The EMBO Journal (2011) 30, 756–769 | © 2011 European Molecular Biology Organization | Some Rights Reserved 0261-4189/11
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THE EMBO JOURNAL

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

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Notch signalling is crucial for the correct development and growth of numerous organs and tissues, and when subverted it can cause cancer. Loss of miR-8/200 microRNAs (miRNAs) is commonly observed in advanced tumours and correlates with their invasion and acquisition of stem-like properties. Here, we show that this miRNA family controls Notch signalling activation in *Drosophila* and human cells. In an overexpression screen, we identified the *Drosophila* miR-8 as a potent inhibitor of Notch-induced overgrowth and tumour metastasis. Gain and loss of *mir-8* provoked developmental defects reminiscent of impaired Notch signalling and we demonstrated that miR-8 directly inhibits Notch ligand Serrate. Likewise, miR-200c and miR-141 directly inhibited JAGGED1, impeding proliferation of human metastatic prostate cancer cells. It has been suggested that JAGGED1 may also be important for metastases. Although in metastatic cancer cells, JAGGED1 modestly regulated *ZEB1*, the miR-200c's target in invasion, studies in *Drosophila* revealed that only concurrent overexpression of *Notch* and *Zfh1/ZEB1* induced tumour metastases. Together, these data define a new way to attenuate or boost Notch signalling that may have clinical interest.

The EMBO Journal (2011) **30,** 756–769. doi:10.1038/emboj.2010.358; Published online 11 January 2011
Subject Categories: signal transduction; molecular biology of disease
Keywords: cancer; development; microRNA miR-8; miR-200c; Notch

Introduction

Cancer often involves the inappropriate activation of signalling pathways used during normal development. As such, key modulators of developmental pathways (e.g. the Notch pathway) are of considerable clinical interest for their potential therapeutic value (Pannuti *et al*, 2010). Indeed, activation of the Notch pathway is an ancient mechanism to control the growth of numerous tissues and organs (Artavanis-Tsakonas *et al*, 1999; Chiba, 2006), and recent evidence indicates this pathway is often recruited to stimulate growth of many solid tumours and leukaemic stem cells and to orchestrate angiogenesis and/or the reprogramming of cancer cells via epithelial–mesenchymal transition (EMT) (Miele *et al*, 2006; Bailey *et al*, 2007). While the general elements in the Notch signalling pathway are well known (Bray, 2006; Kopan and Ilagan, 2009), it is likely that additional regulatory components that might serve new tools with which to inhibit or activate Notch signalling remain to be discovered.

In *Drosophila*, there is a single Notch receptor and two genes that encode the ligands Delta and Serrate (Ser). Four different Notch receptors (NOTCH1–4) and five canonical ligands of the Delta (DLL1, 2, and 4) and Ser (JAGGED (JAG)1,2) families have been characterized in humans. In all phyla, Notch binding of its ligand triggers receptor activation through a round of two consecutive cleavages, one extracellular and the other intracellular. The latter requires the activity of the γ-secretase complex and it releases the intracellular domain of Notch, which then translocates to the nucleus to form a transcriptional activator in complex with the DNA-binding protein, CSL (CBF1/RBP in mammals, Suppressor of Hairless in *Drosophila*, and LAG-1 in *Caenorhabditis elegans*), and with the co-activator Mastermind-like proteins (Artavanis-Tsakonas *et al*, 1999).

Timely ligand-receptor activation and signal strength requires not only spatiotemporal regulation of ligand genes but also post-transcriptional regulation of ligand levels via ligand endocytosis, ubiquitination, and endosome sorting (Bray, 2006; Kopan and Ilagan, 2009). The importance of ligand regulation is highlighted in humans in which the loss of one gene copy or gain of Notch ligand are directly linked to developmental syndromes and age-related diseases including cancer (Kopan and Ilagan, 2009). Significantly, high JAG1 protein correlates with the metastasis, shorter survival time, and recurrence in human carcinomas, including prostate (Santagata *et al*, 2004). Cell culture studies and xenograph assays suggest that high JAG1 might instigate invasion and migration through EMT in breast and prostate cancer cell lines (Bailey *et al*, 2007; Zang *et al*, 2007; Thiery *et al*, 2009; Wang *et al*, 2010). However, how the tightly regulated NOTCH pathway activation is subverted in carcinogenesis remains poorly understood, particularly since activating mutations of NOTCH pathway components are rarely detected in solid tumours (Miele *et al*, 2006).

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Received: 2 July 2010; accepted: 20 December 2010; published online: 11 January 2011

Notch receptor or ligand overexpression is associated with *Drosophila* eye tumours that exhibit hallmarks of mammalian cancers such as uncontrolled overgrowth, invasion, and metastasis (Pagliarini and Xu, 2003; Ferres-Marco *et al*, 2006; Palomero *et al*, 2007; Martinez *et al*, 2009), providing a powerful model for the genetic dissection of the regulatory circuits controlling tissue homeostasis, growth and cancer by Notch signalling pathway. To this aim, we performed an unbiased forward gain-of-expression screen in *Drosophila* eye (Ferres-Marco *et al*, 2006). We used the Gene Search (GS) transposon system to systematically generate gain-of-expression mutations and the *ey-Gal4* line to drive eye-specific expression (Ferres-Marco *et al*, 2006). Herein, we describe the isolation of a GS line in such a screen that allowed us the identification and characterization of a conserved mechanism of control of ligand-mediated Notch activation by the miR-8 (known as miR-200) family of microRNAs (miRNAs).

miRNAs are conserved short, non-coding RNAs, whose main role is to modulate the expression of target genes by binding, with imperfect base pairing, to target sites in the open reading frame (ORF) and/or 3′untranslated region (UTR) of messenger RNAs (Bushati and Cohen, 2007). *Drosophila* miR-8 is the sole homologue of the human miR-200 family, which includes five members expressed from two loci located on chromosome 12 (the *mir-200c∼141* cluster) and chromosome 1 (the *mir-200b∼429* cluster comprised of miR-200b, miR-200a, and miR-429) (Figure 1). Loss of *mir-8* causes adult lethality associated with neurodegeneration, malformed legs (Karres *et al*, 2007), and overall body size reduction (Hyun *et al*, 2009). *mir-8* is expressed in the epithelial imaginal discs (the precursors of the epidermis and eye of the adult fly) and in the fat body (an organ similar to mammalian liver and adipose tissue), where miR-8 stimulates epithelial growth systemically by repressing a gene *ush* (fly FOG2), which represses, in turn, PI3K signalling, thereby stimulating epithelial disc growth systemically (Hyun *et al*, 2009).

Loss of miRNAs of the miR-200 family is commonly observed in advanced tumours in humans (Valastyan and Weinberg, 2009) and correlates with their invasion (via the release of inhibition upon the EMT-inducers ZEB1 and ZEB2 (Christoffersen *et al*, 2007; Hurteau *et al*, 2007; Bracken *et al*, 2008; Gregory *et al*, 2008; Korpal *et al*, 2008; Park *et al*, 2008)) and the acquisition of stem-like properties (via derepression of ZEB1 and Bmi1 (Shimono *et al*, 2009; Wellner *et al*, 2009)). Since EMT reprogramming is frequently reversed in distant metastases (e.g. colon cancers is a paradigm for this conceptual dilemma; Brabletz *et al*, 2005) and elevated re-expression of *mir-200* is observed in human samples of advanced carcinomas of the colon as well as carcinomas of the pancreas and ovary and in cancer cell lines (Hu *et al*, 2009; Li *et al*, 2010), the role of miR-200 miRNAs in the last step of the metastatic cascade remains somehow obscure.

Here, we describe the identification, through a genetic screen, of *Drosophila* miR-8 as a potent inhibitor of Notch-induced growth, tumour growth, and metastasis. We demonstrated that miR-8 directly inhibits the translation of Notch ligand Ser, an unanticipated direct target of this miRNA, and also validated these interactions in human bone-metastatic prostate cancer cells, which express low endogenous levels of these miRNAs and high JAG1 protein levels. Finally, we established assays in *Drosophila* that show the importance of Notch and Zfh1/ZEB1 co-regulation in the formation of tumour metastasis. Together, these data define a novel, conserved mechanism to attenuate or enhance ligand-mediated Notch signalling activity that may be exploited in future cancer therapy.

Results

Inhibition of Notch-induced growth and tumourigenesis by miRNA miR-8

We isolated the *GS(2)SC1* line in the course of an unbiased forward genetic screen in *Drosophila* aimed to identify genes that enhance or restrict Notch-induced tumourigenesis. The *GS(2)SC1* line completely suppressed incidence of secondary eye-derived growths (metastases) and significantly reduced primary eye tumour growth (Figure 1B–D) in a *Drosophila* cancer paradigm associated with the overexpression of *Dl* and two epigenetic repressors, *pipsqueak* and *lola*, collectively known as '*eyeful*' (Ferres-Marco *et al*, 2006) (Figure 1B). Without the overexpression of *GS(2)SC1*, overexpression of *Dl* together with *eyeful* using the eye-specific driver *ey-Gal4* leads to eye tumour growth (>360% the WT eye disc size) and distant metastasis in the thorax and abdomen in ∼15% of the animals ($n=100$). The introduction of the *GS(2)SC1* line in this background, significantly reduced primary eye tumour size (Figure 1C and D) and caused a high incidence of adult lethality. Examination of adult and late pupa also indicated that the GS(2)SC1 line completely inhibited the incidence of metastases (0/100).

The *GS(2)SC1* line carried an insertion 1.1 kb from the conserved miRNA gene *mir-8* (Figure 1E and F), and we confirmed that the inhibition of growth and tumourigenesis in this *GS(2)SC1* line was due to the overexpression of the miRNA *mir-8*. In the first place, direct overexpression of *mir-8* (*UAS-mir-8*) by the Gal4/UAS system produced defects similar to those in the *GS(2)SC1* flies (Supplementary Figure S1A), while locked nucleic acid (LNA) probe *in situ* hybridization and reverse transcriptase polymerase chain reaction (RT–PCR) demonstrated that endogenous *mir-8* was upregulated in *GS(2)SC1* flies (Supplementary Figure S1B). Moreover, the phenotype of *GS(2)SC1* was suppressed by mutations in *dcr-1*, an enzyme essential to process miRNAs (Supplementary Figure S1C).

Mechanism of growth inhibition by miR-8

To identify the functional target of miR-8 implicated in growth inhibition, we examined the phenotypes when *mir-8* was overexpressed in tissues and at development stages in which endogenous *mir-8* is normally expressed (Karres *et al*, 2007) and (Figure 1J; Supplementary Figure S1D–F). The phenotypes detected included small wing and eye (Figures 1G and H and 2), wing notches (Figure 1I), neurogenic defects (data not shown), and embryonic cuticle defects (Figure 1K and L), all reminiscent of the phenotypes produced by reduced Notch signalling and in particular, when Ser is impaired (Thomas *et al*, 1991; Diaz-Benjumea and Cohen, 1995; Walters *et al*, 2005).

To confirm that miR-8 blocks growth by inhibiting Notch ligand signalling, we analyzed the reduction in eye size associated with *mir-8* overexpression (Figure 2; Supplementary Figure S2). During *Drosophila* eye development, Ser first promotes growth and cell survival throughout the ventral

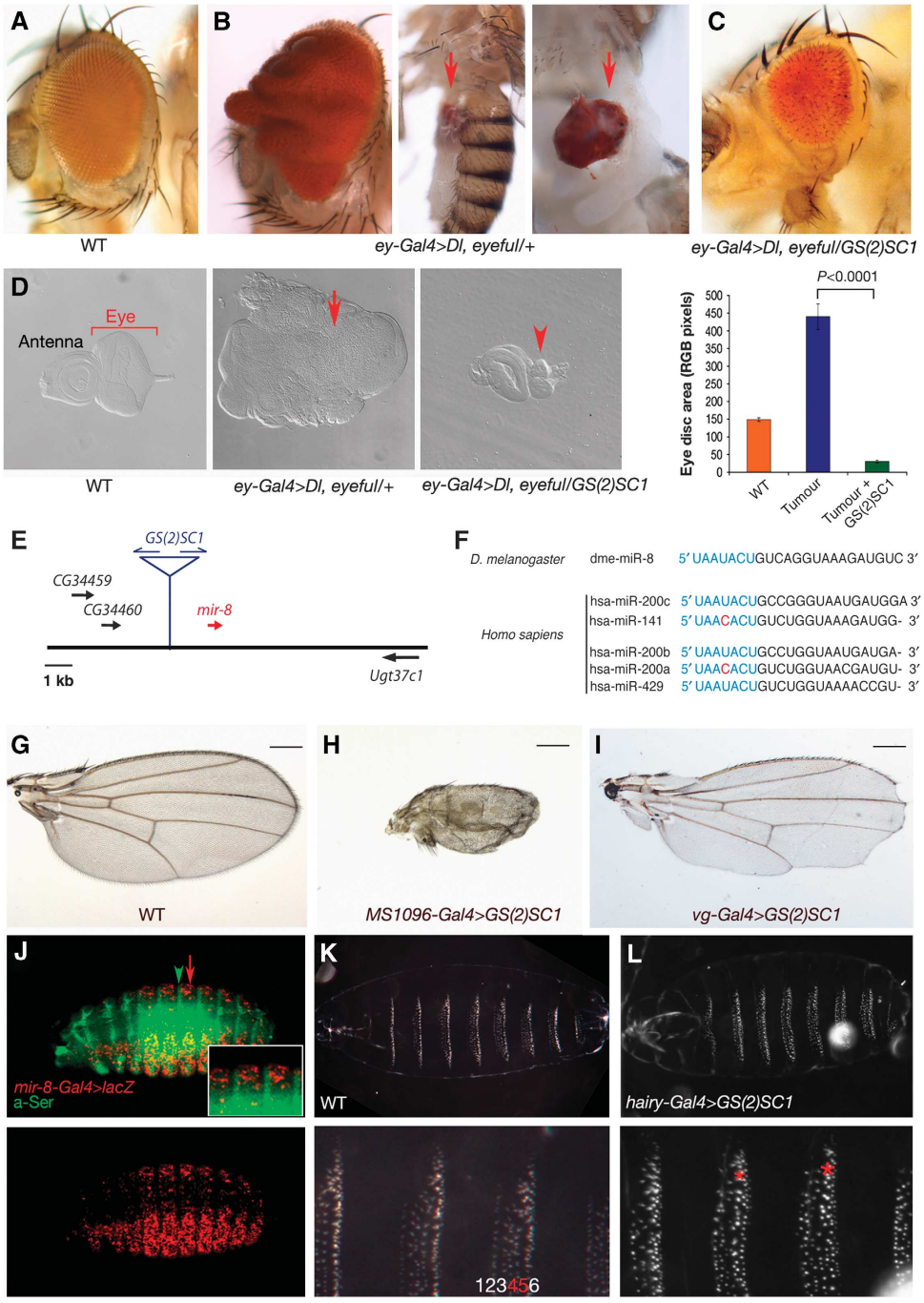

Figure 1 Identification of conserved mir-8 as a negative regulator of growth and tumourigenesis through Notch signalling. (**A**) Control of female wild-type (WT) eye size. (**B**) Representative fly showing eye tumour growth (left) and metastasis in the abdomen (red tumour mass, arrows) in the *Drosophila* cancer paradigm used in the screening that identified the miR-8 microRNA. The bottom image shows the metastatic mass in the open abdomen (arrows) of the same fly. (**C**) A representative of tumour suppression by *GS(2)SC1* (genotype *ey-Gal4>UAS-Dl, eyeful/GS(2)SC1*). Adult flies and late pupa of this genotype scored 0 metastasis (0/100 animals) and strong reduction of eye tumour growth. (**D**) Qualitative and quantitative analyses of the suppression of primary tumour growth by *GS(2)SC1*. Representative images of a WT eye disc, tumour eye-antennal disc (arrow; genotype *ey-Gal>UAS-Dl, eyeful/+*), and suppressed tumour disc (arrowhead; genotype *ey-Gal4>UAS-Dl, eyeful/GS(2)SC1*). Graph shows quantification of the total eye disc areas scored in 20 eye discs from the three genotypes: WT (red bar); tumour (genotype *ey-Gal4>UAS-Dl, eyeful/+*: blue bar); and tumour suppression by *GS(2)SC1* (*ey-Gal4>UAS-Dl, eyeful/GS(2)SC1*: green bar). Error bars represent the standard error of the mean (s.e.m.). *P*-values are obtained using unpaired Students' *t*-test. (**E**) Map of the GS(2)SC1 insertion near *mir-8*. (**F**) The miR-8/200 miRNA precursor family. (**G**) Male wing size control. (**H**) *MS1096-Gal4>GS(2)SC1* and (**I**) *vg-Gal4>GS(2)SC1* wings show typical *Ser*-like phenotypes (small wing, 100% $n=50$; **H**) and (wing nicks, 100% $n=60$; **I**). *MS1096-Gal4* and *vg-Gal4* drive early expression in the dorsal compartment and late expression in the D/V boundary of the wing disc, respectively. (**J**) Stages 14 and 15 embryo showing *mir-8* microRNA segmental expression (*mir-8-Gal4>lacZ*, red) overlapping the *Ser* protein (green). (**K**) Control cuticle embryo and detail (below). (**L**) Overexpressing *mir-8* via GS(2)SC1 by *hairy-Gal4* showed typical Ser^{-} embryonic denticle belt defects.

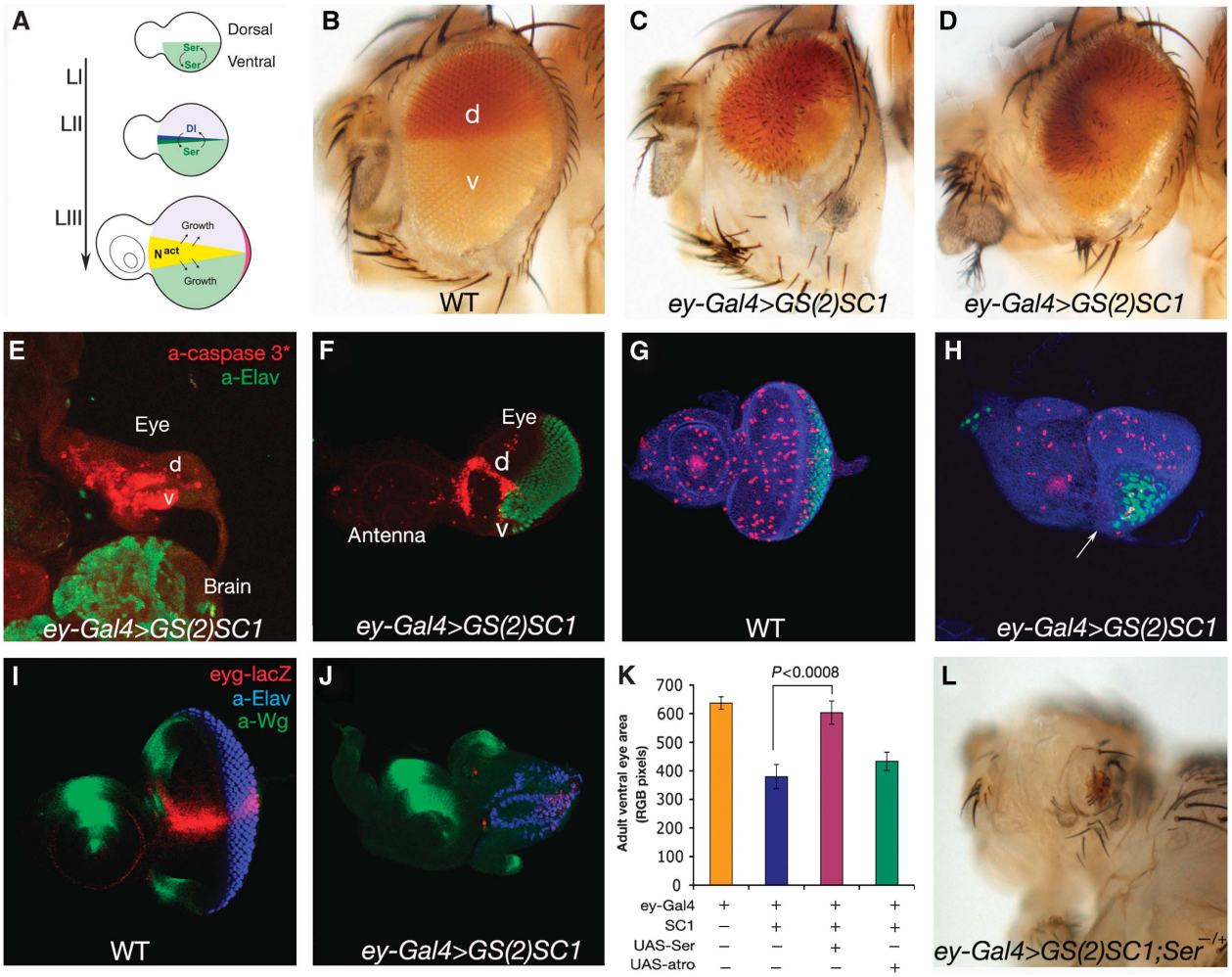

Figure 2 miR-8 represses growth by inducing apoptosis and blocking cell proliferation via Serrate. (**A**) Representation of Ser expression and signalling (green) in first to third larval eye disc development. A D/V growth-promoting organizer is established during the late LII stage by local activation of Notch (yellow) by Dl (blue) and Ser. Retinal differentiation initiates from the posterior end of the disc (red). (**B–D**) The $mirr^{P69Df7}P[w+]$ line marks the subdivision of the eye into dorsal (d) and ventral (v) compartments in WT control eye (**B**) and *mir-8* overexpressing eyes (*ey-Gal4>GS(2)SC1*; **C**, **D**). Note the reduction of the ventral eye region (**C**) and the profound distortion of the D/V boundary (**D**). (**E**) Ubiquitous overexpression of *mir-8* only produced apoptosis in the ventral region of first instar or (**F**) in the organizer region of late third instar discs, as visualized through cleaved caspase-3 (red). (**G**) Mitosis visualized by phospho-histone H3 (red) in control and (**H**) *mir-8* overexpressing eye discs. Note the absence of mitosis in the ventral eye (arrow). Cells are outlined by anti-armadillo staining (blue). (**I**) *eyg-lacZ* (red) marker of Notch induced the D/V organizer in the WT and (**J**) *mir-8* overexpressing eye discs (genotype: *ey-Gal4>GS(2)SC1; eyg-lacZ*). (**K**) Gain of *Ser* ($n=24$) but not *atrophin* ($n=32$) rescued the eye growth defect induced by *miR-8* overexpression. Female eyes were scored and the error bars show the s.e.m. Differences between *ey-Gal4>GS(2)SC1* and *ey-Gal4>GS(2)SC1>UAS-atro* are not statistically significant ($P=0.32$). *P*-values are obtained using unpaired Students' *t*-test. (**L**) Representative adult eye of *ey-Gal4>GS(2)SC1>*$Ser^{-/+}$ flies.

eye region and subsequently, together with Dl it induces a conserved dorsal/ventral (D/V) growth-promoting organizer (Figure 2A). Overexpression of *mir-8* through *ey-Gal4* led to eyes with a very reduced ventral region (17–59% of the wild-type size, $n=194$) and a profoundly distorted D/V boundary (Figure 2B–D). General *mir-8* overexpression throughout the late first instar eye disc resulted in massive cell death of ventral cells alone (Figure 2E–H), the cell population that expresses and requires Ser signalling to survive and proliferate (Singh *et al*, 2006). Apoptotic cells often induce extra compensatory cell divisions to replace the lost cells, commonly leading to normal-sized adult organs. However, miR-8-induced apoptosis was not compensated for by extra cell proliferation (Figure 2G and H) and thus, older eye discs had a strongly reduced ventral region. Later in development, generalized *mir-8* overexpression induced a robust apoptotic response at the D/V interface, the region of the disc that again expresses and requires Ser-Notch signalling, in this case to establish the D/V organizer (Figure 2F). Accordingly, when *mir-8* was overexpressed Notch failed to activate target genes (i.e. *eyegone*; Dominguez *et al*, 2004) (Figure 2I and J) and the D/V boundary did not form adequately (data not shown). Thus, *mir-8* overexpression led to apoptosis and inhibited cell proliferation in specific regions, consistent with the inhibition of Ser signalling.

Serrate is an effector of normal and tumour growth inhibition by miR-8 in vivo

We next tested the capacity of Ser overexpression or down-regulation to rescue or heighten the intrinsic inhibition of eye growth following *mir-8* overexpression. These tests provided

strong evidence that miR-8 regulates Ser *in vivo*. First, the growth defect produced by *mir-8* overexpression was fully rescued by overexpressing a *Ser* transgene (*UAS-Ser*) but not by overexpressing a previously validated target of miR-8, *atrophin* (Figure 2K; Supplementary Figure S2), or the other Notch ligand *Dl* (Supplementary Figure S2) or the predicted target Wg signalling (data not shown). Second, reducing the gene dosage of *Ser* by 50% (Ser^{RX106} null allele) markedly enhanced the effect of *mir-8* on eye growth (Figure 2L). Together, these data indicate that miR-8 negatively regulates Notch signalling by reducing the levels of Ser.

Direct regulation of Serrate by miR-8

Ser is not predicted to be a target of miR-8 in computational analyses or by expression profiling (Karres *et al*, 2007). Nonetheless, we identified two potential binding sites for miR-8 in the *Ser* 3′UTR and therefore, we assessed these two sites for miR-8 activity (Figure 3A). When transfected into *Drosophila* Schneider (S2) cells overexpressing *mir-8*, a luciferase reporter containing the full-length *Ser* 3′UTR yielded 30% less luciferase activity than a UTR with point mutations in each of the two miR-8-binding sites (Figure 3B). Moreover, a tubulin-eGFP sensor construct fused to the full-length *Ser* 3′UTR (Figure 3C) but not that fused to the mutated construct (Figure 3D) was downregulated upon *mir-8* overexpression in the wing disc. These results demonstrate that miR-8 can directly repress *Ser* via the miR-8-binding sites in its 3′UTR region, both *in vitro* and *in vivo*.

We explored the mechanism of Ser inhibition by analyzing Ser protein in wing discs with altered *mir-8* expression

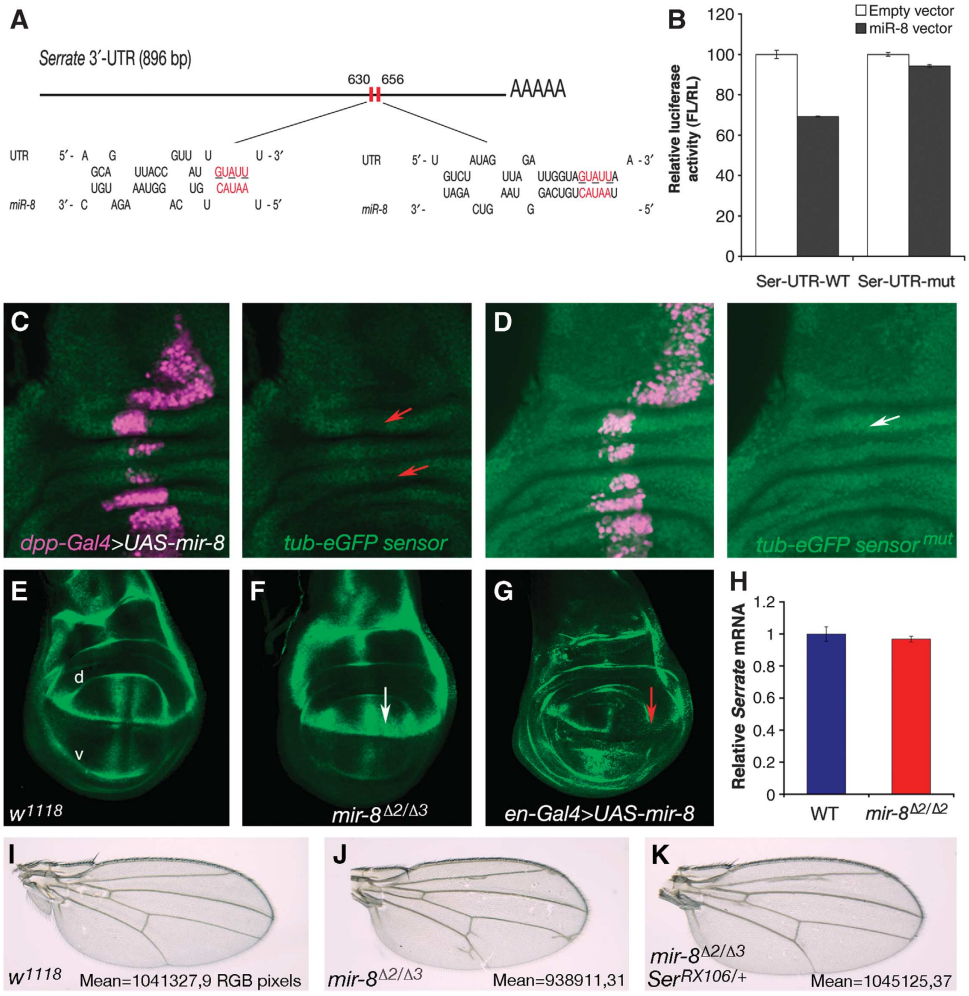

Figure 3 Serrate is directly repressed by miR-8 via translational inhibition. (**A**) Computer prediction of two miR-8-binding sites in the *Ser* 3′UTR (seed sequence in red). (**B**) Luciferase assay in *Drosophila* Schneider (S2) cells co-transfected with *mir-8* or the empty vector, together with a firefly luciferase vector containing the *Ser* 3′UTR (Ser-UTR-WT) or a luciferase vector with mutations in the seed sequence (underlined bases in (**A**): Ser-UTR-mut). (**C**, **D**) Overexpression of *mir-8* by *dpp-Gal4* caused a modest but reproducible downregulation of *GFP* in a tub-eGFP:*Ser*UTR sensor (**C**), but not in the tub-eGFP:*Ser*UTR mutated control in the stripe of cells within the *dpp* domain (arrows, **D**). Nineteen tub-eGFP:*Ser*UTR and nine tub-eGFP:SerUTR^{mut} independent transgenic lines were studied. Wing discs showing Ser protein (green) in (**E**) WT, (**F**) *mir-8* mutant ($mir\text{-}8^{\Delta 2/\Delta 3}$), or (**G**) *mir-8* overexpressing mutants driven by *en-Gal4*. The *en-Gal4* drives expression in the posterior compartment of the wing disc (right in the image, red arrow). (**H**) *Ser* mRNA levels of WT and $mir\text{-}8^{\Delta 2}$ mutant larvae assayed by qPCR were not statistically different ($P = 0.457$; $n = 3$, mean ± s.e.m.). (**I**) Adult male wings (all to scale) from males of w^{1118} (control), (**J**) w^{1118}; $mir\text{-}8^{\Delta 2/\Delta 2}$, and (**K**) w^{1118}; $mir\text{-}8^{\Delta 2/\Delta 3}$; $Ser^{RX106}/+$. In the complete absence of *mir-8*, wing size is reduced by 10% and had extra veins. Reducing by 50% gene dosage of *Ser* fully rescued wing size and partly the extra veins. Mean areas of adult wings ($n = 16$) in each genotype expressed in RGB pixels are shown. Rescued by *Ser* mutation is statistically significant (two-tailed, $P < 0.0001$).

(Figure 3E–G). The loss of endogenous *mir-8* in the mutant condition *mir-8*$^{\Delta 2/\Delta 3}$ (Karres *et al*, 2007) or the overexpression of *miR-8* under the control of *en-Gal4* augmented or depleted the accumulation of Ser protein (Figure 3F and G), without markedly effecting *Ser* mRNA levels (Figure 3H). Thus, miR-8 repressess Ser by translational inhibition.

A target mRNA and its cognate miRNA gene must be co-expressed in order for the regulation by miRNA to be biologically relevant. Indeed, analyses of *mir-8* and *Ser* expression during development showed that both genes are frequently expressed in at least partially complementary patterns (Figure 1J; Supplementary Figure S1F). Moreover, excess Ser in the wing in *mir-8*$^{-}$ animals (Figure 3F) led to extra veins and smaller size in the adult wing that was fully rescued by reducing *Ser* gene dosage by 50% (Figure 3I–K). Notably, two other targets of miR-8, *ush* and *atrophin* did not rescue the wing defects (Karres *et al*, 2007; Hyun *et al*, 2009). Together, the gain- and loss-of-function analysis of *mir-8* and functional validation *in vivo* unveil this miRNA as a negative regulator of *Ser*, expanding the mechanisms that fine tuning Notch signalling activation *in vivo*.

Direct inhibition of human JAGGED1 by miR-200c∼141 in metastatic prostate cancer cells

Thus, the findings in *Drosophila* prompted us to explore whether analogous interactions of human miR-8 and Ser orthologs occur in metastatic human cancer cells. High levels of JAG1 are correlated with a more aggressive disease course and recurrence in human prostate cancers (Santagata *et al*, 2004) and indeed, a metastatic human prostate cancer cell line (PC-3 cells) expressed high levels of JAG1 protein (Figure 4A, upper blot). Human miR-200s are expressed from two loci located on chromosome 12 (the *mir-200c∼141* cluster) and chromosome 1 (the *mir-200b∼429* cluster comprised of miR-200b, miR-200a, and miR-429). When assayed in a quantitative stem-loop PCR (qPCR) the miRNAs on the *mir-200c∼141* cluster were more weakly expressed in PC-3 cells than in non-transformed prostate PNT1A cells (Figure 4B, left). Conversely, the other members of the miR-200 family, produced by the *mir-200b∼429* cluster, were highly expressed in the PC-3 cells (Figure 4B, right). Thus, only the expression of the *mir-200c∼141* cluster is inversely related to the elevated JAG1 expression. Significantly, *mir-200c∼141* cluster is specifically silenced in clinical samples of advanced prostate cancers and highly metastatic prostate cancer PC-3 (Porkka *et al*, 2007).

The *JAG1* gene is predicted to be a target of miR-200a and miR-141 (TargetScan, Pictar), which share a similar seed region (Figure 1F), but not of miR-200c. Nonetheless, we identified two potential miR-200c and one potential miR-200b-binding sites in the *JAG1* 3′UTR (Figure 4C). Indeed, through *in vitro* gain- and loss-of-function analyses, we established a causal connection between the loss of *mir-200c* and *mir-141* but not *mir-200b*, and JAG1 upregulation in prostate cancer. We prepared PC-3 cell lines that stably expressed *mir-200c*, *mir-141*, or both, and the overexpression of *mir-200c* in PC-3 cells caused complete growth arrest and the culture collapsed 48 h after antibiotic addition (see Supplementary data). Remarkably, co-expression of *mir-200c* with *mir-141*-expressing vectors at concentrations identical to the individual *mir-200c* vector allowed stable cell lines to be generated, although we do not yet understand why. Thus, overexpressing *mir-200c* and *mir-141* in the PC-3 cells, or *mir-141* alone, produced a 60% and 30% decrease in the JAG1 protein, respectively (Figure 4A, upper blot), without significantly altering *JAG1* mRNA levels (Supplementary Figure S3A). Conversely, inhibiting endogenous *mir-200c*, *mir-141*, or both miRNAs with LNA-modified antisense oligonucleotides caused a 42, 28, and 52% increase in JAG1 protein in the non-transformed PNT1A cells, respectively (Figure 4A, lower blot).

To demonstrate that miR-200c∼141 directly inhibits JAG1, as in *Drosophila*, we generated *luciferase* constructs containing the full-length 3′UTR region of the human JAG1 and a version with mutations in the three predicted miR-200c/141-binding sequences (Figure 4C). The overexpression of *mir-200c* and *mir-141* in PC-3 cells decreased *luciferase* activity from the *JAG1* 3′UTR reporter by 25% but expression of *luciferase* was unaltered in the mutant sensor construct (Figure 4D). Moreover, expression from the *JAG1* 3′UTR reporter was lower in PNT1A cells, which naturally express the endogenous *mir-200c∼141* cluster, compared with the expression from the mutant sensor construct (Supplementary Figure S3B). Thus, JAG1 appears to be a direct target of endogenous miR-200c∼141. Another *Serrate-like* ligand, *JAG2* also has miR-200c sites in its 3′UTR but we did not experimentally validate these sites because we did not observe complementary expression of *mir-200c* and JAG2 protein in PC-3 cells (data not shown).

Differential regulation of JAGGED1 by miR-200 family members in metastatic prostate cancer cells

To extend the analysis of the miR-200 miRNAs, we next investigated the role of endogenous miRNAs of the miR-200b∼429 cluster, which are highly expressed in correlation with high JAG1 expression in the PC-3 cells (Figure 4B). miR-200b and miR-429 have the same seed match to miR-200c and they potentially may target the same element in the *JAG1* 3′UTR. As overexpression assays might not mimic the endogenous mechanism of miRNAs of a common family, we assessed the endogenous roles of *mir-200b* and *mir-429* by using loss-of-function approach based on LNA-modified antisense molecules, to specifically block each of the endogenous activity of the miRNAs of the *mir-200b∼429* cluster. We assayed effects on both the *JAG1* 3′UTR luciferase sensor constructs as well as endogenous JAG1 protein levels. Co-transfection of the *JAG1* 3′UTR luciferase reporters (WT and mutant sensor construct) with a combination of the three LNAs anti-miRs (*mir-200a*, *mir-200b*, and *mir-429*) together, did not enhance expression of the sensor constructs, indicating that endogenous *mir-200b∼429* cluster do not inhibit significantly JAG1 (Supplementary Figure S3C), consistent with the concurrent co-overexpression of *mir-200b∼429* and JAG1 in these cells (Figure 4A and B). Importantly, depletion of these miRNAs with the LNAs anti-miR-200b and the anti-miR-429 increased expression levels of endogenous *ZEB2* (also known as *SIP1*), which is a validated target mRNA of miR-200b in other cancer cell lines (Bracken *et al*, 2008; Gregory *et al*, 2008; Korpal *et al*, 2008; Park *et al*, 2008) (Supplementary Figure S3D). This demonstrates the specificity and efficacy of the LNA-anti-miR-200b and -429 in the PC-3 cells, and indicates that within the miR-200 family of miRNAs with the same seed sequence, miR-200c (and

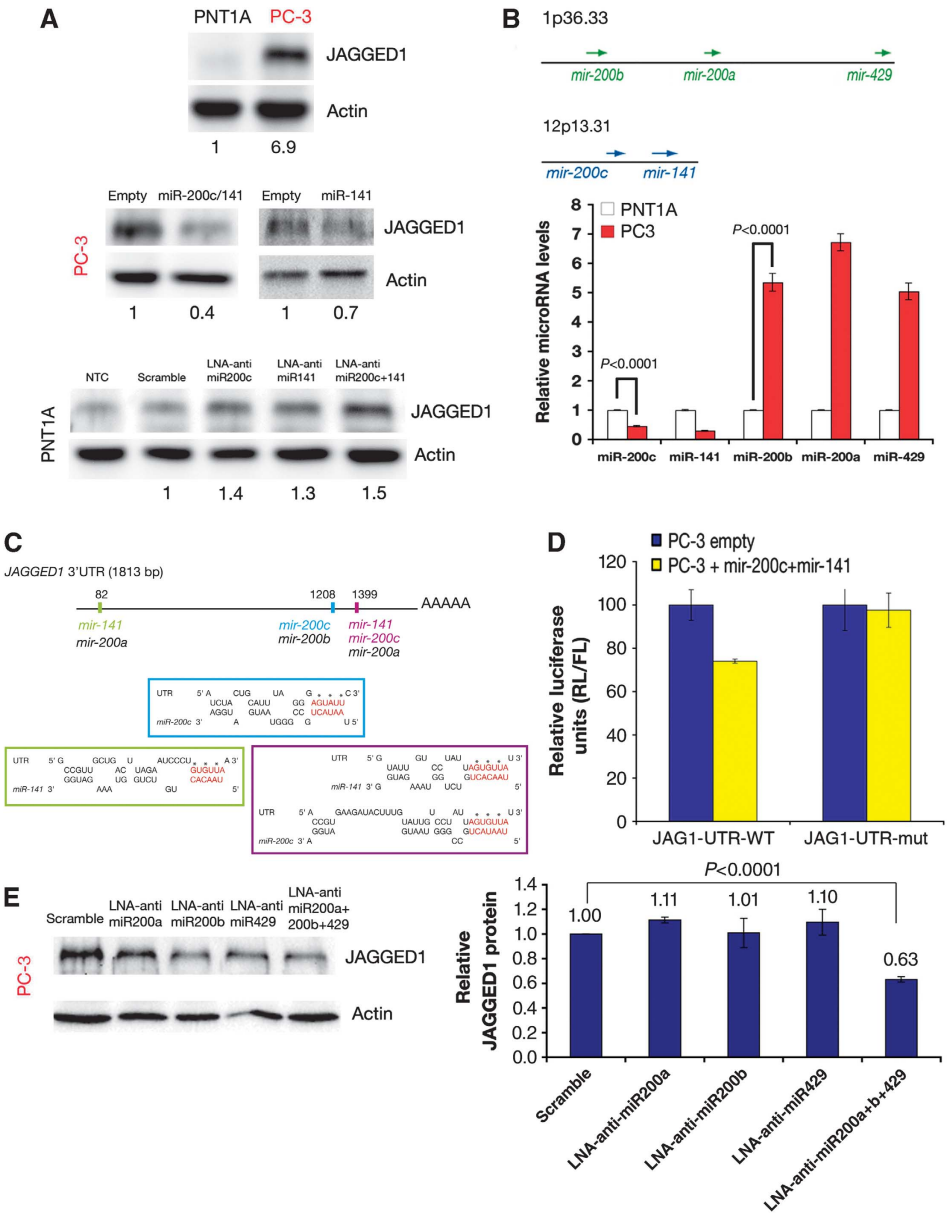

Figure 4 Direct inhibition of JAGGED1 by miR-200c∼141 in metastatic prostate cancer mesenchymal cells. (**A**) Immunoblot analysis of JAG1 protein in human non-tumourigenic epithelial prostate cells PNT1A and bone-derived metastatic prostate cancer cells PC-3 (top). JAG1 protein levels decreased or increased in the presence of miR-200c and miR-141 overexpressing vectors (PC-3 cells, middle blot) or the presence of LNAs in the transformed epithelial prostate cells (bottom blot). The controls are PC-3 cells transfected with the empty vector (middle blot) and the presence or absence (NTC) of scramble LNAs (bottom blot). Equal loading of samples was confirmed by blotting with anti-actin mAb. A representative western blot is shown and quantification below show JAG1 staining relative to control cells in triplicate (**B**) qPCR of microRNAs of the *mir-200c∼141* cluster (*mir-200c* and *mir-141*) and *mir-200b∼429* cluster (*mir-200b*, *mir-200a*, and *mir-429*) in the PC-3 and PNT1A cells. Data shown were normalized to RNU6 (**C**) *JAG1* 3′UTR showing the miR-200-binding sites and the JAG1-miR-200c or -miR-141 duplexes (boxes). (**D**) Reduced *luciferase* activity of *pRL-TK* vector containing the human *JAG1–3′UTR-WT* in PC-3 cells stably transfected with *mir-200c/141* expressing or empty vectors and compared with *JAG1-3′UTR-mut* reporter with mutations in the three miRNA-binding sites (asterisks in **C**). (**E**) JAG1 protein levels are reduced in the presence of three LNAs of the *mir-200b∼429* cluster. PC-3 cells were treated with LNA anti-sense oligonucleotides of miR-200a, miR-200b, miR-429, a mixture of the three or scramble LNAs and a representative immunoblot (left) and histogram from three independent experiments (right) are shown. *P*-values are obtained using unpaired Students' *t*-test in (**B**, **D**, **E**), $n=3$, mean ± s.e.m.

miR-141 to a lesser extent) has a predominant role in the regulation of JAG1 in the prostate cells. We noted that although mature miR-200c and miR-200b sequences are nearly identical, their precursor (pre-miRNA loop) sequences are divergent. In the miR-181 and let-7 families, nucleotides in the pre-miRNA loop outside the common seed domain are known to regulate target specificity by family members with nearly identical mature sequence (Vella *et al*, 2004; Liu *et al*, 2008).

Curiously, depletion of the three miRNAs of the *mir-200b∼429* cluster by a combination of the three LNAs markedly reduced JAG1 protein levels (37%; Figure 4E), without major effect on the endogenous *JAG1* mRNA expression levels (data not shown). The mechanism behind this effect is presently unknown, but the effect was specific and not related to transfection efficiency as we did not detect a decrease in the miR-200b target *ZEB2* in the condition in

which PC-3 cells were transfected with the three LNAs (Supplementary Figure S3D). We reasoned that the decrease in JAG1 protein levels upon depletion of the three *mir-200b*, *200a*, and *-429* miRNAs must be indirect, through an intermediate target that represses JAG1 (see Discussion).

miR-200c∼141 inhibits JAGGED1-mediated metastatic prostate cancer cell proliferation

Having identified a conserved regulation of JAG1 by miR-200c/141 miRNAs, we next confirmed that the depletion of JAG1 protein indeed reduced the activity of the NOTCH pathway in PC-3 cells, as occurred *in vivo* in *Drosophila* (Figures 1–3). We assayed the levels of *HES1* (*HAIRY and ENHANCER OF SPLIT homologue 1*), a conserved NOTCH target that is upregulated in PC-3 cells (Scorey *et al*, 2006). Pharmacological inhibition of NOTCH signalling by the γ-secretase inhibitor DAPT (*N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester) strongly downregulates *HES1* (Supplementary Figure S3F) and similarly, the overexpression of *mir-200c∼141* or directly downregulating *JAG1* expression with siRNAs (>70% mRNA levels of *JAG1*; Supplementary Figure S3A and E) depleted *HES1* (Supplementary Figure S3F), reflecting the failure in NOTCH signalling.

Inhibition of NOTCH signalling typically impairs the growth of many cancer cells, including PC-3 cells (Zhang *et al*, 2006; Wang *et al*, 2010). We confirmed that direct downregulation of *JAG1* by specific siRNA caused also growth inhibition in PC-3 (Figure 5A) and, significantly, observed that proliferation and survival was also greatly impaired in PC-3 cells stably transfected with the *mir-200c*- and *mir-141*-expressing vectors (Figure 5A). Above we have shown that generalized overexpression of *mir-8 in vivo* provoked a robust apoptotic response and cell proliferation inhibition precisely in the cell population that express and require *Serrate* for proliferation and survival (Figure 2). Moreover, a *Serrate* (Figure 2K; Supplementary Figure S2A), but not *Delta* (Supplementary Figure S2C), transgene rescued growth inhibition by miR-8. Therefore, to functionally link the JAG1–miR-200c interactions to growth regulation in human cells, we conducted rescue experiments in the metastatic prostate cancer cells.

For this assay, we used transient transfections (Supplementary data) of the miRNA-expressing vectors alone or in combination with a pcDNA3 vector that contains full-length *JAG1* ORF with (3′UTR+) or without (3′UTR–) the endogenous 3′UTR of *JAG1* mRNA, the later construct yields the expression of *JAG1* resistant to regulation by miR-200c and miR-141. Transient transfection with the miR-200c- and miR-141-expressing vectors efficiently inhibited cell proliferation of PC-3 cells (Figure 5B, red line) when compared with control PC-3 cells transfected with empty vectors (Figure 5B, blue line). Expressing *JAG1* with its endogenous 3′UTR (3′UTR+ construct) did not significantly rescue cell proliferation defect by the miRNAs (no increase in cell proliferation 3 days after transfection; Figure 5B). By contrast, PC-3 cells transiently co-transfected with the miR-200c- and miR-141-expressing vectors and JAG1-expressing vector without its 3′UTR (3′UTR–) (Figure 5B, green line) completely rescued cell proliferation when compared with PC-3 cells transfected with empty vectors (Figure 5B, blue line). We verified overexpression of mature miR-200c and miR-141 (qPCR; Supplementary Figure S4A and B) and confirmed that overexpression of the miRNAs decreased JAG1 protein levels by immunoblot analysis (Supplementary Figure S4C) in the transient transfection experiments. Together, these data suggest that, like its *Drosophila* counterpart, human miR-200c∼141 inhibits cell growth of PC-3 cells at large extent by restricting ligand-mediated activation of NOTCH signalling.

ZEB1 is positively regulated by JAGGED1

Overexpression of *mir-200∼141* fully reverted the mesenchymal phenotype of PC-3 cells (Figure 5C and D), as well as increasing in *E-cadherin* accumulation (>7-fold) while decreasing that of *Vimentin* (three-fold, Figure 5E). These effects are consistent with those of overexpressing *mir-200* genes in other types of cancer cells (Valastyan and Weinberg, 2009). Of note, the mesenchymal-like phenotype of PC-3 cells is associated with high levels of the *mir-200b∼429* cluster. Importantly, although most reports put emphasis on redundant and cooperative roles of miR-200 miRNAs in MET, we noted that the expression of *mir-200c∼141*, but not that of the miRNAs of the *mir-200b∼429* cluster, reflects a consistent relationship between mesenchymal state and loss of the *mir-200c∼141* expression among the panel of 60 cell lines used in the National Cancer Institute's drug screening (NCI60) (http://dtp.nci.nih.gov) representing nine different cancers (Park *et al*, 2008). Thus, while the subgroup of NCI60 cell lines previously classified as mesenchymal state have little or undetectable expression of *mir-200c∼141*, the expression of the *mir-200b∼429* cluster varied among the group of mesenchymal cell lines (Park *et al*, 2008).

Transient downregulation of *JAG1* by siRNA or by blocking NOTCH signalling in the presence of DAPT (20 μM, 72 h) did not revert the mesenchymal phenotype of PC-3 cells, although PC-3 cells deprived of *JAG1* had reduced cell motility in an *in vitro* surrogate assay of invasion (wound-healing assay) (Scorey *et al*, 2006). Hence, we reasoned that inhibition of tumour growth of PC-3 cells by miR-200c could be largely attributed to JAG1, but additional targets may mediate elements of the mesenchymal–epithelial transition.

High levels of *ZEB1* serve as a biomarker for prostate metastasis (Schmalhofer *et al*, 2009) and this factor is a key effector of EMT in these cancer cells (Drake *et al*, 2009, citations therein). Interestingly, we found that in the presence of low *mir-200c∼141* expression, the knockdown of *JAG1* in PC-3 cells with siRNAs reduced the expression of *ZEB1* (Figure 5F), but not that of *ZEB2* or of *TWIST*, *SNAIL1*, or *SNAIL2*, other E-cadherin repressors (data not shown). Similarly, exposing PC-3 cells to DAPT also downregulated *ZEB1* mRNA expression (Figure 5F), without affecting the expression of *mir-200* genes (Supplementary Figure S5 and data not shown). The effect on *ZEB1* is modest, which may explain why the JAG1–NOTCH pathway is insufficient to produce a full EMT without the loss of *mir-200c*.

High JAGGED1, ZEB1, and mir-200c∼141 expression in metastatic colon cancer epithelial cells

We were intrigued by clinical evidence that the *mir-200* genes are re-expressed in advanced colorectal carcinogenesis (Xi *et al*, 2006), where the NOTCH pathway is an essential effector of tumour growth (Qiao and Wong, 2009). Like human colon cancer samples, high levels of *mir-200* were found in metastasis-prone colon cancer cells (e.g. HCT-116; Spaderna *et al*, 2008) relative to Caco-2 cells (Figure 6A). The epithelial-like morphology, gain of *E-cadherin*, and loss of

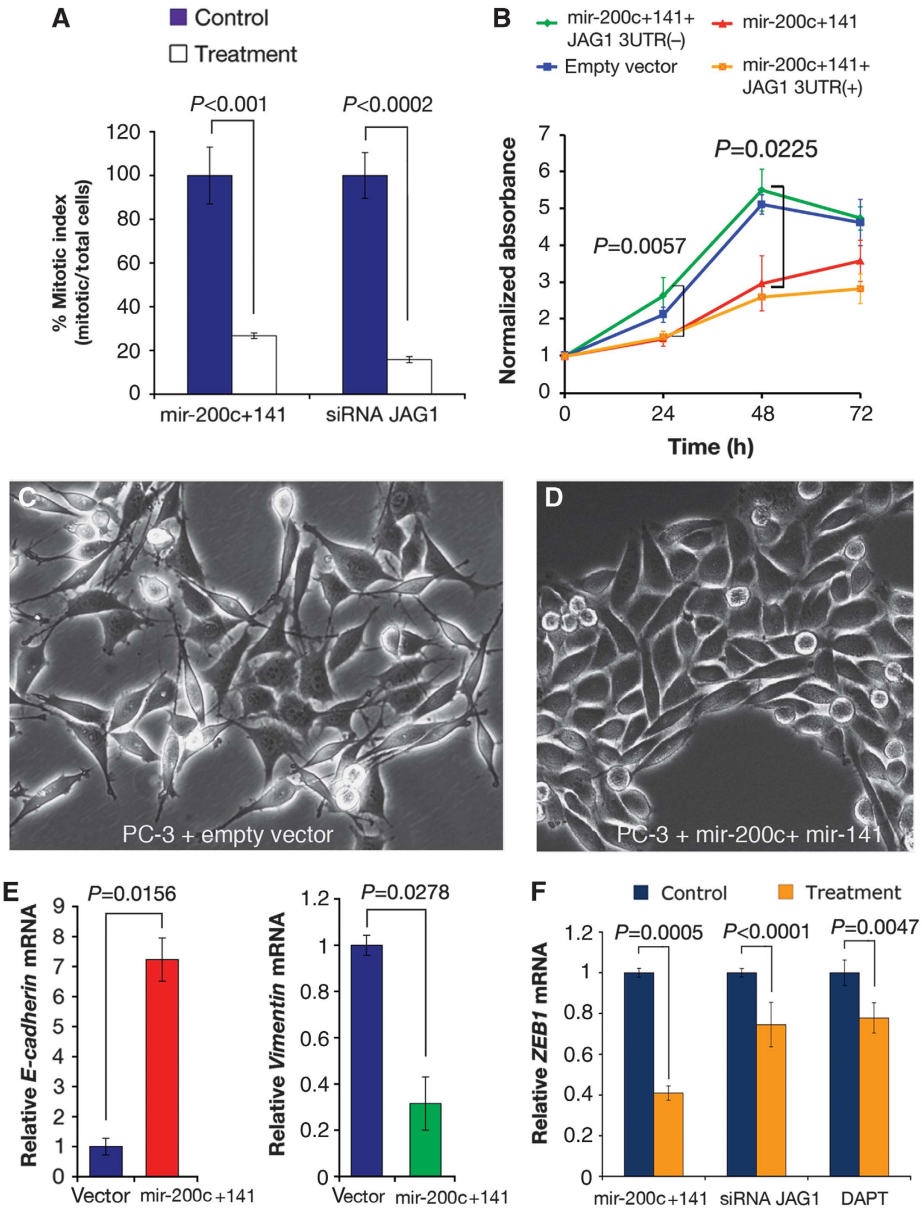

Figure 5 miR-200c∼141 inhibit cell proliferation of metastatic prostate cancer cells by repressing JAG1. (**A**) Significant reduction of cell proliferation of PC-3 cells stably transfected with *mir-200c*- and *mir-141*-expressing vectors, or transiently treated with *JAG1* siRNAs, relative to PC-3 control cells empty vector or treated with scramble siRNAs. The mitotic index was quantified as the ratio between the phosphohistone H3 positive cells and the total number of cells in four independent assays. Error bars show s.e.m. ($n=3$). (**B**) Time course of PC-3 cells expressing the microRNAs and/or JAG1 with or without its 3′UTR as determined by the WST-1 proliferation assay (Roche). Cells transiently transfected with the microRNA miR-200c and miR-141 vectors and/or the indicated *JAG1* vectors, or control empty vectors, were seeded in quadruplicate and viable cells were counted at specified time points after transfection using WST-1 assays. The data are plotted as the mean absorbance values at 415 nm minus those at 595 nm from three independent experiments and error bars represent s.e.m. *P*-values based on unpaired *t*-test at 24 and 48 h indicate statistically significant differences between PC-3 cells co-expressing the microRNAs and *JAG1* without it endogenous 3′UTR (3′UTR–) (green line) and PC-3 cells expressing only the microRNAs (red line). (**C**, **D**) Overexpression of *mir-200c∼141* but not the loss of *JAG1* by siRNAs (data not shown) triggered the mesenchymal–epithelial transition. Representative microphotographs of PC-3 cells transfected with empty vector (**C**) or stably transfected with vectors expressing *mir-200c* and *mir-141* (**D**). PC-3 cells stably transfected with *mir-200c∼141* grew in a monolayer and adopted an epithelial morphology. (**E**) Expression of human *E-cadherin* and *Vimentin* mRNA in PC-3 cells as in (**C**, **D**). Errors show s.e.m. ($n=3$). (**F**) *ZEB1* mRNA expression in stably transfected PC-3 cells with the *mir-200c*- and *mir-141*-expressing vectors, and PC-3 treated with *JAG1* siRNA or DAPT. Control cells were transfected with an empty vector, scramble siRNAs or treated with DMSO. Error bars represent s.e.m. of three independent experiments. *P*-values are obtained using unpaired Student's *t*-test (**E**, **F**).

Vimentin in HCT-116 (Supplementary Figure S6A) is consistent with the presence of *mir-200*. However, the high expression of miR-200c targets, *JAG1* and *ZEB1* (Figure 6B–D) is unanticipated by current models and our above results. Nevertheless, high JAG1 and ZEB1 are consistent with earlier observations demonstrating that colon cancer cells deprived of *JAG1* (Rodilla *et al*, 2009) or *ZEB1* (Spaderna *et al*, 2008) fail to grow or they have reduced metastatic potential,

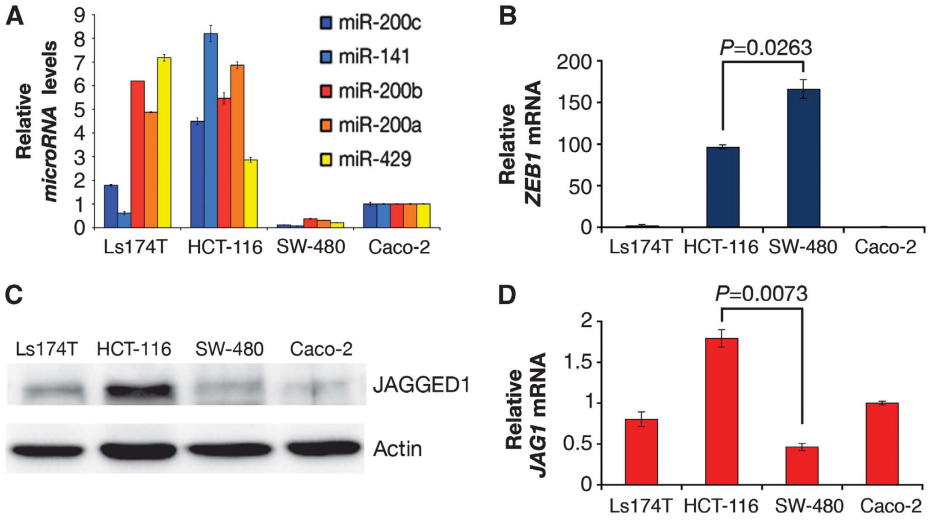

Figure 6 Concurrent expression of *mir-200c∼141* and their targets *JAGGED1* and *ZEB1* in metastatic colon cancer epithelial cells. (**A**) Relative expression of components of the *mir-200* family genes and (**B**) *ZEB1* mRNA in colonic cancer cells (Ls174T, HCT-116, SW-480, and Caco-2) measured by qPCR. (**C**, **D**) Differential expression of JAG1 assayed by immunoblot analysis of JAG1 protein levels (**C**) or mRNA by qPCR (**D**) in the colonic cancer cells. The levels of expression in Caco-2 cells (**A**, **B**, **D**) were used for normalization and the error bars show s.e.m., $n=3$ in each case. *P*-values are obtained using unpaired *t*-test in (**B**, **D**).

respectively. As shown in Supplementary Figure S6B, *ZEB1* mRNA was significantly suppressed by reducing endogenous *JAG1* by siRNAs in the metastatic colon cancer cells HCT-116. By contrast, endogenous *mir-200c∼141* was unable to silence the *JAG1* 3′UTR *luciferase* reporter (Supplementary Figure S6C). We hypothesized that colon epithelial cancer cells may need to produce both miR-200c∼141 and JAG1 to re-gain their epithelial status while safeguarding the cells' capacity for cell proliferation.

Concurrent overexpression of Zfh1/ZEB1 with Notch drives tumour metastasis in Drosophila

Our data suggest that miR-200c may serve as a node in a cancer network, facilitating enhancement of a Notch-Zeb1 axis to foster proliferation with invasion capacity. *Drosophila* homologue of ZEB1, the *zinc-finger homeodomain 1* (*zfh1*) gene (Postigo *et al*, 1999) is a direct target of Notch (Krejci *et al*, 2009) and a predicted target of miR-8 (http://www.flyrnai.org/cgi-bin/DRSC_MinoTar.pl), although this remains to be validated. By analogy to ZEB1, fly *zfh1* regulates myogenic differentiation, cell migration, and somatic stem-cell self-renewal (Broihier *et al*, 1998; Postigo, 2003; Leatherman and Dinardo, 2008). Overexpression of *Delta* and, at less extent *Ser* (Dominguez *et al*, 2004) causes eye growth and none induces dissemination (i.e. benign growth). By contrast, we found that concurrent overexpression of *Ser* (or *Delta*) with *Zfh1/ZEB1* was capable to provoke tumour metastasis. Alone, *zfh1* induced scattered micrometastasis (2/82 flies) and inhibition of endogenous eye/head growth (Supplementary Figure S7A). Co-expressing *Ser* with *zfh1/ZEB1*, animals with distant metastases were seen in 10.41% (5/48 flies), these secondary eye-derived outgrowth were in addition three to five times bigger than the micrometastasis found in flies only expressing *zfh1*. Co-overexpression of *zfh1* with the other Notch ligand, *Dl*, also induced metastasis (∼30% of animals, $n=87$; see also Supplementary Figure S7B–G).

Discussion

We used an unbiased forward genetic screening strategy to identify factors that restrain or boost Notch-induced tumour growth and metastasis in *Drosophila* and uncovered a growth-inhibitory function for the conserved miRNA miR-8 via an unanticipated target Serrate. miRNA target mRNAs are often predicted with significant confidence *in silico*, yet key biologically relevant target genes can be missed because most algorithms rely heavily on the presence of evolutionarily conserved 8mer and 7mer sites that match the seed region of the miRNAs, essentially ignoring all non-conserved sites. Although it has been postulated that non-conserved targeting may represent inconsequential or evolutionarily neutral dampening of gene expression (Bartel, 2009), our results with *Ser* and its human homologue *JAG1*, which were not predicted targets of the *Drosophila* miR-8 or human miR-200c, refute this idea and instead exemplify one of the rare cases of conservation of functional regulatory interactions involving conserved miRNA families.

Intriguingly, miR-200c and miR-200b display complete sequence conservation in the seed region, yet experiments that reduced endogenous expression of the miRNAs failed to validate *JAG1* 3′UTR as a direct target for silencing by miR-200a, miR-200b, and miR-429 in metastatic prostate cancer cells (PC-3 cells). Thus, the presence of conserved seed sites does not guarantee a biological relevant interaction, suggesting that sequences outside the common miRNA seed domain may have a critical role in miRNA target specificity. In addition, our study together with the studies of miR-200b in pancreatic cancer cells suggests that the miR-200c and miR-200b are not fully functionally equivalent in cell proliferation control. miR-200b overexpression stimulates pancreatic cancer cell proliferation via positive regulation of PI3K pathway (Hyun *et al*, 2009). We observed that endogenous depletion of miR-200a, miR-200b, and miR-429 in metastatic prostate cancer cells decreased JAG1 protein (Figure 4E).

We hypothesize that high miR-200b∼429 expression in these cancer cells may influence JAG1 levels indirectly by permitting high PI3K/AKT activity, which is known to stimulate JAG1/NOTCH signalling in prostate cancer cells (Wang *et al*, 2011). Cooperation between Notch and PI3K is known to contribute to uncontrolled proliferation in leukaemia and prostate cancer cells and fly tumourigenesis (Palomero *et al*, 2007; Wang *et al*, 2010). Thus, we speculate that enhanced JAG1 might contribute, to some extent, to the growth-promoting effects of miR-200b miRNA (Gibbons *et al*, 2009; Hyun *et al*, 2009), while JAG1 and Ser would mediate at a large extent the growth-inhibitory function of the miR-200c/8 miRNA in metastatic prostate cancer cells and *Drosophila* epithelial imaginal discs.

The formation of tumours and distant metastases require that tumour cells acquire a capacity to proliferate exhaustively and to colonize foreign tissues. In *Drosophila*, aberrant activation of Notch signalling in the epithelial eye discs, in combination with a cooperating oncogene, triggers massive tumour growth and the spreading of eye-derived tumour cells throughout the body of the fly (Figure 1A and B; Ferres-Marco *et al*, 2006). Using this *Drosophila* cancer paradigm, we unveiled that miR-8 miRNA can function as a potent inhibitor of Notch-mediated tumourigenesis (Figure 1C and D). Moreover, we demonstrate that endogenous *mir-8* expression in the epithelial discs negatively regulates Ser, which induces cell proliferation and survival of eye and wing disc cells. Consistently, eye growth inhibition by *mir-8* overexpression (e.g. Figure 2C) was fully abrogated by a *Ser* transgene (Figure 2K; Supplementary Figure S2A). Similarly, wing size defect of *mir-8*$^{-/-}$ flies (Figure 3J) was rescued by reducing endogenous *Serrate*, implying that the wing size defect is largely caused by elevated Serrate (Figure 3F and G). More significantly, overexpression of human homologues of *mir-8*, *mir-200c*, and *mir-141*, in metastasis-prone prostate cancer cells, which express low endogenous levels of these miRNAs and high JAG1 protein levels, decreased aberrant *JAG1* expression accompanied by a potent antiproliferation effect (Figures 4 and 5B). Overexpression of *JAG1* cDNA without its 3′UTR restored growth in the prostate cancer cells overexpressing the miRNAs. Interestingly, *mir-200c* expression is silenced by epigenetic mechanisms in various human malignancies, including metastatic prostate cancers and cell lines (Vrba *et al*, 2010). Thus, restoration of *mir-200c* expression through the application of demethylating epigenetic drugs might represent a new way to inhibit NOTCH signalling in some metastatic cancers.

Nonetheless, our study also emphasizes that restoring mir-200 family gene expression in some cancer types may be unsafe. Metastatic epithelial cancers such as colon cancer re-activate expression of mir-200c gene in growing metastases concurrently with high expression of the targets ZEB1 and JAG1. We did not find evidence that endogenous miR-200c∼141 silences the *JAG1* 3′UTR in the metastasis-prone colon cancer cell HCT-116 and hence speculate that a mechanism exists in these epithelial cancer cells to protect the *JAG1* 3′UTR from silencing by these miRNAs, as proposed for the differential regulation of *nanos1* expression in the soma and germline by miR-430 (Mishima *et al*, 2006), or the stress-dependent modulation of human *CAT-1* mRNA by miR-*122* via the binding of HuR to AU-rich elements in the *CAT-1* 3′UTR (Bhattacharyya *et al*, 2006). Although we do not exclude the possibility that silencing occurs in a small subset of cells in the population of HCT-116 cells, we reason that protecting JAG1 3′UTR from a silencing may allow cancer cells safeguarding cell proliferation capacity while re-gaining epithelial status by re-expressing miR-200c.

The identification of JAG1–miR-200 interactions has several other implications that may be relevant to cancer metastasis. JAG1 is a transmembrane ligand that signals NOTCH receptors *in trans*, we hypothesize that high JAG1 caused by loss of miR-200 in metastatic cell likely facilitates interactions between tumour cells and between the tumour and the host cells (see model in Supplementary Figure S8). In this context, it is noteworthy that advanced prostate cancer almost always metastasizes to the bone (Gupta and Massague, 2006), although the mechanisms underlying this tendency remain unclear. The seed-and-soil hypothesis predicts that metastatic cells only colonize compatible target tissues and that specific signals from the microenvironment (the soil) sustain cancer cell growth (the seed) (Fidler, 2003). Given that endogenous activation of Notch signalling is critical for normal bone homeostasis (Hilton *et al*, 2008), the influence of JAG1 on tumour cells may aid metastatic colonization of the bone, while signals from the bone metastasis (e.g. TGFβ), known to inhibit *mir-200c* expression in tumour cells (Gregory *et al*, 2008), along with feedforward and negative feedback loops between the miR-200/ZEB1/JAG1 (Burk *et al*, 2008; Wellner *et al*, 2009; this study) would establish a 'vicious circle' in the metastatic bone microenvironment (model Supplementary Figure S8), as may also apply to the organ tropism of other cancers.

Finally, the recent pioneering work by the Weinberg group (Mani *et al*, 2008) has shown that the molecular circuits that promote EMT also confer stem-cell properties to the resulting mesenchymal primary tumour cells (Mani *et al*, 2008). Importantly, the loss of *mir-200* genes, particularly *mir-200c*, seems to be a crucial route in normal and cancer cells to promote EMT and stem-cell regulation (Shimono *et al*, 2009; Wellner *et al*, 2009) by modulating the expression of target genes such as ZEB1 (Wellner *et al*, 2009). Our study shows that concurrent, but not individual, overexpression of Notch ligand Ser (or Delta) and Zfh1/ZEB1 triggers massive metastatic outgrowths *in vivo*. Thus, it is tempting to speculate that an important consequence of the loss of *mir-200c* during cancer progression is to enhance a JAG1/ZEB1 axis, thereby facilitating initiation or maintenance of metastatic growth following dissemination.

Materials and methods

Genetics and fly husbandry

The *GS(2)SC1* line was isolated in a genetic screen for enhancer/suppressors of a large eye phenotype caused by *Dl* overexpression, *GS(2)88A8* (*eyeful*) (Ferres-Marco *et al*, 2006). *mir-8-Gal4*, *mir-8*$^{\Delta 2}$, and *mir-8*$^{\Delta 3}$ (gift from SM Cohen: Karres *et al*, 2007), *atrophin*^{35} (also known as *Gug*^{35}), *UAS-Zfh1*, *UAS- UAS-zhf1*^{CIDm} (Postigo, 2003), and *UAS-atro* (gift from S Kerridge), *dicer-1*^{Q1147X} (Lee *et al*, 2004), *P{lacW*Eq-1 (gift from H Sun). A detailed description of the following stocks and transgenic flies used in this study can be found at http://flybase.org/: *Ser*^{RX106}, *P{lacW*mirr^{P69Df7}, *ey-Gal4*, *dpp-Gal4*, *vg-Gal4*, *MS1096-Gal4*, *en-Gal4*, *omb-Gal4*, *GMR-Gal4*, *hsp70-Gal4*, *hairy-Gal4*, *UAS-Dl*, *UAS-Ser*, *UAS-lacZ*, *UAS-Flp*, and *Act>stop>lacZ*. All combinations of *Gal4*, and the different GS and UAS lines and mutants were raised at 25°C.

Immunofluorescence, antibodies, and in situ hybridization

Imaginal discs were fixed and stained by standard procedures using the following primary antibodies/dilutions/sources: anti-Serrate (1:100), anti-β-galactosidase (1:500; Cappel), anti-cleaved Caspase-3 (1:200; Cell Signaling Technologies), 4D4 (anti-Wg; 1:100), 4F3 (anti-Arm; 1:100), and anti-Elav (1:50; all from the Developmental Studies Hybridoma Bank). The secondary antibodies used were conjugated to Cy3, FITC, or Cy5 (Jackson Immunoresearch), all diluted at 1:200. Images were captured on a Leica TCS-NT confocal microscope.

In situ hybridization was performed as described (Li and Carthew, 2005), and the *miR-8* probe (Exiqon) was end-labelled with a DIG oligonucleotide 3′-end labelling kit (Roche) and hybridized at 42°C.

miRNA-mRNA 3′UTR aligment

Binding sites for miR-8 in the *Ser* 3′UTR, and for human miR-141, miR-200a, miR-200b, miR-200c, miR-429 in the 3′UTRs of human *JAG1*, were identified using the BiBiServ server (Rehmsmeier *et al*, 2004). Seed sequences with G:U wobbles or mismatches within the 7-6-mer seed sequence were not considered, and only miRNA: target-mRNA duplexes with a free energy below −15 Kcal/mol were considered.

Luciferase reporter assays

For *Drosophila* S2 cell luciferase assays, cells were co-transfected in 24-well plates with the *tub-mir-8* plasmid (250 ng; Karres *et al*, 2007), the *luciferase-Ser-UTR* or *luciferase-Ser-UTR-mut* constructs (25 ng) and the *Renilla luciferase* plasmid (25 ng) for normalization. The relative luciferase activity was measured after 60 h post-transfection. Two human prostatic cell lines, immortilized non-malignant prostatic cell line PNT1A (Mitchell *et al*, 2000) and metastatic PC-3 cells, and the colon cancer epithelial cell line HCT-116 were used for human *JAG1* luciferase assays. The *JAG1* 3′UTR sequence was inserted into the pRL-TK plasmid (Promega). Target sites were mutated using the QuickChange Site-Directed Mutagenesis kit (Stratagene; see Figure 4C). Cells were co-transfected in 24-well plates with the *pRL-TK-JAG1-UTR* or *pRL-TK-JAG1-UTR-mut* construct (200 ng) and the pGL3-control plasmid (20 ng; Promega) for normalization. The relative luciferase activities were determined 48 h after transfection and in all cases, lucifesase activity was measured using Dual-Glo Luciferase Assay system (Promega).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We especially thank SM Cohen for reagents and support, R Carthew for help with the *in situ* hybridization of *mir-8*, M Malumbres for providing the miR-141- and miR-200c-expressing vectors, E Ballesta and D Ferres-Marco for technical assistance. We thank F Viana for the prostate cell lines, Ll Espinosa and A Bigas for colon cancer cell lines and pcDNA3-human JAG1 vector, and A Ferrando, C Lopez-Otin, L Garcia-Alonso, E Herrera, A Carmena, J Bolivar, A Nieto, and the members of the Dominguez laboratory for their critical comments on this work. The work in the laboratory of MD is funded by the Ministerio de Ciencia e Innovación (BFU2009-09074 and MEC-CONSOLIDER CSD2007-00023), the Asociación Española Contra el Cáncer, Generalitat Valenciana (PROMETEO 2008/134), and an European Union Research Grant UE-HEALH-F2-2008-201666. DMV was a FPI fellow. DMV is supported by Fundación Marcelino Botín.

Author contributions: DMV performed all the experiments and analyses and contributed to the design and writing of the manuscript; EC made the initial discovery of Zfh1's metastatic capacity and helped with Zfh1/Notch experiments; MD contributed by defining the general concept, by designing the study, as well as interpretation of the data, supervising the work, and by writing the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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