Università degli Studi di Padova
Dipartimento di Biologia
Corso di Laurea Magistrale in Biologia Molecolare

Analysis of the role of the Fused kinase in Hedgehog signalling during *Drosophila melanogaster* development: phenotypic characterization of the first *fused* knock-out mutant

Relatore: Prof. Francesco Argenton
Dipartimento di Biologia

Laureanda: Lisa Barbuglio

Anno Accademico 2016/2017

ñ*Se sapessimo (esattamente) quel che stiamo facendo, non si chiamerebbe ricerca****ò****.*

Albert Einstein

Riassunto

Questa tesi è il risultato del mio stage di sei mesi all'Institut Jacques Monod di Parigi, presso il laboratorio della professoressa Anne Plessis, équipe ñDéveloppement, Signalisation et Traficò.

In molti processi biologici che avvengono durante lo sviluppo di un organismo, ma anche nell'omeostasi del organismo adulto, il signalling cellulare ha un ruolo chiave. Esso infatti regola il differenziamento, la proliferazione, la morte e la migrazione cellulare.

Il mio laboratorio studia una delle più importanti vie di signalling responsabile dello sviluppo negli organismi animali: la via Hedgehog (HH). Si tratta di una via di signalling altamente conservata nei metazoi ed il cui mal-funzionamento può portare allo sviluppo di malformazioni congenite e, nell' adulto, di tumori.

Per studiare la via HH, il mio laboratorio utilizza come organismo modello *Drosophila melanogaster*, la quale presenta una serie di vantaggi. La drosophila è infatti un organismo piccolo e poco costoso, ha un ciclo vitale breve, produce abbondante progenie, ha un genoma piccolo e organizzato in quattro cromosomi e inoltre sono disponibili molti tools genetici.

HH è una lipoproteina che viene secreta da cellule produttrici e che in molti tessuti agisce come un morfogeno. Ciò significa che HH forma un gradiente di concentrazione che induce l'attivazione di una risposta genica specifica da parte delle cellule bersaglio, regolando in questo modo il destino cellulare.

Se il ñcoreò principale della via HH risulta ad oggi ben caratterizzato, al contrario, non è ancora del tutto chiaro come diverse dosi di HH possano determinare una specifica risposta cellulare. Interesse del mio laboratorio è capire come diversi livelli di concentrazione di segnale HH siano trasdotti all'interno della cellula e inducano infine l'espressione di specifici geni.

Fused (FU) sembra avere un ruolo chiave in questo meccanismo, dal momento che la sua mutazione riduce la risposta ad alti livelli di HH. FU è una Ser/Thr chinasi. Essa agisce su targets a valle che regolano l'attività di CI, il fattore di trascrizione che media la risposta al segnale HH. Inoltre, FU ha pure un'azione retroattiva che aumenta l'attività di Smoothened (SMO), la proteina trans-membrana necessaria per la trasduzione del segnale.

Fino ad ora lo studio del ruolo di *fu* è stato limitato dalla mancanza di un vero allele nullo. Gli unici alleli nulli disponibili consistevano in grandi delezioni che includevano anche altri geni oltre a *fu*.

Il primo allele fu^{KO}, loss of function specifico per il gene *fu*, è disponibile nel mio laboratorio. È stato prodotto grazie alla tecnica di genome editing CRISP/Cas. In

questo modo, sono state rimosse 2,6 Kb del locus *fu*, includendo il 5' UTR ma non il 3' UTR, che sono state sostituite con una sequenza codificante per la proteina marcatrice DsRed.

Sotto la supervisione della dottoressa Isabelle Bécam, ho partecipato ad un nuovo progetto: la caratterizzazione fenotipica del mutante fu^{KO} di drosophila.
Ho sviluppato questo studio in tre punti principali:

1) analisi fenotipica della mutazione fu^{KO},
2) analisi comparativa dell'effetto dei diversi mutanti *fu* sul HH signalling,
3) analisi dell'effetto della mutazione fu^{KO} su SMO.

Nelle pagine seguenti è riportato in maniera dettagliata il mio lavoro. Ciò include una breve introduzione alla tematica oggetto di studio, i materiali e i metodi utilizzati, i risultati ottenuti e infine la discussione che questi ultimi aprono.

Table of contents

Summary

The Hedgehog (HH) signalling pathway is highly conserved and has a role in many cellular processes during development. Its deregulation leads to developmental defects and cancers.

In many tissues, HH acts in a graded manner to differentially activate genes expression and to regulate cell fate. While the core of this pathway is well characterized, how different HH doses lead to different outcomes is poorly understood.

Fused (FU) seems to have a pivotal role in this process, as its mutation reduces the responses to high HH levels. FU is a Ser/Thr kinase. It acts on downstream targets that regulate the activity of CI, the transcription factor that mediates HH responses. Moreover, FU has a retroaction activity that enhances the activity of the transmembrane protein Smoothened (SMO) which is required for HH signal transduction.

However, the study of the role of *fu* was limited due to the lack of a real null allele. The goal of my M2 project was the characterization of the first null allele that was obtained by knock-out. I thus have performed a phenotypic analysis of this novel allele. This included the comparison with known alleles and the effect on HH target genes and SMO.

This first characterization of the null allele revealed its potential utility as a genetic tool to better understand *fu* function in the HH pathway.

Abbreviations list

Genes or transcripts abbreviations are written in small letters and italic, while protein abbreviations are written in capital letters. For example: *hh* for the gene and HH for the protein.

A: Anterior

A/P: Antero/Posterior

BCC: Basal Cell Carcinoma

CK1: Casein Kinase 1

CI: Cubitus Interruptus

CIF: CI Full lenght

CIL: CI Labile

CIR: CI Repressor

COL: Collier

C-term: C-terminal

COS2: Costal 2

Cyto-tail: Cytoplasmic tail

DHH: Desert Hedgehog

DPP: Decapentaplegic

EN: Engrailed

FU: Fused

FU-KD: Fused-Kinase Domain

GPCR: G-Protein Coupled Receptor

HH: Hedgehog

HTC: HH Transducing Complex

IHH: Indian hedgehog

IRO: Iroquois

LV: Longitudinal Vein

PKA: Protein Kinase A

NBCCS: Naevoid Basal Cell Carcinoma Syndrome

PC: Primary Cilium

P: Posterior

PKC: Protein Kinase C

PTC: Patched

SMO: Smoothened

SHH: Sonic Hedgehog

SUFU: Suppressor of Fused

TF: Transcription Factor

TGFβ: Transforming Growth Factor-β

List of figures

List of tables

I. INTRODUCTION

Preamble: Cell signalling is implicated in many biological processes during development but also in the adult homeostasis. It regulates cell differentiation, proliferation, cell death and migration. Our laboratory studies the Hedgehog (HH) signalling pathway, one of the major pathway involved in animal development, using the fly as a model organism. Fly presents many advantages: it is small and cheap, has a short life cycle, produces a great number of progeny, has a small genome organized in four chromosomes and many tools for genetic studies are available.
More precisely, the interest of the lab is to understand how different levels of HH signals are intracellularly transduced to finally lead to the expression of specific genes. We are particularly interested in the Ser/Thr kinase Fused (FU), that seems to be important for the transduction of a high HH concentration signal. Under the supervision of I. Bécam, I participated in a new project whose aim was the phenotypic characterization of a fly that carries the first null allele for *fu*.

A. Hedgehog roles in fly and vertebrates

The HH pathway is one of the major signalling pathway involved in metazoans development. *hh* gene was first isolated in *Drosophila melanogaster* [1] and later it was discovered to be conserved in vertebrates [2].
HH is a secreted lipoprotein that in many cases, for example in fly wing imaginal disc and vertebrate neural tube, acts as a morphogen. It forms a gradient across receiving cells that respond in a dose-concentration dependent manner. During development HH signalling controls many processes such as cell proliferation, differentiation, migration and death. In the adult, HH signalling regulates stem cell homeostasis.
Reduced functioning of the HH pathway has been associated with developmental abnormalities, such as holoprosencephaly. While abnormal activation of the pathway is involved in cancer [3]. For instance, inactivating mutations in the human patched (*ptc*) gene, a negative regulator of the pathway, are responsible for the naevoid basal cell carcinoma syndrome (NBCCS), an inherited cancer predisposition disorder also known as Gorlin, or as well as sporadic basal cell carcinomas (BCCs), the most common form of skin cancer.

B. The HH pathway in drosophila and vertebrates

Genetic studies in drosophila permitted to identify the main components of the HH pathway. HH acts on its target genes by the regulation of the bi-functional transcription factor (TF) Cubitus Interruptus (CI). The transduction of HH signal

relies on Smoothened (SMO). SMO is a member of the G-protein coupled receptor (GPCR) superfamily, that regulates the cytoplasmic HH Transducing Complex (HTC), which includes the kinesin protein Costal2 (COS2), FU, Supressor of Fused [SU(FU)] and CI. In absence of HH, SMO is inhibited by the twelve-transmembrane protein PTC, the receptor of the pathway. As a consequence, CI is processed into its repressor form (CIR), leading to the repression of the target genes (fig. 1A). HH binding to PTC releases its inhibition on SMO. As a result, SMO is phosphorylated in its C-terminal (C-term) cytoplasmic tail (cyto-tail) successively by Protein Kinase A (PKA) and Casein Kinase 1 (CK1) and accumulates at the plasma membrane [4]. Phosphorylation activates SMO by inducing a conformational switch and dimerization of its cytosolic C-terminal parts (cyto-tails) [5]. SMO activation is then transduced to the HTC which inhibits CI processing and leads to the release of the full length transcriptional activator CI (CIF) (fig. 1B).

The molecular mechanisms of HH signalling are mainly conserved in vertebrates. Mammals have three *hh* homologs, sonic hedgehog (*shh*), indian hedgehog (*ihh*) and desert hedgehog (*dhh*), two *ptc* homologs and one single *smo* that regulates the three *ci* homologs, *gli1*, *gli2* and *gli3*. Nevertheless, one essential difference between drosophila and vertebrates is the requirement of the Primary Cilium (PC), a structure which is absent in drosophila (fig. 1C). In presence of HH, PTC moves out of the cilium while SMO enters and accumulate there (fig. 1D).

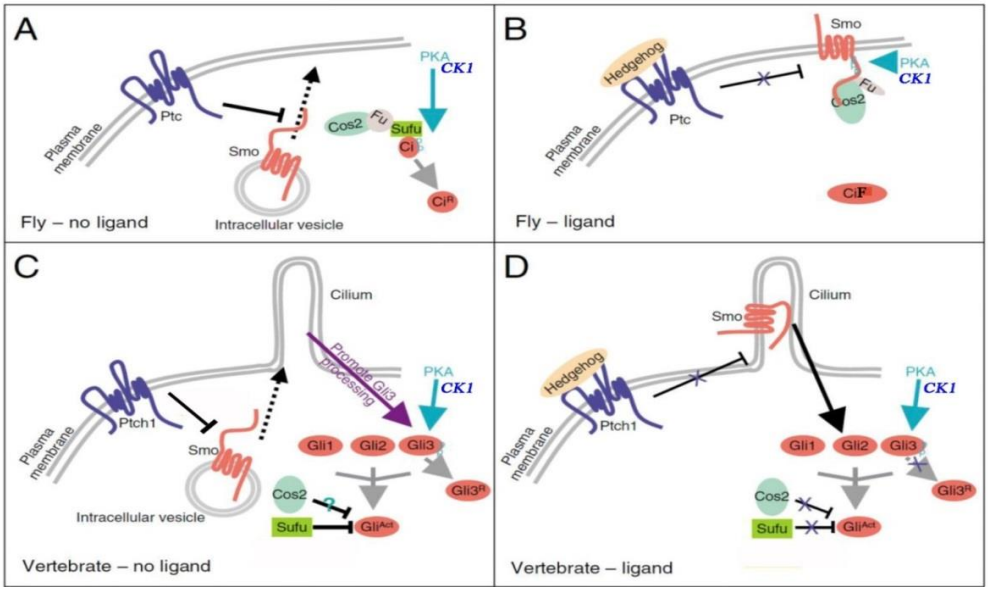

Figure 1: Key players within the HH pathway in drosophila and vertebrates.
In drosophila (A) in the absence of HH, PTC prevents the cell-surface localization of SMO. CI forms a complex with COS2, FU and SUFU and it is phosphorylated by PKA and CK1. This promotes CI proteolytic processing into the repressor form, CIR.

Not cleaved CIF is retained in the cytoplasm by SUFU. (B) In the presence of HH, PTC inhibition is released. SMO is hyper-phosphorylated at its C-terminal tail by PKA and CK1. It accumulates at the plasma membrane where it recruits COS2 and FU. CIF is released. In vertebrates (C) in the absence of HH, PTCH1 prevents the accumulation of SMO in cilia. GLI3 is processed into a repressor form GLI3R in a cilia-dependent manner. The activation of all GLI proteins is inhibited by SUFU and probably COS2. (D) In the presence of HH, PTCH1 inhibition is relieved. SMO is targeted to cilia and activates GLI proteins in a cilia-dependent manner. GLI3 processing is also inhibited. (Figure readapted from[2]).

C. HH role in wing patterning in fly wing imaginal disc

The drosophila wing disc provides a model system to study HH signalling and its role as a morphogen. The wing disc is composed by 50,000 cells that will develop into the adult wing and the body structure, the notum, to which the wing is attached. These cells are organized in two juxtaposed layers separated by a lumen: the ñcolumnarò and the ñperipodialò layers, which are organized in an anterior (A) and posterior (P) compartments, separated by the A/P border [6].

HH is secreted from the P compartment of the disc and it diffuses in the A one producing a gradient that controls the expression of different CI targets in a dose dependent manner (fig. 2B). These genes are responsible for the growth and patterning of the wing, especially of the region between longitudinal vein (LV) 3 and 4 (fig. 2A).

At intracellular level, HH gradient is translated into different level of SMO phosphorylation (fig. 2C). The major contributors to the HH-induced hyperphosphorylation are PKA/CK1 phosphosites. This in turn leads to the production of CIR and CIF gradients with opposite directions. Toward the A/P boundary, CIR is progressively suppressed, while CIF is accumulated. Moreover, at A/P boundary CIF is activated into a labile form (CIL) by a high HH concentration, but the mechanisms are not known. In this way the expression of CI target genes is spatially regulated. Thus, in presence of low level of HH, CI processing is blocked and low levels of CIF promote decapentaplegic *(dpp)* expression. In presence of intermediate levels of HH, the levels of CIF are increased, leading to *ptc* and collier (*col*) expression. Finally, in the A cells adjoining the P compartment cells CIL enables engrailed (*en*) expression (fig. 2 C).

How SMO graded phosphorylation and activation are transduced to the HTC is not well understood. In the fly wing disc, the transduction of high HH signal involves the protein FU.

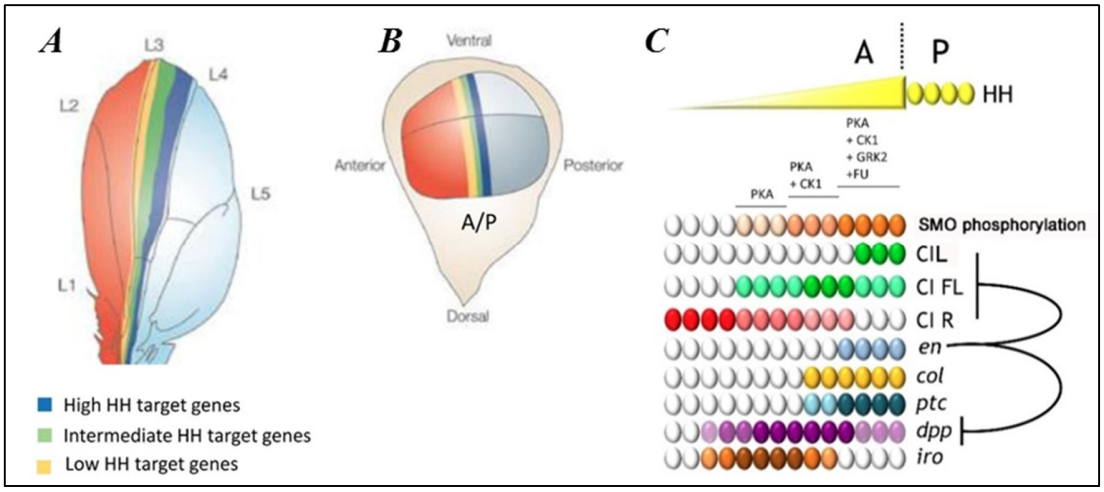

Figure 2: HH roles in fly wing development.

In drosophila, the wing (A) is characterized by longitudinal veins (L) and is derived from the wing disc (B). The disc is structured into A/P axes. The HH morphogen is secreted by cells of the posterior compartment and forms a gradient in the anterior region which is required for the A/P patterning of the developing wing. HH gradient is intracellularly translated in SMO phosphorylation and CIF and CIR opposite gradients (C). This spatially regulates the expression of target genes en, col, ptc, dpp and iro. At the A/P border CIF is converted into CIL but the mechanism is not known.

D. Fused and its role in the signal transduction

fu is a segment polarity gene located on the X chromosome. It encodes for a Ser/Thr kinase that contains an N-terminal catalytic domain (Fu-KD) and a C-term regulatory domain. FU interacts with COS2, SU(FU) and SMO proteins through different binding domains. In particular, FU has two binding sites for COS2, one in the KD and the other in the C-term domain (fig. 3).

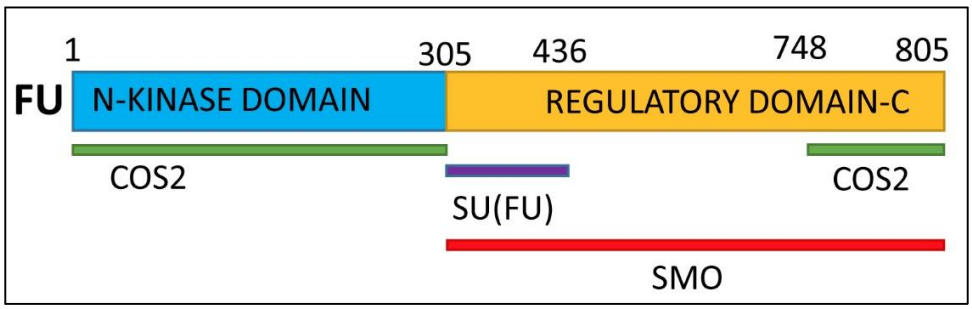

Figure 3: FU protein domains and interaction regions.

FU kinase and regulatory domains are shown in blue and yellow respectively. COS2, SU(FU) and SMO biding sites are shown.

Genetic studies showed that FU acts as positive regulator of the HH pathway. Different recessive *fu* alleles are known which all have both maternal and zygotic effects. Thus homozygous fu^{-} mothers lead to embryos that cannot develop (maternal embryonic lethality) independently of their genotype. In contrast, homozygous (or hemyzygous) *fu* mutant flies born from heterozygous $fu^{-}/+$ mothers, and therefore receiving maternal *fu* RNA/protein, are able to develop, leading to adults with several zygotic defects such as LV3-LV4 wing fusion and ovarian tumorigenesis.

The wing phenotype in *fu* mutants is due to a reduction of high levels of HH signalling in the wing disc, with a reduction in *ptc* expression and a loss of *en* expression. This in turn, leads to the expansion of the domains of CI accumulation toward the A/P boundary (loss of the ñlabile CIF) and of the domain of *dpp* expression [7]. FU directly binds SMO and is recruited by SMO at the plasma membrane in response to HH. FU activation also depends on the phosphorylation of putative FU and CK1 sites present in its activation loop. FU phosphorylation is promoted by dimerization or clustering of FU molecules. In turn, SU(FU) and COS2 are phosphorylated in response to HH. As both SU(FU) and COS2 bind directly to CIF limiting its nuclear accumulation and activity, and FU overcome these inhibitory actions, it has been suggested that FU might act by phosphorylating them. Finally, my lab provided recent evidences that FU is not just an effector of SMO, but can also act on it. They showed that FU phosphorylates SMO and controls its accumulation in the wing disc. This positive feedback loop in which SMO and FU enhance each otherôs activity could sustain high level of signalling [8, 9].

Finally, former genetics studies revealed complex interactions between *fu* and its antagonist *Su(fu)*. In both *fu* allele classes, all embryonic and adult *fu* phenotypes are suppressed in presence of the amorphic mutation $Su(fu)^{LP}$. However, class II alleles display in presence of $Su(fu)^{LP}$ new embryonic and adult phenotypes that are due to an ectopic activation of the pathway. Class I alleles bear alteration in the kinase domain and the C-term domain is intact, while in class II alleles the C-term domain, which normally binds another negative regulator of CI, COS2, is at least partially deleted [10]. The molecular basis of the genetic interaction between $Sufu^{LP}$ and the class II *fu* alleles is not understood yet. For instance, it could be due to a direct negative effect on the HTC of the truncated FU class II mutant or to the removal of COS2 from the HTC.

E. Presentation of the Master project

So far, the *fu* null alleles available consisted in large deletions, including several other genes beside *fu*. In our lab the first fu^{KO} loss of function specific allele for *fu* was available. It was produced by CRISP/Cas genome editing. 2.6 Kb of the *fu* locus were

removed, including the 5ðUTR but not the 3ðUTR, and replaced with a DsRed coding sequence.

My master project consisted in the characterization of this novel mutant. I centred my study on the following points:

1) phenotypic analysis of fu^{KO} mutation,
2) comparative analysis of different *fu* mutants on HH signalling,
3) analysis of fu^{KO} on SMO.

II. MATERIAL AND METHODS

A. Drosophila lines and crosses

Table 1: fly lines that were used in the experiments.

Genotype	Characteristic	Source
W1118	control	Lab stocks
	Mutant lines	
yw fu^{1}*/FM7* and *yw* fu^{1}*/FM7GFP*	fu^{1} mutant (class I)	Lab stocks
fu^{RX2}*/FM3* and fu^{RX2}*/FM7GFP*	fu^{RX2} mutant (class II)	Lab stocks
y fu^{KO} *crispr/y FM0Bar*	fu^{KO} Crispr/CAS mutant	Lab stocks
	Reporter lines	
yw Hs Flp; cyo y+/sp; TM6 tb/irolacZ	Iro transcription lacZ reporter	Lab stocks
yw; dpplacZ/cyo y+; TM6 tb/ ry Dp	dpp transcription lacZ reporter (enhancer trap)	Lab stocks
yw Hs Flp; cyo/sp; dppZ/TM2	dpp transcription lacZ reporter (construct)	Lab stocks
MS1096, hhlacZ	Hh transcription lacZ reporter	Lab stocks
	Rescue experiment	
y fu^{KO} *crispr/FM7GFP;;71BGal4/TM6B*	Established lines	
UASGFPfu		
	Genetic interaction experiment	
$Su(fu)^{LP}$/$Su(fu)^{LP}$	Suppressor of Fused mutant	Lab stocks

The lacZ reporter lines in the table were used to investigate gene transcription activity.

Females fu^{KO}*/FM7GFP;;71BGal4/TM6B* used for the rescue experiment were established crossing fu^{KO}*/FM7GFP;;sb/TM6B* x *FM7GFP/Y;;sb/71BGal4*.

Then fu^{KO}*/FM7GFP;;71***b***Gal4/TM6* females were crossed with males *UASGFPfu*.

All the crosses were kept at 23°C.

The crosses for the genetic interaction with $Su(fu)^{LP}$ were kept at 25°C.

In every cross, 5 males were crossed with 10 females and the tubes were flipped every two days to avoid overcrowding.

B. Mutant male larva selection

Male larva were selected with Leica Z16 stereomicroscope. *fu* mutant male larva were selected using the NiKON SMZ1500 binocular loupe stereomicroscope. I selected fu^{KO}/Y DsRed positive larva (see the first paragraph of the results). To select fu^{1} and fu^{RX2} males, I established two lines: fu^{1}*/FM7GFP* and fu^{RX2}*/FM7GFP*. FM7GFP is a balancer chromosome for the X chromosome that carries a dominant green fluorescent marker. To selected the mutant male, I kept just the not GFP larva.

C. Larva dissection and fixation

Larva were dissected in PBS 1X and fixed in 4% paraformaldehyde for 20 minutes. The samples were quickly washed two times in PBS 0,3% Triton (PBT) and then washed three times for 10 minutes in PBT. At this point the samples can be stored in the fridge at 4°C.

D. Wing imaginal discs immuno-staining

Dissected and fixed larva were washed for 30 minutes in BBT (PBS 1X, triton 0,3%, BSA 0,1%, NaCL 25mM) and incubated overnight at 4°C with the following primary antibody: Ũ-EN mouse (DSHB), Ũ-SMO mouse (DHSB, clone 20C6), Ũ-PTC rabbit (DSHB), Ũ-PKC rabbit and Ũ-ßgalactosidase (Santa Cruz Biotechnology) 1:1000, Ũ-COL mouse 1:25 (M. Crozatier) and Ũ-CI Rat 1:5 (Gift from Robert Holmgren). Then, the samples were washed three times for 10 minutes in BBT and incubated with the secondary antibody for two hours in agitation in the dark, at room temperature. The Secondary antibodies Alexa Fluor 488 Goat anti-Rabbit (Invitrogen), Alexa Fluor 555 Goat anti-Mouse (Invitrogen) and Alexa Fluor Goat anti-Rat (Invitrogen) were used at a dilution of 1:100. Finally, the samples were washed four times for 10 minutes in PBT and stored in Citifluor© at 4°C.
Wing imaginal disc images were acquired with a Leica SP5 confocal microscope with a 20x and 40x magnification oil objective lens and Apotome.2 microscope, analysed with ImageJ (National Institute of Health), and assembled with Photoshop (Adobe, San Jose, CA).

To look at SMO localization fu^{KO}/Y and wild type larva were stained in the same tube and treated exactly in the same conditions.

E. Wings

The males were conserved in ethanol 70%. The right wings were removed, rinsed in water, and mounted in Hoyerôs medium. Wing images were acquired at a 150x magnification using the Keyence VHX-2000 Digital Microscope, and assembled with Photoshop (Adobe, San Jose, CA).

F. Statistics

The *p-Values* concerning the wing experiments were calculates using Excel G^2 test or an online Fisherôs test at https://marne.u707.jussieu.fr/biostatgv/?module=tests/fisher, May 18th, 2017).

III. RESULTS

A. *fu^{KO}/FM0Bar* line production

fu^{KO} mutant was produced with CRISPR/Cas9 mediated genome editing by inDROSO company. With this technique it is possible to perform precise and targeted changes in the genome. Two guide RNAs (gRNA) and a double-strand DNA (dsDNA) plasmid donor containing the fluorescent marker DsRed, were used to lead homology directed repair in *fu* locus. The gRNA1 anneals 545 bp upstream the ATG, while the gRNA2 anneals 26 bp downstream the TAG. In this way about 2.6 Kb were removed, including the 5ðUTR but not the 3ðUTR, and replaced with a DsRed coding sequence. It provides a marker to distinguish *fu^{KO}* mutant adult flies and larva. The Ds-Red signal is mainly visible in adult eyes and in larva entire body.

To maintain *fu^{KO}* mutation the FM0Bar balancer for X chromosome was used. It carries the dominant marker mutation ñBarò which is responsible for kidney shaped eyes in heterozygosis and slit-shaped eyes in homo/hemizygosis. In this way we can distinguish wild type eyes *fu^{KO}* hemizygous male and homozygous females from not mutant Bar/semiBar flies.

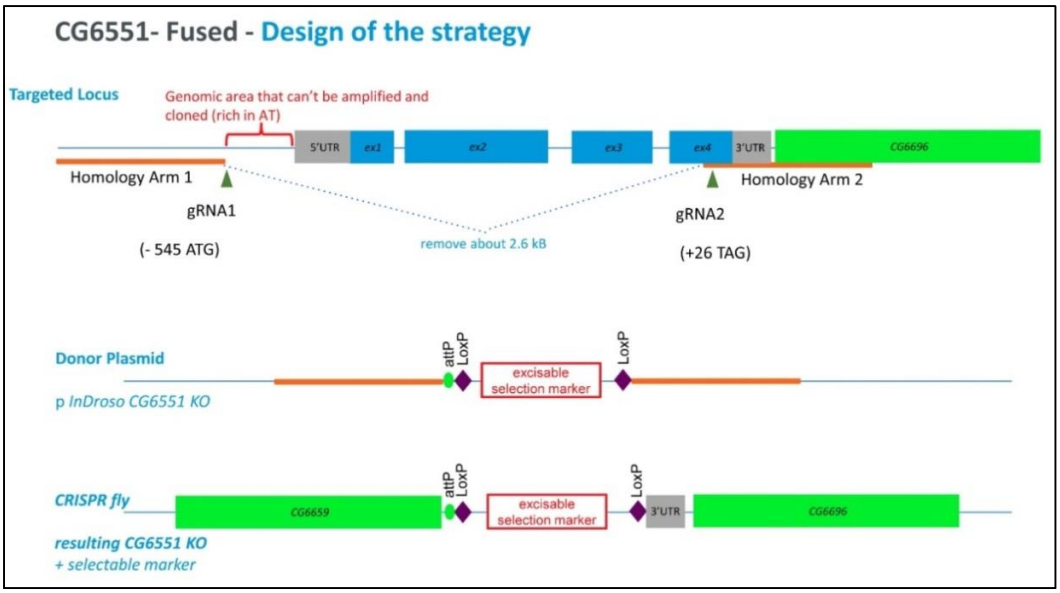

Figure 4: CRISPR/Cas9 mediated genome editing by homology directed repair using two gRNAs and a dsDNA donor plasmid.

On the top, the targeted fu locus and the region that was removed (blue dash line). In the middle, the donor plasmid carrying the DsRed marker with the homology sequences (in orange). At the bottom, the resulting edited locus conserving fu locus 3ðUTR and holding the DsRed coding sequence (figure from inDROSO®).

B. *fu^KO/FM0Bar* line initial characterization: effect on lethality and thermo-sensitivity

1. Lethality

The *fu^KO/FM0Bar* line was kept at 23°C. I thus expected to observe the following genotypes: *fu^KO/Y* and *FM0Bar/Y* males and *fu^KO/fu^KO*, *fu^KO/FM0Bar* and *FM0Bar/FM0Bar* females. Interestingly, I observed that homozygous *fu^KO/fu^KO* females were almost absent. To explain this, I advanced two hypothesis: 1) *fu^KO/Y* in the parental generation can't have progeny. 2) *fu^KO/fu^KO* homozygous genotype is lethal. To test these hypothesis, I crossed *fu^KO/FM0Bar* females with both *fu^KO/Y* and *FM0Bar/Y* males. The progeny genotypes observed at 23°C are reported in the table.

Table 2: crosses to test if fu^KO/Y can have progeny.
fu^KO/FM0Bar x FM0Bar/Y cross was used as control.

Cross at 23°C	F1 genotype	Expected proportion	Observed number	Observed proportion
fu^KO/FM0B x *FM0Bar/Y*	***fu^KO/Y***	**0.5**	**17**	**17/97=0.175**
	FM0Bar/Y	0.5	80	80/97=0.824
	fu^KO/FM0Bar	0.5	152	152/173=0.878
	FM0Bar/FM0Bar	0.5	21	21/173=0.121
	Tot		270	
fu^KO/FM0Bar x *fu^KO/Y*	***fu^KO/Y***	**0.5**	**21**	**21/104=0.201**
	FM0Bar/Y	0.5	83	83/104=0.798
	fu^KO/fu^KO	**0.5**	**9**	**9/145=0.062**
	FM0Bar/fu^KO	0.5	136	136/145=0.937
	Tot		249	

Both *fu^KO/Y* and *FM0Bar/Y* had progeny. For this reason, I excluded the first hypothesis. From this experiment emerged also that the observed proportions for *fu^KO/Y* and *fu^KO/fu^KO* genotypes were lower than the expected (table 2). This result suggests that *fu^KO* allele is somehow affecting the viability when hemizygous in the males and even more when homozygous in females. I concluded that *fu^KO/Y* and *fu^KO/fu^KO* genotypes show an incomplete penetrance for the lethality phenotype. Moreover, the penetrance of the lethality phenotype for *fu^KO* allele seems to be lower for hemizygous males than for homozygous female.

I wanted then to compare the lethality saw in *fu^KO/fu^KO* females with that of another *fu* mutant, *fu^RX2*. *fu^RX2* is a class II allele that bears a 16-bp deletion that affects the C-

term part of the coding sequence. The resulting putative protein lacks the 57 last amino acids of the normal protein that are replaced by 89 news one [10].
As reported in the table, in this case fu^{RX2}/fu^{RX2} females were observed. This means that for fu^{RX2} the homozygous genotype is viable.

Table 3: fu^{RX2}/FM3 line genotypes observed at 23°C.

Males		Females		
fu^{RX2}/Y	*FM3/Y*	$fu^{RX2}/FM3$	fu^{RX2}/fu^{RX2}	*FM3/FM3*
20	0	27	33	2

In conclusion, fu^{KO} allele shows a partial lethality in homozygosity, while fu^{RX2} allele does not.

2. Thermo-sensitivity

Some previous observations done in the lab suggested that *fu* alleles phenotype could be thermo-sensitive. To address this point, I kept the fu^{KO}/*FM0Bar* line at 29°C. The genotypes that I observed are reported in the table:

Table 4: fu^{KO}/FM0Bar line genotypes observed at 29°C.

Males		Females		
fu^{KO}/Y	*FM0Bar/Y*	$fu^{KO}/FM0Bar$	fu^{KO}/fu^{KO}	*FM0Bar/FM0Bar*
2	58	729	0	3

At this temperature fu^{KO}/fu^{KO} females were absent, as it was recorded also at 23°C. In addition, at 29°C I observed very few fu^{KO} males. The proportion of fu^{KO}/Y over the total number of males turned out to be statistically different if compared with the same proportion at 23°C (Fisher exact test p-value < 0,01).

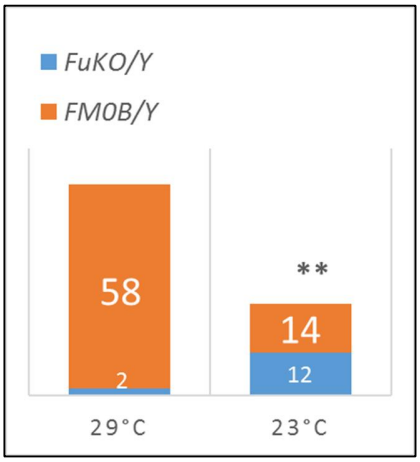

Figure 5: fu^{KO} allele lethality phenotype is thermo-sensitive.
Phenotype distribution according to the genotype at 29°C and 23°C. fu^{KO}/Y males proportion at 29°C is lower if compared with that at 23°C (p-value < 0,01).

In conclusion, fu^{KO}/Y absence at 29°C shows that *fu* null allele lethality phenotype is thermo-sensitive.

C. Analysis of *fu^{KO}/Y* wing phenotype

1. Direct observation of the wing phenotype

I pursued the analysis of *fu^{KO}/Y* flies at 23°C. As it was previously observed for all *fu* alleles, *fu^{KO}/Y* showed a wing phenotype. The proximal fusion between LV3 and LV4, was always present (fig. 6 A, B, C, D). In addition to this, a variable phenotype was observed in the distal region of the wing. This phenotype ranges from a weak phenotype (arrow in fig. 6A), to a stronger one (arrow in fig. 6B), reaching the almost total LV3-LV4 fusion (fig. 6C). Anastomosis, a partial junction between LV3-LV4 (arrow in fig. 6D), were also observed. So, *fu^{KO}* male wings showed at 23°C a variable phenotype for LV3 and LV4 fusion, including anastomosis.

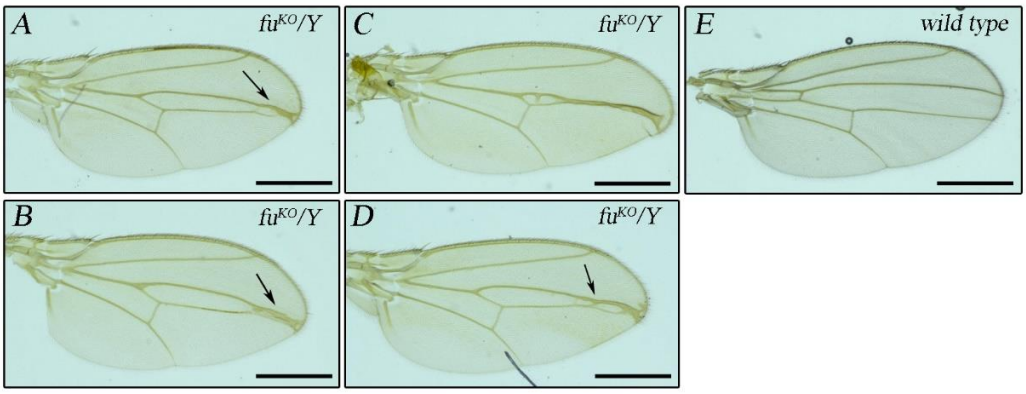

Figure 6: fu^{KO}/Y wings phenotype at 23°C.
fu^{KO}/Y wings showing LV3 and LV4 proximal fusion, (A, B, C and D) and weak distal fusion (arrow in A), stronger distal fusion (arrow in B), almost total fusion (C) and anastomosis (arrow in D). (E) wild type wing. In the figure and in the following wing figures the scale bar represents 500 µm.

2. Rescue experiment

To confirm that these effects were due to the loss of *fu*, I for that purpose examined if the expression of wild type *fu* is sufficient to suppress the wing phenotype. I used the UAS/Gal4 system to express a wild type *fu* transgene. In this system, Gal4 binding to UAS (Upstream Activation Sequence) drives the expression of the downstream gene. The transcription factor Gal4 can be expressed in specific tissues thanks to specific promoters. I used 71BGal4 driver to express UAS-GFP-FU in the wing pouch. The expression of wild type *fu* suppressed the fusion phenotype, both proximally and distally (fig. 7C compared with fig. 7A). In absence of Gal4, *UASGFPfu* expression is not induced and there is no rescue of the wild type phenotype (fig. 7B).

In conclusion, this result shows that *fu^{KO}/Y* wing phenotype is specifically due to the loss of *fu*.

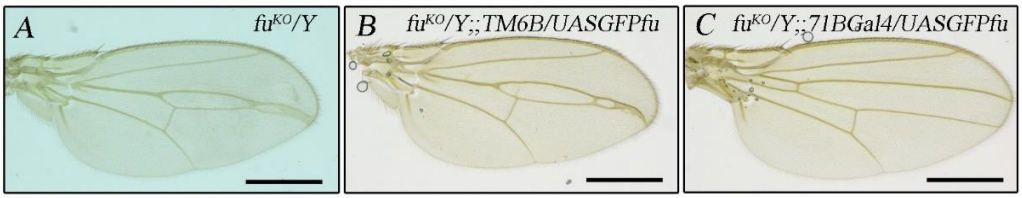

Figure 7: Gal4/UAS driven wild type fu expression in fu^{KO}/Y suppresses the fusion phenotype.

*(A) fu^{KO}/Y wing. (B) fu^{KO}/Y wing not expressing fu because 71**b**Gal4 is absent. (C) fu^{KO}/Y wing expressing wild type fu under the control of 71**b**Gal4.*

D. Comparison between *fu*^{KO} and other *fu* alleles wing phenotype

To better characterize the *fu*^{KO} allele, I compared its effects with the phenotypes of known *fu* alleles, *fu*^{1} and *fu*^{RX2}. *fu*^{1} is the classical allele used in all the labs for class I mutations, even if the nature of the mutation is not known. *fu*^{RX2} is a class II allele that I presented above.

At 23°C *fu*^{1}*/Y* and *fu*^{RX2}*/Y* show a variable wing fusion phenotype, as it was observed for *fu*^{KO}*/Y* (data not shown). To be able to compare the three *fu* alleles wing phenotype, I took in consideration three different criteria: the proximal fusion (fig.8 A, B, C), the distal fusion (fig. 8 D, E, F) and the presence or absence of the total fusion (fig. 8 G and H) between LV3-LV4. For the proximal and distal fusion, we defined three categories: very weak (star in fig. 8 A and D respectively), weak (fig. 8 B and E respectively) and strong (fig. 8 C and F respectively). The three mutant wings showed also anastomosis. I didnôt take this criterion into consideration to compare the wing phenotype because it was hard to quantify.

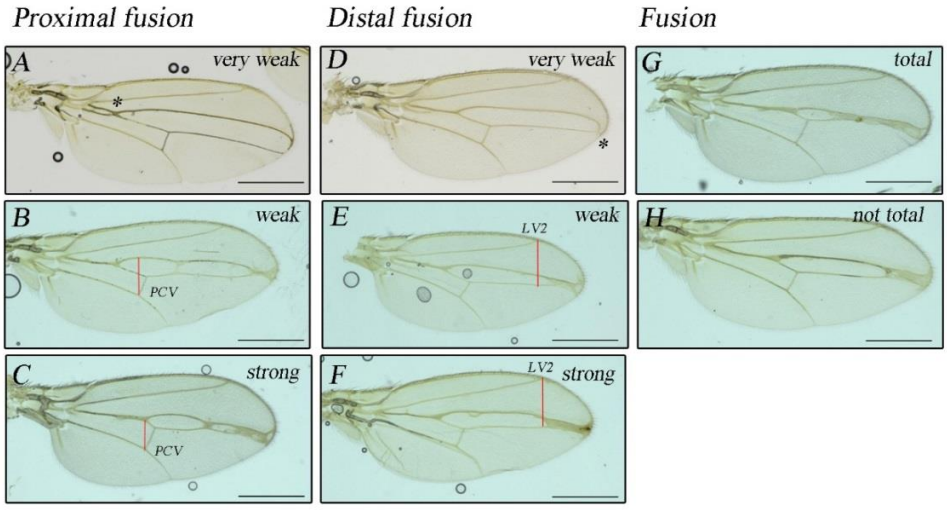

Figure 8: wing phenotype classification.

Proximal fusion phenotype (A, B and C). Distal fusion phenotype (D, E and F). Total or not-total fusion phenotype (G and H). For each phenotype some categories were defined: very weak (A and D), weak (B and E) and strong (C and F). For the proximal fusion, if the perpendicular line joining the Posterior Crossvein (PCV) and LV3 (red line fig. C) fell in the fusion, the wing was classified as ***ñ****strong phenotype****ò****, otherwise as* ***ñ****weak****ò*** *(red line in fig. B). For the distal fusion it was done the same way considering LV2 extremity and LV4 (red line in fig. F and E).*

For the proximal fusion phenotype, I observed that fu^{1}/Y wings showed only the weak fusion, fu^{RX2} wings showed almost only the strong fusion and fu^{KO}/Y showed both. The proportion of wings with ñweakò and ñstrongò fusion turned out to be significantly different between fu^{1}/Y and fu^{KO}/Y (G^2 test p-value <0,001, left graph) and also between fu^{KO}/Y and fu^{RX2}/Y (G^2 test p-value <0,001, right graph). So, fu^{KO}/Y proximal fusion phenotype seems to be intermediate between fu^{1}/Y and fu^{RX2}/Y phenotype.

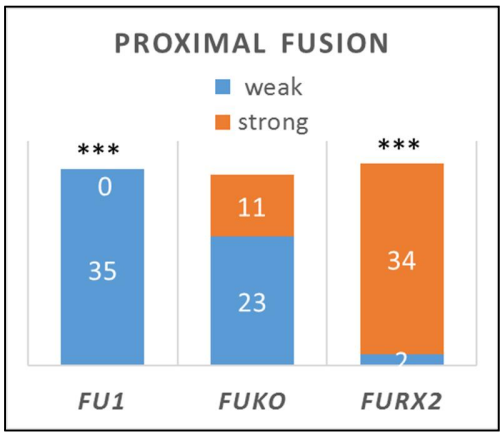

Figure 9: proximal fusion phenotype wing distribution according to the genotype at 23°C.
fu^{KO}/Y wings show an intermediate phenotype between fu^{1} and fu^{RX2} males (G^2 test p-value <0,001).

Similarly, for the distal fusion phenotype, I observed that fu^{1}/Y wings showed almost only the weak fusion, fu^{RX2}/Y wings showed almost only the strong fusion and fu^{KO}/Y showed both. The proportion of wings with ñweakò and ñstrongò fusion turned out to be significantly different between fu^{1}/Y and fu^{KO}/Y (G^2 test p-value <0,01, left graph) and also between fu^{KO}/Y and fu^{RX2}/Y (G^2 test p-value <0,001, right graph). So, also for the distal fusion, fu^{KO}/Y phenotype seems to be intermediate between fu^{1}/Y and fu^{RX2}/Y phenotype.

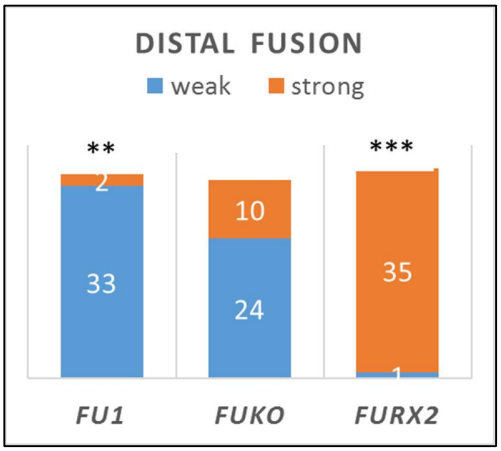

Figure 10: distal fusion phenotype wing distribution according to the genotype at 23°C.
fu^{KO}/Y wings show an intermediate phenotype between fu^{1} and fu^{RX2} males (G^2 test p-value <0,01, left graph. G^2 test p-value <0,001, right graph).

For the total/not-total fusion phenotype I observed that fu^{1}/Y wings never showed the total fusion while fu^{RX2}/Y and fu^{KO}/Y wings did. The proportion of wings with total and not-total fusion turned out to be significantly different between fu^{1}/Y and fu^{KO}/Y (g^2 test p-value <0,001, left graph) and between fu^{KO}/Y and fu^{RX2}/Y, even if with a less significant p-value (g^2 test p-value <0,01, right graph). Even for the total/not-total fusion, fu^{KO}/Y phenotype seems to be intermediate between fu^{1}/Y and fu^{RX2}/Y.

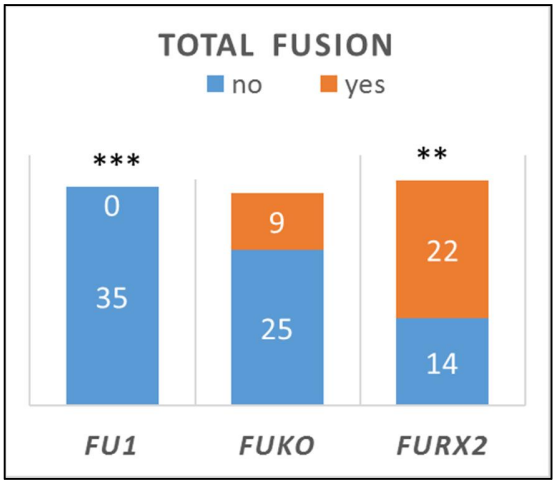

Figure 11: total/not-total fusion phenotype wing distribution according to the genotype at 23°C.
fu^{KO}/Y wings show an intermediate phenotype between fu^{1} and fu^{RX2} males (g^2 test p-value <0,001, left graph. g^2 test p-value <0,01, right graph).

In summary I can say that, for the three criteria considered above, fu^{KO}/Y wing phenotype is intermediate between class I fu^{1}/Y and class II fu^{RX2}/Y mutants.

E. Genetic interaction with $Su(fu)^{LP}$

As presented in the introduction, the genetic interaction with *Su(fu)* has been used to classify the different *fu* alleles. Itôs known that *fu* wing fusion phenotype is totally suppressed by $Su(fu)^{LP}$ mutation when homozygous, partially when heterozygous. To further characterize the fu^{KO} allele, I studied the effect of $Su(fu)^{LP}$ heterozygous mutation on fu^{KO}/Y wing phenotype.
I crossed $fu^{KO}/FM0Bar$ females with $Su(fu)^{LP}$ homozygous males and I looked at the wing phenotype of $fu^{KO}/Y;;Su(fu)^{LP}/+$ progeny. I used fu^{1}/Y as a control.
As expected, fu^{1}/Y wings weak distal fusion (fig. 12B) was always totally suppressed by $Su(fu)^{LP}$ heterozygous mutation (fig. 12C). fu^{KO}/Y wing strong and weak distal fusion (fig. 12D) were always partially suppressed by $Su(fu)^{LP}$ heterozygous mutation, leading to a very weak distal fusion (fig. 12E).
The graph shows the number of wings that were counted and the phenotype distribution according to the genotype.

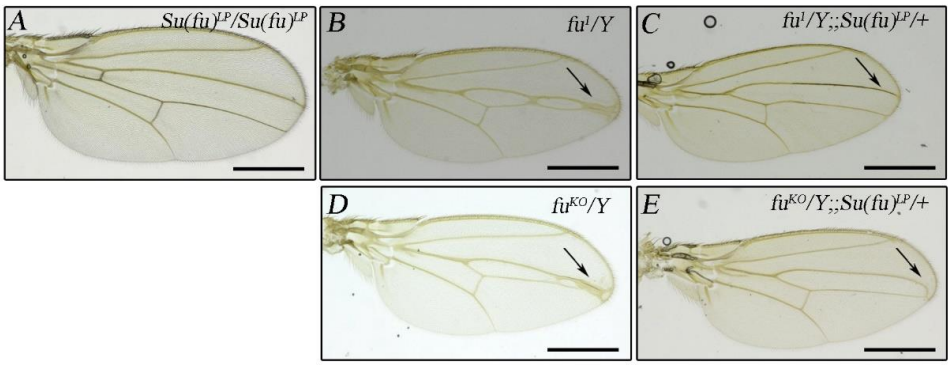

Figure 12: Su(fu)^{LP} heterozygous mutation partially suppress fu^{1}/Y and fu^{KO}/Y distal fusion phenotype at 25°C.

(A) Su(fu)^{LP} homozygous amorphic mutation doesnâ cause any wing phenotype. (B and C) fu^{1}/Y wing weak distal fusion (arrow in B) is totally supressed by Su(fu)^{LP} heterozygous mutation, (arrow in C). (D and E) fu^{KO}/Y wing weak or strong distal fusion (arrow in D) is partially supressed by Su(fu)^{LP} heterozygous mutation (arrow in E).

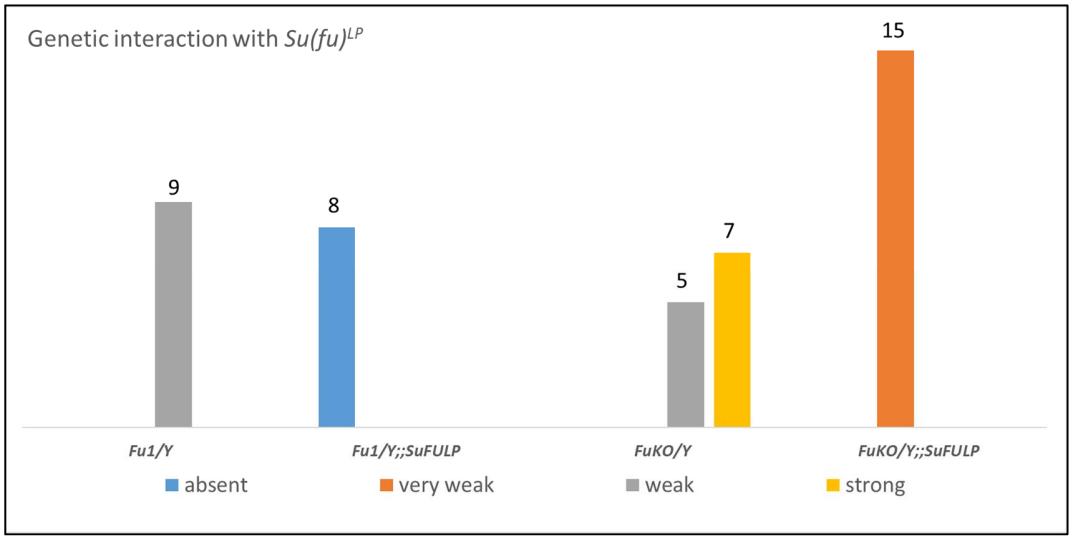

Figure 13: wing phenotype distribution according to the genotype at 25°C.

The graph shows the number of wings that were counted for each genotype and the distal fusion phenotype distribution according to the genotype.

In conclusion, *Su(fu)^{LP}* heterozygous mutation leads to a partial suppression of *fu^{KO}/Y* wing phenotype. This suggests that *fu^{KO}* behaves as a class I allele.

F. Analysis of fu^{KO}/Y wing imaginal disc phenotype

I continued to compare the effects of fu^{KO} with those of fu^{1} and fu^{RX2} by studying their effect on CI and the HH target genes expression in the wing disc.

1. Analysis of high HH target genes

Since FU is involved in HH high signal transduction, I first looked at high target genes expression, *col* and *ptc*.

In wild type wing disc, *col* and *ptc* are expressed in 6 rows of anterior cells abutting the A/P (fig. 14 A and E). *col* expression is affected in a very similar way in fu^{KO}/Y, fu^{1}/Y and fu^{RX2}/Y. In all cases COL fails to accumulate in the central region of the wing pouch and accumulates in a wider region if compared with the wild type (fig. 14 B, C and D compared with A).

ptc expression is more spread toward the A compartment of the disc in *fu* mutants if compared with the wild type (fig. 14 F, G, H compared with E). In addition, *ptc* expression in fu^{KO}/Y and fu^{RX2}/Y is weaker and more spread if compared with fu^{1}/Y.

In conclusion, in *fu* mutants showing a very different wing phenotype high target gene *col* is affected in a similar way. However, *ptc* expression is more affected in fu^{KO}/Y than in other *fu* mutants.

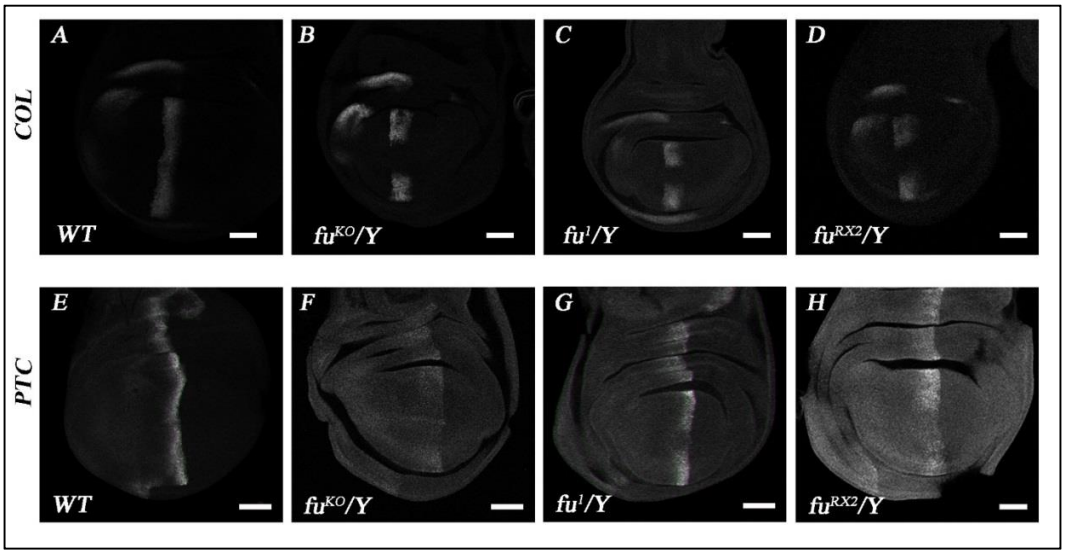

Figure 14: COL and PTC protein accumulation in wild type and fu mutants wing discs at 23°C.

Discs labelled with COL and PTC antibody. (A and E) Wild type, (B and F) fu^{KO}/Y (n=5 and 15), (C and G) fu^{1}/Y (n=12 and 11) and (D and H) fu^{RX2}/Y (n=14 and 10) wing discs. In the figure and in the following wing disc figures the scale bar represents 50 µm and ***ñnò*** *is the number of discs observed.*

2. Analysis of low HH target genes

fu[1] and *fu*[RX2] mutations affect HH high signal transduction but not low signal transduction. For this reason, I investigated whether it was also the case for *fu*[KO] by following low target gene *dpp* expression. *dpp* transcription was followed using two different reporters: a *dppZ* enhancer trap and *dppZ* BS3.0 construct.

dppZ enhancer trap fly line was built by insertional mutagenesis using a P-element construct carrying the *lacZ* reporter gene. In this case, *lacZ* gene is expressed under the control of *dpp* enhancer in the cells containing an activator protein for that enhancer. *LacZ* product can be detected using Ŭ*b*gal antibody.
In wild type wing disc, *dpp* is expressed between 5 and 12 anterior cell rows after the A/P boundary (fig. 15A). In *fu*[KO]*/Y* and *fu*[1]*/Y* wing discs, *dppZ* enhancer trap is expressed in a wider region if compared with the wild type (fig. 15 B and C compared with A). This is in accordance with the weaker *ptc* expression that I observed in *fu* mutants. Indeed, a weaker PTC level will lead to a lower quantity of HH molecules trapped by PTC. This allows HH to diffuse more toward the A compartment, forming a larger intermediate/low concentration region and inducing a wider expression of its low target genes.

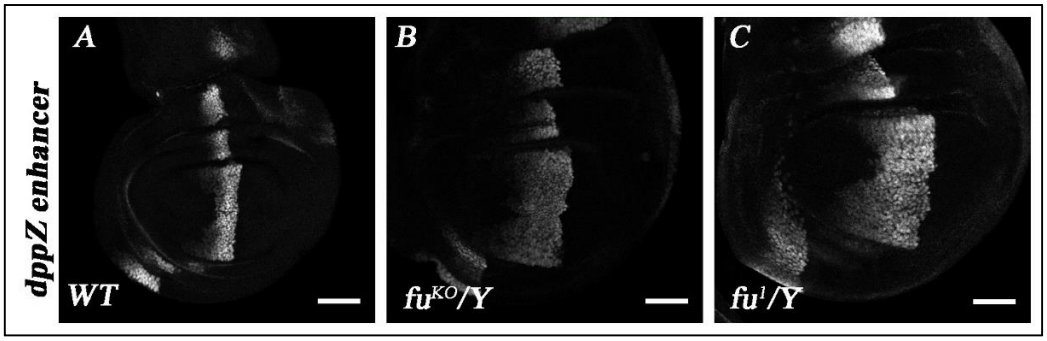

Figure 15: dppZ enhancer trap expression in wild type and fu mutants wing discs at 23°C.
Discs labelled with ***b****gal. Dpp transcription activity in (A) wild type, (B) fu*[KO]*/Y (n=5) and (C) fu*[1]*/Y (n=1) wing discs. fu*[RX2]*/Y phenotype is not available.*

Interestingly, using *dppZ* BS3.0 construct the phenotypes observed were different. *dppZ* BS3.0 is a synthetic construct carrying a region of *dpp* promotor and *lacZ* reporter gene [11].
In the wild type wing disc, BS3.0 directs *dpp* expression in a non-continuous band of cells that extends the length of the disc, parallel to the A/P boundary. *dppZ* BS3.0 expression is absent in *fu*[KO] and *fu*[RX2] mutants if compared with the wild type (fig. 16 B and D compared with A).

For *fu*^{1} I observed no *dpp* expression in 8 out of 13 discs. However, in 5 discs, *dppZ* BS3.0 was still expressed in a wider region and its expression was partially disrupted in the central region of the wing disc (fig. 16 C^{I} C^{II} compared with A).

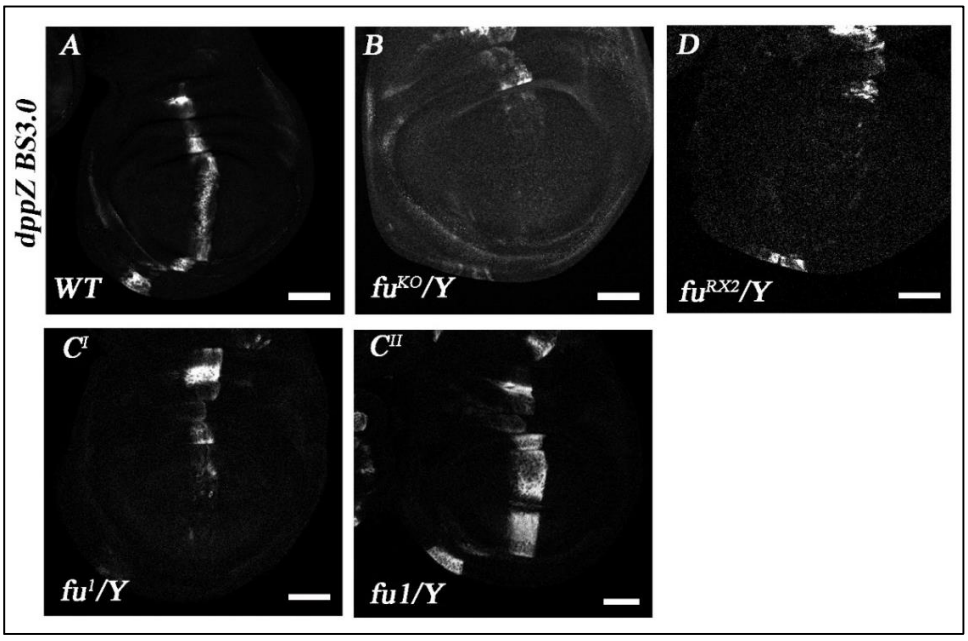

Figure 16: dppZ BS3.0 construct expression in wild type and fu mutants wing discs at 23°C.

Discs labelled with **b***gal. Dpp transcription activity in (A) wild type, (B) fu*^{KO}*/Y (n=16), (C*^{I} *and C*^{II}*) fu*^{1}*/Y (n=14) and (D) fu*^{RX2}*/Y (n=8) wing discs.*

In conclusion, as previously published, in *fu*^{KO} *and fu*^{1} mutants *dpp* followed with *dppZ* enhancer was expressed in a wider region. Unexpectedly, *dpp* analysis with the enhancer trap and BS3.0 construct produced different results. In addition, *fu*^{1}*/Y* mutants showed different phenotypes when analysed with BS3.0.

3. Analysis of CIF accumulation

Since I observed that high CI target genes expression was affected in *fu* mutants, I looked at CIF accumulation in the wing disc.

In wild type wing disc, CIF forms a gradient from the A/P border toward the A compartment and it is more accumulated close to the A/P border (fig. 17A). In the wild type, labile CIL (arrow in fig. 17A) at the A/P border is visible. In the three *fu* mutants, CIF accumulation is more spread out toward the A compartment (fig. 17 B, C and D). Regarding CIL, it is not present at the A/P border in *fu* mutants.

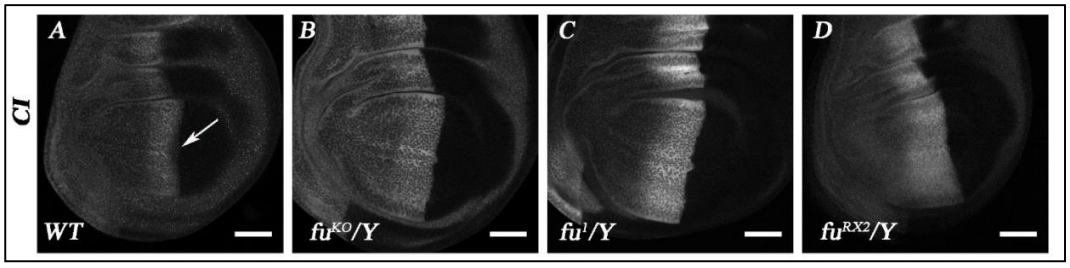

Figure 17: CIF accumulation in wild type and fu mutants wing discs at 23°C.
Discs labelled with CIF antibody. CIF accumulation in (A) wild type, (B) fu^{KO}/Y (n=10), (C) fu^{1}/Y (n=10) and (D)fu^{RX2}/Y (n=10) wing discs. In the three fu mutants CIL (arrow in A) is missing.

4. Analysis of *hh* expression in fu^{KO}

To exclude the possibility that the effect on CI target genes in fu^{KO}/Y was due to a problem in HH production, I looked at *hh* expression in the wing disc. To do this I used a *hhZ* reporter line.
In wild type wing disc, *hhZ* is expressed in the posterior compartment (fig. 18A). In fu^{KO}/Y *hh* transcription is active in the posterior compartment of the wing disc and there are not substantial differences with the wild type (fig. 18D).

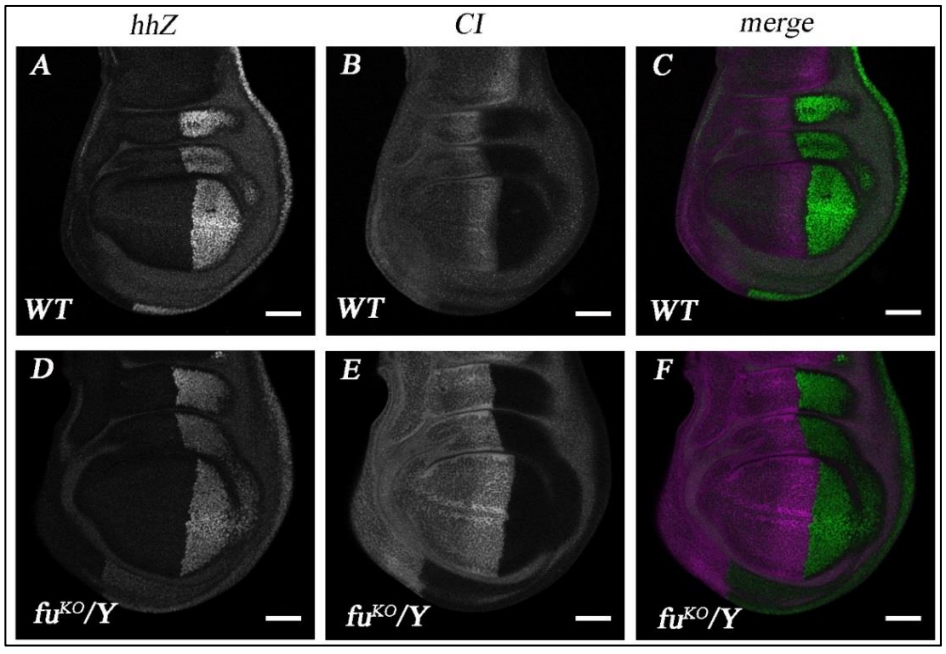

Figure 18: hhZ expression and CIF accumulation in wild type and fu^{KO}/Y mutant wing discs at 23°C.
hhZ transcription activity and CIF accumulation in (A and B) wild type and (D and

E) fu^{KO}/Y (n=9) wing discs. CIF staining was used to mark the A compartment of the disc.

In conclusion, I can say that the effect on CI target genes that I observed in fu^{KO}/Y is cell-autonomous and specifically due to the loss of *fu* in the receiving cells.

G. Regulation of SMO localization

Previous observations done in the lab showed that the phosphorylation of SMO C-term sites by FU promotes its apico-basal distribution in the columnar layer of the wing disc (Sanial et Bécam, 2017). In these experiments, the fraction of SMO present at the plasma membrane was overexpressed and labelled with the SNAP-Tag technology.

I was then interested to see if the loss of *fu* could somehow affect SMO localization. To do this, I performed a SMO and CIF immunostaining in fu^{KO}/Y larva wing disc. I used wild type disc as control. CIF immunostaining allowed me to distinguish fu^{KO}/Y discs, which miss CIL at the A/P border, from wild type ones and to visualize the A and P compartments of the disc. Moreover, to visualize the apical-basal polarity of the cell, I marked the disc with an antibody against Protein Kinase C (PKC), which is an apical marker.

In wild type wing disc (fig. 19A), SMO is visible in the P compartment, where there is no PTC inhibiting it. SMO accumulates at the A/P boundary in correspondence of CIL. In fu^{KO}/Y wing disc (fig. 19D) SMO accumulates in a wider region after the A/P boundary, toward the A compartment if compared with the wild type. In addition, there is not a stronger accumulation of SMO in the A/P in correspondence of CIL.

I also looked at SMO in wing disc XZ-section. The XZ section was performed with SP5 confocal microscope scanning in the wing pouch, perpendicularly to the A/P border (red line in fig. 19A). In the wild type, SMO is mainly localized in the latero-basal domain (fig. 19G). In fu^{KO}/Y SMO seems to be equally distributed between the apical and the basal domains (fig. 19M).

In summary, these preliminary results, that need to be repeated, suggest that the absence of FU influences the apico-basal distribution of SMO in the columnar epithelium of the wing disc.

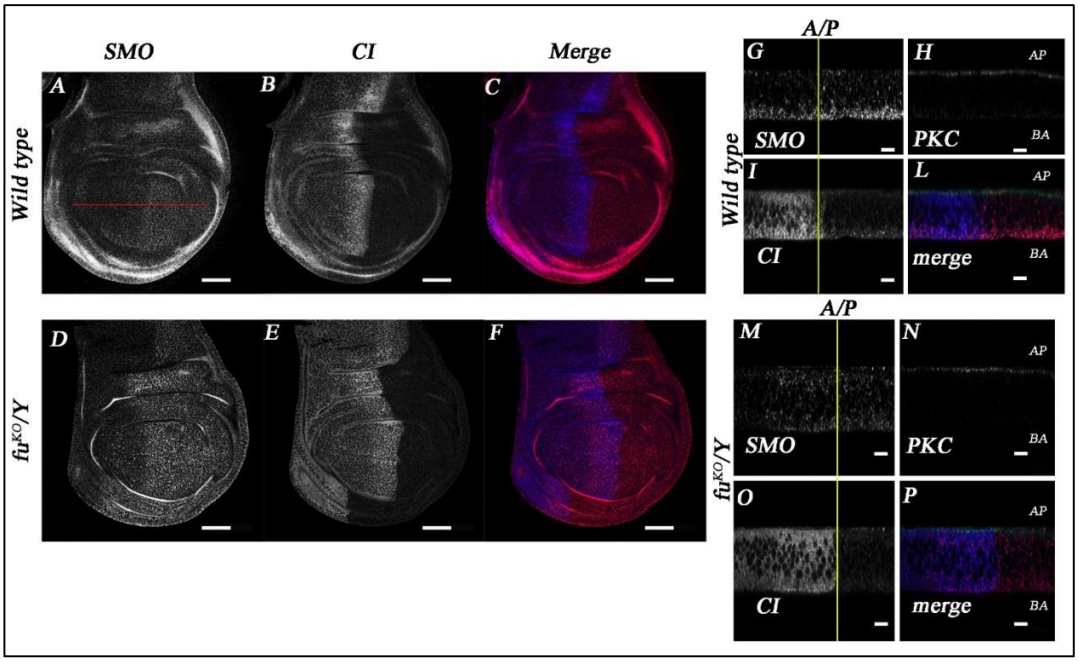

Figure 19: SMO and CIF accumulation in wild type and fu^{KO}/Y mutant wing discs at 23°C.

SMO and CIF accumulation in (A and B) wild type and (D and E) fu^{KO}/Y (n=3) wing discs. (C and F) Merge. SMO and CIF accumulation in (G and I) wild type and (M and O) fu^{KO}/Y wing disc XZ section. (H and N) Apical marker PKC staining. (L and P) Merge.

AP: apical. BA: basal. The scale bar in XZ section picture represents 10 µm.

IV. DISCUSSION

In my lab, we have obtained the first null allele for *fused*, which codes for a positive regulator of the HH pathway. In this study, I have:

1) characterized the different phenotypes of this mutant and compared them to those of different *fu* mutants,
2) shown that FU is in part required in the wing disc for the expression of the low target *dpp*,
3) obtained evidence that FU controls the apico-basal distribution of SMO in the epithelial cells of the wing disc.

A. Different phenotypes are induced by fu^{KO} allele

fu^{KO} allele analysis revealed that at 23°C fu^{KO}/Y and fu^{KO}/fu^{KO} genotypes are partially lethal. Moreover, the effect of the lack of *fu* on viability is stronger in fu^{KO}/fu^{KO} homozygous females than in hemizygous males. It shows that (1) *fu* is important during the development of fly and (2) its role is more crucial in female. To better understand how the loss of *fu* affects viability, it could be useful to understand at which stage (embryo, larva, pupa) fu^{KO}/Y males and fu^{KO}/fu^{KO} females die. Interestingly, fu^{RX2} homozygous and hemizygous mutation didn't affect fly viability.
fu^{KO}/Y wing show the LV3-LV4 fusion phenotype that was already observed in the other *fu* mutants. By comparing fu^{KO}/Y wing phenotype with fu^{1} and fu^{RX2}, I observed that fu^{KO}/Y wings share some phenotypical traits generally observed in fu^{1}/Y wings and at the same time present also the phenotypical traits typical of fu^{RX2}/Y wings. Thus, surprisingly the wing phenotype of fu^{KO} males is less strong than the one of fu^{RX2} which is not a null allele.
This suggests that fu^{RX2} mutation has a negative effect on HH signalling. However, it seems that this negative effect doesn't occur during fly development as the fu^{RX2} homozygous and hemizygous mutation didn't affect fly viability. fu^{RX2} mutation affects the last 57 amino-acids corresponding to the COS2 binding site. Thus, a possible explanation is that this FU-COS2 interaction is necessary for the correct patterning of the wing but is not essential during fly development.

B. fu^{KO} allele: a class I, II or 0 mutation?

fu^{KO} wing phenotype was partially supressed by $Su(fu)^{LP}$ heterozygous mutation and no additional zygotic phenotype were observed. So, it seems that fu^{KO} behaves as a class I allele rather than a class II.

The molecular characterization of *fu* alleles defined a new class of allele: the class 0. Class 0 alleles identified so far have very large deletions encompassing the whole *fu* gene and some neighbour genes. Class 0 alleles behave as class I alleles in genetic interaction with $Su(fu)^{LP}$. So, shall we consider fu^{KO} as a class 0 allele rather than a class I? To address this question additional experiments could be done, considering that class I mutants are dominant over class II in *fuI/fuII;Su(fu)* flies, whereas class 0 mutants are recessive over class II in *fu0/fuII;Su(fu)* flies.

C. How is *dpp* expression regulated?

By studying *dpp* expression, I observed that in fu^{KO}/Y and fu^{1}/Y wing discs the *dppZ* enhancer trap reporter is expressed in a wider region if compared with the wild type. These results are consistent with previous studies using class I and class II mutant alleles.

Surprisingly, we couldnôt detect *dpp* expression using the transgenic BS3.0-*dppZ* construct for fu^{KO} and fu^{RX2} mutants. In BS3.0, *lac-Z* expression is driven by a sub-region of the *dpp* promotor, the ñdisk enhancer regionò, which is required for the development of the wings. These results suggest for the first time that FU activity could be required, at least in part, for *dpp* expression.

Only fu^{1} mutant showed sometimes expression of *dpp* BS3.0 construct. This suggests that the regulation of this enhancer could be in part independent from FU kinase domain and that FU could have another function beyond its kinase activity.

D. Regulation of SMO localization by FU

Results obtained in my lab by overexpressing different form of SMO (phosphomimetic or phosphodeficient for FU phosphorylation) suggest that SMO phosphorylation by FU promotes its localization in the basal region of the polarized epithelial cells of the wing disc [9]. Looking at endogenous SMO, I have observed that in wild type disc SMO is mainly localized in the latero-basal domain, while in fu^{KO}/Y SMO seems to be equally distributed between the apical and the basal domains. These preliminary results confirm the one observed by overexpression and suggest that FU activity is important for SMO localization in the latero-basal domain of the epithelial cells of the wing disc.

Whatôs the meaning of SMO latero-basal distribution? It has been shown that in P cells HH is secreted both basally and apically, producing two different gradients [12]. The basal gradient causes the short range induction of high-threshold target genes. As FU is required for the expression of high HH targets, we could propose that FU could regulate SMO through phosphorylation, increasing its basal localisation.

In addition, to better understand how phosphorylation controls SMO localization, it could be interesting to study if other kinases, such as PKA or CK1, are involved in this apico-basal localisation of SMO.

E. fu^{KO} is a new tool to study FU function and role

The production of KO organisms, is one of the basic approach to understand the role of a specific gene or DNA region. So far, the only KO of *fu* were not locus-specific since they consisted in very large deletions including also neighbour genes. Nowadays, genome editing techniques such as CRISP/Cas allow to delete or replace specific region of DNA, producing gene specific mutants. The *fu* mutant that I studied and characterized is the first real null mutant for *fu*. It will thus be possible to study *fu* function in a real loss of function context for instance by expression of different forms of FU (kinase dead form, tag form or form mutated for specific binding sites) without endogenous FU.

In conclusion, further analysis on the fu^{KO} can reveal interesting features of FU function and better characterize it as a basic tool for genetic studies.

V. REFERENCES

1. Nusslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. Nature *287*, 795-801.

2. Huangfu, D., and Anderson, K.V. (2006). Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from Drosophila to vertebrates. Development *133*, 3-14.

3. Briscoe, J., and Therond, P.P. (2013). The mechanisms of Hedgehog signalling and its roles in development and disease. Nat Rev Mol Cell Biol *14*, 416-429.

4. Jia, J., Tong, C., Wang, B., Luo, L., and Jiang, J. (2004). Hedgehog signalling activity of Smoothened requires phosphorylation by protein kinase A and casein kinase I. Nature *432*, 1045-1050.

5. Zhao, Y., Tong, C., and Jiang, J. (2007). Hedgehog regulates smoothened activity by inducing a conformational switch. Nature *450*, 252-258.

6. Hartl, T.A., and Scott, M.P. (2014). Wing tips: The wing disc as a platform for studying Hedgehog signaling. Methods *68*, 199-206.

7. Alves, G., Limbourg, B.B., Tricoire, H., Brissard, Z.J., Lamour, I.C., and Busson, D. (1998). Modulation of Hedgehog target gene expression by the Fused serine- threonine kinase in wing imaginal discs. Mech Dev *78*, 17-31.

8. Claret, S., Sanial, M., and Plessis, A. (2007). Evidence for a novel feedback loop in the Hedgehog pathway involving Smoothened and Fused. Curr Biol *17*, 1326-1333.

9. Sanial, M., Becam, I., Hofmann, L., Behague, J., Arguelles, C., Gourhand, V., Bruzzone, L., Holmgren, R.A., and Plessis, A. (2017). Dose-dependent transduction of Hedgehog relies on phosphorylation-based feedback between the G-protein-coupled receptor Smoothened and the kinase Fused. Development *144*, 1841-1850.

10. Therond, P., Alves, G., Limbourg-Bouchon, B., Tricoire, H., Guillemet, E., Brissard-Zahraoui, J., Lamour-Isnard, C., and Busson, D. (1996). Functional domains of fused, a serine-threonine kinase required for signaling in Drosophila. Genetics *142*, 1181-1198.

11. Blackman, R.K., Sanicola, M., Raftery, L.A., Gillevet, T., and Gelbart, W.M. (1991). An extensive 3' cis-regulatory region directs the imaginal disk expression of decapentaplegic, a member of the TGF-beta family in Drosophila. Development *111*, 657-666.

12. D'Angelo, G., Matusek, T., Pizette, S., and Therond, P.P. (2015). Endocytosis of Hedgehog through dispatched regulates long-range signaling. Dev Cell *32*, 290-303.

Acknowledgements

I want to thank the people that shared with me these great six months in the Institut Jacques Monod.

Marina, thank you for your "amizade". The moments we shared were always fun and rich of surprises. I wish you good luck for your scientific career!

Lucia, your presence always influenced me positively. Thank you for your "consulta", not only related to science.

Camilla, your energy and your passion were a gift for me. Thanks for sharing with me your knowledge of drosophila but also great evenings eating empanadas!

Matthieu, you welcomed me the first day. We shared together just a few weeks but it was enough to understand that you are the colleague that everyone would like to have!

Anne, thank you for hosting me in your lab. Your determination and your positive attitude thought me that if I really want something I can get it.

Isabelle, you followed me for six months. I learned a lot. Thank you for your patience, your advice, your answers and your questions, and also for your daily funny anecdotes. It was a pleasure for me to be your M2 ñstagiaireò.

I want to thank *Yann*, *Michael*, *Andrea*, *Alexis*, *Olga*, *Bénédicte* that helped me and encouraged me. Thanks *Kahina*, *Gregoire* and *Ioannis* for the lunch times and the beers that we shared.

Thank you, each of you thought a bit how to ñflyò.

Voglio ringraziare anche la mia famiglia, in particolare *Martina*, *Andrea*, *Chiara* e *Marco*. Il vostro sostegno a distanza (e non!) non mi è mai venuto meno.

Grazie a chi mi è venuto a trovare durante il mio soggiorno a Parigi. Grazie a *Cristina, Giacomo, Pierangelo, Leonardo, Mara, Ludovico, Leonardo, Michele* e *Lina*. I momenti che abbiamo condiviso hanno reso ancora più speciale la mia avventura nella Ville Lumière!

Grazie a tutti quelli che ci sono stati con un messaggio o una telefonata, *Maria Francesca, Maria Grazia, Marisa, Luca, Sara, Lucia, Pino, Giordano*.

Grazie ad *Helena* e *Sebastian*, perché siete sempre stati una certezza su cui poter contare. Vi auguro il meglio.

Grazie *Italo*, per aver sempre domandato di me. Anche ora che sono tornata.

Grazie al *prof. Argenton* per la sua disponibilità e il suo aiuto.

Un grazie speciale ad *Elena*. Grazie di esserci, sempre, comunque e (ora più che mai ne ho avuto la prova) ovunque.

Merci à mes amis ñparisiensò *Séréna, Jérémy, Nadine* et *Parpathan*, pour les beaux moments passés ensemble.

Et merci à mon ma'Ere de français, *Rodolphe*. Cô était un plaisir danser avec toi.

Grazie. Thank you. Gracias. Merci.

Lisa