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.

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 (JAGGED1, JAG1, JAG2) families have been characterized in humans. As 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).
Notch receptor or ligand overexpression is associated with Drosophila eye tumours that exhibit hallmarks of mammalian cancers such as uncontrolled overgrowth, invasion, and metastasis (Paglarini 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 eyespecific 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 deregression of ZEB1 and Bmi1 (Shimoto 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% of 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-Gal4 > 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.
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 signalling. 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% (SerRX106 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...
The loss of endogenous mir-8 in the mutant condition mir-8Δ2/Δ3 (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 represses 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 mutants caused complete growth arrest (Karres et al., 2007; Hyun et al., 2009). Together, the gain- and loss-of-function analysis of mir-8 and functional validation in vivo unveiled 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 in PC-3 cells, and indicates that within the miR-200 family, produced by the endogenous activity of the miRNAs of a common family, mir-200b in other cancer cell lines (Bracken et al., 2008; Korpal et al., 2008) with the concurrent co-overexpression of mir-200b ~ 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 this 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
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

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/miR-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.
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, 200c, and -141 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 up-regulated in PC-3 cells (Scorey et al., 2006). Pharmacological inhibition of NOTCH signalling by the γ-secretase inhibitor DAPT (N-[N-(3,5-difluorphenacetyl-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 over-expression 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-200c~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
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 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 its 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).
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 least 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 micrometastases (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 tumors and distant metastases require that tumor 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 (Bhattacharya 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 which 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)SCI line was isolated in a genetic screen for enhancer/suppressors of a large eye phenotype caused by Di overexpression, GS(2)88A8 (eyeful) (Ferres-Marco et al., 2006). mir-8-Gal4, mir-8S66, and mir-8GH (gift from SM Cohen: Karres et al., 2007), atrophin (also known as Gag (Bhattacharyya et al., 2006), UAS-Zh1, UAS- UAS-zh1126 (Postigo, 2003), and UAS-atro (gift from S Kerridge), dicer-1O14TX (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: SER3500, P{lacW}mirr8660, ey-Gal4, dpp-Gal4, vg-Gal4, MS1095-Gal4, en-Gal4, omb-Gal4, GMR-Gal4, hsp70- Gal4, hairy-Gal4, UAS-DI, 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, FTTC, 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 alignment

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 (250 ng; Karres et al., 2007). The relative luciferase activity was measured after 60 h post-transfection. Two human prostatic cell lines, immortalized non-malignant PCA3 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, luciferase 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).

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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 Zhl1’s metastatic capacity and helped with Zhl1/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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The EMBO Journal VOL 30 | NO 4 | 2011
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