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# Regulation of lozenge transcription factor activity and blood cell development by MLF and its partner DnaJ-1

Aichun Chen

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# THÈSE

En vue de l'obtention du

## DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)



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**Présentée et soutenue par :**

**Aichun CHEN**

**le** 27 Juin 2017

**Titre :**

Regulation of Lozenge transcription factor activity and blood cell development by MLF and its partner DnaJ-1

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**Directeur/trice(s) de Thèse :**

Dr. Lucas WALTZER

Dr. Marc HAENLIN

**Jury :**

Pr. Christiane BIERKAMP, président

Dr. Bruno LEMAITRE, rapporteur

Dr. Marie-Bérengère TROADEC, rapportrice

Dr. Magalie LECOURTOIS, rapportrice

Dr. Anne PLESSIS, examinatrice

Dr. Lucas WALTZER, directeur de thèse

Dr. Marc HAENLIN, directeur de thèse



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## Abstract

Hematopoiesis is the process of formation of fully differentiated blood cells from hematopoietic stem cells (HSCs). This process is tightly controlled by the integration of developmental and homeostatic signals to ensure the generation of an appropriate number of each blood cell type. At the molecular level, the regulation of this developmental process is mediated by a number of transcription factors, especially by members of the RUNX family, and mutations affecting these factors are at the origin of numerous hemopathies, including leukemia. Intriguingly, many transcriptional regulators and signaling pathways controlling blood cell development are evolutionarily conserved from humans to *Drosophila melanogaster*. Hence, the fruit fly has become a potent and simplified model to study the mechanisms underlying the specification of blood cell lineages and the regulation of blood cell homeostasis.

Members of the Myeloid Leukemia Factor (MLF) family have been implicated in hematopoiesis and in oncogenic blood cell transformation, but their function and molecular mechanism of action remain elusive. Previous work in *Drosophila* showed that MLF stabilizes the RUNX transcription factor Lozenge (LZ) and controls the number of LZ<sup>+</sup> blood cells. During my PhD, I sought to further decipher the molecular mechanism of action of MLF on Lozenge during blood cell development.

Using a proteomic approach in *Drosophila* Kc167 cells, we identified the Hsp40 co-chaperone family member DnaJ-1 and its chaperone partner Hsc70-4 as two partners of MLF. These interactions were confirmed by co-immunoprecipitations and *in vitro* pull-down assays. Importantly, we found that knocking down DnaJ-1 or Hsc70-4 expression in Kc167 cells caused a reduction in the level of Lozenge protein and a concomitant decrease in Lozenge transactivation activity, which were very similar to those caused by MLF knock-down. Similarly, over-expression of two DnaJ-1 mutants that are unable to stimulate the chaperone activity of Hsc70-4 also decreased Lozenge level and impaired its capacity to activate transcription. These results suggest that MLF could act within a chaperone complex composed of DnaJ-1 and Hsc70-4 to control Lozenge stability and activity. Along that line, we showed by co-immunoprecipitation that Lozenge interacts with MLF, DnaJ-1 and Hsc70-4, respectively. Using various truncated mutants of MLF or DnaJ-1, we showed that MLF and DnaJ-1 interact and together with Lozenge through their conserved MLF



homology domain (MHD) and C-terminal region, respectively. Furthermore, *in vitro* GST pull-down assays suggested that the interactions between MLF, DnaJ-1 and Lozenge are direct. Thus, we propose that MLF and DnaJ-1 control Lozenge protein level by interacting with it and by promoting its folding and/or solubility via the Hsc70 chaperone machinery.

In parallel, we assessed DnaJ-1 function in *Drosophila* blood cells *in vivo* using a null allele of *dnaj-1* generated by CRISPR/Cas9 technique. We found that, like *mlf*, *dnaj-1* mutation leads to an increase in the number and size of LZ<sup>+</sup> blood cells, as well as to an over-activation of the Notch signaling pathway in these cells. Moreover, our data suggested that high levels of active Lozenge are required to control the number and size of LZ<sup>+</sup> blood cells, and to down-regulate Notch expression. We propose that the MLF/DnaJ-1 complex controls LZ<sup>+</sup> blood cell development *in vivo* by regulating Lozenge protein level/activity and thereby Notch pathway activation.

In sum, our results establish a functional link between MLF, the Hsp40 co-chaperone DnaJ-1 and the RUNX transcription factor Lozenge, which could be conserved in other species.

## Résumé

L'hématopoïèse est le processus de formation des cellules sanguines différenciées à partir de cellules souches hématopoïétiques. Ce processus est étroitement contrôlé par l'intégration de signaux de développementaux et homéostatiques pour assurer une production équilibrée des différents types de cellules sanguines. Au niveau moléculaire, la régulation de ce processus est médiée par un certain nombre de facteurs de transcription, en particulier par les membres de la famille RUNX. Ainsi, des mutations affectant les membres de cette famille peuvent entraîner une déréglementation du programme de différenciation hématopoïétique et causer des hémopathies, dont des leucémies. D'une manière intrigante, de nombreux régulateurs de la transcription et des voies de signalisation contrôlant le développement des cellules sanguines sont évolutivement conservés des humains à *Drosophila melanogaster*, qui est donc utilisée comme organisme modèle pour étudier les mécanismes sous-jacents à la spécification des lignages sanguins et au contrôle de l'homéostasie des cellules sanguines.

Les membres de la famille Myeloid Leukemia Factor (MLF) ont été impliqués dans l'hématopoïèse et dans la transformation oncogénique des cellules sanguines, mais leur fonction et leur mécanisme d'action moléculaire restent insaisissables. Des travaux précédents chez la Drosophile ont montré que MLF stabilise le facteur de transcription de type RUNX Lozengue (LZ) et contrôle le nombre de cellules sanguines LZ<sup>+</sup>. Au cours de ma thèse, j'ai cherché à déchiffrer le mécanisme moléculaire d'action de MLF sur Lozengue dans les cellules sanguines.

Par une approche protéomique puis par des expériences de co-immunoprécipitation dans les cellules de Drosophile Kc167, nous avons identifié le co-chaperon de type Hsp40 DnaJ-1, et son partenaire le chaperon Hsc70-4, comme deux partenaires de MLF. De façon importante, nous avons montré que l'inhibition de l'expression de DnaJ-1 ou de Hsc70-4 dans les cellules Kc167 induit une réduction du niveau de protéine Lozengue et une diminution de sa capacité à activer la transcription très semblable à celles observées suite à l'inhibition de l'expression de MLF. De plus, la sur-expression de mutants de DnaJ-1 incapables d'activer le chaperon Hsc70-4 entraîne aussi une réduction du niveau de Lozengue et de sa capacité de transactivation et des expériences de coimmunoprécipitation montrent que Lozengue interagit avec MLF, DnaJ-1 et Hsc70-4. Nos résultats suggèrent donc que MLF agit au sein d'un complexe chaperon composé de DnaJ-1 et Hsc70-4 pour contrôler le

niveau de Lozenge. En utilisant différents mutants de MLF ou DnaJ-1, nous avons montré que MLF et DnaJ-1 interagissent ensemble et avec Lozenge *via* des domaines phylogénétiquement conservés. D'autre part, des expériences de GST « pull down » *in vitro* suggèrent que ces trois protéines peuvent interagir ensemble directement. Nous proposons donc que MLF et DnaJ-1 contrôlent le niveau de protéine Lozenge en interagissant avec elle et en favorisant son repliement et/ou sa solubilité *via* l'activité chaperon de Hsc70-4.

En parallèle, nous avons étudié la fonction de DnaJ-1 *in vivo* dans le développement des cellules sanguines de la Drosophile. Nos résultats montrent que, comme *mlf*, la perte de *dnaj-1* s'accompagne d'une augmentation de la taille et du nombre des cellules sanguines  $LZ^+$ , ainsi que d'une hyperactivation de la voie de signalisation Notch dans ces cellules. Nos résultats suggèrent que des hauts niveaux de Lozenge sont nécessaires pour contrôler le nombre et la taille des cellules  $LZ^+$  et pour inhiber l'expression de Notch. Nous proposons que le complexe MLF/DnaJ-1 contrôle le développement du lignage  $LZ^+$  en régulant le niveau de protéine Lozenge, et ainsi le niveau d'activité de la voie Notch.

En conclusion, nos résultats ont mis à jour un lien fonctionnel entre MLF, le co-chaperon de type Hsp40 DnaJ-1 et un facteur de transcription de type RUNX, qui pourrait être conservé dans d'autres espèces.

## Foreword

Cancers are a large family of diseases that involve abnormal cell growth with the potential to invade or spread to other parts of the body. They have some common hallmarks, including continuous cell growth and division absent the proper signals, limitless number of cell divisions, avoidance of programmed cell death, and invasion of tissue and formation of metastases. It is estimated that there are over 100 types of cancers that affect human health, with signs and symptoms including a lump, abnormal bleeding, prolonged cough, unexplained weight loss and a change in bowel movements. Many factors could contribute to the emergence of cancers, such as tobacco use, which accounts for about 22% of cancer deaths, and obesity, poor diet, excessive drinking of alcohol, exposure to ionizing radiation, environmental pollutants. At the molecular level of pathophysiology, cancers are driven by progressive genetic or chromosomal abnormalities and epigenetic alterations. In genetic abnormalities, two broad categories of genes that regulate cell growth and differentiation are affected, which are oncogenes and tumor suppressor genes. Malignant transformation can occur through the formation of new oncogenes, the inappropriate over-expression of normal oncogenes, or the under-expression or disabling of tumor suppressor genes. Epigenetic alterations are functionally relevant modification to the genome, such as changes in DNA methylation, histone modification or changes in chromosomal architecture. They don't change the underlying DNA sequence, but regulate the expression of some particular genes, for instance, DNA repair genes, whose reduced expression disrupts DNA repair.

Similarly, leukemia is a group of cancers that usually start in the bone marrow of human beings and other warm-blooded animals. It is characterized by an abnormal increase in the number of immature white blood cells in the tissues and often in the blood. According to how quickly the disease develops and which type of blood cells is affected, there provides a total of four most common types of leukemia, which are acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoid leukemia (ALL) and chronic lymphoid leukemia (CLL). Among them, acute myeloid leukemia is the most common malignant myeloid disorder in adults, which frequently results in hematopoietic insufficiency, such as anemia, thrombocytopenia and granulocytopenia and other symptoms. Chromosomal translocations, for instance, t(8;21) translocation in core-binding factor RUNX or t(3;5) translocation in myeloid leukemia factor, have been believed to be involved in the pathogenesis of AML. The t(8;21) translocation produces a fusion protein

RUNX1-ETO, and the t(3;5) translocation produces a fusion protein NPM1-MLF. Although the properties and function of RUNX1 have been studied extensively, the function and mode of action of MLF have been remained rather elusive. Recently, it was shown that MLF is a conserved regulator of RUNX transcription factor activity in a *Drosophila* model, which sheds a new light on the relationship between MLF and RUNX in AML as well as in other cancers

In this PhD thesis, I first made a brief introduction to the hematopoiesis in human and in *Drosophila melanogaster* and its associated three families of genes, which are *RUNX*, *MLF* and *DnaJ* families. Then I presented the results of my PhD project that is entitled *Regulation of Lozenge transcription factor activity and blood cell development by MLF and its partner DnaJ-1*. Finally, I discussed the significance of this study and made a perspective about the future research on this topic.

## List of abbreviations

AGM: Aorta-Gonad-Mesonephros  
AIF: Apoptosis-Inducing Factor  
ALL: Acute Lymphoid Leukemia  
AML: Acute Myeloid Leukemia  
APL: Acute Promyelocytic Leukemia  
Bc: Black cell  
Bgb: Big brother  
Bro: Brother  
CASA: Chaperone-Assistant Selective Autophagy  
CBF: Core-binding Factor  
C/EBP: CCAAT Enhancer-Binding Protein  
CLL: Chronic Lymphoid Leukemia  
CLP: Common Lymphoid Progenitors  
CMA: Chaperone Mediated Autophagy  
CML: Chronic Myeloid Leukemia  
CMP: Common Myeloid Progenitors  
Col: Collier  
Crq: Croquemort  
CSC: Cancer Stem Cell  
CZ: Cortical Zone  
DC: Dendritic Cell  
DnaJ-1: DnaJ-1 like protein  
DREF: DNA Replication Enhancer Factor  
dsRNA: double strand RNA  
EBF: Early B-cell Factor  
ECM: Extra-Cellular Matrix  
EGFR: Epidermal Growth Factor Receptor  
Epo: Erythropoietin  
ERK: Extracellular signal-Regulated Kinase  
ESC: Embryonic Stem Cell  
ETO: Eight Twenty One  
FAB: French-American-British  
FL: Fetal Liver  
FOG: Friend of GATA  
FPD: Familial Platelet Disorder  
Gcm: Glial cell missing

GFP: Green Fluorescent Protein  
GM-CSF: Granulocyte/Macrophage Colony-Stimulation Factor  
GMP: Granulocyte/Macrophage Progenitors  
HT: Huntington's Disease  
HDAC: Histone Deacetylase  
Hh: Hedgehog  
HIPK2: Homeodomain-Interacting Protein Kinase 2  
HLS7: Hematopoietic Lineage Switch 7  
HSC: Hematopoietic Stem Cell  
HSP: Heat Shock Protein  
HSPC: Hematopoietic Stem/Progenitor Cell  
HSR: Heat Shock Response  
HTT: Huntingtin  
InR/TOR: Insulin/Target Of Rapamycin  
JAK/STAT: Janus Kinase/Signal Transducer and Activator of Transcription  
JNK: Jun N-terminal Kinase  
LSC: Leukemia Stem Cell  
LZ: Lozenge  
M-CSFR: Macrophage Colony-Stimulating Factor Receptor  
MDS: Myelodysplastic Syndrome  
MEP: Megakaryocyte/Erythrocyte Progenitors  
MHC: Major Histocompatibility Complex  
MHD: MLF Homology Domain  
MLF: Myeloid Leukemia Factor  
MPD: Myeloproliferative Disorder  
MS: Mass Spectrometry  
MTG8: Myeloid Tumor Gene 8  
MZ: Medullary Zone  
NBD: Nucleotide-Binding Domain  
NECD: Notch Extracellular Domain  
NEF: Nucleotide Exchange Factor  
NES: Nuclear Export Signal  
NHR: Nerve Homology Region  
NICD: Notch Intracellular Domain  
NK cells: Natural Killer cells  
NLS: Nuclear Localization Signal  
NMTS: Nuclear-Matrix-attachment Signal  
NPM: Nucleophosmin

OLIGOM: NPM Oligomerization Domain  
PDGF: Platelet-Derived Growth Factor  
PO: phenoloxidase  
PPO: prophenoloxidase  
PSC: Posterior Signalling Centre  
PTM: Post-translational Modification  
PVR: PDGF/VEFG-related receptor  
RBC: Red Blood Cell  
RHD: Runt Homology Domain  
RNAi: RNA Interference  
ROS: Reactive Oxygen Species  
RUNX: Runt-related binding factor  
SBD: Substrate-Binding Domain  
SCL: Stem Cell Leukemia  
SEM: Scanning Electron Microscope  
Ser: Serrate  
SRP: Serpent  
Su(H): Suppressor of Hairless  
TLE: Transducing-Like Enhancer  
 $\beta$ -TM:  $\beta$ -Thalassemia Major  
TNF: Tumor Necrosis Factor  
Upd: Unpaired  
UPS: Ubiquitin-Proteasome-System  
Ush: U-shaped  
VEGF: Vascular Endothelial Growth Factor  
WBC: White Blood Cell  
Wg: Wingless  
WHO: World Health Organization  
YS: Yolk Sac





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## **Chapter I Introduction**

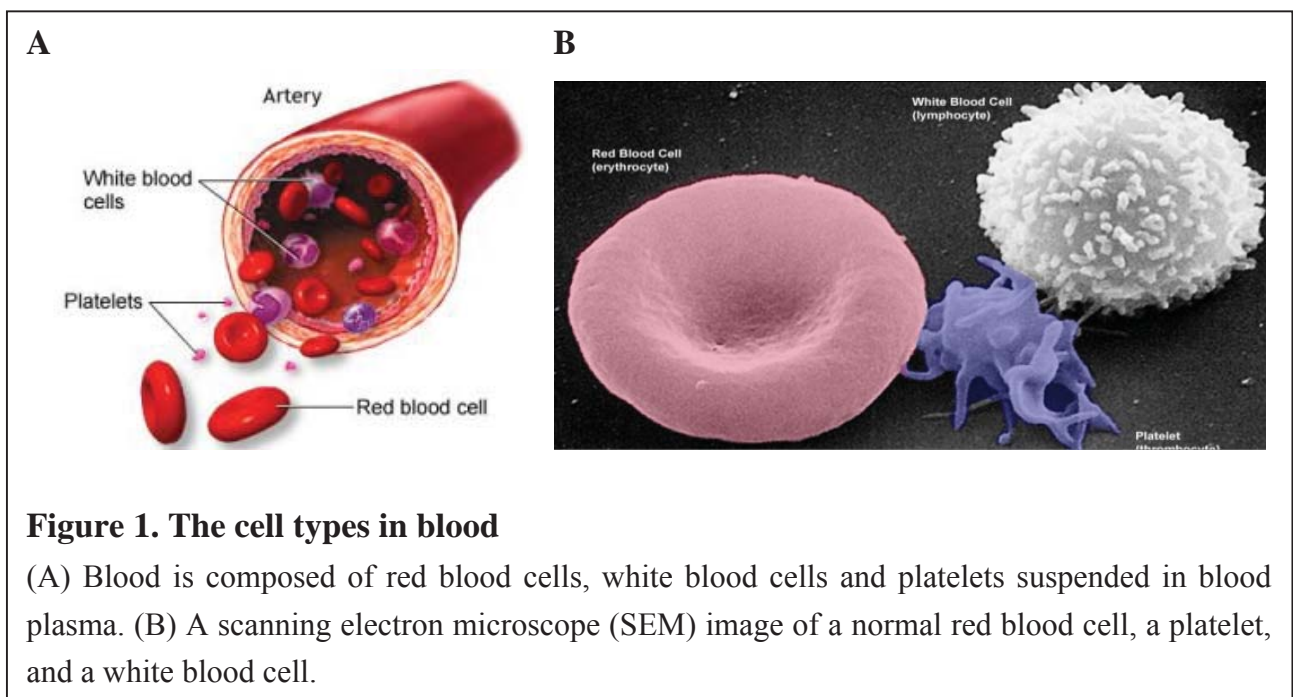


# 1. A general description of hematopoiesis and leukemia

## 1.1. Hematopoiesis in mammals

### 1.1.1. Ontogeny of the mammalian hematopoiesis

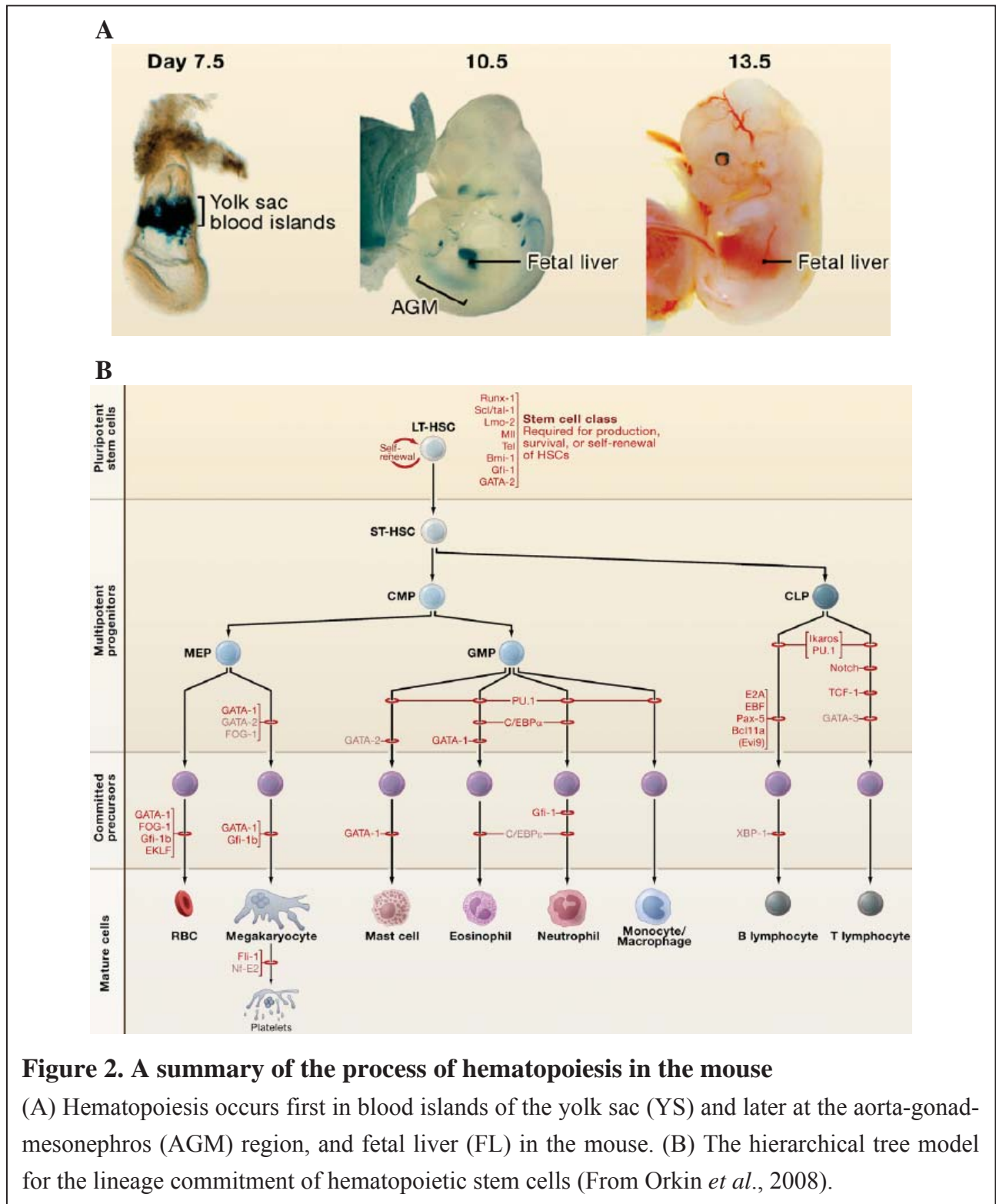
Blood is a bodily fluid in animals that delivers necessary substances to the cells and transports metabolic waste products away from those same cells, which is considered as one of the most highly regenerative tissues with approximately one trillion cells producing daily in adult bone marrow. It performs many important functions within the body, including supply of oxygen and nutrients to tissues, removal of carbon dioxide, immunological functions such as detection of foreign materials by antibodies, regulation of body pH and temperature, coagulation and so on. In vertebrates, blood is mainly composed of blood cells suspended in blood plasma. The cellular components of the blood can be roughly classified into three types, which are red blood cells (RBCs), white blood cells (WBCs) and platelets (**Figure 1**). These three highly specialized cell types are involved in gas transport, immune responses, and blood clotting, respectively. In a word, blood and its cellular components are essentially vital for human health and normal life, so it is of much significance to determine where these blood cells come from and how they are generated.



To answer these important questions, the concept of hematopoiesis would be introduced formally: hematopoiesis is the process of the generation and formation of appropriate numbers of fully differentiated blood cells, which is controlled by the integration of developmental and homeostatic signals (Orkin *et al.*, 2008). In mammals,



diverse types of blood cells are produced from rare hematopoietic stem cells (HSCs) that reside in adult bone marrow. This blood cell development occurs in at least two distinct waves in mammals, which are called primitive hematopoiesis for the first wave and definitive hematopoiesis for the second wave (Jagannathan-Bogdan *et al.*, 2013). The primitive hematopoiesis takes place within the blood islands of the yolk sac (YC) at embryonic day 7.5 in the mouse (**Figure 2.A**) and gives rise to primitive erythrocytes,

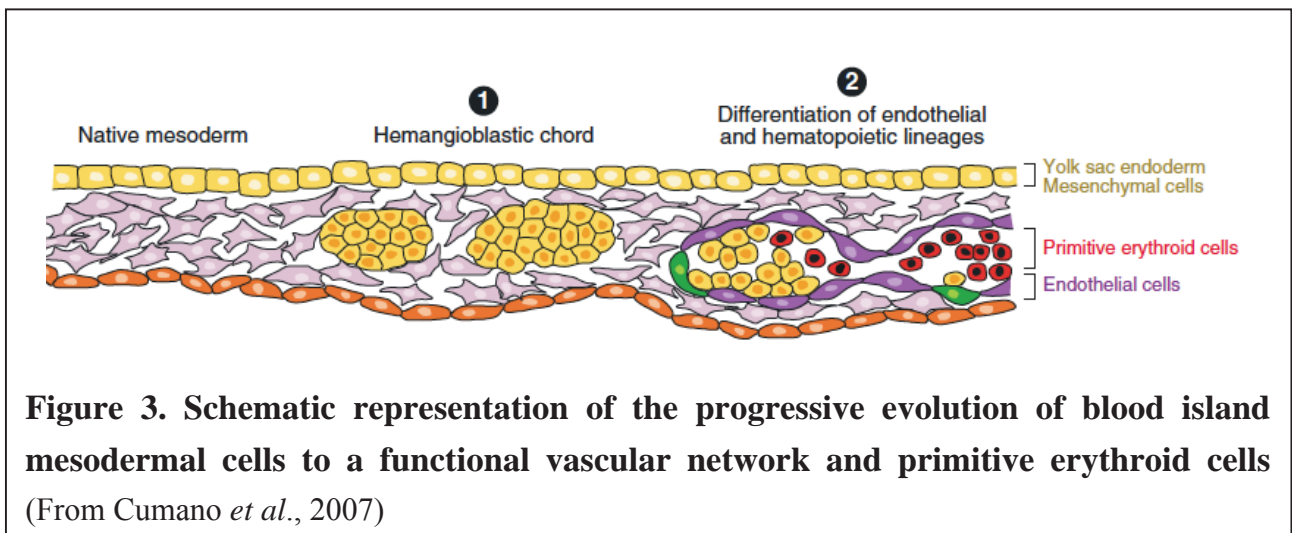


megakaryocytes and macrophages (Dzierzak *et al.*, 2008). The primary purpose of this primitive hematopoiesis is the production of erythrocytes that can facilitate tissue oxygenation as the embryos grow very rapidly (Orkin, 2000). This primitive hematopoietic wave is transient and it is rapidly replaced by the second adult-type wave of hematopoiesis. By contrast, the definitive hematopoiesis first occurs in the aorta-gonad-mesonephros (AGM) region during later embryonic development (**Figure 2.A**) with multipotent hematopoietic stem cells (HSCs) arising (Dzierzak, 1999). These definitive HSCs can give rise to all blood cell lineages of the adult organism and subsequently they migrate to the fetal liver and thymus as the embryo develops. At the end of fetal development, hematopoietic stem cells migrate to the bone marrow, which is the major site of postnatal hematopoiesis in adults. Of note, it is now established that some of the adult macrophages are derived from the primitive wave of hematopoiesis (Perdiguero *et al.*, 2016).

HSCs in the bone marrow ensure continuous hematopoietic cell production throughout life thanks to their dual capacities: they have the capacity to self-renew to maintain their number, and the potential to differentiate into all cell lineages of the blood and immune system. HSC differentiation is a complex and dynamic hierarchical process (**Figure 2.B**) (Orkin *et al.*, 2008). The hematopoietic stem cells first give rise to two distinct groups of multipotent progenitors: the common myeloid progenitors (CMPs) and the common lymphoid progenitors (CLPs). Then common myeloid progenitors further differentiate into megakaryocyte/erythrocyte progenitors (MEPs) and granulocyte/macrophage progenitors (GMPs). From these progenitors, committed precursors for the various lineages arise and can be further specified into diverse mature myeloid blood cells with different specific functions, including erythrocytes, megakaryocytes, granulocytes, monocytes/macrophages. Similarly, common lymphoid progenitors will give rise to B lymphocytes, T lymphocytes, and natural killer cells.

This hierarchical tree model assumes that hematopoiesis is usually governed by binary cell fate choices, in which the lymphoid system is completely separated from the myeloid system (Ceredig *et al.*, 2009). However, some evidence suggests that under some special conditions, lymphoid progenitors retain their ability to give rise to myeloid lineage cells, and thymic progenitors have both lymphoid and myeloid potentials. So this separation may not be as absolute as thought earlier, and a need for new models is in demand. Some years ago, a simple pairwise relationships model of hematopoiesis emerged, which depicts

hematopoiesis as a continuum of lineage relationships between hematopoietic stem cells and their oligopotent progeny (Ceredig *et al.*, 2009). For instance, dendritic cells could be derived from both megakaryocyte-monocyte progenitors and B-cell-T-cell progenitors. This reflects that a final cell fate could be reached through more than one type of intermediate progenitor as well as the high complexity of the hematopoietic system. More recently, single cell-based gene expression profiling analyses have suggested a much revised version of the hematopoietic tree and have questioned the existence of several previously described ‘common progenitors’ (Moignard *et al.*, 2016). Thus, although mammalian blood cell differentiation has been extensively studied and considered as a paradigmatic differentiation process, much remains to be discovered in this field.



In addition, a close relationship between vascular endothelium and hematopoietic stem cells during ontogeny provides us more insights into the origin of blood cells. It has been proposed that during primitive hematopoiesis, blood cell progenitors arise from hemangioblast, a common mesodermal progenitor for both endothelial and hematopoietic cells (**Figure 3.**). However, most recent studies rather suggest that it is a hemogenic endothelium (i.e. an endothelial cell that has the potential to give blood cells) that give rise to blood cell progenitors in the yolk sac and to definitive HSCs (Lacaud *et al.*, 2017). For instance, in the aorta-gonad-mesonephros (AGM) region, the endothelium in the ventral wall of the aorta undergoes an endothelial to hematopoietic transition to produce HSCs. All together, the origin of hematopoietic development can be depicted as a single linear developmental process, which originates from mesoderm, through stages of hemangioblast and/or hemogenic endothelium, hematopoietic stem cells, multipotent progenitors, committed precursors, and finally mature blood cells (Cumano *et al.*, 2007).

### 1.1.2. Functions of mature blood cells

The different mature blood cell types produced in the bone marrow then leave this hematopoietic organ and enter into the blood circulatory system, performing several specialized functions (Hartenstein, 2006; Orkin, 2000).

**Erythrocytes** (also called **red blood cells**) are the most common blood cell type and occupy 40% to 45% of the blood volume. In humans, mature erythrocytes look like small flexible and oval biconcave disks lacking nucleus and most organelles, but are rich in hemoglobin, an iron-containing protein that reversibly binds oxygen and greatly increases its solubility in blood. They take up oxygen in the lungs and deliver it to the body tissue via blood flow through the circulatory system.

**Thrombocytes** (also called **platelets**) are cytoplasmic fragments without nucleus that are derived from the megakaryocytes, giant and polyploid cells from the bone marrow. Their main function is to stop bleeding at the site of interrupted endothelium by clumping and clotting blood vessel injuries through the three steps of adhesion, activation and aggregation.

**Granulocytes** are a category of white blood cells that have a segmented nucleus with varying shapes and are packed with granules filled with a variety of enzymes in their cytoplasm. Distinguished by their appearance under Wright's staining, granulocytes are classified into three principal types, which are neutrophils, eosinophils and basophils. These cells are professional phagocytes that are dedicated to the ingestion and destruction of bacteria and other pathogens invading the body, and they release their granule contents to these pathogens by exocytosis to help fight infection and inflammation.

**Monocytes** are mononuclear professional phagocytes like granulocytes that invade the tissue at the infection sites. They undergo further differentiation into macrophages that can divide and multiply at the needed sites. Macrophages respond to foreign materials (such as bacteria, protozoa or tumour cells) and phagocytose them. Besides their role in phagocytosis and thus in the innate immune response, macrophages process proteins of the pathogens to present them to T lymphocytes that help initiate the adaptive immunity.

**Lymphocytes** are one of the subtypes of white blood cells in a vertebrate immune system, including B-lymphocytes, T-lymphocytes and natural killer cells (NK cells). B-lymphocytes and T-lymphocytes constitute the major cellular components of the adaptive

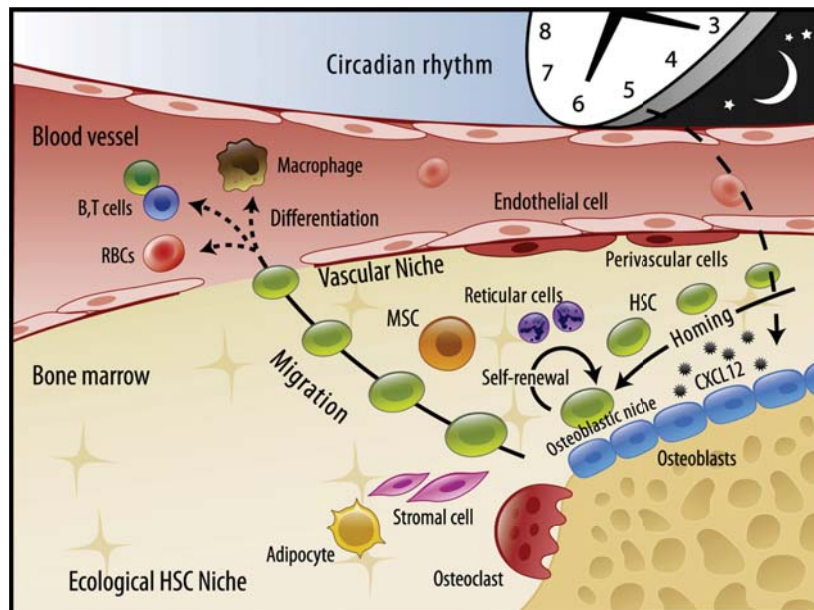
immunity while natural killer cells play a role in the cell-mediated cytotoxic innate immunity. B-lymphocytes are primarily responsible for humoral immunity and they respond to a specific pathogen by producing large quantities of antibodies that then neutralize these foreign substances. T-lymphocytes are involved in cell-mediated immunity and they recognize antigen/major histocompatibility complex (MHC) complexes presented by macrophages with the receptors on their surfaces, which triggers direct cytotoxic effects on the recognized infected cells.

### **1.1.3. Genetic control of the mammalian hematopoietic system**

#### **1.1.3.1. The hematopoietic niches**

Based on numerous studies on hematopoiesis in mammals, including the successful detection of hematopoietic stem cells in the bone marrow of live mice using real-time imaging technology (Xie *et al.*, 2009), it has been showed that there are two subpopulations of hematopoietic stem cells, one that is quiescent and the other that is more active. The decisions of hematopoietic stem cells to become quiescent or differentiate further are tightly controlled by their microenvironment that is termed as niche. The concept of the niche was first proposed in the late 1970s by Schofield who proposed that the fate of a blood stem cell itself is controlled by its interaction with other cells/a particular microenvironment (Schofield, 1978). The niche is composed of subsets of cells and extracellular substrates that can provide structural and trophic support as well as appropriate signals to regulate stem cell functions. This model gained solid experimental and conceptual support from *Drosophila* germ-line stem cell studies. Over the years, a body of experimental evidence revealed that there are three main niches regulating hematopoietic stem cells: the osteoblastic niche, the vascular niche and the perivascular niche (Nakamura-Ishizu *et al.*, 2013). Osteoblasts that align bone surfaces (the osteoblastic niche) interact with hematopoietic stem cells *in vivo* and influence their functions notably through the activation of the Notch signalling pathway to maintain their long-term quiescence. The sinusoidal endothelial cells that align the lumen of sinusoid in the central bone marrow (the vascular niche) can express Notch ligand Jagged-1 and Jagged-2 to control the proliferation of hematopoietic stem cells, which is similar to the osteoblasts. Besides, two perivascular cell groups that possess mesenchymal cells properties also function as niche cells (the perivascular niche). A study on deletion of a chemokine Cxcl12 in mice revealed that hematopoietic stem cells occupy a perivascular niche whereas early lymphoid progenitors occupy an endosteal niche, suggesting different

stem and progenitor cells reside in distinct cellular niches in the bone marrow (Morrison *et al.*, 2014).



**Figure 4. The model of ecological hematopoietic stem cell niche**

HSC homing, maintenance, and differentiation depend on their specific microenvironment (or niche). In the bone marrow, there are at least two major HSC niches, the vascular niche and the osteoblastic niche. In addition, there are growing indications that other types of cells. (From Shiozawa *et al.*, 2012)

The osteoblastic niche and the vascular niche have different roles in the maintenance and differentiation of hematopoietic stem cells in mouse bone marrow, which might be related to oxygen availability. The osteoblastic niche is hypoxic, a low oxygen microenvironment essential for the maintenance and survival of hematopoietic stem cells and their protection from the harmful reactive oxygen species (ROS) accumulation (Croizatier *et al.*, 2011). Meanwhile, the distinct metabolic profile of hematopoietic stem cells also reflects their location in a hypoxic niche, which is revealed by the observation that long-term hematopoietic stem cells utilize glycolysis instead of mitochondrial oxidative phosphorylation to meet their energy demands. Notably, by regulating the response to physiological oxidative stress, the *ataxia telangiectasia mutated (ATM)* gene and the *forkhead O (FoxO)* gene play pivotal roles in the maintenance and survival of hematopoietic stem cells in bone marrow (Morrison *et al.*, 2014). Furthermore, similar to normal tissue stem cells, cancer stem cells (CSCs) in some tumours also contain low ROS levels and enhanced ROS defences, which may contribute to the resistance of tumours to radiation therapy. Therefore, the link between the survival of hematopoietic stem cells and ROS

levels can improve our understanding of the resistance and therapy failure of tumours in the clinical treatments. However, different from the osteoblastic niche, the vascular niche probably has higher oxygen levels that could favour the proliferation and differentiation of hematopoietic stem cells.

### **1.1.3.2. Lineages specific transcription factors and signaling pathways controlling blood cell differentiation**

Genetic and molecular studies in a mouse model have uncovered several signaling pathways that are involved in the communication between hematopoietic stem cells and their niches as well as in the subsequent stages of blood cell proliferation and differentiation, such as Notch, Hedgehog, Wnt or JAK/STAT signaling pathways. Similarly, a plethora of transcription factors and co-factors are involved in the combinatorial transcriptional control of the self-renewal or lineage commitment of hematopoietic stem and progenitor cells, including GATA binding factor (GATA), Friend of GATA co-factors (FOG), runt-related binding factor (RUNX), or Early B-cell factor (EBF) (Orkin *et al.*, 2008). Notably, the development of differentiated blood cells involves a series of alternate cell fate decisions that are driven by the antagonistic activity of “lineage restricted” transcription factors. For example, during primitive hematopoiesis, the transcription factors GATA-1 and Pu.1 exhibit a cross-inhibitory relationship to regulate erythroid versus myeloid cell fate choice: the erythroid factor GATA-1 activates the erythroid program and inhibits myeloid cell fate by interacting with the myeloid factor Pu.1, and conversely, Pu.1 activates the myeloid program and represses the erythroid cell fate (Cantor *et al.*, 2002).

Interestingly, most of these transcription factors and signaling pathways are conserved through evolution and also control blood cell fate in other organisms, as shown by studies in Zebrafish or *Drosophila* (Hartenstein, 2006). Thus, studies in model organisms like *Drosophila*, which exhibit a simpler hematopoietic system and less genetic redundancy, can help to understand some important conserved aspects of the control of blood cell development.

Importantly, the deregulation of these transcription factors and signaling pathways that control normal blood cell development is often at the origin of blood cell cancer (and other hemopathies) in human (Orkin *et al.*, 2008). Accordingly, a number of transcription factors or signaling pathways implicated in hematopoiesis are the targets of recurrent chromosomal translocations or of point mutations that alter (inhibit or activate) their functions. A better

understanding of the normal functions and mode of action of these genes is thus of utmost importance.

## **1.2. Leukemia in human**

Leukemia is a group of cancers that usually start in the bone marrow of human beings and other warm-blooded animals, which is characterized by an abnormal increase in the number of white blood cells in the tissues and often in the blood. These white blood cells are not fully developed and are called leukemic cells. Unlike normal blood cells, leukemic cells don't die when they become old or damaged, so they can build up and crowd out normal blood cells. Due to the low level of normal blood cells, some typical symptoms will appear, including bleeding, bruising problems, feeling tired, fever and an increased risk of infections. So far, the etiology of leukemia remains unknown, but both inherited and environmental factors are believed to be involved in these malignancies. Some risk factors, including smoking, ionizing radiation, chemicals (such as benzene), prior chemotherapy, could contribute to leukemia.

### **1.2.1. Classification of leukemia**

Based on different criteria, leukemia can be subdivided into a variety of large groups. According to how quickly this disease develops, leukemia can be classified into acute leukemia and chronic leukemia clinically and pathologically. **Acute leukemia** usually develops quickly and is characterized by a rapid increase in the number of leukemic cells. This rapid progression and accumulation of leukemic cells, which then spill over into the bloodstream and spread to other organs of the body, out compete normal blood cells, making the bone marrow unable to produce healthy normal blood cells. **Chronic leukemia** usually develops slowly and is characterized by the excessive build up of relatively mature but still abnormal white blood cells. Typically, these cells are produced at a higher rate than normal blood cells, resulting in many abnormal white blood cells over months or years. It is often asymptomatic but if not treated, chronic leukemia can progress into a blast phase, which resembles acute leukemia.

In addition, according to which type of blood cells is affected, leukemia can be classified into myeloid leukemia and lymphoid leukemia, in which the leukemic cells are either derived from a myeloid or a lymphoid cell lineage, respectively. Combining these two classifications, one can distinguish four most common types of leukemia: **acute myeloid leukemia (AML)**, **chronic myeloid leukemia (CML)**, **acute lymphoid leukemia (ALL)**



and **chronic lymphoid leukemia (CLL)**. And within each of these four main types, there are typically several subtypes.

### **1.2.2. Acute myeloid leukemia**

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of hematopoietic progenitor cells, which is the most common malignant myeloid disorder in adults. These hematopoietic progenitor cells are designated as AML blasts that have lost the ability to differentiate normally and to respond to normal proliferation regulators. AML is characterized by the infiltration of the bone marrow, blood and other tissues by clonal, proliferative, abnormally differentiated AML blasts of the hematopoietic system and the interference with the production of normal blood cells, frequently resulting in hematopoietic insufficiency, such as anemia, thrombocytopenia and granulocytopenia, and other symptoms including fatigue, shortness of breath and increased risk of infections.

#### **1.2.2.1. Classification of acute myeloid leukemia**

As studies on AML progress, it is revealed that AML has several subtypes based on the diagnostic procedures, such as morphologic assessment of bone marrow specimens and blood smears, analysis of the expression of cell-surface or cytoplasmic markers, identification of chromosomal abnormalities or screening for selected molecular genetic lesions; subsequently, treatment and prognosis vary among these subtypes. There are two common classification systems for AML according to different criteria, which are the French-American-British (FAB) classification and the World Health Organization (WHO) classification. The FAB classification system was introduced in 1976 and defines AML into eight subtypes that are from M0 to M7 based on the morphological and cyto-chemical characteristics of the leukemic cells (Löwenberg *et al.*, 1999). The WHO classification system was introduced in 2001 and revised in 2008 and in 2016 and defines AML into six major subtypes (**Table 1.**) by incorporating genetic information with morphology, immunophenotype and clinical presentation (Kouchkovsky *et al.*, 2016). These AML classifications provide useful information about the biology and clinical features of this malignancy, also are helpful for assessing treatment options to the patients with AML.

## Table 1. WHO classification of AML and related neoplasms

(From De Kouchkovsky *et al.*, 2016)

Types	Genetic abnormalities
AML with recurrent genetic abnormalities	AML with t(8;21)(q22;q22); RUNX1-RUNX1T1 AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFβ-MYH11 APL with PML-RARA AML with t(9;11)(p21.3;q23.3); MLLT3-KMT2A ML with t(6;9)(p23;q34.1); DEK-NUP214 AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); RBM15-MKL1 AML with BCR-ABL1 (provisional entity) AML with mutated NPM1 AML with biallelic mutations of CEBPA AML with mutated RUNX1 (provisional entity)
AML with myelodysplasia-related changes Therapy-related myeloid neoplasms	AML with minimal differentiation AML without maturation AML with maturation Acute myelomonocytic leukemia Acute monoblastic/monocytic leukemia Acute erythroid leukemia Pure erythroid leukemia Acute megakaryoblastic leukemia Acute basophilic leukemia Acute panmyelosis with myelofibrosis
Myeloid sarcoma Myeloid proliferations related to Down syndrome	Transient abnormal myelopoiesis ML associated with Down syndrome

Abbreviations: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ML, myeloid leukemia; WHO, World Health Organization.

### 1.2.2.2. Pathophysiology of acute myeloid leukemia

AML is a highly heterogeneous disease that appears as a *de novo* malignancy. The abnormal proliferation and differentiation of a clonal population of myeloid stem cells are believed to be involved in the pathogenesis of AML. Recurrent chromosomal translocations, such as t(8;21) translocation in AML or t(15;17) translocation in acute promyelocytic leukemia (APL) as well as point mutations affecting key genes that control normal blood cell development, have been implicated in the development of AML (Rowley, 2009).

The most common targets of AML-associated chromosomal translocations are the genes encoding DNA-binding transcription factors or the regulatory components of transcriptional complexes, and the fusion proteins generated from these chromosomal translocations interfere with the functions of the wild-type proteins in a dominant manner. For instance, the RUNX transcription factor AML1 (Acute Myeloid Leukemia 1/RUNX1) and its heterodimerisation partner CBFβ, which regulate many hematopoietic-specific genes and are essential for the normal development of the hematopoietic system, are the target of the t(8;21) and inv(16) translocations, respectively, making this complex the most frequent target of chromosomal rearrangements in human leukemia (see below, **3.2.5**) (Speck *et al.*, 2002). These mutations or translocations impair blood cell differentiation, but are not

sufficient to cause leukemia. It is usually considered that leukemia development requires a second mutation, which will give a growth advantage to the blood cells. These mutations often target components of signaling pathways controlling blood cell proliferation or survival such as c-Kit, Flt3 or Ras (Kelly *et al.*, 2002). The cooperation between these two classes of mutations eventually leads to AML.

Of note, AML is often preceded by a pre-leukemic condition called “myelodysplastic syndrome” (MDS), a chronic disease that progresses to AML upon acquisition of new mutations.

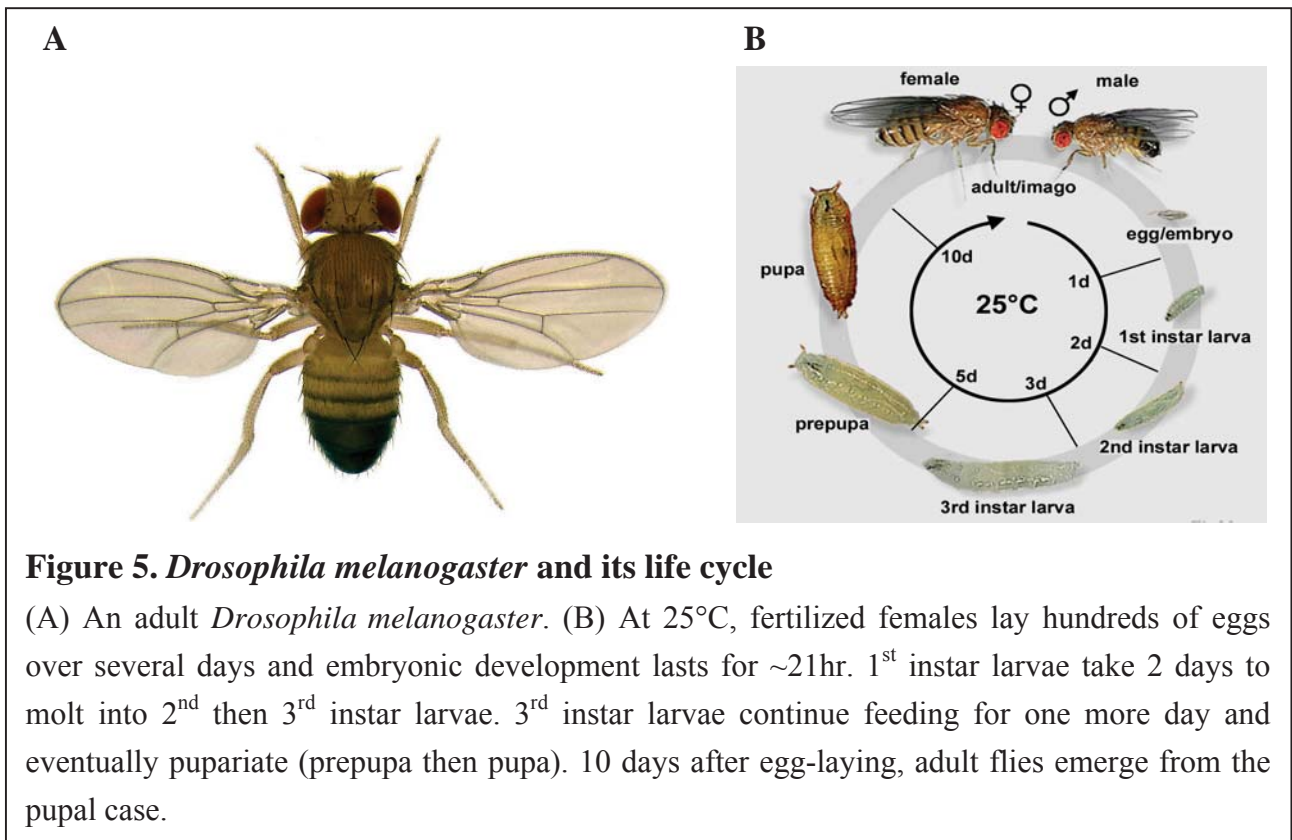
### **1.2.2.3. Current therapy of acute myeloid leukemia**

The primary purpose of treating patients with AML is to induce remission and thereafter prevent relapse. Initially, intensive chemotherapy is the main treatment for patients with AML who can tolerate it, and additional chemotherapy or allogeneic hematopoietic stem cell transplantation could follow. The drugs used in treatments include cytarabine, anthracycline, gemtuzumab ozogamicin, and novel agents under test, such as sarafenib, midostaurin, quizartinib, crenolanib.

While much progress has been made in our understanding of the molecular nature of the events leading to AML and in the refinement of therapeutic strategies, this disease remains of bad prognosis. Thus a better characterization of the function and mode of action of the genes mutated in MDS and AML is of prime interest to be able to develop innovative therapeutic approaches. Besides the study of human blood samples or mouse models, simple genetic model organisms such as *Drosophila* can help not only to decipher the basic principles underlying blood cell development across species but also to better understand the mode of action of conserved genes implicated in AML.

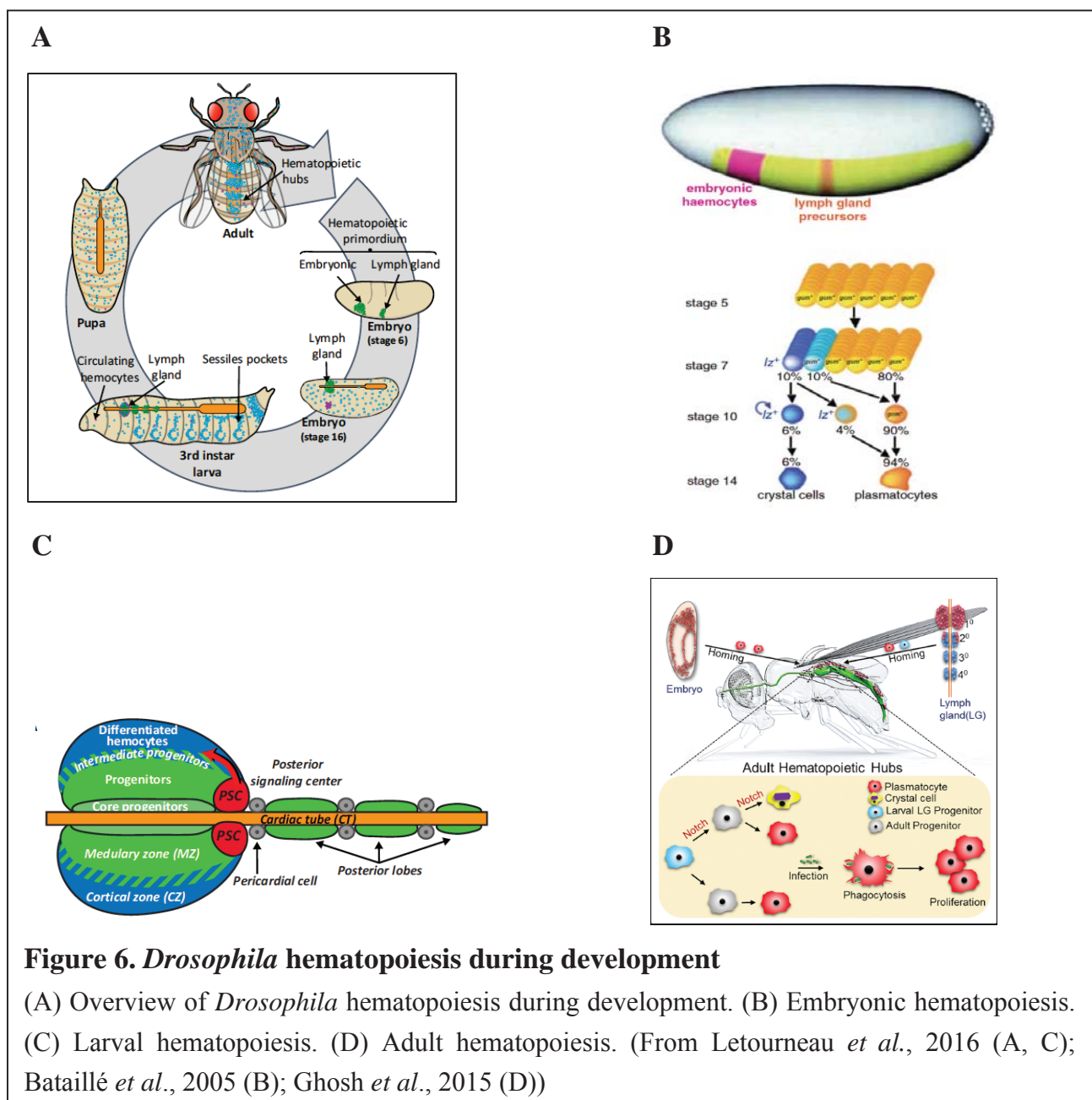
## 2. *Drosophila*: a simplified model to study the mammalian hematopoiesis

*Drosophila melanogaster* is a species in the family *Drosophilidae* of the order *Diptera*, which has multifaceted brick red eyes, a tan thorax studded with arched black bristles, a striped abdomen and a pair of translucent wings (**Figure 5.A**). Since the serendipitous discovery of the white mutation and recognition of its linkage to the X chromosome by Thomas Hunt Morgan in 1910, *D. melanogaster* has been a central model organism in the study of transmission genetics, as well as the study of development, physiology and behaviour. This tiny insect has numerous practical advantages, such as rapid life cycle (ten days for one generation, **Figure 5.B**), low chromosome number, small genome size with low redundancy, easy and cheap stock management, convenient experimental manipulations and observations of cells and tissues, availability of a huge body of knowledge and a rich resource of genetic tools. The conservation of basic signalling pathways and key transcription factors controlling the development and functions of blood cells from *Drosophila* to human, makes *D. melanogaster* a simplified and interesting model to decipher the fundamental mechanisms governing hematopoietic system formation and homeostasis.



## 2.1. Ontogeny of *Drosophila* hematopoietic system

Over the last several decades, due to its apparent simplicity, such as an open circulatory system, fewer blood cell types and significant similarities in blood cell development shared with mammals, *Drosophila melanogaster* has emerged as a powerful genetic model for studying the molecular process that controls hematopoiesis under normal conditions or in pathological situations (Letourneau *et al.*, 2016). These studies notably highlighted how intricate cell communication networks and microenvironment cues regulate blood cell homeostasis and helped revealed the mode of action of key conserved regulators of hematopoiesis.



### 2.1.1. Embryonic hematopoiesis

Similar to the two waves of the vertebrate hematopoiesis, *Drosophila* hematopoiesis takes place in two spatially and temporally distinct waves (**Figure 6.A**). The first wave, which is often compared to the primitive hematopoiesis in vertebrates, occurs in the early embryonic development, when prohemocytes (or hemocyte/blood cell precursors) emerge from the head mesoderm (**Figure 6.B**). These cells are first identified during embryonic stage 5 by the expression of Serpent (Srp), a GATA transcription factor required for hematopoietic development (Rehorn *et al.*, 1996). After four rounds of division, these hemocyte precursors stop proliferation and differentiate into either plasmatocytes or crystal cells. Upon maturation in the head mesoderm, the majority of plasmatocytes that account for 95% of all embryonic hemocytes migrate out of the head region under the influence of chemo-attracting signals along stereotypical routes (Letourneau *et al.*, 2016). In contrast, crystal cells that account for 5% of all embryonic hemocytes generally remain localized as two groups of cells around the anterior part of the gut, which is near their point of origin in the embryo, and they will disperse subsequently during the larval stages. Totally, by the end of embryogenesis, the prohemocytes in the head mesoderm give rise to 600-700 plasmatocytes and approximately 36 crystal cells.

These embryo-derived hemocytes populate the larvae where they can be found in two destinations. One fraction persists as patches of cells attached to the inner epidermis of the body cavity, which is designated as sessile hemocytes; the other fraction circulates in the hemolymph (the circulating fluid in the body cavity of *Arthropods*), which is designated as circulating hemocytes.

The molecular control of this embryonic hematopoiesis has been well characterized. Briefly, prohemocytes generation requires the pan-hematopoietic Serpent (Srp), a *Drosophila* homolog of human GATA transcription factors, which are also implicated in hematopoiesis. The activity of Serpent in the hemocytes is notably controlled by its corepressor U-shaped (Ush), a *Drosophila* homolog of human Friend of GATA (FOG) cofactor, which directly binds Srp to inhibit crystal cell fate choice and regulate plasmatocytes differentiation (Waltzer *et al.*, 2003; Fossett *et al.*, 2003). The lineage commitment of prohemocytes to plasmatocytes or crystal cells depends on the expression of the lineage-specific transcription factors Glide/Glial cell missing (Gcm and Gcm2) and the RUNX transcription factor Lozenge (Lz), respectively. Specifically, the expression of Gcm

factors, together with Srp and Ush, leads to differentiation of prohemocytes into plasmatocytes. In contrast, Lz, a runt-domain protein that resembles human AML1/RUNX1, is required for the differentiation of hemocyte precursors into crystal cells. When Lz is expressed, the expression of Ush is suppressed and Srp and Lz cooperate to induce the differentiation of prohemocytes into crystal cells, similar to the cooperation of GATA and RUNX in vertebrates that controls several steps of blood cell proliferation and differentiation. How Gcm expression is controlled is unknown, but it has been suggested that Notch signalling pathway participates in the induction of Lz expression in the embryonic crystal cell lineage (Lebestky *et al.*, 2003).

### **2.1.2. Larval hematopoiesis**

The second wave of *Drosophila* hematopoiesis, is initiated during the larval stages in a specialized organ, the lymph gland, which forms during embryogenesis and persists through the onset of metamorphosis. During the last two decades, the lymph gland has been a prevailing model to investigate the hematopoiesis process under normal conditions or in response to immune stress, since it is the dedicated *Drosophila* larval hematopoietic organ (Letourneau *et al.*, 2016). Similar to the hematopoietic stem cell emergence in vertebrates, lymph gland cells derive from hemangioblast precursors specified in the late embryo. At that stage, the lymph gland precursors form a single pair of lobes that are localized along the dorsal vessel. This single pair of lobes only contains about 20 cells each, and they are designated as the primary lobes (or the anterior lobes). At the end of the first larval instar, additional pairs of posterior lobes also emerge along the dorsal vessel, which are designated as the secondary lobes (or the posterior lobes). So in third instar larvae, lymph gland is composed of a large pair of primary anterior lobes followed by several small pairs of posterior lobes, each separated by a pair of pericardial cells. The posterior lobes are mainly composed of prohemocytes, however, the primary lobes are organized into three domains: the cortical zone (CZ), the medullary zone (MZ) and the posterior signalling centre (PSC) (**Figure 6.C**). The peripheral cortical zone contains mature and differentiated hemocytes, whereas the central medullary zone contains quiescent prohemocytes. At the posterior tip of each primary lobe, a cluster of about 30 cells constitutes the posterior signalling centre (PSC), which acts as a niche-like structure that controls prohemocyte maintenance and lymph gland homeostasis. There is also a population of “intermediate progenitors” between the cortical zone and the medullary zone that express both some progenitors and early

differentiation markers. During the first two larval stages, lymph gland progenitors divide actively before entering quiescence and giving rise to plasmatocytes and crystal cells under normal conditions and to lamellocytes in response to immune challenges, such as wasp parasitism. During pupariation, the lymph gland disrupts and releases all hemocytes into the circulation.

Besides the lymph gland, there is also another site of hematopoiesis during the larval stages, which is called hematopoietic pockets or subepidermal and muscular pockets and has only been identified recently. In third instar larvae, sessile hemocytes, which are derived from the differentiated hemocytes of the embryo, are prevalent along the posterior region of the dorsal vessel and in close association with oenocytes on the lateral sides, where they are sandwiched between the epidermal and muscular layers. These so-called hematopoietic pockets, provide a specific microenvironment that attracts plasmatocytes and supports their survival, proliferation and differentiation (Makhijani *et al.*, 2011). These sessile hemocytes can expand through self-renewal in differentiated state in third instar stage and increase their accumulation at the site of injury or in circulation under certain circumstances, such as starvation, wound inflammation.

### **2.1.3. Hematopoiesis in adult *Drosophila***

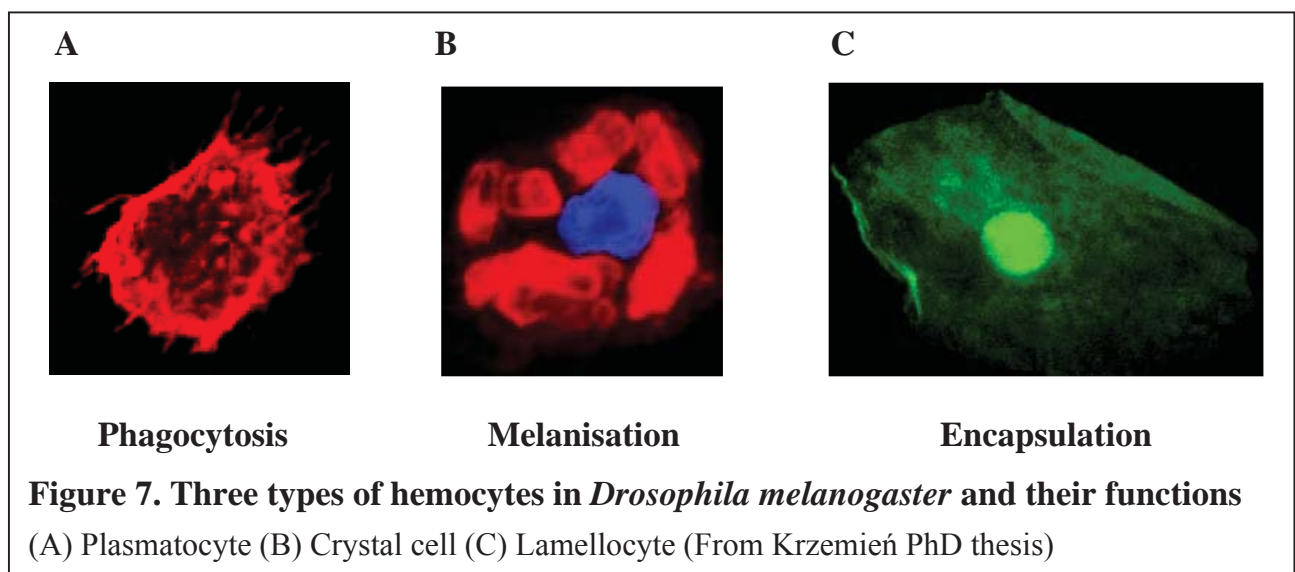
Although the embryonic and larval hematopoiesis in *Drosophila* have been studied extensively, the current knowledge about adult blood cells is much more limited. Adult blood cells are present mostly as sessile hemocytes dispersed under the cuticle and associated with different tissues/organs. It has been shown that they are derived from the embryonic and larval waves of hematopoiesis and it has long been thought that *Drosophila* adults lack a hematopoietic organ. Hence, it is generally assumed that adult flies only rely on a fixed pool of differentiated plasmatocytes and that no blood cell proliferation or differentiation takes place during adulthood. However, recently, a study demonstrated the presence of active hematopoietic sites in the abdomen of adult flies, which are called hematopoietic hubs and can give rise to new blood cells (**Figure 6.D**) (Ghosh *et al.*, 2015). It was shown that these hematopoietic hubs contain some hemocyte progenitors, which originate from the posterior lobes of lymph gland, and it was proposed that these cells can give rise to plasmatocytes and crystal cells. In addition, whereas the total number of hemocytes declines with age in adult flies (Horn *et al.*, 2014), it was shown that adult blood cells can proliferate in response to an immune infection. These exciting observations



suggest that active hematopoiesis could take place in the hematopoietic hubs of adult flies and project this hematopoietic hub as a simple version of the vertebrate bone marrow. However, these findings need to be confirmed and the extend as well as the functional importance of adult blood cell production/differentiation remains unclear.

## 2.2. Functions of *Drosophila* hemocytes

As mentioned above, *Drosophila* hemocyte progenitors can differentiate into three mature blood cell types: plasmatocytes and crystal cells (during embryonic, larval and adult stages) as well as lamellocytes (during larval stage, in response to specific immune challenges).



### 2.2.1. Prohemocytes

Prohemocytes have been described in the embryonic head mesoderm, in embryo-derived larval hemocytes, in the larval lymph gland and in adult hematopoietic hubs. They are generally described as small cells (4-6 um in diameter) with a high nuclear/cytoplasmic ratio but few defined characteristics. Although the GATA factor *Srp* is expressed in all prohemocytes, this factor is also expressed in differentiated blood cells and so far there is no known specific marker universally labelling *Drosophila* prohemocytes. For instance, in the lymph gland, prohemocytes express *tep4* and the reporter gene *dome-meso*, but these two markers do not label embryonic prohemocytes and it is not known if they label adult blood cell progenitors. Therefore, further molecular characterization of the prohemocytes is needed

### 2.2.2. Plasmatocytes

Plasmatocytes are relatively round cells with a diameter of 8-10  $\mu\text{m}$  and contain abundant lysosomes and endoplasmic reticulum (**Figure 7.A**). Their differentiation from prohemocytes requires the expression of transcription factor *Glial/Glide* cells missing (*Gcm*) in the embryo, and that of the GATA transcription factor *Pannier* in the lymph gland (Minakhina *et al.*, 2007). They are the professional phagocytes of the immune system, related to mammalian macrophages, which mediate the cellular immune defence. Phagocytosis is an evolutionarily conserved process that is critical for the removal of invading pathogens and apoptotic cells. In *Drosophila*, plasmatocytes are able to engulf dead cells and debris as well as invading pathogens, and they are important for bacterial clearance and for the resistance to systemic infection. The phagocytic ability of plasmatocytes depends on the expression of scavenger and pattern recognition receptors on their surfaces. *Croquemort* (*Crq*), a member of the CD36 family of receptors, mediates the recognition of apoptotic cells (Franc *et al.*, 1996). *Eater* and *NimC1*, two members of the *Nimrod* family of cell-surface receptors, recognize both Gram-positive and Gram-negative bacteria, leading to rapid engulfment of invading microorganisms (Kocks *et al.*, 2005; Kurucz *et al.*, 2007). *Draper*, the *Ced-1* homologue, recognizes lipoteichoic acid from *Staphylococcus aureus* and mediates the uptake of this bacterium in adult flies (Hashimoto *et al.*, 2009). Additionally, plasmatocytes sculpt various developing tissues and organs through the phagocytic removal of cells. For example, the embryonic nervous system doesn't condense properly if the plasmatocyte-mediated phagocytosis is absent.

Plasmatocytes are also highly motile cells, providing a powerful model to study cell migration and chemotaxis in response to developmental cues or tissue damage (Evans *et al.*, 2014; Ratheesh *et al.*, 2015). This motility requires the expression of *PVR* (PDGF/VEFG-related) receptor, *Rho* GTPase and *fascin*, and it is essential to ensure the homeostatic function of plasmatocytes. Additional functions of plasmatocytes include secretion of extracellular matrix (ECM) components required for proper tissue morphogenesis (Bunt *et al.*, 2010) and for the maintenance of ovarian stem cells (Van De Bor V *et al.*, 2015), controlling the activity of intestinal stem cells during injury-induced regeneration (Ayyaz *et al.*, 2015), regulation of glucose metabolism to modulate life span (Woodcock *et al.*, 2015), and mediating apoptosis-induced proliferation in the imaginal disks (Fogarty *et al.*, 2016). Similar to the mammalian macrophages that have a plethora of functions in addition to their

role in immunity, *Drosophila* plasmatocytes constitute a very helpful model to understand how macrophage diversity of function is generated and regulated.

### 2.2.3. Crystal cells

Crystal cells are megakaryocyte-like cells with the diameter of 10-12  $\mu\text{m}$  (**Figure 7.B**). They derive their name owing to the presence of paracrystalline inclusions composed of prophenoloxidasases (PPO) in the cytoplasm. They are non-phagocytic and function in melanization, an important immune response related to clotting and wound healing in *Arthropods* (Evans *et al.*, 2003). Melanization leads to the blackening of wound sites or the surface of the invading pathogens, due to the local production and deposition of melanin.

As described above, crystal cells contain paracrystalline inclusions consisting of mass quantities of one or more components of the melanisation enzymatic cascade, which are prophenoloxidase enzymes that play a key role in melanin biosynthesis. There are three PPOs in *Drosophila*, which are PPO1 (Black Cell), PPO2 and PPO3. Upon injury, activation of the JNK pathway and the TNF homolog Eiger leads to crystal cells rupture and release of PPO zymogens into the hemolymph (Bidla *et al.*, 2007). PPOs are then cleaved into active phenoloxidase (PO) by a proteolytic cascade (Dudzic *et al.*, 2015), and PO in turn catalyses the oxidation of mono- and di-phenols to ortho-quinones, which subsequently polymerize into melanin and produce reactive oxygen species (ROS) as by-products (Eleftherianos *et al.*, 2011). Although crystal cells are dispensable for fly viability, melanization participates in the innate immune response, and the resistance to infection. Crystal cell differentiation absolutely requires the induction of Lozenge expression both in the embryos and in the larvae (Lebestky *et al.*, 2000; Fossett *et al.*, 2003). The processes regulating crystal cell production are described in detail below.

### 2.2.4. Lamellocytes

Lamellocytes are large, flat and adherent cells (**Figure 7.C**). They are scarcely present in healthy larvae, but their differentiation is massively induced in response to some specific immune challenges, such as parasitization by the wasp *Leptopilina boulardi* (Lanot *et al.*, 2001). Lamellocytes participate both in the cellular response and in melanisation, and they primarily function in the encapsulation and neutralization of pathogens or bodies that are too large to be phagocytosed. The encapsulation of parasitic wasp eggs requires the cooperation of all three types of larval hemocytes (Mortimer *et al.*, 2013). At the early stage of parasitization, circulating plasmatocytes recognize the injected eggs and attach to them,

and subsequently form septate junctions to separate the eggs from the hemolymph circulation. This detection induces the proliferation and differentiation of sessile hemocytes as well as lymph gland hemocytes into lamellocytes. Lamellocytes adhere to the wasp eggs, activate the melanisation cascade, and eventually kill the parasites.

### **2.3. Genetic control of *Drosophila* hematopoiesis**

#### **2.3.1. PSC in *Drosophila* hematopoiesis**

Similar to the mammalian bone marrow niche controlling the balance between hematopoietic stem cells (HSCs) self-renewal and differentiation, the posterior signalling centre (PSC) within *Drosophila* lymph gland, which controls the maintenance and differentiation of prohemocytes non-cell-autonomously and could function as a hematopoietic niche, represents a helpful and genetically tractable model to study signal integration and crosstalk during hemocyte development (Crozatier *et al.*, 2011). It has been shown that the specification of PSC cells in the embryo critically requires the expression of two transcription factors, which are the homeotic protein Antennapedia (Antp) (Mandal *et al.*, 2007) and the *Drosophila* orthologue of early B-cell factors (EBFs), Collier (Col) (Crozatier *et al.*, 2004). Once the Collier activity is absent, the PSC cells are not specified and larval prohemocytes differentiate prematurely, which indicates the critical requirement for Collier activity in PSC cells specification. Besides transcription factors, the Wingless (Wg) signalling pathway also controls both the number of PSC cells and the maintenance of prohemocytes (Sinenko *et al.*, 2009). In addition, PSC cells express the signalling molecule Hedgehog (Hh), and this Hh signalling pathway is required to maintain hemocyte homeostasis in the lymph gland (Mandal *et al.*, 2007). Furthermore, Serrate-mediated Notch signalling from the PSC is required to maintain normal levels of Collier transcription (Krzemień *et al.*, 2007). All together, these signalling pathways, including Wg, Hh, Notch and other signalling pathways, are integrated into PSC cells to control JAK/STAT signalling activity in prohemocytes, preventing their premature differentiation.

#### **2.3.2. ROS in *Drosophila* hematopoiesis**

As many transcription regulators and signalling pathways involved in hematopoiesis are conserved from humans to *Drosophila*, a model for reactive oxygen species (ROS) has also been established in *Drosophila* (Owusu-Ansah *et al.*, 2009). This study showed that the accumulation of ROS in the lymph gland is tightly controlled during development. Specifically, the multipotent hematopoietic progenitors have an increased level of ROS

under *in vivo* physiological conditions. Conversely, once the ROS in the hematopoietic progenitors is increased beyond its basal level, it will trigger precocious differentiation of these progenitors into all three mature hemocyte types, through a signalling pathway that involves JNK and FoxO activation as well as Polycomb down-regulation. In sum, this study provides a helpful model that could be extended to reveal the possible role of ROS in the differentiation of common myeloid progenitors (CMPs) in the mammalian hematopoiesis and oxidative stress response.

### **2.3.3. Genetic control of blood cell fate**

A number of conserved signalling pathways and transcription factors controlling *Drosophila* hematopoiesis have been identified (Letourneau *et al.*, 2016). In particular, many publications have focused on the regulation of blood cell progenitor fate in the larval lymph gland, showing for instance that the Hedgehog, JAK/STAT, Wnt, Insulin/Target of Rapamycin, or Notch signalling pathways, the level of reactive oxygen species (ROS), as well as transcription factors, such as the EBF factor Collier, or the GATA factor Serpent and Pannier, control lymph gland homeostasis during normal development. In contrast, there were fewer studies about the fate of embryo-derived hemocytes that populate the larval hemocoel or of the adult blood cells, for which we only have very limited information. Similarly, while we have a relatively good understanding of crystal cell fate development, how plasmatocyte and lamellocyte differentiation is controlled is less well understood.

Here, I will focus my description on the mechanisms of regulation of crystal cell fate, as this is the blood cell lineage in which the gene that I studied during my PhD (MLF, see below), which was shown to be required (Bras *et al.*, 2012). As detailed below, the RUNX transcription factor Lozenge (LZ) is specifically expressed in the crystal cell lineage and it is absolutely required for crystal cell development (Lebestky *et al.*, 2000). The expression of Lozenge, and thus the induction of crystal cell fate is dependent on the Notch signaling pathway both in circulating larval cells and in lymph gland (Mukherjee *et al.*, 2011; Lebestky *et al.*, 2003; Duvic *et al.*, 2002). The activation of the Notch pathway is mediated by its ligand Serrate (Ser) and requires the transcription factor Suppressor of Hairless (Su(H)). Accordingly, decreasing Notch activity (using for instance a thermo-sensitive allele or over-expression of a dominant negative form of Notch) leads to a decrease in the number

of crystal cells, while over-expression of Notch induces a strong increase in their number (Duvic *et al.*, 2002).

In the lymph gland, the posterior signaling center (PSC) was first characterized thanks to its specific expression of Ser (Lebestky *et al.*, 2003). It was thus initially proposed that the PSC is necessary for the induction of crystal cell fate by contacting the neighboring hemocytes. However, it was then shown that the PSC is not required for crystal cell development, and that it was proposed that some other cells expressing Ser and dispersed in the cortical zone could be responsible for the signal that induces crystal cell fate (Crozatier *et al.*, 2004). Consistent with this hypothesis, a recent study demonstrated that the expression of Yorkie and Scalloped, two components of the Hippo pathway, promotes the expression of Ser in these cells of the cortical zone, and is required for the specification of the adjacent cells into crystal cells (Ferguson *et al.*, 2014a; 2014b). Another related study suggests that the Hippo pathway controls the development of crystal cells in a non-cell-autonomous manner via Ser and the Notch pathway but also in a cell-autonomous manner by directly activating the transcription of *lz* (Milton *et al.*, 2014). In addition, the identification of Notch/Su(H) direct targets in the Kc cells showed that Notch/Su(H) cooperates with LZ in the crystal cells to activate the transcription of certain genes, such as *pebbled/hindsight*, which are involved in the endo-replication of crystal cells, and *klumpfuss*, which represses the alternate (plasmatocytes) fate (Terriente-Felix *et al.*, 2013). In addition, another study showed both in the lymph gland and in circulating larval blood cells that Notch also contributes to crystal cell survival (Mukherjee *et al.*, 2011). Interestingly, this study showed that this function of Notch in  $LZ^+$  cells does not depend on its ligand. Indeed, in  $LZ^+$  cells, high level of the HIF- $\alpha$  homolog Sima stabilizes the internalisation of activated Notch in the endosome even in the absence of activation by its ligand Ser or Delta. This leads to a non-canonical, ligand-independent activation of Notch, which acts with Su(H) to permit crystal cell maturation and to prevent crystal cell rupture. In addition, the relocation and accumulation of Notch in early endosomes was shown to coincide with an increase in the number of crystal cells in the lymph gland in the absence of Asrij, a conserved protein involved in endocytosis. Therefore, it was suggested that Asrij restricts the number of crystal cells by controlling the intracellular trafficking of Notch (Kulkarni *et al.*, 2011). Finally, it has recently been shown that crystal cells can be produced by trans-differentiation of plasmatocytes in the sessile islets in response to the activation of

the Ser/Notch pathway (Leitão *et al.*, 2015). In sum, it appears that the Notch pathway acts at multiple levels in the development of crystal cells. First, Ser is required to activate Notch in blood cell progenitors or plasmacytes to induce the expression of LZ and thus crystal cell fate; then, activated Notch cooperates with LZ to lock crystal cell fate and finally ligand independent activation of Notch increases crystal cell survival.

### 3. RUNX family of transcription factors in hematopoiesis and leukemia

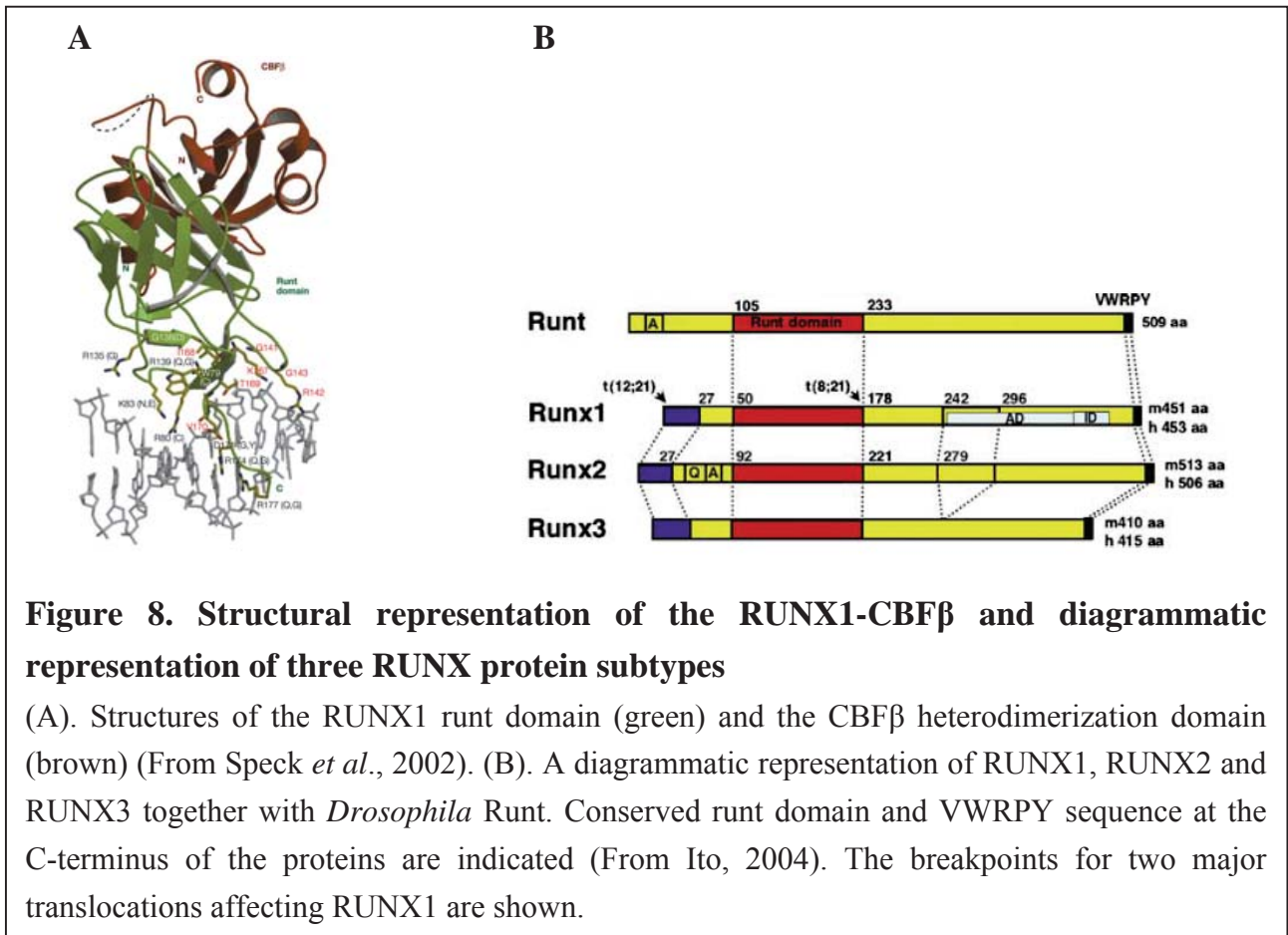
#### 3.1. The Core-binding factors family

As described above, many transcription factors are involved in the regulation of the mammalian hematopoiesis and its associated leukemia. Among these transcriptional regulators, core-binding factors (CBFs) are a class of transcription factors, which are essential for hematopoietic development and are also the frequent targets of mutations or gene rearrangements in human leukemia (Speck *et al.*, 2002). They are heterodimeric transcription factor complexes composed of  $\alpha$  and  $\beta$  subunits (**Figure 8.A**) (Hart *et al.*, 2002). The  $\alpha$  subunit (CBF $\alpha$ ) is the DNA-binding element of the complex, which is able to bind to a specific nucleotide sequence motif *in vitro* even without the  $\beta$  subunit. The  $\beta$  subunit (CBF $\beta$ ) stabilizes the binding of CBF $\alpha$  to the specific DNA sequence motif without direct DNA contact (Ogawa *et al.*, 1993a) and can protect the  $\alpha$  subunit from proteolysis (Huang *et al.*, 2001). Structural analyses have shown that the DNA recognition and binding by CBF $\alpha$  is mediated by an allosteric transition in the runt domain, which is further stabilized by CBF $\beta$  (Tahirov *et al.*, 2001), as well as by the bent-helical conformation of the free DNA target (Bartfeld *et al.*, 2002) (**Figure 8.A**). All CBF $\alpha$  subunits contain an evolutionarily conserved 128 amino acid domain called the Runt domain, in reference to *Drosophila* CBF $\alpha$  protein Runt, the founding member of this family. This domain is responsible both for DNA binding and heterodimerization with CBF $\beta$  (Nimmo *et al.*, 2008; Ogawa *et al.*, 1993a). Runt domain proteins are found throughout the metazoan kingdom (Rennert *et al.*, 2003) and recognize the PyGPyGGT consensus sequence, which seems to be shared by all members of the CBF family. In addition, most RUNX proteins contain a C-terminal VWRPY motif that functions as a binding site for transcriptional co-repressor of the Groucho/TLE family (Ito, 2004) (**Figure 8.B**).

In mammals, there are three CBF $\alpha$  subunits encoded by three corresponding genes, *RUNX1*, *RUNX2* and *RUNX3* (Ito, 2004), and a single ubiquitous  $\beta$  subunit encoded by *CBF $\beta$* . The three *RUNX* genes are required for the development of various tissues. Because they bind the same DNA sequence, their specific role is largely due to their respective spatio-temporal expression pattern. In particular, *RUNX1* is required for hematopoietic stem cell emergence and controls several steps of blood cell maturation, whereas *RUNX2* plays a key role in osteogenesis and *RUNX3* is important for neurogenesis (Bae *et al.*, 2006; de Bruijn *et al.*, 2017; Blyth *et al.*, 2010; Lotem *et al.*, 2015). However, these genes also play a



critical role in other organs, notably *RUNX2* and *RUNX3* participate in T cell development in the hematopoietic system. Of note, because of a series of aliases for CBF $\alpha$  subunits generated in many independent laboratories during the past decades, a unified nomenclature for this exciting class of transcription factors has been established (van Wijnen *et al.*, 2004), as shown in **Table 2**.



**Table 2. Synonyms for mammalian RUNX gene subtypes and locus**  
 (CBFA: Core Binding Factor Alpha; AML: Acute Myeloid Leukemia; PEBP2: Polyomavirus Enhancer Binding Protein 2)

RUNX1	CBFA2	AML1	PEBP2 $\alpha$ B	21q22
RUNX2	CBFA1	AML2	PEBP2 $\alpha$ A	6p21
RUNX3	CBFA3	AML3	PEBP2 $\alpha$ C	1p36

### 3.2. Identification of RUNX1 in mammals

It has been known that specific chromosomal translocations are closely associated with a large number of human blood malignancies. The isolation and subsequent study of many

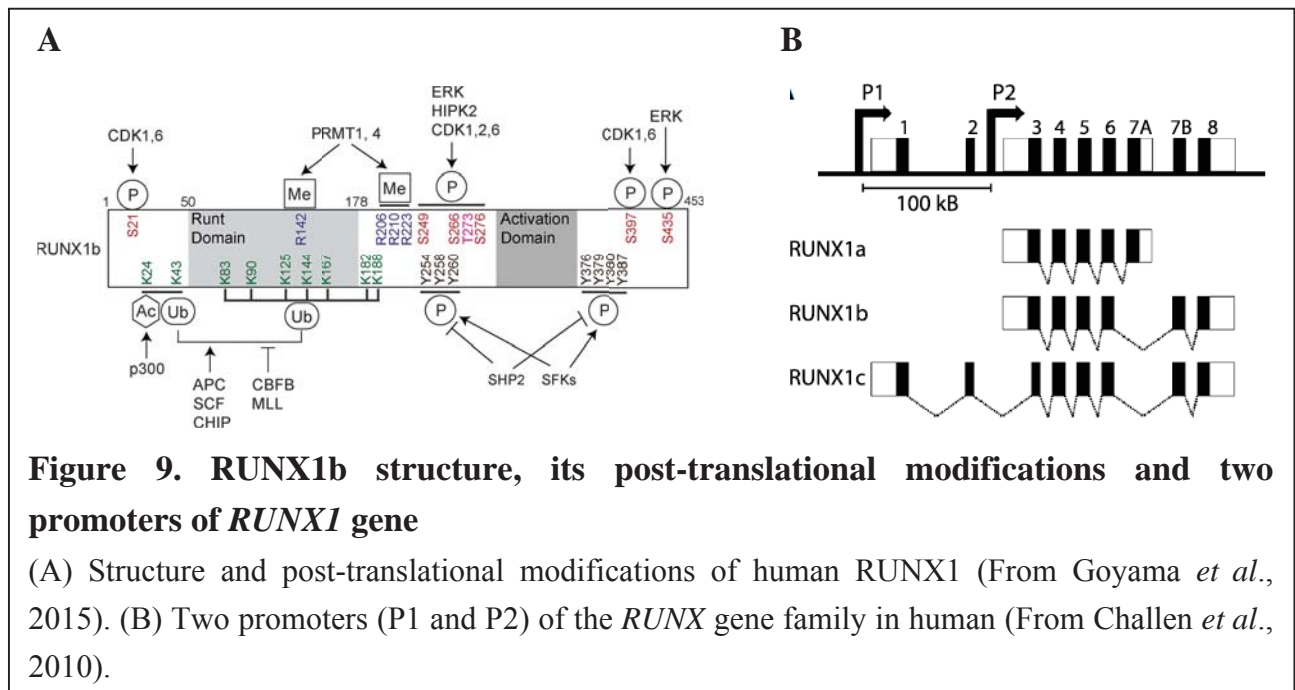
genes located at the breakpoint regions revealed that they often play critical roles in the regulation of proliferation and differentiation of various blood cell lineages. The t(8;21)(q22;q22) translocation is one of the most common and frequent translocations in acute myeloid leukemia (AML), occurring in 12-15% of all cases, especially in the M2 AML subtype according to the French-American-British (FAB) classification system (Sangle *et al.*, 2011). After the successful isolation of important genes involved in the t(8;14) Burkitt lymphoma (*c-myc*), t(9;22) chronic myeloid leukemia (*c-abl*), t(15;17) acute promyelocytic leukemia (*c-erbA*), *RUNX1/AML1*, the first identified mammalian CBF gene, was isolated successfully by virtue of its location on human chromosome 21 at the t(8;21)(q22;q22) breakpoint (Miyoshi *et al.*, 1991), meanwhile its murine homolog, *PEBP2 $\alpha$ /PEA2*, was isolated and cloned successfully very soon after thanks to its capacity to bind the enhancer core sites of the polyomavirus (Ogawa *et al.*, 1993b).

### 3.2.1. Structure and isoforms of RUNX1 transcription factor

Based on the sequence analyses of various forms of *RUNX1* cDNA that reflects a complex pattern of mRNA species, it was revealed that *RUNX1* gene has 12 exons, and two alternative promoters: the distal promoter or P1, and the proximal promoter or P2 (**Figure 9.B**), which are juxtaposed with their corresponding and specific first coding exons (Sroczynska *et al.*, 2009; Bee *et al.*, 2009; Challen *et al.*, 2010). This organization is conserved in mammals and the usage of two alternative promoters is also observed in other mammalian *RUNX* genes. So *RUNX1* gene could generate more than 12 different mRNA isoforms and 3 main protein isoforms (RUNX1a, RUNX1b, RUNX1c) by alternative splicing and alternative promoter usage (Miyoshi *et al.*, 1995; Levanon *et al.*, 2001). Systematic analyses of *RUNX1* P1 and P2 promoter usage during mouse hematopoietic development showed that the proximal P2-mediated *RUNX1* isoform marks a hemogenic endothelium cell population and primitive erythrocytes, whereas the distal P1-mediated *RUNX1* defines fully committed definitive hematopoietic progenitors (Bee *et al.*, 2009). This demonstrates that the differential activities of these 2 *RUNX1* promoters define milestones of hematopoietic development, and suggests that the proximal *RUNX1* isoform is a key regulator in the generation of hematopoietic cells from hemogenic endothelium (Sroczynska *et al.*, 2009).

As mentioned above, *RUNX1* codes for 3 protein isoforms. The P2 promoter regulates the expression of RUNX1a and RUNX1b, whereas the P1 promoter controls RUNX1c

expression (**Figure 9.B**). The two long isoforms, RUNX1b and RUNX1c, contain both the DNA-binding Runt domain at their N-terminus followed by a transactivation domain (**Figure 9.A**), a nuclear-matrix attachment motif, two putative transcriptional repression domains and finally the VWRPY motif. They are generally considered similar in functions (Challen *et al.*, 2010), although RUNX1c has extra 27 amino acids at the N-terminus as compared to RUNX1b. In contrast, RUNX1a lacks the C-terminal transactivation domain and VWRPY motif, and it could act as a competitive inhibitor for RUNX1b (Miyoshi *et al.*, 1995; Zhang *et al.*, 1996).



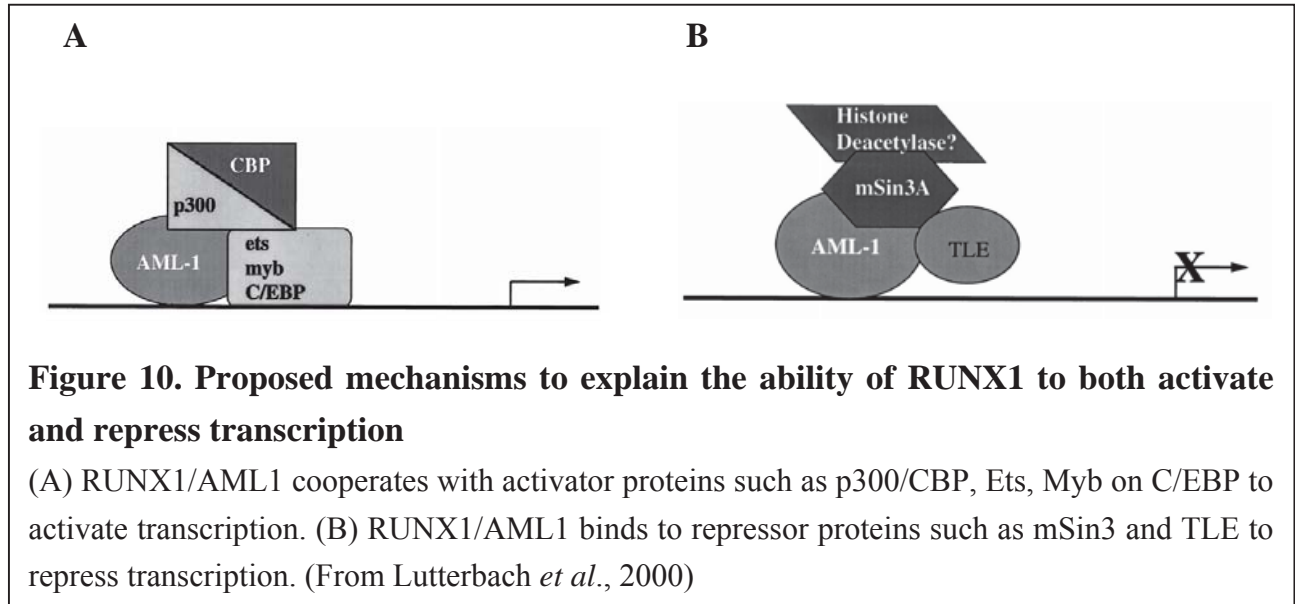
Expression pattern analyses of these RUNX1 isoforms showed that they have differential expression profiles during hematopoietic differentiation in mouse embryos and in human embryonic stem cells (hESCs) (Challen *et al.*, 2010). RUNX1a and RUNX1b isoforms were expressed consistently throughout hematopoietic differentiation, whereas RUNX1c isoform was only expressed at the onset of the emergence of definitive HSCs as well as in the AGM region of E10.5 to E11.5 mouse embryo, which suggested that RUNX1c isoform could be essential for the specification or function of definitive HSCs. However, several other studies indicated that RUNX1a isoform could have more diverse roles than the other two isoforms. Enforced expression of RUNX1a in the mouse primitive hematopoietic cells resulted in enhanced engraftment upon transplantation, which demonstrated that RUNX1a isoform has the capacity to potentiate stem and progenitor cell engraftment (Tsuzuki *et al.*, 2007). Another study revealed that ectopic expression of RUNX1a isoform in mouse HSCs facilitates their expansion (Tsuzuki *et al.*, 2012) and

positively regulates the expression of mesoderm and hematopoietic differentiation-related factors. Similarly, RUNX1a over-expression favoured hematopoietic lineage commitment in human pluripotent stem cells (Ran *et al.*, 2013). In addition, a higher expression level of RUNX1a was found in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML)-M2 patients and RUNX1a antagonized with RUNX1b, which indicated that RUNX1a over-expression could promote leukemogenesis (Liu *et al.*, 2009). Yet, the respective functions of the different RUNX1 isoforms in hematopoiesis and leukemia remain to be firmly established.

### **3.2.2. RUNX1: a master transcriptional regulator of hematopoietic development**

RUNX1 is a master transcription factor that plays a critical role in the development and differentiation of specific cell lineages from hematopoietic stem cells. Initial studies showed that it directly controls the expression of various genes that are essential for blood cell survival and differentiation, such the granulocyte/macrophage colony-stimulating factor (GM-CSF) or the macrophage colony-stimulating factor receptor (M-CSFR) (Asou, 2003). It acts as an activator or repressor of target gene expression depending on the large number of transcription factors as well as transcriptional co-activators (**Figure 10.A**) or co-repressors (**Figure 10.B**) that interact with it. For instance, RUNX1 interacts with CCAAT enhancer-binding protein (C/EBP) to activate synergistically *M-CSFR* expression (Zhang *et al.*, 1996). It also binds PU.1 (Petrovick *et al.*, 1998), p300 (Kitabayashi *et al.*, 1998), Ets-1 (Kim *et al.*, 1999), MOZ (Kitabayashi *et al.*, 2001) or GATA-1 (Elagib *et al.*, 2003) to activate transcription. In contrast, RUNX1 interacts with transducing-like enhancer of split (TLE1), a human homolog of the Groucho family of co-repressors, to repress gene expression (Imai *et al.*, 1998). Other RUNX1-associated co-repressors include mSin3A (Imai *et al.*, 2004) and histone deacetylase (HDAC) (Reed-Inderbitzin *et al.*, 2006). Hence, RUNX1 functions as an organizing protein that facilitates the assembly of transcriptional activation or repression complexes, which can be described that it recruits non-DNA-binding co-activators or co-repressors to initiate the activation or repression of target genes transcription under different circumstances. The effect of RUNX1 on target gene expression is thus highly context dependent, determined by the composition of the transcriptional complexes in which RUNX1 functions at a particular gene. RUNX1 target gene repertoire has now been established at the genome-wide level in different cell types (Lichtinger *et al.*, 2012; Lie-A-Ling *et al.*, 2014; Bevington *et al.*, 2016; Umansky *et al.*, 2015; van Riel *et al.*,

2012; Tijssen *et al.*, 2011; Pencovich *et al.*, 2011). These studies notably highlighted the role of RUNX1 in remodelling the epigenetic landscape and promoting the association of other transcription factors with new set of target genes to induce the hematopoietic fate (Lichtinger *et al.*, 2012).



### 3.2.3. Post-translational modifications of RUNX1

Besides the physical interactions with various co-activators or co-repressors, RUNX1 can be modified by multiple post-translational modifications (PTMs), including phosphorylation, methylation, acetylation, ubiquitination and sumoylation (**Figure 9.A**), which will impact on its activity.

RUNX1 phosphorylation has been studied extensively, since it can promote the transcriptional activation or the degradation of RUNX1. For instance, the extracellular signal-regulated kinase (ERK), which is activated by several hematopoietic cytokines or phorbol ester treatment, phosphorylates RUNX1 at serine (S)249 and S266, thereby potentiating the transactivation and transforming capacities of RUNX1 in fibroblast cells (Tanaka *et al.*, 1996; Zhang *et al.*, 2004). ERK-dependent phosphorylation of RUNX1 disrupts RUNX1 association with the transcriptional co-repressor mSin3A and enhances RUNX1-mediated transactivation but also results in the degradation of RUNX1 by the proteasome (Imai *et al.*, 2004). In addition, RUNX1 is phosphorylated on other residues by homeodomain-interacting protein kinase 2 (HIPK2), Pim-I kinase (Aho *et al.*, 2006), cyclin-dependent kinases (Biggs *et al.*, 2006; Zhang *et al.*, 2008; Guo *et al.*, 2011) or Src family kinase Shp2 (Huang *et al.*, 2012), resulting in a tight regulation of its activity.

RUNX1 is also modified post-translationally by methylation on arginine residues. For example, the protein arginine methyltransferase PRMT1 methylates RUNX1 (Zhao *et al.*, 2008; Shia *et al.*, 2012), and this methylation on two arginines abrogates the interactions between RUNX1 and SIN3A to potentiate the transcriptional activity of RUNX1. Conversely, RUNX1 methylation by PRMT4 promotes the assembly of a DPF2-containing co-repressive complex that blocks myeloid differentiation by repressing miR-233 expression in human cord blood cells (Vu *et al.*, 2013). Similarly, RUNX1 is acetylated on lysine residues by the histone acetyltransferase p300, which causes an increase in its DNA-binding activity and enhances its transcriptional activity (Yamaguchi *et al.*, 2004; Kitabayashi *et al.*, 1998). Moreover, RUNX1 (as well as other RUNX proteins) was found to be sumoylated by PIAS-1, but the functional impact of this modification remains to be determined (Kim *et al.*, 2014).

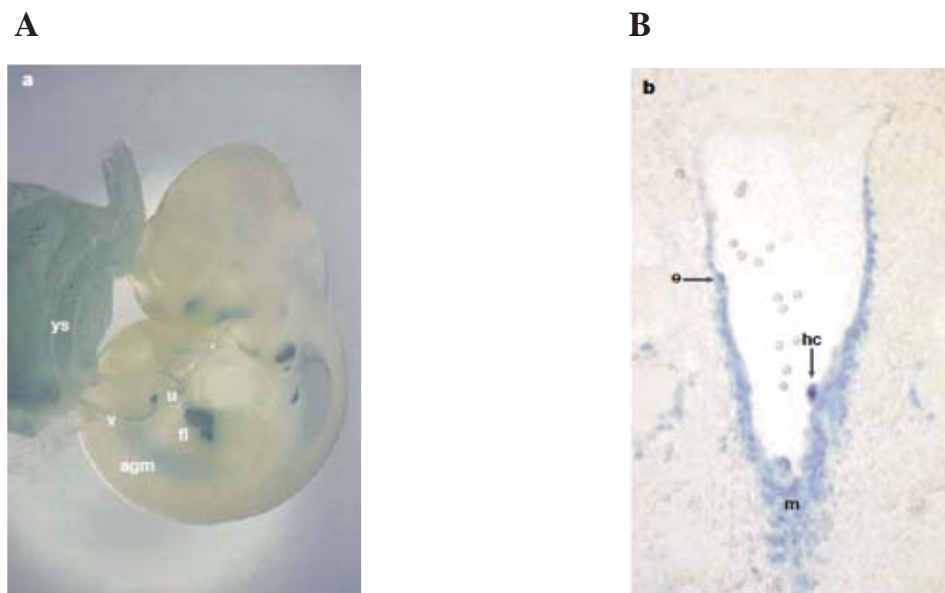
Finally, it is important to note that the stability of RUNX1 is controlled by ubiquitination, notably by the E3 ubiquitin ligase CHIP (Shang *et al.*, 2009) and the SCF/APC/C complex (Biggs *et al.*, 2006), which target RUNX1 to the proteasome degradation pathway. Also CBF $\beta$  was shown to stabilise RUNX1 by preventing its ubiquitination (Huang *et al.*, 2001) whereas RUNX1 phosphorylation promotes its ubiquitination and degradation (Biggs *et al.*, 2005; Biggs *et al.*, 2006; Imai *et al.*, 2004). In sum, RUNX1 post-translational modifications have a profound impact on its activity by controlling its interactions with different partner proteins and by regulating its level.

#### **3.2.4. Role of RUNX1 in hematopoiesis**

RUNX1 plays a key role in the development of the hematopoietic system. Homozygous disruption of *RUNX1* in mouse embryo resulted in the developmental defects, including lack of fetal liver hematopoiesis and mid-gestation embryonic lethality around embryonic day (E) 12.5 (Okuda *et al.*, 1996). These *RUNX1*-deficient embryos still had normal morphogenesis and yolk sac-derived erythropoiesis, but they lacked other yolk-sac derived blood cell types as well as definitive hematopoietic progenitors (Okuda *et al.*, 1996) and no hematopoietic progenitor/stem cell cluster emerged from their aorta-gonad-mesonephros (AGM) region (Mukouyama *et al.*, 2000) (**Figure 11**). So RUNX1 is required for the emergence of all definitive hematopoietic cells, but it is not essential for the formation of primitive erythrocytes. Of note, primitive erythrocytes in null *RUNX1* mice displayed abnormal morphology and reduced expression of Ter119 and GATA, which

indicates that *RUNX1* is also involved in the development of the primitive erythrocytes (Yokomizo *et al.*, 2008). Interestingly, it was shown that *RUNX1* haploinsufficiency resulted in the precocious appearance of HSCs in the AGM region and in the yolk sac, which suggests that *RUNX1* dosage is important for the spatio-temporal control of mouse hematopoiesis (Cai *et al.*, 2000).

Expression pattern analyses showed that *RUNX1* is expressed in definitive hematopoietic progenitor cells as well as in endothelial cells that reside in the yolk sac, the vitelline and umbilical arteries, which contributes directly to the generation of hematopoietic cells through the formation of intra-arterial clusters (North *et al.*, 1999). By conditional deletion study, it was shown that *RUNX1* function in endothelial cells is essential for the formation of hematopoietic stem/progenitor cells (HSPCs) from the vasculature (Yokomizo *et al.*, 2001; Chen *et al.*, 2009). Furthermore, this transition is tightly controlled by the sub-aortic mesenchyme, and *RUNX1* and the Notch signalling pathway are involved in this process (Richard *et al.*, 2013). Altogether, *RUNX1* is required for the definitive hematopoiesis and the endothelial to hematopoietic cell transition in the embryo, as well as involved in the primitive erythropoiesis.



**Figure 11. *RUNX1* expression in hematopoiesis sites in the E10.5 embryo**

(A) *RUNX1* is expressed (blue) in a small population of endothelial cells and hematopoietic cells that are scattered throughout the yolk sac (ys), in endothelial cells lining the vitelline (v) and umbilical (u) arteries, and in endothelial cells, mesenchymal cells and intra-aortic hematopoietic cluster in the ventral portions of the dorsal aorta within the aorta/gonad/mesonephros (agm) region. (B) Detailed view of *RUNX1* expression in endothelial cells (e), mesenchymal cells (m) and a hematopoietic cluster (hc) in the ventral AGM region. (From Speck *et al.*, 2002)

In adult mice, *RUNX1* is expressed in functional hematopoietic stem cells as well as in the majority of myeloid cells and a smaller proportion of lymphoid cells, but its expression decreases substantially during erythroid differentiation (North *et al.*, 2004). Specifically, *RUNX1* is expressed in myeloid, B-lymphoid and T-lymphoid cells, and its expression is regulated in a cell type- and maturation-specific manner in *RUNX1*-IRES-GFP knock-in mice (Lorsbach *et al.*, 2004). Using conditional knock-out mice, it was shown that *RUNX1*-deficient bone marrow exhibited inhibition of megakaryocytic maturation, defective T- and B-lymphocyte development and increased hematopoietic progenitor cells, which demonstrated that *RUNX1* is required for maturation of megakaryocytes and differentiation of T- and B-lymphocytes (Ichikawa *et al.*, 2004). *RUNX1* transcriptionally regulates megakaryocyte development in a cell-autonomous manner (Pencovich *et al.*, 2013) in collaboration with the GATA transcription factor GATA1 (Elagib *et al.*, 2003). Notably, it promotes the megakaryocyte fate in bi-potent erythroid/megakaryocytic precursors by repressing the expression of the erythroid transcription factor KLF1 (Kuvardina *et al.*, 2015) or the erythroid miR144/451 cluster (Kohrs *et al.*, 2016), as well as by activating the expression of megakaryocyte specific genes (Pencovich *et al.*, 2013; Pencovich *et al.*, 2011). And as for T-lymphocyte development, *RUNX1* is required for active repression in CD4<sup>-</sup>CD8<sup>-</sup> thymocytes (Taniuchi *et al.*, 2002) and regulates the two transitions of developing thymocytes from the CD4<sup>-</sup>CD8<sup>-</sup> double-negative stage to the CD4<sup>+</sup>CD8<sup>+</sup> double-positive stage and from the double-positive stage to the mature single-positive stage (Egawa *et al.*, 2007), which indicates that *RUNX1* has critical roles at multiple stages of T-lymphocyte development. Meanwhile, during early B-lymphocyte development, loss of *RUNX1* resulted in a developmental block that was bypassed following retroviral transduction of *Ebfl*, a key transcription factor regulating early B-lymphocyte development, demonstrating that *RUNX1* is essential for B-lymphocyte lineage specification in part through the epigenetic activation of *Ebfl* gene (Seo *et al.*, 2012). In addition, *RUNX1* also regulates the development of Flt3<sup>+</sup> dendritic cell progenitors to facilitate multi-lineage hematopoietic differentiation (Satpathy *et al.*, 2014).

In sum, *RUNX1* is not only required for the emergence of the HSCs, but also for the differentiation of several blood cell lineages in mice. Of note, the findings obtained in mice have been corroborated by studies in human blood cells and *RUNX1* is also a key regulator of hematopoiesis in *Xenopus* or zebrafish, suggesting that it plays a conserved role in blood



cell development in vertebrates.

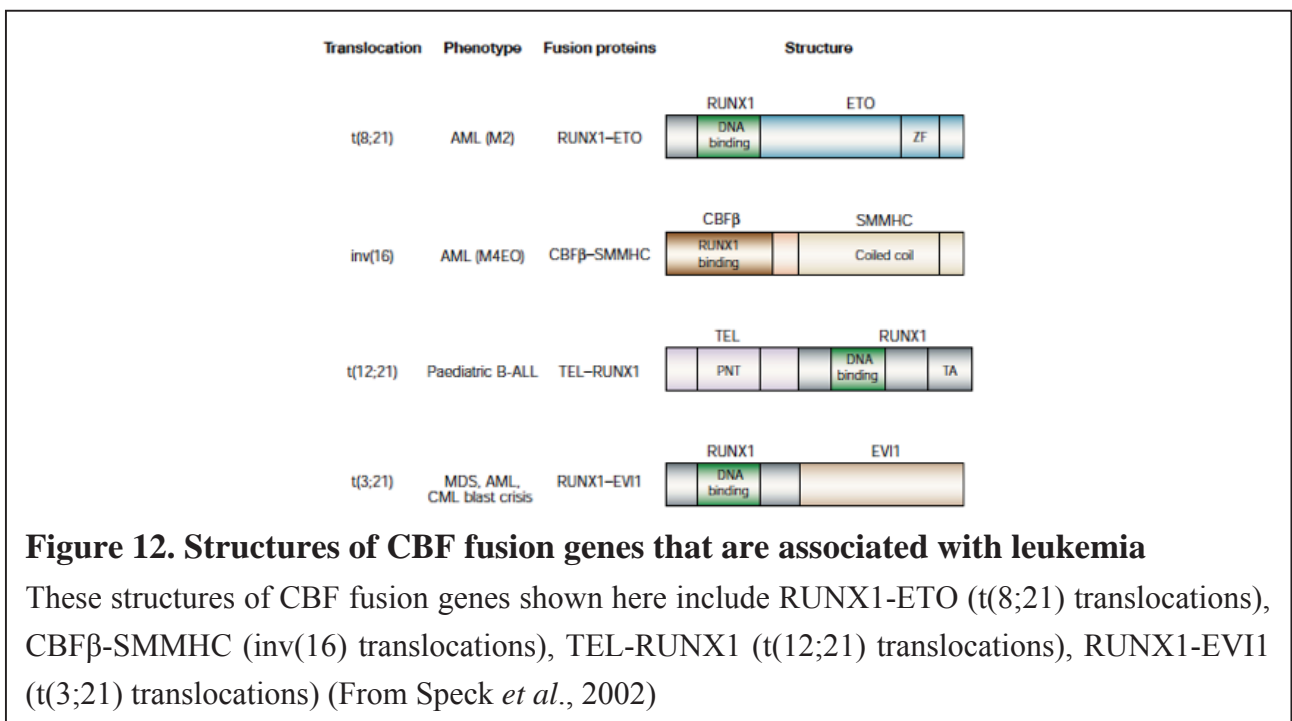
### 3.2.5. RUNX1 in leukemia

The importance of RUNX1 in hematopoiesis is further exemplified by the high number of mutations found in RUNX1 in patients with hematological malignancies. Indeed, as mentioned above, RUNX1 was originally identified as the target of the t(8;21) translocation in human AML (Miyoshi *et al.*, 1991). Since then, a number of translocations or point mutations affecting RUNX1 have been identified in diverse blood cell cancers, making *RUNX1* one of the most frequently altered genes in these malignancies.

Thus far, more than 50 different chromosomal translocations that involve *RUNX1* have been discovered in acute leukemia cases (De Braekeleer *et al.*, 2011), among which the best studied are the t(8;21)(q22;q22) (Downing *et al.*, 1993; Erickson *et al.*, 1992), t(12;21)(p13;q22) (Golub *et al.*, 1995; Romana *et al.*, 1995) and t(3;21)(q26;q21) (Mitani *et al.*, 1994; Nucifora *et al.*, 1994) translocations (**Figure 12**). These translocations are associated with the development of either myeloid or lymphoid leukemia. For instance, the t(8;21)(q22;q22) is the most frequent chromosomal translocation in AML patients ( $\pm 12\%$  of all cases) whereas t(12;21)(p13;q22) is the most frequent one in ALL patients ( $\pm 17\%$  of all cases). In addition, the inv(16)(p13;q22) that affects RUNX1 dimerization partner CBF $\beta$  accounts for approximately 15% of all AML cases. Thus modification of RUNX1 activity is implicated in a large fraction of human acute leukemia. Generally, these chromosomal translocations lead to the production of RUNX1 or CBF $\beta$  fusion proteins that act as dominant inhibitors of the wild-type RUNX1 to promote blood cell transformation.

In most cases, the translocations affecting *RUNX1* led to the production of a fusion protein between RUNX1 DNA-binding domain and (part of) another protein that brings along a transcriptional repressor domain. For instance, in the t(8;21) translocation, RUNX1 C-terminal transactivation domain is replaced by the almost entire ETO (Eight Twenty One) protein, which provides an oligomerisation domain as well as a binding interface for different co-repressors such as N-CoR (Gelmetti *et al.*, 1998), SMRT (Hildebrand *et al.*, 2001), Sin3 (Lutterbach *et al.*, 1998) and numerous HDACs (Wang *et al.*, 1998). The resulting RUNX1-ETO fusion protein can repress transcription from RUNX1-responsive genes by competing with wild type RUNX1 (Miyoshi *et al.*, 1993; Ptasinska *et al.*, 2012). Yet, RUNX1-ETO mode of actions is more complex: it was shown that it also forms a complex with wild-type RUNX1 on chromatin and it was proposed that the relative binding

signals of RUNX1 and RUNX1-ETO determine whether RUNX1-ETO activates or represses its targets (Li *et al.*, 2016; Minucci, 2016). In addition, RUNX1-ETO also binds and regulates other transcription factors such as the myeloid factors C/EBP $\alpha$  or PU.1 (Pabst *et al.*, 2001; Vangala *et al.*, 2003). Thus, RUNX1-ETO expression has a profound impact on blood cell development by repressing myeloid differentiation and promoting leukemic cell self-renewal (Ptasinska *et al.*, 2014). However, it is not sufficient to induce malignant blood cell transformation and, as for other translocations affecting *RUNX1*, other mutations are required for the progression towards leukemia. Thus, an important issue is to identify the genes that cooperate or interfere with RUNX1 or its oncogenic derivatives during hematopoiesis and leukemia.



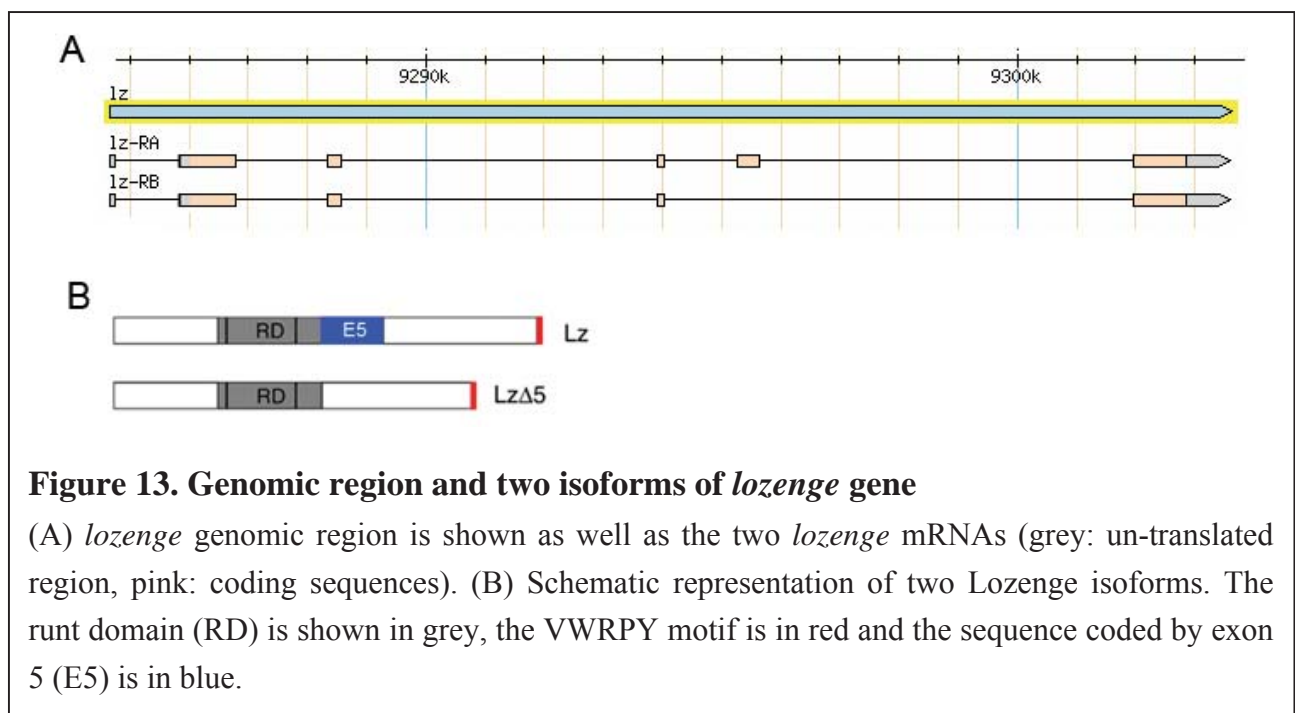
Besides translocations, a number of point mutations affecting RUNX1 are associated with human blood cell diseases. Notably, rare cases of haploinsufficient germ-line mutations in *RUNX1* lead to the development of familial platelet disorder with predisposition to acute myeloid leukemia (Song *et al.*, 1999). There are also numerous examples of somatic point mutations of *RUNX1* in patients with myelodysplastic syndromes, acute myeloid leukemia but also in acute lymphoid leukemia and chronic myelomonocytic leukemia (Sood *et al.*, 2017). Rare cases of bi-allelic “null” mutations have been found in AML-M0 subtypes, but mutations affecting *RUNX1* are mostly mono-allelic and could affect RUNX1 activity by different mechanisms that remain to be established.

Nonetheless, these findings highlight the critical role of RUNX1 in normal blood cell development in humans and indicate that a tight regulation of its activity and expression level is crucial for human health.

### 3.3. Lozenge in *Drosophila* hematopoiesis

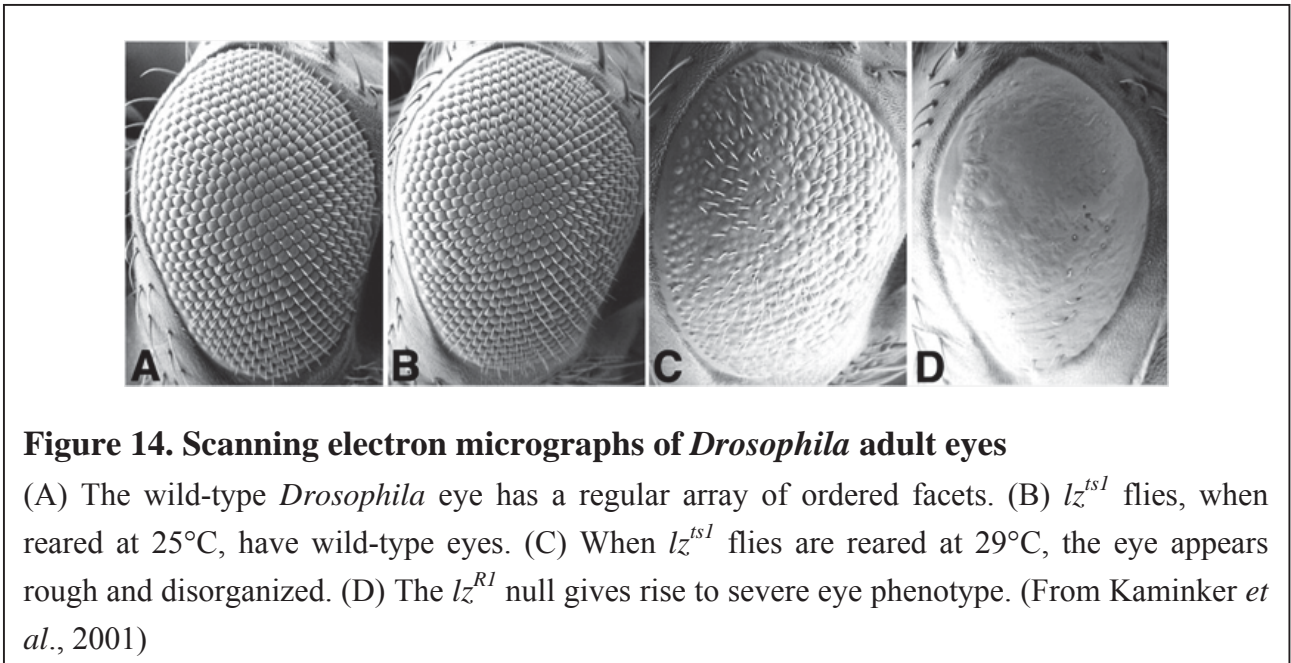
*Drosophila* genome encodes four *RUNX* genes: *runt*, *lozenge*, *RunxA* and *RunxB*. Lozenge is known to be expressed in *Drosophila* blood cells and I will thus focus on this factor. As a member of the RUNX family, Lozenge (Lz) harbors a highly conserved runt domain that is essential for the DNA-binding and protein-protein interactions, and a C-terminal motif VWRPY that is capable of recruiting Groucho family of co-repressors. It is worth mentioning that *Drosophila* has two CBF $\beta$  homologs: Brother and Big-Brother that can interact with Lozenge (Li *et al.*, 1999; Kaminker *et al.*, 2001), but their function in hematopoiesis has not been investigated.

*lozenge* gene is located on the X chromosome and it is transcribed from a single promoter into two different mRNAs that are generated by alternative splicing of the exon 5. There are thus two protein isoforms (**Figure 13**): Lz (826 amino acids long) and Lz  $\Delta$  5 (705 amino acids long), which contain or not an interaction domain for ETS family of transcription factors just after the runt homology domain (RHD) (Jackson Behan *et al.*, 2005). As for mammalian RUNX1, the relative contribution of these two isoforms to *Drosophila* hematopoiesis remains to be determined.



Lozenge is involved in several different developmental processes, including the development of eye, antennal and tarsal claw, female fertility and hematopoiesis (Canon *et al.*, 2000). It is known to have a major role in cell fate determination, and the most characterized example of this function of *lozenge* is during eye and crystal cell development.

*lozenge* mutant alleles were first identified by Morgan and Bridges in 1920 thanks to their effects on the morphology of the *Drosophila* eye (**Figure 14**).



*Drosophila* has a compound eye composed of 800 identical units called ommatidia. In each ommatidium, there are eight light-absorbing photoreceptor neurons (R1-R8) and four non-neuronal cone cells, which produce the lens (Batterham *et al.*, 1996). A number of transcription factors are involved in the cell-specific patterning in the eye, which marks the identity of individual cell types. Among such transcription factors are Seven-up (Svp), a member of the steroid hormone receptor superfamily expressed in R1, R3, R4 and R6 (Mlodzik *et al.*, 1990), Bar, a homeobox protein required in R1 and R6 (Higashijima *et al.*, 1992), and DPax-2, the *Drosophila* Pax-2 homolog required for the development of the cone cells (Fu *et al.*, 1997). Genetic analyses have revealed that *lozenge* positively or negatively regulates all of the known transcription factors required for the cell lineage specification. It plays a crucial role in governing the fate of two groups of cells that are born in a single round of mitosis in the larval fly eye disc (Daga *et al.*, 1996). By negatively regulating *seven-up* (*svp*), Lozenge helps to define a subset of these cells as an equipotential

group that is competent to respond to the EGFR/Sevenless developmental signal. In contrast, by positively regulating *Bar*, Lozenge confers proper photoreceptor identity in a second group of cells (Daga *et al.*, 1996). Another study showed that Lozenge directly activates *argos* (which encodes an inhibitor of EGFR) and *klumpfuss* (the homolog of apoptotic regulator WT1), to regulate programmed cell death in *Drosophila* eye (Wildonger *et al.*, 2005). Therefore, Lozenge patterns multiple cell types in the *Drosophila* eye through the control of cell-specific transcription factors (Flores *et al.*, 1998). It is important to note that Lozenge can act both as a transcriptional activator and as a transcriptional repressor in the same cell. Its capacity to repress transcription of *deadpan* not only requires the co-repressor Groucho but also the transcription factor Cut, whose expression is activated by Lozenge, and which binds next to Lozenge on *deadpan* regulatory region to stabilize the formation of a repressor complex (Canon *et al.*, 2003). Conversely, it was proposed that Lozenge cooperates with the ETS factor Pointed to activate the expression of *prospero* and *D-Pax2* (Jackson Behan *et al.*, 2005). This duality of Lozenge function in the eye was actually used to show that the human RUNX1-ETO oncogenic fusion protein behaves as a constitutive repressor: when RUNX1-ETO is expressed in the fly eye, it represses both *deadpan* and *D-Pax2* transcription (Wildonger *et al.*, 2005).

Lozenge is also absolutely required for crystal cell differentiation both in the embryo and in the larva during *Drosophila* hematopoiesis (Lebestky *et al.*, 2000). It is specifically expressed in the crystal cell lineage, and in a *lozenge* null background no crystal cells is formed. Moreover, using a thermo-sensitive allele of *lozenge*, it was shown that its function is continuously required for crystal cell development.

In the embryo, *lozenge* is expressed first in the anterior-most row of prohemocytes slightly after the onset of Serpent expression (Bataillé *et al.*, 2005). A fraction of these cells maintains *lozenge* expression and differentiates into crystal cells, whereas the remaining progenitors give rise to plasmacytes (Bataillé *et al.*, 2005). In the larvae, *lozenge* expression is activated in scattered cells in response to the Serrate/Notch signaling pathway both in the lymph gland and in the sessile hematopoietic pockets (Duvic *et al.*, 2002; Lebestky *et al.*, 2003). Recently, it was proposed that the Serrate/Notch signaling in the lymph gland leads to the up-regulation of the transcriptional co-factor Yorkie, which acts together with its DNA-binding partner Scalloped to directly activate *lozenge* transcription in the crystal cell lineage (Milton *et al.*, 2014). Whether Scalloped and Yorkie are also

involved in the activation of *lozenge* transcription in the embryo and in the larval sessile hematopoietic pocket is not known. So, it is still unknown how *lozenge* expression is initiated in the crystal cell precursors. However, it has been shown that *Lozenge* can activate its own transcription, suggesting that its expression in the crystal cell lineage could be maintained via an auto-activation loop (Ferjoux *et al.*, 2007).

At the molecular level, *Lozenge* can trigger the expression of crystal cell specific markers in any tissues that express the GATA factor *Serpent*, indicating that *Lozenge* synergizes with *Serpent* to induce crystal cell differentiation (Waltzer *et al.*, 2003; Fossett *et al.*, 2003). This functional cooperation is mediated by a direct interaction between *Serpent* and *Lozenge* (Waltzer *et al.*, 2003) as well as a conserved *Serpent/Lozenge*-responsive *cis*-regulatory module present in many crystal cell specific genes (such as the prophenoloxidasases *PPO1* and *PPO2* or *lozenge* itself), which is composed of at least one *Serpent* and one *Lozenge* binding site in close association (Ferjoux *et al.*, 2007). Furthermore, the interaction between *Serpent* and *Lozenge* has been conserved through evolution (Waltzer *et al.*, 2003), so the cooperation between *Serpent* and *Lozenge* might be used as a paradigm to study how GATA/RUNX complexes regulate transcription and blood cell development from *Drosophila* to vertebrates. The *Serpent/Lozenge* complex notably acts with several subunits of the mediator transcription complex to activate gene expression (Gobert *et al.*, 2010). In addition, it was shown that *Lozenge* (and potentially *Serpent*) facilitates the recruitment of the Notch/Su(H) complex to some of its target genes and thus collaborates with the Notch signaling pathway during crystal cell differentiation (Terriente-Felix *et al.*, 2013; Skalska *et al.*, 2015).

## 4. Myeloid Leukemia Factor (MLF) family

Myeloid leukemia factors are a novel family of proteins in the course of evolution, and MLF1, the founding member, is associated with the emergence of blood cell cancers in human (Yoneda-Kato *et al.*, 1996). However, their functions and molecular mechanisms of action remain still largely unknown.

### 4.1. Human myeloid leukemia factor (hMLF)

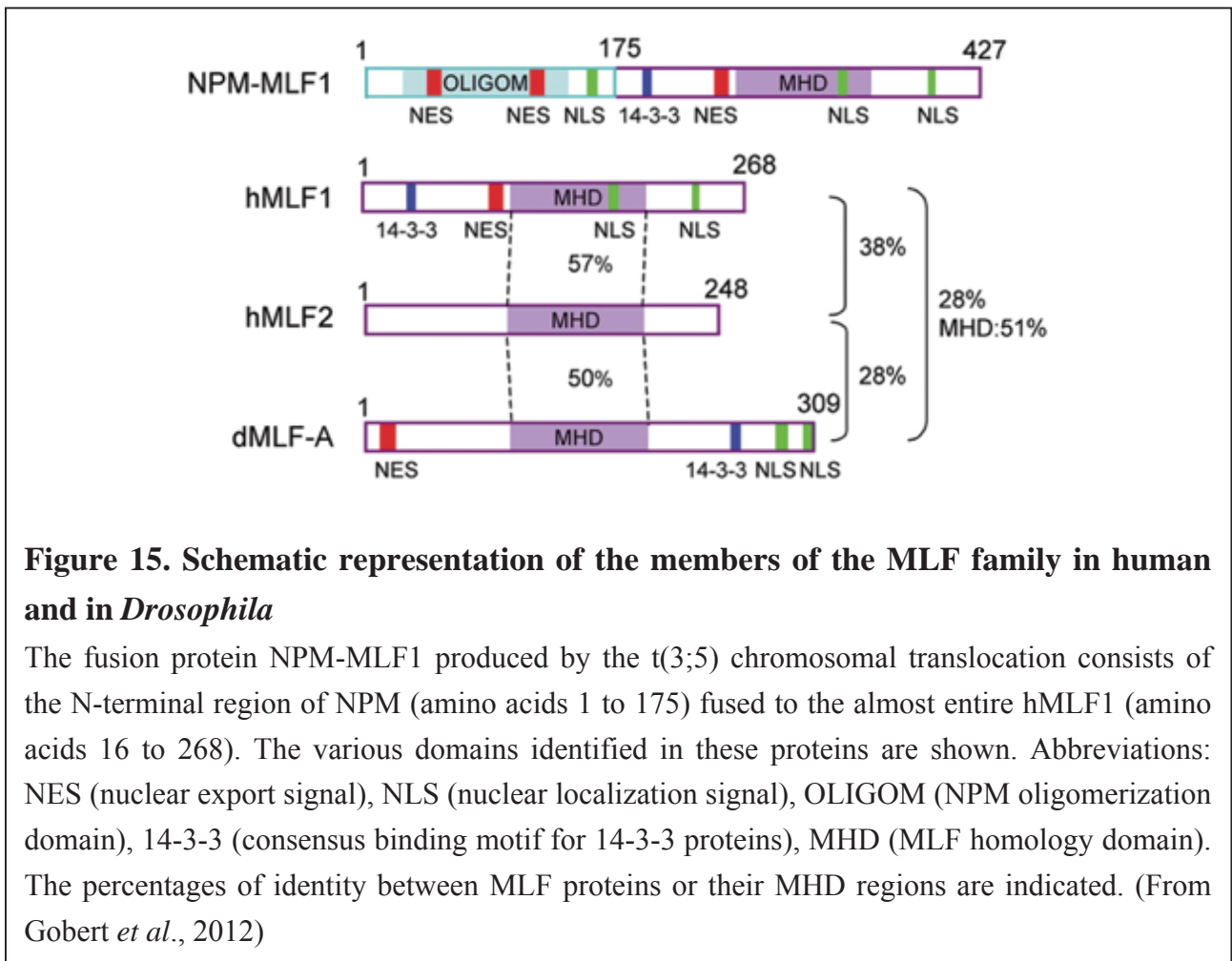
#### 4.1.1. Discovery and structure of hMLF1 and hMLF2

Myeloid leukemia factor 1 (MLF1), the founding member of the MLF family, was identified by cloning the breakpoints of the t(3;5)(q25.1;q34) translocation, which is a chromosomal rearrangement found in rare (less than 0.5%) cases of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) in human (Yoneda-Kato *et al.*, 1996) (**Figure 15**). It exhibits marked variability in expression, with high levels in testis, ovary, skeletal and cardiac muscle, colon and kidney, but low or apparently absent transcription in other tissues. Soon after the discovery of MLF1, *MLF2* cDNA was cloned, which encodes a protein highly homologous to MLF1 (Kuefer *et al.*, 1996). The *MLF2* gene locus was mapped to chromosome 12q13 in human, which is a chromosomal region involved frequently in the translocations and deletions in acute myeloid leukemia or acute lymphoid leukemia, although no evidence indicates so far that MLF2 directly participates in blood cell cancers and/or hematopoiesis.

The members of the MLF family appear to be present in all metazoans (Martin-Lannerée *et al.*, 2006). In human, the MLF family comprises two members, hMLF1 and hMLF2, which share nearly 40% of identity between them. At the molecular level, hMLF1 and hMLF2 are relatively small-sized proteins (about 270 amino acids). hMLF1 has an N-terminal nuclear export signal (NES) and two C-terminal nuclear localization signals (NLS) (**Figure 15**), which allow hMLF1 to shuttle between the nucleus and the cytoplasm (Yoneda-Kato *et al.*, 2008). hMLF1 and hMLF2 do not have specific known functional domains that could help ascribe their biochemical activity, and they only have a central domain preserved within the MLF family and a 14-3-3 protein binding domain (Gobert *et al.*, 2012).

In addition to the adaptor protein 14-3-3 $\zeta$ , which can bind MLF1 (Lim *et al.*, 2002; Molzan *et al.*, 2012) and regulate its interaction with the apoptotic regulator Bcl-XL (Sun *et*

*al.*, 2015), only a few proteins are known to interact with mammalian MLF proteins, including the COP9 signalosome subunit CSN3 which is required for MLF1-induced degradation of p53 (Yoneda-Kato *et al.*, 2005), the HOP survival complex components Hax1 and HtrA2 (Sun *et al.*, 2017) (see below), the centromere protein CENPU/MLF1IP (Hanissian *et al.*, 2004), the adaptor protein MADM (Lim *et al.*, 2002), and the hnRNP-U like protein MANP (Winteringham *et al.*, 2006). Also, in many cases, the physiological relevance of these interactions has not been known, so we only have a limited understanding of MLF protein possible mode of action.



#### 4.1.2. Roles of hMLF1 in hematopoiesis

So far, the roles of hMLF1 in hematopoiesis in human have still not been characterized. However, its expression profile suggests that it regulates blood cell progenitor fate. Indeed, hMLF1 is strongly expressed in CD34<sup>+</sup> progenitor cells and its expression decreases as soon as the specification of these cells towards myeloid and erythroid lineage progresses (Matsumoto *et al.*, 2000).



In a mouse model, MLF1/HLS7 (Hematopoietic Lineage Switch 7) was identified during a screening for genes controlling the transition of erythroleukemic cells (J2E)/immature myeloid cells (J2E-m2) (Williams *et al.*, 1999). Indeed, the ectopic expression of MLF1 in J2E cells reduces their ability to differentiate into mature erythrocytes in response to erythropoietin and causes the acquisition of immature monocyte-type phenotypes. Therefore, MLF1 can induce the reprogramming of erythroleukemic cells to monocytes, which is called “lineage switching”. In addition, over-expression of MLF1 in primary hematopoietic cells of the fetal liver, as well as in different blood cell lines, confirmed that MLF1 promotes the differentiation of myeloid cells and inhibits erythropoietin-induced cell differentiation (Williams *et al.*, 1999; Winteringham *et al.*, 2004). While the consequences of *MLF1* invalidation on mice hematopoiesis remains to be established, it was shown that *MLF1* knock-out lymphocytes are more resistant to apoptotic stimulation than wild-type cells, suggesting that MLF1 controls lymphocyte fate (Sun *et al.*, 2015). However, further analyses will be required to better characterize MLF1 function and mode of action during mammalian blood cell development.

#### **4.1.3. Roles of hMLF in cancer, cell proliferation and apoptosis.**

As mentioned above, hMLF1 was identified by cloning the breakpoints of the chromosomal translocation t(3;5)(q25.1;q34) associated with MDS and AML (Yoneda-Kato *et al.*, 1996). This rare translocation results in the expression of the NPM-MLF1 fusion protein, which is composed of the N-terminal portion of nucleophosmin (NPM) containing a nuclear localization signal and a dimerization domain, fused to the almost entire protein sequence of hMLF1 (**Figure 15**). NPM is a nucleolar protein with many functions, such as control of ribosomal protein transport, regulation of cell cycle progression, maintenance of genome stability or assembly/disassembly of nucleosomes (Colombo *et al.*, 2011).

Many evidences indicate that the NPM-MLF1 fusion protein plays a direct role in malignant transformation. In particular, NPM-MLF1 expression increases the proliferative potential of hematopoietic progenitors *in vitro* and facilitates the oncogenic transformation induced by RasV12 in murine embryonic fibroblast (Lee *et al.*, 2012). Moreover, *NPM* is very frequently mutated in AML and it is targeted by two other chromosomal translocations found in leukemia, to generate fusion proteins with ALK or RAR (Falini *et al.*, 2007). It has been proposed that NPM converts its partners into onco-proteins by providing an oligomerization domain as well as a nuclear localization signal (NLS) in the case of

translocations. In fact, MLF1 is mostly localized in the cytoplasm, but NPM-MLF1 is nuclear in cell culture after transfection (Falini *et al.*, 2007; Ohno *et al.*, 2000). It also should be noted that the expression of *MLF1* is deregulated because it is under the control of the regulatory regions of *NPM1* in the chromosomal translocation t(3;5). Furthermore, it has been observed that the expression of *MLF1* increases during the progression of MDS to AML and that high expression levels of *MLF1* are associated with a poor prognosis in MDS patients without the t(3;5) translocation. Thus, deregulation of *MLF1* expression could participate in the malignant transformation of myeloid cells (Matsumoto *et al.*, 2000). All together, the roles and the molecular mechanisms of action of MLF1 in leukemic transformation remain rather elusive.

In addition, *MLF1* may have an oncogenic role in other tissues, for instance, *MLF1* is over-expressed in squamous lung carcinomas (Sun *et al.*, 2004) and esophageal carcinomas (Chen *et al.*, 2008). Moreover, MLF2 was shown to contribute to cancer cell metastasis and potential gain of function point mutations in *MLF2* have been identified in lung and breast metastatic cells (Dave *et al.*, 2014). Strikingly, a recent study identified a bi-allelic null mutation of *MLF1* in infants with T-cell acute lymphoid leukemia (Mansur *et al.*, 2015). Therefore, *MLF* family members could play a role as a tumor suppressor or as an oncogene depending on the cell types.

Along that line, it was shown that MLF1 overexpression impairs cell cycle exit in erythrocytes by promoting the degradation of the cell cycle regulator p27<sup>Kip1</sup> (Winteringham *et al.*, 2004), but inhibits the proliferation of fibroblasts through preventing p53 degradation (Yoneda-Kato *et al.*, 2005). More recently, a study showed that MLF1 is a novel modulator of neonatal rat cardiomyocyte proliferation (Rangrez *et al.*, 2017). MLF1 overexpression in cardiomyocytes inhibited their proliferation and promoted apoptosis, whereas MLF1 knockdown protected them from apoptosis and hypoxia-induced cell death and promoted proliferation. Interestingly, MLF1 is highly expressed in the heart in human, but at significantly reduced levels in patients with dilated cardiomyopathy and it is profoundly down-regulated in an *in vivo* mouse model of cardiomyopathy, suggesting that MLF1 could be implicated in this pathology. Finally, another recent study showed that MLF1 is a pro-apoptotic antagonist of HOP complex-mediated survival (Sun *et al.*, 2017). By interacting with HAX1 and HtrA2, two components of the HAX1/HtrA2-OMI/PARL (HOP) mitochondrial protein complex, MLF1 inhibits HtrA2 cleavage and activation, which leads

to apoptotic cell death. Interestingly, *mlf1* deletion reverses lymphopenia and significantly ameliorates the progressive neurodegeneration observed in Hax null mutant mice. Thus, MLF1 could control cell survival in the hematopoietic and nervous system by regulating HOP function. However, only a small fraction of MLF1 is present in the mitochondria (Sun *et al.*, 2017) and thus its function is probably not restricted to that cell compartment.

## **4.2. *Drosophila* myeloid leukemia factor (dMLF)**

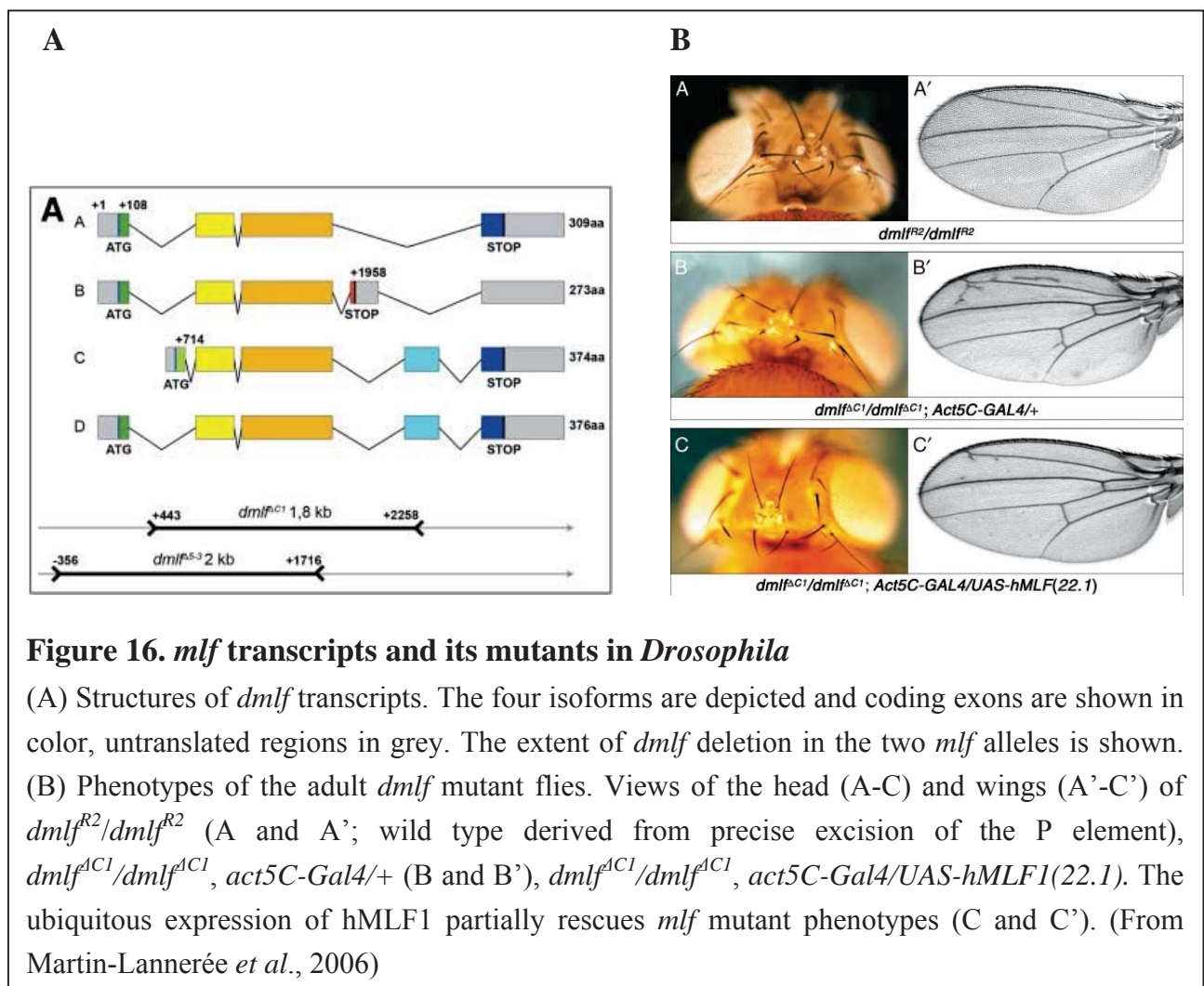
### **4.2.1. Isoforms and structure of dMLF**

In *Drosophila*, there is a single *mlf* gene, which is located on chromosome II (Ohno *et al.*, 2000). *dmlf* codes for four distinct isoforms that are generated by alternative splicing, which are dMLF-A, dMLF-B, dMLF-C and dMLF-D, and these MLF isoforms that range from 273 to 376 amino acids long, differ by their N-terminal or C-terminal region (Martin-Lannerée *et al.*, 2006) (**Figure 16**). dMLF-A, the most abundant isoform of dMLF, has 28% identity with hMLF1 and hMLF2 (Gobert *et al.*, 2012). The central region of dMLF-A (amino acids 96 to 202), which corresponds to the MLF homology domain (MHD), has about 50% identity with the corresponding domain of hMLF1 or hMLF2 (**Figure 15**). dMLF contains a nuclear export signal (NES) and two nuclear localization signals (NLS), and a study showed that these two NLS are required to enable nuclear localization of dMLF-A in *Drosophila* cell culture (Sugano *et al.*, 2007). MLF-C and D also carry these NLS but MLF-B does not, and it was reported to be located both in the cytoplasm and in the nucleus (Martin-Lannerée *et al.*, 2006). Finally, except a binding site for the 14-3-3 family of proteins at the C-terminus, dMLF does not possess any homology with other proteins.

### **4.2.2. Expression profile and mutants of dMLF**

The expression profile of *mlf* gene reveals that it has a strong maternal contribution and it is ubiquitously expressed during the early embryonic stage. At later stage, its expression level increases in the central nervous system, gonads, digestive tract and crystal cells (Martin-Lannerée *et al.*, 2006; Bras *et al.*, 2012). Furthermore, *mlf* gene is expressed ubiquitously at different larval stages in the imaginal discs. dMLF is localized in both the nucleus and the cytoplasm in different ratios depending on the tissues and stages of development (Martin-Lannerée *et al.*, 2006). In the larval hemocytes, dMLF is expressed predominantly in the crystal cell lineage where it is mainly localized in the nucleus (Bras *et al.*, 2012).

The imprecise mobilization of a transposable element located in the first intron of the *mlf* gene allowed to generate two *mlf* null mutant alleles (Martin-Lannerée *et al.*, 2006), and both alleles resulted in a deletion of almost entire *mlf* coding region (**Figure 16.A**). *mlf* null mutation is associated with a strong lethality during development. Some *mlf* mutant individuals can survive to the adult stage, but they do not exhibit obvious morphological defects apart from the loss of some interocellar bristles, frequent shortening of head macrochaetes and ectopic vein formation in the wings (**Figure 16.B**) (Martin-Lannerée *et al.*, 2006). Therefore, the phenotypes of these *mlf* mutants do not provide strong hints concerning *mlf* functions *in vivo*.



#### 4.2.3. dMLF partners and involvement in apoptosis

*Drosophila* MLF was first identified in a two-hybrid screen in yeast as a partner of the *Drosophila* transcription factor DREF (DNA Replication Enhancer Factor), which regulates the expression of genes involved in DNA replication and cell proliferation (Ohno *et al.*, 2000). Moreover, dMLF over-expression was found to induce a reduction in eye and

wing size (Sugano *et al.*, 2007; Yanai *et al.*, 2014). Importantly, a recent publication showed that MLF is recruited to chromatin on a DREF-responsive enhancer in basket, a gene coding for the Jun N-terminal Kinase (JNK) whose activation promotes apoptosis, and genetic experiments indicate that gain of MLF causes a reduction in wing size by activating the JNK pathway and apoptosis (Yanai *et al.*, 2014). Together with previous findings showing that MLF is associated with polytene chromosomes in *Drosophila* (Fouix *et al.*, 2003) and that mouse Mlf1 can bind DNA (Winteringham *et al.*, 2006), this report demonstrated for the first time that MLF could act directly on chromatin to regulate gene expression. This was very recently confirmed at the genome-wide level in S2 cells by another study (Dyer *et al.*, 2016) (see Discussion).

Independently, another two-hybrid screen showed that MLF is a partner of Suppressor of Fused, a negative regulator of the Hedgehog signalling pathway, and gain of function experiments showed that dMLF genetically interacts with Su(fu) in the eye disc (Fouix *et al.*, 2003). In addition, dMLF was shown to physically interact with another negative regulator of Hedgehog, dCostal2 (Fouix *et al.*, 2003), and as its mammalian counterpart, with the COP9 subunit CSN3 (Sugano *et al.*, 2007). However, the importance of MLF interaction with these proteins *in vivo* remains largely unknown.

#### **4.2.4. Roles of dMLF in hematopoiesis**

In *Drosophila*, dMLF is strongly expressed in crystal cells, one of the three main blood cell lineages, at the embryonic stage and the larval stage (Martin-Lannerée *et al.*, 2006; Bras *et al.*, 2012). Actually, *mlf* expression in the crystal cell lineage seems to be directly activated by the SRP/LZ complex (Bras *et al.*, 2012; Ferjoux *et al.*, 2007). In addition, *mlf* was identified as a positive regulator of the activity of the SRP/LZ complex in a genome-wide RNA interference (RNAi) screen in cell culture. Indeed, the down-regulation of dMLF expression by RNAi technology in Kc167 cells resulted in a decrease in the transactivation activity of a target reporter gene activated by the SRP/LZ complex (Gobert *et al.*, 2010). It has been shown that MLF regulates the activity of this complex through ensuring LZ stable expression both in Kc167 cells and in crystal cells *in vivo* (Bras *et al.*, 2012).

Thanks to its capacity to control transcription factor LZ stability, dMLF also regulates the development of the embryonic and the larval LZ<sup>+</sup> cell lineage in *Drosophila* (Bras *et al.*, 2012). During embryonic hematopoiesis, dMLF is required for the maintenance of LZ<sup>+</sup> cells. These cells are specified normally, but their number decreases gradually in absence of

dMLF. This is probably due to a lack of LZ accumulation/maintenance, which prevents the self-regulation of LZ expression and the maintenance of embryonic crystal cells. Indeed, the enforced expression of LZ in the LZ<sup>+</sup> cells can restore crystal cell number in *mlf* mutant embryos. On the contrary, an increase in the number of circulating LZ<sup>+</sup> cells is observed in the *mlf* mutant larvae. This phenotype in the larvae is also associated with a decrease in LZ protein level and is rescued by the enforced expression of LZ, suggesting that this unexpected increase in larval crystal cell number caused by the loss of dMLF is due to a decrease in LZ level. However, the precise mechanism by which dMLF regulates LZ stability and why there is an increase in larval crystal cell number still remain unknown. During my PhD, I tried to tackle these two important questions to better understand MLF function and molecular mechanism of action during hematopoiesis.

It is worth mentioning that the defects in crystal cell number observed in *mlf* mutants were rescued by re-expressing dMLF specifically in the LZ<sup>+</sup> cells using the *lz-GAL4* driver, demonstrating that these defects are cell autonomous and caused after the induction of LZ expression (Bras *et al.*, 2012). In addition, hMLF1 expression also rescued *mlf* mutant defects in crystal cell number, indicating a conservation of MLF function. Finally, RUNX1-ETO accumulation in Kc167 cells as well as in *Drosophila* crystal cells or in human leukemic cells was also dependent on dMLF or hMLF1, respectively, suggesting that MLF factors could regulate the stability of different Runt-domain containing transcription factors. This could be particularly relevant for hMLF1 function in the development of MDS and AML in human.

#### **4.2.5. Roles of dMLF in suppression of polyglutamine aggregates and neurodegeneration**

Huntington's disease (HD), an autosomal dominant neurodegenerative disease, is caused by the expansion of normally polymorphic polyglutamine (polyQ) stretches at the N-terminus of the protein Huntingtin (HTT). If the polyQ stretches in Huntingtin is expanded beyond 36, it will lead to misfolding of the protein, which causes the formation of cytoplasmic and nuclear/perinuclear aggregates that are also known as intracellular inclusions. It has been known that Huntingtin interacts with several proteins and these proteins are recruited to the aggregates. However, the contributions of these proteins to the pathogenesis of Huntington's disease have not been identified fully. Several *Drosophila* models have been developed to model Huntington's disease as well as other polyQ-

associated neurodegenerative diseases (McGurk *et al.*, 2015). Indeed, the expression of polyQ in the *Drosophila* eye is toxic and produces phenotypes (eye depigmentation and cell degeneration) that can be used as readout to screen for enhancers or suppressors of polyQ-associated neurodegeneration. Thereby, it was shown that over-expression of dMLF can ameliorate the cellular toxicity of the polyQ proteins expressed in the eye and in the central nervous system (Kazemi-Esfarjani *et al.*, 2000). In particular, the endogenously or ectopically expressed dMLF co-localizes with polyQ aggregates in the retina, suggesting that dMLF alone or through an intermediary molecular partner sequesters polyQ and/or its aggregates to suppress toxicity. Moreover, in transfected primary rat neuronal culture, dMLF also co-localizes with the polyQ inclusions and suppresses their toxicity, reducing the morphological phenotypes and inclusions (Kim *et al.*, 2005). Interestingly, similar suppression was observed with hMLF1 or hMLF2, suggesting that suppression of polyQ toxicity is a conserved function of MLF proteins. At the molecular level, dMLF was found to reduce the recruitment of the histone acetyltransferase CBP and the chaperone Hsp70 into the inclusions, two essential proteins trapped in polyQ aggregates. In addition, hMLF1 and hMLF2 were recently found to preferentially interact with the mutated N-terminal Huntingtin. Both of them significantly reduced the number of cells containing mutant Huntingtin aggregates and subsequent apoptosis in Neuro2A cells model (Banerjee *et al.*, 2017). In presence of hMLF1 and hMLF2, the mobile fraction of mutant Huntingtin aggregates was increased, and hMLF1 could release some transcription factors from mutant Huntingtin aggregates. These data suggest that MLF proteins could modulate the formation of aggregates as well as the induction of apoptosis, resulting in a decrease in polyQ associated toxicity. Thus, beyond their role in hematopoiesis and leukemia, MLF proteins could be important regulators in neurodegenerative diseases.

## 5. The Hsp40/DnaJ chaperone family

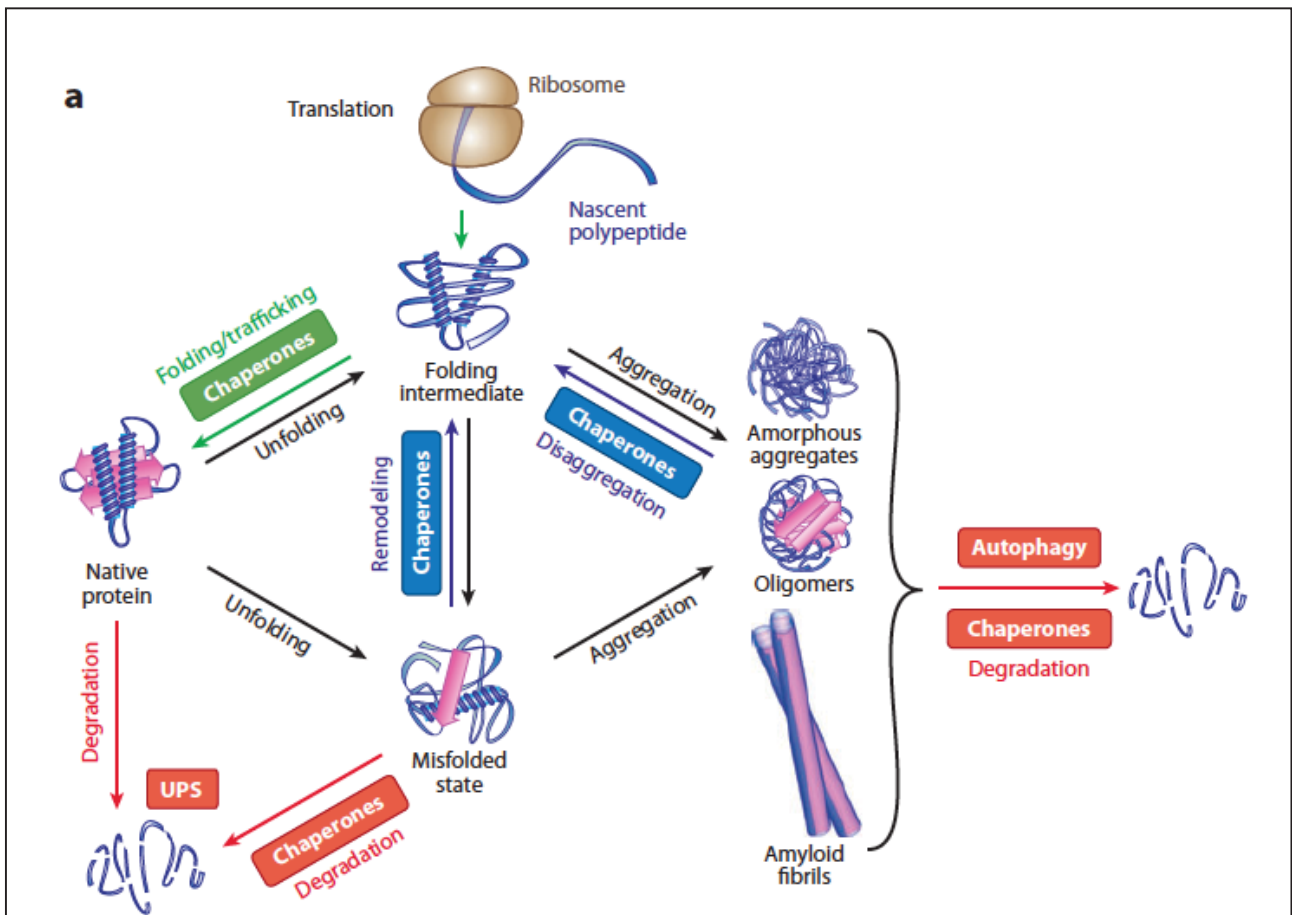
The 40kDa heat shock protein (Hsp40/DnaJ) co-chaperones are the largest and the most diverse sub-group of the heat shock protein (HSP) family. They are widely accepted as regulators of the Hsp70 chaperones, but also have roles as co-chaperones in the Hsp90 chaperone machine. However, a growing number of evidences show that their biological functions may be independent of either of these two chaperone machines.

### 5.1. General description of molecular chaperones

The successful execution of cellular processes is dependent on the coordinated interactions of proteins. After synthesis as linear sequence of amino acids on ribosomes, the large majority of proteins must fold into well-defined three-dimensional structures to obtain their functions. Although some newly translated proteins are capable of folding spontaneously, a large number of proteins are less efficient to fold properly and easy to mis-fold, leading to the formation of protein aggregates, which can be toxic and cause diseases. To settle these problems, cells have a network of molecular chaperones that assist in *de novo* folding and maintain preexisting proteins in their native states (Hartl *et al.*, 2011).

Molecular chaperones are any proteins that interact with and aid in the folding or assembly of another protein without being part of its final structure (Kim *et al.*, 2013). They are found in bacteria, plants, insects and other animals, and represent the most preserved system of the living kingdom. They are main players of protein homeostasis in cells under physiological or stress conditions (Saibil, 2013), modulating the integrity or activity of their protein substrates (called clients) with different mechanisms. They participate into many biological processes, ranging from folding of newly synthesized proteins or refolding of mis-folded protein aggregates to the regulation of their stability and subcellular location (**Figure 17**). Under physiological conditions, they recognize the hydrophobic regions of proteins during synthesis, and thus preventing the specific interactions with other proteins as well as the formation of lethal insoluble aggregates in cells. Under cellular stress conditions, the chaperones interact with denatured or poorly folded proteins and then try to refold their substrates properly. If the client protein cannot be refolded properly, it is usually delivered to the degradation pathways. As main cyto-protective players, molecular chaperones protect cells against different types of stresses and ensure organism survival under adverse conditions.





**Figure 17. Protein fates in the proteostasis network**

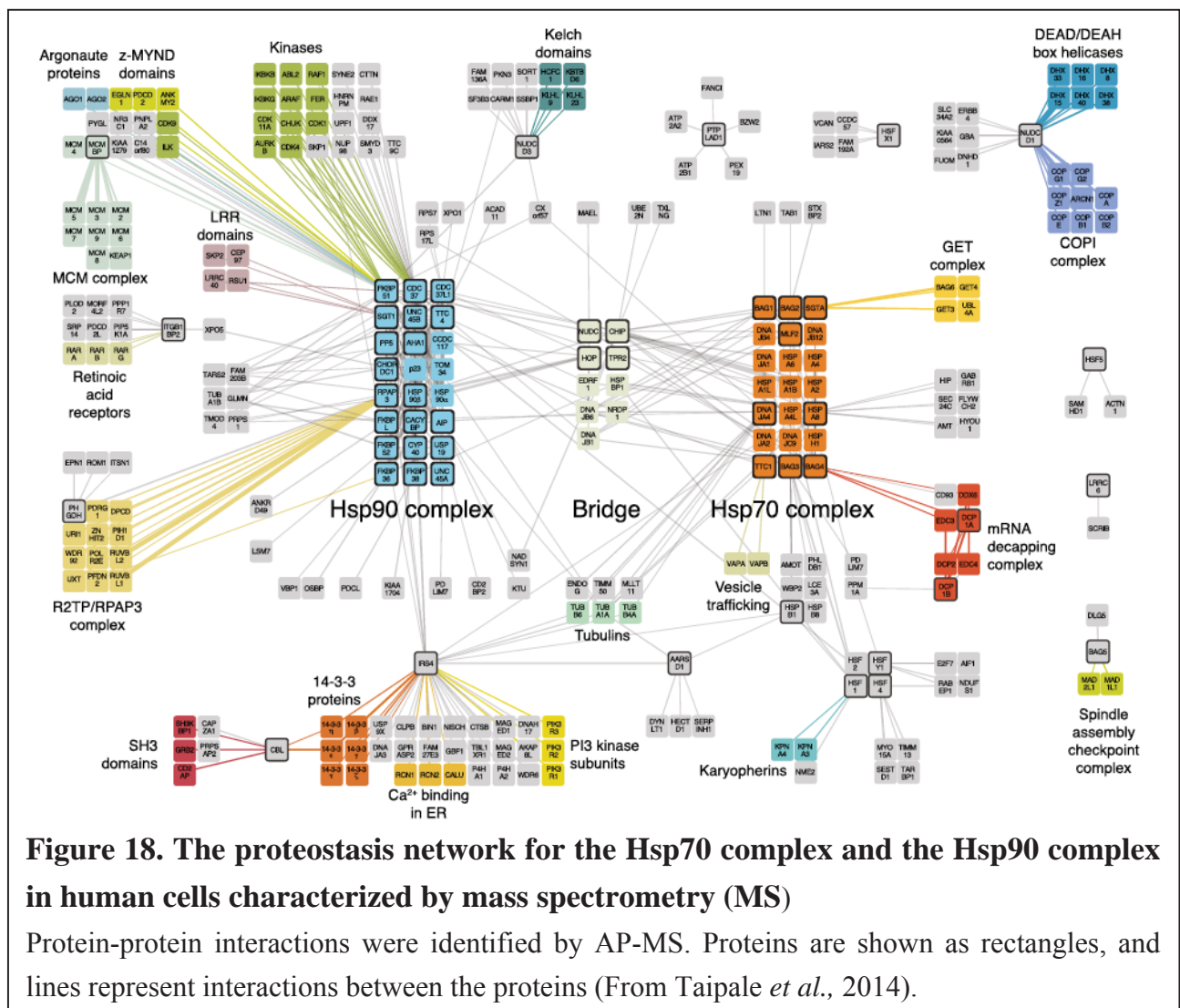
The proteostasis network integrates chaperone pathways for the folding of newly synthesized proteins, for the remodeling of misfolded states and for disaggregation with the protein degradation mediated by the ubiquitin-proteasome-system (UPS) and the autophagy system. (From Kim *et al.*, 2013)

## 5.2. Heat shock proteins

Up to now, the largest and best-characterized group of molecular chaperones is composed of heat shock proteins (Hsps). The heat shock response (HSR) is an ancient and highly conserved molecular response to disruption of protein homeostasis (Morimoto, 2011), which was discovered in 1962 and characterized with an abnormal transcription of certain loci on polytene chromosomes of salivary glands in *Drosophila* following an elevation of temperature (Jamrich *et al.*, 1977). Since then, many studies showed that other stresses, including endoplasmic reticulum stress, nutrient deficiencies or viral infections, can induce or increase the expression of Hsps. In addition, some Hsps are constitutively expressed and have household functions (Vos *et al.*, 2008). Notably, many HSPs are chaperones and play an important role in protein homeostasis by binding to newly

synthesized polypeptides, catalyzing their conformational maturation or participating in protein quality control.

In mammals, Hsps are grouped into six main families depending on their molecular weight: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the family of small stress proteins (small Hsps, or sHsps) (Hartl *et al.*, 2011). These families have some common specificity with respect to their subcellular localization (cytoplasm, mitochondria, endoplasmic reticulum), their mechanisms of action, their dependence on ATP. Here, it should be noted that, historically, the inducible chaperones are denoted as Hsp (Heat shock protein), while the constitutively expressed chaperones are denoted as Hsc (Heat shock cognate protein). These chaperones form a vast network of molecular chaperones that can be schematically subdivided into four families of ATP-dependent chaperones (Hsp60, Hsp70, Hsp90 and Hsp100), whose activities are regulated by their respective co-chaperones, for instance, Hsp40/DnaJ for the Hsp70 chaperone system.



### 5.3. The DnaJ proteins family

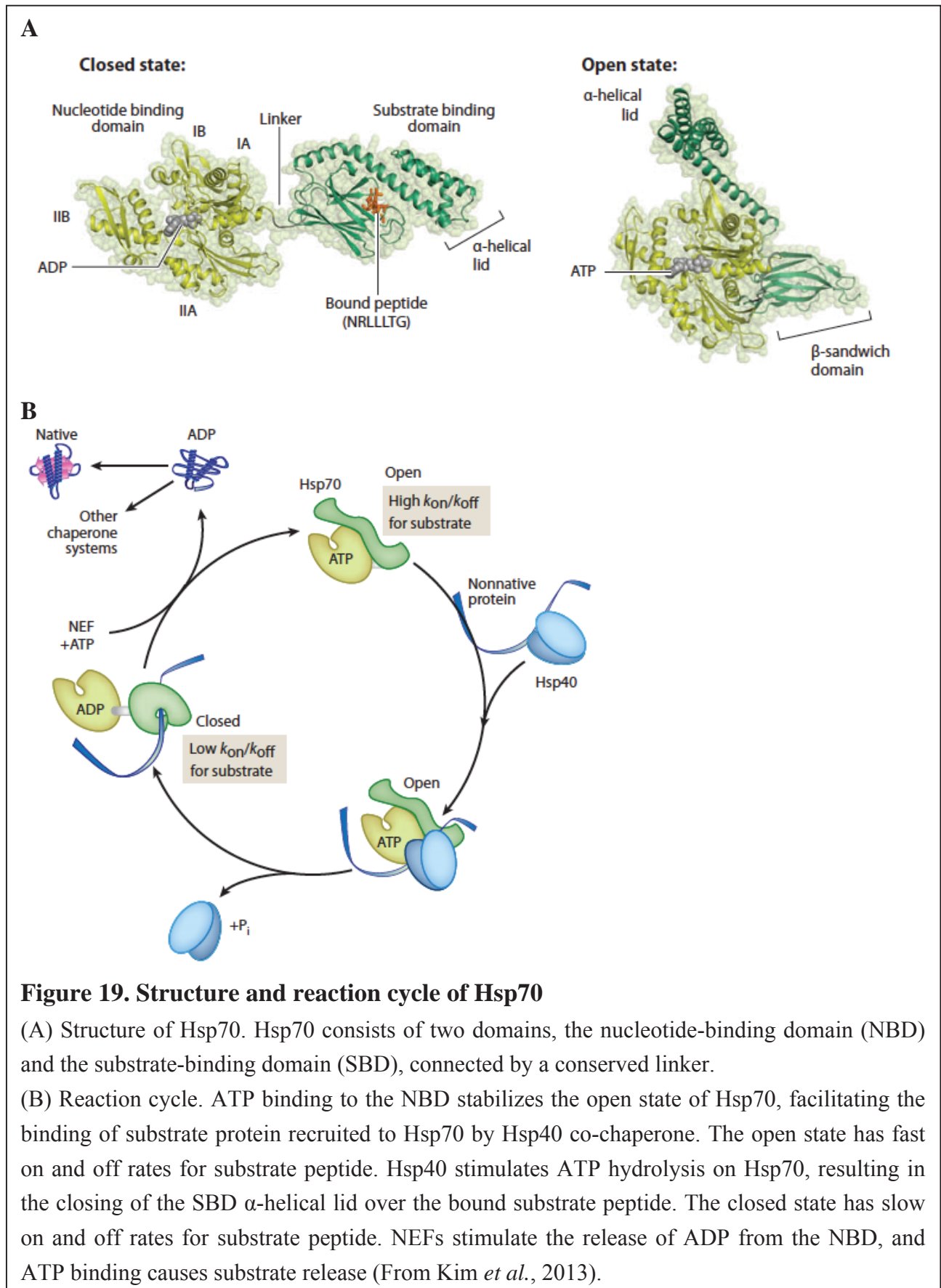
#### 5.3.1. The Hsp70/DnaJ chaperone system

Generally, there are two common chaperone families, the Hsp70 system and the Hsp90 system, which participate broadly in *de novo* protein folding and refolding (**Figure 18**). They are multicomponent molecular chaperone machines that promote protein folding through ATP- and co-factor-regulated binding and release cycles.

The founding member of the Hsp40 family, DnaJ, was identified as a regulator of the ATPase activity of DnaK, the Hsp70 homolog in *E.coli* (Yochem *et al.*, 1978). In this bacterium, DnaK consists of two domains connected by a highly conserved hydrophobic linker region, an N-terminal nucleotide-binding domain (NBD) that binds ATP and carries the ATPase activity, and a C-terminal substrate-binding domain (SBD) that binds and fixes substrates and then refolds them (Doyle *et al.*, 2013) (**Figure 19.A**). Its reaction cycle for the folding of proteins can be described as follows (Kim *et al.*, 2013) (**Figure 19.B**): following ATP binding to the NBD, DnaK adopts an open conformation with the exposed substrate binding domain. In parallel, the co-chaperone DnaJ transiently interacts with a particular substrate via its C-terminal substrate-binding domain. Then DnaJ binds to DnaK via its N-terminal J domain, presents the substrate to DnaK and stimulates DnaK ATPase activity. The hydrolysis of ATP to ADP stimulated by DnaJ induces a change in the conformation of the substrate-binding domain, resulting in the closing of DnaK's  $\alpha$ -helical lid over the bound substrate peptide, which makes it possible to modify the non-native substrate. DnaJ is then released from DnaK as it has a reduced affinity for ADP-bound DnaK. The attachment of nucleotide exchange factor (NEF) stimulates the release of ADP from the nucleotide-binding domain, and ATP binding induces the opening of DnaK C-terminal substrate-binding domain, hence the modified substrate is released. The same substrate may undergo several successive cycles if necessary, or be delivered to the proteasome degradation system if this folding/refolding process fails. In sum, the Hsp40/DnaJ co-chaperones are canonically involved in the presentation of the substrate peptides to the Hsp70 chaperones as well as in the stimulation of the Hsp70 ATPase activity.

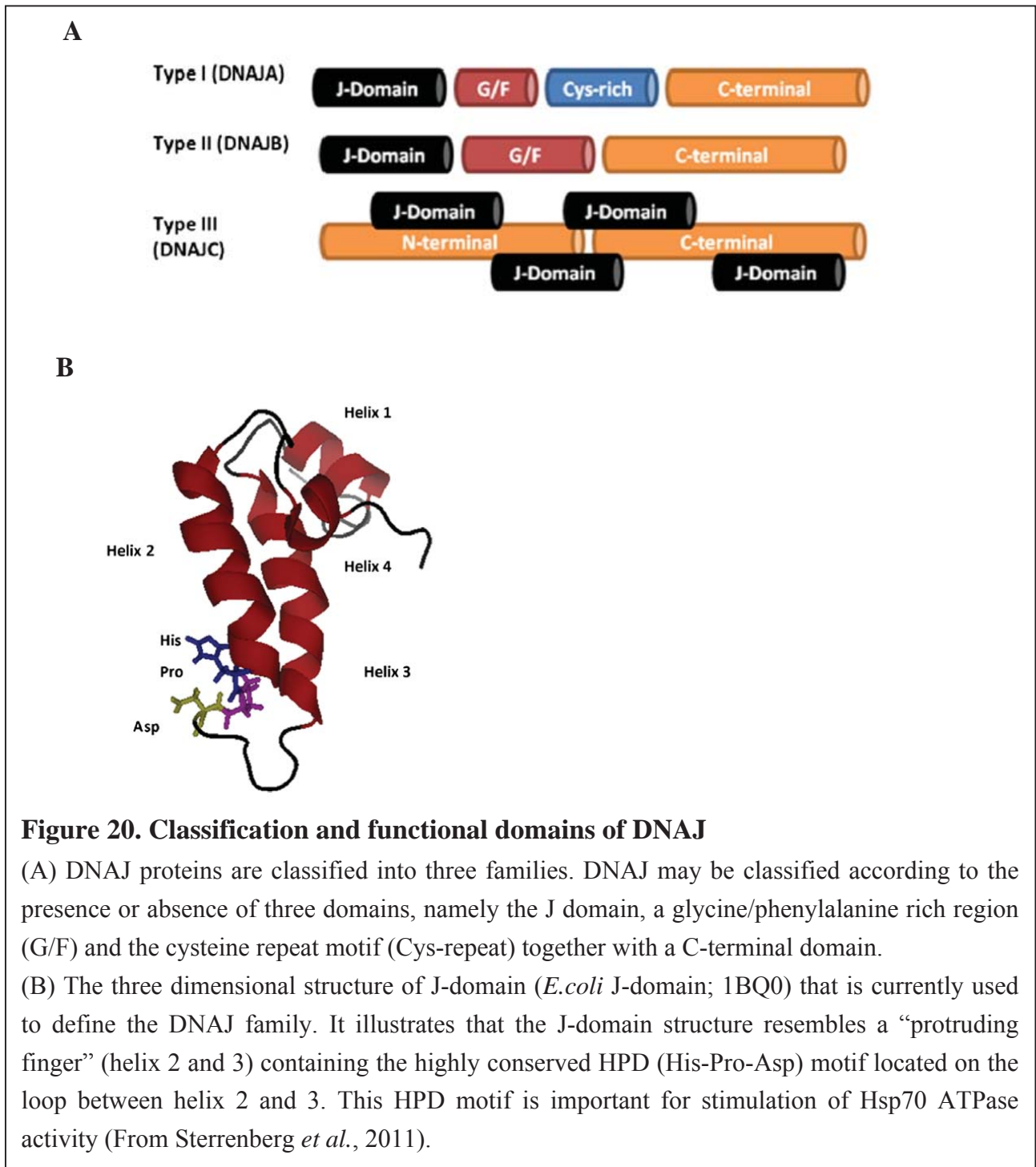
Although the major effect of DnaJ on DnaK reaction cycle is the stimulation of DnaK ATPase activity, DnaJ also has a chaperone activity by itself; for example, it is able to

renature the denatured luciferase *in vitro* (Fink, 1999). In addition, some DnaJ proteins could be direct or indirect regulators of the Hsp90 chaperones (Sterrenberg *et al.*, 2011).



### 5.3.2. Structure and classification of Hsp40/DnaJ proteins family

Generally, members of the Hsp40/DnaJ proteins family possess a common N-terminal J-domain allowing them to bind to Hsp70 chaperones and a conserved C-terminal domain (**Figure 20.A**). This J-domain is composed of approximately 70 amino acids that are organized into a structure consisting of four helices and a loop located between the helices II and III. It also contains a highly conserved HPD (His-Pro-Asp) motif, which is essential for the stimulation of the Hsp70 ATPase activity (Sterrenberg *et al.*, 2011) (**Figure 20.B**).



**Figure 20. Classification and functional domains of DNAJ**

(A) DNAJ proteins are classified into three families. DNAJ may be classified according to the presence or absence of three domains, namely the J domain, a glycine/phenylalanine rich region (G/F) and the cysteine repeat motif (Cys-repeat) together with a C-terminal domain.

(B) The three dimensional structure of J-domain (*E.coli* J-domain; 1BQ0) that is currently used to define the DNAJ family. It illustrates that the J-domain structure resembles a “protruding finger” (helix 2 and 3) containing the highly conserved HPD (His-Pro-Asp) motif located on the loop between helix 2 and 3. This HPD motif is important for stimulation of Hsp70 ATPase activity (From Sterrenberg *et al.*, 2011).

The Hsp40/DnaJ proteins family in human is composed of 49 members and 44 members in *Drosophila*. These proteins are classified into three types according to the presence or absence of two other domains in addition to the N-terminal J-domain and the conserved C-terminal domain (Cheetham *et al.*, 1998): Type I (or Type A) has a glycine/phenylalanine (G/F) rich domain as well as a cysteine repeat domain, Type II (or Type B) has a glycine/phenylalanine (G/F) rich domain and type III (or Type C) does not have any of these extra domains (**Figure 20.A**). Hsp40/DnaJ proteins are found in the cytosol, mitochondria, nucleus, endoplasmic reticulum, endosomes or ribosomes, and their expression may be ubiquitous or restricted to a particular tissue. Some Hsp40/DnaJ proteins of type I and II could interact with unfolded polypeptides and have a chaperone function independently of Hsp70 proteins (Hageman *et al.*, 2010). Besides the domains described above, some Hsp40/DnaJ proteins possess additional regions that enable them to exert a specific function (Lu *et al.*, 2006; Cunnea *et al.*, 2003; Shi *et al.*, 2005).

Finally, DnaJ-1, the protein that we identified in our proteomic screen as a partner of dMLF, belongs to type II (or type B) and its homologs in human are DnaJB1, DnaJB4 and DnaJB5. It was first identified in an yeast two-hybrid screen as a partner of the transcription factor HSF and it was shown to be a nuclear protein that delays the onset of the heat shock response in SL2 cells (Marchler *et al.*, 2001). However, its function during *Drosophila* development and hematopoiesis still remained unknown.

#### **5.4. Hsp70/DnaJ and hematopoiesis**

So far, the role of DnaJ proteins in hematopoiesis has barely been studied. However, some studies revealed the major role of the Hsp70 chaperones family in erythropoiesis (Weiss *et al.*, 2009). Indeed, it has been shown that Hsp70 regulates erythroblast viability by preventing their death induced by apoptosis. On the one hand, erythropoietin (Epo) treatment induces the translocation of Hsp70 to the nucleus where it binds GATA-1 and performs essential functions in erythroid differentiation, since Hsp70 inhibits the cleavage of GATA-1 by Caspase 3 (Ribeil *et al.*, 2007). Conversely, during Epo deprivation, Hsp70 is excluded from the nucleus and GATA-1 is cleaved by Caspase 3. In fact, it has been shown that during erythropoiesis, caspases activation results in GATA-1 cleavage and cessation of erythrocyte maturation or apoptosis (De Maria *et al.*, 1999). Moreover, the phosphorylation of Hsp70 in response to Epo is necessary for its relocation in the nucleus and for erythroblast differentiation. On the other hand, AIF (Apoptosis-Inducing Factor) is

also involved in erythropoiesis. Following Epo treatment, transient mitochondrial depolarization is observed, which leads to the release of procaspases, the caspase activator cytochrome C and AIF. AIF can then translocate to the nucleus and induce DNA fragmentation independently of the caspases to trigger full-blown apoptotic death (Lui *et al.*, 2007; Zermati *et al.*, 2001). In this case, it has been proposed that Hsp70 limits the apoptotic activity of AIF by sequestering it in the cytoplasm (Lui *et al.*, 2007). In addition, a study carried out in zebrafish showed the involvement of a conserved mitochondrial matrix chaperone Hspa9b during the development of erythrocytes. Indeed, in this model, the loss of the Hspa9b chaperone induces a myelodysplastic phenotype, producing oxidative stress and apoptosis in blood cells (Craven *et al.*, 2005). These animals are anemic, and blood cells do not differentiate into mature erythrocytes and die by apoptosis.

The chaperone proteins are also involved at different stages of the hemoglobin production (Arlet *et al.*, 2014). In particular, it has recently been shown that the formation of hemoglobin aggregates in patients with  $\beta$ -thalassemia major ( $\beta$ -TM) causes Hsp70 sequestration in the cytoplasm, where it binds to the  $\alpha$ -chain of the globin. This prevents Hsp70 from protecting GATA-1, and leads to a maturation termination of erythrocytes and their death by apoptosis (Arlet *et al.*, 2014).

The role of DnaJ proteins in hematopoiesis remains largely unknown. However, DnaJB9/hTid1 was shown to interact with the transcription factor STAT5b and to inhibit its expression, thereby suppressing STAT5b-induced hematopoietic cell growth (Dhennin-Duthille *et al.*, 2011). In addition, a recent study showed that over-expression of the co-chaperone ERDJ4 (also called DNAJB9), by enhancing the folding of an endoplasmic reticulum protein, promotes hematopoietic stem cell survival (van Galen *et al.*, 2014).

### **5.5. Molecular chaperones and polyglutamine aggregation**

In mammals, the importance of molecular chaperones is underlined by the consequences of the toxicity of the specific aggregations of poorly folded proteins, which is at the origin of large numbers of neurodegenerative diseases (Borrell-Pagès *et al.*, 2006; Muchowski *et al.*, 2005). Due to their roles in the fight against aggregated proteins, molecular chaperones play a central role in the prevention of neurodegenerative diseases that are caused by the aggregation of polyglutamine (polyQ) proteins. Indeed, many studies have demonstrated the involvement of the protein quality control systems, like the Hsp70 chaperones system and the co-chaperones Hsp40/DnaJ, in different neurodegenerative

diseases (Bilen *et al.*, 2007; Fernandez-Funez *et al.*, 2000). Moreover, molecular chaperones are also involved in some other neurodegenerative diseases, such as Alzheimer's disease (Dou *et al.*, 2003) and Parkinson's disease (Huang *et al.*, 2006). Notably, over-expression of DnaJ-1 can suppress the polyQ cytotoxicity in different *Drosophila* neurodegeneration models (Fayazi *et al.*, 2006; Kazemi-Esfarjani *et al.*, 2000; Chan *et al.*, 2000), and DnaJ-1 cooperates with Hsp70 in this process (Chan *et al.*, 2000). In addition, mutations in different genes encoding for DnaJ-like proteins are at the origin of pathologies associated with proteostasis problems in human, such as neuropathy, muscular dystrophy or Parkinson's disease (Koutras *et al.*, 2014).



## 6. Objective of this project

MLF factors constitute a small family of poorly characterized proteins and they have been implicated in hematopoiesis and in oncogenic blood cell transformation. However, their function and molecular mechanism of action still remain elusive. Previous work in *Drosophila melanogaster* showed that dMLF controls the number of crystal cells, one blood cell lineage related to megakaryocyte, and stabilizes the RUNX transcription factor Lozenge that is indispensable for the development of crystal cells. Moreover, our results suggested that regulation of RUNX transcription factor stability and activity is a conserved characteristic of MLF factors, which could therefore play a role in many cancers caused by a change in RUNX activity in human. During my PhD study, I used *Drosophila melanogaster* as a model to decipher the molecular mechanism of action of MLF on Lozenge stability and activity during blood cell development by searching for MLF interacting partners and characterizing the role of these MLF partners in the regulation of the homeostasis of the hematopoietic system and particularly in the control of proliferation and differentiation of LZ<sup>+</sup> cell lineage.

## **Chapter II Results**

During my PhD, I focused my work on the characterization of MLF mode of action in relation with the RUNX transcription factor stability and hematopoiesis. To gain insights into the possible mechanism of action of MLF proteins, we carried out a proteomic approach to identify *Drosophila* MLF partners in the *Drosophila* Kc167 blood cell line. This allowed us to identify the small chaperone DnaJ-1 as a partner of MLF. We then proceeded with the characterization of *Drosophila* MLF/DnaJ-1 interactions and the functional analysis of DnaJ-1 in cell culture and *in vivo*, as described thereafter.

## 1. Article:

### **Control of RUNX-induced repression of Notch signaling by myeloid leukemia factor (MLF) and its partner DnaJ-1 during *Drosophila* hematopoiesis**

Marion Miller<sup>1\*</sup>, Aichun Chen<sup>1\*</sup>, Vanessa Gobert<sup>1</sup>, Benoit Augé<sup>1</sup>, Mathilde Beau<sup>2</sup>,  
Odile Burlet-Schiltz<sup>2</sup>, Marc Haenlin<sup>1#</sup> and Lucas Waltzer<sup>1#</sup>

1: Centre de Biologie du Développement (CBD), Centre de Biologie Intégrative (CBI), Université de Toulouse, CNRS, UPS, 118 route de Narbonne, F-31062 Toulouse, France

2: Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, UPS, France

# Corresponding authors, E-mail: [lucas.waltzer@univ-tlse3.fr](mailto:lucas.waltzer@univ-tlse3.fr) ; [marc.haenlin@univ-tlse3.fr](mailto:marc.haenlin@univ-tlse3.fr)

\* These authors contributed equally to this work.



## Abstract

A tight regulation of transcription factor activity is critical for proper development. For instance, modifications of RUNX transcription factor dosage are associated with several diseases, including hematopoietic malignancies. In *Drosophila*, myeloid leukemia factor (MLF) has been shown to control blood cell development by stabilizing the RUNX transcription factor Lozenge (LZ). However, the mechanism of action of this conserved family of proteins involved in leukemia remains largely unknown. Here, we further characterized MLF mode of action in *Drosophila* blood cells using proteomic, transcriptomic and genetic approaches. Our results show that the Hsp40 co-chaperone family member DnaJ-1 is a partner of MLF and demonstrate that like MLF, DnaJ-1 binds LZ and promotes its expression in cell culture, suggesting that MLF and DnaJ-1 form a chaperone complex to regulate LZ level and activity. Importantly, *dnaj-1* loss causes an accumulation of  $LZ^+$  blood cells similar as in *mlf* mutant larvae, and we find that *dnaj-1* genetically interacts with *mlf* to control LZ level and  $LZ^+$  blood cell development *in vivo*. In addition, we show that *mlf* or *dnaj-1* loss alters  $LZ^+$  cell differentiation and causes increased Notch expression and over-activation of the Notch signaling pathway. Finally, using different conditions to manipulate LZ activity, we show that high levels of LZ are required to repress Notch transcription and signaling. Our findings thus establish a functional link between MLF and the co-chaperone DnaJ-1 to control RUNX transcription factor activity and Notch signaling during blood cell development *in vivo*. We propose that MLF/DnaJ-1-dependent increase in RUNX level allows the repression of Notch expression and signaling to prevent aberrant blood cell development.

## Author Summary

A tight regulation of protein expression level is required for proper development. Notably, the abnormal expression of key transcription factors or signaling pathway components controlling blood cell development contributes to hematological diseases, such as leukemia. In this report, we used *Drosophila* as a model to study the function and mode of action of a family of conserved but poorly characterized proteins implicated in leukemia called myeloid leukemia factors (MLF). By combining proteomic, transcriptomic and genetic approaches, we show that MLF acts in concert with an Hsp40 co-chaperone to control the level and activity of a RUNX transcription factor and therefore *RUNX*<sup>+</sup> blood cell number and differentiation. Furthermore, we show that RUNX dosage directly impinges on the activity of Notch signaling pathway, which is critical for *RUNX*<sup>+</sup> cell survival and differentiation, by regulating the transcription of the Notch receptor. These findings shed light on a new mode of regulation of RUNX level and Notch activity to prevent abnormal blood cell accumulation, which could be involved in leukemogenesis.

## Introduction

Proper blood cell development requires a fine-tuned regulation of transcription factors and signaling pathways activity. Consequently mutations affecting key regulators of hematopoiesis such as members of the RUNX transcription factor family or components of the Notch signaling pathway are associated with several blood cell disorders including leukemia [1, 2]. Also, leukemic cells often present recurrent chromosomal rearrangements that participate in malignant transformation by altering the function of these factors [3]. The functional characterization of these genes is thus of importance not only to uncover the molecular basis of leukemogenesis but also to decipher the regulatory mechanisms controlling normal blood cell development. Myeloid leukemia factor 1 (MLF1) was identified as a target of the t(3;5)(q25.1;q34) translocation associated with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) more than 20 years ago [4]. Further findings suggested that MLF1 could act as an oncogene [5-8] or a tumor suppressor [9] depending on the cell context and it was shown that MLF1 over-expression either impairs cell cycle exit and differentiation [10], promotes apoptosis [11, 12] or inhibits proliferation [13, 14] in different cell types *ex vivo*. Yet, its function and mechanism of action remains largely unknown.

MLF1 is the founding member of a small evolutionarily conserved family of nucleocytoplasmic proteins present in all metazoans but lacking recognizable domains that could help ascribe their biochemical activity [15]. Whereas vertebrates have two closely related MLF paralogs, *Drosophila* has a single *mlf* gene encoding a protein that presents around 50% of identity with human MLF in the central conserved domain [16, 17]. In fly, MLF was identified as a partner of the transcription factor DREF (DNA replication-related element-binding factor) [16], for which it acts as a co-activator to stimulate the JNK pathway and cell death in the wing disc [18]. MLF has been shown to bind chromatin [18-20], as its mouse homolog [21], and it can either activate or repress gene expression by a still unknown mechanism [18, 20]. MLF also interacts with Suppressor of Fused, a negative regulator of the Hedgehog signaling pathway [19], and, like its mammalian counterpart [13], with CSN3, a component of the COP9 signalosome [22], but the functional consequences of these interactions remain elusive. Interestingly, the overexpression of *Drosophila* MLF or that of its mammalian counterparts can suppress polyglutamine-induced



cytotoxicity in fly and in cellular models of neurodegenerative diseases [17, 23-25]. Moreover phenotypic defects associated with MLF loss in *Drosophila* can be rescued by human MLF1 [17, 26]. Thus MLF function seems conserved through evolution and *Drosophila* appears as a genuine model organism to characterize MLF proteins [15].

Along this line, we recently analyzed the role of MLF during *Drosophila* hematopoiesis [26]. Indeed, a number of proteins regulating blood cell development in human, such as RUNX and Notch, also controls *Drosophila* blood cell development [27]. In *Drosophila*, the RUNX factor Lozenge (LZ) is specifically expressed in and required for the development of the crystal cells [28], one of the three hematopoietic lineages, which accounts for  $\pm 4\%$  of the circulating larval blood cells [27]. The Notch pathway also controls the development of this lineage: it is required for the induction of LZ expression and it contributes then to LZ<sup>+</sup> cell survival and differentiation [28-31]. Interestingly, our previous analysis revealed a functional and conserved link between MLF and RUNX factors [26]. Indeed MLF controls LZ activity and prevents its degradation in cell culture and the stabilization of LZ by MLF appears to be critical to control crystal cell number *in vivo* [26]. Intriguingly, though while LZ is required for crystal cell development, *mlf* mutation caused a decrease in LZ expression and an increase in crystal cell number. In humans, deregulation of RUNX protein level is associated with several pathologies. For instance, haploinsufficient mutations in RUNX1 are associated with MDS/AML in the case of somatic mutations, and with familial platelet disorders with associated myeloid malignancy for germline mutations [1]. In the opposite, RUNX1 overexpression can promote lymphoid leukemia [32, 33]. Understanding how RUNX protein level is regulated and how this affects specific developmental processes is thus of particular importance.

Here, we further studied MLF function and mode of action in *Drosophila* blood cells by using proteomic, transcriptomic and genetic approaches. We show that MLF interacts with the Hsp40 co-chaperone protein DnaJ-1 to stabilize LZ as well as to control LZ<sup>+</sup> cell number and differentiation. In addition, consistent with the analysis of *mlf* or *dnaj-1* mutant, it appears that high levels of LZ are required to tune-down Notch expression and signaling to prevent aberrant blood cell accumulation. These findings thus establish a functional link between the MLF/DnaJ-1 complex and the regulation of a RUNX-Notch axis required for blood cell homeostasis *in vivo*.

## Results

### MLF interacts with DnaJ-1

To better characterize MLF molecular mode of action, we sought to identify its partners. Accordingly, we established a *Drosophila* Kc167 cell line expressing a V5-tagged version of MLF close to endogenous level of MLF in a copper-inducible manner (Fig 1A). After anti-V5 affinity purification from whole cell extracts of control or MLF-V5-expressing cells, isolated proteins were identified by mass spectrometry (see M&M for details). Five proteins reproducibly co-purified with MLF and were either absent or present at more than 4 folds lower levels in control purifications (Fig 1B): the Hsp40 co-chaperone DnaJ-1 (also known as DROJ1, [34]), the constitutively expressed Hsp70 chaperones Hsc70-4 and Hsc70-3, the RNA binding protein Squid (Sqd), and the retrotransposon-encoded protein Copia.

As DnaJ-1 was the strongest hit in our proteomic approach, we focused our analysis on this candidate. To confirm the interaction between MLF and DnaJ-1, we performed co-immunoprecipitation experiments in Kc167 cells transfected with expression plasmids for HA-DnaJ-1, GFP or GFP-MLF. We found that GFP-MLF (but not GFP alone) co-precipitated HA-DnaJ-1 and that conversely HA-DnaJ-1 co-precipitated GFP-MLF as well as endogenous MLF (Fig 1C and 1D), indicating that these proteins specifically interacted. Moreover, *in vitro* translated MLF and DnaJ-1 interacted with GST-DnaJ-1 and GST-MLF respectively but not with GST alone in pull-down assays (Fig 1E and 1F). Of note, immunostaining also showed that DnaJ-1 and MLF co-localized in the nuclei of Kc167 transfected cells (S1A Fig). We then mapped the domains required for their interactions. Hsp40/DnaJ co-chaperones play a crucial role in the regulation of protein folding and degradation; they chiefly act by delivering client proteins to Hsp70/DnaK chaperones and stimulating their ATPase activity [35, 36]. DnaJ-1 belongs to the DnaJB/class II subfamily of Hsp40/DnaJ proteins, which are characterized by an N-terminal J domain required to stimulate Hsp70 ATPase activity (amino acids 4 to 57 in DnaJ-1), a central glycine/phenylalanine (G/F)-rich region (amino acids 64 to 144 in DnaJ-1), and a conserved C-terminal region (amino acids 157 to 320 in DnaJ-1) that contains the client binding domain followed by a dimerization interface [36]. Immunoprecipitations of GFP-MLF expressed with different HA-tagged DnaJ-1 variants indicated that DnaJ-1 C-terminal region mediates MLF binding (Fig 1G). In contrast, a point mutation in the highly

conserved HPD loop (P32S) crucial for Hsc70 activation [36], deletion of the J-domain or deletion of the J and G/F domains did not affect DnaJ-1 binding to GFP-MLF. MLF does not harbor characteristic domains besides a central “MLF homology domain” (MHD, amino acids 96 to 202) conserved between MLF family members [15]. Using GFP-DnaJ-1 as bait and deletion mutants in MLF as preys, we found that the MHD was sufficient for binding to DnaJ-1, while MLF N and C-terminal regions were dispensable (Fig 1H). Finally, consistent with the above results, the C terminal region (amino acids 157 to 334) of DnaJ-1 fused to GFP but not the GFP moiety alone co-precipitated the HA-tagged MHD (S1B Fig). In sum, these data indicate that MLF and DnaJ-1 specifically bind to each other through their conserved central and C-terminal region, respectively.

### **MLF acts in a chaperone complex with DnaJ-1 to control LZ activity and stability**

We have previously shown that MLF is required for LZ-induced transactivation and stable expression [26]. We thus asked whether DnaJ-1 also controlled LZ function. As shown in Fig 2A, transfection of LZ expression plasmid in Kc167 cells induced a robust activation of the 4xPPO2-Fluc reporter gene [37], which was significantly decreased when either MLF or DnaJ-1 expression was knocked down by dsRNA treatment. Furthermore, Western blot analyses revealed that, like *mlf*, *dnaj-1* knockdown caused a drop in LZ protein expression (Fig 2B). Importantly RT-qPCR experiments showed that *mlf* and *dnaj-1* knockdown did not affect the expression of each other or decrease *lz* transcript level, while they caused a significant reduction in the expression of LZ target gene *ppo2* (Fig 2C-F). Hence, like MLF, DnaJ-1 controls LZ protein activity by regulating its stability.

We then asked whether MLF or DnaJ-1 could bind LZ. Upon transfection of the corresponding expression plasmids, both HA-MLF and HA-DnaJ-1 were co-immunoprecipitated by GFP-tagged LZ but not by GFP alone (Fig 2G and 2H). Furthermore, *in vitro* translated LZ bound to *E. coli*-purified GST-MLF and GST-DnaJ-1 but not to GST alone in pull down assays (S2A Fig). These results strongly suggest that MLF and DnaJ-1 specifically interact with LZ. Using different MLF variants in co-immunoprecipitation assays, we found that the N-terminal part of MLF homology domain (amino acids 96 to 147) was crucial for the interaction with LZ (S2B Fig). Similarly, DnaJ-1 C-terminal domain was required for binding LZ, while its J domain was dispensable (S2C Fig). Therefore it appears that LZ interacts with conserved domains of MLF and DnaJ-1 and

our results suggest that the MLF/DnaJ-1 complex regulates LZ stability by interacting with it.

Reminiscent of our previous results with MLF [26], we observed that DnaJ-1 over-expression was associated with an increase in LZ-induced transactivation and LZ expression level (Fig 2I and 2J). The over-expression of C-terminus-truncated DnaJ-1 proteins did not affect LZ-induced transcription or LZ expression. In contrast, the over-expression of DnaJ-1 carrying the P32S point mutation or deleted of its J domain caused a decrease in LZ-induced transactivation and a drop in LZ level (Fig. 2I and 2J), suggesting that Hsc70 activation by DnaJ-1 is required for LZ stable expression. Since we identified Hsc70-4 as a potential partner of MLF, we further tested this hypothesis. Immunoprecipitation experiments confirmed that MLF and DnaJ-1 interacted with Hsc70-4 (S3A and S3B Fig). Moreover, knocking down *hsc70-4* by dsRNA caused a strong decrease in LZ-induced transactivation of the 4xPPO2-Fluc reporter gene and a concomitant reduction in LZ expression (S3C and S3D Fig). In sum, our results support the idea that MLF acts in a chaperone complex with DnaJ-1 and Hsc70-4 to control LZ stability and activity.

### **DnaJ-1 cell-autonomously controls LZ<sup>+</sup> cell number and differentiation *in vivo***

Since DnaJ-1 interacts with MLF and controls LZ level *ex vivo*, we sought to analyze DnaJ-1 function during larval crystal cell development, using the *lz-GAL4/+* driver and the *UAS-mCD8GFP* reporter to monitor this LZ<sup>+</sup> blood cell lineage. Given that no mutant for *dnaj-1*, we used a CRIPR/Cas9 strategy to generate *dnaj-1* null alleles (See Materials and Methods and S4 Fig) [38]. In the following experiments, we used an allelic combination between two mutant lines obtained from independent founder flies (*dnaj-1<sup>A</sup>* and *dnaj-1<sup>C</sup>*), which harbor a complete deletion of *dnaj-1* coding sequence (S4 Fig). Around 65% of the *dnaj-1<sup>A/C</sup>* mutants reached larval stage and 15% emerged as adult flies, but they did not show obvious morphological defect. Notwithstanding and reminiscent of *mlf* phenotypes, bleeding of third instar larvae revealed that *dnaj-1* mutants exhibited a  $\pm 1.8$ -fold increase in circulating *lz>GFP<sup>+</sup>* cells as compared to wild type (Fig 3A). In addition, as in *mlf* mutants, crystal cells from *dnaj-1* mutant larvae still expressed the differentiation marker PPO1 and were capable of melanization upon heat treatment (Fig 3C-H). A closer examination also revealed the presence of unusually large *lz>GFP<sup>+</sup>* cells in *dnaj-1* mutants and quantitative analyses confirmed that *dnaj-1* loss caused a significant increase in *lz>GFP<sup>+</sup>* cell size whereas

*lz>GFP* cells were unaffected (Fig 3B). Interestingly, a similar phenotype is observed in *mlf* mutant larvae, suggesting that both genes not only control crystal cell number but also their differentiation (see below). Importantly, *lz>GFP*<sup>+</sup> cell number and size was restored to wild-type when DnaJ-1 was re-expressed in the crystal cell lineage of *dnaj-1<sup>AC</sup>* mutant larvae using the *lz-GAL4* driver (Fig 3A and 3B), demonstrating that these phenotypes are specifically caused by *dnaj-1* mutation and that DnaJ-1 acts cell autonomously after the onset of *lz* expression in the crystal cell lineage. Furthermore, the increase in crystal cell number and size was also observed when we monitored crystal cell presence by immunostaining against PPO1 in larvae carrying *dnaj-1<sup>A</sup>* or *dnaj-1<sup>C</sup>* homozygous mutation or over a deficiency covering *dnaj-1* locus (Def(3L)BSC844) (S4 Fig). Overall, these results demonstrate that, like *mlf*, *dnaj-1* controls larval crystal cell number and size.

We then assessed whether DnaJ-1 affected LZ stability *in vivo* as it does in cell culture. Unexpectedly, immunostaining against LZ did not reveal a decrease in LZ expression in *dnaj-1* mutant crystal cells, while LZ level was clearly lower in *mlf* mutant (Fig 3I-K). Actually, quantitative analyses revealed a slight (30%) but significant (p=0.006) increase in LZ level in *dnaj-1* mutant as compared to wild type, whereas LZ level dropped by more than 2 folds in *mlf* mutant (Fig 3L). Thus, unlike *mlf*, *dnaj-1* loss is not sufficient to destabilize LZ *in vivo*.

### **DnaJ-1 and MLF act together to control LZ accumulation and crystal cell development**

One potentially important difference between Kc167 cells, in which DnaJ-1 is required to stabilize LZ, and crystal cells, in which it is not, is MLF expression. Indeed, in Kc167 cells, MLF is chiefly detected in the cytoplasm and is expressed at low levels in the nucleus (S5A Fig). In contrast, MLF is present at high levels in the nucleus of larval crystal cells (S5B Fig). Moreover, its expression in this lineage is not affected by *dnaj-1* loss (S5C and 5D Fig). We thus supposed that the presence of high levels of nuclear MLF might prevent LZ degradation in the absence of DnaJ-1.

To test this hypothesis, we designed two complementary experiments. On the one hand, we assessed whether MLF over-expression in Kc167 cells could protect LZ from degradation following *dnaj-1* knockdown. On the other hand, we asked whether LZ protein

would still be stable in *dnaj-1* mutant crystal cells if MLF level is decreased. As shown in Figure 4, LZ expression was reduced when Kc167 cells were treated with a dsRNA targeting *dnaj-1* and increased upon over-expression of MLF. Strikingly though, and reminiscent of the above observations in *dnaj-1* mutant crystal cells, LZ level was not reduced but further increased when *dnaj-1* was knocked down in MLF overexpressing cells (Fig 4D and 4E). Conversely, *in vivo*, the expression of a dsRNA against *mlf* in *lz>GFP<sup>+</sup>* cells caused a drop in LZ expression that was significantly enhanced in *dnaj-1* deficient larvae, while *dnaj-1* mutation alone increased LZ level (Fig 4F-J). Hence, it appears that high levels of MLF can prevent LZ degradation in the absence of DnaJ-1.

Then, since chaperones are important for proper protein folding [35, 36], we postulated that LZ protein accumulating in crystal cells in the absence of DnaJ-1 might be less active. Thus increasing LZ expression should be sufficient to rescue *lz>GFP<sup>+</sup>* cell number and size. Consistent with this hypothesis, and as observed in *mlf* mutant larvae, *lz>GFP<sup>+</sup>* cell number and size was restored to wild-type when we enforced LZ expression in this lineage (Fig 5A and 5B). Finally, since MLF and DnaJ-1 bind to each other and jointly control LZ stability, we tested whether they genetically interacted to regulate crystal cell development. While heterozygous mutation in either *mlf* or *dnaj-1* did not efficiently alter circulating *lz>GFP<sup>+</sup>* cell number or size, *mlf<sup>dC1</sup>/+*, *dnaj-1<sup>A</sup>/+* transheterozygote larvae displayed a significant increase of both parameters (Fig 5C and 5D). We thus conclude that DnaJ-1 and MLF act together to control LZ expression and crystal cell development *in vivo*.

### **MLF and DnaJ-1 control crystal cell differentiation**

In parallel, to gain further insights into the function of MLF in the control of crystal cell development, we established the transcriptome of circulating *LZ<sup>+</sup>* blood cells in wild type and *mlf* mutant larvae. Heterozygous *lz-GAL4*, *UAS-mCD8GFP* L3 larvae carrying or not *mlf* null mutation were bled, *lz>GFP<sup>+</sup>* cells were collected by FACS and their gene expression profile was determined by RNA sequencing (RNAseq) from biological triplicates (see M&M for details). Using *Drosophila* reference genome dm3, we detected the expression of 7399 genes (47% of the total fly genes) in each of the 6 samples (Fig 6B and S6 Table). Consistent with the role of the crystal cells as the main source of phenoloxidasases [39], the two most strongly expressed genes were *PPO1* and *PPO2*. In addition, *lz* expression as well as that of several other crystal cell markers (see below) was readily

detected. It was recently shown that larval circulating  $LZ^+$  cells derive from plasmatocytes, which express Hemolectin (Hml) and Nimrod C1 (NimC1) and transdifferentiate into mature crystal cells [40]. Accordingly, we detected the expression of these genes as well as other “plasmatocytes” markers such peroxidasin and croquemort (which were actually shown to be also expressed in crystal cells [41, 42]) in  $l_z>GFP^+$  cells.

Using DESeq2 to identify differentially expressed genes between wild type and *mlf* mutant  $l_z>GFP^+$  cells, we found 779 genes with significantly altered expression (adjusted p-value <0.01): the transcript level of 469 genes was decreased and that of 310 genes was increased in the absence of MLF (Fig 6A, 6B and S7 Table). In line with our previous *in situ* hybridization results [26], RNAseq analysis did not reveal a significant modification of *PPO1* or *PPO2* expression in the absence of *mlf*. However, *l\_z* transcript level was reduced by  $\pm 2$  folds (p-value=0.0018), which could be due to defective maintenance of *l\_z* auto-activation loop [43]. To assess whether other crystal cell markers were affected by *mlf* loss, we established a compilation of genes expressed in (embryonic or larval) crystal cells based on Flybase data mining and re-examination of Berkeley *Drosophila* Genome Project *in situ* hybridizations (<http://insitu.fruitfly.org/cgi303bin/ex/insitu.pl>) (S8 Table). Among these 129 genes (i.e. excluding *mlf* itself), 44 (34%) were differentially expressed in the absence of *mlf* (19 repressed and 25 activated) (Fig 6C), indicating a strong over-representation of deregulated genes in the “crystal cell” gene set as compared to all expressed genes (p-value= $2.6 \times 10^{-13}$ , hypergeometric test) and showing that *mlf* plays a crucial role in proper crystal cell differentiation.

To substantiate these results, we assessed by *in situ* hybridization the expression of 4 genes that were either down-regulated (CG7860 and Oscillin) or up-regulated (CG6733 and Jafrac1) in *mlf* mutant. CG7860 and Oscillin were specifically expressed in  $l_z>GFP^+$  but not in the surrounding  $l_z>GFP^-$  hemocytes in wild-type conditions (Fig 6D and 6G). Consistent with our RNAseq data, the expression of CG7860 and Oscillin was strongly reduced in *mlf* mutant larvae. Although it is expressed in embryonic crystal cells [43], CG6733 was not detectably expressed by *in situ* hybridization in circulating hemocytes of wild-type larvae, but it was expressed in the  $l_z>GFP^+$  lineage in *mlf* mutant larvae (Fig 6J and 6K). Finally, Jafrac1 expression increased in  $l_z>GFP^+$  cells of *mlf* mutant larvae as compared to wild-type, whereas its (lower) expression in  $l_z>GFP^-$  blood cells seemed

similar (Fig 6M and 6N). These data thus confirm the RNAseq results and demonstrate that MLF controls the expression of several crystal cell markers. Since MLF acts together with DnaJ-1, we also tested whether these four genes were deregulated in *dnaj-1* mutant larvae. As for *mlf*, we observed that *dnaj-1* mutation caused a down-regulation of CG7860 and Oscillin expression and an up-regulation of CG6733 and Jafrac1 expression in *lz>GFP<sup>+</sup>* blood cells (Fig 6F, 6I, 6L and 6O). Therefore, the loss of *mlf* or *dnaj-1* leads to the deregulation of crystal cell gene marker expression, indicating that both genes are required for proper differentiation of the *LZ<sup>+</sup>* blood cell lineage.

### **MLF and DnaJ-1 repress Notch signaling**

Interestingly, the levels of Notch receptor transcripts were significantly higher in *mlf* mutant ( $p=1.3 \times 10^{-6}$ ) (Fig 6C). Notch signaling plays a key role in crystal cell development [27]: Notch is first activated by its ligand Serrate to specify crystal cell precursors and subsequently Notch activation is maintained in *LZ<sup>+</sup>* cells in a ligand independent manner to promote crystal cell growth and survival [29-31, 40, 44]. The rise in *lz>GFP<sup>+</sup>* cell number and size observed in *mlf* and *dnaj-1* mutants could thus be due to increased ligand-independent Notch signaling [30, 31]. Hence, we further investigated the level of Notch expression and activation. Immunostaining using an antibody against Notch extracellular domain (NECD) showed that Notch was expressed at higher levels in *lz>GFP<sup>+</sup>* cells of *mlf* and *dnaj-1* mutant larvae than in wild-type condition (Fig 7A-C). Quantitative analyses confirmed that *mlf* loss caused a significant increase of Notch expression in *lz>GFP<sup>+</sup>* cell, whereas the (lower) expression of Notch in *lz>GFP<sup>-</sup>* blood cells was not affected (Fig 7D). Similar results were obtained when we measured Notch protein levels using an antibody directed against its intra-cellular domain (NICD) (Fig 7E). These data strongly suggest that Notch expression is specifically increased in *lz>GFP<sup>+</sup>* cells of *mlf* and *dnaj-1* mutants. We then tested whether this resulted in increased signaling by monitoring the expression of two Notch signaling pathway reporter genes expressed in larval crystal cells: Klumpfuss-Cherry [31] and NRE-GFP [45]. Both *mlf* and *dnaj-1* loss were associated with a strong increase in the expression of these reporters (Fig 7F-J). Thus *mlf* and *dnaj-1* are required to tune down Notch signaling in the crystal cell lineage.

Crystal cells tend to increase their size as they mature in response to Notch signaling [31, 40]. To better characterize the defects associated with *mlf* or *dnaj-1* loss, we analyzed



the distribution of  $lz>GFP^+$  cells as well as Notch expression level according to  $lz>GFP^+$  cell size category. Whereas cells more than 1.3-fold larger than the mean wild-type cell size represented a small fraction ( $\pm 10\%$ ) of the  $lz>GFP^+$  population in wild-type larvae, they constituted the prevalent population in *mlf* or *dnaj-1* mutant (respectively 49.6% and 37%) (Fig 7K). In addition, while Notch expression was maximum in the population of  $lz>GFP^+$  cells of mean cell size and lower in larger cells of wild-type larvae, it was maintained at higher levels in the larger cell populations of *mlf* or *dnaj-1* mutants (Fig 7L-N).

All together, these results show that MLF/DnaJ-1 loss causes the accumulation of large and mis-differentiated crystal cells exhibiting abnormal Notch signaling activation.

### **High levels of LZ are required to prevent accumulation of $lz>GFP^+$ cells and to repress Notch expression/signaling**

LZ is absolutely required for crystal cell formation and differentiation [27]. The above data show that the increase in crystal cell number and size observed in *mlf* or *dnaj-1* mutant is rescued by enforcing LZ expression. This is intriguing since it suggests that decreasing LZ activity causes an expansion of the crystal cell population associated with aberrant differentiation. We thus tested what happens when LZ activity is reduced. Accordingly, we introduced the  $lz^{r1}$  null allele in the  $lz^{GAL4}$  context. This hypomorphic allelic combination caused a decrease in LZ expression (Fig 8B) and resulted in an increase in  $lz>GFP^+$  cell number and size (Fig 8E and 8F). Interestingly,  $lz^{GAL4}/Y$  hemizygote larvae displayed similar phenotypes (Fig 8C, 8E and 8F), indicating that this P{GAL4} insertion in *lz* alters its expression in the crystal cell lineage. As an alternate strategy, we interfered with LZ activity by expressing a fusion protein between LZ partner Brother (*Drosophila* CBF $\beta$  homolog) and the non-muscular myosin heavy chain SMMHC [46]. This chimera mimics the CBF $\beta$ -MYH11 fusion protein generated by the inv(16) translocation in human AML and can sequester RUNX factors in the cytoplasm [1, 47]. Bro-SMMHC expression in  $lz>GFP^+$  cells titrated LZ from the nucleus and also caused an increase in  $lz>GFP^+$  cell number and size (Fig 8D-F). Furthermore, consistent with the analyses of *mlf* and *dnaj-1* mutants, the expression of the Notch signaling pathway reporters NRE-GFP and Klu-Cherry was strongly increased in  $lz^{GAL4}/lz^{R1}$  mutant or upon Bro-SMHCC expression in the  $LZ^+$  blood cell lineage (Fig 8G, H).

In contrast to Notch (Fig 7L), we observed that LZ expression increased with  $lz>GFP^+$  cell size (S9 Fig). This suggested that LZ level rises as crystal cells mature and, in view of the above results, we surmised that this increase might down-regulate Notch signaling by repressing Notch receptor expression. Accordingly, we found that Notch level significantly augmented in  $lz>GFP^+$  cells of hypomorphic  $lz^{GAL4}/Y$  hemizygote larvae whereas it was reduced when LZ was over-expressed (Fig 9 A-E). In addition, the increase in Notch expression observed in  $lz^{GAL4}/Y$  larvae was suppressed by enforcing LZ expression. We hypothesized that Notch might be a transcriptional target of LZ. By analyzing the expression of different GAL4 lines that cover potential Notch regulatory regions [48], we identified two lines that drive expression in circulating  $LZ^+$  blood cells (Fig 9F and S10 Fig). Interestingly, the regulatory elements carried by these two lines (GMR30A01 and GMR30C06) overlapped on a 668bp DNA segment that contains two consensus binding sites for RUNX transcription factors conserved in other *Drosophila* species (S10A Fig), suggesting that Notch might be a direct target gene of LZ. We thus tested the effect of LZ dosage manipulation on the activity of this enhancer-GAL4 line. Strikingly, a hypomorphic lozenge mutation ( $lz^g/Y$ ) [49] or the expression of Bro-SMMHC caused an increase in the expression of this enhancer, whereas the over-expression of LZ resulted in its down-regulation (Fig 9G-K). These findings strongly argue that LZ directly represses Notch expression

All together, these results demonstrate that high levels of LZ are required to prevent the accumulation of over-grown  $lz>GFP^+$  cells as well as over-activation of the Notch pathway and we propose that LZ-mediated repression of Notch transcription is critical for this process.

## Discussion

Members of the RUNX and MLF families have been implicated in the control of blood cell development in mammals and *Drosophila* and deregulation of their expression is associated with human hemopathies including leukemia [1, 9, 15, 50]. Our results establish the first link between MLF, DnaJ-1 and the regulation of RUNX transcription factor *in vivo*. In addition, our data suggest that the stabilization of LZ by the MLF/DnaJ-1 complex is critical to control Notch signaling and thus blood cell growth and survival. These findings pinpoint the specific function of the Hsp40 chaperone DnaJ-1 in hematopoiesis, reveal a potentially

conserved mechanism of regulation of RUNX activity and highlight a new layer of control of Notch signaling at the transcriptional level.

In line with results published as this manuscript was in preparation [20], we found that MLF binds DnaJ-1 and Hsc70-4 and that these two proteins, like MLF, are required for LZ stable expression in Kc cells. In addition, we show that MLF and DnaJ-1 bind to each other via evolutionarily conserved domains and also interact with LZ, suggesting that LZ is a direct target of a chaperone complex formed by MLF, DnaJ-1 and Hsc70-4. Of note, a systematic characterization of Hsp70 chaperone complexes in human cells identified MLF1 and MLF2 as potential partners of DnaJ-1 homologs, DNAJB1, B4 and B6 [51], a finding corroborated by Dyer et al. [20]. Therefore, the MLF/DnaJ-1/Hsc70 complex could play a conserved role in mammals, notably in the regulation of RUNX transcription factors stability. How MLF acts within this chaperone complex remains to be determined. *In vivo*, we demonstrate that *dnaj-1* mutation leads to defects in crystal cell development strikingly similar to those observed in *mlf* mutant larvae and we show that these two genes act together to control LZ<sup>+</sup> cell development by impinging on LZ activity. Our data suggest that in the absence of DnaJ-1, high levels of MLF lead to the accumulation of defective LZ proteins, whereas lower levels of MLF allow its degradation. We thus propose that MLF stabilizes LZ and, together with DnaJ-1, promotes its proper folding/conformation. In human, DnaJB4 stabilizes wild-type E-cadherin but induces the degradation of mutant E-cadherin variants associated with hereditary diffuse gastric cancer [52]. Thus the fate of DnaJ client proteins is controlled at different levels and MLF might be an important regulator in this process.

In this work, we present the first null mutant for a gene of the DnaJB family in metazoans and our results demonstrate that a DnaJ protein is required *in vivo* to control hematopoiesis. There are 16 DnaJB and in total 49 DnaJ encoding genes in mammals and the expansion of this family has likely played an important role in the diversification of their functions [53, 54]. DnaJB9 overexpression was found to increase hematopoietic stem cell repopulation capacity [55] and Hsp70 inhibitors have anti-leukemic activity [56], but the participation of other DnaJ proteins in hematopoiesis or leukemia has not been explored. Actually DnaJ molecular mechanism of action has been fairly well studied but we only have limited insights as to their role *in vivo*. Interestingly though both DnaJ-1 and MLF suppress polyglutamine protein aggregation and cytotoxicity in *Drosophila* models of neurodegenerative diseases [17, 23, 24, 33, 57-61], and this function is conserved in

mammals [24, 25, 62, 63]. It is tempting to speculate that MLF and DnaJB proteins act together in this process as well as in leukemogenesis and thus a better characterization of their mechanism of action may help develop new therapeutic approaches for these diseases.

As shown here, *mlf* or *dnaj-1* mutant larvae harbor more crystal cells than wild type larvae and display a higher fraction of the largest *lz>GFP*<sup>+</sup> cell population, which likely corresponds to the more mature crystal cells [31, 40]. It is thus tempting to speculate that *mlf* or *dnaj-1* loss promotes the survival of fully differentiated crystal cells. Our RNAseq data demonstrate that *mlf* is critical for expression of crystal cell associated genes, but we observed both up-regulation and down-regulation of crystal cell differentiation markers in *mlf* or *dnaj-1* mutant *LZ*<sup>+</sup> cells. In addition, our transcriptome did not reveal a particular bias toward decreased expression for “plasmacyte” markers in *LZ*<sup>+</sup> cells from *mlf* mutant larvae (for instance, *hml*, *peroxidasin*, *viking* or *Cg25C* expression was unaffected, *croquemort* was down-regulated and *NimCI* was up-regulated). Thus, it appears that MLF and DnaJ-1 loss do lead to the abnormal accumulation of mis-differentiated crystal cells.

Our data support a model whereby MLF and DnaJ-1 act together to promote *LZ* expression, which in turn represses Notch transcription and signaling pathway to control crystal cell size and number. Indeed, we observe an over-activation of the Notch pathway in *LZ*<sup>+</sup> blood cells of *mlf* or *dnaj-1* mutant larvae as well as when we interfere with *LZ* activity, and it has been shown that Notch activation increases crystal cell growth and survival [30, 31, 40]. Our results suggest that *LZ* directly represses Notch transcription as we identified a *LZ*-responsive Notch *cis*-element that contains a conserved RUNX binding site. Activation of Notch pathway in circulating *LZ*<sup>+</sup> cells is ligand-independent and mediated through stabilization of the full length Notch protein in endocytic vesicles [30, 64]. Hence, a tight control of Notch expression is of particular importance to keep Notch pathway activation in check and prevent abnormal development of the *LZ*<sup>+</sup> blood cell lineage. By stabilizing *LZ*, MLF and DnaJ-1 thus provide a cell-autonomous mechanism to inhibit Notch signaling. Further experiments will now be required to establish how *LZ* represses Notch transcription. RUNX factors can act as transcriptional repressors by recruiting co-repressors such as members of the Groucho family [65]. Whether MLF and DnaJ-1 directly contribute to *LZ*-induced repression in addition to regulating its stability is an open question. MLF and DnaJ-1 were recently found to bind and regulate a common set of genes in cell culture [20]. They may thus provide a favorable chromatin environment for *LZ* binding or be recruited with *LZ*

and/or favor a conformational change in LZ that allows its interaction with co-repressors. The scarcity of  $lz>GFP^+$  cells precludes a biochemical characterization of LZ, MLF and DnaJ-1 mode of action notably at the chromatin level, but further genetic studies should help decipher their mode of action. While the post-translational control of Notch has been extensively studied, its transcriptional regulation seems largely overlooked [66]. Our findings indicate that this is nonetheless an alternative entry point to control the activity of this pathway. Given the importance of RUNX transcription factor and Notch signaling in hematopoiesis and blood cell malignancy [1, 2], it will be of particular interest to further study whether RUNX factors can regulate Notch expression and signaling during these processes in mammals.

In conclusion, our study shows that MLF and DnaJ-1 act together to regulate RUNX transcription factor activity, which in turn controls Notch signaling during hematopoiesis *in vivo*. We anticipate that the extraordinary genetic toolbox available in *Drosophila* will help shed new light on the mechanism of action of these evolutionarily conserved proteins and will bring valuable insights into the control of protein homeostasis by MLF and DnaJ-1 during normal or pathological situations.

## Materials and Methods

### Fly strains

The following *Drosophila melanogaster* lines were used:  $mlf^{dCl}$ ,  $UAS-mlf$  [17],  $UAS-ds-mlf$  (National Institute of Genetics, NIG),  $UAS-lz$ ,  $lz^{GAL4}$ ,  $UAS-mCD8-GFP$ ,  $lz^g$ ,  $lz^{r1}$ ,  $P\{EPgy2\}DnaJ-1EY04359$ ,  $UAS-dnaj-1$ ,  $Def(3L)BSC884$ ,  $vas-Cas9$ ,  $UAS-GFPnls$ ,  $NRE-GFP$ ,  $GMR30C06$ ,  $GMR30A01$  (Bloomington *Drosophila* Stock Center),  $Bc-GFP$  [67],  $Klu-mCherry$  [31],  $UAS-Bro-SMMHC$  [46]. To generate  $dnaj-1$  deficient flies, we designed two guide RNA targeting  $dnaj-1$  locus (S4 Fig) and the corresponding DNA oligonucleotides (g2: GTCGACCACAACGCGCCGGATCAA; g3: GTCGCATCACAGTCACGCTTTCCT) were cloned in pCFD3 (Addgene, [68]).  $vas-cas9$  females were crossed to  $P\{EPgy2\}DnaJ-1EY04359$  males and the resulting embryos were injected using standard procedures with both pCFD3-g2 and pCFD3-g3 plasmids (500ng/ $\mu$ l). Deletion of the  $P\{EPgy2\}EY04359$  transposon, as revealed by loss of the  $w^+$  marker, was screened for at the F2 generation, and deletion of  $dnaj-1$  locus was assessed by PCR and sequencing. All crosses were conducted on standard food medium as described in [69].

### **Immunostainings and in situ hybridizations**

For each sample, four third instar larvae were bled (or  $5.0 \times 10^3$  Kc167 cells were dispensed) in 1ml of PBS in 24-well-plate containing a glass coverslip. Unless mentioned otherwise, only female larvae were used. The hemocytes were centrifuged for 2 min at 900g, fixed for 20 min with 4% paraformaldehyde in PBS and washed twice in PBS. For immunostainings: cells were permeabilized in PBS-0.3% Triton (PBST) and blocked in PBST-1% Bovine Serum Albumin (BSA). The cells were incubated with primary antibodies at 4°C over-night in PBST-BSA, washed in PBST, incubated for 2h at room temperature with corresponding Alexa Fluor-labeled secondary antibodies (Molecular Probes), washed in PBST and mounted in Vectashield medium (Eurobio-Vector) following incubation with Topro3 (ThermoFisher). The following antibodies were used: anti-LZ, anti-Notch intracellular domain, anti-Notch extracellular domain (Developmental Studies Hybridoma Bank, DSHB), anti-MLF [19], anti-PPO1 [70], anti-GFP (Fisher Scientific), anti-HA (Sigma).

For in situ hybridizations: after fixation, the cells were washed and permeabilized in PBS-0.1% Tween20 (PBSTw), pre-incubated for 1h at 65°C in HB buffer (50% formamide, 2xSSC, 1 mg/ml Torula RNA, 0.05 mg/ml Heparin, 2% Roche blocking reagent, 0.1% CHAPS, 5 mM EDTA, 0.1% Tween 20) and 535 incubated over-night with anti-sense DIG-labeled RNA probes (against CG6733, CG7860, Jafrac or Oscillin) diluted in HB. The samples were washed in HB for 1h at 65°C, in 50% HB- 50% PBSTw for 30 min at 65°C and three times in PBSTw for 20 min at room temperature. Then the cells were incubated for 30 min in PBSTw- 1% BSA before being incubated with anti-DIG antibody conjugated to alkaline phosphatase (Roche, 1/2000 in PBSTw) for 3h. After 4 washes in PBSTw, in situ hybridization signals were revealed with FastRed (Roche). The cells were then processed for immunostaining against GFP as described above, incubated in Topro3, washed in PBS and mounted in Vectashield medium for analysis.

Experiments were performed using at least biological triplicates. Samples were imaged with laser scanning confocal microscopes (Leica) and images were analyzed with ImageJ. Cell size and protein expression levels were measured on maximal intensity projections of Z-sections through the whole cell on a minimum of 25 cells per genotype. Crystal cell

counts were performed as described in [25]. Most statistical tests and graphs were performed using Prism v5 (GraphPad Software).

## **Plasmids**

The following previously described plasmids were used: pAc-LZ-V5, 4xPPO2-Firefly luciferase (originally named 4xPO45-Fluc, [37]), pET-3c-LZ [71], pAc-MLF [17]. We generated the following *Drosophila* expression plasmids for C-terminally tagged or N-terminally tagged proteins using standard cloning techniques: pAc-LZ-EGFP, pAc-MLF-EGFP, pMT-MLF-V5-His, pAc-DnaJ-1-EGFP, pAc-Hsc70-4-EGFP, pAc-3xHA-DnaJ-1(2-334), pAc-3xHA-DnaJ-1(P32S), pAc-3xHA-DnaJ-1(58-334), pAc-3xHA-DnaJ-1(2-156), pAc-3xHA-DnaJ-1(2-191), pAc-3xHA-DnaJ-1(2-269), pAc-3xHA-DnaJ-1(157-334), pAc-3xHA-MLF(2-309), pAc-3xHA-MLF(2-147), pAc-3xHA-MLF(2-202), pAc-3xHA-MLF(202-309), pAc-3xHA-MLF(148-309), pAc-3xHA-MLF(96-309), pAc-3xHA-MLF(96-202). DnaJ-1 and MLF cDNA were also cloned into pBlueScript II to generate pBS-DnaJ-1 and pBS-MLF and in pGEX-2T to generate pGEX-DnaJ-1 and pGEX-MLF. All constructs were verified by sequencing.

## **Cell culture, dsRNA treatments and transfections**

*Drosophila* Kc167 cells were grown at 25°C in Schneider medium (Invitrogen) supplemented with 5% fetal bovine serum (FBS) and 50 µg/ml of penicillin/streptomycin (Invitrogen). For RNAi experiments, double stranded RNA duplexes (dsRNA) corresponding to 400-600bp exonic regions were produced using T7 promoter containing primers and MEGAscript T7 transcription kit (Ambion). After an annealing step, dsRNA probes were purified using the RNeasy clean-up protocol (Qiagen). Independent dsRNA targeting different regions of *dnaj-1* and *hsc70-4* were produced. Cells were seeded at  $2 \times 10^6$ /ml on dsRNA (16 µg/well for 6-well-plate, 8 µg for 12-well plate and 1 µg for 96-well-plate) and incubated in Schneider medium without FBS for 40min before being transferred to 5% FBS containing medium. 24h later, cells were transfected with the plasmids of interest using Effectene (Qiagen) and they were collected 72h later for subsequent analyses.

## **Luciferase reporter assays**

For luciferase assays, 50 ng of 4xPPO2-Firefly luciferase reporter plasmid [37] were co-transfected with 20 ng of pAc-*Renilla* luciferase plasmid, 10 ng of pAc-LZ-V5 and/or 10 ng of pAc expression plasmid for the protein of interest in 96-well plate. Firefly and *Renilla* luciferases activities were measured 72h after transfection using Promega Dual luciferase reporter assay. Three biological replicates were performed for each transfection assay.

### **Real-time quantitative PCR**

For RT-qPCR, RNA was from Kc167 cells using RNeasy kit (Qiagen) with an additional on-column DNase treatment step. 1 µg of total RNA was used for reverse transcription using Superscript II and random primers (Invitrogen). 10 µl of a 1/300 dilution of cDNA was used as template for real time PCR using HOT Pol Evagreen qPCR mix (Bio-rad) to analyse *dnaj-1*, *mlf*, *lz*, *PPO2*, *renilla luciferase* and *rp49* expression. All experiments were performed using biological triplicates or quadruplicates.

### ***In vitro* pull down assays**

pET-3c-LZ, pBS-MLF and pBS-DnaJ-1 plasmids were used as template to produce 35S-methionine-labeled proteins *in vitro* using Rabbit Reticulocyte Lysate coupled transcription-translation system (Promega). pGEX-2T, pGEX-MLF and pGEX-DnaJ-1 were used to produce GST, GST-MLF and GST-DnaJ-1 in *Escherichia coli* (BL21). Equivalent amounts of GST purified proteins immobilized on Glutathione-Sepharose beads were used to pull down LZ, MLF or DnaJ-1. Proteins were incubated for 2h at 4°C in buffer A (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.1 mg/ml BSA, 1 mM DTT, 0.05% NP40). After extensive washing in buffer B (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% NP40), bound proteins were eluted in SDS-loading buffer, separated by SDS-PAGE and visualized by autoradiography.

### **Protein extraction, immunoprecipitations and western blots**

Kc167 cells were collected, washed in PBS and incubated for 30 min on IP buffer (150 mM NaCl, 0.5% NP40, 50 mM Tris-HCl, pH8.0, 1mM EGTA) supplemented with protease inhibitor cocktail (Roche). The extracts were cleared by centrifugation at 13 000g for 15 min at 4°C and subjected to SDS-PAGE (50 µg of proteins par lane) or immunoprecipitation (1 mg per point). For immunoprecipitation, proteins were pre-absorbed with 100 µl of



Sepharose beads slurry for 1h at 4°C before being incubated with 20 µl of anti-GFP (Chromotek), anti-V5 (Sigma-Aldrich) or anti-HA (Covance) antibody coupled to Sepharose beads for 4h at 4°C. The beads were spun down and washed in IP buffer and immunoprecipitated proteins were processed for SDS-PAGE and Western Blot analyses. Western blots were performed using standard techniques and the blots were developed by photoluminescence procedure using Lumi-Light<sup>PLUS</sup> Western Blotting Substrate (Roche) and Amersham Hyperfilm<sup>TM</sup> ECL (GE Healthcare) or Chemidoc<sup>TM</sup> Touch Imaging System (BioRad). The following antibodies were used for western blots: anti-V5 (Invitrogen), anti-HA (BioLegend), anti-GFP, anti-tubulin (Sigma-Aldrich), anti-*Renilla* luciferase (MBL) and anti-MLF [19].

### **Affinity purification and mass spectrometry analysis**

Stable Kc167 cells carrying an inducible expression vector for MLF were obtained by co-transfecting pMT-MLF-V5-His and pCoBlast (Thermo Fisher Scientific) expression plasmids and selecting individual clones with 25µg/ml blasticidin. For affinity purification, MLF-inducible or parental Kc167 cells were seeded at 10<sup>6</sup>/ml and cultivated for 24h in the presence of 50 mM CuSO<sub>4</sub> to induce MLF expression. 20 mg of proteins extracted in IP buffer were then incubated on 200 µl of anti-V5 coupled Sepharose beads (Sigma-Aldrich) or 400 µl of anti-V5 coupled magnetic beads (MBL). After several washes in IP buffer, affinity purified proteins were eluted in Laemmli buffer, reduced in 30 mM DTT and alkylated with 90 mM Iodoacetamide before being loaded on 12% SDS-PAGE. The single band of proteins was cut and digested overnight at 37°C with 1 µg of Trypsin (Promega) in 50 mM NH<sub>4</sub>CO<sub>3</sub>. Digested peptides were extracted from the gel by incubating 15 min at 37°C in 50 mM NH<sub>4</sub>CO<sub>3</sub> and twice for 15 min at 37°C in 5% formic acid/acetonitrile (1:1). The dried peptide extracts were dissolved in 17 µl of 2% acetonitrile, 0.05% trifluoroacetic acid and the peptide mixtures were analysed by nanoLC-MS/MS using an Ultimate3000-RS system (Dionex) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). 5µl of each peptide extract were loaded on a 300 µm ID x 5 mm PepMap C18 precolumn (LC Packings, Dionex,) at 20 µl/min in 5% acetonitrile, 0.05% trifluoroacetic acid. After 5 minutes desalting, peptides were online separated on a 75µm IDx50 cm C18 Reprosil C18 column. The flow rate was set at 300 nl/min. Peptides were eluted using a 0 to 50% linear gradient of solvent B (solvent A: 0.2% formic acid in 5% acetonitrile, solvent B: 0.2% formic acid in 80% acetonitrile) for 80 min at 300 nl/min. The LTQ Orbitrap was

operated in data-dependent acquisition mode with the XCalibur software (version 2.0 SR2, Thermo Fisher Scientific), on the 350-1800 m/z mass range with the resolution set to a value of 60 000. The twenty most intense ions per survey scan were selected for CID-MS/MS fragmentation and the resulting fragments were analyzed in the linear ion trap (parallel mode). A 60 s dynamic exclusion window was used to prevent repetitive selection of the same peptide. The Mascot Daemon software (version 2.2.0, Matrix Science, London, UK) was used for protein identification against a non-redundant SwissProt database. Mascot results were parsed with Mascot File Parsing and Quantification (MFPaQ) version 4.0 [72]. Quantification of proteins was performed using the label-free module of the MFPaQ software, where a protein abundance index based on the average of peak area values for the three most intense tryptic peptides of the protein was calculated [73]. Triplicate injections were performed.

### **RNA-seq experiments**

RNAseq experiments were performed using independent biological triplicates. For each sample, around 150 third instar larvae of control (*lz-GAL4, UAS-mCD8GFP/+*) or *mlf* mutant (*lz-GAL4, UAS-mCD8GFP/+, mlf<sup>ΔC1</sup>/mlf<sup>ΔC1</sup>*) genotypes were bled in ice-cold PBS. The hemocytes were centrifuged through a 40 μm mesh at 1000 rpm for 1 min and *lz>GFP<sup>+</sup>* cells were collected by FACS (FacsAria II) under a pressure of 20 psi. A fraction of the collected cells were used to control GFP<sup>+</sup> cell purification specificity by examination under an epifluorescent microscope after fixation and mounting in Vectashield medium with DAPI. RNA was extracted from sorted cells using Arcturus PicoPure RNA kit (Applied Biosystems). RNA samples were run on Agilent Bioanalyzer to assess RNA integrity and concentration. The NuGEN Ovation RNA-Seq system with Ribo-SPIA technology was used to prepare the cDNA according to the manufacturer instruction. Library preparation was performed using the Illumina TruSeq RNA-Seq library preparation kit. The resulting libraries were sequenced using a 1x50-bp on Illumina HiSeq 2500. Initial sequence data QC was done using FASTQC. Reads were filtered and trimmed to remove adapter-derived or low quality bases using Trimmomatic and checked again with FASTQC. Illumina reads were aligned to *Drosophila* reference genome (BDGP R5/dm3) with TopHat and Bowtie2. Read counts 679 were generated for each annotated gene using HTSeq-Count. RPKM (Reads Per Kilobase of exon per Megabase of library size) values were calculated using Cufflinks. Read normalization, variance estimation and pair-wise differential expression

analysis with multiple testing correction was conducted using the R Bioconductor DESeq2 package. Heatmaps and hierarchical clustering were generated with R Bioconductor. The RNAseq data were deposited on GEO under the accession number GSE93823.

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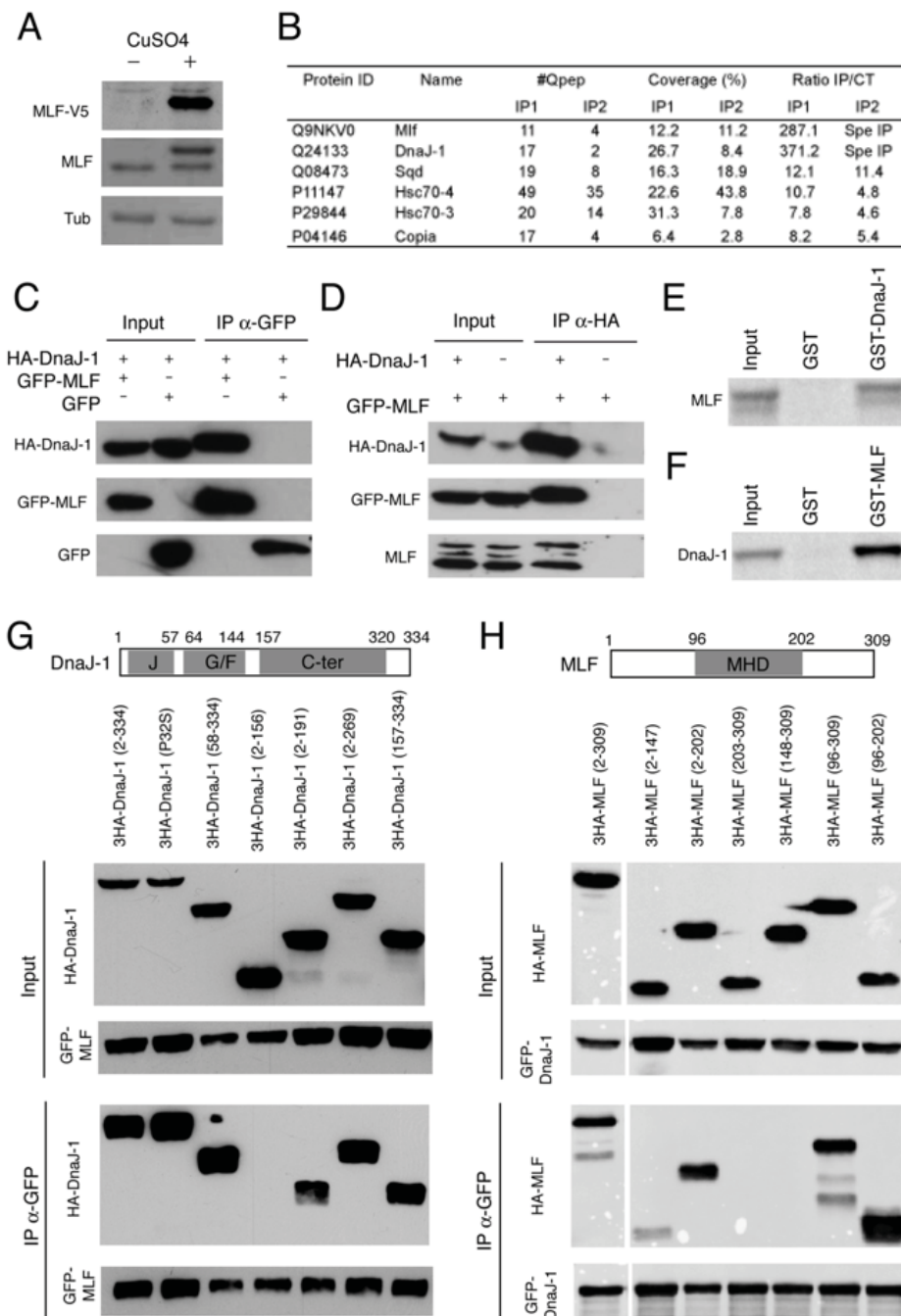
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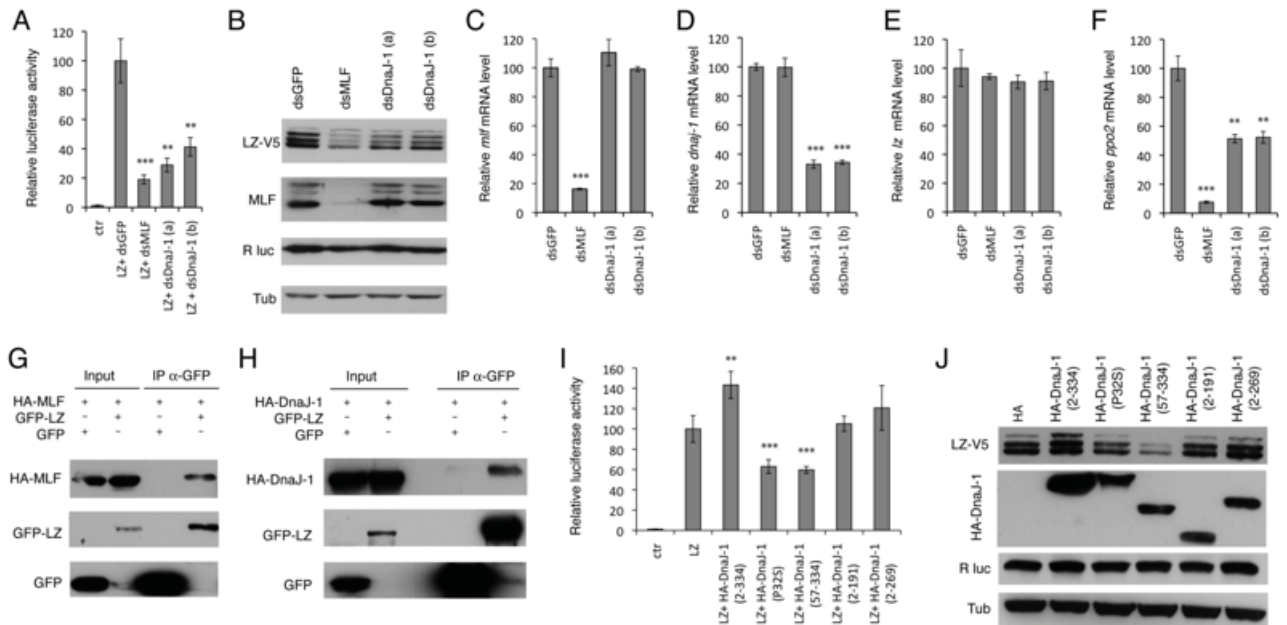
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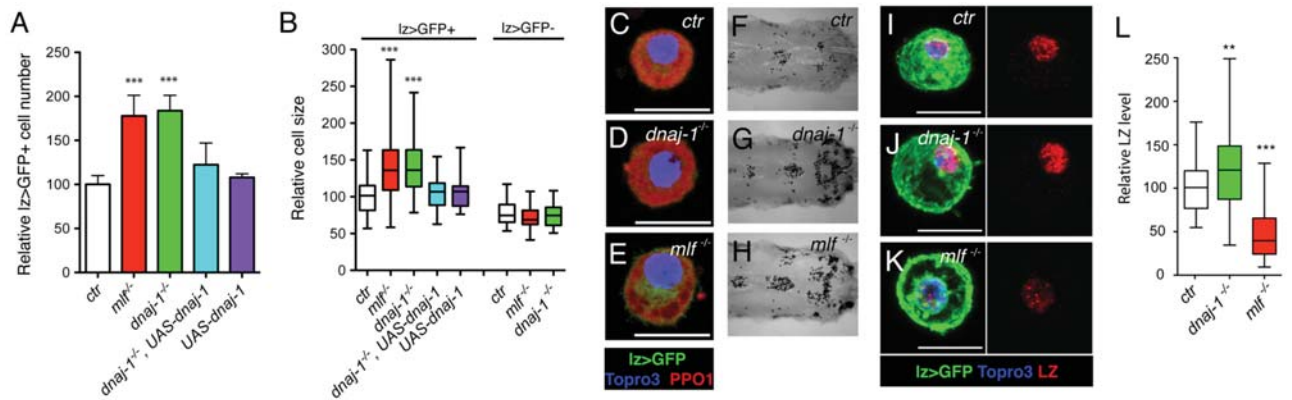
## FIG 1. MLF interacts with the co-chaperone DnaJ-1.

(A) Western blots showing MLF and MLF-V5 expression in Kc167 cells stably transfected with the copper-inducible pMT-MLF-V5 expression vector and treated or not with 50  $\mu$ m CuSO<sub>4</sub> for 24h. Tubulin (Tub) was used as an internal loading control. (B) Proteins identified by mass spectrometry from CuSO<sub>4</sub>-induced Kc167-pMT-MLF-V5 cells using anti-V5 antibody coupled to Sepharose (IP1) or magnetic (IP2) beads for purification. The number of quantified peptides (#Qpep), sequence coverage and fold enrichment in comparison to control (parental Kc167 cells) are indicated for each experiment. Spe IP: not detected in control condition. (C, D) Western blots showing the results of immunoprecipitation experiments against GFP (C) or HA (D) performed in Kc167 cells transfected with expression vectors for the indicated proteins. (E, F) Autoradiograms showing the results of pull down assays between in vitro translated <sup>35</sup>S methionine labeled MLF (E) or DnaJ-1 (F) and the indicated GST fusion proteins produced in *E. coli*. (G, H) Schematic representation of DnaJ-1 (G) and MLF protein domains (H) and western blots showing the results of immunoprecipitation experiments against GFP performed in Kc167 cells transfected with expression vectors for GFP-MLF and various HA-DnaJ-1 mutants (G) or GFP-DnaJ-1 and different HA-MLF mutants (H). Conserved domains are highlighted in grey. J: J domain. G/F: glycine/phenylalanine-rich region. C-ter: C-terminal domain. MHD: MLF homology domain.



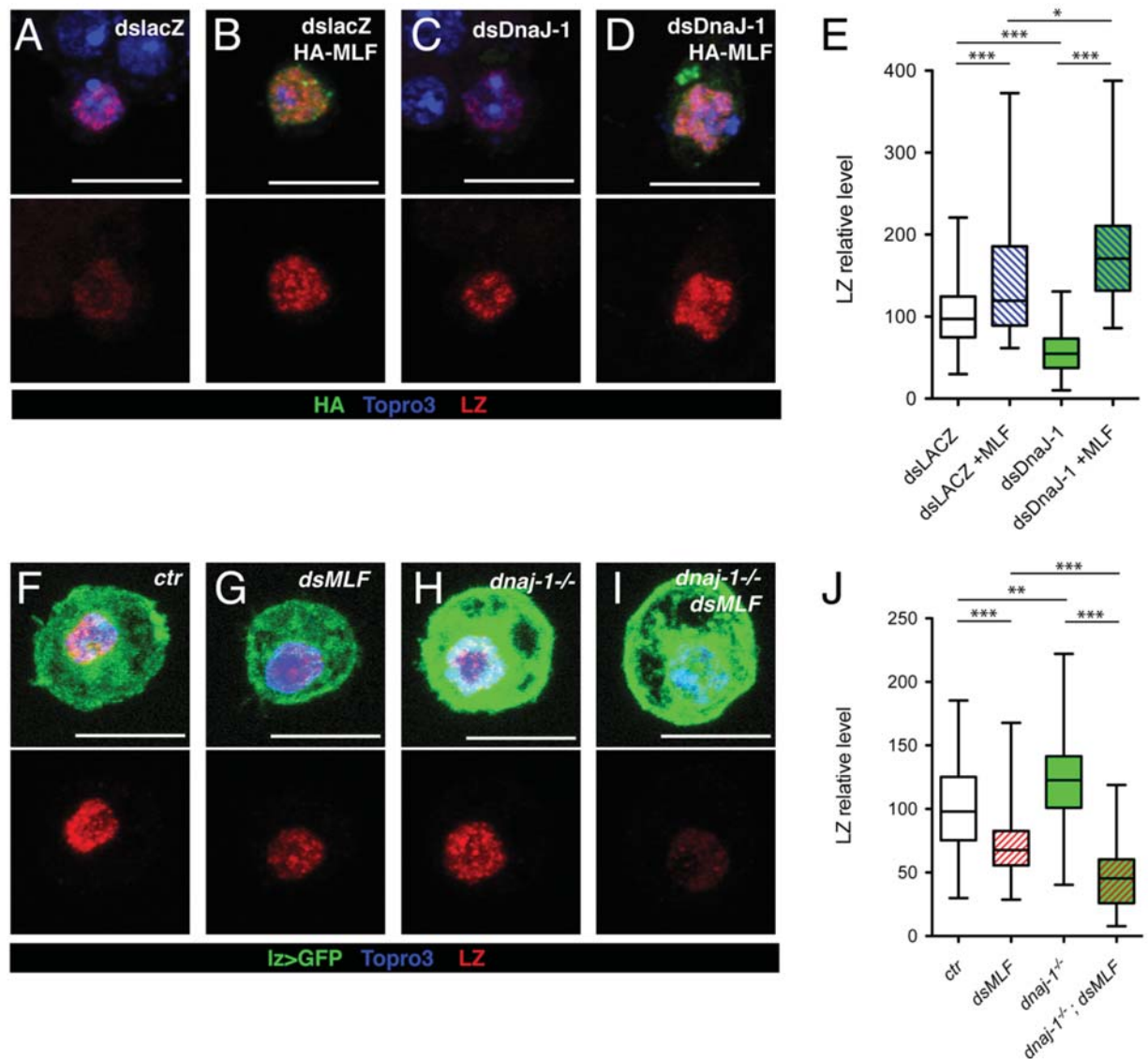
**FIG 2. MLF and DnaJ-1 bind LZ and control its stability and activity.**

(A) Luciferase assays in Kc167 cells treated with the indicated dsRNA and transfected with 4xPPO2-Fluc reported plasmid in the presence or not (ctr) of pAc-LZ-V5 expression plasmid. pAc-Rluc was used as an internal normalization control. (B) Western blots showing LZ-V5, MLF, *Renilla* luciferase (R luc) and Tubulin (Tub) expression in Kc167 cells treated with the indicated dsRNA and cotransfected with pAc-LZ-V5 and pAc-Rluc expression vectors. (C-F) Results of RT-qPCR assays showing the relative expression of *mlf*, *dnaj-1*, *lz* and *ppo2* transcripts in Kc167 cells transfected with pAc-LZ-V5 and pAc-Rluc and treated with the indicated dsRNA. (G, H) Western blots showing the results of immunoprecipitation experiments against GFP performed in Kc167 cells transfected with expression vectors for HA-MLF (G) or HA-DnaJ-1 (H) and GFP or GFP-LZ as indicated in the upper part of the panels. (I, J) Luciferase assays (I) and western blots (J) performed on Kc167 cells transfected with 4xPPO2-Fluc reported plasmid and pAc-based expression plasmids for LZ and for different DnaJ-1 variants as indicated. pAc-Rluc and Tubulin were used as internal controls. (A-F): dsDnaJ-1 (a) and (b) correspond to two distinct dsRNA targeting *dnaj-1*. (A, C-F and I) For luciferase assays and RT-qPCR, means and standard deviations of results from biological triplicates are shown. \*\*\*: p-value<0.001, \*\*: p-value<0.01 (Students t-tests) as compared to LZ with dsGFP condition.



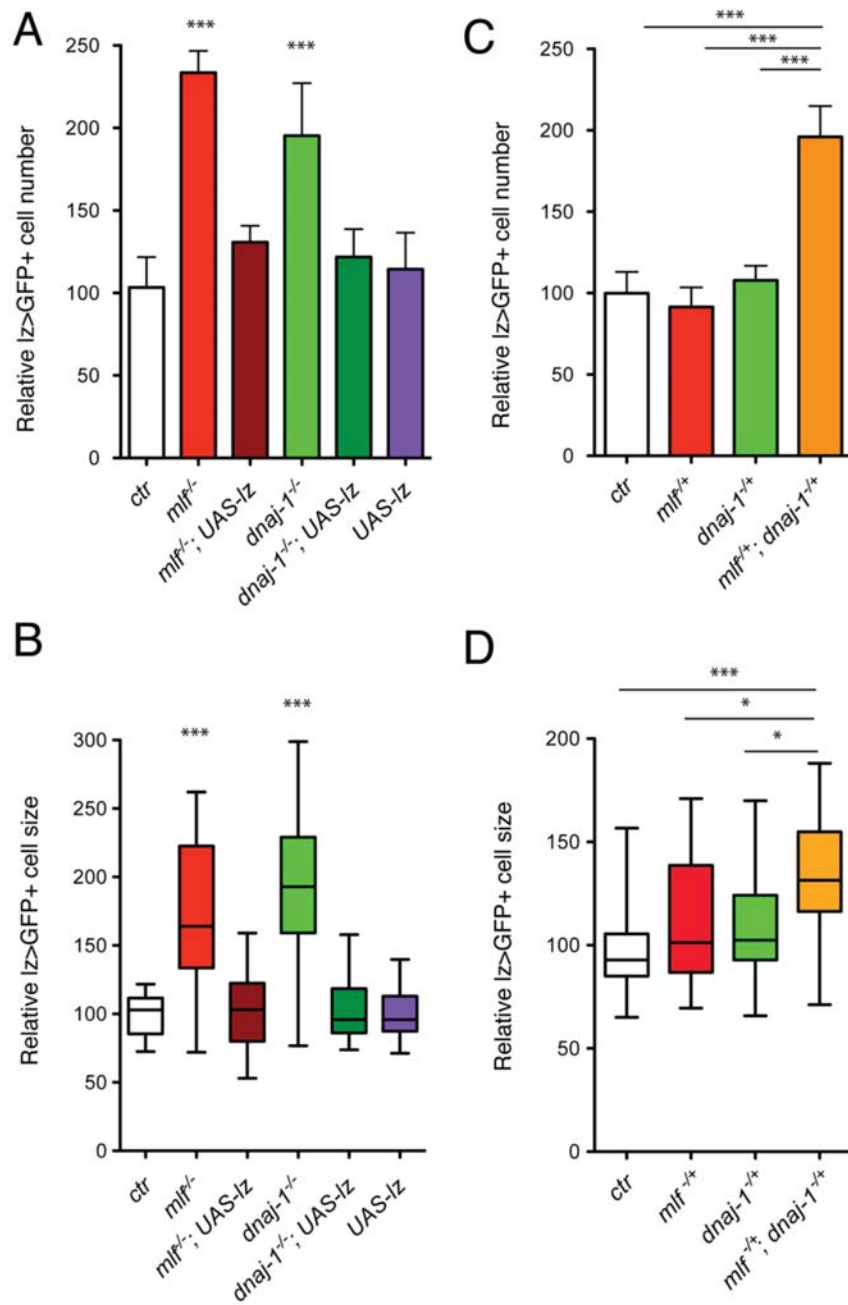
**FIG 3. *dnaj-1* controls crystal cell development.**

(A, B) Quantification of circulating *lz>GFP*<sup>+</sup> cell number (A) and *lz>GFP*<sup>+</sup> or *lz>GFP*<sup>-</sup> cell size (B) in *lz-GAL4*, *UAS-mCD8GFP/+* third instar larvae of the indicated genotypes. (C-E) Fluorescent immunostainings against the crystal cell differentiation marker PPO1 in third instar *lz>GFP*<sup>+</sup> hemocytes. (F-H) Bright field images of the posterior segments of third instar larvae heat-treated at 65°C for 10 min to induce crystal cell melanization. (I-K) Fluorescent immunostainings against LZ in *lz>GFP*<sup>+</sup> blood cells of third instar larvae from the indicated genotypes. LZ expression alone is shown on the right panels. (L) Corresponding quantification of LZ expression level. (A, B, L) \*\*: p-value<0.01 and \*\*\*: p-value<0.001 compared to control. (C-E, I-K): nuclei were stained with Topro3. Scale bar: 10 μm.



**FIG 4. High levels of MLF prevent LZ degradation in the absence of DnaJ-1.**

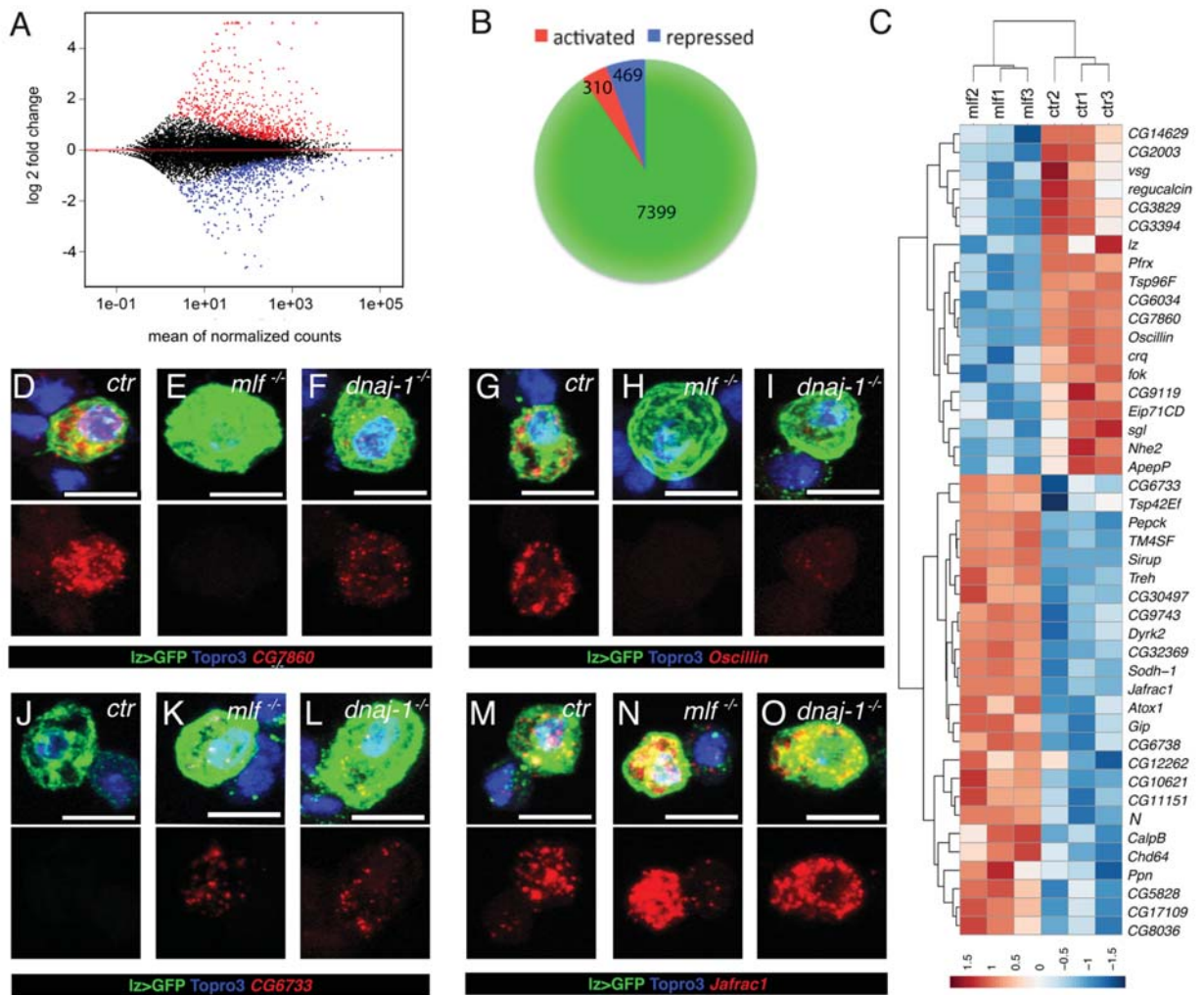
(A-D) Immunostainings against LZ (red) and HA-MLF (green) in Kc167 cells treated with the indicated dsRNA and transfected with pAc-LZ-V5 alone (A, C) or in combination with pAc-3HA-MLF (B, D). (E) Quantification of LZ expression level in Kc167 cells transfected with pAc-LZ-V5 alone or in combination with pAc-3HA-MLF and treated with the indicated dsRNA. (F-I) Immunostainings against LZ in circulating blood cells from *lz>GAL4, UAS-mCD8GFP/+* control (F), *UAS-dsMLF* (G), *dnaj-1<sup>-/-</sup>* (H) and *UAS-dsMLF; dnaj-1<sup>-/-</sup>* (I) third instar larvae. (J) Quantification of LZ expression level in *lz>GFP<sup>+</sup>* circulating blood cell from third instar larvae of the indicated genotypes. (A-D, F-I) Nuclei were stained with Topro3. LZ expression only is shown in the lower panels. Scale bar: 10 μm. (E, J) \*: p-value<0.05, \*\*: p-value<0.01, \*\*\*: p-value<0.001.



**FIG 5. DnaJ-1 and MLF interfere with LZ activity and act together to control LZ<sup>+</sup> blood cell development**

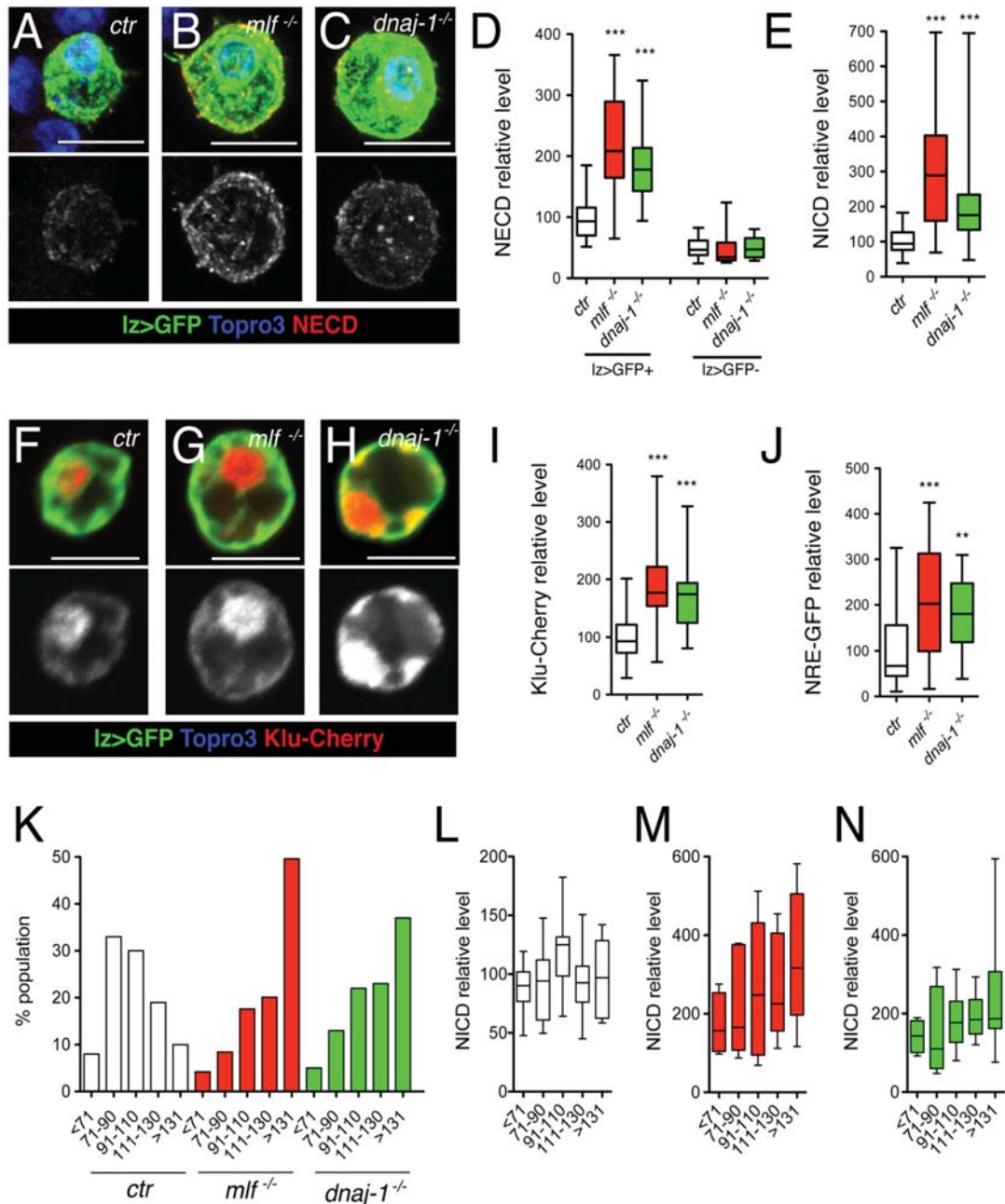
Relative *lz>GFP*<sup>+</sup> blood cell number (A, C) and size (B, D) in *lz-GAL4*, *UAS-mCD8GFP/+* third instar larvae of the indicated genotypes. \*: p-value<0.05, \*\*\*: p-value<0.001.





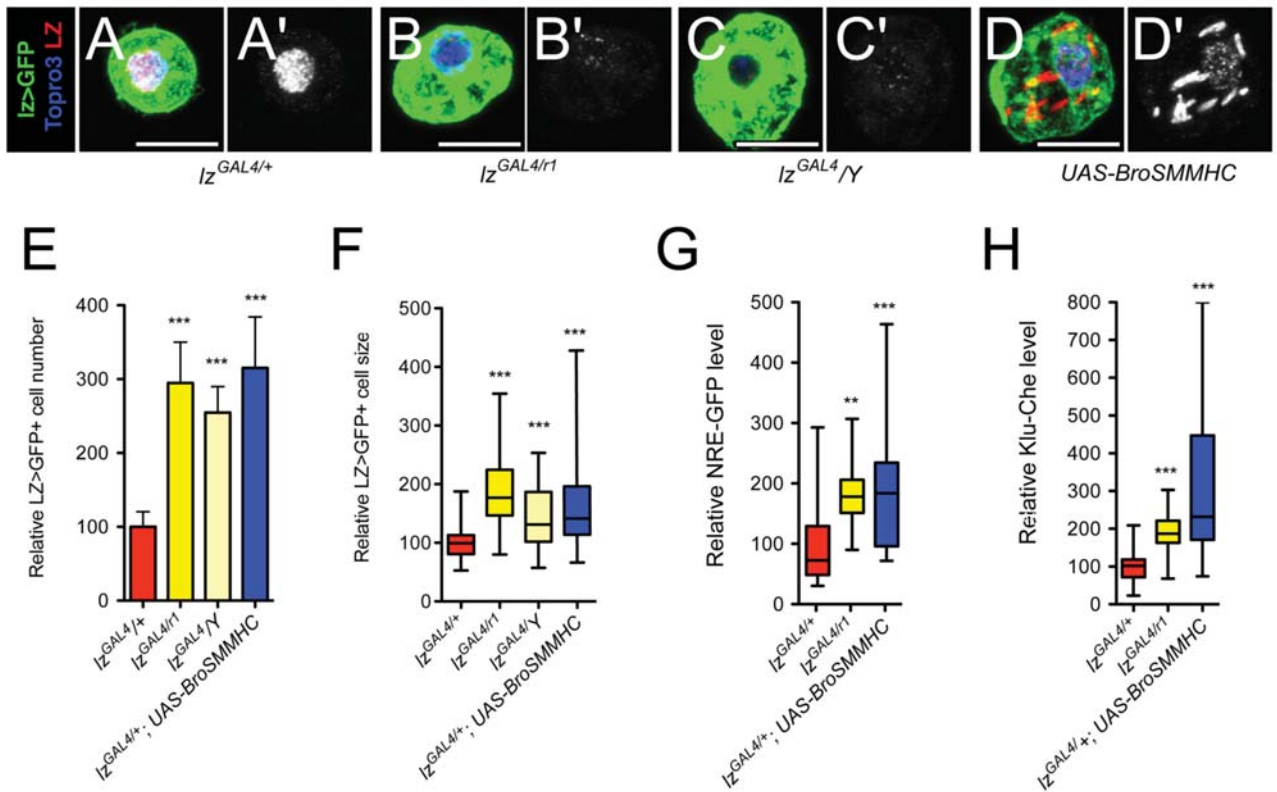
**FIG 6. MLF and DnaJ-1 control crystal cell differentiation.**

(A) MA-plot of DESeq2 results for RNAseq data comparison between control and *mlf*<sup>-/-</sup>, *lz>GFP*<sup>+</sup> blood cells sorted by FACS from third instar larvae. Genes that are significantly (adjusted p-value < 0.01) up-regulated or down-regulated in *mlf* mutant are highlighted in red or blue, respectively. Red triangles: genes with log<sub>2</sub> fold change > 5. (B) Pie chart showing the number of expressed genes in *lz>GFP*<sup>+</sup> cells and the number of up-regulated (red) or down-regulated (blue) genes in *mlf* mutant. (C) Heat map of differentially expressed (p-value < 0.01) “crystal cell”-associated genes between control and *mlf* mutant *lz>GFP*<sup>+</sup> cells. Differential gene expression as per comparison to the mean of 6 samples is displayed as log<sub>2</sub> scale. Hierarchical clustering was performed using R-Bioconductor. (D-O) Immunostainings against GFP and *in situ* hybridization against *CG7860* (D-F), *Oscillin* (G-I), *CG6733* (J-L) and *Jafrac1* (M-O) in blood cells from *lz-GAL4*, *UAS-mCD8GFP/+* control (D, G, J, M), *mlf*<sup>-/-</sup> (E, H, K, N) or *dnaj-1*<sup>-/-</sup> (F, I, L, O) third instar larvae. RNA expression only is shown in the lower panels. Nuclei were stained with Topro3. Scale bar: 10 μm.



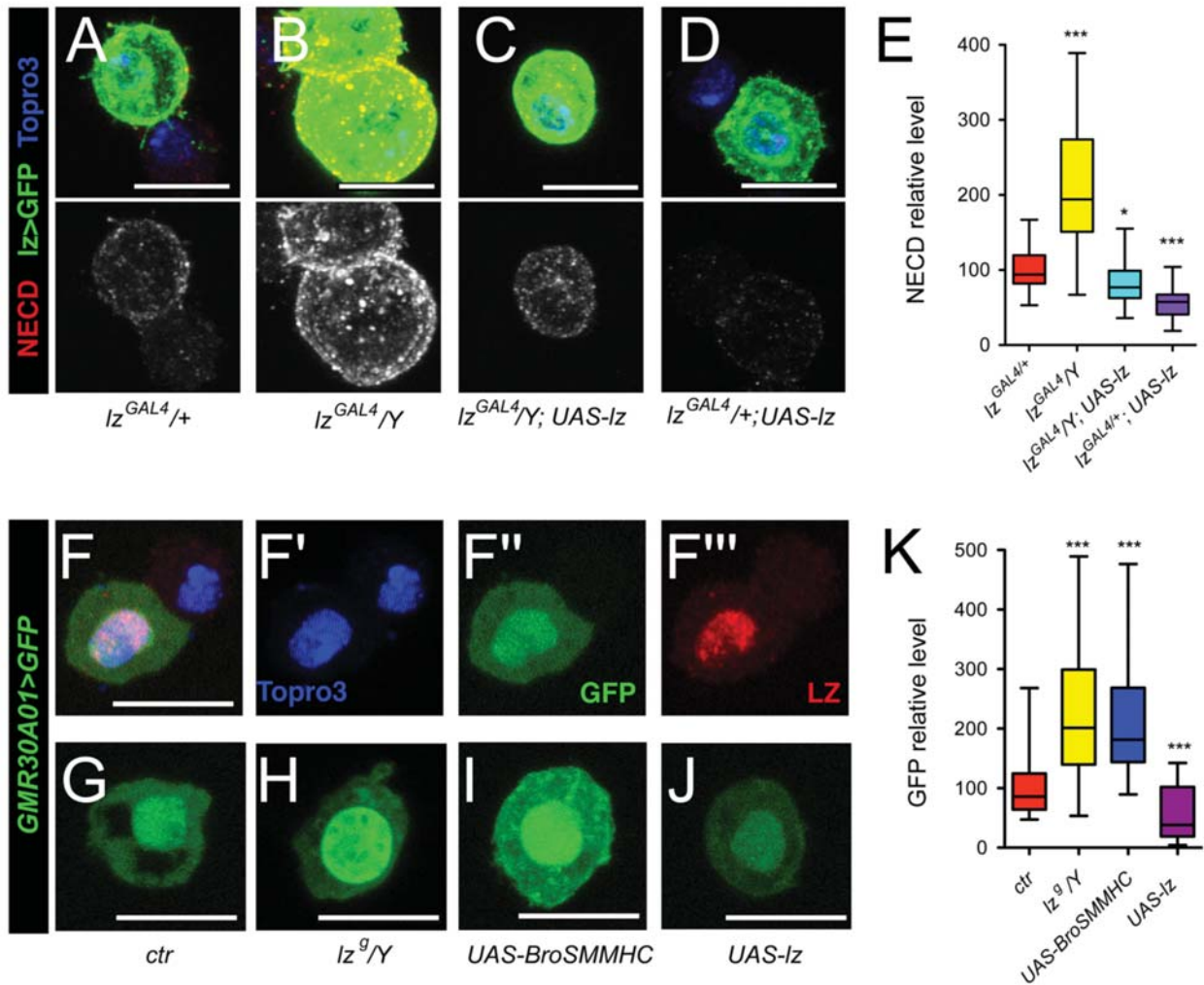
**FIG 7. MLF and DnaJ-1 are required to prevent Notch overexpression and overactivation of Notch signaling in the crystal cell lineage.**

(A-C) Immunostainings against Notch (NECD: Notch extracellular domain) in blood cells from *lz>GAL4, UAS-mCD8GFP/+* control (A) *mlf<sup>-/-</sup>* (B) and *dnaj-1<sup>-/-</sup>* (C) larvae. The expression of Notch protein only is shown in the lower panels. Nuclei were stained with Topro3. (D) Quantification of NECD immunostainings in *lz>GFP+* and *lz>GFP-* blood cells from control, *mlf<sup>-/-</sup>* and *dnaj-1<sup>-/-</sup>* larvae. (E) Quantification of NICD (Notch intracellular domain) immunostainings in *lz>GFP+* blood cells from control, *mlf<sup>-/-</sup>* and *dnaj-1<sup>-/-</sup>* larvae. (F-H) Expression of the Notch pathway reporter Klu-Cherry in *lz>GFP+* blood cells from control, *mlf<sup>-/-</sup>* or *dnaj-1<sup>-/-</sup>* larvae. Klu-Cherry expression only is shown in the lower panels. (I) Corresponding quantification of Klu-Cherry expression levels. (J) Quantification of the expression level of the Notch pathway reporter NRE-GFP in PPO1-expressing cells from control, *mlf<sup>-/-</sup>* or *dnaj-1<sup>-/-</sup>* larvae. (A-C, F-H) Scale bar: 10µm. (K) Quantification of the proportion of *lz>GFP+* cells according to their size in control, *mlf<sup>-/-</sup>* or *dnaj-1<sup>-/-</sup>* larvae. Cells were grouped into 5 categories as compared to the mean size of *lz>GFP+* cells in wild type condition. (L-N) Quantification of NICD expression level (relative to control) in each of the five *lz>GFP+* cell size categories in control (L), *mlf<sup>-/-</sup>* (M) and *dnaj-1<sup>-/-</sup>* (N) larvae.



**FIG 8. High levels of LZ prevent accumulation of  $lz>GFP^+$  cells and overactivation of Notch signaling.**

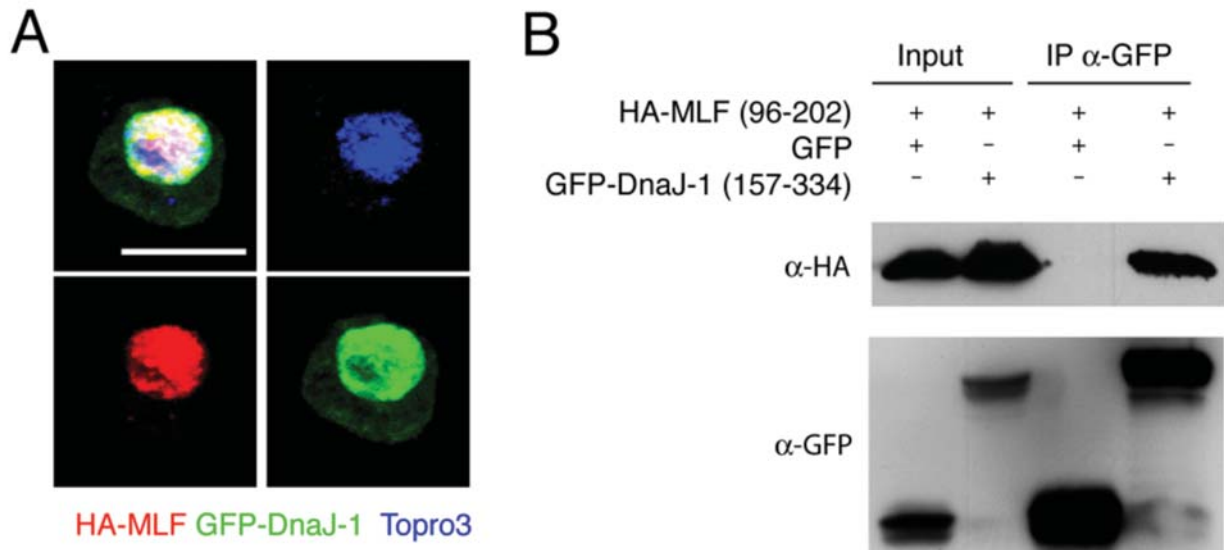
(A-D) Fluorescent immunostainings against LZ in circulating blood cells from *lz-GAL4, UAS-mCD8GFP/+* (A, control), *lz-GAL4, UAS-mCD8GFP/lz<sup>r1</sup>* (B), *lz-GAL4, UAS-mCD8GFP/Y* (C) and *lz-GAL4, UAS-mCD8GFP/+, UAS-BroSMMHC* (D) third instar larvae. Nuclei were stained with Topro3. Scale bar: 10 $\mu$ m. (A'-D'): LZ expression only is shown. (E-H) Relative quantification of  $lz>GFP^+$  cell number (E) and size (F) as well as NRE-GFP (G) and Klu-Cherry (H) expression levels in third instar larvae of the indicated genotypes. \*\*: p-value<0.01, \*\*\* p-value<0.001.



**Fig 9. LZ represses Notch expression.**

(A-D) Immunostainings against NECD (Notch extracellular domain) in blood cells from *lz-GAL4*, *UAS-mCD8GFP/+* (A), *lz-GAL4*, *UAS-mCD8GFP/Y* (B), *lz-GAL4*, *UAS-mCD8GFP/Y; UAS-lz* (C) and *lz-GAL4*, *UAS-mCD8GFP/+; UAS-lz* (D) third instar larvae. The expression of Notch protein only is shown in the lower panels. Nuclei were stained with Topro3. (E) Corresponding quantification of NECD level in *lz>GFP*<sup>+</sup> blood cells. (F-F''') Immunostaining against LZ in circulating blood cells from Notch *GMR30A01-GAL4*, *UAS-GFPnls* third instar larvae. Nuclei were stained with Topro3. (F'-F'''): single channel images. (G-J) Notch *GMR30A01-GAL4*-driven expression of GFP in circulating blood cells from larvae of the indicated genotypes. (K) Corresponding quantification of GFP expression level. (A-D, F-J) Scale bar: 10µm. (E, K) \*: p-value<0.05, \*\*\* p-value<0.001.

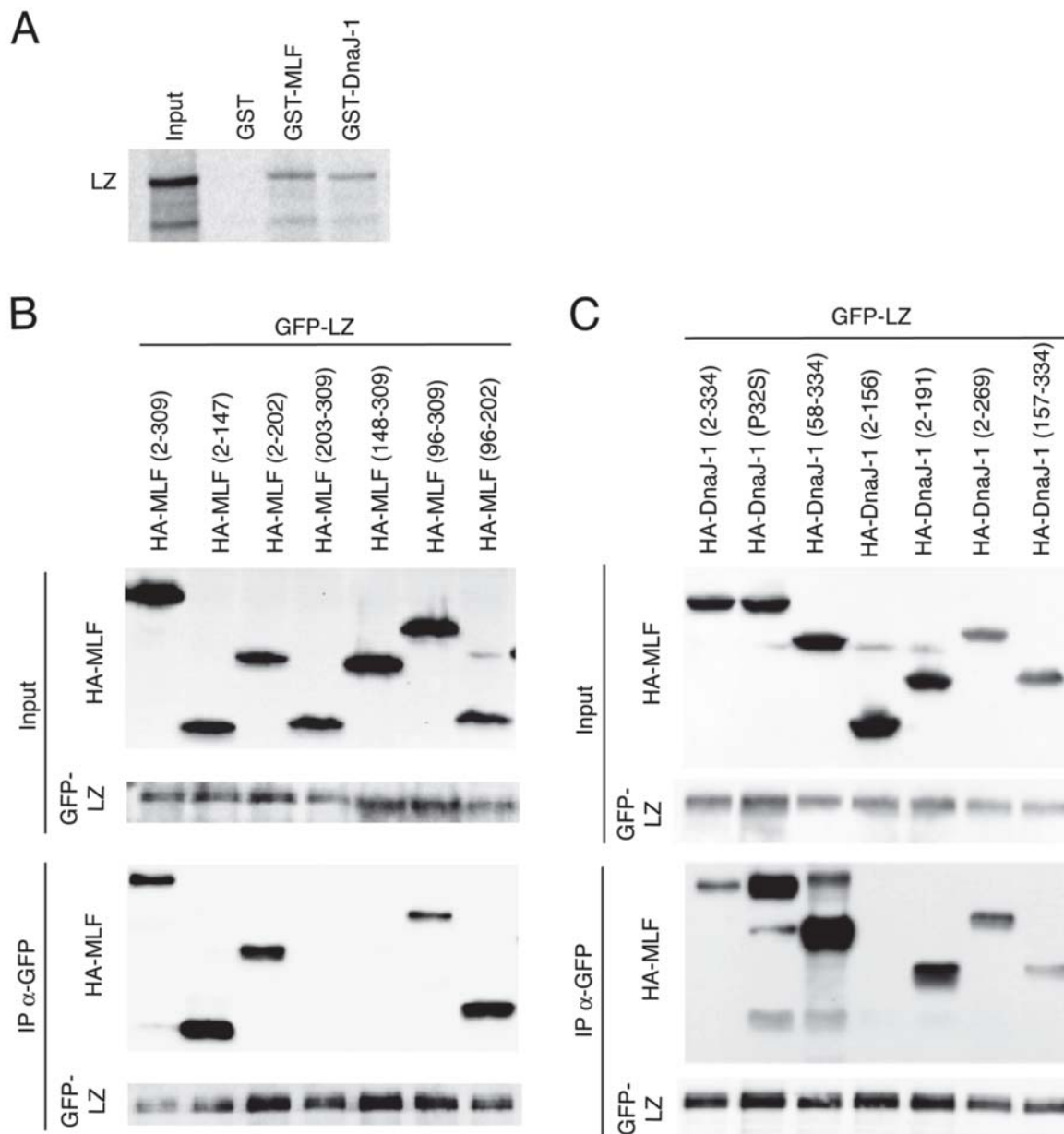
## Supporting information



### S1 Fig. DnaJ-1 and MLF interact in Kc167 cells.

(A) Confocal images of fluorescent immunostainings against GFP (green) and HA (red) in Kc167 cells transfected with expression plasmids for GFP-DnaJ-1 and HA-MLF. Nuclei were stained with Topro3. Merged and individual channels are displayed. Scale bar: 10  $\mu$ m.

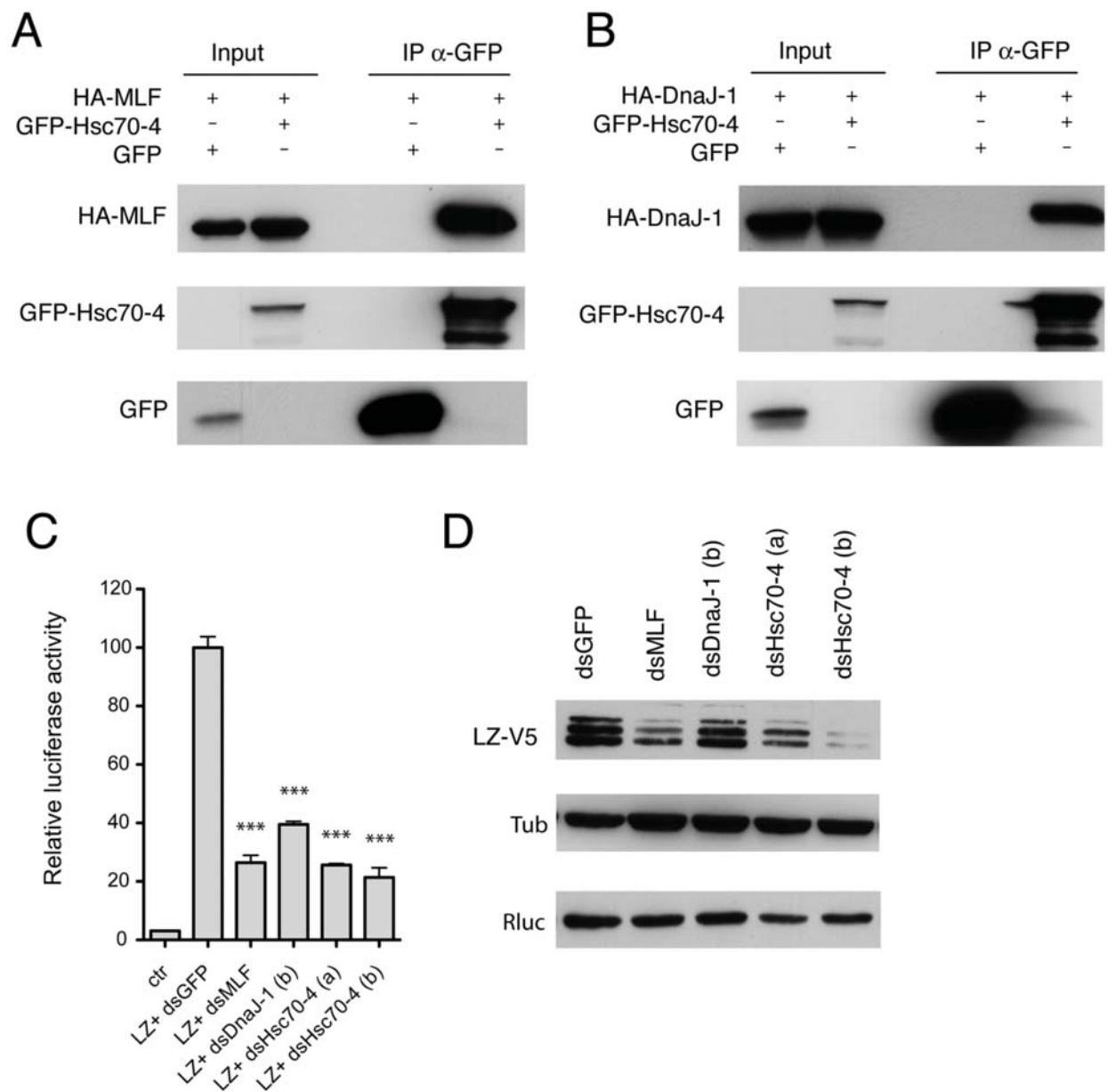
(B) Western blots showing the results of an immunoprecipitation experiment against GFP in Kc167 cells transfected with expression plasmids for the indicated proteins.



**S2 Fig. LZ interacts with MLF and DnaJ-1.**

(A) Autoradiogram showing the results of pull down assays between *in vitro* translated <sup>35</sup>S-methionine-labeled LZ and the indicated GST fusion proteins produced in *E. coli*.

(B, C). Western blots showing the results of immunoprecipitation experiments against GFP performed in Kc167 cells transfected with expression vectors for GFP-LZ and various HA-MLF (B) or HA-DnaJ-1 (C) mutants.

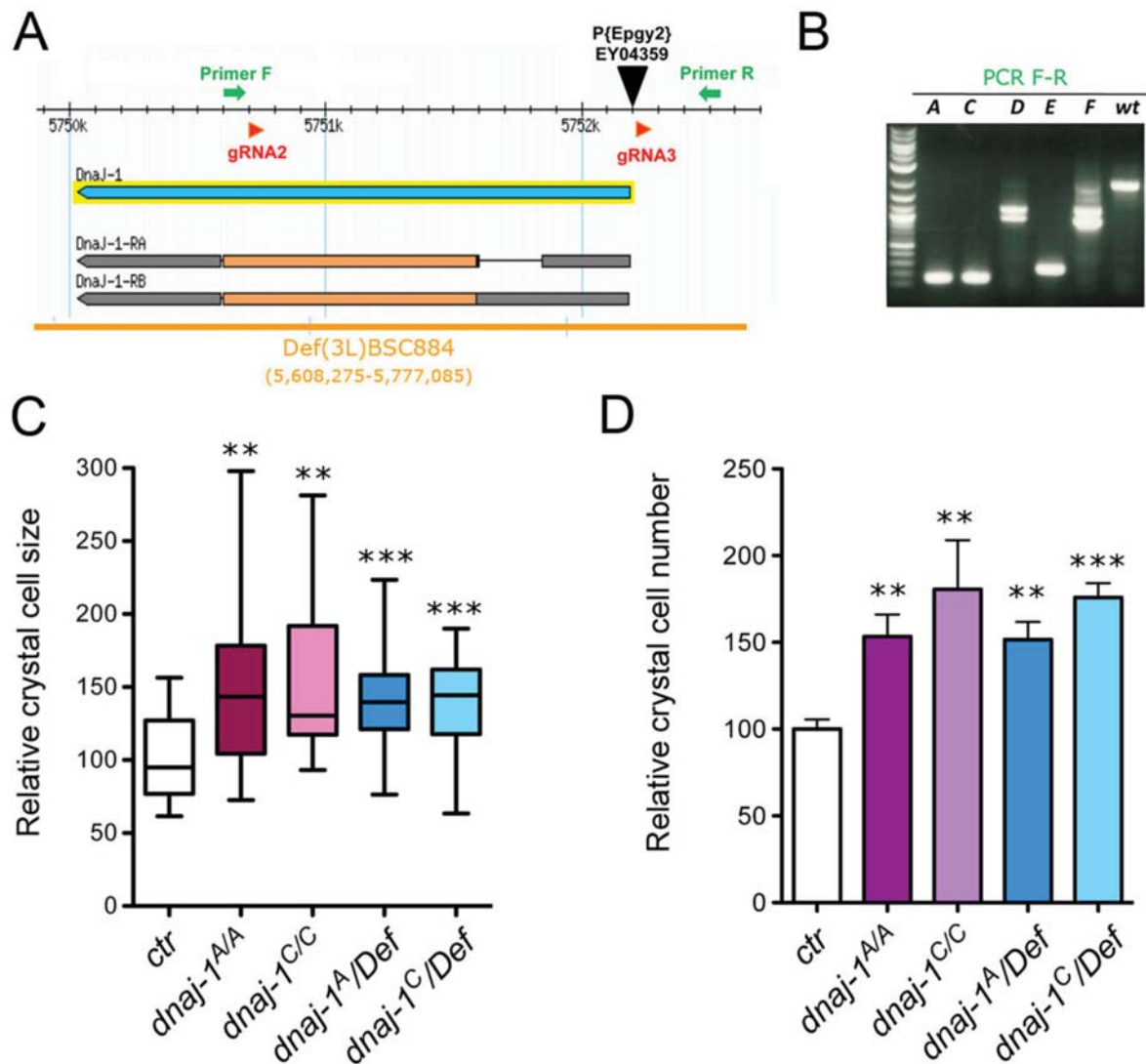


**S3 Fig. Hsc70-4 interacts with MLF and DnaJ-1 and controls LZ activity and expression.**

(A, B) Western blots showing the results of immunoprecipitation experiments against GFP performed in Kc167 cells transfected with expression vectors for GFP or GFP-Hsc70-4 and either HA-MLF (A) or HA-DnaJ-1 (B).

(C) Luciferase assays in Kc167 cells treated with the indicated dsRNA and transfected with 4xPPO2-Fluc reported plasmid in the presence or not (ctr) of pAc-LZ-V5 expression plasmid. pAc-Rluc was used as an internal normalization control. Means and standard deviations from biological triplicates are represented. \*\*\*: p-value < 0.001 as compared to pAc-LZ-V5+dsGFP.

(D) Western blots showing LZ-V5, Renilla luciferase (Rluc) and Tubulin (Tub) expression in Kc167 cells treated with the indicated dsRNA and cotransfected with pAc-LZ-V5 and pAc-Rluc expression vectors. (B, C) dsHsc70-4 (a) and (b) correspond to two distinct dsRNA targeting Hsc70-4.



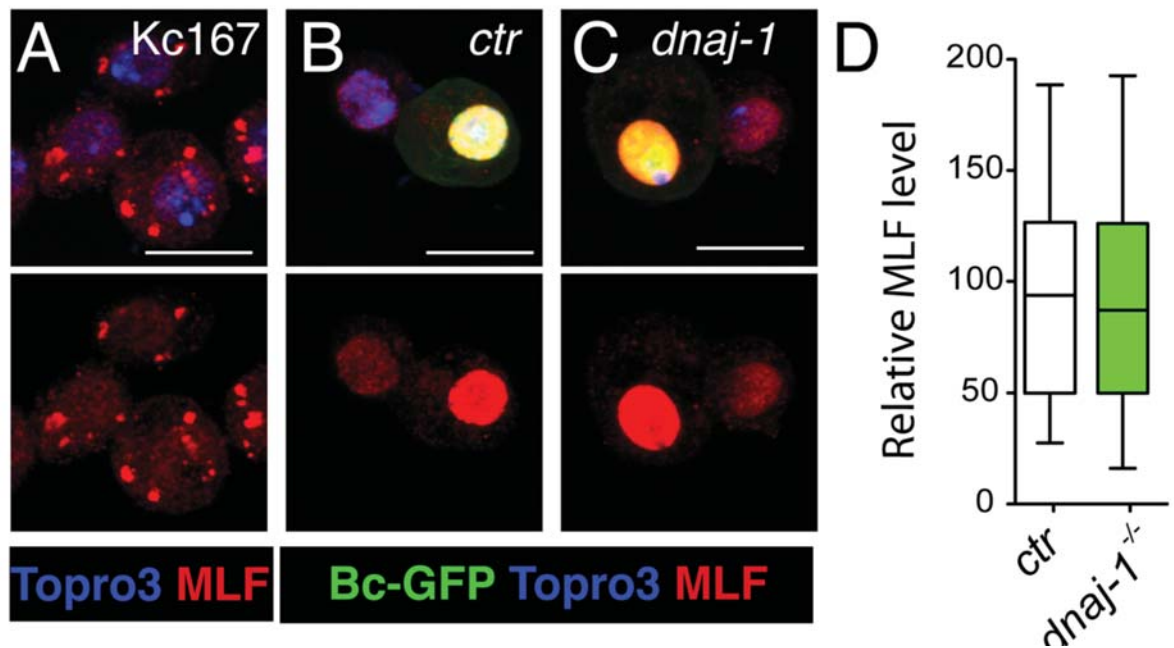
#### S4 Fig. Generation and characterization of *dnaj-1* mutants.

(A) Schematic representation of *dnaj-1* locus. *dnaj-1* transcripts and coding sequence(orange) are shown. The location of the sequences targeted by the 2 guide RNAs (gRNA2 and gRNA3), of the P(EPgy2) element used to select CRISPR/Cas9-mediated deletion events, and of the primers (F and R) used for PCR validation are indicated. Part of the region covered by the deletion Def(3L)BSC884 is also indicated.

(B) Results of PCR amplification on genomic DNA from wild-type (wt) and putative *dnaj-1* deletion mutants (A, C, D, E and F) using the F and R primers displayed in (A). The mutant lines A and C exhibit a complete deletion of the region located between the two gRNAs, as confirmed by sequencing. Other mutants carried deletion of *dnaj-1* associated with more complex rearrangements.

(C-D) Immunostaining against the crystal cell differentiation marker PPO1 was used to assess crystal cell size and number in different *dnaj-1* mutant backgrounds. (C) Relative size of the PPO1<sup>+</sup> blood cells in bleeds from third instar larvae of the indicated genotypes. (D) Relative number of PPO1<sup>+</sup> blood cells in bleeds from third instar larvae of the indicated genotypes. \*\*: p-value<0.01; \*\*\*: p-value<0.001.

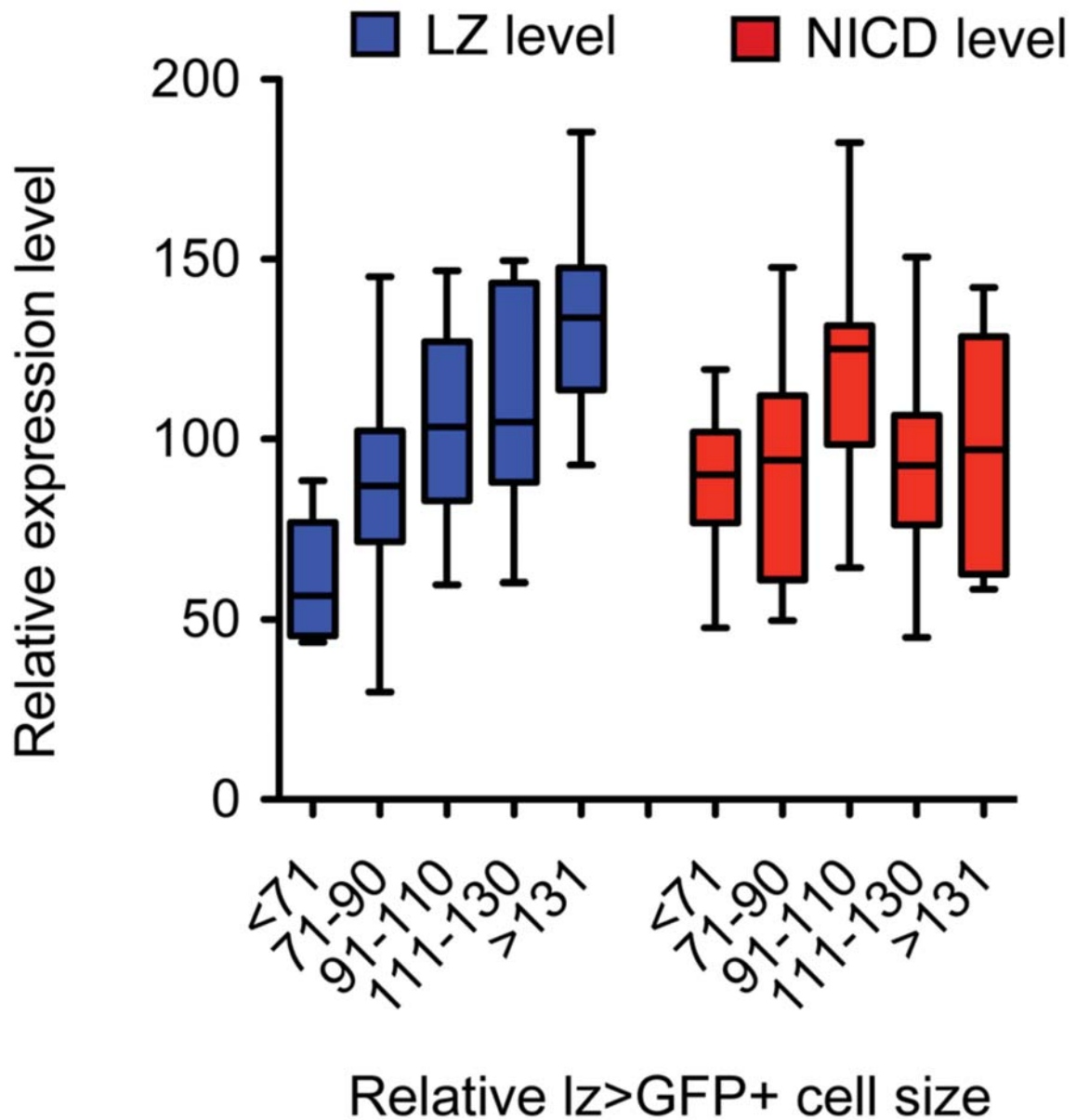




**S5 Fig. MLF expression in Kc167 cells and in larval crystal cells.**

(A-C) Fluorescent immunostainings against MLF in Kc167 cells (A) or in circulating blood cells from *Bc-GFP/+* control (B) or *dnaj-1<sup>-/-</sup>* (C) third instar larvae. Nuclei were stained with Topro3. Only MLF staining is shown in the lower panels. Scale bar: 10  $\mu$ m.

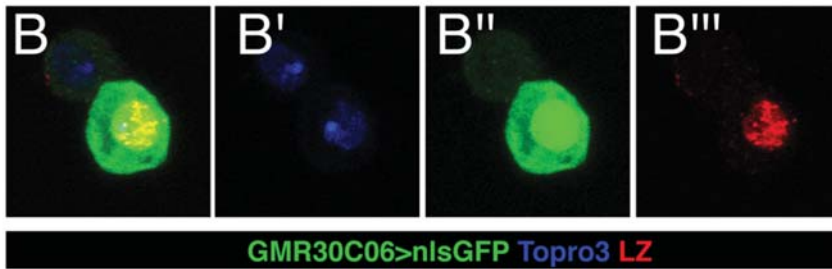
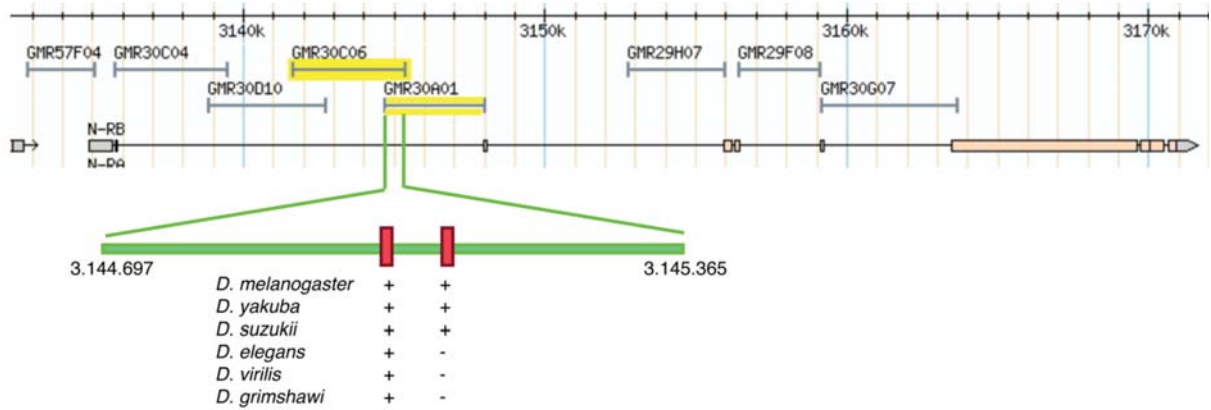
(D) Quantification of MLF expression level in *lz>GFP<sup>+</sup>* circulating blood cells from control or *dnaj-1<sup>-/-</sup>* third instar larvae.



**S9 Fig. LZ expression increases with *lz>GFP*<sup>+</sup> cell size.**

Quantification of LZ and NICD expression levels in *lz>GFP*<sup>+</sup> circulating blood cells of *lz-GAL4, UAS-mCD8GFP/+* third instar larvae. Cells were pooled into 5 categories according to their size (% of the mean cell size) and LZ or NICD expression level in each pool was plotted.

A



**S10 Fig. Characterization of *Notch-Gal4* lines**

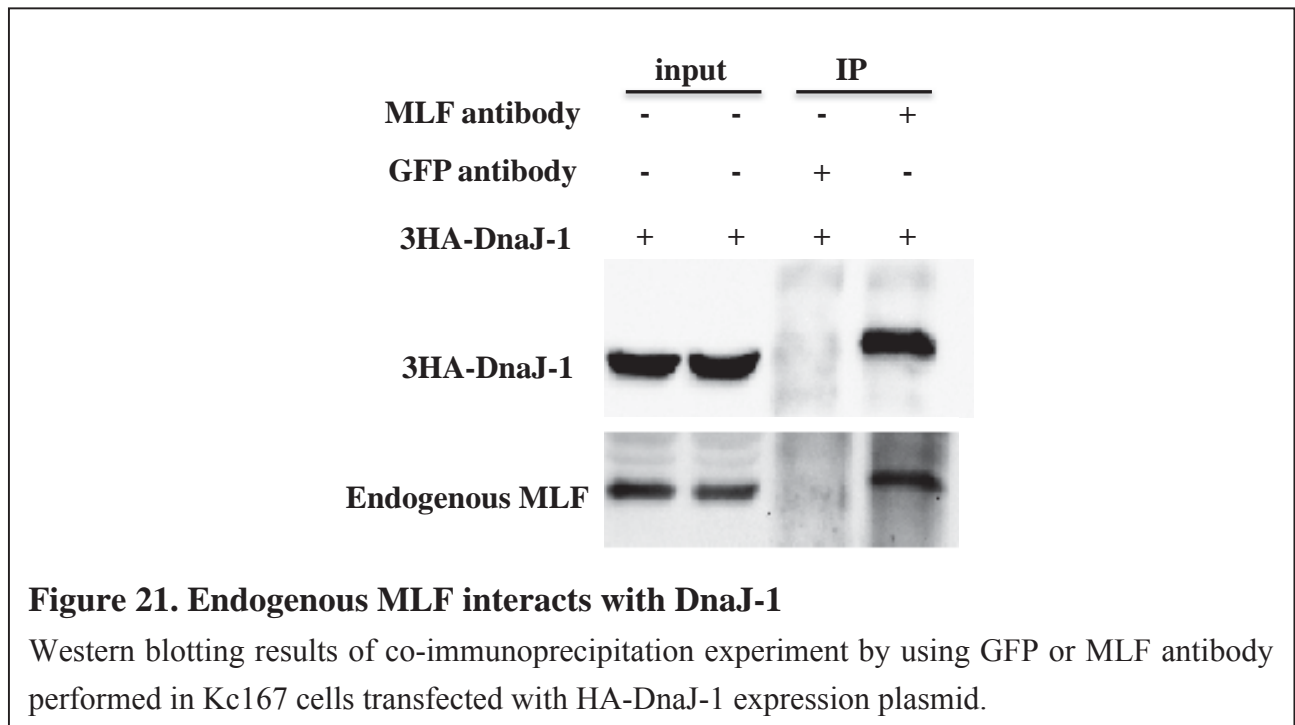
(A) Schematic representation of Notch locus with the position of the two GMR lines that drive expression in *LZ*<sup>+</sup> blood cells. The putative RUNX binding site and their conservation in different *Drosophila* species are indicated.

(B) *LZ* and GFP expression in *NotchGMR30C06-GAL4*, *UAS-GFPnls* circulating blood cells from third instar larvae. Nuclei were stained with Topro3.

## 2. Supplementary results (not present in the article)

### 2.1. Endogenous MLF interacts with DnaJ-1

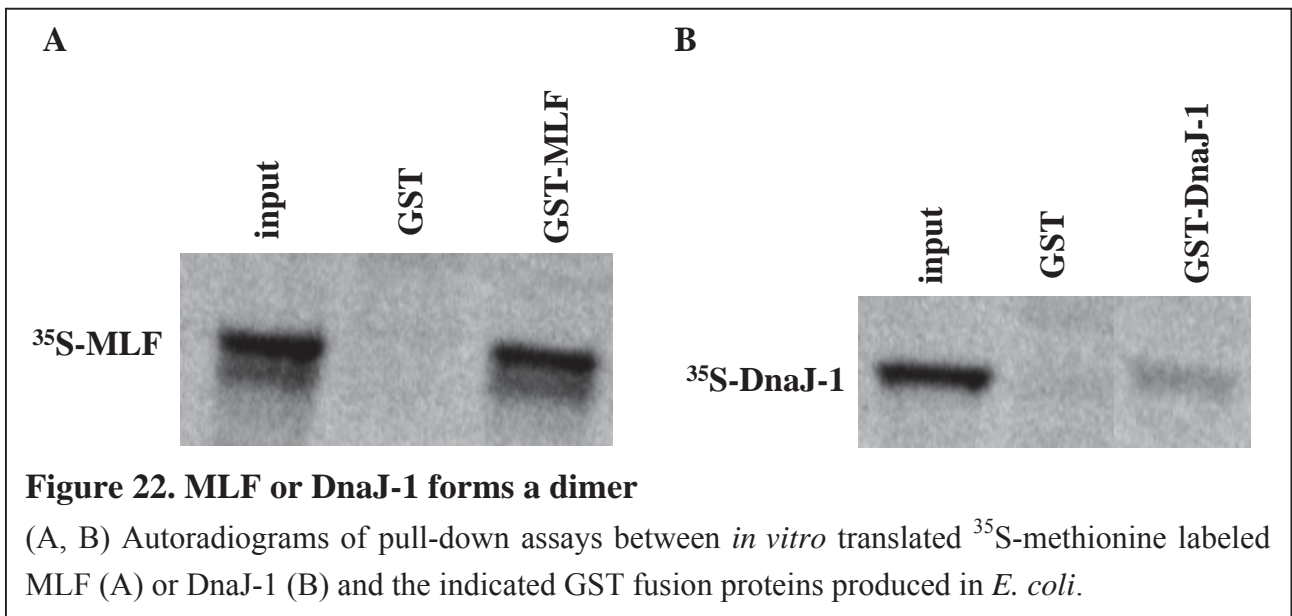
Our results show that MLF and DnaJ-1 interact with each other specifically in co-immunoprecipitation experiments following co-transfection of epitope-tagged version of these two proteins as well as in *in vitro* GST pull-down assays, suggesting that the interaction between them is direct. In addition, we found that 3HA-DnaJ-1 co-precipitated GFP-MLF as well as the endogenous MLF (see above; **Figure 1.D** of the submitted manuscript). To strengthen our conclusions, we asked whether the endogenous MLF could also co-precipitate 3HA-DnaJ-1 in Kc167 cells. So we performed a co-immunoprecipitation experiment with extracts of Kc167 cells transfected with pAc-3HA-DnaJ-1 expression plasmid by using rabbit anti-MLF antibody to precipitate the endogenous MLF and rabbit anti-GFP antibody as a negative control. Western blot analysis showed that MLF antibody precipitated the endogenous MLF and co-precipitated 3HA-DnaJ-1, while GFP antibody did not precipitate either protein (**Figure 21**), which further confirmed the interaction between MLF and DnaJ-1. Of note, since we do not have the antibody against DnaJ-1, we could not test the interaction between both endogenous DnaJ-1 and MLF.



### 2.2. MLF or DnaJ-1 can form a dimer

DnaJ-1 is a member of the DnaJB/class II subfamily of Hsp40/DnaJ proteins, which contains an N-terminal J-domain required for the stimulation of Hsp70 ATPase activity, a

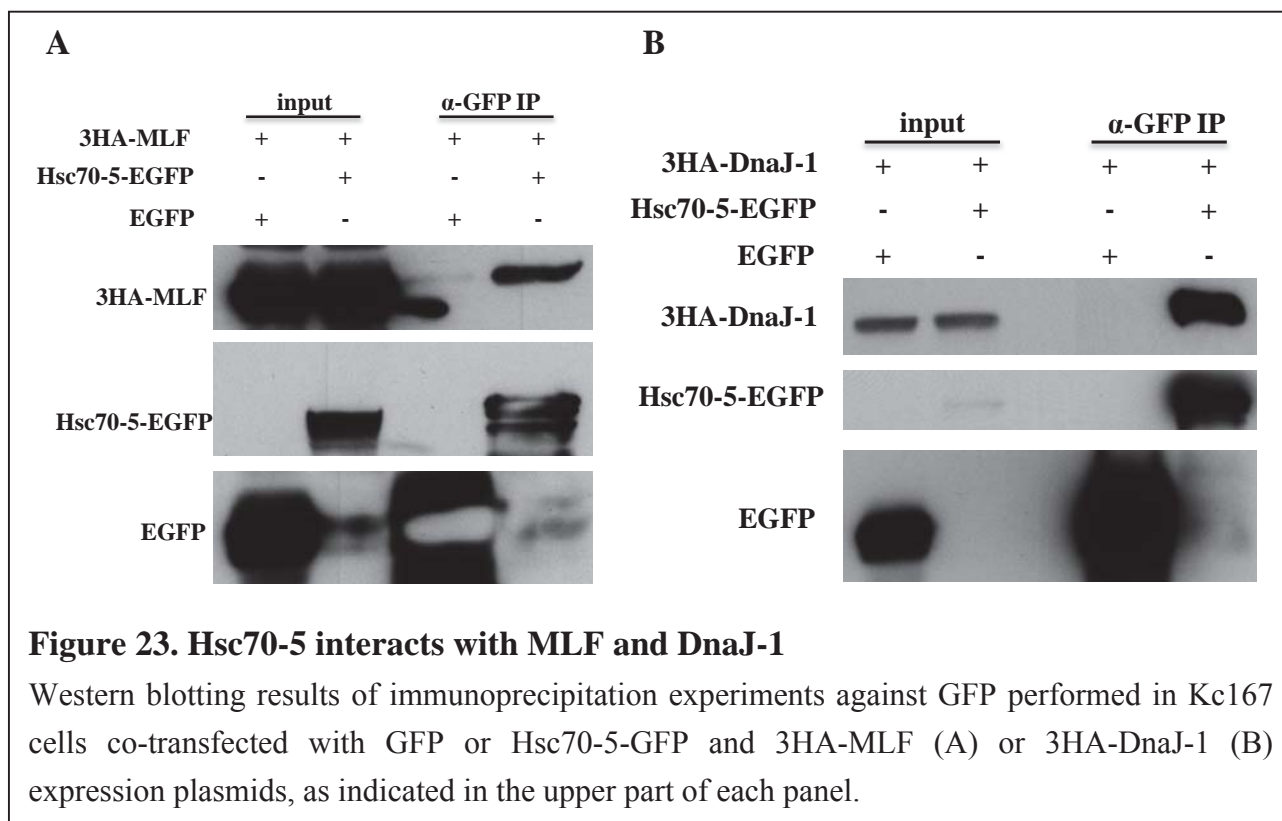
central glycine/phenylalanine (G/F) rich region, and a conserved C-terminal region that contains a client binding domain followed by a dimerization interface (Kampinga *et al.*, 2010). Interestingly, when we performed *in vitro* GST pull-down assays, we found that *in vitro* translated <sup>35</sup>S-labeled DnaJ-1 weakly interacted with GST-DnaJ-1, but not with GST alone (**Figure 22.B**), suggesting that DnaJ-1 can directly interact with itself, probably due to the existence of a dimerization interface in its conserved C-terminal region. Similarly, we also found that *in vitro* translated MLF strongly interacted with GST-MLF, but not with GST alone (**Figure 22.A**), indicating that MLF interacts with itself.



### 2.3. Hsc70-5 interacts with MLF and DnaJ-1

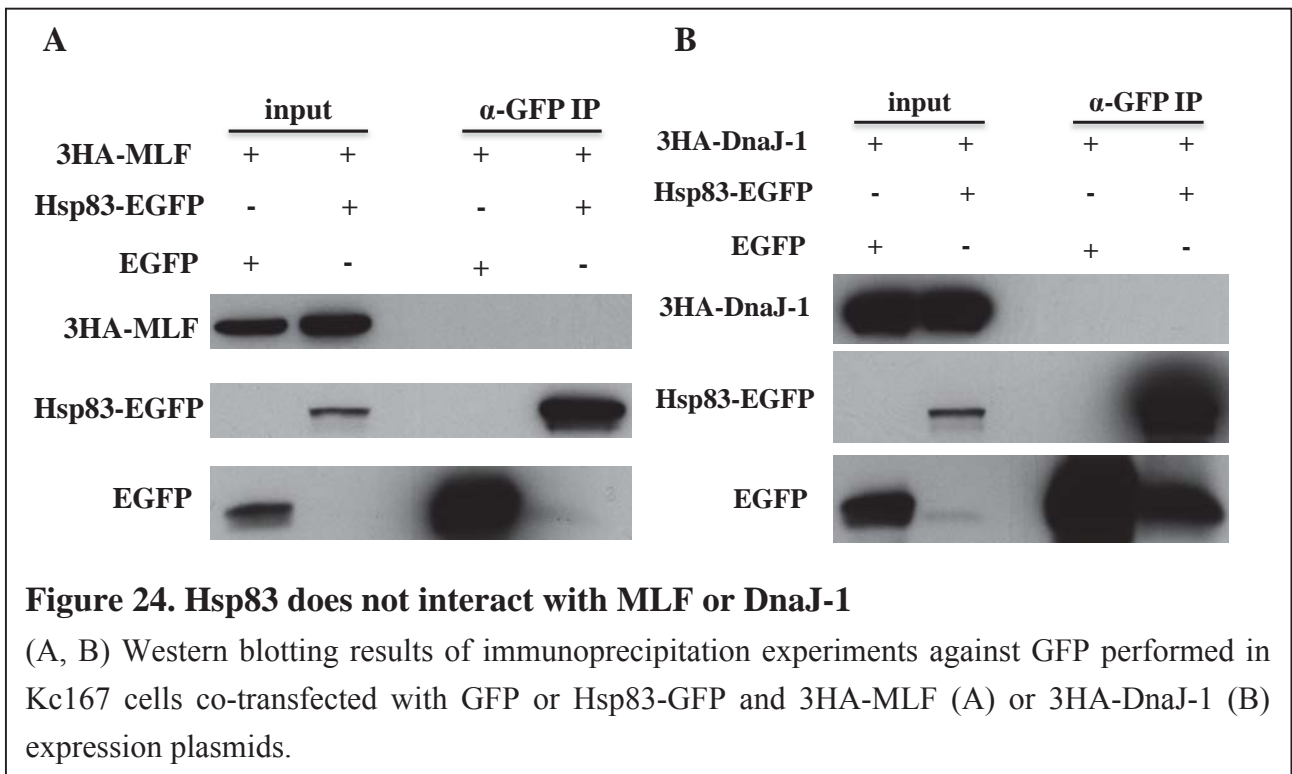
Previous work has revealed that Hsc70-4 interacts specifically with MLF and DnaJ-1 (see above and Dyer *et al.*, 2016). Hsc70-4 belongs to the family of constitutively expressed Hsp70 chaperones, which are composed of 5 paralogs in *Drosophila melanogaster* (Hsc70-1 to Hsc70-5). Considering the similarity in protein structure of the various Hsc70s (the 5 Hsc70 proteins exhibit ±60% of identity), it is interesting to know whether the interaction between Hsc70-4 and MLF or between Hsc70-4 and DnaJ-1 is conserved among other Hsc70s. Actually, we also identified Hsc70-3 as a potential partner of MLF in our proteomic analysis (see above; **Figure 1.B** of the submitted manuscript). To further test this hypothesis, we expressed a GFP-tagged version of Hsc70-5 (the most divergent Hsc70 family member, which is 52% identical to Hsc70-4) together with HA-MLF or HA-DnaJ-1 in Kc167 cells and we performed immunoprecipitation using GFP-Trap resin. Our results showed that GFP-tagged Hsc70-5, but not GFP alone, co-precipitated both MLF (**Figure**

23.A) and DnaJ-1 (**Figure 23.B**), suggesting that Hsc70-5 specifically interacts with MLF and DnaJ-1. These data suggest that the interactions between Hsc70s and MLF or DnaJ-1 are conserved across members of the Hsc70 family and support the idea that MLF acts within the Hsp70 chaperone system.



#### 2.4. Hsp83 does not interact with MLF or DnaJ-1

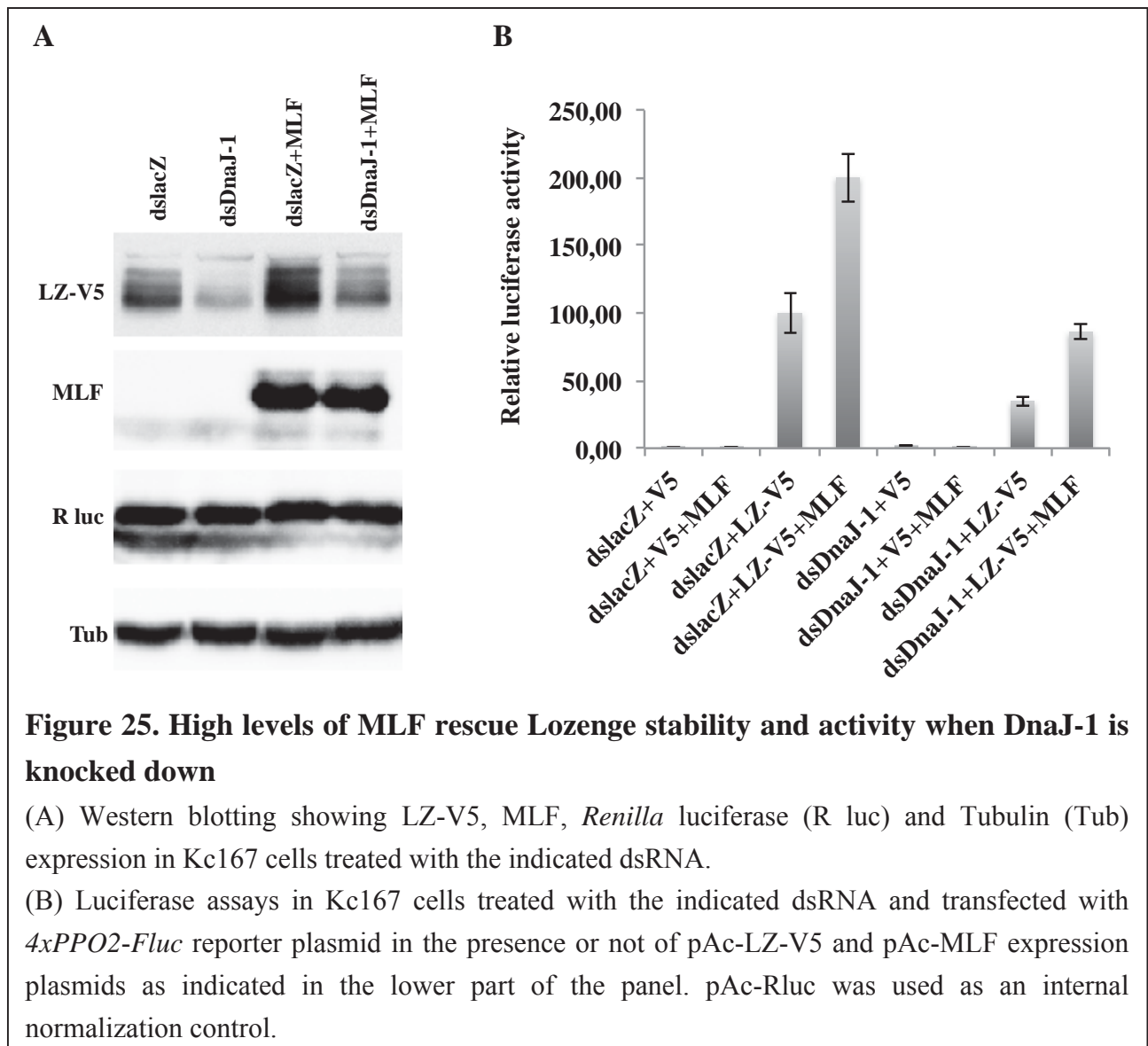
There are two main chaperone systems, the Hsp70 system and the Hsp90 system, both of which participate broadly in *de novo* protein folding and refolding (Hartl *et al.*, 2011). Our data show that MLF can act through DnaJ-1 and the Hsp70 system to control Lozenge stability and activity. However, it is still unknown whether MLF or DnaJ-1 might also interact with the Hsp90 chaperone machinery. To answer this question, we cloned Hsp83, a *Drosophila* homolog of human Hsp90 chaperones, and performed immunoprecipitation experiments between Hsp83 and MLF or DnaJ-1 after co-transfection of the corresponding expression plasmids in Kc167 cells. As Western blotting results showed, Hsp83 co-precipitated neither MLF (**Figure 24.A**) nor DnaJ-1 (**Figure 24.B**), suggesting that MLF and DnaJ-1 do not act in the Hsp90 chaperone system. These data also further confirm the specificity of the interactions that we observed between MLF, DnaJ-1 and Hsc70 proteins.



## 2.5. High levels of MLF rescue Lozenge stability and activity when DnaJ-1 is knocked down

DnaJ-1 was identified as a partner of MLF. When it was knocked down in Kc167 cells, we observed a decrease in Lozenge protein level and a reduction in Lozenge transactivation activity (**Figure 2.A-F** of the submitted manuscript). In contrast, a slight but significant increase in Lozenge protein level was observed in *dnaj-1<sup>-/-</sup>* mutant larvae (**Figure 3.I-L** of the submitted manuscript). Yet, the endogenous MLF level in Kc167 cells is much lower than that in larval crystal cells (**Figure 4** of the submitted manuscript), and our data suggest that the level of MLF could explain the differences in Lozenge sensitivity to DnaJ-1 knockdown between Kc167 cells and larval crystal cells. To further test this hypothesis, we performed Western blotting and transactivation assays in Kc167 cells over-expressing MLF or not following DnaJ-1 knockdown. Our results showed that knockdown of DnaJ-1 caused a decrease in Lozenge level that was rescued when MLF was over-expressed (**Figure 25.A**). Of note, knockdown of DnaJ-1 had no effects on the expression of MLF, indicating that it does not impact on Lozenge level by modifying MLF level (**Figure 25.A**). Moreover, DnaJ-1 knockdown caused a reduction in Lozenge-induced activation of the *4xPPO2-Fluc* reporter gene and this drop was rescued upon MLF over-expression (**Figure 25.B**). All

together, these data indicate that high levels of MLF can rescue Lozenge stability and activity when DnaJ-1 is knocked down.

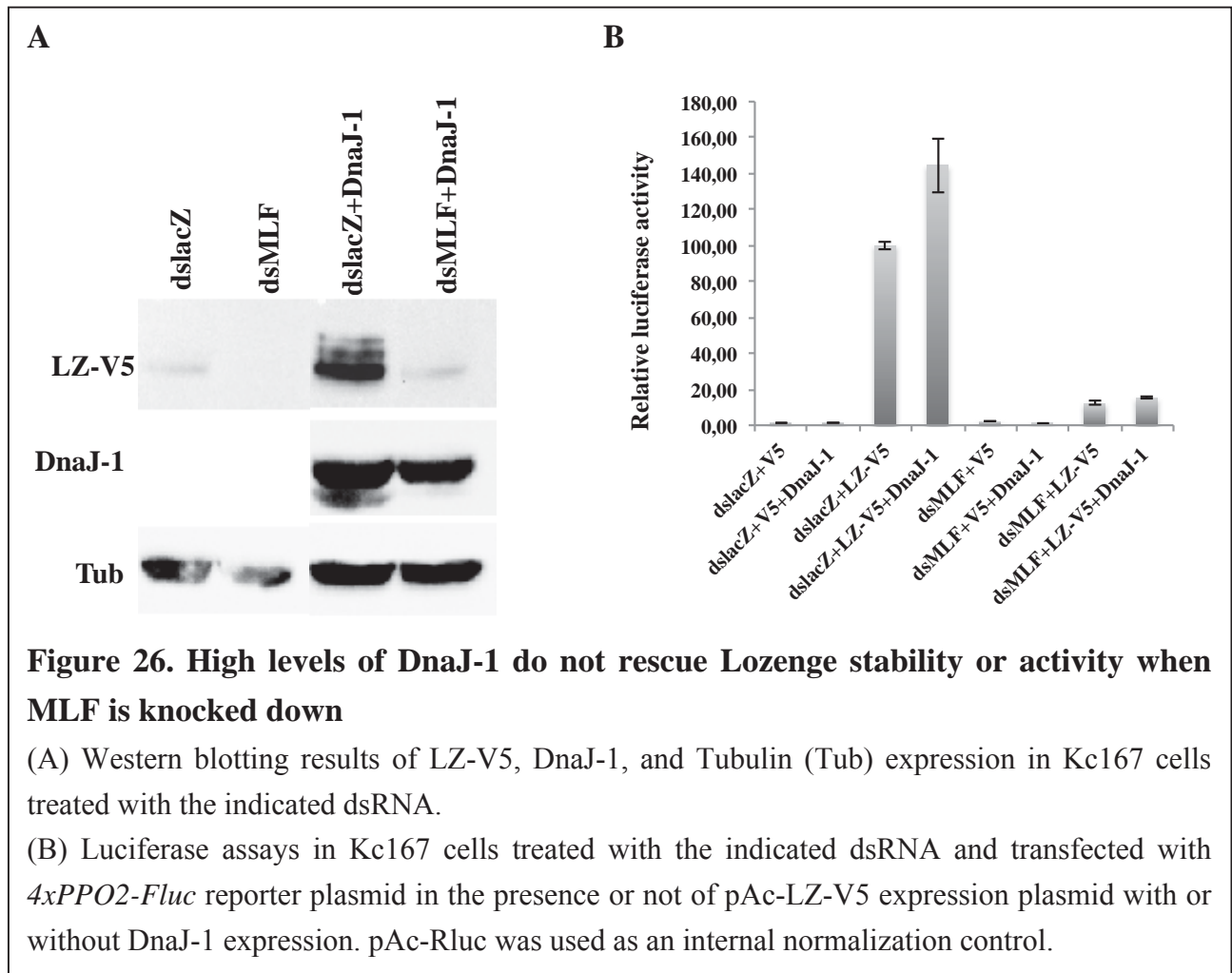


## 2.6. High levels of DnaJ-1 do not rescue Lozenge stability or activity when MLF is knocked down

Similarly, it was shown that MLF knockdown caused a dramatic decrease in Lozenge level and a concomitant reduction in transactivation activity in Kc167 cells (Bras *et al.*, 2012). As described above, high levels of MLF rescue Lozenge stability and transactivation activity in the absence of DnaJ-1 in Kc167 cells. Here, we asked whether the converse is true. Our results showed that DnaJ-1 over-expression didn't increase Lozenge level following MLF knockdown (**Figure 26.A**), and similar results were observed in the luciferase assays (**Figure 26.B**). In addition, knockdown of MLF had no effects on DnaJ-1 level in Kc167 cells (**Figure 26.A**). Taken together, these data indicate that high levels of



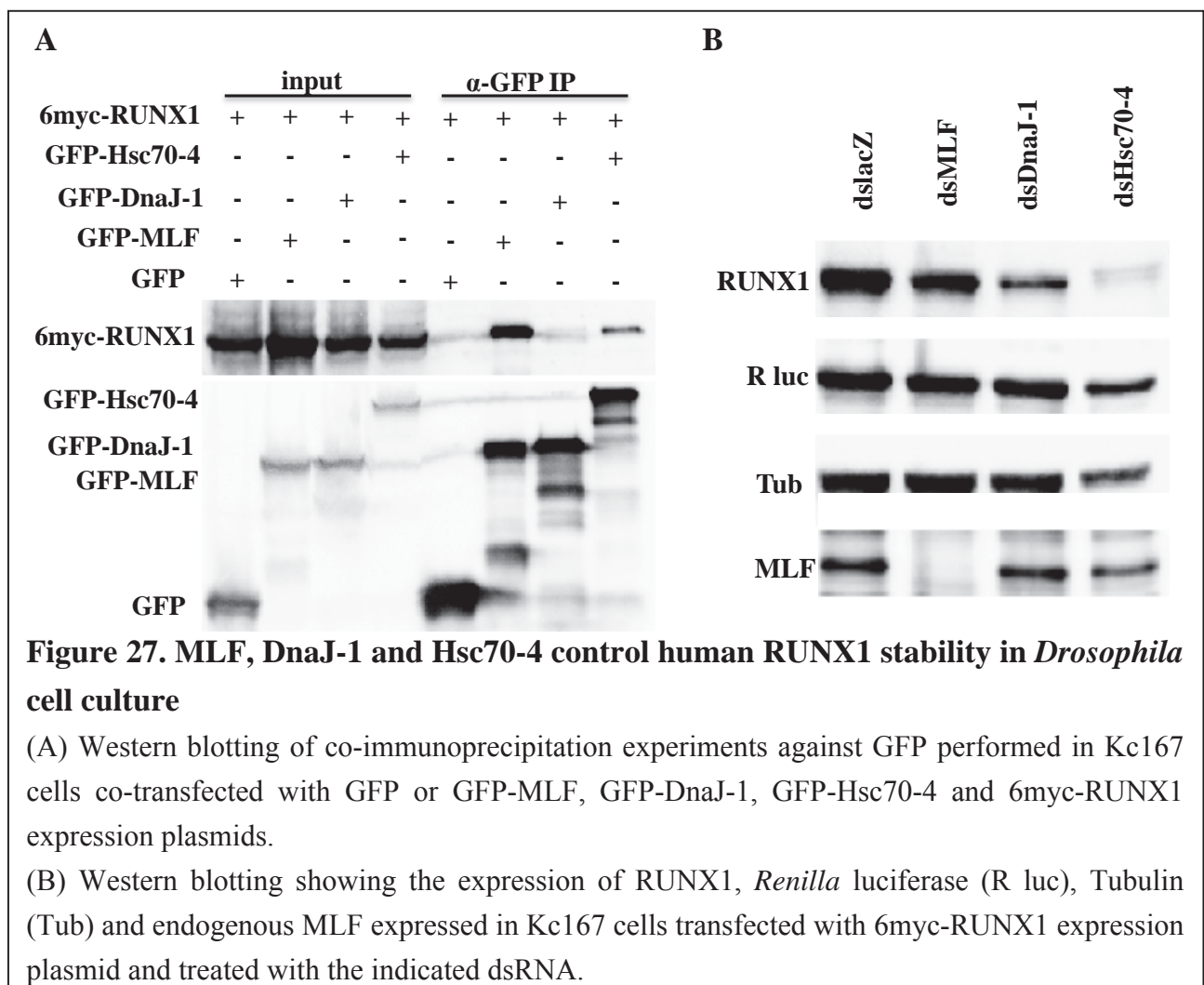
DnaJ-1 are not sufficient to rescue Lozenge stability or activity when MLF is knocked down.

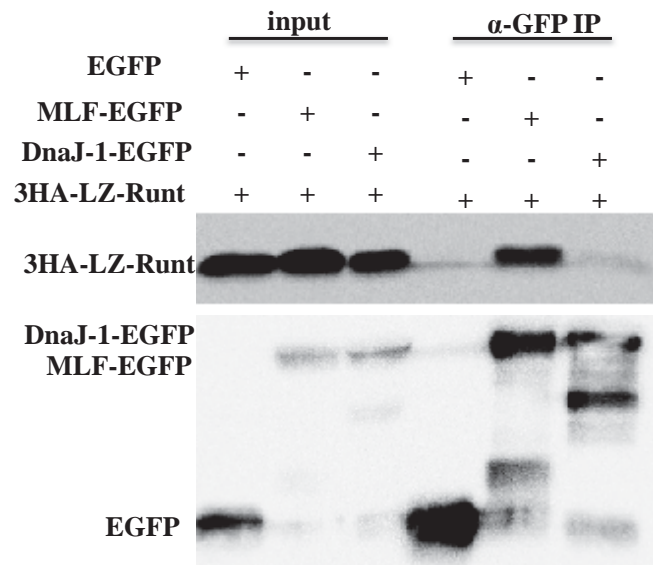


## 2.7. MLF, DnaJ-1 and Hsc70-4 control human RUNX1 stability

As we know, members of the RUNX family of proteins contain a highly evolutionarily conserved 128 amino acid domain at their N-terminus, which is designated as the runt homology domain (RHD) and mediates the interactions between RUNX family proteins with DNA but also with some other proteins. We have demonstrated that Lozenge, a *Drosophila* homolog of human RUNX1 transcription factor, interacts with MLF, DnaJ-1 and Hsc70-4, and that its activity and stability are regulated by these three proteins to control *Drosophila* blood cell development. Here, we asked that whether the MLF/DnaJ-1/Hsc70-4 chaperone complex might also regulate other RUNX factors. First, we tested whether human RUNX1 interacts with MLF, DnaJ-1 or Hsc70-4. Accordingly, we expressed RUNX1 in Kc167 cells together with GFP-tagged versions of MLF, DnaJ-1 or Hsc70-4 and we performed co-immunoprecipitation experiments. Our results showed that

MLF and Hsc70-4 co-precipitated RUNX1. However, we did not observe a co-immunoprecipitation of RUNX1 with GFP-DnaJ-1 (**Figure 27.A**). Then we asked whether these proteins are required for the stable expression of RUNX1 in Kc167 cells. Interestingly, the knockdown of MLF, or DnaJ-1 or Hsc70-4 caused a decrease in the level of RUNX1 (**Figure 27.B**). Thus, even though we could not detect yet a physical interaction between DnaJ-1 and RUNX1 in our assays, these data indicate that RUNX1 stability can be regulated by MLF, DnaJ-1 and Hsc70-4 from *Drosophila*, and we propose that RUNX1 stability might also be controlled by the homologs of these proteins in human. These data also suggest that the runt homology domain, which is conserved between Lozenge and RUNX1, is an important determinant of the regulation by the MLF/DnaJ/Hsc70 chaperone machinery. In line with this hypothesis, co-immunoprecipitation results showed that Lozenge RHD is sufficient for the interaction between Lozenge and MLF as MLF co-precipitated the runt domain, but DnaJ-1 didn't (**Figure 28**). Thus, other RUNX proteins might also be regulated by these factors.





**Figure 28. MLF interacts with Lozenge runt domain**

Western blotting of co-immunoprecipitation experiments against GFP performed in Kc167 cells co-transfected with GFP or GFP-MLF, GFP-DnaJ-1 and 3HA-LZ-Runt expression plasmids.

## **Chapter III Discussion**



## **1. The interactions between LZ, MLF, DnaJ-1 and Hsc70-4**

### **1.1. MLF/DnaJ-1 complex**

MLF is known to be a conserved regulator of RUNX transcription factor Lozenge (LZ) stability and activity, but the molecular mechanism of action of MLF on LZ stability has not been well understood (Bras *et al.*, 2012). To answer this key question, an affinity purification approach followed by mass spectrometry was applied to search for MLF interacting partners, which could provide some valuable clues to this mystery. This approach allowed us to discover that MLF interacts with DnaJ-1 in Kc167 cells, which is in accordance with the results published by Dyer *et al.* who used a similar strategy in *Drosophila* S2 cells. We confirmed these results by co-immunoprecipitation experiments in Kc167 cells and our *in vitro* GST pull-down assay results further suggest that the interaction between MLF and DnaJ-1 is direct, which is consistent with the findings using purified recombinant proteins (Dyer *et al.*, 2016). Furthermore, our domain mapping experiments show that MLF homology domain (MHD) interacts with DnaJ-1's conserved C-terminus, suggesting that the interaction between MLF and DnaJ-1 is conserved. In line with this idea, Dyer *et al.* showed that over-expressed hMLF1 or hMLF2 interacts with DnaJB6, a homolog of DnaJ-1, in human 293T cells. This provides a possible mechanism for MLF-directed transcription factor stabilization, since DnaJ-1 co-chaperone plays a crucial role in the regulation of protein folding and degradation, and MLF is likely to participate in the chaperone complex and exerts its function in the regulation process of a particular molecular chaperone complex. These findings open a new direction to decipher MLF molecular mode of action in the *Drosophila* model organism. However, it is still difficult to assess the interaction between MLF and DnaJ-1 *in vivo*, due to the small amount of crystal cells and the technical difficulties of their successful isolation from *Drosophila*. Technologies like BiFc (Bimolecular Fluorescence Complementation) (Hudry *et al.*, 2011) using transgenically expressed MLF and DnaJ-1 proteins fused to the N- or C-terminal domain of GFP, or Proximity Ligation Assays (Weibrecht *et al.*, 2010) using antibody directed against each protein, could be an alternative option to try to validate MLF/DnaJ-1 physical interaction *in vivo*.

### **1.2. MLF/Hsc70-4 complex**

DnaJ-1, a member of the Hsp40/DnaJ co-chaperone family, chiefly acts by delivering client proteins to the Hsp70 chaperones and stimulating the Hsp70 ATPase activity by

interacting with them via their N-terminal J-domain. Interestingly, we also recovered Hsc70-4 and Hsc70-3 as potential MLF partners in our proteomic approach, two Hsp70 paralogs in *Drosophila*. Our co-immunoprecipitation experiments confirmed that MLF and DnaJ-1 interact with Hsc70-4, respectively. Similarly, Dyer *et al.* also found that MLF interacts with Hsc70-4. In addition, our co-immunoprecipitation results showed that MLF (and DnaJ-1) can also interact with Hsc70-5, which is only  $\pm 50\%$  identical to Hsc70-4, suggesting that MLF very probably interacts with several Hsc70 proteins and participates in diverse Hsp70 chaperone complexes in association with DnaJ-1. Yet, whether MLF directly interacts with Hsc70 proteins or indirectly interacts with them via DnaJ-1 remains to be determined. In *in vitro* GST pull-down assays, I only observed a weak interaction between *in vitro* translated Hsc70-4 and GST-MLF, but a strong interaction with GST-DnaJ-1 (data not shown). However, these results need to be confirmed. In addition, there is another common chaperone complex, the Hsp90 system, which participates broadly in *de novo* protein folding and refolding (Hartl *et al.*, 2011). So we tested whether MLF or DnaJ-1 could also interact with this Hsp90 chaperone. Our co-immunoprecipitation results showed that neither MLF nor DnaJ-1 interacts with Hsp83, a *Drosophila* homologue of human Hsp90 chaperones, indicating that MLF and DnaJ-1 are not part of the Hsp90 chaperone system.

Of note, Dyer *et al.* also identified the nucleotide exchange factor (NEF) BAG2, which stimulates the release of client proteins from the Hsp70 chaperones, as one of the main partners of MLF. However, we did not retrieve this protein in our proteomic approach, which could be due to its weak association with the MLF/DnaJ-1/Hsc70-4 complex. Furthermore, a systematic characterization of Hsp70 chaperone complexes in human cells has identified hMLF1 and hMLF2 as potential partners of DnaJ-1 homologs, DnaJB1, DnaJB4 and DnaJB6 (Taipale *et al.*, 2014). All together, these publications and our work strongly support the conclusion that MLF is a conserved component of the Hsp70 chaperone system that interacts with DnaJB co-chaperones.

### **1.3. MLF/DnaJ-1/Hsc70-4 complex and LZ**

Results from our team suggested that MLF proteins act as a conserved regulator of RUNX transcription factor stability and activity. In particular, *Drosophila* MLF controls LZ activity and prevents its degradation by the proteasome in cell culture (Bras *et al.*, 2012). However, it was unknown whether MLF physically interacts with LZ to control its stability.

Our co-immunoprecipitation results showed that MLF interacts specifically with LZ and our *in vitro* GST pull-down assays suggested that the interaction between MLF and LZ is direct. In addition, we demonstrated that DnaJ-1 and Hsc70-4 also interact specifically with LZ, respectively, suggesting that LZ is a direct target of the chaperone complex formed by MLF, DnaJ-1 and Hsc70-4. Importantly, we found that knockdown of DnaJ-1 or Hsc70-4 expression by RNAi in Kc167 cells leads to a reduction in LZ protein level and activity without affecting its mRNA level, similar to what was observed following MLF knockdown. Dyer *et al.* also observed similar results on LZ protein level in S2 cells. In addition, we found that over-expression of DnaJ-1 mutants unable to stimulate Hsp70 chaperone activity also causes a reduction in LZ level and activity, suggesting that the effect of DnaJ-1 on LZ is strictly dependent on its capacity to activate Hsc70-4. Thus, the MLF/DnaJ-1/Hsc70-4 chaperone complex is required for the stable expression of LZ in cell culture. Yet, how each component acts within this chaperone complex still remains to be determined.

## **2. MLF in the Hsp70 chaperone complex: a chaperone or a co-chaperone?**

Many studies have shown that over-expression of MLF, DnaJ-1 and some Hsp70s can suppress the cytotoxicity associated with polyglutamine (polyQ) protein aggregations in *Drosophila* (Kuo *et al.*, 2013; Kim *et al.*, 2005). In view of these publications and our results, it is tempting to speculate that the chaperone complex formed by MLF, DnaJ-1 and Hsc70-4 could play a role in the progression of neurodegenerative pathologies due to protein aggregation. This also further provides clues concerning the possible mode of action of MLF.

DnaJ-1 is known to act as a co-chaperone to assist the Hsp70 chaperones by delivering client proteins to their C-terminal substrate binding domain and stimulating their ATPase activity, but also as a chaperone by itself to mediate protein folding and refolding (Kampinga *et al.*, 2010). On one hand, given the strong interaction between MLF and DnaJ-1, MLF could act as a chaperone when DnaJ-1 acts as a co-chaperone, or MLF could act as a co-chaperone when DnaJ-1 acts as a chaperone by itself. On the other hand, MLF interacts with Hsc70-4 (directly or via DnaJ-1), an Hsp70 chaperone involved in a wide range of protein quality control functions, including *de novo* protein folding and protein degradation. So it would be interesting to test whether (1) MLF holds an intrinsic chaperone activity, (2) MLF modulates DnaJ-1 chaperone activity, (3) MLF modulates the activity of the



DnaJ/Hsc70 complexes. In that sense, we tried to produce highly purified MLF, DnaJ-1 and Hsc70-4 recombinant proteins in *E. coli* to assess the effects of MLF on the stimulation of Hsc70-4 ATPase activity and on Hsc70-4 dependent refolding of a denatured model substrate protein (*i.e.*, firefly luciferase), as well as the effects of MLF on DnaJ-1 intrinsic chaperone activity in an assay based on the prevention of luciferase aggregation (Perrody *et al.*, 2012). However, MLF was highly insoluble and we could not produce enough proteins to perform these experiments. MLF could also act by regulating the client specificity of DnaJ proteins, and it would be interesting to identify other factors whose stability is regulated by MLF and/or DnaJ-1. According to the reaction cycle of the Hsp70 chaperone system, MLF could bind to LZ to prevent it from forming aggregates and then, together with DnaJ-1, present LZ to Hsc70-4 for proper protein folding. Our preliminary data suggest that an important fraction of LZ is not soluble when expressed in Kc167 cells and might form aggregates. In this model, MLF could act as a co-chaperone to prevent LZ but also polyglutamine (polyQ) protein aggregations. It would be interesting to further investigate the impacts of MLF, DnaJ-1 or Hsc70-4 knockdown on LZ soluble and insoluble fractions.

### **3. Regulation of LZ stability and activity by MLF and DnaJ-1**

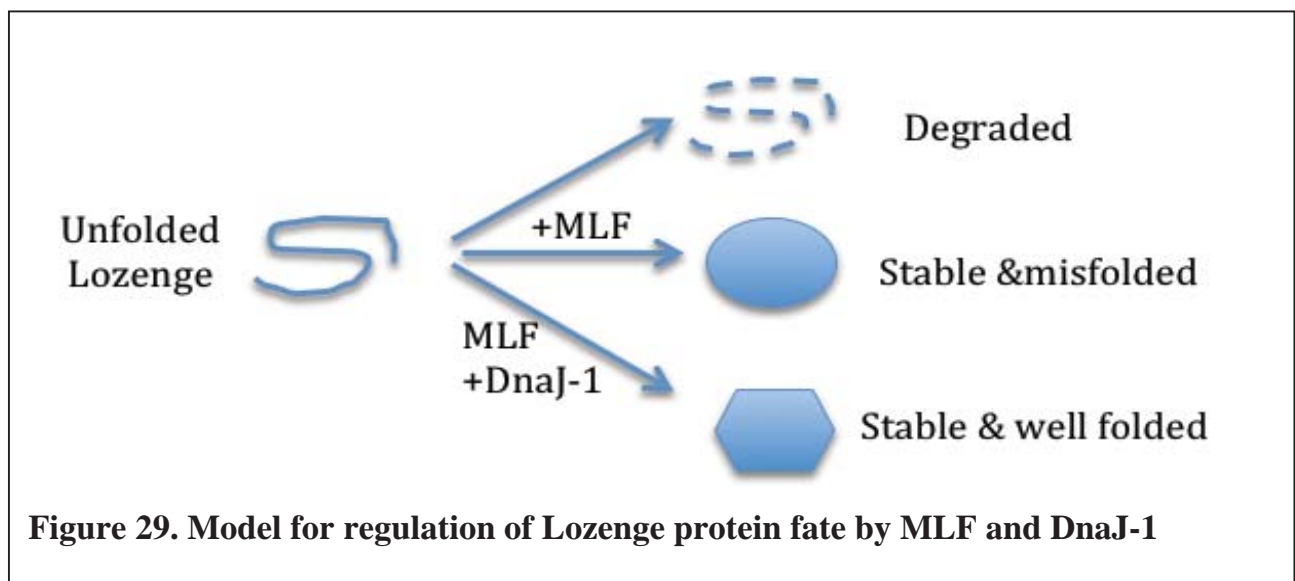
It has been demonstrated that MLF stabilizes RUNX transcription factor Lozenge, as the endogenous nuclear Lozenge is degraded in LZ<sup>+</sup> cells from *mlf* null mutant larvae and the Lozenge expressed in Kc167 cells is decreased when the endogenous MLF is knocked down (Bras *et al.*, 2012). Due to loss of Lozenge, a key transcription factor for crystal cell development, phenotypic defects in LZ<sup>+</sup> cells emerge, such as changes in the number and the size of LZ<sup>+</sup> cells, indicating that the regulation of Lozenge stability by MLF is vital for the normal development of LZ<sup>+</sup> cells (Bras *et al.*, 2012). However, how MLF regulates Lozenge stability remains largely unknown. Understanding this process might provide valuable information as to MLF functions and mode of action but also concerning the regulation of RUNX transcription factors. Both MLF and RUNX are implicated in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) in human, and thus our findings might be of interest in terms of human health. Based on our findings and those of Dyer *et al.*, the prevailing hypothesis is that MLF acts together with the chaperone complex DnaJ-1/Hsc70-4 to control Lozenge level post-translationally by preventing its degradation.

The human homolog of Lozenge, RUNX1 is essential for hematopoiesis and it is subjected to proteolytic degradation by the ubiquitin-proteasome pathway in absence of CBF $\beta$  subunit (Huang *et al.*, 2001), suggesting that RUNX1 could be unstable in the nucleus without its  $\beta$  subunit and this instability could be an intrinsic property of RUNX1. It has been shown that RUNX1 is modified by a chain of ubiquitin and then the 70S proteasome can recognize this ubiquitylated RUNX1 to degrade it (Huang *et al.*, 2001). However, when we tested whether Lozenge could be ubiquitylated, we didn't detect ubiquitylation of Lozenge in Kc167 cells (data not shown). Nonetheless, Lozenge is degraded in the absence of *mlf* in LZ<sup>+</sup> cells as well as in Kc167 cells and its degradation in Kc167 cells is partly inhibited by MG132 treatment, suggesting that the proteasome is implicated in Lozenge degradation (Bras *et al.*, 2012). There is also a proteasome pathway independent of ubiquitylation (Erales *et al.*, 2014), and it could be interesting to check whether Lozenge is degraded by this pathway. Besides degradation mediated by proteasome, some specific proteases and certain lysosomes also cause the degradation of particular proteins. We propose that MLF might act at three different levels. Firstly, MLF binding to Lozenge could block its interaction with the proteolytic chamber of the 20S proteasome or interfere with its recognition by the 70S proteasome. Secondly, MLF could block Lozenge ubiquitylation and its subsequent targeting to the proteasome, even though we have not obtained direct evidence that Lozenge can be ubiquitylated. Thirdly, MLF could inhibit the proteasome-independent degradation of Lozenge. An important challenge will be to further define MLF mode of action and the pathway that mediates Lozenge degradation.

We have demonstrated that MLF is involved in the Hsp70 chaperone system, and Hsp70 chaperones can also mediate the degradation of some proteins that are not refolded by Hsp70 chaperones through chaperone mediated autophagy (CMA) or chaperone-assisted selective autophagy (CASA), both of which are dependent or independent on ubiquitylation (Kettern *et al.*, 2010). So, even though our results argue that Hsc70-4 is required for Lozenge stability, it could be interesting to test whether Lozenge degradation is caused by Hsp70 chaperones-mediated autophagy in the absence of MLF.

Intriguingly, we found that in the presence of high levels of MLF (LZ<sup>+</sup> larval blood cells or Kc167 cells over-expressing MLF), the absence or the knockdown of DnaJ-1 does not result in a decrease in Lozenge level, in contrast to what we observed in the presence of

low level of MLF (naive Kc167 cells or LZ<sup>+</sup> larval blood cells with MLF knockdown) (**Figure 2** and **4** in the manuscript). It is possible that MLF and DnaJ-1 somehow compete for binding to Lozenge or, alternatively, that the association of MLF with DnaJ-1 changes the fate of Lozenge protein. MLF could act first to stabilize Lozenge (prevent its degradation), and then the MLF/DnaJ-1 complex could favor proper Lozenge folding and thus its activity. In view of our results, we propose that in the absence of MLF, Lozenge is degraded whereas in the absence of DnaJ-1, Lozenge is not properly folded and either it is degraded if there is a low level of MLF or it accumulates in a unfolded/inactive state if there is enough MLF to prevent its degradation (**Figure 29**).



Our preliminary results indicate that an important fraction of Lozenge is insoluble when it is expressed in Kc167 cells (data not shown). This suggests that Lozenge is prone to form aggregates and that MLF or DnaJ-1, which has been involved in the regulation of polyQ aggregates, might participate in the solubilization of Lozenge. It would be interesting to further test this hypothesis by systemically assessing the effects of MLF, DnaJ-1 and Hsc70-4 knockdown or over-expression on the soluble and insoluble fraction of Lozenge as well as by testing *in vitro* whether these factors modify the solubility of purified recombinant Lozenge protein.

Finally, since DnaJ-1 and MLF can also bind chromatin and regulate transcription (Dyer *et al.*, 2016), it would be interesting to test whether Lozenge recruits these proteins to chromatin or whether these two proteins are also involved in the recruitment of Lozenge to chromatin. Indeed, even though our data show that the defects caused by MLF or DnaJ-1 loss can be rescued by the re-expression of Lozenge *in vivo*, we can not exclude the

possibility that these two proteins somehow participate directly in the regulation of Lozenge capacity to regulate gene expression. However, the limited amount of crystal cells present in the larva precludes our possibility to perform ChIP experiments *in vivo* to test these hypotheses.

#### **4. MLF, DnaJ-1, LZ and the control of crystal cell size and number**

Based on the data collected from the *dnaj-1*<sup>-/-</sup> mutant larvae, we showed that this gene is required for the normal development of the circulating crystal cells and that it genetically interacts with *mlf* during this process. The loss of DnaJ-1 notably caused an increase in the number and the size of LZ<sup>+</sup> blood cells, which was rescued by the re-expression of DnaJ-1 in this lineage. This demonstrates that these phenotypes are specifically due to the absence of DnaJ-1 and that DnaJ-1 acts after the induction of crystal cell fate and in a cell-autonomous way. So far, the function of DnaJB proteins in hematopoiesis has barely been studied and thus our results provide the first evidence that this conserved family of co-chaperones controls blood cell development *in vivo*. Besides the circulating larval blood cells, it would be interesting now to study the role of DnaJ-1 in the development of the crystal cells in the embryo, the lymph gland and the adult but also to assess its function in other blood cell types during *Drosophila* hematopoiesis.

In addition, like in *mlf*<sup>-/-</sup> mutant larvae, we found that the increase in LZ<sup>+</sup> blood cell size and number observed in *dnaj-1*<sup>-/-</sup> larvae is rescued when we enforced the expression of Lozenge in this lineage. Together with our results in Kc167 cells, this strongly suggests that MLF and DnaJ-1 control LZ<sup>+</sup> cell development by promoting Lozenge activity. Consistent with this hypothesis, we found that reducing Lozenge expression or interfering with its activity causes an increase in the number and the size of LZ<sup>+</sup> cells, a phenotype that could be described as “preleukemic”. This not only reinforces our conclusion but also establishes an interesting parallel with the situation in mammals where a reduction in RUNX1 activity has been associated with oncogenic blood cell transformation.

Moreover, we could link the decrease in Lozenge level/activity with an over-activation of the Notch signaling pathway and our data suggest that high levels of Lozenge directly repress Notch expression and thus activation of the Notch pathway to control LZ<sup>+</sup> blood cells number and size. It will be interesting to decipher how Lozenge represses Notch transcription and to explore further how the Notch pathway controls LZ<sup>+</sup> cells growth and

survival. Again, this could be particularly interesting to study the role of Notch pathway activation in human leukemia.

## **5. Conservation of regulation of RUNX transcription factor stability and activity by MLF/DnaJ-1**

Given the conservation of the RUNX, MLF, DnaJ and Hsp70 families through evolution, it will be particularly interesting to study the relationships between the MLF/DnaJ/Hsc70 complex and RUNX factors in other species. RUNX1 and Lozenge share a highly conserved runt domain, and we showed that RUNX1 stability is regulated by MLF, DnaJ-1 as well as Hsc70-4 in Kc167 cells. In addition, we found that MLF and Hsc70-4 (but not DnaJ-1) bind to RUNX1. Moreover, it was shown previously in the lab that RUNX1-ETO protein level is also regulated by MLF both in *Drosophila* and in human leukemia cells (Bras *et al.*, 2012). Thus, the MLF/DnaJ/Hsc70 complex could play a conserved role in the regulation of RUNX transcription factors stability and it would be worth investigating whether homologs of MLF, DnaJ-1 and Hsc70-4 also control the level and/or the activity of RUNX transcription factors in human, particularly in normal and leukemic blood cells. One limitation of these study is that mammals could have the high level of gene redundancy between members of the DnaJ family, Thus, studies in simpler model organisms like *Drosophila* still offer a good opportunity to study *in vivo* the function of these factors.

## **6. Perspectives**

We have demonstrated that MLF, DnaJ-1 and Hsc70-4 interact with Lozenge, and regulate the stability as well as the activity of this RUNX transcription factor. Yet, we still don't know whether all these proteins can be in the same complex and how they organize topologically. Therefore, more biochemical approaches are needed to gain insights into the relationships among these proteins and to decipher how MLF and DnaJ-1/Hsc70-4 control Lozenge stability.

In our study, biochemical experiments were carried out in Kc167 cells, an embryonic blood cell line. These cells have lower levels of endogenous MLF than crystal cells, which lead to differences in Lozenge expression and regulation between these two cell types. Notably, a reduction in Lozenge expression was observed in Kc167 cells when DnaJ-1 was knocked down, while Lozenge accumulation was present (and stronger) in LZ<sup>+</sup> cells in

*dnaj-1* null mutant larvae. In addition, we cannot exclude the possibility that other important proteins implicated in Lozenge regulation by the MLF/DnaJ/Hsc70 complex are differentially expressed between Kc167 cells and LZ<sup>+</sup> larval blood cells. Thus Kc167 cells are not ideal to characterize the regulation of Lozenge stability by MLF and DnaJ-1. It would be best if we could isolate and purify sufficient amounts of LZ<sup>+</sup> cells from *Drosophila* embryos or larvae to perform these experiments, or if we could obtain a cell line that contains endogenous MLF level equivalent to those observed *in vivo* in crystal cells.

We have revealed that MLF and DnaJ-1 interact with each other via their highly evolutionarily conserved domains and that Lozenge also associates with each one of them, establishing a functional link between RUNX transcription factors and the Hsp70 chaperone complex formed by MLF, DnaJ-1 and Hsc70-4. Based on our study of RUNX1 regulation in Kc167 cells, we propose that a complex homologous to the MLF/DnaJB/Hsc70 complex could regulate the stability of RUNX in human. More generally, it seems that MLF1 and MLF2 could regulate the Hsp70 chaperone machinery in human. Hence, besides their impact on RUNX transcription factors, it will be interesting to identify other proteins whose stability/activity is regulated by MLF and DnaJ and that could be implicated for instance in neurodegenerative diseases, in which these two families of proteins are involved. This would provide valuable insights into the molecular mechanism of action of MLF and DnaJ protein families in normal development and in pathological situations.



## **Chapter IV References**





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