Supplemental Figures and Legends, Tables, Methods, and References

RASAL2 Activates RAC1 and Promotes Triple Negative Breast Cancer Progression

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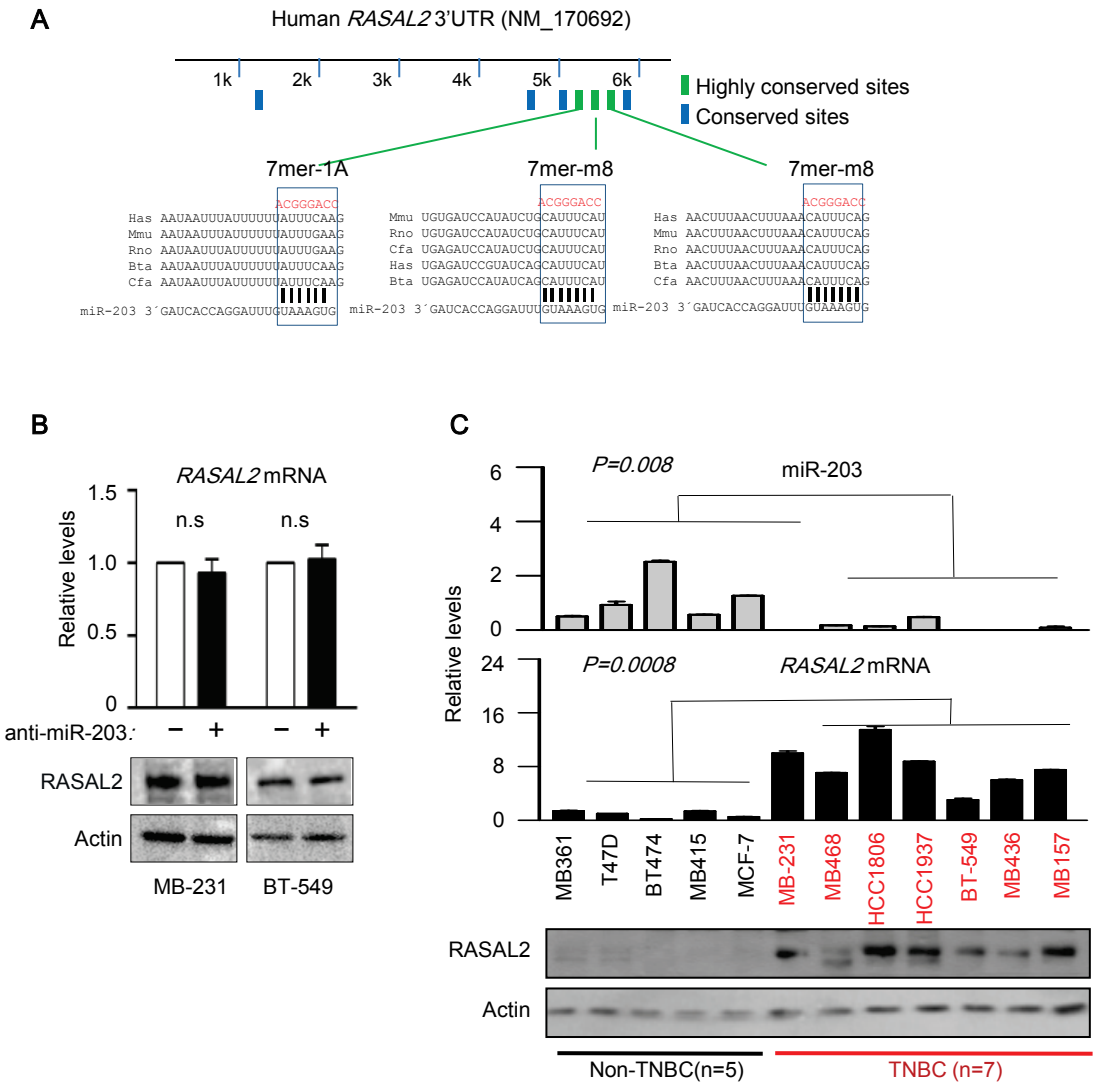
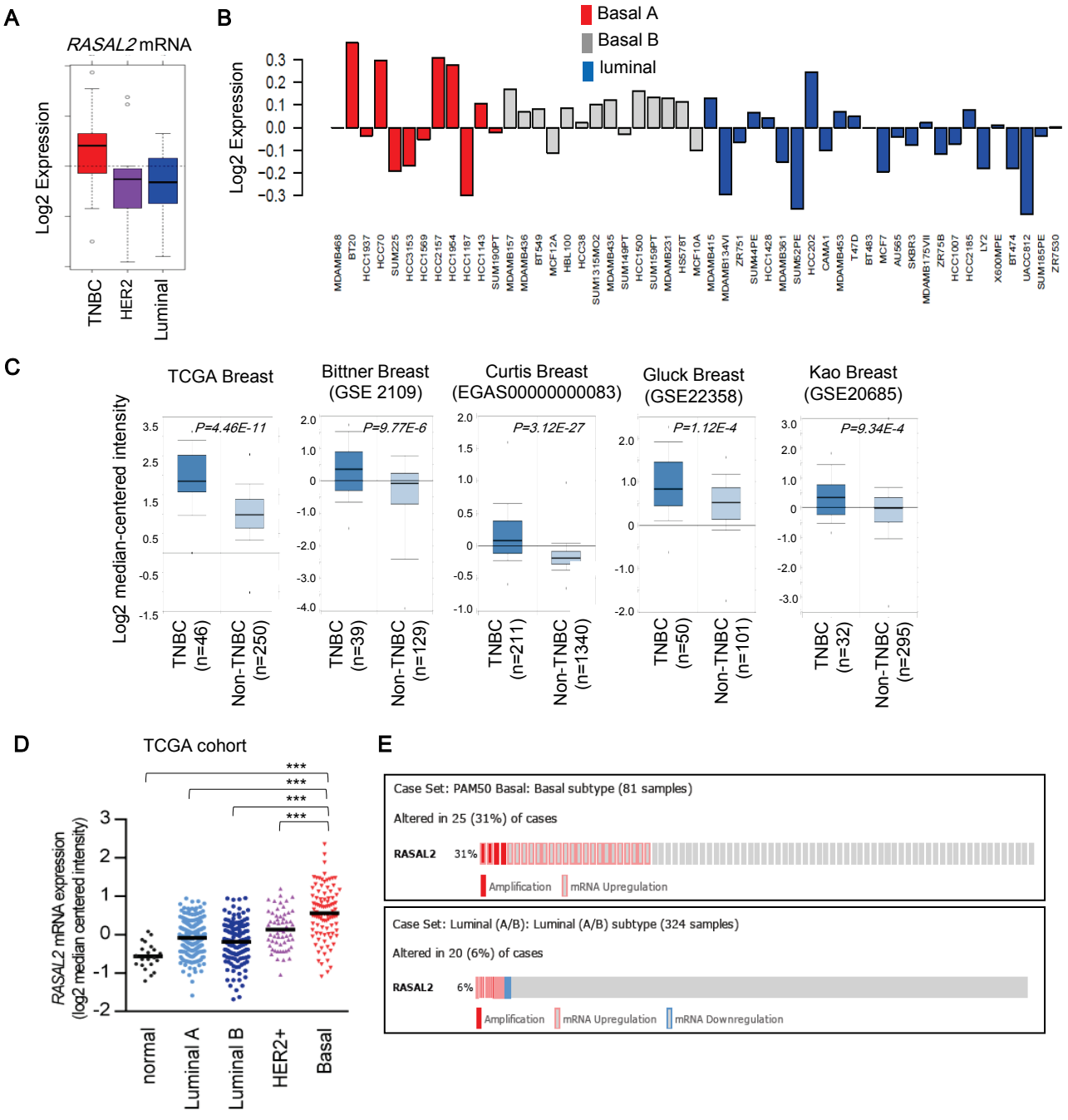
Supplemental Figure 1



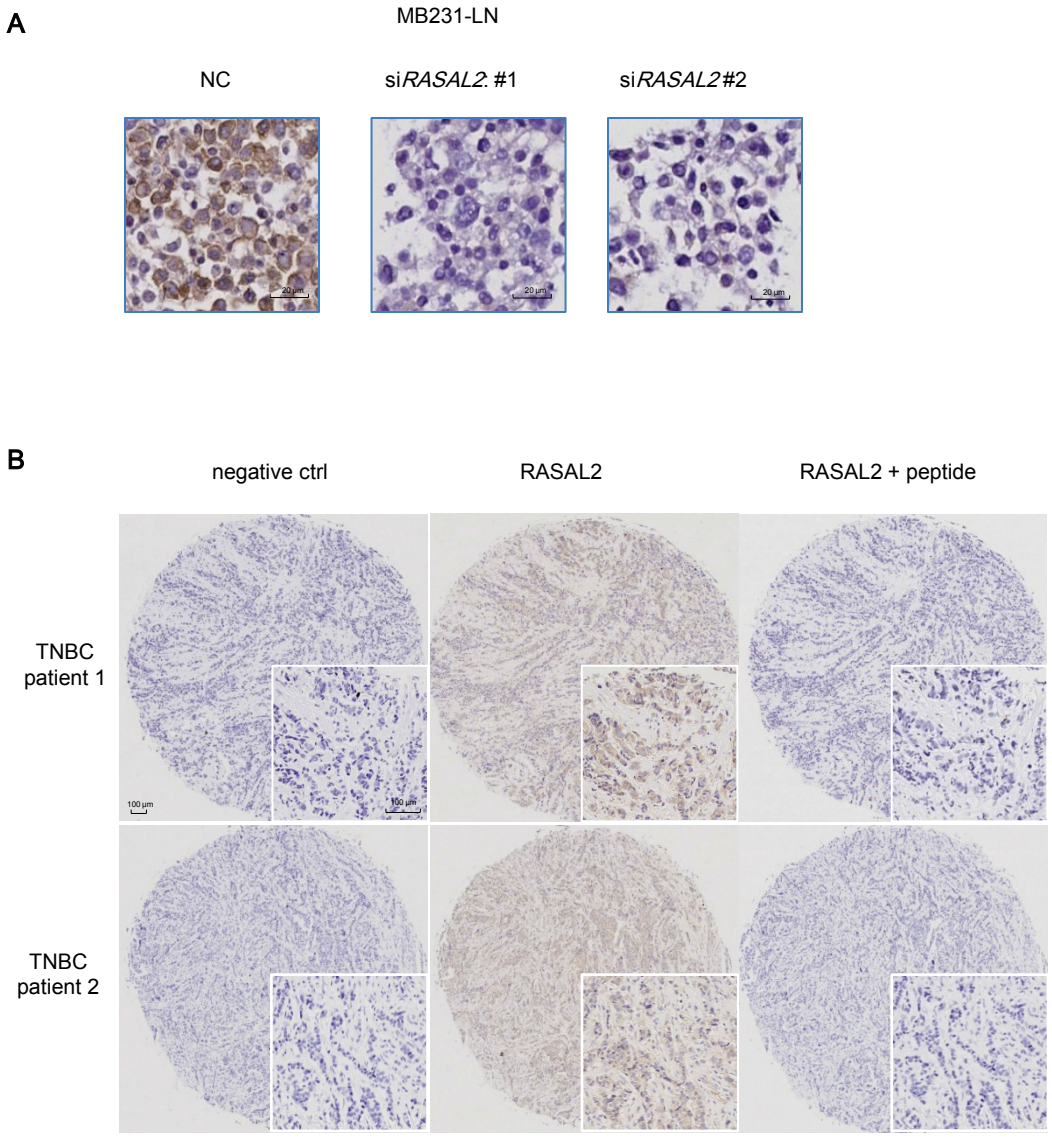
Supplemental Figure 1. RASAL2 is a direct target of miR-203. (**A**) Analysis of evolutionary conservation of 3'UTR of *RASAL2* transcript. miR-203 MRE (micorRNA response element) sites in indicated species is shown in bottom panel. Short sequence in red denotes the mutated MRE to disrupt the MRE-miRNA pairing utilized in reporter assay in Figure 1C. (**B**) Q-PCR and Western blot of indicated cells line with anti-miR-203 treatment. (**C**) Top panel: Q-PCR analysis of miR-203 in a panel of TNBC and non-TNBC cell lines. Lower panels: Q-PCR and Western blot analysis of *RASAL2* expression in the same set of cell lines.

Supplemental Figure 2



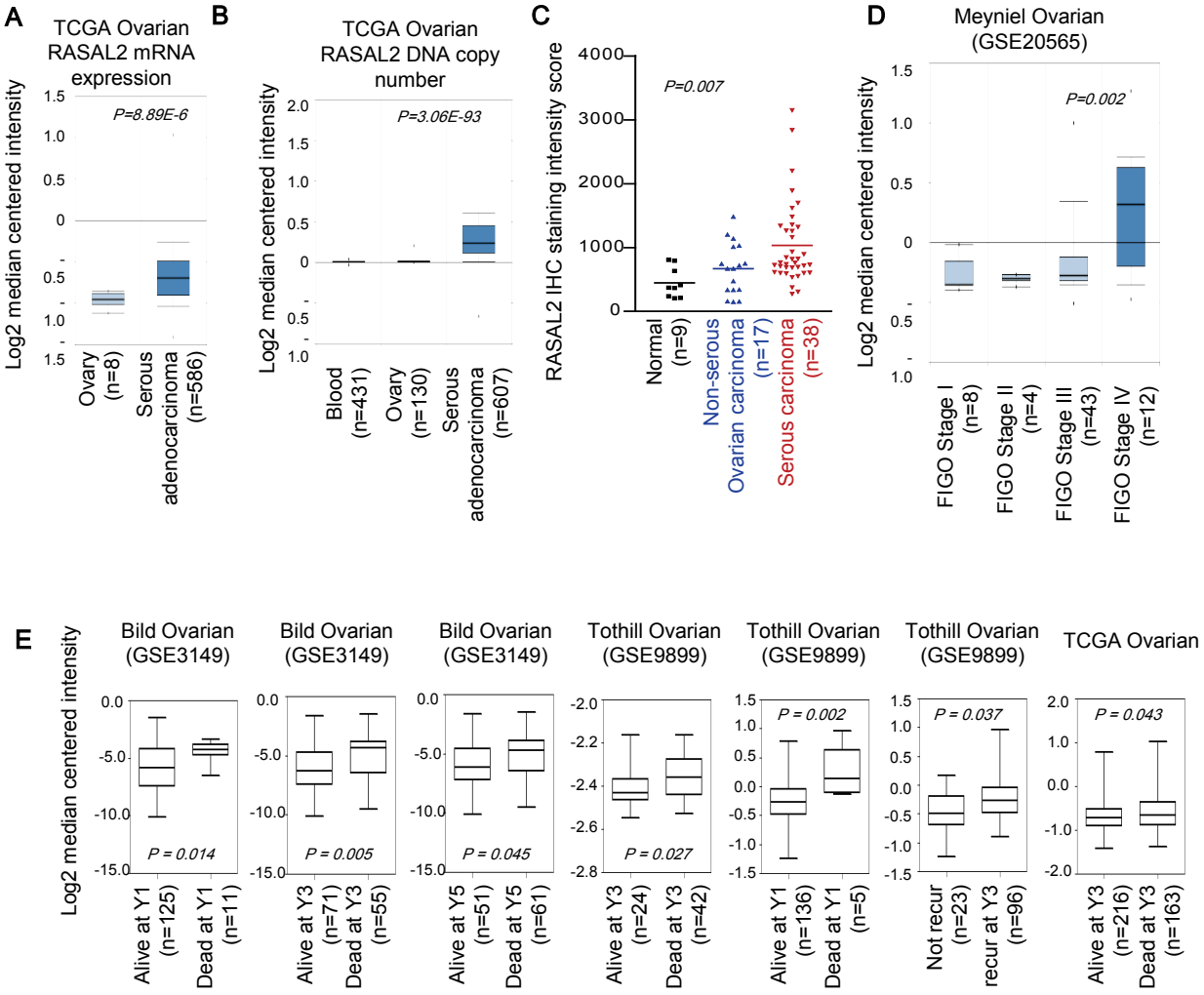
Supplemental Figure 2. RASAL2 is overexpressed in TNBC. (**A**) Box plots of *RASAL2* expression levels in the Neve breast cancer cell line panel using GOBO online analysis tools. (**B**) *RASAL2* expression in individual cell lines of the Neve panel. (**C**) Box plots showing differential *RASAL2* mRNA expression levels between TNBC and non-TNBC patients in 5 Oncomine datasets. Error bars denote mean ± SEM. (**D**) Scatter plot showing the RASAL2 mRNA levels in different subtypes of TCGA breast cancer cohort. (**E**) *RASAL2* gene amplification, mRNA expression analysis in basal (top) and luminal (bottom) breast cancer samples in TCGA dataset.

Supplemental Figure 3



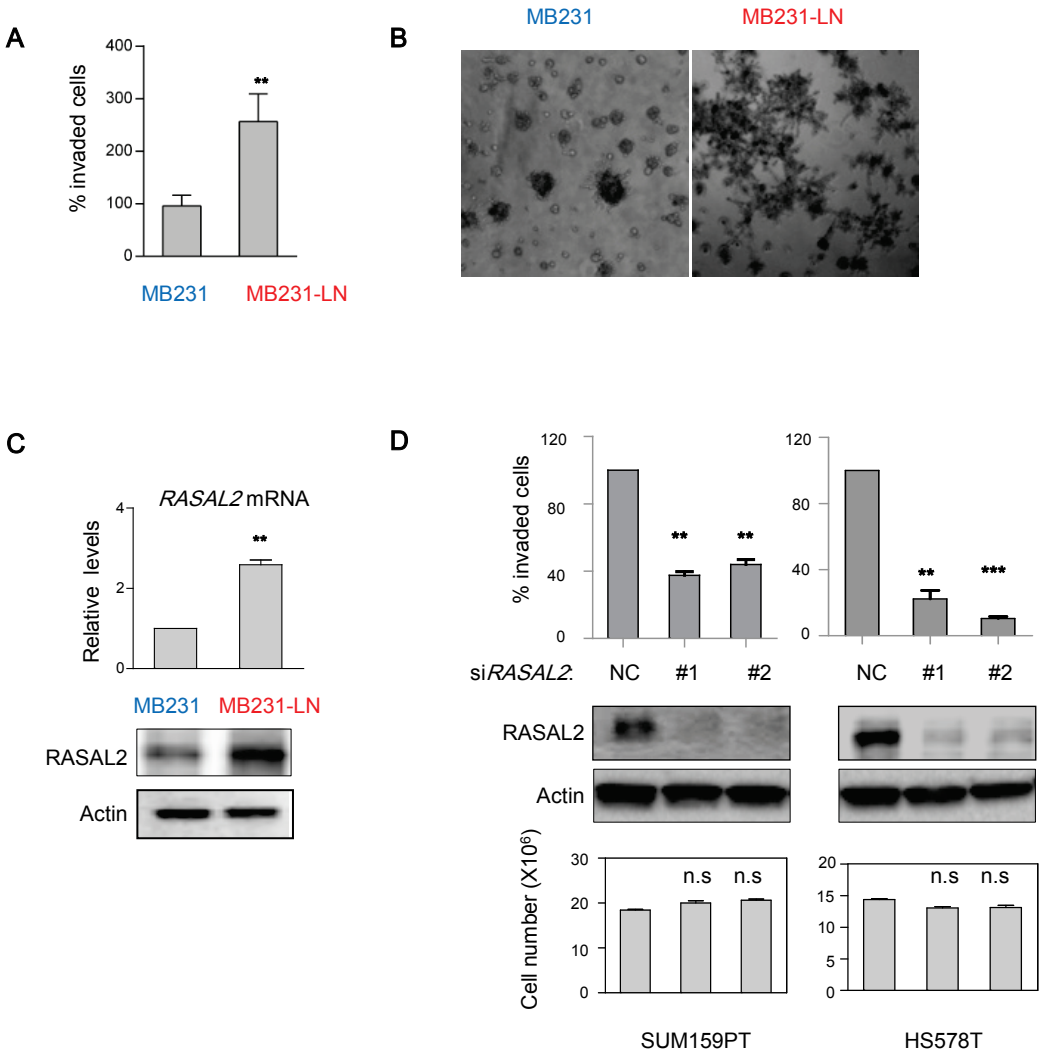
Supplemental Figure 3. RASAL2 antibody NBP1-82579 recognizes RASAL2 protein specifically in IHC . (**A**) : IHC staining of RASAL2 on paraffin sections of MB231-LN cells with indicated siRNA treatment. (**B**) IHC staining of identical TMA slides with rabbit IgG isotope control, RASAL 2 antibody NBP1-82579 alone, or antibody with an epitope peptide blocker.

Supplemental Figure 4



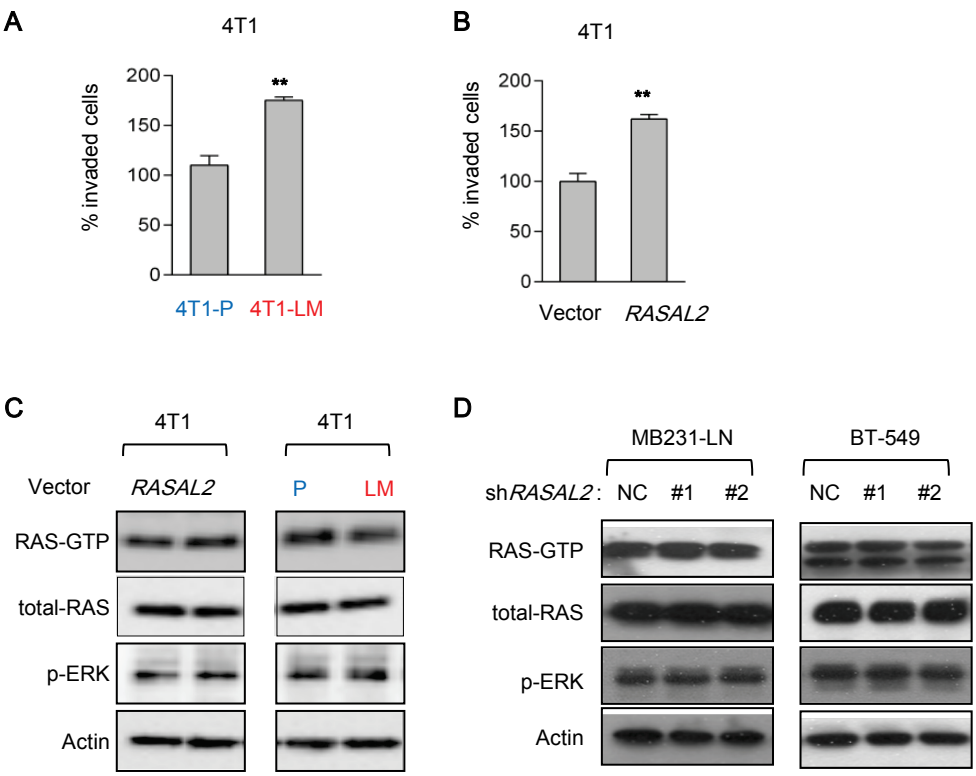
Supplemental Figure 4. *RASAL2* is overexpressed in high grade ovarian cancer and correlates with poorer disease outcomes. (**A**) Box plots showing mRNA expression of *RASAL2* in Oncomine TCGA ovarian cancer dataset. (**B**) Box plots showing *RASAL2* gene copy number in Oncomine TCGA ovarian cancer DNA dataset. (**C**) IHC staining intensities of RASAL2 protein in paraffin embedded primary ovarian tissues of 55 cancer patients and 9 normal controls as indicated. (**D**) Box plots showing mRNA expression of *RASAL2* in the Oncomine Meyniel Ovarian cancer dataset. Patient tumors were graded according to FIGO stage. (**E**) Box plots showing the correlation of the mRNA expression of *RASAL2* with patient disease outcome as indicated in 7 Oncomine ovarian cancer dataset analysis. Error bars, mean ± SEM.

Supplemental Figure 5



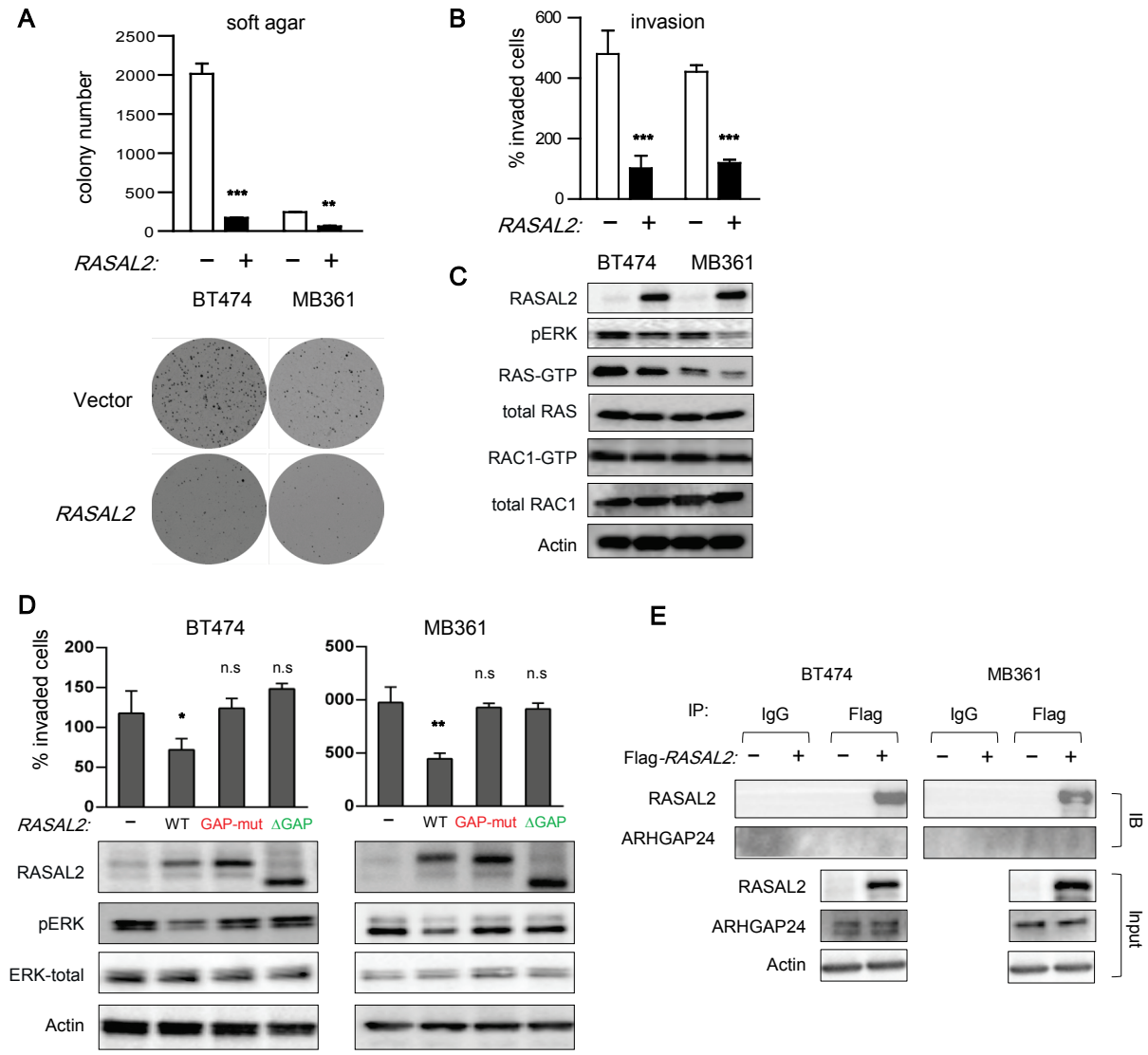
Supplemental Figure 5. MB231-LN is an aggressive subline harboring elevated RASAL2 expression. (**A**) Transwell Matrigel invasion assay comparing parental MB231 and MB231-LN subline. (**B**) Representative images comparing parental MB231 and MB231-LN cell growth in 3D Matrigel. (**C**) Q-PCR and Western blot analysis of RASAL2 expression in MB231 and MB231-LN cell lines. (**D**) Effects of *RASAL2* knockdown on invasion (top panel), RASAL2 protein expression (middle) and proliferation (bottom) of SUM159PT and HS578T cells. ***P<0.001; **P<0.01; *P<0.05. Error bars: mean ± SEM.

Supplemental Figure 6



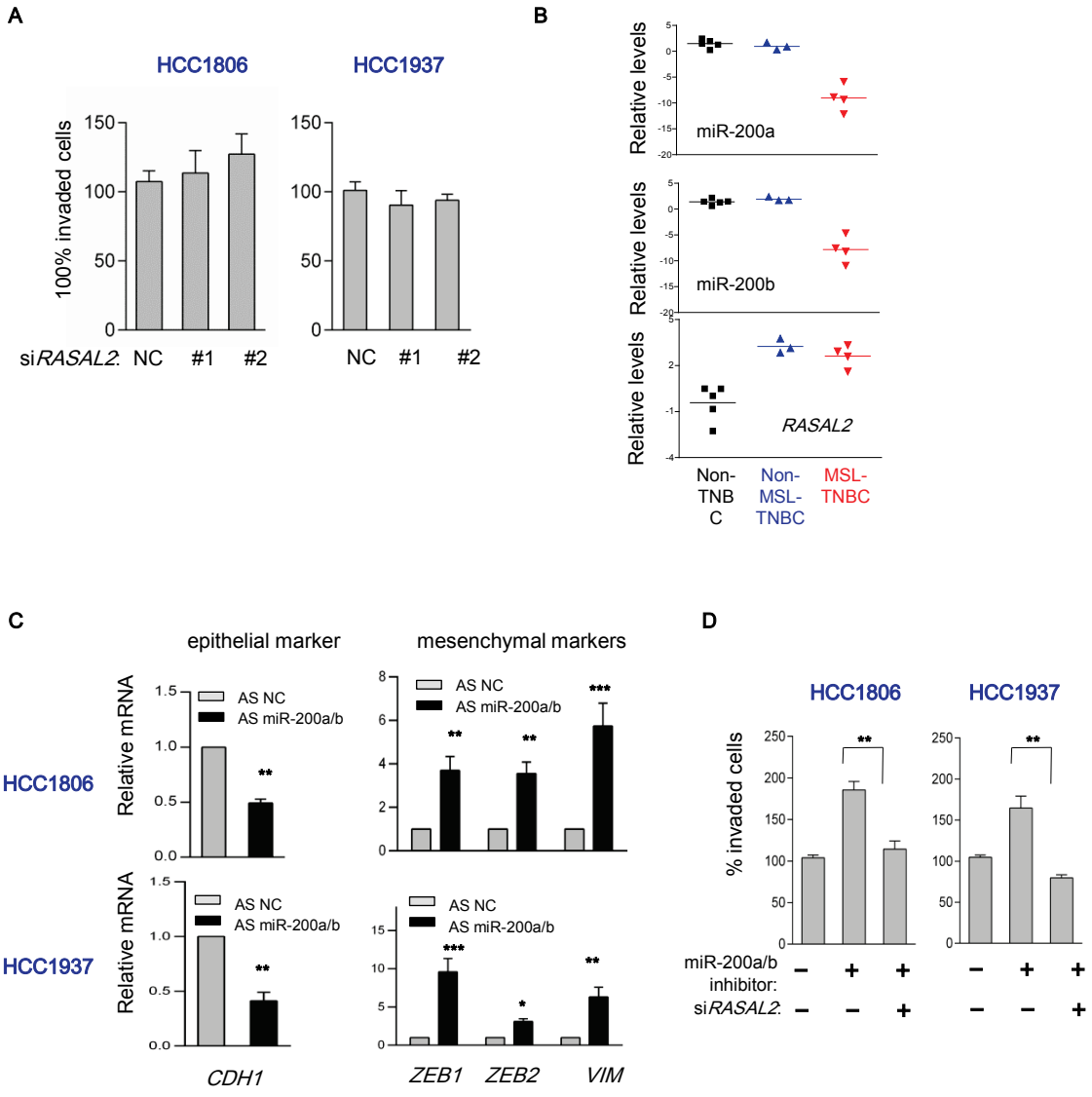
Supplemental Figure 6. Manipulation of RASAL2 expression does not affect RAS activity in basal breast cancer cells. (**A**) and (**B**) Transwell Matrigel invasion assay comparing invasiveness of 4T1-lung metastasis subline (4T1-LM) or ectopic *RASAL2* expressing with respective controls. (**C**) and (**D**) Western blot analysis showing the levels of GTP-bound RAS, total RAS, and p-ERK following manipulation of RASAL2 levels in indicated cell lines. **P<0.01) . Error bars: mean ±SEM.

Supplemental Figure 7



Supplemental Figure 7. Effect of ectopic *RASAL2* on luminal B breast cancer cells. (**A**) Soft agar colony growth of BT474 and MB361 cells with or without ectopic *RASAL2* respectively. (**B**) and (**C**)Transwell invasion assay and western blot analysis showing the levels of GTP-bound RAS, total RAS, GTP-bound RAC1, total RAC1 and p-ERK upon ectopic *RASAL2* expression. (**D**) Effects of *RASAL2* wild type or indicated mutants on invasion (top panel) and p-ERK levels (bottom panel) in BT474 and MB361 cells. (**E**). Co-IP of Flag-RASAL2 in BT474 and MB361 cells showing no association between RASAL2 and ARHGAP24. **P<0.01; *P<0.05; n.s. not significant. Error bars: mean ± SEM.

Supplemental Figure 8



Supplemental Figure 8. RASAL2 selectively regulates mesenchymal TNBC invasion. (A) Effects of two independent RASAL2 siRNAs on cell invasion in non-mesenchymal like TNBC cell lines: HCC1806 and HCC1937. (B) Scatter plots of the mRNA expression of miR-200a, mir-200b and *RASAL2* in breast cancer panel grouped into non-TNBC, non-mesenchymal like TNBC and mesenchymal like TNBC. (C) Q-PCR analysis showing the expression level changes of EMT markers *CDH1, ZEB1, ZEB2 and Vim* in indicated cells treated with miR200a/b inhibitors . (D) Transwell Matrigel invasion assay on non-mesenchymal like TNBC HCC1806 and HCC1937 cells treated with miR200a/b synthetic inhibitors and *RASAL2* siRNAs as indicated.

Supplemental Table 1: SAM analysis of miRNAs in TNBC and non-TNBC cell lines

Gene Name	BT-549	MB-231	MCF-7	BT474	Score(d)	Numerator(r)	Denominator(	Fold Chang	q-value(%)	localfdr(%)
hsa-miR-141	-3.26862	-3.1449666	6.425307	6.553509	15.50312	9.6961999	0.625435354	829.5585	0	9.734205
hsa-miR-200c	-3.26862	-3.4485896	5.626307	5.770781	13.89621	9.0571474	0.65177115	532.6882	0	9.604344
hsa-miR-203	-3.26862	-3.4485896	3.761867	4.175101	9.618887	7.3270874	0.761739629	160.5732	0	8.962422
hsa-miR-200b	-3.26862	-1.2161376	4.541641	5.143928	4.411986	7.0851618	1.605889323	135.7833	1.99765	7.744532
hsa-miR-200a	-3.26862	-3.4485896	2.332005	3.135531	6.42591	6.0923709	0.94809464	68.23173	0	8.93467
hsa-miR-429	-3.26862	-3.4485896	2.319111	3.034121	6.668504	6.03521915	0.905033457	65.5816	0	8.982459
hsa-miR-375	-3.26862	-3.4485896	1.442952	1.740135	6.971131	4.95014685	0.710092401	30.91311	0	9.014119
hsa-miR-489	-3.26862	-3.4485896	0.573268	1.544806	4.287262	4.41764012	1.030410695	21.37185	1.99765	7.656044
hsa-miR-342-3p	3.165062	-3.4485896	4.70288	3.737937	1.124789	4.3621725	3.878214665	20.56576	7.86309	25.4926
hsa-miR-205	-3.26862	-3.4485896	0.269076	0.10397	5.383685	3.545126	0.658494298	11.67318	1.99765	8.485644
hsa-miR-432*	-3.111	-3.4485896	-0.64228	0.435787	2.884563	3.17654797	1.101223115	9.041411	3.9953	8.863005
hsa-miR-554	-3.26862	-3.4485896	-0.37057	-0.1241	4.515819	3.11126725	0.688970827	8.641413	1.99765	7.824356
hsa-miR-211	-3.26862	-3.4485896	-1.43275	0.818577	1.832051	3.05151903	1.665629952	8.290844	7.828628	16.23329
hsa-miR-34a	1.128275	2.4397976	3.726934	5.478789	1.728729	2.818825	1.630576894	7.055875	7.828628	17.39377
hsa-miR-326	-2.09404	-3.4485896	-0.82006	0.660257	1.748083	2.6914157	1.539638663	6.45947	7.828628	17.17051
hsa-miR-1236	-2.21412	-3.4485896	-0.54896	0.209678	2.111049	2.6617154	1.260849383	6.32785	7.828628	13.49446
hsa-miR-675b	-2.40824	-1.4235501	0.024665	1.398125	1.901954	2.62729118	1.381364027	6.178648	7.828628	15.49241
hsa-miR-342-5p	-2.30217	-3.4485896	0.090021	-0.58651	2.185724	2.62713772	1.20195317	6.177991	7.828628	12.8618
hsa-miR-346	-2.00185	-3.4485896	-0.39587	0.181416	1.990559	2.61799625	1.315206402	6.138968	7.828628	14.60548
hsa-miR-204	-3.26862	-3.4485896	-1.43695	-0.05927	2.120503	2.61049076	1.23107131	6.107114	7.828628	13.41198
hsa-miR-195*	-1.13463	-3.4485896	-0.06893	0.546917	1.459715	2.53060675	1.733630541	5.778146	7.86309	20.76267
hsa-miR-602	-1.77122	-3.4485896	0.279499	-0.50357	1.708589	2.49787355	1.461950952	5.648523	7.828628	17.62895
hsa-let-7g*	-2.26591	-3.4485896	-0.90579	0.0596	1.872841	2.43415334	1.299711781	5.404471	7.828628	15.79659
hsa-miR-183	2.57933	0.6501334	4.026337	4.011466	1.601708	2.40417005	1.501004181	5.29331	7.828628	18.92483
hsa-miR-657	-3.26862	-3.4485896	-1.77422	-0.16227	1.774105	2.39035886	1.3473603	5.242878	7.828628	16.87458
hsa-miR-21*	2.806849	4.044633	6.379594	5.229953	1.722645	2.3790328	1.381034751	5.201879	7.828628	17.46451
hsa-miR-1227	-1.10118	-2.591556	-0.05961	1.058878	1.598008	2.34599817	1.468077037	5.08412	7.828628	18.97109
hsa-let-7e*	-3.26862	-3.4485896	-2.05721	-0.00928	1.486525	2.3253563	1.564289569	5.011895	7.86309	20.4061

hsa-miR-33a*	-3.26862	-3.4485896	-1.7438	-0.38166	1.876685	2.29587285	1.223366307	4.91051	7.828628	15.75607
hsa-miR-193b	1.9404	1.5685351	5.10765	2.979678	1.416155	2.2891969	1.616487244	4.887839	7.86309	21.3508
hsa-miR-130a*	-2.64515	-3.4485896	-0.97258	-0.59296	2.308703	2.26410258	0.980681712	4.803555	6.849085	11.91243
hsa-miR-1538	-2.61179	-3.4485896	-1.22406	-0.38567	1.971679	2.22532391	1.128644164	4.676159	7.828628	14.78954
hsa-miR-1207-3p	-2.6539	-3.4485896	-1.32249	-0.40853	1.914036	2.18573064	1.141948262	4.549571	7.828628	15.36802
hsa-miR-149	0.466493	-0.3558376	2.525307	1.949277	2.101309	2.18196391	1.038383082	4.537708	7.828628	13.58015
hsa-miR-411*	-1.61599	-0.6120877	0.254178	1.620058	1.482114	2.05115575	1.383939307	4.144378	7.86309	20.46448
hsa-miR-95	-3.26862	-3.4485896	-0.30922	-2.31106	1.329021	2.04846683	1.541335391	4.136661	7.86309	22.55627
hsa-miR-515-3p	-3.26862	-3.4485896	-1.90976	-0.75946	1.809523	2.02399185	1.118522173	4.067076	7.828628	16.4797
hsa-miR-943	-3.26862	-3.4485896	-2.52277	-0.14788	1.1714	2.02327826	1.727231574	4.065065	7.86309	24.81245
hsa-miR-92b	-1.9241	-3.4485896	-0.99467	-0.33413	1.479009	2.02194752	1.367096093	4.061317	7.86309	20.50564
hsa-miR-139-5p	-3.26862	-3.4485896	-2.47268	-0.30421	1.2129	1.97016245	1.624340323	3.918122	7.86309	24.21097
hsa-miR-502-5p	-3.26862	-3.4485896	-1.12907	-1.73378	2.262375	1.9271796	0.851839331	3.80311	6.849085	12.25677
hsa-miR-613	-2.02525	-3.4485896	-1.77045	0.08577	1.110583	1.89458109	1.705933366	3.71814	7.86309	25.70069
hsa-miR-34b	0.684378	0.36164784	2.130718	2.697082	2.192822	1.89088703	0.862307755	3.708632	7.828628	12.8039
hsa-miR-1910	0.101533	-1.0690019	1.167865	1.565398	1.602775	1.85036561	1.154476327	3.605916	7.828628	18.91151
hsa-miR-1909*	0.72835	-0.5209622	1.793563	2.078496	1.556687	1.83233524	1.177073982	3.56113	7.86309	19.49377
hsa-let-7d*	-0.14983	-0.6558695	1.073864	1.688124	1.909272	1.78384473	0.934306458	3.443426	7.828628	15.41695
hsa-miR-200c*	-3.26862	-3.4485896	-1.61088	-1.61154	2.789736	1.74739095	0.626364284	3.357508	6.849085	9.22054
hsa-miR-631	-0.34771	-0.3713841	0.98646	1.735825	1.888282	1.72069121	0.91124678	3.295943	7.828628	15.63448
hsa-miR-1296	0.191509	0.01611781	1.410603	2.176385	1.818459	1.68968047	0.929182842	3.225852	7.828628	16.38152
hsa-miR-182	0.052539	-1.555476	0.818176	0.990221	1.231003	1.65566708	1.344973679	3.150688	7.86309	23.95005
hsa-miR-1539	-1.1272	-1.0408399	0.68839	0.432891	2.45023	1.64466082	0.671227245	3.126743	6.849085	10.95709
hsa-miR-34b*	-3.26862	-1.5716033	-1.38583	-0.17028	1.039211	1.64205783	1.580100088	3.121107	7.86309	26.7501
hsa-miR-1229	-0.74458	-1.7470405	0.168261	0.558707	1.498014	1.60929346	1.074284996	3.051024	7.86309	20.25463
hsa-miR-526b*	-3.26862	-3.4485896	-2.56448	-0.96552	1.188451	1.5936047	1.340909054	3.018025	7.86309	24.56479
hsa-miR-744*	-0.83173	0.22686505	0.58263	1.97755	1.120815	1.58251988	1.411936688	2.994925	7.86309	25.55078
hsa-miR-129*	-1.90567	-1.8164656	-0.76604	0.184136	1.549112	1.57011382	1.013557189	2.969281	7.86309	19.59077
hsa-miR-18b*	-2.28242	-3.4485896	-1.59533	-1.04946	1.307517	1.54310705	1.180180867	2.914214	7.86309	22.85899
hsa-miR-223	-1.93163	-3.4485896	-1.42127	-0.90596	1.141363	1.5264907	1.337427467	2.880842	7.86309	25.25025
hsa-miR-1237	-0.78793	-2.5699663	0.003578	-0.36856	1.034454	1.49645839	1.446616025	2.821492	7.86309	26.82021
hsa-miR-200b*	-2.90206	-3.0555577	-1.33403	-1.71568	1.959347	1.45395515	0.742061189	2.739581	7.828628	14.91121
hsa-miR-96	4.446384	2.895262	5.095236	5.154247	1.107748	1.4539189	1.31249927	2.739512	7.86309	25.74225
hsa-miR-652	-1.17695	-1.1977038	1.021689	-0.56203	1.066892	1.41715695	1.328304505	2.670587	7.86309	26.34247

hsa-miR-634	-1.35569	-1.279665	-0.36192	0.525184	1.425606	1.39931047	0.981554826	2.637755	7.86309	21.22232
hsa-miR-557	-1.95178	-1.8734082	-0.79526	-0.23722	1.706757	1.39635575	0.818134024	2.632358	7.828628	17.6505
hsa-miR-629*	0.61845	0.8022201	2.134596	2.070229	2.19662	1.39207755	0.633736039	2.624564	7.828628	12.77307
hsa-miR-636	-0.738	-2.0133824	-0.1819	0.149198	1.137328	1.35934101	1.195205304	2.56568	7.86309	25.30921
hsa-miR-19b-1*	-1.10146	-0.5219564	0.130487	0.962197	1.301784	1.35804926	1.043221544	2.563383	7.86309	22.94001
hsa-miR-335*	-0.42051	0.01768541	0.899305	1.413263	1.553289	1.35769759	0.874079163	2.562759	7.86309	19.53723
hsa-miR-937	-0.09116	0.21807599	1.219909	1.601457	1.722925	1.34722422	0.781940332	2.544221	7.828628	17.46125
hsa-let-7b*	-0.25833	-0.240767	0.612592	1.570618	1.320718	1.3411515	1.015471154	2.533535	7.86309	22.67292
hsa-miR-339-3p	-1.35465	-1.8435173	0.427198	-0.99223	1.022969	1.3165659	1.28700519	2.490725	7.86309	26.98955
hsa-miR-498	-3.01985	-2.1668973	-0.87326	-1.69174	1.162692	1.3108725	1.127446328	2.480915	7.86309	24.93917
hsa-miR-1200	-3.26862	-3.4485896	-2.81309	-1.35973	1.00283	1.27219535	1.268604833	2.415288	7.86309	27.28658
hsa-miR-1253	-3.26862	-3.4485896	-2.26964	-1.91621	1.722748	1.26567505	0.734683731	2.404397	7.828628	17.46331
hsa-miR-647	0.400137	0.17518353	1.357127	1.746807	1.660606	1.26430643	0.76135251	2.402117	7.828628	18.20094
hsa-miR-1224-3p	0.826683	0.666044	1.929736	2.083561	1.946135	1.26028503	0.647583642	2.395431	7.828628	15.04283
hsa-miR-30d*	-3.26862	-2.6796727	-2.1222	-1.35025	1.211444	1.23792205	1.021856528	2.358586	7.86309	24.232
hsa-miR-615-3p	0.132276	0.17449284	1.121936	1.616866	1.549577	1.21601637	0.78474101	2.323044	7.86309	19.58481
hsa-miR-885-5p	1.337701	1.1923783	2.252032	2.708386	1.566254	1.21516955	0.775844669	2.321681	7.828628	19.37177
hsa-miR-328	1.845821	1.7224047	2.898424	3.095002	1.858585	1.21260035	0.652431888	2.31755	7.828628	15.94782
hsa-let-7f-1*	-1.10372	-0.5769568	0.057484	0.663713	1.280406	1.20093765	0.937935196	2.29889	7.86309	23.24323
hsa-miR-593	-3.26862	-2.3444476	-1.51737	-1.77022	1.145052	1.16273695	1.015444273	2.238818	7.86309	25.19638
hsa-miR-576-5p	-3.26862	-2.570084	-2.08365	-1.44871	1.1436	1.1531682	1.008366992	2.224018	7.86309	25.21759
hsa-miR-133a	-3.26862	-3.4485896	-2.21343	-2.20507	1.834672	1.1493501	0.626460724	2.218139	7.828628	16.20486
hsa-miR-1323	-1.1397	-1.6406784	-0.33253	-0.16844	1.424701	1.13970579	0.799961346	2.203361	7.86309	21.2346
hsa-miR-671-3p	-3.26862	-3.4485896	-2.68361	-1.79537	1.130968	1.11911695	0.989521113	2.17214	7.86309	25.40219
hsa-miR-296-5p	0.119503	-0.7630193	0.514941	1.033269	1.045554	1.09586322	1.048117251	2.137409	7.86309	26.65664
hsa-miR-766	1.987869	2.697489	3.3365	3.499373	1.194182	1.07525745	0.900413372	2.107098	7.86309	24.48172
hsa-miR-625*	-0.86017	-0.9495168	0.205318	0.123136	1.79051	1.0690694	0.597075315	2.09808	7.828628	16.69054
hsa-miR-605	0.097859	0.32376075	0.917263	1.634978	1.167348	1.06531071	0.912590774	2.092621	7.86309	24.87139
hsa-miR-425*	-1.95336	-1.1484101	-0.53136	-0.44124	1.130895	1.06458645	0.941366095	2.09157	7.86309	25.40326
hsa-miR-566	-3.10956	-3.4485896	-2.65771	-1.80233	1.0528	1.0490521	0.996440459	2.06917	7.86309	26.54991
hsa-miR-1225-3p	0.050952	-0.2536295	1.005374	0.887252	1.497245	1.04765179	0.699719547	2.067162	7.86309	20.26474
hsa-miR-485-3p	1.38294	1.4010746	2.391352	2.421403	1.831234	1.01437015	0.553927058	2.020021	7.828628	16.24216
hsa-miR-877*	2.1766	2.1298177	3.166223	3.163586	1.807226	1.0116957	0.559805752	2.01628	7.828628	16.50503
hsa-miR-324-5p	3.44501	3.452603	1.80187	2.277812	-1.81948	-1.40896565	0.774378558	0.376582	7.86309	22.2896

hsa-let-7c*	-1.68224	-0.929385	-2.7663	-3.04589	-1.70619	-1.60027978	0.937925125	0.329813	7.86309	23.70262
hsa-miR-18a	2.559681	3.4058678	0.981907	1.318742	-1.84768	-1.83245025	0.991758955	0.280787	7.86309	21.94707
hsa-miR-543	-1.61218	-0.5614972	-2.98142	-3.04589	-1.81312	-1.9268163	1.062704694	0.263009	7.86309	22.36733
hsa-miR-18b	0.546302	1.6409681	-1.12938	-0.5448	-1.66893	-1.93072615	1.15686624	0.262297	7.86309	24.17883
hsa-miR-320d	5.245348	4.4022474	2.72933	2.769085	-2.16465	-2.0745904	0.958395907	0.237403	6.849085	18.40767
hsa-miR-23a	7.093152	8.109778	5.387523	5.545748	-2.0316	-2.1348293	1.050810266	0.227694	7.828628	19.82007
hsa-miR-320c	4.633553	3.7463543	2.028614	2.039682	-2.19978	-2.15580565	0.980011347	0.224408	6.849085	18.05331
hsa-miR-181c	-0.56492	-0.250844	-2.21797	-3.04589	-2.27148	-2.22404875	0.979119704	0.21404	6.849085	17.35436
hsa-miR-27a	7.309567	8.256939	5.574056	5.529547	-2.20808	-2.2314515	1.010585961	0.212944	6.849085	17.97072
hsa-miR-33a	-1.0125	-0.4146822	-2.98142	-3.04589	-2.74793	-2.30006308	0.837017995	0.203054	3.841634	13.54713
hsa-miR-181a-2*	-0.55384	-0.8428416	-2.98142	-3.04589	-3.38284	-2.31530985	0.684427766	0.20092	1.737087	10.54251
hsa-miR-30e*	-0.47942	1.0113075	-2.35338	-1.7543	-1.7316	-2.31978648	1.339677231	0.200297	7.86309	23.38094
hsa-miR-29b-1*	0.882032	1.39205	-0.58599	-1.8012	-1.9498	-2.33063827	1.195324451	0.198796	7.828628	20.74202
hsa-miR-582-5p	-0.22908	-1.0247157	-2.98142	-3.04589	-2.55131	-2.3867573	0.935501472	0.191212	3.841634	14.94141
hsa-miR-767-5p	-0.74601	-0.4699271	-2.98142	-3.04589	-3.5475	-2.40568227	0.678134305	0.18872	1.737087	10.07271
hsa-miR-218	1.004854	0.61663795	-2.24617	-1.24561	-2.38271	-2.55663788	1.072996703	0.169971	3.9953	16.33503
hsa-miR-370	-0.21003	-0.4915459	-2.98142	-3.04589	-3.91149	-2.66286535	0.680779601	0.157906	1.737087	9.375154
hsa-miR-22	5.97814	6.217947	3.763845	3.04641	-2.94435	-2.6929164	0.914603848	0.154651	1.737087	12.38898
hsa-miR-409-3p	1.554668	1.7227333	-0.25129	-2.7263	-1.76025	-3.12749862	1.776732827	0.114427	7.86309	23.02137
hsa-miR-125b	5.327297	7.592721	2.942785	3.482081	-1.9095	-3.2475755	1.700742917	0.105289	7.828628	21.21053
hsa-miR-135b	2.808188	0.95713735	-1.72085	-1.68973	-2.45408	-3.58795103	1.462033383	0.083161	3.9953	15.72272
hsa-miR-132	2.408417	0.96707463	-0.91084	-3.04589	-2.0095	-3.66610787	1.824389805	0.078776	7.828628	20.06526
hsa-miR-221*	0.106812	1.2582943	-2.98142	-3.04589	-3.32088	-3.69620578	1.113020366	0.077149	1.737087	10.74823
hsa-miR-29b	6.343899	8.148369	3.138746	3.553639	-2.66726	-3.89994155	1.462153851	0.066989	3.841634	14.08958
hsa-miR-630	1.535065	0.39808416	-2.93437	-3.04589	-3.57233	-3.95670233	1.107595653	0.064404	1.737087	10.01097
hsa-miR-10a	1.871671	2.469231	-2.59132	-1.53799	-3.70885	-4.23510805	1.141891628	0.053101	1.737087	9.710696
hsa-miR-29a	8.296694	8.632475	3.579658	3.617016	-6.8995	-4.86624735	0.70530388	0.034286	1.737087	9.92131
hsa-miR-30a*	1.770021	3.5765831	-2.98142	-1.8783	-3.19999	-5.1031602	1.594742263	0.029093	1.737087	11.19894
hsa-miR-30a	4.400636	6.5628757	0.135942	0.562426	-3.13281	-5.13257181	1.638326795	0.028506	1.737087	11.47924
hsa-miR-222	2.264451	4.0716515	-2.98142	-3.04589	-4.2912	-6.181704	1.440552472	0.013776	1.737087	9.032313
hsa-miR-221	3.810996	5.810259	-1.37518	-3.04589	-3.81772	-7.02115855	1.839098445	0.007699	1.737087	9.51519
hsa-miR-100	4.755602	8.149007	-0.85975	-1.79354	-3.38783	-7.77894992	2.296148113	0.004553	1.737087	10.52667
hsa-miR-130a	4.610727	6.1186085	-2.98142	-3.04589	-6.48976	-8.37832065	1.291006815	0.003005	1.737087	9.71837

Supplemental Table 2: Primers & siRNA sequences

RASAL2 **Gene cloning primer**

Rasal2-LF-EcoRV-F1	5′-CATGGATATCACCATG GAGCTCTCTCCGTCGTCC
Rasal2-XhoI-R2	5′-CATGCTCGAGTAGCAGCTGCTGTTTTTGAATT

RASAL2 **mutant construction primer**

RASAL2-435-MluI-Rev1	**CATG** acgcgt tttaatccgaatagaagg
RASAL2-760-MluI-For	**CATG** acgcgt cctacgccaatacaacag
RASAL2-K435E-Rev	gtttggaaacgtgactcaatccgaatagaaggtcctcctgtct
RASAL2-K585E-Rev	gtaagagttgatgatctcgcagaaagccagctcac
RASAL2-K435E-For	agacaggaggaccttctattcggattgagtcacgtttccaaac
RASAL2-K585E-For	gtgagctggctttctgcgagatcatcaactcttac

RAC1 **Gene cloning primer**

fu-Rac1For-BamHI	5′-CTAGACTGCCGGATCggatccACCATGCAGGCCATCAAGTGT
fu-Rac1Rev-XhoI	5′-GAATTTAAATGGATCCTCGAGTTACAACAGCAGGCATTT

ARHGAP24 **Gene cloning primer**

ARHGAP24-F1	5′-CATGGAATTCACCATGGAGGAGAACAATGACTCCACG
ARHGAP24-R2	5′-TGACCTCGAGTA CTGAATCCATATTGTGTT

pMIR REPORT-RASAL2-miR203 **-MRE1-P1 cloning primers**

Ras2-UTR-1-For	5′-GCTAG ACT AGT ACTTCTTCCCCTTATCCCCTCA
Ras2-UTR-1-Rev	5′-GCTAG GAG CTC TGTCTGTTTAACATGTAACCTTCC

pMIR REPORT-RASAL2-miR203 **-MRE2to7-P2 cloning primers**

Ras2-UTR-2to7-For	5′-GCTAG ACT AGT AGCCTCAAGTGTTTGAGTAACCA

Ras2-UTR-2to7-Rev	5′-GCTAG GAG CTC TCTGCAGAATTCGAAAGTAACAGT

***pMIR REPORT-RASAL2-miR203* -MRE1to7 mutation primers**

Ras2UTR-mut1	5′-GCTCGTAGATCTGACAATAATGACCCACGGGACCCACTTGTCATCCATAGCCTTATTATTATAGAAC
Ras2UTR-mut2	5′-AGGAAAGTGGTAGTCTTTGCCTGACGGGACGCATGCATTTTCCTTTCCTACTCAGTTGC
Ras2UTR-mut3	5′-CATTTTATGTGATTTCCAAATTTTCGAGGGAAAACGGGACATTCCCAGTTCAACAAAAGGTTAAATAC
Ras2UTR-mut4	5′-CAATGATAGCTCTAAATAATTTATTTTTCGGGACCGAAAGAGAAATACACCTTAGAAAAACAAAACCC
Ras2UTR-mut5	5′-GATTATTCCGTATGTGAGATCCGTATCAGACGGGACTTACCTGGGTTTGTTCTAAAGAAGATTTATTTTTG
Ras2UTR-mut6	5′-CCTACAAAGAATACAAATAAACTTTAACTTTAAAACGGGACGACTAAAGTTGCTACTATATTTGACATATTGC
Ras2UTR-mut7	5′-CCTAGAATTTCCCAACTTCCATGTAATAAGCGGGACCTTTAGGAAAAATAACTAGCATTAGCATGTATTG

sh*RASAL2* construct

shRasal2-1-T	5'-gatccGCCCTCGTGTTCTTGCTGATTTCAAGAGAATCAGCAAGAACACGAGGGTTTTTTACGCGTg-----3'
shRasal2-1-B	5'-aattcACGCGTAAAAAACCCTCGTGTTCTTGCTGATTCTCTTGAAATCAGCAAGAACACGAGGGCg-----3'
shRasal2-2-T	5'-gatccGCAGGACAGTTCAACCTAATATTCAAGAGATATTAGGTTGAACTGTCCTGCTTTTTTACGCGTg-----3'
shRasal2-2-B	5'-aattcACGCGTAAAAAAGCAGGACAGTTCAACCTAATATCTCTTGAATATTAGGTTGAACTGTCCTGCg-----3'

Real time primers

RASAL2-P2-F	5′-TGGGCCGAGAGCTTTCAGTTTTGC
RASAL2-P2-R	5′-TTGCCACGGTCGCCTGTAGGA
Rac1-F1	5′-CTGATGCAGGCCATCAAGT
Rac1-R1	5′-TCTCCAGGAAATGCATTGGT
ARHGAP24-right	5′-CTGGTTCTGGAAGTTCTCGG
ARHGAP24-left	5′-CTTTGACTGTGGGGAGAAGC
MMP1-F	5′-CCTCGCTGGGAGCAAACA
MMP1-R	5′-TTGGCAAATCTGGCGTGTAAT
MMP-7-F	5′-GTTGTATGGGGAACTGCTGA
MMP-7-R	5′-GTTTCCTGGCCCATCAAATG
MMP9-F	5′-GACGCAGACATCGTCATCCA

MMP9-R	5′-AACTCGTCATCGTCGAAATGG
SNAI1-F1	5′-CCCCAATCGGAAGCCTAACT
SNAI1-R1	5′-GCTGGAAGGTAAACTCTGGATTAGA
SNAI2-F1	5′-CTTGCCCTCACTGCAACAGA
SNAI2R1	5′-TCTGCAGATGAGCCCTCAGA
CDH1F1	5′-TGCCCAGAAAATGAAAAAGG
CDH1R1	5′-GTGTATGTGGCAATGCGTTC-3′
VIM-F	5′-TCGAGCAGCTCAAGGGCCAA-3′
VIM-R	5′-CCTGCAGCTCCTGGATTTCC-3′
ZEB1-F1	5′-GCCAATAAGCAAACGATTCTG-3′
ZEB1-R1	5′-TTTGGCTGGATCACTTTCAAG-3′
ZEB2-PF:	5′-CCCCCACACTTCGCGGCTTC-3′
ZEB2-PR:	5′-AGCACGCAGGCTCGATCTGC-3′
18S-F:	5′-CGAACGTCTGCCCTATCAACTT-3′
18S-R:	5′-ACCCGTGGTCACCATGGTA-3′

***siRASAL2* sequence**

si*RASAL2* #1	5′-CCCUCGUGUUCUUGCUGAUUU-3′
si*RASAL2* #2	5′-GGACAGTTCAACCTAATAAG-3′
si*RASAL2* #3	5′-GCACUAUCACCAAGGUACUGUUGGG-3′

***siARHGAP24* sequence**

si*ARHGAP24* #1	5′-TGGATGTACAGAAGTCTAACTGGTG-3′
si*ARHGAP24* #2	5′-AGGATAAAGAGCTTAGAACAGCGAA-3′

Supplemental Method

Survival analysis and molecular subtype association analysis

Meta-analysis of patient overall survival (OS) and distant metastasis free survival mixed with relapse free survival (DMFS & RFS) on a total of 10 breast cancer cohorts (Chin breast, GSE11121, GSE12093, GSE1456, GSE2034, GSE2603, GSE3494, GSE5327, GSE6532 and GSE7390) comprising 1789 patients were performed using the intrinsic settings of the GOBO algorithm after normalization cross cohorts and platforms (http://co.bmc.lu.se/gobo/) (1). Patients were divided into three groups based on low (0-33%), intermediate (34-66%) and high (67-100%) gene expression after stratification based on ER (estrogen receptor) status or PAM50 subtypes. Subtype specific *RASAL2* gene expression analysis on five breast cancer data sets, Bos Breast (GSE12276), Desmedt Breast (GSE7390), Ma Breast (GSE1379), Curtis Breast (EGAS 00000000083), Kao Breast (GSE20685) and the four ovarian cancer cohorts, Bild Ovarian (GSE31499), Tothill Ovarian (GSE9899), TCGA Ovarian and Meyniel Ovarian (GSE20565) were obtained using default oncomine settings (2). The 5-year recurrence free survival on Esserman Breast (GSE22226) analysis was performed on Oncomine data sets after patient stratification based on ER status according to author's original documentations. For all the Oncomine analysis patients were grouped into two groups according to high or low expression of indicated genes based on the mean of Log2 median centered expression ratio. For RASAL2 and miR-203 association analysis in clinical specimens, after q-RT PCR analysis, difference of Ct values between target gene and referenced control genes (dCq) were further normalized by calculating the z-score for each independent data set. Then patients were grouped by dividing the expression of miR-203 in quartiles, breast tumors expressing high (Upper quartile) and low (lower quartile) levels of miR-203 were compared. Correlation between miR-203 and RASAL2 was accessed by linear regression in either TNBC context or Luminal context.

Immunohistochemistry (IHC) staining of Paraffin-embedded tissue slides

Paraffin-embedded tissue microarray (TMA) slides were purchased from US Biomax (Rockville, MD, cat: BR1505, BRM961, BC110118). Staining and image analysis of TMA were performed by the Histopathology Department of the Institute of Molecular and Cell Biology, Agency for Science, Technology, and Research (A*STAR), Singapore, using standardized protocol. Briefly, Four-micrometer-thick histology sections on slides were deparaffinized using Bond^{TM} Dewax solution and rehydrated through 100% ethanol to 1X Bond^{TM} Wash solution. Antigen retrieval was done in Bond^{TM} Epitope retrieval solution for 40 min at 100 C^{o}. Immunohistochemical staining was performed using polyclonal antibodies against RASAL2 (Novus Biologicals, Littleton, CO, cat: NBP1-82579) or ER (Santa Cruz Biotechnology, Santa Cruz, CA, cat: sc-7207) for 45 min. Cell nuclei were counterstained with haematoxylin. Stained slides were scanned at 20x magnification using Leica SCN400 slide scanner then the resulted images were analyzed using Measured Stain Area algorithms of SlidePath Tissue IA software (Leica Microsystems, Germany). The final staining concentration, reflecting the sum of the staining absorbance for all positively stained areas in pixels, divided by the total analyzed area were used to evaluate respective protein levels for each sample. Duplicate specimens were assessed and average staining intensity was analyzed for each individual patients.

For the peptide blocking experiment, the same procedure was adapted except that the antibody was incubated with an epitope peptide (provided by the same company PrEST Antigen for Anti-RASAL2 NBP1-82579 (HPA018805) at antibody: peptide ratio 1:2 for 30 minutes at room temperature prior to exposure to TMA slides.

Mouse pulmonary metastasis models and bioluminescence measurement

All animal experiments were performed in accordance with protocols approved by Biopolis Institutional Animal Care and Use Committee (IACUC) of Singapore. Each group of animal experiment comprises at least 10 mice. For the 4T1-Luc orthotopic xenograft model, approximately $5x10^4$ 4T1-Luc or subline cells were mixed with Matrigel (BD Biosciences, Singapore, cat: 354234) at 1:1 ratio in a 20 µl total volume. Then the cell/Matrilgel mix was injected into the mammary fat pads of 13-week female NOD-SCID mice. The primary tumors were removed at day 12 and the lungs were harvested and fixed in the Bouin's solution (Sigma-Aldrich, St Louis, MO, cat: HT10132) 15 days later. The primary tumor volume was calculated with formulation width x width x length x $\pi/6$. The lung nodules were blinded between control group and *RASAL2* over expression group then manually counted. For MB231-LN (MDA-MB-231-Luc-D3H2LN) orthotopic xenograft models to evaluate the in vivo effects *RASAL2* knock down on spontaneous lung metastasis, approximately $1x10^6$ cells mixed with 1:1 Matrigel were injected into the mammary fat pads of 4-6 week-old female athymic NOD/SCID mice. For late stage lung colonization models, approximately $2x10^6$ cells of MB231-LN control and sh*RASAL2* cells were injected into NOD/SCID mice via lateral tail vein intravenous injection. Primary tumour and lung metastasis development were monitored weekly by BLI measurement with IVIS imaging System (Xenogen, Alameda, CA). Comparisons of primary tumour and BLI lung metastasis growth between groups were performed using two tailed student t-test analysis. Animal survival curve was generated using Kaplan-Meier analysis and the statistical parameters were calculated by Log-Rank (mantel-Cox) test using Graphpad Prism software as described previously (3).

RNA isolation and Q-RT-PCR

Snap frozen human breast cancer tissues were disrupted using TissueLyser II (Qiagen). Total RNA including small RNAs from both human breast cancer tissue as well as the cell lines were isolated using miRNeasy Mini Kit (Qiagen, cat. 217004) according manual. FFPE

sample RNAs were extracted using Qiagen All Prep DNA/RNA FFPE Kit (Cat. No. 80234). Agilent Human miRNA Microarray Kit V3 (G4470C) which contains probes for 866 human from the Sanger database v12.0 was utilized in profiling the miRNA expression in cell lines as described previously (4). In brief, 100 ng total RNA was dephosphorylated with calf intestine alkaline phosphatase, denatured with dimethyl sulfoxide and labeled with pCp-Cy3 using T4 RNA ligase. The labeled RNAs were hybridized to Agilent human miRNA microarray slides for 22 hours. After extensive washing, the arrays were scanned with Agilent microarray scanner and the raw intensity data were further analyzed using GeneSpring GX software (Agilent Technologies, Santa Clara, CA). Differentially expressed miRNAs were analyzed by significant analysis of microarray (SAM) protocol. To quantify mature form miRNA expression levels in patient samples the TaqMan MicroRNA assays were utilized following the manufacturer's protocol (Thermo Scientific, Waltham, MA). RNU6B levels were used to normalize the data. To quantify protein coding gene mRNA expression levels the KAPA SYBR® FAST qPCR Kits (KAPA Biosystems, Wilmington, MA) were applied following c-DNA conversion using High Capacity cDNA Archive kits (Applied Biosystems). For normalization purpose 18S level was used as internal control. All Q-RT PCR reactions were analyzed in an ABI 7500HT Fast Real-Time PCR system in 96-well plate format. Q-PCR Primer sequences can be found in Supplemental Table 2.

Plasmids construction

RASAL2 (NM_170692.2), *ARHGAP24* (NM_001025616.2) and *RAC*1(NM_006908.4) c-DNAs were amplified from normal breast tissue controls by PCR and inserted into either GFP based expression vector pBabeMNires (pMN, a gift from LZ Penn, University of Toronto, Canada) or pBabe-PURO vector (addgene) after adding appropriate expression tags –myc or –Flag. For co-expression analysis in HEK293T cells *RASAL2* and *ARHGAP24* c-DNAs were sub-cloned into pcDNA4 vector (Invitrogen). The sh*CDH1* plasmid was obtained

from Addgene (Plasmid 18801: pLKO.1 puro shRNA E-cadherin). *RASAL2* shRNA constructs were generated by inserting two independent short hairpin oligo-nucleotides into pSIREN-RetroQ vector (Clontech). RASAL2 GAP deficient mutant construct (GAP-mut) identical to K417 and K567 of MGC Human RASAL2 Sequence-Verified cDNA (CloneId:40028914) (5) was generated using QuikChange Multi Site-Directed Mutagenesis Kits (Agilent) using the primers computed by manufacture's recommended tool QuikChange Primer Design incorporating desired mutation. RASAL2 GAP deletion mutant (ΔGAP) was constructed by ligating two PCR product spanning RASAL2 sequence omitting the GAP domain in frame. For the 3'UTR repression reporter assays the *RASAL2* 3' UTR fragments surrounding the miR-203 MREs (micro RNA response element) approximately 400 base pair in length including MRE site 1 or 1173 base pair in length including 6 clustered MRE site 2 to site 7 were cloned between *Spe*I and *Sac*I restriction sites of pMIR-REPORT Luciferase vector immediately downstream of firefly luciferase gene (Thermo scientific, Waltham, MA). Site-directed mutagenesis reactions were carried out using QuikChange Multi Site-Directed Mutagenesis Kits (Agilent). All plasmids were verified by sequencing. The sequences of all primers are provided in Supplemental Table 2.

Cell culture, siRNA transfection and stable cell line construction

MDA-MB231, BT-549, MCF-7, T47D, MB468, HCC1806, HCC1937, HS578T, SUM159PT and HEK293T cells were purchased from American Type Culture Collection. BPLE and BPLER cells are generous gift from Dr. Robert Weinberg (Whitehead Institute for Biomedical research, Cambridge, USA). HMLE and derivatives were cultured in MEGM (Lonza, Portsmouth, NH, Cat No. CC-3150). BPLE and BPLER were cultured in WIT-T™ Culture Medium (Stemgent). HCC1806, HCC1937 and 4T1 cell lines were maintained in RMPI1650 medium while all other cancer cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Oligo miRNA

mimics and antagomir inhibitors were purchased from Dharmacon (Lafayette, CO) were transfected into appropriate cells using Lipofectamine RNAimax (Invitrogen) at 10 nM (for mimics) and 100 nM (for antagomirs) final concentrations. All siRNAs were pre-designed by IDT-DNA (Singapore) and were transfected into cells using Lipofectamine RNAimax (Invitrogen, Carlsbad, CA) at 30 nM. Sequences of siRNAs are listed in Supplemental Table 2. Stable cell lines were generated via retroviral infection using Platinum-A Retroviral Packaging Cell Line, a derivative of HEK293T cells with engineered retrovirus packaging proteins (Cell Biolabs, San Diego, CA, cat: RV-102), as has been previously described (6). Briefly, approximately 4 µg of pMN-*RASAL2*, pSIREN-RetroQ-sh*RASAL2* or respective controls were transfected into Plat A cells. Forty-eight hours later the replication defective retrovirus harboring target genes were recovered and added to actively dividing recipient cells at Multiplicity of infection (MOI) of 3 for additional 48 hours. Then FACS sorting was employed to eliminate non-expressing cells for pMN vector based gene expression while 2 µg/ml puromycin was added to growth medium to select cells with target gene expression for pSIREN-RetroQ based gene knockdown cell lines.

Cell viability assay

Cell viability was determined using CellTiter-Glo™ Luminescent Cell Viability Assay kit (Promega, Madison, WI, cat: G7570) as instructed by manufacturer's manual. Briefly equal number of 1000 cells of stable cell lines or transfected cells 48 hours post microRNA mimics, antagomirs or siRNA treatment were plated in triplicates in 96-well plate. The ATPase readings were taken daily up to 7 days and the readings were normalized to the day zero readings as of the ATPase activity 4 hours post cell seeding.

Transwell invasion assay

Transwell invasion assay were performed using 24-well FluoroBlok transwell insert (Falcon, Dallas, TX) with a pore size of 8 µm according to manufacturer suggested protocol. In brief the inserts were pre-coated with growth factor-reduced Matrigel (BD Biosciences, cat: 354230 Falcon) for 6 hrs at 37˚C at the concentration of 600 µg/ml for the mesenchymal like TNBC cell lines MB231-LN or BT-549 and 200 µg/ml for the non-mesenchymal like TNBC cell lines HCC1806 or HCC1937 respectively. Then $5x10^4$ of cells were seeded into each insert in MEM containing 0.25% FBS as serum starvation medium. The insert were subsequently immersed in DMEM medium supplemented with 10% FBS as a chemoattractant in the outer chamber. Invaded cells were fixed after 48 hours of incubation by using 3.7 % formaldehyde and stained with 5 µg/ml propidium iodide (Sigma-Aldrich). The invaded cells were scanned and counted with CELLOMICS ARRAYSCAN® VTI HCS Reader facility (Thermo Scientific) using standard settings.

Wound Healing Migration Assays

Equal number of indicated MB231-LN cells and sublines were seeded on BioCoat™ collagen I coated 6-well tissue culture dishes (BD Biosciences, cat: 354400) and allowed to grow to confluent after 48 hours. Then scratches were made using p200 pipette tips and floated cells were carefully washed away with fresh growth medium. The wound-healing of the scratch regions were monitored and imaged at designated time points (0, 4, 6, and 8 hours respectively).

Three dimensional Matrigel growth assays

The 8-well chamber slides (Falcon, cat: 354656, Falcon) were pre-coated with 7.6 mg/ml growth factor-reduced Matrigel (Falcon, cat: 354230, Falcon) for 30 min at 37˚C. Approximately $5x10^3$ MB231 and BT-549 with indicated treatments were seeded in each well

in DMEM medium containing 10% FBS and 150 µg/ml Matrigel. Medium were replenished every 3 days and cell growth was monitored every 3 days by imaging.

Tumorsphere assay

Active growing MB231-LN and BT-549 cells were treated with 0.05% trypsin for 10 minutes then passed through 0.4 µm cell strainer to achieve single cell suspension. Approximately $1x10^4$ cells were re-suspended in 2 ml of MammoCult medium supplemented with 0.5µg/ml hydrocortisone and 4µg/ml heparin (STEMCELL Technologies, Vancouver, BC, cat: 05621). After washing the cells were seeded in 6-well ultra-low attachment plates (Corning, Corning, NY, cat: CLS3471). Tumor spheres formation was monitored daily up to day 7 until being stained with 4 µg/ml p-Iodonitrotetrazolium Violet (INT) overnight. Imaging and quantification were done using GelCount™ apparatus and associated software (Oxford Optronix, Abingdon, UK).

Flow cytometry

Mammospheres of MB231-LN and derivative cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS) then treated with 0.05% trypsin to obtain single cells. The resulting cell suspension were span down and washed with PBS containing 1% FBS (wash buffer), and resuspended in the wash buffer to the density of c.a. 10^6 cells in 100 µl volume. Combinations of fluorochrome-conjugated monoclonal antibodies against human CD44-FITC (BD Biosciences, cat. #555478) and CD24-APC (BD Biosciences, cat.559942) or the respective isotype controls were added to the cell suspension at concentrations recommended by the manufacturer and incubated at 4°C in the dark for 40 minutes. The resulting labeled cells were washed again in the wash buffer, then fixed in PBS containing 1% paraformaldehyde, and then analyzed on a FACSVantage cell sorter (BD Biosciences).

Immunoblotting analysis, Co-immunoprecipitation (co-IP) and activate GTPase Pull down.

Immunoblotting assays were performed using standard protocol following protein extraction using RIPA buffer as described previously (6). Antibodies used in this paper include RASAL2 (NBP1-82579, Novus Biologicals); ARHGAP24 (ab84046, Abcam); myc-tag (c-Myc (9E10), Roche Diagnostics), FLAG (F3165, Sigma-Aldrich), Anti-Phosphoserine antibody (ab9332, Abcam) and Actin (A5441, Sigma-Aldrich). For Co-immunoprecipitation (Co-IP) assays the whole cell lysates of transfected HEK293T cells or BT-549 cells or derivatives with indicated ectopically expressed genes were extracted using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) then subjected to immunoprecipitation using anti-Flag M2 affinity gel (A2220, Sigma-Aldrich), anti-myc-tag affinity agarose (sc-40 AC, Santa Cruz biotechnology), anti-RASAL2 (sc-67935, Santa Cruz biotechnology) or anti ARHGAP24 (ab76898, Abcam) antibodies. After extensive washing the precipitated proteins were eluted with SDS sample buffer in the presence of 3 mM DTT reducing reagent and separated by SDS-PAGE gel electrophoresis. For detection appropriate antibodies from different species were used. Active GTPase pull down assays were performed using affinity binding kits according to the manufacturer's protocol (RhoA / Rac1 / Cdc42 Activation Assay Combo Biochem Kit, cat: BK030, cytoskeleton; Ras Activation Assay Kit, Cat. # 17-218 – Millipore). In brief, approximately 10 million cells from a 15cm culture dish were washed with cold PBS twice. Then the cells were lysed on plates with 600 µl cell lysis buffer. After clearing by centrifugation at 12000g for 1 minute, the supernatant was snap frozen immediately in liquid nitrogen. For active GTPase pull down assays approximately 400 µg of cell lysate were incubated with GST-tagged bait protein conjugated agarose beads for 1 hour at 4 C^{o} (Pak1 for RAC1-GTP, Rhotekin for RHOA-GTP and p21 for RAS-GTP respectively). Then the beads were washed twice and the precipitated proteins

were eluted with SDS sample buffer in the presence of 3 mM DTT reducing reagent and separated by SDS-PAGE gel electrophoresis. Detection was performed by using appropriate antibodies supplied in the kits.

Luciferase 3'UTR repression assay

HEK293T cells were plated at a density of 5×10^4 cells/well in 24-well plate format. Twenty-four hours later attached cells were co-transfected with 10 ng of pMIR REPORT containing respective MREs (micro RNA response element) and 40 nM of microRNA mimics together with the internal control Renilla luciferase vector pRL null using FuGENE® HD Transfection Reagent (Promega). Twenty-four hours after transfection, the luciferase activities were measured using the Dual Luciferase system (Promega, cat: E1910). The pRL null Renilla luciferase reading was employed as an internal control all through our reporter assays.

Immunofluorescent cell staining assay

MB231-LN cells were seeded at 4 X 10^5 cells / well in 6-well culture plates. Twenty-four hours later the attached cells were transfected with 30 μM microRNA mimics and allowed to grow further for 72 hours. Then the post treatment cells were trypsinized and re-seeded at a density of 1.5 X 10^5 cells / well on 8 mm cover slips in 12-well plates. After additional 48 hours, cover slips with cells grown on were fixed in methanol, and probed with primary E-cadherin (BD biosciences, cat: BD 610182,), or Vim (Santa Cruz biotechnology, cat: SC-6260,) antibodies in 1/100 to 1/1,000 dilution then subsequently florescent labeled secondary antibodies. Cell nuclei were stained with DAPI. After cell staining the cover slips were mounted with FluorSave reagent (CALBIOCHEM). Cells were imaged using Zeiss Meta upright microscope under 63X oil objective.

Supplemental References

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