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A crosstalk between the RNA binding protein Smaug and the Hedgehog pathway links cell signaling to mRNA regulation in drosophila

Lucía Bruzzone

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Thèse de doctorat
de l'Université Sorbonne Paris Cité
Préparée à l'Université Paris Diderot
Ecole doctorale HOB n° 561

Institut Jacques Monod / Equipe Développement, Signalisation et Trafic

A crosstalk between the RNA binding
protein Smaug and the Hedgehog pathway
links cell signaling to mRNA regulation in
Drosophila

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Thèse de doctorat de Biologie

Dirigée par Anne Plessis

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Titre: Des régulations réciproques entre la protéine de liaison aux ARNm Smaug et la voie Hedgehog lient la signalisation cellulaire à la régulation des ARNm chez la drosophile.

Résumé: La régulation post-transcriptionnelle de l'expression génique joue un rôle essentiel dans divers processus cellulaires pendant le développement. Les protéines de liaison à l'ARN (RBP) sont des médiateurs fondamentaux des régulations post-transcriptionnelles qui contrôlent l'expression de l'ARNm en reconnaissant des séquences spécifiques dans les transcrits cibles. Smaug est une protéine de liaison à l'ARN conservée de la levure jusqu'à l'homme qui est essentielle pendant l'embryogenèse précoce de la drosophile. Smaug reconnaît et lie des éléments de reconnaissance de Smaug (SRE) dans ses ARNm cibles et recrute des facteurs supplémentaires, via des interactions protéine-protéine, qui régulent l'ARNm lié. Un concept qui émerge est celui des voies de signalisation pouvant moduler l'activité des RBP par des modifications post-traductionnelles, en ajoutant ainsi une couche supplémentaire dans le contrôle de l'expression des gènes.

Au cours de mon travail de thèse, j'ai cherché à mettre en évidence que la voie de signalisation Hedgehog régule Smaug en favorisant sa phosphorylation. Mon travail montre que la signalisation HH diminue les niveaux de protéines Smaug affectant sa capacité à réprimer la traduction de l'ARNm. Cet effet négatif semble dépendre de l'interaction entre Smaug et le transducteur de signal HH, Smoothened. De plus, Smaug est constitutivement phosphorylée dans son domaine de liaison à l'ARN, ce qui semble être nécessaire pour la formation des foci cytoplasmiques de Smaug.

Mots clefs: Smaug, régulation post-transcriptionnelle, drosophile, voie de signalisation Hedgehog, Smoothened, phosphorylation, Smaug foci

Title: A crosstalk between the RNA binding protein Smaug and the Hedgehog pathway links cell signaling to mRNA regulation in *Drosophila*.

Abstract: Post-transcriptional regulation of gene expression plays a critical role in a variety of cellular processes during development. RNA binding proteins are fundamental mediators of post-transcriptional regulations that control mRNA expression by recognizing specific *cis* acting elements within the target transcripts. Smaug is a highly conserved sequence specific RNA-binding protein that is essential during *Drosophila* early embryogenesis. Smaug binds Smaug Recognition Elements (SRE) in the target mRNA and recruits additional factors, via protein-protein interactions, that regulate the bound mRNA. An emergent concept that signaling pathways can modulate RBP activity by post-translation modifications adds a new layer in the control of gene expression.

During my thesis work, I sought to understand how the Hedgehog pathway regulates Smaug by promoting its phosphorylation. My work shows that HH signaling downregulates Smaug protein levels affecting its ability to repress mRNA translation. This negative effect seems to be dependent on the interaction between Smaug and the HH signal transducer Smoothed. Moreover, Smaug is constitutively phosphorylated in its RNA binding domain, which appears to be necessary for cytoplasmic Smaug foci formation.

Keywords: Smaug, post-transcriptional regulations, *Drosophila*, Hedgehog pathway, Smoothed, signaling, phosphorylation, Smaug foci, development

A mis padres.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	5
TABLE OF FIGURES	12
LIST OF ABBREVIATIONS	14
PREAMBLE	16
INTRODUCTION	19
Chapter I. Cytoplasmic mRNA post-transcriptional regulations play a key role in development	19
1. An overview of RNA metabolism in eukaryotic cells	19
2. Roles of cytoplasmic mRNA post-transcriptional regulations during <i>Drosophila melanogaster</i> early development	21
2.1 <i>Drosophila</i> as model organism to study mRNA post-transcriptional regulation	21
2.1.1 The life cycle of <i>Drosophila</i>	21
2.1.2 <i>Drosophila</i> early embryogenesis as model to study mRNA regulation	22
2.2 Maternal mRNA localization determines the anterior-posterior axis during <i>Drosophila</i> early development	24
2.3 The maternal-to-zygotic transition is a period of maternal mRNA clearance	26
3. Molecular mechanisms of cytoplasmic mRNA post-transcriptional regulations.....	27
3.1 Targeting mRNA to specific cytoplasmic locations	28
3.2 Translational regulation	30
3.3 Regulation of mRNA degradation	33
Chapter II: RNA binding proteins are versatile and fundamental mediators of gene expression	35
1. Properties and functions of RNA binding proteins	35

1.1	RBPs bind RNA via a repertoire of RNA binding domains.....	37
1.1.1	RNA Recognition Motif (RRM).....	38
1.1.2	K-Homology (KH) domain.....	39
1.1.3	Double-Stranded (ds) RNA Binding Domain (dsRBD).....	39
1.1.4	Zinc Finger (ZnF) domain.....	40
1.1.5	Pumilio (PUF) domain.....	40
1.1.6	PAZ and PIWI domain.....	40
1.1.7	Sterile α Motif (SAM) domain.....	41
1.2	RBPs serve as scaffolds to recruit other proteins.....	42
1.3	RBPs possess intrinsically disordered regions that promote aggregation.....	42
2.	Ribonucleoprotein granules are dynamic complexes containing both RNA binding proteins and RNA.....	43
2.1	Cytoplasmic ribonucleoprotein granules.....	44
2.1.1	Processing bodies.....	44
2.1.2	Stress granules.....	44
2.1.3	Neuronal granules.....	46
2.2	How do ribonucleoprotein granules form?.....	47
Chapter III: RNA binding proteins are regulated by post-translational modifications.....		49
1.	Proteins are regulated by a plethora of post-translational modifications.....	49
2.	Post-translational modifications regulate multiple aspects of RBP function.....	50
2.1	Effect on the mRNA binding activity.....	51
2.2	Effect on the formation of RNPs.....	52
Chapter IV: Smaug, a multifunctional RNA binding protein conserved from yeast to human.....		53
1.	Smaug is a conserved RNA regulator with multiple roles in eukaryotes.....	53
1.1	Smaug plays critical roles during <i>Drosophila</i> development.....	53
1.1.1	Smaug expression in the embryo.....	53
1.1.2	Smaug plays a key role during maternal-to-zygotic transition.....	55
1.1.3	Smaug establishes antero-posterior (AP) embryonic polarity by spatial regulation of <i>nos</i> mRNA during early embryogenesis.....	55

1.1.4	Smaug regulates dendritic arborization in the larval peripheral nervous system (PNS).....	56
1.2	Properties and functions of mammalian Smaug.....	57
1.2.1	The translational repressor Smaug1 is involved in many different biological processes.....	57
1.2.2	Smaug2 regulates <i>nos1</i> mRNA during neuronal differentiation.....	59
2.	Smaug regulates mRNA through diverse mechanisms	60
2.1	Smaug represses <i>nos</i> mRNA translation via specific binding to SRE	60
2.1.1	Smaug forms a stable complex with the eIF4E binding protein Cup	60
2.1.2	Smaug recruits Ago1 in a microRNA independent manner	61
2.2	Smaug promotes mRNA poly(A) tail deadenylation and decay.....	62
2.2.1	Smaug recruits the CCR4-NOT deadenylase complex.....	62
3.	Smaug protein sequence and structure	63
3.1	Smaug RNA binding structure	64
3.2	Smaug contains intrinsically disordered regions	66
Chapter V: The Hedgehog signaling pathway		67
1.	Roles of the HH pathway during <i>Drosophila</i> development	67
1.1	HH establishes segmental polarity in the embryo	67
1.2	HH regulates wing growth and patterning.....	67
2.	Mechanisms of the HH signal transduction in the wing imaginal disc	69
2.1	HH activates gene expression via the transcription factor Cubitus Interruptus.....	69
2.2	The HH signal is transduced via activation of the G protein-coupled receptor Smoothened	69
3.	The activity of the signal transducer Smoothened is regulated by PTMs	71
3.1	SMO is downregulated by ubiquitin-mediated endocytosis and degradation	72
3.2	Phosphorylation induces SMO conformational switch and stabilizes the protein at the plasma membrane	72
4.	The HH pathway is conserved in mammals	74
THESIS RATIONALE.....		75
I.	General context.....	75

1. Identification of the RNA binding protein Smaug as a novel partner of Smoothened	75
2. Smaug upregulates SMO levels in a mRNA dependent manner in <i>Drosophila</i> wing imaginal disc	76
3. HH signaling induces Smaug phosphorylation	76
II. Thesis objectives	76
SUMMARY OF THESIS WORK.....	78
RESULTS	81
PART 1: MANUSCRIPT	82
ABSTRACT.....	83
INTRODUCTION	84
RESULTS	86
DISCUSSION AND CONCLUSION.....	92
MATERIAL AND METHODS	94
PART 2: OTHER RESULTS	120
I. Characterization of Smaug phosphorylation induced by HH signaling and the kinases in play	121
1. Identification of Smaug phosphorylated sites and protein partners.....	121
1.1 Identification of Smaug phosphosites by mass spectrometry	121
1.2 Identification of Smaug partners by quantitative mass spectrometry	123
1.3 Study of the FU kinase implication in Smaug phosphorylation	125
1.4 Analysis of DOP kinase involvement in Smaug phosphorylation.....	126
2. Identification of Smaug phosphorylated regions by systematic site directed mutagenesis	127
2.1 Design of Smaug synthetic gene as strategy.....	128
2.2 Characterization of Smaug phosphorylated regions in response to HH signaling.	131

3. Smaug is constitutively phosphorylated	132
II. Functional characterization of Smaug constitutive phosphorylation in cultured Cl8 cells.....	133
1. Study of the role of Smaug constitutive phosphorylation	134
1.1 Phosphorylation of the SAM domain downregulates Smaug protein levels and upregulates Smaug mRNA repressive activity	134
1.2 Phosphorylation of Smaug SAM domain regulates S-foci formation	137
DISCUSSION.....	139
I. SMO/Smaug interaction	139
1. How is the interaction between Smaug and SMO regulated?.....	139
2. What is the nature and the dynamics of the foci containing Smaug and SMO?	140
3. Development of the SNAP-tagged reporter assay to measure Smaug repressive activity	141
4. HH/SMO signaling downregulates Smaug accumulation levels and Smaug repressive activity	141
II. Smaug regulation by phosphorylation	142
1. Identification of Smaug phosphorylated sites	142
2. Characterization of the kinases implicated in Smaug phosphorylation	144
3. What is the function of Smaug SAM domain constitutive phosphorylation?	145
III. Model of Smaug regulation by phosphorylation in response to HH/SMO signaling ..	148
IV. Concluding remarks and future perspectives	149
REFERENCES	150

TABLE OF FIGURES

FIGURE 1. RNA METABOLISM IN EUKARYOTIC CELLS.	20
FIGURE 2. LIFE CYCLE OF DROSOPHILA MELANOGASTER AT 25°C	22
FIGURE 3. REPRESENTATION OF DROSOPHILA OOCYTE AND THE FIRST STAGES OF EMBRYONIC NUCLEAR CLEAVAGE	23
FIGURE 4. MODEL OF ANTERIOR-POSTERIOR PATTERN FORMATION IN DROSOPHILA.....	25
FIGURE 5. MRNA REGULATION DURING DROSOPHILA EARLY EMBRYOGENESIS.....	27
FIGURE 6. EXAMPLES OF MRNA LOCALIZATION BY THREE DISTINCT MECHANISMS IN DROSOPHILA.....	29
FIGURE 7. FORMATION OF THE CLOSED-LOOP STRUCTURE DURING MRNA TRANSLATION INITIATION.....	31
FIGURE 8. MULTIPLE MECHANISMS INHIBIT FORMATION OF THE TRANSLATION CLOSED- LOOP STRUCTURE IN LOCALIZED MRNAS.	32
FIGURE 9. DEADENYLATION-DEPENDENT MRNA DECAY.....	34
FIGURE 10. RNA INTERACTOME CAPTURE IN VIVO.	36
FIGURE 11. RNA BINDING DOMAINS ARE DIVERSE.....	37
FIGURE 12. REMODELING OF CYTOPLASMIC STRESS GRANULES AND PROCESSING BODIES. 46	
FIGURE 13. LINKING INTRACELLULAR FORMATION OF RNP GRANULES BY PHASE SEPARATION AND DISEASE.	48
FIGURE 14. EXAMPLES OF POST-TRANSLATIONAL MODIFICATIONS.....	49
FIGURE 15. SIGNAL INTEGRATION AND EFFECTS OF PTM ON RBP FUNCTION	50
FIGURE 16. SMAUG IS SYNTHESIZED AFTER FERTILIZATION AND IS UNIFORMLY DISTRIBUTED ACROSS THE EMBRYO DURING EARLY EMBRYOGENESIS	54
FIGURE 17. SMAUG1 FOCI ARE DYNAMIC STRUCTURES IN EQUILIBRIUM WITH TRANSLATING POLYSOMES.....	58
FIGURE 18. SMAUG BINDS NOS MRNA VIA RECOGNITION OF A STEM-LOOP STRUCTURE CALLED SRE IN THE 3' UTR	60
FIGURE 19. DROSOPHILA SMAUG IS EXPRESSED IN FIVE ISOFORMS (A TO E)	63
FIGURE 20. SMAUG PROTEIN STRUCTURE AND SEQUENCE IN DROSOPHILA.	64

FIGURE 21. SMAUG RNA BINDING DOMAIN STRUCTURE AND SAM DOMAIN HOMOLOGUES.	65
FIGURE 22. PROTEIN DISORDERED PREDICTION OF DROSOPHILA SMAUG.	66
FIGURE 23. THE HEDGEHOG MORPHOGEN CONTROLS WING DEVELOPMENT IN DROSOPHILA.....	68
FIGURE 24. MODEL OF THE HEDGEHOG SIGNALING PATHWAY IN DROSOPHILA.....	70
FIGURE 25. SMO PHOSPHORYLATION INDUCES ITS CONFORMATIONAL SWITCH.....	73
FIGURE 26. IDENTIFICATION OF SMAUG PHOSPHOSITES BY TANDEM MASS SPECTROMETRY	122
FIGURE 27. STUDY OF THE IMPLICATION OF THE FU KINASE IN SMAUG PHOSPHORYLATION BY WESTERN BLOT	125
FIGURE 28. STUDY OF DROP OUT KINASE IMPLICATION IN SMAUG PHOSPHORYLATION PROMOTED BY HH SIGNALING.....	127
FIGURE 29. SMAUG GENE SYNTHESIS STRATEGY	129
FIGURE 30. TEST OF SMAUG DOWNREGULATION BY RNAI IN CL8 CELLS	130
FIGURE 31. IDENTIFICATION OF SMAUG PHOSPHORYLATED REGIONS BY SITE-DIRECTED MUTAGENESIS.....	131
FIGURE 32. SMAUG IS CONSTITUTIVELY PHOSPHORYLATED	133
FIGURE 33. PREVENTION OF SMAUG SAM DOMAIN PHOSPHORYLATION DECREASES SMAUG LEVELS AND UPREGULATES SMAUG MRNA REPRESSIVE ACTIVITY.	136
FIGURE 34. PREVENTION OF SMAUG SAM DOMAIN PHOSPHORYLATION PROMOTES THE FORMATION OF SMALLER AND MORE ABUNDANT CYTOPLASMIC FOCI.	137
FIGURE 35. REPRESENTATION OF DIFFERENT SCENARIOS OF SMAUG REGULATION BY PHOSPHORYLATION.	144
FIGURE 36. SEQUENCE ALIGNMENT OF SAM DOMAIN OF SMAUG HOMOLOGUES.....	146
FIGURE 37. SUBCELLULAR FRACTIONATION OF SMAUG IN CL8 CELLS.....	147
FIGURE 38. MODEL OF SMAUG REGULATION VIA SMO UPON HH SIGNALING ACTIVATION	148
TABLE 1. COMMON RNA BINDING DOMAINS AND EXAMPLES OF RBPS CONTAINING THEM.....	38
TABLE 2. IDENTIFICATION OF SMAUG INTERACTORS BY LABEL FREE QUANTITATIVE MASS SPECTROMETRY.....	124

LIST OF ABBREVIATIONS

3D: Three dimension
aa: amino acid
Ago: Argonaute
ALS: Amyotrophic lateral sclerosis
AP: Anterior Posterior
Brat: Brain Tumor
CamKII α : calmodulin kinase II α
CCR4: Carbon Catabolite Repression 4
Dcp: decapping protein
Da: dendritic arborization
eIF: eukaryotic initiation factor
EJC: Exon-Junction Complex
FDR: false discovery rate
FRAP: Fluorescence recovery after photobleaching
FRET: Fluorescence resonance energy transfer
FTLD: Fronto-temporal lobe degeneration
FUS: Fused in sarcoma
GPCR: G protein coupled-receptor
HH: Hedgehog
hnRNP: Heterogenous nuclear ribonucleoprotein
hsp83: heat-shock protein-83
HuR: human antigen R
IDR: Intrinsically disordered region
MBT midblastula transition
MEG: maternal-effect germline defective
mGluR: Metabotropic glutamate receptor
miRNA: micro RNA

MZT: Maternal-to-Zygotic Transition
Nos: Nanos
NOT: Negative on TATA less
Osk: Oskar
PABP: Poly(A)-binding protein
P bodies: processing bodies
P granules: polar granules
PAN: PolyA Nuclease
PHAT: pseudo-HEAT repeat analogous topology
PNG: Pan Gu Ser/Thr kinase
PTM: Post-translational modification
PUM: Pumilio
RBP: RNA binding protein
RISC: RNA-induced silencing complex
RNA: ribonucleic acid
RNP: Ribonucleoprotein particle
SAM: Sterile Alpha Motif
SG: stress granules
Smg: Smaug
SMO: Smoothened
SRE: Smaug Recognition Element
SSR: Smaug Similarity Region
TIA-1: T-cell intracellular antigen-1
TRiP: Transgenic RNAi Project
UV: Ultraviolet
WB : western-blotting
WT : wild-type
Xrn1

PREAMBLE

In eukaryotes, the regulation of gene expression is fundamental to diverse biological processes, including cell proliferation, adaptation to environmental signals, as well as cell differentiation and development. Control of gene expression occurs at different levels and can be classified into two main kinds: transcriptional and post-transcriptional regulations. In addition, after the proteins are expressed they can still be regulated by post-translational modifications. Dysregulation at any of these levels of gene expression can be implicated in many diseases.

Throughout the last couple of decades, the attention was mainly focused on understanding how transcriptional control takes place and, consequently, many advances were made thanks to genome-wide approaches such as gene expression profiling. On the other hand, post-transcriptional regulations have been less extensively studied. This difference becomes evident when one compares the number of scientific publications that appear in PubMed by searching for the terms ‘transcriptional regulation’ (over 222 000 hits) versus ‘post-transcriptional regulation’ (merely 15 000 hits). Nevertheless, elucidating the roles and mechanisms of post-transcriptional gene expression regulation has recently become a widely relevant task among the scientific community.

Post-transcriptional regulations involve multiple and complex processes such as mRNA processing in the nucleus (capping, splicing and polyadenylation), mRNA export and localization in the cytoplasm, mRNA translation and final mRNA degradation. Today we know that transcripts are not found in the cell as naked macromolecules but bound to different proteins called RNA binding proteins (RBP) forming dynamic ribonucleoprotein (RNP) complexes. These RBPs regulate every step of an mRNA lifespan in a spatio-temporal fashion and are crucial effectors in the control of gene expression (Marchese, de Groot et al. 2016). *In silico* approaches plus the development of genome-wide techniques, such as RNA interactome capture in human cells combined with mass spectrometry analysis, have allowed to successfully identify proteins harboring canonical RNA binding domains as well as

hundreds of novel RBPs with unorthodox binding activity (Beckmann, Castello et al. 2016, Castello, Horos et al. 2016).

Recently, there has been increasing evidence that external signals and internal cues trigger post-translational modifications of RBPs that alter their RNA binding activity by influencing their ability to bind RNAs and/or protein partners as well as their subcellular localization (Lee 2012, Lovci, Bengtson et al. 2016). Importantly, disruption of RBP function is becoming widely recognized as a major cause of disease, especially in neurodegenerative diseases as well as developmental disorders and cancer (Kechavarzi and Janga 2014, Brinegar and Cooper 2016, Fan and Leung 2016). Thus, connecting signaling pathways with post-transcriptional regulations is a topic that awaits further exploration. Understanding how the two are linked will certainly provide novel and invaluable insights into the finely tuned regulatory network that defines growth, development and disease.

My thesis project focused on understanding the link, discovered prior to my arrival in the lab, between the RNA binding protein Smaug and the Hedgehog (HH) pathway. A preliminary observation that HH signaling regulates Smaug by inducing its phosphorylation led me to pursue the characterization of Smaug regulation by the HH pathway and determine its biological function.

In the first chapter, I will introduce the diversity of post-transcriptional regulations with a special focus on the roles and mechanisms of cytoplasmic control of gene expression. I will present relevant examples of these regulatory processes that have been extensively studied during *Drosophila melanogaster* (*Drosophila* hereafter) early development.

In chapter II, I will discuss the general characteristics of RBPs and their properties to bind RNA as well as proteins which lead to the formation of dynamic ribonucleoprotein complexes.

Next, recent efforts to study how post-translational regulations modulate RBP function and its connection to signal transduction will be presented in chapter III.

In chapter IV, I will address the conserved RNA binding protein Smaug, which is the subject of my thesis project, and describe its major roles as a translational repressor and promoter of mRNA decay during *Drosophila* early embryogenesis, as well as its mechanisms of action and the protein structure.

Finally, in chapter V, I will present the conserved Hedgehog (HH) signaling pathway which plays a fundamental role during many developmental processes including cell proliferation, tissue polarity and cell differentiation to mention a few. A detailed description on how the levels of HH regulate the stability and activity of the transducer protein Smoothed (SMO) by multiple post-translational modifications will be given.

INTRODUCTION

Chapter I. Cytoplasmic mRNA post-transcriptional regulations play a key role in development

This chapter introduces the different stages of RNA metabolism focusing on the post-transcriptional regulations that a messenger RNA undergoes once exported into the cytoplasm. Despite the existing variety of regulatory mechanisms, they all share one thing in common in the sense that they ultimately control whether a given mRNA will be translated into a protein. I will present the functions and mechanisms of these processes by focusing on specific examples of cytoplasmic genetic control in *Drosophila*.

1. An overview of RNA metabolism in eukaryotic cells

Before mRNAs are exported into the cytoplasm where they are available for translation, nascent transcripts undergo a series of processing steps in the nucleus. Indeed, addition of the cap structure at the 5' end of the transcript and a long chain of adenine nucleotides (poly(A) tail) at the 3' end as well as intron removal (splicing) are crucial and necessary mRNA maturation steps. Both the 5' cap and the 3' poly(A) tail structures influence mRNA stability and translational regulation (see below). The processes of splicing by the spliceosome, the use of alternative exons (alternative splicing) and 3' end polyadenylation were first considered as post-transcriptional modifications of the nascent mRNA but it has been shown that most mRNA processing steps occur co-transcriptionally (Proudfoot, Furger et al. 2002). It is important to note that, despite presenting RNA processing and nuclear export as sequential events, these processes can influence each other and are not independent from one another (Moore 2005).

Once the functional mRNAs are shuttled to the cytoplasm by mRNA export proteins through the nuclear pores, the transcripts undergo multiple cytoplasmic post-transcriptional regulations. These include mRNA localization to specific subcellular compartments where translation can be triggered or repressed as well as mRNA (de)stabilization. Finally, mRNA

degradation include different decay machineries such as the surveillance pathway known as non-sense mediated decay (NMD), gene silencing by small non-coding RNAs (such as piwi RNAs, small interfering RNAs and microRNAs) and sequestering transcripts into processing bodies (Fig. 1). Hence, these cytoplasmic regulatory events are central to post-transcriptional gene expression regulation and some of their roles and molecular mechanisms will be described in detail below.

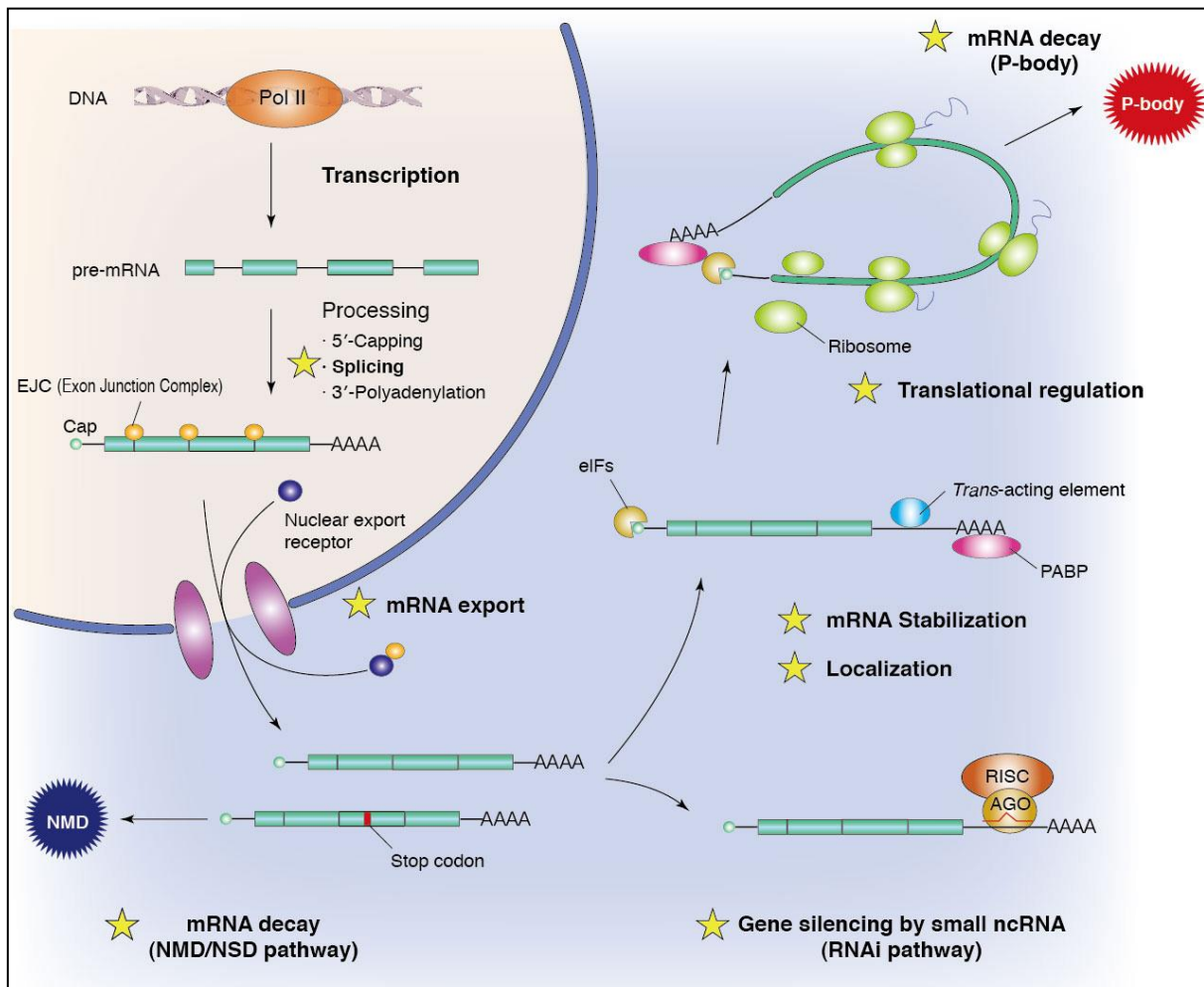


Figure 1. RNA metabolism in eukaryotic cells.

A broad range of regulatory post-transcriptional mechanisms (shown with stars) control the fate of mRNAs. In the nucleus, nascent transcripts are modified co-transcriptionally by a series of maturation steps that involve the addition of the 5' cap structure and the 3' poly(A) tail as well as the removal of introns, a process known as splicing. To be available for translation, mRNAs are exported out of the nucleus into the cytoplasm where they can also be stabilized or localized in specific compartments as well as undergo degradation by different mRNA decay pathways (see below). Trans-acting elements recognize and interact with the target transcripts and play a primary role in these regulating processes.

Note that RNA processing events are shown to occur after transcription for simplicity purposes. (<http://ruo.mbl.co.jp/bio/g/product/epigenetics/RNAworld.html>)

2. Roles of cytoplasmic mRNA post-transcriptional regulations during *Drosophila melanogaster* early development

2.1 *Drosophila* as model organism to study mRNA post-transcriptional regulation

Drosophila's genome contains over 15 000 genes that are distributed in four pair of chromosomes: the X/Y pair and three autosomal chromosomes numbered 2, 3 and 4 (Adams, Celniker et al. 2000). Comparative genomics have shown that *Drosophila* shares up to 60% of its genes with humans which contribute to the conservation of multiple physiological and developmental processes (Pandey and Nichols 2011). Importantly, the use of classical genetics and molecular biology in *Drosophila* has helped unlock the post-transcriptional mechanisms that control development from the egg to the adult, making the fly an ideal model for studying mRNA regulation during development.

First, an overview of *Drosophila's* life cycle and early embryogenesis will be given in order to better understand the context of the post-transcriptional control of gene expression that takes place during fly development.

2.1.1 The life cycle of *Drosophila*

The life cycle of *Drosophila* is rapid and temperature dependent, taking about 10 days to be completed at 25°C (Fig.2). Development of the fruit fly consists in four different stages during the life cycle, each with a very distinct body plan: embryo, larva, pupa and adult. Twenty-four hours after the adult female flies lay eggs, the motile larvae hatch from the embryo and undergo three molting stages known as instars. The future adult structures of the fly are contained within the larvae as imaginal discs, which are primarily composed of undifferentiated epithelium. For this reason, larvae (and in particular the wandering third instar larva) are commonly used to study developmental processes. After proceeding to the pupal phase, imaginal discs undergo massive morphological changes that give rise to the final adult structures such as the wings, legs, eyes, mouthparts and genital ducts. The pupa stage is a stationary phase during which the larvae are metamorphosing into the adult fly. Although male flies are sexually active hours after hatching from the pupal case, female flies become sexually mature within 48h after emerging, allowing the cycle to begin again.

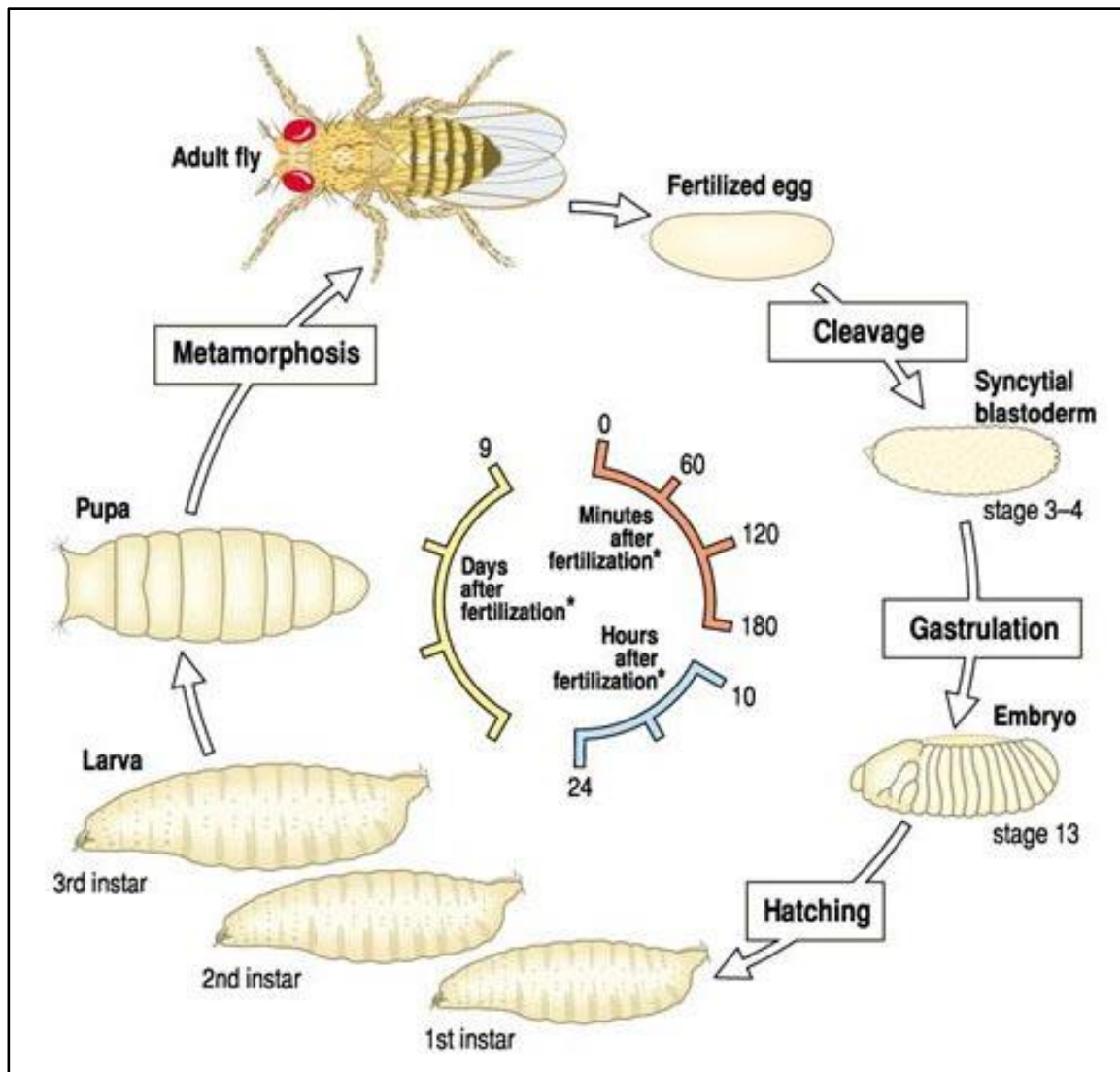


Figure 2. Life cycle of *Drosophila melanogaster* at 25°C

2.1.2 *Drosophila* early embryogenesis as model to study mRNA regulation

In the ovary of female flies, a structure called egg chamber contains follicular epithelial cells surrounding the transcriptionally silent oocyte (Fig. 3 A) as well as nurse cells that are highly active in transcription and translation. During oogenesis, many maternal mRNAs and proteins expressed in the nurse cells are transferred to the oocyte through connecting channels.

By the end of oogenesis, the nurse cells discharge the content of their cytoplasm into the oocyte and undergo apoptosis. An approximate number of 5 000 maternal transcripts are found in the early embryo (Tadros, Goldman et al. 2007). Then, the maternal mRNAs and protein factors are transported through the cytoskeleton to different regions of the oocyte, a process that is essential for the establishment of the embryonic polarity and the maternal-to-zygotic transition (MZT) that will be described later (Bastock and St Johnston 2008, Lasko 2012).

Upon oocyte fertilization, the egg is activated and nuclear divisions are triggered. A unique characteristic of *Drosophila* is that nuclear cleavage occurs in a multinucleate syncytium and divisions do not undergo cytokinesis (Fig. 3 B). After ten rapid and synchronous cleavages, hundreds of nuclei migrate to the periphery of the egg, leading to the formation of the syncytial blastoderm. Moreover, at the posterior of the embryo, pole

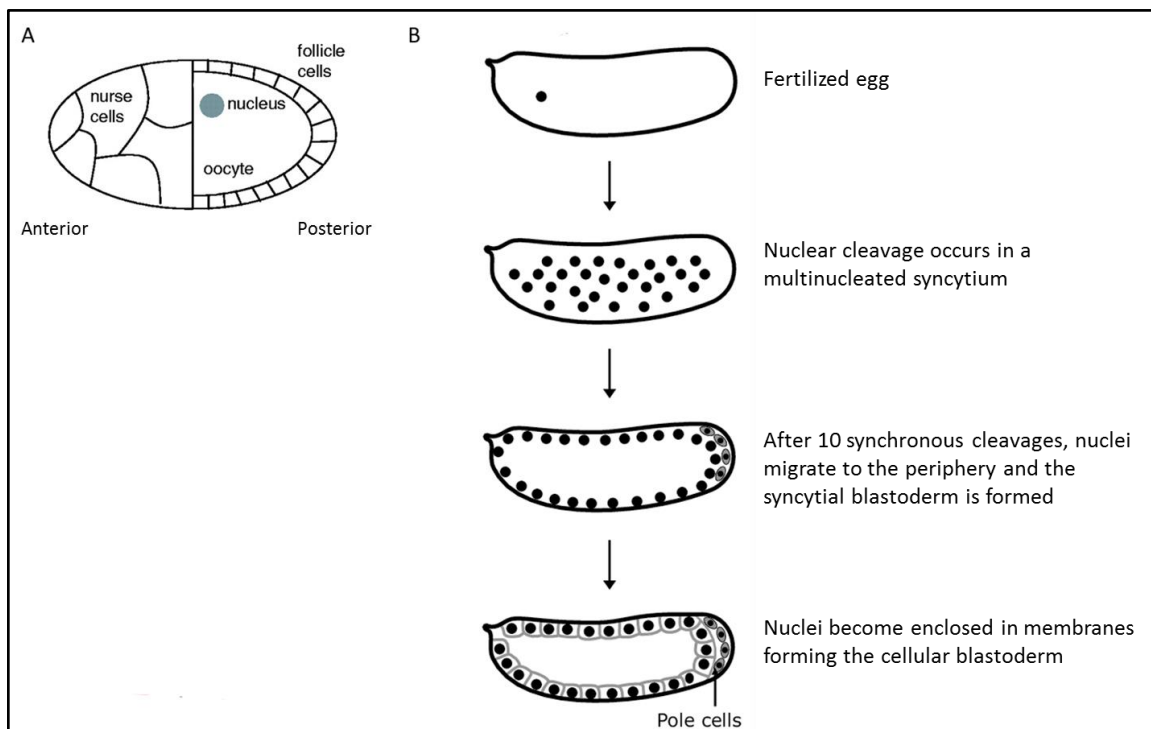


Figure 3. Representation of Drosophila oocyte and the first stages of embryonic nuclear cleavage (A) Representative Drosophila oocyte surrounded by follicle cells and nurse cells. The anterior and posterior poles are shown. (B) After fertilization, the egg undergoes rapid and synchronous nuclear cleavage in a multinucleated syncytium. After 10 divisions, nuclei migrate to the periphery leading to the formation of a syncytial blastoderm. After the 13th division, cell membranes are formed, which derives in the formation of the cellular blastoderm.

cells are formed containing posterior determinants in polar granules. Hence, the egg is polarized by differential localization of maternal mRNAs which is crucial for pattern formation of the embryo (see next section). After the 13th division and about 3-4 hours after fertilization, the midblastula transition (MBT) takes place. During this phase, around 6000 nuclei will undergo a process called “cellularization” which derives in the formation of the cellular blastoderm. These cells will later undergo the process of gastrulation where the three primordial tissue layers (ectoderm, mesoderm and endoderm) are formed (Bastock and St Johnston 2008).

Consequently, since the first stages of *Drosophila* embryogenesis depend exclusively on the maternal mRNAs and proteins loaded into the transcriptionally silent oocyte, it is an ideal model to study specifically post-transcriptional regulations of gene expression such as mRNA localization coupled with translation as well as transcript degradation.

2.2 Maternal mRNA localization determines the anterior-posterior axis during *Drosophila* early development

One of the best understood examples of pattern formation is the anterior-posterior (AP) axis of the *Drosophila embryo*. Its formation is the consequence of a cascade of post-transcriptional regulations during oogenesis and later in the embryo.

Basically, three different and crucial types of genes are responsible for the AP patterning of the fly embryo: the maternal genes, which I will focus on in this and the upcoming section, the zygotically produced segmentation genes (which include the gap, pair-rule and segment polarity genes) and the homeotic genes. Specific localization of maternal mRNAs in the oocyte and their controlled expression upon fertilization, establishes a morphogen gradient across the embryo that is absolutely required for determining the anterior-posterior axis (Fig. 4) (Palacios 2007, Lasko 2012).

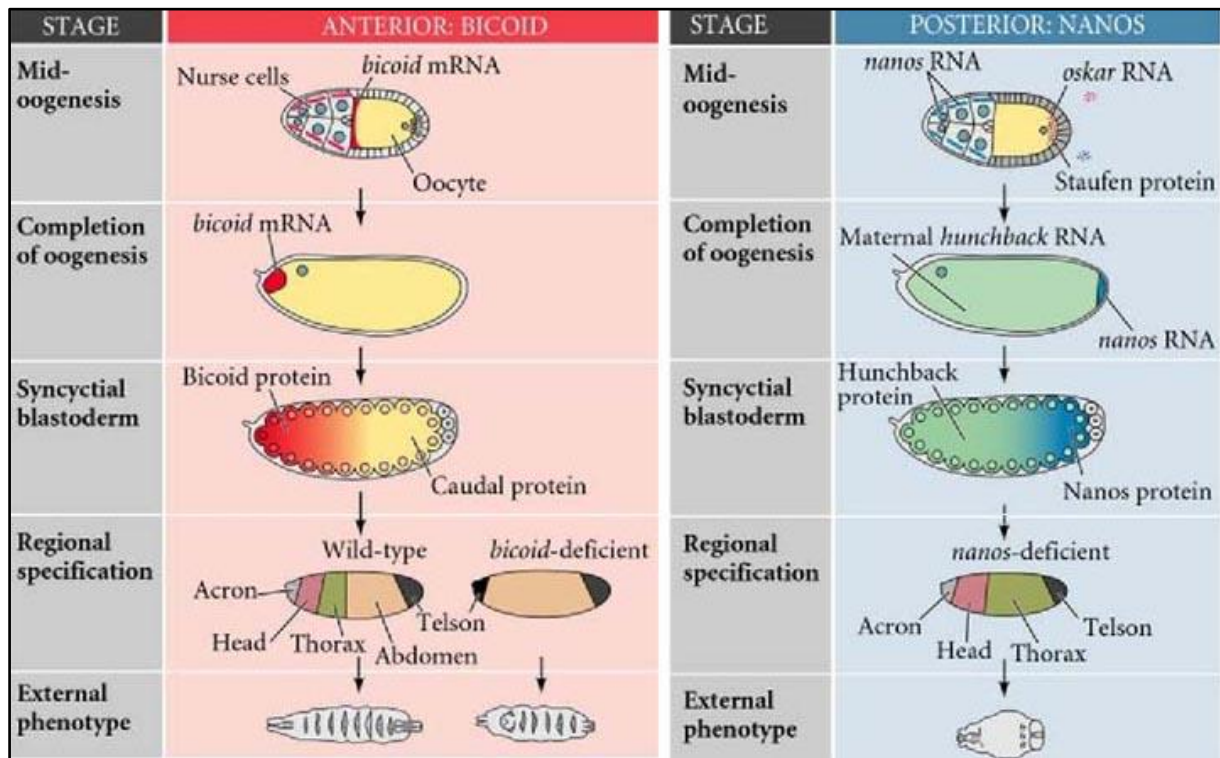


Figure 4. Model of anterior-posterior pattern formation in *Drosophila*.

In the early embryo, the anterior-posterior axis is established by asymmetrically distributed maternal genes (such as *bicoid*, *hunchback*, *caudal* and *nos* mRNAs) that encode morphogenetic proteins. These determinants create gradients which differentially activate the segmentation genes, thus leading to the division of the embryo into segments along the anterior-posterior axis. In embryos from *bicoid* mutant females, there is no head or thorax, only posterior structures on both ends. On the other hand, in embryos from *nos* mutant mothers the abdominal segments are not developed. (Adapted figure from *Developmental Biology*, 6th edition, 2000).

Most importantly, maternal *bicoid* (*bcd*) and *hunchback* (*hb*) are the determinants responsible for the patterning of the anterior parts (head and thorax) while *nanos* (*nos*) and *caudal* (*cad*) mRNAs determine the posterior abdominal segments of the embryo. Mutations for any of these genes induce embryonic lethality. (Driever and Nusslein-Volhard 1988, Lehmann and Nusslein-Volhard 1991, Rivera-Pomar and Jackle 1996).

Studies on *oskar* (*osk*) mRNA localization by *in situ* hybridization showed it localizes at the posterior pole of the oocyte (Ephrussi, Dickinson et al. 1991). Osk protein is required for *nos* mRNA localization at the posterior pole later during *Drosophila* embryogenesis. Localized *nos* mRNA is then translated only at the posterior, generating a morphogen gradient

towards the anterior pole (Ephrussi, Dickinson et al. 1991, Gavis and Lehmann 1992). I will further describe *nos* mRNA regulation in chapter IV.

2.3 The maternal-to-zygotic transition is a period of maternal mRNA clearance

The maternal-to-zygotic transition (MZT) is the stage in which all the maternal mRNAs that allowed the early development of the embryo are degraded and the transcription of the zygotic genome is activated. This transfer of genetic control is a major developmental process and is widely conserved in multiple organisms including mammals (Tadros and Lipshitz 2009). Multiple studies have therefore focused on understanding the underlying molecular mechanisms of mRNA decay regulation as well as the control of spatial and temporal mRNA localization and protein synthesis.

In *Drosophila*, the MZT begins during the 11th syncytial division just prior to the cellularization of the embryo. In the case that the maternal mRNAs are not degraded the embryo fails to undergo cellularization and therefore it ceases to develop. During this period, 35% of the maternal mRNAs are degraded (De Renzis, Elemento et al. 2007) which occurs in several stages (Fig. 5). First, there is an early clearance of 20% of the maternal transcripts followed by the transcriptional activation of the zygotic genome (Tadros and Lipshitz 2009). The key players of this first wave are the maternal RNA binding proteins Pumilio (PUM), Brain Tumor (Brat) and Smaug. Then, a second wave takes place in which zygotic factors, primarily microRNAs, destabilize the other 15% of the maternal transcripts degraded (Laver, Li et al. 2015, Luo, Li et al. 2016).

Finally, the MZT ends with the midblastula transition which represents the first morphological change that solely depends on the transcription of the embryo's genome (Tadros and Lipshitz 2009, Walser and Lipshitz 2011).

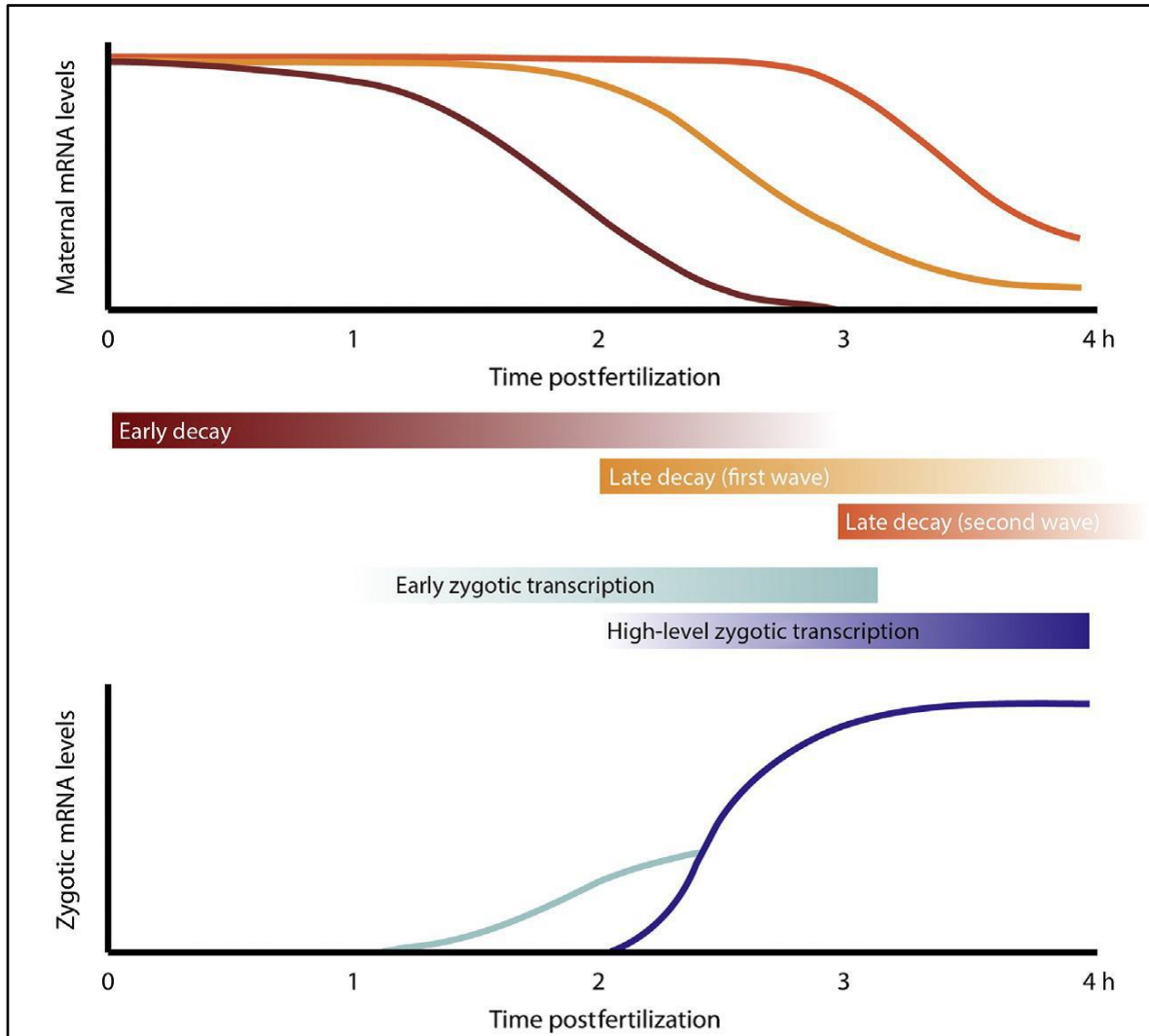


Figure 5. mRNA regulation during *Drosophila* early embryogenesis

Upon activation of the egg after fertilization, the only available mRNAs are the maternal transcripts deposited during oogenesis. During the maternal-to-zygotic transition, which occurs around two hours after fertilization, a global degradation of the maternal mRNAs takes place (brown curve) and part of the zygotic genome is activated (light blue curve). Subsequently, there is a late decay phase of the maternal mRNA that occurs in two successive waves (yellow and orange curves) and the zygotic genome is highly activated (dark blue curve). Figure from (Laver, Marsolais et al. 2015)

3. Molecular mechanisms of cytoplasmic mRNA post-transcriptional regulations

In this section, I will focus on the molecular aspects of how different cell types traffic mRNAs to specific destinations in a variety of organisms as well as the mechanisms regulating mRNA translation and degradation.

3.1 Targeting mRNA to specific cytoplasmic locations

Selection of the target mRNAs consists in recognition of determined localization sequences known as *cis-acting* elements by *trans-acting* factors such as RNA binding proteins which will be described in the following chapter. Large-scale genomic analyzes in various organisms have revealed that a large number, up to 70% in *Drosophila*, have a specific subcellular localization (Lecuyer, Yoshida et al. 2007). Localization of target mRNAs to specific compartments in the cell is a fundamental and complex step involved in several developmental processes such as neuronal morphogenesis and maturation, asymmetric cell division, cell migration, cell differentiation and axis patterning to name a few (Holt and Bullock 2009, Medioni, Mowry et al. 2012).

Three different mechanisms have been proposed regarding the asymmetric distribution of mRNAs within cells: 1) active transport by motor proteins along the cytoskeleton, 2) diffusion-coupled local entrapment and 3) localized protection from mRNA degradation (Medioni, Mowry et al. 2012). However, most of the cases of asymmetric mRNA localization are driven by directed transport along the cytoskeleton which is a polarized network of filaments and microtubules that maintains the structure of the cell. Molecular motors such as kinesin, dynein and myosin, move directionally along the cytoskeleton and are responsible for the trafficking of various organelles and ribonucleoprotein complexes in the cytoplasm (Palacios 2007, Lasko 2012, Medioni, Mowry et al. 2012).

Trans acting factors specifically recruit motor proteins that enable the directional traffic of the ribonucleoprotein complex along the cytoskeleton. In *Drosophila*, transport of *bcd* and *osk* mRNAs from the nurse cells to the oocyte occurs by formation of distinct RNP complexes which are actively transported via the microtubule cytoskeleton. On the one hand, the *bcd* transcript is targeted at the anterior part of the embryo via dynein motors moving towards the microtubules minus-end while *osk* mRNA is localized at the posterior compartment via kinesin motors that move towards the microtubules plus-end (Fig. 6 A) (Becalska and Gavis 2009). Moreover, targeting *nos* mRNA at the posterior pole during *Drosophila* early embryogenesis is an example of mRNA localization via diffusion and cortical actin-dependent entrapment and anchoring (Fig. 6 B) (Forrest and Gavis 2003, Shahbadian and Chartrand 2012).

Finally, mRNA degradation coupled with local protection has been illustrated by *heat-shock protein-83 (hsp83)* mRNA localization at the posterior compartment of the *Drosophila* embryo although the mechanisms of selective protection from degradation remain unknown (Ding, Parkhurst et al. 1993). In this case, *hsp83* transcript is uniformly distributed during embryogenesis but becomes localized at the posterior pole as the embryo develops (Fig. 6 C). If the degradation machinery is impaired, *hsp83* mRNA ceases to be selectively localized and becomes stable across the embryo (Medioni, Mowry et al. 2012).

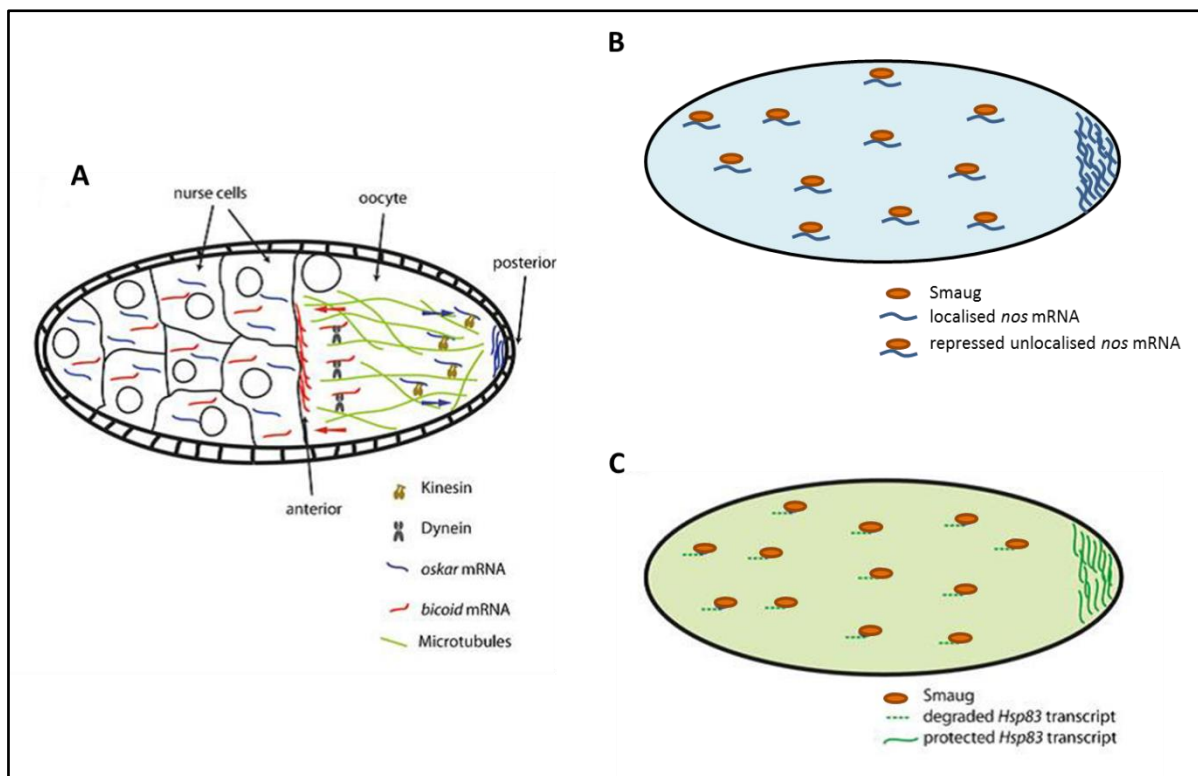


Figure 6. Examples of mRNA localization by three distinct mechanisms in *Drosophila*.

(A) The *osk* mRNA is transported from the nurse cells to the posterior pole of the oocyte through active transport via kinesins. On the contrary, *bicoid* mRNA is located at the anterior pole via active transport mediated by the dyneins. (B) *nos* mRNA diffuses across the embryo and is locally entrapped at the posterior end. However, *nos* localization at the posterior is inefficient and unlocalized *nos* mRNA is translationally repressed at the anterior pole of the embryo by the RBP Smaug. (C) The *hsp83* mRNA is selectively stabilized at the posterior of the embryo while the non-localized mRNA is degraded by Smaug. Figure adapted from (Shahbadian and Chartrand 2012)

Targeting mRNAs to specific locations is not only relevant during early embryogenesis in the fly but rather a widespread and conserved mechanism from yeast to human. Other

classical examples of localized mRNAs are the targeting to the bud tip of the mRNA of the transcriptional repressor *ASH1* which inhibits mating type switching during asymmetric cell division in yeast, or the localization of β -actin mRNA to the lamellipodia in mammalian fibroblasts allowing cytoskeletal motility. Importantly, a critical advantage of localized mRNA translation is that it allows a quick cellular response upon reception of external signals. This is particularly relevant in the case of neurons which are highly polarized cells and where local translation of silenced mRNAs is induced upon stimuli (Martin and Ephrussi 2009). In humans, alterations in mRNA localization have been implicated in pathologies such as mental retardation and cancer.

Nevertheless, in order to achieve protein synthesis of a localized mRNA, translation must also be spatially regulated. Thus, combining intracellular mRNA targeting and localized translation is a commonly used strategy by various types of cells. It is generally thought that localizing mRNAs are translationally repressed during transport and that they are stabilized once they reach their final destination. The mechanisms of translational regulation are presented next.

3.2 Translational regulation

Translation of a given mRNA can be divided in three major steps: initiation, elongation and termination. During the initiation step, binding of the complex eukaryotic translation initiation factor (eIF) 4F to the 5' methylated guanosine cap structure mediates the assembly of ribosomal subunits 40S and 60S at the transcript AUG start codon (Standart and Minshall 2008). The process of recruiting the eIF4F complex on the target mRNA is the rate-limiting step of translation initiation. The eIF4F complex is composed of the cap binding protein eIF4E, the RNA helicase eIF4A and the scaffold protein eIF4G that contains binding sites of eIF4E, eIF4A, eIF3 and poly(A)-binding protein (PABP). The interaction between eIF4G and PABP promotes the formation of a closed-loop mRNA structure that activates cap-dependent translation by enhancing the binding affinity of eIF4E for the 5' cap and facilitating the recruitment of ribosomes to the mRNA (Fig. 7) (Sonenberg and Hinnebusch 2009).

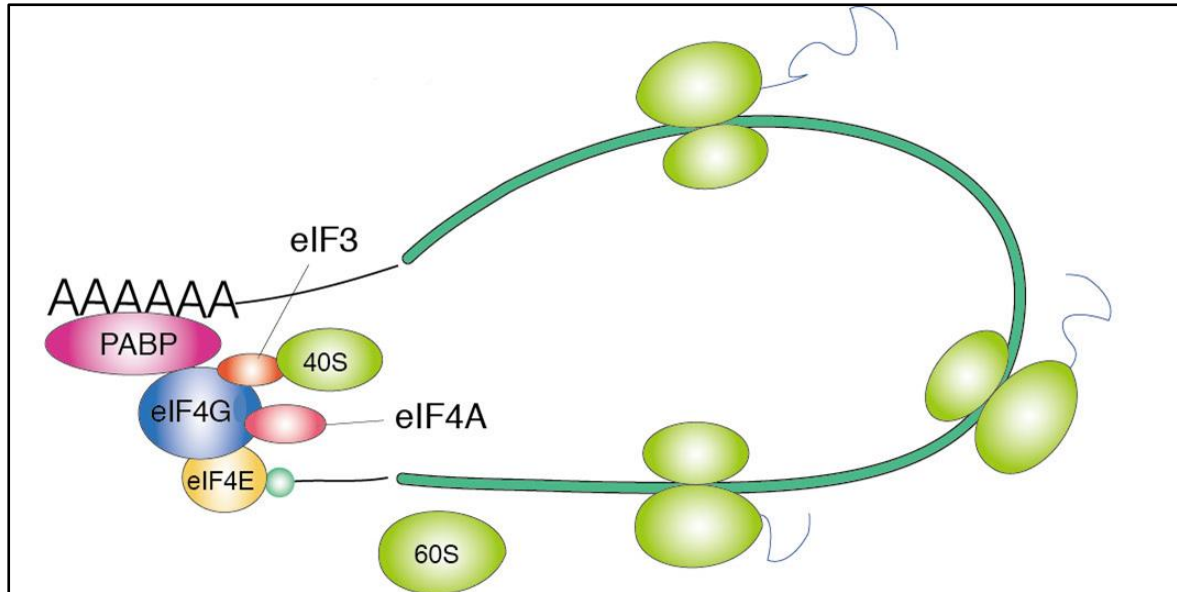


Figure 7. Formation of the closed-loop structure during mRNA translation initiation. *eIF4E* binds the cap structure and interacts with *eIF4G*, which in turn interacts with *PABP*, *eIF3* and *eIF4A*. The combination of these interactions leads to the formation of a closed-loop mRNA structure and the assembly of ribosomal subunits 40S and 60S at the mRNA start codon.

Considering that initiation of translation employs a larger number of factors compared to the steps of elongation and termination it does not come as a surprise that most translational regulation occurs at the level of initiation, particularly at the level of cap-dependent initiation of translation (Gallie 2002, Besse and Ephrussi 2008). For instance, specific *eIF4E* binding proteins (*eIF4E*-BP) target the formation of the *eIF4F* complex by preventing association between *eIF4G* and *eIF4E* (Fig. 8). This is the case of *Drosophila* *eIF4E*-BP Cup that is recruited by the translational repressor Bruno to the *osk* 3' UTR and competes with *eIF4G* for binding during early embryogenesis (Nakamura, Sato et al. 2004, Besse and Ephrussi 2008). Derepression seems to be achieved by a decrease of affinity of the translational repressor for its target mRNA, but the mechanism by which silencing is relieved when *osk* mRNA reaches the posterior compartment remains unknown (Bastock and St Johnston 2008). Another well-known mechanism in translational control is the prevention of the assembly of ribosomal subunits 40S and 60S (Fig. 8). Such is the case of *ash1* mRNA, which is translationally repressed by *trans* acting factors that inhibit the assembly of the ribosomal subunits, during transport along actin filaments in yeast (Besse and Ephrussi 2008).

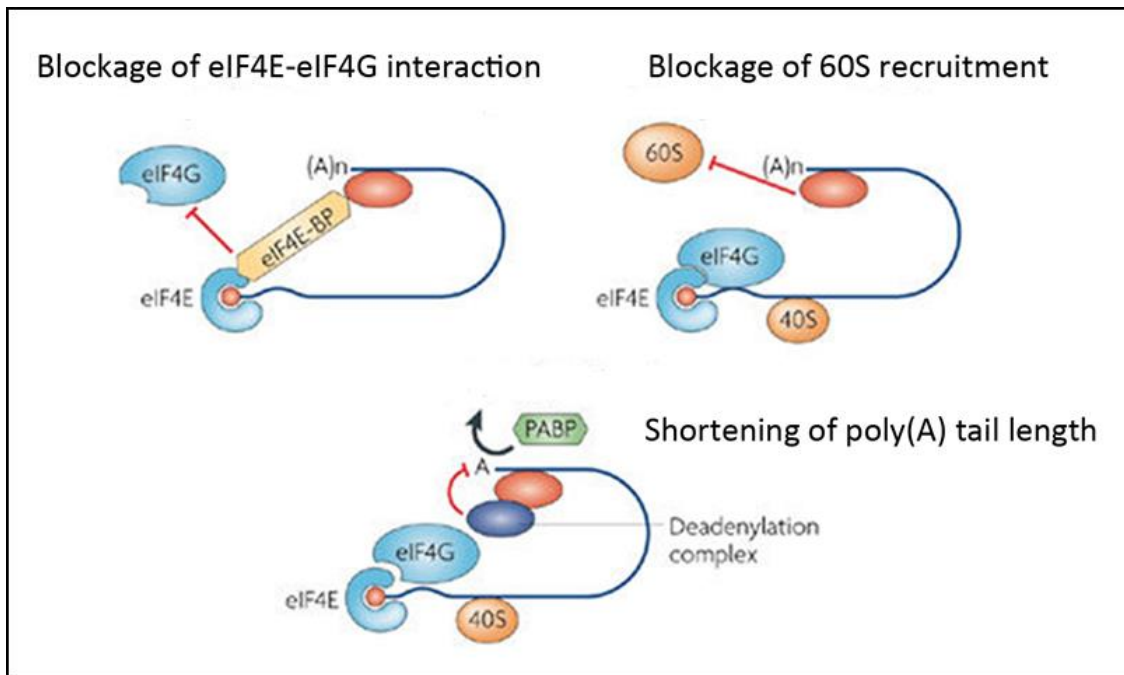


Figure 8. Multiple mechanisms inhibit formation of the translation closed-loop structure in localized mRNAs.

mRNA translation blockage can take place when formation of the eIF4G complex is prevented by eIF4E-binding proteins. In addition, translation can also be blocked when the assembly of ribosomal subunits 40S and 60S is inhibited or when poly(A) tail undergoes shortening. The different inhibitory mechanisms can be combined by multifunctional translational repressors ensuring a highly precise regulation of gene expression. Adapted figure from (Besse and Ephrussi 2008)

Last but not least, initiation of translation can be regulated by the poly(A) tail length and recruitment of PABP (Fig. 8). Indeed, in *Xenopus* oocytes modulating the mRNA poly(A) tail length plays a crucial role in translation efficiency and mRNAs with short poly(A) tails are translationally repressed. Furthermore, cytoplasmic poly(A) polymerases can regulate the stability of a transcript as well as its translational state by elongating the poly(A) tail (Stevenson and Norbury 2006). On the other hand, shortening of the poly(A) tail by cytoplasmic deadenylases usually precedes mRNA decay which mechanisms will be introduced below.

3.3 Regulation of mRNA degradation

A large proportion of regulated gene expression in a cell is due to changes in the mRNA decay rates. As previously mentioned, eukaryotic mRNAs present two stability structures: the 5' cap and the 3' poly(A) that protect the transcript from degradation by exonucleases via interaction with the cytoplasmic proteins eIF4E and PABP, respectively. Degradation is triggered when either the 5' cap or the 3' poly(A) are removed, leading to an unstable mRNA, or when the mRNA is cleaved internally by endonucleases. The mechanisms involved in mRNA decay are diverse and involve deadenylase complexes, decapping enzymes, such as decapping proteins 1 and 2 (Dcp1, Dcp2) and exonucleases like XRN1 (also known as Pacman in *Drosophila*) among others (Garneau, Wilusz et al. 2007). Furthermore, nearly all major eukaryotic mRNA decay pathways (such as the RNA-induced silencing complex (RISC), the surveillance mechanism of nonsense-mediated mRNA decay (NMD) and the exosome pathways) are initiated by deadenylation (Fig. 9) (Chen and Shyu 2011).

In *Drosophila*, the two major cytoplasmic deadenylase complexes implicated in mRNA decay are the conserved CCR4-NOT (Carbon Catabolite Repression 4, also known as Twin, and Negative On TATA less) and PAN2-PAN3 (PolyA Nuclease 2 and PolyA Nuclease 3) (Temme, Simonelig et al. 2014). On the other hand, transcript deadenylation can also be promoted by microRNAs (miRNAs) which are RNA sequences of around 20 nucleotides that bind complementary sequences in the target mRNA 3'UTR. These endogenous short non-coding RNAs induce silencing by recruiting and guiding the RISC complex, which contains Argonaute (Ago) and Gawky 182 (GW182), to the target transcript. Then, the direct interaction between the GW182 proteins of the RISC complex and the NOT subunit of the deadenylase complex promotes the shortening of the target mRNA poly(A) tail and the consequent destabilization and degradation by exonucleases (Braun, Huntzinger et al. 2011, Fabian, Cieplak et al. 2011).

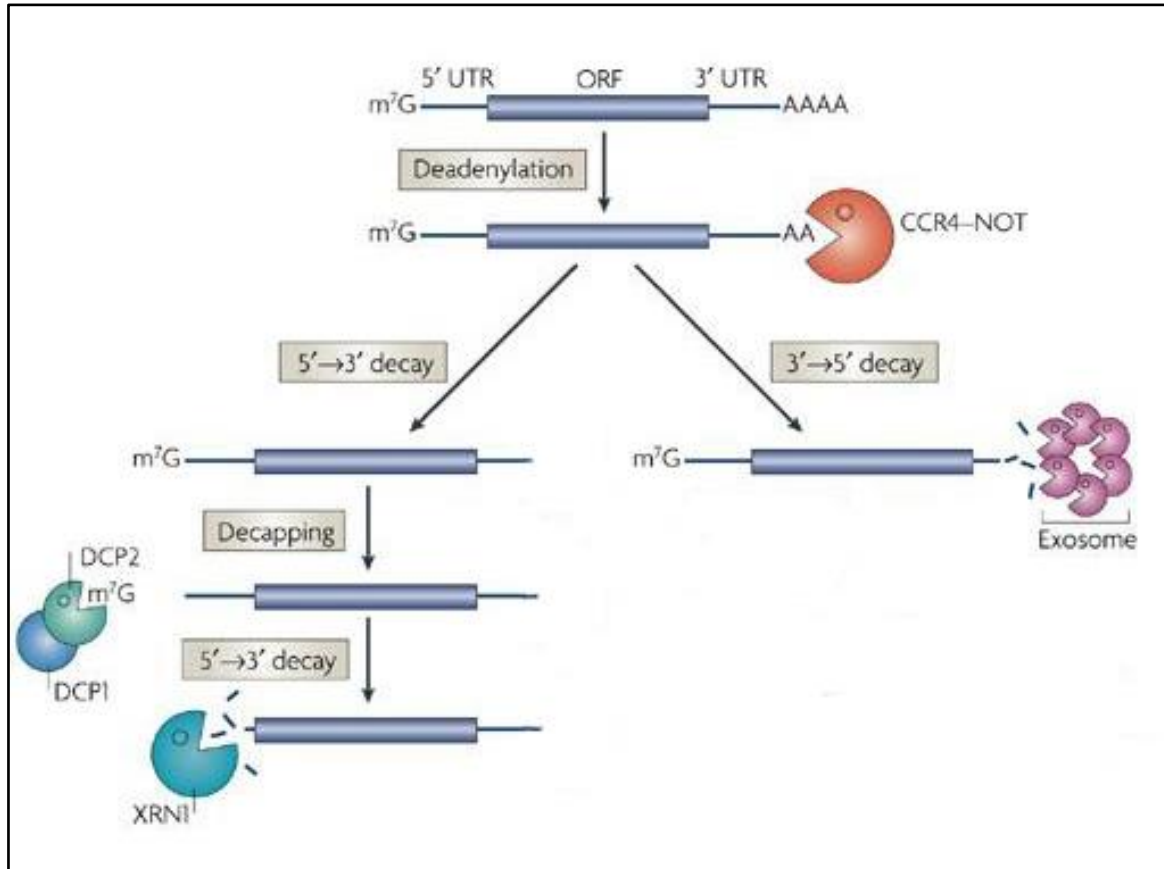


Figure 9. Deadenylation-dependent mRNA decay.

The majority of mRNAs undergo decay by the deadenylation-dependent pathway. The deadenylase complex, shown here as CCR4-NOT, removes the poly(A) tail and this process can be followed by either decapping and 5'→3' decay or 3'→5' decay. In the decapping pathway, the mRNA m⁷G cap structure is removed by the DCP1–DCP2 complex which makes the mRNA susceptible to decay by the 5'→3' exoribonuclease XRN1. Alternatively, the deadenylated mRNA can be degraded in the 3'→5' direction by the exosome. Figure adapted from (Garneau, Wilusz et al. 2007)

In conclusion, the mechanisms of post-transcriptional regulations are complex and diverse, combining interplay between mRNAs, non-coding RNAs, RNA binding proteins and multiple other factors. A critical question in RNA biology is how RNA binding proteins target and regulate mRNAs. I will focus on the properties and functions of the RNA binding proteins involved in these processes and their ability to form ribonucleoprotein particles in the following chapter.

Chapter II: RNA binding proteins are versatile and fundamental mediators of gene expression

1. Properties and functions of RNA binding proteins

As mentioned above, the regulation of mRNAs occurs via specific recognition of *cis*-regulatory sequences by RBPs that orchestrate, according to the context, the outcome of the mRNAs. RBPs constitute a very complex and diverse class of regulatory factors that coordinate the fate of mRNAs and interact with multiple protein factors. In eukaryotes, they are involved in every stage of RNA metabolism including biogenesis, processing, nuclear export, localization, stability, translation and decay (Marchese, de Groot et al. 2016). For this reason, mRNAs cannot be seen as single macromolecules that are transported from the nucleus to the cytoplasm but as ribonucleoprotein complexes containing RBPs that can recruit other associated factors (Gebauer, Preiss et al. 2012).

Most often, the *cis* elements recognized by these RBPs are present in the 5' and/or 3' untranslated region (UTR) sequence of the transcripts. However, some cases show that they can be found in the protein coding sequence (Zhang, Pierce et al. 1999, Semotok, Luo et al. 2008). The heterogeneous localization of these regulatory sequences suggests distinct mechanisms used by RBPs to regulate mRNA targets. Moreover, the specificity and affinity with which an RBP binds its target transcripts are variable, depending on conditions such as the length and/or the secondary structure of the *cis* sequences as well as the amount of RNA binding domains present in the RBP (see below) (Lunde, Moore et al. 2007, Helder, Blythe et al. 2016). Alterations in RBP expression levels and/or localization as well as mutations in the *cis* elements they recognize can lead to different types of human pathologies including neurodevelopmental disorders and cancer (Kechavarzi and Janga 2014, Brinegar and Cooper 2016).

A combination of biochemical methods and genome-wide approaches, such as crosslinking and immunoprecipitation followed by deep sequencing, have proved that individual RBPs can bind hundreds to thousands mRNA targets and that a single transcript can be regulated by many different RBPs (Hogan, Riordan et al. 2008, Anko and Neugebauer 2012, Ascano, Hafner et al. 2012). Furthermore, these methodologies have allowed the

mapping of the binding sites of specific RBPs at high resolution and transcriptome-wide (Hafner, Landthaler et al. 2010).

Recently, hundreds of novel RBPs have been identified as a result of methodological advances such as RNA interactome capture *in vivo* (Castello, Horos et al. 2013). This new method has been successfully applied using different mammalian cell lines, budding yeast, *C. elegans* and *Drosophila* embryos (Baltz, Munschauer et al. 2012, Matia-Gonzalez, Laing et al. 2015, Beckmann, Castello et al. 2016, Castello, Horos et al. 2016, Sysoev, Fischer et al. 2016). RNA interactome capture technique involves *in vivo* UV crosslinking followed by purification of polyadenylated RNA bound to proteins on oligo(dT)-coated beads and subsequent analysis of the RBPs associated by mass spectrometry (MS) (Fig. 10).

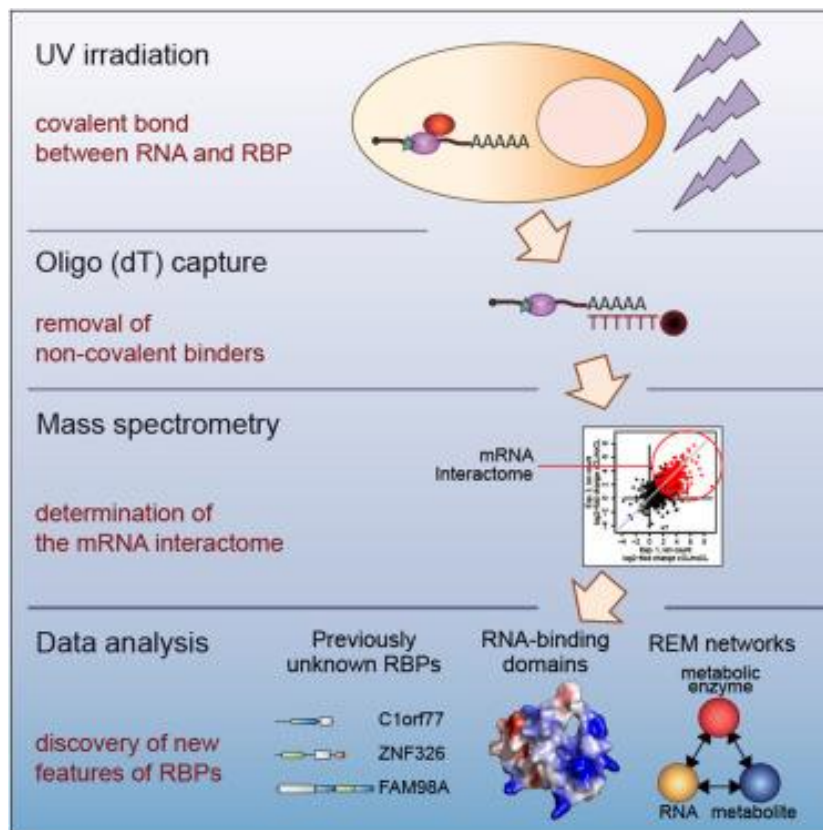


Figure 10. RNA interactome capture *in vivo*.

In vivo UV cross-linking of RBPs to polyadenylated RNAs leads to covalently bound proteins that are captured with oligo(dT) magnetic beads. After stringent washes, the mRNA interactome is determined by quantitative mass spectrometry. After data analysis, previously unknown RBPs can be discovered, novel RNA binding domains can be identified and new links between RBPs and signaling pathways be highlighted. A particular advantage of interactome capture over other *in vitro* and *in silico* approaches is that only RBPs bound to RNA in physiological conditions are identified. Figure taken from (Castello, Fischer et al. 2012).

1.1 RBPs bind RNA via a repertoire of RNA binding domains

Up-to-date, the discovery of RBPs has led to the identification of over 40 different RNA binding domains (RBD). Strikingly, sequence analysis has revealed that many RBPs contain several different RBDs. Combinatorial arrangements of distinct RBDs along with other functional domains convey structural and functional diversity to RBPs, enabling them to regulate every step of RNA metabolism (Lunde, Moore et al. 2007). Quantitative analysis showed that the number of RBDs in a protein inversely correlates with the number of nucleotides usually recognized by that type of RBD (Mitchell and Parker 2014). Multiple RNA binding modules in one RBP can increase the specificity and affinity for its target transcript.

In order to better comprehend how RNA binding proteins recognize and interact with their target mRNAs, I will describe the structure of some of the best-characterized RBDs known to date and give examples of RBPs containing them (Fig. 11 and Table 1).

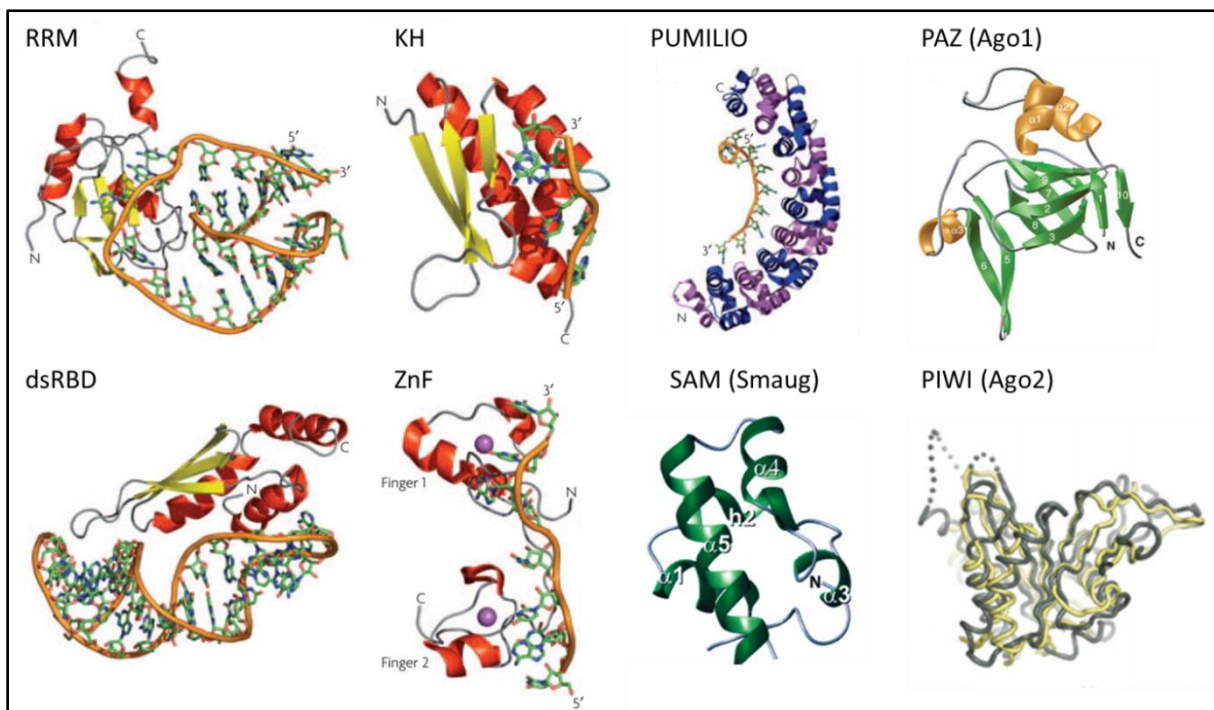


Figure 11. RNA binding domains are diverse.

Structure examples of the RRM, HK, dsRBD, Zinc Finger and Pumilio domains recognizing their target RNA (represented with an orange ribbon) (Lunde, Moore et al. 2007); SAM domain of Smaug, adapted figure from (Green, Gardner et al. 2003); PAZ domain of Ago1 (Yan, Yan et al. 2003); PIWI domain of Ago2 (Schirle and MacRae 2012).

Protein	RNA binding domain	Domain Topology	Example of mRNA target	Function on the mRNA	Reference
Glorund	RRM	$\alpha\beta$	<i>nos</i>	Translational repression during oogenesis	(Kalifa, Huang et al. 2006)
FMR1	KH	$\alpha\beta$	<i>futsch</i>	Translational repression during development of the nervous system	(Zhang, Bailey et al. 2001)
Staufen	dsRBD	$\alpha\beta$	<i>osk</i>	mRNA localization during oogenesis	(Irion, Adams et al. 2006)
Nanos	ZnF	$\alpha\beta$	<i>hunchback</i>	Translational repression during early embryogenesis	(Sonoda and Wharton 1999)
Argonaute	PAZ and PIWI	$\alpha\beta$	<i>orb</i>	mRNA destabilization during oogenesis	(Li, Maines et al. 2012)
Pumilio	Puf	α	<i>hunchback</i>	translational repression during early embryogenesis	(Murata and Wharton 1995)
Smaug	SAM	α	<i>nos</i>	translational repression during early embryogenesis	(Smibert, Wilson et al. 1996)

Table 1. Common RNA binding domains and examples of RBPs containing them.

1.1.1 RNA Recognition Motif (RRM)

The RRM domain is one of the first identified domains for RNA interaction and is, by far, the most common RBD and the best characterized one. It is found in many splicing factors and heterogeneous nuclear RNP (hnRNP) proteins that are involved in pre-mRNA processing in the nucleus. An example of cytoplasmic RBP containing RRM domains is the translational repressor Glorund which plays a relevant role repressing *nos* translation during early oogenesis in the fly (Table 1).

The RRM domain is composed of 80-90 amino acids (aa) that fold in a $\beta\alpha\beta\beta\alpha\beta$ structure forming a four-stranded anti-parallel β -sheet with two helices packed against it (Fig. 11) (Lunde, Moore et al. 2007). Furthermore, it is via stacking interactions involving aromatic and basic residues in the β -sheet surface that the RRM interacts with the target RNA nucleotides (usually between 4 to 8 nucleotides). This RBD is often present in multiple copies in the protein and the specificity of the binding depends on the number of copies contained in the protein, ranging from 2 to 6 (Maris, Dominguez et al. 2005). Moreover, RRM can also function as a protein-protein interaction domain allowing RBP hetero-dimerization (Fribourg, Gatfield et al. 2003).

1.1.2 K-Homology (KH) domain

The K-Homology domain got its name after it was first identified in the nuclear protein hnRNP K which is involved in mRNA processing in the nucleus (Matunis, Michael et al. 1992). Importantly, mutations in the HK domain of the RNA binding protein FMR1 cause fragile-X mental retardation syndrome (see Table 1) (De Boulle, Verkerk et al. 1993).

The KH domain is composed of approximately 70 residues that bind both single stranded (ss)DNA and ssRNA. All KH domains form a three-stranded β -sheet packed against three α -helices (Grishin 2001). Nonetheless, there are two different subfamilies of KH domains depending on the basis of their topology: $\alpha\beta\beta\alpha\alpha\beta$ (type I fold) and $\beta\alpha\alpha\beta\beta\alpha$ (type II fold). KH domains recognize four RNA nucleotides that form a consensus loop and, unlike the RRM, RNA recognition occurs through hydrogen bonding, electrostatic interactions and shape complementarity (Fig. 11) (Lunde, Moore et al. 2007).

1.1.3 Double-Stranded (ds) RNA Binding Domain (dsRBD)

One of the first dsRBP to be identified was the *Drosophila* Staufen which is responsible for mRNA localization of various transcripts during oogenesis in fly (see Table 1) (St Johnston, Beuchle et al. 1991).

The dsRBD is a $\alpha\beta$ domain, containing approximately 70 aa, that binds dsRNA in a non-specific fashion (Fig. 11). It is usually present in one to five copies and this multiplicity imparts higher specificity to the RBPs (Saunders and Barber 2003).

1.1.4 Zinc Finger (ZnF) domain

Nanos is a particularly well characterized, evolutionarily conserved, ZnF RNA binding protein known to repress the translation of specific mRNAs (see Table 1).

ZnF proteins are a class of DNA and RNA binding proteins that possess a domain rich in cysteine (C) and histidine (H) that chelates a zinc ion (Clemens, Wolf et al. 1993). They can be classified in three distinct subtypes according to the residues used to coordinate the zinc ion: CCHH, CCCH and CCHC. The ZnF RBD contains 30 aa and it was originally discovered in the transcription factor TFIIIA where it is present in nine CCHH copies. The CCHH domain interacts primarily with DNA while CCCH and CCHC interact mostly with AREs in the mRNA 3' UTR which are sequences rich in adenylate and uridylate nucleotides. RNA recognition of, generally, 2 to 4 nucleotides occurs mainly by hydrogen bonding to the protein backbone (Fig. 11) (Hall 2005, Lunde, Moore et al. 2007).

1.1.5 Pumilio (PUF) domain

Pumilio (Pum) is a conserved sequence specific RBP that is ubiquitously expressed in the *Drosophila* embryo (Macdonald 1992) and acts as a post-transcriptional repressor by binding the 3'UTR of mRNA targets. The PUF domain (named after Pumilio and the *C. elegans* FBF protein) typically consists of 8 α -helical repeats, each of which recognizes one RNA base (Fig. 11). All the members of the PUF family recognize similar RNA sequences of 8 nucleotides (see Table 1). More precisely, they all target the consensus sequence UGUR (where R represents a purine) followed by recognition of other sequences that are specific to each PUF protein (Miller, Higgin et al. 2008) (Miller and Olivas 2011).

1.1.6 PAZ and PIWI domain

The PAZ and PIWI domains are RBDs exclusively found in proteins involved in gene silencing, especially in processing microRNA (miRNA) and small interfering RNA (siRNA)

precursors. For instance, the Argonaute proteins contain both: an amino-terminal PAZ domain and a carboxi-terminal PIWI domain.

The PAZ domain is composed of 110 aa containing a β -barrel motif juxtaposed to a $\alpha\beta$ domain that forms a clamp-like structure in which RNA binds (Yan, Yan et al. 2003). PAZ domains in Ago proteins promote cleavage of the target strand by the RNA-induced silencing complex (RISC), which is responsible for degradation of the target mRNA. The associated PIWI domain is in charge of anchoring the guide strand, thus allowing degradation of the target strand (Table 1 and Fig. 11) (Parker, Roe et al. 2005).

1.1.7 Sterile α Motif (SAM) domain

Finally, the SAM domain (Fig. 11), which is present in Smaug RBP and therefore I am particularly interested in, has originally been characterized as a protein-protein interaction domain. However, biochemical and genetic approaches have demonstrated that, in the case of the Smaug (*Drosophila*)/SAMD4 (mammals) proteins, this domain is also involved in RNA binding (see Table 1) (Smibert, Wilson et al. 1996, Dahanukar, Walker et al. 1999, Smibert, Lie et al. 1999). I will further describe Smaug SAM domain in chapter IV.

In summary, RBPs play multiple roles in RNA metabolism and they can do so by interacting with the target transcripts via a wide variety of RNA binding domains. The RBP architecture has been classically viewed as a combination of RBD modules. However, recent RNA interactome capture experiments using different human cell lines showed that only 45% of the known RBPs are capable of binding mRNAs through canonical RNA binding domains (Beckmann, Castello et al. 2016, Castello, Fischer et al. 2016). The remaining 55% of RBPs do not present a known RBD which suggests the existence of uncharacterized RBDs requiring further structural studies. Surprisingly, these large-scale studies have also found that hundreds of the newly identified RBPs lacking canonical RBDs contain low-complexity regions raising the possibility of RNA binding through disordered regions (see below) (Castello, Fischer et al. 2012, Mitchell and Parker 2014, Beckmann, Castello et al. 2016). Moreover, some of the discovered RNA binders have protein domains with both enzymatic and RNA binding activity which challenges the general concept that RBPs lack enzymatic

activity and only act as an adaptor (Walden, Selezneva et al. 2006, Beckmann, Horos et al. 2015, Beckmann, Castello et al. 2016).

1.2 RBPs serve as scaffolds to recruit other proteins

A characteristic of RBPs is that they can serve as a recruitment scaffold for other protein factors forming dynamic ribonucleoprotein particles that will modify the target mRNA. For instance, the multiprotein EJC (exon-junction complex) is an RNA binding protein complex that recognizes and assembles with mRNAs during the splicing reaction in the nucleus. The EJC remains bound to the spliced transcripts and then serves as a platform to recruit nuclear and cytoplasmic factors that will influence their cytoplasmic fate. Such is the case of *osk* mRNA, which splicing process at the nucleus is coupled to its cytoplasmic localization at the posterior pole of the *Drosophila* oocyte (Hachet and Ephrussi 2004, Lunde, Moore et al. 2007). It is important to note that even small changes in the RBD sequence and/or structure can be sufficient to alter protein-protein interaction and therefore, indirectly affect recognition of the target transcript.

1.3 RBPs possess intrinsically disordered regions that promote aggregation

Generally, RBPs contain intrinsically disordered regions (IDR) that are sequences of low amino acid complexity that lack a defined 3D structure. IDRs can undergo disordered to ordered transition after binding with interactors and can, therefore, modulate the formation of RNPs. These disordered regions, generally rich in basic sequences such as arginine/serine (R/S) or arginine/glycine (R/G), are capable of mediating protein-RNA interactions. Hence, IDRs can regulate RNA metabolism through direct interaction with the target transcript. In addition, it has been shown that IDRs contribute to RBP assembly and formation of RNPs such as processing bodies and stress granules that will be presented below (Calabretta and Richard 2015, Jarvelin, Noerenberg et al. 2016).

RNA binding proteins often contain low complexity sequences rich in glutamine-asparagine (Q/N) that are prion-like domains capable of self-assembly that contribute to RNP formation. Initially without structure, these Q/N-rich sequences can undergo conformational changes that induce structural alterations in other proteins with similar

domains, leading to macromolecular assembly (Alberti, Halfmann et al. 2009, Alberti 2013). For instance, prion-like IDRs of mammalian RBPs TIA-1 (T-cell antigen intracellular 1) and FUS (fused in sarcoma) modulate their targeting to stress granules after cellular damage (Gilks, Kedersha et al. 2004, Kato, Han et al. 2012). Importantly, formation of prion-like and amyloid-like structures has emerged as causes of protein misfolding neuropathologies like Alzheimer's and Parkinson's diseases (Malinowska, Kroschwald et al. 2013).

2. Ribonucleoprotein granules are dynamic complexes containing both RNA binding proteins and RNA

RBPs assemble with their target mRNAs both in the nucleus and/or in the cytoplasm and form ribonucleoprotein (RNP) complexes that are non-membranous structures that control mRNA fate. A functional advantage of RNP formation would be that components are concentrated and/or stored in space in an efficient way (Anderson and Kedersha 2006, Kishore, Lubner et al. 2010).

The specific composition of RNPs (mRNP code) depends on the sequence of the mRNAs that are being targeted, their processing as well as the activity of the RBPs that bind them (Glisovic, Bachorik et al. 2008, Mitchell and Parker 2014). Moreover, it can also be subjected to the cellular context. Components such as ribosomal subunits, translation factors, decay enzymes, helicases and scaffold proteins are usually found in RNA granules. Also, molecules like small non-coding (nc) RNA (miRNA and PIWI-interacting RNAs) as well as long ncRNAs are present in RNP structures (Muller-McNicoll and Neugebauer 2013).

RNPs can remodel themselves in a time and space dependent manner, according to the developmental, signaling or environmental context. They act as regulatory hubs and this is due to both changes in the composition of the proteins present in the different subcellular compartments where mRNAs are targeted, as well as to post-translational modifications (see next chapter) (Kedersha, Ivanov et al. 2013). Understanding how mRNPs dynamics are regulated is crucial to comprehend their role and functioning. In this section, I will focus on the principles and properties of cytoplasmic RNPs.

2.1 Cytoplasmic ribonucleoprotein granules

Various classes of RNP granules have been described to date. Distinctions are made whether these structures are present in somatic cells (processing bodies and stress granules), neurons (neuronal granules) or germinal cells (polar granules). In the section, I will describe the different RNP granules and highlight their differences.

2.1.1 Processing bodies

Processing bodies (P bodies) are cytoplasmic granules that are constitutively present in eukaryotic cells. They contain mRNAs associated with translational repressors as well as factors of the mRNA decay machinery such as decapping enzymes and deadenylases. Transcripts present in P bodies are considered to be either stored and/or targeted for degradation. However, recent studies using novel methods of P-bodies purification from human epithelial cells have shown that the P-bodies condense and segregate thousands of translationally repressed, but not decayed, mRNAs (Hubstenberger, Courel et al. 2017).

In addition, subcellular localization studies by immunolabeling of P-body components showed that P-bodies can increase in size and number under stress conditions. However, even though P body components are crucial mediators of RNA-mediated gene silencing, their spatial aggregation is not required for their function in mRNA decay. Thus, P body formation is a consequence and not the cause of repression of gene expression (Eulalio, Behm-Ansmant et al. 2007).

2.1.2 Stress granules

Stress granules (SG) form when translation initiation is impaired due to external stress or lack of translation initiation factors. Assembly of SGs is a conserved cellular strategy that minimizes stress-related damage and promotes cell survival. This has been shown by using translation initiation inhibitory drugs or by inducing cellular stress which result in the formation of SGs. On the other hand, mRNAs that are trapped in polysomes and, therefore, are being translated, fail to form SGs. Thus, these types of granules are transient and their existence depends on the cellular context (Mitchell and Parker 2014, Protter and Parker

2016). Notably, mutations that dysregulate stress granule formation can give rise to neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) (Fan and Leung 2016).

Remarkably, biochemical purification of SGs granules suggests that SGs are composed of stable cores surrounded by a phase-separated shell that is more dynamic and open for exchange with the cytoplasmic *milieu*. Recent fluorescence recovery after photobleaching (FRAP) studies prove how dynamic SGs can be since most of its components exchange rapidly, with half-times for recovery of less than 30 seconds (Protter and Parker 2016). Considering that SGs form under stress conditions, it has been hypothesized that these RNPs act as signaling centers. The transient formation would modulate signaling pathways by intercepting and sequestering signaling components (Kedersha, Ivanov et al. 2013).

Interestingly, it has been shown that SGs and P bodies can colocalize in the cell (Kedersha, Stoecklin et al. 2005). Altogether these data suggest a dynamic mRNA cycle where mRNPs can be remodeled within these assemblies and exchange components between them (see Fig. 3). Finally, SGs can disassemble and resume translation if the cellular conditions become favorable or they can undergo autophagy which provides another way for stress granule clearance (Fig. 12) (Buchan, Kolaitis et al. 2013).

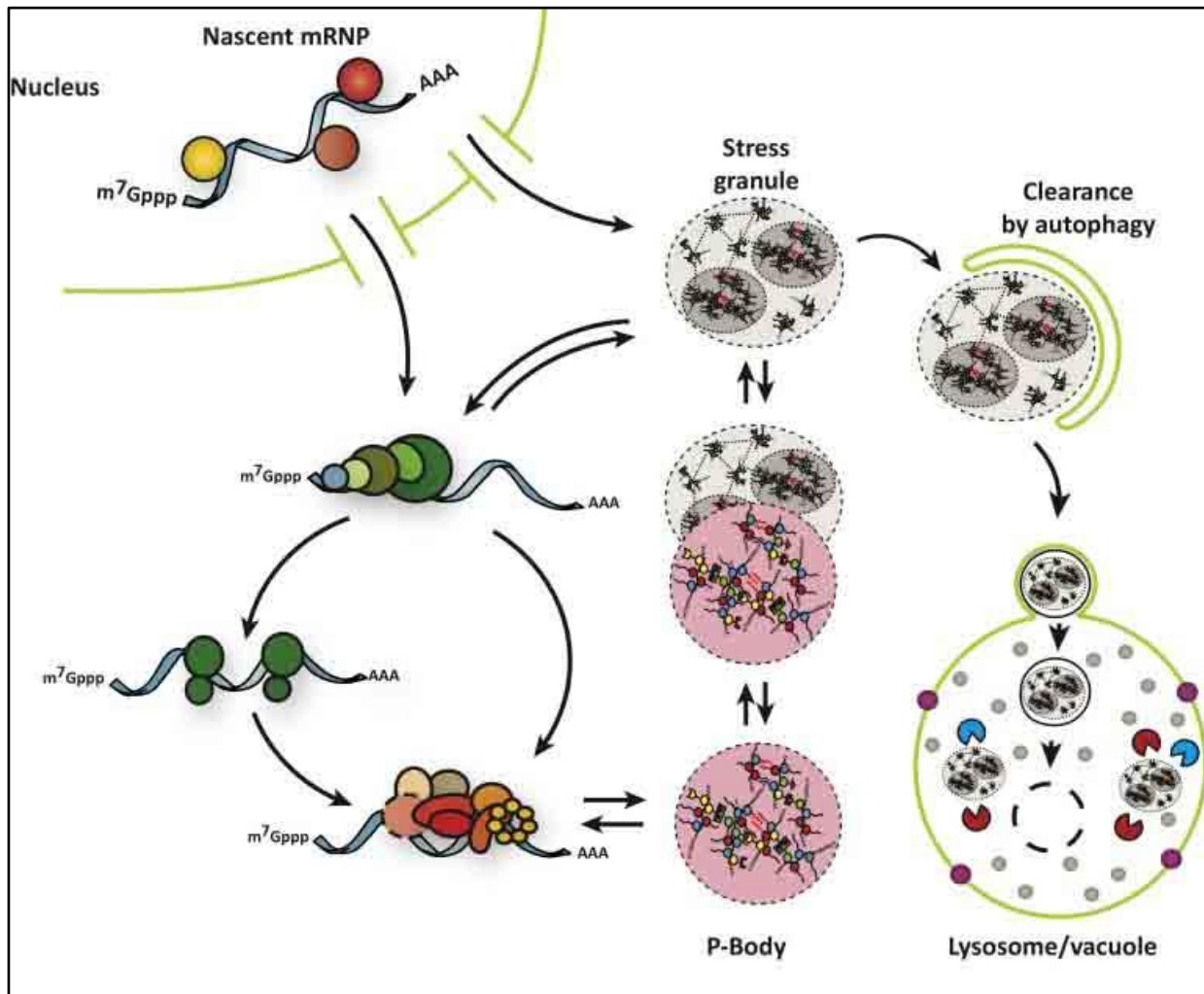


Figure 12. Remodeling of cytoplasmic stress granules and processing bodies.

SGs and P bodies are foci that are highly concentrated in RNA and proteins involved in translational repression (SGs) and mRNA degradation (P bodies). SGs can dynamically exchange mRNP components with P bodies, disassemble or be cleared by autophagy according to the cellular context (Protter and Parker 2016).

2.1.3 Neuronal granules

First described as transport granules in the neurons, these RNPs contain mRNAs that are translationally arrested during their transport from the neuronal body until the final destination, at the growth cone or dendrites, is reached. Upon synaptic stimuli, the localized mRNAs are released to actively translated pools (Krichevsky and Kosik 2001). Staufen and the fragile X mental retardation protein (FMRP) are key RNA binding proteins commonly found in neuronal granules involved in traffic of translationally repressed RNAs (Kiebler and Bassell 2006). The RBP Imp (IGF-II mRNA-binding protein) also accumulates in neuronal

granules, albeit being distinct from Staufen and FMRP granules. Imp is essential for neural stem cell maturation and has recently been identified as a major player in neuron remodeling (Medioni, Ramialison et al. 2014).

2.2 How do ribonucleoprotein granules form?

A central issue in the field of RNA biology has been to understand how these dynamic ribonucleoprotein structures form. It has traditionally been viewed that a combination of RNA-protein and protein-protein interactions promotes a growing RNP granule assembly. Almost a decade ago, FRAP and stress application experiments have shown that P granules in *C. elegans* embryos behave like liquid droplets (Brangwynne, Eckmann et al. 2009). Liquid droplet formation takes place when RNA molecules, RBPs and the proteins associated transition from a soluble state to a condensed phase (Brangwynne, Eckmann et al. 2009, Hubstenberger, Noble et al. 2013). The liquid state relies on the fact that the molecular interactions that are involved are weak and permanently changing while aggregates are based on more rigid molecular interactions.

Since then, pioneering studies have proposed that P bodies and SGs also have liquid-like properties and form by liquid-liquid phase separation. The latter takes place after high concentrations of RNP components reach a critical threshold and start to assemble spontaneously through weak and multivalent interactions between multidomain RBPs and RNA molecules (Brangwynne, Eckmann et al. 2009, Li, Banjade et al. 2012, Hubstenberger, Noble et al. 2013, Aguzzi and Altmeyer 2016). Further studies on RNP granules have confirmed a liquid-like behavior of RNP fusion and evidence show that RBPs intrinsically disordered regions can promote phase separation *in vitro* (Kato, Han et al. 2012, Li, Banjade et al. 2012, Malinowska, Kroschwald et al. 2013, Lin, Protter et al. 2015).

Finally, phase separated droplets can stabilize over time forming hydrogel structures that can lead to further aggregation and form amyloid-like fibers (Fig. 13). However, the proteins that drive the phase transitions *in vivo* remain to be elucidated. As mentioned above, amyloid-like structures are tightly associated with neurological disease.

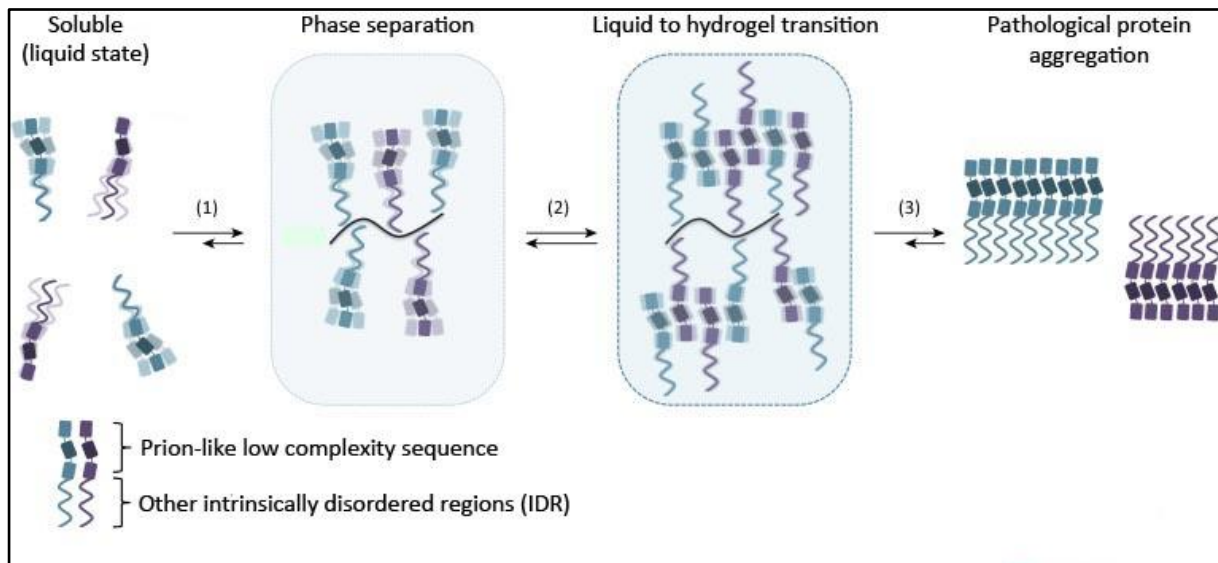


Figure 13. Linking intracellular formation of RNP granules by phase separation and disease. Proteins with intrinsically disordered regions (often low complexity sequences such as prion-like domains) present a high degree of structural flexibility which allows them to dynamically undergo heterotypic interactions with other molecules. 1) Local enrichment of RNP components generates a liquid droplet by phase separation compartmentalizing the RNP granule. Due to the weak nature of these interactions, RNPs granules are permanently rearranging their binding surface. 2) Maturation of the liquid droplet leads to the formation of a hydrogel-like structure with reduced fluidity and protein movement. 3) Higher concentration of aggregation-prone proteins that contain prion-like domains increases the risk of pathological protein aggregation. Adapted figure from (Aguzzi and Altmeyer 2016)

In conclusion, RBPs are critical and often multifunctional mediators of mRNA post-transcriptional regulations and their activity is tightly tuned to the cellular context. Considering the primary role that RBPs play in the regulation of gene expression, coordinating every step since the synthesis of mRNAs in the nucleus to their degradation in the cytoplasm, it comes as no surprise that RBP related malfunctions are a major cause of disease. In order to be able to identify the pathogenic mechanisms and consequences of the altered RBPs (which can be sequestered, hyperactive or aggregated in the disease conditions) it is fundamental to know more about the structure of RBPs as well as their mode of interaction with RNA and other protein factors. Finally, recent studies have demonstrated that RBPs activity can be altered by post-translational modifications, which I will describe more in detail in the next chapter.

Chapter III: RNA binding proteins are regulated by post-translational modifications

Studies from different research groups have demonstrated that RBPs are regulated by a large variety of mechanisms, including environmental signals, induced RBP conformational changes, changes in the RBP expression levels, competition with other RBPs for target transcripts, differential subcellular localization or the availability of the target mRNAs (Strein, Alleaume et al. 2014).

In this chapter, I will discuss some specific examples of RBP regulation by post-translational modifications (PTMs) and their effects on the RBP subcellular localization, RNA binding affinity and ability to interact with other associated proteins.

1. Proteins are regulated by a plethora of post-translational modifications

A wide range of PTMs exist and regulate all types of eukaryotic proteins, during or after their synthesis, by altering their activity state, localization, turnover, and interactions with other proteins. These covalent, and usually reversible, modifications play a crucial role in cell signaling. PTMs include the addition or the removal of chemical groups, lipids, carbohydrates or amino-acids (Fig. 14).

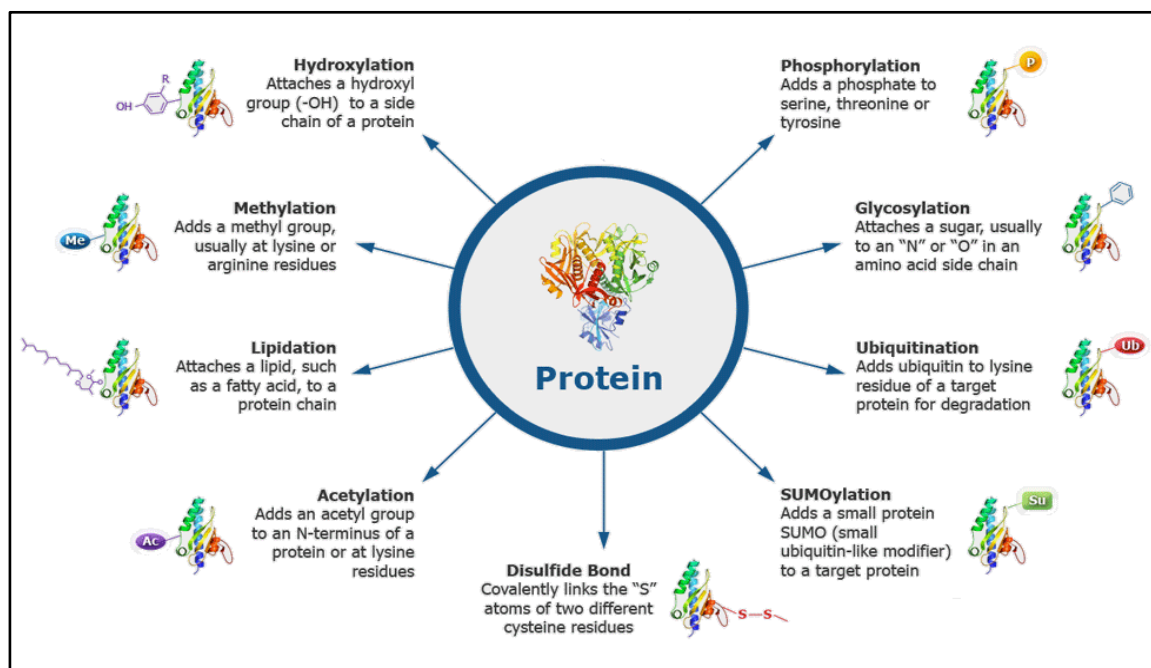


Figure 14. Examples of post-translational modifications

In order to understand the role of protein post-translational modifications, it is first necessary to identify them. Major advances in proteomic techniques, particularly in mass spectrometry, have allowed mapping a protein's post-translational modifications with high accuracy. Two of the most common PTMs are phosphorylation and ubiquitination, being widely used as cellular mechanisms to regulate the activity of proteins. On the one hand, protein phosphorylation plays a major role in the activation/deactivation of multiple enzymes and receptors via the action of protein kinases and phosphatases. On the other hand, cytoplasmic protein modification by ubiquitination generally marks the proteins for degradation via the proteasome pathway. However, ubiquitination of several different types of transmembrane proteins can also act as a signal for their entry into the endocytic pathway, leading to their intracellular trafficking into lysosomes where they will be degraded (MacGurn, Hsu et al. 2012).

2. Post-translational modifications regulate multiple aspects of RBP function

Signaling transduction processes depend on reversible PTMs events to rapidly reprogram individual protein functions. Particularly, PTMs have the potential to affect RBP activity by altering their expression levels, stability, subcellular localization and structural conformation (Fig. 15) (Thandapani, O'Connor et al. 2013, Lovci, Bengtson et al. 2016).

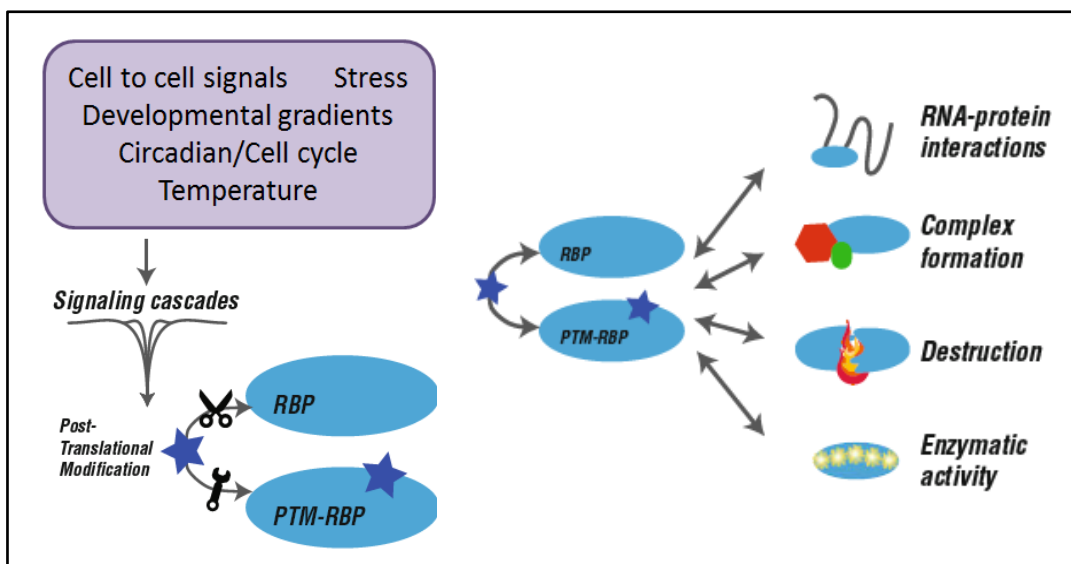


Figure 15. Signal integration and effects of PTM on RBP function

Upon different external or internal signals, signaling cascades are activated and RBPs are modified post-translationally. These PTMs can activate or deactivate a given RBP by influencing the interactions with target mRNAs or protein partners as well as by altering the expression levels or the enzymatic activity. Figure from (Lovci, Bengtson et al. 2016)

In addition, post-translational modifications can also impact RBP binding affinity for specific RNA sequences or for other protein partners. Note that, if a modification (or the lack of it) is necessary to facilitate RBP binding, then the pool of free protein is not really equivalent to the pool of functionally available protein. Thus, this functional alteration can be used to regulate binding globally or in specific subcellular compartments (Mitchell and Parker 2014).

2.1 Effect on the mRNA binding activity

Local variations in the concentration of active RBP due to pre-localized kinases and phosphatases is an example of RBP regulation by phosphorylation (Besse and Ephrussi 2008). For Instance, it has been reported that a ‘phosphogradient’ of the RNA-binding protein Mex5 is created by localization of the kinase Par-1 at the posterior compartment of the *C. elegans* embryo (Griffin, Odde et al. 2011). Mex-5 phosphorylation in S⁴⁰⁴ and S⁴⁵⁸ by Par-1 reduces its mRNA binding activity, leading to Mex5 release from mRNP complexes and higher diffusion at the posterior pole.

Another example of RBP regulation by phosphorylation concerns the translational repressor FMR1 which is phosphorylated *in vitro* and *in vivo* in S⁴⁰⁶ by Casein Kinase II (CKII) in *Drosophila* neurons. RNA binding assays showed that FMR1 phosphorylation increases its ability to bind its target transcripts (Siomi, Higashijima et al. 2002). On the other hand, FMR1 dephosphorylation promotes mRNA release and activation of translation thus inducing a translational switch at synapsis.

Similarly, recognition of AREs by ARE RBPs is also regulated by multiple intracellular signals that trigger protein methylation, acetylation, phosphorylation or ubiquitination to name a few. Such is the case of the conserved neuron specific RBP HuR (human antigen R) which binds to multiple transcripts controlling their stability and translation. HuR function is primarily regulated by post-translational modifications that alter its ability to bind target RNAs as well as its nucleo-cytoplasmic shuttling (Grammatikakis, Abdelmohsen et al. 2017).

2.2 Effect on the formation of RNPs

RNP granules can be modified by a wide variety of PTMs that affect RNP properties, assembly and dissolution. For instance, it has been shown that granule formation can be favored by (de)phosphorylation, methylation and deacetylation (Kedersha, Ivanov et al. 2013, Lovci, Bengtson et al. 2016). More specifically, the MEG (maternal-effect germline defective) proteins, which are found in the pole plasm of the *C. elegans* embryo where they control fertility, have IDRs that regulate polar granules dynamics upon phosphorylation and dephosphorylation events. Genetic analysis of mutant embryos showed that phosphorylation of Meg1 and Meg3 by kinase MBK-2/DYRK promotes granules dissolution while dephosphorylation by phosphatase PP2A induces granules assembly (Wang, Smith et al. 2014).

Hence, post-translational modification of RBPs is a widely used mechanism to control gene expression by regulating RBP function and physiological assembly of RNP granules in eukaryotes. It constitutes an additional layer for control of gene expression and although multiple examples have been described so far, it is likely that we are only seeing the tip of the iceberg and that many other RBPs are being functionally regulated by PTMs. Lastly, despite the fact that RNA granules components and dynamics have been extensively studied, the signaling pathways that regulate them are largely unknown.

Chapter IV: Smaug, a multifunctional RNA binding protein conserved from yeast to human

My thesis focuses on the RNA binding protein Smaug which was first discovered in 1996, through a combination of genetic and biochemical analysis in *Drosophila* embryos. Smaug is the founding member of a novel group of post-transcriptional regulators that is conserved from yeast to humans. Smaug homologues are defined by the presence of a unique Sterile Alpha Motif (SAM) domain through which they bind defined stem-loop structures in their mRNA targets (Smibert, Lie et al. 1999).

Smaug is known to act as a multifunctional repressor by blocking the translation of unlocalized *nos* mRNA and by promoting mRNA decay of hundreds of maternal transcripts during early embryogenesis in the fly (Dahanukar, Walker et al. 1999, Smibert, Lie et al. 1999, Semotok, Cooperstock et al. 2005). Throughout this chapter, I will describe the advances made during the past two decades on determining Smaug multiple functions, its known mechanisms of action and protein structure.

1. Smaug is a conserved RNA regulator with multiple roles in eukaryotes

1.1 Smaug plays critical roles during *Drosophila* development

1.1.1 Smaug expression in the embryo

Western Blot experiments from embryonic extracts at different developmental stages indicate that Smaug protein accumulates in high levels throughout the embryo during the first 3h after egg activation (Dahanukar, Walker et al. 1999, Smibert, Lie et al. 1999). About 4h after fertilization, Smaug protein levels drop during the cellularization phase (Fig. 16). However, detection of *smaug* mRNA by RNA *in situ* hybridization and detection of Smaug protein by immunohistochemistry showed that both the transcript and the protein are still found in the pole cells (Smibert, Lie et al. 1999).

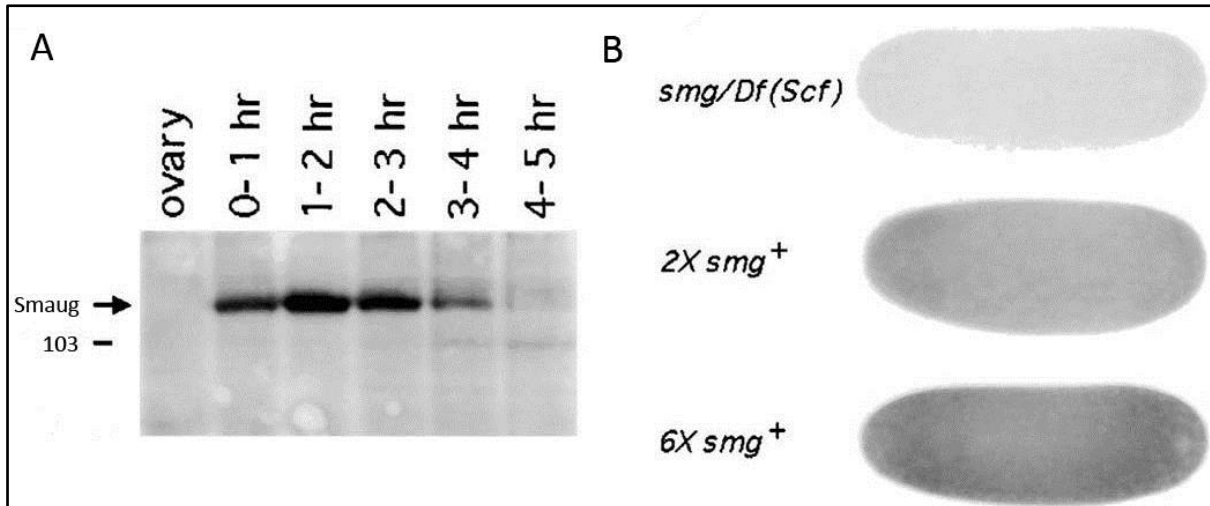


Figure 16. *Smaug* is synthesized after fertilization and is uniformly distributed across the embryo during early embryogenesis

(A) Western blot of extracts prepared from ovaries (lane 1) and hourly collections from wild-type embryos (lanes 2–6). A protein of around 120 kDa is recognized by using antibodies against *Smaug* RNA binding domain. (B) Bright-field photographs of embryos derived from *smaug* mutant embryos (*smg/Df(Scf)* females) (top), wild-type females bearing two copies of the endogenous *smg⁺* gene (center), and transgenic females bearing four additional copies of the *smg⁺* gene (bottom). The anterior of each embryo is to the left and the dorsal surface is at the top. Figure adapted from (Dahanukar, Walker et al. 1999)

During *Drosophila* oogenesis, *smaug* maternal mRNA is deposited ubiquitously in the oocyte but its translation only takes place upon egg activation by fertilization (Smibert, Lie et al. 1999). This indicates that *smaug* mRNA is itself a target of post-transcriptional regulation. Genome-wide analysis showed that maternal RBP Pumilio can form a complex with *smaug* mRNA although its repression has not been tested (Gerber, Luschnig et al. 2006). Analysis of Pan Gu (PNG) embryo mutants showed that PNG allows the translation of *smaug* mRNA after fertilization (Tadros, Goldman et al. 2007).

Finally, subcellular localization studies by immunolabeling showed that Smaug forms discrete cytoplasmic foci of variable sizes in the embryo. Moreover, *Drosophila* Smaug still forms granules when expressed in mammalian cells and so does its mammalian orthologue Smaug1, indicating the conservation of Smaug assembly properties (Baez and Boccaccio 2005).

1.1.2 Smaug plays a key role during maternal-to-zygotic transition

Egg activation induces the destabilization of over 1600 maternal transcripts. As mentioned in chapter I, one of the major key players in the MZT is the RBP Smaug which is responsible for the degradation of two-thirds of the destabilized maternal transcripts (Tadros, Goldman et al. 2007). Consequently, embryos from *smaug* mutant female flies are unable to proceed with cellularization due to the absence of maternal mRNA degradation which derives in embryonic lethality. Thus, Smaug accumulation acts as a trigger for the MZT (Dahanukar, Walker et al. 1999, Smibert, Lie et al. 1999, Benoit, He et al. 2009).

Moreover, loss of Smaug has a major negative effect on more than 70 species of zygotic microRNAs (miRNAs) synthesis which are required for the second wave of maternal mRNA clearance. In particular, Smaug is required for the expression of miRNA-309-cluster, which is responsible for the destabilization of 410 maternal transcripts (Benoit, He et al. 2009). Smaug is also required for the synthesis and stability of RISC component Ago1 with which it physically interacts (Pinder and Smibert 2013, Luo, Li et al. 2016). Thus, Smaug controls the MZT through direct regulation of its maternal mRNA targets and indirectly by blocking miRNA synthesis which regulates other maternal transcripts.

RNA co-immunoprecipitation followed by hybridization to DNA microarrays (RIP-chip) showed that Smaug binds to mRNAs involved in many biological processes during the MZT. Gene ontology analysis of Smaug-bound mRNAs resulted in enriched transcripts that play a role in regulation of protein folding (chaperonins), mRNAs involved in degradation (proteasome particles) and metabolism (Chen, Dumelie et al. 2014).

1.1.3 Smaug establishes antero-posterior (AP) embryonic polarity by spatial regulation of *nos* mRNA during early embryogenesis

Localized *nos* mRNA translation is crucial for the proper organization of abdominal segmentation as well as for germ cell development. Northern blot experiments allowed determination of the relative amounts of unlocalized and localized *nos* in wild-type embryos. Results showed that only 4% of *nos* mRNA is found at the posterior compartment whereas the majority remains dispersed and translationally repressed in the bulk cytoplasm of the

embryo (Bergsten and Gavis 1999). A series of RNA binding assays led to the identification of Smaug as *nos* mRNA translational repressor during early embryogenesis. Smaug recognizes and binds specifically two *cis* acting sequences present in *nos* 3'UTR, called hereafter Smaug Recognition Elements (SRE). Studies of embryos from *smaug* mutant female flies showed that unlocalized *nos* mRNA translation is no longer repressed which leads to the repression of the anterior determinant *hunchback*. Ergo, Smaug blocks unlocalized *nos* mRNA translation (Smibert, Wilson et al. 1996, Dahanukar, Walker et al. 1999, Smibert, Lie et al. 1999). Further analysis demonstrated that Smaug also promotes unlocalized *nos* mRNA decay.

Interestingly, *nos* mRNA translation is not repressed at the posterior pole despite Smaug being expressed across the embryo. This is due to inhibition of Smaug binding with *nos* 3' UTR at the posterior pole by Osk. Moreover, GST pull-down assays showed direct interaction between Smaug and Osk suggesting that Osk binding releases Smaug from *nos* transcript (Zaessinger, Busseau et al. 2006). Subsequent studies of *nos* mRNA regulation demonstrated that Osk prevents both *nos* translational repression and mRNA decay at the posterior pole (Jeske, Moritz et al. 2011).

1.1.4 Smaug regulates dendritic arborization in the larval peripheral nervous system (PNS)

There is one report indicating that *nos* mRNA is essential for dendrite morphogenesis in *Drosophila* larval peripheral neurons where it regulates higher order dendritic arborization (da) (Ye, Petritsch et al. 2004). Da neurons innervate the larval epidermis and 4 different classes exist based on the complexity of their dendritic arbors. Similarly to *nos* regulation in the embryo, point mutations of *nos* SREs are sufficient to disrupt *nos* dendritic localization in the PNS (Brechbiel and Gavis 2008). Particularly, unlocalized *nos* mRNA leads to a decrease in dendritic branching complexity of class IV da neurons. Thus, localization and translational repression of unlocalized *nos* mRNA is required for higher order dendritic arborization. Interestingly, *smg* mutant larvae show decreased branching complexity as well, suggesting that Nos protein synthesis is spatially regulated and under control of Smaug in larval peripheral neurons.

1.2 Properties and functions of mammalian Smaug

Two Smaug homologous genes are present in mammals, Smaug1 (SAM4A) and Smaug2 (SAM4B) (Baez and Boccaccio 2005). Smaug 1 is located in human and mouse chromosome 14 while Smaug2 is located in human chromosome 19 and mouse chromosome 7.

Smaug1 is mostly expressed in neuronal dendrites where it seems to control synapsis morphogenesis and function, through regulation of specific mRNAs (Baez and Boccaccio 2005, Baez, Luchelli et al. 2011). Smaug2 has been less studied, although it is known to be widely expressed in neural embryonic and adult tissues (Luo, Li et al. 2010) and recent studies have linked it to neurogenesis regulation (Amadei, Zander et al. 2015). Note that only Smaug2 is expressed during embryonic cortical neurogenesis while Smaug1 is expressed later in hippocampal neuron development, suggesting that both proteins are relevant at different stages during neuron differentiation and maturation.

1.2.1 The translational repressor Smaug1 is involved in many different biological processes

Subcellular localization studies by immunolabeling and confocal microscopy, showed that Smaug1 forms neuron specific cytoplasmic foci of 0,5-2 μm that are distinct to P-bodies. Also, when *Drosophila* Smaug was coexpressed with mammalian Smaug1, both proteins were found colocalizing in the cytoplasmic granules (Baez and Boccaccio 2005), suggesting the ability to form granules is conserved. These granules, called S-foci hereafter, form 20S particles containing polyadenylated RNA, as deduced by the presence of PABP, as well as some stress granules markers such as Staufen and TIA-1 (Baez and Boccaccio 2005). Treatment with translation inhibitors, like cyclohexymide and puromycin, proved that Smaug1 foci are dynamic granules in equilibrium with polysomes (Fig. 17) (Baez and Boccaccio 2005).

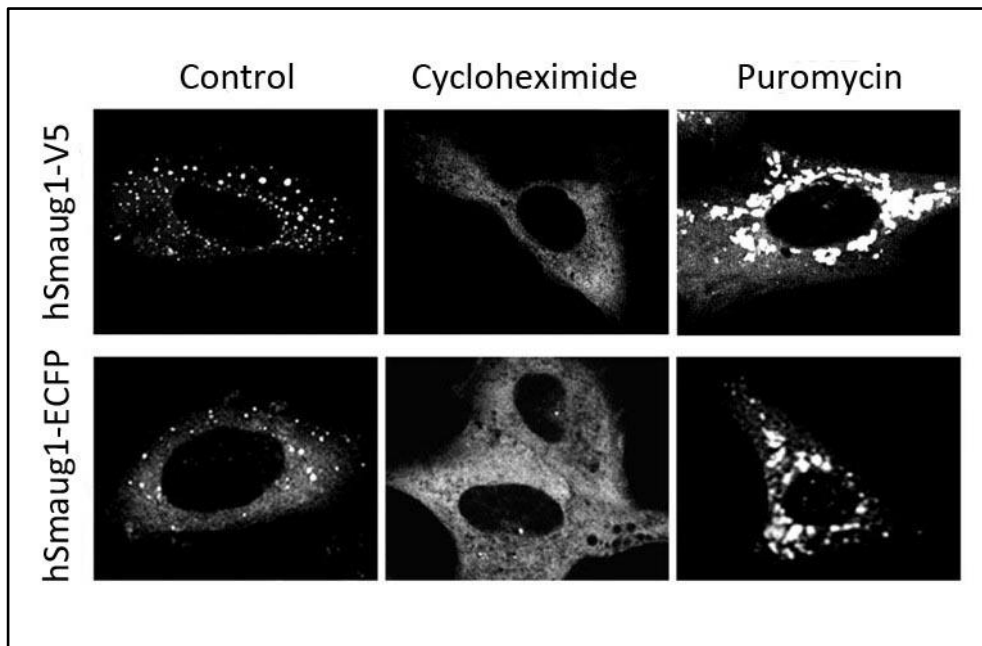


Figure 17. Smaug1 foci are dynamic structures in equilibrium with translating polysomes. Mammalian fibroblast cells transiently expressing tagged forms of human Smaug1 (hSmaug1-V5 or hSmaug1-ECFP) for 8h were exposed to 0,25mg/ml cycloheximide or 0,25 mg/ml puromycin. Smaug1 granules dissolve after 2h of treatment with the polysome stabilizing drug cycloheximide. On the contrary, Smaug1 granules formation is enhanced when cells are treated with puromycin, a translation initiation inhibitor. Non-treated cells are shown as control. Figure adapted from (Baez and Boccaccio 2005)

In order to determine whether Smaug1 functions as a translational repressor like its *Drosophila* homologue, a luciferase reporter assay was performed showing that Smaug1 represses translation of SRE-containing mRNAs in fibroblast cells (Baez and Boccaccio 2005). However, no changes in the reporter mRNA stability were observed by real time PCR, suggesting that Smaug1 does not induce degradation of this reporter in these cells. The mechanisms by which mammalian Smaug1 represses mRNA translation are yet unknown and the possibility that Smaug1 promotes mRNA decay in a different cell context remains to be explored (Baez and Boccaccio 2005).

Another study showed that Smaug1 forms mRNA-silencing foci at post-synapses of hippocampal neurons that dissolve upon stimulation with glutamate receptor agonist N-methyl-D-aspartic (NMDA). S-foci dissolution leads to localized translation of specific silenced mRNAs such as the *CamKII α* (*calmodulin kinase II α*) mRNA (Baez, Luchelli et al.

2011). Furthermore, Smaug seems to respond specifically to this activation which paradoxically induces a global arrest of translation (Baez, Luchelli et al. 2011, Pascual, Luchelli et al. 2012). Metabotropic glutamate receptor (mGluR) activation also induces transient S-foci dissolution and subsequent translation of the sequestered mRNAs. Knockdown experiments by siRNA against Smaug1 resulted in the formation of smaller and more numerous synapses provoking a defective response to stimuli (Baez, Luchelli et al. 2011).

In addition to its role during synaptogenesis, Smaug1 was recently reported to be a key regulator of osteoblastogenesis and bone development by repressing translation of *mig6* (mitogen-inducible gene 6) mRNA in mice (Niu, Xiang et al. 2017). Furthermore, Smaug1 has a positive function in myoblasts since it is able to restore impaired CUG binding protein 1 (CUGBP1) translational functions and suppress CUG-induced miopathy (de Haro, Al-Ramahi et al. 2013). Studies using a *Drosophila* and mouse model that recapitulates a genetic condition characterized by muscle weakness showed that Smaug1 is involved in mitochondrial dysfunction in the muscle via promotion of mRNA decay (Chartier, Klein et al. 2015). Finally, a missense recessive mutation of Smaug1, designated ‘supermodel’, induces metabolic disorders of homozygous mice resulting in exceptionally thin, sterile individuals that live shorter lives (Chen, Holland et al. 2014). This phenotype is related to a malfunction in glucose metabolism. *Supermodel* mice produce little insulin but are extremely sensitive to it, leading to an excessive use of energy resources. Biochemical analysis of mutant mice showed that mTORC1 signaling pathway was also affected, due to decreased phosphorylation of two mTORC1 complex targets implicated in the control of mRNA translation. These results suggest that Smaug1 plays a role in the control bone development and muscle metabolism via inhibition of translation.

1.2.2 Smaug2 regulates *nos1* mRNA during neuronal differentiation

A recent study has shown that, similarly to *nos* mRNA regulation in the *Drosophila* embryo, Smaug2 is involved in the regulation of *nos1* transcript in murine precursor neurons (Amadei, Zander et al. 2015). *Nos1* is an activator of differentiation and its translation is negatively regulated by Smaug2.

2. Smaug regulates mRNA through diverse mechanisms

Smaug can either block translational repression and/or promote poly(A) tail deadenylation of hundreds of target mRNAs by forming multiple complexes through interaction with different protein factors in *Drosophila* embryo (Nelson, Leidal et al. 2004, Semotok, Cooperstock et al. 2005, Tadros, Goldman et al. 2007, Pinder and Smibert 2013). In this section, I will describe Smaug regulatory mechanisms by focusing on the two best characterized Smaug targets known to date: *nos* and *hsp8* mRNAs.

2.1 Smaug represses *nos* mRNA translation via specific binding to SRE

A series of point mutations demonstrated that Smaug recognizes specifically two stem-loop structures with CUGGN loop sequences (N being any kind of base) in *nos* mRNA 3'UTR (Smibert, Wilson et al. 1996, Dahanukar, Walker et al. 1999, Smibert, Lie et al. 1999). These SREs are required and sufficient to induce Smaug-mediated translational repression (Fig. 18) (Smibert, Wilson et al. 1996).

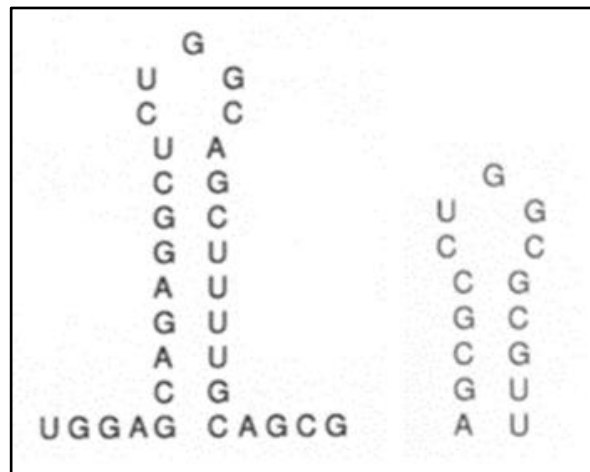


Figure 18. Smaug binds *nos* mRNA via recognition of a stem-loop structure called SRE in the 3' UTR

2.1.1 Smaug forms a stable complex with the eIF4E binding protein Cup

A GST pull-down of a form of Smaug containing the RNA binding domain (Smaug⁵⁸³⁻⁷⁶³) coupled with mass spectrometry analysis led to the identification of the protein Cup as a direct interactor of Smaug (Nelson, Leidal et al. 2004). More *in vitro* assays showed that Cup mediates an indirect interaction between Smaug and the cap binding protein eIF4E, which

was later confirmed *in vivo* by co-immunoprecipitation experiments using extracts from embryos collected 0-3h post egg-laying. In addition, genetic analysis showed that Smaug ability to repress *nos* translation requires Cup. Thus, Cup would prevent eIF4E interaction with eIF4G and consequently block formation of the mRNA closed loop structure required for initiation of *nos* mRNA translation. More recent work has shown that the slow step is forming the repressor complex. Once assembled, the repressed mRNA/protein complex is very stable (Jeske, Moritz et al. 2011).

As mentioned above, Osk expression at the posterior pole is sufficient for activation of *nos* mRNA translation (Zaessinger, Busseau et al. 2006). It should be noted that both Osk and Cup proteins interact with the same region of Smaug that contains the RNA binding domain (Smaug⁵⁸⁴⁻⁸⁵⁹ and Smaug⁵⁸³⁻⁷⁶³ respectively). We can thus hypothesize that the recruitment of Cup by Smaug is blocked by competition with Osk, which would allow the correct interaction between eIF4E and eIF4G and the subsequent translation of *nos* mRNA.

Importantly, the regulatory mechanisms seem to be conserved in mammals since Smaug2 binds to the *nos1* transcript, probably via its SRE, and recruits a eIF4E binding protein blocking translation initiation (Amadei, Zander et al. 2015).

2.1.2 Smaug recruits Ago1 in a microRNA independent manner

Argonaute proteins are known to target mRNAs for translational repression and transcript decay through association with small RNAs such as microRNA (miRNA). Biochemical and genetic analysis showed that Smaug interacts with Ago1 which is necessary for unlocalized Smaug-mediated translational repression of *nos* mRNA (Pinder and Smibert 2013). Furthermore, Ago1 immunoprecipitation from early embryo extracts, coupled with RT-qPCR, proved that Ago1 binds *nos* mRNA in a Smaug dependent manner. Surprisingly, it appears that Ago1 recruitment by Smaug to *nos* transcript occurs in the absence of a targeting miRNA (Pinder and Smibert 2013). Furthermore, recruitment of Ago1 does not affect *nos* mRNA stability which suggests that Smaug can promote mRNA decay and inhibits translation by forming functionally distinct protein complexes.

2.2 Smaug promotes mRNA poly(A) tail deadenylation and decay

2.2.1 Smaug recruits the CCR4-NOT deadenylase complex

In addition to its ability to repress translation, Smaug can also recruit the CCR4-Not deadenylase complex which leads to the deadenylation and decay of hundreds of target mRNAs during early embryogenesis (Semotok, Cooperstock et al. 2005).

For instance, Smaug recognizes eight SREs located in the open reading frame (ORF) in *hsp83* transcripts which leads to unlocalized mRNA destabilization (Semotok, Luo et al. 2008). In embryos from female *smaug* mutants, unlocalized maternal *hsp83* transcripts fail to be destabilized by poly(A) tail shortening and remain uniformly distributed in the bulk cytoplasm of the embryo. Interestingly, Smaug does not repress *hsp83* mRNA translation which suggests that Smaug could use different regulatory mechanism according to the target transcript (Semotok, Cooperstock et al. 2005).

Finally, it has been proposed that Smaug-mediated deadenylation and degradation involves the RNA binding protein Aubergine (Aub), a piwi-type Ago (Rouget, Papin et al. 2010). Subcellular localization studies showed that Smaug and CCR4 partially colocalize in the early embryo and this cytoplasmic distribution is strongly affected in *aub* mutant embryos (Rouget, Papin et al. 2010).

To conclude, Smaug regulates its target transcripts through diverse molecular mechanisms involving the formation of functionally distinct protein complexes. Importantly, Smaug ability to repress translation as well as to induce mRNA decay seems to be conserved in the mammalian homologues (Amadei, Zander et al. 2015, Chartier, Klein et al. 2015, Niu, Xiang et al. 2017).

3. Smaug protein sequence and structure

In *Drosophila*, there is one *smaug* gene (CG5263) which is located on the left arm of chromosome 3 at locus 66F1 and is expressed in five different isoforms (A to E) (Fig. 19). *smaug* isoform A encodes a 999 amino acid (aa) protein, with a predicted molecular weight of 109 kDa (Smibert, Lie et al. 1999).

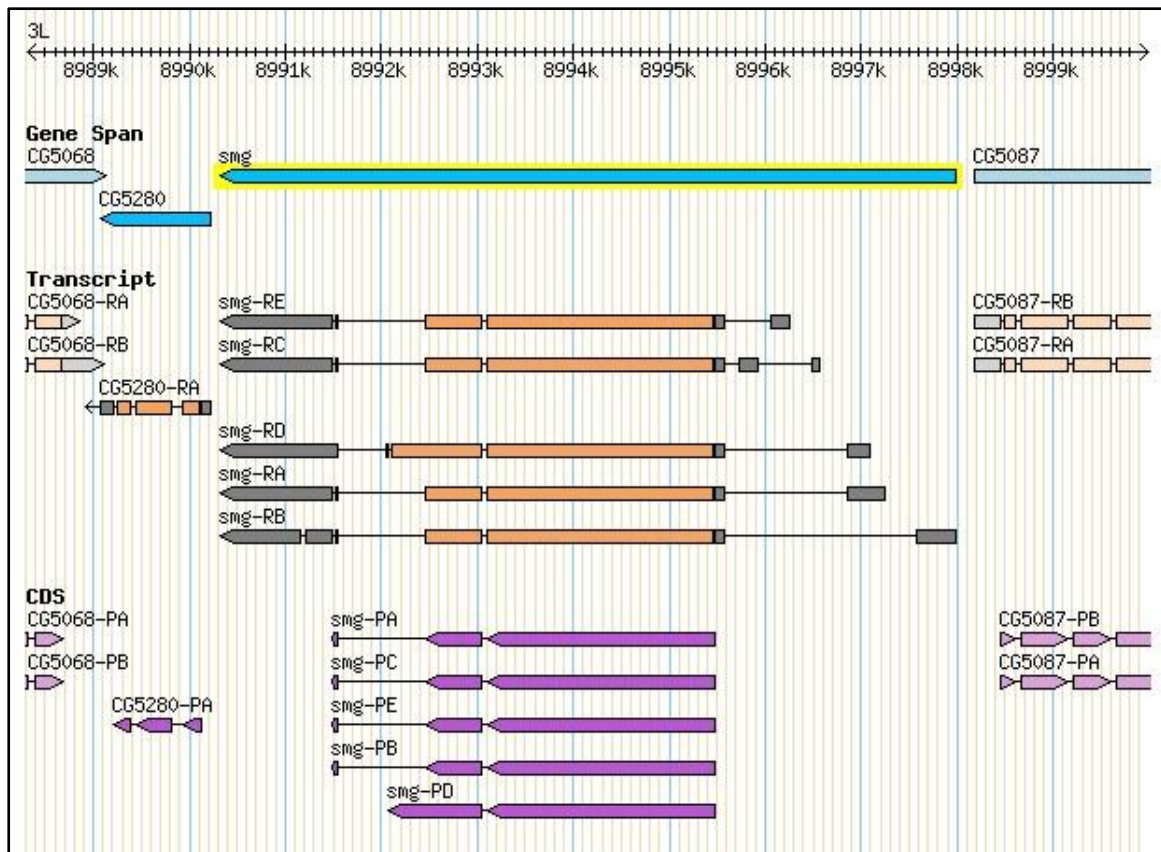


Figure 19. *Drosophila Smaug* is expressed in five isoforms (A to E)

The genomic location of *smaug* gene is shown. Five *smaug* isoforms give rise to two distinct *Smau* proteins. Figure taken from flybase (<http://flybase.org/reports/FBgn0016070>)

Smaug binds its target mRNAs via a Sterile Alpha Motif (SAM) domain, as previously introduced in chapter II, which is conserved from yeast to humans (Fig. 20). *Drosophila Smaug* also contains a pseudo-HEAT repeat analogous topology (PHAT) domain which is not necessary for RNA recognition but it seems to play a role in high affinity mRNA binding (Green, Edwards et al. 2002, Green, Gardner et al. 2003). In *Drosophila* and mammalian *Smaug* homologues, two conserved *Smaug* Similarity Regions (SSR1, SSR2) are found

(Smibert, Lie et al. 1999, Aviv, Lin et al. 2003, Green, Gardner et al. 2003). However, the functional significance of SSR1 and SSR2 remains unknown.

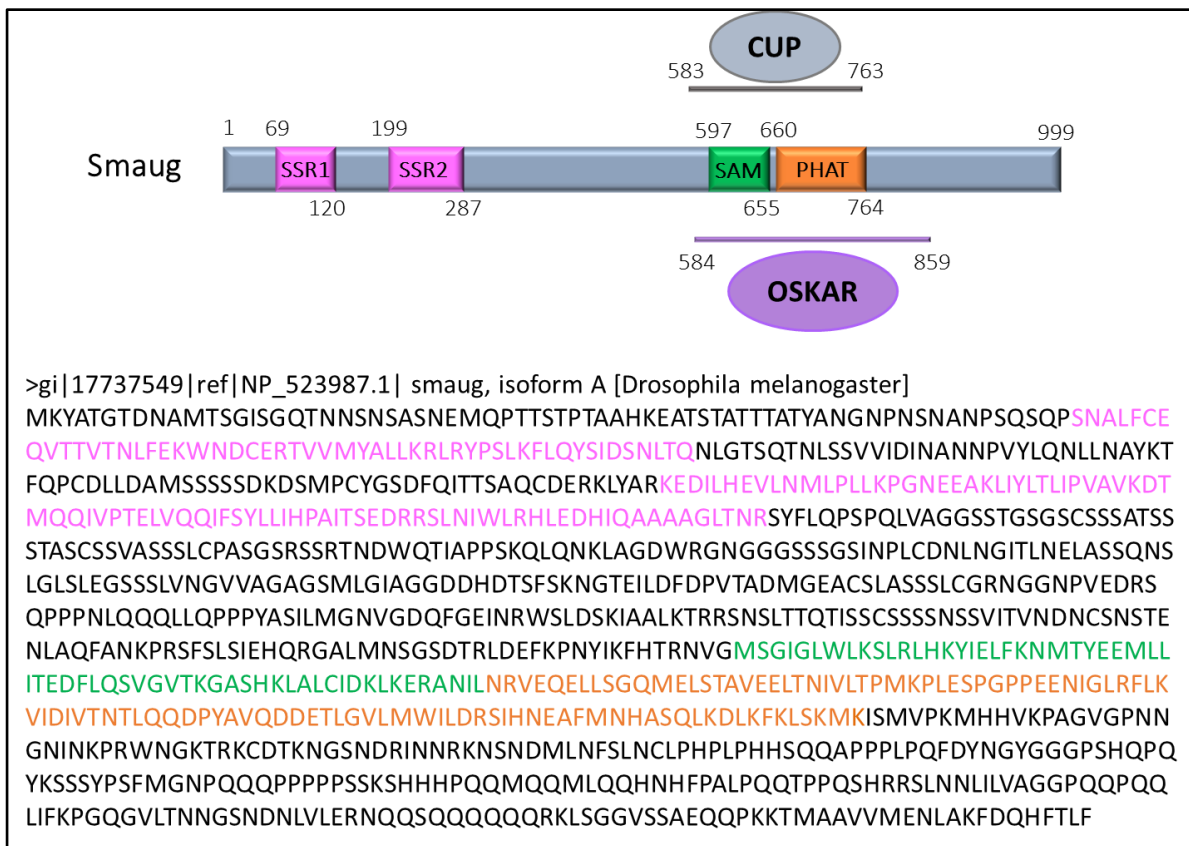


Figure 20. Smaug protein structure and sequence in Drosophila.

Representation of Drosophila Smaug isoform A which contains 999 amino acids. The conserved domains are represented by the colored rectangles that correspond to the color of the sequence presented. Regions of interaction with Oskar and the eIF4E-binding protein CUP are shown. SSR1: 69-120; SSR2: 199-287; SAM: 597-655; PHAT: 660-764.

3.1 Smaug RNA binding structure

Crystallization analysis of Smaug allowed characterizing the SAM domain as a new motif for RNA recognition. The SAM domain is approximately 65 aa long and is organized into 5 α -helices that recognize specific stem-loop RNA structures (SREs) and constitutes a hydrophobic core. In addition, it is abundant in basic residues (lysine and arginine) which convey an electropositive potential that is crucial for the RNA interaction to happen (Aviv, Lin et al. 2003, Green, Gardner et al. 2003, Aviv, Lin et al. 2006).

Connected to the electropositive SAM domain is the PHAT domain, which is remarkably electronegative and also forms α -helices (Fig. 21) (Green, Gardner et al. 2003). Using a fluorescence-polarization assay, which allows to measure changes in mRNA binding, a putative RNA binding surface was proposed for regions containing the C terminus of helix α_1 , the connecting fragment between helix α_1 and α_2 and the N terminus of helix α_5 (Aviv, Lin et al. 2003).

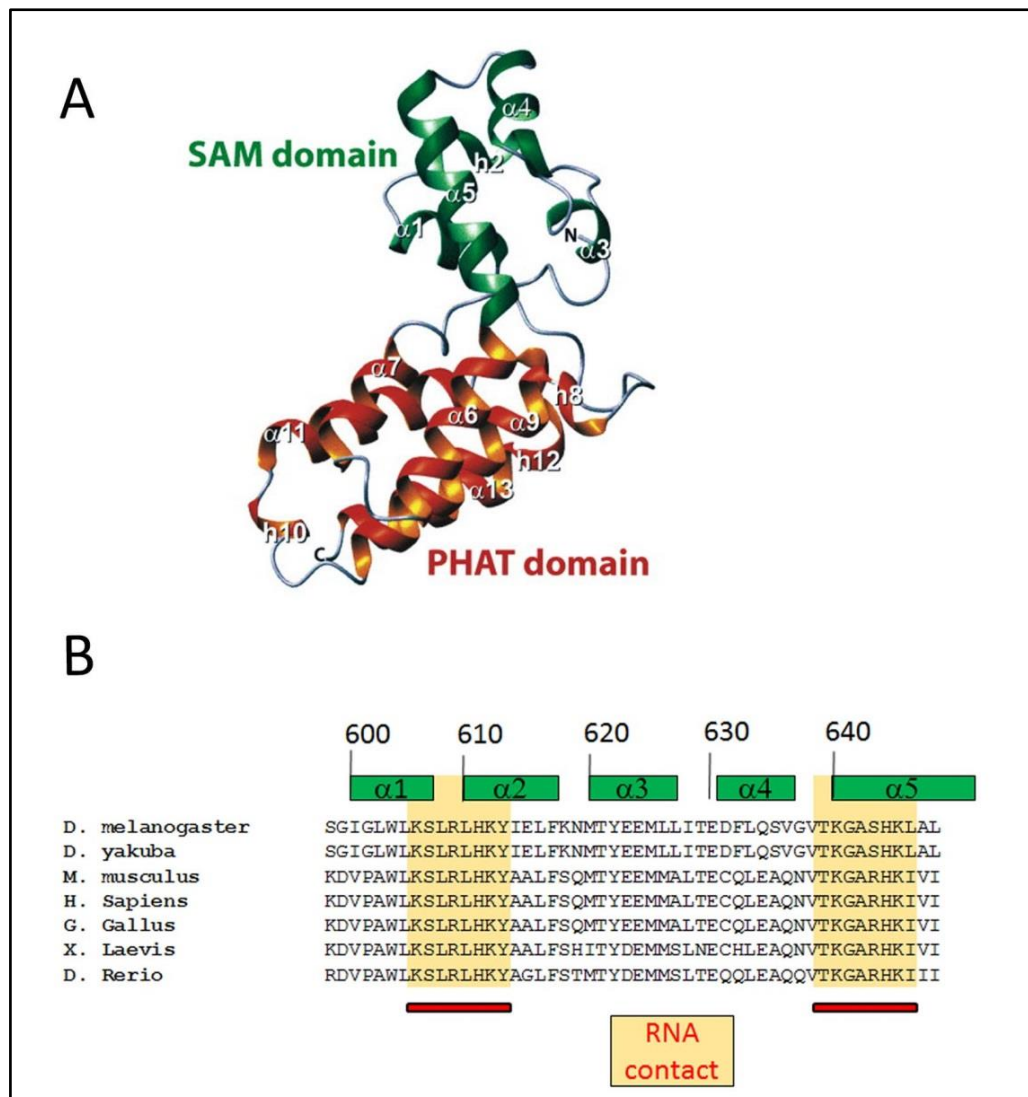


Figure 21. Smaug RNA binding domain structure and SAM domain homologues.

(A) Smg RBD contains a SAM domain (green) connected to a PHAT domain (orange). Both domains consist predominantly of α helices. (B) Sequence alignment of SAM domain of Smaug homologues. Location of the different α -helices is shown above the sequence. Red bars and sequences highlighted in yellow indicate the RNA binding contact regions. The stars indicate conserved amino acids. Figure adapted from (Aviv, Lin et al. 2003)

3.2 Smaug contains intrinsically disordered regions

In silico protein disorder predictive analysis allowed to identify putative Smaug IDRs (Fig. 22). In particular, the last 250 amino acids do not present a defined structural motif and represent a low complexity region that is highly rich in glutamine (Q). As previously mentioned, these poly-Q regions promote protein aggregation which is associated with neurodegenerative diseases. However, to date, the molecular mechanisms of S-foci assembly remain unknown.

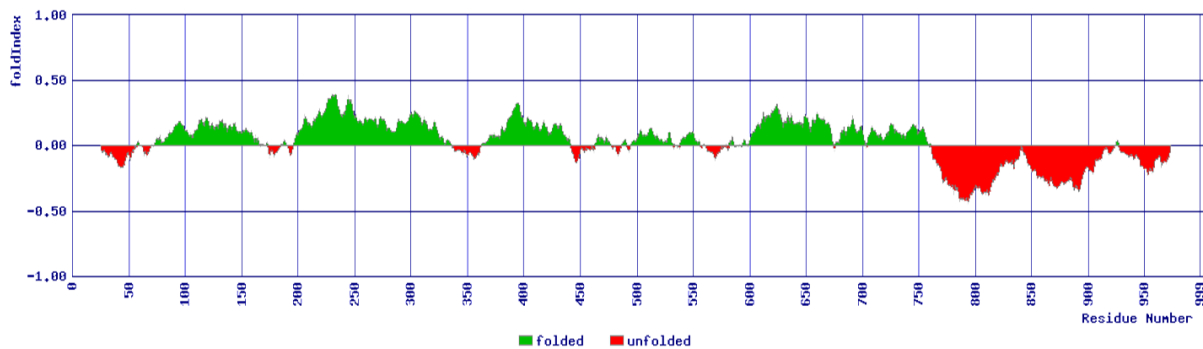


Figure 22. Protein disorder prediction of *Drosophila Smaug*.

Using a protein disorder prediction software, several putative IDRs (shown in red) were identified in Smaug protein. (<https://bip.weizmann.ac.il/>)

In summary, Smaug plays a particularly relevant role during *Drosophila* MZT in the embryo, as well as in tissue development in both *Drosophila* and mammals. Smaug is a multifunctional RNA regulator that binds specific sequences in target mRNAs via its SAM domain. SRE recognition leads to mRNA translational repression and/or destabilization and decay. Finally, despite the advances made on understanding Smaug multiple functions, further studies are necessary to better comprehend Smaug mechanisms of action and identify its associated protein partners.

Chapter V: The Hedgehog signaling pathway

To finish, I will briefly introduce the Hedgehog (HH) signaling pathway, as its effect on Smaug is at the heart of this thesis project. The HH pathway was first discovered in a genetic screen which goal was to identify essential genes for embryonic development in *Drosophila*. This pathway was christened with the name ‘Hedgehog’ due to the ‘spiked’ cuticle phenotype observed in *hh* mutant embryos, resembling the spines of a hedgehog. Since then, the HH pathway has been extensively studied and today it is known to be involved in multiple biological processes such as cell proliferation, migration, differentiation as well as specification of cell fate and tissue polarity. Hence, HH is a major morphogen during development of embryonic and adult structures in metazoans, which is why its deregulation is the underlying cause of diverse developmental disorders and cancer in humans (Briscoe and Therond 2013).

1. Roles of the HH pathway during *Drosophila* development

1.1 HH establishes segmental polarity in the embryo

The HH pathway was first described to play a key role in the formation of AP polarity of each of the fourteen body segments in the developing *Drosophila* embryo. Indeed, genetic studies have revealed that the AP patterning of each segment involves reciprocal cell-cell communications that relies on a positive feedback loop between HH and Wingless (WG) producing cells (Martinez Arias, Baker et al. 1988, Ingham 1993). HH signaling is also involved in the development of different fly appendages and organs such as the wing, which I will describe next.

1.2 HH regulates wing growth and patterning

The larval wing imaginal disc (WID) is the epithelial structure that will give rise to the adult wing (Fig. 23 B). In this structure, downregulation of HH production or transduction are easily spotted by analyzing morphogenesis of the wing, which makes the WID a widely used model system to study HH signaling.

During the WID development HH act as a morphogen which is absolutely required for the WID growth since without HH there is no wing. HH is secreted by cells at the posterior region of the WID and diffuses towards the anterior compartment forming a concentration gradient that will induce transcription of its target genes in a dose-dependent manner in the neighboring cells (Fig. 23 A).

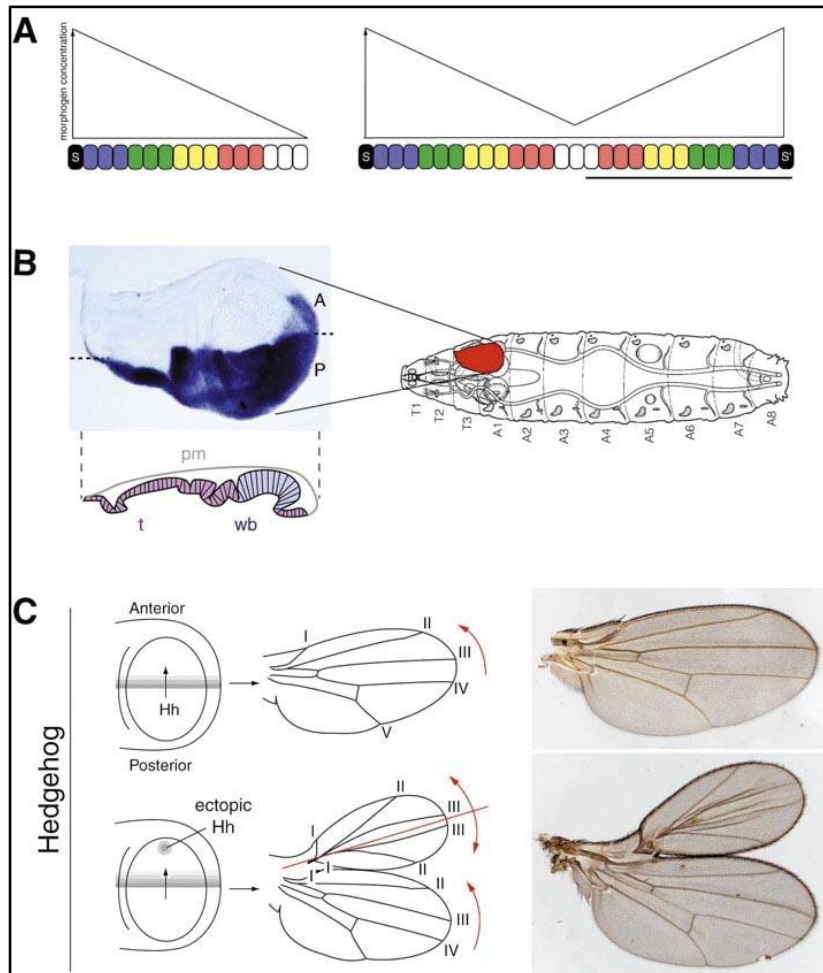


Figure 23. The Hedgehog morphogen controls wing development in *Drosophila*.

(A) A model for morphogen signaling: a morphogen is expressed in cell (S) and sets the positional identity of a cell by forming a concentration gradient across a field of receiving cells (left). Deregulations due to an ectopic source (S') of morphogen can induce mirror image duplication (right). (B) The wing imaginal disc of third instar *Drosophila* larva (red) is compartmentalized into anterior (A) and posterior (P) compartments along the AP axis. It is a two-sided sac containing a columnar cell layer that comprise the wing blade (wb) and thorax (t) regions, and an overlying squamous peripodial membrane (pm). *hh* mRNA is visualized by *in situ* hybridization in the posterior compartment where it is expressed (left). (C) In physiological conditions, Hh is produced in the posterior compartment and diffuses into the anterior one (top). Ectopic expression of *hh* (ectopic Hh) induces a mirror image duplication of the anterior wing structure (bottom). Wing veins I to V are indicated. Figure adapted from (Tabata and Takei 2004).

The HH pathway specifically defines the space between veins 3 and 4 of the adult wing. It has been shown that the ectopic expression of HH in the anterior region of the WID induces a mirror image duplication of the wing due to the formation of a second gradient, demonstrating its morphogenetic effect (Fig. 23 C) (Tabata and Takei 2004).

2. Mechanisms of the HH signal transduction in the wing imaginal disc

2.1 HH activates gene expression via the transcription factor Cubitus Interruptus

Most of the proteins involved in the HH signal transduction have been first identified in *Drosophila*. The HH pathway regulates and act through the zinc finger transcription factor Cubitus Interruptus (CI) of the Gli family (Aza-Blanc and Kornberg 1999). In the absence of HH, CI is partially degraded which leads to a truncated repressor form (CI-R). Higher HH dose at the AP boundary inhibits CI proteolysis leading to the accumulation of full length CI (CI-FL). Hence, in the presence of low levels of HH, CI processing is prevented, which promotes the transcription of ‘low level’ target genes such as *decapentaplegic (dpp)* and *iroquois (iro)*. In the presence of intermediate levels of HH, CI-FL levels are augmented, leading to the expression of *patched (ptc)* and *collier (col)* genes. Finally, in the anterior cells that are adjacent to the posterior compartment, the HH dose is maximal and CI-FL becomes hyperactive (CI-A) leading to the anterior expression of the ‘high level’ HH target *engrailed (en)*.

2.2 The HH signal is transduced via activation of the G protein-coupled receptor Smoothened

In HH responding cells, reception of the HH signal is mediated by the twelve transmembrane domains protein Patched (PTC) and its two co-receptors named Interference of Hedgehog (IHOG) and Brother Of IHOG (BOI) (Zheng, Mann et al. 2010).

In the absence of HH, PTC antagonizes the G protein-coupled receptor (GPCR) Smoothened (SMO) leading to SMO internalization and subsequent degradation (Fig. 24). This inhibition is alleviated upon reception of the HH signal leading to endocytic internalization of PTC, which is destined for degradation.

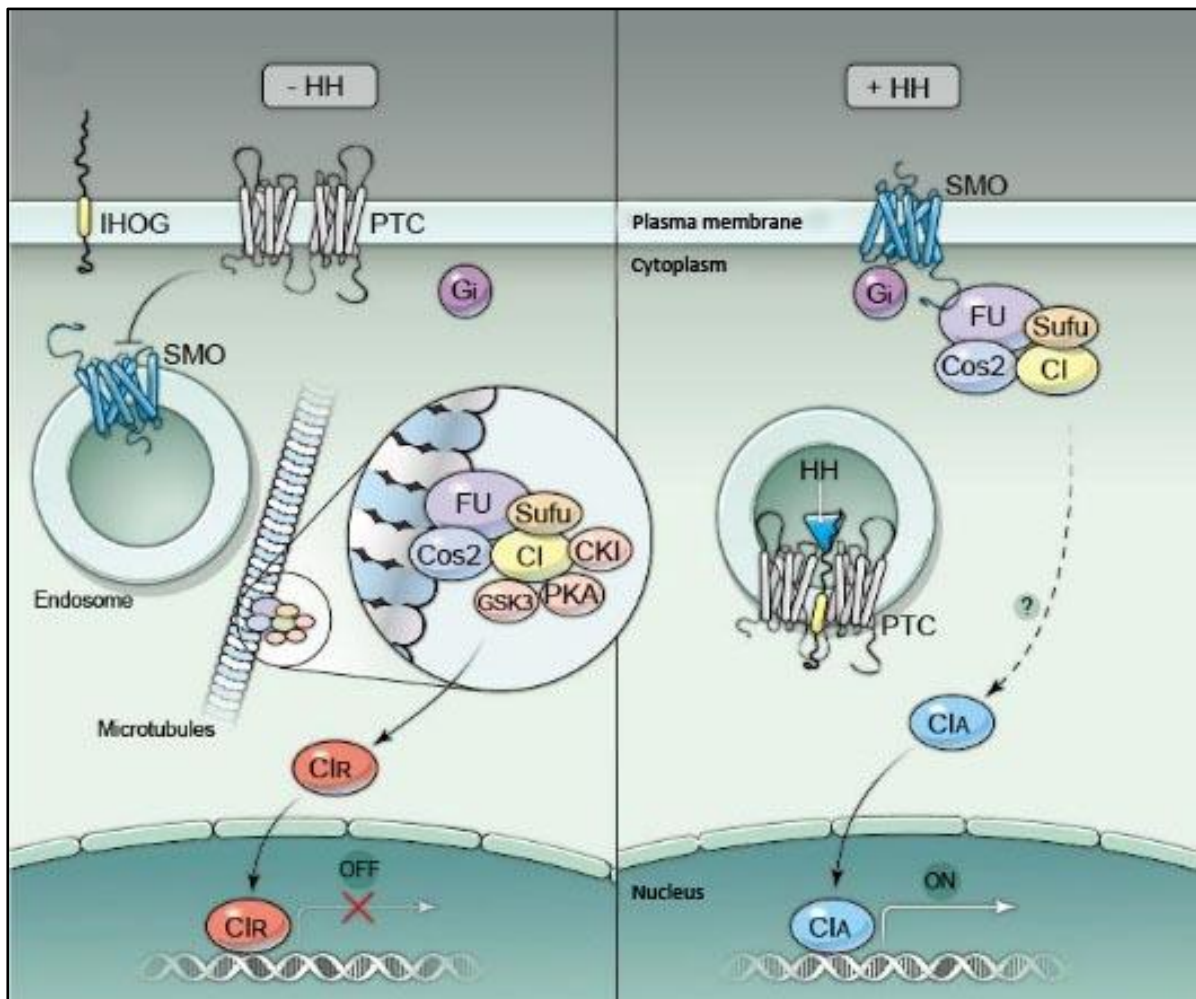


Figure 24. Model of the Hedgehog signaling pathway in *Drosophila*.

In the absence of HH (left), PTC receptor inhibits the activity of SMO and promotes its internalization. The transcription factor CI is linked to a complex formed by kinesin COS2, the kinase Fused (FU) and three other kinases: protein kinase A (PKA), CK1 (casein kinase 1) and the GSK3 (Glycogen Synthase Kinase 3). Phosphorylation of CI leads to its partial degradation and the formation of the repressive form CI-R which then translocates to the nucleus and inhibits the expression of HH target genes.

Upon HH reception (right) by PTC and its co-receptors, IHOG (Interference Hedgehog) and BOI (Brother of IHOG, not shown), the antagonist effect on SMO is released, allowing the intracellular signal transduction to occur. PTC receptor undergoes internalization and degradation and CI cleavage is blocked, thus leading to CI activation (CI-A) and promotion of HH target genes in the nucleus. Figure adapted from (Robbins, Fei et al. 2012).

On the contrary, when SMO inhibition by PTC is released upon HH reception, SMO relocates from internal cytoplasmic vesicles to the plasma membrane (Zhu, Zheng et al. 2003). Importantly, a critical issue is how PTC inhibits SMO in absence of HH. Recent studies on HH signaling in vertebrates showed that a transmembrane flux of sodium ions controls the activity of the mammalian homologue PTCH1 (Myers, Neahring et al. 2017) and that cholesterol regulates SMO via its transmembrane domain (Huang, Nedelcu et al. 2016), but the link between these two effects remains to be understood.

SMO activation is required for signal transduction by the HH transduction complex (HTC) which contains many different protein factors such as the kinesin Costal 2 (COS2) which is involved in HTC transport along microtubules, the kinase Fused (FU), the protein Suppressor of Fused (SUFU), Protein kinase A (PKA), casein kinase 1 (CK1), glycogen synthase kinase 3 (GSK3) and CI (Robbins, Fei et al. 2012).

In the absence of HH, kinases PKA, CK1 and GSK3 phosphorylate CI at its C-terminal region, which induces CI ubiquitination and subsequent partial degradation by the proteasome machinery, leading to the truncated transcriptional repressor CI-R (Robbins, Fei et al. 2012).

3. The activity of the signal transducer Smoothed is regulated by PTMs

SMO is a seven transmembrane domains protein belonging to the heterotrimeric G protein-coupled receptors family. Like many GPCRs, SMO contains a Cysteine Rich Domain (CRD) at its extracellular amino-terminal (N-terminal) region necessary for pathway activation (Nakano, Nystedt et al. 2004).

SMO is found to localize in vesicles that are positive for endosomal markers such as Rab5, Dynamin and Rab7. Analysis of *dynammin* mutants showed that SMO can accumulate at the plasma membrane even in the absence of HH (Zhu, Zheng et al. 2003). However, the underlying mechanisms of how SMO is trafficked within the cell are poorly understood (Li, Chen et al. 2012). SMO subcellular localization seems to be tightly connected to its activation state. Numerous studies have shown that SMO undergoes various post-translational modifications as well as conformational changes in response to HH signal reception (Jia, Tong et al. 2004, Li, Chen et al. 2012, Sanial, Becam et al. 2017).

3.1 SMO is downregulated by ubiquitin-mediated endocytosis and degradation

Multiple studies suggest that, in the absence of HH signal, SMO is ubiquitinated on various lysine residues in its cytoplasmic region leading to SMO vesicular internalization and degradation by the lysosome pathway (Zhu, Zheng et al. 2003, Li, Chen et al. 2012, Xia, Jia et al. 2012).

3.2 Phosphorylation induces SMO conformational switch and stabilizes the protein at the plasma membrane

In the intracellular region SMO also contains a SMO auto-inhibitory domain (SAID) which comprises several regulatory modules that restrict SMO concentration at the plasma membrane and inhibit its activity. In absence of HH, several positively charged arginine residues in SMO SAID interact with the most carboxyl-terminal (C-terminal) end that is negatively charged promoting SMO closed conformation (Fig. 25 A).

SMO SAID contains three clusters of serines (S) and threonines (T) phosphorylated by PKA and CKI. Reception of HH signal inhibits SMO ubiquitination by inducing phosphorylation of its intracellular tail by PKA and CKI protein kinases. FRET (fluorescence resonance energy transfer) analysis in cultured cells and *in vivo* indicate that phosphorylation changes SMO C-terminal to an open conformation promoting its dimerization which leads to a stabilized SMO protein at the cell surface, even without HH activation (Fig. 25 B) (Jia, Tong et al. 2004, Zhang, Williams et al. 2004, Zhao, Tong et al. 2007)

Experiments of phosphomimetic mutations of PKA and CKI clusters in *Drosophila* led to a constitutive activation of the pathway. However, these phosphomimetic SMO variants did not possess full pathway activity and were still stimulated by HH. These results suggest that full activation of SMO by HH involves additional phosphorylation events (Zhang, Williams et al. 2004). Indeed, several other kinases including GPRK2 (G protein-coupled receptor kinase 2), CKII (casein kinase II), aPKC (atypical protein kinase C), and Gish (Gilgamesh) have been shown to phosphorylate SMO C-terminal region (Cheng, Maier et al. 2010, Jia, Liu et al. 2010, Jiang, Liu et al. 2014, Li, Li et al. 2016). Our team has recently identified four phosphorylation

clusters of kinase FU in SMO C-terminal as necessary for its full activation (Sanial, Becam et al. 2017)

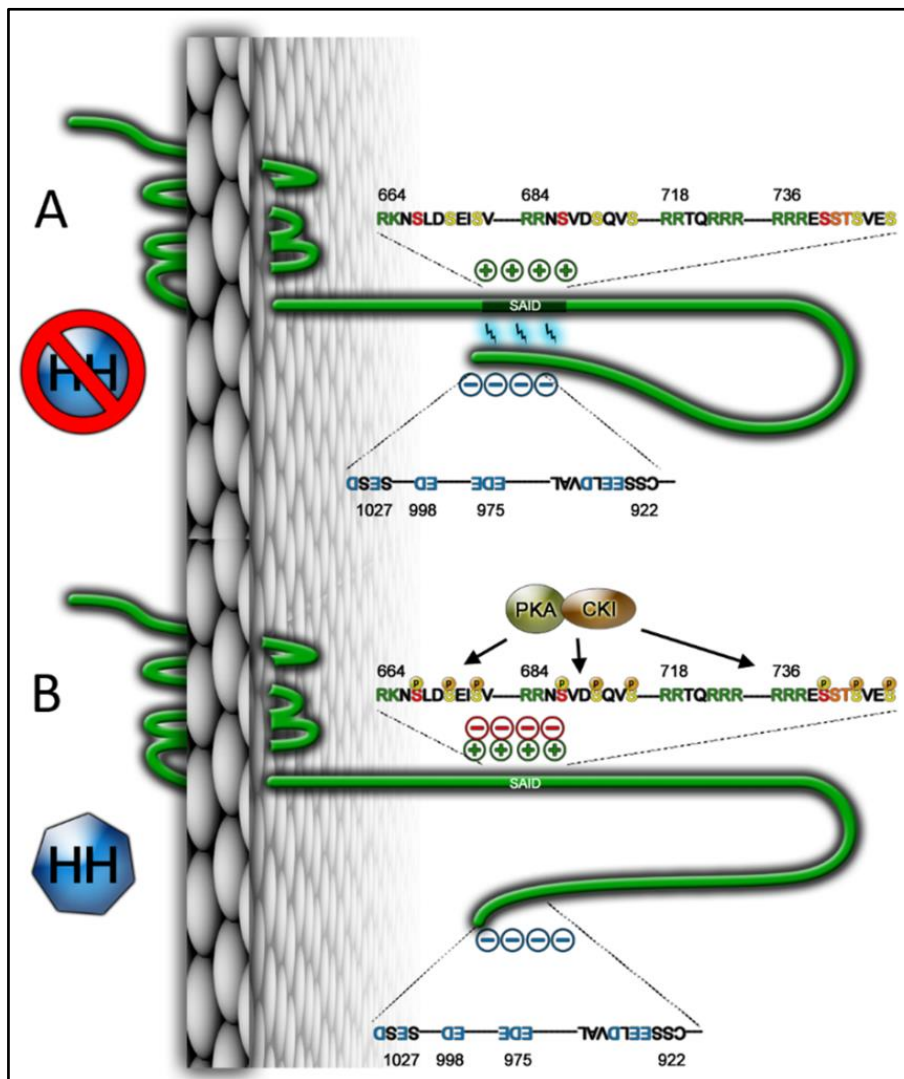


Figure 25. SMO phosphorylation induces its conformational switch.

(A) In the absence of HH signal, the SAID domain that contains the phosphorylation sites for kinases PKA (red), CKI (yellow), GPRK2 (orange) and positively charged residues (green) can form electrostatic bonds with the negatively charged residues (blue) in the C-terminal region leading to SMO closed conformation. (B) In the presence of HH signal, phosphorylation by kinases PKA/CKI provides negative charges which neutralize the positive charges of arginines. The electrostatic bonds between the two domains disappear allowing the protein to switch to an open conformation. Adapted from Matthieu Sanial from (Zhao, Tong et al. 2007).

Finally, recent findings showed that sumoylation might act in parallel with phosphorylation blocking SMO ubiquitination and degradation, thus promoting its localization at the plasma membrane and activation (Ma, Li et al. 2016).

4. The HH pathway is conserved in mammals

The HH pathway is conserved in mammals where it is involved in the development of multiple processes, as for example, in vasculogenesis, tissue repair, development of the neural tube as well as tissue homeostasis and the polarization of the distal limbs (Briscoe and Therond 2013). Multiple orthologues of members of the HH pathway have been identified, including HH, SMO, PTC, COS2, SUFU and CI. Three *hh* orthologues exist in vertebrates: Sonic hedgehog (Shh), Desert hedgehog (Dhh), and Indian hedgehog (Ihh); being each of them implicated in different developmental processes (Pereira, Johnson et al. 2014). Two *ptc* orthologues are known (*patched1* and *patched2*) and three CI orthologues have been identified (*gli1*, *gli2*, *gli3*).

Despite conservation of many components of the HH pathway, HH signaling in mammals differs from *Drosophila*'s in several aspects. One of the most striking is the dependence in mammals on the presence of a structure that is absent in the fly: the primary cilium. The primary cilium is a microtubule-based antenna like structure that is present in the surface of nearly all mammalian cells with the exception of red blood cells.

Similarly to what happens in *Drosophila*, phosphorylated mammalian SMO relocates to the plasma membrane of the primary cilium (Robbins, Fei et al. 2012). Remarkably, in spite of the low sequence homology between *Drosophila* and mammalian SMO, the presence of self-interacting domains as well as the phosphoregulatory motifs regulating SMO conformational switch are conserved. However, the role of and FU, which is a critical member of the pathway in fly, does not seem to be conserved suggesting that the mechanisms of HH signal transduction from the receptor to the CI/GLI transcription factors have evolved differentially (Varjosalo and Taipale 2008).

Finally, it is worth mentioning that in the past decade, numerous studies have identified 'non-canonical' HH signaling pathways which function independently of transcriptional changes mediated by CI. Non-canonical HH pathways can be classified in two distinct types: those independent of Ci and those not requiring SMO. Hence, HH signaling is now proposed to act via a variety of different context-dependent mechanisms making the HH pathway a highly dynamic network (Robbins, Fei et al. 2012).

THESIS RATIONALE

I. General context

Our team is interested in the transduction of the Hedgehog signal in *Drosophila melanogaster*. In particular, one of the main focuses in the lab is to characterize the underlying mechanism of SMO activation by phosphorylation, which is required for signal transduction via the HH transduction complex. In order to better understand how SMO activation is regulated, our team looked, prior to my arrival in the lab, for new SMO intracellular partners through a yeast two-hybrid screen.

1. Identification of the RNA binding protein Smaug as a novel partner of Smoothened

This yeast two-hybrid screen was performed using SMO cytoplasmic C-terminal tail leading to the identification of several potential partners (manuscript, supplementary material). Among these was the post-transcriptional repressor Smaug which plays a critical role during *Drosophila* early development. Next, a secondary RNAi screen based on the two-hybrid screen results was conducted by I. Bécam, a MCU of our lab with R. Holmgren, one of our collaborators from Northwestern University. This screen was based on the analysis of changes in the spacing between vein 3 and 4 in the fly wing, which reflects deregulation of the HH signaling, and was conducted in a *fu* mutant background which led to a narrowing of this region. It revealed that Smaug genetically interacts with the kinase FU (data not shown). This genetic interaction was also confirmed using mutants of *fu* and *smaug*. Furthermore, co-immunoprecipitation (co-IP) assays using extracts from WID cultured cells confirmed SMO and Smaug interaction. In addition, subcellular localization analysis by confocal microscopy showed that SMO and Smaug colocalize in cytoplasmic punctuate structures and that HH reception promotes the recruitment of Smaug by SMO at the plasma membrane in cultured cells (manuscript, Fig. 2). Finally, *in vivo* studies showed that Smaug is expressed throughout the wing imaginal disc where it partially colocalizes with SMO.

2. Smaug upregulates SMO levels in a mRNA dependent manner in *Drosophila* wing imaginal disc

To further study this unexpected relationship between the RNA regulator Smaug and SMO, C. Argüelles also studied the effect of Smaug in the wing imaginal disc. It led to an upregulation of SMO protein levels in the WID. Interestingly, the overexpression of a form of Smaug unable to bind mRNA did not affect SMO levels. These results, which are currently being further studied in the lab, suggest that Smaug may modulate SMO levels in an mRNA binding domain dependent manner via the regulation of one or several mRNAs.

3. HH signaling induces Smaug phosphorylation

Finally, a preliminary observation, which was made upon my arrival in the lab, showed that HH signaling promotes Smaug phosphorylation. This result suggested that the HH pathway could regulate Smaug post-translationally.

II. Thesis objectives

In continuation with this work, my thesis project has been centered on the following two questions: what is the biological function of SMO and Smaug interaction and how is the post-transcriptional repressor Smaug regulated by the HH signaling pathway? More specifically, my main goals have been:

- 1) To understand how the proteins SMO and Smaug interact and what is the role of their interaction. This required to first map the interaction regions which will allow assessing their role on SMO and Smaug respective activity *in vivo* after fly transgenesis.
- 2) To tackle how Smaug is phosphorylated in response to HH and what are the functional consequences. This implied to, first, identify the sites that are phosphorylated as well as to characterize the kinase(s) involved. Secondly, I aimed to assess the effect of HH signaling on Smaug ability to repress mRNA translation, by using a reporter system that allows measuring Smaug repressive activity.

3) Finally, during this work, I obtained unexpected results that suggested that Smaug is constitutively phosphorylated. Therefore, to understand the role of this constitutive phosphorylation I sought to test the effect of Smaug mutant variants, unable to undergo phosphorylation, on its ability to repress translation as well as to form cytoplasmic foci.

SUMMARY OF THESIS WORK

In order to achieve my thesis goals, I have undertaken a combination of proteomic and biochemical approaches using Cl8 cultured cells, which are cells derived from the wing imaginal disc known to respond to HH. Part of my research work is included in the manuscript presented in the next section, which I will refer to when necessary. Altogether, my research efforts have led to the following findings:

SMO and Smaug physical interaction involves a conserved N-terminal region of Smaug and the most C-terminal region of SMO.

In order to understand the role of the Smaug/SMO interaction, it was necessary to map the regions involved. To this aim, I built along with C. Argüelles and G. Alvisi, an M2 student that I co-supervised, a series of tagged deletions of Smaug and SMO. We thus tested 20 different constructions by co-IP from transfected Cl8 cultured cells (manuscript, Fig. 1). For Smaug, the smallest SMO binding region includes the two conserved Smaug Similarity Regions (SSR) of so far unknown function. For SMO, we could reduce the Smaug binding region down to the last 78 residues of the C-terminal region.

HH signaling promotes Smaug phosphorylation which requires interaction with SMO.

Preliminary results obtained in Cl8 cells indicated that Smaug undergoes phosphorylation upon activation of HH signaling as seen by reduced electrophoretic mobility in a polyacrylamide gel. Consequently, I sought to optimize the experimental conditions in order to best analyze the retarded mobility shift of Smaug phosphorylated isoform (manuscript, Fig. 3B).

Then, I showed that Smaug phosphorylation requires both the activation of the cells by HH and the presence of SMO (manuscript, Fig. 3A). Moreover, a constitutively activated form of SMO can promote Smaug phosphorylation in absence of HH. Finally, I showed that Smaug

phosphorylation in presence of HH is directly dependent on SMO interaction (manuscript, Fig. 3 C).

Smaug contains multiple regions required for HH induced phosphorylation

Our data on Smaug phosphorylation constitute the first indication that Smaug could be regulated by a post-translational modification. I therefore decided to pursue the study of HH induced phosphorylation of Smaug in order to understand how it occurred as well as its biological function.

By a combination of mass spectrometry analysis and systematic site directed mutagenesis, I showed that Smaug contains three regions required for promotion of phosphorylation by HH signaling. Moreover, it seems that preventing the phosphorylation of a small region that precedes the SAM domain induces Smaug constitutive phosphorylation in absence of HH. Last but not least, my results also indicated that Smaug SAM domain is constitutively phosphorylated in absence of HH.

Identification of the kinases involved in HH promotion of Smaug phosphorylation

In order to identify the kinases involved in Smaug phosphorylation I analyzed Smaug interactors by mass spectrometry after co-IP and found potential kinases candidates, some of which play a role in the HH pathway. A knock-down of these candidates will be achieved by RNAi and the implication of the kinases known to regulate SMO activation will also be tested in a similar fashion.

Activation of HH signaling downregulates Smaug repressive activity.

Smaug function has been associated to its ability to repress bound mRNAs by promoting their degradation or blocking its translation. I therefore wanted to assess the effect of HH signaling in Smaug ability to repress mRNA. To this aim, we have constructed and set up in collaboration with M. Sanial a reporter gene that allows quantification of the repression of Smaug bound mRNA in Cl8 cells, as well as quantification of Smaug levels. We thus found that activation of HH signaling inhibits Smaug repressive activity and downregulates Smaug protein levels (manuscript, Fig. 4). Furthermore, it seems that Smaug downregulation

requires interaction with SMO. Altogether, these results provide the first evidence that Smaug activity could be regulated by a signaling pathway.

Inhibition of Smaug SAM domain constitutive phosphorylation upregulates Smaug repressive activity and downregulates its protein levels

By a combination of mass spectrometry and WB analysis, we observed that Smaug is constitutively phosphorylated in C18 cells. I thus decided to determine the function of this phosphorylation by studying Smaug repressive activity. Inhibition of phosphorylation in the SAM domain led to a highly repressive Smaug mutant which presented decreased accumulation levels.

Smaug SAM domain phosphorylation is implicated in S-foci formation

Since prevention of phosphorylation in the SAM domain of Smaug led to higher repressive activity, I wanted to check whether Smaug ability to form S-foci was also affected. By confocal microscopy, I showed that the inhibition of SAM domain phosphorylation disrupts S-foci formation leading to an increase of the number of punctuate structures, which are of smaller size. Moreover, it seemed that a small proportion of Smaug protein could be located in the nucleus when we blocked phosphorylation of the SAM domain. Further characterization of Smaug phosphorylated residues in the SAM domain is currently underway in the lab.

RESULTS

PART 1: MANUSCRIPT

The GPCR Smoothened binds and regulates the RNA-binding protein Smaug/SAMD4

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Running title: Smaug interacts with Smoothened

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ABSTRACT

Regulation of the localization, stability and translation of cytoplasmic mRNAs is used by cells for spatio-temporal regulation of gene expression. The conserved RNA binding protein Smaug/Samd4 thus controls the fate of many mRNAs during fly embryonic development. In mammals, it is involved in synapse biology, muscle development and in osteogenesis. Smaug/Samd4 proteins recognize stem-loop structures in their target mRNAs, and repress their expression via the recruitment of protein partners that destabilize these transcripts, prevent their translation or both. Whereas Smaug/Samd4 controls the fate of numerous transcripts by various mechanisms in a variety of developmental and cellular context, the mechanisms that regulate its activity and ensure its specificity remain to be understood.

We show here that Smaug interacts and colocalizes with the seven transmembrane domain protein Smoothed (SMO), a key member of the Hedgehog (HH) signalling pathway which controls metazoan development and is a central player in oncogenesis. Moreover, activated SMO is able to recruit Smaug at the plasma membrane. We also demonstrate that binding of activated SMO to Smaug controls Smaug fate as it induces its phosphorylation, reduces its accumulation levels and downregulates its repressing activity. By highlighting an unexpected relationship between HH/SMO signaling and Smaug, our data provide evidence for novel regulation of Smaug by a signaling pathway.

INTRODUCTION

Cytoplasmic regulation of the stability, localization and/or translation of mRNAs is used by cells for dynamic spatio-temporal regulation of proteins and is central for the development of multicellular organisms and numerous cellular functions (Bullock, 2011). Accordingly, improper control of these processes is involved in diseases, notably cancer and neurological disorders (Degrauwe et al., 2016; Lenzken et al., 2014; Thomas et al., 2011). This post-transcriptional regulation of mRNAs involves specific RBPs (RNA binding proteins) which both bind specific mRNAs and recruit proteins regulating their localization, their stability or their expression. These mRNA-protein complexes form microscopically visible and non-membranous discrete cytoplasmic foci where the mRNAs are stored in a silent state, thereby providing a RNA-centric regulation hub (Bullock, 2011; Thomas et al., 2011). Understanding how these RBPs are regulated, especially the spatio-temporal control of their interaction with their mRNA and protein partners and their ability to aggregate into foci is crucial to understand their role and functioning.

Drosophila melanogaster has been instrumental in the identification of such regulators, mainly through genetic analysis of pattern formation in the *Drosophila* embryo and neurogenesis (see for instance (Barckmann and Simonelig, 2013; Besse and Ephrussi, 2008; Pinder and Smibert, 2013b; Vardy and Orr-Weaver, 2007)). Among these RBPs, the Smaug/Smad4 protein stands as a key regulator of the fate of mRNAs during the early development of the fly embryo (Gotze and Wahle, 2014; Pinder and Smibert, 2013b). Thus, zygotic translation of maternal *smaug* mRNA controls both the establishment of the fly embryo's anteroposterior polarity by repressing the translation of a key determinant of the posterior identity, *nos* (*nos*) (Jeske et al., 2006; Zaessinger et al., 2006) and is required for the clearance of two-thirds of the unstable maternal mRNAs that occurs during the maternal-to-zygotic transcription transition (Benoit et al., 2009; Tadros et al., 2007). Very little is known on the role of Smaug during later stages of development, except that its zygotic expression controls the morphogenesis of periphery larval neurons via the regulation of *nos* mRNA translation (Brechtel and Gavis, 2008). In mammals, two Smaug genes are both expressed in the nervous system. Smaug1/Samd4a seems to control synapse morphogenesis and function, likely through the control of specific mRNAs

(Baez and Boccaccio, 2005; Luchelli et al., 2015) and is also involved in muscle growth as well as in osteoblastogenesis and bone development (Chen et al., 2014b; de Haro et al., 2013; Niu et al., 2017). *Smaug2/Samd4b*, was recently shown to restrict neurogenesis by silencing *nos* mRNA (Amadei et al., 2015).

The *Smaug/Samd4* proteins binds to their target transcripts via a Sterile Alpha Motif (SAM) domain that recognizes short stem/loop RNA structures called *Smaug Recognition Elements (SRE)* (Aviv et al., 2003). Numerous studies in fly have shown that *Smaug/Samd4* can silence its target mRNAs by multiple, non-exclusive, mechanisms, depending on the mRNA regulatory proteins that it recruits (Pinder and Smibert, 2013b). It can thus inhibit of the translation of target mRNAs via the recruitment of the translational repressor CUP, likely by preventing the formation of the elongation initiation factor 4F (eIF4F) complex (Jeske et al., 2011; Nelson et al., 2004; Pinder and Smibert, 2013a) and/or promote deadenylation by recruiting a complex that includes the CCR4–NOT deadenylase complex (Baez and Boccaccio, 2005; Chen et al., 2014a; de Haro et al., 2013; Nelson et al., 2004; Pinder and Smibert, 2013a; Rouget et al., 2010; Semotok et al., 2005; Semotok et al., 2008; Zaessinger et al., 2006). Proteins of the Argonaute family such as Ago1 and Aubergine (a piwi-type Ago) have also been implicated in these processes (Pinder and Smibert, 2013a; Rouget et al., 2010).

The multiplicity of *Smaug/Samd4* targets, mechanisms of action, and roles points to the importance of the spatio-temporal regulation of its activity during development and in neurons. Thus, the local stabilization and translation of *nos* mRNA at the posterior pole of the embryo occurs by its dissociation from *Smaug* in response to the posterior located *Osk* protein binding to *Smaug* at or near its SAM domain- (Zaessinger et al., 2006). It was also reported that the foci which include *Smaug1/Samd4A* at the post-synapse are transiently dissolved upon synaptic stimulation of the N-methyl-D-aspartic acid receptor (NMDAR), an event associated with the transitory release and translation of mRNAs encoding Calcium/calmodulin-dependent protein kinase II α (CaMKII α) (Baez et al., 2011; Pascual et al., 2012).

The present data reveal an unexpected connection between fly *Smaug* and Hedgehog (HH) signaling, a key pathway in the development of many animals, in tissue repair and in carcinogenesis. We demonstrate that *Smaug* can directly interact with *Smoothed (SMO)*, a seven transmembrane domain protein required for the

transduction of the HH signal (for revue see (Ayers and Therond, 2010)). Although this interaction is found both in the absence and presence of HH, it is negatively regulated by the highest levels of phosphorylation of SMO induced by HH. Moreover, we show that SMO and Smaug co-localize in foci and that SMO activation leads to the recruitment of Smaug to the plasma membrane. Finally, we also provide evidence that the activation of SMO by HH promotes the phosphorylation of Smaug and downregulates both Smaug levels and its repressive activity. Together these results shed light on the unexpected regulation of the RBP Smaug by the GPCR SMO.

RESULTS

Smaug interacts with SMO

We identified the mRNA binding protein Smaug in a two-hybrid screen as the most frequent partner (137/258 of the positive hits, see Sup Table 1) of a SMO cytoplasmic C-terminal tail (aa 558-1036, called thereby cytotail) that harbored mutations known to mimic the activating phosphorylation induced by HH at protein kinase A (PKA) sites (SMO^{PKA-SD} cytotail). We confirmed this unexpected interaction between SMO and Smaug by testing their ability to coimmunoprecipitate using Cl8 *Drosophila* cultured cells that are known to respond to HH (Chen et al., 1999). The entire SMO^{WT}-HA and SMO^{PKA-SD}-HA proteins and Myc-Smaug were expressed either alone or together. Note that the epitope tags are known not to interfere with the normal functions of SMO and Smaug, respectively (Semotok et al., 2005; Zhu et al., 2003). The protein complex immunoprecipitated with an anti-HA antibody was analyzed by Western blot with anti-HA and anti-Myc antibodies, respectively (Fig. 1A). Myc-Smaug coimmunoprecipitated with SMO^{PKA-SD}-HA as expected from the two-hybrid data in yeast. Moreover, Myc-Smaug also interacted with SMO^{WT}-HA both in presence and in absence of HH, indicating that this interaction occurred independent of HH or of the phosphorylation of the PKA sites. Note that reciprocally, SMO^{WT}-HA also coimmunoprecipitated with Myc-Smaug (see for instance Fig.1E).

The two-hybrid screen identified the region of Smaug that covers aa 74-291 as sufficient to bind SMO. It contains two conserved sequences, called respectively Smaug Similarity Regions 1 and 2 (SSR1 from aa 69 to 120 and SSR2 from aa 199 to 287, blue boxes in Fig. 1B) (Smibert et al., 1999) separated by a 79 aa long non conserved region (called here M). Accordingly, both Myc-Smaug⁶⁹⁻²⁸⁷ and Myc-Smaug¹⁻³⁷⁴ coimmunoprecipitated with SMO^{WT}-HA (Fig. 1C and S1A). However, deletion of the SSR regions alone (Myc-Smaug⁶⁹⁻¹⁹⁹ or Myc-Smaug¹²¹⁻²⁸⁷, respectively) or together (Myc-Smaug¹²¹⁻¹⁹⁹) led to the loss of the interaction with SMO (Fig. 1C and S1B), indicating that both the SSR1 and SSR2 regions are required for the interaction of Myc-Smaug with SMO-HA.

To identify the region of SMO cytotail (Fig. 1D) involved in its interaction with Smaug, we perform a deletion analysis. As shown in Fig. 1E and S1C-E, the interaction of SMO-HA with Myc-Smaug was reduced by the deletion of the last 58 aa acids of SMO (SMO^{Δ978-1003}-HA) and totally lost when twenty more aa were removed (SMO^{Δ958}-HA). Moreover, the last 79 aa of SMO fused to the GFP (GFP-SMO⁹⁵⁸⁻¹⁰³⁶), but not the last 59 aa (GFP-SMO⁹⁷⁸⁻¹⁰³⁶), was sufficient to interacted with Smaug (Fig. 1F). This region, partially overlaps a sequence that binds the protein kinase Fused (FU) and is embedded in four clusters of S/T (green boxes) whose phosphorylation is induced by FU, an event required for high levels of HH signaling (Sanial et al., 2017a).

The activation of SMO by HH is associated with its hyperphosphorylation which can be easily followed as it leads to slower electrophoretic migration. Careful examination of input (In) and immunoprecipitated (IP) fractions of Myc-SMO^{WT} revealed that only the forms of Myc-SMO^{WT} that are not or are partially phosphorylated were associated with Myc-Smaug. In contrast, the most hyper phosphorylated forms of SMO remained in the supernatant of the immunoprecipitation, ruling out their specific degradation during the immunoprecipitation process (Fig. 1G). This effect is not due to phosphorylation of the region of SMO that interacts with Smaug, as the replacement of the five S/T by A (called here SMO^{5S-A}) present in or near that region did not reduced the HH induced phospho-shift nor prevented its inhibitory effect (Fig. S2A-C). We also tested and excluded the phosphosites (altogether more than thirty sites) known to be targeted by PKA, CKI FU, GPRK2, Gish and aPKC in response to HH (Fig. S2C-E).

In summary, these data show that Smaug and SMO interact together both in yeast and in fly cells. This interaction takes place between the region between aa 69-287 of Smaug that includes the two conserved SSR1 and 2 regions and the region between aa 958-1003 of SMO that partially overlaps the FU binding site and is flanked by FU phosphorylation sites. Moreover, HH downregulates this interaction, probably via novel phosphorylation of SMO that remains to be identified.

Smaug and SMO colocalize in foci and activated SMO can recruit Smaug at the cell plasma membrane

Smaug was shown to be a constituent of mRNA storage bodies, called Smaug-foci (S-foci) (Baez and Boccaccio, 2005; Baez et al., 2011; Parker and Sheth, 2007). Patel/ Todd Blankenship 2016) (Baez and Boccaccio, 2005; Zaessinger et al., 2006), SMO can also be associated with membranes as its unphosphorylated form is modestly localized to intracellular endocytic vesicular structures in the absence of HH, and following HH reception, hyperphosphorylated forms accumulate at the plasma membrane (Nakano et al., 2004; Zhu et al., 2003).

To analyze whether SMO and Smaug colocalize and whether they affect each other localization, we expressed SMO-mCherry (SMO-mCh) and GFP-Smaug fusions alone or together in C18 cells (Fig. 2A). As expected, SMO-mCh is present in vesicular intracellular structures and is partially relocated at the plasma membrane in response to HH (Fig. 2A1 and 2A2) and GFP-Smaug alone is present on foci, (Fig. 2A3 and 2A4). The number and the size of these Smaug structures were not be significantly affected by HH (Fig. S1F and data not shown). When coexpressed, SMO-mCh and GFP-Smaug always strongly colocalized in dot-like structures in absence of HH with all cotransfected cells having more of 90% foci that are colabelled in 25/25 cells (Fig. 2A5"). Moreover, in presence of HH, GFP-Smaug also always (45/45 cells) localizes with SMO-mCh at or near the cell surface (Fig. 2A6-6"). A similar colocalization at the cell surface was also seen with SMO^{PKA-SD FU-SD}-GFP and SMO^{PKA-SD}-GFP, that are constitutively hyperactive forms which accumulates at the cell surface independent of HH (Fig. 2B1-1" and data not shown). We confirmed that Smaug interacted with the fraction of SMO that is present at the plasma membrane by specifically labelling this fraction using a SNAP tag that was fused to the N-

terminal extracellular part of SMO^{PKA-SD FU-SD} (SNAP-SMO^{PKA-SD FU-SD}) (Fig. 2B2-2” and C).

Last, to test whether the colocalization of Smaug and SMO was affected by the loss of their interaction, we examined the colocalization of GFP-Smaug with SMO^{Δ1004}-mCh and SMO^{Δ958}-mCh (Fig. 2D). Both SMO proteins responded to HH as shown by their cell surface localization in presence of signal (Fig. 2D4” and 2D8”). However, SMO^{Δ1004}-mCh, but not SMO^{Δ958}-mCh, still colocalized with Smaug (Fig. 2D3” and 2D7”) and promoted its localization at the plasma membrane in response to HH (Fig. 2D4” and 2D8”). This confirmed that the colocalization of SMO and Smaug results from their physical association.

In summary, our data show that SMO and Smaug can colocalize together in an interaction-dependent manner and that activated SMO can directly recruit Smaug at the cell membrane in response to HH.

HH/SMO activation promotes the phosphorylation of Smaug

The known partners of SMO, including the kinase FU and the kinesin Costal2 are phosphorylated upon SMO activation by HH (Aikin et al., 2008). We therefore tested whether this was the case for Smaug. In presence of HH, SMO^{WT}-GFP induced an electrophoretic mobility shift of HA-Smaug and this effect is absent when SMO^{WT}-GFP is not co-expressed or in absence of HH, respectively (Fig. 3A). A similar retardation shift was seen when Smaug-HA was coexpressed with activated SMO^{PKA-SD}-GFP. In all cases, treatment of the extracts with a phosphatase abolished the slower migrating forms induced by activated SMO-GFP, demonstrating that these forms are phosphorylated (Fig.3B). These effects depends on the ability of SMO^{WT}-GFP (and SMO^{PKA-SD}-GFP) to interact with Smaug as SMO^{PKA-SD Δ958}-GFP and SMO^{Δ978}-GFP which do not or poorly interact with HA-Smaug, respectively (see Fig. 1) are unable to induce the phosphorylation of HA-Smaug (Fig. 3C). This is not likely due to a lack of activity of these SMO constructs since SMO^{Δ978}-GFP is known to be constitutively active (Malpel et al., 2007).

We then searched to identify the Smaug phosphosites involved. Myc-Smaug⁶⁹⁻²⁸⁷, that binds to SMO and includes the SSR1-M-SSR2 regions, is still phosphorylated in presence of activated SMO (Fig. S3A). This effect is lost when the intermediate M

region is deleted (Fig. S3B), suggesting that it contains one or several sites that are necessary. The SSR1-M-SSR2 region contains 33 S/T. To identify the relevant ones, we then performed Liquid Chromatography–tandem MS (LC-MS/MS) on extracts that expressed Smaug with SMO in presence of HH. We obtained 48% peptide coverage of Smaug which includes 84 of the 181 T/S present in Smaug) (Fig. S3C). It allowed the identification of nine phosphopeptides, three of which with a PTM score above 89% (Fig. 3D and S3C). Three potential phosphosites are present in the SSR1-M-SSR2 region. However, the combined mutation of two candidates, S126 and S127 in Myc-Smaug did not affect the phosphorylation induced shift in gel migration indicating that they play little or no role or in the phosphorylation of Smaug induced by SMO/HH (data not shown).

In summary, these data reveal that HH-activated SMO promotes an interaction-dependent phosphorylation of Smaug in its N-terminal region.

Activation of HH/SMO signaling reduces the levels of Smaug and upregulates its repressing activity

The phosphorylation of Smaug induced by HH/SMO activation raises the possibility that HH/SMO controls the levels of Smaug accumulation and/or its mRNA repression activity. To explore these possibilities, we developed an assay in C18 cells that allowed us to simultaneously measure Smaug levels and analyze its repressing activity (Fig. 4A). This test is based (i) on a λ N/5BoxB protein-mRNA dual system (derived from the bacteriophage λ) in which λ N protein binds with high affinity a series of five B Box B (called here 5BoxB) inserted in a mRNA reporter (Behm-Ansmant et al., 2006; Gehring et al., 2005; Rehwinkel et al., 2005) and (ii) on the SNAP self-labelling peptide to allow the simultaneously quantification of the levels of the different partners.

First, we validated our system by verifying that the λ N-SNAP-Smaug could specifically decrease SNAP-GUS protein expression of the 5BoxB containing reporter (Fig. 4B and S4A). Its expression led to two to three fold downregulation of SNAP-GUS levels. This effect increases with λ N-SNAP-Smaug levels and is observed with different ratios between the Smaug expressing vector and the reporter

vector (data not shown). Importantly, no effect was seen when Smaug was absent (λ N-SNAP control) or not anchored to the reporter mRNA (SNAP-Smaug control) (Fig. 4C and S4A).

Next, we monitored the effects of SMO/HH on protein expression of the *snap-gus-5BoxB* reporter (Fig. 4D). While SMO or HH alone had almost no effect, coexpression of SMO in presence of HH led to an increase of reporter protein expression. Notably, SMO^{PKA-SD} had a similar effect in absence of HH than SMO and HH together. The effect of HH/SMO reflected a reduction the inhibitory effect of Smaug as HH/SMO had no effect on the reporter in absence of Smaug (Fig. S4A). It also depended on Smaug-SMO association as it was not seen with SMO ^{Δ 958} (Fig. 4D).

The effect of activated SMO on SNAP-GUS reporter levels could be due a downregulation of Smaug levels, of its mRNA repressing activity or both. As shown in Fig. 4E, either SMO coexpression or the presence of HH alone had a weak negative effect on λ N-SNAP-Smaug levels. Note that this effect was reproducible (in two independent biological triplicates) but not statistically significant. However, in the presence of HH, SMO coexpression led to a strong and significant decrease (40-60%, depending on the experiment) in Smaug levels (Fig. 4E). This effect depended on the ability of SMO to bind Smaug, as it was not seen with SMO ^{Δ 958} which has lost its ability to bind Smaug. Notably, SMO^{PKA-SD} had a strong negative effect on Smaug levels and this effect was not significantly increased by HH. Finally, we analyzed the contribution of the reduction of Smaug levels to the HH/SMO induced decrease in Smaug repressing activity. For that purpose, we repeated the repression assays using different doses of the Smaug expressing vector and analyzed the levels of reporter expression in relation to the levels of Smaug proteins (Fig. 4F). These experiments show that, for the same amount of Smaug protein, the level of reporter expression was systematically higher in presence of HH/SMO. Thus, HH/SMO reduced the intrinsic repressing activity of Smaug.

In summary, our data reveal SMO can have two cumulative negative effects on Smaug: (i) it reduces its accumulation levels and (ii) it downregulates its mRNA repressing activity. Both effects depend on SMO activation and on SMO ability to interact with Smaug.

DISCUSSION AND CONCLUSION

Our molecular and genetic experiments reveal an unexpected relationship between HH signaling and the mRNA post-transcriptional regulator Smaug. They provide the first evidence that the fly Smaug protein can be post-translationally regulated and support a potential novel role of HH/SMO signaling in the regulation of the fate of cytoplasmic mRNA.

First, our results bring evidence for a physical interaction between Smaug and SMO. The mapping of the regions mediating their association sheds light on two poorly characterized regions of these proteins. First, a 118 aa long region present in N-terminal part of Smaug which includes two conserved regions is sufficient for the interaction with SMO. Notably, the SSR1 (aa 69-120) which is necessary to bind SMO contains two hydrophobic regions separated by a positively charged conserved motif (LLKRL/V(N)5K/RFLQ) while the region of SMO that interacts with Smaug contains mainly polar and acidic aa, suggesting that an electrostatic interaction may take place between these two regions. Our data also reveal that the interaction between SMO and Smaug is negatively regulated by HH signaling via its action on SMO. We ruled out phosphorylation of the region that binds Smaug and almost all of the characterized phosphosites of SMO, included those targeted by PKA/CKI, FU, and GPRK2/GRK2, Gish and aPKC. Although we cannot exclude that that the simultaneous phosphorylation of these different sites might be required this suggests that phosphorylation sites that remain to be determined might be involved. Finally, another possibility is that HH may block the interaction of SMO with Smaug by promoting another type of post-translation modification that is associated with the hyperphosphorylated form of SMO.

Moreover, our study shows that SMO and Smaug colocalize in cultured cells and that his colocalization reflects their interaction as it is lost with a mutant of SMO unable to interact with Smaug. While HH does not seem to affect the subcellular localization of Smaug in absence of SMO, it promotes its recruitment at the plasma membrane via its interaction with SMO. Note that paradoxically, HH does not prevent SMO to interact or to localize with Smaug while it promotes the hyperphosphorylation of SMO which blocks its interaction with Smaug. This probably reflects that, at least

under our experimental conditions, only a subfraction of SMO undergoes hyperphosphorylation in response to HH and that sufficient amounts of SMO with low to intermediate phosphorylation levels are present at the cell surface to recruit Smaug. This recruitment of Smaug at the plasma membrane by activated SMO raises the possibility that SMO could thus finely tune the spatial regulation of one or several mRNAs bound to Smaug. Note that such localized regulation might be conserved as Smad4 is also found at or near the plasma membrane in several mammalian cell lines (<https://www.proteinatlas.org/ENSG0000020577-SAMD4A/cell>).

Finally, our results also provides novel evidence that SMO/HH signaling can in turn regulate Smaug as it reduced its Smaug levels, increase its activity and promote its phosphorylation. This latter effect could be due to the recruitment of one or several kinase(s) associated to the activated form of SMO. Note that little is known on a potential regulation of Smaug by phosphorylation, except for a global proteomic study (Zhai et al., 2008) and of a paper reporting that the Akt/PKB kinase could phosphorylate Smaug1/Samd4a *in vitro* (Chen et al., 2014b). Given the high numbers of potential S/T phosphosites presents in Smaug (181 over 999 aa), the identification of the sites targeted in response to HH will be challenging. Whatever, an attractive hypothesis is that the effects of HH/SMO on Smaug phosphorylation could affect its stability and its ability to repress target mRNA.

In conclusion, this work elucidates a novel connection between mRNA post-transcriptional regulation and HH signaling. We propose that SMO binding to Smaug allows HH/SMO signaling to finely regulate the fate of one or several mRNAs bound to Smaug, both by decreasing Smaug levels and its repressive activity. Moreover, the inhibition of the SMO-Smaug interaction by very high levels of SMO activation, suggests that such regulation may be transient, as this was shown for the dissolution of Smaug foci upon synaptic activation of the MNDAR (Baez et al., 2011; Pascual et al., 2012). The biological context of this regulation and the identification of the mRNA(s) involved will be the next challenge given the multiplicity of roles for SMO/HH and the high number of mRNA targeted by Smaug. SMO and Smaug are conserved proteins and it is particularly interesting to note that Samd4 has been recently reported as being a frequent insertion site in a transposon based screen for

genes involved in medulloblastoma formation in a mouse model. A HH signaling can promote MB, this suggests that the relationship undercover here between SMO/HH signaling and Smaug might be conserved (Badodi et al., 2017). Given the importance of these proteins in various pathologies, especially such as degenerative diseases and cancer, our study could provide a better insight on the pathophysiology of these diseases and guide searches for novel therapeutic targets.

MATERIAL AND METHODS

Two hybrid screen

86 x10⁶ clones from a 0-24 embryonic polyA c-DNA library were screen as described in (Formstecher et al., 2005) using as bait the cytotail of SMO^{PKA-S_D} in which the cAMP-dependent protein kinase (PKA) phosphosites (symbolized by S) were replaced by Aspartic Acid (D) to mimic activating phosphorylations (Jia et al., 2004).

Plasmids

All genes on plasmids were expressed under a *pAct.1C* promotor, written here *pAct. pActC.smo^{WT}-HA*, *pAct.SNAP-smo^{WT}*, *pAct.smo^{PKA-S_D}-HA* and *pAct.smo^{PKA-S_D}^{FU-S_D}-HA* were described in (Sanial et al., 2017b). Wild type *smo* or *smaug* coding sequences was introduced into *pENTR/D-TOPO* by directional TOPO Cloning (Invitrogen) before being transferred using the Gateway Technology (GW) (Invitrogen following the manufacturer's instructions) the vectors *pAct.GW-HA*, *pAct.GW-mCherry*, *pAct-Myc-GW-HA*, *pAct.GFP-GW-HA* (gifts from T. Murphy). Mutated forms of *smaug* and *smo* were made in *pENTR/D-TOPO-smo* by sub-cloning (for the deletions) or site directed mutagenesis (for replacement of S/T codons by A codons). *pAct.λN-SNAP-smaug* was built by introducing the *smaug* ORF(from *pENTR/D-TOPO-smaug*) into *pAct.λN-SNAP-GW* which was built by cloning the *λN sequence* from *pAc.1B-λN-HA* (Behm-Ansmant et al., 2006) into *pAct.SNAP-GW*. The *pAct.SNAP-GUS-Stop-5BoxB* plasmid was built in two steps. First, we replaced the Fluc sequence in *pAct.Fluc-Stop-5BoxB* (Rehwinkel et al., 2005) by a SNAP-GW sequence, leading to *pAct.SNAP-GW-Stop-5BoxB* in which the glucuronidase (GUS)

(from *Arabidopsis thaliana*) coding sequence was inserted. *pAct.GFP-SNAP* was built by insertion of the SNAP coding regions (in *pENTR SNAP*) into *pAct.GFP-GW*. All constructs were checked by restriction mapping, all the fragments produced by PCR and their junctions were sequenced.

C18 cell culture, transfection, immunoprecipitation and immunodetection

C18 cells were cultured as in (Claret et al., 2007) in 2% CFS (Hyclone). Transient transfections were done with Transit Insect Reagent (Mirus) using a total of 0,5 to 1 µg of plasmid DNA/ for 2 µl reactant. 48h post transfection, cells were washed twice in PBS1X. After centrifugation the pellet was lysed RIPA buffer with the “Complete EDTA free antiprotease mix” (Roche) and the phosphatase inhibitor mix Phostop (Roche), before centrifugation (12000 rcf) 10 minutes at 4°C, and mix with Laemmli sample buffer (Bio-Rad) and 0.1M DTT. Protein concentrations were estimated with the Bradford Ultra reagent (Expedeon). For direct immunodetection, 60µg of protein was warmed 5 minutes at 25°C before loading on a 10% Anderson gel (ratio acrylamide/bis-acrylamide=77) (Anderson et al., 1973). Gels were run 90 minutes at 150 volts in a Miniprotean (Bio-Rad). The subsequent steps were performed as in (Sanial et al., 2017b). Primary antibodies: 1:1000 rat monoclonal anti-HA (Roche), 1:5000 rabbit anti-GFP (Torrey Pines Biolabs), 1:2000 rabbit anti-GMAP (Sigma, gift from Laurent Ruel), 1:1000 mouse anti-Myc (clone 4A6, Millipore). Secondary antibodies conjugated with HRP: anti-rat (JacksonImmuno), anti-mouse (Sigma) and anti-rabbit (JacksonImmuno). The enhanced chemiluminescence detection system (ECL Select, Amersham) was used on a LAS-3000 imager (Fujifilm).

Coimmunoprecipitation on cell lysates: 50 µg of lysate (Input , corresponds to 1/20 of IP, see below) was removed, mixed with loading buffer and frozen in liquid nitrogen before conservation overnight at 80 °C. 1 mg of protein was mixed with 0,5 mg of antibody against the protein tag mouse: anti-HA 12CA5 (Sigma-Aldrich and rabbit) and rabbit anti Myc 51 c (Euromedex) in 500 µl of RIPA buffer with the “Complete EDTA free antiprotease mix” (Roche) and Phostop (Roche), before incubation with gentle rocking overnight at 4°C. Pre-washed Protein A/G Magnetic beads (ThermoScientific) were added for 2h at 4°C. The beads (IP) were then

separated on a magnetic rack, washed 3 times with cell lysis buffer and re-suspended with loading buffer before heating at 95 °C for 3 min and electrophoresis. For the GFP fusions, coimmunoprecipitation was done using anti GFP nanobodies cross-linked to NHS resin (1 µg/µl from ChromoTek) and beads were pelleted by centrifugation before loading. Phosphatase assay were done with Lambda phosphatase (NEB) in absence of phosphatase inhibitor.

Cells transfection and fluorescent imaging

10⁶ C18 cells were plated 24 hr before transfection with 300 ng of *pAct.smo-GFP/mCh* or *pAct.SNAP-smo* and/or *pAct.smaug-GFP/mCh* constructs alone or together. Varying amounts of *pAct.GAL4* were added to ensure a total DNA concentration at 1000 ng (Sanial et al., 2017a). Cells were analysed 48hr after transfection. The extracellular SNAP labelling was done by incubation with SNAP-Surface 488 (NEB) (1/800 dilution in C18 medium) for 10 minutes at room temperature before being briefly rinsed 3 times in PBS, fixed for 15 min in PFA 4% and finally washed with PBS three times. Nuclei were labelled with Hoechst. Images were taken with a CSU-W1 (Yokogawa - Andor) spinning disc (Leica DMI8 microscope) with a 63x oil objective.

LC-MS/MS acquisition: Proteins on beads were digested overnight at 37°C by sequencing grade trypsin (12.5 µg/ml; Promega Madison, WI, USA) in 20 µl of 25 mM NH₄HCO₃. Peptides mixtures from biological replicates were analyzed by an Orbitrap Velos ETD, an Orbitrap Fusion Tribrid or a Q-Exactive plus coupled each to a Nano-LC Proxeon 1000 equipped with an easy spray ion source (all from Thermo Scientific). Peptides were separated by chromatography with the following parameters: Acclaim PepMap100 C18 pre-column (2 cm, 75 µm i.d., 3 µm, 100 Å), Pepmap-RSLC Proxeon C18 column (50 cm, 75 µm i.d., 2 µm, 100 Å), 300 nl/min flow rate, gradient from 95 % solvent A (water, 0.1% formic acid) to 35% solvent B (100 % acetonitrile, 0.1% formic acid) over a period of 97 minutes, followed by column regeneration for 23 min, giving a total run time of 2 hours. Peptides were analyzed in the Orbitrap cell, in full ion scan mode, at a resolution of 120,000 (at *m/z* 200), with a mass range of *m/z* 350-1550 and an AGC target of 4x10⁵. Fragments

were obtained by high collision-induced dissociation (HCD) activation with a collisional energy of 30%, and a quadrupole isolation window of 1.6 Da. MS/MS data were acquired in the Orbitrap cell (Q-Exactive plus), or in the ion trap (Orbitrap Fusion, Orbitrap Velos). Precursor priority was highest charge state, followed by most intense. Peptides with charge states from 2 to 8 were selected for MS/MS acquisition. The maximum ion accumulation times were set to 100 ms for MS acquisition and 60 ms for MS/MS acquisition. All MS and MS/MS data for protein samples were processed with the Proteome Discoverer software (Thermo Scientific, version 2.1) and with the Mascot search engine (Matrix Science, version 2.5.1). The mass tolerance was set to 7 ppm for precursor ions and 0.5 (Orbitrap Fusion, Orbitrap Velos) or 0.02 Da for fragments (Q-Exactive plus). A *Drosophila melanogaster* protein database was extracted from the NCBI database and used for all identifications. The following variable modifications were allowed: oxidation (M), phosphorylation (ST) (2 by peptide maximum in the parameter modifications by peptide). All results were 1% FDR (False Discovery Rate) filtered before exporting.

Repressing assay

Unless indicated otherwise, the following plasmid concentrations were used for transfection: 300ng *pAct.SNAP-GUS-Stop-5BoxB*, 50ng *pAct.λN-SNAP-smaug*, 50 ng *pAct.GFP-SNAP*, 50ng *pAct.smo-HA*, 50ng *pAct-hhN*; the total levels of DNA were adjusted to 500ng using *pAct.GAL4*. Cells were lysed in 1% Triton, 50mM Tris pH8, 150mM NaCl, 1mM DTT with complete EDTA-free antiprotease mix (Roche) before labelling for 30 min at 37° with SNAP-Cell Oregon Green (NEB, diluted at 1/600) in 0.5% Triton, 50mM Tris pH8, 150mM NaCl, 1mM DTT. At least three independent experiments were performed. All gels of a triplicate were run and scanned together. Images were analyzed and quantified using Imagemol (Biorad) software. After quantification, the λN-SNAP-Smaug and SNAP-GUS were normalized to the levels of GFP-SNAP. Statistical analysis were done using Kruskal-Wallis rank test followed by Dunn test were estimated using Graph Pad Prism.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Figure 1: Smaug and SMO physically interact.

(A) Coimmunoprecipitation of SMO-HA and Myc-Smaug proteins. Extracts of C18 cells expressing SMO^{WT}-HA or SMO^{PKA-SD}-HA, Myc-Smaug alone or together, in absence or presence of HH, as indicated, were immunoprecipitated (IP) with anti-HA. The input (lower panel) and the IP complexes (upper panel) were analyzed by Western blot with anti-Myc or anti-HA antibodies as indicated. Here and in the other Figures, the name of the proteins detected are indicated on the left and the molecular weights on the right, in Kda; the sample loaded in input is equivalent to a twentieth of volume loaded for the IP.

(B) Schematic representation of the domain structure of the Smaug protein. SSR1 and SSR2 (Smaug similarity regions 1 and 2) are shown in blue, the SAM domain (sterile alpha motif domain) is in green and the PHAT domain (pseudo heat analogous topology) in yellow. The dashed double-arrow line at the top represent the smallest interacting region (called SID for Smallest Interacting Domain) found according the two hybrid screen. The truncated constructs used are presented below. The amino acid numbers correspond to Smaug-PA (<http://flybase.org/reports/FBgn0016070.html>). The full red line below represents the smallest SMO binding region (BR) region that we could identify. The ability to interact with SMO is indicated on the left: in green for yes, red for no. See also Fig. S1A-B.

(C) Mapping of the SMO interaction domain in Smaug. Extracts of C18 cells expressing (or not) SMO^{WT}-HA with Myc-Smaug⁶⁹⁻²⁸⁷ or Myc-Smaug⁶⁹⁻¹⁹⁹ were analyzed after immunoprecipitation as in 1C.

(D) Schematic representation of the C-terminal cytotoil of SMO. The PKA/CKI and FU phosphorylations regions are indicated as orange and green boxes, respectively. FU interaction region is indicated by a full green double arrow on the top. The truncated constructs used are presented below and their ability to coimmunoprecipitate with Myc-Smaug indicated on the right. The full red line at the bottom represents the smallest Smaug binding region (BR) region that we identified.

(E-F) Mapping of the Smaug interaction domain in SMO. Extracts from C18 cells transfected with Myc-Smaug with various forms (as indicated) of SMO fused to either HA (E) or to the GFP (F) before being IP with anti-Myc (E) or anti-GFP (F) and analyzed by Western blotting with anti-Myc (lower panel in E and upper panel in F), anti-HA (E, upper panel) or anti-GFP (F, lower panel). In: Input. See also Fig. S1C-E.

(G) Hyperphosphorylated forms of SMO does not interact with Smaug. Extracts of C18 cells expressing wild-type SMO-HA with or without Myc-Smaug, in absence or presence of HH, as indicated, were IP with an anti-Myc antibody before analysis by Western blot with anti-HA (upper panel), or anti-Myc antibodies (lower panel). The black arrows indicate the unphosphorylated form of SMO and the brackets indicate the phosphorylated forms of SMO-HA that have slower migration properties.

Here and in the Fig. 3, all the Western blot data were independently reproduced at least twice.

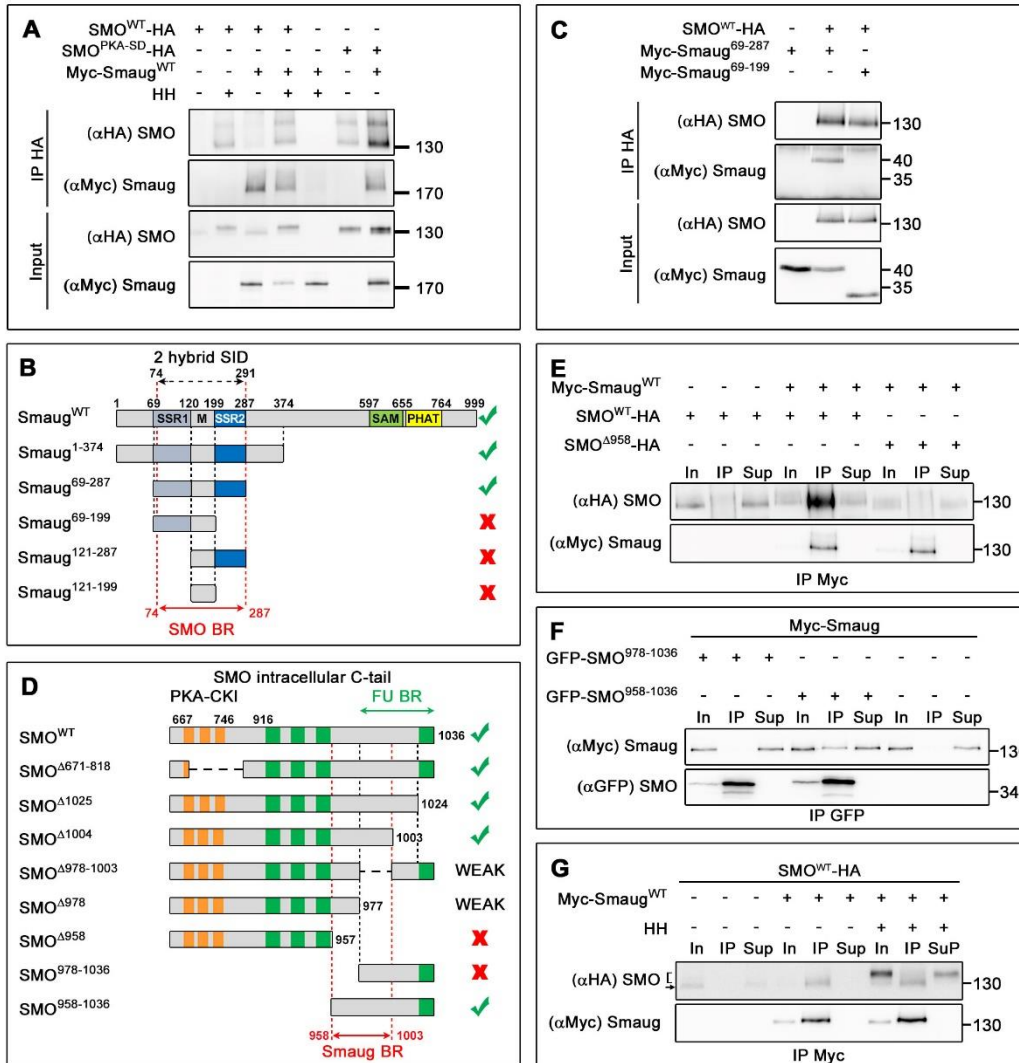


Figure 1 Argüelles, Bruzzone et al.

Figure 2: SMO and Smaug colocalize in an interaction dependent manner.

(A) SMO and Smaug colocalize Fluorescent images of Cl8 transfected with GFP-SMG (3-6) and SMO^{WT}-mCherry (SMO^{WT}-mCh, 1, 2, 5 and 6) alone (1-4) or together (5, 5', 6 and 6') without (1, 3 and 5) or with HH (2, 4, and 6), as indicated. The merge images in 5" and 6" show GFP-Smaug in green and SMO^{WT}-mCh in red. See also Fig.S1 for Smaug foci analysis. Note also that same results were seen with different fluorescent tags as well as in S2 cells (data not shown). A least 20 cells were seen for each conditions.

(B-C) Smaug colocalize with cell surface activated SMO. Fluorescent images of Cl8 cotransfected with mCh-SMG (1, 2, red in 1" and 2") and SMO^{PKA-SD FU-SD}-GFP (1', green in 1") or SNAP- SMO^{PKA-SD FU-SD} (2', green in 2"). Merge images: 1" and 2". For SNAP-SMO^{PKA-SD FU-SD}, the SNAP tag was self-labelled with an extracellular fluorescent substrate as shown in (C). No HH. A least 20 cells were seen for each conditions.

(D) Smaug colocalization with SMO depends on their interaction. Fluorescent images of Cl8 transfected with SMO^{Δ1004}-mCh (1, 2, 3-3" and 4-4") or SMO^{Δ958}-mCh (5, 6, 7-7", 8-8") alone (1, 2, 5, 6) or with GFP-SMG (3,-3", 4-4", 7-7" and 8-8"); with or without HH, as indicated. Merge images in 3" and, 4", 7" and 8" with Smaug in green and SMO in red. A least 10 cells were seen for each conditions.

Scale bar (shown in A1, identical for all cells): 10μm.

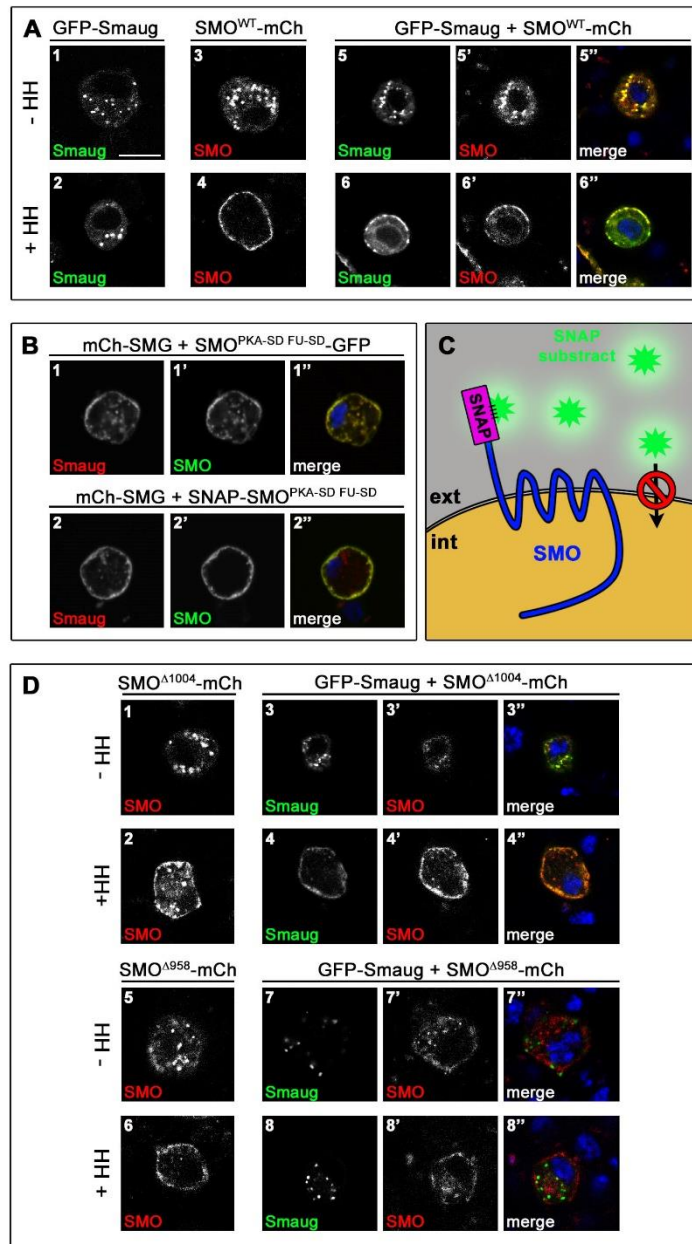


Figure 2 Argüelles, Bruzzone et al.

Figure 3: SMO/HH activation promotes the phosphorylation of Smaug.

(A) HH/SMO promote slow migrating forms of Smaug. Western blotting analysis of C18 cells that transiently express HA-Smaug alone, with SMO^{WT}-GFP or SMO^{PKA-SD}-GFP, in presence or in absence of HH, as indicated. (U): untransfected cells. GMAP serves as a loading control. Here and in (B,C), The black arrows indicate the unphosphorylated form of Smaug and the brackets indicate the phosphorylated forms of SMO-HA that have slower migration properties.

(B) Phosphatase treatment suppresses the Smaug slow migrating bands. Extracts of C18 cells expressing either HA-Smaug alone, or with SMO^{WT}-GFP / SMO^{PKA-SD}-GFP (as indicated) in the presence of HH, were analyzed by Western blotting after being treated with a λ phosphatase (λ phos) or with a phosphatase inhibitor (Phos Inh) under the same conditions.

(C) Smaug phosphorylation requires its interaction with SMO. Western blotting analysis of C18 cells expressing HA-Smaug, with SMO^{PKA-SD}-HA, SMO^{PKA-SD} Δ 978-HA or with SMO ^{Δ 958}-HA as indicated, in presence of HH.

(D) Identification of potential phosphosites. Potential phosphorylation sites identified by LC-MS/MS of C18 cells transfected with HA-Smaug with SNAP-SMO^{PKA-SD} in presence of HH. Only hits in a peptide with high confidence level (FRD>1%) and with a PTM score above 30% are shown. Among them three are in the M region (grey boxes). No site was identified in the SSR1 and SSR2 region. In red: phosphosites identified in this study, in blue: phosphosites also reported in a large scale analysis of the fly embryo proteome (Zhai et al., 2008). See also Fig. S3C.

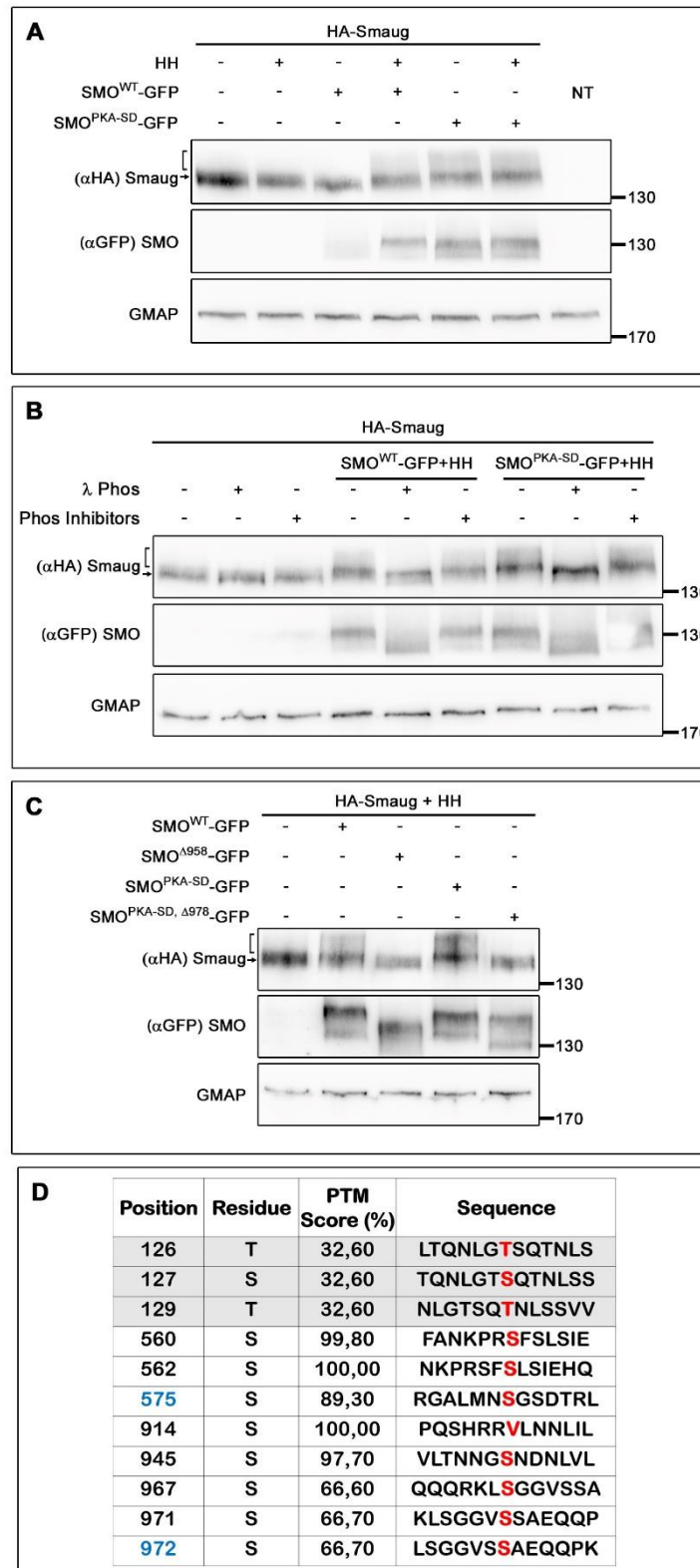


Figure 3 Argüelles, Bruzzone et al.

Figure 4: SMO/HH regulates Smaug levels and activity.**(A) Smaug repression assay.**

This assay is based on the dual expression of a construct encoding a λ N-SNAP-HA-Smaug chimeric protein (written here λ N-SNAP-Smaug for simplicity) and a second which transcription leads to an mRNA (called *SNAP-GUS-5BoxB*) carrying a translational fusion between the SNAP and the glucuronidase (GUS, from *A. thaliana*) coding regions followed by five Box B hairpins (5BoxB) inserted in the 3' untranslated region (UTR). A plasmid encoding a GFP-SNAP fusion is used as transfection and loading control. See also Fig. S3A-B

(B-C) Smaug downregulates the reporter expression.

Relative levels of the reporter expression (SNAP-GUS/GFP-SNAP ratio) in absence (black) and in presence of λ N-SNAP-Smaug (red), λ N-SNAP (pale grey) or SNAP-Smaug (dark grey) in transfected Cl8 cells. See also Fig. S3A-B.

(D-E-F) HH/SMO reduces the effect of Smaug repressing effects Smaug levels.

Relative levels of the reporter expression (estimated as above) (D) or of Smaug λ N-SNAP-Smaug (E), both reported to GFP-SNAP in transfected Cl8 cells in absence of SMO constructs (red) or in presence of SMO^{WT}-HA (green), SMO^{PKA-SD}-HA (blue) or SMO ^{Δ 958}-HA (yellow), Plain boxes: without HH, striped boxes in presence of HH. In (F) the reporter's levels were plotted against the levels of Smaug.

All assays were done as biological triplicates and were independently reproduced at least twice. Statistical analysis was done by a Kruskal-Wallis test by *ranks* followed by a Dunn test after pooling two independent triplicates; p values as indicated,

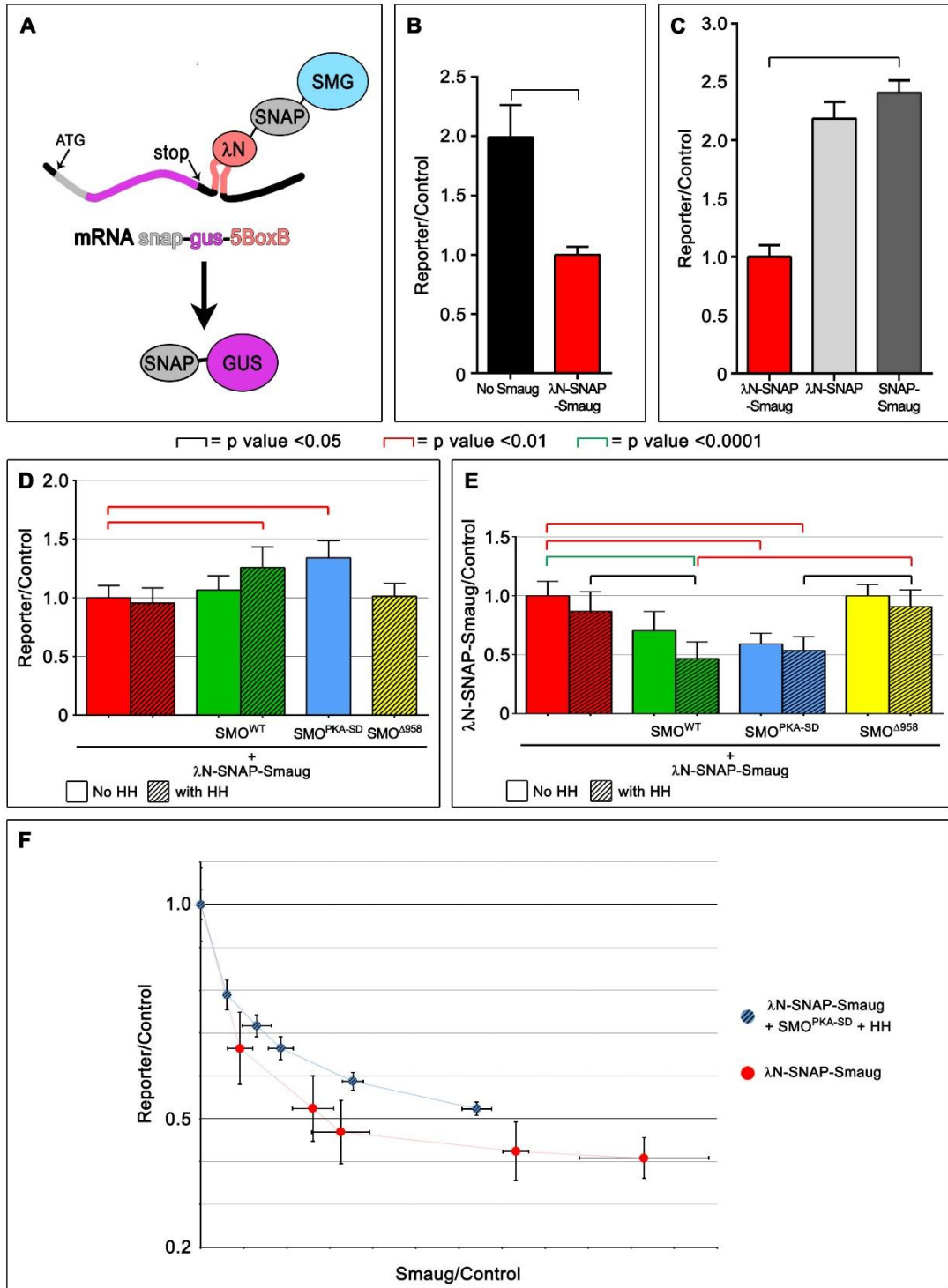


Figure 4 Argüelles, Bruzzone et al.

BIBLIOGRAPHY

- Aikin, R. A., Ayers, K. L. and Therond, P. P.** (2008). The role of kinases in the Hedgehog signalling pathway. *EMBO Rep* **9**, 330-336.
- Amadei, G., Zander, M. A., Yang, G., Dumelie, J. G., Vessey, J. P., Lipshitz, H. D., Smibert, C. A., Kaplan, D. R. and Miller, F. D.** (2015). A Smaug2-Based Translational Repression Complex Determines the Balance between Precursor Maintenance versus Differentiation during Mammalian Neurogenesis. *J Neurosci* **35**, 15666-15681.
- Anderson, C. W., Baum, P. R. and Gesteland, R. F.** (1973). Processing of adenovirus 2-induced proteins. *J Virol* **12**, 241-252.
- Aviv, T., Lin, Z., Lau, S., Rendl, L. M., Sicheri, F. and Smibert, C. A.** (2003). The RNA-binding SAM domain of Smaug defines a new family of post-transcriptional regulators. *Nat Struct Biol* **10**, 614-621.
- Ayers, K. L. and Therond, P. P.** (2010). Evaluating Smoothed as a G-protein-coupled receptor for Hedgehog signalling. *Trends Cell Biol* **20**, 287-298.
- Badodi, S., Dubuc, A., Zhang, X., Rosser, G., Da Cunha Jaeger, M., Kameda-Smith, M. M., Morrissy, A. S., Guilhamon, P., Suetterlin, P., Li, X. N., et al.** (2017). Convergence of BMI1 and CHD7 on ERK Signaling in Medulloblastoma. *Cell Rep* **21**, 2772-2784.
- Baez, M. V. and Boccaccio, G. L.** (2005). Mammalian Smaug is a translational repressor that forms cytoplasmic foci similar to stress granules. *J Biol Chem* **280**, 43131-43140.
- Baez, M. V., Luchelli, L., Maschi, D., Habif, M., Pascual, M., Thomas, M. G. and Boccaccio, G. L.** (2011). Smaug1 mRNA-silencing foci respond to NMDA and modulate synapse formation. *J Cell Biol* **195**, 1141-1157.
- Barckmann, B. and Simonelig, M.** (2013). Control of maternal mRNA stability in germ cells and early embryos. *Biochim Biophys Acta* **1829**, 714-724.
- Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P. and Izaurralde, E.** (2006). mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev* **20**, 1885-1898.
- Benoit, B., He, C. H., Zhang, F., Votruba, S. M., Tadros, W., Westwood, J. T., Smibert, C. A., Lipshitz, H. D. and Theurkauf, W. E.** (2009). An essential role for the RNA-binding protein Smaug during the Drosophila maternal-to-zygotic transition. *Development* **136**, 923-932.
- Besse, F. and Ephrussi, A.** (2008). Translational control of localized mRNAs: restricting protein synthesis in space and time. *Nat Rev Mol Cell Biol* **9**, 971-980.
- Brechbiel, J. L. and Gavis, E. R.** (2008). Spatial regulation of nos is required for its function in dendrite morphogenesis. *Curr Biol* **18**, 745-750.
- Bullock, S. L.** (2011). Messengers, motors and mysteries: sorting of eukaryotic mRNAs by cytoskeletal transport. *Biochem Soc Trans* **39**, 1161-1165.
- Chen, C. H., von Kessler, D., Park, W., Wang, B., Ma, Y. and Beachy, P. A.** (1999). Nuclear trafficking of Cubitus interruptus in the transcriptional regulation of Hedgehog target gene expression. *Cell* **98**, 305-316.
- Chen, L., Dumelie, J. G., Li, X., Cheng, M. H., Yang, Z., Laver, J. D., Siddiqui, N. U., Westwood, J. T., Morris, Q., Lipshitz, H. D., et al.** (2014a). Global regulation of mRNA translation and stability in the early Drosophila embryo by the Smaug RNA-binding protein. *Genome Biol* **15**, R4.
- Chen, Z., Holland, W., Shelton, J. M., Ali, A., Zhan, X., Won, S., Tomisato, W., Liu, C., Li, X., Moresco, E. M., et al.** (2014b). Mutation of mouse Samd4 causes leanness, myopathy, uncoupled mitochondrial respiration, and dysregulated mTORC1 signaling. *Proc Natl Acad Sci U S A* **111**, 7367-7372.
- Claret, S., Sanial, M. and Plessis, A.** (2007). Evidence for a novel feedback loop in the Hedgehog pathway involving Smoothed and Fused. *Curr Biol* **17**, 1326-1333.
- de Haro, M., Al-Ramahi, I., Jones, K. R., Holth, J. K., Timchenko, L. T. and Botas, J.** (2013). Smaug/SAMD4A restores translational activity of CUGBP1 and suppresses CUG-induced myopathy. *PLoS Genet* **9**, e1003445.

- Degrauwe, N., Suva, M. L., Janiszewska, M., Riggi, N. and Stamenkovic, I.** (2016). IMPs: an RNA-binding protein family that provides a link between stem cell maintenance in normal development and cancer. *Genes Dev* **30**, 2459-2474.
- Formstecher, E., Aresta, S., Collura, V., Hamburger, A., Meil, A., Trehin, A., Reverdy, C., Betin, V., Maire, S., Brun, C., et al.** (2005). Protein interaction mapping: a Drosophila case study. *Genome Res* **15**, 376-384.
- Gehring, N. H., Kunz, J. B., Neu-Yilik, G., Breit, S., Viegas, M. H., Hentze, M. W. and Kulozik, A. E.** (2005). Exon-junction complex components specify distinct routes of nonsense-mediated mRNA decay with differential cofactor requirements. *Mol Cell* **20**, 65-75.
- Gotze, M. and Wahle, E.** (2014). Smaug destroys a huge treasure. *Genome Biol* **15**, 101.
- Jeske, M., Meyer, S., Temme, C., Freudenreich, D. and Wahle, E.** (2006). Rapid ATP-dependent deadenylation of nos mRNA in a cell-free system from Drosophila embryos. *J Biol Chem* **281**, 25124-25133.
- Jeske, M., Moritz, B., Anders, A. and Wahle, E.** (2011). Smaug assembles an ATP-dependent stable complex repressing nos mRNA translation at multiple levels. *Embo J* **30**, 90-103.
- 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.
- Lenzken, S. C., Achsel, T., Carri, M. T. and Barabino, S. M.** (2014). Neuronal RNA-binding proteins in health and disease. *Wiley Interdiscip Rev RNA* **5**, 565-576.
- Luchelli, L., Thomas, M. G. and Boccaccio, G. L.** (2015). Synaptic control of mRNA translation by reversible assembly of XRN1 bodies. *J Cell Sci* **128**, 1542-1554.
- Malpel, S., Claret, S., Sanial, M., Brigui, A., Piolot, T., Daviet, L., Martin-Lannere, S. and Plessis, A.** (2007). The last 59 amino acids of Smoothened cytoplasmic tail directly bind the protein kinase Fused and negatively regulate the Hedgehog pathway. *Dev Biol* **303**, 121-133.
- Nakano, Y., Nystedt, S., Shivdasani, A. A., Strutt, H., Thomas, C. and Ingham, P. W.** (2004). Functional domains and sub-cellular distribution of the Hedgehog transducing protein Smoothened in Drosophila. *Mech Dev* **121**, 507-518.
- Nelson, M. R., Leidal, A. M. and Smibert, C. A.** (2004). Drosophila Cup is an eIF4E-binding protein that functions in Smaug-mediated translational repression. *Embo J* **23**, 150-159.
- Niu, N., Xiang, J. F., Yang, Q., Wang, L., Wei, Z., Chen, L. L., Yang, L. and Zou, W.** (2017). RNA-binding protein SAMD4 regulates skeleton development through translational inhibition of Mig6 expression. *Cell Discov* **3**, 16050.
- Parker, R. and Sheth, U.** (2007). P bodies and the control of mRNA translation and degradation. *Mol Cell* **25**, 635-646.
- Pascual, M. L., Luchelli, L., Habif, M. and Boccaccio, G. L.** (2012). Synaptic activity regulated mRNA-silencing foci for the fine tuning of local protein synthesis at the synapse. *Communicative & integrative biology* **5**, 388-392.
- Pinder, B. D. and Smibert, C. A.** (2013a). microRNA-independent recruitment of Argonaute 1 to nos mRNA through the Smaug RNA-binding protein. *EMBO Rep* **14**, 80-86.
- (2013b). Smaug: an unexpected journey into the mechanisms of post-transcriptional regulation. *Fly (Austin)* **7**, 142-145.
- Rehwinkel, J., Behm-Ansmant, I., Gatfield, D. and Izaurralde, E.** (2005). A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. *RNA* **11**, 1640-1647.
- Rouget, C., Papin, C., Boureux, A., Meunier, A. C., Franco, B., Robine, N., Lai, E. C., Pelisson, A. and Simonelig, M.** (2010). Maternal mRNA deadenylation and decay by the piRNA pathway in the early Drosophila embryo. *Nature* **467**, 1128-1132.
- Sanial, M., Becam, I., Hofmann, L., Behague, J., Arguelles, C., Gourhand, V., Bruzzone, L., Holmgren, R. A. and Plessis, A.** (2017a). 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.
- (2017b). Dose dependent transduction of Hedgehog relies on phosphorylation-based feedback between the GPCR Smoothened and the kinase Fused. *Development*.

- Semotok, J. L., Cooperstock, R. L., Pinder, B. D., Vari, H. K., Lipshitz, H. D. and Smibert, C. A.** (2005). Smaug recruits the CCR4/POP2/NOT deadenylase complex to trigger maternal transcript localization in the early *Drosophila* embryo. *Curr Biol* **15**, 284-294.
- Semotok, J. L., Luo, H., Cooperstock, R. L., Karaiskakis, A., Vari, H. K., Smibert, C. A. and Lipshitz, H. D.** (2008). *Drosophila* maternal Hsp83 mRNA destabilization is directed by multiple SMAUG recognition elements in the open reading frame. *Mol Cell Biol* **28**, 6757-6772.
- Smibert, C. A., Lie, Y. S., Shillinglaw, W., Henzel, W. J. and Macdonald, P. M.** (1999). Smaug, a novel and conserved protein, contributes to repression of nos mRNA translation in vitro. *Rna* **5**, 1535-1547.
- Tadros, W., Goldman, A. L., Babak, T., Menzies, F., Vardy, L., Orr-Weaver, T., Hughes, T. R., Westwood, J. T., Smibert, C. A. and Lipshitz, H. D.** (2007). SMAUG is a major regulator of maternal mRNA destabilization in *Drosophila* and its translation is activated by the PAN GU kinase. *Dev Cell* **12**, 143-155.
- Thomas, M. G., Loschi, M., Desbats, M. A. and Boccaccio, G. L.** (2011). RNA granules: the good, the bad and the ugly. *Cell Signal* **23**, 324-334.
- Vardy, L. and Orr-Weaver, T. L.** (2007). Regulating translation of maternal messages: multiple repression mechanisms. *Trends Cell Biol* **17**, 547-554.
- Zaessinger, S., Busseau, I. and Simonelig, M.** (2006). Osk allows nos mRNA translation in *Drosophila* embryos by preventing its deadenylation by Smaug/CCR4. *Development* **133**, 4573-4583.
- Zhai, B., Villen, J., Beausoleil, S. A., Mintseris, J. and Gygi, S. P.** (2008). Phosphoproteome analysis of *Drosophila melanogaster* embryos. *J Proteome Res* **7**, 1675-1682.
- Zhu, A. J., Zheng, L., Suyama, K. and Scott, M. P.** (2003). Altered localization of *Drosophila* Smoothed protein activates Hedgehog signal transduction. *Genes Dev* **17**, 1240-1252.

SUPPLEMENTARY MATERIAL

Global PBS	Prey genes (258 hits)
A	2 ^{§ *} ,
B	3 ^{**}
C	7 ^{***}
D	26 ^{***}
Total	38

Table Sup: Yeast two-hybrid screen of potential SMO interactors

A total 258 hits were found that corresponding to 44 genes that were classified following their PBS score (column 4) A in red, B in blue, C in green, D in black. Note that the E preys are not shown in this table as considered being false positive.

§ includes Smaug which was found as a prey 137 times.

* Several partners were previously shown to interact with SMO as Cut up (CTP) (*) which was also found as an interactor of SMO in an independent proteomic screen (Giot et al., 2003) and FU (**) which is a known partner of SMO (Monnier et al., 1998) *** includes proteins found in previous two-hybrid screens with other members of the HH pathway as baits (Fused, Cubitus interruptus or Patched) screens were also found (3 in C and 3 in D) (Formstecher et al., 2005)

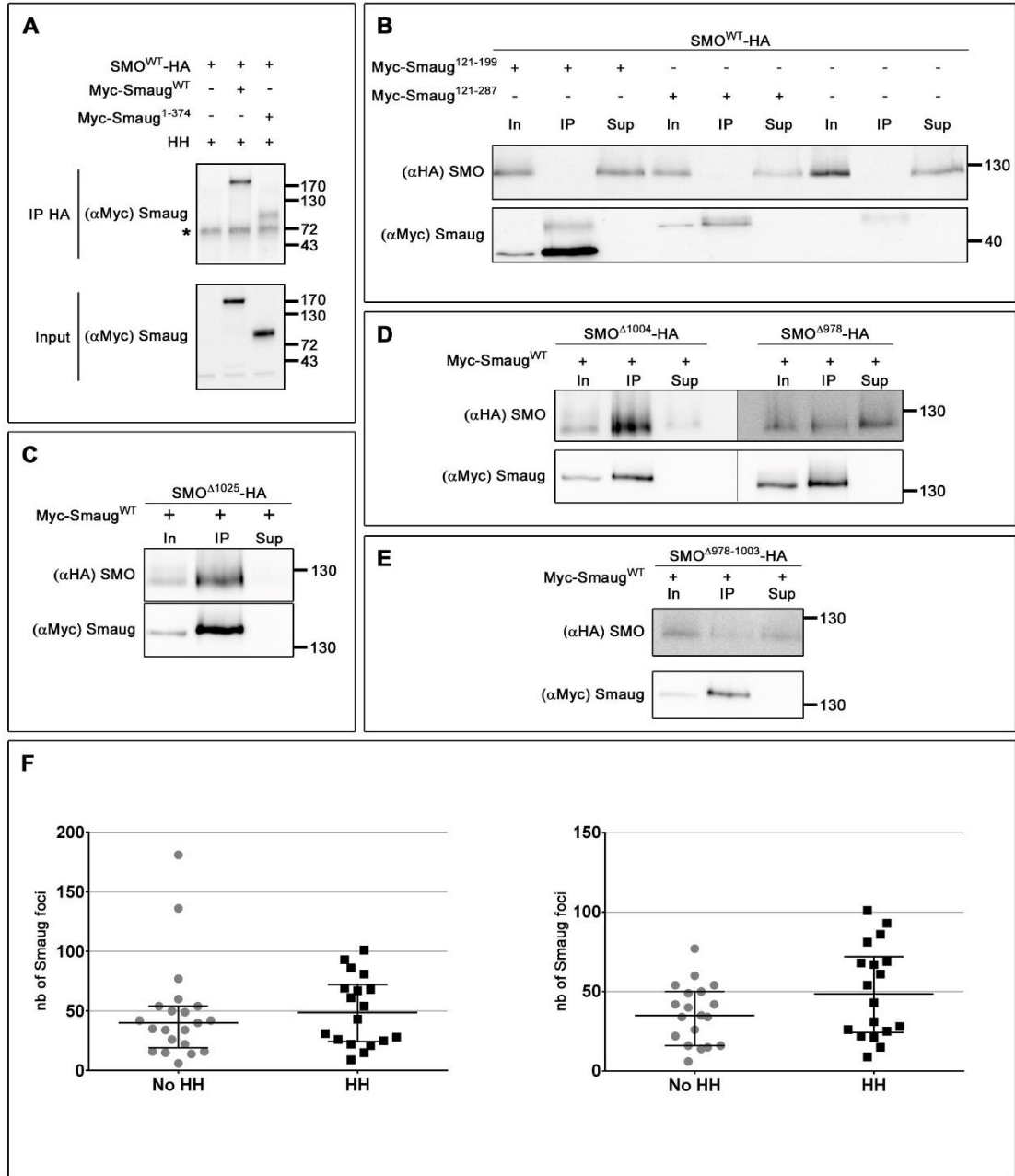


Figure S1 Argüelles, Bruzzone et al.

Figure S1: Smaug-SMO interaction and localization.

(A-B) Extracts from transfected Cl8 cells expressing SMO^{WT}-HA with Myc-Smaug constructs (Myc-Smaug¹⁻³⁷⁴, Myc-Smaug¹²¹⁻²⁸⁷ or Myc-Smaug¹²¹⁻¹⁹⁹) were immunoprecipitated with an anti-HA (A) or an anti-Myc (B) antibody prior to their analysis by Western blotting with anti-Myc (A) or anti-HA (B) antibodies respectively. In: input (In, before immunoprecipitation), IP: immunoprecipitated (beads), Sup: supernatant (after immunoprecipitated). The samples loaded in the In and Sup lanes are equivalent to a twentieth of volume loaded for the IP. * indicates background due to the detection of the primary antibody used for the IP. Myc-Smaug¹⁻³⁷⁴ and Myc-Smaug¹²¹⁻²⁸⁷ interacted with SMO^{WT}-HA but not Myc-Smaug¹²¹⁻¹⁹⁹.

(C-E) Extracts from transfected Cl8 cells expressing Myc-Smaug with different SMO-HA constructs (SMO^{Δ1025}-HA, SMO^{Δ1004}-HA, SMO^{Δ978}-HA or SMO^{Δ978-1003}-HA) immunoprecipitated with an anti-Myc antibody before Western blotting with anti-HA (upper panels) or anti-Myc antibodies (lower panels). SMO^{Δ978-1003}-HA and SMO^{Δ978}-HA poorly coimmunoprecipitated with Myc-Smaug but the interaction was not affected with SMO^{Δ1004}-HA.

(F) Analysis of the foci distribution.

Left: The distribution of the number of RFP-Smaug foci per cell was estimated using Imaris after confocal 3D imaging of the entire cell volume (left). n=21 cells (-HH) and n= 18 (+HH). The median was slightly higher in the presence of HH. Right: The distribution after elimination of the outlayers using reiterative CRUBS tests (with $\alpha=0.05$), shows that the median value (50 percentile) in presence of HH corresponds to the 75 first percentile in absence of HH. However this effect did not seem to be statistically significant (using a Mann Witheny two tailed test with $p<0,05$)

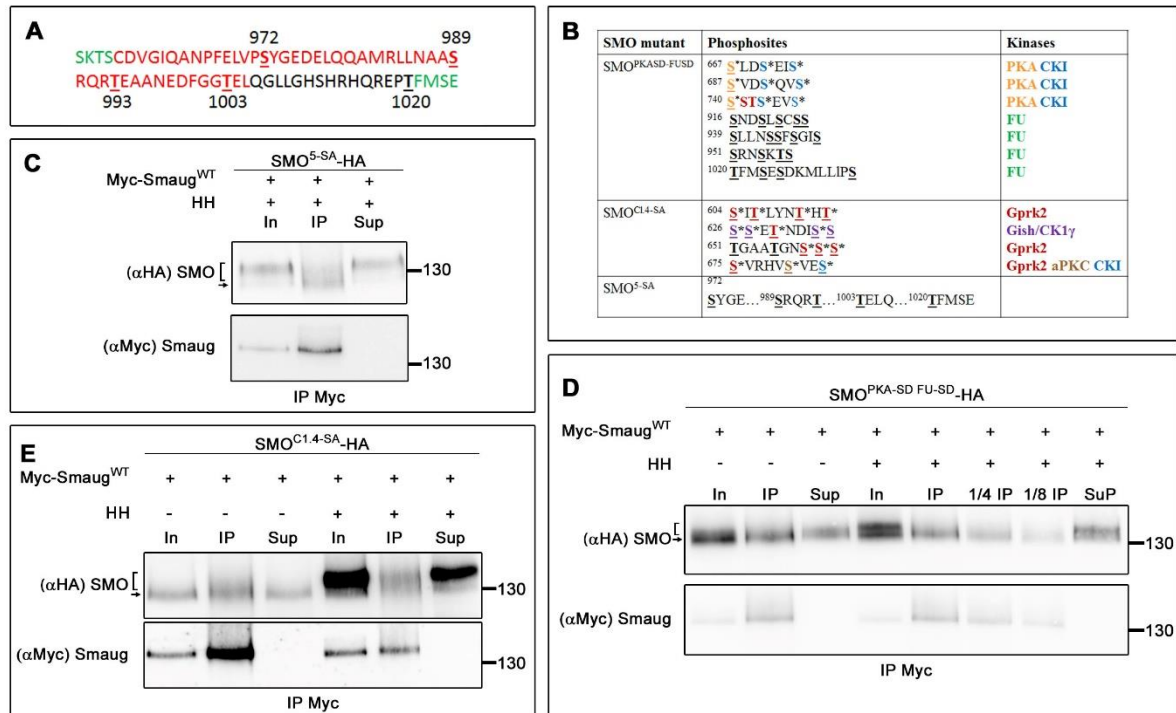


Figure S2 Argüelles, Bruzzone et al.

Figure S2: Interaction of SMO phosphomimetic mutant with Smaug.

(A) The sequence of the region of SMO that interacts with Smaug (red) contains 4 putative S/T phosphosites and is imbedded in two of the four clusters of S/T that we previously showed to be phosphorylated in response to HH, likely by FU (Sanial et al., 2017). The residues that were replaced by A in SMO^{c1.4-SA} are underlined.

(B) SMO phosphomutants with the corresponding phosphosites and kinases.

The underlined S/T residues were mutated into D (for SMO^{PKA-SD FU-SD}) or into A (SMO^{5-SA} and SMO^{c1.4-SA}) as indicated.

In orange: PKA sites, blue: CK1, green: FU sites, purple: Gish sites, brown: GrprK2 sites, grey: apKC sites. * phosphosites previously identified by Mass Spectrometry (Maier et al., 2014; Zhang et al., 2004).

(C-E) Extracts of Cl8 cells expressing, SMO^{5S-A}-HA **(C)**, SMO^{PKA-SD FU-SD}-HA (with 21 S/T replacements) **(D)** or SMO^{c1.4-SA} (with 17 S/T into A) with Myc-Smaug, in absence (-) or presence (+) of HH were IP with an anti-Myc antibody before analysis by Western blot with anti-HA (upper panel), or anti-Myc antibodies (lower panel). SMO^{PKA-SD FU-SD}-HA still coimmunoprecipitated with Myc-Smaug. However, its HH-induced phosphorylation (on other sites) still precluded its interaction with Smaug. SMO^{5-SA}-HA and SMO^{c1.4-SA}-HA were also mostly present as phosphorylated forms that poorly coimmunoprecipitated with Myc-Smaug. The black arrows indicate the unphosphorylated form of SMO and the brackets indicate the phosphorylated forms of SMO-HA that have slower migration properties.

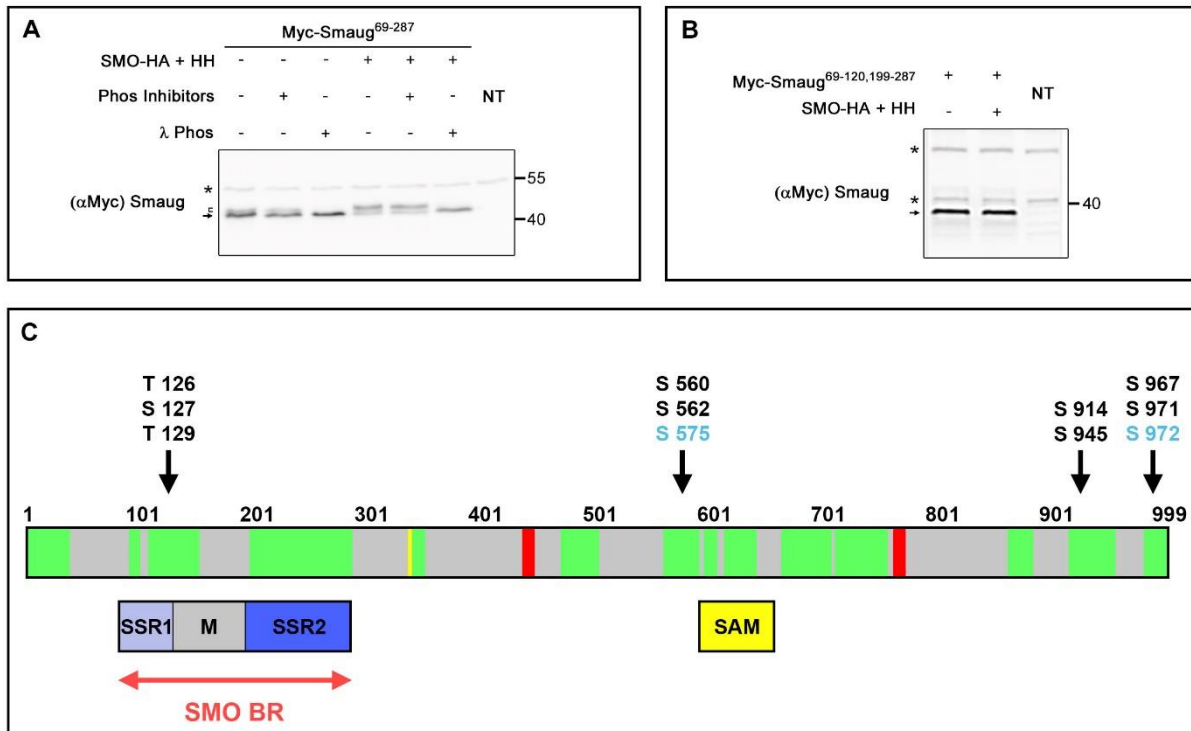


Figure S3 Argüelles, Bruzzone et al.

Figure S3: Smaug phosphorylation

(A-B) Western blotting of extracts of C18 cells that transiently express Myc-Smaug^{69-120, 200-287} or Myc-Smaug⁶⁹⁻²⁸⁷ alone or with SMO^{SD}-HA as indicated in presence of HH. Note that in presence of HH, the members of the HH signaling pathway (SNAP-SU(FU), COS2-CFP, GFP-FU, GFP-CI) were coexpressed. NT: non transfected.

(C) The regions of Smaug that were found in the LC-MS/MS analysis, are indicated in green (high confidence, FDR<0.01) and red (low confidence FDR<0.1). The SSR1 and 2 regions are indicated as blue boxes, the SAM domain as a yellow box and of the potential phosphosites are indicated on the top. The phosphosites found in this study are shown on the top with in blue the phosphosites that were reported in a large scale analysis of the fly embryo proteome (Zhai et al., 2008).

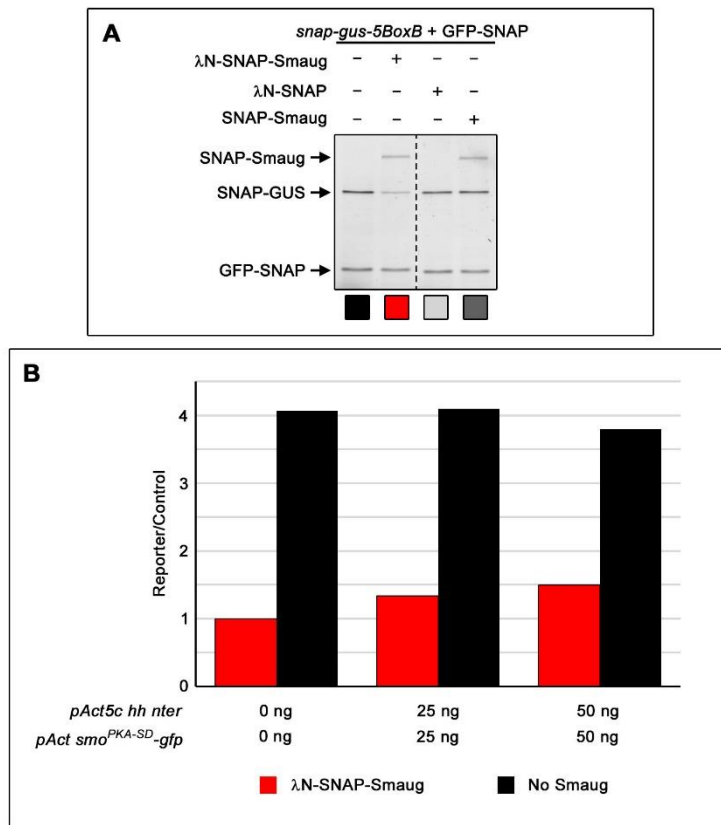


Figure S4 Argüelles, Bruzzone et al.

Figure S4: Smaug repression assay.

(A) Direct fluorescent imaging of an electrophoretic gel with extracts (labelled with a fluorescent SNAP substrate) of C18 cells that express the *snap-gus-5BoxB* reporter (encoding the SNAP-GUS protein), in absence (black box) or in presence of λ N-SNAP-Smaug (red), λ N-SNAP (pale grey) or SNAP-Smaug (dark grey). GFP-SNAP is used as a control for transfection and protein extraction efficiency to normalize the amounts of SNAP-GUS and SNAP-Smaug/ λ N-SNAP-Smaug. λ N-SNAP-Smaug, SNAP-GUS, GFP-SNAP simultaneously produced in this assay have distinct molecular weight (of respectively 137, 91 and 50 kDa, see Fig. S4A) and can therefore be simultaneously detected and quantified. The dashed line indicates that the central lanes of the gel were spliced.

(B) Relative levels of the reporter expression (estimated as above) or of Smaug λ N-SNAP-Smaug (reported to the levels of the GFP-SNAP control) in presence of λ N-SNAP-Smaug (red, levels of transfection) and different doses of HH/SMO as indicated.

SUP BIBLIOGRAPHY

- Formstecher, E., Aresta, S., Collura, V., Hamburger, A., Meil, A., Trehin, A., Reverdy, C., Betin, V., Maire, S., Brun, C., et al.** (2005). Protein interaction mapping: a Drosophila case study. *Genome Res* **15**, 376-384.
- Giot, L., Bader, J. S., Brouwer, C., Chaudhuri, A., Kuang, B., Li, Y., Hao, Y. L., Ooi, C. E., Godwin, B., Vitols, E., et al.** (2003). A protein interaction map of Drosophila melanogaster. *Science* **302**, 1727-1736.
- Maier, D., Cheng, S., Faubert, D. and Hipfner, D. R.** (2014). A broadly conserved g-protein-coupled receptor kinase phosphorylation mechanism controls Drosophila smoothed activity. *PLoS Genet* **10**, e1004399.
- Monnier, V., Dussillol, F., Alves, G., Lamour, I. C. and Plessis, A.** (1998). Suppressor of fused links fused and Cubitus interruptus on the hedgehog signalling pathway. *Curr Biol* **8**, 583-586.
- 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 GPCR Smoothed and the kinase Fused. *Development*.
- Zhai, B., Villen, J., Beausoleil, S. A., Mintseris, J. and Gygi, S. P.** (2008). Phosphoproteome analysis of Drosophila melanogaster embryos. *J Proteome Res* **7**, 1675-1682.
- Zhang, C., Williams, E. H., Guo, Y., Lum, L. and Beachy, P. A.** (2004). Extensive phosphorylation of Smoothed in Hedgehog pathway activation. *Proc Natl Acad Sci U S A* **101**, 17900-17907.

PART 2: OTHER RESULTS

As shown in the manuscript above, our results indicate that Smaug undergoes phosphorylation upon activation of HH signaling and that this event requires the interaction with an activated form of SMO in cultured Cl8 cells (manuscript, Fig 3). I thus decided to pursue the study of Smaug regulation by phosphorylation in order to understand how it occurs and what its role is. To this aim, I first sought to identify (i) the phosphorylated sites and (ii) the kinases involved in the process. To achieve my first goal, I used two approaches: tandem mass spectrometry and site directed mutagenesis. To achieve my second goal, I took advantage of the mass spectrometry approach, which also allows the identification of Smaug partners. Thus, I set up experimental conditions in order to identify simultaneously both Smaug phosphosites as well as its associated proteins.

I will first start by presenting my other results obtained on the identification of Smaug phosphosites and the partners found by mass spectrometry analysis. In addition, I will show my efforts on further identifying the kinase(s) implicated by testing the effect of two kinase candidates on Smaug phosphorylation by WB. I will then continue by presenting the obtained results on the characterization of Smaug phosphorylation by site directed mutagenesis. Finally, I will show my preliminary efforts on characterizing the effect of Smaug phosphorylation on (i) its ability to repress mRNA translation and (ii) its subcellular localization in cultured Cl8 cells.

I. Characterization of Smaug phosphorylation induced by HH signaling and the kinases in play

1. Identification of Smaug phosphorylated sites and protein partners

1.1 Identification of Smaug phosphosites by mass spectrometry

In order to characterize which are the phosphorylated residues upon HH induction, I overexpressed HA-tagged Smaug in Cl8 cells and purified it via HA-immunoprecipitation (IP). Then, in collaboration with the IJM institute's proteomics facility, we analyzed by tandem mass spectrometry (MS-MS) the immunoprecipitated fractions obtained from Cl8 cells expressing either HA-Smaug alone or with SMO^{PKA-SD} in presence of HH. More specifically, purified Smaug was digested with trypsin, which is a widely used protease that cleaves specifically at the carboxyl side of lysine (K) and arginine (R) residues. The resulting peptides were then separated by reverse-phase high-performance liquid chromatography (HPLC) and matched to UniProt sequence database. A second analysis (hence the term MS-MS), during which isolated peptides were fragmented and analyzed one at a time, led to the identification of Smaug peptides that are phosphorylated or not.

Smaug protein sequence contains 181 S/T, representing 18% of the protein residues. After testing different experimental conditions in 5 independent MS-MS experiments, we obtained a maximal coverage of 48% of Smaug protein (Fig. 26 A), including 84 out of the 181 S/T. I tried to obtain higher protein coverage by using different and/or multiple proteases but the resulting peptides were too small for MS-MS analysis. Altogether, our results led to the preliminary identification of 9 different Smaug peptides that contain potentially phosphorylated residues. Three of the nine phosphopeptides found (T³⁴⁵, T⁴⁴³, and S⁷⁶⁶) were identified only when Smaug was cotransfected with HH and SMO^{PKA-SD}, albeit being identified in low or medium confidence peptides (shown in blue, Fig. 26 B). Interestingly, the rest of the phosphopeptides were found either in absence or in presence and presence of HH/SMO^{PKA-SD}. These results indicate that Smaug is constitutively phosphorylated. Consequently, Smaug could be regulated by phosphorylation independently of the HH pathway or its activation.

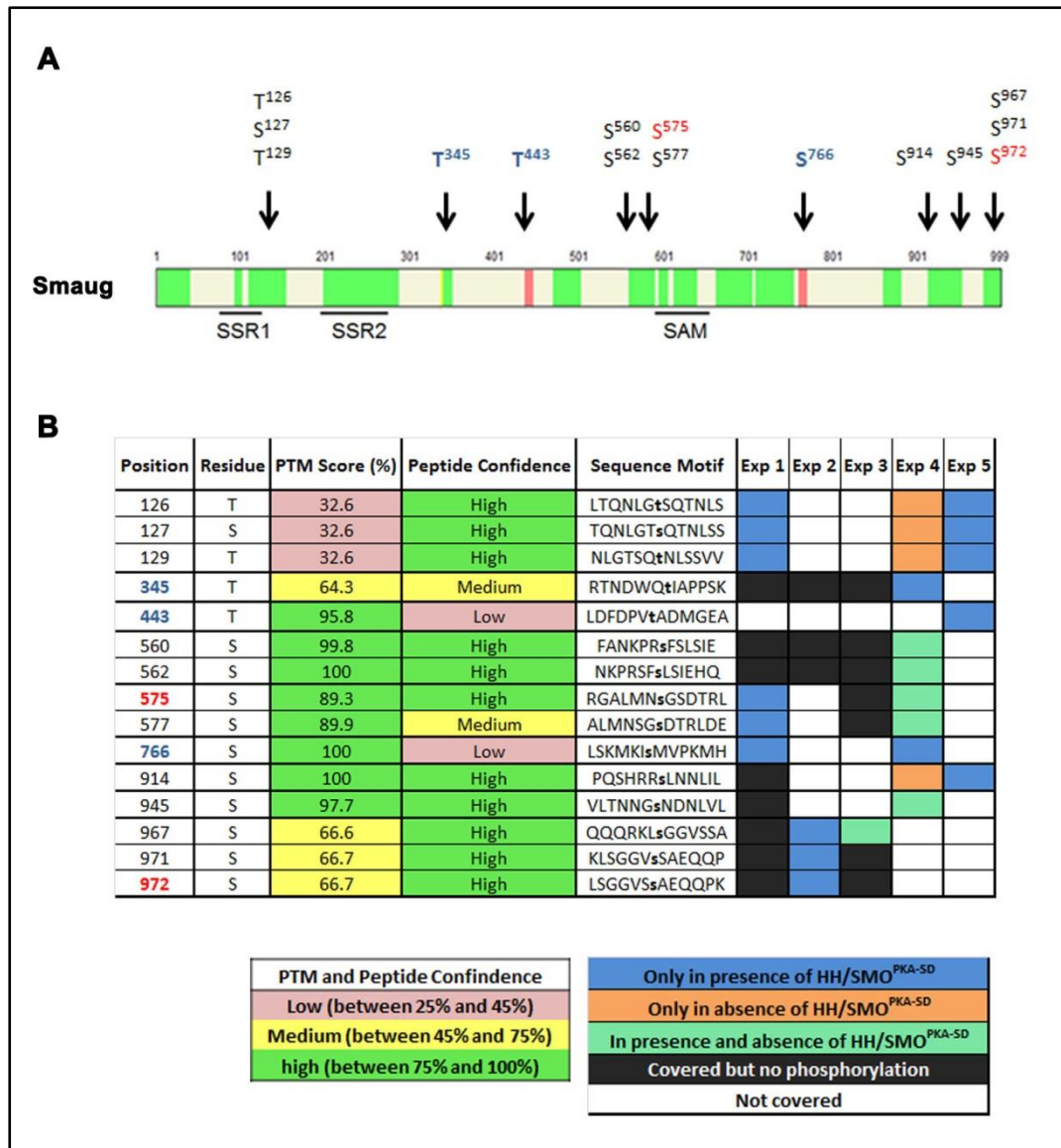


Figure 26. Identification of *Smaug* phosphosites by tandem mass spectrometry. *C18* cells were transfected with HA-*Smaug* in presence and absence of HH/SMO^{PKA-SD}. After HA-*Smaug* purification by IP and proteolytic digestion with trypsin, the resulting peptides were analyzed by MS-MS leading to the identification of *Smaug* phosphopeptides. (A) Schematic representation of *Smaug* protein coverage obtained by MS. Indicated above are the residues presenting the highest PTM score in each peptide found. (B) Table showing the totality of the phosphopeptides found in absence and/or presence of HH/SMO^{PKA-SD} after performing five independent MS experiments in which I tested different experimental conditions. Low, medium and high confidence peptides correspond to peptides found with a FDR (false discovery rate) lower than 10%, 5% or 1% respectively. Note that low and medium confidence peptides were not removed from the list since phosphorylations were identified in these peptides with a high score and could therefore be worth to investigate further. Residues present a PTM score that indicates the probability of the amino acid to carry the phosphorylation. Therefore, although the MS-MS can identify phosphorylated peptides with high accuracy, the exact location of the phosphorylated residue is not certain. Position and type of residue, peptide confidence and the sequence of the peptides found are shown. S⁵⁷⁵ and S⁹⁷² (shown in red) have previously been reported as phosphorylated residues in a large scale analysis of the fly embryo proteome (Zhai, Villen et al. 2008).

1.2 Identification of Smaug partners by quantitative mass spectrometry

As mentioned above, I also sought to identify Smaug protein partners and, more in particular, the kinases implicated in Smaug phosphorylation by quantitative mass spectrometry (MS) in collaboration with the IJM proteomics facility. I thus performed three independent biological replicates of HA-Smaug immunoprecipitation in presence and absence of HH/SMO^{PKA-SD}, as described above. As negative control, I used non-transfected cells, which allowed identification, and removal from the analysis, of the proteins that represented background noise from the coIP. Then, with Samia Miled and our collaborators, we identified Smaug interactors by label free quantitative MS analysis. After statistical analysis and filtration of the identified proteins that presented fold-enrichment higher than 2, we obtained 21 highly enriched proteins considered to be Smaug interactors (Table 2). Among these proteins was Not1, found both in presence and in absence of HH/SMO^{PKA-SD}, which is part of the CCR4-NOT deadenylase complex and a known partner of Smaug.

In addition, we found four Smaug interactors belonging to the Serine/Threonine kinase family, under conditions of HH pathway activation compared to the control. These protein kinases are Polo, Casein kinase alpha I (CKI α), Fused and Discs overgrown (Dco). The Polo kinase is known to regulate cell cycle and to play a role during mitosis and cytokinesis. CKI α is known to play a critical role in the HH pathway by regulating SMO activation by phosphorylation of its C-terminal region. Recently, our team has identified the kinase FU as necessary for the hyper-phosphorylation of SMO and the full activation of the HH pathway (Sanial, Becam et al. 2017). Dco, which belongs to the casein kinase I family, is involved in the regulation of circadian rhythms and it has also been linked to CI regulation (Price and Kalderon 2002). Furthermore, Dco was also found as an interactor of Smaug in absence of HH/SMO^{PKA-SD}, raising the possibility that it could regulate Smaug phosphorylation, independently of the activation of the HH pathway.

Finally, when we compared the proteins found in presence of HH/SMO^{PKA-SD} against the ones found in absence of HH/SMO^{PKA-SD}, we observed that FU was significantly enriched. Thus, FU could be bound to Smaug or interact indirectly via SMO. We tested these possibilities by performing a coIP from transfected Cl8 cells and we observed that FU

physically interacts with Smaug only in the presence of SMO (data not shown). Ergo, Smaug and FU interaction occurs via SMO.

HA-Smaug vs Control	HA-Smaug + HH/SMO ^{PKA-SD} vs Control	HA-Smaug + HH/SMO ^{PKA-SD} vs HA-Smaug
Abnormal spindle (Asp)	Abnormal spindle (Asp)	
	AP-2 complex alpha (AP-2 α)	
	AP complex 1-2 beta (AP-1-2 β)	AP complex 1-2 beta (AP-1-2 β)
Belphegor (Bor)	Belphegor (Bor)	
	Casein Kinase alpha I (CKI α)	
	Coatomer subunit beta (β COP)	
	Cytoplasmic FMR1-interacting protein (Sra-1)	Cytoplasmic FMR1-interacting protein (Sra-1)
Discs overgrown (Dco)	Discs overgrown (Dco)	
	Fused (FU)	Fused (FU)
Failed axon connections (Fax)		
G protein alpha i subunit	G protein alpha i subunit	
Histone H2A		
Not1	Not1	
Nucleoporin 358kD		
	Polo	
Rab1		
Regulatory particle non-ATPase 5 (Rpn5)	Regulatory particle non-ATPase 5	
Smaug (Smg)	Smaug	
	Smoothened (SMO)	Smoothened
Spectrin alpha (α -spec)	Spectrin alpha	
	40S ribosomal protein S5a (RpS5a)	
	CG12112-RA	CG12112-RA

Table 2. Identification of Smaug interactors by label free quantitative mass spectrometry
Extracts from Cl8 cells transiently transfected with HA-Smaug in presence and absence of HH/SMO^{PKA-SD} were immunoprecipitated with an anti-HA antibody. Smaug protein partners were analyzed by label free quantitative mass spectrometry. Proteins showing a fold-enrichment higher than 2 were considered to be associated with Smaug. Protein kinase candidates are shown in red and protein symbols are shown in brackets. Non-transfected cells were used as control. Statistical test: T-student, p-value<0,01. FDR<1%

In conclusion, any of the kinase candidates found could be playing a role in HH induced phosphorylation of Smaug. In order to further identify the kinase(s) implicated, a small RNAi screen against the candidates found using cultured Cl8 cells will be performed in the lab.

Given that the FU kinase is a known interactor of SMO, which has been well studied by our team, I took advantage of the tools we had in the lab and decided to assess FU involvement in Smaug phosphorylation.

1.3 Study of the FU kinase implication in Smaug phosphorylation

We have showed that Smaug binding region in SMO cytoplasmic tail is next to FU phosphorylation clusters and even overlaps with FU binding region (manuscript Fig. 1 D). Therefore, an attractive hypothesis is that the kinase FU could be implicated in Smaug phosphorylation upon interaction with SMO and activation of the HH pathway. To assess if FU is involved in Smaug phosphorylation, I decided to test the effect of a form of FU that is constitutively active (GAP-FU) as well as the effect of a form that lacks kinase activity (FU DANA) on Smaug phosphorylation by WB.

I thus cotransfected Cl8 cells with HA-Smaug in presence of the members of the HH transduction complex with Myc-FU WT, Myc-GAP-FU or RFP-FU-DANA and compared Smaug phosphorylated shift (Fig. 27).

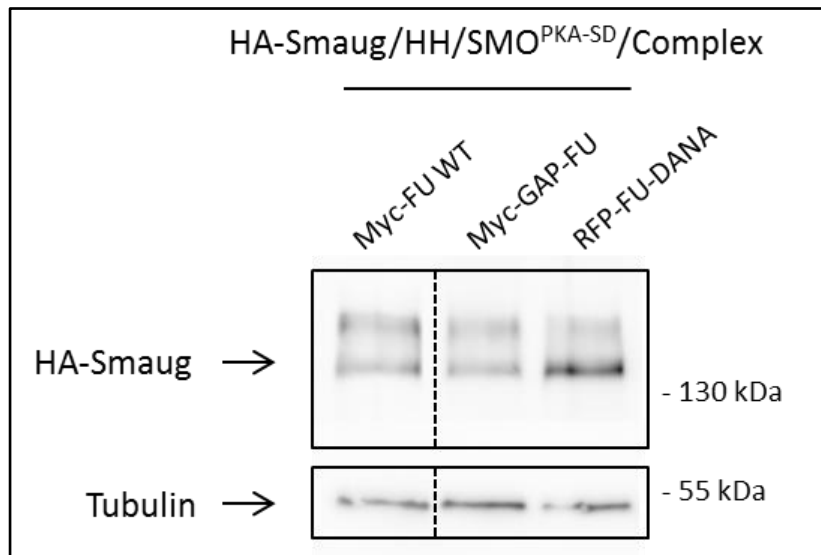


Figure 27. Study of the implication of the FU kinase in Smaug phosphorylation by Western Blot
 Extracts from transiently transfected Cl8 cells with HA-Smaug were analyzed by Western Blot using an anti-HA antibody. Smaug phosphorylated shift does not seem to be influenced by the constitutive activation of the FU kinase (Myc-GAP-FU) (lane 2). Nevertheless, when a kinase dead form of FU (RFP-FU-DANA) is coexpressed (lane 3), the ratios between Smaug phosphorylated and non-phosphorylated forms seem to be altered. Dot lines indicate that the different lanes belong to the same gel, which was cut for visualization purposes. Tubulin was used as loading control.

Similar Smaug retardation in migration was observed when we compared the effect of GAP-FU and FU WT. Thus, an active form of FU does not seem to induce changes in Smaug phosphorylated shift. However, when cells were cotransfected with FU-DANA, the ratio between phosphorylated and non-phosphorylated forms of Smaug varied, increasing the accumulation of the non-phosphorylated form. This could be due to the fact that FU-DANA has a negative effect on SMO by reducing its full activation (Claret, Sanial et al. 2007), which is in turn required for promotion of Smaug phosphorylation as described in the manuscript. Consequently, our results indicate that FU activation is not sufficient to induce Smaug phosphorylation, although it could affect it indirectly via regulation of SMO activation. In order to avoid a negative effect on SMO activation by FU-DANA, the experiment should be repeated cotransfecting cells with a form of SMO that mimics phosphorylation both in PKA/CKI and FU clusters (SMO^{PKA-SD FU-SD}) (Sanial, Becam et al. 2017).

1.4 Analysis of DOP kinase involvement in Smaug phosphorylation

Despite not being found as a partner of Smaug by MS analysis, another kinase candidate is the conserved kinase Drop out (DOP) which was identified, by our collaborators from the University of Toronto, as associated with Smaug by coIP using extracts from *Drosophila* embryos (unpublished data). Moreover, their results suggest that DOP kinase activity is required for Smaug-mediated recruitment of Ago1 to its target mRNAs (https://tspace.library.utoronto.ca/bitstream/1807/67310/3/Pinder_Benjamin_D_201211_PhD_Thesis.pdf).

To determine whether DOP is involved in Smaug phosphorylation, I studied the effect of DOP kinase on HH induced Smaug phosphorylated shift by WB in cultured Cl8 cells. HA-Smaug was cotransfected either with a wild-type form of DOP-HA or with a DOP-HA mutant that lacks the kinase domain (DOP-HA Δ KIN), in conditions of HH pathway activation or not (Fig. 28).

After WB analysis, we observed that overexpressing Smaug in presence of DOP-HA does not induce Smaug retarded shift (compare lanes 1 and 3 of first gel). Thus, we conclude that DOP-HA WT alone is not sufficient to promote Smaug phosphorylation. Nevertheless, it seems that DOP-HA overexpression has an effect on the ratio of Smaug isoforms in absence

or presence of HH (compare lanes 5 vs 7 and 6 vs 8 of first gel respectively). On the other hand, when the kinase dead form of DOP-HA was cotransfected, Smaug phosphorylated shift was still observed (Fig. 28, second gel).

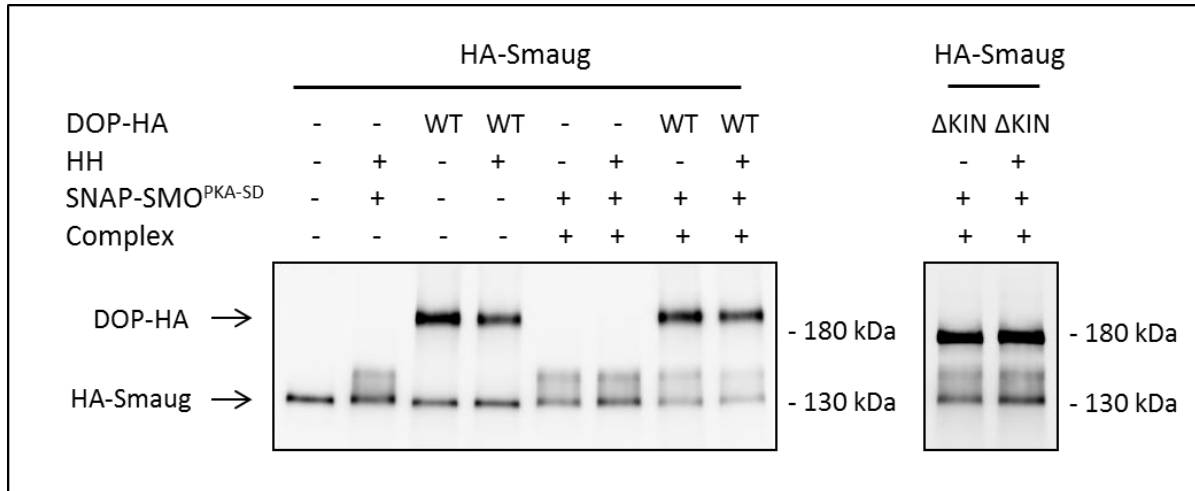


Figure 28. Study of Drop Out kinase implication in Smaug phosphorylation promoted by HH signaling
 Extracts from transiently transfected Cl8 cells were analysed by Western blot using an anti-HA antibody. HA-Smaug was cotransfected with two forms of DOP-HA kinase (WT and Δ KIN which lacks the kinase domain) in absence and presence of HH and/or SNAP-SMO^{PKA-SD} as well as other members of the HH pathway. Smaug phosphorylated shift is not induced by DOP overexpression (compare lanes 1 and 3 of first gel). When cells are cotransfected with DOP-HA Δ KIN, Smaug phosphorylated shift was still observed (shown in second gel). Members of the complex: Myc-FU, SUFU-Myc, Ci-GFP.

However, visualizing the difference between the ratios of Smaug isoforms can be limited by WB analysis. For this reason, in order to be able to quantify differences in the ratios, we should repeat the experiment overexpressing SNAP-tagged Smaug. Labeling of the SNAP-tag is irreversible and quantitative and can be easily detected by scanning in-gel fluorescence after gel electrophoresis.

2. Identification of Smaug phosphorylated regions by systematic site directed mutagenesis

Given that the MS-MS analysis led to an incomplete (only 48%) coverage of Smaug protein, I decided to pursue the identification of Smaug phosphosites by site directed mutagenesis.

2.1 Design of Smaug synthetic gene as strategy

As mentioned above, Smaug protein sequence is highly rich in S/T residues. Consequently, the high amount of S/T made the implementation of classical approaches of site-directed mutagenesis, such as by PCR, very difficult to implement. We therefore designed, in collaboration with M. Sanial, a synthetic gene in which all the codons encoding S or T were replaced by A codons.

Importantly, in order to prevent protein expression problems, the systematic S/T-A mutations were designed respecting the proportion of *Drosophila's* codon usage as well as avoiding mutations that would encode rare codons. As 181 S/T-A mutations would likely disrupt Smaug protein structure and folding, we decided to assess Smaug phosphorylation by mutating different fragments and therefore analyse a smaller number of residues at a time. To this aim, we generated multiple restriction sites by inserting silent mutations in Smaug WT and S/T-A Smaug mutant sequences. Thus, the amount of Smaug chimeres obtained was the result of the availability of suitable restriction sites.

I then built 11 different Smaug S/T-A chimeres in which short regions with S/T-A mutations were inserted in Smaug WT sequence by molecular cloning (Fig. 29 A). Note that the number of S/T differs among the different Smaug regions, being regions 1, 5 and 7 the ones with a higher number of S/T (containing 28, 38 and 26 S/T respectively) (Fig. 29 B and C).

In order to study the function of Smaug phosphorylation in response to HH signaling first in cultured C18 cells and then in the fly, I decided to build a Smaug construct resistant to RNAi. To do so, I used RNAi target sequences that had been created and validated by the TRiP (Transgenic RNAi Project) fly stocks (fgr.hms.harvard.edu). I thus selected two different RNAi target sequences (GL00406 and HSM04335) that did not present any RNAi off targets and built two different pUAS vectors encoding them. RNAi GL00406 targets 21 nucleotides located between Smaug SSR1 and SSR2 while RNAi HSM04335 targets 21 nucleotides present in the SAM domain (Fig. 30 A). In order to confer RNAi resistance to either GL00406 or HSM04335 RNAi, I inserted silent mutations in Smaug WT sequence by PCR and confirmed them by sequencing.

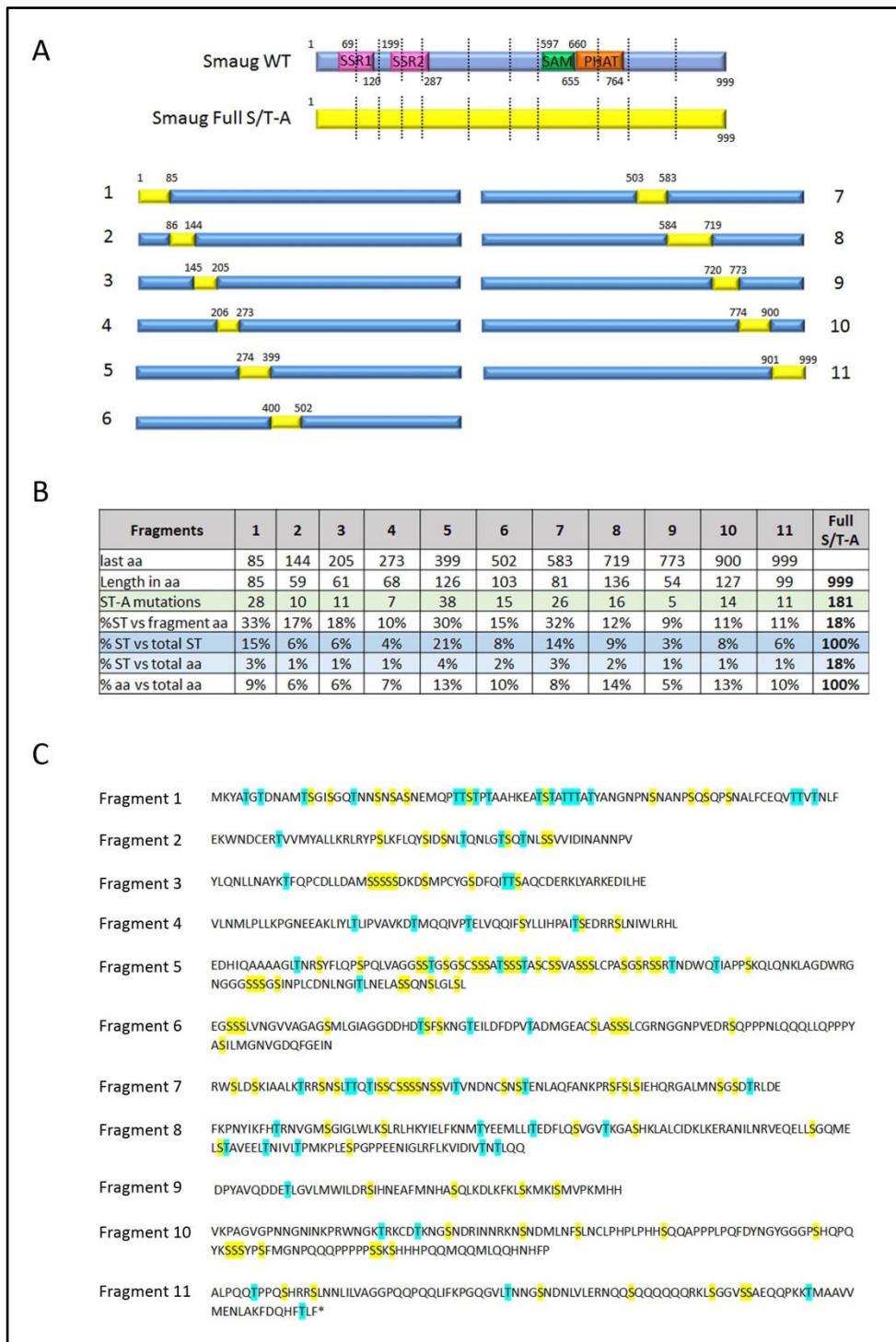


Figure 29. Smaug gene synthesis strategy

(A) Schematic representation of Smaug S/T-A chimeric constructs. All Smaug S/T residues were systematically mutated into A codons in Smaug S/T-A. The resulting 11 chimers (numbered from 1 to 11) are shown, each of them containing a different mutated region after cloning the corresponding S/T-A sequences in Smaug WT sequence. All Smaug chimers were sequenced. Blocks in yellow and blue represent the S/T-A mutations and wild-type (WT) sequence respectively. (B) Table showing the length of the different chimers as well as the amount of S/T-A mutations, and the percentage it represents. (C) Sequences of the 11 Smaug fragments obtained after enzymatic restriction are shown. All the S and T that were mutated into A codons are highlighted. Fragments 1, 5 and 7 contain stretches highly rich in S/T.

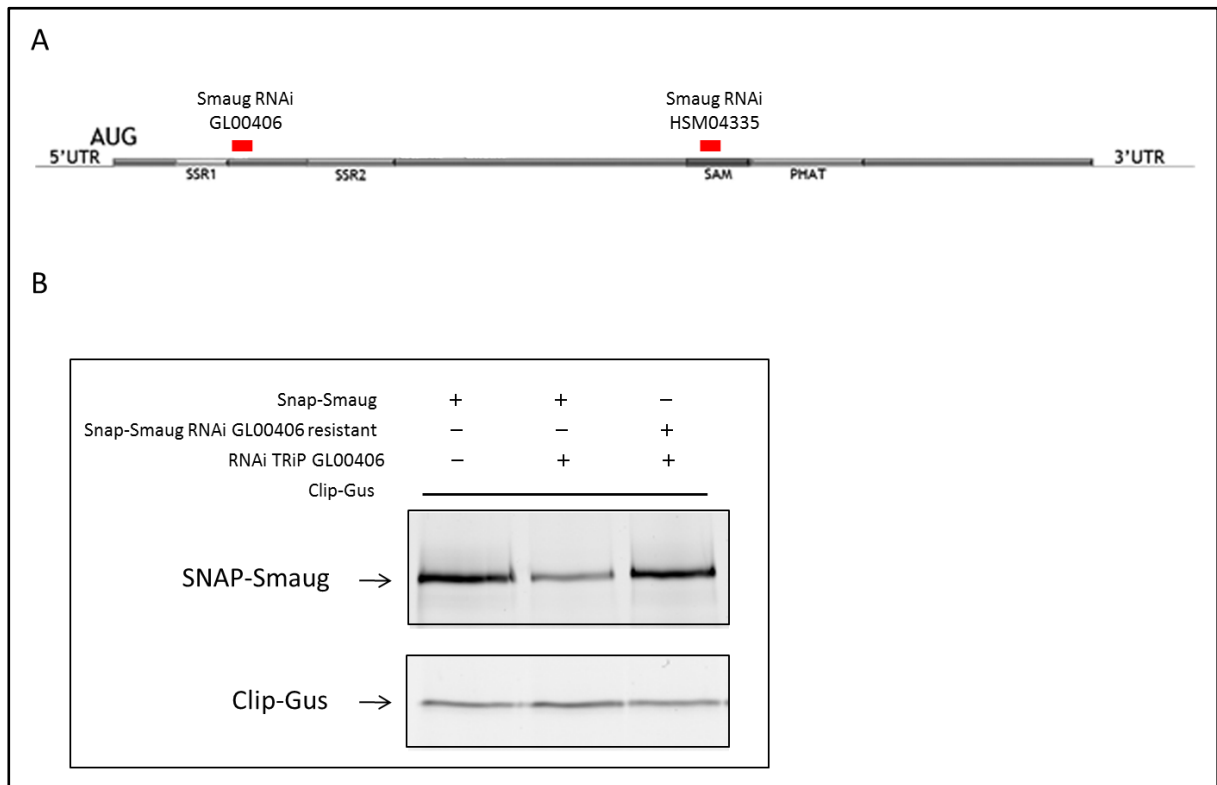


Figure 30. Test of Smaug downregulation by RNAi in Cl8 cells

(A) Representation of Smaug mRNA sequence and the target sequences of RNAi GL00406 and RNAi HSM04335. (B) Extracts from transiently transfected Cl8 cells with SNAP-Smaug in presence and absence of GL00406 RNAi were analysed by gel electrophoresis. SNAP-Smaug expression is decreased when cells express RNAi GL00406 (compare lanes 1 and 2). Expression of SNAP-Smaug that contains GL00406 target sequence mutated is not altered after RNAi treatment, thus confirming RNAi resistance (lane 3). Clip-Glucuronidase (Clip-Gus) was used as loading control.

I first tested whether SNAP-Smaug WT expression was downregulated by the RNAi TriP GL00406 encoding vector in Cl8 cells (Fig. 29 B). We observed that Smaug WT expression was decreased when cells were cotransfected with the RNAi encoding vector (lane 2). In addition, I sought to see whether the Smaug vector that contains RNAi GL00406 target sequence mutated was resistant to RNAi treatment. Expression of SNAP-Smaug mutant from cells treated with GL00406 RNAi was similar to the one from cells expressing only Smaug WT (compare lanes 1 and 3). These results indicate that SNAP-Smaug carrying the silent mutations is indeed resistant to GL00406 RNAi. Note that expression of HSM04335 RNAi also downregulates Smaug expression (data not shown). Smaug resistance to HSM04335 will be tested in a similar fashion.

Finally, I also built a Smaug construct that contains both GL00406 and HSM04335 RNAi target sequences mutated (called Smaug R2R) as well as all the inserted restriction sites. Importantly, Smaug R2R ability to repress mRNA translation was not altered compared to Smaug WT (data not shown). Having this tool will allow us to test the function of Smaug phosphorylated regions in response to HH signaling *in vitro* and *in vivo*, while removing the effect of endogenous Smaug.

2.2 Characterization of Smaug phosphorylated regions in response to HH signaling

In order to identify the phosphorylated regions involved, I analyzed HH induced phosphorylation of the Smaug chimers by WB. I thus cotransfected Cl8 cells with the different chimers tagged with HA, in absence and presence of HH and SMO^{PKA-SD} (Fig. 31).

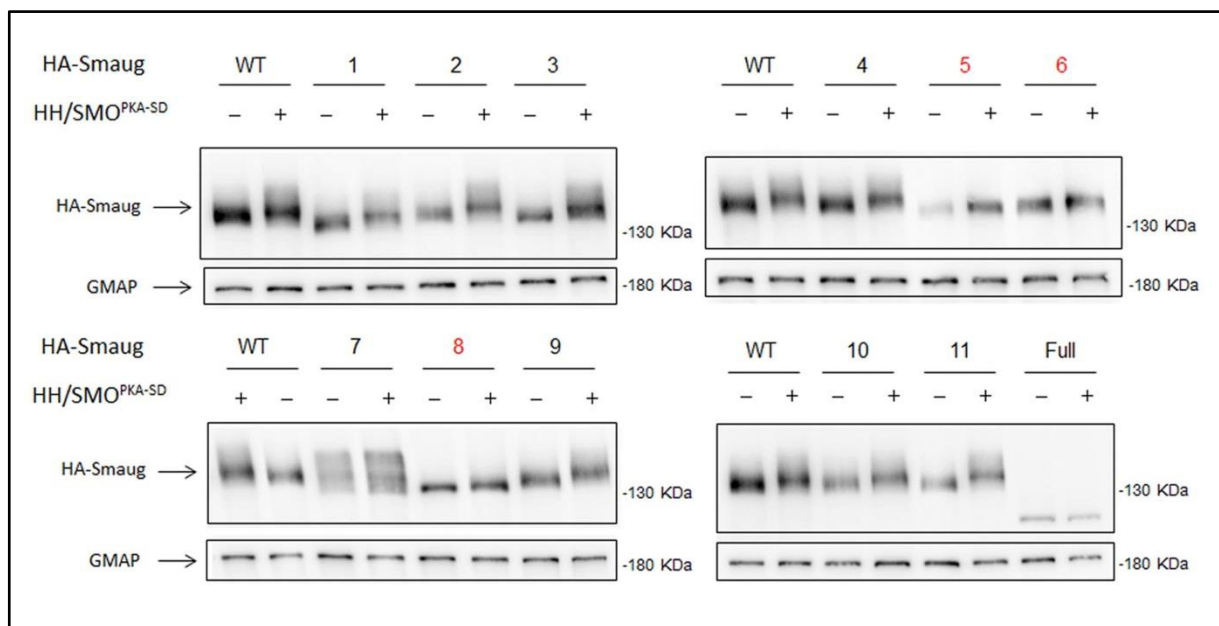


Figure 31. Identification of Smaug phosphorylated regions by site-directed mutagenesis
 Extracts from transfected Cl8 cells overexpressing HA-Smaug WT or each of the HA-tagged Smaug chimers with (+) or without (-) HH/SMO^{PKA-SD} were analyzed by WB. Smaug regions 5, 6 and 8 (shown in red) show a total loss of HH induced phosphorylated shift. Note that region 7 has gained a constitutive phosphorylated shift in absence of HH/SMO^{PKA-SD}. Smaug full, which represents the global mutations of Smaug S/T into A, has a theoretical molecular weight (MW) of 105 kDa and migrates as a protein of lower MW compared to Smaug WT or the rest of the chimers. Gmap was used as loading control.

On the one hand, we observed that S/T-A mutations in regions 5, 6 and 8 led to a total loss of Smaug phosphorylated shift in presence of HH/SMO^{PKA-SD}. Therefore, we can infer that regions 5, 6 and 8 are required for HH promotion of Smaug phosphorylation. On the other hand, Smaug region 7 S/T-A mutations led to the appearance of a shift in absence and in presence of HH/SMO^{PKA-SD}. This constitutive shift was lost after treatment with λ phosphatase (data not shown), which is an enzyme that removes phosphates. Thus, this result indicates that phosphorylation in region 7 normally blocks other Smaug phosphorylation events. Refining of the mapping of these phosphorylated regions is currently underway by a new M1 student in the lab.

What is more, protein migration of Smaug S/T-A mutants 5, 6 and 8 was also altered in absence of HH/SMO^{PKA-SD}, when compared to Smaug WT. Smaug mutant 8 migrates as a protein of considerably lower molecular weight compared to Smaug WT. This is particularly interesting since region 8 corresponds to the SAM domain and part of the PHAT, which are involved in RNA binding and protein interaction. This could mean that Smaug is constitutively regulated by phosphorylation in absence of HH pathway activation, although we cannot exclude at this stage that these results may reflect a folding problem promoted by the mutations inserted.

3. Smaug is constitutively phosphorylated

First, I sought to determine whether Smaug is constitutively phosphorylated by WB analysis. In order to achieve this, extracts from transfected Cl8 cells with Myc-Smaug were treated either with λ -phosphatase or with phosphatase inhibitors under the same experimental conditions of temperature (30°C) and incubation time (30 min). We thus compared the retardation in the migration of each condition to the untreated condition. Extracts were then run in a polyacrylamide gel containing Phos-Tag (Fig. 32), which allows specific separation of phosphorylated proteins based on the levels of phosphorylation (Kinoshita, Kinoshita-Kikuta et al. 2009).

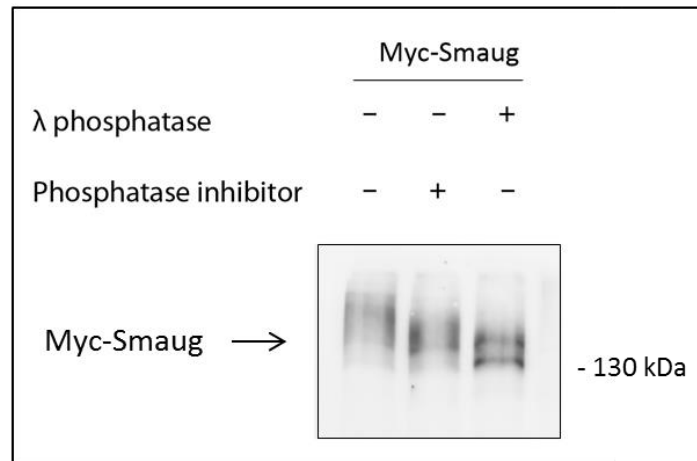


Figure 32. Smaug is constitutively phosphorylated

Extracts from transiently transfected Cl8 cells with Myc-Smaug were treated with λ phosphatase, phosphatase inhibitor or not treated. Samples were run in a 20 μ M Phos-tag gel and analysed by Western Blot. After phosphatase treatment, Smaug smear disappears (lane 3) and Smaug appears to migrate as a doublet.

We observed that, in absence of treatment, Myc-Smaug presents a smear of bands (lane 1). After treating the extract with phosphatase, Smaug smear disappeared (lane 3) thus confirming Smaug constitutive phosphorylation. Moreover, the treatment with phosphatase led to a clear visualization of Smaug doublet. This doublet might be due to a different post-translational modification of Smaug or it could also be the result of an impediment of the phosphatase to access the phosphate groups. Finally, prevention of dephosphorylation by phosphatase inhibitors showed a smear of bands, albeit less important than the one observed in the non-treated extract. This could be the result of degradation after the incubation at 30°C. Thus, given our current results, it seems that Smaug is constitutively phosphorylated independently of the HH pathway activation.

II. Functional characterization of Smaug constitutive phosphorylation in cultured Cl8 cells

In this section, I will present my preliminary results on the functional characterization of Smaug constitutive phosphorylation in cultured Cl8 cells. I focused on studying the effect in Smaug ability to repress mRNA translation as well as in Smaug capacity to form cytoplasmic structures known as S-foci.

1. Study of the role of Smaug constitutive phosphorylation

Smaug constitutive phosphorylation could play a role in the regulation of Smaug mRNA repressive activity and/or control Smaug accumulation levels. I therefore decided to test these possibilities by studying the Smaug S/T-A chimeric mutants.

1.1 Phosphorylation of the SAM domain downregulates Smaug protein levels and upregulates Smaug mRNA repressive activity

To this aim, I used the SNAP-tagged reporter system developed in the lab, which is described in the manuscript present above (Fig. 4 A). This *in vitro* repression assay is based on the tethering of a λ N/5BoxB dual system in which the λ N protein, that is fused to SNAP-Smaug, recognizes and binds the 5BoxB sequences located in the *snap-glucuronidase-5BoxB* (*snap-gus-5BoxB*) mRNA reporter 3' UTR. SNAP-tag covalently binds to a fluorescent dye leading to its self-labeling which can be easily detected and quantified after gel electrophoresis without Western Blotting. Thus, this assay allows us to measure both, Smaug repressive activity as well as to quantify Smaug protein levels, by detecting SNAP-tag emission of fluorescence. An advantage that the SNAP-tagged assay presents compared to a regular luciferase reporter assay is that it correlates the repressive activity measured to the protein repressor amounts in the system. Importantly, experimental conditions were set up in order to respect the linear range of the repression assay, leading to a reporter repression directly proportional to Smaug levels. After quantification, the reporter levels and Smaug levels were normalized to GFP-SNAP, which was used as transfection and loading control.

I thus transfected cultured Cl8 cells with Smaug WT and the different S/T-A chimeric Smaug mutants -all fused to λ N-SNAP at the N-terminal region and analyzed their expression levels as well as their repressive activity by measuring SNAP-GUS reporter expression (Fig. 33 A and B). After performing the experiment in triplicate, no significant difference was observed between the reporter levels in Smaug WT condition versus Smaug chimers 1 to 11. However, SNAP GUS levels were significantly higher when the Smaug S/T-A full mutant was expressed compared to Smaug WT, reaching a reporter expression similar to the one obtained in absence of Smaug. Moreover, Smaug S/T-A full accumulation levels were

significantly reduced presenting a 3-fold downregulation compared to Smaug WT levels. Hence, these results indicate that the totality of 181 S/T-A mutations in Smaug S/T-A full leads to an low levels of Smaug protein which lacks any repressive activity.

Interestingly, after building a new construct carrying the S/T-A only in the SAM domain (Smaug^{SAM}), we observed that Smaug levels were significantly reduced compared to Smaug WT. However, the reporter mRNA continued to undergo translational repression (compare grey and red bars in Fig. 33 A and B). I then decided to test the effect of the reduction of Smaug^{SAM} levels in Smaug repressive activity. To do so, I repeated the repression assay using increasing doses of Smaug WT and Smaug^{SAM} expressing vectors, and quantified the levels of reporter expression in function of the levels of Smaug proteins variant (Fig. 33 C). Surprisingly, we observed that, for the same amount of Smaug protein, the expression levels of the reporter were systematically lower in Smaug^{SAM} mutant condition, indicating that Smaug^{SAM} possess a higher repressive activity than Smaug WT. Thus, prevention of phosphorylation in the SAM domain leads to lower Smaug^{SAM} protein levels but Smaug^{SAM} is more repressive in comparison to Smaug WT.

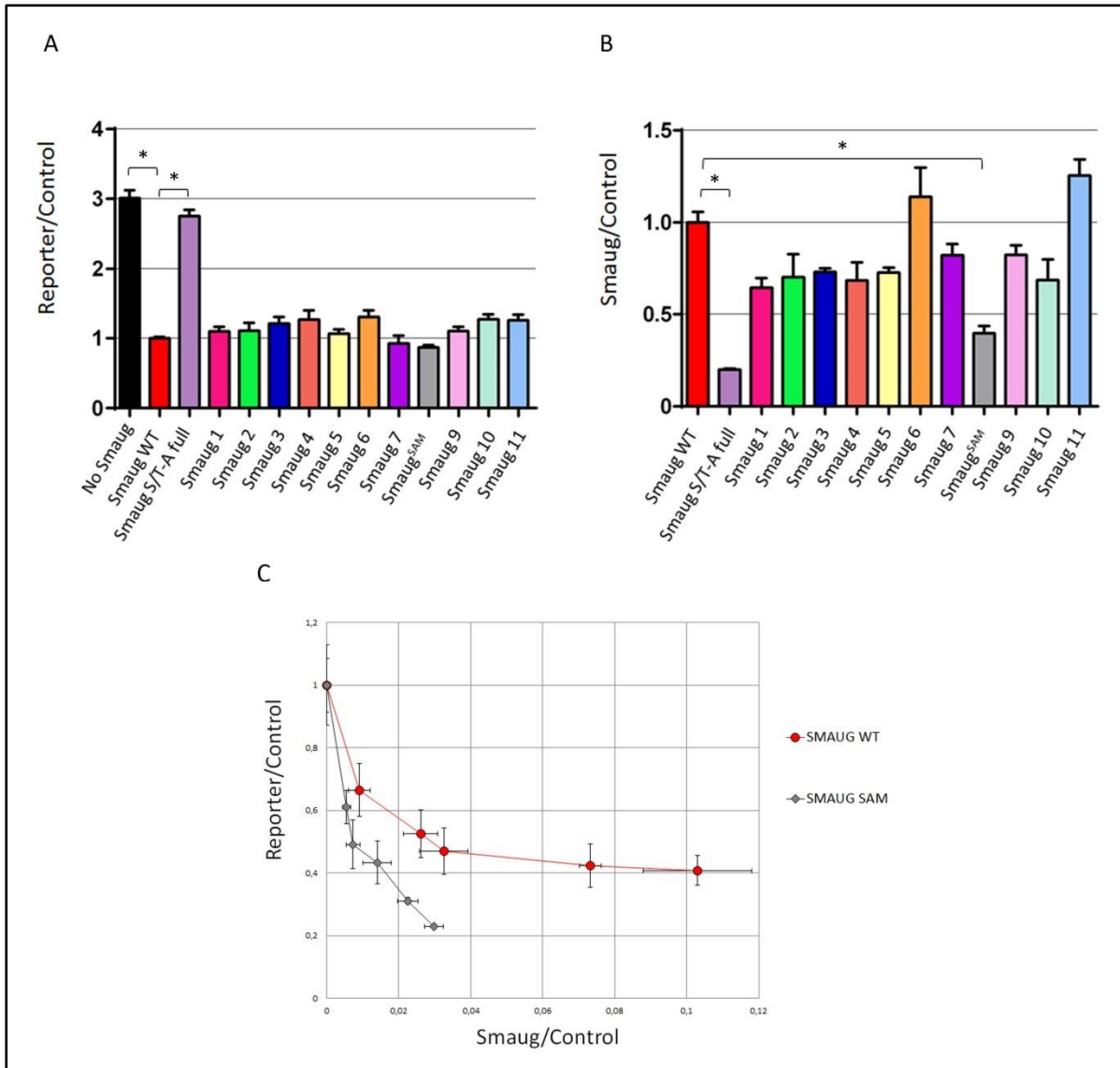


Figure 33. Prevention of Smaug SAM domain phosphorylation decreases Smaug levels and upregulates Smaug mRNA repressive activity.

Results from three independent biological replicas are shown. C18 cells overexpressing SNAP-GUS reporter were cotransfected with Smaug WT and with different S/T-A Smaug chimeres (S/T-A full, and Smaug chimeres 1 to 11). (A) Relative levels of the reporter expression (measured as the ratio of SNAP-GUS on the control GFP-SNAP) in different Smaug chimeric overexpression conditions. Smaug WT expression led to a three-fold downregulation of SNAP-GUS reporter levels (red) when compared to the reporter expression in absence of Smaug (black) and the one of Smaug S/T-A full (light purple). (B) Relative Smaug expression levels (measured as the ratio of Smaug on the control GFP-SNAP) in different Smaug chimeric overexpression conditions. Smaug S/T-A and Smaug^{SAM} protein levels are significantly lower than Smaug WT levels. (C) Reporter expression levels were plotted against Smaug WT and Smaug^{SAM} protein levels.

All Smaug proteins are fused to λ N-SNAP at their N-terminal region, albeit not being shown in the graphs for visualization purposes. GFP-SNAP was used as loading and transfection control. Statistic analysis was performed by the non-parametric one-way ANOVA Kruskal Wallis test and comparison of ranks was achieved by Dunn's test. P -value < 0,05

To test whether the blockage of SAM domain phosphorylation induces protein instability, Smaug^{SAM} half-life will be tested and compared to Smaug WT. On the other hand, we will look for a transcriptional effect by measuring *ΔN-snap-smaug wt* and *ΔN-snap-smaug^{SAM}* mRNA levels by quantitative PCR. Further characterization of the residues that are constitutively phosphorylated within the SAM domain is currently underway in the lab.

1.2 Phosphorylation of Smaug SAM domain regulates S-foci formation

We have shown that Smaug forms cytoplasmic foci, known as S-foci, in cultured Cl8 cells (manuscript, Fig. 2). Thus, a possible scenario is that constitutive phosphorylation in Smaug SAM domain could influence Smaug subcellular localization. To test this possibility, I decided to study the subcellular localization of GFP-Smaug^{SAM} by confocal microscopy.

I thus transfected Cl8 cells with GFP-tagged versions of Smaug WT as well as Smaug chimeras that carry S/T-A mutations in the regions 5, 6, 7 and the SAM domain (Fig. 34).

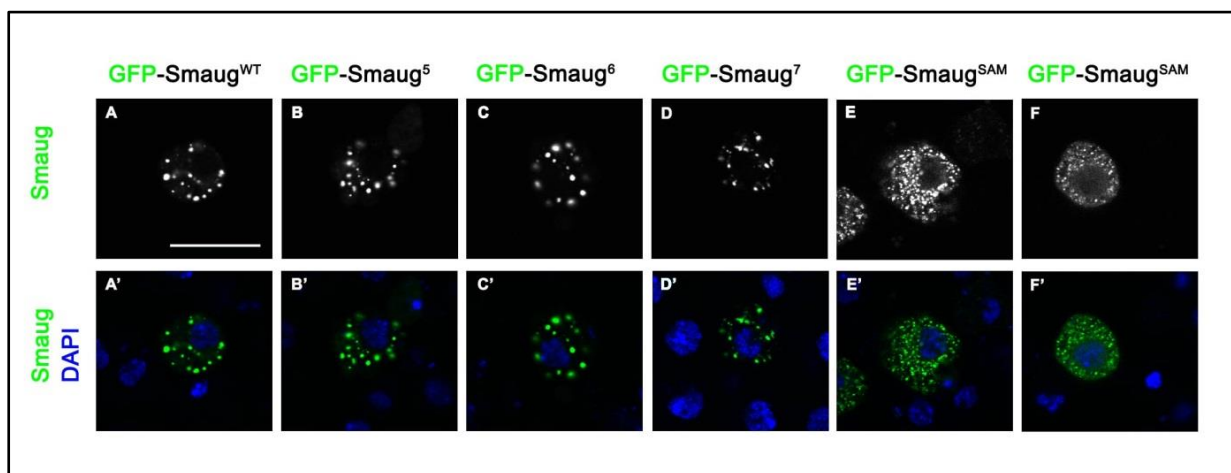


Figure 34. Prevention of Smaug SAM domain phosphorylation promotes the formation of smaller and more abundant cytoplasmic foci.

63X confocal images of Cl8 cells transfected with GFP-SMG WT (A), GFP-Smaug 5 (B), GFP-Smaug 6 (C), GFP-Smaug 7 (D) and GFP-Smaug^{SAM} (E and F) are shown. The merge images showing the nuclei staining by DAPI are presented below (A' to F'). Smaug WT, 5, 6 and 7 form discrete cytoplasmic foci while Smaug^{SAM} induces the formation of smaller and more abundant structures. GFP staining in the nucleus is observed in cells overexpressing GFP-Smaug^{SAM} (F and F'). The scale bar represents 20 μ m. Experiments were done twice with similar results.

Results showed that GFP-Smaug 5 and 6 form similar S-foci as the GFP-Smaug WT. On the other hand, it appears that GFP-Smaug 7 induces the formation of smaller punctuate structures when compared to Smaug WT form. In order to confirm such effect, further studies quantifying the number and size of S-foci will be done. Finally, GFP-Smaug^{SAM} induced the formation of smaller and highly abundant cytoplasmic foci compared to GFP-Smaug WT, indicating that constitutive phosphorylation in the SAM domain could play a role in Smaug assembly properties. If so, regulation could take place by modulating the interaction with Smaug target mRNAs and/or protein partners. Refining of the phosphosites implicated in Smaug S-foci formation is currently underway in the lab.

Last but not least, confocal images of GFP-Smaug^{SAM} showed some GFP staining in the nucleus (Fig. 34 F and F'). This result raises the possibility that phosphorylation of the SAM domain could play a role in Smaug subcellular localization.

DISCUSSION

I. SMO/Smaug interaction

We provided the first evidence for a physical interaction between Smaug and SMO cytoplasmic tail based on the combination of a yeast two-hybrid screen and colP in cultured Cl8 cells. This interaction is the first example of a partner of Smaug that is not an mRNA regulatory protein. Moreover, it is the first indication that Smaug could be regulated by a signaling pathway and that HH signaling could be connected to post-translational regulations. Whatever, this interaction involves the N-terminal conserved regions SSR1 and SSR2 of Smaug, and the C-terminal region of SMO, near where FU binds and phosphorylates SMO. Note that, although Smaug and SMO partially colocalize in punctuate structures in the WID, it will be important to confirm their interaction *in vivo*, by performing colP from extracts of embryos or WID.

1. How is the interaction between Smaug and SMO regulated?

We have shown that Smaug interacts with the non or low phosphorylated forms of SMO. However, it seems that Smaug/SMO interaction is negatively regulated by hyperphosphorylation of SMO C-terminal region in response to HH. By testing various phosphodeficient and phosphomimetic mutants, we concluded that none of the residues of SMO that are known to be phosphorylated by PKA/CKI, FU, GRK2, Gish or aPKC were responsible for the inhibition of Smaug/SMO interaction. It would however be interesting to test a SMO mutant that mimics all these phosphorylations to determine whether the combination of all these phosphorylation events inhibits the interaction with Smaug. It could also be due to phosphorylation at unidentified residues of the kinases mentioned or by a novel kinase that remains to be characterized. Thus, other kinase candidates that could be tested are Dco or the Polo kinase, which were found to be associated with Smaug during our mass spectrometry analysis in conditions of HH pathway activation. Furthermore, we could evaluate whether hyperphosphorylation of Smaug regulates interaction with SMO.

In addition, given that both Smaug and SMO are constitutively phosphorylated proteins, it would also be interesting to test whether their constitutive phosphorylation is required for Smaug/SMO interaction. We could therefore test whether interaction is affected when both proteins are dephosphorylated by treating cell extracts with phosphatase prior to the coIP.

2. What are the nature and the dynamics of the foci containing Smaug and SMO?

Subcellular localization studies indicate that Smaug and SMO colocalize in cytoplasmic foci, both in absence and presence of HH, in a manner that depends on the interaction between Smaug and SMO. Remarkably, upon reception of HH signal, SMO recruits Smaug at the plasma membrane in CI8 cultured cells.

SMO has been described to be localized in cell trafficking vesicles positive for endocytic markers such as Rab5 or Rab7 and ESCRT (Yang, Mao et al. 2013) whereas Smaug is localized in granules containing untranslated mRNAs as well as multiple RNA binding proteins such as Aub (Rouget, Papin et al. 2010) and the CCR4-NOT deadenylase complex (Semotok, Cooperstock et al. 2005, Zaessinger, Busseau et al. 2006). Another interactor of Smaug involved in mRNA silencing is Ago1 (Pinder and Smibert 2013). Consequently, SMO and Smaug are not expected to have the same subcellular localization and yet they colocalize in cytoplasmic foci. Smaug/SMO interaction thus raises the question whether SMO recruits Smaug alone or Smaug and its known interactors in these foci. In order to determine this, we could analyze colocalization between Smaug, SMO and other proteins related to P-bodies that are present in S-foci such as Ago1, Aub, CCR4, DCP1A, and XRN1.

In addition, mammalian Smaug1 is known to form reversible RNP granules which contain translationally repressed mRNAs in neurons (Baez and Boccaccio 2005). Smaug1 responds to NMDA receptor activation (Baez, Luchelli et al. 2011) leading to a rapid and reversible dissolution of Smaug foci thus allowing translation of the silenced mRNAs. In order to further investigate the function of Smaug/SMO interaction, we could analyze the effect of HH/SMO signaling on S-foci dynamics by FRAP experiments in CI8 cells. We could also study the effect of HH/SMO on S-foci dynamics by using translation inhibitors such as cycloheximide and puromycin which are known to regulate S-foci dissolution and formation, respectively.

3. Development of the SNAP-tagged reporter assay to measure Smaug repressive activity

We have developed a novel SNAP-tagged reporter assay, inspired by the λ N/5BoxB dual system of a luciferase reporter assay used to measure translational repression (Behm-Ansmant, Rehwinkel et al. 2006). In our system, Smaug is forced to interact with the 3' UTR of a reporter mRNA by λ N/5BoxB recognition and binding. In this manner, by forcing a heterologous interaction, we are studying the functional repressive activity of Smaug independently of its intrinsic ability to recognize and bind mRNAs. The major advantage our reporter assay provides is that it allows to simultaneously quantify repression of the mRNA reporter and to correlate it to the amount of Smaug protein levels in the cells.

Nevertheless, mRNA repression is tied to the binding affinity of our system and the concentration of available Smaug to bind the mRNA reporter. Moreover, we cannot discriminate whether Smaug could also bind endogenous mRNAs that contain SREs. If such competition exists, then the mRNA reporter would be only repressed by the amount of available Smaug protein in the cell. In order to distinguish between these possibilities, we could use a Smaug variant that carries a point mutation in its SAM domain (Smaug^{K612Q}) causing its inability to bind mRNA (Aviv, Lin et al. 2003).

4. HH/SMO signaling downregulates Smaug accumulation levels and Smaug repressive activity

We have shown that activation of the HH/SMO signaling downregulates both Smaug accumulation levels and its repressing activity in Cl8 cells. Moreover, these effects require physical interaction between Smaug and SMO.

It is important to identify at which level HH/SMO signaling is regulating Smaug protein accumulation. Indeed it could be due to changes in its stability but also to changes in its encoding mRNA synthesis, translation or stability. Consequently, RT-qPCR experiments on *smaug* mRNA would discriminate if the effect takes place at the mRNA or protein level. However, if there were an effect on *smaug* mRNA, we would not distinguish if it is

transcriptional or due to mRNA instability. We could therefore measure *smaug* mRNA decay rates over time by RT-qPCR in presence of a transcription inhibitor like actinomycin D. If the effect concerns only Smaug protein levels, we will need to discriminate whether it is an effect on its synthesis or stability. We are currently setting up experimental conditions to measure Smaug protein stability by studying half-life of a SNAP-Smaug fusion. Thus, it would be interesting to evaluate if any of these parameters change upon HH pathway activation.

In addition, our results raise the question whether activation of HH signaling affects Smaug ability to bind mRNA and/or its associated proteins involved in mRNA repression. First, in order to study whether HH/SMO signaling regulates Smaug ability to bind mRNA, we will use an mRNA reporter that contains multiple *hsp83* SREs at the 3'UTR (Semotok, Luo et al. 2008) and is currently being built by M. Sanial. Secondly, it would be interesting to test by coIP whether Smaug physical interaction with its known partners is affected upon HH pathway activation. We could also study their subcellular distribution in response to HH and analyze their colocalization by confocal imaging in Cl8 cells.

II. Smaug regulation by phosphorylation

Our MS-MS analyses of Smaug phosphopeptides, as well as the studies of Smaug protein migration by WB in *Drosophila* Cl8 cells, constitute the first evidence that Smaug is constitutively phosphorylated and hyperphosphorylated in presence of HH via its interaction with SMO.

1. Identification of Smaug phosphorylated sites

MS-MS analysis offers a rapid and highly sensitive way to map post-translational modifications but this approach also presents limitations, especially when it comes to cover the entire protein sequence and confident phosphosite localization. After studying the impact of S/T-A mutations in multiple Smaug fragments on HH induced Smaug phosphorylation, we identified three regions (5, 6, and 8) required for Smaug phosphorylation in response to the HH pathway.

Smaug regions 5 and 6 are intrinsically disordered regions, as often found in RNA binding proteins where they play a role in RNP assembly. Phosphorylation events in Smaug IDRs could affect mobility to adopt a certain structural conformation and could thus play a role in Smaug mRNA binding or its ability to interact with protein partners. Consequently, we cannot exclude that the S/T-A changes that we introduced in the regions 5 and 6 do not affect Smaug IDRs mobility. It would be therefore important to mutate them into Glycine which should provide more flexibility. Region 8 corresponds to the SAM domain and part of the PHAT domain, raising the possibility that HH induced phosphorylation could regulate interaction between Smaug and its mRNA targets and/or with its associated proteins. In addition, prevention of phosphorylation in region 7, which is highly rich in S/T and locates near the SAM domain, induces Smaug hyperphosphorylation. An attractive hypothesis is that these regions could be regulating each other's phosphorylation upon arrival of HH signal (Fig. 35). For instance, we could imagine the possibility that HH/SMO signaling induces phosphorylation of one of these regions triggering a cascade of subsequent phosphorylation events, similarly to the activation process of SMO (Chen and Jiang 2013). Another possibility, given that protein phosphorylation is a reversible process coordinated by opposing kinases and phosphatases, could be that HH promotes Smaug phosphorylation by inducing dephosphorylation in region 7.

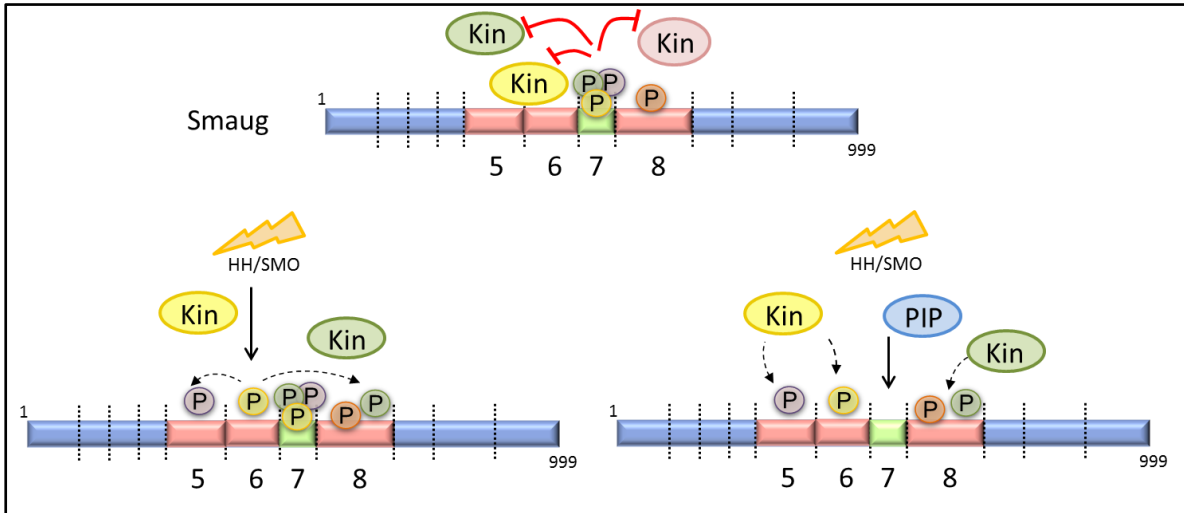


Figure 35. Representation of different scenarios of Smaug regulation by phosphorylation. In absence of HH/SMO signaling, Smaug is constitutively phosphorylated both in region 7, which blocks hyperphosphorylation events, and in region 8 which contains Smaug SAM domain. Upon activation of the HH/SMO pathway, we can imagine that one or multiple kinases are activated leading to phosphorylation of regions 5, 6 and/or 8. On the other hand, HH/SMO signaling could promote dephosphorylation events in region 7 by inducing phosphatase(s) (shown as PIP) which derives in the phosphorylation of Smaug regions by one or multiple kinases.

In order to explore the relationship between the phosphorylation of the different regions of Smaug (5, 6, 7 and 8), it would be instrumental to combine phosphodeficient and/or phosphomimicking mutations in these regions. However, whether the downregulation of Smaug repressive activity and decreased Smaug protein levels that we observe in response to HH/SMO is due to induced phosphorylation of Smaug remains to be studied. To shed light on this topic, more precise identification of HH induced phosphosites is required.

2. Characterization of the kinases implicated in Smaug phosphorylation

When searching for Smaug partners by mass spectrometry analysis, we obtained a list of kinase candidates that could be implicated in Smaug regulation by phosphorylation. In order to determine if any of these kinases is responsible for Smaug phosphorylation, a mini RNAi screen against the protein kinases found is currently underway. After testing the involvement of the FU and DOP kinase in Smaug phosphorylation, we concluded that neither is sufficient to induce Smaug hyperphosphorylation in response to HH. Phosphorylation of Smaug could take place by interaction of kinases that form a complex with SMO or that are bound to Smaug and become active upon activation of the HH pathway. Another possibility

is that the phosphorylation event occurs rapidly and the transient interaction with the kinase involved is lost, and therefore not found as a Smaug partner. It would thus be worth to test if the protein kinases known to play a role in the HH pathway could be acting on Smaug phosphorylation. Note that, despite not having found phosphatase proteins in our MS analysis, it would be interesting to test whether phosphatases PP4 and PP2A, which regulate SMO and CI phosphorylation respectively (Jia, Liu et al. 2009), are involved in Smaug regulation by phosphorylation.

3. What is the function of Smaug SAM domain constitutive phosphorylation?

Given our current results, prevention of phosphorylation in the SAM domain of Smaug (i) upregulates Smaug ability to repress bound mRNA and (ii) disrupts cytoplasmic S-foci distribution in Cl8 cells. Thus, we can hypothesize that introduction of negative charges in the SAM domain could lead to conformational changes that are required for Smaug binding to its associated protein factors.

In parallel, even though our actual reporter assay does not measure Smaug ability to bind mRNA, phosphorylation in the SAM domain of Smaug could regulate its ability to bind its target transcripts. In order to determine if Smaug ability to bind mRNA is affected by phosphorylation, first we need to identify which residue(s) is(are) responsible for the changes observed. Therefore, refining of the mapping of the phosphorylated sites within the SAM domain is currently underway in the lab. Preliminary results using the SNAP-tagged reporter assay show that the implicated phosphosites could be T⁶²⁹, S⁶³⁵, T⁶³⁹ or S⁶⁴³, and importantly, both T residues are highly conserved (Fig. 36). Particularly, T⁶³⁹ and S⁶⁴³ are located in the α -helix 5 which is required in mRNA binding. Furthermore, S⁶⁴³ is located next to A⁶⁴² which mutation completely abolishes Smaug ability to bind mRNA (Aviv, Lin et al. 2003). Thus, once we have identified the phosphosite(s) in the SAM domain, we could test the effect of phosphomimetic and phosphodeficient Smaug mutants on its ability to interact with mRNAs and/or its known partners.

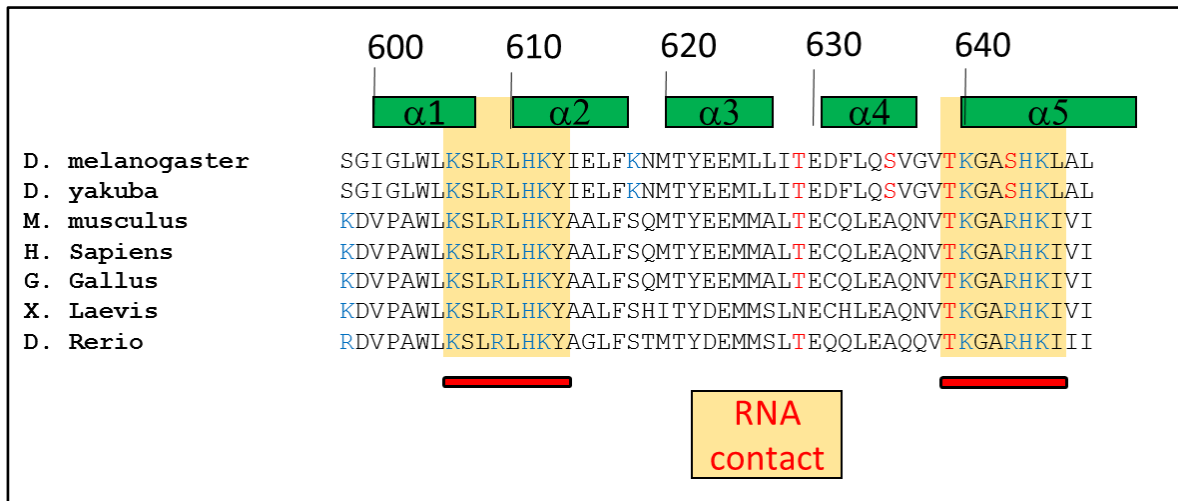


Figure 36. Sequence alignment of SAM domain of Smaug homologues.

Smaug SAM domain sequence alignment from different species (left) is shown. Location of the different α -helices is represented above the sequence. Red bars and sequences highlighted indicate the RNA binding contact regions which are highly rich in basic residues (shown in blue). Potential S/T residues involved in Smaug constitutive phosphorylation are shown in red.

What is more, subcellular localization studies by confocal imaging from two independent experiments showed that a small fraction of Smaug^{SAM} mutant is present in the nucleus. Moreover, we found the Nucleoporin 358 (Nup358) protein in our MS analysis indicating it could be an interactor of Smaug. Nup358 is part of the nuclear pore complex and is embedded at the nuclear membrane where it plays a role in nucleo-cytoplasmic shuttling of proteins (Forler, Rabut et al. 2004). It would be interesting to test whether phosphorylation in Smaug SAM domain plays a role in Smaug subcellular localization by blocking nuclear protein export using Leptomycin B (LMB) and see if Smaug accumulates in the nucleus. To this aim, differential fractionation conditions are currently being set up by M. Sanial in the lab. Remarkably, a preliminary analysis showed that a small fraction of Smaug WT and Smaug^{SAM} protein is found in the nucleus (Fig. 37). Furthermore, inhibition of nuclear protein export did not seem to have an effect on Smaug accumulation in the nucleus. Nevertheless, we should repeat the analysis under optimal experimental conditions and add a nuclear protein known to respond to LMB as control.

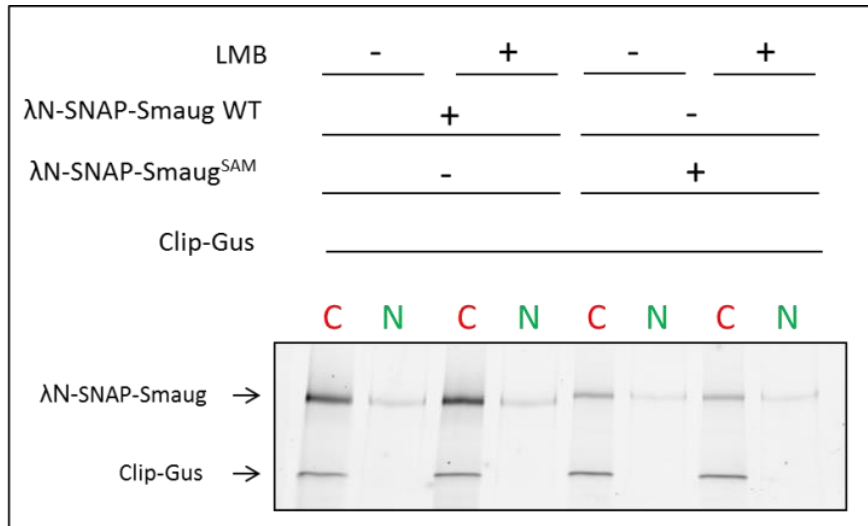


Figure 377. Subcellular fractionation of Smaug in C18 cells

C18 cells were cotransfected with λ N-SNAP-Smaug WT or λ N-SNAP-Smaug^{SAM} and treated in presence or absence of a nuclear protein export inhibitor (LMB). Nuclear (N) and cytoplasmic (C) fractions were separated by differential centrifugation. Clip-Gus was used as the cytoplasmic protein control.

Last but not least, it would be interesting to study if Smaug SAM domain undergoes phosphorylation *in vivo* in *Drosophila* and what its role is. For example, it is not known how Smaug is regulated during early embryogenesis. It seems that MZT is activated by the progressive accumulation of Smaug protein in the embryo but how it is destabilized after the MZT remains unknown. Different possibilities can be envisioned. First, Smaug could autoregulate its mRNA levels by inducing its mRNA decay via promotion of deadenylation. This would result in the inhibition of newly synthesized Smaug protein and therefore a gradual reduction of Smaug protein levels. Another scenario could be that Smaug undergoes phosphorylation which induces a downregulation of Smaug protein levels. We could check first if Smaug constitutive phosphorylation is preserved in the embryo and study its role during the MZT by comparing the effect between Smaug^{SAM} mutant and Smaug WT.

III. Model of Smaug regulation by phosphorylation in response to HH/SMO signaling

Smaug is known to act as an mRNA post-transcriptional repressor by recruiting complexes that inhibit translation or that promote mRNA decay by deadenylation of its target mRNAs. Our data allow us to propose a model in which the RBP Smaug is regulated post-translationally by the HH pathway in *Drosophila* (Fig. 38). In absence of HH, Smaug colocalizes in cytoplasmic foci with SMO. In addition, Smaug could be bound to its target mRNAs promoting silencing. In presence of HH, SMO is activated by phosphorylation which leads to its localization, and recruitment of Smaug, at the plasma membrane. One possibility is that one or more prelocalized protein kinases that form a complex with SMO could act on Smaug by promoting its hyperphosphorylation. Such event would cause the release of the bound mRNA leading to its subsequent translation. Finally, when higher levels of SMO activation are achieved after additional post-translational modifications, interaction with Smaug is inhibited causing its release and relocation to the cytoplasm.

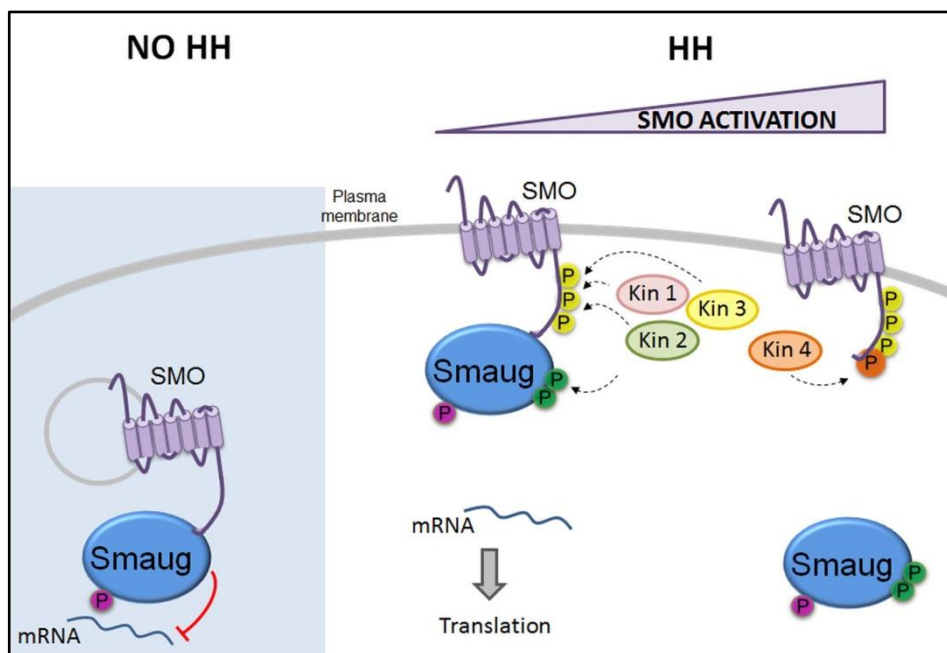


Figure 38. Model of Smaug regulation via SMO upon HH signaling activation

In absence of HH, SMO and Smaug are found in cytoplasmic foci and Smaug is constitutively phosphorylated and bound to an unknown target mRNA. In the presence of HH, activated SMO and Smaug are relocated to the plasma membrane which results in the release of the target mRNA that is translated. Both SMO and Smaug undergo phosphorylation. When higher activated SMO levels are reached, a new post-translational modification of SMO (P in orange) inhibits interaction with Smaug. Phosphorylated Smaug is in the cytoplasm associated or not with a target mRNA.

An important question that our model raises is which are the mRNA targets of Smaug? One possibility is that Smaug regulates mRNAs that encode proteins of the HH pathway. Interestingly, an *in silico* analysis conducted by our collaborators in Argentina, revealed that SMO contains four putative SREs in the ORF region. We can thus hypothesize that HH signaling could regulate a pool of *smo* mRNA that is silenced in absence of HH and released upon HH activation leading to a rapid SMO protein synthesis. Another possibility is that Smaug could regulate HH target genes which expression depends on CI. Finally, Smaug could also regulate other mRNAs independently of CI which is a characteristic of non-canonical HH pathway.

IV. Concluding remarks and future perspectives

For a number of RBPs, post-translational modifications have been well studied and are known to play a major role in the regulation of their physiological function. So far, however, very little is known about the PTMs of Smaug protein and their effect at the functional level. Here we provide the first evidence that Smaug protein is constitutively phosphorylated which seems to modulate Smaug repressive activity as well as its ability to form S-foci in cultured CI8 cells. Moreover, activation of the HH pathway induces its hyperphosphorylation which could regulate Smaug ability to bind mRNA and/or its known associated proteins.

My thesis work raises several other important issues related to post-transcriptional control. What are the mRNAs targets that are regulated by HH signaling through its effects on Smaug? Moreover, is Smaug regulation by phosphorylation a general mechanism used by cell signaling pathways to regulate gene expression or is it specific to the HH pathway? Could the phosphorylation of Smaug control which protein complexes are being recruited by Smaug to specific mRNA targets? Importantly, is Smaug phosphorylation conserved in mammals? Lastly, my data shed light on novel ways that could regulate Smaug multiple functions. Moreover, the development of our reporter assay can be useful to study the function of other key RNA binding proteins.

REFERENCES

Adams, M. D., S. E. Celniker, R. A. Holt, C. A. Evans, J. D. Gocayne, P. G. Amanatides, S. E. Scherer, P. W. Li, R. A. Hoskins, R. F. Galle, R. A. George, S. E. Lewis, S. Richards, M. Ashburner, S. N. Henderson, G. G. Sutton, J. R. Wortman, M. D. Yandell, Q. Zhang, L. X. Chen, R. C. Brandon, Y. H. Rogers, R. G. Blazej, M. Champe, B. D. Pfeiffer, K. H. Wan, C. Doyle, E. G. Baxter, G. Helt, C. R. Nelson, G. L. Gabor, J. F. Abril, A. Agbayani, H. J. An, C. Andrews-Pfannkoch, D. Baldwin, R. M. Ballew, A. Basu, J. Baxendale, L. Bayraktaroglu, E. M. Beasley, K. Y. Beeson, P. V. Benos, B. P. Berman, D. Bhandari, S. Bolshakov, D. Borkova, M. R. Botchan, J. Bouck, P. Brokstein, P. Brottier, K. C. Burtis, D. A. Busam, H. Butler, E. Cadieu, A. Center, I. Chandra, J. M. Cherry, S. Cawley, C. Dahlke, L. B. Davenport, P. Davies, B. de Pablos, A. Delcher, Z. Deng, A. D. Mays, I. Dew, S. M. Dietz, K. Dodson, L. E. Doup, M. Downes, S. Dugan-Rocha, B. C. Dunkov, P. Dunn, K. J. Durbin, C. C. Evangelista, C. Ferraz, S. Ferriera, W. Fleischmann, C. Fosler, A. E. Gabrielian, N. S. Garg, W. M. Gelbart, K. Glasser, A. Glodek, F. Gong, J. H. Gorrell, Z. Gu, P. Guan, M. Harris, N. L. Harris, D. Harvey, T. J. Heiman, J. R. Hernandez, J. Houck, D. Hostin, K. A. Houston, T. J. Howland, M. H. Wei, C. Ibegwam, M. Jalali, F. Kalush, G. H. Karpen, Z. Ke, J. A. Kennison, K. A. Ketchum, B. E. Kimmel, C. D. Kodira, C. Kraft, S. Kravitz, D. Kulp, Z. Lai, P. Lasko, Y. Lei, A. A. Levitsky, J. Li, Z. Li, Y. Liang, X. Lin, X. Liu, B. Mattei, T. C. McIntosh, M. P. McLeod, D. McPherson, G. Merkulov, N. V. Milshina, C. Mobarry, J. Morris, A. Moshrefi, S. M. Mount, M. Moy, B. Murphy, L. Murphy, D. M. Muzny, D. L. Nelson, D. R. Nelson, K. A. Nelson, K. Nixon, D. R. Nusskern, J. M. Pacleb, M. Palazzolo, G. S. Pittman, S. Pan, J. Pollard, V. Puri, M. G. Reese, K. Reinert, K. Remington, R. D. Saunders, F. Scheeler, H. Shen, B. C. Shue, I. Siden-Kiamos, M. Simpson, M. P. Skupski, T. Smith, E. Spier, A. C. Spradling, M. Stapleton, R. Strong, E. Sun, R. Svirskas, C. Tector, R. Turner, E. Venter, A. H. Wang, X. Wang, Z. Y. Wang, D. A. Wassarman, G. M. Weinstock, J. Weissenbach, S. M. Williams, WoodageT, K. C. Worley, D. Wu, S. Yang, Q. A. Yao, J. Ye, R. F. Yeh, J. S. Zaveri, M. Zhan, G. Zhang, Q. Zhao, L. Zheng, X. H. Zheng, F. N. Zhong, W. Zhong, X. Zhou, S. Zhu, X. Zhu, H. O. Smith, R. A. Gibbs, E. W. Myers, G. M. Rubin and J. C. Venter (2000). "The genome sequence of *Drosophila melanogaster*." Science **287**(5461): 2185-2195.

Aguzzi, A. and M. Altmeyer (2016). "Phase Separation: Linking Cellular Compartmentalization to Disease." Trends Cell Biol **26**(7): 547-558.

Alberti, S. (2013). "Aggregating the message to control the cell cycle." Dev Cell **25**(6): 551-552.

Alberti, S., R. Halfmann, O. King, A. Kapila and S. Lindquist (2009). "A systematic survey identifies prions and illuminates sequence features of prionogenic proteins." Cell **137**(1): 146-158.

Amadei, G., M. A. Zander, G. Yang, J. G. Dumelie, J. P. Vessey, H. D. Lipshitz, C. A. Smibert, D. R. Kaplan and F. D. Miller (2015). "A Smaug2-Based Translational Repression Complex Determines the Balance between Precursor Maintenance versus Differentiation during Mammalian Neurogenesis." J Neurosci **35**(47): 15666-15681.

Anderson, P. and N. Kedersha (2006). "RNA granules." J Cell Biol **172**(6): 803-808.

Anko, M. L. and K. M. Neugebauer (2012). "RNA-protein interactions in vivo: global gets specific." Trends Biochem Sci **37**(7): 255-262.

- Ascano, M., M. Hafner, P. Cekan, S. Gerstberger and T. Tuschl (2012). "Identification of RNA-protein interaction networks using PAR-CLIP." Wiley Interdiscip Rev RNA **3**(2): 159-177.
- Aviv, T., Z. Lin, G. Ben-Ari, C. A. Smibert and F. Sicheri (2006). "Sequence-specific recognition of RNA hairpins by the SAM domain of Vts1p." Nat Struct Mol Biol **13**(2): 168-176.
- Aviv, T., Z. Lin, S. Lau, L. M. Rendl, F. Sicheri and C. A. Smibert (2003). "The RNA-binding SAM domain of Smaug defines a new family of post-transcriptional regulators." Nat Struct Biol **10**(8): 614-621.
- Aza-Blanc, P. and T. B. Kornberg (1999). "Ci: a complex transducer of the hedgehog signal." Trends Genet **15**(11): 458-462.
- Baez, M. V. and G. L. Boccaccio (2005). "Mammalian Smaug is a translational repressor that forms cytoplasmic foci similar to stress granules." J Biol Chem **280**(52): 43131-43140.
- Baez, M. V., L. Luchelli, D. Maschi, M. Habif, M. Pascual, M. G. Thomas and G. L. Boccaccio (2011). "Smaug1 mRNA-silencing foci respond to NMDA and modulate synapse formation." J Cell Biol **195**(7): 1141-1157.
- Baltz, A. G., M. Munschauer, B. Schwanhauser, A. Vasile, Y. Murakawa, M. Schueler, N. Youngs, D. Penfold-Brown, K. Drew, M. Milek, E. Wyler, R. Bonneau, M. Selbach, C. Dieterich and M. Landthaler (2012). "The mRNA-bound proteome and its global occupancy profile on protein-coding transcripts." Mol Cell **46**(5): 674-690.
- Bastock, R. and D. St Johnston (2008). "Drosophila oogenesis." Curr Biol **18**(23): R1082-1087.
- Becalska, A. N. and E. R. Gavis (2009). "Lighting up mRNA localization in Drosophila oogenesis." Development **136**(15): 2493-2503.
- Beckmann, B. M., A. Castello and J. Medenbach (2016). "The expanding universe of ribonucleoproteins: of novel RNA-binding proteins and unconventional interactions." Pflugers Arch **468**(6): 1029-1040.
- Beckmann, B. M., R. Horos, B. Fischer, A. Castello, K. Eichelbaum, A. M. Alleaume, T. Schwarzl, T. Curk, S. Foehr, W. Huber, J. Krijgsveld and M. W. Hentze (2015). "The RNA-binding proteomes from yeast to man harbour conserved enigmRBPs." Nat Commun **6**: 10127.
- Behm-Ansmant, I., J. Rehwinkel, T. Doerks, A. Stark, P. Bork and E. Izaurralde (2006). "mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes." Genes Dev **20**(14): 1885-1898.
- Benoit, B., C. H. He, F. Zhang, S. M. Votruba, W. Tadros, J. T. Westwood, C. A. Smibert, H. D. Lipshitz and W. E. Theurkauf (2009). "An essential role for the RNA-binding protein Smaug during the Drosophila maternal-to-zygotic transition." Development **136**(6): 923-932.
- Bergsten, S. E. and E. R. Gavis (1999). "Role for mRNA localization in translational activation but not spatial restriction of nanos RNA." Development **126**(4): 659-669.
- Besse, F. and A. Ephrussi (2008). "Translational control of localized mRNAs: restricting protein synthesis in space and time." Nat Rev Mol Cell Biol **9**(12): 971-980.

- Brangwynne, C. P., C. R. Eckmann, D. S. Courson, A. Rybarska, C. Hoegge, J. Gharakhani, F. Julicher and A. A. Hyman (2009). "Germline P granules are liquid droplets that localize by controlled dissolution/condensation." Science **324**(5935): 1729-1732.
- Braun, J. E., E. Huntzinger, M. Fauser and E. Izaurralde (2011). "GW182 proteins directly recruit cytoplasmic deadenylase complexes to miRNA targets." Mol Cell **44**(1): 120-133.
- Brechbiel, J. L. and E. R. Gavis (2008). "Spatial regulation of nanos is required for its function in dendrite morphogenesis." Curr Biol **18**(10): 745-750.
- Brinegar, A. E. and T. A. Cooper (2016). "Roles for RNA-binding proteins in development and disease." Brain Res **1647**: 1-8.
- Briscoe, J. and P. P. Therond (2013). "The mechanisms of Hedgehog signalling and its roles in development and disease." Nat Rev Mol Cell Biol **14**(7): 416-429.
- Buchan, J. R., R. M. Kolaitis, J. P. Taylor and R. Parker (2013). "Eukaryotic stress granules are cleared by autophagy and Cdc48/VCP function." Cell **153**(7): 1461-1474.
- Calabretta, S. and S. Richard (2015). "Emerging Roles of Disordered Sequences in RNA-Binding Proteins." Trends Biochem Sci **40**(11): 662-672.
- Castello, A., B. Fischer, K. Eichelbaum, R. Horos, B. M. Beckmann, C. Strein, N. E. Davey, D. T. Humphreys, T. Preiss, L. M. Steinmetz, J. Krijgsveld and M. W. Hentze (2012). "Insights into RNA biology from an atlas of mammalian mRNA-binding proteins." Cell **149**(6): 1393-1406.
- Castello, A., B. Fischer, C. K. Frese, R. Horos, A. M. Alleaume, S. Foehr, T. Curk, J. Krijgsveld and M. W. Hentze (2016). "Comprehensive Identification of RNA-Binding Domains in Human Cells." Mol Cell **63**(4): 696-710.
- Castello, A., R. Horos, C. Strein, B. Fischer, K. Eichelbaum, L. M. Steinmetz, J. Krijgsveld and M. W. Hentze (2013). "System-wide identification of RNA-binding proteins by interactome capture." Nat Protoc **8**(3): 491-500.
- Castello, A., R. Horos, C. Strein, B. Fischer, K. Eichelbaum, L. M. Steinmetz, J. Krijgsveld and M. W. Hentze (2016). "Comprehensive Identification of RNA-Binding Proteins by RNA Interactome Capture." Methods Mol Biol **1358**: 131-139.
- Chartier, A., P. Klein, S. Pierson, N. Barbezier, T. Gidaro, F. Casas, S. Carberry, P. Dowling, L. Maynadier, M. Bellec, M. Oloko, C. Jardel, B. Moritz, G. Dickson, V. Mouly, K. Ohlendieck, G. Butler-Browne, C. Trollet and M. Simonelig (2015). "Mitochondrial dysfunction reveals the role of mRNA poly(A) tail regulation in oculopharyngeal muscular dystrophy pathogenesis." PLoS Genet **11**(3): e1005092.
- Chen, C. Y. and A. B. Shyu (2011). "Mechanisms of deadenylation-dependent decay." Wiley Interdiscip Rev RNA **2**(2): 167-183.
- Chen, L., J. G. Dumelie, X. Li, M. H. Cheng, Z. Yang, J. D. Laver, N. U. Siddiqui, J. T. Westwood, Q. Morris, H. D. Lipshitz and C. A. Smibert (2014). "Global regulation of mRNA translation and stability in the early Drosophila embryo by the Smaug RNA-binding protein." Genome Biol **15**(1): R4.
- Chen, Y. and J. Jiang (2013). "Decoding the phosphorylation code in Hedgehog signal transduction." Cell Res **23**(2): 186-200.

- Chen, Z., W. Holland, J. M. Shelton, A. Ali, X. Zhan, S. Won, W. Tomisato, C. Liu, X. Li, E. M. Moresco and B. Beutler (2014). "Mutation of mouse *Samd4* causes leanness, myopathy, uncoupled mitochondrial respiration, and dysregulated mTORC1 signaling." Proc Natl Acad Sci U S A **111**(20): 7367-7372.
- Cheng, S., D. Maier, D. Neubueser and D. R. Hipfner (2010). "Regulation of smoothed by *Drosophila* G-protein-coupled receptor kinases." Dev Biol **337**(1): 99-109.
- Claret, S., M. Sanial and A. Plessis (2007). "Evidence for a novel feedback loop in the Hedgehog pathway involving Smoothed and Fused." Curr Biol **17**(15): 1326-1333.
- Clemens, K. R., V. Wolf, S. J. McBryant, P. Zhang, X. Liao, P. E. Wright and J. M. Gottesfeld (1993). "Molecular basis for specific recognition of both RNA and DNA by a zinc finger protein." Science **260**(5107): 530-533.
- Dahanukar, A., J. A. Walker and R. P. Wharton (1999). "Smaug, a novel RNA-binding protein that operates a translational switch in *Drosophila*." Mol Cell **4**(2): 209-218.
- De Boulle, K., A. J. Verkerk, E. Reyniers, L. Vits, J. Hendrickx, B. Van Roy, F. Van den Bos, E. de Graaff, B. A. Oostra and P. J. Willems (1993). "A point mutation in the FMR-1 gene associated with fragile X mental retardation." Nat Genet **3**(1): 31-35.
- de Haro, M., I. Al-Ramahi, K. R. Jones, J. K. Holth, L. T. Timchenko and J. Botas (2013). "Smaug/SAMD4A restores translational activity of CUGBP1 and suppresses CUG-induced myopathy." PLoS Genet **9**(4): e1003445.
- De Renzis, S., O. Elemento, S. Tavazoie and E. F. Wieschaus (2007). "Unmasking activation of the zygotic genome using chromosomal deletions in the *Drosophila* embryo." PLoS Biol **5**(5): e117.
- Ding, D., S. M. Parkhurst, S. R. Halsell and H. D. Lipshitz (1993). "Dynamic Hsp83 RNA localization during *Drosophila* oogenesis and embryogenesis." Mol Cell Biol **13**(6): 3773-3781.
- Driever, W. and C. Nusslein-Volhard (1988). "The bicoid protein determines position in the *Drosophila* embryo in a concentration-dependent manner." Cell **54**(1): 95-104.
- Ephrussi, A., L. K. Dickinson and R. Lehmann (1991). "Oskar organizes the germ plasm and directs localization of the posterior determinant nanos." Cell **66**(1): 37-50.
- Eulalio, A., I. Behm-Ansmant, D. Schweizer and E. Izaurralde (2007). "P-body formation is a consequence, not the cause, of RNA-mediated gene silencing." Mol Cell Biol **27**(11): 3970-3981.
- Fabian, M. R., M. K. Cieplak, F. Frank, M. Morita, J. Green, T. Srikumar, B. Nagar, T. Yamamoto, B. Raught, T. F. Duchaine and N. Sonenberg (2011). "miRNA-mediated deadenylation is orchestrated by GW182 through two conserved motifs that interact with CCR4-NOT." Nat Struct Mol Biol **18**(11): 1211-1217.
- Fan, A. C. and A. K. Leung (2016). "RNA Granules and Diseases: A Case Study of Stress Granules in ALS and FTLD." Adv Exp Med Biol **907**: 263-296.
- Forler, D., G. Rabut, F. D. Ciccarelli, A. Herold, T. Kocher, R. Niggeweg, P. Bork, J. Ellenberg and E. Izaurralde (2004). "RanBP2/Nup358 provides a major binding site for NXF1-p15 dimers at the nuclear pore complex and functions in nuclear mRNA export." Mol Cell Biol **24**(3): 1155-1167.

- Forrest, K. M. and E. R. Gavis (2003). "Live imaging of endogenous RNA reveals a diffusion and entrapment mechanism for nanos mRNA localization in *Drosophila*." *Curr Biol* **13**(14): 1159-1168.
- Fribourg, S., D. Gatfield, E. Izaurralde and E. Conti (2003). "A novel mode of RBD-protein recognition in the Y14-Mago complex." *Nat Struct Biol* **10**(6): 433-439.
- Gallie, D. R. (2002). "Protein-protein interactions required during translation." *Plant Mol Biol* **50**(6): 949-970.
- Garneau, N. L., J. Wilusz and C. J. Wilusz (2007). "The highways and byways of mRNA decay." *Nat Rev Mol Cell Biol* **8**(2): 113-126.
- Gavis, E. R. and R. Lehmann (1992). "Localization of nanos RNA controls embryonic polarity." *Cell* **71**(2): 301-313.
- Gebauer, F., T. Preiss and M. W. Hentze (2012). "From cis-regulatory elements to complex RNPs and back." *Cold Spring Harb Perspect Biol* **4**(7): a012245.
- Gerber, A. P., S. Luschnig, M. A. Krasnow, P. O. Brown and D. Herschlag (2006). "Genome-wide identification of mRNAs associated with the translational regulator PUMILIO in *Drosophila melanogaster*." *Proc Natl Acad Sci U S A* **103**(12): 4487-4492.
- Gilks, N., N. Kedersha, M. Ayodele, L. Shen, G. Stoecklin, L. M. Dember and P. Anderson (2004). "Stress granule assembly is mediated by prion-like aggregation of TIA-1." *Mol Biol Cell* **15**(12): 5383-5398.
- Glisovic, T., J. L. Bachorik, J. Yong and G. Dreyfuss (2008). "RNA-binding proteins and post-transcriptional gene regulation." *FEBS Lett* **582**(14): 1977-1986.
- Grammatikakis, I., K. Abdelmohsen and M. Gorospe (2017). "Posttranslational control of HuR function." *Wiley Interdiscip Rev RNA* **8**(1).
- Green, J. B., T. A. Edwards, J. Trincao, C. R. Escalante, R. P. Wharton and A. K. Aggarwal (2002). "Crystallization and characterization of Smaug: a novel RNA-binding motif." *Biochem Biophys Res Commun* **297**(5): 1085-1088.
- Green, J. B., C. D. Gardner, R. P. Wharton and A. K. Aggarwal (2003). "RNA recognition via the SAM domain of Smaug." *Mol Cell* **11**(6): 1537-1548.
- Griffin, E. E., D. J. Odde and G. Seydoux (2011). "Regulation of the MEX-5 gradient by a spatially segregated kinase/phosphatase cycle." *Cell* **146**(6): 955-968.
- Grishin, N. V. (2001). "KH domain: one motif, two folds." *Nucleic Acids Res* **29**(3): 638-643.
- Hachet, O. and A. Ephrussi (2004). "Splicing of oskar RNA in the nucleus is coupled to its cytoplasmic localization." *Nature* **428**(6986): 959-963.
- Hafner, M., M. Landthaler, L. Burger, M. Khorshid, J. Hausser, P. Berninger, A. Rothballer, M. Ascano, Jr., A. C. Jungkamp, M. Munschauer, A. Ulrich, G. S. Wardle, S. Dewell, M. Zavolan and T. Tuschl (2010). "Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP." *Cell* **141**(1): 129-141.

- Hall, T. M. (2005). "Multiple modes of RNA recognition by zinc finger proteins." Curr Opin Struct Biol **15**(3): 367-373.
- Helder, S., A. J. Blythe, C. S. Bond and J. P. Mackay (2016). "Determinants of affinity and specificity in RNA-binding proteins." Curr Opin Struct Biol **38**: 83-91.
- Hogan, D. J., D. P. Riordan, A. P. Gerber, D. Herschlag and P. O. Brown (2008). "Diverse RNA-binding proteins interact with functionally related sets of RNAs, suggesting an extensive regulatory system." PLoS Biol **6**(10): e255.
- Holt, C. E. and S. L. Bullock (2009). "Subcellular mRNA localization in animal cells and why it matters." Science **326**(5957): 1212-1216.
- Huang, P., D. Nedelcu, M. Watanabe, C. Jao, Y. Kim, J. Liu and A. Salic (2016). "Cellular Cholesterol Directly Activates Smoothed in Hedgehog Signaling." Cell **166**(5): 1176-1187 e1114.
- Hubstenberger, A., M. Courel, M. Benard, S. Souquere, M. Ernoult-Lange, R. Chouaib, Z. Yi, J. B. Morlot, A. Munier, M. Fradet, M. Daunesse, E. Bertrand, G. Pierron, J. Mozziconacci, M. Kress and D. Weil (2017). "P-Body Purification Reveals the Condensation of Repressed mRNA Regulons." Mol Cell **68**(1): 144-157 e145.
- Hubstenberger, A., S. L. Noble, C. Cameron and T. C. Evans (2013). "Translation repressors, an RNA helicase, and developmental cues control RNP phase transitions during early development." Dev Cell **27**(2): 161-173.
- Ingham, P. W. (1993). "Localized hedgehog activity controls spatial limits of wingless transcription in the Drosophila embryo." Nature **366**(6455): 560-562.
- Irion, U., J. Adams, C. W. Chang and D. St Johnston (2006). "Miranda couples oskar mRNA/Staufen complexes to the bicoid mRNA localization pathway." Dev Biol **297**(2): 522-533.
- Jarvelin, A. I., M. Noerenberg, I. Davis and A. Castello (2016). "The new (dis)order in RNA regulation." Cell Commun Signal **14**: 9.
- Jeske, M., B. Moritz, A. Anders and E. Wahle (2011). "Smaug assembles an ATP-dependent stable complex repressing nanos mRNA translation at multiple levels." EMBO J **30**(1): 90-103.
- Jia, H., Y. Liu, R. Xia, C. Tong, T. Yue, J. Jiang and J. Jia (2010). "Casein kinase 2 promotes Hedgehog signaling by regulating both smoothed and Cubitus interruptus." J Biol Chem **285**(48): 37218-37226.
- Jia, H., Y. Liu, W. Yan and J. Jia (2009). "PP4 and PP2A regulate Hedgehog signaling by controlling Smo and Ci phosphorylation." Development **136**(2): 307-316.
- Jia, J., C. Tong, B. Wang, L. Luo and J. Jiang (2004). "Hedgehog signalling activity of Smoothed requires phosphorylation by protein kinase A and casein kinase I." Nature **432**(7020): 1045-1050.
- Jiang, K., Y. Liu, J. Fan, G. Epperly, T. Gao, J. Jiang and J. Jia (2014). "Hedgehog-regulated atypical PKC promotes phosphorylation and activation of Smoothed and Cubitus interruptus in Drosophila." Proc Natl Acad Sci U S A **111**(45): E4842-4850.
- Kalifa, Y., T. Huang, L. N. Rosen, S. Chatterjee and E. R. Gavis (2006). "Glorund, a Drosophila hnRNP F/H homolog, is an ovarian repressor of nanos translation." Dev Cell **10**(3): 291-301.

- Kato, M., T. W. Han, S. Xie, K. Shi, X. Du, L. C. Wu, H. Mirzaei, E. J. Goldsmith, J. Longgood, J. Pei, N. V. Grishin, D. E. Frantz, J. W. Schneider, S. Chen, L. Li, M. R. Sawaya, D. Eisenberg, R. Tycko and S. L. McKnight (2012). "Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels." *Cell* **149**(4): 753-767.
- Kechavarzi, B. and S. C. Janga (2014). "Dissecting the expression landscape of RNA-binding proteins in human cancers." *Genome Biol* **15**(1): R14.
- Kedersha, N., P. Ivanov and P. Anderson (2013). "Stress granules and cell signaling: more than just a passing phase?" *Trends Biochem Sci* **38**(10): 494-506.
- Kedersha, N., G. Stoecklin, M. Ayodele, P. Yacono, J. Lykke-Andersen, M. J. Fritzler, D. Scheuner, R. J. Kaufman, D. E. Golan and P. Anderson (2005). "Stress granules and processing bodies are dynamically linked sites of mRNP remodeling." *J Cell Biol* **169**(6): 871-884.
- Kiebler, M. A. and G. J. Bassell (2006). "Neuronal RNA granules: movers and makers." *Neuron* **51**(6): 685-690.
- Kinoshita, E., E. Kinoshita-Kikuta and T. Koike (2009). "Separation and detection of large phosphoproteins using Phos-tag SDS-PAGE." *Nat Protoc* **4**(10): 1513-1521.
- Kishore, S., S. Luber and M. Zavolan (2010). "Deciphering the role of RNA-binding proteins in the post-transcriptional control of gene expression." *Brief Funct Genomics* **9**(5-6): 391-404.
- Krichevsky, A. M. and K. S. Kosik (2001). "Neuronal RNA granules: a link between RNA localization and stimulation-dependent translation." *Neuron* **32**(4): 683-696.
- Lasko, P. (2012). "mRNA localization and translational control in *Drosophila* oogenesis." *Cold Spring Harb Perspect Biol* **4**(10).
- Laver, J. D., X. Li, D. Ray, K. B. Cook, N. A. Hahn, S. Nabeel-Shah, M. Kekis, H. Luo, A. J. Marsolais, K. Y. Fung, T. R. Hughes, J. T. Westwood, S. S. Sidhu, Q. Morris, H. D. Lipshitz and C. A. Smibert (2015). "Brain tumor is a sequence-specific RNA-binding protein that directs maternal mRNA clearance during the *Drosophila* maternal-to-zygotic transition." *Genome Biol* **16**: 94.
- Laver, J. D., A. J. Marsolais, C. A. Smibert and H. D. Lipshitz (2015). "Regulation and Function of Maternal Gene Products During the Maternal-to-Zygotic Transition in *Drosophila*." *Curr Top Dev Biol* **113**: 43-84.
- Lecuyer, E., H. Yoshida, N. Parthasarathy, C. Alm, T. Babak, T. Cerovina, T. R. Hughes, P. Tomancak and H. M. Krause (2007). "Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function." *Cell* **131**(1): 174-187.
- Lee, E. K. (2012). "Post-translational modifications of RNA-binding proteins and their roles in RNA granules." *Curr Protein Pept Sci* **13**(4): 331-336.
- Lehmann, R. and C. Nusslein-Volhard (1991). "The maternal gene nanos has a central role in posterior pattern formation of the *Drosophila* embryo." *Development* **112**(3): 679-691.
- Li, P., S. Banjade, H. C. Cheng, S. Kim, B. Chen, L. Guo, M. Llaguno, J. V. Hollingsworth, D. S. King, S. F. Banani, P. S. Russo, Q. X. Jiang, B. T. Nixon and M. K. Rosen (2012). "Phase transitions in the assembly of multivalent signalling proteins." *Nature* **483**(7389): 336-340.

- Li, S., Y. Chen, Q. Shi, T. Yue, B. Wang and J. Jiang (2012). "Hedgehog-regulated ubiquitination controls smoothed trafficking and cell surface expression in *Drosophila*." *PLoS Biol* **10**(1): e1001239.
- Li, S., S. Li, Y. Han, C. Tong, B. Wang, Y. Chen and J. Jiang (2016). "Regulation of Smoothed Phosphorylation and High-Level Hedgehog Signaling Activity by a Plasma Membrane Associated Kinase." *PLoS Biol* **14**(6): e1002481.
- Li, Y., J. Z. Maines, O. Y. Tastan, D. M. McKearin and M. Buszczak (2012). "Mei-P26 regulates the maintenance of ovarian germline stem cells by promoting BMP signaling." *Development* **139**(9): 1547-1556.
- Lin, Y., D. S. Protter, M. K. Rosen and R. Parker (2015). "Formation and Maturation of Phase-Separated Liquid Droplets by RNA-Binding Proteins." *Mol Cell* **60**(2): 208-219.
- Lovci, M. T., M. H. Bengtson and K. B. Massirer (2016). "Post-Translational Modifications and RNA-Binding Proteins." *Adv Exp Med Biol* **907**: 297-317.
- Lunde, B. M., C. Moore and G. Varani (2007). "RNA-binding proteins: modular design for efficient function." *Nat Rev Mol Cell Biol* **8**(6): 479-490.
- Luo, H., X. Li, J. M. Claycomb and H. D. Lipshitz (2016). "The Smaug RNA-Binding Protein Is Essential for microRNA Synthesis During the *Drosophila* Maternal-to-zygotic Transition." *G3 (Bethesda)*.
- Luo, N., G. Li, Y. Li, X. Fan, Y. Wang, X. Ye, X. Mo, J. Zhou, W. Yuan, M. Tan, H. Xie, K. Ocorr, R. Bodmer, Y. Deng and X. Wu (2010). "SAM4B, a novel SAM-containing protein, inhibits AP-1-, p53- and p21-mediated transcriptional activity." *BMB Rep* **43**(5): 355-361.
- Ma, G., S. Li, Y. Han, S. Li, T. Yue, B. Wang and J. Jiang (2016). "Regulation of Smoothed Trafficking and Hedgehog Signaling by the SUMO Pathway." *Dev Cell* **39**(4): 438-451.
- Macdonald, P. M. (1992). "The *Drosophila pumilio* gene: an unusually long transcription unit and an unusual protein." *Development* **114**(1): 221-232.
- MacGurn, J. A., P. C. Hsu and S. D. Emr (2012). "Ubiquitin and membrane protein turnover: from cradle to grave." *Annu Rev Biochem* **81**: 231-259.
- Malinowska, L., S. Kroschwald and S. Alberti (2013). "Protein disorder, prion propensities, and self-organizing macromolecular collectives." *Biochim Biophys Acta* **1834**(5): 918-931.
- Marchese, D., N. S. de Groot, N. Lorenzo Gotor, C. M. Livi and G. G. Tartaglia (2016). "Advances in the characterization of RNA-binding proteins." *Wiley Interdiscip Rev RNA* **7**(6): 793-810.
- Maris, C., C. Dominguez and F. H. Allain (2005). "The RNA recognition motif, a plastic RNA-binding platform to regulate post-transcriptional gene expression." *FEBS J* **272**(9): 2118-2131.
- Martin, K. C. and A. Ephrussi (2009). "mRNA localization: gene expression in the spatial dimension." *Cell* **136**(4): 719-730.
- Martinez Arias, A., N. E. Baker and P. W. Ingham (1988). "Role of segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo." *Development* **103**(1): 157-170.

- Matia-Gonzalez, A. M., E. E. Laing and A. P. Gerber (2015). "Conserved mRNA-binding proteomes in eukaryotic organisms." Nat Struct Mol Biol **22**(12): 1027-1033.
- Matunis, M. J., W. M. Michael and G. Dreyfuss (1992). "Characterization and primary structure of the poly(C)-binding heterogeneous nuclear ribonucleoprotein complex K protein." Mol Cell Biol **12**(1): 164-171.
- Medioni, C., K. Mowry and F. Besse (2012). "Principles and roles of mRNA localization in animal development." Development **139**(18): 3263-3276.
- Medioni, C., M. Ramialison, A. Ephrussi and F. Besse (2014). "Imp promotes axonal remodeling by regulating profilin mRNA during brain development." Curr Biol **24**(7): 793-800.
- Miller, M. A. and W. M. Olivas (2011). "Roles of Puf proteins in mRNA degradation and translation." Wiley Interdiscip Rev RNA **2**(4): 471-492.
- Miller, M. T., J. J. Higgin and T. M. Hall (2008). "Basis of altered RNA-binding specificity by PUF proteins revealed by crystal structures of yeast Puf4p." Nat Struct Mol Biol **15**(4): 397-402.
- Mitchell, S. F. and R. Parker (2014). "Principles and properties of eukaryotic mRNPs." Mol Cell **54**(4): 547-558.
- Moore, M. J. (2005). "From birth to death: the complex lives of eukaryotic mRNAs." Science **309**(5740): 1514-1518.
- Muller-McNicoll, M. and K. M. Neugebauer (2013). "How cells get the message: dynamic assembly and function of mRNA-protein complexes." Nat Rev Genet **14**(4): 275-287.
- Murata, Y. and R. P. Wharton (1995). "Binding of pumilio to maternal hunchback mRNA is required for posterior patterning in Drosophila embryos." Cell **80**(5): 747-756.
- Myers, B. R., L. Neahring, Y. Zhang, K. J. Roberts and P. A. Beachy (2017). "Rapid, direct activity assays for Smoothed reveal Hedgehog pathway regulation by membrane cholesterol and extracellular sodium." Proc Natl Acad Sci U S A **114**(52): E11141-E11150.
- Nakamura, A., K. Sato and K. Hanyu-Nakamura (2004). "Drosophila cup is an eIF4E binding protein that associates with Bruno and regulates oskar mRNA translation in oogenesis." Dev Cell **6**(1): 69-78.
- Nakano, Y., S. Nystedt, A. A. Shivdasani, H. Strutt, C. Thomas and P. W. Ingham (2004). "Functional domains and sub-cellular distribution of the Hedgehog transducing protein Smoothed in Drosophila." Mech Dev **121**(6): 507-518.
- Nelson, M. R., A. M. Leidal and C. A. Smibert (2004). "Drosophila Cup is an eIF4E-binding protein that functions in Smaug-mediated translational repression." EMBO J **23**(1): 150-159.
- Niu, N., J. F. Xiang, Q. Yang, L. Wang, Z. Wei, L. L. Chen, L. Yang and W. Zou (2017). "RNA-binding protein SAMD4 regulates skeleton development through translational inhibition of Mig6 expression." Cell Discov **3**: 16050.
- Palacios, I. M. (2007). "How does an mRNA find its way? Intracellular localisation of transcripts." Semin Cell Dev Biol **18**(2): 163-170.

- Pandey, U. B. and C. D. Nichols (2011). "Human disease models in *Drosophila melanogaster* and the role of the fly in therapeutic drug discovery." Pharmacol Rev **63**(2): 411-436.
- Parker, J. S., S. M. Roe and D. Barford (2005). "Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex." Nature **434**(7033): 663-666.
- Pascual, M. L., L. Luchelli, M. Habif and G. L. Boccaccio (2012). "Synaptic activity regulated mRNA-silencing foci for the fine tuning of local protein synthesis at the synapse." Commun Integr Biol **5**(4): 388-392.
- Pereira, J., W. E. Johnson, S. J. O'Brien, E. D. Jarvis, G. Zhang, M. T. Gilbert, V. Vasconcelos and A. Antunes (2014). "Evolutionary genomics and adaptive evolution of the Hedgehog gene family (Shh, Ihh and Dhh) in vertebrates." PLoS One **9**(12): e74132.
- Pinder, B. D. and C. A. Smibert (2013). "microRNA-independent recruitment of Argonaute 1 to nanos mRNA through the Smaug RNA-binding protein." EMBO Rep **14**(1): 80-86.
- Price, M. A. and D. Kalderon (2002). "Proteolysis of the Hedgehog signaling effector Cubitus interruptus requires phosphorylation by Glycogen Synthase Kinase 3 and Casein Kinase 1." Cell **108**(6): 823-835.
- Protter, D. S. and R. Parker (2016). "Principles and Properties of Stress Granules." Trends Cell Biol **26**(9): 668-679.
- Proudfoot, N. J., A. Furger and M. J. Dye (2002). "Integrating mRNA processing with transcription." Cell **108**(4): 501-512.
- Rivera-Pomar, R. and H. Jackle (1996). "From gradients to stripes in *Drosophila* embryogenesis: filling in the gaps." Trends Genet **12**(11): 478-483.
- Robbins, D. J., D. L. Fei and N. A. Riobo (2012). "The Hedgehog signal transduction network." Sci Signal **5**(246): re6.
- Rouget, C., C. Papin, A. Boureux, A. C. Meunier, B. Franco, N. Robine, E. C. Lai, A. Pelisson and M. Simonelig (2010). "Maternal mRNA deadenylation and decay by the piRNA pathway in the early *Drosophila* embryo." Nature **467**(7319): 1128-1132.
- Sanial, M., I. Becam, L. Hofmann, J. Behague, C. Arguelles, V. Gourhand, L. Bruzzone, R. A. Holmgren and A. Plessis (2017). "Dose-dependent transduction of Hedgehog relies on phosphorylation-based feedback between the G-protein-coupled receptor Smoothed and the kinase Fused." Development **144**(10): 1841-1850.
- Saunders, L. R. and G. N. Barber (2003). "The dsRNA binding protein family: critical roles, diverse cellular functions." FASEB J **17**(9): 961-983.
- Schirle, N. T. and I. J. MacRae (2012). "The crystal structure of human Argonaute2." Science **336**(6084): 1037-1040.
- Semotok, J. L., R. L. Cooperstock, B. D. Pinder, H. K. Vari, H. D. Lipshitz and C. A. Smibert (2005). "Smaug recruits the CCR4/POP2/NOT deadenylase complex to trigger maternal transcript localization in the early *Drosophila* embryo." Curr Biol **15**(4): 284-294.

- Semotok, J. L., H. Luo, R. L. Cooperstock, A. Karaiskakis, H. K. Vari, C. A. Smibert and H. D. Lipshitz (2008). "Drosophila maternal Hsp83 mRNA destabilization is directed by multiple SMAUG recognition elements in the open reading frame." Mol Cell Biol **28**(22): 6757-6772.
- Shahbadian, K. and P. Chartrand (2012). "Control of cytoplasmic mRNA localization." Cell Mol Life Sci **69**(4): 535-552.
- Siomi, M. C., K. Higashijima, A. Ishizuka and H. Siomi (2002). "Casein kinase II phosphorylates the fragile X mental retardation protein and modulates its biological properties." Mol Cell Biol **22**(24): 8438-8447.
- Smibert, C. A., Y. S. Lie, W. Shillinglaw, W. J. Henzel and P. M. Macdonald (1999). "Smaug, a novel and conserved protein, contributes to repression of nanos mRNA translation in vitro." RNA **5**(12): 1535-1547.
- Smibert, C. A., J. E. Wilson, K. Kerr and P. M. Macdonald (1996). "smaug protein represses translation of unlocalized nanos mRNA in the Drosophila embryo." Genes Dev **10**(20): 2600-2609.
- Sonenberg, N. and A. G. Hinnebusch (2009). "Regulation of translation initiation in eukaryotes: mechanisms and biological targets." Cell **136**(4): 731-745.
- Sonoda, J. and R. P. Wharton (1999). "Recruitment of Nanos to hunchback mRNA by Pumilio." Genes Dev **13**(20): 2704-2712.
- St Johnston, D., D. Beuchle and C. Nusslein-Volhard (1991). "Staufen, a gene required to localize maternal RNAs in the Drosophila egg." Cell **66**(1): 51-63.
- Standart, N. and N. Minshall (2008). "Translational control in early development: CPEB, P-bodies and germinal granules." Biochem Soc Trans **36**(Pt 4): 671-676.
- Stevenson, A. L. and C. J. Norbury (2006). "The Cid1 family of non-canonical poly(A) polymerases." Yeast **23**(13): 991-1000.
- Strein, C., A. M. Alleaume, U. Rothbauer, M. W. Hentze and A. Castello (2014). "A versatile assay for RNA-binding proteins in living cells." RNA **20**(5): 721-731.
- Sysoev, V. O., B. Fischer, C. K. Frese, I. Gupta, J. Krijgsveld, M. W. Hentze, A. Castello and A. Ephrussi (2016). "Global changes of the RNA-bound proteome during the maternal-to-zygotic transition in Drosophila." Nat Commun **7**: 12128.
- Tabata, T. and Y. Takei (2004). "Morphogens, their identification and regulation." Development **131**(4): 703-712.
- Tadros, W., A. L. Goldman, T. Babak, F. Menzies, L. Vardy, T. Orr-Weaver, T. R. Hughes, J. T. Westwood, C. A. Smibert and H. D. Lipshitz (2007). "SMAUG is a major regulator of maternal mRNA destabilization in Drosophila and its translation is activated by the PAN GU kinase." Dev Cell **12**(1): 143-155.
- Tadros, W. and H. D. Lipshitz (2009). "The maternal-to-zygotic transition: a play in two acts." Development **136**(18): 3033-3042.
- Thandapani, P., T. R. O'Connor, T. L. Bailey and S. Richard (2013). "Defining the RGG/RG motif." Mol Cell **50**(5): 613-623.

- Varjosalo, M. and J. Taipale (2008). "Hedgehog: functions and mechanisms." Genes Dev **22**(18): 2454-2472.
- Walden, W. E., A. I. Selezneva, J. Dupuy, A. Volbeda, J. C. Fontecilla-Camps, E. C. Theil and K. Volz (2006). "Structure of dual function iron regulatory protein 1 complexed with ferritin IRE-RNA." Science **314**(5807): 1903-1908.
- Walser, C. B. and H. D. Lipshitz (2011). "Transcript clearance during the maternal-to-zygotic transition." Curr Opin Genet Dev **21**(4): 431-443.
- Wang, J. T., J. Smith, B. C. Chen, H. Schmidt, D. Rasoloson, A. Paix, B. G. Lambrus, D. Calidas, E. Betzig and G. Seydoux (2014). "Regulation of RNA granule dynamics by phosphorylation of serine-rich, intrinsically disordered proteins in *C. elegans*." Elife **3**: e04591.
- Xia, R., H. Jia, J. Fan, Y. Liu and J. Jia (2012). "USP8 promotes smoothed signaling by preventing its ubiquitination and changing its subcellular localization." PLoS Biol **10**(1): e1001238.
- Yan, K. S., S. Yan, A. Farooq, A. Han, L. Zeng and M. M. Zhou (2003). "Structure and conserved RNA binding of the PAZ domain." Nature **426**(6965): 468-474.
- Yang, X., F. Mao, X. Lv, Z. Zhang, L. Fu, Y. Lu, W. Wu, Z. Zhou, L. Zhang and Y. Zhao (2013). "Drosophila Vps36 regulates Smo trafficking in Hedgehog signaling." J Cell Sci **126**(Pt 18): 4230-4238.
- Ye, B., C. Petritsch, I. E. Clark, E. R. Gavis, L. Y. Jan and Y. N. Jan (2004). "Nanos and Pumilio are essential for dendrite morphogenesis in *Drosophila* peripheral neurons." Curr Biol **14**(4): 314-321.
- Zaessinger, S., I. Busseau and M. Simonelig (2006). "Oskar allows nanos mRNA translation in *Drosophila* embryos by preventing its deadenylation by Smaug/CCR4." Development **133**(22): 4573-4583.
- Zhai, B., J. Villen, S. A. Beausoleil, J. Mintseris and S. P. Gygi (2008). "Phosphoproteome analysis of *Drosophila melanogaster* embryos." J Proteome Res **7**(4): 1675-1682.
- Zhang, C., E. H. Williams, Y. Guo, L. Lum and P. A. Beachy (2004). "Extensive phosphorylation of Smoothed in Hedgehog pathway activation." Proc Natl Acad Sci U S A **101**(52): 17900-17907.
- Zhang, M., R. A. Pierce, H. Wachi, R. P. Mecham and W. C. Parks (1999). "An open reading frame element mediates posttranscriptional regulation of tropoelastin and responsiveness to transforming growth factor beta1." Mol Cell Biol **19**(11): 7314-7326.
- Zhang, Y. Q., A. M. Bailey, H. J. Matthies, R. B. Renden, M. A. Smith, S. D. Speese, G. M. Rubin and K. Broadie (2001). "Drosophila fragile X-related gene regulates the MAP1B homolog Futsch to control synaptic structure and function." Cell **107**(5): 591-603.
- Zhao, Y., C. Tong and J. Jiang (2007). "Hedgehog regulates smoothed activity by inducing a conformational switch." Nature **450**(7167): 252-258.
- Zheng, X., R. K. Mann, N. Sever and P. A. Beachy (2010). "Genetic and biochemical definition of the Hedgehog receptor." Genes Dev **24**(1): 57-71.
- Zhu, A. J., L. Zheng, K. Suyama and M. P. Scott (2003). "Altered localization of *Drosophila* Smoothed protein activates Hedgehog signal transduction." Genes Dev **17**(10): 1240-1252.