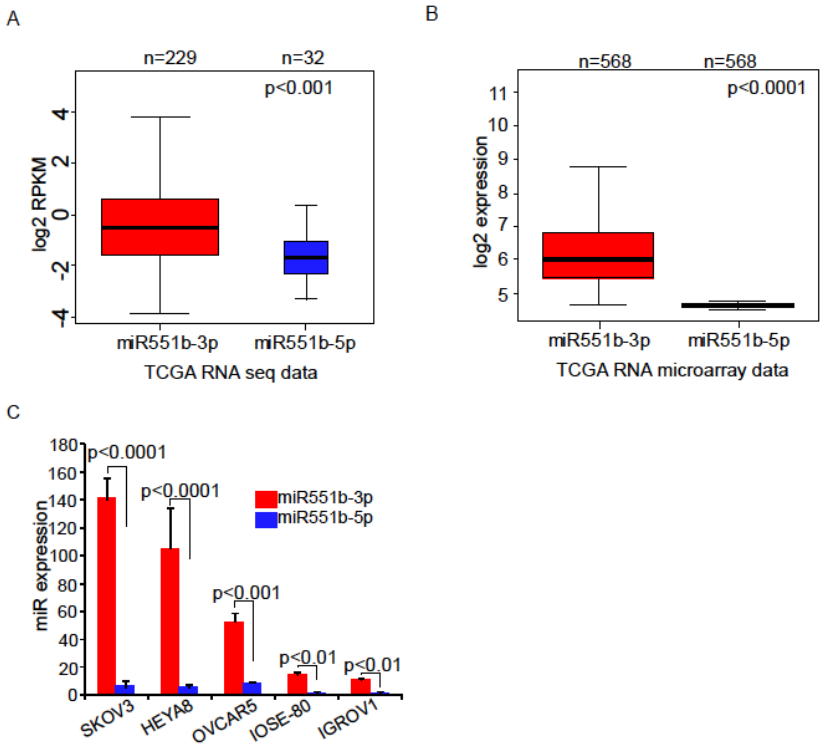
Supplemental Information

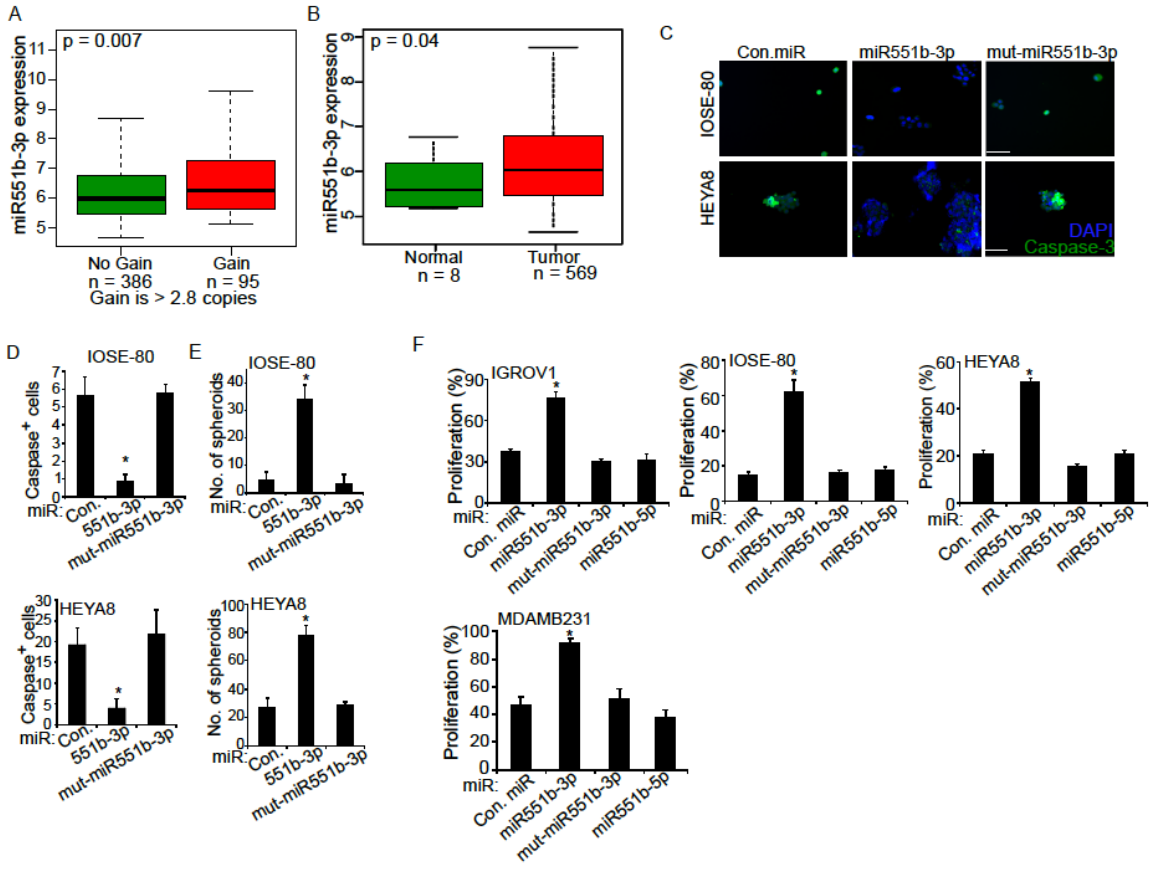
Direct upregulation of STAT3 by microRNA-551b-3p deregulates growth and metastasis of ovarian cancer

Pradeep Chaluvally-Raghavan, Kang Jin Jeong, Sunila Pradeep, Andreia Machado Silva, Shuangxing Yu, Wenbin Liu, Tyler Moss, Cristian Rodriguez-Aguayo, Dong Zhang, Prahlad Ram, Jinsong Liu, Yiling Lu, Gabriel Lopez-Berestein, George Calin, Anil K Sood and Gordon B. Mills

Supplemental Data



Supplemental Figure S1, related to Figure 1. Mature microRNA miR551b-3p is highly expressed in ovarian cancer. A-B, miR551b-3p or miR551b-5p expression was assessed in the HGSEOC TCGA RNASeq and microarray data. **C,** RNA was extracted in triplicate from the indicated cells and qPCR performed to quantify the miRs. Bars indicate s.d. of three independent experiments. p values were determined by Student's t-test.



Supplemental Figure S2, related to Figure 1. miR551b amplification is associated with miR551b-3p expression and provides growth advantage to ovarian cancer cells. A, miR551b-3p expression was assessed in 481 TCGA ovarian cancer samples and plotted based on copy number status. Samples with miR551b copy number more than 2.8 were considered as gain of copy number, greater than 4 was considered amplification. **B,** miR551b-3p expression was assessed in normal vs tumor samples in the TCGA data set. **C,** IOSE-80 or HEYA8 cells were grown on low attachment culture plates and cells transfected with control (Con.) miR, miR551b-3p or mutated miR551b-3p. Spheroids formed were fixed and stained with DAPI or anti-cleaved caspase 3. Scale bar represents 50 μm. **D-E**, Cleaved caspase-3 positive cells were counted from three

different fields from three different experiments as described in (C). * indicates $p<0.001$ compared to control or mutated miR transfected group. Significance was determined by Student's t test. **F,** IOSE80, MDAMB231 or HEYA8 cells were transfected with indicated miRs and cell proliferation assessed on day 0 and day 3 using MTT. Cell proliferation (%) was assessed, by normalizing MTT value of day 3 to day 0. Bars represent standard deviation (s.d.) of three independent experiments. p values were determined by Student's t test. *$p<0.005$ compared to control, mutated or 5p form of miR treatment.

Clinical information of the patients' whose samples included in Tissue Micro Array

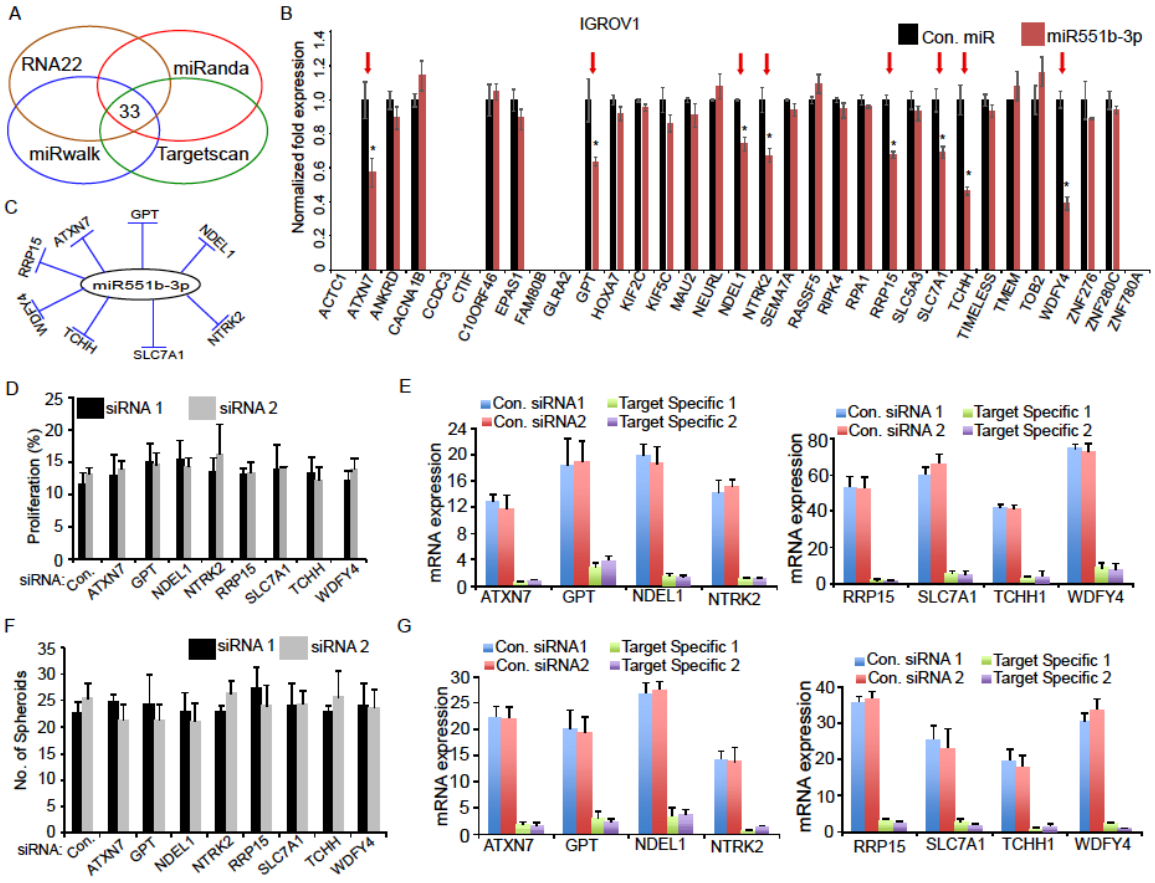
Age	Mean (Range)	58.8 (35-88)
Grade	All high-grade serous	
Stage	I or II	9
	III or IV	136
Cytoreduction	Optimal	35
	Suboptimal	107
	Data NA	3
Preoperative CA125 level	Mean (Range)	2342 (8-49000)

Table S1, related to Figure 1. Summary of clinical information of the patients whose samples were used in the tissue microarray (TMA) for miR551b RNA hybridization and STAT3 immunohistochemistry.

Incidence of ascites formation and miR551b-3p expression in HGSEOC patients

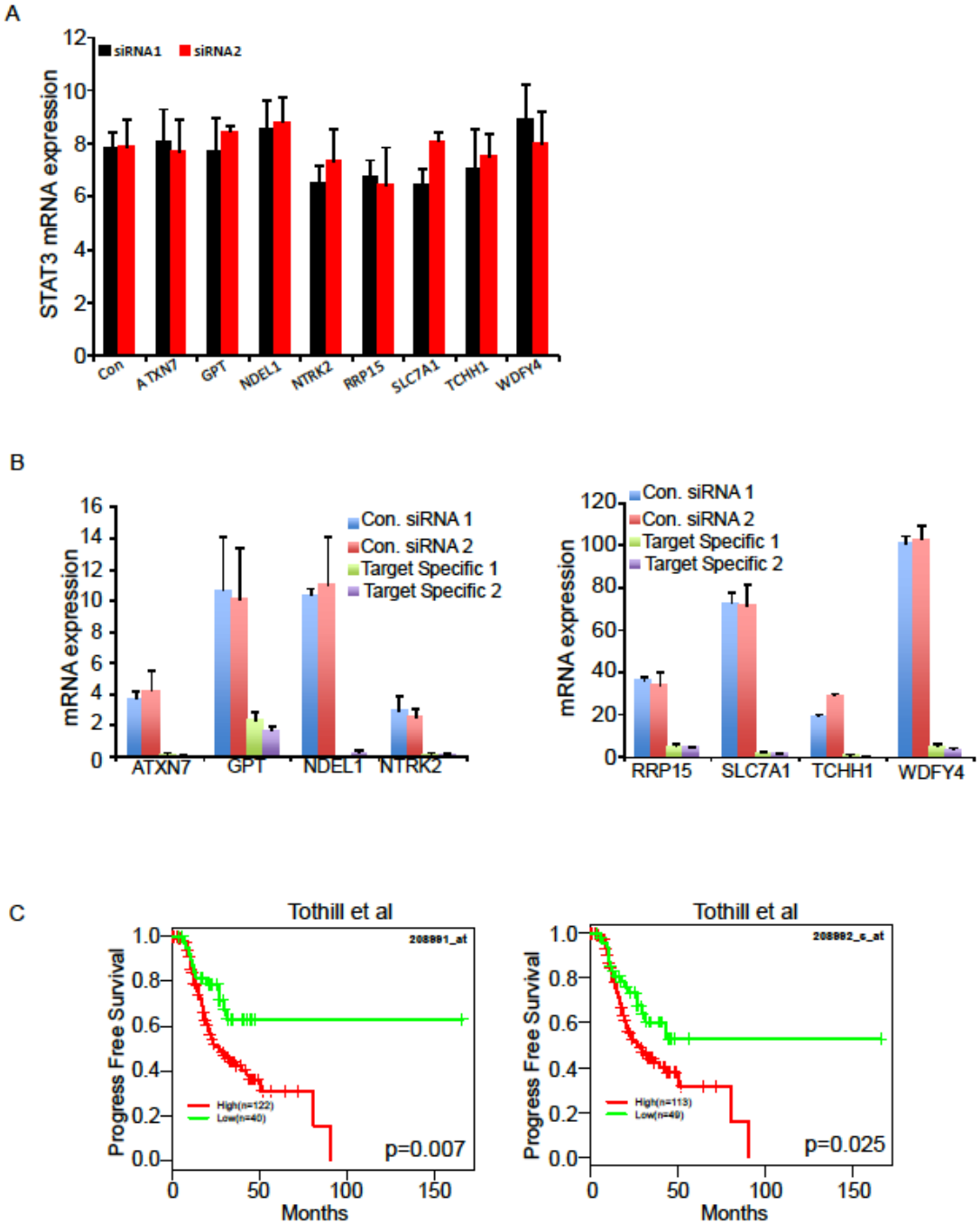
	miR551b-3p expression		
Ascites	1	2	Total
0	35	18	53
1	35	55	90
Total	70	73	143

Table S2, related to Figure 2. microRNA expression in the TMA samples were evaluated and the incidence of ascites formation in the same patients tabulated. Significance was determined by Chi-square test; $p<0.01$



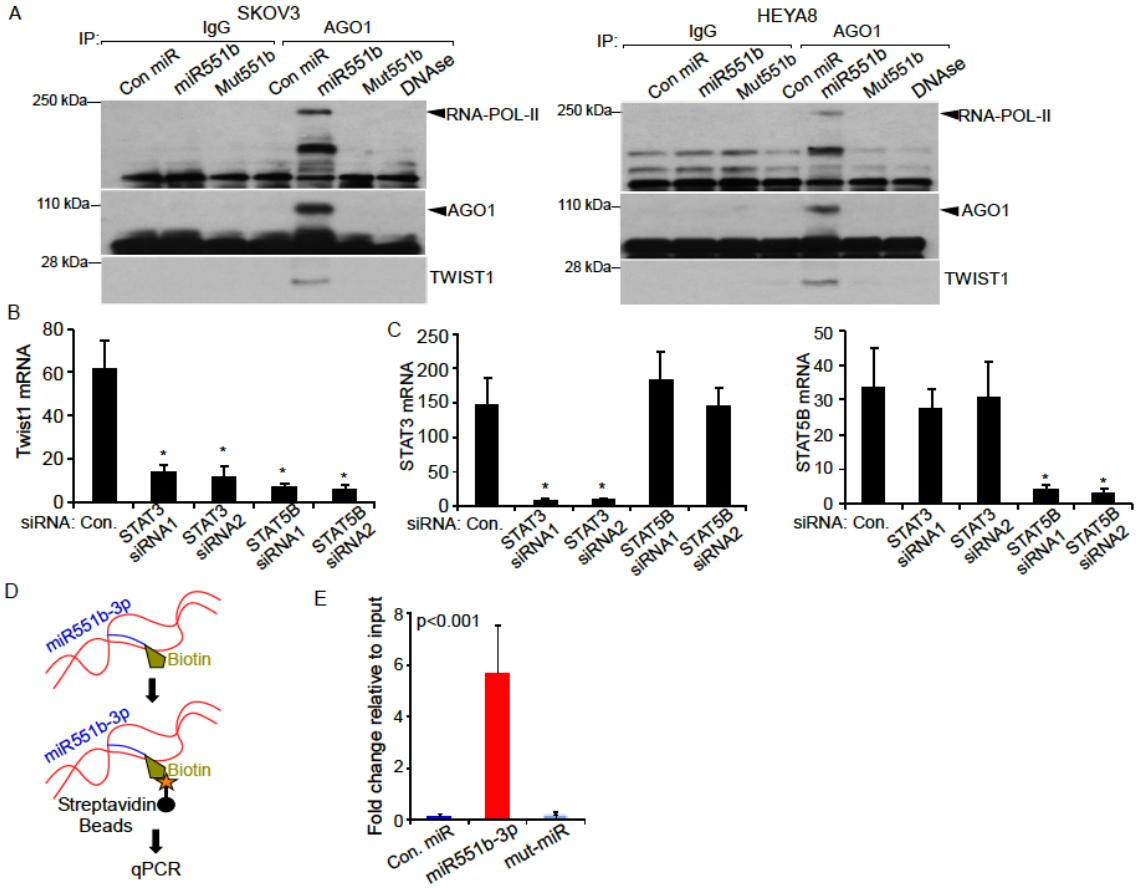
Supplemental Figure S3, related to Figure 3. **A**. Venn diagram presents the number of putative miR551b-3p targets predicted by miRwalk, RNA22, miRanda or TargetScan. **B,** IGROV1 cells were transfected with control (con.) miR or miR551b-3p. RNA was extracted 48h after transfection, cDNA was synthesized and qPCR performed to quantify the mRNAs listed. Bars indicate s.d. of three independent experiments. p values were determined by Student's t test. *indicates p≤0.001 compared to the respective control. **C,** Scheme of the targets downregulated upon miR551b-3p expression described in **(B)** are indicated. **D,** IGROV1 cells were transfected with indicated siRNAs. Cell viability was quantified on day 0 and day 3 by MTT assay. Cell proliferation (%) was assessed, by normalizing MTT value of day 3 to day 0. Bars indicate s.d. of three independent

experiments. **E,** RNA was extracted from (D). Relative expression of the indicated genes was analyzed by qPCR. Samples were normalized to β-Actin. Bars indicate s.d. of three independent experiments. **F,** IGROV1 cells were grown under non-adherent culture conditions, and transfected with the indicated siRNAs. Cells were grown on non-adherent plates for five days after transfection. The number of spheroids formed was quantified. Bars indicate s.d. of three independent experiments. **G,** RNA was extracted from (F). Relative expression of the indicated genes was analyzed by qPCR. Samples were normalized to β-Actin. Bars indicate s.d. of three independent experiments.



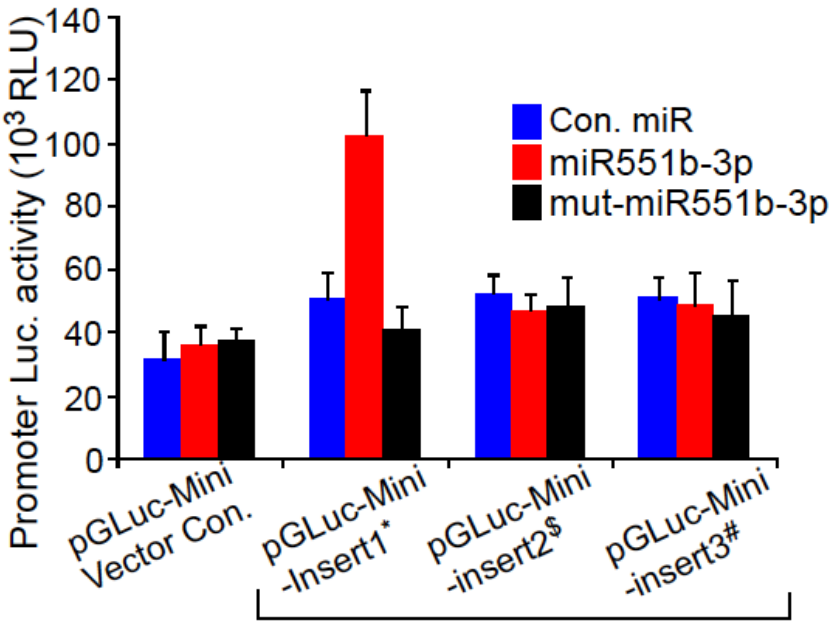
Supplemental Figure S4, related to Figure 4. STAT3 expression predicts the outcome of ovarian cancer patients. A, IGROV1 cells were transfected with the indicated siRNAs. RNA was extracted 48h after transfection. Relative expression of

STAT3 was analyzed by qPCR. Samples were normalized to β-Actin. Bars indicate s.d. of three independent experiments. B, RNA was extracted from (D). Relative expression of the indicated genes was analyzed by qPCR. Samples were normalized to β-Actin. Bars indicate s.d. of three independent experiments. **C,** Kaplan-Meier plot of progression free survival (PFS) of 162 ovarian cancer patient samples stratified by average high or low expression of two different STAT3 probes in Tothill et al data set. Log-rank test was used to compare differences between groups.



Supplemental Figure S5, related to Figure 5. miR551b-3p facilitates the interaction of TWIST1 with AGO1 complex on the STAT3 promoter. A, Biotinylated STAT3 promoter fragments were hybridized with con. miR, miR551b or mutant miR 551b. Promoter-miRNA complexes were incubated with either SKOV3 or HEYA8 cell lysates and immunoprecipitated 72h later with control IgG or anti-AGO1. DNAase was used to degrade the complex where indicated. AGO1 immunoprecipitates were western blotted with the indicated antibodies. **B-C,** IGROV1 cells were transfected with indicated siRNAs. RNA was extracted 48h after transfection. Relative expression of Twist1, STAT3 or STAT5B was analyzed by qPCR. Samples were normalized to β-Actin. p values were determined by Student's t test. Bars represent s.d. of three independent

experiments. *indicates p≤0.001 compared to the respective control. **D-E,** Scheme of assay used to pull down biotinylated con. miR, miR551b-3p or mutant miR551b-3p hybridized with sonicated IGROV1 DNA fragments. qPCR analysis of the STAT3 promoter normalized to input from the assay described in (D). Bars represent s.d. of three independent hybridization assays. Significance was determined by Student's t test.



*172 bases of STAT3 promoter includes miR551b-3p binding sequences

$mutation on the miR551b-3p binding sequences in insert1

#STAT3 promoter without Twist1 binding sequences

Supplemental Figure S6, related to Figure 5. **miR551b-3p interacts with the complement sequence on STAT3 promoter**. 172bp fragment of STAT3 promoter (-591bp to -419bp upstream to TSS) containing the miR551b complementary site and Twist1 binding site cloned into the minimal promoter in pGLuc-Mini-TK reporter vector were co-transfected with control miR or miR551b-3p or mutated miR551b-3p into IGROV1 cells and luciferase activity assessed 48h after transfection. Bars represent s.d. of triplicates. Insert 2 and insert 3 are mutated fragments of 172bp fragment of STAT3 cloned in pGLuc-Mini-TK vector. $ indicates the mutation on the miR551b binding sequence marked as Mut2 in Fig4C. # indicates the 172bp fragment of STAT3 promoter (-591bp to -419bp upstream to transcription start site (TSS)) without Twist1 binding seq (-591bp to -464bp upstream to TSS).

Supplemental Experimental Procedures

Cell viability assay

Cell viability (survival) was determined by a 2-hr incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), followed by lysis in acidic isopropanol (0.35% HCl in isopropanol) and measurement of absorbance at 570 nm. Cell viability was calculated as increase in MTT signal compared to MTT signal on Day 0 prior to treatment.

Oligonucleotide Transfection

The miRIDIAN microRNA mimics and siRNAs are from Dharmacon (Lafayette, CO) and antimiR from Ambion were transfected using Lipofectamine RNAi Max (Invitrogen).

List of siRNA used

Gene Symbol	Sequence#1	Sequence#2
STAT3	GAGAUUGACCAGCAGUAUA	CAACAUGUCAUUUGCUGAA
TWIST1	UGAGCAACAGCGAGGAAGA	GGAGUCCGCAGUCUUACGA
STAT5B	GAAUUUACCAGGACGGAAU	GGACACAGAGAAUGAGUUA
ATXN7	AAAGAAACCUGAAGACAAU	CGAAGGCGAUGACAAAGA
GPT	GAAGAAGCCUUUCACCGAG	GAACAUGGACGCUGCAGUG
NDEL1	GAAGCUAGAGCAUCAAUAU	GCUAGGAUAUCAGCACUAA
NTRK2	GAACAGAAGUAAUGAAAUC	GUAAUGCUGUUUCUGCUUA
RRP15	GAAAUGAUGUGCAGAGUAA	AAUGGUAACUGGAGCCGUA
SLC7A1	AGACCAAGCUCUCAUUUAA	GAACGUCUAUCUCAUGAUG
TCHH	GUAAUGGGCGUGUCGAUUU	CAAUGGGUCGGUUUGUUUA
WDFY4	CGAAUGAGGAAACGCAUCA	CUCAUGAGAUCCUGCGAAA
Non Specific	UGGUUUACAUGUCGACUAA	UGGUUUACAUGUUGUGUGA

Morphogenesis assay

Morphogenesis assays and anchorage independent growth assays were performed as described previously (Pradeep et al., 2012a; Pradeep et al., 2012b). For 3D culture assays, trypsinized cells were re-suspended in DMEM/F12 medium supplemented with 2% horse serum.

Reverse-phase protein arrays (RPPA)

RPPA analysis was performed as described previously (Hennessy et al., 2010) and detailed at http://www.mdanderson.org/education-and-research/resources-for-professionals/scientific-resources/core-facilities-and-services/functional-proteomics-rppa-core/index.html.

Indirect immunofluorescence

Immunofluorescence analysis of acini was performed as described previously (Pradeep et al., 2012a; Pradeep et al., 2012b). Spheroids were fixed in methanol-acetone and blocked in 10% goat serum. Secondary blocking was performed in buffer containing goat anti-mouse $F(ab')_2$ fragments (20 µg/ml). Primary antibodies were incubated for 15-18 hr at 4°C and secondary antibodies for 1 hr before confocal microscopy.

Western blotting and Immunoprecipitation

Whole-cell lysates for western blotting were extracted with RIPA (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, protease, and phosphatase inhibitor cocktail). Cell lysates (10 µg to 25 µg) were loaded onto SDS-PAGE and transferred to a polyvinylidene fluoride membrane. After blocking with 5% (w/v) milk, membranes were cut into two or three pieces based on the molecular weight of the proteins to be assessed, then incubated overnight at 4°C with primary antibody and 1 hr with appropriate horseradish peroxidase-conjugated secondary antibodies.

Protein expression was depicted with an enhanced chemiluminescence western blot detection kit (Amersham Biosciences, Pittsburgh, PA).

Whole-cell lysates for immunoprecipitation were prepared using lysis buffer containing 0.5% NP-40, 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA pH 8, and protease inhibitors. Nuclear extracts for immunoprecipitation was prepared using Nuclear Complex IP Kit (Active motif, Carlsbad, CA). The lysates were pre-cleared by incubating with 1:1 slurry of protein A/G agarose (Santa Cruz Biotechnology, Dallas, TX) for 1 hr at 4^{o}C, and then immunoprecipitated using RNA-POL-II antibodies (Millipore, Billerica, MA).

Luciferase reporter assays

Cells were transfected with reporter plasmids or control vector using Lipofectamine 2000 (Invitrogen). Twenty-four hr after transfection, cells were washed twice with PBS and then lysed and luciferase activity measured using luciferase assay substrate (Promega) according to manufacturer's protocol using an Opticom II luminometer (MGM Instruments, Hamden, CT).

Animal Studies

Female athymic nude mice were from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). Animals were cared for according to guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the U.S. Public Health Service policy on Human Care and Use of Laboratory Animals. All mouse studies were approved and supervised by the MD Anderson Cancer Center Institutional Animal Care and Use Committee.

All animals used were between 8-12 weeks of age at the time of injection. IGROV1 Cells (1×10^6 cells/animal) or HEYA8 (2.5×10^5 cells/animal) cells were trypsinized, washed and resuspended in Hanks' balanced salt solution (Gibco, Carlsbad, CA) and injected

into mice. Seven days after injection, mice were randomly divided and treated with antimiR or miR551b as indicated. In the therapeutic approach antimiR or miR551b incorporated in neutral nanoliposomes (intraperitoneal [IP] administration) was injected in two groups (n = 10/group): control antimiR/DOPC, anti-miR551b/DOPC, intraperitoneally twice in a week. Treatment continued for 5 weeks, at which point, all mice were sacrificed, necropsied, and tumors harvested. Tumor weight and number and location of tumor nodules were recorded. Tumor tissue was either fixed in formalin for paraffin embedding, frozen in optimal cutting temperature (OCT) media to prepare frozen slides, or snap frozen for lysate preparation. The mice were imaged once weekly for bioluminescence signal using a Xenogen IVIS system.

Liposomal preparation

Anti-miR or miR for in vivo delivery was incorporated into DOPC as previously described (Landen et al., 2005; Pecot et al., 2013). DOPC and miR were mixed in the presence of excess tertiary butanol at a ratio of 1:10 (w/w) miR/DOPC. Tween 20 was added to the mixture in a ratio of 1:19 Tween 20:miR/DOPC. The mixture was vortexed, frozen in an acetone/dry ice bath and lyophilized. Before in vivo administration, the preparation was hydrated with PBS at room temperature at a concentration of 200 μg/kg per injection.

Immunohistochemistry

Tissue specimens were analyzed as previously described (Sood et al., 2004). Briefly, STAT3 expression was determined by assessing semi-quantitatively the percentage of stained tumor cells and the staining intensity. The percentage of positive cells was rated as follows: 0 points, no staining; 1 points, 0 to 25%; 2 points, 26 to 50%; 3 points, 51-75%; 4 points, 75-100%. The staining intensity was rated as follows: 0 points, none 1 points, weak; 2 points, moderate; 3 points, strong. Points for expression and percentage

of positive cells were added and an overall score (OS) was assigned. Tumors were categorized into four groups: negative (OS = 1), 1 to 2 points; weak expression (OS = 2), 3 to 4 points; moderate expression (OS = 3), 5 to 6 points; strong expression (OS = 4), 7 and above.

Proliferation was assessed by immunohistochemistry using antibodies against Ki67 (Abcam, Cambridge, MA) and angiogenesis assessed using antibodies against CD31 (PharMingen, San Diego, CA). Paraffin-embedded sections were deparaffinized, heated in a microwave in 0.01 M sodium citrate buffer for antigen retrieval, treated with 3% H_2O_2 for 10 min, and rinsed in H_2O and PBS. Sections were blocked in 5% goat serum in PBS followed by incubation with primary antibodies. Goat anti-rabbit horseradish peroxidase–conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was used to amplify anti-CD31 and anti-Ki67 signals (1h, room temperature). Signal was visualized after incubating with 3,3′-diaminobenzidine and counterstaining with Gill's no. 3 hematoxylin (Sigma-Aldrich, St Louis, MO).

RNA Pull-Down Assay

Biotin-labeled RNAs were transcribed in vitro and purified. Cell nuclear lysates were harvested, sonicated and resuspended in freshly prepared polysome lysis buffer. Biotinylated microRNA (10 pmol) was mixed with 200 µg of nuclear lysate and incubated at 75^{o}C for 2h then mixed with prewashed streptavidin-agarose beads for 1 h at room temperature. DNA was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA).

Biotin End Labeling

microRNA was biotinylated using Thermo Scientific Pierce RNA 3' End labeling kit (Waltham, MA USA). 50 pmol of RNA was biotinylated using T4 RNA ligase for 30 min

reaction at 37°C. DNA fragment was biotinylated using 3' End labeling kit from PureBiotech (Middlesex, NJ, USA). 40 pmol of DNA was biotinylated Terminal deoxynucleotidyl transferase (TdT) enzyme for 30 min reaction at 37°C.

Supplemental References

Hennessy, B.T., Lu, Y., Gonzalez-Angulo, A.M., Carey, M.S., Myhre, S., Ju, Z., Davies, M.A., Liu, W., Coombes, K., Meric-Bernstam, F*., et al.* (2010). A Technical Assessment of the Utility of Reverse Phase Protein Arrays for the Study of the Functional Proteome in Non-microdissected Human Breast Cancers. Clinical proteomics *6*, 129-151.

Landen, C.N., Jr., Chavez-Reyes, A., Bucana, C., Schmandt, R., Deavers, M.T., Lopez-Berestein, G., and Sood, A.K. (2005). Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery. Cancer Res *65*, 6910-6918.

Pecot, C.V., Rupaimoole, R., Yang, D., Akbani, R., Ivan, C., Lu, C., Wu, S., Han, H.D., Shah, M.Y., Rodriguez-Aguayo, C*., et al.* (2013). Tumour angiogenesis regulation by the miR-200 family. Nature communications *4*, 2427.

Pradeep, C.R., Kostler, W.J., Lauriola, M., Granit, R.Z., Zhang, F., Jacob-Hirsch, J., Rechavi, G., Nair, H.B., Hennessy, B.T., Gonzalez-Angulo, A.M*., et al.* (2012a). Modeling ductal carcinoma in situ: a HER2-Notch3 collaboration enables luminal filling. Oncogene *31*, 907-917.

Pradeep, C.R., Zeisel, A., Kostler, W.J., Lauriola, M., Jacob-Hirsch, J., Haibe-Kains, B., Amariglio, N., Ben-Chetrit, N., Emde, A., Solomonov, I*., et al.* (2012b). Modeling invasive breast cancer: growth factors propel progression of HER2-positive premalignant lesions. Oncogene *31*, 3569-3583.

Sood, A.K., Coffin, J.E., Schneider, G.B., Fletcher, M.S., DeYoung, B.R., Gruman, L.M., Gershenson, D.M., Schaller, M.D., and Hendrix, M.J. (2004). Biological significance of focal adhesion kinase in ovarian cancer: role in migration and invasion. Am J Pathol *165*, 1087-1095.