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A conserved function of the chromatin ATPase Kismet in the regulation of *hedgehog* expression

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ABSTRACT

The development of the *Drosophila melanogaster* wing depends on its subdivision into anterior and posterior compartments, which constitute two independent cell lineages since their origin in the embryonic ectoderm. The anterior–posterior compartment boundary is the place where signaling by the Hedgehog pathway takes place, and this requires pathway activation in anterior cells by ligand expressed exclusively in posterior cells. Several mechanisms ensure the confinement of *hedgehog* expression to posterior cells, including repression by *Cubitus interruptus*, the co-repressor Groucho and Master of thick veins. In this work we identified Kismet, a chromodomain-containing protein of the SNF2-like family of ATPases, as a novel component of the *hedgehog* transcriptional repression mechanism in anterior compartment cells. In *kismet* mutants, *hedgehog* is ectopically expressed in a domain of anterior cells close to the anterior–posterior compartment boundary, causing inappropriate activation of the pathway and changes in the development of the central region of the wing. The contribution of Kismet to the silencing of *hedgehog* expression is limited to anterior cells with low levels of the repressor form of *Cubitus interruptus*. We also show that knockdown of CHD8, the *kismet* homolog in *Xenopus tropicalis*, is also associated with ectopic *sonic hedgehog* expression and up-regulation of one of its target genes in the eye, *Pax2*, indicating the evolutionary conservation of Kismet/CHD8 function in negatively controlling *hedgehog* expression.

Introduction

The Drosophila wing imaginal disc is subdivided into anterior and posterior compartments that grow since their specification as clonally segregated territories (Garcia-Bellido et al., 1973). The identity of the posterior compartment depends on the activity of *engrailed* (*en*), which is expressed in posterior cells (Kornberg et al., 1985). In these cells, *en* represses the transcription of *Cubitus interruptus* (*Ci*), restricting *Ci* expression to the anterior compartment (Dominguez et al., 1996; Chanas et al., 2004; Bejarano and Milan, 2009). The absence of *Ci* in posterior cells allows the expression of *hedgehog* (Hh), the ligand of the Hh signaling pathway (reviewed in Ingham and McMahon, 2001; Hooper, 2003). Complementary, the presence of *Ci* in anterior cells limits the transcriptional responses to Hh signaling to anterior cells exposed to the ligand (Dominguez et al., 1996). The subdivision of the wing into posterior, *hh*-expressing cells, and anterior, Hh-transducing cells, is paramount to 1) maintain the clonal segregation between compartments (Rodriguez and Basler, 1997), and 2) establish nested domains of Hh-target gene expression in anterior cells (Crozatier et al., 2002).

Hh transduction in anterior cells prevents the proteolytic processing of Ci from a full-length form that function as a transcriptional activator (Ci^{155}), to a shorter form of the protein (Ci^{75}) that represses the expression of target genes (Aza-Blanc et al., 1997; Méthot and Basler, 1999). Thus, Ci^{155} accumulation is maximal in cells closer to the antero-posterior compartment boundary, the cells exposed to higher amounts of Hh, and Ci^{75} is the only form present in cells located away from this boundary and consequently out of reach of the Hh protein (Aza-Blanc et al., 1997; Alexandre et al., 1996). In this manner, the ratio between Ci^{155} and Ci^{75}, which depends on the amount of Hh available to the cell, determines the transcriptional output of the pathway (Methot and Basler, 2001). Ci regulates in anterior cells the expression of several target genes such as *patched* (*ptc*), *master of thick veins* (*mtv*), *knot* (*kn*), *decapentaplegic* (*dpp*), *engrailed* (*en*) and the *araucan* and *caupolican* genes of the *Iroquois* complex (*iro*). All these genes are expressed in stripes several cells wide parallel to the antero-posterior boundary (Alexandre et al., 1996; Méthot and Basler, 1999; Vervoort et al., 1999; Tanimoto et al., 2000; Mohler et al., 2000; Crozatier et al., 2002), and their expression is critical to establish the pattern of the central region of the wing, including the L3 and L4 longitudinal veins and the L3/L4 intervein (Hidalgo, 1994; Basler and Struhl, 1994; Mullor et al., 1997; Vervoort et al., 1999; Crozatier et al.,

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2002). Furthermore, because *dpp* is required for patterning and growth of the entire wing, Hh signaling indirectly is also needed for the development of the most anterior and posterior regions of the wing (Posakony et al., 1990; Sanicola et al., 1995; Zecca et al., 1995).

There are several mechanisms contributing to the restriction of *hh* expression to posterior cells. First, *en* and *hh* transcription are repressed in anterior cells by the activity of Polycomb complexes (Busturia and Morata, 1988; Maurange and Paro, 2002; Chanas et al., 2004; Chanas and Mashat, 2005; Schwartz and Pirrotta, 2007). Second, Ci^{75} acts as a direct repressor of *hh* throughout the anterior compartment (Dominguez et al., 1996; Méthot and Basler, 1999; Methot and Basler, 2001). Finally, Mtv, a direct target of Ci^{155}, in combination with the co-repressor Gro represses *hh* in anterior cells close to the dorso-ventral boundary (Funakoshi et al., 2001; Bejarano et al., 2007; de Celis and Ruiz-Gomez, 1995). The existence of several mechanisms to repress *hh* in anterior cells close to the compartment boundary is in part a necessity imposed by the presence of En and low levels of Ci^{75} in these cells. Thus, the expression of *en* in anterior cells during the third larval instar (Blair, 1992) implies that *Ci* expression is reduced in these cells. Furthermore, the level of Ci^{75} here is very low or absent due to high levels of Hh signaling, and consequently Ci^{75}-independent mechanisms of *hh* transcriptional repression are needed to prevent *hh* expression in these cells.

In this work we identify Kismet (Kis) as a novel component of the mechanism that represses *hh* expression in anterior cells. Kis is a member of the *trithorax* group (trxG) of genes, and is required for segmentation and for the determination of body segment identities (Daubresse et al., 1999). Initially, *kis* was identified in a screen for suppressors of *Polycomb* (*Pc*) (Kennison and Tamkun, 1988), but it has also been identified in other genetic screens searching for genes involved in the Notch and EGFR signaling pathways (Go and Artavanis-Tsakonas, 1998; Therrien et al., 2000). Kis encodes three protein isoforms, two long (KisL, named Kis-PA and Kis-PC), of 5322 and 5517 amino acids respectively, and one shorter form (KisS or Kis-PB) of 2151 amino acids (Daubresse et al., 1999; Therrien et al., 2000). The long isoforms contain an SNF2-like ATPase domain that is conserved in chromatin remodeling proteins such as Brahma, ISWI, Mi-2 and CHD1 (Daubresse et al., 1999) and two chromodomains that are conserved in proteins that interact with chromatin, including Pc, heterochromatin-associated protein 1 (HP1) and the chromodomain helicase DNA binding domain (CHD) family of chromatin remodeling enzymes (Cavalli and Paro, 1998). The three Kis isoforms share a 45 amino acid domain named BRK that is also found in the CHD6–9 proteins and in the Brahma/BRG1 families. The vertebrate Kis homolog CHD8 binds to the protein insulator CTCF, and this interaction mediates transcriptional insulation (Allen et al., 2007; Ishihara et al., 2006). In addition, mutations in the BRK domain of human CHD7 cause the CHARGE syndrome, which is characterised by congenital abnormalities caused by the abnormal development of the neural crest (Vissers et al., 2004; Bajpai et al., 2010).

We suggest that Kis chromatin remodeling activity represses the transcription of *hh* by making its regulatory region accessible to repressors in anterior cells with low levels of Ci^{75}. In this manner Kis contributes to the silencing of *hh* in these anterior cells and to the maintenance of the boundary between cells expressing and not-expressing *hh*. This function of Kis seems evolutionarily conserved in vertebrates, as we found that *CHD8*, a *kis* homolog, is required *in X. tropicalis* embryos for the repression of *Sonic hedgehog* (*Shh*).

Methods

Drosophila stocks

We have used the following stocks: *nub-Gal4*, *hh-Gal4* and *ap-Gal4* (Calleja et al., 1996), *UAS-GFP* (Ito et al., 1997), *UAS-hh* (Ingham and Fietz, 1995), *UAS-iKis* (3696R-1; NIG-fly), *UAS-iCi* (2125R-1; NIG-fly), *UAS-iSmo* (9542; VDSC), *UAS-*Ci^{Cell}*-HA* (Méthot and Basler, 1999), *P{PZ}*hh^{P30}, *hh-lacZ* (*P{PZ}P2023-44*), kis^{1} (Daubresse et al., 1999), kis^{172A3}, kis^{165A1}, kis^{61C}, kis^{29D4}, and kis^{59C3} (Terriente-Felix et al., 2010) and the deficiencies *Df(2L)net-PMF*, *Df(2L)Exel6001* and *Df(2L)ED21*. Unless otherwise stated, crosses were done at 25 °C. Wings were mounted in lactic acid-ethanol (1:1) and photographed with a Spot digital camera and a Zeiss Axioplan microscope. Lines not described in the text can be found in flybase (Gelbart et al., 1997).

Isolation of novel kis alleles by ENU mutagenesis

kis^{172A3}, kis^{165A1}, kis^{61C}, kis^{29D4}, and kis^{59C3} were isolated by ethyl nitrous urea (ENU) treatment of an *al dp b pr FRT40A* chromosome following standard procedures (Ashburner, 1989).

Generation of FLP-FRT clones

We induced clones of *kis* mutant cells in larvae of the following genotypes:

- *w* $hsFLP^{1.22}$ f^{36a}; kis^{1} *FRT40A / ck* $P[f^{+}]^{30A}$ *FRT40A* (100).
- *w* $hsFLP^{1.22}$ f^{36a}; kis^{1} *FRT40A / M(2)z* $P[f^{+}]^{30A}$ *FRT40A* (190).
- *w* $hsFLP^{1.22}$ f^{36a} */ +; kis*^{61C} *FRT40A / 2X[tubGFP] FRT40A; hh-lacZ*^{P30} */ +* (25).
- *w* $hsFLP^{1.22}$ */ +; kis*^{61C} *FRT40A / 2X[tubGFP] FRT40A; hh-lacZ*^{l32} */ +* (19).
- *w* sal^{EPv}*-Gal4 / +; kis*^{61C}*al dp b FRT40A / M(2)z FRT40A; UAS-FLP / +* (30).
- *w 638-Gal4 / +; kis*^{61C} *al dp b FRT40A / M(2)z FRT40A; UAS-FLP / +* (30).
- *w; kis*^{61C} *FRT40A / M(2)z FRT40A; hh-Gal4 / UAS-FLP* (15).
- $hsFLP^{1.22}$ *actGal4 UAS-GFP; kis*^{1} *FRT40A / tubGal80 FRT40A* (117).
- $hsFLP^{1.22}$ *actGal4 UAS-GFP; FRT40A / tubGal80 FRT40A; UAS-iCi / +* (56).
- $hsFLP^{1.22}$ *actGal4 UAS-GFP;* kis^{1} *FRT40A / tubGal80 FRT40A; UAS-iCi / +* (65).
- $hsFLP^{1.22}$ *actGal4 UAS-GFP; FRT40A / tubGal80 FRT40A; UAS-ismo / +* (61).
- $hsFLP^{1.22}$ *actGal4 UAS-GFP;* kis^{1} *FRT40A / tubGal80 FRT40A; UAS-ismo / +* (99).
- $hsFLP^{1.22}$ *actGal4 UAS-GFP; FRT40A / tubGal80 FRT40A; UAS-*Ci^{Cell}*HA / +* (58).
- $hsFLP^{1.22}$ *actGal4 UAS-GFP;* kis^{1} *FRT40A / tubGal80 FRT40A; UAS-*Ci^{Cell}*HA / +* (73).

In brackets we indicate the number of clones analysed for each genotype. In average we studied at least 20 discs each containing from two to seven independent clones.

Immunocytochemistry

We used rabbit anti-KisL (Srinivasan et al., 2005) and anti-activated Cas3 (Cell Signalling), rat anti-Iro (Gómez-Skarmeta and Modolell, 1996) and anti-Ci (Aza-Blanc and Kornberg, 1999) and mouse anti-βGalactosidase (Promega). The anti-Iro antibody recognises two proteins of the Iro-C, Araucan and Caupolican, which are expressed in the same pattern during wing development. From the Hybridoma bank at Iowa University we used the mouse monoclonals anti-En, anti-Ptc, anti-Smo and anti-HA. Secondary antibodies were from Jackson Immunological Laboratories (used at 1/200 dilution). Imaginal wing discs were dissected, fixed and stained as described in (de Celis, 1997).

In situ hybridization

We used dygoxigenin-labelled RNA probes synthesise from the *hh* cDNA clone LD10183. Third instar larvae were dissected in PBS and fixed 25 min in 4% paraformaldehide, washed three times for 5 min in

PBT-0.1% Tween20, and re-fixed 20 min in paraformaldehide 4% + 0.1% Tween20. After several washes in PBT-0.1% Tween20, the carcases were kept at −20 °C in hybridisation solution (HS: 50% Formamide, SSC 5X, 100 μg/ml DNA salmon sperm, 50 μg/ml heparine, 0.1% Tween20). The hybridization was carried out overnight at 55 °C with 2 μl of probe in 100 μl of HS (previously de-naturalised by 10 min incubation at 80 °C). Excess of probe was washed at 55 °C in HS, discs washed several times in PBT-0.1% Tween20, and incubated for 2 h with anti-Digoxigenine antibody (Roche) in a 1:4000 dilution in PBT-0.1% Tween20. The colour reaction was carried out in 100 mM NaCl, 50 mM MgCl2, 100 mM Tris-HCl pH 9.5, 0.1% Tween20, nitroblue tetrazolium chloride and bromo-chlro-indolyl-phosphate (Roche). After the colour developed, the discs were rinsed several times in PBT-0.1% Tween20, dissected in 30% Glycerol and mounted in 70% Glycerol.

Xenopus tropicalis morpholino injections and in situ hybridization

A *Xenopus tropicalis* morpholino to knockdown the translation of CHD8 (MOCHD8) was designed to bind to the donor splice site between exon 12 and intron 12 (5′-ATGACTGTCAAATTTCTCACCTATT-′3). A total of 7.5 ng of this morpholino was injected into one cell at the two cell-stage embryos. To evaluate the inhibition of MOCHD8 on *CHD8* mRNA splicing, we designed primers in exons 10 and 14 (5′-CACAGCACTGACAAGGACAATGG-3′ and 5′-CTGTTTTTGAGCCGAT-GAGCC-3′). A band of 1127 bp should be produced only if the morpholino inhibits correct removal of intron 12. The inclusion of this intron in the mRNA introduces several precocious stops codons. For RT-PCR total RNA was extracted from 5 stage 25 morphants and control embryos and amplification was carried out for 30 cycles. For in situ hybridization, specimens were prepared, hybridized and stained as described (Harland, 1991). X-Gal staining was carried out according to (Coffman et al., 1993).

Results

The phenotype of Kismet mutant wings

We isolated in a loss-of-function mutagenesis screen a complementation group composed by five alleles that were lethal in combination with the deficiencies *Df(2L)net-PMF* and *Df(2L)Exel6001* (Terriente-Felix et al., 2010). These alleles also failed to complement the kis^{1} mutation, and therefore were classified as novel alleles of *kis*. To identify the genetic requirements of *kis* during wing development, we studied the phenotype of mosaic wings composed of *kis* mutant cells. These wings were generated by driving the expression of FLP using two different Gal4 drivers, sal^{EPv}*-Gal4* (Cruz et al., 2009) and *638-Gal4* (Molnar et al., 2006), in a genetic background bearing *kis* alleles in a *FRT40A* chromosome. Homozygous *kis FRT40A* clones were, in addition, associated with the loss of a *Minute* allele to increase the size of the clones (see Material and methods). We obtained similar phenotypes using kis^{1} and the novel alleles kis^{172A3}, kis^{165A1}, kis^{61C} and kis^{59C3}. Thus, when the clones were induced in the domain of sal^{EPv}*-Gal4* expression (L2–L5 interval), we found the differentiation of ectopic veins close to the position of the longitudinal veins in both the anterior and posterior compartments and a broadening of the L3/L4 intervein region (Figs. 1A–F). When mutant *kis* territories occupy the entire wing we found a reduction of wing size, loss of the L2 vein and increase in the distance between the L3 and L4 veins (Figs. 1A′–F′). Taken together, these phenotypes indicate a role of Kis in the regulation of wing size and in the generation of the vein pattern.

We also reduced Kis levels by expressing an interference *kis* RNA (*ikis*) directed against the KisS and KisL isoforms. The expression of *ikis* is very effective in reducing the levels of Kis (Figs. 2C–C′), and we found the formation of ectopic veins in adult *ikis* wings (Figs. 2A–B). However, the size of the wing and the distance between L3 and L4 were normal in these wings, indicating that the use of *kis* alleles uncovers several requirements for the gene that are not revealed in *ikis*-expressing wings. To analyse the cell autonomy of the *kis* phenotype we analysed labelled *kis* clones induced in different regions of the wing. The phenotype of *kis* clones was striking, because *kis* mutant cells caused different phenotypes depending on the wing region where they appear. Thus, the reduction of Kis in the posterior compartment causes the formation of ectopic veins of normal thickness and dorso-ventral characteristics (Fig. 2H). A similar phenotype was observed when the entire posterior compartment was made homozygous for the kis^{61C} allele (Fig. 2G). In the anterior compartment, *kis* clones located in the L2 vein or in the region

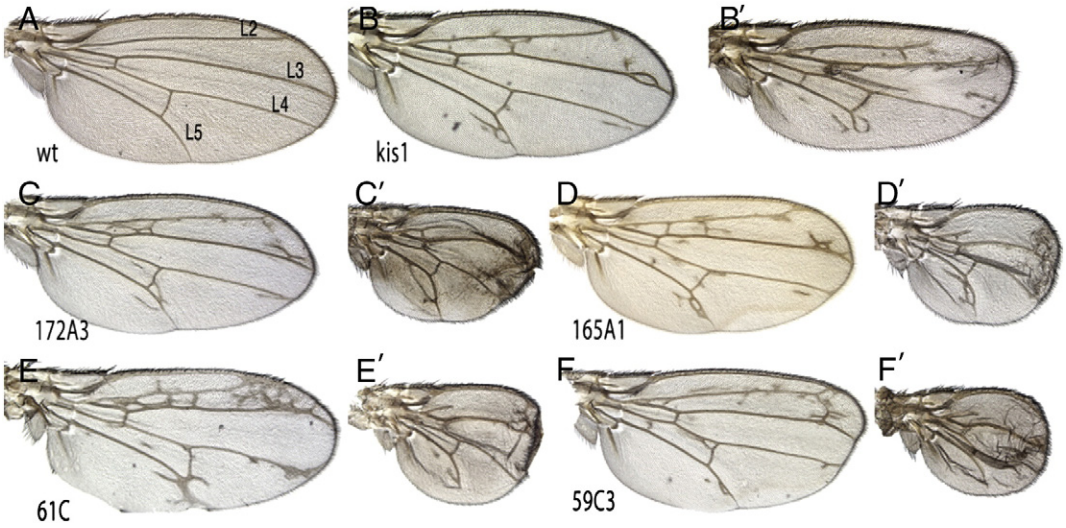

Fig. 1. Loss-of-function phenotypes of *kis* mosaic wings. (A) Wild type wing showing the longitudinal veins (L2–L5). (B–B′) Wings of sal^{EPv}*-Gal4 / +; kis^{1} FRT40A / M(2)z FRT40A; UAS-FLP / +* (B) and *638-Gal4 / +; kis^{1} FRT40A / M(2)z FRT40A; UAS-FLP / +* (B′) genotype. (C–C′) Wings of sal^{EPv}*-Gal4 / +; al dp kis^{172A3}FRT40A / M(2)z FRT40A; UAS-FLP / +* (C) and *638-Gal4 / +; al dp kis^{172A3} FRT40A / M(2)z FRT40A; UAS-FLP / +* (C′) genotype. (D–D′) Wings of sal^{EPv}*-Gal4 / +; al dp kis^{165A1} FRT40A / M(2)z FRT40; UAS-FLP / +* (D) and *638-Gal4 / +; al dp kis^{165A1}FRT40A / M(2)z FRT40A; UAS-FLP / +* (D′) genotype. (E–E′) Wings of sal^{EPv}*-Gal4 / +; al dp kis^{61C} FRT40A / M(2)z FRT40A; UAS-FLP / +* (E) and *638-Gal4 / +; al dp kis^{61C} FRT40A / M(2)z FRT40A; UAS-FLP / +* (E′) genotype. (F–F′) Wings of sal^{EPv}*-Gal4 / +; al dp kis^{59C3} FRT40A / M(2)z FRT40A; UAS-FLP / +* (F) and *638-Gal4 / +; al dp kis^{59C3} FRT40A / M(2)z FRT40A; UAS-FLP / +* (F′) genotype. In all cases, *kis* clones were induced in the domain of sal^{EPv}*-Gal4* expression (B–F) or in the entire wing (B′–F′). The phenotype of mutant *kis* mosaic wings consists in the differentiation of ectopic veins (B–F′), the loss of L2 stretches (B′, C′, D′, E′ and F′) and the increase in the distance between the L3 and L4 veins (B–F′).

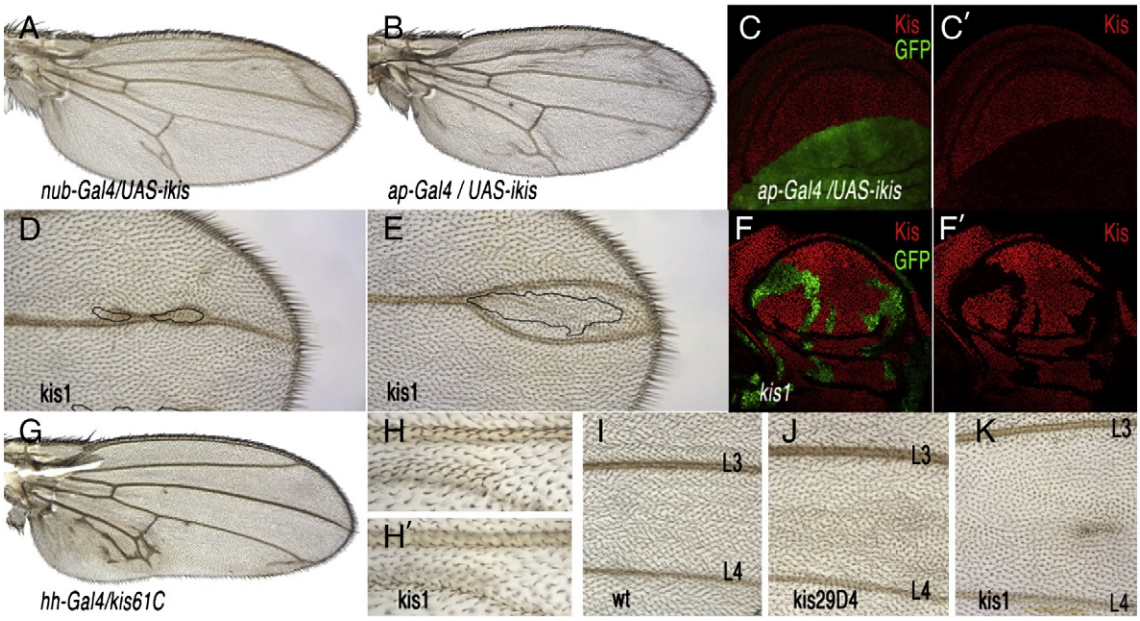

Fig. 2. Phenotype of *kis* loss-of-function clones in the wing. (A–C) Effects of the expression of interference RNA against *kis* (*ikis*). Expression of *ikis* in the wing blade and hinge (*nub-Gal4/UAS-ikis*; A) or in the dorsal compartment (*ap-Gal4/UAS-ikis*; B) cause the formation of ectopic longitudinal veins. (C–C′) Expression of Kis (red) in *ap-Gal4 UAS-GFP/UAS-ikis* third instar wing disc. Kis (red in C–C′) is not detected in the dorsal compartment (labelled in green in C). (D–E) Example of two kis^{1} clones (enclosed by black lines) in the dorsal side of the L3 vein, causing non-autonomous formation of L3 vein by wild type cells. (F–F′) Loss of Kis expression (red) in kis^{1} clones located in the wing blade. Clones are labelled by the expression of GFP (green), and were induced in *hsFLP1.22 act-Gal4 UAS-GFP*; kis^{1}*FRT40A/tubGal80 FRT40A* larvae. Kis is not detected in kis^{1}mutant cells. (G) Mosaic wing in which the posterior compartment is formed by kis^{61C} mutant cells generated in kis^{61C} *FRT40A/M(2)z FRT40A; hh-Gal4/UAS-FLP* flies. The posterior compartment is smaller and differentiates ectopic veins. (H–H′) High magnification of the dorsal (H) and ventral (H′) side of the L3–L4 intervein showing the formation of ectopic veins by kis^{1} mutant cells (labelled with *forked*). (I–K) High magnification of the L3–L4 intervein in a wild type wing (I), and in wings carrying *kis* clones occupying a small part of the intervein (kis^{29D4}; J) and a large fraction of the same intervein (kis^{1}; K). In both cases there is an increase in the distance between the L3 and L4 veins.

anterior to this vein were normal (data not shown). In contrast, *kis* clones located adjacent or in the L3 vein cause the duplication of this vein. *kis* cells contributed to the L3 when the size of the clones was very small (Fig. 2D), but in most cases *kis* cells differentiate as intervein, and the ectopic L3 vein was composed by wild type cells surrounding the mutant clone (Fig. 2E). Finally, *kis* clones located between the L3 and L4 veins cause a variable increase in the width of the L3/L4 intervein (Figs. 2I–K). This increase is proportional to the fraction of *kis* mutant cells occupying the L3/L4 intervein (Figs. 2I–K). We confirmed that the expression of Kis is strongly reduced or absent in kis^{1} mutant cells (Figs. 2F–F′). In summary, we found that Kis has a dual requirement during wing development. On one side, Kis is required to antagonize the formation of veins in some intervein regions of the anterior and posterior compartments, and on the other Kis is needed for the correct patterning of the L3 vein and L3/L4 intervein. In what follows, we will focus in the analysis of this second aspect of Kis function.

Loss of Kis causes ectopic Hh signaling in the anterior compartment

The patterning of the central region of the wing depends on Hh signaling (Gómez-Skarmeta and Modolell, 1996; Mullor et al., 1997; Vervoort et al., 1999; Crozatier et al., 2002), and in this manner some of the defects observed in *kis* mutant clones could be caused by alterations in the transcriptional response to Hh. We looked at the expression of three Hh target genes in anterior compartments bearing *kis* mutant clones. We chose *ptc*, *en* and the genes of the *Iroquois* complex (*Iro*), because their expression is activated by different levels of Hh signaling (Blair, 1992; Sanicola et al., 1995; de Celis and Ruiz-Gomez, 1995; Gómez-Skarmeta and Modolell, 1996; Methot and Basler, 2001). The anterior expression of *en* is required for the formation of the L3/L4 intervein (Hidalgo, 1994), and the genes of the Iroquois complex (Iro) promote the formation of the L3 vein (Gómez-Skarmeta et al., 1996). In wild type discs the expression of Ptc and En is regulated by high levels of Hh signaling, and is restricted to narrow stripes of cells abutting the A/P boundary (Figs. 3A and C). We found that these two genes are ectopically expressed in a cell-autonomous manner in *kis* mutant clones located in anterior cells close to the A/P boundary (Figs. 3B–B′ and D–D′). The expression of Iroquois, which in wild type discs is activated by low levels of Hh signaling and occurs in cells anterior to the domain of En expression (Fig. 3E), is also detected in *kis* clones, but in this case Iro is expressed both by *kis* mutant cells and by wild type cells abutting the clones (Figs. 3F–F′). This effect most likely explains the non-autonomous appearance of ectopic L3 veins observed in *kis* mutant clones. We also studied the expression of Smo, which accumulation in the cell membrane implies high levels of Hh signaling (Denef et al., 2000; Ingham et al., 2000; Zhu et al., 2003; Nakano et al., 2004), but did not detect any change in Smo accumulation in *kis* mutant clones (Figs. 3G, H–H′). Taken together, these observations are compatible with the generation of low levels of Hh activity in both *kis* mutant cells and in its neighboring wild type cells, but only when the clones are located in close proximity to the normal domain of Hh signaling.

Kis regulates Hh expression in the anterior compartment

The non-autonomous effects of *kis* mutant cells suggest that the expression of *hh* might be affected in these clones. The expression of *hh* is restricted to the posterior compartment in wild type discs (Figs. 4A, C and G). We found that *kis* mutant cells located in the anterior compartment and close to the A/P boundary express low levels of two *hh* lacZ reporters (Figs. 4B, E and F). The ectopic expression of *hh* was detected even in very small clones, but only when they were in close proximity to the A/P boundary (Figs. 4E and F). Interestingly, the heterozygosity of *kis* also causes ectopic expression of the *hh-lacZ* reporter in this territory, although at very low levels (white bracket in Fig. 4D). We confirmed that *hh* is ectopically expressed in *kis* mutant conditions by making *in situ*

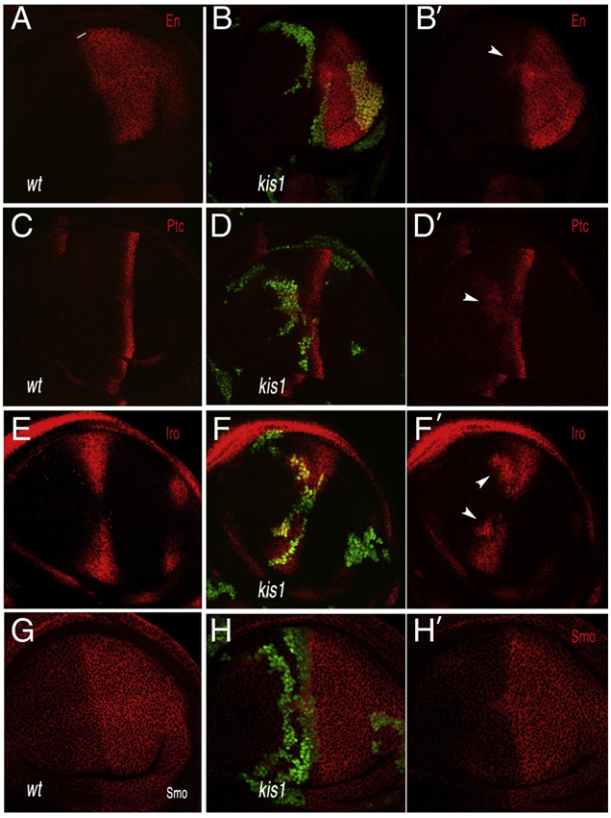

Fig. 3. Expression of Hh-target genes in *kis* mutant clones. (A–B′) Expression of En (red) in a third instar wild type wing disc (A) and in discs bearing *kis*^{1} clones (B–B′; labelled in green). The white line in (A) labels the extent of En expression in the anterior compartment. (C–D′) Expression of Ptc (red) in a third instar wild type wing disc (C) and in discs bearing *kis*^{1} clones (D–D′; labelled in green). (E–F′) Expression of Iro in a third instar wild type wing disc (E) and in discs bearing *kis*^{1} clones (F–F′; labelled in green). En, Ptc and Iro are ectopically expressed in *kis* mutant cells in a cell autonomous manner (arrowheads in B′, D′ and F′). Iro is also expressed in the surrounding wild type cells (arrowhead in F′). (G–H′) Expression of Smo in a third instar wild type wing disc (G) and in discs bearing *kis*^{1} clones (H–H′; labelled in green). Smo is not ectopically expressed in anterior *kis* mutant cells.

hybridization with *hh* probes (Figs. 4G–I). In this manner, not only the *lacZ* reporters are very sensitive to Kis levels, but also the *hh* gene is de-repressed close to the A/P boundary in *kis* mutant cells. We conclude that the ectopic expression of *hh* observed in anterior *kis* mutant clones causes inappropriate activation of Hh signaling in the anterior compartment.

Ci^{75}*-mediated repression of Hh does not require Kis function*

The repression of *hh* transcription in the anterior compartment is to a large extent mediated by the repressor form Ci^{75} (Dominguez et al., 1996; Methot and Basler, 2001). In cells that receive high levels of Hh the amount of Ci^{75} is low, and these cells require the participation of the Hh-target gene Mtv and the co-repressor Gro to repress *hh* transcription (de Celis and Ruiz-Gomez, 1995; Bejarano et al., 2007). We studied whether the repression of *hh* by Kis involves some contribution of the different forms of Ci by analysing the expression of Hh target genes in *kis*^{1} mutant cells with modified levels of Ci^{155} and Ci^{75}. The expression of these targets serves as a sensitive read-out to detect ectopic expression of *hh*. To reduce the total level of Ci, we generated clones of cells expressing interference RNA directed against *Ci* (*iCi*). In these clones, where the amount of both Ci^{155} and Ci^{75} is reduced (Supplementary Fig. 1), we observed the loss of *en* and *ptc* expression in anterior cells (Figs. 5A–A′, E–E′). We also observed ectopic and cell-autonomous expression of Iro (Figs. 5I–I′), most likely due to low levels of ectopic *hh* expression caused by the reduction of Ci^{75}. When *iCi* clones were simultaneously mutant for *kis*^{1}, we found similar results within the clones, i.e. loss of *en* and *ptc* and ectopic Iro expression (Figs. 5B–B′, F–F′ and J–J′, respectively). However, these clones also caused non-autonomous expression of Ptc and Iro throughout the anterior compartment (Figs. 5F–F′ and J–J′). These effects suggest a strong and synergistic de-repression of *hh* in *kis*^{1} anterior cells that express *iCi*. The expression of *en* was similarly affected in *iCi* and *kis*^{1} / *iCi*, suggesting that the levels of ectopic *hh* are not sufficient to activate *en* in cells abutting the clones (Figs. 5B–B′).

To increase the levels of Ci^{75}, we generated clones of cells expressing interference RNA directed against *smo* (*ismo*), because in this background most Ci will be cleavage to its repressor form. These clones show, as expected, the loss of En, Ptc and Iro expression in a cell autonomous manner (Figs. 5C–C′, G–G′ and K–K′, respectively). These effects of *ismo* expressing clones were not modified by the homozygosity of *kis*^{1} (Figs. 5D–D′, H–H′ and L–L′). In addition, the ectopic expression of En, Iro and Ptc characteristic of *kis*^{1} mutant clones was suppressed in *ismo* cells, indicating that *kis* function is not required to repress *hh* in the presence of high levels of Ci^{75}. Finally, we also observed that the form Ci^{Cell}, which mimics the Ci^{75} repressor (Méthot and Basler, 1999), also made irrelevant the function of Kis to repress *hh*. Thus, the repression of En, Iro and Ptc in anterior cells expressing Ci^{Cell} is still observed when these cells are also mutant for *kis* (Figs. 6A–F). Taken together these data indicate that Kis participates in a repression mechanism of *hh* transcription that is only effective when the levels of Ci^{75} are low, i.e. in anterior cells near the A/P boundary. The effects of *kis* clones on En, Ptc and Iro expression are summarized in Fig. 6G.

Knockdown of Kis function in Xenopus tropicalis is associated with increased Hh signaling and expression

To determine whether the function of Kis in repressing *hh* is conserved in vertebrates, we studied the loss of function of CHD8, one of the Kis homologs in *X. tropicalis*. CHD8 is a member of the CHD subfamily of chromatin remodelers, and contains the SNF2-like/ATPase domain, the chromodomain and the BRK domain. We knocked-down CHD8 function in *X. tropicalis* using a specific splicing morpholino, *MOCH8* (see Material and methods for details). RT-PCR with primers from exons 10 and 14 show an extra band of 1127 bp in the morphant embryos, indicating that *MOCHD8* partially inhibits the correct removal of intron 12 (Fig. 7A, red arrow). The inclusion of this intron in the mRNA introduces several precocious stops codons that eliminate the key domains of the protein. Therefore, in these injected embryos the function of CHD8 should be partially impaired. We evaluated the consequences of this knockdown by examining the expression of *Pax2* in the morphant embryos. *Pax2* is expressed, among other territories, in the ventral eye, and this expression is positively controlled by Hh (Lupo et al., 2005). In embryos injected with *MOCHD8*, we observed a dorsal expansion of *Pax2* (Figs. 7B–D, red arrows) in a way reminiscent to that observed in embryos in which Hh has been over-expressed (Lupo et al., 2005). Moreover, in these morphant embryos *Pax2* expression is down-regulated in the phronephros (Figs. 7B and C, green arrowhead), as has been shown for other kidney markers in embryos with increased Hh signaling (Urban et al., 2006). To determine if the changes in *Pax2* were a consequence of ectopic *Shh* expression, we examined this gene in the *MOCHD8* morphant embryos. Indeed this was the case, an in the injected side of embryos we observed a clear dorsal expansion of *Shh* expression (Fig. 7E, arrows). We also notice that in *CHD8* morphant embryos the expression of *Pax2* in other tissues, such as the hindbrain or the otic vesicle was also reduced (Fig. 7B and C, red and blue arrowheads), but we did not analyse whether these effects were also due to alterations in Hh signalling.

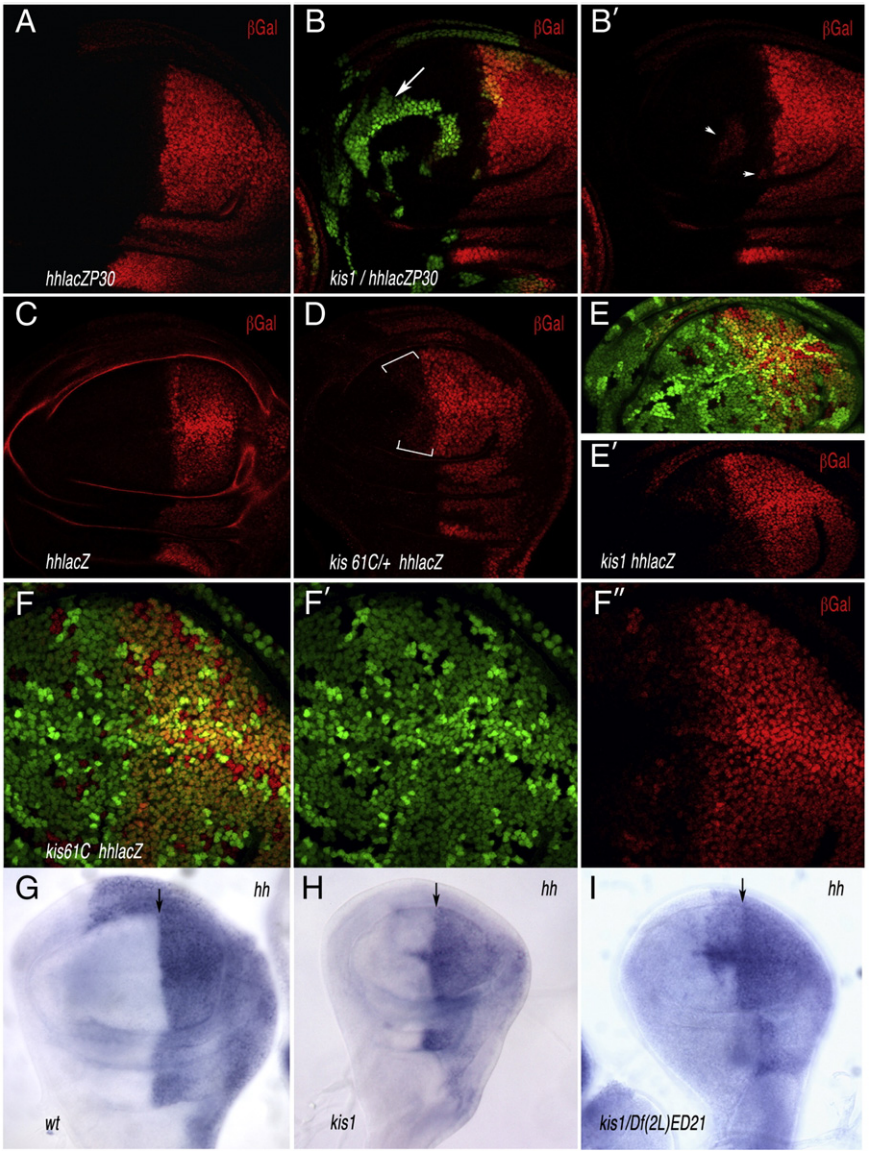

Fig. 4. Expression of *hh* in *kis* mutant cells. (A) Expression of βGal (red) in the posterior compartment of *hh-lacZ*^{P30} / + third instar wing disc. (B–B′) Ectopic expression of *hh-lacZ*^{P30} (red) in *kis*^{1} clones located close to the antero-posterior compartment boundary (arrowheads in B′). *hh-lacZ*^{P30} is not expressed in *kis* mutant cells located in more anterior regions of the anterior compartment (arrow in B). (C) Expression of βGal (red) in the posterior compartment of *hh-lacZ* / + third instar wing disc. (D) Expression of βGal (red) in the anterior compartment of *kis*^{61C} / +; *hh-lacZ* / + third instar wing disc. *hh-lacZ* is now also detected at low levels in an anterior domain close to the A/P boundary (bracketed). (E–E′) Expression of βGal (red) at higher than background levels in the anterior compartment of *hh-lacZ* / + third instar wing discs bearing *kis*^{1} clones (labelled by the absence of green). (F–F″) Cell autonomous de-repression of *hh-lacZ* in small *kis* mutant clones induced in *hsFLP1.22* / +; *kis*^{61C} *FRT40A* / *tubGFP FRT40A; hh-lacZ* / + larvae. In E–F′ LacZ is detected at higher levels in all *kis* mutant cells located close to the A/P compartment boundary. (G–I) Expression of *hh* mRNA in the posterior compartment of wild type discs (G) and de-repression of *hh* in anterior cells of *kis*^{1} homozygous (H) and *kis*^{1} / *Df*(*2L*)*ED21* (I) mutant discs. The position of the A/P boundary is indicated by a black arrow in G–I.

Discussion

In this work we have used a genetic approach to analyse the role of Kis in the patterning of the Drosophila wing. Our main finding is that Kis is required, among other processes, for the repression of *hh* in anterior cells close to the A/P boundary. This conclusion is based in the phenotype of *kis* clones in the wing, the changes in the expression of Hh-target genes in these mutant cells, and more directly, in the observation of *hh* ectopic expression in wing discs of *kis* loss-of-function alleles. We also identified a similar requirement for CHD8, a Kis homolog in *X. tropicalis*, suggesting conservation in the mechanisms of *hh*/*Shh* regulation during evolution. Finally, we determined that the repression of *hh* mediated by Kis is not needed when the repressor form of Ci, Ci^{75}, is present in the cell.

Kis functions during vein differentiation

As a way to identify the functional requirements of Kis, we studied the phenotype caused by several *kis* loss-of-function alleles. For six of these alleles we found similar results, and consequently we will refer to all of them together. We identified two main alterations in wings mutant for *kis*: the formation of ectopic veins and defects in the patterning of the central region of the wing. These phenotypes are diagnostic of failures in the regulation of the level or domain of activity of two signaling pathways, EGFR and Hh (Cruz et al., 2009), and it is likely that they identify independent requirements for Kis in the modulation of these pathways during wing development. The formation of ectopic veins is observed in all situations when the activity of the EGFR pathway is not correctly restricted to the positions

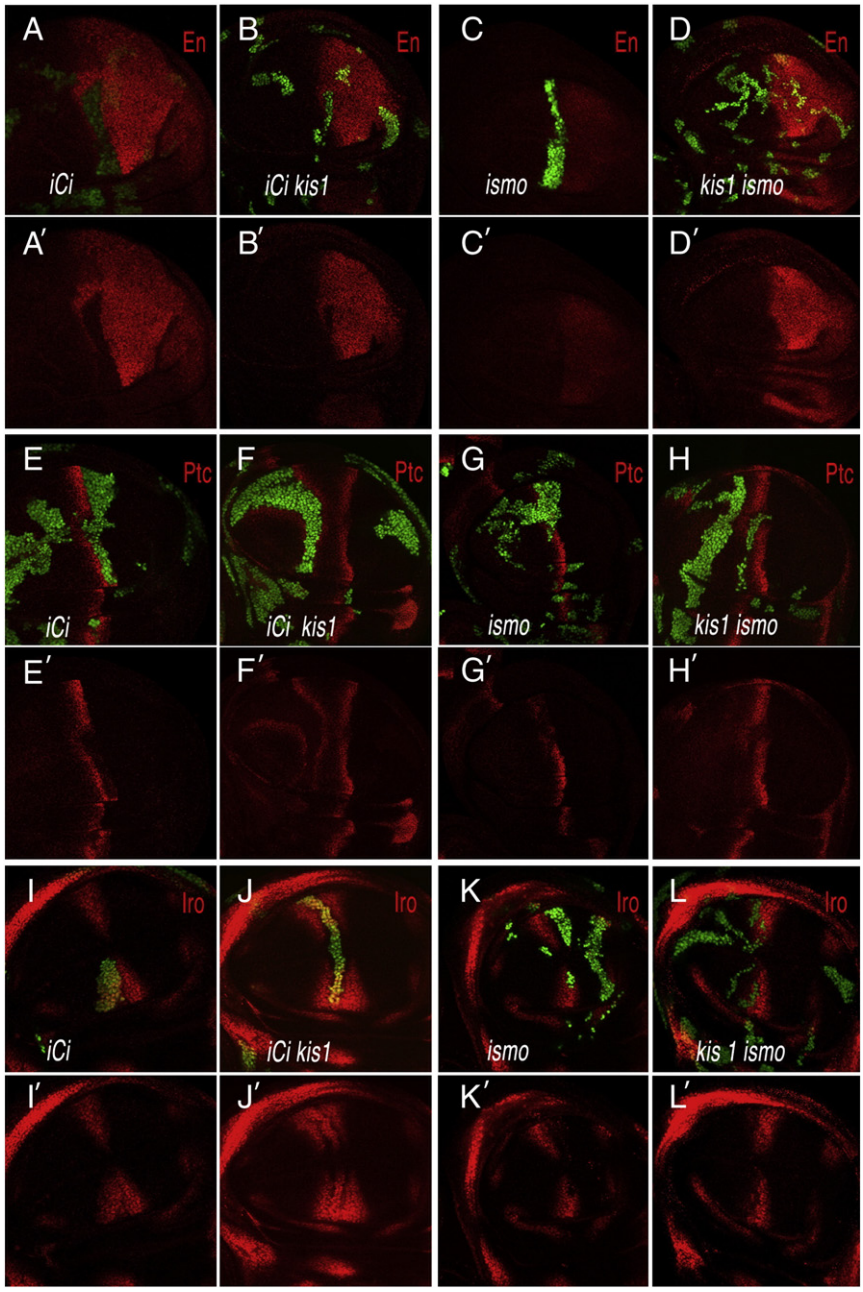

Fig. 5. Interactions between Kis and Ci in the regulation of Hh-target gene expression. (A–L) Expression of En, Ptc and Iro proteins in *kis* clones induced 48–72 AEL in larvae of the following genotypes:

- *hsFLP1.22 actGal4 UAS-GFP; FRT40A / tubGal80 FRT40A; UAS-iCi / +* (*iCi*: A, E and I).
- *hsFLP1.22 actGal4UAS-GFP; kis^{1}FRT40A / tubGal80 FRT40A;UAS-iCi / +* (*iCi kis^1*: B, F and J).
- *hsFLP1.22 actGal4 UAS-GFP; FRT40A / tubGal80 FRT40A; UAS-ismo / +* (*ismo*: C, G and K).
- *hsFLP1.22 actGal4UAS-GFP; kis^{1}FRT40A / tubGal80FRT40A;UAS-ismo / +* (*ismo kis^1*: D, H and L). In all cases, mutant clones were labelled by the expression of GFP (green). The expression of En (A–D and A′–D′), Ptc (E–H and E′–H′) and Iro (I–L and I′–L′) is shown in red. The red channels of A–L are shown in A′–L′, respectively. The reduction of Ci (*iCi*) causes loss of En (A–A′) and Ptc (E–E′) and cell-autonomous ectopic expression of Iro (I–I′). When *kis* is also mutated, the main difference is the strong non-autonomous expression of Ptc (F–F′) and Iro-C (J–J′). The reduction in Smo causes loss of En (C–C′), Ptc (G–G′) and Iro (K–K′). The same changes are observed in *ismo* cells mutant for *kis^1* (D–D′, H–H′ and L–L′).

occupied by the normal veins (Sturtevant and Bier, 1995; Sotillos and de Celis, 2005). Thus, over-activation of EGFR and loss-of-function alleles in a variety of the known antagonists of the pathway, such as *MKP3* and *sprouty*, result in the formation of ectopic veins in similar positions to those observed in *kis* alleles (Gomez et al., 2005; Reich et al., 1999; Kramer et al., 1999). The implication of Kis in regulating EGFR signaling is also supported by the identification of *kis* alleles in several genetic screens searching for modifiers of EGFR phenotypes in different developmental stages and tissues. For example, *kis* was found in a screen of *kinase-suppressor of Ras* modifiers in the eye (Therrien et al., 2000) and in a screen of EGFR modifiers affecting border cells migration during oogenesis (Mathieu et al., 2007). *kis* alleles were also identified as modifiers of the Notch phenotype caused by dominant-negative *mastermind* over-expression in the

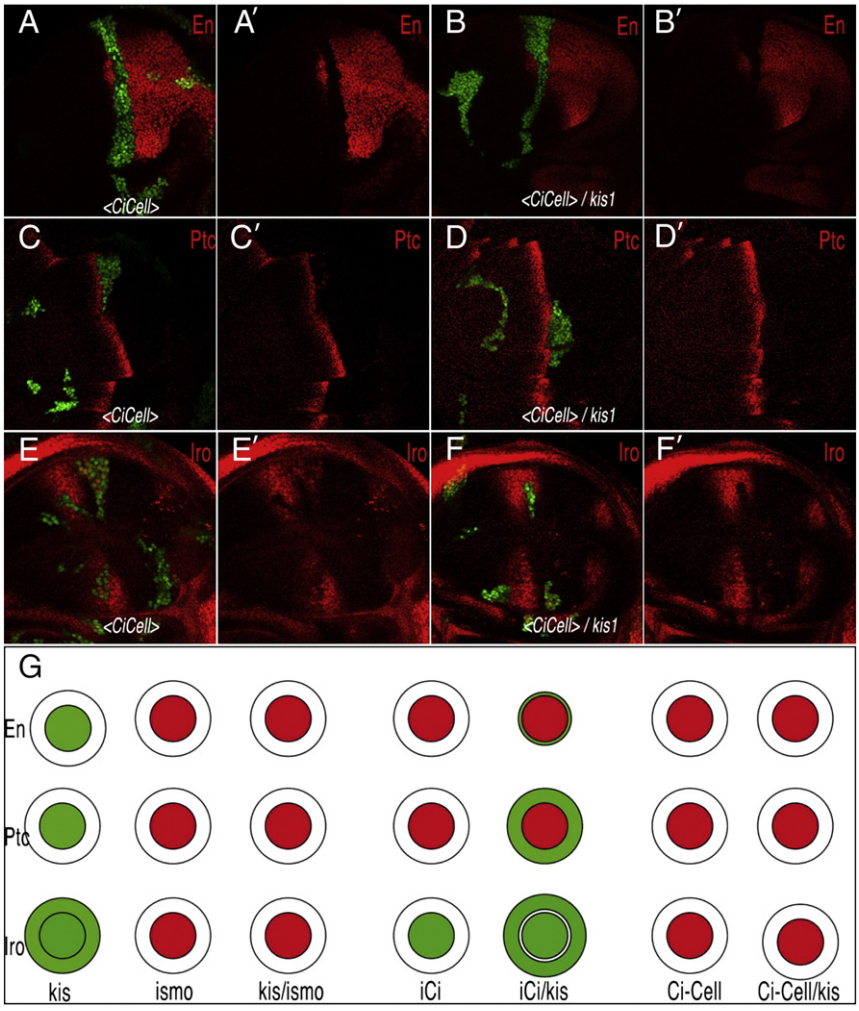

Fig. 6. Suppression of *kis* effects by Ci^{Cell} expression. Expression of En, Ptc and Iro proteins in clones induced 48–72 AEL in larvae of the following genotypes:

- *hsFLP1.22 actGal4 UAS-GFP; FRT40A / tubGal80 FRT40A;UAS-*Ci^{Cell} */ +* (<Ci^{Cell}>: A, C and E).
- *hsFLP1.22 actGal4 UAS-GFP;* kis^1*FRT40A / tubGal80 FRT40A;UAS-*Ci^{Cell} */ +* (<Ci^{Cell}> / kis^1: B, D and F). In all cases, mutant clones were labelled by the expression of GFP (green). The expression of En (A and B), Ptc (C and D) and Iro (E–F) is shown in red. The red channels of A–F are shown in A′–F′, respectively. Ectopic expression of Ci^{Cell} causes loss of En (A–A′), Ptc (C–C′) and Iro (E–E′). These phenotypes are not modified when *kis* is mutant in Ci^{Cell}-expressing cells (B–B′ for En, D–D′ for Ptc and F–F′ for Iro). (G) Schematic representation of the effects on Hh target gene expression of Ci-Kis combinations as shown in Fig. 5 and Fig. 6A–F. Inner circles represents the mutant clones, outer circles the cells surrounding the mutant clone, green implies gene expression and red gene repression.

wing disc (Kankel et al., 2007). However, in our hands the vein phenotype of loss of *kis* does not appear related to Notch signalling, because we never observed Notch-related defects such as thickened veins or loss of wing margin in *kis* mutant wings.

We have not directly analysed the implication of Kis in the modulation of EGFR signalling. However, it is interesting to notice in this context that the two SNF2-familiy chromatin-remodeling complexes containing Brm as the catalytic subunit are also involved in this pathway. In this manner, the BAP (Brahma associated proteins) and PBAP (Polybromo-Brahma associated proteins) (Mohrmann et al., 2004) complexes are required to modulate positively or negatively, respectively, EGFR signaling in the wing (Terriente-Felix and de Celis, 2009; Rendina et al., 2010). Furthermore, Brm and Kis share some domain architecture, as they both have an ATPase domain N-terminal to a BRK (Brahma related to Kismet) domain. They also bind to identical sites in polytenic chromosomes (Srinivasan et al., 2005), and both are part of the Trithorax group of genes (TrxG) (Schuettengruber et al., 2007; Kennison and Tamkun, 1988; Daubresse et al., 1999; Petruk et al., 2008). Therefore, it is possible that BAP, PBAP and Kis participate in the transcriptional regulation of EGFR targets using a conserved mechanism involving chromatin modifications in collaboration with other PcG and TrxG proteins. Similarly, the function of CHD7 in the regulation of neural crest cell identities requires the function of PBAF (Polybromo, Brg1-Associated Factors), the homolog of PBAP (Bajpai et al., 2010), suggesting that Kis and its vertebrate homologs can collaborate with other SNF2-helicases.

Kis represses the expression of Hh in the anterior compartment of the wing blade

In this work we have focussed in the second phenotype observed in *kis* mutant cells, which consists in duplications of the L3 vein and increase in the distance between the L3 and L4 veins. These two defects are limited to the pattern elements regulated by the Hh pathway, and correspond to an enlargement of the domain of Hh signalling. Hh activity and diffusion in anterior cells are linked to each other by the function of the receptor Ptc, because *ptc* expression is activated by Ci^{155} and Hh diffusion is prevented by Ptc (Chen and

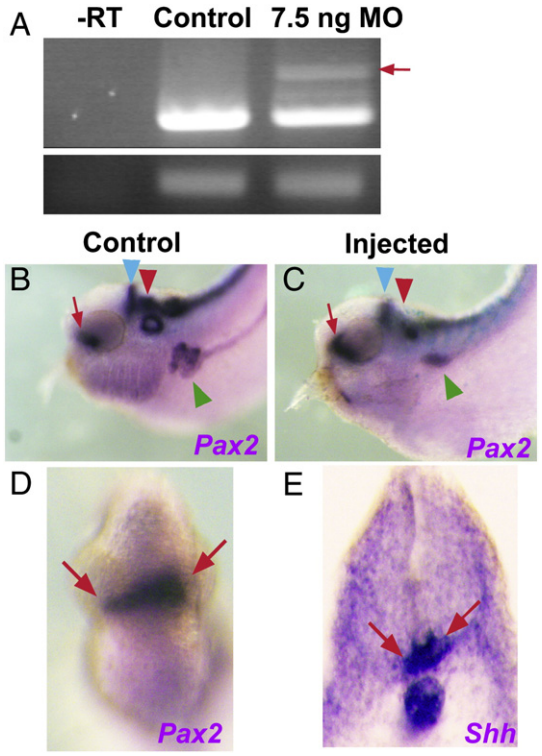

Fig. 7. Expression *Pax2* and *Shh* in stage 35 *Xenopus tropicalis* embryos injected with *MOCHD8*. (A) RT-PCR with primers from exons 10 and 14 reveals an extra band of 1127 bp (red arrow) in the embryos injected with 7.5 ng of *MOCHD8* that is not observed in the control embryos (compare second and third lanes). (B–D) *Pax2* expression in *MOCHD8* morphant embryos. (B) Lateral view of the control un-injected side. (C) Lateral view of the *MOCHD8*-injected side. (D) Frontal view of morphant embryos with the injected side to the right. Note the dorsal expansion of the expression of *Pax2* in the eye (red arrows), and the reduction of its expression in the pronephros and hindbrain (green and red arrowheads, respectively). The expression in the midbrain-hindbrain boundary (blue arrowhead) is unaffected. (E) Transverse section of the spinal cord showing *Shh* expression in embryos injected with 7.5 ng of *MOCHD8*. The injected side is to the right, and the control, uninjected side, to the left. Note the dorsal expansion of *Shh* expression in the injected side (right arrow).

Struhl, 1996; Johnson et al., 1995). In this manner the phenotype of *kis* clones might be caused by a reduction in Hh signaling leading to loss of Ptc and consequently to an increase in the range of Hh diffusion. However, we found that *kis* clones do not affect the normal domain of *ptc* expression, but cause ectopic *ptc* expression in cells localised anterior to this domain. This observation indicates that Hh signaling itself is not affected in *kis* mutant clones, and points towards changes in *hh* expression as a likely cause for the *kis* phenotype. Indeed, we do find ectopic expression of *hh* in *kis* mutant cells localised in the anterior compartment close to the A/P boundary. Furthermore, ectopic and cell autonomous expression of *hh-lacZ* reporters is observed in small *kis* clones (less than 5 cells) localised in this region. These results suggest that Kis is needed during wing imaginal development to maintain *hh* expression turned-off in anterior cells that, due to Hh signalling, don't have enough levels of the repressor Ci^{75}. This requirement for Kis readily explains both the phenotype of *kis* alleles in the central region of the wing and the changes in the expression of *hh* and its target genes observed in *kis* mutant cells. Interestingly, this activity of Kis in the repression of *hh* expression appears conserved in its *Xenopus* homolog CHD8.

Possible mechanisms of Hh repression by Kis

The regulation of *hh* expression relies on a combination of several mechanisms acting in different domains of the wing disc. First, Ci^{75}, a form of Ci that is produced by proteolysis from Ci^{155} when Hh signaling is not active, represses *hh* in anterior cells (Methot and Basler, 2001). In those anterior cells exposed to Hh the levels of Ci^{75} are low, and in this domain a second mechanism involving Mtv and Gro represses *hh* expression (Bejarano et al., 2007). Finally, several genes of the *Polycomb* group (*PcG*), such as Polycomb (Pc) and Polyhomeotic (Ph) are also involved in the repression of *hh* transcription in a tissue-specific manner (Chanas et al., 2004; Chanas and Mashat, 2005). In this way, the PcG is involved in maintaining the repressive transcriptional state of *hh* in anterior cells, while the TrxG maintains the active transcriptional state of *hh* in posterior cells (Randsholt et al., 2000; Maurange and Paro, 2002). This regulation seems direct, because the Pc protein and the TrxG member GAF/Trl bind two regions of the *hh* gene, and one of them, situated upstream of the *hh* transcription start site, exhibits cellular memory module (CMM) characteristics (Maurange and Paro, 2002). The activity of this CMM is also regulated by PcG and TrxG proteins in experimental situations in which *hh* is activated by ectopic En in anterior cells (Maurange and Paro, 2002), or turndown by loss of En in posterior cells (Bejarano and Milan, 2009). Interestingly, ectopic expression of En in anterior cells located along the dorso-ventral boundary induces *hh* in most of the cells of the wing blade except those nearest to the A/P boundary, implying that the activity of this *hh* CMM in the anterior compartment is excluded or less efficient in the territory where Kis represses *hh*.

How Kis regulates *hh* in anterior cells is not known, but several arguments suggest that Kis is related to the repression mediated by Mtv/Gro. Thus, *mtv/gro* and *kis* mutations cause ectopic expression of *hh* in a similar domain of the wing disc, and in both cases they are not required when the repressor Ci^{75} is present. In this scenario, we propose that the chromatin remodeling activity of Kis could make the *hh* regulatory region accessible to the Mtv/Gro repressor complex. The putative function of Kis as part of the Mtv/Gro repressor complex would be independent of other functions assigned for the protein in, for example, the control of transcriptional elongation and Histone methylation (Srinivasan et al., 2005, 2008). Similarly, this function of Kis on *hh* regulation would be independent of its role as a TrxG protein (Kennison and Tamkun, 1988; Daubresse et al., 1999), because it is only effective in a spatial domain complementary to that in which PcG and TrxG regulate *hh* expression (Maurange and Paro, 2002).

Interestingly, heterozygous mutations in human CHD7 result in congenital anomalies called CHARGE syndrome (Vissers et al., 2004), which is caused by the abnormal development of the neural crest (Bajpai et al., 2010). The function of CHD7 in the regulation of neural crest cell identities implies the regulation of several transcription factors expressed in these cells, and requires the function of the PBAF chromatin remodeler. In this manner, Kis and other CHD proteins might form part of different multiprotein complexes regulating different promoters using independent molecular mechanisms. It is remarkable that CHD8 is required for the regulation of *Shh*, as this implies a strong conservation in the mechanism of *hh* and *Shh* transcriptional regulation during evolution. Future experiments should address the mechanisms by which Kis/CHD8 are recruited to the *hh/Shh* regulatory regions.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.12.003.

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