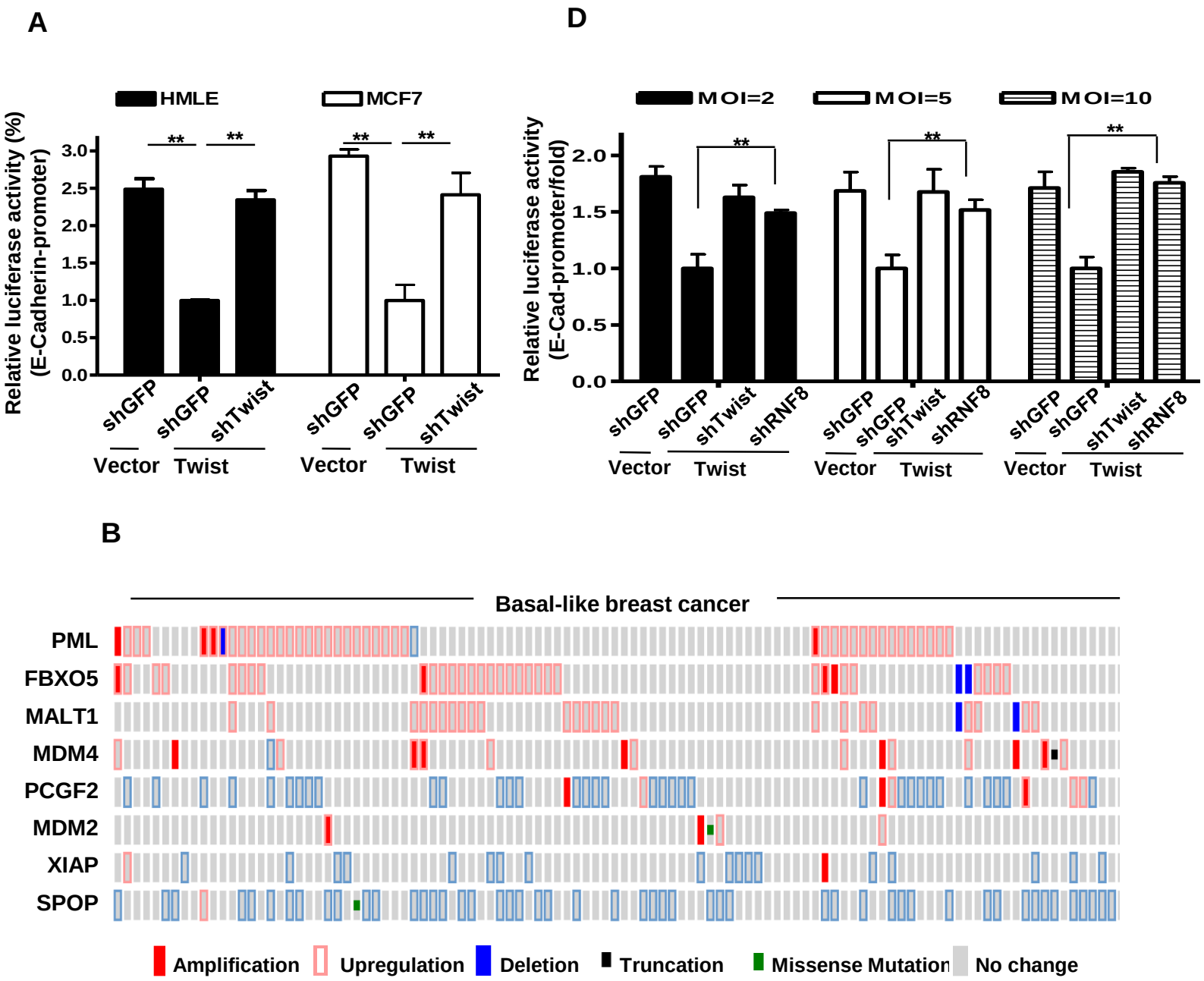

C

%	Ampplification/Upreulation	Deletion/Downregulation	Missense/Truncation Mutation
RNF8	57	0	0
PML	28	1	0
FBXO5	23	2	0
MALT1	13	2	0
MDM4	8	0	1
PCGF2	5	7	0
MDM2	3	0	1
XIAP	2	4	0
SPOP	0	20	1

Figure S1, related to Figure 1

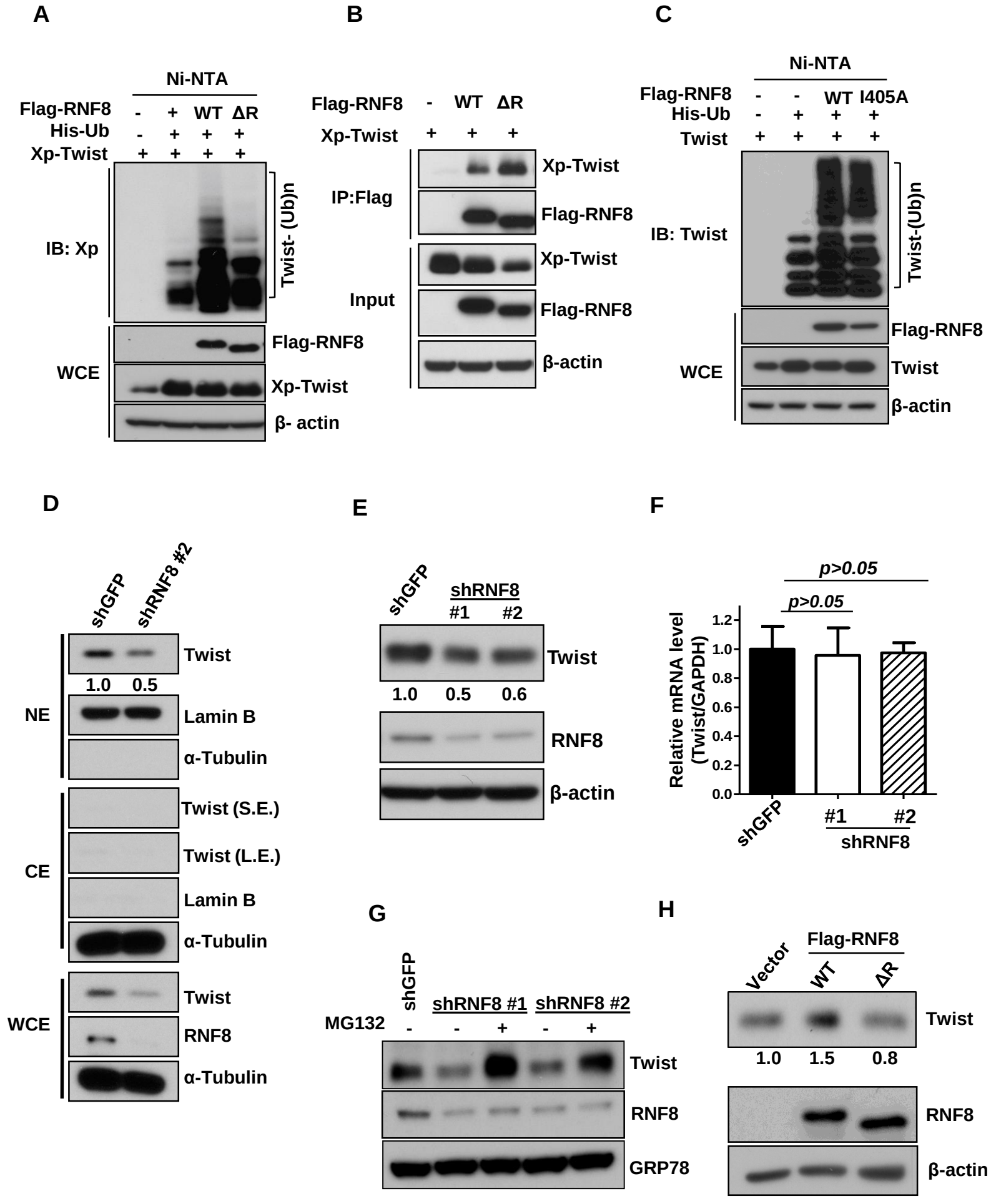

Figure S2, related to Figure 2

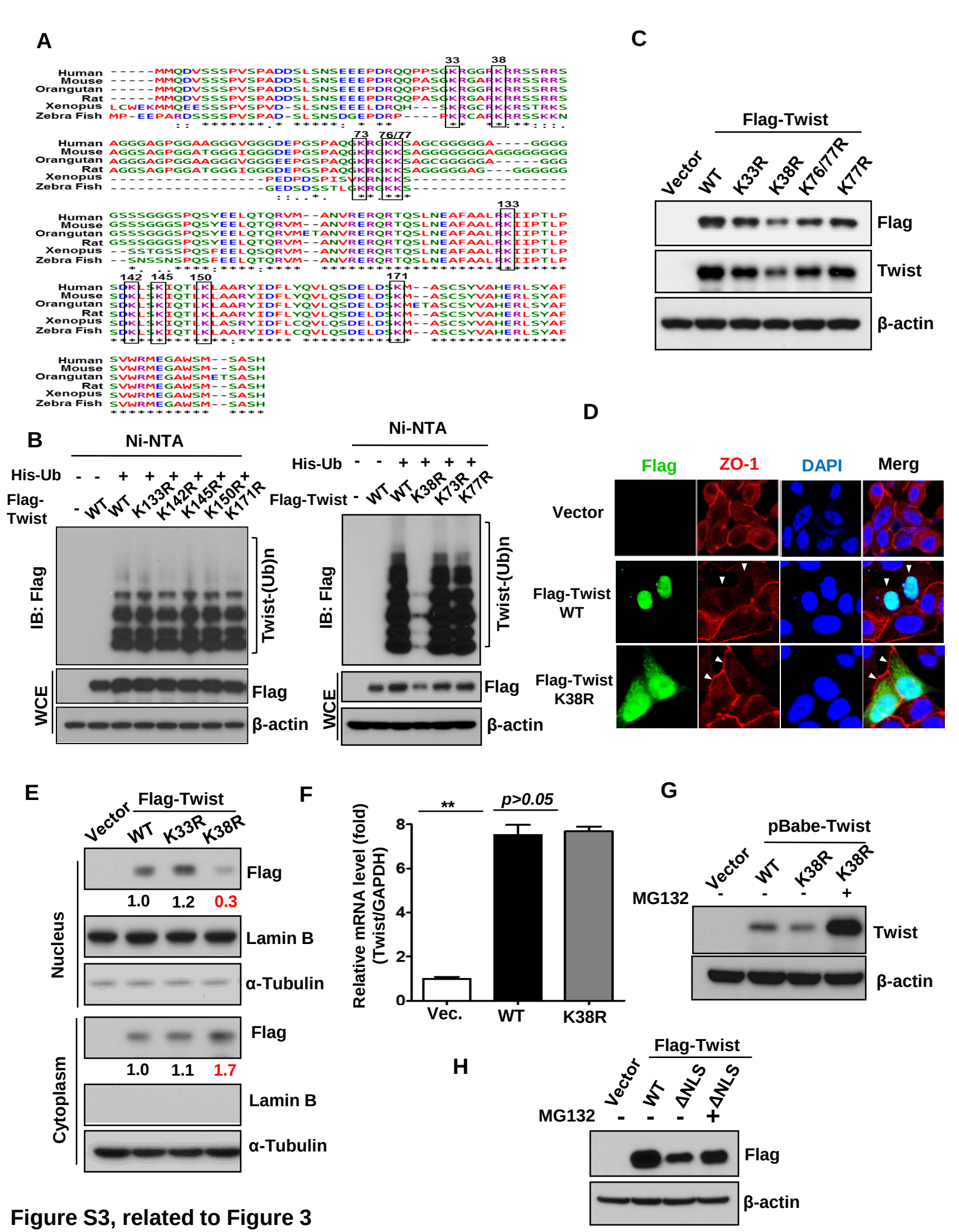

Figure S3, related to Figure 3

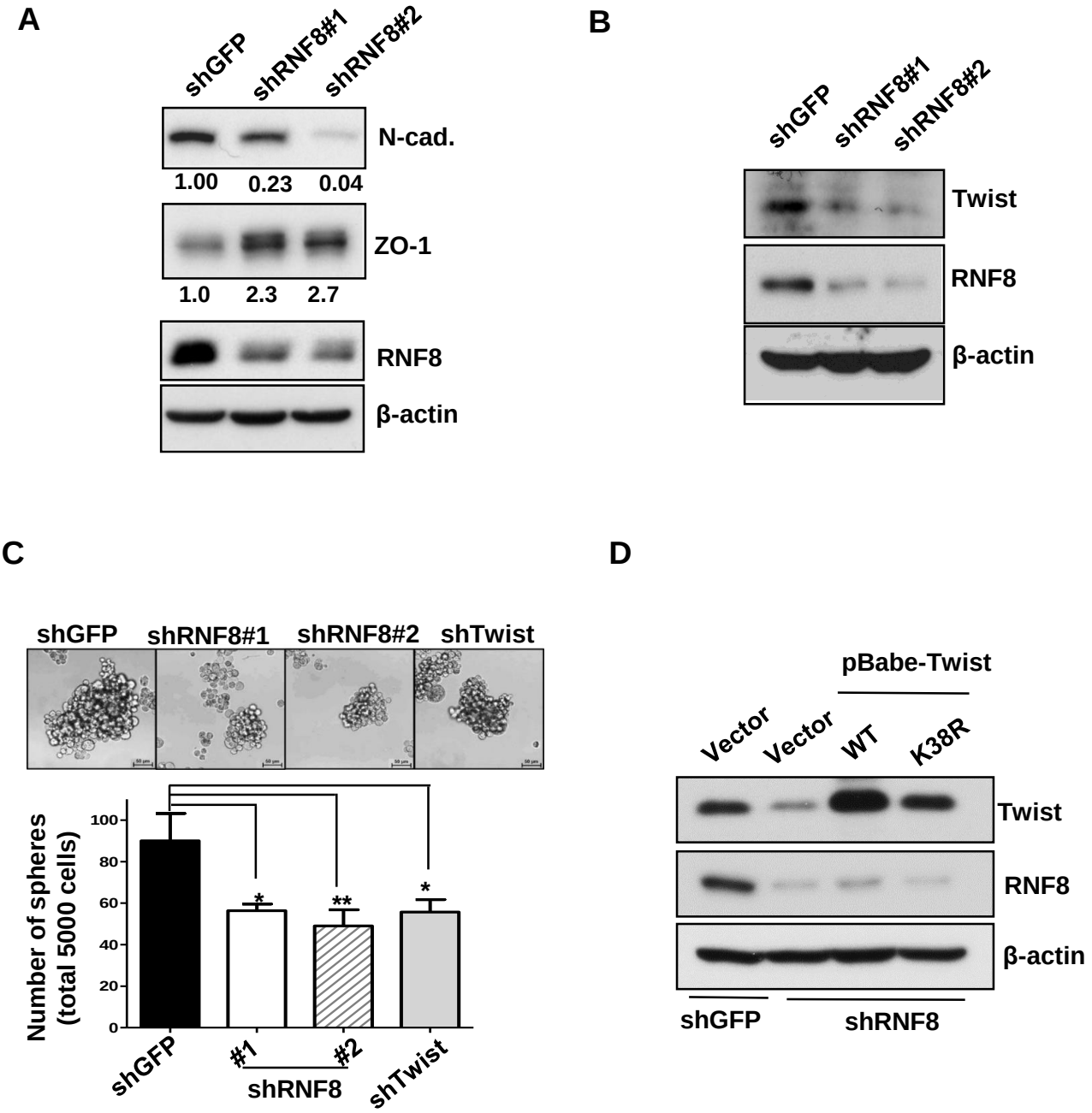

Figure S4, related to Figure 4

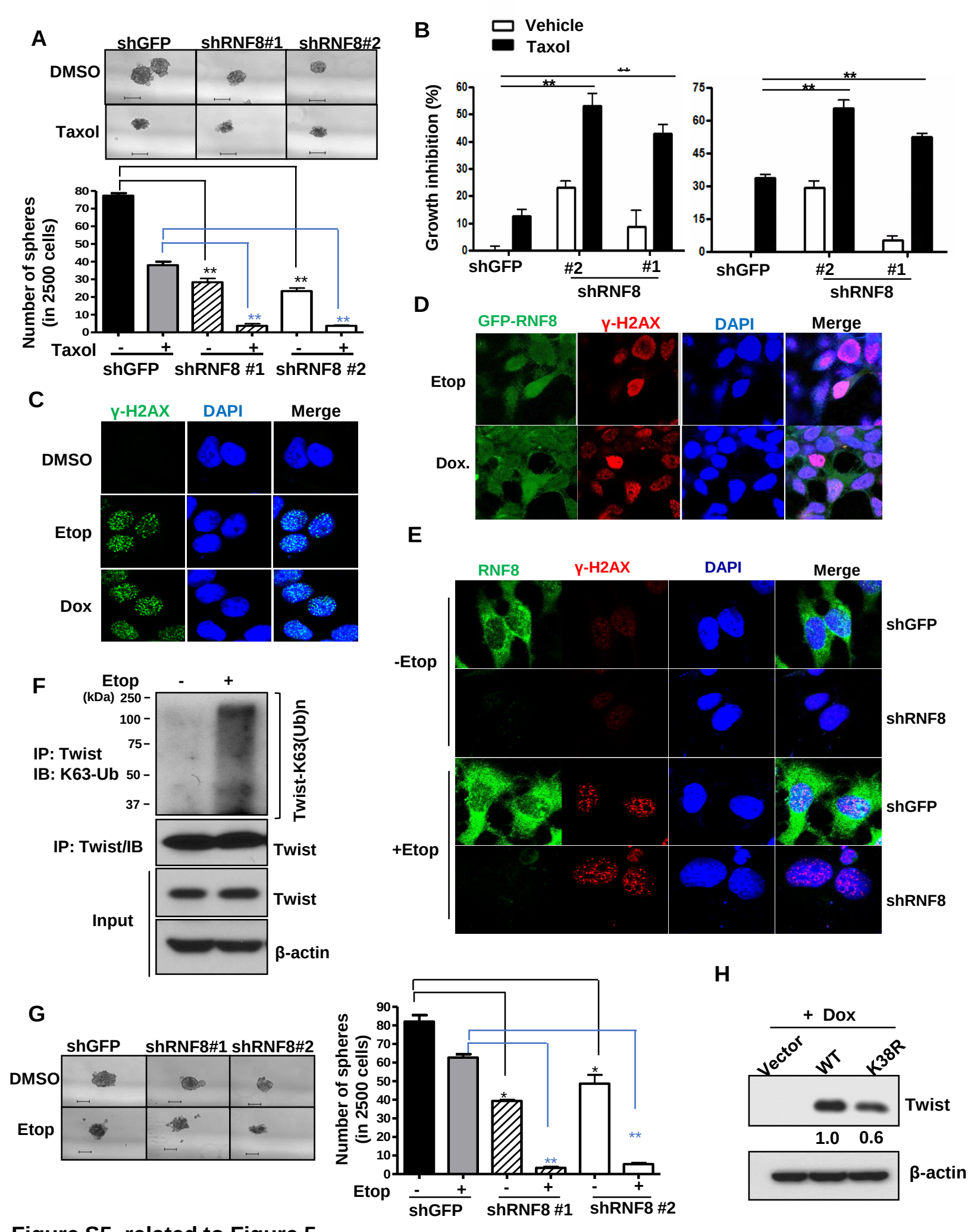

Figure S5, related to Figure 5

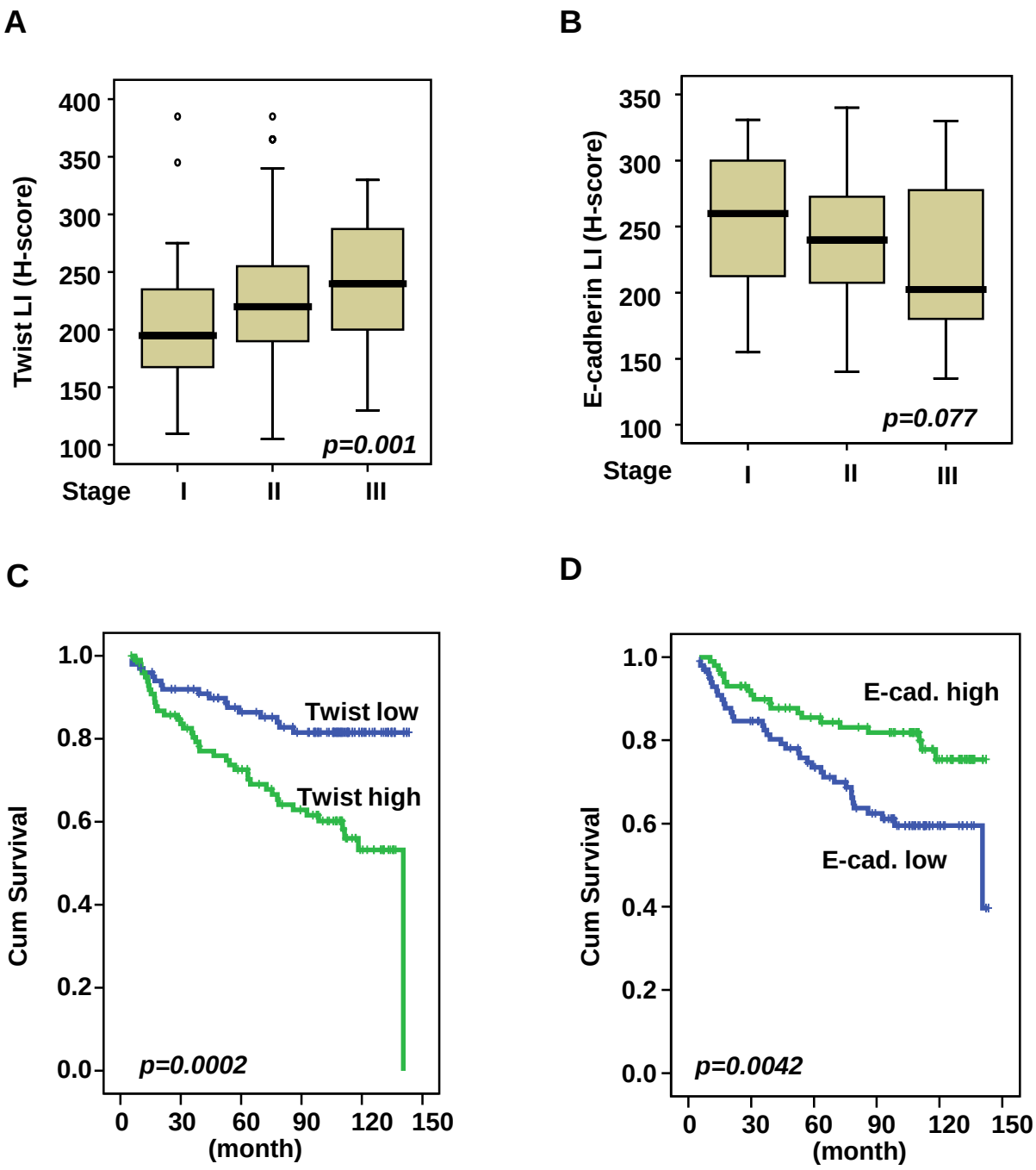

Figure S6, related to Figure 6

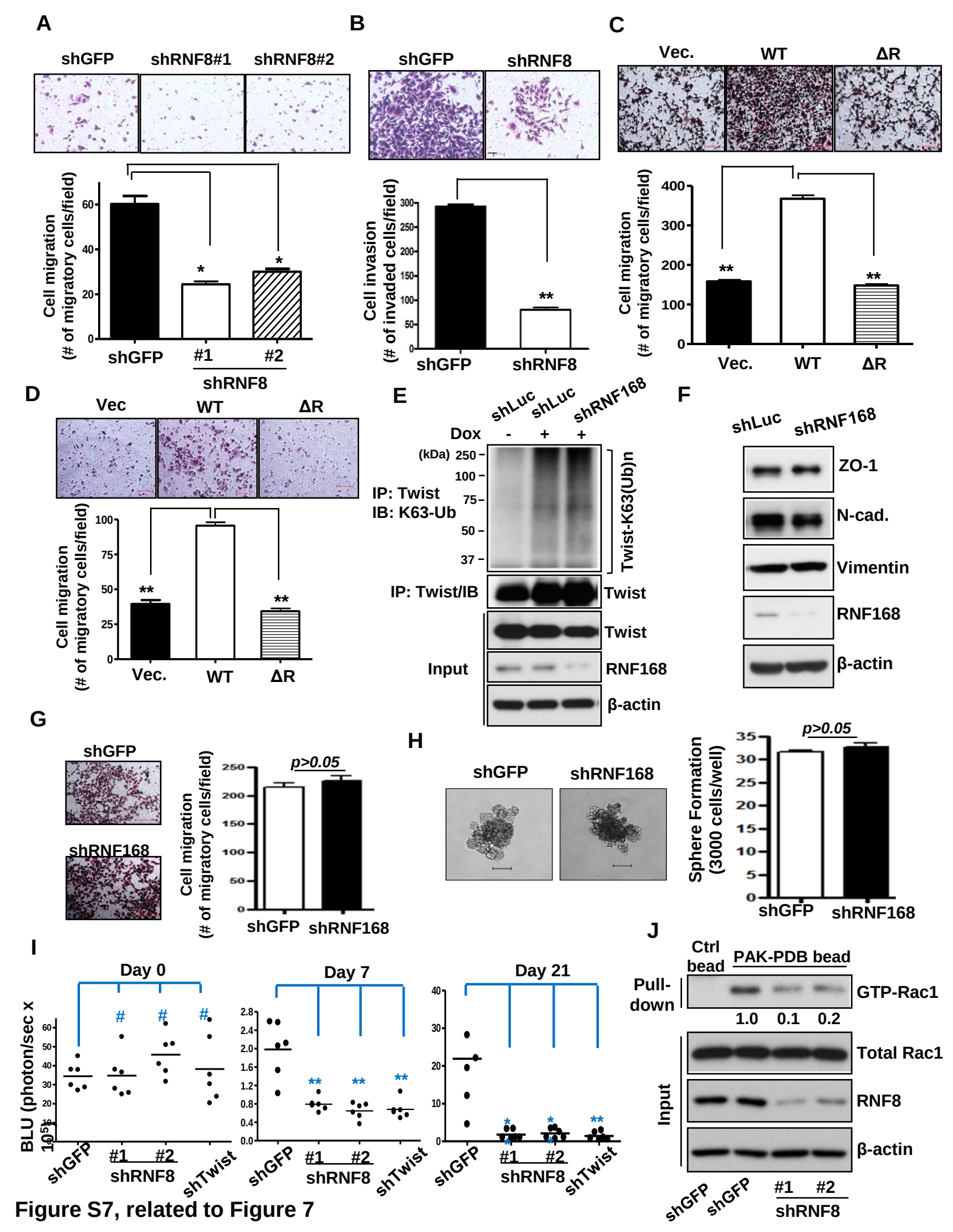

Figure S7, related to Figure 7

Table S1. A list of 150 E3 ligases used for E-cadherin luciferase reporter assay, related to Figure 1.

Table S2. E3 ligases that act as potential Twist activators, related to Figure 1.

Table S3. E3 ligases that act as potential Twist inhibitors, related to Figure 1.

Table S4. Associations between RNF8 expressions with important clinicopathologic variables and Twist1 and E-cadherin expression, related to Figure 6.

Parameter	Category	Case No.	RNF8 LI		Twist1 LI		E-Cadherin LI	
			H-Score	p-value	H-Score	p-value	H-Score	p-value
Primary tumor (T)[#]	T1	82	231.4±52.14	0.090	210.6±53.84	**0.009***	253.0±52.64	0.136
	T2	96	241.3±55.93		220.9±55.67		242.2±49.74	
	T3-T4	24	252.5±11.95		251.0±59.51		229.2±63.75	
Nodal status (N)[&]	N0	117	225.8±52.39	**<0.001***	206.6±53.07	**<0.001***	252.28±50.76	**0.031***
	N1-N2	85	256.4±53.61		239.2±55.85		235.04±54.71	
Stage[#]	I	63	224.4±48.33	**<0.001***	202.1±49.28	**0.001***	255.2±51.12	0.077
	II	115	239.7±53.13		225.6±58.26		242.6±51.12	
	III	24	270.8±66.47		242.29±54.29		230.2±63.44	
TWIST[%]		202	r=0.284	**<0.001***	-	-	-	-
E-cadherin[%]		202	r=-0.163	**0.020***	r=-0.162	**0.021**	-	-

[#], Kruskal-Wallis H test; [&], Mann-Whitney U test; [%], Pearson Correlation test; *, statistically significant; LI: Labeling Index

Table S5. Univariate analyses for distal metastasis-free survival, related to Figure 6.

Parameters	Category	MeFS		
		Case No.	Event No.	p-value
Primary tumor (T)	T1	82	9	**<0.0001***
	T2	96	34	
	T3-T4	24	14	
Nodal status (N)	N0	117	19	**<0.0001***
	N1-N2	85	38	
Stage	Stage I	63	5	**<0.0001***
	Stage II-III	139	52	
RNF8 LI	Low (< median)	101	17	**<0.0001***
	High (≥ median)	101	40	
TWIST LI	Low (< median)	101	17	**0.0002***
	High (≥ median)	101	40	
E-Cadherin LI	High (≥ median)	101	37	**0.0042***
	Low (< median)	101	20	

MeFS, metastasis-free survival; *, statistically significant; LI: Labeling Index

Table S6. Multivariate survival analyses, related to Figure 6.

Parameters	**Category**	**MeFS**	
		HR (95% CI)	**p-value**
Stage	Stage II-III vs. Stage I	6.890 (2.476-19.175)	**<0.001***
RNF8 LI	High (≥ median) vs. Low (< median)	2.040 (1.125-3.698)	**0.019***
TWIST LI	High (≥ median) vs. Low (< median)	1.884 (1.048-3.387)	**0.034***
E-Cadherin LI	Low (<median) vs. High (≥ median)	1.645 (0.937-2.888)	0.083

MeFS, metastasis-free survival; *, statistically significant; LI: Labeling Index

Supplemental Figure Legends

Figure S1. RNF8 recapitulates the effect of Twist on E-cadherin transcriptional repression. Related to Figure 1. (A) An E-cadherin promoter construct with a luciferase reporter gene was integrated in HMLE and MCF7 cells with or without Twist overexpression. These established E-cadherin luciferase reporter cell lines were then infected by lentiviruses containing shRNA targeting GFP or Twist at an MOI of 2. (B) cBioPortal was used to access and to visualize the cancer genome atlas (TCGA) data in 107 cases of invasive carcinoma of basal-like breast cancer. The candidate E3 ligases as EMT activators include RNF8, PML, FBXO5, MALT1, MDM4, PCGF2, MDM2, XIAP and SPOP were used as the query terms. Each column represents an individual sample, with the bottom legend providing information regarding the genomic alterations in particular genes and individuals. (C) A table summarizing the frequency (%) of genetic alterations of the candidate E3 ligases in 107 tumor cases with basal-like breast cancer. (D) Control and Twist-overexpressing MCF7 cells containing the E-cadherin luciferase reporter were infected by lentiviruses with shRNAs targeting GFP, Twist or RNF8 at various MOIs.

Figure S2. RNF8 promotes K63-linked ubiquitination and protein stability of Twist. Related to Figure 2. (A) *In vitro* ubiquitination assay in 293T cells transfected with Twist and His-Ub, along with wild-type (WT) or RING-domain deleted mutant (ΔR) of Flag-RNF8. (B) 293T cells transfected with indicated plasmids were harvested for immunoprecipitation with Flag antibody, followed by IB analysis. (C) *In vivo* ubiquitination assay in 293T cells transfected with Twist, WT and I405A mutant of Flag-RNF8 along with His-Ub and Twist constructs. (D) Control and RNF8-knockdown BT549 cells were harvested for cellular fractionation. The

nuclear and cytosolic fractions were isolated for IB analysis. Laminin B and α-Tubulin served as markers for nuclear and cytosolic fractions, respectively. (E) IB assay for analyzing Twist expression in BT549 cells with GFP or RNF8 knockdown. Two different RNF8 shRNAs were used in this assay. The relative intensity of RNF8 was quantified with ImageJ software and normalized with β-actin. (F) Real-time PCR for gene expression of Twist in GFP and RNF8-silenced BT549 cells. The quantified results are presented as means ± SEM (n=3). (G) IB assay for Twist expression in GFP and RNF8-knockdown BT549 cells in the absence and presence of MG132. Two different RNF8 shRNAs were used in the assays (H) IB assay for analyzing Twist expression in BT549 cells with overexpression of vector, WT and ΔR mutant of Flag-RNF8.

Figure S3. K63-linked ubiquitination regulates Twist proteolysis and function in EMT. Related to Figure 3. (A) Identification of the residues on Twist where K63-linked ubiquitination takes place. A sequence alignment among different species revealed there are 10 evolutionarily conserved lysine residues of Twist (indicated with black boxes). (B) *In vivo* ubiquitination assay in 293T cells transfected with His-Ub and Flag-Twist or various Flag-Twist mutants. NTA indicates nickel bead precipitate; WCE indicates whole-cell extracts. (C) IB assay for Twist expression in MCF7 cells transfected with various plasmids as indicated. (D) MCF7 cells transfected with mock, wild-type and the K38R mutant of Flag-Twist were fixed and stained with the epithelial marker ZO-1, and subjected to confocal microscopy analysis. The arrow indicates the cells harboring ectopic expression of Flag-Twist wild-type or K38R mutant. (E) MCF7 cells transfected with indicated plasmids were harvested for cellular fractionation. The nuclear and cytosolic fractions were isolated for IB analysis. Laminin B and α-Tubulin served as markers for nuclear and cytosolic fractions, respectively. The relative intensity of Twist WT,

K33R and K38R in the nucleus and cytoplasm was quantified with ImageJ software and normalized with expression of Laminin B and α-Tubulin, respectively. (F) Real-time PCR for gene expression of Twist in MCF7 cells with expression of vector control, WT and the K38R mutant of Twist. The quantified results are presented as means ± SEM (n=3). (G) IB assay for Twist expression in MDA-MB-231 cells with stable expression of pBabe vector, WT and the K38R mutant of Twist in the absence and presence of MG132. (H) IB assay for Twist expression in MCF7 cells with expression of vector control, Twist WT and mutant with deletion of nuclear localization sequence (ΔNLS) in the absence and presence of MG132.

Figure S4. RNF8 regulates EMT and CSC self-renewal. Related to Figure 4. (A) MDA-MB-231 cells with GFP or RNF8 knockdown were harvested for IB analysis for expression of the mesenchymal marker N-cadherin and the epithelial marker ZO-1. (B) MDA-MB-231 cells with GFP or RNF8 knockdown were harvested for IB analysis for Twist expression. Two RNF8 lentiviral shRNAs were used in this assay. The relative intensity of N-cadherin and ZO-1 was quantified with ImageJ software and normalized with β-actin. (C) Mammosphere formation assay in MDA-MB-231 cells with GFP, RNF8 or Twist knockdown. The quantified results are presented as means ± SEM (n=3); **, $p < 0.05$, **, $p < 0.01$. (D) IB assays in BT549 cells with GFP, RNF8 knockdown or with RNF8 knockdown plus overexpression of Twist WT or K38R mutant.

Figure S5. F8 deficiency augments CSC and cancer cell sensitivity to chemotherapy. Related to Figure 5. (A) Mammosphere formation assay in BT549 cells with GFP or RNF8 knockdown in response to Taxol treatment. (B) Cell growth inhibition assay in BT549 (upper)

and MDA-MB-231 (bottom) cells with GFP or RNF8 knockdown in the absence or presence of Taxol. Cell numbers were counted using a hemocytometer. (C) Confocal analysis in BT549 cells showed that both etoposide (Etop.) and doxorubicin (Dox) triggered DNA double strand breaks, evident by the formation of γH2AX foci. (D) Confocal analysis in BT549 cells showed that a large amount of RNF8 are dissociated from the γH2AX foci in response to etoposide (Etop) and doxorubicin (Dox) (E) GFP and RNF8-silenced BT549 cells treated with or without etoposide were fixed and stained with RNF8 and γH2AX, and subjected to confocal microscopy analysis. (F) BT549 cells treated with vehicle or etoposide for 2 hrs were harvested for IP with Twist, followed by IB analysis to determine the level of Twist ubiquitination. (G) Mammosphere formation assay in BT549 cells with GFP or RNF8 knockdown in response to etoposide. The quantified results are presented as means ± SEM (n=3); *, $p < 0.05$ and **, $p < 0.01$. Scale bar, 100 μm in (A and G) (H) IB analysis for MDA-MB-231 cells with stable expression of vector alone (pBabe), Twist wild-type and Twist K38R mutant in the presence of doxorubicin treatment.

Figure S6. The association of Twist and E-cadherin expression in disease progression and patient survival rates in breast cancer. Related to Figure 6. (A and B) Quantification analyses of Twist (A) and E-cadherin (B) expressions in 202 breast tumor cases in various tumor stages. (C and D) Kaplan-Meier plot analysis of metastasis-free survival of 202 cases of breast cancer patients with low or high expression of Twist (C) and E-cadherin (D).

Figure S7. RNF8 phenocopies the effect of Twist in cancer cell migration, invasion and metastasis. Related to Figure 7. (A and B) Transwell cell migration (A) and Matrigel cell

invasion (B) assays in MDA-MB-231 cells with GFP and RNF8 knockdown. (C and D) Transwell cell migration assay in BT549 (C) and MDA-MB-231 (D) with overexpression of vector control, WT and ΔR mutant of Flag-RNF8. (E) BT549 cells with GFP or RNF168 knockdown treated with vehicle or doxorubicin for 2 hrs were harvested for IP with Twist, followed by IB analysis to determine the level of Twist ubiquitination. (F) IB analysis for expression of EMT markers in BT549 cells with GFP or RNF168 knockdown. (G) Transwell cell migration assay in BT549 cells with GFP and RNF168 knockdown. (H) Mammosphere formation assay in BT549 cells with GFP or RNF168 knockdown. The quantified results are presented as means ± SEM (n=3). Scale bar, 100 μm (I) MDA-MB-231-Luciferase cells with GFP, RNF8 or Twist knockdown were injected into nude mice, and the kinetics of breast cancer metastasis to the lung were measured by bioluminescence and quantified using the IVIS system. The quantification results of bioluminescent signals in the lung at day 0, 7 and 21 are presented as means ± SEM (n = 6 for each group). *#, $p > 0.05$ and* **, $p < 0.01$*. (J) Active Rac1 pulldown assay in BT549 cells with control or RNF8 knockdown. The relative intensity of GTP-bound (active form) Rac1 was quantified by ImageJ software and normalized with expression of total Rac1.

Supplemental Experimental Procedures

Cell culture and reagents

293T, BT549, MDA-MB-231, MCF7 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) while HMLE were cultured in a 1:1 ratio of DMEM/F12 (Gibco) and MEGM (Lonza); the culture was supplemented with 2.5 μg/ml insulin (Sigma), 5 ng/ml human Epidermal Growth Factor (Sigma), and 250 ng/ml hydrocortisone (Sigma). All the cell lines were incubated at 37˚C in an atmosphere of 5% CO2 and 95% air. $(His)_6$-ubiquitin, $(His)_6$-ubiquitin K48R and $(His)_6$-ubiquitin K63R constructs was described previously (Chan et al., 2012) . Flag-RNF8 is a gift from Dr. Junjie Chen. Flag-Twist, BT549, MDA-MB-231 are gifts from Dr. Mien-Chie Hung. All cells were routinely verified to be free of mycoplasma contamination via the R&D MycoProbe® Mycoplasma Detection Kit (Catalog # CUL001B). Xpress (Xp)-Twist was constructed in pcDNA4-TOPO vector (Invitrogen). GST-Twst1 was constructed in pGEX-5T1 vector (GE Healthcare). Luciferase reporter gene alone and luciferase reporter gene fusion with E-cadherin promoter constructs were constructed in pBabe retroviral vector. Flag-RNF8-ΔRING (Δ403-441 a. a.) and all the Twist mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol.

Viral infection

For retroviral infection, 293T cells were co-transfected with pBabe, pBabe-Twist or pBabe-Twist K38R mutant with envelope plasmid (VSV-G) and packing plasmid (Gag-Pol). For lentiviral shRNA infection, 293T cells were co-transfected with RNF8, Twist, or GFP shRNA with envelope plasmid (VSV-G) and packing plasmid (deltaVPR8.9). Lipofectamine 2000 reagent in accordance with the manufacturer's instructions. RNF8-lentiviral shRNA-1 (5´-

CCAAAGAATGACCAAATGATA-3´), RNF8-lentiviral shRNA-2 (5´-TGGAGCAACTAGAGAAGACTT-3´), RNF168-lentiviral shRNA (5´-CGTGGAACTGTGGACGATAAT-3´) and Twist-lentiviral shRNA (5´-CCTGAGCAACAGCGAGGAAGA-3´), were transfected with packing plasmids into 293T cells for 2 days, and virus particles were then used to infect mammalian cells. All the infected cells were cultured in medium containing appropriate antibiotics.

Immunoblotting and Immunoprecipitation analysis

Cells were lysed with RIPA buffer in the presence of protease inhibitor cocktail (Roche) for immunoblotting analysis. The following antibodies were used for immunoblotting (IB) analysis: anti-RNF8 (Santa Cruz, clone B2), anti-Twist (Abcam, clone 2C1a and Santa Cruz, clone H-81), anti-Flag (Sigma, clone M2), anti-E-cadherin (Cell Signaling, clone 24E10 and BD Biosciences, cat.610404), anti-ZO-1 (Cell Signaling, clone D7D12), anti-N-cadherin (Santa Cruz, clone H-63), anti-Vimentin (Cell Signaling, clone D21H3), anti-K63-Ub (Cell Signaling, clone D7A11), anti-K48-Ub (Cell Signaling, cat. 4289), anti-RNF168 (Santa Cruz, clone B-11) anti-Rac1 (Millipore, clone 23A8), anti-Lamin B (Santa Cruz, clone C5), anti-GRP78 (BD Biosciences, cat. 610978), anti-β-actin (Sigma, clone AC-15) and anti-α-tubulin (Sigma, clone B-5-1-2). For immunoprecipitation, cells were lysed by E1A lysis buffer (250 mM NaCl, 50 mM HEPES, [pH 7.5], 0.1% NP40, 5 mM EDTA), with a protease inhibitor cocktail (Roche). Cell lysates were immunoprecipitated with anti-Twist antibody (Santa Cruz, clone H-81) and subjected for IB analysis.

***In vivo* and *in vitro* ubiquitination assay**

293T cells were transfected with the indicated plasmids for 48 h. After cell harvest, cells were lysed by the denatured buffer (6M guanidine-HCl, 0.1M Na_2HPO_4/NaH_2PO_4, 10 mM imidazole). The cell extracts were then incubated with nickel beads for 3 h, washed, and subjected to IB analysis. For *in vitro* ubiquitination assays, recombinant GST and GST-Twist proteins were expressed and purified from the bacterial lysates of BL21 cells. Flag-RNF8 was expressed in 293T cells, immunoprecipitated by anti-Flag antibody, and eluted from Protein A/G beads using Flag peptides according to manufacturers' standard procedures. Purified GST, GST-Twist and Flag-RNF8 proteins were incubated for 3 hr at 37°C in reaction buffer (20 mM HEPES [pH 7.4], 10 mM MgCl2, 1 mM DTT, 59 mM ubiquitin, 50 nM E1, 850 nM of Ubc13/Uev1a, 1 mM ATP, 30 mM creatine phosphate, and 1 U of creatine kinase). After incubation, protein mixtures were diluted in E1A buffer and the supernatant fluid was precleared with Protein A/G beads for 2 hrs, and immunoprecipitated overnight with anti-Flag antibody, after which Protein A/G beads were added for an additional 2 hrs. Beads were washed four times with E1A Buffer. Proteins were eluted in SDS-sample buffer and subjected to immunoblotting analysis.

Immunofluorescent staining

Cells grown on a 4-well chamber slide (Falcon) were washed with PBS, fixed at -20 °C with pre-chilled methanol for 20 min, rinsed with cold acetone for 3 min and then air dried. For E-Cadherin, cells were fixed with 3% paraformaldehyde/2% sucrose for 30 min, permeabilized with 0.5% Triton X-100 solution for 10 min. After blocking with 10% BSA in PBS, the slides were subjected to stain with anti-Twist (Abcam), anti-N-Cadherin (Santa Cruz), anti-E-Cadherin (BD Biosciences) or anti-RNF8 antibody (Abnova). Alexa-488-conjugated goat anti-mouse IgG (Molecular Probes) or Alexa-594-conjugated goat anti-rabbit IgG (Molecular Probes) were

applied for visualization of images by a Leica SP5 confocal microscope.

Cellular fractionation

Cells were washed with PBS once, harvested and resuspended in lysis buffer (20mM Hepes [pH 7.0], 2mM MgCl2, 10mM KCl, 0.5% (v/v) NP-40, protease inhibitor cocktail) followed by Dounce homogenization. The suspensions were centrifuged at 1,500 x g for 5 minutes at 4 ºC, and then the resulting supernatants and nuclear pellets were further processed for cytosolic and nuclear fractions, respectively. For cytosolic fractions, the supernatants were clarified by centrifugation at 13,500 rpm for 15 minutes at 4 ºC. For nuclear fractions, the nuclear pellets were washed three times with lysis buffer and then resuspended in RIPA buffer followed by sonication and centrifugation.

Real-time PCR

In brief, total RNA was isolated using Trizol (Qiagen) and then reverse transcribed using the SuperScript™ II Reverse Transcriptase (RT) kit (Life Science). SYBR-based real-time PCR was conducted using SYBR Green Fast Master Mix (Applied Biosystems) on a StepOnePlus real-time PCR system (Applied Biosystems). The relative expression of mRNAs was quantified by $2^{-\Delta\Delta Ct}$ method. Primer sequences used for amplifications were as follows: Twist, 5′-TCCATGTCCGCGTCCCACTA-3′ (forward) and 5′-ATTCAAAGAAACAGGGCGTG-3′ (reverse); GAPDH, 5′-GATTCCACCCATGGCAAATTC-3′ (forward) and 5′-CTTCTCCATGGTGGTGAAGAC-3′ (reverse). The gene expression levels were normalized to GAPDH.

Mammosphere formation assay

BT549 and MDA-MB-231 cells, when seeded in ultra-low attachment plates (Corning, Corning, NY) in serum-free media form non-adherent spheroids termed mammospheres, with the ability to self-renew (Fillmore and Kuperwasser, 2008). To examine the effects of various treatments on mammosphere formation, BT549 and MDA-MB-231 cells were seeded in 6-well ultra-low attachment plates in plating medium (MEGM) with and without added treatments at a density of 2500 cells per well. 24h after, chemotherapeutic agents were added into plate. The appearance of spheres was evaluated after 7 (BT-549) or 10-12 (MDA-MB-231) days. Mammospheres with diameters of ≥100 μm (BT-549) and ≥50 μm (MDA-MB-231) were manually counted under microscope.

Immunohistochemistry and Scoring

The procedures of immunohistochemical studies were performed as previously described (Huang et al., 2006). In brief, sections were cut onto an adhesive-coated glass slides at 3-mm thickness. For staining in human samples, the slides were incubated with primary antibodies targeting RNF8 (Santa cruz, sc-271462), Twist (Biorbyt, orb13736), and E-cadherin (Epitomics, EP700Y). Primary antibodies were detected using the ChemMate DAKO EnVision kit (DAKO, K5001). The slides were incubated with the secondary antibody for 30 min and developed with 3,3-diaminobenzidine for 5 min. Incubation without the primary antibody was used as a negative control. Immunoexpression was scored by two pathologists (C.F.L and H.Y.H.) using a multi-headed microscope to reach a consensus for each case. The staining was evaluated based on a combination of both the percentage and intensity of positively stained tumor cells to generate an H-score, which was calculated using the following equation: H-score = $\Sigma Pi\ (i + 1)$, where i is the

intensity of the stained tumor cells (0 to 4 +), and Pi is the percentage of stained tumor cells for each intensity. Patient tissues used in this study were obtained with consent under the protocol approved by the institutional review board (IRB # 10501-012) of Chi-Mei Foundational Medical Center.

Statistical Analysis for human samples.

For human breast samples, the Mann-Whitney U test was used to assess the differential expression level of Twist and E-cadherin expression in relation to RNF8 expression status. The Spearman's rank correlation coefficient was used to clarify the association between RNF8 expression to clinicopathological variables and Twist and E-cadherin expression levels. The endpoint analyzed was distal metastasis-free survival, calculated from the starting date of surgery to the date of event. The median period of follow-up was 103 months (range, 6–143). Survival analysis was performed using the Cox proportional hazards model. Survival curves were plotted using the Kaplan-Meier method, and log-rank tests were performed to evaluate prognostic differences between groups for categorical variables.

Cell migration and invasion assay

The cell migration assay was performed in a 24-well transwell plate with 8-μm polyethylene terephthalate membrane filters (Falcon cell culture insert; Becton-Dickinson) separating the lower and upper culture chambers. In brief, BT549 and MDA-MB-231 cells were seeded in the upper chamber at 8×10^4 cells per well in serum-free DMEM. The bottom chamber contained DMEM with 10% FBS. BT549 cells were allowed to migrate for 12 h, whereas MDA-MB-231 cells were allowed to migrate for 6 h. After the incubation period, the filter was removed, and

non-migrant cells on the upper side of the filter were detached with the use of a cotton swab. Filters were fixed with 4% formaldehyde for 15 min, and cells located in the lower filter were stained with 0.1% crystal violet for 20 min and three random fields were counted. Quantified results are presented as means ± SEM. The cell invasion assay was essentially similar to the cell migration assay, except that the membrane filter was coated with Matrigel (BD Biosciences) with 1: 3 dilution in serum-free medium before the assay.

Rac1 pulldown assay

To determine Rac activity by measuring GST-bound Rac1, 5×10^6 BT549 cells were seeded one day before the experiment. Cells were lysed in 1X MLB buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl2, 1 mM EDTA and 10% glycerol) with 1X protease inhibitor cocktail (Roche) and centrifuged for 15 min at 14,000 rpm and 4°C. The supernatant was then incubated with the glutathione agarose beads with or without GST-fusion the Rac1 (p21) binding domain (PBD) of the human p21 activated kinase 1 protein (PAK) protein (EMD Millipore) at 4°C for 1 h while rotating. Beads were washed five times in 1x MLB buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl2, 1 mM EDTA and 10% glycerol) and resuspended in SDS sample buffer. GST-bound Rac1 protein was determined by immunoblotting analysis.

Supplemental References

Chan, C.H., Li, C.F., Yang, W.L., Gao, Y., Lee, S.W., Feng, Z., Huang, H.Y., Tsai, K.K., Flores, L.G., Shao, Y., *et al.* (2012). The Skp2-SCF E3 ligase regulates Akt ubiquitination, glycolysis, herceptin sensitivity, and tumorigenesis. Cell *149*, 1098-1111.

Fillmore, C.M., and Kuperwasser, C. (2008). Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. Breast cancer research : BCR *10*, R25.

Huang, H.Y., Kang, H.Y., Li, C.F., Eng, H.L., Chou, S.C., Lin, C.N., and Hsiung, C.Y. (2006). Skp2 overexpression is highly representative of intrinsic biological aggressiveness and independently associated with poor prognosis in primary localized myxofibrosarcomas. Clinical cancer research : an official journal of the American Association for Cancer Research *12*, 487-498.