Supplementary Table 1. List of genes co-amplified in cases with NCSTN gene amplification (TCGA, Nature 2012)

Official gene symbol	Number of cases in ``Breast Invasive Carcinoma (TCGA, Nature 2012)'' study where both the gene and NCTSN are amplified	Number of cases in ``Breast Invasive Carcinoma (TCGA, Nature 2012)'' study where the gene is amplified	Number of cases in the study that report amplified genes	p-value corresponds to statistical significance of the value in the second column	p-value in the sixth column corresponds to the statistical significance of number of cases where a gene is amplified in cases when nicastrin is not amplified
ARHGAP30	38	45	825	<< 0.01	~1
CD244	38	42	825	<< 0.01	~1
CD48	38	40	825	<< 0.01	~1
DEDD	38	44	825	<< 0.01	~1
F11R	38	45	825	<< 0.01	~1
ITLN1	38	42	825	<< 0.01	~1
ITLN2	38	44	825	<< 0.01	~1
KLHDC9	38	44	825	<< 0.01	~1
LY9	38	42	825	<< 0.01	~1
NIT1	38	44	825	<< 0.01	~1
PFDN2	38	44	825	<< 0.01	~1
PVRL4	38	45	825	<< 0.01	~1
SLAMF6	38	38	825	<< 0.01	~1
SLAMF7	38	40	825	<< 0.01	~1
TSTD1	38	45	825	<< 0.01	~1
USF1	38	45	825	<< 0.01	~1
PEX19	39	39	825	<< 0.01	~1
COPA	40	40	825	<< 0.01	~1
NHLH1	40	40	825	<< 0.01	~1
SUMO1P3	40	40	825	<< 0.01	~1
VANGL2	40	40	825	<< 0.01	~1

Supplementary Table 2. TP53 and PIK3CA mutation correlation with NCSTN amplification in the TCGA, Nature 2012 dataset

Official gene symbol	Number of cases in ``Breast Invasive Carcinoma (TCGA, Nature 2012)'' study where gene is mutated and NCTSN is amplified	Number of cases in ``Breast Invasive Carcinoma (TCGA, Nature 2012)'' study where the gene is mutated.	Total number of cases in the study where mutated genes are reported	p-value corresponds to statistical significance of the value in the second column	p-value corresponds to statistical significance of number of cases where a gene is mutated in cases when nicastrine is not amplified
TP53	15	187	507	0.0482	0.9799
PIK3CA	4	178	507	0.9971	0.0113

Supplementary Table 3.

Predictors of 10-year disease free survival on multivariate analysis in a Cox regression model

Characteristic	*Cox p* value	Hazard Ratio
Grade	0.411	1.625
Stage	<0.001	1.967
Size	0.291	1.479
Nicastrin RNAScope H-score	**0.044**	1.352

Supplementary Table 4. Correlation of Nicastrin RNAScope H-score with clinical and tumor parameters

Variable/biomarker	Nicastrin RNAScope H-Score		*p*-value
	Low (<120) Number (%)	High (≥120) Number (%)	
Patients age (years)			
≤50	50 (33.1)	83 (51.2)	0.005
>50	98 (64.9)	77 (47.5)	
Grade			
1	1 (0.7)	0 (0.0)	
2	19 (12.6)	12 (7.4)	0.175
3	131(86.8)	150 (92.6)	
Stage			
1	99 (65.6)	93 (58.1)	
2	35 (23.2)	47(29.4)	0.381
3	17 (11.3)	20 (12.5)	
Size			
<1.5cm	46 (30.9)	35(22.9)	0.117
≥1.5cm	103(69.1)	118(77.1)	
Pleomorphism			
2	4 (2.7)	0 (0.0)	0.036
3	144 (97.3)	161 (100)	
Mitosis			
1	16(10.8)	7(4.3)	
2	28(18.9)	21 (13.0)	0.023
3	140(70.3)	133(82.6)	
Nicastrin Membrane Score			
0	14 (13.2)	14 (11.3)	
1	51 (48.1)	45 (37.9)	
2	31 (29.2)	37 (28.2)	0.056
3	10 (9.4)	28 (22.6)	
Nicastrin Cytoplasmic Score			
0	11 (10.3)	7(5.6)	
1	32(29.9)	36 (29.0)	0.414
2	49 (45.8)	56 (45.2)	
3	15 (14.0)	25 (20.2)	
Ki67			
Negative	28 (18.9)	11(7.0)	0.002
Positive	120 (81.1)	146 (93.0)	
E-cadherin			
Negative	30 (24.0)	18 (12.1)	0.01
Positive	95 (76.0)	131 (78.9)	
BRCA1cyto			
Negative	49 (29.9)	44 (29.9)	0.025
Positive	64 (56.6)	103 (70.1)	

Supplementary Figure Legends

Supplementary Figure 1. (a) **FACS analysis of hybridoma supernatants (Genovac).** Antibodies were raised by genetic immunisation of WISTAR rats using the cDNA, encoding for the ECD of nicastrin (34-669 a.a). This cDNA was cloned into a proprietary GENOVAC expression vector developed to maximise an immune response in mammals and to allow screening of the resulting immune sera. The immunisation constructs were introduced into the animals using a Genegun. Following DNA boosts, test sera were taken from the animals and tested for the presence of antibodies in a transient transfection assay in flow cytometry against the corresponding GENOVAC screening vector. Labels bellow the histograms annotate the MAb clone reference number. (b) **FACS analysis assessing the binding efficacy of the 21 anti-nicastrin MAb clones.** MDA-MB-231 cells were grown to 70-80% confluence. Cells were incubated with the supernatants obtained from the first round of hybridoma cloning and with the rat IgG2b as the isotype control antibody at an arbitrary concentration of 50 µg/ml. The concentration of the mAbs in the preparations was at this phase unknown. Secondary anti-Rat-FITC conjugated antibody was used. FACS analysis was performed using the FACSCalibur, BD. The labels in the top left-hand corner of the histograms annotate the MAb clone reference number. **Blue:** Rat IgG2b isotype control; **Green:** MAb supernatants. (c) **Anti-nicastrin monoclonal antibodies affect MDA-MB-231cell invasion.** MDA-MB-231 cells were treated with indicated anti-NCT MAbs and subjected to the transwell invasion assay for 72 h. Bars represent mean number of invaded cells ± SEM from three separate experiments, each in triplicate. Statistical difference from IgG2b isotype control antibody. 1H4, *p = 0.01; 2H6, *p = 0.0005; 6H5, *p = 0.01; 6H11, *p = 0.01; 9G7, *p = 0.01; 10C5, *p = 0.02; 10C11, *p = 0.004. Anti-nicastrin PcAb (100 µg/ml) was used as a positive control,*p < 0.001.

Supplementary Figure 2. K_d determination (a, b) Whole IgG 10C11 (a) and 2H6 (b) were directly immobilised to a BIACore 3000 CM5 chip and NCT-Fc fusion protein was flowed over at a concentration range of 1000-62.5 nM. Replicate injections and double referencing was used and the data fitted to a 1:1 binding model using BIAevaluation v2.0 to determine the kinetic constants and K_d. Clone 10C11 has a significantly better association rate compared to clone 2H6, whereas 2H6 has a slower dissociation rate. Kinetic values are

shown in Figure 1-Table 1. (c,d) IgG clones 10C11 and 2H6 (c) and 1E2 and 10C5 (d) were analyses by flow cytometry on MDA-MB-231 cells at a concentration range of 200 mg/ml to 19 ng/ml. The mean fluorescent intensity (MFI) was fitted to a sigmoidal binding model to determine the cellular binding K_d which are incorporated in Table 1-Figure 1.

Supplementary Figure 3. (a) Binding of anti-NCT MAbs to endogenous cell surface nicastrin of mouse and rat cells. Non-permeabilized mouse melanocytes and rat fibroblast cells were incubated with 50 µg/ml of 2H6 and 10C11 antibodies, followed by the incubation with the secondary anti-rat FITC antibody. Rat IgG antibody was used as control. Binding was assessed by FACS. (b) Mouse weights (grams) during the metastatic in vivo mouse model experiment. (c-f) Biochemistry analysis from mouse serum collected at termination of animals in the metastatic in vivo mouse model experiment. Kidney function was assessed through creatinine and urea measurements, while liver function test were assessed through alanin aminotransferase (ALT) and alkaline phosphatise (Alk Phos).

Supplementary Figure 4. (a-e) Mann-Whitney Test was used to compare all conditions to one another for each time point and all p values summarised, highlighting significant differences ($p<0.05$).

Supplementary Figure 5. Nicastrin gene amplification and high mRNA levels predict worse survival of breast cancer patients. (a) Kaplan Meier overall survival curves showing worse outcome of patients with nicastrin gene amplification in the Nature TCGA 2012 dataset (cBio Portal for Cancer Genomics). (b) Correlation of nicastrin gene amplification and mRNA levels in the Nature TCGA 2012 dataset. (c) Bright field microscopy images (magnification x40) of nicastrin RNAScope analyses on paraffin embedded breast cancer tissue microarrays. Representative images are show for four different levels of the H-score. Cut-off value = 120. (d) Kaplan Meier analysis showing worse disease free survival of breast cancer patients with the Nicastrin RNAScope scope ≥ 120, $p = 0.019$.

Fig S1

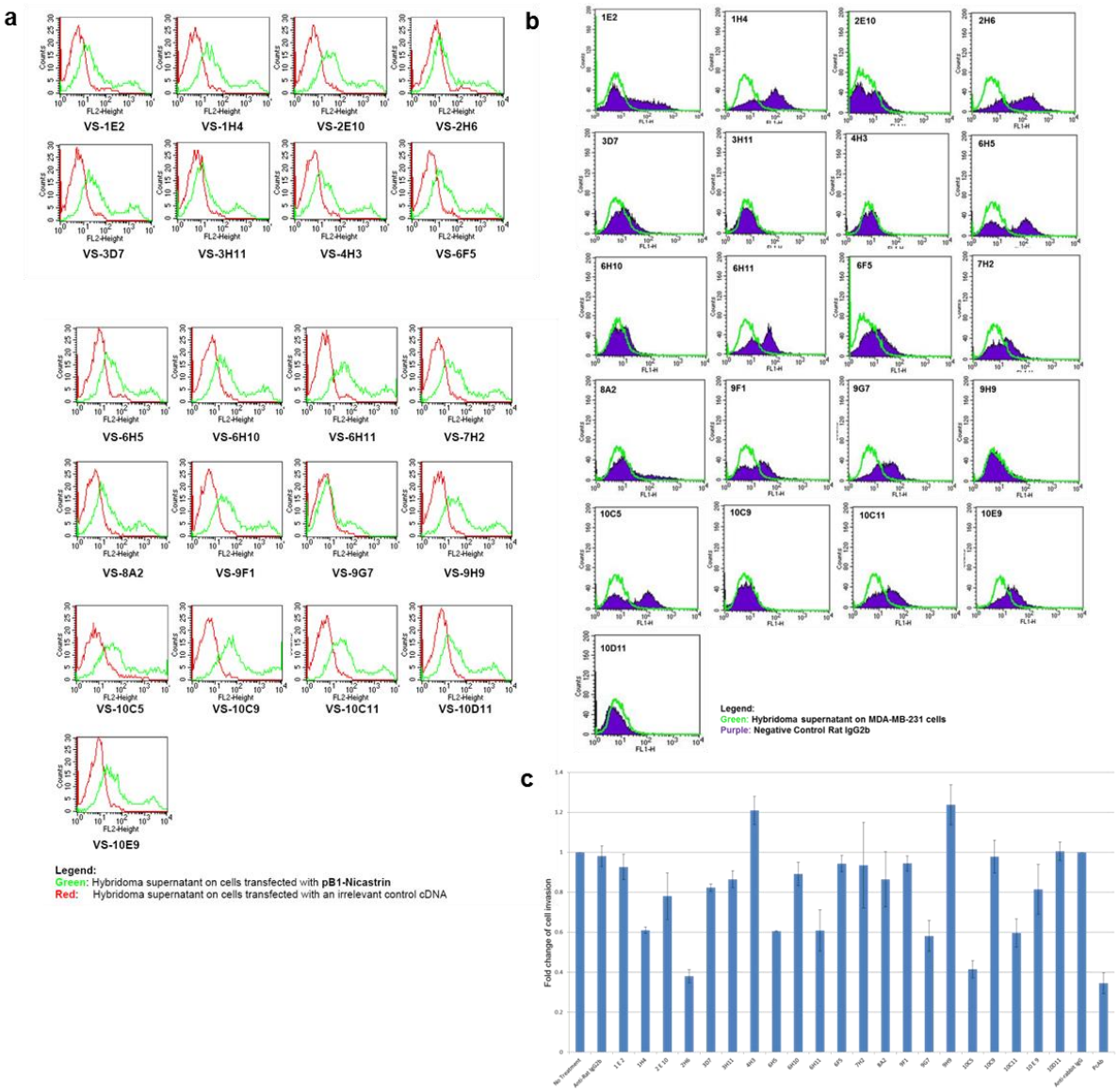
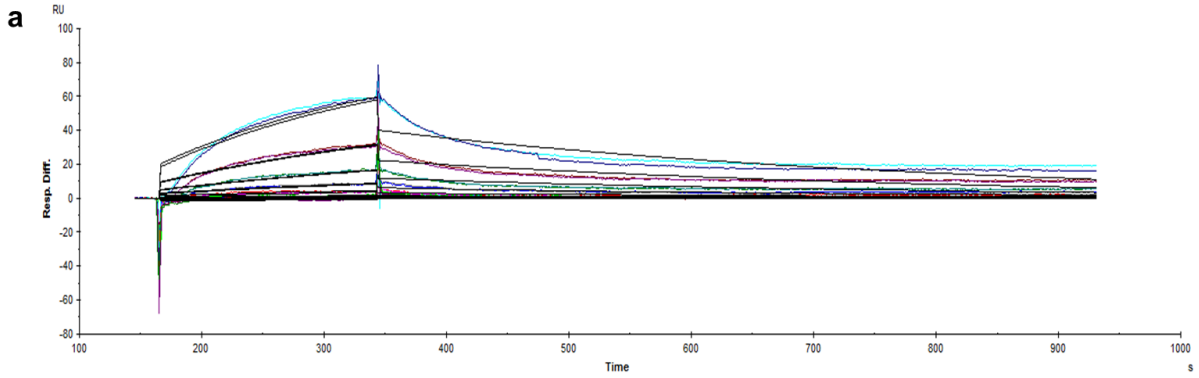
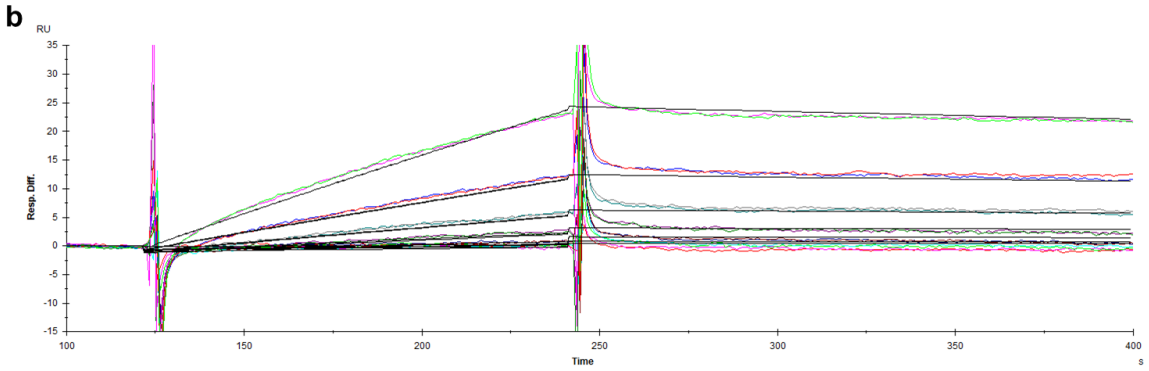
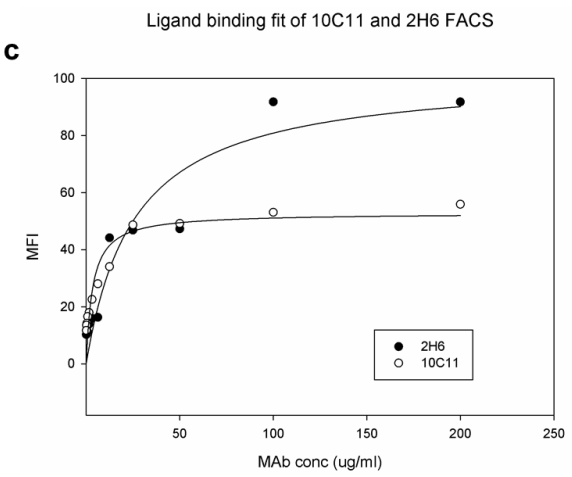

a
VS-1E2
VS-1H4
VS-2E10
VS-2H6
VS-3D7
VS-3H11
VS-4H3
VS-6F5
VS-6H5
VS-6H10
VS-6H11
VS-7H2
VS-8A2
VS-9F1
VS-9G7
VS-9H9
VS-10C5
VS-10C9
VS-10C11
VS-10D11
VS-10E9
Legend:
Green: Hybridoma supernatant on cells transfected with pB1-Nicastrin
Red: Hybridoma supernatant on cells transfected with an irrelevant control cDNA
b
1E2
1H4
2E10
2H6
3D7
3H11
4H3
6H5
6H10
6H11
6F5
7H2
8A2
9F1
9G7
9H9
10C5
10C9
10C11
10E9
10D11
Legend:
Green: Hybridoma supernatant on MDA-MB-231 cells
Purple: Negative Control Rat IgG2b
c
Fold change of cell invasion

Fig S2

Figure S2.







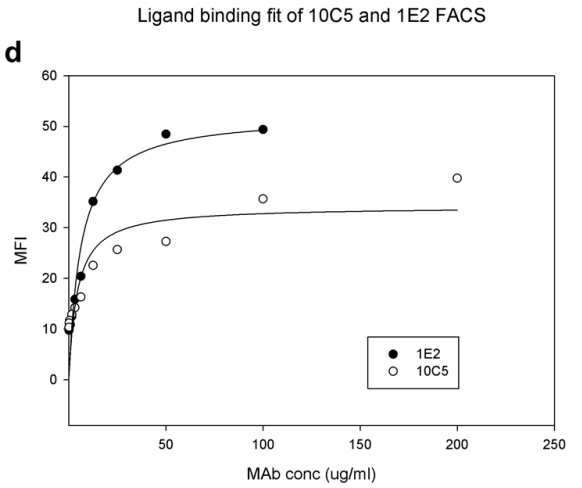


Fig S3.

a

DAY 6	PBS	Isotype	2H6	10C11	GSI
PBS		1.0000	0.6126	**0.0401**	0.7551
Isotype			0.6454	**0.0104**	0.8329
2H6				**0.0148**	0.6601
10C11					**0.0062**
GSI					

b

DAY 12	PBS	Isotype	2H6	10C11	GSI
PBS		**0.0289**	0.0541	**0.0093**	0.1807
Isotype			0.5054	0.3282	0.1419
2H6				0.2345	0.2824
10C11					0.0813
GSI					

c

DAY 18	PBS	Isotype	2H6	10C11	GSI
PBS		0.4557	0.1520	0.2810	0.1014
Isotype			0.3357	0.9551	0.2949
2H6				0.3282	0.4908
10C11					0.2284
GSI					

d

DAY 24	PBS	Isotype	2H6	10C11	GSI
PBS		1.0000	0.2319	0.8665	0.4452
Isotype			0.2345	0.7984	0.2824
2H6				0.1605	0.9497
10C11					0.2448
GSI					

e

DAY 30	PBS	Isotype	2H6	10C11	GSI
PBS		0.6282	**0.0027**	0.0813	0.0931
Isotype			**0.0006**	0.0541	0.0513
2H6				0.0740	1.0000
10C11					0.4136
GSI					

Fig S4.

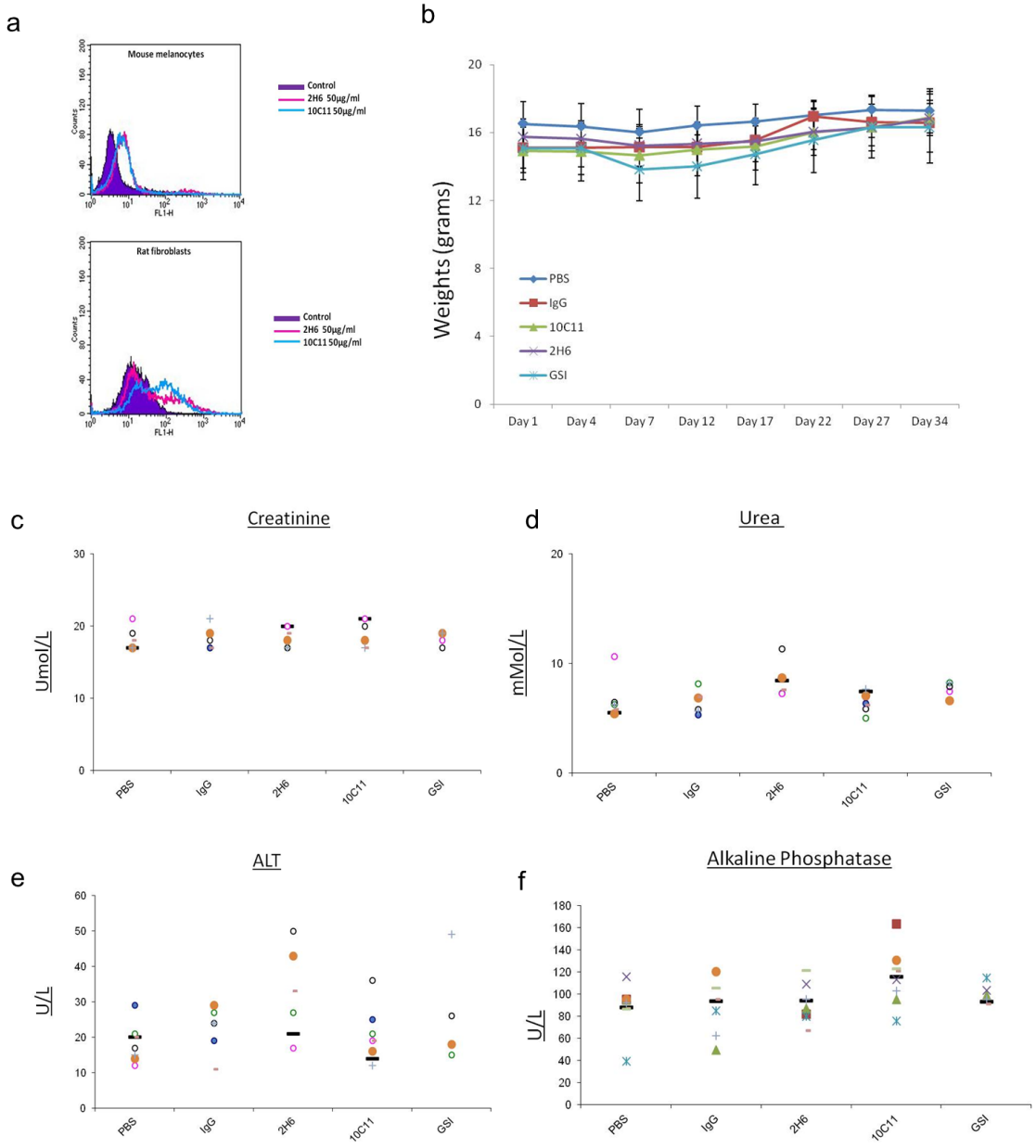
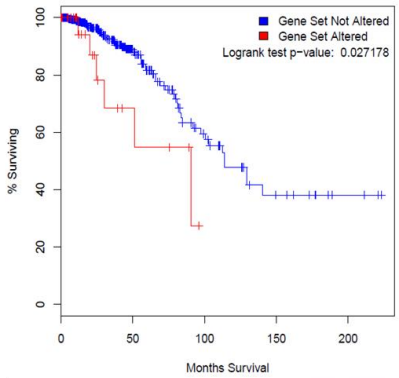
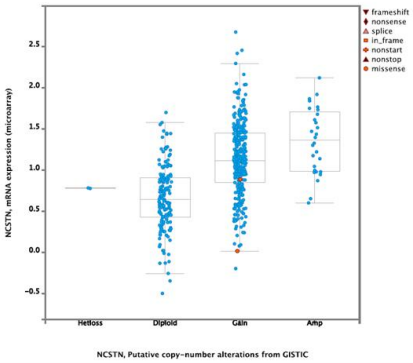

a
Mouse melanocytes
Control
2H6 50µg/ml
10C11 50µg/ml
Count
FL1-H
Rat fibroblasts
Control
2H6 50µg/ml
10C11 50µg/ml
Count
FL1-H
b
Weights (grams)
PBS
IgG
10C11
2H6
GSI
Day 1
Day 4
Day 7
Day 12
Day 17
Day 22
Day 27
Day 34
c
Creatinine
Umol/L
PBS
IgG
2H6
10C11
GSI
d
Urea
mMol/L
PBS
IgG
2H6
10C11
GSI
e
ALT
U/L
PBS
IgG
2H6
10C11
GSI
f
Alkaline Phosphatase
U/L
PBS
IgG
2H6
10C11
GSI

Fig S5

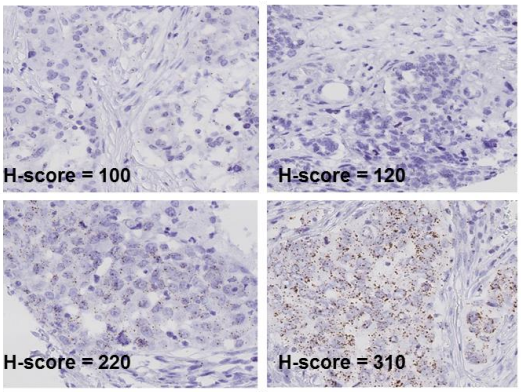
a


Gene Set Not Altered
Gene Set Altered
Logrank test p-value: 0.027178
% Surviving
Months Survival

b


frameshift
nonsense
splice
in_frame
nonstart
nonstop
missense
NCSTN, mRNA expression (microarray)
HetLoss
Diploid
Gain
Amp
NCSTN, Putative copy-number alterations from GISTIC

c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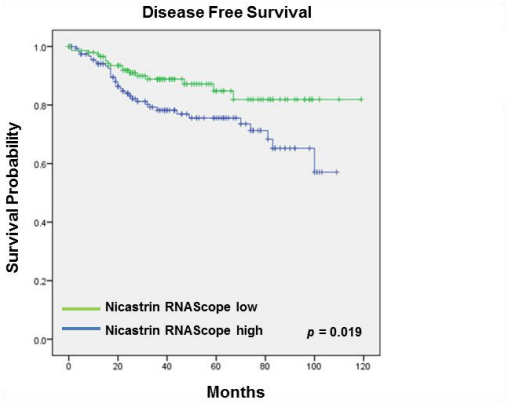
H-score = 120
H-score = 220
H-score = 310

d


Disease Free Survival
Survival Probability
Nicastrin RNAScope low
Nicastrin RNAScope high
p = 0.019
Months