacturer's instructions. For immunoprecipitation, lysate from $5x10^6$ cells and 10 µg anti-TRIM25 mouse monoclonal antibody (BD Biosciences, Franklin Lakes, NJ, USA) or 10 µg mouse monoclonal IgG antibody (R&D Systems, Minneapolis, MN, USA) was used. 1% input was used as a control for subsequent ChIP-seq analysis. For qPCR validation, immunoprecipitated and input DNA were amplified using standard protocols (WGA3, Sigma, St Louis, MO, USA) and analyzed by qPCR.

Primers used:

GAPDH -Forward 5′- CGGGATTGTCTGCCCTAATTAT-3′

GAPDH -Reverse 5′- GCACGGAAGGTCACGATGT-3′

FOXD3-Forward 5′- GGCACTCAAACCCTCTTCC-3′

FOXD3-Reverse 5′- GTCGCTGTCCTTCTCTTCCA-3′

JARID2-Forward 5′- CCGCACTCTGTTGTCATTGT-3′

JARID2-Reverse 5′-AGGAGGAGGGGAAGGTTC-3′

TSC22D2-Forward 5′-GGACCTTCAGCCAGCTCC-3′

TSC22D2-Reverse 5′-TCGGAGATGCCTAATTCAGC-3′

KLF10-Forward 5′-AGAGACGGGGTGGTCTGAG-3′

KLF10-Reverse 5′-GTCACTCGGGGACGAGAAG-3′

RNA Immunoprecipitation

EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (EMD Millipore Billerica, MA, USA) was used for RNA Immunoprecipitation according to manufacturer's instructions. For immunoprecipitation, anti-TRIM25 mouse monoclonal antibody (EMD Millipore Billerica, MA, USA) or mouse monoclonal IgG antibody (EMD Millipore Billerica, MA, USA) was used. EZ-Magna RIP™ positive control antibody was used to control for successful IP.

Primers used:

BACH1 Forward 5′-TGTTGTCGGGAAGTTCAGTG-3'

BACH1 Reverse 5′-GCTCTCGCTTCAGTCAGTCG-3'

FOXP1 Forward 5′-TCACCTCAAAAGGTCACGTC-3'

FOXP1 Reverse 5′-GTTGCAGTCCTGTGGCATTA-3'

KLF12 Forward 5′-CCCATCAAGCATTAACATTCTG-3'

KLF12 Reverse 5′-CTGCTCTGCAGCTTCTGTTC-3'

NCR3 Forward 5′-AAGGCAGAGGATCCTTCCAG-3'

NCR3 Forward 5'-AAGGCAGAGGATCCTTCCAG-3'

NCR3 Reverse 5′-TGGATGCTGTTGCTCATCTT-3'

RPUSD1 Forward 5′-CGGCTCCGGTACACGAT-3'

RPUSD1 Reverse 5′-CTTCCAGGGTCTCTGGGTC-3'

MYCL Forward 5′-GTCGTAGAAATAGTGCTGGTACG-3'

MYCL Reverse 5′-GTGCGTGTGTGCTGGCT-3'

Ubiquitination Assay

BT549-TKO or CAL-51-TKO cells infected with indicated constructs were transfected with plasmid encoding hemagglutinin-tagged ubiquitin (HA-Ub) for 48h. After treatment with MG132 (25μM) for 6 hours, cell lysates were prepared and subjected to immunoprecipitation with antibody to TRIM25 (LifeSpan Biosciences, 5C3). Followed by western blotting. The anti-HA antibody was purchased from Cell Signalling (C29F4).

Quantitative RT-PCR

Total RNA was isolated from cells using RNeasy Plus according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). cDNA was generated using 1 μg RNA and cDNA EcoDry Premix (Clontech, Mountain View, CA, USA). qRT–PCR was performed using a QuantStudio™ 6 Flex Real-Time PCR System (Thermo Scientific, Pittsburgh, PA, USA) and analyzed by standard delta relative quantitation (ΔΔRQ). qRT–PCR parameters were as follows: initial denaturation at 95 °C for 5 min, then 40 cycles of 95 °C 15 s and 60 °C 1 min.

Primers used:

GAPDH-Forward 5′-AATGAAGGGGTCATTGATGG-3′

GAPDH-Reverse5′-AAGGTGAAGGTCGGAGTCAA-3′

TRIM25-Forward 5'-GCCCCGTTGATCTGACTGTA-3'

TRIM25-Reverse 5'-TGGTGGAGCATAAGACCTGC-3

ZNF581-Forward 5'-GTCTCAACGGAGGAAAATGC-3'

ZNF581-Reverse 5'-CTGAGAGGCTGCTGCACTG-3'

PAX3-Forward 5′-GGAGAAGAGGAAGACCTGGAGCAATAAA-3′

PAX3-Reverse 5′-GCACGCACACAAGCAAATGGAA-3′

FOXA2-Forward 5′-CCATTGCTGTTGTTGCAGGGAAGT-3′

FOXA2-Reverse 5′-CACCGTGTCAAGATTGGGAATGCT-3′

BRACHURY-Forward 5′-GGTGTGCCAAAGTTGCCAATACAC-3′

BRACHURY-Reverse 5′-TGTCCCAGGTGGCTTACAGATGAA-3′

NANOG Forward 5’-CAGGGCTGTCCTGAATAAGC -3’

NANOG Reverse 5’-GATTTGTGGGCCTGAAGAAA-3’

POU5F1 Forward 5’-CTTCTGCTTCAGGAGCTTGG-3’

POU5F1 Reverse 5’-GAAGGAGAAGCTGGAGCAAA-3’

SOX2 Forward 5’-GCTTAGCCTCGTCGATGAAC-3’

SOX2 Reverse 5’-AACCCCAAGATGCACAACTC-3’