Supplementary Information

Targeting Notch signalling by the conserved miR-8/200 microRNA family in development and cancer cells

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Supplementary Materials and Methods

GS-element Mapping

Genomic DNA flanking the *P*-element insertions in the *GS(2)SC1* stock were recovered by inverse PCR (http://www.fruitfly.org/about/methods) and sequenced. A BLAST search with the sequence produced perfect matches to the genomic region in 53D11 (2R chromosome), with an insertion point at 12,717,824 chromosome 2R.

Reverse Transcription (RT-PCR)

To assess the levels of the primary *mir-8* precursor (*pri-mir-8*) when the *GS(2)SC1* EP-element was activated by Gal4, we performed RT-PCR experiments using RNA isolated from third instar larvae of the *hsp70-Gal4* genotype crossed with *GS(2)SC1* in the absence of heat shock (—), immediately after heat-shock, after 1 hour, and 1 hour after heat-shock followed by 2 hours at 25ºC. The RNA was extracted with Trizol (Gibco BRL), cDNAs were synthesized using DuraScript RT-PCR kit (Sigma), and the *pri-mir-8* sequence was amplified using specific primers. The *pri-mir-8* levels were normalized against *RP-49* mRNA: *pri-mir-8* forward 5´-aaggggccaatgttctaag-3´, reverse primer: 5´-ccgcttgtcttcgcattatc-3´. *RP-*

49 forward primer: 5´-catccgcccagcatacag-3´, reverse primer: 5´-accgttggggttggtgag-3´.

Transgenic Constructs

The *UAS-mir-8* construct was generated cloning a DNA fragment of 564 bp flanking the miR-8 precursor into pUASt plasmid. The *tub-eGFP:Ser-UTR* and *tub-eGFP:Ser-UTR-mut* constructs were generated by cloning the full length 3' UTR of the *Drosophila* Ser gene, or a mutated sequence, into the 3' end of the *tub-eGFP* reporter vector (a gift from S.M. Cohen). In the *tub-eGFP:Ser-UTR-mut* construct, the two miR-8 target sites were mutated as described in Figure 3A.

Cuticle Preparations

Embryos were collected in agar plates for 3-4 hours at 25ºC and incubated for 16-18 hours at 25ºC. Unhatched embryos were removed, dechorionated using 50% bleach for 2 minutes and rinsed thoroughly with water. Embryos were devitellinized in a heptane:methanol:EGTA 0.5 M (1:1:0.2) solution and rinsed 3 times with 100% methanol. Rehydrated embryos were transferred into Hoyer's solution and incubated overnight at 65ºC on a microscope slide. The cuticles were then examined under microscope.

Quantitative PCR (qPCR)

To assess the levels of mature microRNA, specific primer sets were obtained from Applied Biosystems. Products were amplified from 10 ng of total RNA (extracted from cultured cells using miRNeasy-Mini Kit, Quiagen) with the "TaqMan MicroRNA Assay". Mature miRNA levels were normalized to U6B snRNA. For mRNA levels, total RNA was extracted from cultured cells or *Drosophila* tissue using RNAeasy-Mini Kit (Qiagen), and cDNA was synthesized using an oligo-dT primer and SuperScript RT-III (Invitrogen). *GAPDH*, *β-actin*, *HPRT1* and *PBGD* primers were used for mRNA normalization of human mRNA, whereas *dGAPDH*, *dVih* and *dUba2* primers were used to normalize *Drosophila* mRNA. Quantitative real time PCR (qPCR) for mRNA was performed using the ABI SYBR green system using gene-specific primers designed with *Universal Probe Library Assay Design Center* (ROCHE: see https://qpcr1.probefinder.com).

The qPCRs for microRNAs and mRNA were performed in the ABI7500 apparatus and analyzed using Applied Biosystem software. Comparative qPCRs were performed in triplicate and the relative expression was calculated using the comparative C_t method.

Primer sequences:

Drosophila		
Serrate	forward	5´-gggggaatttgccttgac-3´
	reverse	5´-tccgagcagcgtttacct-3´
Gapdh2	forward	5´-aagctgatctcttggtacgacaac-3´
	reverse	5´-gtagtgttcttggtgttccacatt-3´
vih	forward	5´-ctgtttggacatactgaaggacaa-3´
	reverse	5´-gggattgtatggacagcagaa-3´
uba2	forward	5´-ttcggttcatcctccttgtctt-3´
	reverse	5´-agtcgtagcagatgcctcacag-3´
Human		
JAGGED1	forward	5´-gaatggcaacaaaacttgcat-3´
	reverse	5´-agccttgtcggcaaatagc-3´
HES1	forward	5´-agtgaagcacctccggaac-3´
	reverse	5´-cgttcatgcactcgctga-3´
E-cadherin	forward	5´-tcatgagtgtcccccggtat-3´
	reverse	5´-cagccgctttcagattttcat-3´
Vimentin	forward	5´-aaagtgtggctgccaagaac-3´
	reverse	5´-agcctcagagaggtcagcaa-3´
ZEB1	forward	5´-aactgctgggaggatgacac-3´
	reverse	5´-tcctgcttcatctgcctga-3´
ZEB2	forward	5´-aactgctgggaggatgacac -3´
	reverse	5´-tcctgcttcatctgcctga -3´
GAPDH	forward	5´-agccacatcgctcagacac-3´
	reverse	5´-gcccaatacgaccaaatcc-3´
ß-actin	forward	5´-ccaaccgcgagaagatga-3´
	reverse	5´-ccagaggcgtacagggatag-3´
HPRT-1	forward	5´-tgaccttgatttattttgcatacc-3´
	reverse	5´-cgagcaagacgttcagtcct-3´
PBGD	forward	5´-cgcatctggagttcaggagta-3´
	reverse	5´-ccaggatgatggcactga-3´

Cell Culture and Transfection

Drosophila S2 cells were maintained in Express-Five serum free medium (Invitrogen) at 25ºC. Human prostate cancer cells (PC-3) and human prostate non-transformed epithelial cells (PNT1A: obtained from ATCC) were cultured in RPMI-1640 + Glutamax medium (Gibco) supplemented with 10% FBS. Human colon cancer cells Ls174T, HCT-116, SW-480 and Caco-2 (obtained from A. Bigas) were cultured in DMEM (Sigma) supplemented with 10% FBS. All human cells were incubated at 37ºC in 5% CO_2.

For generation of cells stably expressing *mir-141*, PC-3 cells were transfected with *mir-141*-GFP or empty-GFP vector (Paddison et al, 2004), and then sorted for GFP expression in order to establish a stable cell line. To generate cells stably expressing *mir-200c+mir-141*, PC-3 cells were co-transfected with *mir-141*-mirVec and *mir-200c*-mirVec vectors, or transfected with empty-mirVec vector (miR-Lib, Geneservice). Subsequently, 48 hours after transfection, individual cell clones were harvested and selected by adding 10 μg/mL blasticidine (Fluka). The miR-200c+miR141 clones were identified by qPCR and amplified for further experiments, and experiments were performed on two independent clones. Overexpression of *mir-200c* alone or with the *mir-200c*-mirVec vector in PC-3 cells caused complete growth arrest and the culture collapsed after addition of the antibiotic at 48 hr. Although individual *mir-200c* expression could not produce stable clones, co-expression of *mir-200c* together with *mir-141* expressing vectors at concentrations identical to the individual *mir-200c* vector recovered their viability.

For miRNA knockdown experiments, 30 nM locked-nucleic-acid (LNA)-modified anti-miR-141, anti-miR-200c or Scramble oligonucleotides (Exiqon) were transfected with Lipofectamine 2000 reagent (Invitrogen) into PNT1A cells at a density of $2.5x10^5$ cells per well in 6-well plates. For experiments using PC-3 cells (including Luciferase assay), 30 nM locked-nucleic-acid (LNA)-modified anti-miR-200a, anti-miR-200b, anti-miR-429 or Scramble oligonucleotides (Exiqon) were transfected. Transfections were repeated after a 24-hour incubation and the cells were incubated for a total of 72 hours after first transfection, and then harvested for experiments.

For siRNA JAG1 experiments, 100 nM of siRNA Smart-pool for JAG1 and siRNA Smart-pool Control#2 (Dharmacon) were transfected with Lipofectamine2000 reagent (Invitrogen) into PC-3 cells at a density of $2.5x10^5$ cells per well in 6-well plates. Cells were incubated 72 hours and then collected for experiments.

Western Blotting

Cells were harvested and lysed in RIPA buffer, incubated for 15 minutes on ice and the protein concentration was hen determined using the Bradford assay (BioRad, CA). Subsequently, 30 μg of protein from the cell lysates was separated by SDS-PAGE in 8% gels and transferred onto an Immobilon

polyvinylidene difluoride membrane (PVDF: Millipore, Bedfrod MA). The membrane was probed with the primary JAG1 antibody (1:200: Santa Cruz, CA) and an anti-actin antibody as a loading control (1:200: Sigma), both diluted in 3% BSA in PBS 0.1%-Tween-20. An HRP-conjugated anti rabbit was used as secondary antibody and chemiluminescent detection of proteins was performed with ECL system (Pierce, Rockford, IL).

DAPT Treatment

PC-3 cells ($1x10^5$ cells per well) were plated 12 hours prior treatment in 6-well plates and DAPT dissolved in DMSO was added at a final concentration of 20 μM. The same volume of DMSO alone was added to the control cells. The cells were then incubated for 72 hours before harvesting for analysis.

Mitotic index

PC-3 stably transfected with empty vectors or vectors expressing the miRNAs or PC-3 cells treated with *JAG1* siRNAs or scramble siRNAs were plated on glass coverslips (Becton Dickinson) to carry out mitotic marker assays. The cells were fixed with 4% paraformaldehyde for 20 minutes at 4ºC and permeabilized with blocking solution (0.1% Triton X-100 and 2.5% fetal bovine serum (FBS) in PBS. The phospho-histone H3 (pH3) protein was visualized using a 1:200 dilution of the phospho-H3 antibody (Sigma) and with a 1:200 dilution of a Cy3-conjugated anti-rabbit antiserum (ICN-Cappel), before the cells were mounted in Vectashield containing 1.5 mg/ml DAPI. A minimum of 500 PC-3 cells was counted per sample from four independent treatments, presented as a percentage of the ratio between proliferating cells (pH3-positive cells) and the total cell number.

Cell proliferation assays

For rescue experiments of the anti-proliferative effect of the miR-200c we used a pcDNA-JAG1 open reading frame lacking the entire 3'UTR of endogenous JAG1 except the first 72 nucleotides after the stop codon (pcDNA3-JAG1-3'UTR(—). The pcDNA-JAG1-3'UTR(+) was constructed by introducing the full 3'UTR sequence (nucleotide positions 4089-5890). Cell proliferation of PC-3 cells transiently transfected with mirvec-empty vector, mirvec-mir-141 and mirvec-mir-200c, pcDNA3.1-empty vector or pcDNA3-JAG1-3'UTR(—)and pcDNA3-JAG1-3'UTR(+)was determined by colorimetric assays that detect viable

cells exclusively based on the cleavage of the water soluble tetrazolium salt (WST-1) by mitochondrial dehydrogenases (Roche). Control and transiently transfected PC-3 cells were plated in 96-well plates at 5 $x10^3$ cells per well in 100 μl of medium for WST-1 proliferation assays. Each condition was plated in quatriplicate. The remaining of the transient transfection cells were plated in a 6-well dish, and harvested 48 h after the transfection to determinate JAG1 protein and microRNA expression. Cell counts were estimated at time points 4, 24, 48 and 72 h after transfection by adding 10 μL of the WST-1 reagent to each well and incubating for 0.5 h in a humidified incubator at 37 °C with 5% CO_2 atmosphere. The absorbance of the formazan dye formed, which correlates with the number of metabolically active cells in the culture, was measured at 415 nm (reference at 595 nm) using a Benchmark Microplate Reader (Bio-Rad).

Histology

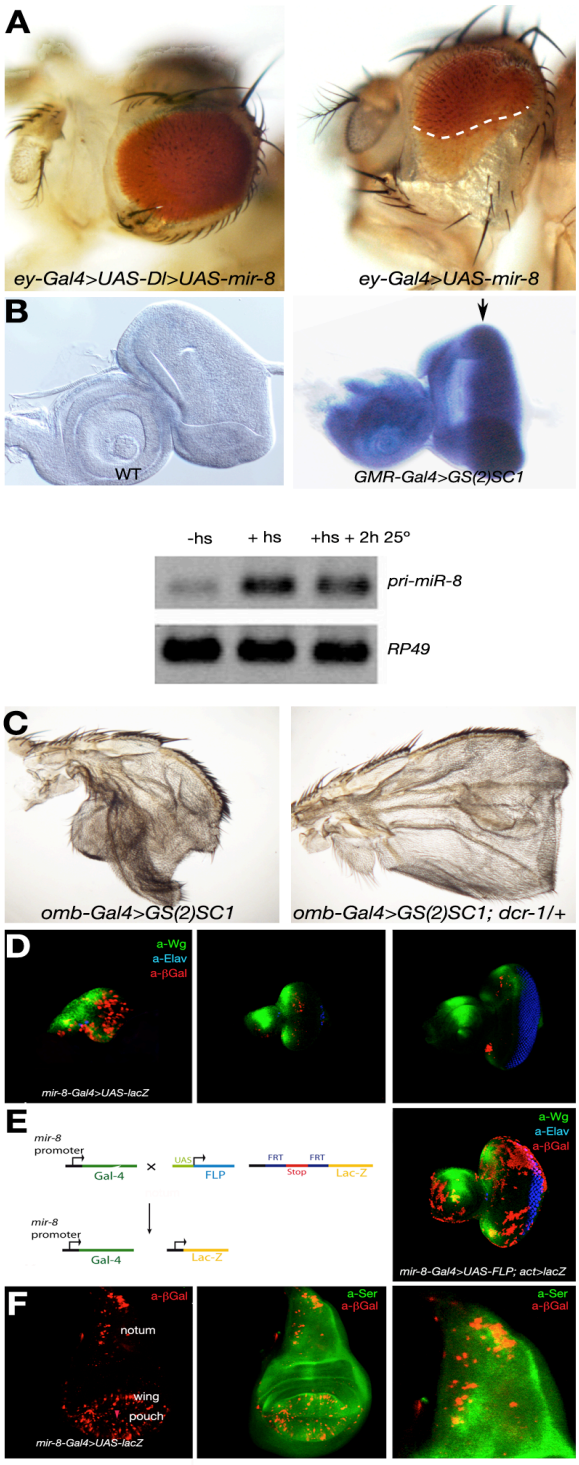
Histological sections of the flies were obtained and studied as described previously (Ferres-Marco et al, 2006).

Image Capture and Analysis

Drosophila images were obtained with a ZEISS Axiophot light microscope using the CoolSnap software. For 3D images, Automontage Essentials software was used and the images were analyzed using ImageJ software (area calculation, density profiles and automated count of cells: http://rsb.info.nih.gov/ij/, 1997-2005).

Supplementary Figures

Figure S1. Overexpression of *mir-8* is responsible for *GS(S)SC1* phenotypes and *mir-8* and *Serrate* show dynamic overlapping expression patterns



(**A**) Overexpression of UAS-*mir-8* by Gal4 produced suppression of *Delta*-induced eye overgrowth (left panel) and a marked reduction in ventral eye size (right panel), similar to that in *GS(2)SC1* flies.

(**B**) LNA-probe *in situ* hybridization reveals *de novo* overexpression of *mir-8* in the cells behind the morphogenetic furrow in animals that overexpress *GS(2)SC1* under the control of *GMR-Gal4*. RT-PCR analysis of pri-miRNA abundance in larvae of the *hsp70-Gal4* genotype crossed with *GS(2)SC1* in the absence of heat shock (—),1 hour after heat-shock (+), or 1 hour after heat-shock followed by two hours at 25ºC, also confirmed that pri-*mir-8* RNA was only elevated in the *Gal4*-induced (+) larvae.

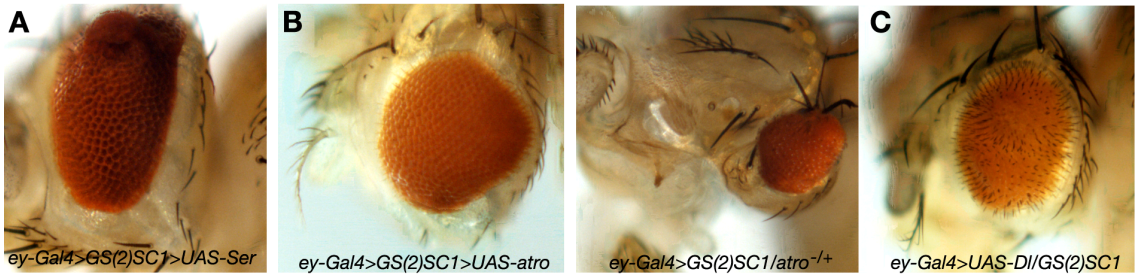
(**C**) *GS(2)SC1* driven by *omb-Gal4* led to smaller wings with nicks at the wing tips that are partially suppressed when a mutant copy of *dcr-1* is introduced (*dcr-1*^{Q1147X}/+).

(**D**) Dynamic expression of microRNA miR-8 revealed by mir-8-Gal4 in late second (left panel), early third (central panel) and mature third instar eye discs (right panel).

(**E**) MicroRNA *mir-8* gene expression assessed in the eye disc with a GAL4/FRT/Flp-on lacZ 'memory' expression assay permanently labels cells descending from those expressing *mir-8* at any early developmental point. This 'memory' expression assay reveals the roughly complementary expression of *mir-8* and late Ser expression along the D/V boundary in the eye disc.

(**F**) Double staining of Ser protein (green) and *mir-8* (*mir-8-Gal4>UAS-lacZ*; red) also highlights the partially complementary pattern of the miRNA and its potential target in the wing disc. Right panel: miR-8 (red) and Ser (green) in partially overlapping patterns.

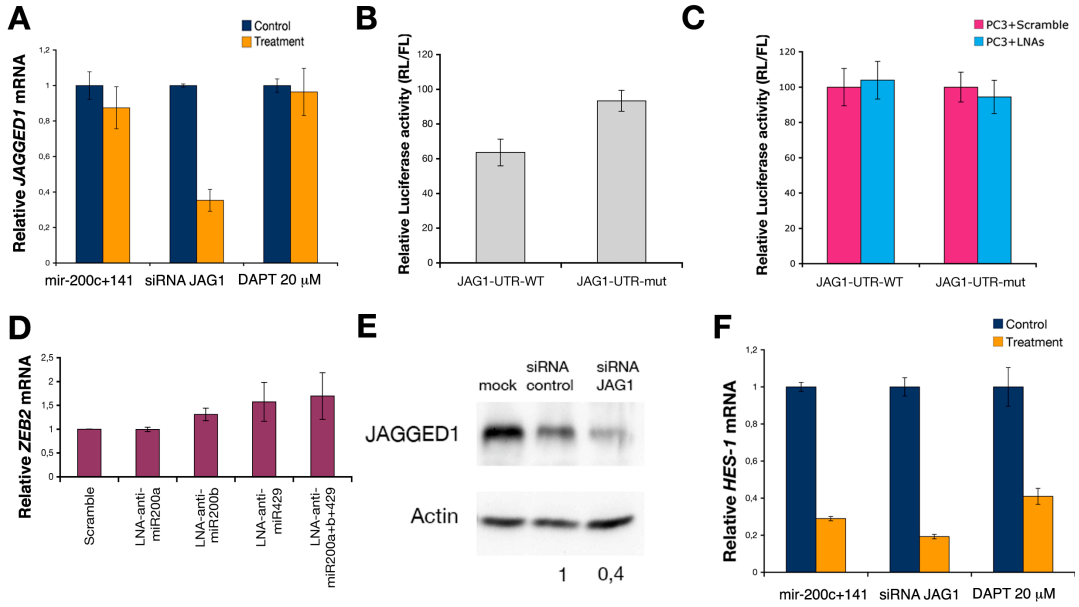
Figure S2. Images of the rescue by Serrate and the failure of Atrophin mutants to modify the inhibition of eye growth by *mir-8* overexpression from representative animals



(**A**) Representative female eye demonstrating the rescue by Serrate gain of function when *mir-8* overexpression is driven by ey-Gal4.

(**B**) Gain (*UAS-atro*, left panel) or loss ($atro^{35/+}$, right panel) of *atrophin* did not modify the growth defects of *mir-8* overexpression when driven by ey-Gal4. Both *Serrate* and *atrophin* mutations enhanced the lethality of *mir-8* overexpression (100% adult lethality in males). The effect is specific of Notch ligand Serrate. *Delta* (*Dl*) overexpression driven by *ey-Gal4* induces eye overgrowth (130% of the WT eye size) and (**C**) *Dl* overexpression with *GS(2)SC1* led to flies with eyes very reduced (0%-48% of the WT eye size, n= 61), reminiscent to the flies expressing *GS(2)SC1* alone.

Figure S3. Specific silencing of JAGGED1 by miR-200c~141



(**A**) *JAG1* mRNA expression in PC-3 cells stably expressing *mir-200~141* cells or transiently transfected with *JAG1* siRNAs, or in the presence of the γ-secretase inhibitor, DAPT (20μM), and their corresponding controls. *JAG1* mRNA levels did not alter significantly in PC-3 cells stably expressing *mir-200c/141*. However, the levels of *JAG1* mRNA began to decrease after four cell passages.

(**B**) Luciferase activity of the pRL-TK vectors containing human *JAG1-UTR-WT* or *JAG1-UTR-mut* in PNTA1 cells.

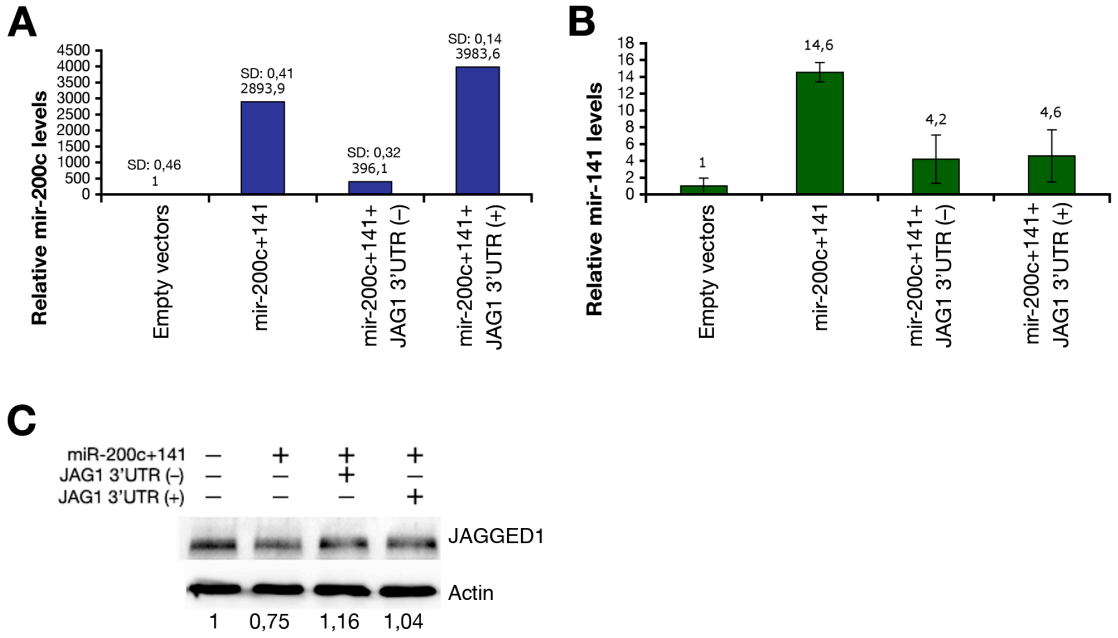
(**C**) Luciferase activity of pRL-TK vectors containing human *JAG1-UTR-WT* or *JAG1-UTR-mut* was unaffected by the presence of LNA anti-sense oligonucleotides against miR-200b, miR-200a and miR429 in PC-3 cells. Error bars represent ± SEM (n=3).

(**D**) *ZEB2* (*SIP1*) mRNA levels determined by qPCR in PC-3 cells treated with LNAs anti-miR-200a, -b, or -429, or the three together, and the control (scramble LNAs). Error bars represent ± SEM (n=3).

(**E**) Immunoblotting of JAG1 protein in PC-3 cells treated with siRNAs against *JAG1* and control scrambled siRNAs. The *JAG1* siRNAs decreased JAG1 protein levels by approximately 60%.

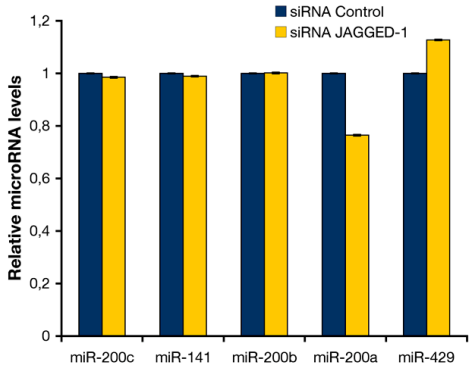
(**F**) *HES1* mRNA levels in PC-3 cells stably expressing *mir-200c* and *mir-141*, which were transiently transfected with *JAG1* siRNAs or treated with the γ-secretase inhibitor, DAPT (20μM), and their corresponding controls.

Figure S4. *mir-200c* and *mir-141* expression levels and JAG1 protein levels in PC-3 transiently transfected with miR-vec and pcDNA3 vectors



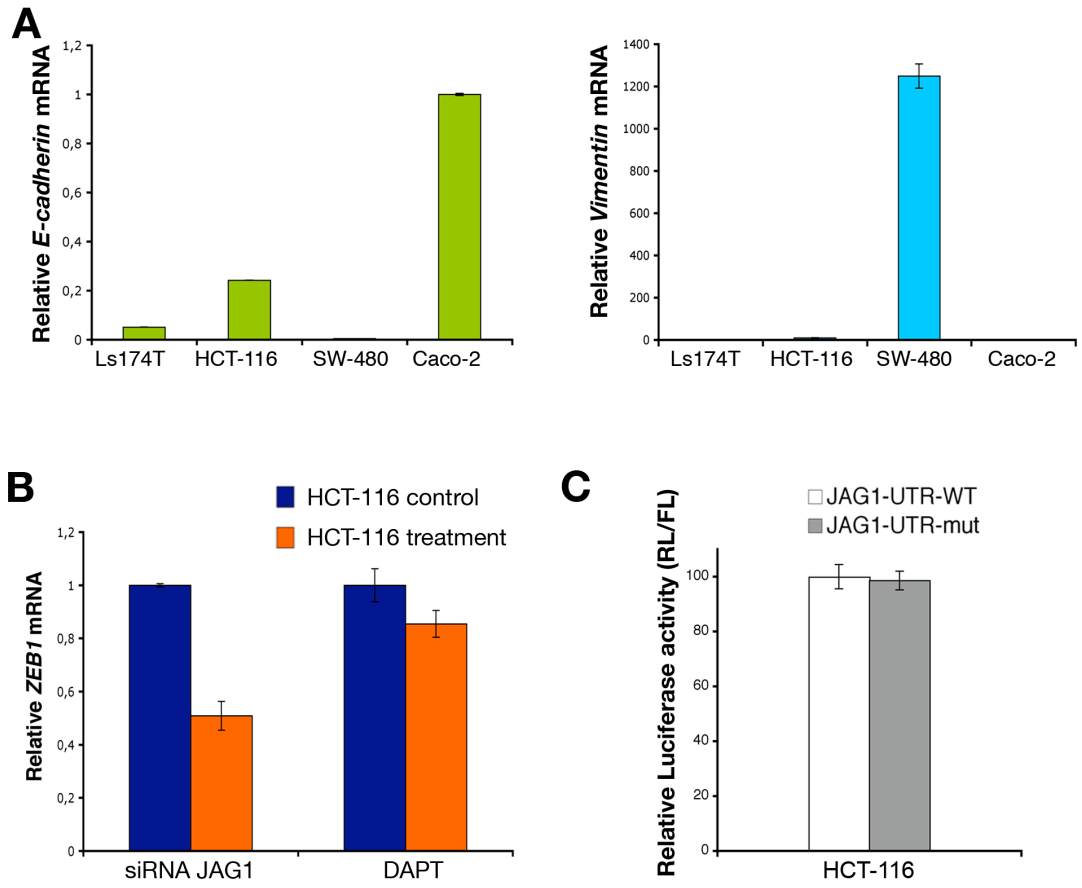
(**A**, **B**) Expression of mature miR-200c (A) and miR-141 (B) in PC-3 cells transduced with the *mir-200c*-mirVec and *mir-141*-mirVec. Measurements of miRNA expression levels were performed by qPCR, 48 hours after transfection. The data represent triplicates from single RNA samples. Levels of miRNAs are normalized to RNU6. Both the transient transfections experiments and the analysis of expression of these microRNAs in the PC-3 stably transfected clones (data not shwon) yielded high expression levels of mature miR-200c and a more modest overexpression of mature miR-141. In (**A**) S.D. (standard deviation) values are shown above the bars. (**C**) Immunoblot of *JAG1* and actin in PC-3 cells transiently transfected with the microRNA precursors from the experiment above. For comparison, the levels of JAG1 in PC-3 cells transfected with empty vectors (—, —, —) are shown. Data are normalized to Actin levels and relative to the levels in PC-3 control (empty vectors).

Figure S5. JAGGED1 knockdown does not change *mir-200c~141* expression



Expression of the two *mir-200c~141* and *mir-200b~429* microRNA paralogues, clusters of the miR-200 family, in PC-3 cells transiently transfected with JAG1 siRNAs (yellow bars) or control siRNA scrambled (blue bars).

Figure S6. JAGGED1-mediated ZEB1 regulation and miR-200c-JAGGED1 interactions in metastatic colon cancer epithelial cells

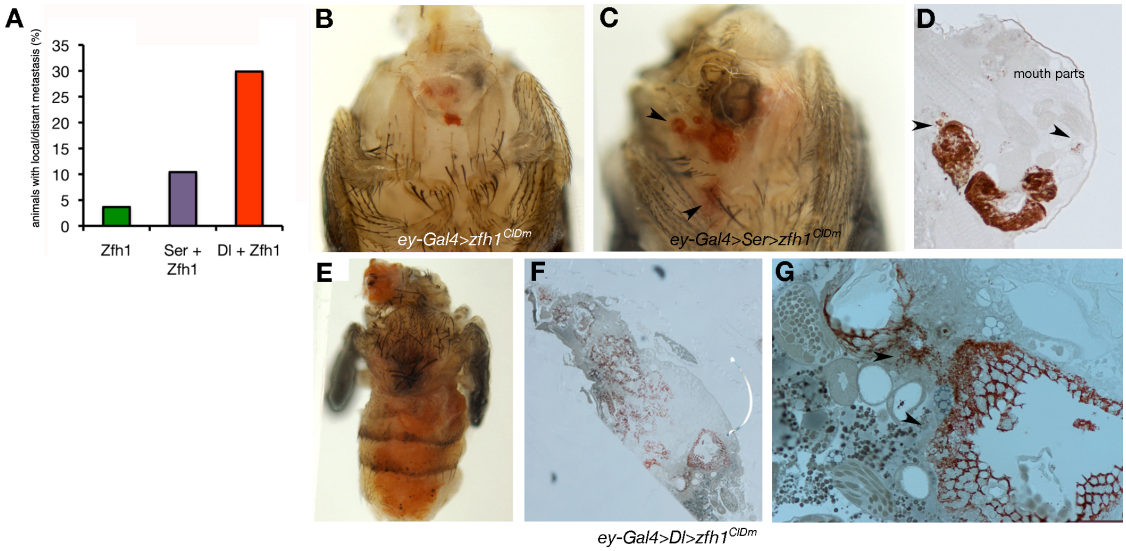


(**A**) Relative levels of expression of human *Vimentin* and *E-cadherin* mRNA in colon cancer cell lines Ls174T, HCT-116, SW-480 and Caco-2 was quantified by qPCR using ß-*actin* and *GAPDH* as normalization controls. The expression of mesenchymal marker *Vimentin* in SW-480 and its absence in HCT-116 is consistent with the mesenchymal and epithelial status of these respective cells and the expression of *mir-200* microRNAs (**Figure 6A**).

(**B**) *ZEB1* mRNA expression in HCT-116 decreased when treated with *JAG1* siRNA or with DAPT (20μM).

(**C**) *JAG1* 3'UTR *luciferase* reporter assay in HCT-116 cells. Luciferase activity in the wild type reporter (JAG1-UTR-wt) is not reduced by the endogenous high expression of *mir-200c* and *mir-141* in the HCT-116 colon cancer cells when compared with activity in the reporter harboring mutations in the three sites (JAG1-UTR-mut). Error bars represent ± SEM (n=3 experiments for each case).

Figure S7. Concurrent overexpression of Zfh1 and Notch ligands induces massive metastasis in *Drosophila*



(**A**) The graph show the quantification of animals with local and/or distant metastases upon overexpression of UAS-zfh1 (fly ZEB1) alone (green bar); UAS-zfh1 and UAS-Ser (violet bar) or UAS-Dl and UAS-zfh1 (red bars).

Human ZEB1 and fly Zfh1 are versatile transcription factors that can function as transcriptional activators or repressors, at large extent by recruiting the co-repressor CtBP. Remarkably, the Zfh1 protein lacking the CtBP interacting domain (when the PLDLS domain between nucleotides 2374 to 2388 is changed to ASASA in the Zfh1^{CIDm}; Postigo et al, 1999) was capable of inducing metastatic-like behavior with *Ser* and *Dl*.

(**B**) Adult pharate overexpressing UAS-*zfh1*^{CIDm} led to local scattered micrometastasis.

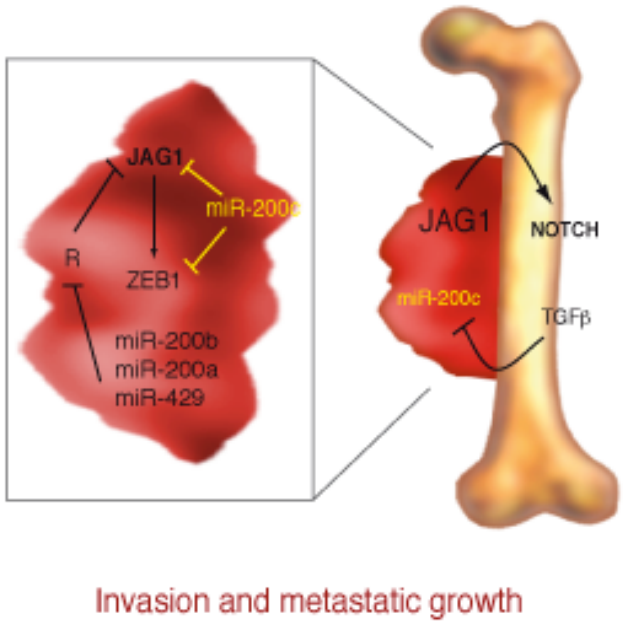
(**C, D**) Co-overexpressing *UAS-zfh1*^{CIDm} with *UAS-Ser* led to distant, larger metastasis in the thorax and (**D**) mouthparts (proboscis). The arrow points to eye-derived (red pigmented cells) metastatic groups of cells.

(**E**) Adult pharate fly co-expressing *UAS-zfh1*^{CIDm} with *UAS-Dl* overexpression produced the largest metastases.

(**F, G**) Histological longitudinal sections of a representative fly and detail of metastases in abdomen (genotype; *ey-Gal4>Dl>zfh1*^{CIDm}. Arrowheads point to examples of second eye-derived (red tissue) overgrowths within the abdomen.

These results suggest that a Notch-Zeb1 axis may facilitate tumor metastasis and may draw attention in the transcriptional activation and/or CtBP –independent repression of ZEB1 in human metastasis.

Figure S8. Model of a 'vicious cycle' in prostatic metastasis by miR-200-JAG1-ZEB1 interactions



Aberrant JAG1 accumulation would be produced by opposite, direct and indirect actions of distinct miR-200 miRNAs. JAG1 produced by tumor cells would stimulate the NOTCH receptor in the bone microenvironment, thereby provoking unbalanced bone resorption/formation. Bone lesions at the site of metastasis release active TGFß (Gupta and Massague, 2006), which would induce the expression of EMT-associated factors such as *ZEB1* that in turn represses *mir-200c* (Park et al, 2008; Wellner et al, 2009), thereby creating a 'vicious circle'. Functional cooperation between JAG1 and ZEB1 would render tumor cells the motility and invasion capacity and the ability to grow. Functional cooperation between JAG1 and ZEB1 would render tumor cells the motility and invasion capacity and the ability to grow. Uncoupling silencing of JAG1 by the miR-200c may allow safeguarding stemness by JAG1 in metastatic tumor cells that re-gaining epithelial state through re-expression of miR-200c.

Supplementary References

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